A BIOTECHNOLOGICAL APPROACH FOR DECAFFEINATION

A thesis submitted to the
University of Mysore for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

By
RENU SARATH BABU VEGESNA

Department of Fermentation Technology and Bioengineering
Central Food Technological Research Institute
Mysore-570020, India

February 2007

Dr. M. S.Thakur,
Scientist,
Fermentation Technology and
Bioengineering Department.

Date:

CERTIFICATE

I hereby declare that the thesis entitled "**A BIOTECHNOLOGICAL APPROACH FOR DECAFFEINATION**" submitted to the University of Mysore for the award of the degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** by **Mr. VEGESNA RENU SARATH BABU**, is the result of the research work carried out by him in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, India, under my guidance during the period June 2002 – February 2007.

(M. S. Thakur)

VEGESNA RENU SARATH BABU,
Senior Research Fellow,
Fermentation Technology and
Bioengineering Department,
Central Food Technological Research Institute,
Mysore-570020.

Date:

DECLARATION

I hereby declare that the thesis entitled **"A BIOTECHNOLOGICAL APPROACH FOR DECAFFEINATION"** submitted to the University of Mysore for the award of the degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** is the result of the research work carried out by me in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, India, under the guidance of **Dr. M. S. Thakur** during the period of June 2002 –February 2007.

I further declare that the work embodied in this thesis had not been submitted for the award of degree, diploma or any other similar title.

(VEGESNA RENU SARATH BABU)

Acknowledgements

I owe much of what I know about care as well as the ability to express it. My first, and most earnest, acknowledgment must go to my guide Dr. M. S. Thakur. Seven years ago, a chance meeting with him started me on the path I traveled at CFTRI. Dr. Thakur has been instrumental in ensuring my academic, professional, and moral well being ever since. In every sense, none of this work would have been possible without him.

I am immensely grateful to the Director CFTRI, for allowing me to take up my Ph.D. at CFTRI and for providing facilities for carrying out my research work.

In particular, I would like to thank Dr. N.G.Karanth, whose guidance is a privilege very few people can have. I owe a huge debt of gratitude to him, who was instrumental in improving my scientific writing skills. I can hardly imagine how my work would have evolved without his many comments, suggestions and clarifications. It is an honour working with a person like him.

Far too many people to mention individually have assisted in so many ways during my work at CFTRI. They all have my sincere gratitude. I would like to thank Dr. Ghildhyal, Dr. M. C. Misra, Dr. S. Divakar, Dr. S. G. Prapulla, Dr. A. Sattur, Mr. Eugene Raj, Dr. Yogesh, Mr. M.A. Kumar and Dr. Somasekhar for their help and support in my work. I also salute the intellectual input of Dr. Appu Rao, Dr. U.J.S. Prasada Rao and Dr. S.G.Bhat.

I am also indebted to the Council of Scientific and Industrial Research for direct financial aid through CISR-JRF and SRF fellowships, awards, and travel grants.

My sincere thanks also go to Dr. Ch. Mohan Rao, Dr. T. Ramakrishna Murthy and Mr. Tirumal at CCMB, Hyderabad, for helping me with the protein characterization work. I also express my gratitude to Dr. Girish Sahni (Director, IMTECH), Dr. G. P. S. Raghava and Mr. Manish (IMTECH, Chandigarh) for helping me predict the protein structure. My sincere thanks to Mr. Ravi Kumar, Executive Director, AVT Natural Products and Mr. Marimuthu, Manager, Tantea, Pandiar tea factory for their help in procuring tea dhool and valuable interactions in development of biodecaffeination process.

Thanks to the concept of ***Biodecaffeination***, because of which I not only enjoyed science but also had been fortunate to see the wonderland of tea and coffee gardens in Ooty and Coorg. I am indebted to the IT and World Wide Web for opening up a plethora of knowledge to carry out my work. Google had always been a shoulder to depend upon for answering my queries.

No words are enough to express my immense gratitude to Sanju and Ashwin who were with me in all my ups and downs in all these years.

I also thank my former seniors and friends at CFTRI, Devraj, Naveen, Mohan, Suresh, Obul Reddy, Vivek, Ravi and Sekhar who were very helpful. The fun and enthusiasm with the group is never forgettable.

My sincere thanks to Sujith, Raghuraj, Abhijit, Dr. Shabana, Praveen and other Research Fellows and Assistants in FTBE department. A special note of thanks goes to Mr. Somanna, who has been helpful during my fermentation studies and his ready to help nature.

Last but not least I thank the staff of CIFS, Accounts and other administrative departments who were ever ready to help me when required.

A penultimate thank-you goes to my wonderful parents, for always being there when I needed them most, and never once complaining about how infrequently I visit, they deserve far more credit than I can ever give them. I owe this thesis to my brother, who is the driving force in my pursuing my Ph.D.

The same also to my In-laws who have been supportive and encouraging in all these years.

My final, and most heartfelt, acknowledgment must go to my wife Bharathi. Her patience, support, encouragement, and companionship have turned my journey through life into a pleasure. For all that, and for being everything I am not, she has my everlasting love.

I thank **THEE,** for without his blessings, I would never have had been able to be able to reach this stage of life. God, the almighty!! I bow in front of you for being with me in all times of need and giving me the strength, will and opportunities I had enjoyed in all walks of my life.

V. Renu Sarath Babu.

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List of Abbreviations:

AA	Amino Acids
AAS	Atomic Absorption Spectroscopy
AMP	Adenosine Monophosphate
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CDM	Caffeine Demethylase
cDNA	complementary DNA
Cf	Caffeine
CFE	Cell Free Extract
CLM	Caffeine Liquid medium
CPDE	Cyclic Phosphodiesterase
CPR	Cytochrome P450 Reductase
CYP	Cytochrome P-450
DCPIP	Dichlorophenol Indophenol
DEAE	Diethyl Amino ethyl
DNA	Deoxyribo Nucleic acid
DO	Dissolved Oxygen
DTT	Dithio Threitol
EDTA	Ethylene Diamine Tetra Acetic Acid
ESI	Electro spray Ionization
FMO	Flavin-containing Monooxygenase
FTIR	Fourier Transform Infra Red Spectroscopy
GABA	Gamma Amino Butyric Acid
GC	Gas Chromatography
gDNA	genomic DNA
Gly	Glycine
GMP	Guanosine Monophosphate
HIC	Hydrophobic Interaction Chromatography
HNN	Hierarchical Neural Network
HPLC	High Performance Liquid Chromatography
IMP	Inosine Monophosphate
KDa	Kilo Daltons
Km	Michael Mentons Constant
LC-MS	Liquid Chromatography-Mass Spectroscopy
LCST	Low Critical Solution Temperature
LD	Lethal Dose
µM	Micro Moles
MB	Molecular Biology
MC	Micro Carriers
MTCC	Microbial Type Culture Collection
MWCO	Molecular Weight Cutoff
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
NMT	N-Methyl transferases
P450	Pigment at 450 nm
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBSA	Protein Based Stabilizing Agent
PHB	Poly Hydroxy Alkanoate
PMS	Phenazine Metho Sulphate
PMSF	Phenyl Methyl Sulphonyl Fluoride
PRP	Proline Rich Protein
PSI	Pounds Per Square Inch.
PVA	Polyvinyl Alcohol

Px	Paraxanthine		Ethyl Diamine
RNA	Ribo Nucleic Acid	TFA	Trifluoro Acetic Acid
SDS	Sodium Dodecyl Sulphate	THF	Tetra hydro furan
		TLC	Thin layer Chromatography
SEM	Scanning Electron Microscope	TMU	Trimethyl Uric Acid
SIDS	Sudden Infant Death Syndrome	Tp	Theophylline
		Vmax	Maximum Velocity of Enzyme Reaction
SRS	Substrate Recognition Sites	XMP	Xanthosine Monophosphaste
Tb	Theobromine		
TEMED	Tetra Ethylene Methyl	XO	Xanthine Oxidase

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Title: A BIOTECHNOLOGICAL APPROACH FOR DECAFFEINATION

Synopsis

Tea and coffee are consumed largely world over. India is one of the largest exporters of coffee and tea and these beverages form a part of the major foreign exchange earner for our country. Presence of caffeine limits the frequent intake of these beverages, the reason being the toxic and addictive effects of caffeine when taken in large doses leading to a need for the decaffeination of coffee and tea. The market for decaffeinated products is increasing at 10-15% Per annum. However conventional decaffeination technologies use toxic solvents, and methods, which not only strip the essential flavor and aroma components, but also are expensive, and environmentally unsafe. The present day shift is towards safe and economical processes for decaffeination through biotechnological route (biodecaffeination) are considered as the best alternative to the existing methods of decaffeination. This thesis reports the development of a process for biodecaffeination of caffeine containing materials by suitable microorganisms and enzymes.

The work involved the isolation, characterization and identification of potent microorganisms capable of degrading caffeine, selection of the efficient organisms for caffeine degradation and optimization of parameters of growth and caffeine degradation. Further work was carried out on the identification of enzyme/enzymes responsible for caffeine degradation, immobilization of whole cells and enzymes for bio-decaffeination and optimization of parameters for efficient bio-decaffeination by the immobilized cell/enzyme system. Work was also carried out on the development of biodecaffeination processes for coffee, tea and caffeine containing solutions. Development of

biodecaffeination process for tea and coffee involved several complex problems to be solved for application to the real samples, which are detailed in the thesis.

Proposed objectives:

1. Isolation, purification, characterization and identification of microbial cultures for decaffeination.
2. Selection of the efficient organisms for caffeine degradation.
3. Optimization of parameters of growth and caffeine degradation by the selected microorganisms.
4. Identification of enzyme/enzymes responsible for caffeine degradation.
5. Immobilization of whole cells for bio-decaffeination.
6. Immobilization of enzymes responsible for bio-decaffeination.
7. Optimization of parameters for efficient bio-decaffeination by the immobilized cell/enzyme system.

Thesis Organization:

Chapter 1:

The first chapter of thesis is on review of the literature on caffeine, its effects on health, existing methods of decaffeination and their advantages and disadvantages, degradation of caffeine in different biological systems and microbial degradation of caffeine.

The first section in the chapter deals with caffeine, in general; availability of caffeine, details on worldwide consumption of caffeine and health problems associated with excess caffeine intake. The second section of the chapter details the importance of decaffeination, the conventional methods of decaffeination and the disadvantages.

The last section is related to the importance of biodecaffeination, its advantages, the constraints existing for developing methods of biodecaffeination and the possibilities of developing biodecaffeination methods. It also deals with the, work carried out by different groups around the world on development of biodecaffeination, biological systems involved in biodecaffeination and the drawbacks and constraints present in the development of efficient biodecaffeination technologies. The chapter concludes with the challenges and opportunities for the development of biodecaffeination technologies at CFTRI and future prospects.

Chapter 2:

The second chapter is organized into three sections and is focused on the isolation, and characterization of caffeine degrading microorganisms and optimization of parameters for biodecaffeination using the cells.

The first section is a brief background of the work done by other groups on isolation of microorganisms capable of degrading caffeine, and includes an introduction to biodecaffeination.

The second section deals with experimental details of isolation, characterization and identification of biodecaffeinating organisms and optimization of several physico-chemical parameters for biodecaffeination by the isolated organisms.

The third section deals with results and discussion of the work on the isolation and characterization of three bacteria capable of degrading caffeine, selection of potent caffeine degrading organism (*Pseudomonas alcaligenes* MTCC 5264), optimization of media, carbon source, nitrogen source and physical conditions for efficient biomass production and caffeine degradation by *P. alcaligenes* MTCC 5264.

Chapter 3:

The third chapter deals with immobilization of whole cells of *P. alcaligenes* MTCC 5264 in different matrices and studies on biodecaffeination with immobilized cells. The chapter details the studies on the selection of a suitable matrix for immobilization and biodecaffeination of pure caffeine and tea and coffee using the immobilized cells. Cells immobilized in calcium alginate were found to be most suitable and used for immobilization of *P. alcaligenes* MTCC 5264. Immobilized cells could do biodecaffeination of a pure caffeine solution, whereas complete biodecaffeination of coffee and tea was not possible due to the utilization of sugars and other nutrients in tea by the organism and not caffeine. Moreover, the process affects the quality of the coffee and tea making it unsuitable for biodecaffeination of coffee and tea. Therefore an enzymatic process for biodecaffeination is viewed as a potent alternative and this chapter ends with suggestions on development of biodecaffeination processes based on immobilized or soluble enzymes.

Chapter 4:

Fourth chapter deals with the isolation, identification and partial characterization of enzymes involved in the biodecaffeination.

The first section of the chapter is a brief introduction to the enzymes involved in degradation of caffeine by microorganisms.

The second sections deals with the methodologies applied in the study. The third section represents the results, discussion and conclusions on the nature of the enzymes involved in caffeine degradation. The enzymes involved in the degradation of caffeine by *P.alcaligenes* MTCC 5264 identified as caffeine demethylase, xanthine oxidase, uricase,

allantoinase, allantoicase, glyoxylate dehydrogenase and urease. Caffeine demethylase has been found to be the rate-limiting enzyme in this process and is highly inducible. The enzyme is also highly unstable and is being reported conclusively for the first time in this thesis. This chapter focuses on the isolation, identification and characterization of caffeine demethylase enzyme and the stabilization of the enzyme.

Caffeine demethylase enzyme (1N-demethylase) was purified and characterized by using LC-MS/MS Analysis and bioinformatics tools and the 3D structure of the enzyme was predicted.

Chapter 5:

This chapter is organized into three sections. The first section is a brief introduction to the concept of biodecaffeination, and a survey of the research work done on biodecaffeination in different parts of the world.

The second section of the chapter represents the methodologies adopted to develop biodecaffeination processes for coffee and tea.

The third section is a detailed analysis of the results and includes discussions on the biochemical aspects of biodecaffeination. In conclusion, the parameters for efficient biodecaffeination using enzymes were optimized and the stabilized enzyme preparation was used for the biodecaffeination of tea dhool and immobilized enzymes were used for the biodecaffeination of coffee beans through an extraction cum biodecaffeination process designed by us. These processes achieved more than 80-90% biodecaffeination in tea and coffee respectively. This chapter ends with recommendations on further work to be carried out on the caffeine demethylase enzyme and other enzymes involved in biodecaffeination.

Chapter 6:

Chapter 6, deals with studies on the development of a whole cell based biosensor for caffeine analysis in food, fermentation and clinical samples. In brief, we have utilized a microbe, which can degrade caffeine to develop an amperometric biosensor for determination of caffeine in solutions. Whole cells of *P. alcaligenes* MTCC 5264 having the capability to degrade caffeine were immobilized by covalent crosslinking method. The biosensor system was able to detect caffeine in solution over a concentration range from 0.1 mg mL^{-1} to 1 mg mL^{-1}. With read-times as short as 3 min, this caffeine biosensor acts as a rapid analysis system for caffeine in solutions. Although a few biosensing methods for caffeine are reported, they have limitations in application for commercial samples. The optimum pH and temperature of measurement were 6.8 and 30 ± 2^{0}C respectively. Interference in analysis of caffeine due to different substrates was not observed. Caffeine content of commercial samples of instant tea and coffee was analysed by the biosensor and the results compared well with HPLC analysis.

Conclusions and Future Recommendations:

The work reported in this thesis has led to the development of biodecaffeination processes for coffee and tea. Although several critical factors, which affect the biodecaffeination process, have been identified and optimized, there is a scope for improvement of the efficiency of this process. Further studies on stabilization of caffeine demethylase, cloning and hyper expression of caffeine demethylase, molecular characterization of the enzyme are to be carried out. Also studies on the scale up of the biodecaffeination process are needed and are being carried out.

In conclusion, this thesis deals with the basic studies on the microbiological, enzymological and biochemical aspects of development of biodecaffeination processes. Several bottlenecks which were found to hinder the process of biodecaffeination were overcome through a detailed study on the factors influencing the activities of enzymes in biodecaffeination has been carried out. An enzymatic process for biodecaffeination of coffee was developed by using an immobilized system and a biodecaffeination process for tea was developed by using soluble enzymes during fermentation of tea. The biodecaffeinated tea and coffee had the same taste and aroma profile and no change in quality was observed. These processes have immense potential in industries and are being pursued.

Dr. M.S. Thakur **V. R. Sarath Babu**

Guide.

CHAPTER 1
REVIEW OF LITERATURE

1.1. SCOPE OF THE REVIEW:

Caffeine as a stimulant molecule has gained very high prominence and is the most widely used molecule without any legal restrictions. This molecule has attracted several researchers around the world and a lot of research has been done around several aspects of this molecule. Apart from its clinical importance, caffeine is also commercially significant in terms of its consumption in the form of caffeinated beverages like coffee, tea and cola drinks. Recent studies show that excess consumption of caffeine has many health implications in humans. Therefore decaffeination technologies have been developed since 1920's through chemical routes. These chemical decaffeination methods have several disadvantages. Biodecaffeination as an alternative route for decaffeination is in the budding stage and knowledge about this area of coffee and tea biotechnology is scarce.

The first part of this review of literature details a brief history of caffeine, its use world over, statistics of caffeine consumption in terms of different beverages and clinical aspects of caffeine consumption. Conventional decaffeination technologies and their disadvantages are detailed in the middle part of the review and the later part of the review deals with the biological aspects of caffeine metabolism by different organisms, the enzyme systems involved in humans, plants, fungi and bacteria and the existing literature on biodegradation of caffeine by these systems. The later part also discusses the developments in the area of biodecaffeination at Central Food Technological Research Institute (CFTRI), Mysore, India. The review ends with conclusions and future perspectives of the development of biodecaffeination technologies using enzymes isolated from microorganisms.

1.2. HISTORY OF CAFFEINE:

Caffeine, a methyl xanthine molecule is the most widely consumed psychoactive substance in the world over, most commonly from the beverages coffee, tea and soda. The English word **caffeine** comes from the French (Spanish & Portuguese) word for **coffee**: ***café***. Because of its stimulatory nature, it was used as a cardiotonic till the end of 19th century (Wijhe, 2002). In the first half of the 20th century, it was used as a stimulant of respiration and circulation in Dutch medicine. The Islamic physicians were the first to exploit the medicinal use of coffee well before second millennium A.D, the first documented use as a beverage was by the Sufis of Yemen. With caffeine being increasingly used as a stimulant, it was prohibited from being used as it was thought that caffeine use was a cause for vices and is seditious. Coffee was introduced to England around 1650's and in Holland a decade later. The Dutch introduced the coffee plant to the island of Java in 1688. The island's association with coffee production led to the use of "Java" as a nickname for high quality coffee.

In 1903 an Italian manufacturer invented the **espresso** (Italian for ***fast***). Espresso topped with equal parts of foamed and steamed milk is called **cappuccino**. Caffeine contributes to a particular proportion of the perceived bitterness of a cup of coffee and makes a small contribution to espresso's strength and body (Illy and Viani, 1995).

1.2.1. Discovery of caffeine and related compounds:

Kihlman (1974) has excellently reviewed the discovery of caffeine in his book 'Caffeine and Chromosomes'. Although not called caffeine or 1,3,7-trimethylxanthine at the time, German and French workers discovered the compound independently in

the early 1820s. In the book 'Neueste Phytochemische Entdeckungen zur Begründung einer wissenschaftlichen Phytochemie', Ferdinand Runge (1820) described a substance with basic properties which he had isolated from green coffee beans, and which he termed 'Kaffebase'. This publication appears to contain the first detailed description of caffeine. However, during the same year his German colleague F. Von Giese (1820) reported in a letter to Scherer's 'Allgemeine nordische Annalen der Chemie für die Freunde der Naturkunde und Arzneiwissenschaft' that he had found a new alkaloid in extracts of coffee beans. He called the alkaloid 'Kaffeestoff', but subsequently declared it to be identical with Runge's 'Kaffebase' (Giese, 1821). Independent of the German discoveries, the French workers Robiquet (1823) and Pelletier (1826) (in collaboration with Caventou) discovered caffeine in extracts of coffee beans, and described it as a white crystalline volatile substance remarkable for its very high content of nitrogen, without referring to it by any particular name. Who first named the compound caffeine is difficult to say. However, in the *Dictionnaire des termes de Médicine* of 1823, the substance is listed under the name 'cafiéne' or 'cofeina' and Fechner (1826) described it in his 'Repertorium der organischen Chemie' under the heading 'Caffeine oder Coffein'.

Table 1.2.1. lists plants, which contained a new substance, given a specific name by the discoverer, but which was subsequently shown to be caffeine. An analogous alkaloid, theobromine, was identified in cacao beans (*Theobromina cacao*) by Woskresensky (1842) and another one, theophylline, in tea leaves by Kossel (1888). Both these compounds are dimethylxanthines.

Table 1.2.1: Plants reported to contain a new substance subsequently identified as caffeine.

Beverage/Food (Plant)	New Compound's Original Name	Reference
Coffee (*Coffea arabica*)	Kaffebase Kaffeestoff	Runge (1820) Giese (1820) Robiquet (1823) Pelletier (1826)
Tea (*Camellia sinensis*)	Thein	Oudry (1827) Mulder (1838) Jobst (1838a, 1838b)
Guarana (*Paulina sorbilis*)	Guaranine	Martius (1840) Berthemot and Dechastelus (1840)
Paraguay tea or Mate (*Ilex paraguanensis*)	No name	Stenhouse (1843a, 1843b)
Kola nuts (*Cola acuminate*)	No name	Danielle (1865) Attifeld (1865)

1.3. SOURCES OF CAFFEINE:

Caffeine is found in about a hundred species of plants, but the most highly cultivated sources are the coffee beans, (*Coffea arabica* or *Coffea canephora*, variety *robusta*), the leaves & leaf-buds of tea (*Thea sinensis* or *Camellia sinensis*), cola nuts (*Cola acuminata*) and cacao beans (*Theobroma cacao*).

Coffee and tea plants are the major sources of natural caffeine and related compounds such as theophylline and theobromine are produced by a large number of plant species belonging to numerous genera, families, and orders (Table 1.3.1). It is believed that methylxanthine-producing plants accumulate these substances as part of a chemical defence system against pests and herbivores.

Interestingly, a very large proportion of the non-alcoholic beverages used in social settings contain caffeine. The most important beverages and foods containing caffeine are coffee, tea, guarana, maté, cola nuts, cola drinks, cocoa, chocolate,

yaupon and yoco. The amount of caffeine found in these products varies, but is generally high. Based on dry weight, the highest amounts are found in guarana (4-7%). Tea leaves contain approximately 3-5% caffeine, coffee beans 1.1-2.2% (Saldana et.al 2000), cola nuts 1.5%, and cacao beans 0.03% *(*Bogo and Mantle, 2000; Kretschmar and Baumann, 1999). Cacao beans in addition contain about 1.8-2.5% theobromine. Caffeine also occurs in certain soft drinks, energy drinks, and so called “smart” drinks, as well as in medicinal drugs. In these cases, however, purified or synthesized caffeine has often been added to the products.

Table 1.3.1. Caffeine-containing plants (Willaman and Schubert, 1961 O'Connel, 1969).

Family	Species	Number of	
		Genera	Species
Annonaceae	*Annona cherimolia*	1	1
Aquifoliaceae	*Ilex cassine*		
	Ilex cuiabensis		
	Ilex paraguariensis		
	Ilex vomitora	1	4
Cactaceae	*Cereus jamacaru*		
	Harrisia adscendens		
	Leocereus bahiensis		
	Pilocereus gounellei		
	Trichocereus sp.	5	>5
Celastraceae	*Maytenus sp.*	1	>1
Combretaceae	*Combretum jaquinii*		
	Combretum loeflingii	1	2
Dilleniaceae	*Davilla rugosa*	1	1
Geraniaceae	*Erodium cicutarium*	1	1
Icacinaceae	*Villaresia congonha*		
	Villaresia mucronata	1	2

Liliaceae	*Scilla maritime*	1	1
Malpighiaceae	*Banisteriopsis inebrians*	1	1
Nyctaginaceae	*Neea theifera*	1	1
Phytolaccaceae	*Gallesia gorazema*	1	1
Rubiaceae	*Coffea abeokutae*		
	Coffea Arabica		
	Coffea bengalensis		
	Coffea canephora		
	Coffea congensis		
	Coffea exelsa		
	Coffea liberica		
	Coffea perrieri		
	Coffea quillon		
	Coffea robusta		
	Coffea schumanniana		
	Coffea stenophylla		
	Coffea ugandae		
	Genipa Americana		
	Oldenlandia corymbosa	3	15
Sapindaceae	*Paullinia cupana*		
	Paullinia scarlatina		
	Paullinia sorbilis		
	Paullinia triantennata		
	Paullinia yoco	1	5
Sterculiaceae	*Cola acuminate*		
	Cola ballayi		
	Cola johnsoni		
	Cola nitida		
	Cola verticillata		
	Guazuma ulmifolia		
	Helicteres ovata		
	Sterculia chichi		
	Sterculia elata		
	Sterculia platanifolia		
	Sterculia pruriens		
	Sterculia speciosa		
	Theobroma bicolor		
	Theobroma cacao		

	Theobroma grandiflora		
	Theobroma microcarpa		
	Theobroma obovata		
	Theobroma speciosa		
	Theobroma spruceana		
	Theobroma subincana	5	20
Theaceae	*Camellia sinensis*	1	1
Turneraceae	*Piriqueta ulmifolia*		
	Turnera ulmifolia	2	2
Total	**17**	**28**	**>63**

1.4. CAFFEINE CONSUMPTION PATTERNS AROUND THE WORLD:

The type of coffee beans used and the method of preparation of the drink influence the caffeine content of coffee drinks. The average caffeine content of instant coffee, percolated coffee, and filter coffee as calculated was 53, 84 and 103 mg/cup (150 ml), respectively (Andersson et. al., 2004). There are great differences between individuals and cultures regarding the consumed quantities of methylxanthine-containing beverages and foods. This is obvious for coffee but the consumption of tea varies even more than coffee from one country to another.

In the United States, the average daily caffeine intake was estimated to be between 186 and 227 mg. Corresponding intakes in Canada, Australia, Brazil, Sweden, and Denmark are 238 mg, 240 mg, 171 mg, 425 mg and 490 mg, respectively (Andersson et. al., 2004). It is obvious that the intake is higher in the Nordic countries than elsewhere.

The caffeine content of some beverages is given in Table 1.4.1. These beverages are largely consumed by many people world over and account for most of the caffeine consumed by humans.

Table 1.4.1. Caffeine content of some beverages (Barone and Roberts, 1996).

Product	Serving Size [a]	Caffeine (mg)[b]
Coffees		
Coffee, brewed	8 ounces	135
General Foods International Coffee, Orange Cappuccino	8 ounces	102
Coffee, instant	8 ounces	95
General Foods International Coffee, Cafe Vienna	8 ounces	90
Maxwell House Cappuccino, Mocha	8 ounces	60-65
General Foods International Coffee, Swiss Mocha	8 ounces	55
Maxwell House Cappuccino, French Vanilla or Irish Cream	8 ounces	45-50
Maxwell House Cappuccino, Amaretto	8 ounces	25-30
General Foods International Coffee, Viennese Chocolate Cafe	8 ounces	26
Maxwell House Cappuccino, decaffeinated	8 ounces	3-6
Coffee, decaffeinated	8 ounces	5
Teas		
Celestial Seasonings Iced Lemon Ginseng Tea	16-ounce bottle	100
Bigelow Raspberry Royale Tea	8 ounces	83
Tea, leaf or bag	8 ounces	50
Snapple Iced Tea, all varieties	16-ounce bottle	48
Lipton Natural Brew Iced Tea Mix, unsweetened	8 ounces	25-45
Lipton Tea	8 ounces	35-40
Lipton Iced Tea, assorted varieties	16-ounce bottle	18-40
Lipton Natural Brew Iced Tea Mix, sweetened	8 ounces	15-35
Nestea Pure Sweetened Iced Tea	16-ounce bottle	34
Tea, green	8 ounces	30
Arizona Iced Tea, assorted varieties	16-ounce bottle	15-30

Lipton Soothing Moments Blackberry	Tea 8 ounces	25
Nestea Pure Lemon Sweetened Iced Tea	16-ounce bottle	22
Tea, instant	8 ounces	15
Lipton Natural Brew Iced Tea Mix, diet	8 ounces	10-15
Lipton Natural Brew Iced Tea Mix, decaffeinated	8 ounces	< 5
Celestial Seasonings Herbal Tea, all varieties	8 ounces	0
Celestial Seasonings Herbal Iced Tea, bottled	16-ounce bottle	0
Lipton Soothing Moments Peppermint Tea	8 ounces	0
Soft Drinks		
Josta	12 ounces	58
Mountain Dew	12 ounces	55
Surge	12 ounces	51
Diet Coke	12 ounces	47
Coca-Cola	12 ounces	45
Dr. Pepper, regular or diet	12 ounces	41
Sunkist Orange Soda	12 ounces	40
Pepsi-Cola	12 ounces	37
Barqs Root Beer	12 ounces	23
7-UP or Diet 7-UP	12 ounces	0
Barqs Diet Root Beer	12 ounces	0
Caffeine-free Coca-Cola or Diet Coke	12 ounces	0
Caffeine-free Pepsi or Diet Pepsi	12	0

	ounces	
Minute Maid Orange Soda	12 ounces	0
Mug Root Beer	12 ounces	0
Sprite or Diet Sprite	12 ounces	0
Caffeinated Waters		
Java Water	16.9 ounces	125
Krank 20	16.9 ounces	100
Aqua Blast	16.9 ounces	90
Water Joe	16.9 ounces	60-70
Aqua Java	16.9 ounces	50-60
Juices		
Juiced	10 ounces	60
Frozen Desserts		
Ben & Jerry's No Fat Coffee Fudge Frozen Yogurt	1 cup	85
Starbucks Coffee Ice Cream, assorted flavors	1 cup	40-60
Häagen-Dazs Coffee Ice Cream	1 cup	58
Häagen-Dazs Coffee Frozen Yogurt, fat-free	1 cup	40
Häagen-Dazs Coffee Fudge Ice Cream, low-fat	1 cup	30
Starbucks Frappuccino Bar	1 bar (2.5 ounces)	15
Healthy Choice Cappuccino Chocolate Chunk or Cappuccino Mocha Fudge Ice Cream	1 cup	8
Yogurts, one container		
Dannon Coffee Yogurt	8 ounces	45
Yoplait Cafe Au Lait Yogurt	6 ounces	5
Dannon Light Cappuccino Yogurt	8 ounces	< 1

Stonyfield Farm Cappuccino Yogurt	8 ounces	0
Chocolates or Candies		
Hershey's Special Dark Chocolate Bar	1 bar (1.5 ounces)	31
Perugina Milk Chocolate Bar with Cappuccino Filling	1/3 bar (1.2 ounces)	24
Hershey Bar (milk chocolate)	1 bar (1.5 ounces)	10
Coffee Nips (hard candy)	2 pieces	6
Cocoa or Hot Chocolate	8 ounces	5

a— Serving sizes are based on commonly eaten portions, pharmaceutical instructions, or the amount of the leading-selling container size. For example, beverages sold in 16-ounce or half-liter bottles were counted as one serving.

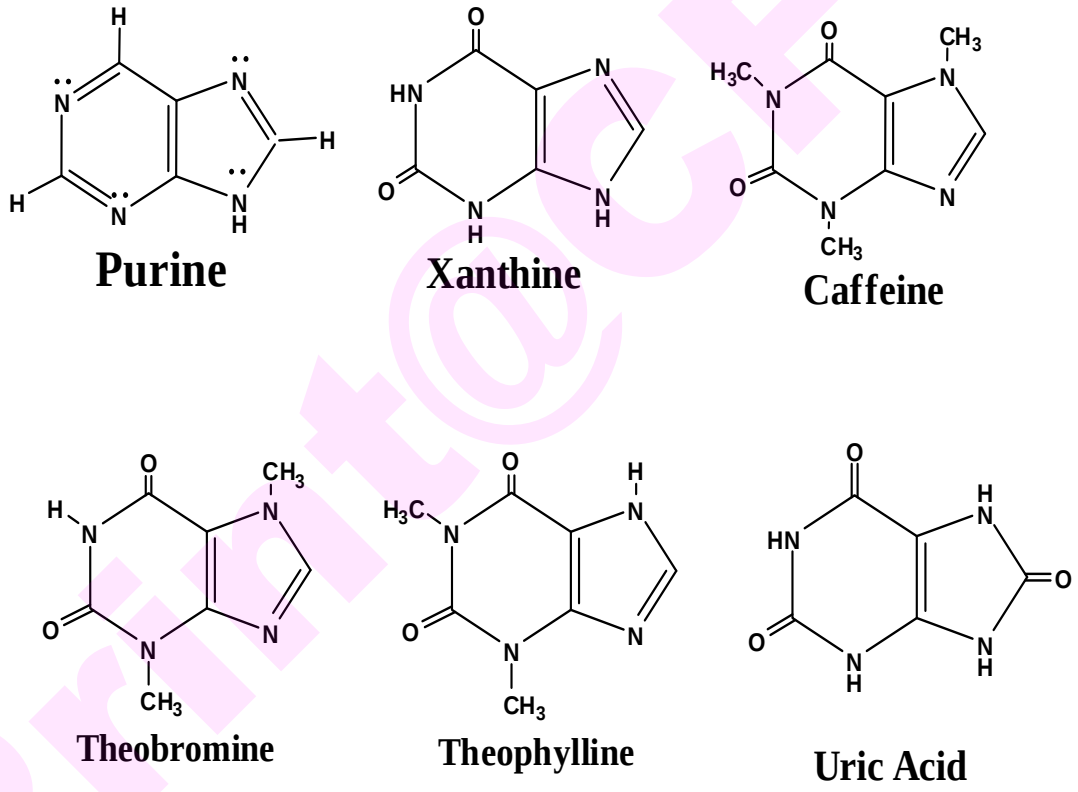
b — Sources: National Coffee Association, National Soft Drink Association, Tea Council of the USA, and information provided by food, beverage, and pharmaceutical companies.

Cocoa is another caffeine rich material, which is widely used in the world in the form of chocolates and other beverages. Use of the cacao (cocoa) bean for a beverage originated with the Indians of South America, possibly earlier than 1,000 B.C. The Spanish were introduced to the drink by the Aztecs. In 1886 the Georgia pharmacist John Pemberton (www.wikipedia.com) created a beverage flavored by caffeine-containing kola nuts and fortified by cocaine from the coca plant (*Erythroxylum coca*) of Peru and Bolivia. He called his beverage Coca-Cola in honor of the plants of origin. Near the turn of the century cocaine was removed from Coca-Cola and replaced with more caffeine.

1.5. CAFFEINE CHEMISTRY:

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6 dione), a purine alkaloid, is a key component in most popular drinks especially tea and coffee. It is a white compound, moderately soluble in water and organic solvents like methylene chloride, chloroform, ethanol, ethyl acetate, methanol, benzene, etc.

Figure 1.5.1. Structures of purine, xanthine, caffeine, theobromine, theophylline and uric acid.



Caffeine (Fig.1.5.1) is 1,3,7-trimethylxanthine, meaning it is a **xanthine** molecule with methyl groups replacing all of the three hydrogens bound to nitrogens in the xanthine ring. The molecular weight of caffeine is 194.2 and structurally it is related to uric acid and contains imidazole and a uracil ring (Tarnopolsky, 1994).

Besides its stimulatory effects for which it is consumed, caffeine has no nutritional value (Clarkson, 1993; Tarnopolsky, 1994; MacIntosh and Wright, 1995).

Theobromine and Paraxanthine (Fig. 1.5.1.) are dimethyl xanthines derived from the removal of methyl groups at 1 and 3 N positions of the xanthine ring of caffeine. Caffeine, paraxanthine and theobromine have stimulatory effects on humans, but caffeine due to its three methyl groups is associated with highest stimulatory activity and other ill effects on health. Dimethyl xanthines are known to possess anti asthmatic, anti cancer and antioxidant properties (Persson, 1984; Yuji, et. al., 1998; Omar et. al., 2005). Removal of methyl groups reduces the ill effects of these molecules and increases the therapeutic values of the derivatives.

1.6. MECHANISMS OF ACTION OF CAFFEINE:

Caffeine is responsible for the stimulant action of coffee (Europaisches, 1978). It stimulates the Central nervous system, increases the contraction power of the heart, widens the vessels of heart, kidney and the skin and exhibits broncholytical and diuretical actions (Europaisches, 1978).

In mammals, ingested caffeine is rapidly absorbed, metabolized, and excreted in the urine as methyl xanthine derivatives. Apart from being a stimulant to the central nervous system, if consumed in excess it causes mutation; it is teratogenic, causes inhibition of DNA repair, inhibition of cyclic AMP phoshodiesterase activity and inhibits seed germination (Friedman and Waller, 1983a and b). It is the major cause of cancer, heart diseases, and complications in pregnant women and aging (Green and Suls, 1996; Infante et. al., 1993; Srisuphan and Bracken, 1986, Dlugosz et.al., 1996; Fenster et. al., 1991).

The stimulant effect of caffeine is thought to be due to an increase in adrenaline release, which may stimulate the sympathetic nervous system, but the mechanism is not wholly understood (Clarkson, 1993). Caffeine enhances Acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors (Carter, et al, 1995).

It is known that caffeine is responsible for many effects on the human body. Details of the mechanism of caffeine at cellular and organ level can give an insight into how this molecule affects different functions in the body and the reasons for adverse effects on the body.

1.6.1. Cellular Effects:

There are four main mechanisms of caffeine action at the cellular level. These are intracellular mobilisation of calcium from the sarcoplasmic reticulum of the skeletal muscle, inhibition of phosphodiesterases, adenosine antagonism and Sodium/Potassium ATPase pump activity changes (Tarnopolsky, 1994).

1.6.1.1. Mobilization of extra cellular calcium:

In vitro, caffeine increases the release of calcium and inhibits its uptake from the sarcoplasmic reticulum. After ingestion of coffee the circulating plasma concentration of caffeine is less than 100 μM and caffeine becomes toxic at a concentration above 200 μM and lethal at 500 μM (Tarnopolsky, 1994).

1.6.1.2. Phosphodiesterase Inhibition:

Phosphodiesterase breaks down cAMP which is the second messenger in most of the cell signaling pathways in humans and animals. Caffeine is known to inhibit the phosphodiesterase in vitro and invivo (Fredholm, 1995). When this process is

inhibited increase in cAMP results, which enhances the stimulation of catecholamines.

1.6.1.3 Adenosine antagonism:

Caffeine being structurally similar to adenosine competes for adenosine receptors. Adenosine reduces the spontaneous firing of neurons, suppresses synaptic transmission and causes the release of neurotransmitters. The net effect of this process includes vasoconstriction, increased diuresis and central nervous system stimulation (Tarnopolsky, 1994). This can occur at less than 100 mM, which can be achieved by drinking one to three cups of coffee. Thus this mechanism may be feasible as a mechanism of action of caffeine in vivo (Nehlig and Debry, 1994).

1.6.1.4 Sodium/Potassium ATPase Pump Activity:

Caffeine is known to increase the levels of adrenalin in the body, which in turn increases the sodium/potassium ATPase pump activity. This leads to the accumulation of low levels of potassium in the muscle during exercise leading to fatigue. Therefore caffeine affects performance by varying the sodium/potassium ATPase pump activity (Tarnopolsky, 1994).

1.6.1.5. Catecholamines:

A number of studies report the increase in plasma adrenaline levels after caffeine ingestion (Graham, et. al., 1994). Caffeine shows a direct specific action on the adrenal medulla, which is a stress mechanism. Excessive caffeine consumption therefore increase stress in the body (Graham, et. al., 1994).

1.6.2. Side Effects:

Caffeine stimulates the central nervous system and can produce a variety of effects elsewhere in the body. The symptoms of a caffeine overdose ("caffeinism") will vary, according to individual differences and the amount consumed. Doses ranging from 250 to 750 mg (2 to 7 cups of coffee) can produce restlessness, nausea, headache, tense muscles, sleep disturbances, and irregular heart beats (Tarnopolsky, 1994). Doses of over 750 mg (7 cups of coffee) can produce a reaction similar to an anxiety attack, including delirium, ringing ears, and light flashes. These amounts of caffeine may come from a single dose or from multiple doses at short intervals (Shirlow and Mathers, 1985).

1.6.2.1. Effects on sleep:

Adenosine is a potentiator of sleep by increasing potassium ion influx thereby hyperpolarizing (inhibiting) neurons. Adenosine also reduces the activity of GABA neurons in the brain promoting sleep (Strecker et. al., 2000). Caffeine promotes wakefulness by opposing the actions of adenosine, blocking the adenosine receptors. Caffeine increases attention & vigilance and lessens feelings of weariness.

1.6.2.2. Toxic dose:

The LD-50 of caffeine is estimated at 10 grams for *oral* administration. The lethal dosage varies from individual to individual according to weight. Ingestion of 150mg/kg of caffeine seems to be the LD-50 for all people (Kerrigan and Lindsey, 2005; Holmgren et.al., 2004; Walsh et.al., 1987; Mrvos et.al., 1989). In small children ingestion of 35 mg/kg can lead to moderate toxicity.

1.6.3. Caffeine and health problems:

1.6.3.1. Gastrointestinal problems:

Many people experience a burning sensation in their stomach after drinking coffee because coffee increases the secretion of hydrochloric acid leading to an increased risk for ulcers (James and Stirling, 1983). Coffee reduces the pressure on the valve between the esophagus and the stomach so that the highly acidic contents of the stomach pass up to the esophagus leading to heartburn and gastro-esophageal reflux disease.

1.6.3.2. Heart disease:

Researchers at Johns Hopkins Medical Institute (LaCroix et al., 1986) found heavy coffee drinkers (defined as five or more cups per day) were two to three times more likely to have coronary heart disease than were nondrinkers (Onrot et.al., 1985). This relationship was true even when accounting for other important risk factors such as age, smoking habits, serum cholesterol and blood pressure (James, 1997; Waring et. al., 2003; Leviton and Cowan, 2002).

If coffee drinking does increase the risk of heart disease, it may do so through its effect on cholesterol. A few studies have linked heavy coffee consumption to elevated total serum cholesterol, although caffeine alone does not seem to be responsible (Thelle et. al., 1983). Coffee in excess of 8 cups per day may aggravate cardiac arrhythmias (Meyers et.al., 1991) and raise plasma homo-cysteine (Petra et. al., 2002). Caffeine is also linked to coronary vasospasms, the cause for 20% of all fatal heart attacks, which kill otherwise perfectly healthy people.

1.6.3.3. Cancer:

In the period between the 1950s and 1970s many believed that caffeine could be a serious cause of cancer in humans because of studies in plants showing chromosome breaks, inhibition of mitosis and formation of chromatin bridges after high-dose caffeine treatment (Brogger, 1979). More recent evidence does show a capacity for caffeine to worsen the mutagenicity of ionizing radiation and other carcinogenic agents through interference with cell cycle control (Kaufmann et. al., 1997).

1.6.3.4. Addiction and withdrawal:

Caffeine is addictive according to several definitions of 'addiction' (Kaufmann et.al., 1997; Daly and Fredholm, 1995; Greenberg et. al., 1999). It causes withdrawal symptoms after cessation of heavy use (most commonly headaches) and regular users develop tolerance and experience cravings when ceasing use. Regular users can also become emotionally and mentally dependent upon their daily caffeine (coffee, soda, etc). The withdrawal effects of caffeine in humans are headache, fatigue, apathy and drowsiness (Nehlig, 1999; Lorist and Tops, 2003).

1.6.3.5. Effects on pregnant women:

Caffeine has long been suspected of causing mal-formations in fetus, and that it may reduce fertility rates (Hatch and Bracken, 1993; Mills, et.al, 1993; Eskenazi, 1999; Cnattingius et. al., 2000; Christian and Brent, 2001). A recent study found a weak link between Sudden-Infant-Death-Syndrome (SIDS) and caffeine consumption by the mother, which reinforces the recommendation for moderation -possibly even abstinence- above. On men, it has been shown that caffeine reduces rates of sperm

motility, which may account for some findings of reduced fertility (Christian and Brent, 2001).

1.6.3.6. Osteoporosis:

There was a significant association between (drinking more) caffeinated coffee and decreasing bone mineral density at both the hip and the spine, independent of age, obesity, years since menopause, and the use of tobacco, estrogen, alcohol, thiazides, and calcium supplements (in women) (Barrett, et, al., 1994).

1.6.3.7. Metabolism:

Caffeine increases the level of circulating fatty acids. This has been shown to increase the oxidation of these fuels, hence enhancing fat oxidation. Caffeine has been used for years by runners and endurance in people to enhance fatty acid metabolism. It's particularly effective in those who are not habitual users (Acheson et. al., 2004; Graham et.al., 1994)

1.6.3.8. Blood sugar swings:

Caffeine mobilizes intracellular sugars and induces a temporary surge in blood sugar which is then followed by an overproduction of insulin that causes a blood sugar crash within hours (Pizziol et. al., 1998). Its use as a weight loss agent infact leads to the increase in weight due to its hyperglycemic effect which stimulates insulin's message to the body to store excess sugar as fat (Dam and Hu, 2005; Lee et.al., 2005).

1.6.3.9. Nutritional deficiencies:

Caffeine inhibits the absorption of some nutrients and causes the urinary excretion of calcium, magnesium, potassium, iron and trace minerals, all essential

elements necessary for good health. Coffee drinking is associated with decreased absorption of magnesium resulting in lower blood levels of magnesium (Johnson, 2001). Caffeine reduces the reabsorption of calcium and magnesium in the kidney, causing minerals to be excreted in the urine (Massey and Wise, 1984; Massey et.al., 1994). Although caffeine and coffee intake does not directly influence potassium absorption, caffeine has a diuretic effect, and diuretics increases excess excretion of potassium as well as magnesium from the kidneys (al'Absi et. al., 1998, Lovallo et.al., 1996)

1.6.3.10. Other effects of caffeine:

Caffeine administered acutely increases diuresis (urination). Caffeine regularly increases energy metabolism throughout the brain while decreasing cerebral blood flow and there is no tolerance for these effects. Vasoconstriction due to 250 milligrams of caffeine can decrease central blood flow by 20-30%, which is why caffeine has been used to treat migraine headache. Because blood glucose is usually more than ample for cerebral metabolism the combination of increased metabolism & decreased blood flow would be more likely to induce hypoxia than ischemia. But if caffeine increases oxygen intake by bronchodilation or increases sensitivity to carbon dioxide in the medulla, then there may be compensation. (Both hypoxia and caffeine elevate plasma adenosine.) The consumption of fewer than four cups of coffee daily during pregnancy is not deemed to endanger the child (Leviton and Cowan, 2002).

1.7. CAFFEINE CONTAINING AGRO WASTES: An environmental problem:

Coffee pulp is the most abundant waste produced during the pulping operation of the coffee cherry (Cabezas et. al., 1979; Braham, 1987). Wet Coffee processing results in discharge of waste-water rich in fermentable sugars and caffeine

(Elias, 1986). Coffee pulp is improperly utilized and, therefore, it is considered the most abundant pollutant material of lakes and rivers located near the coffee-processing sites (Adams and Dougan, 1981). The utilization of coffee pulp is limited by anti-physiological factors like high caffeine, polyphenolic, potassium, and fiber content naturally occurring in the material (Bressani, 1987a). Several technologies to detoxify coffee pulp have been investigated by several authors, but have met with little success (Bressani, 1987b). Decaffeination through chemical solvents has proved to be an alternative process to detoxify the material for animal feeding (Molina et.al., 1974). However, it is considered a relatively high-cost technology to be implemented at the coffee-processing sites.

In view of the ill effects of excessive consumption of caffeine by humans and the pollution problems posed by coffee and tea processing wastes to water and soil, development of methods for removal of caffeine from caffeine containing foods, beverages and agricultural wastes was considered very important from both health and environmental point of view. Since the start of the 20th century several methods for the removal of caffeine were developed and the area of decaffeination still remains an active area of research owing to the increasing demand for decaffeinated foods and beverages.

1.8. DECAFFEINATION:

Decaffeination is defined as the act of removing caffeine from coffee beans and tea leaves (http://en.wikipedia.org/wiki/Decaffeination). Most decaffeination processes are performed on unroasted (green) coffee beans, but the methods vary somewhat. It generally starts with the steaming of the beans. They are then dipped

into solvent for several hours. The process is repeated for 8 to 12 times until it meets either the international standard of having removed 97 % of the caffeine in the beans or the EU standard of having the beans 99.9 % caffeine free by mass.

The first commercially successful decaffeination process was invented by Ludwig Roselius and Karl Wimmer in 1903. It involved steaming coffee beans with a brine (salt water) solution and then using benzene as a solvent to remove the caffeine. Coffee decaffeinated this way was sold as *Cafe sanka* in France and later as Sanka brand coffee in the US. Due to health concerns regarding benzene, this process is no longer used commercially and Sanka is produced using a different process.

Three different methods of decaffeination, widely used, are; 'Water decaffeination', 'Solvent decaffeination' and 'Carbon dioxide decaffeination'. Although caffeine is water soluble above 175^{o} F, water alone is generally not used to decaffeinate coffee because it strips away too many of the essential flavor and aroma elements.

Decaffeination by solvents can be through two methods: direct and indirect contact. In the first the beans come directly in contact with the decaffeinating agents, after being softened by steam. In the latter method, a water/coffee solution is normally used to draw off the caffeine; after being separated from the beans, the solution containing the caffeine is then treated with a decaffeinating agent. In both methods, the agent is removed from the final product.

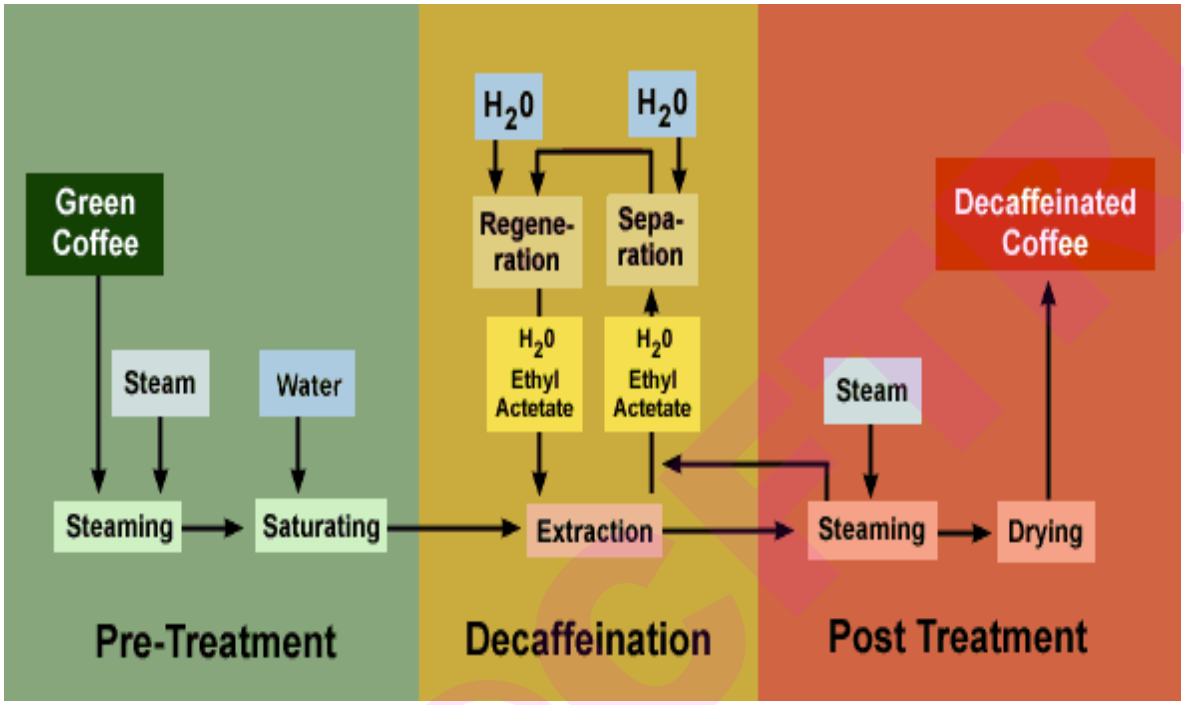
1.8.1. Solvent decaffeination:

A solvent is used for decaffeination in this technique. There are criteria in choosing the right solvent for this process. According to Katz (1987), some of the

criteria include: Safety, cost, caffeine solubility, ease of solvent removal and recovery, toxicity and chemical reactivity, and environmental effects. The common solvents used are methylene chloride and ethyl acetate. However, methylene chloride is mostly used in the industry. This chemical is more selective to remove caffeine without removing the taste and aroma of coffee. According to the United States Food and Drug Administration (FDA), most decaffeinated coffee has less than 0.1 parts per million residual methylene chloride.

The process of solvent decaffeination involves steaming, pre-wetting, caffeine extraction, steam stripping, and drying (Fig. 1.8.1.1). Green coffee beans are transferred into an extractor, steamed to make the surface more permeable so that the caffeine can be easily extracted when the solvent comes in contact with the caffeine. After steaming the beans are steeped in water to increase their moisture content to 40 % by weight. Prewetting water and solvent (methylene chloride or ethyl acetate) are added together in this step. The ratio of solvent to beans is 4:1 (Pintauro, 1975). Caffeine in the beans is extracted by heating the solvent, at a temperature of 150°F. The caffeine extraction step takes about 10 hours to be completed. About 97 % of the caffeine in the green coffee beans is extracted in this step. Solvent stripping or steam stripping is then done on the green coffee beans. The main purpose of this step is to get rid of any residual methylene chloride or solvent. The coffee beans are then dried and stored. However, due to health concerns the use of solvent decaffeination has greatly decreased in recent years.

Figure 1.8.1.1: Schematic process for decaffeination using solvents.

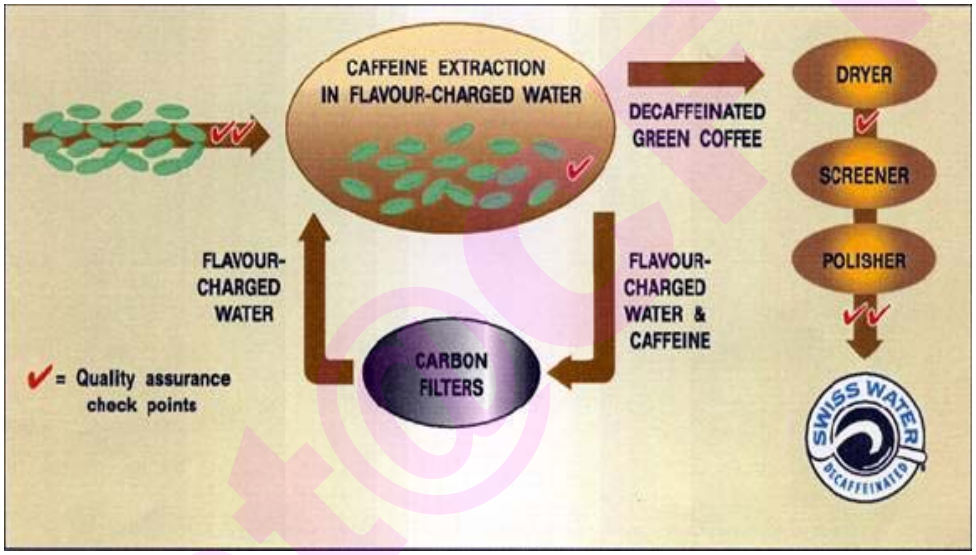


1.8.2. Water decaffeination:

Water decaffeination, uses water to extract caffeine from the green coffee beans. The water decaffeination is probably the most widely accepted method used to decaffeinate coffee. This method is based on the natural ability of water to make caffeine soluble. However, in this process the water acts non-selectively on the raw coffee, extracting all of the soluble components, like the aromas and the flavor. In order to prevent the extraction of all water-soluble components of coffee beans, the extraction water contains essentially equilibrium quantities of the non-caffeine soluble solids (Katz, 1987). The coffee beans are kept in the extractor for about 8 hours to remove about 98 % of the original caffeine. The extract water with caffeine, coffee solids, coffee aroma and flavor is subjected to caffeine extraction by solvents. The organic solvent also extracts the flavor and aroma of coffee. Since the extract water is recycled, the organic solvent must be removed. After removal of the

solvent, the coffee extract is returned to the beans to reabsorb the flavor components. The decaffeinated coffee beans are then washed, dried and stored. Another form of water decaffeination is the Swiss Water Decaf method (Fig. 1.8.2.1). This method is also based on the theory of the caffeine being soluble in water, however it is not necessary to return the other soluble components to the bean.

Figure 1.8.2.1. Schematic diagram of swiss water decaffeination process. **(adapted from http://www.hollandcoffee.com/images/swissprocess.jpg)**



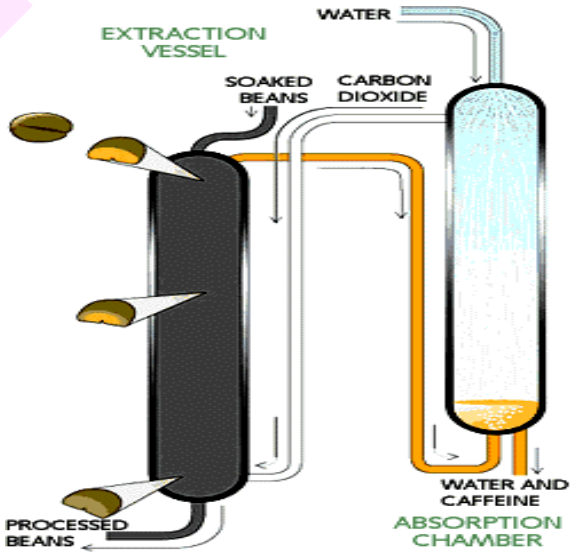
Swiss water decaffeination is a relatively simple process. The coffee beans are extracted in hot water, removing the caffeine and the flavor components of the bean into the water. After the water has been saturated, the caffeine is removed by passing the water through carbon filters. Caffeine is adsorbed on to the carbon filters and the caffeine free extract is reabsorbed by the beans, which are dried and roasted.

1.8.3. Supercritical carbon dioxide:

The supercritical carbon dioxide decaffeination is considered to be a safer process than the solvent decaffeination. By using only carbon dioxide and water this method has gained acceptance as being a natural method of decaffeination. The

supercritical carbon dioxide decaffeination uses carbon dioxide gas that has been compressed and subjected to high temperature. The combination of high temperature and pressure enables carbon dioxide to become a solvent. Figure 1.8.3.1, represents a schematic representation of the set up of a super critical decaffeination plant. The decaffeination process begins with prewetting the beans with steam, loading of the prewetted coffee beans into an extractor and at the same time solid absorbent (activated carbon adsorber) is loaded into a vessel. Moist carbon dioxide is also loaded into the vessel that contains coffee beans and the solid absorbent. The supercritical carbon dioxide is then circulated between the extractor and solid adsorber vessel. As the carbon dioxide passes through the extractor, caffeine is extracted and the caffeine rich carbon dioxide flows to the adsorber where the caffeine is adsorbed. The caffeine-free carbon dioxide then goes through the cycle again (Fig.1.8.3.1). This process is continued till the desired level of decaffeination is achieved. The beans are then dried and stored.

Figure1.8.3.1: Schematic of super critical carbon dioxide decaffeination process
(adapted from Katz.1987).



The advantage of this process is that no flavor elements are lost from the coffee and 98% of caffeine can be removed from the coffee beans. However, there are a couple of disadvantages. The first disadvantage is that due to the high pressure used, the equipment is costly and only batch processing can be done (Katz, 1987). The second disadvantage is that the average concentration of caffeine in the carbon dioxide is low; therefore a large quantity of carbon dioxide is needed. This might also be costly. The disadvantages might cause the price of the decaffeinated coffee beans to be higher than solvent decaffeinated and water decaffeinated coffee beans. Moreover, the use of membranes or carbon filters in caffeine removal processes will be very expensive and the commercialization of the process becomes less viable.

In lieu of the disadvantages of the existing processes of decaffeination research there has been an increasing emphasis of developing greener and economic methods of decaffeination.

Biotechnological decaffeination methods are the only alternatives, which offer safe, economical and greener routes of decaffeination of beverages. Moreover biological means of decaffeination have a wider reach of application even to pollution abatement due to coffee and tea processing wastes.

1.9. BIODECAFFEINATION: A natural route of decaffeination:

Biodecaffeination can be defined as the removal of caffeine from coffee, tea and other caffeine containing materials by the action of externally added microbial cells or enzymes.

The concept of biodecaffeination is a relatively new area of decaffeination and there is a growing interest in this area of biotechnology due the advantages it offers

like being environmentally safe, economical and in preserving the quality of the beverages. Development of biological or enzymatic methods of decaffeination demands a deep understanding of the caffeine metabolism in microbial, plant and animal systems. A thorough knowledge of the caffeine metabolism, the enzymes involved and various factors involved in the caffeine degradation in different living systems will give deep insights into the development of efficient biodecaffeination processes. Detailed information on different enzymes involved in the degradation of caffeine in different organisms could help in developing an enzymatic process for caffeine removal. Caffeine degrading systems in humans, plants, fungi and bacteria and the prospects of developing biodecaffeination processes are dealt with in the following sections.

1.9.1. Caffeine degradation in eukaryotes:

In plants, degradation of caffeine occurs through sequential demethylation that finally results in the formation of xanthine. The demethylation reactions have been found to be catalyzed by demethylase enzymes, viz., *N*-1 demethylase, *N*-7 demethylase and *N*-3 demethylase. Xanthine is then converted into CO_2 and ammonia by purine catabolism (Mazzafera, 1994a; Ashihara et.al., 1996a&b; Ashihara et.al., 1997; Vitoria and Mazzafera, 1998; Koyama et.al., 2003). In contrast to plants, the degradation pathway in mammals results in the formation of methyl xanthines and methyl uric acids by cytochrome P450 enzymes (CYP1A2, CYP3A4, CYP2E1), xanthine oxidase and *N*-acetyl transferase (Cornish and Christman, 1957; Khanna et.al., 1972; Wreck and Feyereisen, 2000). Methylated xanthines and the respective uric acid formed due to degradation are excreted from the body through urine

(Rao, et.al., 1973). Caffeine is metabolized (demethylated) in the liver by Cytochrome P450 enzymes known as 1A2 CYP1A2s (Shirley et. al., 2003). The half-life for caffeine metabolism is typically 5-6 hours in an adult. In yeast and fungi, caffeine is degraded via theophylline to respective methyl xanthines, further degraded to ammonia and carbon dioxide. The key enzymes involved are reported to be cytochrome P-450s.

1.9.1.1. Cytochrome p 450 and related enzymes:

NADPH-cytochrome P450 reductase (CPR) is the electron donor protein for several oxygenase enzymes found on the endoplasmic reticulum of most eukaryotic cells. These oxygenases include the cytochromes P450, a family of enzymes involved in the metabolism of many drugs and dietary substances, and in the synthesis of steroid hormones and other extracellular lipid signaling molecules; heme oxygenase, a hemeprotein that catalyzes the first step in the degradation of heme to bilirubin; and squalene monooxygenase, the second enzyme in the committed pathway for sterol biosynthesis. CPR may also donate electrons to 7-dehydrocholesterol reductase in the sterol synthesis pathway, and to cytochrome b_5, which supports both sterol synthesis and the fatty acid desaturase and elongase pathways.

The name **P450** refers to the "pigment at 450 nm", so named for the characteristic Soret peak formed by absorbance of light at wavelengths near 450 nm when the heme iron is reduced (with sodium dithionite) and complexed to carbon monoxide. The cytochrome P450s (P450) are exceptional, not only because they are ubiquitous throughout nature, but also because of their ability to effect the

metabolism of multiple unrelated, exogenous, and endogenous compounds. The cytochrome P450 (CYP) super family consists of more than 2500 members joined into 281 families (Nelson, 1995). While the majority of CYPs have unknown function because they have been sequenced during genome projects, others are known to catalyze mono-oxygenation reactions of more than 10,000 organic compounds (Danielson, 2002; Newcomb et al., 2003). In accordance with their functions, these P450s can be divided into two groups, those which metabolize Xenobiotics (drugs, pollutants, agrochemicals, etc.) and those participating in key biosynthetic pathways (steroidogenesis, biosynthesis of sterols, Vitamin D, etc.). The families from the first group metabolize a broad variety of different structures and biosynthesis of these P450s is often substrate inducible and their gene knockouts sometimes do not show any obvious phenotype (Gonzalez and Kimura, 2003).

Camphor (cytochrome P450) 5-monooxygenase (Fig. 1.9.1.1), originally isolated from the bacterium *Pseudomonas putida* PgG 786, catalyzes the degradation of camphor (Zurek, et.al., 2006), an organic pollutant into non toxic forms. It is also known to convert essentially stereospecific conversion of tetralin (1,2,3,4-tetrahydronaphthalene) to (R)-1-tetralol (Grayson et.al., 1996). It is the first bacterial Cytochrome P450, whose crystal structure could be resolved.

Figure 1.9.1.1: 3-D Structure of cytochrome P-450 cam (*Pseudomonas putida*) with thiocamphor bound to active site (Zurek et.al., 2006).

Within a given gene family these P450 sequences are known to have the highest variability in the regions comprising the six predicted substrate recognition sites (SRS) (Gotoh, 1992) where substitution of a key residue usually does not lead to enzyme inactivation but often to remarkable changes in catalytic preferences (Wachenfeld and Jonson, 1995). This feature was suggested as an origin for P450 evolutionary bio-diversification aimed to accommodate the increasing number of organic compounds appearing in nature (Negishi et al., 1996). For example, cytochrome P450 46A1 (P450 46A1) is known to catalyze 24(*S*)-hydroxylation of cholesterol. This reaction produces biologically active oxysterol, 24(*S*)-hydroxycholesterol, and is also the first step in enzymatic degradation of cholesterol

in the brain. Mast et. al., (2003) report that P450 46A1 can further metabolize 24(*S*)-hydroxycholesterol, giving 24,25- and 24,27-dihydroxycholesterols. In addition, P450 46A1 is able to carry out side chain hydroxylations of two endogenous C27-steroids with and without a double bond between C5-C6 (7α-hydroxycholesterol and cholestanol, respectively) and introduce a hydroxyl group on the steroid nucleus of the C21-steroid hormones with the C4-C5 double bond (progesterone and testosterone). Also, P450 46A1 was found to metabolize xenobiotics carrying out dextromethorphan O- and N-demethylations, diclofenac 4'-hydroxylation, and phenacetin O-deethylation. Thus, it was concluded that substrate specificities of P450 46A1 are not limited to cholesterol and include a number of structurally diverse compounds. Activities of P450 46A1 suggest that, in addition to the involvement in cholesterol homeostasis in the brain, this enzyme may participate in metabolism of neurosteroids and drugs that can cross the blood-brain barrier and are targeted to the central nervous system.

The P450s from the second group have narrow substrate specificity. Catalyzing a particular regio- and stereospecific reaction, they are designed to preserve metabolic pathways of endogenous compounds. This group is a potential subject for study of the basis of P450 functional conservation. Essential residues in this group of P450s, however, are usually masked under a high degree of identity of the entire sequence, because the families predominantly consist of enzymes from evolutionarily close species (Nelson et al., 1996).

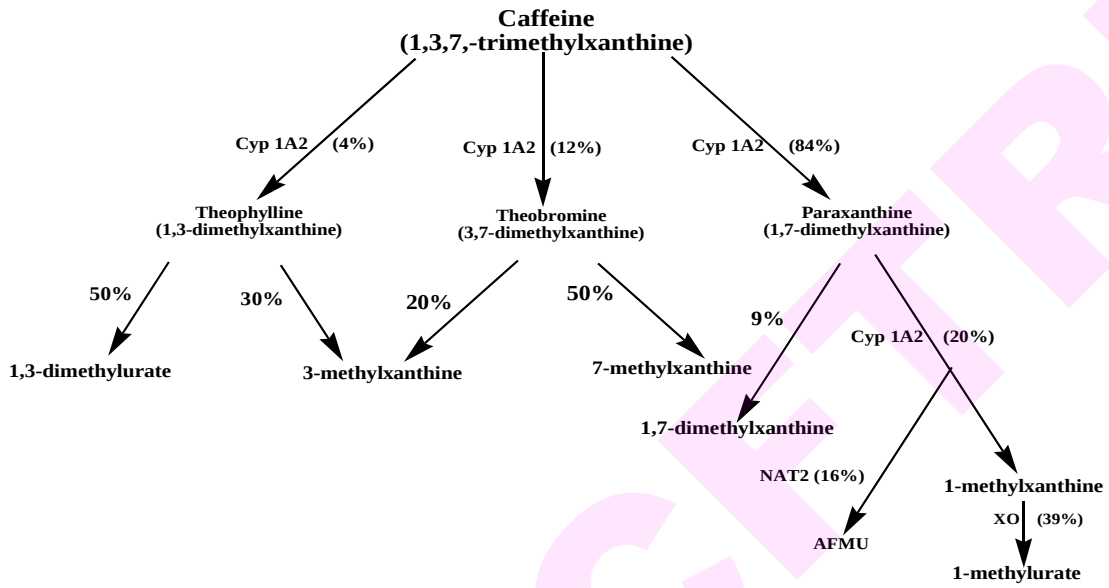
Cytochrome P-450 (CYP) 1A2 is constitutively expressed in liver in mice and humans (Sesardic et. al., 1988; Ikeya et. al., 1989). CYP1A2 is involved in the

metabolism of a number of clinically significant drugs, as well as the bioactivation of heterocyclic and arylamine procarcinogens (Shimada et al., 1989). In general, the active site(s) are believed to be hydrophobic in nature. The overall broad specificity of the P450s is due to the existence of several subfamilies, each possessing different, sometimes overlapping, substrate specificities. P450 1A2 is a significant constituent of the P450s found in the average human liver (Relling, et.al., 1992; Lewis and Lake, 1996). Most P450s are generally involved in the deactivation and detoxification of Xenobiotics (Guengerich and Shimada,1991; Lewis et al., 1994). Remarkably, there is a striking similarity in the structures of the substrates, inducers, and inhibitors of the P450 1A subfamily; i.e., structures tend to be planar, aromatic, lipophilic, and generally polycyclic (Lewis et. al., 1986).

1.9.1.2. Caffeine degradation in humans: Role of cytochrome P450:

In humans caffeine is rapidly metabolized by the Cytochrome P450 enzymes. The half life of caffeine in humans is usually 4-5 hours and excreted into urine. The first products of metabolism are all dimethyl xanthines: paraxanthine (84% 1,7-dimethylxanthine), theobromine (12%, 3,7-dimethylxanthine), and theophylline (4% , 1,3-dimethylxanthine) (Fig. 1.9.1.2). Caffeine (1,3,7-trimethylxanthine) has been extensively studied as an in vivo metabolic probe for CYP1A2 activity in mice (Buters et. al., 1996) and humans (Kalow and Tang, 1991; Fuhr et.al., 1996; Rostami et al., 1996).

Figure 1.9.1.2. Caffeine degradation pathway in human liver.

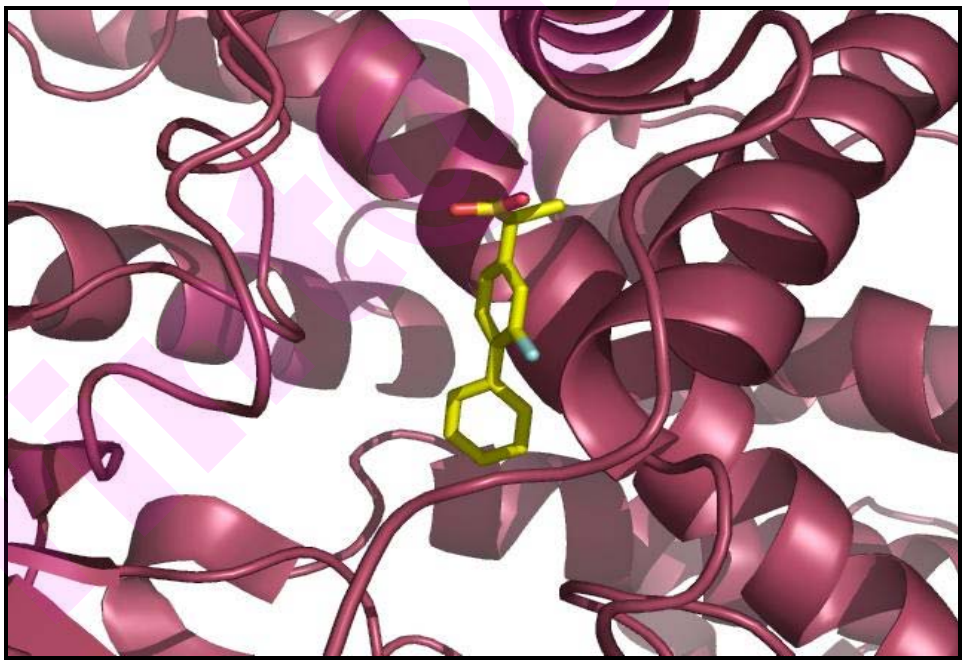


The 3-demethylation of caffeine to form paraxanthine (1,7-dimethylxanthine) accounts for 80% of the clearance of caffeine in humans and mice (Lelo et al., 1986; Buters et al., 1996). This activity has been attributed almost exclusively to CYP1A2 activity in humans, based on in vivo inhibition of caffeine metabolism by the CYP1A2-specific inhibitor furafylline (Tarrus et al., 1987) and by in vitro experiments with individual human CYP isoforms expressed from cDNAs (Gu et al., 1992). A *Cyp1a2* (2/2) knockout line was used to demonstrate that 87% of the clearance of caffeine was attributable to CYP1A2 activity in mice (Buters et al., 1996). The study of genetic variation of CYP1A2 expression in human populations is somewhat confounded by the environmental responsiveness of the expression of the gene. Because numerous environmental exposures, including foods, drugs, smoking, and industrial pollutants may influence gene expression, it is extremely difficult to discriminate these factors from genetic variation in expression (Casley et.al., 1999)

Until recently, the only available 3D structures representing the P450 superfamily came from the crystal structures for four soluble bacterial P450s, namely P450 101 (P450cam) (Fig. 1.9.1.1), P450 102 (P450 BM3), P450 108 P450terp), and P450eryF (Poulos et al., 1995).

Recent advantages in genetic engineering and biotechnology have enabled the cloning and expression of the human cytochrome P450 in suitable expression systems. CYP 1A2 is involved in the metabolism of caffeine and its crystal structure has been resolved (Fig.1.9.1.3).

Figure 1.9.1.3. Crystal structure of human cytochrome P450 1A2 (Berthou et.al.,1992).



It is known that caffeine can sit within the active site in three different orientations which would lead to three different *N*-demethylation products. The rat isoform of P450 1A2 is known to oxidize these three methyl groups at nearly equal rates (Berthou et al., 1992).

The activity of CYP1A2, assessed by the caffeine metabolite ratio, has large inter individual difference (Kalow and Tang, 1991; Butler et. al., 1992; Nakajima et. al., 1994; Ou-Yang et. al., 2000). The remarkable inter individual variations of activity of CYP1A2 may affect the disposition and response of some drugs, such as phenacetin (Kobayashi , 1998), acetaminophen (Patten et. al., 1993), theophylline (Gu et. al., 1992), mexiletine (Abolfathi et. al., 1995), imipramine (Lemoine et. al., 1993), propranolol (Yoshimoto et. al., 1995), clozapine (Pirmohamed et. al., 1995) and tacrine (Xu et. al., 1996; Fontana, 1998). Some inducers of CYP1A2, such as omeprazole, may increase enzyme activity in a dose-dependent manner, leading to certain drug interactions (Rost et al., 1994a,b). CYP1A2 also plays an important role in the metabolisms of many procarcinogens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx) and aflatoxin B1. In humans, large interindividual differences have been reported in the activity of CYP1A2-mediated phenacetin O-deethylation (Alvares et al., 1979), carcinogen metabolism (Butler et al., 1989), the protein content of CYP1A2 (Sesardic et. al., 1988), and the mRNA content of CYP1A2 (Farin and Omiecinske, 1993), which were due more to environmental factors than to heredity (Tantcheva et. al., 1999).

Upon consumption of caffeine containing beverages like coffee and tea, the caffeine present in it undergoes metabolic oxidation by N-demethylation at each of the 3 tertiary amine nitrogen atoms by hepatic microsomal enzymes and generates theobromine (Tb, 3,7-dimetylxanthine), paraxanthine (Px, 1,7-dimethylxanthine) and theophylline (Tp, 1,3-dimethylxanthine,) (Carrillo, et.al., 2000; Chung and Cha,

1997) (Figure 1.9.1.2). Additionally, the caffeine is also hydroxylated and forms 1,3,7-trimethylurate (TMU) by the liver microsomal enzymes. Among these microsomal metabolites, the N-3 demethylation of caffeine producing paraxanthine is known to be catalyzed by CYP1A2 in human liver (Butler et.al., 1989). Ingested caffeine undergoes extensive biotransformation in human and generates at least 17 detectable urinary metabolites including those mentioned above (Nakajima et. al, 1994). Attempts have been made to identify the specific microsomal enzymes responsible for productions of Theobromine (Tb), Theophylline (Tp) and Trimethyluric acid (TMU) from caffeine for the purpose of finding additional methods of phenotyping drug metabolizing enzymes in human (Rostami, et. al., 1997) and the results indicated that CYPs 1A2, 2A6, 2D6, 2E1, 3A4 and 3A5 as well as xanthine oxidase are involved (Rostami, et. al., 1997).

1.9.1.3. Role of flavin containing monooxygenase:

Flavin-containing monooxygenase (FMO) is known to be present in liver microsomes together with the CYP (Ziegler, 1988). Based on the fact that multiple forms of FMO are known to exist and that FMO is responsible for the oxidation of many clinically useful drugs bearing nitrogen- and sulfur- atoms, interest on FMO has been fast developing (Cherrington et.al, 1998). Studies on caffeine metabolism in rat and human liver microsomes at various pH conditions suited either for the hepatic microsomal CYP or FMO, as well as in the presence of inhibitors for each of these hepatic oxidases, present the evidence that FMO is responsible primarily for productions of Tb and Tp from caffeine. While FMO is known to be present in human liver microsomes together with CYP and to share substrate specificities with CYP, it is known to catalyze preferentially the oxidation of nitrogen- and sulfur-containing

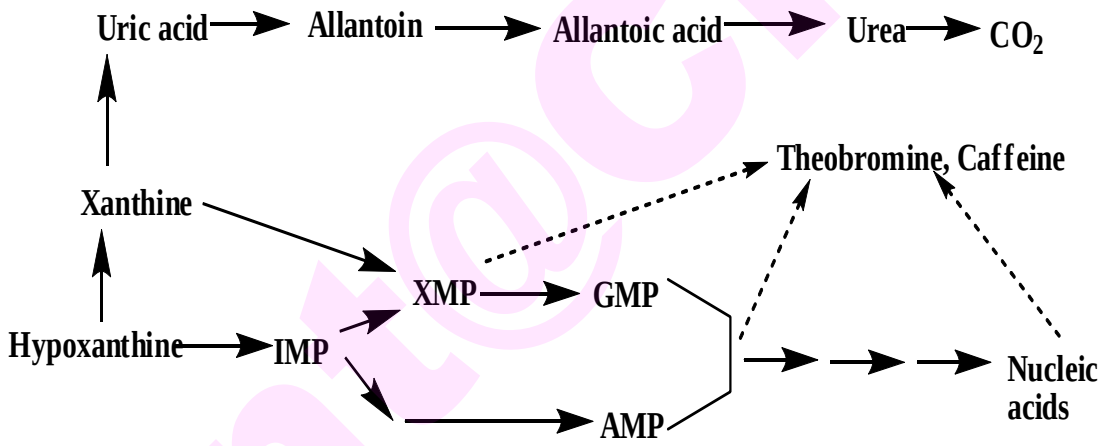
medicines and alkaloids. The FMO is known to be much more sensitive to thermal degradation than the CYP (Ziegler, 1988). It was also found that both CYP and FMO together are capable of catalyzing the N-demethylations of caffeine. Attempts to determine the contributions of CYP and FMO in liver microsomes of rat and human by inhibiting the caffeine metabolism with their respective inhibitors like SKF525A (CYP) and methimazole (FMO) showed clearly that the CYP played a primary role in the production of Paraxanthine (Px) and the FMO in the production of Tb and Tp. Unlike the CYP reaction mechanism sequences which require initial binding of an acidic substrate to receive the electron input into the heme iron and the oxygen input for eventual drug oxidation, the FMO is kept under reactive state having the 4a-hydroperoxy flavin form at a high pH to oxidize the drugs with high pKa immediately upon contact (Ziegler, 1993). The hydroperoxy flavin moiety contained in FMO is known to make an electrophilic attack on the nitrogen atom and oxidizes numerous tertiary amine type drugs to produce their N-oxide metabolites. The N-oxides formed from tertiary amines are known to be relatively stable and thus, these have served as reliable markers for the FMO catalyzed oxidation for many substrates. In the case of 1-N- and in particular N-demethylation of caffeine, apart from CYP1A2, other cytochrome P-450 isoenzymes play a considerable role.

1.9.2. Caffeine catabolism in plants:

The metabolic path of xanthine and hypoxanthine in buds of tea is given in Figure 1.9.2.1, below. Hypoxanthine is converted to xanthine, which is converted to xanthosine monophosphate through inosine monophosphate as the intermediate. This molecule is further converted to guanosine monophosphate or adenosine

monophosphate (AMP) which serve as substrates for the methyl transferases which sequentially add methyl groups at the 1,3, and 7-N positions of the xanthine ring producing theobromine and caffeine. Alternatively xanthine and hypoxanthine are metabolized through the salvage pathway of purine degradation where they are converted to urea and carbon dioxide through theophylline. The plants always maintain a certain amount of caffeine by balancing the synthetic and degradation pathways of caffeine.

Figure 1.9.2.1: Metabolism of hypoxanthine and xanthine in buds of tea leaves.



1.9.2.1. Degradation of caffeine:

Caffeine is produced in young leaves and immature fruits, and continues to accumulate gradually during the maturation of these organs. However, it is very slowly degraded with the removal of the three methyl groups, resulting in the formation of xanthine, which is further degraded by the purine catabolism pathway to CO_2 and NH_3 via uric acid, allantoin and allantoate (Kalberer, 1964 and 1965; Hartman, 1970; Konishi and Oishi, 1973; Suzuki and Takahasi, 1975).

A detailed review of catabolism of caffeine in plants and microorganisms has been published by Mazzafera (2004). He found that exogenously supplied [8-14C] theophylline is degraded to CO_2 far more rapidly than [8-14C] caffeine indicating that the initial step, i.e., the conversion of caffeine to theophylline, seems to be the major rate-limiting step of caffeine catabolism (Ashihara et. al., 1996b; Ito et. al., 1997). Ashihara and Crozier (1999a) have found that in the leaves of *Coffea eugenioides*, a low caffeine containing species, [8-14C] caffeine was degraded rapidly, and much of the radioactivity was recovered as $14CO_2$. *Coffea eugenioides* therefore possessed far higher levels of caffeine demethylase activity, and is able to convert endogenous caffeine efficiently to theophylline, which is rapidly degraded further.

1.9.3. Caffeine metabolism in prokaryotes:

In bacteria (*Pseudomonas*), caffeine is initially converted into theobromine and paraxanthine parallely by demethylases. Further demethylation forms xanthine with 7-methyl xanthine as the intermediate. There is also an evidence for oxidation of xanthine, mono and dimethyl xanthines to uric acid, which enter the purine catabolic pathway (Blecher and Lingens, 1977). In *Serratia marcescens*, the caffeine catabolic pathway is similar to *Pseudomonas* sp. except for the formation of methyl uric acid intermediate (Mazzaffera et.al., 1994a).

Caffeine is incorporated into the soil by leaching from the coffee tree canopy, as well as from litter and coffee beans. In the soil, caffeine might be degraded by microorganisms (Hagedorn et. al., 2003a&b) absorbed by minerals and humic compounds, or transported through the soil profile. It is believed that caffeine-degrading microorganisms utilizing caffeine as the sole source of carbon and nitrogen

have enzymes that bring about the actual degradation of the substrate (Mazzafera et. al., 1994a). Silva et. al. (2000) studied the diversity of microbial populations during the maturation and natural processing (sun-dried) of coffee fruits during two consecutive years. A total of 754 isolates of bacteria, yeast and fungi were obtained. They found that bacteria were the predominant microorganisms and they detected a large variation of microorganisms depending on the farm where the coffee was collected, the maturation stage, and the processing method, but no consistent pattern of variation was observed. Fermentative bacteria and yeast, cellulolytic bacteria, and pectinolytic bacteria, yeast and filamentous fungi were identified among 626 microorganisms. They found that caffeine degradation was very slow in the coffee pulp and proposed that ensiling of the coffee pulp would enhance the quality of the pulp for use as cattle feed. But they could not trace the caffeine degrading capability to any of these microorganisms.

1.9.3.1. Caffeine degradation by bacteria:

Till the 1970s it was believed that caffeine is toxic to bacteria and no studies on caffeine degradation by microorganisms were reported till 1970 (Sundarraj and Dhala, 1965; Putrament et. al., 1972; Kihlman, 1974). A few studies have established that caffeine can be mutagenic through inhibition of DNA repair in bacteria (Grigg, 1972; Kihlman, 1974; Frischknecht et. al., 1985). It was also shown that caffeine at 0.1% concentration also reversibly inhibits protein synthesis in bacteria and yeast. However the reports state that the inhibition of protein synthesis is post translational since caffeine does not affect RNA translation (Putrament et. al., 1972). Although high concentrations are required for bactericide action, caffeine is regarded as toxic

for bacteria and fungi (Nehlig and Derby, 1994; Denis et. al., 1998; Bogo and Mantle, 2000). However, some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid (Sundarraj and Dhala, 1965). Actually, it is not rare to find bacterial strains resistant to caffeine (Woolfolk, 1975). Some microorganisms, e.g., *Klebsiella pneumoniae*, can utilize purines as carbon or nitrogen sources (Vogels and Drift, 1976.). First report on caffeine degradation by microorganisms was in the early 1970s (Kurtzman and Schwimmer, 1971). Since then progress has been achieved on using caffeine as source for microbial growth (Schwimmer and Khurtzman, 1971; Vogels and Drift, 1976; Roussos et. al., 1995).

A few reports in the literature have already described the isolation of bacteria strains from soil with the ability to degrade caffeine (Wool folk, 1975; Blecher and Lingens, 1977; Gluck and Lingens, 1987; Mazzafera et.al, 1994a). Bacterial strains capable of degrading caffeine belonged to *Pseudomonas* and *Serratia* genus. Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. Synergistic effect has been observed when caffeine is added to antimicrobial agents like chloramphenicol (Sundarraj and Dhala, 1965). Attempts were made for biological production of caffeine catabolic intermediates with the help of inhibitors. Asano et. al., (1993) reported the production of theobromine using *Pseudomonas* strain for the first time. Theobromine was accumulated at different levels ranging from 5 g/l and above in the presence of 1mM of Zn2+. Fructose and tryptone were found to be the most suitable carbon and nitrogen sources (Asano et. al., 1993).

Bacteria can be used in reducing the caffeine content in caffeine bearing plants. It has been found that leaf surface play a vital role in *Agrobacterium* infection in tea plants (Kumar et. al., 2004). A method has been proposed for producing tea leaves with less caffeine content by growing caffeine degrading bacteria on the surface of the leaf. Ramarethinam and Rajalakshmi (2004) found in situ lowering of caffeine in tea leaves without affecting the quality of the other tea components when tea plants were sprayed with a suspension of *Bacillus licheniformis*.

Anaerobic fermentation of coffee pulp resulted in about 13–63% reduction of caffeine in 100 days (Porres et. al., 1993). In contrast, aerobic fermentation resulted in 100% degradation of caffeine in 14 days (Rojas, et. al., 2003). Several studies were carried out to investigate the use of purines, including caffeine, as a source of energy for microorganism growth (Schwimmer and Kurtzman., 1971; Woolfolk, 1975; Woolfolk and Downard, 1977; Middelhoven and Bakker, 1982; Mazzafera et. al., 1994a). A comprehensive review on purine utilization by microorganisms was published by Vogels and Drift (1976). Although fungi growing on caffeine have been isolated, most of the studies were done with bacteria isolated from soil, mainly those belonging to the *Pseudomonads* group, with particular attention to *Pseudomonas putida* (Burr and Caesar, 1985). Madyastha et. al., (1998, 1999) have reported the degradation of caffeine by a consortium of bacteria belonging to *Klebsiella* and *Acinetobacter species.* They have reported that the caffeine degradation in this consortium is through a novel route of oxidation at C-8 position in carbon, unlike the normal N-demethylation reported in other bacteria. They have also reported that the enzyme involved in the first step of caffeine degradation is a caffeine oxidase and the

product is 1,3,7-trimethyl uric acid. Gokulakrishnan et. al., (2005), have reviewed the caffeine degradation by bacteria and fungi. They report the degradation of caffeine by a strain of *Pseudomonas.* NCIM 5235.

Dickstein et al. (1957) and Bergmann et al. (1964) studied the degradation of 3-monomethylxanthine mediated by dehydrogenase activity in *Pseudomonas fluorescens*. They did not find activity with 1-monomethylxanthine as substrate. However, Woolfolk (1975) used a *P. fluorescens* strain with ability to grow on caffeine to demonstrate dehydrogenase activity against both mono methyl xanthines. Hydrolytic enzyme degrading caffeine, with the methyl groups being removed by sequential hydrolysis was suggested. Methanol and xanthine were the final reaction products, and indications were that methanol was further oxidized to CO_2. Blecher and Lingens (1977) studied degradation of caffeine by *P. putida* strains isolated from soil. They identified 14 catabolites: theobromine, paraxanthine, 7-monomethylxanthine, xanthine, 3,7-dimethyluric acid, 1,7-dimethyluric acid, 7-methyluric acid, uric acid, allantoin, allantoic acid, ureidoglycolic acid, glyoxylic acid, urea and formaldehyde. The overall pathway of caffeine degradation in bacteria and fungi is given in figure 1.9.3.1.

Caffeine is first converted to respective dimethyl xanthines (theobromine, paraxanthine and theophylline) by a caffeine demethylase enzyme. The diemthyl xanthines are either converted to the respective dimethyluric acids by xanthine oxidase or directly demethylated to their monomethyl uric acids by the action of xanthine dehydrogenases present in the system. The monomethylxanthines are converted to xanthine which is oxidized by xanthine oxidase to uric acid. By the

action of uricase uric acid is degraded to allantoin. Allantoin is further degraded down the pathway and ends up in ammonia and CO_2 by the action of urease.

Middelhoven and Lommen (1984) studied degradation of caffeine as influenced by oxygen. They concluded that the first enzymatic steps in caffeine degradation in a *P. putida* strain were the successive removal of the three methyl groups, probably mediated by mono-oxygenases. However, they failed to demonstrate the mono-oxygenase activities.

Enzymological aspects of caffeine degradation by *Pseudomonas putida* were reported by Hohnloser et al. (1980) in detail. Using NADPH as cofactor in enzyme assays, they observed that only theobromine was formed from caffeine, but when they used paraxanthine, theobromine or 7-monomethylxanthine, they did not detect any activity. The authors suggested that there was only a single enzymatic system responsible for the sequential demethylation of caffeine. Regarding the lack of activity against theobromine and other substrates, they also suggested that although not detected *in vitro*, enzymatic degradation might occur *in vivo* but at very low rates.

It was also argued that the slow and poor growth of the bacteria on caffeine as the sole source of carbon was due to a limiting demethylation of caffeine as well as other methylxanthines. A limiting demethylation rate of caffeine was observed by Mazzafera et al. (1994a) in a *Serratia marcescens* strain isolated from soil collected under coffee trees. By cultivating the bacteria on different substrates as the sole source of carbon and nitrogen, they could establish that caffeine was degraded to paraxanthine and/or theobromine, and subsequently to 7-monomethylxanthine and xanthine.

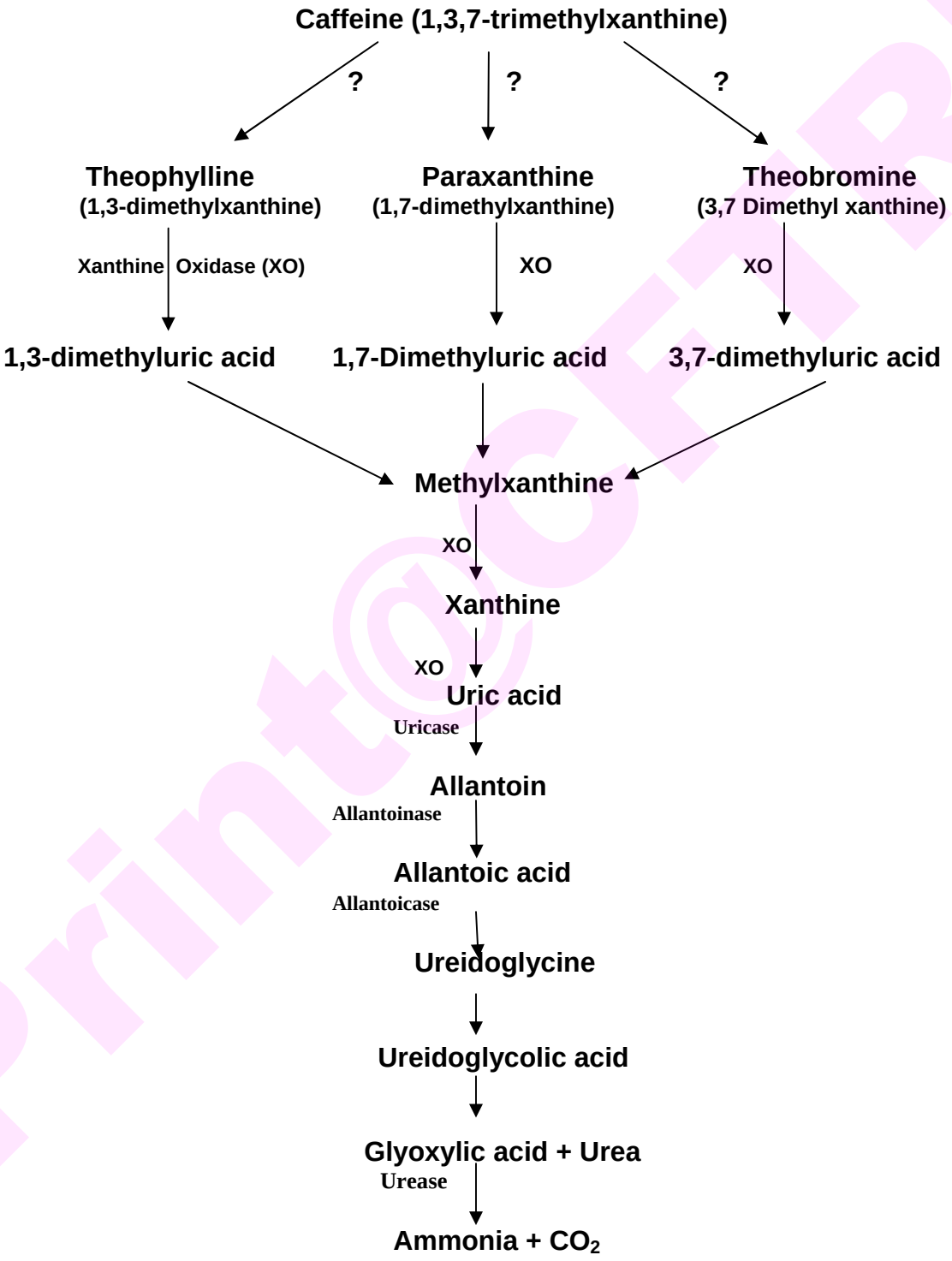
Sauer (1982) obtained indications that caffeine in yeast was degraded by cytochrome P450, suggesting that the catabolic pathway might be similar to animals. In humans, several cytochrome P-450 isoforms are responsible for caffeine degradation (Berthou et al., 1992).

However, data obtained by Schwimmer et al. (1971), who studied the degradation of caffeine to theophylline in fungi, and Blecher and Lingens (1977), who studied degradation of caffeine to theobromine in bacteria; do not indicate participation of P-450 on caffeine degradation mechanism.

Gluck and Lingens (1987) by *P. putida* mutants obtained a mixture of theobromine and paraxanthine as degradation products of caffeine. These results support inferences of Blecher and Lingens (1977), who suggested that caffeine can be degraded either via theobromine or via paraxanthine. Similar conclusion was reached by Mazzafera et. al., (1994a) with *Serratia marcescens.*

Figure 1.9.3.1. Caffeine degradation pathway in microorganisms.

(Blecher and Lingens, 1977)



Most studies on caffeine degradation by *Pseudomonas* use bacterial strains obtained through a procedure known as enrichment. Caffeine was added to the soil (Woolfolk, 1975) or culture medium (Blecher and Lingens, 1977; Hohnloser et. al., 1980; Middelhoven and Lommen, 1984) to induce the appearance of mutants. In the case of soil enrichment, caffeine was mixed to the soil and incubated for several months. In the second case, using artificial media, caffeine was added in low concentrations and the bacteria sub cultured several times until mutants were obtained. After that, bacteria were maintained in media containing caffeine as the sole source of carbon. Gluck and Lingens (1987) isolated a *P. putida* strain by culturing the bacteria with 2.0% caffeine as the sole source of carbon and nitrogen. Blecher and Lingens (1977) added caffeine up to 5.0% in the culture media. Middelhoven and Bakker (1982) grew the strain C 3204 of *P. putida* at 20 g L^{-1} of caffeine. However, Mazzafera et al. (1994a) and Yano and Mazzafera (1998) used a different approach, and collected mutants in soil samples taken under coffee plants. Water was added to the soil samples and after shaking for a few hours, aliquots were plated in solid medium containing caffeine as the sole source of carbon and nitrogen. In the first case, they isolated a *S. marcescens* strain (Mazzafera et al., 1994a) and in the second (Yano and Mazzafera, 1998), several *P. putida* strains and other bacteria.

A strain of *P. putida* isolated by Yano and Mazzafera (1998) showed an impressive ability to grow in high concentrations of caffeine. Growth was observed at 25 g L^{-1} in liquid medium and at 50 g L^{-1} in solid medium. The direct isolation from the soil without any enrichment is a strong indication that because of competition for organic nutrients, bacteria growing in soil under coffee plants have developed

mechanisms to degrade the caffeine released by the plants (leaves, fruits and litter). In other words, there was a natural enrichment.

Yano and Mazzafera (1999) studied the caffeine degradation pathway in this *P. putida* strain, and in agreement with results previously obtained by Blecher and Lingens (1987), suggested the degradation pathway showed in Figure. 1.9.3.1. Yano and Mazzafera (1999) also purified a xanthine oxidase, which is responsible for the conversion of methylxanthines to their respective uric acids. Attempts to purify the demethylase involved in the first step of caffeine degradation were not successful (Yano and Mazzafera, 1998). The activity was labile in partially purified extracts. The enzyme was NADH or NADPH- dependent producing theobromine and paraxanthine from caffeine. Activity was higher for paraxanthine, as observed in previous studies (Yano and Mazzafera, 1998).

Blecher and Lingens (1977) and Gluck and Lingens (1987) isolated *P. putida* mutants with the ability to degrade caffeine and attempted to block its degradation in order to produce caffeine derivatives like theobromine, paraxanthine and other monomethyl xanthines for commercial application. In contrast to these authors, Asano et al. (1993) were successful in isolating a *P. putida* strain where the route of caffeine degradation could be blocked by addition of Zn to the culture medium, accumulating theobromine. This dimethylxanthine was excreted in the medium, and precipitated at the bottom of the flask because of its low solubility. This was the first time that a caffeine derivative was selectively produced using a bacterium.

Yano and Mazzafera (1998) isolated more than 20 bacteria strains from soil collected under coffee plants, observing predominance of *Pseudomonas* sp., which

was also the most efficient caffeine degrader. Mazzafera (2002) used the same *P. putida* strain, used by Yano and Mazzafera (1996 and 1998), to study coffee husk decaffeination. Different proportions of inoculum and husk were incubated during 30 days, resulting in a reduction of up to 80% of caffeine. At shorter incubation periods (9 days) a 40% reduction was observed.

1.9.3.2. Caffeine degradation by fungi:

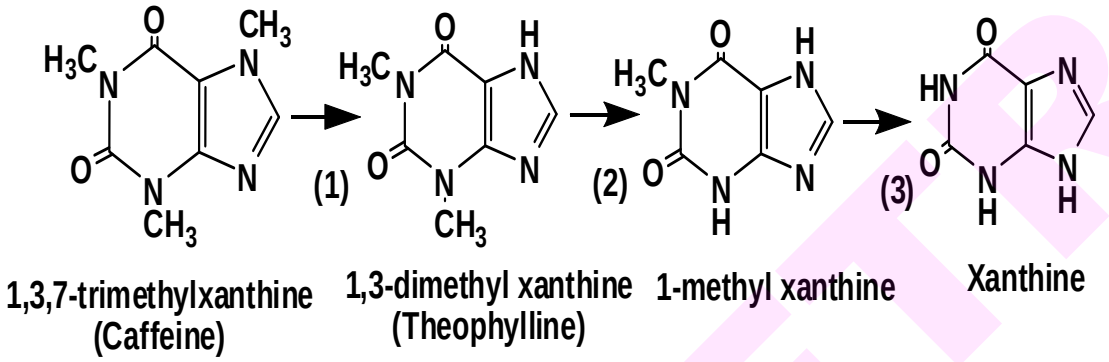
Several studies have shown that coffee fruits are a rich source of microorganisms. Boccas et. al., (1994) isolated 248 fungal cultures from coffee plants and soil collected from plantation areas. Roussos et. al., (1995) isolated 272 strains of filamentous fungi from soil, fruits and leaves using a culture media containing coffee extract, coffee extract plus sucrose, and coffee pulp extract. The fungi strains with the highest ability to degrade caffeine were identified as *Aspergillus* and *Penicillium.*

In studies in which the addition of coffee pulp was made at relatively high amounts in animal diets, it was suggested that its success was related to the pulps processing method which would allow a decrease of caffeine and tannin contents by microorganisms (Braham, 1987; Cabezas et. al., 1987; Jarquin, 1987). Actually, Murillo (1987) showed a decrease of these substances in ensiled coffee husk.

Caffeine degradation has been observed in fungal species like *Stemphyllium* sp., *Penicillium* sp. (Kurtzmann and Schwimmer, 1971), and *Aspergillus* sp. (Roussos et. al., 1995), *A. tamari*, *A. niger*, *A. fumigatus* and *P. commune* showed appreciable growth when caffeine was used as the sole source of nitrogen (Hakil et. al., 1998) *A. tamari* and *P. commune* showed good caffeine degrading ability (about 60%) whereas others had less than 20% caffeine degradation (Hakil et. al., 1998). Bioremediation of

coffee pulp to reduce the caffeine content has been studied more in fungal systems. Among the microbial community present in coffee pulp, only a few species like *Aspergillus*, *Penicillium* and *Rhizopous* could degrade caffeine (Roussos et. al., 1995). *Aspergillus* and *Penicillium* species degraded caffeine almost with 100% efficiency at 25^0C, whereas the efficiency of degradation decreased to 30% at 30^0C (Roussos et. al., 1995). *Rhizopous* sp. produced a higher quantity of biomass, whereas *Aspergillus* sp. showed more efficient caffeine degradation (92%). The degradation of caffeine in coffee pulp and coffee husk has been studied by solid-state fermentation with *Aspergillus*, *Rhizopus* and *Phanerochaete*. In *Rhizopus* and *Phanerochaete*, the critical parameters affecting caffeine degradation were pH and moisture. The critical values of pH and moisture content for *Rhizopus* and *Phanerochaete* were found to be 5.5, 65 and 6, 60%, respectively. For *A. niger* the critical parameters affecting caffeine degradation were temperature and pH and the optimal values were 28^0 C and 4.0, respectively (Brand et. al., 2000). An effective method has been reported for utilizing the caffeine using coffee pulp and husk as the substrate for the growth of molds (Leifa et. al, 2000; Salmones et. al., 2005). Caffeine was degraded during the growth of *Lentinus edode* whereas caffeine was accumulated in the fruiting bodies of *Pleurotus* sp. Hence, *Pleurotus* sp. could be used to recover caffeine from coffee and tea waste. The first steps of caffeine degradation by *Rhizopus delemar* LPB 34 by solid state fermentation of coffee husk consist of demethylation reactions (Fig. 1.9.3.2.). According to Hakil (1998), theophylline is the major degradation product of caffeine by various filamentous fungi.

Figure.1.9.3.2. First steps in the degradation pathway of caffeine by *Rhizopus delemar (Brand et.al.,2000).*



1.10. BIOTECHNOLOGICAL PROCESSES FOR DECAFFEINATION:

Several studies have been conducted by various groups since 1970s on the degradation of caffeine by microorganisms. A few groups (Kurtzman and Schwimmer, 1971; Mazzafera, et.al., 1994a) have attempted the development of biotechnological processes for decaffeination of coffee extracts, coffee pulp etc. The enzymes involved in the caffeine degradation pathway have also been identified and the caffeine degradation pathway in bacteria and fungi was elucidated (Blecher and Lingens, 1977; Mazzafera et.al., 1994a; Brand et.al., 2000). A few attempts were made to purify the enzymes involved in caffeine degradation in the microorganisms. The development of biotechnological processes for decaffeination still remained a far reality due to several factors like the extreme lability of the enzymes, requirement of co factors and the complex problems like inhibition of enzyme activities in involved in the decaffeination pathway. Due to these factors processes for biological decaffeination of caffeine containing materials could not be developed for commercial applications. Our group at CFTRI has isolated a strain of *Pseudomonas*

alcaligenes MTCC 5264, having a high caffeine degrading capacity (Sarath et. al., 2005). We have successfully developed a process for biodecaffeination of tea, coffee and beverages by using enzymes from *P. alcaligenes* MTCC 5264 strain isolated in our laboratory and this will be described in detail in the subsequent chapters.

The important parameters for the development of technological process for microbial degradation of caffeine are: (i) rate of caffeine degradation; (ii) initial caffeine content; (iii) nitrogen source and (iv) pH. The rate of caffeine degradation by important microorganisms is given in Table 1.10.1. The bacterial degradation of caffeine was better than fungi because the rate of degradation was higher in bacteria. Since caffeine is toxic to microorganisms (Sundarraj et. al., 1965; Putrament et. al., 1972; Kihlman 1974), the initial concentration of caffeine in fermentation is crucial. In *S. marcescens*, the critical inhibitory concentration has been found to be 1.2 mg/ml (Mazzafera et. al., 1994a). *P. putida* has been found to be the best organism for degrading caffeine at higher concentration. In fungi, the presence of an external nitrogen source inhibits caffeine degradation completely till the external source is depleted. The presence of urea and ammonium sulphate prevents the uptake of caffeine in *A. niger* and *Penicillium verrucosum* (Hakil et. al., 1999; Roussos et. al., 1994). However, in contrast to fungi, the presence of an external organic nitrogen source did not prevent the degradation of caffeine in bacteria (Asano et.al., 1993). Packed bed fermentation of *R. delemar* with coffee pulp as a substrate has been reported to produce theophylline and 3-methyl xanthine as the major metabolites due to the degradation of caffeine in the pulp (Tagliari et.al., 2003; Hakil et.al., 1998). No reports are available on bacterial degradation of caffeine in bioreactor.

Table 1.10.1. Comparison of caffeine degradation rates in various microorganisms.

Micro-organism	Initial caffeine conc.(g/l)	Carbon source (g/l)	Caffeine degradation (%)	Rate of caffeine degradation	Reference
Klebsiella and *Rhodococcus*	0.5	Glucose (1)	100% in 10 h	0.05	Madhyasta et. al., 1998
S. marcescens	0.6	-	100% in 72 h	0.008	Mazzafera et.al, 1994a
Stemphyllium sp	0.19	Sucrose (30.1)	100% in 29 h	0.0053	Kurtzman and Schwimmer, 1971
P. putida	5	-	95% in 50 h	0.095	Woolfolk, 1975
Aspergillus tamarii	1.2	Sucrose (28.4)	67.2% at 48 h	0.0536	Hakil et. al., 1998
Penicillium commune	1.2	Sucrose (28.4)	61.6% at 48 h	0.0521	Hakil et. al., 1998

1.10.1. Enzymatic methods of caffeine degradation:

It is imperative that biodecaffeination is a safe and economic alternative to existing decaffeination processes. Therefore a strong need exists for the development of biodecaffeination processes and all the possible sources of caffeine degrading enzymes need to be surveyed and the possibilities of their application, their advantages and disadvantages need to be weighed for selecting suitable enzymes/enzyme systems for the development of biodecaffeination processes. The following sections deal in detail about the different sources of enzymes, their activities and pros and cons of employing these enzymes for biodecaffeination systems.

1.10.1.1. Plant enzymes:

The most studied enzymes are plant caffeine anabolic enzymes, which are aimed at producing decaffeinated plants. The caffeine synthesis in plants comprises of sequential methylations at *N*-7,*N*-3 and*N*-1 of xanthosine ring which are catalyzed by different *N*-methyl transferases (NMT), viz., 7-methyl transferase, 3-methyl transferase and 1-methyl transferase (Suzuki and Takahasi, 1975; Magalhaes et. al., 1991; Mazzafera et., al 1994b). Caffeine synthesis in plants can be stopped if xanthosine synthesis in plants is inhibited since caffeine is synthesized from xanthosine. It has been proposed that by deactivation of inosine monophosphate dehydrogenase using inhibitor ribavarin, caffeine deficient tea and coffee plant can be produced (Keyaa et. al., 2003). Recently, cDNA of theobromine synthase (methyl transferase enzyme which produces theobromine) enzyme was isolated. The repression of theobromine synthase and caffeine synthase genes in a coffee plant using RNA interference technique resulted in a plant with 50% lower caffeine (Ogita et. al., 2004). A naturally decaffeinated coffee plant was also recently reported (Silvarolla et. al., 2004). Chemical defense theory proposes that caffeine may give protection to young leaves, fruits and flower buds from predators like larvae (Harborne, 1993). It was seen that solutions of caffeine are effective in killing or repelling slugs and snails when applied to foliage or the growing medium of plants (Hollingsworth et. al., 2002). Allelopathic theory proposes that caffeine may prevent the growth of other species of plants in its surroundings (Waller, 1989). So, aiming for decaffeinated plants with the knowledge of the genes involved in caffeine production may not be advantageous than microbial and enzymatic degradation of

caffeine source as and when required. Caffeine is known to be degraded via theophylline to ammonia and carbon dioxide, a pathway similar to fungal metabolism. It is imperative that all the enzymes involved are the same as those of microorganisms. The possibility of using caffeine degrading enzymes from coffee and tea plants offers the advantage of abundant fresh leaf material for enzyme extraction and isolation. Ashihara et.al, (1999a and b), have identified coffee plants with very low caffeine. They report that the low caffeine content of the plant is not due to a defective mechanism in caffeine synthesis, but a high caffeine degrading activity in the plants. However, studies on the extraction of caffeine demethylase enzyme from coffee plants were not successful till date (Ashihara, 2006). Kato, et. al., (1999) attempted the purification of caffeine synthase enzyme from leaves of coffee and found that the enzyme was highly labile for conducting experiments in vitro. It is also well known that demethylases are highly instable (Sideso, et. al., 2001). Even the presence of caffeine demethylase enzyme in coffee plants is not established well. Therefore development of bio-decaffeination processes using plant enzymes is still not very feasible. Alternative sources of the enzymes are microorganisms which have the capability to degrade caffeine.

1.10.1.2. Microbial enzymes:

The enzymes involved in the degradation of caffeine in microorganisms are demethylases and oxidases (Hohnloser et. al., 1980; Asano et. al., 1993; Yano and Mazzafera, 1998; Yano and Mazzafera, 1999). Yano and Mazzafera (1998) attempted to purify caffeine demethylase but found that the purified enzyme was labile and it rapidly lost its activity. It has been observed that the use of cryo-protectants and

freeze-drying to low moisture contents improved the stability of the enzyme (Sideso et. al., 2001). In general, the caffeine degrading enzymes are very labile and more studies are required to improve the stability of the enzymes, which will help in developing a specific process for caffeine degradation in reactors. In a mixed culture consortium belonging to *Klebsiella* sp. and *Rhodococcus* sp., caffeine was directly oxidized by the enzyme caffeine oxidase at the C-8 position leading to the formation of 1,3,7-trimethyluric acid and this process did not have demethylation steps. Only partial characterization of this enzyme is available (Madyastha et. al., 1998, Madyastha et. al., 1999). The oxidative degradation of caffeine to trimethyluric acid (single step) appears to be efficient for development of enzymatic degradation of caffeine. Studies on enzyme stability, cloning and over expression of this enzyme in suitable hosts will lead to development of technological process for efficient caffeine degradation. Sauer (1982) obtained indications that caffeine in yeast was degraded by cytochrome P450, suggesting that the catabolic pathway might be similar to animals. Sauer (1982) reported that degradation of caffeine in fungi is brought about by Cytochrome P450. However, the application of cytochrome P450s for biodecaffeination process is not feasible as their cofactor requirement is very high and the caffeine degradation rates are too slow. Moreover, these enzymes are expressed at very low quantities and are microsome bound requiring the separation of these enzymes in the form of microsomes which would increase the costs of a commercial process to inhibitory levels. Bacterial enzymes are known to degrade caffeine more efficiently, and their co-factor requirements are less. Moreover, extraction and purification of these enzymes is relatively simpler and crude cell free extracts contain

relatively higher enzyme activities. But the disadvantage of enzyme lability still exists in these processes and efforts were made at CFTRI to address these problems.

Work at CFTRI:

Three strains of caffeine degrading bacteria belonging to *Alcaligenes fecalis*, *Pseudomonas alcaligenes* and *Acetobacter* species were isolated. *P. alcaligenes* MTCC 5264, was found to have a very high caffeine degrading capability and this strain was found to completely degrade caffeine (1 g/l) within 12 hours of incubation (Sarath et. al., 2005). This strain was also found to grow at caffeine concentrations as high as 10g/L of caffeine. This strain was used for the development of biodecaffeination processes for coffee and tea extracts. The enzymes involved in biodecaffeination were isolated and identified. These enzymes were found to be highly labile and were stabilized using different stabilizing agents. The enzymes were immobilized into suitable matrices and process for biodecaffeination of coffee and tea extracts were developed. A process for the biodecaffeination of tea has also been developed for use during tea fermentation and the results were promising (Sarath et. al., 2003 (428/Del/03); Sarath et. al., 2006a, 2006b). Further work is underway on the cloning and hyper expression of the caffeine demethylase enzyme into a suitable vector for the production of enzymes at a larger scale, increasing the stability of the enzymes in vitro and application of these enzymes for biodecaffeination processes.

1.11. CONCLUSION AND FUTURE PERSPECTIVES:

Present day scenario around the globe demands for more safe products produced through green route. Coffee and tea being the most widely consumed beverages all around the world, and the risks associated with high intake of these

beverages have led to the development of the existing decaffeination processes. Each process has its own disadvantage either in the form of solvent residues or loss of essential flavors or inhibitory costs of decaffeination as in the case of super critical process. Moreover these processes are capable of catering only coffee and tea, but are not feasible for application to the huge amounts of coffee and tea wastes generated. These processes also have added up to the existing environmental problems of the industrialized world. Alternative green technologies are the need of the day and biological means of decaffeination is the best alternative.

Though the search for caffeine degrading microorganisms began nearly 35 years ago, studies conducted in this area are inadequate. In bacteria, *Pseudomonas* species and in fungi *Aspergillus* and *Penicillium* species are reported to be efficient degraders of caffeine. Degradation in bacteria occurs predominantly through demethylation route but oxidative route occurs predominantly in mammals and the degradation pathway in fungi is not clearly known. The area of fungal decaffeination is limited by insufficient literature and knowledge generated. Even in microbial systems, studies have to be performed to ensure that toxic metabolites are not formed by the strain during its growth. More microorganisms, which could degrade caffeine, need to be isolated. Use of whole cells might be an alternative, due to the advantages of the stability of the biomolecules and reusability. Coffee and tea are rich in nutrients and the organisms would prefer the simple nutrients for caffeine (Sarath et. al., 2005) leading to a compromise in the quality of the finished products.

Enzymatic methods of biodecaffeination are the only alternative and research needs to be focused in this area. The limitation of the use of

biodecaffeinating enzymes is their high lability. Stabilization of the enzymes, improving the yields of the enzymes through biochemical and genetic engineering of the caffeine degraders, improved process conditions etc. are the key areas which need a lot of research. Focus is now on the development of coffee and tea plants devoid of caffeine. But this alternative is not a very suitable method as the plant defense system would be completely inactivated and would be detrimental to the plant. This would demand more pesticides and herbicides to protect the plant leading to environmental pollution problems. Moreover this would demand a replanting of at least a part of the 206 million hectares of tea and coffee plantations all over the world which would amount to huge expenditure and loss of crop and it is not necessary that the products should be completely decaffeinated. The caffeine levels in the beverages should be brought to levels safe for human consumption to get the benefits of caffeine also. Caffeine degradation by microbes and enzymes is more advantageous than creating decaffeinated plants with the help of genes of the plant anabolic enzymes. An alternative will be cloning and hyper expression of the genes coding the caffeine and its metabolite degrading enzymes into suitable expression vectors, increasing the yields of the enzymes, stabilizing the enzymes and application of these enzymes for the development of biodecaffeination processes. Once this is done, the enzymes may be purified, immobilized in a bioreactor and its caffeine degrading ability can be studied and optimized. Biological production of theobromine and other methyl xanthines from caffeine need to be optimized. Such studies will lead to development of process for caffeine degradation as well as production of value added compounds and this work is going on in our laboratory. An integrated approach of basic science

with biochemical engineering will help in developing a successful process for caffeine degradation using microorganisms or enzymes associated with it. Success was achieved in our laboratory at CFTRI on the development of enzymatic biodecaffeination of coffee and tea using immobilized and free enzymes. Work is also underway in our laboratory on the cloning and hyper expression of the genes coding for the caffeine degrading enzymes in a suitable expression vector. Also work is underway on the scaling up of the biodecaffeination process for industrial applications. Several problems like protein polyphenol interactions, caffeine catechin complexes, inhibition of the caffeine degrading enzymes etc have been addressed but need an in-depth study and industry applicable solutions so that the biodecaffeination process would be converted to an industrial success from being a mere scientific discovery.

1.12. REFERENCES:

1. Abolfathi Z., Pakdel, H., Beaune, P., (1995), CYP1A2 is the major enzyme involved in the N-oxidation of mexiletine in man. *Clin. Pharml. Ther***.** **57**:210.
2. Acheson, K., Gremaud, G., Meirim, I., (2004), Metabolic effects of caffeine in humans: lipid oxidation or futile cycling? *Am. J. Clin. Nutr*. **79**: 40-46.
3. Adams, M.R., Dougan, J. (1981), Biological management of coffee processing. *Tropic*. *Sci.* **123**: 178-196.
4. al'Absi, M., Lovallo, W.R., McKey, B., Sung, B.H., Whitsett, T.L., Wilson, M.F., (1998), Hypothalamic-pituitary-adrenocortical responses to psychological stress and caffeine in men at high and low risk for hypertension. *Psychosom. Med.* **60(4):** 521-7.
5. Alvares, A.P., Kappas, A., Eiseman, J.L., Anderson, K.E., Pantuck, C.B., Pantuck, E.J., (1979), Intra individual variation in drug disposition. *Clin. Pharmacol. Ther*. **26:**407- 419.
6. Andersson, H. C., Hallström, H., Kihlman, B.A, (2004), Intake of caffeine and other methylxanthines during pregnancy and risk for adverse effects in pregnant women and their fetuses. *TemaNord.* **:**565
7. Asano, Y., Komeda, T., Yamada, H., (1993), Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.* **57**: 1286–89.
8. Asano, Y., Komeda, T., Yamada, H., (1994), Enzymes involved in theobromine production from caffeine by a *Pseudomonas putida* No. 352. *Biosci. Biotech. Biochem.* **58**:2303–2304.
9. Ashihara, H., Crozier, A., (1999a), Biosynthesis and catabolism of caffeine in low-caffeine-containing species of Coffea. *J. Agric. Food Chem*. **47:** 3425-3431.
10. Ashihara, H., Crozier, A., (1999b), Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Adv. Bot. Res.* **30**: 118-205.

11. Ashihara, H., Gillies, F.M., Crozier, A., (1997), Metabolism of caffeine and related purine alkaloids in leafs of tea (*Camellia Sinensis*). *Plant Cell Physiol.* **38**: 413–19.

12. Ashihara, H., Monteiro, A.M., Gillies, F.M., Crozier, A., (1996a), Biosynthesis of caffeine in leaves of coffee. *Plant Physiol.* **111:** 747-753.

13. Ashihara, H., Monteiro, A.M., Moritz, T., Gillies, F.M., Crozier, A., (1996b), Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L., *Planta.* **198**: 334-339.

14. Ashihara, H., (2006), Metabolism of alkaloids in coffee plants. *Braz. J. Plant Physiol.* **18(1):** 1-8.

15. Attifeld, J., (1865). On the food-value of the cola-nut- a new source of thein. *Pharm. J.* **6**: 457-460.

16. Barone, J.J., Roberts, H.R., (1996), "Caffeine Consumption." *Food Chem. Toxicol.*. **34**: 119-129.

17. Barrett, C., Chang, J. C., Edelstein, S. L., (1994), Coffee-associated osteoporosis offset by daily milk consumption. The Rancho Bernardo Study, *J. Amer. Med. Ass.* **271:** 280-283.

18. Bergmann, F., Ungar-Waron, H., Kwietny-Govrin H., (1964), Some specific reactions of the purine oxidizing system of *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta,* **79**: 512-522.

19. *Berthemot, Dechastelus (1840), Chemische Untersuchung des Guarana. *Liebig´s Ann. Chem*. **36**: 90-93.

20. Berthou, F., Guillois, B., Riche, C., Dreano, Y., Jacqz-Aigrain, E., and Beaune, P. (1992), *Xenobiotica,* **22:** 671–680.

21. Blauch, J.L., Tarka J.R., Stanley, M., (1983), HPLC determination of caffeine and theobromine in coffee, tea and instant hot cocoa mixes. *J. Food Sci.* **48**: 745-748.

22. Blecher, R., Lingens, F., (1977), Metabolism of caffeine by *Pseudomonas putida*. *Hoppe Seyler's Z Physiol Chem*. **358:**807–17.

23. Boccas F., Roussos S., Gutierrez M., Serrano L., Viniegra G.G., (1994), Production of pectinase from coffee pulp in solid-state fermentation system - selection of wild fungal isolate of high potency by a simple 3-step screening technique. *J. Food Sci. Tech*. (Mysore). **31:**.22-26.

24. Bogo, A., Mantle, P.G., (2000), Caffeine: also a fungal metabolite, *Phytochem*; **54**: 937–939.

25. Braham, J.E., (1987), Coffee pulp in other species. In: Brahan, J.E., Bressani, R. (Ed.) Coffee pulp**:** composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, 51-54.

26. Brand, D., Pandey, A., Roussos, S., Soccol, C.R., (2000) Biological detoxification of coffee husk by filamentous fungi using a solid-state fermentation system. *Enz. Microb. Technol.* **27**:127–33.

27. Bressani, R., (1987a), Antiphysiological factors in coffee pulp. In: Brahan, J.E.; Bressani, R. (Ed.) Coffee pulp**:** composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama. 83-88.

28. Bressani, R., (1987b), Potential uses of coffee berry byproducts. In: Brahan, J.E.; Bressani, R. (Ed.) Coffee pulp**:** composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, 17-25.

29. Brogger, A., (1979), Caffeine-induced enhancement of chromosome damage in human lymphocytes treated with methyl-methane-sulphonate, mitomycin C and X-rays, *Mut.Res./Gen. Toxicol.* **23(3):** 353-360.

30. Burr, T.J., Caesar, A., (1985), Beneficial plant bacteria. *CRC Critic. Rev. Plant Sci*., **2**:120.

31. Buters, J. T., Tang, B.K., Pineau, T., Gelboin, H,V., Kimura, S., Gonzalez, F.J., (1996), Role of CYP1A2 in caffeine pharmacokinetics and metabolism: Studies using mice deficient in CYP1A2. *Pharmacogen.* **6:** 291-296.

32. Butler, M. A., Lang, N.P., Young, J.F., Caporaso, N.E., Vineis, P., Hayes, R.B., (1992), Determination of CYP1A2 and NAT2 phenotypes in human population by analysis of caffeine urinary metabolites. *Pharmacogen.* **2**:116-127.

33. Butler, M. A., Iwasaki, M., Guengerich, F. P., Kadlubar, F. F. (1989), *Proc. Natl. Acad. Sci. USA,* **86:** 7696–7700.

34. Cabezas, M. T., Flores, A., Egana, J. I., (1987), Use of coffee pulp in ruminant feeding. In: Braham, J.E.; Bressani, R. (Ed.) *Coffee pulp: composition, technology, and utilization.* Guatemala City: Institute of Nutrition of Central America and Panama. 25-38.

35. Carrillo, J.A., Christensen, M., Ramos, S., Alm, C., Dahl, M.L., Benitez, J., (2000), Evaluation of caffeine as an *in vivo* probe for CYP 1A2 using measurements in plasma, saliva and urine. *Ther. Drug. Monit.* **22**: 409-417.

36. Carter, A.J., O'Connor, W.T., Carter, M.J., Ungerstedt, U., (1995), Caffeine enhances Acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors. *J. Pharmacol. Exp. Ther.* **273(2):** 637-42.

37. Casley, W.L., Menzies,J.A., Whitehouse,L.W., Moon,T.W., (1999), Detection Of Quantitative Trait Loci Affecting Caffeine Metabolism By Interval Mapping In A Genome-Wide Scan Of C3h/Hej 3 Apn F2 Mice, *Drug. Meta. Disp*. **27(12):** 1375–1380.

38. Cherrington, N.J., Cao, Y., Cherrington, J.W., Rose, R.L., Hodgson, E., (1998), Physiological factors affecting protein expression of flavin-containing monooxygenases 1, 3, and 5. *Xenobiotica,* **28:**673–682.

39. Christian, M., Brent, R., (2001), Teratogen Update: Evaluation of the Reproductive and Developmental Risks of Caffeine. *Teratology*, **64**: 51-78.

40. Chung W.G., Cha, Y.N., (1997), Oxidation of Caffeine to Theobromine and Theophylline Is Catalyzed Primarily by Flavin-Containing Monooxygenase in Liver Microsomes, *Biochem. Biophys. Res. Comm,* **235:** 685–688.

41. Clarkson, (1993), Nutritional Ergogenic Aids: Caffeine. *Int.J. Sport Nutr*. **3:**103-111.

42. Cnattingius, S., Signorello, L.B., Anneren, G., Clausson, B., Ekbom, A., Ljunger, E., Blot, W.J., McLaughlin, J.K., Petersson, G., Rane, A., Granath, F., (2000),

Caffeine Intake and the Risk of First-Trimester Spontaneous Abortion. *New Eng. J. Med.* **343**: 1839-1845.

43. Cornish, H. H., Christmanm A. A., (1957), A study of the metabolism of theobromine, theophylline and caffeine in man. *J. Biol. Chem.* **228**:315–23.

44. Daly, J.W., Fredholm, B.B., (1998), Caffeine—an atypical drug of dependence. *Drug. Alcohol. Depend.* **51**:199–206.

45. Dam, R. M. V., Hu**,** F. B., (2005), Coffee Consumption and Risk of Type 2 Diabetes: A Systematic Review, *J. Amer. Med. Assoc*. **294(1):** 97 - 104.

46. *Danielle, W.F. (1865), On the cola-nut of tropical West Africa (The guru nut of Sudan*). Pharm. J*.* **6**: 450-457.

47. Danielson, P.B., (2002), The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. *Curr. Drug. Metab*. **6:** 561–597.

48. Denis, S., Augur, C., Marin, B., Roussos S., (1998), *Biotechnol. Techn.* **12(5):**359-362.

49. Dickstein, S., Bergmann, F., Henis, Y., (1957), Studies on uric acid and related compounds. IV. The specificity of bacterial xanthine oxidases. *J. Biol. Chem*. **224**: 6777.

50. Dlugosz, L., Belanger, K., Hellenbrand, K., et al., (1996), Maternal caffeine consumption and spontaneous abortion: a prospective cohort study. *Epidemiol.* **7**:250–5.

51. Elias, P.S. (1986), Current biological problems with coffee and caffeine. In: Colloque International Sur La Chimie Du Cafe. 12., Lome, 1985. **Proceedings.** Paris: *Assoc. Scientifi*.-112.

52. Eskenazi, B., (1999), Caffeine- filtering the facts. N*ew Eng. J. Med.* **341**:1688-1689.

53. *Europaisches, A., (1978), *Coffeinum*, p 670: *Theophyllinum*, 1213; Deutscher Apotheker Verlag Stuttgart.

54. Farin, F,M,, Omiecinske, C,J., (1993), Region-specific expression of cytochrome P450s and microsomal epoxide hydrolase in human brain tissue. *J Toxicol. Environ. Health.* **40**:323-341.

55. *Fechner, M.G.T., (1826), Repertorium der organischen Chemie, (Leipzig).

56. Fenster, L., Eskenazi, B., Windham, G.C., (1991), Caffeine consumption during pregnancy and fetal growth. *Am. J. Public Health*. **81**:458–61.

57. Fontana, R. J., (1998), Caffeine based measures of CYP1A2 activity correlate with oral clearance of tacrine in patients with Alzheimer's disease. *Br. J. Clin. Pharmacol.* **46:**221-228.

58. Fredholm, B., (1995), Adenosine, adenosine receptors and actions of caffeine. *Pharmacol. Toxicol.* **76**:93-101.

59. Friedman, J., Waller, G.R., (1983a), Caffeine hazards and their prevention in germinating seeds of coffee (*Coffea arabica* L.). *J. Chem. Ecol*. **9**:1099-1106.

60. Friedman, J., Waller, G.R., (1983b), Seeds as allelopathic agents. *J. Chem. Ecol.* **9:**1107-1115.

61. Frischknecht, P. M., (1985), Purine alkaloid formation in buds and developing leaflets of *Coffea arabica*: expression of an optimal defense strategy?? *Phytochem.* **3**:613-616.

62. Fuhr, U., Rost, K.L., Engelhardt, R., Rachs, M., Liermann, D., Belloc, C., (1996), Evaluation of caffeine as a test drug for CYP1A2, NAT2 and CYP2E1 phenotyping in man by in vivo versus correlations. *Pharmacogenetics*. **6**:159-176.

63. *Giese, F.V., (1820), Vermischte Notizen .1. Kaffestoff und Salzgehalt des Quassia Extrakts. *Allg. Nord. Ann. Chem. Freunde Naturkd. Arzneiwiss*. **4:** 240-241.

64. *Giese, F.V. (1821) Verschiedene Beobachtungen. *J. Chem. Phys*. **31**, 203-209.

65. Gluck, M., Lingens, F., (1987), Studies on the microbial production of thebromine and heteroxanthine from caffeine. *Appl. Microb. Biotech.* **25**: 334-340.

66. Gokulakrishnan, S., Chandraraj, K., Gummadi, S.N., (2005), Microbial and enzymatic methods for the removal of caffeine, *Enz. Microb. Tech*. **37**, 225-232.

67. Gonzalez, F.J., Kimura, S., (2003), Study of P450 function using gene knockout and transgenic mice. *Arch. Biochem. Biophys*. **409**: 153– 158.

68. Gotoh, O., (1992), Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem*. **267:** 83–90.

69. Graham, T.E., Rush, J.W.E., Soeren, M.H., (1994), Caffeine and exercise: Metabolism and performance. *Can. J. App. Physiol*. **19**: 111-138.

70. Grayson, D. A., Tewari, Y. B., Mayhew, M. P., Vilker, V. L., Goldberg, R. N., (1996), Tetralin as a substrate for camphor (cytochrome P450) 5-monooxygenase. *Arch. Biochem. Biophys*. **332(2):** 239-47.

71. Green, P.J., Suls, J., (1996), The effects of caffeine on ambulatory blood pressure, heart rate, and mood in coffee drinkers. *J. Behavior. Med*. **19(2)**:111-28.

72. Greenberg, J. L., Lewis, S. E., Dodd, D. K., (1999), Overlapping addictions and self-esteem among college men and women. *Addict. Behav*. **24**:565–71.

73. Grigg, C.W., (1972), Effects of coumarin, pyronine Y, 6,9-dimethyl-2 methylthiopurine and caffeine on excision repair and recombination in *Escherichia coli*., *J. Gen. Microb.* **70**;221230.

74. Gu, L., Gonzalez, F. J., Kalow, W., Tang, B. K., (1992), Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. *Pharmacogenetics* **2**: 73-77.

75. Guengerich, F. P., Shimada, T., (1991) *Chem. Res. Toxicol.* **4,** 391–407.

76. Hagedorn, C. J. B., Crozier, K. A., Mentz, A. M., Booth, A. K., Graves, N. J., Nelson, R. B. Reneau, Jr. (2003a), Carbon source utilization profiles as a method to identify sources of fecal pollution in water. *J. Appl. Microb*. **94(5**): 792-799.

77. Hagedorn, C. (2003b). BST Methodologies. Internet address: http://soils1.cses.vt.edu/ch/biol_4684/bst/BSTmeth.html .

78. Hakil, M., Denis, S., Gonzalez G.V., Augur, C., (1998), Degradation and product analysis of caffeine and related dimethyl xanthines by filamentous fungi. *Enz. Microb. Technol*. **22**:355–9.

79. Hakil, M., Voisinet, F., Gonzalez, G.V., Augur, C., (1999), Caffeine degradation in solid-state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources. *Process Biochem.* **35**:103–9.

80. Harborne, J. B., (1993), Introduction to ecological biochemistry. Academic Press.

81. Hartman, S. C., (1970), *Metab. Pathways*. **4**, 1-68.

82. Hatch, E., Bracken, M., (1993), Association of Delayed Conception with Caffeine Consumption. *Amer. J. Epidem.* **138:** 1082-1091.

83. Hiroshi, Ashihara,. (2006), Metabolism of alkaloids in coffee plants, *Braz. J. Plant Physiol.* **18(1):**1-8.

84. Hohnloser, W., Osswalt, B., Lingens, F., (1980), Enzymological aspects of caffeine demethylation and formaldehyde oxidation by *Pseudomonas putida* C1. *Hoppe seyler's Z. Physiol. Chem.* **361**:1763–76.

85. Hollingsworth, R. G., Armstrong, J., Wi, Campbell, E., (2002), Pest control: caffeine as a repellent for slugs and snails. *Nature.* **417(6892**): 915–6.

86. Holmgren, P., Norden-P. L., Ahlner, J., (2004). "Caffeine fatalities — four case reports". *Forensic Sci. Int.* **139 (1):** 71-3.

87. Ikeya, K., Jaiswal, A. K., Owens, R. A., Jones, E., Nebert, D.W., Kimura, S., (1989), Human CYP1A2: sequence, gene structure, comparison with the mouse and rat orthologous gene, and differences in liver 1A2 mRNA expression. *Mol. Endocrinol.* **3**: 1399-1408.

88. Illy, A., Viani, R., (1995), Definition of quality. In *Espresso Coffee: the Chemistry,of quality*, Ed by Illy A and Viani R, Academic press, London., 24-26.

89. Infante, R. C., Fernandez, A., Gauthier, R., David, M., Rivard, G.E., (1993), Fetal loss associated with caffeine intake before and during pregnancy. *J. Am. Med. Assoc*. **270:**2940-2943.

90. Ito. E., Crozier. A., Ashihara, H., (1997), Theophylline metabolism in higher plants. *Biochim. Biophys. Acta.* **1336**: 323-330.

91. James, J.E., (1997), Is habitual caffeine use a preventable cardiovascular risk factor? *Lancet*. **349**:279–81.

92. James, J.E., Stirling, K.P., (1983), Caffeine: A summary of some of the known and suspected deleterious effects of habitual use. *Brit. J. Addict.* **78(3):** 251-8.

93. Jarquín, R ., (1987), Coffee pulp in swine feeding. In: Braham, J.E.; Bressani, R. (Ed.) *Coffee pulp: composition, technology, and utilization*. Guatemala City: Institute of Nutrition of Central America and Panama, 39-49.

94. *Jobst, C., (1838a), Thein identisch mit Caffein. *Ann. Chem. Pharm*. **25**: 63-66.

95. *Jobst, C., (1838b), Analyse des Theins. *Arch. Pharm*. **65**: 85-86.

96. Johnson, S., (2001), The multifaceted and widespread pathology of magnesium deficiency. *Medical Hypothesis*. **56(2):**163-70.

97. *Kalberer, P., (1964) *Ber. Schweiz. Bot. Ges*. **74:** 62-107.

98. Kalberer, P., (1965) *Nature (London***) 205:** 597-598.

99. Kalow, W., Tang, B.K., (1991), Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase activities. *Clin. Pharmacol. Ther*. **50**:508-519.

100. Kato, M., Mizuno, K., Fujimura, T., Iwama, M., Irie, M., Crozier, A., Ashihara, H., (1999), Purification and characterization of caffeine synthase from tea leaves. *Plant Physiol.* **120**: 597-586.

101. Katz, S.N., (1987), Decaffeination of Coffee, Coffee Technology. Ed. Clarke R.J. and R. Macrae. New York: Elsevier Applied Science.

102. Kaufmann, W. F., Heffernan, T. P., Beaulieu, L. M., Doherty, S., Frank, A. R., Zhou, Y., Bryant, M. F., Zhou, T., Luche, D. D., Feinberg, N. N., Schuh, K. J., Griffiths, R. R., (1997), Caffeine reinforcement: the role of withdrawal. *Psychopharmacology*; **130**:320–6.

103. Kerrigan, S., Lindsey, T., (2005), Fatal caffeine overdose: two case reports. *Forensic Sci. Int.* **153 (1):** 67-9.

104. Keyaa, C. A., Crozier, A., Ashihara, H., (2003), Inhibition of caffeine biosynthesis in tea (*Camellia sinensis*) and coffee (*Coffea arabica*) plants by ribavirin. *FEBS Lett.* **554:**473–7.

105. Khanna, K. L., Rao. G.S., Cornish, H.H., (1972), Metabolism of caffeine-3H in the rat. *Toxicol. Appl. Pharmacol*. **23**:720–30.

106. Kihlman, B. A., (1974), Effects of caffeine on the genetic material. *Mutat. Res.* **26:**53–71.

107. Kobayashi, K., (1998), Inhibitory effects of antiarrhythmic drugs on phenacetin O-deethylation catalysed by human CYP1A2. *Br. J. Clin. Pharmacol*. **45**:361-368.

108. Konishi, S., Oishi, F., (1973), Nippon Chagyo-Gijutsu, Kyokai Kenkyu-Happyokai Abstr. 38.

109. *Kossel, A. (1888) Ueber eine neue Base aus dem Pflanxenreich. Ber. Dtsch. Chem. Ges., 21:2164-2167.

110. Koyama, Y., Tomoda, Y., Kato, M., Ashihara, H., (2003), Metabolism of purine bases, nucleosides and alkaloids in theobromine-forming *Theobroma cacao* leaves. *Plant Physiol. Biochem.* **41**:977–84.

111. Kretschmar, J.A., Baumann, T.W., (1999), Caffeine in citrus flowers. *Phytochemistry*. **52**:19–23.

112. Kumar, N., Pandey, S., Bhattacharya, A., Ahuja, P.S., (2004), Do leaf surface characteristics affect *Agrobacterium* infection in tea [*Camellia sinensis* (L.) O. Kuntze]. *J. Biosci*. **29(3):**309–17.

113. Kurtzman Jr., R.H., Schwimmer, S., (1971), Caffeine removal from growth media by microorganism. *Experientia*. **27**:481–2.

114. LaCroix, A. Z., Mead, L. A., Liang, K., Thomas, C. B., Pearson, T. A. (1986), Coffee consumption and the incidence of coronary heart disease. *New Eng. J. Med*. **315**: 977-982.

115. Lee, S., Hudson, R., Kilpatrick, K., Graham, T. E., Ross, R. (2005), Caffeine Ingestion Is Associated With Reductions in Glucose Uptake Independent of Obesity and Type 2 Diabetes Before and After Exercise Training. *Diabetes Care*, **28(3**): 566 - 572.

116. Leifa, F., Pandey, A., Soccol, CR., (2000), Solid-state cultivation—an efficient method to use toxic agro-industrial residues. *J. Basic Microbiol*. **40(3**):187–97.

117. Lelo, A., Birkett, D.J., Robson, R.A., Miners, J. O., (1986), Comparative pharmacokinetics of caffeine and its primary demethylated metabolites paraxanthine, theobromine and theophylline in man. *Br. J. Clin. Pharmacol*. **22:** 177-182.

118. Lemoine, A., Gautier, J. C., Azoulay, D., Kiffel, L., Belloc, C., Guengerich, F. P., (1993), Major pathway of imipramine metabolism is catalysed by cytochromes P450 1A2 and P450 3A4 in human liver. *Mol. Pharmacol*. **43**:827-832.

119. Leviton, A., Cowan, L., (2002), A review of the literature relating caffeine consumption by women to their risk of reproductive hazards. *Food Chem. Toxicol*. **40**:1271–310.

120. Lewis, D. F. V., Lake, B. G., (1996), *Xenobiotica*.. **26:** 723–753.

121. Lewis, D. F. V., Ioannides, C., Parke, D. V., (1986), *Biochem. Pharmacol.* **35:** 2179–2185.

122. Lewis, D. F. V., Moereels, H., Lake, B. G., Loannides, C., Parke, D. V., (1994), *Drug Metab. Rev.* **26:** 261–285.

123. Lorist, M. M., Tops, M., (2003), Caffeine, fatigue, and cognition. *Brain Cogn*. **53**:82–94.

124. Lovallo, W.R., Al'Absi, M., Blick, K., Whitsett, T.L. and Wilson, M.F. (1996), Stress-like adrenocorticotropin responses to caffeine in young healthy men. *Pharmacol. Biochem. Behavior*. **55(3)**:365-9.

125. MacIntosh, B.R., Wright, B.M.., (1995), Caffeine ingestion and performance of a 1500 m swim. *Can. J. Appl. Physiol.*, **20**: 168-177.

126. Madyastha, K.M., Sridhar, G.R., Vadiraja, B.B., Madhavi, Y.S., (1999), Purification and partial characterization of caffeine oxidase-A novel enzyme from a mixed culture consortium. *Biochem. Biophys. Res. Commun.* **263:**460–4.

127. Madyastha, K.M., Sridhar, G.R., (1998), A Novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem. Biophys. Res. Commun.* **249**:178–81.

128. *Martius, T., (1840) , Ueber die Zusammensetzung des Guaranins. *Ann. Chem.* **36:** 93-95.

129. Massey, L. K., Wise, K. J., (1984), The effect of dietary caffeine on urinary excretion of calcium, magnesium, sodium and potassium in healthy young females. *Nutr. Res*. **4**: 43–50.

130. Massey, L. K., Bergman, E. A., Wise, K. J. and Sherrard, D. J. (1994) Interactions between dietary caffeine and calcium on calcium and bone metabolism in older women. *J. Am. Coll. Nutr*. **13:** 592–596.

131. Mast, N., Norcross, R., Andersson, U., Shou, M, Nakayama, K., Bjorkhem, I., Pikuleva, I.A., (2003), Broad Substrate Specificity of Human Cytochrome P450 46A1 Which Initiates Cholesterol Degradation in the Brain, *Biochemistry*. **42 (48):**14284 -14292.

132. Mazzafera, P., (2004), Catabolism of caffeine in plants and microorganisms. *Front. Biosci*. **9**: 1348-1359.

133. Mazzafera, P., Crozier, A., Magalhaes. A.C., (1991), Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochemistry*. **30**:3913–6.

134. Mazzafera, P., Crozier, A., Sandbergs, G., (1994a), Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei*. *J. Agric. Food Chem*. **42**:7423–77.

135. Mazzafera, P., Olsson, O., Sandberg, G., (1994b), Degradation of caffeine and related methyl xanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microb. Ecol*. **31**:199–207.

136. *Meyers, M.G. et al. (1991) *Ann. Intern. Med.*, **114**: 147-150.

137. Middelhoven, W. J., Bakker, C. M., (1982), Degradation of caffeine by immobilized cells of P*seudomonas putida* strain C 3024. *Euro. J. Appl. Microbial. Biotechnol*. **15:** 214-217.

138. Middelhoven, W. J., Lommen, A., (1984), Degradation of caffeine by *Pseudomonas putida* C3024. *Anton. van Leeuwenho.* **50:**298-30.

139. Mills, J.L., Holmes, L.B., Aarons, J.H., Simpson, J.L., Brown, Z.A., Jovanovic-Peterson, L.G., Conley, M.R., Graubard, B.I., Knopp, R.H. and Metzger, B.E., (1993), Moderate caffeine use and the risk of spontaneous abortion and intrauterine growth retardation. *J. Am. Med.Assoc*. **269:**593-597.

140. Molina, M. R., de la Fuente, G., Batten, M. A., Bressani, R., (1974), Decaffeination. A process to detoxify coffee pulp. *J. Agric. Food Chem*. **22(6):**1055-59.

141. Mrvos, R. M., Reilly, P. E., Dean, B. S., Krenzelok, E. P., (1989), Massive caffeine ingestion resulting in death. *Vet. Hum. Toxicol*. **31 (6):** 571-2.

142. Mulder, Ch. J., (1838), Chemische Untersuchung des chinesischen und des javanischen Threes. *Arch. Pharm*. **65:** 68-84.

143. Murillo, B., (1987), Coffee pulp silage. In: Braham, J.E.; Bressani, R. (Ed.) *Coffee pulp: composition, technology, and utilization*. Guatemala City: Institute of Nutrition of Central America and Panama. 55-62.

144. Nakajima, M., Yokoi, T., Mizutani, M., Shin, S., Kadlubar, F.F., Kamataki, T., (1994), Phenotyping of CYP1A2 in a Japanese population by analysis of

caffeine urinary metabolites: absence of mutation prescribing the phenotype the CYP1A2 gene. *Cancer Epidemiol. Biomark. Prev.* **55**:413-421.

145. Negishi, M., Iwasaki, M., Juvonen, R. O., Sueyoshi, T., Darden, T. A., Pedersen, L. G., (1996), Structural flexibility and functional versatility of cytochrome P450 and rapid evolution. *Mutat. Res*. **350:** 43– 50.

146. Nehlig, A., Debry, G., (1994), Potential genotoxic, mutagenic and antimutagenic effects of coffee: a review. *Mutat. Res*. **317(2):**145-62.

147. Nehlig, A., (1999), Are we dependent upon coffee and caffeine? A review on human and animal data. *Neurosci. Biobehav. Rev*. **23:**563–76.

148. Nelson, D. R., (1995), *in "Cytochrome P450: Structure, Mechanism and Biochemistry*, Ortiz de Montellano, P., Ed., 575–606, Plenum Press, New York.

149. Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W., (1996), P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **1**: 1–42.

150. Newcomb, M., Hollenberg, P. F., Coon, M. J., (2003), Multiple mechanisms and multiple oxidants in P450-catalyzed hydroxylations. *Arch. Biochem. Biophys*. **409:** 72–79.

151. O'Conell, F. D., (1969), Isolation of caffeine from *Banisteriopsis inebrians* (Malpighiaceae). *Naturwissenschaften.* **56**: 139.

152. Ogita, S., Uefuji, H., Yamaguchi, Y., Koizumi, N., Sano, H., (2003), Producing decaffeinated coffee plants. *Nature*. **423**: 823.

153. Omar, S., Usmani, Maria, G., Belvisi, Hema, J. P., Natascia, C., Mark, A. B., Marta, K., Dezso, K., Peter, J. B., (2005), Theobromine inhibits sensory nerve activation and cough, *FASEB. J.* **19**: 231-233.

154. Onrot, J., Goldberg, M. R., Biaggioni, I., Hollister, A. S., Kingaid, D., Robertson D., (1985), Hemodynamic and humoral effects of caffeine in

autonomic failure. Therapeutic implications for postprandial hypotension., *N. Engl. J. Med*. **313(9):**549-54.

155. Oudry, V., (1827), Thein, eine organische Salzbase im Three (Thea chinesis). *Mag. Pharm.* **19:** 49-50.

156. Ou-Yang, D. S., Huang, L., Wang, W., Xie, H. G., Xu, Z. H., Zhou, H. H., (2000), Phenotypic polymorphism and gender-related differences of CYP1A2 activity in a Chinese population. *Br. J. Clin. Pharmacol.* **49**:141-151.

157. Pablos, M. J., Gonzalez. A. G. F., (1998), Discrimination between arabica and robusta green coffee varieties according to their chemical composition. *Talanta.* **46**:1259–64.

158. Patten, C. J., Thomas, P.E., Guy, R.L,, Lee, M., Gonzalez, F.J., Guengerich, F.P., (1993), Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem. Res. Toxicol.* **6**:511-518.

159. *Pelletier, M. J., (1826), Note sur la caféine. *J. Pharm*. **12**: 229-233.

160. Persson, C. G. A., (1985), On the medical history of xanthines and other remedies for asthma: a tribute to H. H. Salter. *Thorax* ; **40**:881-6.

161. Petra.V., Pasman, W.J., Vliet, T.V., Urgert, R., Katan, M.B., (2002), Contribution of caffeine to the homocysteine-raising effect of coffee: a randomized controlled trial in humans, *Amer. J. Clin. Nut*. **76 (6):** 1244-1248.

162. Pintauro, N.D., (1975), Coffee Solubilization: Commercial Process and Techniques. Park Ridge: Noyes Data Corporation.

163. Pirmohamed, M., Williams, D., Madden, S., Templeton, E., Park, B. K., (1995), Metabolism and bioactivation of clozapine by human liver in vitro. *J. Pharmacol. Exp. Ther.* **272:** 984-990.

164. Pizziol, V., Tikhonoff, C.D., Paleari, (1998), Effects of Caffeine on Glucose Tolerance: A Placebo Controlled Study, *European J. Clin. Nut.* **52:** 846-849.

165. Porres, C., Alvarez, D., Calzada, J,. (1993), Caffeine reduction in coffee pulp through silage. *Biotechnol. Adv*. **11(3):**519–23.

166. Poulos, T. L., Cupp-Vickery, J., Li, H. (1995), *in* "*Cytochrome P450: Structure, Mechanism and Biochemistry*" Ortiz de Montellano, P., Ed., 125–150, Plenum Press, New York.

167. Preusser, E., (Proiser, E.), Serenkov, G. P., (1963*), Biokhimiya* **28**: 857-861.

168. Putrament, A., Baranowska, H., Bilinsky, T., Prazmo, W., (1972), On the specificity of caffeine effects. *Mol. Gen.* **118**:373–9.

169. Ramarethinam, S., Rajalakshmi, N., (2004), Caffeine in tea plants [*Camellia sinensis* (L.) O. Kuntze]: in situ lowering by *Bacillus licheniformis* (Weigmann) Chester. *Indian J. Exp. Biol*. **42(6):**575–80.

170. Rao, K.L., Khanna, H.H., Cornish, (1973), Identification of two new metabolites in rat urine. *Experientia*; **29:**953–5.

171. Relling, M.V., Lin, J.S., Ayers, G. D., Evans, W. E., (1992), Racial and gender differences in N-acetyltransferase, xanthine oxidase, and CYP1A2 activities. *Clin. Pharmacol. Ther*. **52**:643-658.

172. *Robiquet, (1823), Cafe. In: Dictionnarie technologique, Vol.4, (Thomine et Fortic, Paris).

173. Rojas, J. B. U., Verreth, J. A. J., Amato, S., Huisman, E. S., (2003), Biological treatments affect the chemical composition of coffee pulp. *Bioresour. Technol.* **89:**267–74.

174. Rost, K. L., Brosicke, H., Heinemeyer, G., Roots, I., (1994a) Specific and dose dependent enzyme induction by omeprazole in human beings. *Hepatology*. **20**:1204-1212.

175. Rost, K. L., Roots, I., (1994b), Accelerated caffeine metabolism after omeprazole treatment is indicated by urinary metabolite ratios: coincidence with plasma clearance and breath test. *Clin Pharmacol Ther*. **55**:402-411.

176. Rostami, H. A., Nurminen, S., Jackson, P. R., Tucker, G. T., (1996), Caffeine urinary metabolite ratios as markers of enzyme activity: A theoretical assessment. *Pharmacogenetics.* **6:** 121-149.

177. Roussos, S., Angeles, A. M. D. L., Trejo, H. M. D. R., Gaime, P. I., Favela, E., Ramakrishna, M., (1995), Biotechnological management of coffee pulp-isolation, screening, characterization, selection of caffeine degrading fungi and natural microflora present in coffee pulp and husk. *Appl Microbiol Biotechnol.* **42**:756–62.

178. *Runge, F., (1820), Phytochemische Entdeckungen, (Berlin) 204 .

179. Saldana, M. D. A., Mohamed, R. S. and Mazzafera, P., (2000), Supercritical carbon dioxide extraction of methylxanthines from mate tea leaves, *Braz. J. Chem. Engg*. **17 (3):** 251-259.

180. Salmones, D., Mata, G., Waliszewski, K. N., (2005), Comparative culturing of *Pleurotus* spp. on coffee pulp and wheat straw: biomass production and substrate biodegradation. *Bioresour Technol*. **93**:537–44.

181. Sarath Babu, V. R., Thakur, M. S., Karanth, N. G., (2003), A process for the bio-decaffeination of solutions containing caffeine (428/Del/03, Columbia patent applied).

182. Sarath Babu, V. R., Thakur, M. S., Karanth, N. G., (2006a), A bioreactor design for enzymatic decaffeination (Patent Applied).

183. Sarath Babu, V. R., Thakur, M. S., Karanth, N. G., (2006b), A process for enzymatic decaffeination of tea (Patent Applied).

184. Sarath Babu, V. R., Patra, S., Karanth, N. G., Varadaraj, M. C., Thakur M. S., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708, *Enz, Microb. Tech*. **37:** 617-624.

185. Sauer, M., (1982), Comparison of the cytochrome P450 containing monooxygenases originating from two different yeasts. *Dev. Biochem.* **23**:.452-457.

186. Schwimmer, S., Kurtzman, Jr., R. H., Heftmann, E., (1971), Caffeine metabolism by *Penicillium roqueforti*. *Arch Biochem Biophys*; **147**:109–13.

187. Sesardic, D., Boobis, A. R., Edwards, R. J., Davis, D. S., (1988), A form of cytochrome P450 in man, orthologous to form in the rat, catalyse the O-deethylation of phenacetin and is inducible by cigarette smoking. *Br. J. Clin. Pharmacol.* **26**:363-372.

188. Shilo, L., Sabbah, H., Hadari, R., Kovatz, S., Weinberg, U., Dolev, S., (2002), The effects of coffee consumption on sleep and melatonin sectretion. *Sleep Med*. **3**:271–3.

189. Shimada, T., Martin, M. V., Pruess, S., D., Marnett, L. J., Guengerich, F. P., (1989), Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res*. **49:** 6304-6312.

190. Shirley, K. L., Hon, Y. Y., Penzak, S. R., Lam, Y. W. F., Spratlin, V., Jann, M. W., (2003), Correlation of cytochrome P450 (CYP) 1A2 activity using caffeine phenotyping and olanzapine disposition in healthy volunteers, *Neuropsychopharmacology*, **28:** 961-966.

191. Shirlow, M. J., Mathers, C. D., (1985), A study of caffeine consumption and symptoms; indigestion, palpitations, tremor, headache and insomnia. *Int. J. Epidemiol*. **14(2):**239-48.

192. Sideso, O. F. P., Marvier, A. C., Katerelos, N. A., Goodenough, P. W., (2001), The characteristics and stabilization of a caffeine demethylase enzyme complex. *Int. J. Food Sci. Technol.* **36:**693–8.

193. Silva, C. F., Scwan, R. F., Dias, E. S., Wheals, A. E., (2000), Microbial diversity during maturation and natural processing of coffee cheries of *Coffea arabica* in Brazil. *Int. J. Food Microbiol*. **60:** 251-260.

194. Silvarolla, M. B., Mazzafera. P., Fazuol, L. C., (2004), Plant biochemistry: a naturally decaffeinated arabica coffee. *Nature*. **429(6994):**826.

195. Srisuphan, W., Bracken, M. B., (1986), Caffeine consumption during pregnancy and association with late spontaneous abortion. *Am. J. Obstet. Gynecol.* **154**:14–20.

196. *Stenhouse, J., (1843a), Ueber Thein und seine Dartstellung. *Liebig´s Ann. Chem*. **45**:366-372.

197. *Stenhouse, J., (1843b), Nachträgliches über das Thein. *Lieglig´s Ann. Chem*. **46**: 227-230.

198. Strecker, R. E., Morairty. S., Thakkar, M. M., Porkka, H. T., Basheer, R., Dauphin, L. J., Rainnie, D. G., Portas, C. M., Greene, R.W., McCarley, R.W., (2000), Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state? *Behavior Brain Res.*, **115:** 183-204.

199. Sundarraj, C. V., Dhala, S., (1965), Effect of naturally occurring xanthines on bacteria (I). Antimicrobial action and potentiating effect on antibiotic spectra. *Appl. Microbiol*. **13**:432–6.

200. Suzuki, T., Takahasi, E., (1975), Biosynthesis of caffeine by tea leaf extracts, enzyme formation of theobromine from 7-methyl xanthine and of caffeine from theobromine. *Biochem. J*. **146**:87–96.

201. Tagliari, C. V., Sanson, R. K., Zanette, A., Franco, T.T., Soccol, C. R., (2003), Caffeine degradation by *Rhizopus delemar* in packed bed column bioreactor using coffee husk as substrate. *Braz. J. Microbiol.* **34**:102–4.

202. Tantcheva, P. I., Zaigler, M., Rietbrock, S., Fuhr, U., (1999), Estimation of cytochrome P450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine test. *Pharmacogenetics.* **9**:131-144.

203. Tarnopolsky, M. A., (1994), Caffeine and endurance performances. *Sports Med.* **18 (2):** 109 – 125.

204. Tarrus, E., Cami, J., Roberts, D.J., Spickett, R.G., Celdran, E., Segura, J., (1987), Accumulation of caffeine in healthy volunteers treated with furafylline. *Br. J. Clin. Pharmacol.* **23:** 9-18.

205. Thelle, D. S., Arnesen, E., Forde, O. H., (1983), The TromsÃ¸ heart study. Does coffee raise serum cholesterol? *New Eng. J. Med.* **308(24)**:1454-7.

206. Vitoria, A. P., Mazzafera, P., (1998), Caffeine degradation in fruits and leaves of *Coffea arabica* and *Coffeea dewevrei*. *Pesq. Agropec. Bras.* **33**:1957–61.

207. Vogels, G. D., Van der Drift, C., (1976), Degradation of purines and pyrimidines by microorganisms. *Bacteriol*. *Rev*. **40**: 403–468.

208. Wachenfeld, V. C., Jonson, E. F., (1995), Structures of eukaryotic P450s. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd Ed. Plenum Publishing Corp., New York, pp. 183–225.

209. Waller, G. R., (1989), Biochemical frontiers of allelopathy. *Biol. Plant.* **31**: 418–447.

210. Walsh, I., Wasserman, G. S., Mestad, P., Lanman, R. C., (1987), Near-fatal caffeine intoxication treated with peritoneal dialysis". *Pediatr. Emerg. Care* **3(4):** 244-9.

211. Waring, W. S., Goudsmith, J., Marwick, J., Webb, D. J., Maxwell, S. R. J., (2003), Acute caffeine intake influences central more than peripheral blood pressure in young adults. *Am. J. Hypertens.* **16:** 919–24.

212. Wijhe, M. V., (2002), The history of caffeine as used in anaesthesia. *Int. Congr. Ser*. **1242**:101–3.

213. Willaman, J. J., Schubert, B. G., (1961), Alkaloid-bearing plants and their contained alkaloids. *Agric. Res. Serv., U.S. Dept. Agric., Tech. Bull*. **1234:** 1-287.

214. Woolfolk, C. A., Downard, J. S., (1977), Distribution of xanthine oxidase and xanthine dehydrogenase specificity types among bacteria. *J. Bacteriol*. **130**: 1175-1191.

215. Woolfolk, C. A., (1975), Metabolism of N- methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. *J. Bacteriol*. **123**:1088-1106.

216. *Woskresensky, A. (1842) , Ueber das Theobromin. *Liebig´s Ann. Chem*. **41**: 125-127.

217. Wreck, R. D., Feyereisen, R., (2000), Cytochrome P-450 a success story. *Genome Biol*. **6(3003):** 1–9.

218. Xu, Z. H., Zhou, H. H., (1996), Cytochrome P4501A2 in drug metabolism. *Chin. J. Clin. Pharmacol.* **12:**115-121.

219. Yano, Y. D. M., Mazzafera, P., (1998), Degradation of caffeine by *Pseudomonas putida* isolated from soil. *Allel. J.* **5:** 23–34.

220. Yano, Y. D. M., Mazzafera, P., (1999), Catabolism of Caffeine and Purification of xanthine oxidase responsible for methyluric acids production in *Pseudomonas putida* L. *Revista de Microbiologia*. **30:** 62-70.

221. *Yano, Y. D. M., Mazzafera, P., (1996), Descafeinação da palha de café Por bactérias. In: congresso brasileiro de pesquisas, Cafeeiras, 22., águas de lindóia, 1996. *Resumos. Rio de janeiro: Ministério da agricultura e reforma agrarian* .36-39.

222. Yoshimoto, K., Echizen, H., Chiba, K., (1995), Identification of human CYP isoforms involved in the metabolism of propranolol enantiomers- N-desisopropylation is mediated by CYP1A2. *Br. J. Clin. Pharmacol.* **39:**421-431.

223. Yuji, T., Nakahara, H., Kubo, H., Muraki, M., Fukuoka, M., Nakajima, S., (1998), Theophylline suppresses the release of interleukin-4 by peripheral blood mononuclear Cells, *Int. Arch. Aller. Immunol.* **115**:42-46.

224. Ziegler, D. M., (1988), *Drug Metab. Rev.* **19:** 1–32.

225. Ziegler, D. M., (1993), *Ann. Rev. Pharmacol. Toxicol.* **33:**179–199.

226. Zurek, J., Nicolas, F., Jeremy N. H., Adrian J.M., (2006), Mechanisms of reaction in cytochrome P450: hydroxylation of camphor in P450cam, *Org. Biomol. Chem.* **4:** 3931-3937.

*** Original reference not seen.**

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF CAFFEINE DEGRADING BACTERIA

2.0. SCOPE OF THE WORK:

Caffeine (1,3,7-trimethylxanthine) was a molecule discovered by serendipity and has been consumed worldwide since hundreds of years. The coffee plant has evolved a defense mechanism to ward off pests, insects and to prevent competition due to other plants by leaching the compound into the surrounding soil and water. Many microorganisms have evolved mechanisms of surviving the inhibitory effect of caffeine either by developing resistance to caffeine or by developing mechanisms to use caffeine as a source of nitrogen and carbon. Several bacteria, fungi and a few yeasts are known to degrade caffeine and have been growing in coffee and tea plantation soils.

Harnessing the caffeine degrading potential of such organisms is of importance in developing processes for biodecaffeination and production of methylxanthine intermediates which have therapeutic value. This chapter deals with the isolation, identification and characterization of bacteria capable of degrading caffeine. The first section of the chapter presents a survey of literature on the isolation of caffeine degrading microorganisms and the potential of these organisms in developing microbial process for caffeine degradation. The second section of the chapter deals with the methodologies used for the isolation, identification and characterization of caffeine degrading bacteria, optimization of media, and physical conditions for efficient biodegradation of caffeine by the selected isolates and the third section of the chapter deals with studies on caffeine degradation by *Pseudomonas alcaligenes* MTCC 5264, and the optimum conditions for the degradation of caffeine by the isolate.

2.1. INTRODUCTION:

The purine alkaloid caffeine belonging to xanthines or methyl xanthines is found in more than sixty plant species, with significant levels in coffee beans, tea, cocoa, etc (Mazzafera et.al., 1991; Suzuki et al., 1992). Other two important alkaloids of the xanthine derivative group are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) and are found in coco beans, tea leaves, mate leaves, cola nuts and guarana seeds.

2.1.1. Caffeine and microorganisms:

Caffeine production by plants is known to be an evolutionary adaptation to ward off predators, insects and microorganisms. Caffeine found in plants at doses found in *C. arabica* is toxic to a variety of insects and fungi (Baumann and Gabriel, 1984; Nathanson, 1984; Frischknecht et al. 1986). Caffeine is known to be powerfully allelopathic to many plants, insects and microorganisms. Caffeine is known to inhibit the growth of plants and bacteria near the germinating seeds. Friedman and Waller (1983a) showed that caffeine inhibits mitosis in the roots of many plants and thereby reduces access to nutrients.

Since caffeine reduces root growth and kills most bacteria, it effectively kills off any nearby competition for vital nutrients as well as protects itself from being consumed by microbes (Friedman and Waller, 1983b). Caffeine's solubility in water provides for simple transport into the nearby soil where inhibition of nearby organisms will occur. High concentrations are required for bactericide action, however, some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid (Sundarraj and

Dhala, 1965). Actually, it is not rare to find bacterial strains resistant to caffeine (Woolfolk, 1975).

The oxidation of purine forms xanthine which is the parent molecule for the methylxanthines. There are three pyrrole type nitrogens which can be *N*-methylated to form methylxanthines. If all three available pyrrole type nitrogen atoms are methylated, the product is caffeine.

Several studies are reported in literature on the use of purines, including caffeine, as a source of energy for microorganism growth (Mazzafera et. al., 1994a; Middelhoven and Bakker, 1982; Schwimmer et. al., 1971; Woolfolk, 1975; Woolfolk and Downard, 1977). A comprehensive review on purine utilization by microorganisms was published by Vogels and Van der Drift (1976). Although fungi growing on caffeine have been isolated, most of the studies were done with bacteria isolated from soil, mainly those belonging to the *Pseudomonads* group, with particular attention to *Pseudomonas putida* (Burr and Caesar, 1985).

Woolfolk (1975) used a *Pseudomonas fluorescens* strain with ability to grow on caffeine to demonstrate dehydrogenase activity against both monomethylxanthines. Blecher and Lingens (1977) studied degradation of caffeine by *P. putida* strains isolated from soil. They identified 14 catabolites: theobromine, paraxanthine, 7-monomethylxanthine, xanthine, 3,7-dimethyluric acid, 1,7-dimethyluric acid, 7-methyluric acid, uric acid, allantoin, allantoic acid, ureidoglycolic acid, glyoxylic acid, urea and formaldehyde.

Mazzafera et. al., (1994b) isolated a strain of *Serratia marcescens* from coffee plantation soil having the capability to degrade caffeine. They observed that

demethylation of caffeine was the rate limiting step in the degradation of caffeine by the microorganism. They found that caffeine was degraded to paraxanthine and/or theobromine, and subsequently to 7-monomethylxanthine and xanthine.

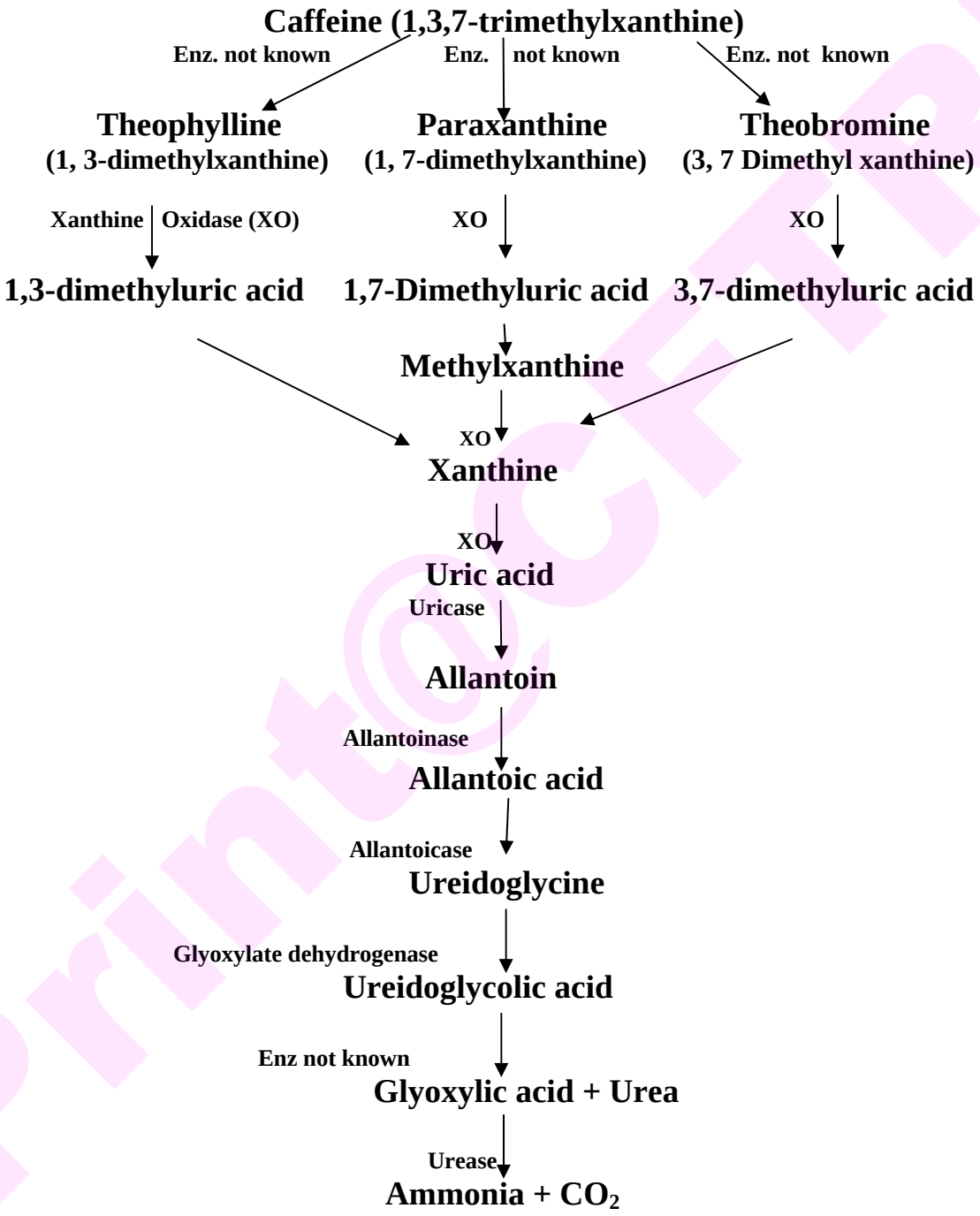
Most studies on caffeine degradation by *Pseudomonas* use bacterial strains obtained through a procedure known as enrichment. Caffeine was added to the soil (Woolfolk, 1975) or culture medium (Blecher and Lingens, 1977; Hohnloser et. al., 1980; Middlhoven and Lommen, 1984) to induce the appearance of mutants. In the case of soil enrichment, caffeine was mixed to the soil and incubated for several months. In the second case, using artificial media, caffeine was added in low concentrations and the bacteria sub-cultured several times until mutants were obtained. After that, bacteria were maintained in media containing caffeine as the sole source of carbon. Gluck and Lingens (1987) isolated a *P. putida* strain by culturing the bacteria with 20 $g.L^{-1}$ of caffeine as the sole source of carbon and nitrogen. Blecher and Lingens (1977) reported the growth of a *P. putida* strain in media containing up to 50 $g.L^{-1}$ caffeine in the culture media. Middelhoven and Bakker (1982) grew the strain C32024 of *P. putida* at 20 $g.L^{-1}$ of caffeine. However, Mazzafera et. al., (1994b) and Yano and Mazzafera (1998) used a different approach, and collected mutants in soil samples taken under coffee plants. Water was added to the soil samples and after shaking for a few hours, aliquots were plated in solid medium containing caffeine as the sole source of carbon and nitrogen. In the first case, they isolated a *S. marcescens* strain (Mazzafera et. al., 1994b) and in the second (Yano and Mazzafera, 1998), several *P. putida* strains and other bacteria.

A strain of *P. putida* isolated by Yano and Mazzafera (1998) showed an impressive ability to grow in high concentrations of caffeine. Growth was observed at 25 $g.L^{-1}$ in liquid medium and at 50 $g.L^{-1}$ in solid medium. Yano and Mazzafera (1999) studied the caffeine degradation pathway in this *P. putida* strain, and in agreement with results previously obtained by Blecher and Lingens (1977), suggested the degradation pathway showed in Figure 2.1.1.

Blecher and Lingens (1977) and Gluck and Lingens (1987) isolated *P. putida* mutants with the ability to degrade caffeine to theobromine and attempted to block the degradation of theobromine for commercial application. In contrast to these authors, Asano et. al., (1993) were successful in isolating a *P. putida* strain where the route of caffeine degradation could be blocked by addition of Zn to the culture medium, accumulating theobromine.

Through inhibition of DNA repair in bacteria it has been shown that caffeine can be mutagenic (Grigg, 1972; Kihlman, 1974). Caffeine at 0.1% concentration also reversibly inhibits protein synthesis in bacteria and yeast. This effect is post translational since caffeine does not affect RNA translation (Putrament et. al., 1972). Sauer (1982) obtained indications that caffeine in yeast was degraded by cytochrome P450, suggesting that the catabolic pathway might be similar to animals. In humans, several cytochrome P-450 isoforms are responsible for caffeine degradation (Berthou et. al., 1992). Schwimmer et. al., (1971), reported that the degradation of caffeine to theophylline in fungi, and Blecher and Lingens (1977), who studied degradation of caffeine to theobromine in bacteria, do not indicate participation of P-450 on caffeine degradation mechanism.

Figure 2.1.1. Caffeine degradation pathway in microorganisms.



Boccas et. al., (1994) isolated 248 fungal cultures from coffee plants and soil collected from plantation areas. Roussos et. al., (1995) isolated 272 strains of

filamentous fungi from soil, fruits and leaves using a culture media containing coffee extract, coffee extract plus sucrose, and coffee pulp extract. The fungi strains with the highest ability to degrade caffeine were identified as *Aspergillus* and *Penicillium*. Yano and Mazzafera (1998) isolated more than 20 bacteria strains from soil collected under coffee plants, observing predominance of *Pseudomonas* sp., which was also the most efficient caffeine degrader. Silva et. al., (2000) studied the diversity of microbial populations during the maturation and natural processing (sun-dried) of coffee fruits during two consecutive years. A total of 754 isolates of bacteria, yeast and fungi were obtained. Bacteria were the predominant microorganisms. Fermentative bacteria and yeast, cellulolytic bacteria, and pectinolytic bacteria, yeast and filamentous fungi were identified among 626 microorganisms. Porres et. al., (1993) have studied the ensilation of coffee pulp and the effect of addition of sugar cane molasses on caffeine degradation. In the case of treatment with 5% molasses 63% caffeine was degraded in the pulp and in the absence of the treatment only 13% of the caffeine was degraded. Reduction in caffeine from 13-63% was observed, with the highest reduction in the treatment with 5% of molasses. In the case of compressed silage very low caffeine degradation was observed. Coffee pulp is considered to be the major polluting agent of rivers and lakes located near the coffee-processing regions (Roussos et. al., 1995). For environmental protection and economic gain, attempts have been made in the past to utilize coffee pulp, but were not successful due to the presence of anti-physiological factors such as caffeine, tannins, chlorogenic acid and high levels of potassium (Bressani 1987a, 1987b). Work has been carried out in our laboratory at CFTRI on the biodecaffeination of

coffee processing wastes such as coffee pulp, coffee hulls and spent coffee. Caffeine could be completely degraded in these coffee wastes and edible mushrooms could be grown on these substrates with high protein and free amino acid content. The coffee processing wastes were also used for the production of biofertilizers through vermicomposting and bioferttilizer from these wastes was found to have high NPK and organic carbon content. These wastes after the growth of mushrooms and earthworms were found to contain less antinutritional factors like tannins.

Accurate knowledge about the nature and characteristics of coffee pulp natural microflora is of utmost importance for its further utilization in the development of biodecaffeination process for caffeine containing materials and in the isolation and identification of potent caffeine degrading strains.

The consumption of decaffeinated products like coffee, tea and chocolate is increasing world over. Several processes based on organic solvents and super critical carbon dioxide have been developed for the decaffeination of coffee and tea. These methods are either unsafe to the human health and environment or too expensive. The need for safe and eco-friendly decaffeination processes has given rise to the novel concept of Biodecaffeination, where enzymes are employed for the decaffeination. These processes are safe, preserve the quality of the decaffeinated products and are eco friendly. The development of biodecaffeination processes necessitates the isolation of enzymes capable of degrading caffeine and its metabolites from microorganisms capable of degrading caffeine. Several microorganisms present in the vicinity of the caffeine containing plants like coffee and tea have developed mechanisms to overcome the toxic effects of caffeine by

producing enzymes responsible for degrading caffeine. Screening for microorganisms and selection of potent biodecaffeinating microorganisms is important to develop processes for biodecaffeination. The optimum conditions for the growth and production of biodecaffeinating enzymes by the selected isolates have to be determined. This chapter reports the isolation, purification and characterization of caffeine degrading bacteria from coffee plantation soils. Studies on the degradation of caffeine by the organisms and the selection of a caffeine degrader are also reported. This chapter also describes the optimization of media and physical conditions for the production of biodecaffeinating enzymes by the selected isolate.

2.2. MATERIALS AND METHODS:

Caffeine, theobromine, paraxanthine and methyl xanthines were purchased from Sigma Chemicals, St. Louis, USA. Nutrient broth, nutrient agar, citrate agar, peptone, yeast extract, and other chemicals for identification of bacterial isolates were procured from Hi Media labs, Mumbai, India. Sodium dihydrogen orthophosphate, di sodium hydrogen ortho phosphate, ferric chloride, zinc sulphate, sodium molybdate, manganese tetra oxide, magnesium oxide, sodium hydroxide, hydrochloric acid and other chemicals were of analytical grade and procured from Qualigenes Fine Chemicals, Mumbai. HPLC grade acetonitrile and methanol were procured from Merck, Germany. All other chemicals were of the highest purity and were procured from standard sources.

2.2.1. Isolation of caffeine degrading bacteria from soil:

Soil was collected under tea plants growing at the tea plantations at Gudalur, Udhagamandalam District, Tamilnadu and from Chamundi Coffee Curing Works, Mysore, Karnataka. Leaves and debris were removed from the soil surface, and the topmost 15-cm layer was collected. A medium designated as M9 (Table 2.2.7.1) after modification with supplementation of 0.3 $g.L^{-1}$ caffeine was used for screening. For enrichment of isolates capable of degrading caffeine as well as for induction of the organism, the following medium designated as Caffeine Liquid Medium (CLM) was used which contained the following constituents in ($g.L^{-1}$) $Na_2HPO_4.7H_2O$, 6.4; KH_2PO_4, 1.5; NaCl, 0.25; NH_4Cl, 0.5, and Caffeine, 0.3. The pH of the medium was adjusted to 7.2 with 0.1N NaOH. CLM was used as the growth medium with caffeine as the sole source of carbon and nitrogen.

In the laboratory 1 g of soil was mixed with 50 ml of CLM in a 250-ml flask and agitated for 72 hrs (150 rpm), at room temperature (28±2°C). Caffeine agar medium was prepared by addition of 2.5% agar to CLM and poured into petri plates. Serial dilutions (10^{-1} to 10^{-9}) of the above grown culture were then made and 0.1 ml of the same was inoculated on to each petri plate and incubated at 37^{o}C for 2 days. Isolated single colonies growing on the above plates were purified further to obtain pure cultures. The caffeine degrading isolates were initially designated as T1, T2 and T3. These purified cultures were inoculated on to fresh slants of caffeine agar medium with subsequent sub culturing.

Single colonies were transferred to new caffeine agar plates, and colonies growing on these plates were transferred once more to a new set of plates.

2.2.2. Identification of bacteria:

The bacterial isolates were characterized by biochemical tests according to Bergey's manual of determinative bacteriology. The following tests were conducted for the identification of the bacterial isolates.

Catalase, oxidase, nitrate reduction, indole production, methyl red test, voges proskauer test, starch hydrolysis, citrate utilization, oxidation, fermentation, motility, malonate utilization, arginine dihydrolase, gelatin hydrolysis, growth at 41^{0}C, growth at 4^{0}C, acid slant, acid butt, alkaline slant, gas production, urease, dextrose utilization, mannitol utilization and polyhydroxy alkanoate (PHA) accumulation.

The results of these tests were reported as either positive or negative and identification was based on these tests.

2.2.3. Studies on bacterial growth in caffeine as the sole source of carbon and nitrogen:

The ability of the bacteria to grow in liquid medium containing caffeine was tested. Pure, isolated single colonies from caffeine containing plates were used as inoculum. Bacterial growth was also investigated in caffeine at 1000, 2000, 4000, and 5000 $mg.L^{-1}$. Three replicates were made for each concentration. Bacterial growth was followed by measuring the optical density at 600 nm in 1-ml aliquots taken from the flasks. These samples were centrifuged at 14,000 rpm in a bench-top centrifuge, and the supernatant was stored at -21°C until analysis for methylxanthine concentrations.

2.2.4. Estimation of methylxanthines by high performance liquid chromatography (HPLC):

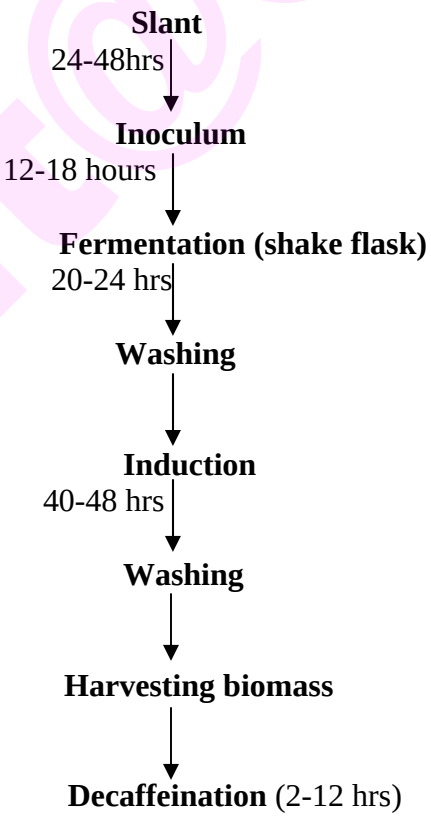
HPLC analysis of caffeine was performed in a Shimadzu LC 10 A- HPLC system, and the methyl xanthine compounds were separated on a C18 ODS-Luna column under isocratic conditions with 15 % acetonitrile in water at a flow rate of 1.0 ml/min. Compounds eluting from the column were detected at 273 nm, and the peak areas were compared with those obtained with standards of known concentration.

2.2.5. Growth of cells and induction for caffeine degradation:

A loop full of actively growing culture of the isolate from the culture slant was transferred to 100ml of nutrient broth containing 0.3 $g.L^{-1}$ caffeine and incubated at $30^{o}C$ in an orbital shaker for 24 hrs. A 5% v/v of the 24 hrs grown pre inoculum was transferred to 100ml of nutrient broth containing 0.3 $g.L^{-1}$ caffeine and grown under the same conditions.

Samples were drawn at known intervals of time for the measurement of cell growth. Biomass accumulated after 24 hours was harvested by centrifugation in a bench top centrifuge (Kubota, Japan) at 16,000g for 20 min at 0-4^{0}C to form a pellet. The biomass pellet was aseptically transferred into a 500ml flask containing 100ml of CLM containing 1g.L^{-1} caffeine and incubated at 30^{o}C on the orbital shaker for a period of 48hrs for inducing the cells to degrade caffeine. These induced cells were harvested by centrifugation as before. The cells were washed several times to remove caffeine. 10 gms of these induced cells were suspended in 100 ml of phosphate buffer containing caffeine, which were used for caffeine degradation experiments. The scheme of preparation of cells is given below.

Scheme of preparation of bacterial cells for biodecaffeination:



2.2.6. Caffeine degradation experiments:

10 ml of the induced cell suspension was aseptically transferred to 90ml of CLM containing 1g.L^{-1} caffeine and incubated on a rotary shaker at 30^{o}C for 24 hours. 0.5 ml aliquot samples were drawn at 2, 4, 6 and 24 hours, centrifuged and the supernatants were analyzed for caffeine content. Studies on the effect of pH and temperature on caffeine degradation by the isolate were carried out by incubating the induced cells in CLM containing 1g.L^{-1} caffeine, which was adjusted to different pH and temperature.

For studying the effect of inoculum on caffeine degradation, induced cell suspension was added to CLM containing 1g.L^{-1} caffeine so as to obtain inoculum levels of 1.2, 2.5, 5.0 and 7.5%w/v (based on wet weight of cells). Studies on the effect of caffeine concentration on the efficiency were carried out by incubating the induced cells in CLM containing caffeine in the concentration range of 1-5 g.L^{-1}. Samples were drawn at known intervals of time and analyzed for caffeine content and calculations of the residual caffeine content were made on the basis of the final volume after drawing the sample. Percentage caffeine degradation was calculated as follows:

% Caffeine degradation = (a- b) x 100/a ---------- Eqn.1

where**, a** = initial caffeine concentration, and

b = residual caffeine concentration

2.2.7. Optimization of parameters for growth and caffeine degradation by isolate T2:

The development of Biodecaffeination processes demands high enzymatic activity expressed by the organism during the course of the fermentation. So,

selection of a suitable media, with carbon, nitrogen and other nutrient sources, optimum conditions like pH, temperature, inoculum age, volume etc need to be determined for maximum enzyme production in the cells. Therefore studies on the optimization of conditions for growth and decaffeination by the organism were carried out.

2.2.7.1. Screening of media:

The following 21 different media were initially screened for the growth and caffeine degrading efficiency by the isolate *P. alcaligenes* MTCC 5264.

The composition of media is given in Table 2.2.7.1, below ($g.L^{-1}$). The pH of all the media was adjusted to 7.0, and sterilized at 121^0C for 15 min at 15 PSI, cooled and inoculated with a loop full of actively growing culture of *P. alcaligenes* MTCC 5264.

Table 2.2.7.1. Composition of media screened for caffeine degradation and growth of *P. alcaligenes* MTCC 5264.

Media Composition ($g.L^{-1}$), pH 7.0			
M1. Nutrient Broth		**M2. Czepek-Dox broth**	
Peptones	5.0	Sucrose	30.0
Yeast extract	3.0	$NaNO_3$	3.0
Sodium chloride	6.0	K_2HPO_4	1.0
Beef Extract	15.0	$MgSO_4.7H_2O$	0.5
		KCl	0.5
		$FeSO_4.7H_2O$	0.01
M 3. Defined Medium		**M4. O-Brien Synthetic media**	
Sucrose	10.0	Glucose	20.0
K_2HPO_4	2.5	Glycine	2.6
KH_2PO_4	2.5	Sodium acetate	1.36
$(NH_4)_2HPO_4$	1.0	$(NH_4)_2SO_4$	0.54
$MgSO_4$ $7H_2O$	0.20	$K_2HPO_4.3H_2O$	0.05
$FeSO_4$ $7H_2O$	0.01	$ZnSO_4.7H_2O$	0.03
$MnSO_4$ H_2O	0.007	$FeSO_4.7H_2O$	0.025
		$CuSO_4.5H_2O$	0.016
		$MnSO_4.4H_2O$	0.012
		$CaCl_2$. $2H_2O$	0.05
		$MgSO_4.7H_2O$	0.05

M 5. Glucose- NH_4SO_4 Medium :		**M6. Dulaney's medium**	
Glucose	50.0	Glucose	10.0
$Na_2HPO_4.12\ H_2O$	16.0	NaCl	5.0
KH_2PO_4	2.96	K_2HPO_4	2.0
NH_4SO_4	5.0	$MgSO_4.7H_2O$	0.4
$MgSO_4$	1.0	$CaCl_2$	0.4
$FeSO_4$	0.0002	$FeSO_4.7H_2O$	0.02
		$ZnSO_4.7H_2O$	0.01
		$(NH_4)_2HPO_4$	4.0
M 7. Complex organic media		**M8. Hobb's medium**	
Glucose	25.0	Glucose	20.0
Yeast extract	3.0	NaCl	5.0
$(NH_4)_2SO_4$	2.0	Na_2SO_4	5.0
$CaCO_3$	2.0	$NaNO_3$	4.5
NaCl	2.0	K_2HPO_4	1.2
KH_2PO_4		Trisbase	1.2
		$MgSO_4.7H_2O$	1.0
		$ZnSO_4$	0.01
M 9. Gause Mineral Salt Medium		**M 10. Lindenberg Synthetic Media**	
Starch	20.0	Glycerol	30.0
KNO_3	1.0	$NaNO_3$	2.0
K_2HPO_4	0.5	K_2HPO_4	1.0
$MgSO_4$ x $7H_2O$	0.5	$MgSO_4.7H_2O$	0.5
NaCl	0.5	$FeSO_4.7H_2O$	0.4
$FeSO_4$	0.01		
M11. Tryptone Commercial Sugar medium		**M12. Tryptone Yeast Extract Medium:**	
Tryptone	1.5	Tryptone	1.5
Yeast extract	3.0	Yeast extract	3.0
Sucrose	10.0	Peptone	6.0
KH_2PO_4	1.5	Glucose	1.0
M 13. Production media II:		**M14. Production media III**	
Soyabean Meal	20.0	Yeast extract	4.0
$(NH_4)_2SO_4$	5.0	Glucose	20.0
Meat Extract	4.0	Peptone	4.0
Yeast Extract	2.5	$(NH_4)_2SO_4$	5.0
Glucose	6.0		
KCl	4.0		
$CaCO_3$	0.1		
K_2HPO_4	0.1		

M 15. Synthetic Medium II:		**M16. Synthetic medium III**	
Fructose	5.0	D-glucose	12.0
NH_4Cl	0.52	yeast extract	1.5
KH_2PO_4	0.28	malt extract	1.5
$MgSO_4$ $7H_2O$	0.25	peptone	2.5
$CaCl_2$ $2H_2O$	0.07	$(NH_4)_2$ HPO_4	1.5
		K_2 HPO_4	2.5
		$MgSO_4$	0.05
M 17. Growth Medium		**M18. MS Medium**	
Sucrose	20.0	KH_2PO_4	2.3
Yeast Extract	2.6	$NaHPO_4$.$2H_2O$	2.9
Peptone	3.00	NH_4Cl	1.0
$(NH_4)_2SO_4$	1.54	$MgSO_4$.$7H_2O$	0.005
$FeSO_4$.$7H_2O$	0.03	$NaHCO_3$	0.005
$CuSO_4$.$5H_2O$	0.5	Fe(NH_4) citrate	0.05
K_2HPO_4	0.5	Trace element solution	5.0 ml
M 19. Xanthine MS Medium		**M 20. M9-medium**	
Xanthine	1.0	Na_2HPO_4	6.0
KH_2PO_4	2.3	KH_2PO_4	3.0
$NaHPO_4$. $2H_2O$	2.9	NaCl	0.5
NH_4Cl	1.0	NH_4Cl	1.0
$MgSO_4$. $7H_2O$	0.005		
$NaHCO_3$	0.005		
Fe(NH_4) citrate	0.05		
Trace element solution	5.0 ml		

M 21. Trypticase Soy Broth

Trypticase peptone	17.0
Phytone peptone	3.0
NaCl	5.0
K_2HPO_4	2.5
Glucose	2.5

All the above media were modified by the addition of 1.0 $g.L^{-1}$ of caffeine. A loop full of the actively growing culture of the bacteria was inoculated in to the media and incubated on a shaker at 150rpm at a temperature of 30±2^oC for 96 hours. Samples were drawn at 12 hours intervals and the growth was recorded as an increase in the absorbance of the medium at 600nm. The sample was then centrifuged, and the

pellet was dried on a Whatman filter paper (No.1) in an oven at 60^{o}C for 8 hrs and the dry weight was recorded. Caffeine degradation was followed by HPLC analysis of the residual caffeine present in the medium.

2.2.7.2. Effect of carbon source on caffeine degradation isolate T2:

Carbon sources are required for the basic metabolic activities, growth and modulating the organisms' metabolic activities either enabling them to use a substrate such as caffeine or regulating the uptake of the substrate itself. Therefore careful selection of a suitable carbon source is very important in the development of a bioprocess like decaffeination. Fructose, sucrose, glucose, inulin, sorbose, cellobiose, raffinose and starch were checked for their suitability as the carbon sources for growth as well as caffeine degradation by the organism. The carbon sources were added individually at 10g.L^{-1} concentrations into caffeine liquid medium, which served as the basal medium for the studies. The media was sterilized and a loopfull of actively growing culture of *P. alcaligenes* MTCC 5264 was inoculated into the medium and incubated on a shaker at 150rpm at a temperature of 30± 2^{o}C for 96 hours. Samples were drawn at 12 hours intervals and the growth was recorded as an increase in the biomass by weight. Caffeine degradation was followed by HPLC analysis of the residual caffeine present in the medium.

2.2.7.3. Effect of nitrogen source on caffeine degradation isolate T2:

The various organic nitrogen sources tried were yeast extract, tryptone, peptone and beef extract. The inorganic nitrogen sources tried were ammonium nitrate, ammonium sulphate and urea. The nitrogen sources were added individually at 10 g.L^{-1} concentrations into caffeine liquid medium, which served as the basal

medium for the studies. The media was sterilized and a loop full of actively growing culture of *P. alcaligenes* MTCC 5264 was inoculated into the medium and incubated on a shaker at 150rpm at a temperature of 30± 2^{o}C for 96 hours. Samples were drawn at 12 hours intervals and the growth was recorded as an increase in the biomass by weight. Caffeine degradation was followed by HPLC analysis of the residual caffeine in the medium. The optimum concentration of each of the suitable nitrogen source was also determined.

2.2.7.4. Effect of pH on caffeine degradation isolate T2:

pH plays an important role on the growth and activity of different enzymes required for caffeine degradation. The optimization studies were carried out with respect to the maximum rate of caffeine degradation where biomass growth and caffeine utilization were maximum. 100 ml of the CLM was adjusted to pH in the range of 4.0-9.0 in 500ml Erlenmeyer flasks was inoculated with a loop full of actively growing culture of *P. alcaligenes* MTCC 5264 and incubated at 30^{o}C for 96 hrs on an incubator shaker at 150 rpm. The growth and caffeine degradation were recorded as increase in the biomass by weight and residual caffeine analysis of the samples drawn at 12 hr intervals by HPLC.

2.2.7.5. Effect of temperature on caffeine degradation by isolate T2:

Temperature is an important parameter for achieving efficient biodecaffeination and has a drastic effect on the activities of the enzymes involved in biodecaffeination. 100 ml of the CLM was adjusted to pH 7.0, was inoculated with a loop full of actively growing culture of *P. alcaligenes* MTCC 5264 and incubated at different temperatures in the range of 20-60^{o}C for 96 hrs on an incubator shaker at

150 rpm. The growth and caffeine degradation were recorded as increase in the biomass by weight and residual caffeine analysis of the samples drawn at 12 hr intervals by HPLC.

2.2.7.6. Effect of caffeine concentration on caffeine degradation by isolate T2:

Caffeine is known to be toxic to micro-organisms at high concentrations and the efficient biodecaffeination demands the selection of a threshold level of caffeine which the organisms can tolerate and degrade efficiently. Therefore the optimum caffeine concentration for biodecaffeination was checked by incubating the organism in CLM containing caffeine in the concentration range of 0.5 $g.L^{-1}$ to 5 $g.L^{-1}$ and recording the growth and caffeine degradation according to the above experiments.

2.2.7.7. Effect of inoculum volume on caffeine degradation by isolate T2:

Initial Inoculum volume gives a measure of the number of cells at the start of the Biodecaffeination process in the liquid culture. An optimum initial cell concentration is essential for overcoming the initial lag phase by the organism in CLM. Therefore a 24 hrs old inoculum was prepared and inoculated into fresh CLM flask in the range of 1-10 % v/v, incubated at 30±2 ^{o}C for 96 hours and the growth and caffeine degradation were followed as above.

2.3. RESULTS AND DISCUSSION:

2.3.1. Isolation of caffeine degrading bacteria:

Initially the screening was carried out in nutrient medium containing caffeine at a lower concentration ($0.3g.L^{-1}$). Active growth of bacteria was observed in caffeine plates receiving the soil filtrate. When transferred to a new set of caffeine plates containing $1.0g.L^{-1}$ caffeine, most of the bacterial colonies did not grow, indicating that residues from the soil filtrate were supplying nutrients for growth in the first plating.

Further selection of efficient caffeine degraders was achieved by enrichment technique and only three bacterial strains were found to grow on the caffeine enriched plates indicating that these organisms are resistant to caffeine and have the capability to degrade caffeine.

The three bacterial strains (initially designated as T1, T2 and T3) capable of growing on caffeine agar were selected for decaffeination studies. Based on the biochemical tests and by referring the results with Bergey's manual of determinative Bacteriology, they were identified as *Alcaligenes fecalis* (T1), *Pseudomonas alcaligenes* MTCC 5264 (T2) and *Acetobacter* sps. (T3) (Table 2.3.1.1).

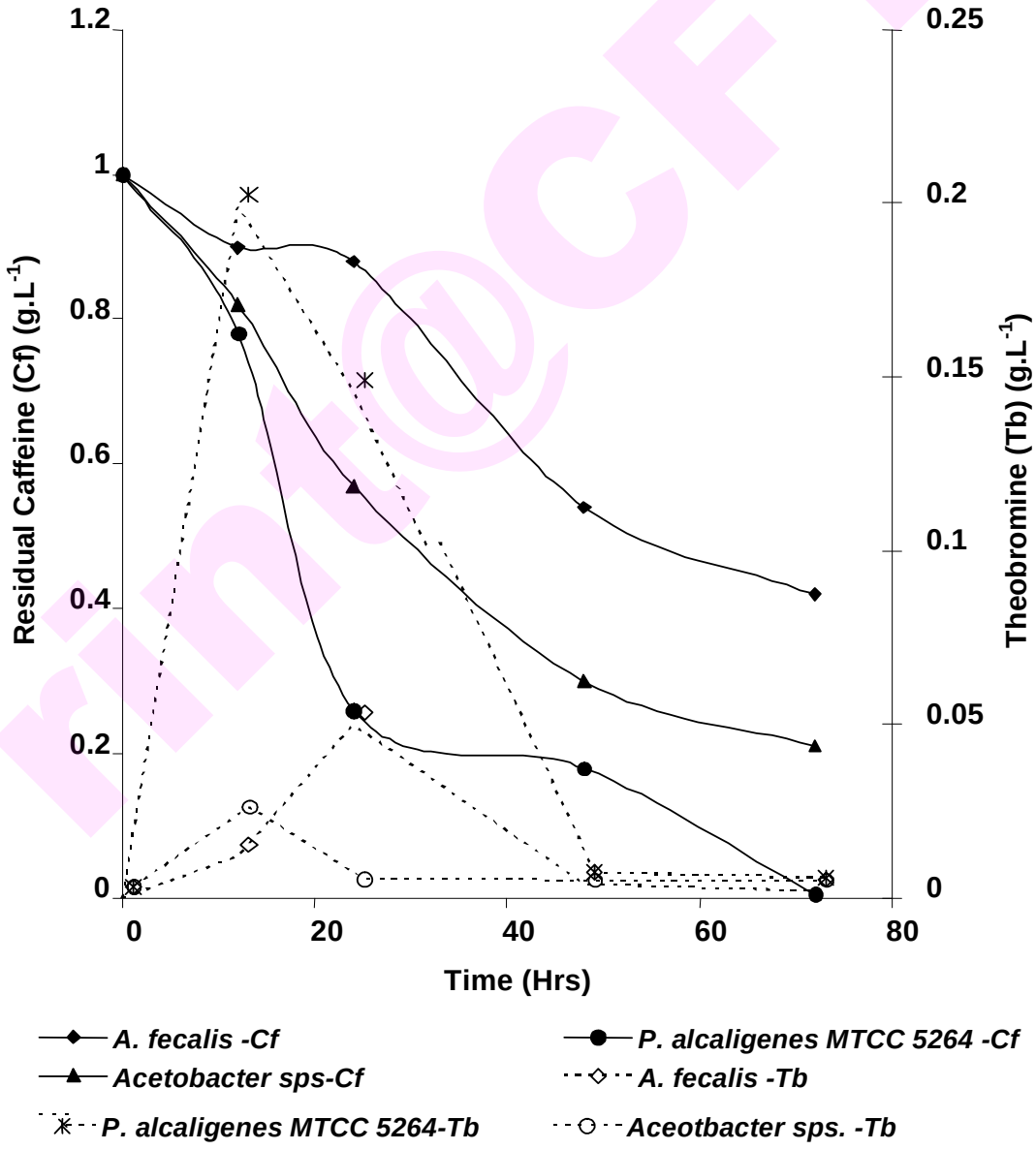
Table 2.3.1.1. Characterization of caffeine degrading isolates.

Biochemical Test	T1	T2	T3
Catalase	+	+	+
Oxidase	-	+	+
Nitrate reduction	-	+	-
Indole Production	-	-	-
Methyl red test	-	-	-
Voges Proskauer test	-	-	-
Starch hydrolysis	-	-	-
Citrate utilization	+	+	+
Oxidation	+	+	+
Fermentation	+	-	-
Motility	+	+	+
Malonate Utilization	+	-	+
Arginine dihydrolase	+	+	+
Gelatin hydrolysis	-	-	-
Growth at 41^0c	+	+	-
Growth at 4^0c	-	-	-
Acid slant	-	-	-
Acid butt	-	-	-
Alkaline slant	+	+	+
Gas production	-	-	-
Urease	+	+	+
Dextrose utilization	-	-	-
Mannitol utilization	-	-	-
Polyhydroxy alkanoate (PHA) accumulation	-	-	-
Identified as	*Alcaligenes fecalis*	*Pseudomonas alcaligenes*	*Acetobacter* sp.

+ = Positive for Test, - = Negative for test

All the three strains were found to be gram-negative rods. Figure 2.3.1.1., represents the caffeine degradation ability of the three bacterial isolates incubated in a medium containing caffeine. All the three isolates showed an initial delay in degrading caffeine.

Figure 2.3.1.1. Degradation of caffeine by bacteria isolated from tea plantation soil.



Only 11% of the initial caffeine was degraded by *A. fecalis* within the first 12 hours of incubation, whereas *P. alcaligenes* MTCC 5264 and *Acetobacter* sps. degraded 22 and 18% of the initial caffeine within 12 hours of incubation.

In the case of *P. alcaligenes*, the degraded caffeine was found to accumulate as theobromine (199mg.L^{-1}) whereas the corresponding values for the other two strains were below 20mg.L^{-1} within the same time of incubation. The rate of degradation of caffeine as well as theobromine increased with an increase in time of incubation and 74% of the initial caffeine was degraded by *P. alcaligenes* within 24 hrs of incubation. The corresponding caffeine degradation rates in the other two isolates were 12 and 43% for *A. fecalis* and *Acetobacter* sps. respectively. 99% of the initial caffeine was degraded by *P. alcaligenes* within 72 hours of incubation whereas only 40% caffeine was degraded by *A. fecalis* and 79% caffeine was degraded by *Acetobacter* Sp. within the same time.

Of the three isolates *P. alcaligenes* MTCC 5264 *(*strain T2) was found to be the best caffeine degrader and was selected for further studies (Fig.2.3.1.1). Purified culture of *P. alcaligenes* MTCC 5264 growing on caffeine agar plate is shown in Figure 2.3.1.2a., and on Nutrient agar is shown in Figure 2.3.1.2b. The isolate could grow in caffeine agar plates containing 50bg.L^{-1} of caffeine. Zones of clearance are found around the colonies indicating that the organism has utilized the caffeine in the plates.

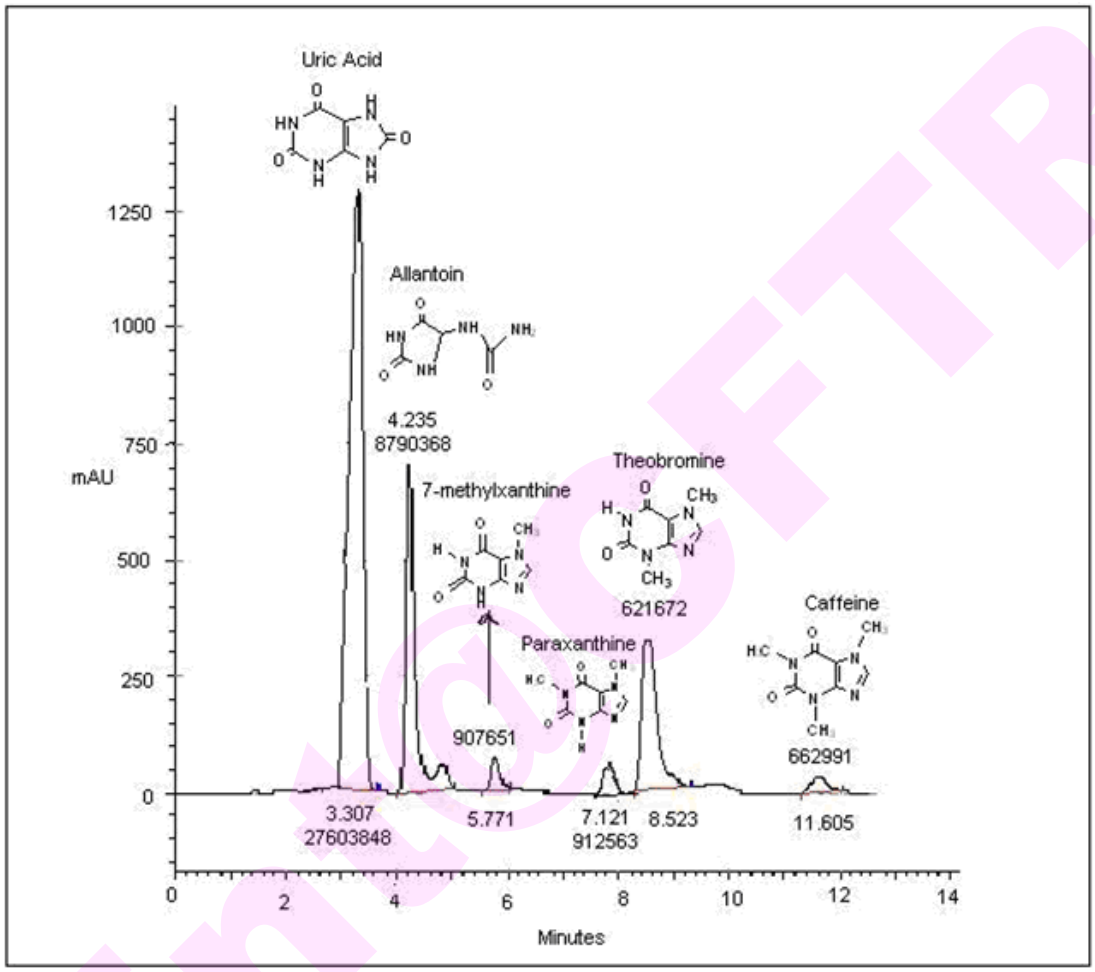
Figure 2.3.1.2 a. Pure culture of *P. alcaligenes* MTCC 5264 growing on caffeine agar plate containing 50 g.L^{-1} of caffeine.

Figure 2.3.1.2 b. Pure culture of *P. alcaligenes* MTCC 5264 growing on nutrient agar plate containing 0.3 g.L^{-1} of caffeine.

Caffeine has been mentioned as a potent allelopathic compound, inhibiting selectively, several fungi and bacteria under coffee trees (Fries and Kihlman, 1948; Waller et al. 1986). Several studies were conducted on the isolation and characterization of microorganisms capable of degrading caffeine (Woolfolk, 1975; Asano et.al., 1993; Roussos et al., 1995). Mazzafera et al (1996) reported the isolation of a strain of *Serratia marcescens* from soil from coffee plantations. However most of the caffeine degrading strains reported till date belong to *Pseudomonas putida* (Bergmann et.al., 1964; Woolfolk, 1975; Asano et.al., 1993; Mazzafera et.al., 1996). There are no reports on the isolation or degradation of caffeine by *Alcaligenes fecalis, Pseudomonas alcaligenes* and *Acetobacter* sps. We have isolated for the first time isolates belonging to the fore mentioned strains. All the caffeine degrading bacteria are reported in literature are known to require long acclimatization times (Asano et. al., 1993; Woolfolk, 1975; Mazzafera et. al., 1994). The bacterial strains isolated in our laboratory required short acclimatization times and induction times for the caffeine degradation activity. The caffeine degradation times in shake flask cultures are also shorter compared to the earlier reports (Asano et al., 1993). HPLC analyses of the fermentation broth showed that catabolites of caffeine and theobromine were released in the medium (Fig. 2.3.1.3), and their reabsorption could explain the continued growth after the disappearance of the original substrates in the medium.

7-Methylxanthine, uric acid and allantoin were detected in CLM (Fig.2.3.1.3). Xanthine and 7-methylxanthine were detected in the medium after 48 hrs. After 72 hrs of incubation, uric acid, allantoin, allantoic acid, urea and ammonia were released into the medium. The formation of ammonia was monitored by Nessler's Reagent.

Figure 2.3.1.3: HPLC analysis of caffeine degradation products produced by *P. alcaligenes* MTCC 5264 after 72 hrs of incubation in CLM.



2.3.2. Optimization of parameters for growth and caffeine degradation by *P. alcaligenes* MTCC 5264:

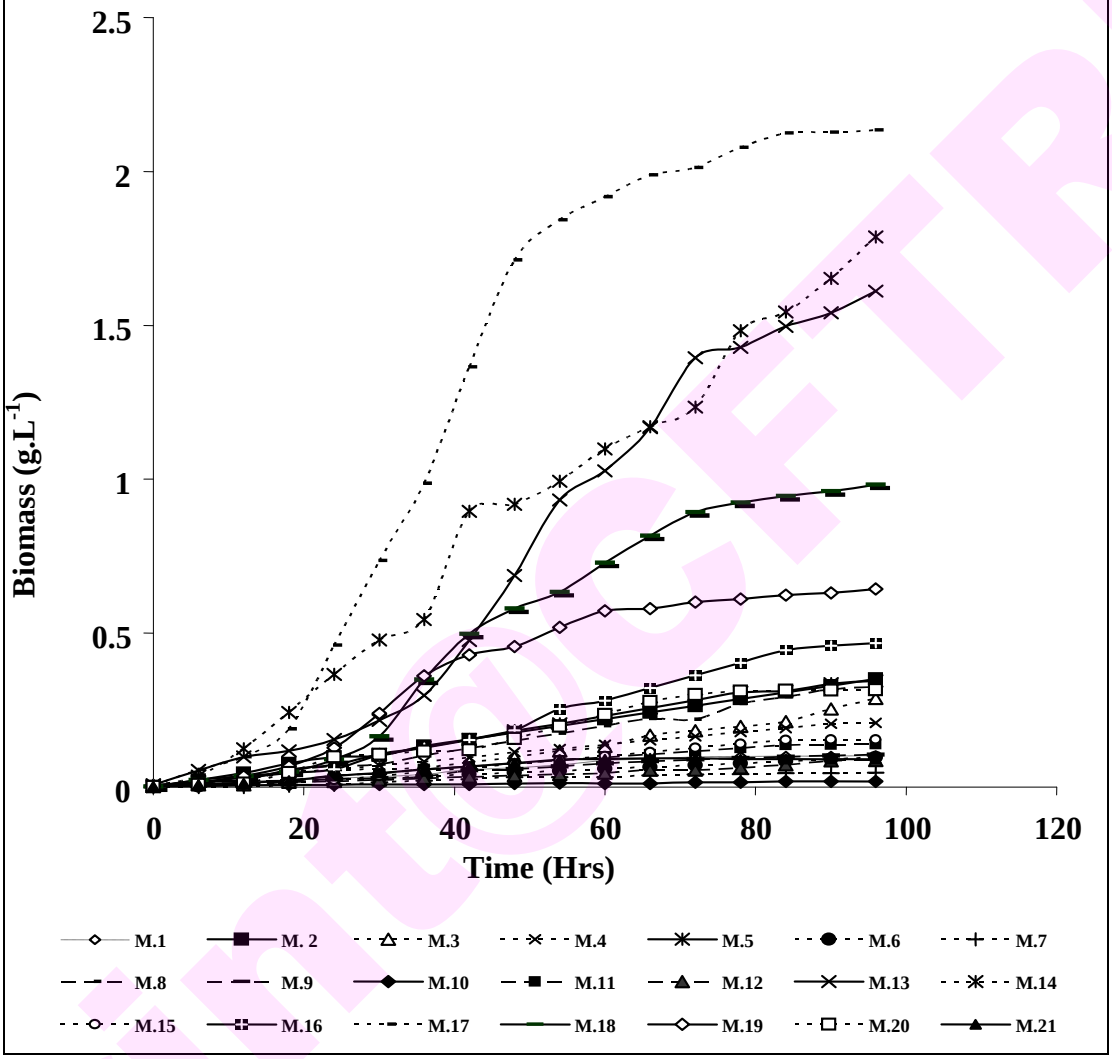
Several parameters for growth and efficient decaffeination by *P. alcaligenes* MTCC 5264 were optimized and the results are as follows:

2.3.2.1. Screening of media:

Microorganisms have specific growth requirements in terms of substrates for growth and utilization of different media components. An initial screening in different complex media was carried out to check for the specific nutritional requirements of

P. alcaligenes MTCC 5264. Therefore 21 different media were initially screened for the growth and caffeine degrading efficiency by *P. alcaligenes* MTCC 5264. Figure 2.3.2.1, represents the growth of *P. alcaligenes* in different media. In Growth medium containing sucrose (M17), maximum biomass accumulation of 2.137 $g.L^{-1}$ was observed. Production media II (M13) and III (M14), MS (M18) medium and Xanthine MS medium (M19) also supported good growth of the organism and the biomass accumulated in these media was 1.611, 1.787, 0.982 and 0.643 $g.L^{-1}$ respectively. Growth was minimum in Complex organic medium, Tryptone Yeast extract medium (M12), Tryptone commercial sugar medium (M11), Guase Mineral Medium (M9), Dulaney's medium (M6), Lindenberg Synthetic medium (M10) and Trypticase Soy Broth (M21). Moderate growth was observed in the remaining media. Inhibition of growth was observed in the tryptone containing media probably due to the inhibitory effect of tryptone on the organism. Caffeine degradation was observed only in Growth medium containing sucrose (M17), Production media II (M13) and III (M14), MS (M18) medium and Xanthine MS medium (M19). Complete degradation of caffeine was observed in the sucrose growth medium (M17) within 48 hrs of incubation. Caffeine degradation in Production media II (M13) and III (M14), MS medium (M18) and Xanthine MS medium (M19) was 27%, 33%, 54% and 62% respectively (Table 2.3.2.1).

Figure 2.3.2.1: Growth of *P. alcaligenes* MTCC 5264 in different media.



Key to legend of figure 2.3.2.1

Legend Key	Medium Name	Legend Key	Medium Name
M.1	Nutrient Broth	M.11	Tryptone Commercial Sugar Medium
M.2	Czapex Dox Broth	M.12	Tryptone Yeast Extract Medium
M.3	Defined Medium	M.13	Production Medium II
M.4	O-Brien Synthetic Medium	M.14	Production Medium III
M.5	Glucose NH4SO4 Medium	M.15	Synthetic medium II
M.6	Dulaney's Medium	M.16	Synthetic Medium III
M.7	Complex Organic Medium	M.17	Growth Medium
M.8	Hobbs Medium	M.18	MS Medium
M.9	Gause Mineral Salt Medium	M.19	Xanthine MS Medium
M.10	Lindenberg Synthetic Medium	M.20	M-9 Medium
M.21 Trypticase Soy Broth			

Table 2.3.2.1. Caffeine degradation by *P. alcaligenes* MTCC 5264, grown in different media.

S.No.	Medium	Caffeine degradation (%) (48 Hrs)
1	Growth medium containing sucrose (M17)	100
2.	Production media II (M13)	27
3.	Production media III (M14)	33
4.	MS Medium (M18)	54
5.	Xanthine MS Medium (M19)	62

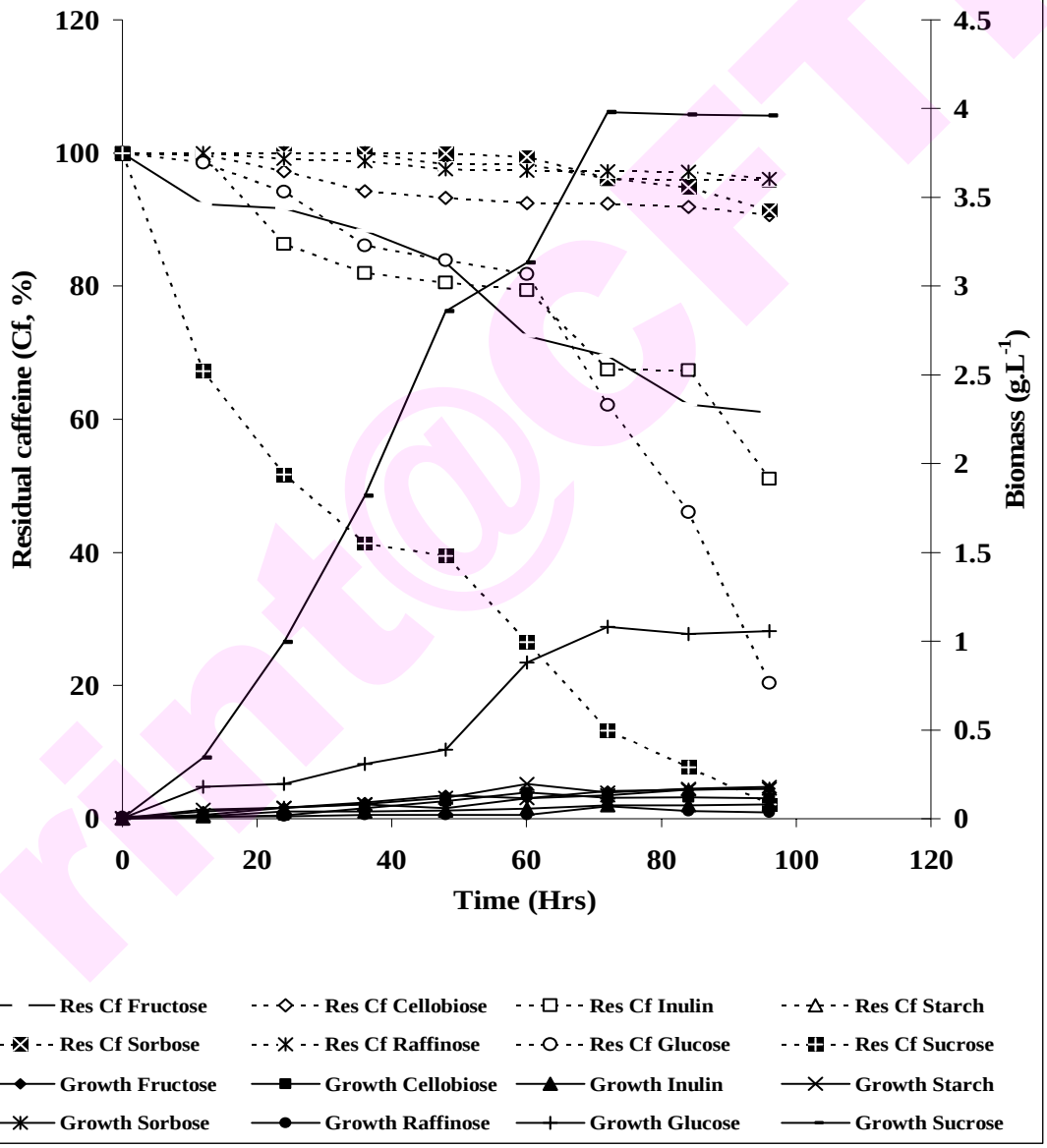
Media containing sucrose as the carbon source and inorganic salts (Growth medium (M17), Production media II (M13) and III (M14), Xanthine MS medium (M19) exerted a positive influence on the growth of the organism. Growth in media containing monosaccharides like glucose and fructose {Complex organic medium (M7), Nutrient Broth (M1), Gause Mineral Salt Medium (M9), Dulaney's medium (M6), Lindenberg Synthetic medium (M10) and Trypticase Soy Broth (M21)} was slow and low biomass accumulation was observed in the media (Fig. 2.3.2.1). These results indicate that *P. alcaligenes* MTCC 5264 shows optimal growth in media containing disaccharides as carbon sources, peptones, yeast extract and salts like ammonium sulphate as nitrogen sources. Similar observations were also reported by Woolfolk (1975) for growth of *Pseudomonas putida* capable of growing in a media containing caffeine, where the organism showed a slow growth in media containing simple sugars like glucose. From the results it was concluded that Growth Medium containing Sucrose (M.17) was the best medium (Fig.2.3.2.1) and was selected for further studies. In all the studies it was also found that there was a direct relationship between the growth and caffeine degradation by the organism. Maximum caffeine degradation was observed in the media enabling high biomass accumulation (M17).

2.3.2.2. Effect of carbon source on growth and caffeine degradation by *P. alcaligenes* MTCC 5264:

Carbon sources are required for the basic metabolic activities, growth and modulating the organisms' metabolic activities either enabling them to use a substrate or regulating the uptake of the substrate itself. Therefore screening and selection of a suitable carbon source is very important in the development of a bioprocess like decaffeination. A carbon source which is suitable for one organism would not necessarily be suitable for another closely related species capable of degrading caffeine. Moreover, different carbon sources exert different activities on the degradation of a substrate like caffeine. Of all the carbon sources tested sucrose was found to be the best both in terms of growth and caffeine degradation (Fig.2.3.2.2a). In the presence of Sucrose as the sole source of carbon, 3.98 $g.L^{-1}$ of biomass was accumulated in the medium after 72 hrs of growth and 87% of the initial caffeine was degraded within 72 hrs. Glucose and fructose although were simpler substrates showed a lower growth and caffeine degradation. Biomass accumulation was only 1.08 and 0.169 $g.L^{-1}$ for glucose and fructose respectively. Caffeine degradation was also slow with on 38 and 31 % caffeine degraded in 72 hrs by glucose and fructose respectively. Other sugars were found to have an inhibitory effect both on the growth and caffeine degradation by *P. alcaligenes* MTCC 5264 (Fig.2.3.2.2a). Cellobiose, Inulin, Raffinose and Sorbose are complex sugars and the organism might not be having the necessary enzymes required to utilize these substrates. The inhibitory effect of the monosaccharides is thought due to metabolite repression, the organism preferring utilization of these sugars rather than caffeine as a source of carbon, thereby lowering the expression of enzymes responsible for caffeine degradation.

Sucrose being a disaccharide is metabolized relatively slowly leading to a slow release of monosaccharides providing energy for growth as well as enabling the expression of enzymes involved in the degradation of caffeine.

Figure 2.3.2.2 a: Effect of carbon source on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.



Based on the above results sucrose was selected as the carbon source for further experiments.

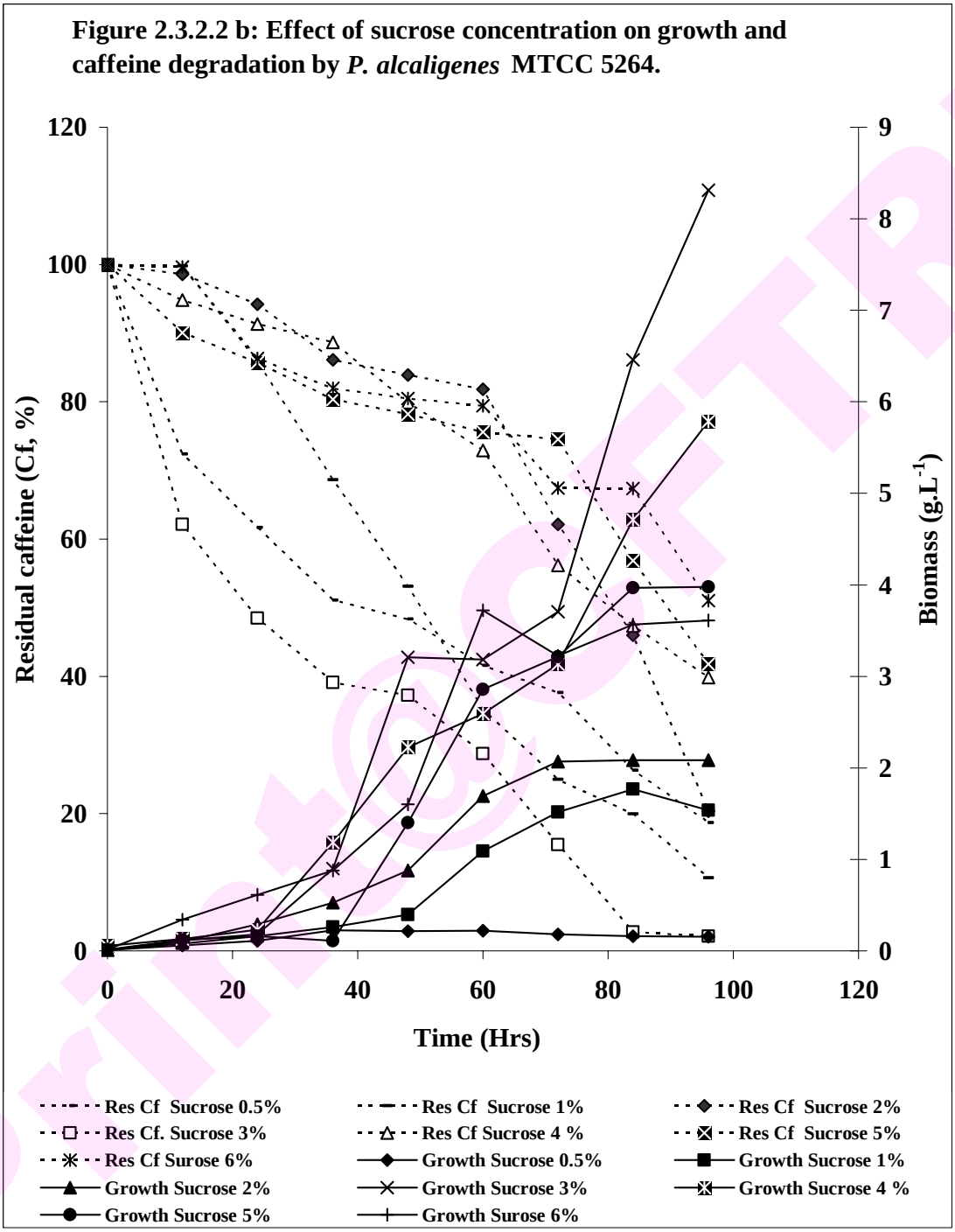
Figure 2.3.2.2b, represents the effect of different concentrations of sucrose on growth and caffeine degradation by *P. alcaligenes* MTCC 5264. Maximum biomass (8.312 $g.L^{-1}$) was observed in medium containing 30 $g.L^{-1}$ of sucrose. The optimum sucrose concentration was found to be 30 $g.L^{-1}$ (Fig.2.3.2.2b). The growth and caffeine degradation was found to be dependent on the sucrose concentration in the medium.

There is a gradual increase in the biomass from 0.156 $g.L^{-1}$ to 8.312 $g.L^{-1}$ and 73 to 98% caffeine degradation by the organism as the concentration of sucrose was increased from 10 to 30 $g.L^{-1}$ of sucrose. The growth dropped to 5.785, 3.976 and 3.612 $g.L^{-1}$ at 40, 50 and 60 $g.L^{-1}$ of sucrose respectively (Fig.2.3.2.2b, Table 2.3.2.2). There was a decrease in the caffeine degradation to 60, 58 and 49% at 40, 50 and 60 $g.L^{-1}$ of sucrose respectively. Sugar at higher concentration will increase the osmolarity of the medium, thereby effecting osmotic stress on the organism. This could lead to inhibition of growth of the organism as the organism has to spend more energy on dealing with the external stress leading to low growth and caffeine degradation.

Table. 2.3.2.2. Effect of carbon source on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

Carbon Source	Biomass ($g.L^{-1}$)	Caffeine Degradation (%)
Fructose	0.169	39.02
Glucose	1.056	79.64
Sucrose	3.961	97.87
Cellobiose	0.116	8.34
Starch	0.179	4.13
Sorbose	0.169	8.64
Inulin	0.081	8.94
Raffinose	0.036	3.01

Figure 2.3.2.2 b: Effect of sucrose concentration on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

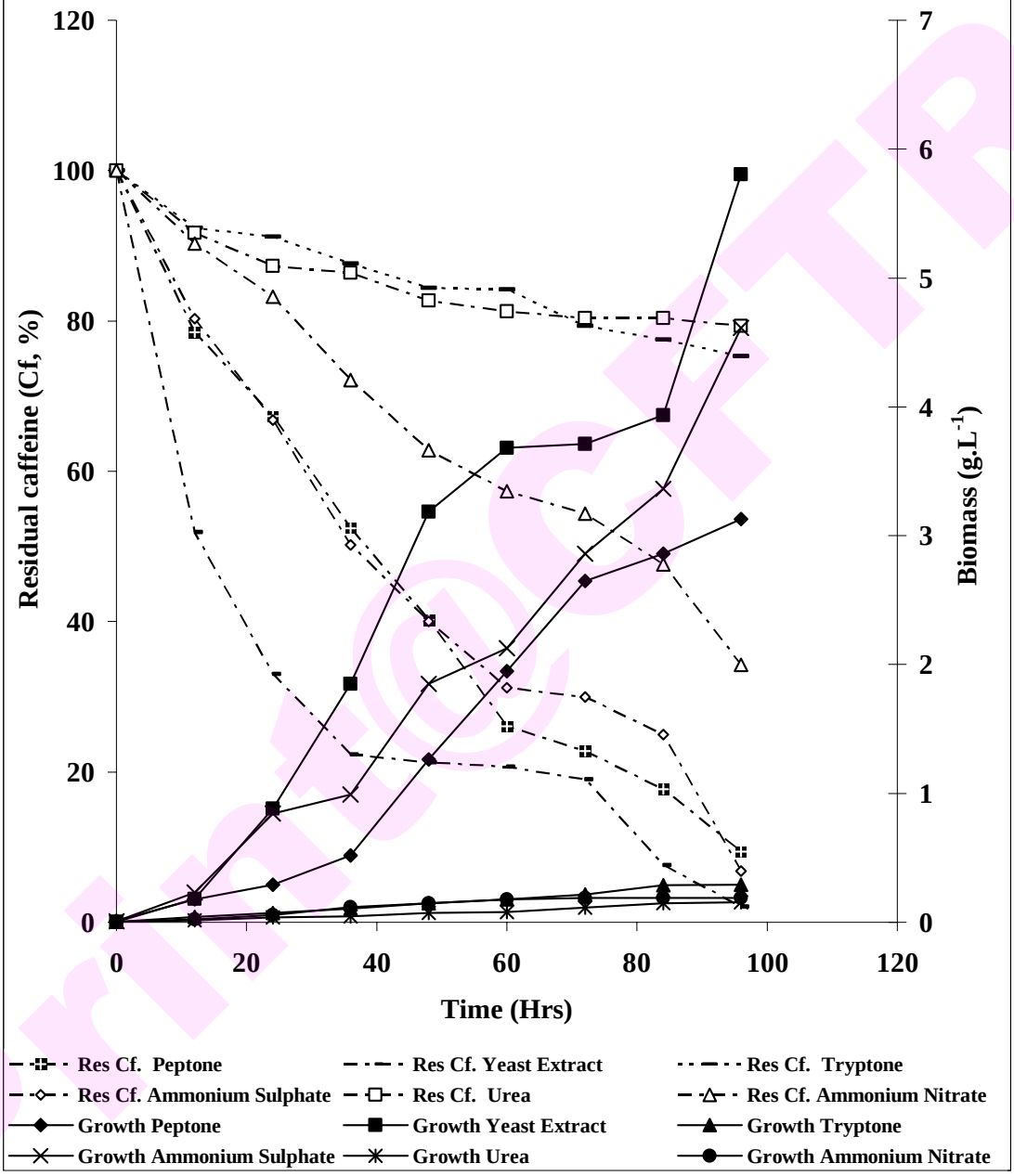


2.3.2.3. Optimization nitrogen source:

Nitrogen is a key component of the proteins and also is important for the growth of the organism. Nitrogen sources exert a significant effect on the caffeine degradation by microorganisms (Blecher, 1976; Roussos et.al, 1994; Hakil, et.al.,

1999; Sánchez et.al 2004) have reported that nitrogen source and concentration play an important role in the degradation of caffeine by bacteria and fungi. Figure 2.3.2.3, represents the effect exerted by different nitrogen sources on growth and caffeine degradation by *P. alcaligenes* MTCC 5264. Maximum caffeine degradation (97.9 %) and biomass accumulation (5.805 $g.L^{-1}$) was observed in medium containing yeast extract as the external carbon source. Peptone and ammonium sulphate also supported growth (3.131 and 4.612 $g.L^{-1}$ respectively), whereas growth was low in tryptone, urea and ammonium nitrate (0.291, 0.156 and 0.189 $g.L^{-1}$ respectively). Bacterial growth was low in media containing tryptone, and the same effect was observed in our earlier experiments too (M1, M21, M12) (Fig.2.3.2.1, Fig.2.3.2.3). Tryptone also exerted an inhibitory effect on the caffeine degradation by the isolate and only 25% of the initial caffeine was degraded after 96 hrs of growth. Urea also did not support growth of the organism and the caffeine degradation was only 19.67% after 96 hrs of growth. This is because urea is a product of the decaffeination pathway and is at the end of the pathway. Peptone, yeast extract and ammonium sulphate were found to be the best nitrogen sources and were used for further studies on degradation of caffeine by *P. alcaligenes* MTCC 5264.

Figure 2.3.2.3: Effect of nitrogen sources on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.



2.3.2.4. Optimization of pH:

pH plays an important role on the growth and activity of different enzymes required for caffeine degradation. Figure 2.3.2.4 represents the effect of pH on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

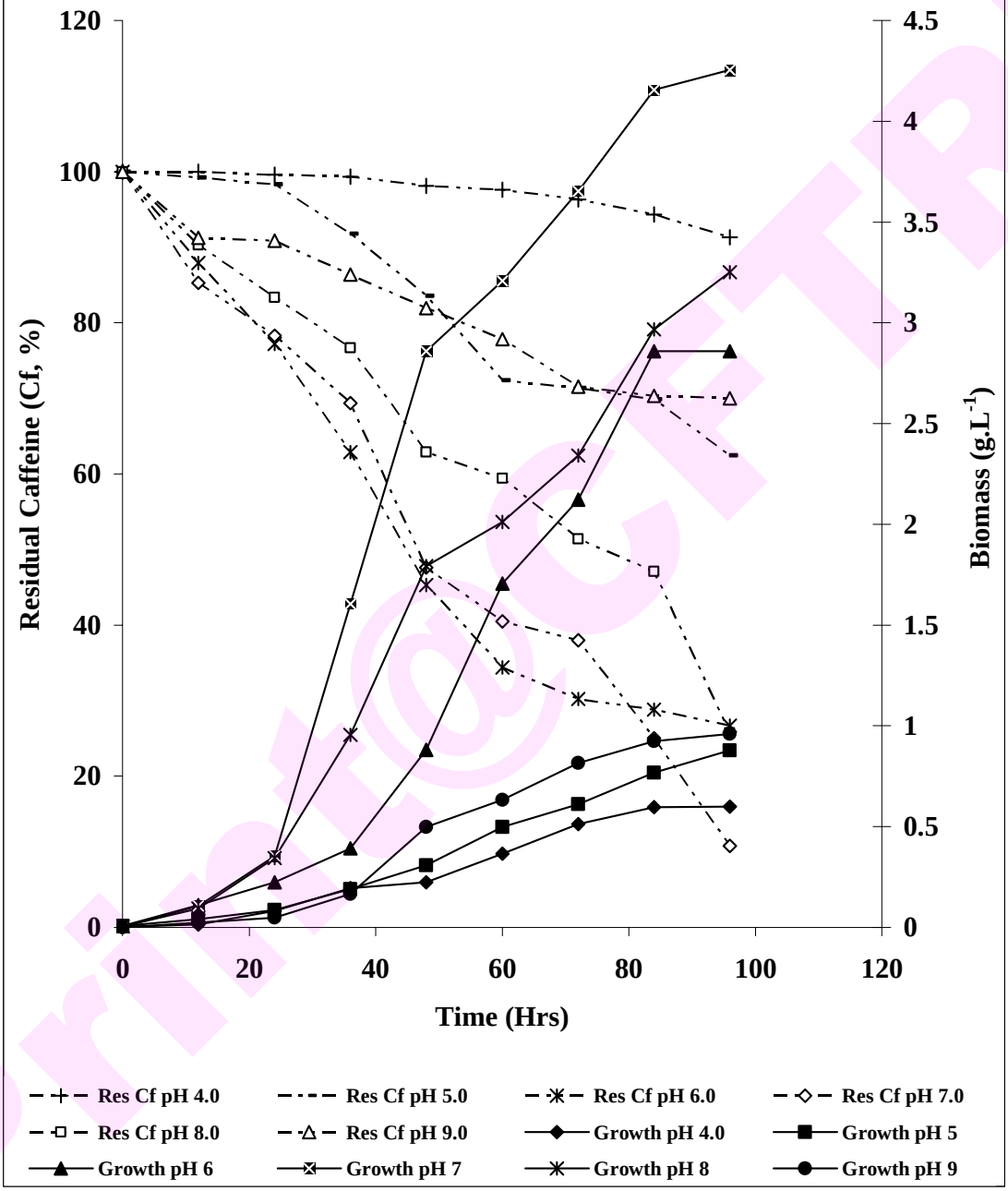
Biomass accumulation was only 0.598 $g.L^{-1}$ when *P. alcaligenes* was grown at pH 4.0. Less than 10% of the initial caffeine was degraded by the isolate at pH 4.0. Biomass accumulation as well as efficiency of caffeine degradation increased as the pH of the medium was increased till 7.0. Maximum biomass accumulation (4.254 $g.L^{-1}$) was observed at pH 7.0 and 90 % of the initial caffeine was degraded within 96 hrs of incubation.

Biomass accumulation as well as caffeine degradation efficiency decreases as the pH of the medium increased from 7.0 to 9.0. At pH 9.0, only 30 % of the initial caffeine was degraded after 96 hrs and the biomass accumulated was 0.961$g.L^{-1}$.

From the studies it can be concluded that the optimum pH for caffeine degradation and growth of *P. alcaligenes* MTCC 5264 was found to be 7.0 (Fig.2.3.2.4). Woolfolk (1975), Blecher (1976), Asano et.al., (1993), Mazzaffera et. al., (1994) and Sarath et.al., (2005) also report the degradation of caffeine by bacterial strains at pH 7.0±0.2.

Highly acidic or alkaline pH was found to inhibit the growth of the organisms as well as caffeine degradation (Fig. 2.3.2.4) indicating that the optimum pH for the enzymes involved in caffeine degradation is near 7.0.

Figure 2.3.2.4: Effect of pH on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

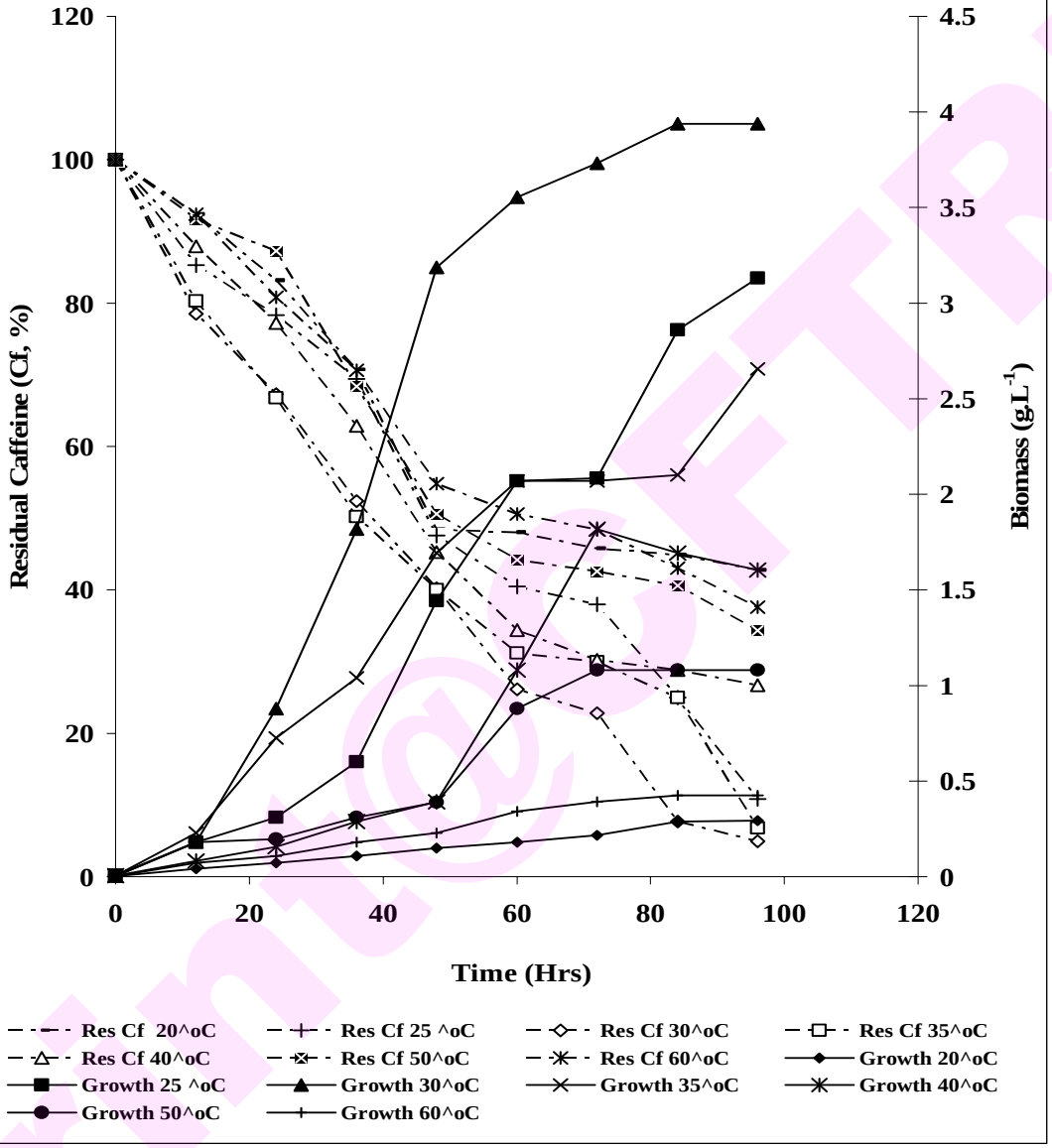


2.3.2.5. Optimization of temperature:

Temperature is an important parameter for achieving an efficient Biodecaffeination and has a drastic effect on the activities of the enzymes involved in Biodecaffeination. From Figure 2.3.2.5, it can be observed that maximum caffeine

degradation (95%) was achieved when the organism was grown at 30^0C and the biomass accumulation was also high at this temperature (3.937 $g.L^{-1}$). Good Growth was observed at 25^oC (3.131 $g.L^{-1}$) and the organism also degrades around 90% of the initial caffeine at 25^oC. Caffeine degradation as well as biomass accumulation was high till the temperature of the medium was increased upto 35^oC (Fig. 2.3.2.5) (3-4 $g.L^{-1}$ biomass and 90-95% Caffeine degradation). Increase in the temperature above 40^oC led to decrease in the growth as well as caffeine degradation by the isolate. These results indicate that the organism is a mesophile and thrives well at 25-35^oC with maximum efficiency of caffeine degradation. Maximum caffeine degradation efficiency of the isolate in the mesophilic temperature range is due to the enzymes acting more efficiently in this temperature range. Interestingly the organism degraded around 50% of the initial caffeine at 60^o indicating a possible use of *P. alcaligenes* MTCC 5264 at higher temperatures also. Usually bacteria belonging to *Pseudomonas* sps. are known to be mesophilic living in almost any substrate at these temperatures (Mennet and Nakayama, 1971). However they are known to adapt themselves to extreme temperatures by developing protective mucilage layer around them which would protect them from the effects of temperature (Gügi et.al., 1991; Hebraud et.al., 1994). This mechanism may probably be involved in the survival and degradation of caffeine by *P. alcaligenes* observed in this study.

Figure 2.3.2.5: Effect of temperature on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.



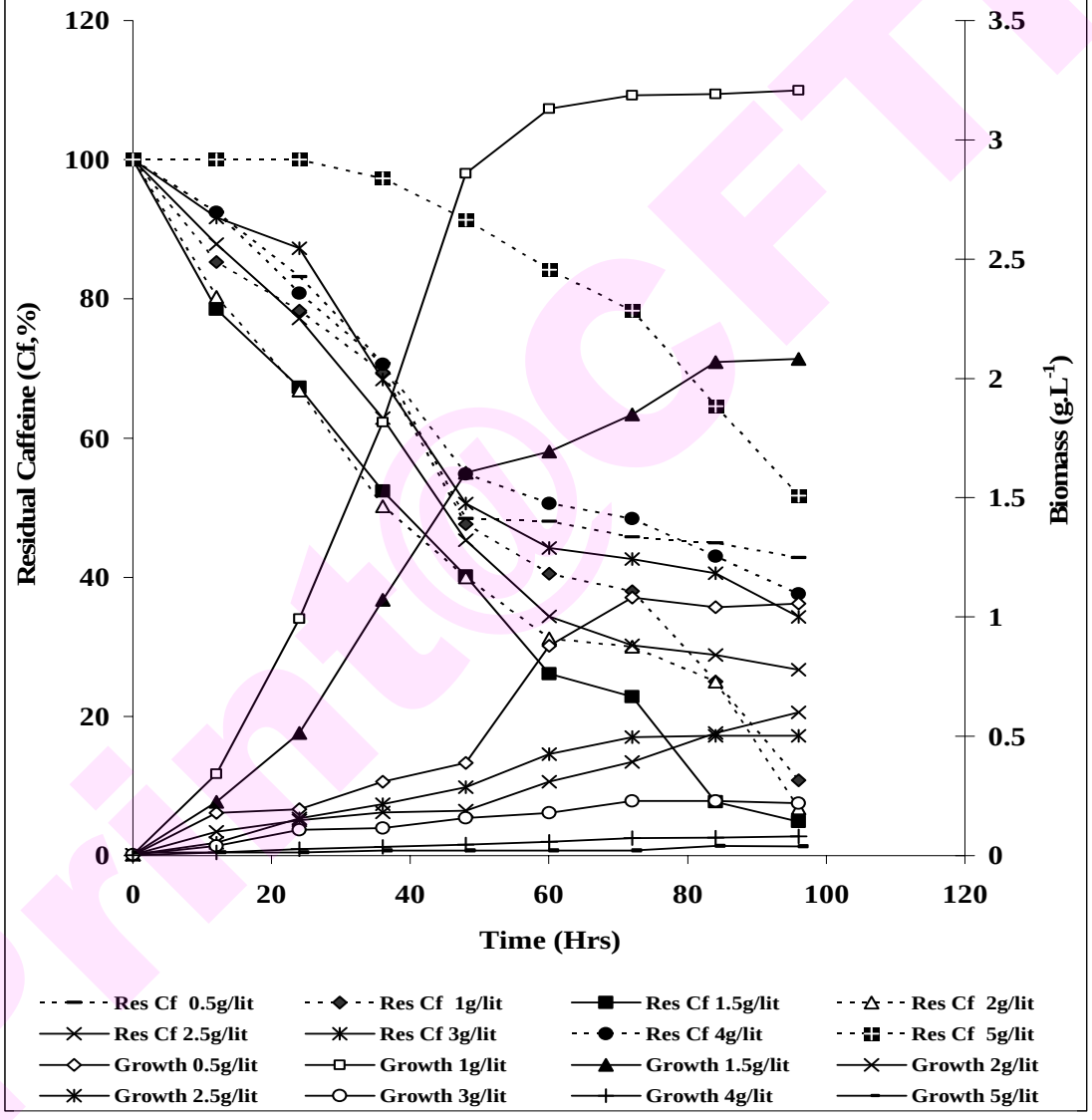
2.3.2.6. Optimization of caffeine concentration:

Caffeine is known to be toxic to organisms and the efficient Biodecaffeination demands the selection of a threshold level of caffeine which the organisms can tolerate and degrade efficiently. *P. alcaligenes* MTCC 5264 exhibited high biomass of 3.208 $g.L^{-1}$ and 90 % of the initial caffeine was degraded in liquid medium containing 1.0 g.L-1 of caffeine (Fig. 2.3.2.6). As the caffeine concentration

increased to 2.0 $g.L^{-1}$, there was a slight decrease in the growth but more than 95 % of the initial caffeine was degraded within 96 hrs of incubation. At the beginning of the incubation period, a slight growth inhibition of growth and caffeine degradation was observed at 2.0 $g.L^{-1}$ of caffeine, which was probably due to the initial toxic effect of caffeine. The organism could however degrade more than 95% of the caffeine within 96 hrs and this trend was observed till a concentration of 2.0 $g.L^{-1}$. Initial concentration of caffeine above 2.0 $g.L^{-1}$ were highly inhibitory for the organism and only 70-75 % of the initial caffeine was degraded after 96 hrs of incubation owing to the inhibitory effect of caffeine on the organism. At a caffeine concentration of 5.0 $g.L^{-1}$, 49 % of the initial caffeine was degraded although the biomass accumulated was only 0.041 $g.L^{-1}$ (Fig. 2.3.2.6). Although caffeine is known to be toxic at high concentrations, *P. alcaligenes* MTCC 5264 appears to have adapted itself to survive the high concentrations of caffeine by expressing enzymes capable of degrading the caffeine. The low efficiency of caffeine degradation is due to the inhibitory effect of caffeine on the enzymes. Similar effect of caffeine was also observed by several authors like Asano et.al., (1993), wherein they have reported the growth of a strain of *P. putida* capable of growing on caffeine at 5.0 $g.L^{-1}$. The organism could degrade only 40-45 % of the initial caffeine within 8 weeks after a long acclimatization time of 2 years. Studies on caffeine degradation by bacteria upto concentrations of 50.0 $g.L^{-1}$ have been reported earlier (Blecher and Lingens, 1977). But the caffeine degradation rates reported in all thee studies are low and require very long acclimatization times and partial degradation at high initial caffeine concentrations (Woolfolk, 1975; Asano et.al., 1993; Mazzaffera et.al., 1994a). The

strain of *P. alcaligenes* MTCC 5264 used in this study requires low induction times and has high efficiency of degrading caffeine even at 5.0 $g.L^{-1}$ making it a promising strain for use in biodecaffeination processes.

Figure 2.3.2.6: Effect of caffeine concentration on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

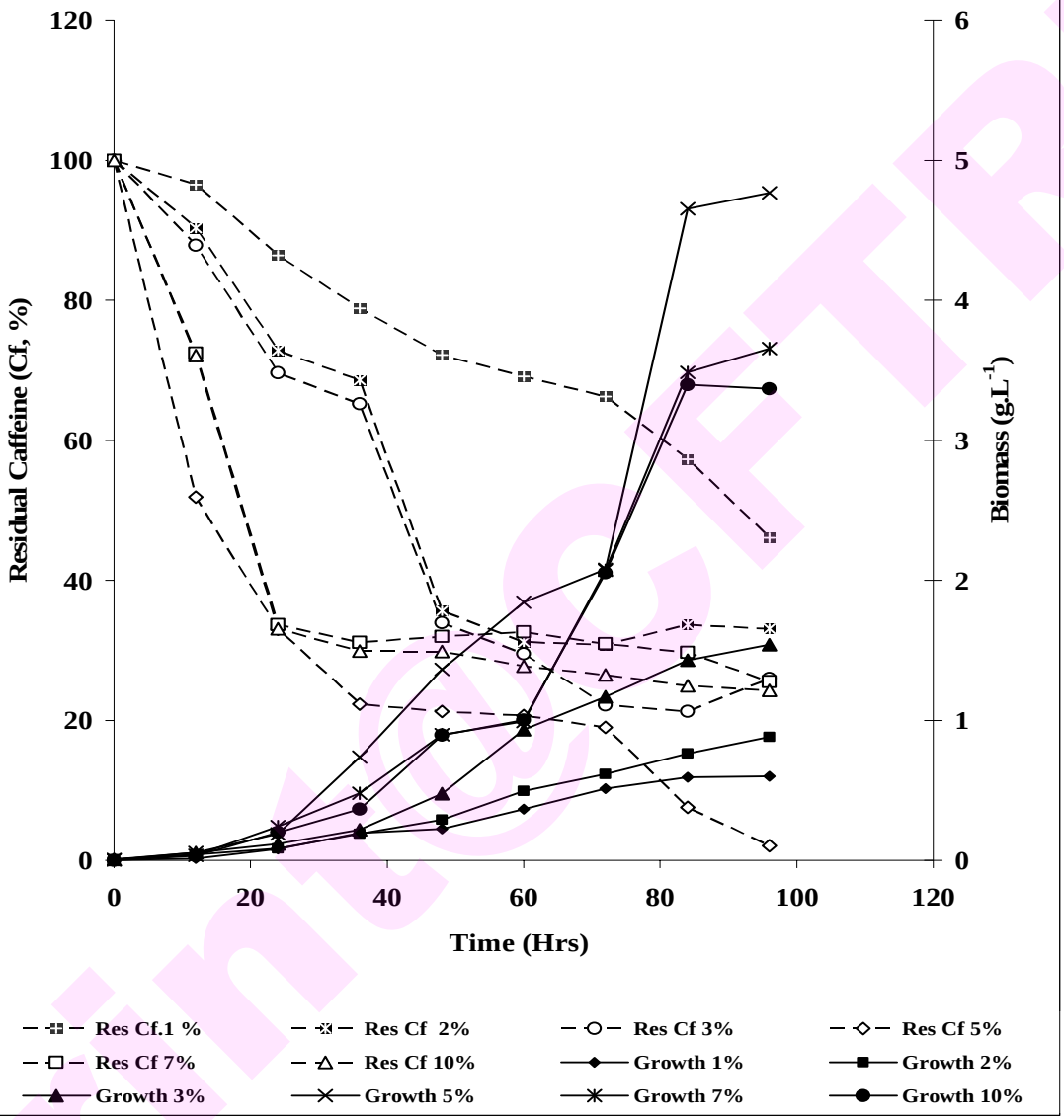


2.3.2.7. Optimization of inoculum volume:

Initial inoculum volume gives a measure of the number of cells at the start of the Biodecaffeination process in the liquid culture. An optimum initial cell

concentration is essential for overcoming the initial lag phase by the organism in CLM. Figure 2.3.2.7 represents the effect of the initial inoculum volume on caffeine degradation by *P. alcaligenes* MTCC 5264. At an initial inoculum level of 1 % w/v the biomass accumulated was 0.601 $g.L^{-1}$ and 54% of the initial caffeine was degraded within 96 hrs. Caffeine degradation efficiency increased with an increase in the initial inoculum volume and 81 % caffeine was degraded within 96 hrs. Inoculum volumes above 5 % w/v showed no further improvement in the caffeine degradation efficiency. At 10 % w/v of inoculum volume 70 % of the initial caffeine was degraded in the medium. The optimum inoculum volume was found to be 5 %v/v (Fig.2.3.2.7) inoculum volumes above this did not increase the caffeine degradation ability. There was a slight decrease in the degradation probably owing to the death of the cells. At inoculum volumes above 5 % w/v, decrease in growth and caffeine degradation was observed (Fig. 2.3.2.7). Increase in the inoculum volume will lead to increase in the density of cells in the medium. Above a certain level of inoculum, there will be intense competition among the cells for nutrients leading to death of many cells. Further due to the increased metabolism of the cells in the initial hours of incubation nutrient depletion occurs adding up to the increasing number of dead cells in the medium. This leads to low biomass accumulation as well as decreased efficiency of caffeine degradation.

Figure 2.3.2.7: Effect of inoculum volume on growth and caffeine degradation by *P. alcaligenes* MTCC 5264.

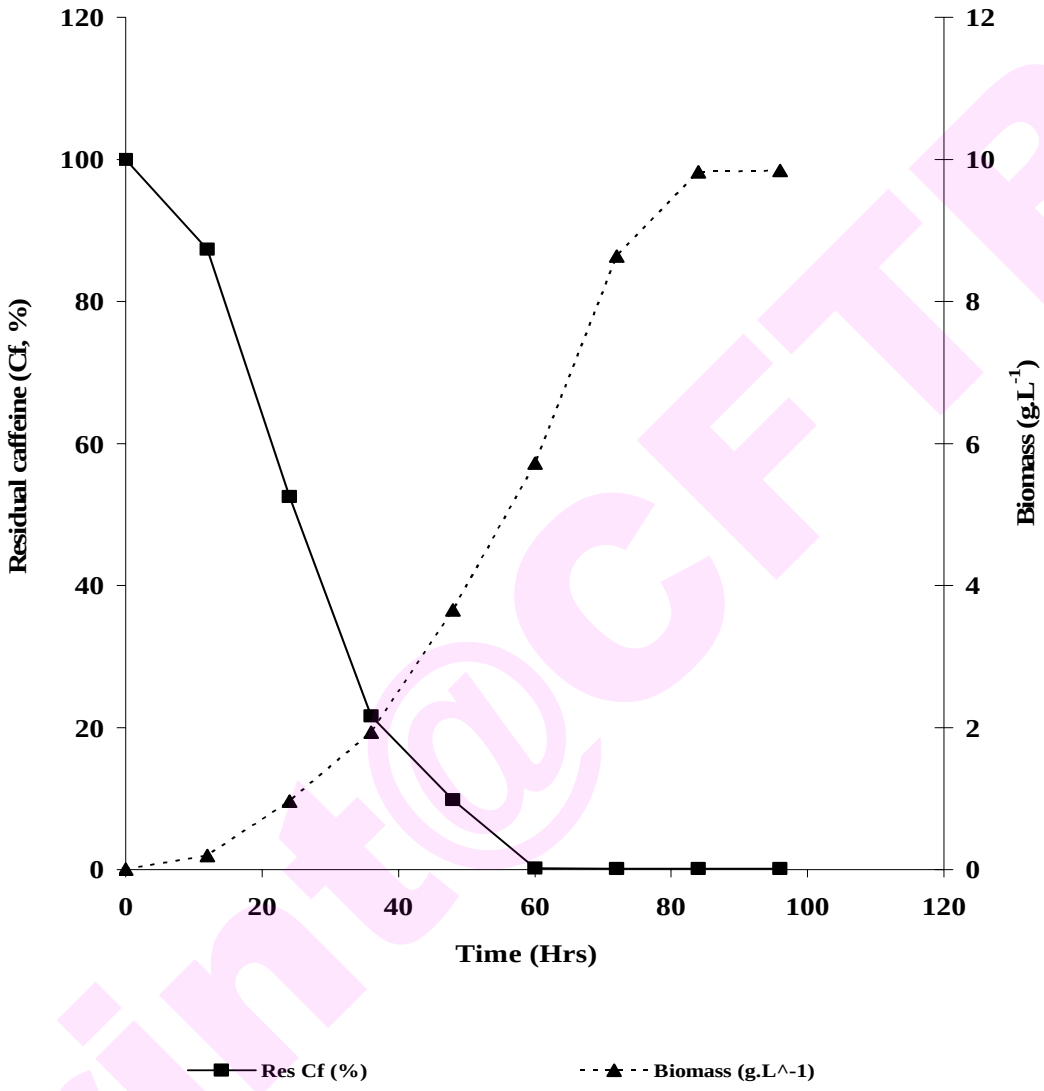


2.3.2.8. Degradation of caffeine by *P. alcaligenes* MTCC 5264 under optimized conditions:

P. alcaligenes MTCC 5264 was grown in the optimized growth medium containing sucrose (30 $g.L^{-1}$), caffeine (1.5 $g.L^{-1}$), yeast extract (15 $g.L^{-1}$), peptone (30 $g.L^{-1}$) and ammonium sulphate (15 g.L-1). The temperature of the medium was set at 30^{o}C, and pH adjusted to 7.0 and an inoculum of 5 %w/v was added to the medium

and incubated by shaking at 150rpm for 96 hrs. Figure 2.3.2.8 represents the growth and caffeine degradation by *P. alcaligenes* MTCC 5264 under optimum conditions. Under these conditions, 9.6 $g.L^{-1}$ of biomass was accumulated after 96 hrs with a growth rate of 0.031 $g.L^{-1}.h^{-1}$. Caffeine was almost completely (99.8%) degraded within 60 hrs of incubation. High caffeine degradation rates were observed under these conditions. The caffeine degradation rate was 0.024 $g.L^{-1}.h^{-1}$. Under optimized conditions the acclimatization time for caffeine was reduced to 24hrs and around 50% of the initial caffeine was degraded within 24 hrs of incubation (Fig. 2.3.2.8). Both the caffeine degradation rate as well as growth rate were high in the initial 48 hours of incubation. 90% of the initial caffeine was degraded within 48 hrs of incubation and the biomass accumulated at this point of time was 3.654 $g.L^{-1}$. Maximum caffeine degradation rate was also observed during this period as the cells were in active phase of growth. Growth rate was high after 48 hrs of growth. By 48 hrs 90% of the caffeine was degraded (Fig. 2.3.2.8) and no inhibition due to caffeine will be present in the medium. The organism would utilize the methyl xanthines produced during the degradation of caffeine as sources of nitrogen and carbon along with the external carbon and nitrogen sources added to the medium thereby increasing the biomass in the medium.

Figure 2.3.2.8: Growth and caffeine degradation by *P. alcaligenes* MTCC 5264 under optimum conditions.



2.4. CONCLUSIONS:

Three bacterial isolates capable of degrading caffeine were isolated and characterized. *P. alcaligenes* MTCC 5264 was found to be highly efficient in degrading caffeine. This isolate was selected for use in degradation of caffeine from solutions. The growth conditions and media composition for maximum caffeine degradation by the isolate were optimized. This organism was used for the

decaffeination of caffeine containing liquids. *P. alcaligenes* MTCC 5264 is an efficient caffeine degrader requiring little acclimatization times and having the ability to resist high concentrations of caffeine in the medium. Although several studies have been reported on the degradation of caffeine by bacteria and fungi, they have been largely limited by the long acclimatization times, low caffeine tolerances and low caffeine degradation efficiency of the organisms. The isolate reported in this study is advantageous over the other strains reported in literature and has immense potential of development of enzymatic processes for decaffeination which will be dealt in the later chapters.

2.5. REFERENCES:

1. Asano, Y., Komeda, T, Yamada, H. (1993), Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem*. **57:**1286–89.
2. Baumann, T. W., Gabriel, H., (1984), Metabolism and excretion of caffeine during germination of *Coffea arabica* L. *Plant Cell Physiol*. **25:** 1431–1436.
3. Bergmann, F., Ungar-Waron, H., Kwietny-Govrin H., (1964), Some specific reactions of the purine oxidizing system of *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, **79:** 512-522.
4. Berthou, F., Guillois, B., Riche, C., Dreano, Y., Jacqz-Aigrain, E., Beaune, P., (1992), *Xenobiotica*, **22:** 671–680.
5. Blecher, (1976), Microbial breakdown of caffeine (author's transl), Zentralbl *Bakteriol [Orig B]*. **162(1-2):** 180-3. [Article in German]
6. Blecher, R., Lingens, F., (1977), The metabolism of caffeine by a *Pseudomonas putida* strain. *Hoppe Seylers Z Physiol Chem*. **358(7):** 807-17.
7. Boccas, F., Roussos, S., Gutierrez, M., Serrano, L., Viniegra, G.G., (1994), Production of pectinase from coffee pulp in solid-state fermentation system - selection of wild fungal isolate of high potency by a simple 3-step screening technique. *J. Food Sci. Tech. (Mysore)*, **31:** 22-26.
8. Bressani, R., (1987a), Antiphysiological factors in coffee pulp. In: Brahan, J.E.; Bressani, R. (Ed.) Coffee pulp: composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, 83-88.
9. Bressani, R., (1987b), Potential uses of coffee berry byproducts. In: Brahan, J.E.; Bressani, R. (Ed.) Coffee pulp: composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, 17-25.
10. Burr, T.J., Caesar, A., (1985), Beneficial plant bacteria. *CRC Critic. Rev. Plant Sci*., **2:**120.

11. Friedman, J., Waller, G.R., (1983a), Caffeine hazards and their prevention in germinating seeds of coffee (*Coffea arabica* L.) *J. Chem. Ecol*. **9:**1099-1106.

12. Friedman, J., Waller, G.R., (1983b), Seeds as allelopathic agents. *J. Chem. Ecol*. **9:**1107-1115.

13. Fries, N., Kihlman, B., (1948), Nature, **162:** 573

14. Frischknecht, P.M., Ulmer, D.J., Baumann, T.W., (1986), Purine alkaloid formation in buds and developing leaflets of *Coffea arabica*: expression of an optimal defense strategy?? *Phytochemistry*. **25:**613-616.

15. Gluck, M., Lingens, F., (1987), Studies on the microbial production of thebromine and heteroxanthine from caffeine. *Appl. Microb.Biotech.* **25:** 334-340.

16. Grigg, C.W., (1972), Effects of coumarin, pyronine Y, 6,9-dimethyl-2methylthiopurine and caffeine on excision repair and recombination in *Escherichia coli*. *J. Gen. Microb.,* **70:** 221230.

17. Gügi, B., Orange, N., Hellio, F., Burini, J.F., Guillou, C, Leriche F., Guespin-Michel J.F., (1991), Effect of growth temperature on several exported enzyme activities in the psychrotrophic bacterium Pseudomonas fluorescens. J Bacteriol. **173(12):** 3814-3820

18. Hakil, M., Voisinet, F., Gonz´alez, G.V., Augur, C., (1999), Caffeine degradation in solid-state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources. *Process Biochem*. **35**:103–9.

19. Hebraud, M., Dubois, E., Potier, E., Labadie**,** J., (1994), Effect of growth temperatures on the protein levels in a psychrotrophic bacterium, *Pseudomonas fragi*. J Bacteriol. **176(13):** 4017-4024

20. Hohnloser, W., Osswalt ,B., Lingens, F., (1980), Enzymological aspects of caffeine demethylation and formaldehyde oxidation by *Pseudomonas putida* C1. *Hoppe seyler's Z Physiol Chem.* **361:** 1763–76.

21. Kihlman, B.A., (1974), Effects of caffeine on the genetic material. *Mutat Res.,* **26:**53–71.

22. Mazzafera, P., Crozier, A., Magalhaes, A.C., (1991), Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochem.* **30:**3913–16.

23. Mazzafera, P., Crozier, A., Sandbergs, G., (1994a), Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei*. *J Agric Food Chem*. **42:**7423–77.

24. Mazzafera, P., Olsson, O., Sandberg, G., (1994b), Degradation of caffeine and related methyl xanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microb Ecol*. **31:**199–207.

25. Mennett, R.H., Nakayama, T.O.M., (1971), Influence of Temperature on Substrate and Energy Conversion in *Pseudomonas fluorescens, Appl Microbiol*. **22(5):** 772–776.

26. Middelhoven, W. J., Bakker, C. M., (1982), Degradation of caffeine by immobilized cells of P*seudomonas putida* strain C 3024. *Europ. J. Appl. Microbial Biotechnol*. **15:** 214-217.

27. Middelhoven, W.J.; Lommen, A., (1984), Degradation of caffeine by *Pseudomonas putida* C3024. *Anton. van Leeuwenho.* **.50:** 298-30.

28. Nathanson, J. A., (1984), Caffeine and related methylxanthines: possible naturally occuring pesticides. *Science*, **226**: 184–187.

29. Porres, C., Alvarez, D., Calzada, J., (1993), Caffeine reduction in coffee pulp through silage. *Biotechnol. Adv.* **11(3):** 519–23.

30. Putrament, A., Baranowska, H., Bilinsky, T., Prazmo, W., (1972) On the specificity of caffeine effects. *Mol. Gen.***118**: 373–9.

31. Roussos, S., Hannibal, L., Aquiahuatl, M. A., Trejo Hernandez, M. R., Marakis, S., (1994), Caffeine degradation by *Penicillium verrucosum* in solid state fermentation of coffee pulp: critical effect of additional inorganic and organic nitrogen sources. *J. Food Sci. Technol*., **31:** 316-319.

32. Roussos, S., Angeles-Aquiahuatl, M.D.L., Trejo-Hernandez, M.D.R., Gaime-Perraud, I., Favela, E., Ramakrishna, M., (1995), Biotechnological management

of coffee pulp-isolation, screening, characterization, selection of caffeine degrading fungi and natural microflora present in coffee pulp and husk. *Appl Microbiol Biotechnol*. **42:**756–62.

33. Sanchez, G. G., Roussos, S., Augur, C., (2004), Effect of the nitrogen source on caffeine degradation by *Aspergillus tamarii*. *Letts. Appl. Microbiol.* **38(1):** 50-55.
34. Sarath Babu, V.R., Patra, S, Karanth, N.G., Varadaraj, M.C., Thakur M.S., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708, *Enz, Microb. Tech*. **37:** 617-624.
35. Sauer, M., (1982), Comparison of the cytochrome P450 containing monooxygenases originating from two different yeasts. *Dev. Biochem*. **23:** 452-457.
36. Schwimmer, S., Kurtzman Jr. R.H., Heftmann E., (1971), Caffeine metabolism by *Penicillium roqueforti*. *Arch Biochem Biophys*; **147:**109–13.
37. Silva, C.F.; Scwan, R.F.; Dias, E.S.; Wheals, A.E. (2000), Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. *Int. J. Food Microbiol*. **60:** 251-260.
38. Sundarraj C. V., Dhala, S., (1965), Effect of Naturally Occurring Xanthines on Bacteria I. Antimicrobial Action and Potentiating Effect on Antibiotic Spectra, Appl. *Microbiol*. **13(3):** 432–436.
39. Suzuki T, Ashihara H., Waller G. R., (1992), Purine and purine alkaloid metabolism in *Camellia* and Coffea plants, *Plant Physiol.*, **31(8):** 2575-2584.
40. Vogels, G. D. and Van der Drift C., (1976), Degradation of purines and pyrimidines by microorganisms. *Bacteriol*. *Rev*. **40:** 403–468.
41. Waller, G. R., Kumari D., Friedman, J., Friedman, N., Chou, C. H., (1986), Caffeine autotoxicity in *Coffea arabica* L. *In* Advances in Allelopathy. Eds. C. S . Tang and A. R. Putnam. J. Wiley and Sons, New York.

42. Woolfolk, C.A. (1975), Metabolism of N- methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. *J. Bacteriol*, **123:** 1088-1106.

43. Woolfolk, C.A., Downard, J.S. (1977), Distribution of xanthine oxidase and xanthine dehydrogenase specificity types among bacteria. *J. Bacteriol*. **130:** 1175-1191.

44. Yano, Y.D.M., Mazzafera, P., (1998), Degradation of caffeine by *Pseudomonoas putida* isolated from soil. *Allel. J*; **5:** 23–34.

45. Yano, Y.D.M., Mazzafera, P., (1999), Catabolism of Caffeine and Purification of xanthine oxidase responsible for methyluric acids production in *Pseudomonas putida* L. ; *Revista de Microbiologia*, **30:** 62-70.

CHAPTER 3

IMMOBILIZATION: AN APPLICATION ORIENTED METHOD FOR BIODECAFFEINATION.

3.1. SCOPE OF THE WORK:

Biodecaffeination of tea, coffee and other caffeine containing materials is gaining importance due to the increasing demand for the decaffeinated products and the consumer preference towards naturally biodecaffeinated products. Development of biodecaffeination processes for caffeine containing materials requires the employment of microorganisms or enzymes capable of degrading caffeine. Immobilized cells have advantages over free cells due to the retention of the cells in the matrix enabling reuse of the immobilized cells and multi enzymes involved in sequential degradation of caffeine to NH_3 and CO_2. The conversion of caffeine to its metabolites is primarily brought about by N-demethylases (such as caffeine 1N-demethylase and 3N-demethylase), xanthine oxidase, uricase, urease etc., that are produced by several caffeine-degrading bacterial species such as *Pseudomonas putida, Serratia, Alcaligenes sp., Rhodococcus, Klebsiella*, etc. Development of biodecaffeination techniques using whole cells offers an attractive alternative to the present existing chemical and physical methods removal of caffeine, which are costly, toxic and non-specific to caffeine. This chapter mainly focuses on the microbial caffeine degradation of caffeine in pure solutions as well as real samples.

3.2. IMMOBILIZATION:

The technique used for the physical or chemical fixation of cells, organelles, enzymes, or other proteins (e.g. antibodies), Nucleic acids (DNA, RNA) onto a solid support, into a solid matrix or retained by a membrane, in order to increase their stability and make possible their repeated or continued use making the process economical.

The industrial biotechnology processes using microorganisms generally involve the cells suspended in the fermentation medium. The classical fermentations suffer from various constrains such as low cell density, nutritional limitations, and batch-mode operations requiring high power input. It has been well recognized that the microbial cell density is of prime importance to attain higher volumetric productivities. The continuous fermentations with free-cells and cell recycle options aim to enhance the cell population inside the fermentor. During the last 20–25 years, the cell immobilization technology, with its origins in enzyme immobilization, eliminates most of the constrains faced with free-cell systems and has attracted the attention of several research groups. The remarkable advantage of immobilized cell based system is the freedom it has to determine the cell density prior to fermentation. It also facilitates operation of microbial fermentation on continuous mode without cell washout. Since the early 70s, when Chibata's group (Chibata et.al., 1974 a&b) announced successful operation of continuous fermentation of l-aspartic acid, numerous research groups have attempted various microbial fermentations with immobilized cells. Several process based on immobilized microbial cells have been developed.

3.2.1. Rationale for whole-cell immobilization:

Immobilization commonly is accomplished using a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose, etc. In these cases, the cells are immobilized by entrapment in the pertinent gel by a drop-forming procedure. The major factors which determine the applicability of an immobilized cell based bioprocess include cost of immobilization, mass transport limitations, applicability to a specific end-product, etc. These factors are to be carefully examined before choosing any particular methodology.

Many processes have been practised traditionally, embodying the basic principle of microbial conversions offered by cells bound to surfaces. Some examples of use of immobilized cells include waste treatment in trickling filters and ethanol oxidation to produce vinegar. Immobilization of cells is the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrates, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. Therefore it is expected that the microenvironment surrounding the immobilized cells is not necessarily the same experienced by their free-cell counterparts.

3.2.2. Advantages of immobilized cells (Webb, 1989):

a) The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes where all the required enzymes are concentrated in the immobilized cells.

b) It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation.

c) It enables the ease of conversion of batch processes into a continuous mode and maintenance of high cell density without washout conditions even at very high dilution rates.

d) Immobilized cells are advantageous over immobilized enzymes where co-factors are necessary for the catalytic reactions. Since co-factor regeneration machinery is an integral function of the cell, its external supply is uneconomical.

e) The bound-cell systems are far more tolerant to perturbations in the reaction environment and similarly less susceptible to toxic substances present in the liquid medium.

f) Immobilized cells enable higher retention of plasmids and can be used for recombinant product formation.

3.2.3. Immobilization methods:

Many methods namely adsorption, covalent bonding, cross-linking, entrapment, and encapsulation are widely used for immobilization (Groboillot, et.al., 1994). Every method has its own advantages and disadvantages and the immobilization method varies from process to process.

3.2.3.1. Adsorption:

Adsorption of cells to surfaces is a mild process, and suitable for obtaining viable cells and the adsorption is based on non-covalent forces such as ionic

interactions. Ion-exchange materials such as Dowex-1 and DEAE-cellulose have proved useful (West and Strohfus, 1996). Cell immobilization by this method depends on a number of factors and one of the most important is the charge on the support material.

The disadvantage of adsorption is cell leaching, and this would cause serious problems if the cell continues growing downstream of the reaction or provides a source for bacterial growth or releases contaminating proteins and biochemicals when the cell is disrupted (Yaskovich, 1998). Microcarriers (MC) are known to be the best supports for adsorption of cells. They are very small (0.2mm) so provide a large surface area for cell growth. One gram of MC provides more than 6000 cm^2. MC are manufactured from dextran, polyacrylamide, or polystyrene and binding of cells is by ionic attraction (Van Wezel, 1967, Dixit, et.al., 1992). MC are small and would pack down to much in a column so are always used in a stirred apparatus with gentle agitation. Salter et. al., (1990) have reported a novel method of cell immobilization called as Hydrodynamic deposition on ceramic microspheres. The spheres are hollow and cell immobilization is carried out by passing a cell suspension through a column of such particles. The advantage of this method is ability to achieve high biomass densities, while the hydrodynamic properties of columns containing such immobilized cells are excellent. (Kanasawud, et. al., 1989; Guoqiang et.al., 1992)

3.2.3.2. Covalent bonding:

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is

necessary. Cells of *S. cerevisiae* were immobilized by coupling silanized silica beads (Novarro and Durand, 1977). The reaction requires introduction of reactive organic group on inorganic silica surface for the reaction between the activated support material and yeast cells. a -amino propyl triethoxy silane is generally used as the coupling agent (Marek et. al., 1986). This inorganic functional group condenses with hydroxyl group on silica surface. As a result, the organic group is available for covalent bond formation on the surface of silica. Covalent bonding can also be achieved by treating the silica surface with glutaraldehyde and isocyanate (Kennedy and Cabral, 1985). A system of more general interest has been developed by Kennedy and Cabral (1985), using inorganic carrier system. The addition of Ti^{4+} or Zr^{4+} chloride salts to water results in pH-dependent formation of gelatinous polymeric metal hydroxide precipitates wherein the metals are bridged by hydroxyl or oxide groups. By conducting such a precipitation in a suspension of microbial cells, the cells have been entrapped in the gel-like precipitate formed. In continuous operation, titanium hydroxide-immobilized cells of *Acetobacter* were employed to convert alcohol to acetic acid.

3.2.3.3. Cross linking:

Microbial cells can be immobilized by cross-linking each other with bi- or multifunctional reagents such as glutaraldehyde (Novarro, and Durand, 1977), toluenediisocyanate (Kennedy and Cabral, 1985) was used for cross-linking obviously imposes limitations for the general applicability of these procedures. Apart from chemical cross-linking, procedures employing physical processes, such as flocculation (Lee and Long, 1974) and pelletization (Mcginis, 1985), also benefit the

immobilization techniques because of strong mutual adherence forces of some microbial cell cultures. Bacterial cells can be cross-linked using agents such as glutaraldehyde to join cells together to provide viable cells. If only interested in the enzyme then a non-viable cell can be produced by heating and has been used to aggregate bacterial cells directly after fermentation and used for glucose isomerase activity. Fungal cells have been dried to produce an aggregated mass still have alpha-galactosidase activity used to refine sucrose. A different type of aggregation uses Ti^{4+} and Zr^{4+} chlorides, which produce a polymer metal hydroxide precipitate that aggregates the cells. Animal cells have not been immobilized extensively by this method as many procedures involve toxic chemicals.

3.2.3.4. Entrapment:

The most extensively studied method in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. Among the above matrices, polyacrylamide has been widely used by several workers (Martinsen, et.al., 1989). The entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. The other procedures for network formation for cell entrapment are precipitation, ion exchange gelation, and polymerization. The precipitation techniques are exemplified by collagen (Kurosawa, et al., 1989), cellulose and carrageenan (Axelsson, et.al., 1994). Entrapment of cells in alginate gel

is popular because of the requirement for mild conditions and the simplicity of the used procedure. Several reports on alginate gel are available (Jamuna, et.al., 1992).

κ -carrageenan is one of the earliest gel materials used for cell immobilization for continuous production of l-lactic acid by *Escherichia coli (*Ogbonna et.al., 1989). The immobilization procedure is similar to alginate. Using k -carrageenan, Takata *et al*. (1978) reported that the immobilized *Brevibacterium flavum* attained high stability against several denaturing chemicals. The rate of cell leakage could be lowered by hardening the gel with potassium cations. Similarly several other natural polymers such as agar, agarose, pectin and gelatin were also employed for cell immobilization. The reversible network formed is affected by certain calcium-chelating agents like phosphates, Mg^{2+}, K^{+} and EDTA and the gel integrity was poor.

3.2.4. Immobilization matrices:

Immobilization matrices must prevent the bacteria from dislodging from the matrix and flowing downstream. An ideal immobilization matrix would be functional at ambient temperatures, survive harsh wastewater conditions including contaminated water and turbidity, and allow the flow of nutrients and oxygen and analytes through the matrix along with wastes out. It would also prevent cell flow within the matrix. There are several types of immobilization matrices used for whole cells studied today.

3.2.4.1. Alginates:

Common immobilization matrices include naturally occurring alginates. Alginates are formed by converting mannuronic and guluronic acid into their salt forms of mannuronate (M) and guluronate (G). They are copolymers consisting of (1-4) linked β-D-mannuronic acid and β-L-guluronic acid (Smidsrod and Skajk., 1990).

Alginates are linear polymers comprised of blocks of M and G, or alternating GM blocks. Alginates are often used in the food industry as gelling compounds, such as in the production of the meat-like chunks found in pet food. Alginates are ionically crosslinked between the carboxylic acid elements through divalent ions like Ca+2. Because their crosslinks are ionic as opposed to covalent, they are easily broken apart by cationic scavengers such as sodium citrate and chelators such as ethylene diamine tetra acetic acid (EDTA) (Smeds and Grinstaff, 2001, Lu *et al.*, 2000). In addition to the weak bonding structure, natural hydrogels are also susceptible to biodegradation, making their use somewhat limited depending upon the cell type being immobilized.

3.2.4.2. Photopolymers:

Another commonly used class of immobilization matrices is photopolymers. Most photopolymers utilize visible or ultraviolet light to crosslink the monomers used in the formation of the matrices. Some photopolymers utilize harsh chemical initiators to facilitate the polymerization. A photon from the light source breaks the photo initiator into groups of highly energized radicals. The radicals then react with the resident monomer in solution and initiate the thermoset polymerization (Bryant *et al.*, 2000). This method has a major drawback of loss of cells.

3.2.4.3. Sol gels:

Sol gels are hybrid organic-inorganic compounds that are a bridge between glasses and polymers (Livage, 1997). Sol gels are rigid, thermally and structurally stable, and transparent, making them very useful for the immobilization of cells that are luminescent or show other visible changes when sensing the environment. Most commonly used sol-gels are silica gels which are made by the hydrolysis and

condensation of silicon alkoxides that produces alcohol, a cell killer (Nassif *et al.,* 2002). Recently, aqueous silica gels formed at room temperature have been found to effectively immobilize viable bacterial cells (Brinker and Scherrer, 1990, Yu, *et al.,* 2004). The aqueous silica provides a non-toxic and biologically inert environment that shows an increase in overall cell viability when formed in the presence of glycerol (Nassif, et.al., 2003).

3.2.4.4. Thermally reversible gels:

Thermally reversible gels, while most commonly used in drug delivery systems, are quickly being viewed as a potential matrix for immobilization. In aqueous solutions, thermally reversible gels undergo volume-phase transitions about a certain temperature (Sun, et *al.,* 2003). They form collapsed, dehydrated, hydrophobic gels above a lower critical solution temperature (LCST) and swollen, hydrated, hydrophilic dispersed solutions below the LCST. Cells can be easily immobilized by mixing cells with the aqueous thermally reversible gel solution at low temperatures. When the solution temperature is raised above its LCST, the cells will be immobilized within the hydrogel matrix.

3.2.4.5. Modified methods of cell immobilization:

Entrapment of microbial cells within the polymeric matrices is preferred by many researchers. Among the various methods, alginate gels have received maximum attention. There are several studies on the composition of alginate and their suitability for cell immobilization (Nguyen and Luong, 1986; Kurosawa et al., 1989; Mignot, and Junter, 1990). Studies on the diffusional characteristics of the immobilized system are being carried out to provide a better understanding on the

microenvironment prevailing near the immobilized cells (Axelsson, et. al, 1994; Westrin, 1990; Willaert, and Baron, 1994; Teixeira, et.al., 1994; Jamuna et.al., 1992; Ogbonna et.al., 1989; Vorlop, and Klein, 1981) which will enable researchers to optimize the immobilization protocols (Nishida et.al., 1977; Takata et.al., 1978) and to improve the stability of the gel beads by modifying the protocols like hardening the beads by glutaraldehyde treatment (Ruggeri et.al., 1991; Yamagiwa et.al., 1993).

Incorporation of additional component into the gel matrix to improve the mechanical strength has been tried. Several components such as silica (Chu et.al., 1995), sand, alumina, and various gums are generally used. In addition, the gel particles are further strengthened by treating with various cross-linking agents, such as glutaraldehyde. Chu *et al*. (1995) reported the polyelectrolyte complex gel prepared from xanthan and chitosan for immobilization of *Corynebacterium glutamicum* having fumarase activity. By mixing two opposite-charged electrolytes, a complex resulted due to electrostatic interactions. Generally, these complexes were obtained as precipitates, but Sakiyama *et al* (1993) and Chu *et al*. (1995) obtained moldable chitosan/ k -carrageenan and xanthan/chitosan complex gels in the presence of NaCl. It has been observed that the cells immobilized in these complexes were very stable and exhibited 5-fold higher activity compared to free-cells. The pore size was found to be similar to that of polysaccharide gel. In a similar study, Pandya and Knorr (1991) used low molecular weight compounds which were immobilized in complex coacervate capsules consisting of water-soluble chitosan salts or acid-soluble chitosan cross-linked with k -carrageenan or alginate. This type of coacervate

capsules could be used for cell immobilization and simultaneously the presence of chitosan salts in the capsule will affect permeabilization of the cells.

3.2.5. Biotransformations by immobilized microbial cells:

The field of biotransformations using immobilizing cells has been expanding in recent years. Schmauder *et al*. (1991) reviewed the state of art of this area and have summarized the research output so far available.

3.2.5.1. Emerging trends:

Whole-cell immobilization as a tool to intensify microbiological processes has been well established. Several examples of production of a variety of biochemicals by immobilized cells have been successfully demonstrated and are represented in Table. 3.2.5.1.

Table 3.2.5.1. Production of biochemicals and enzymes by immobilized whole cells.

Product	Organism	Support	References
A) Antibiotics			
Actinomycin D	*Streptomyces parvullus*	Ca-Alginate	Dalili and Chau, 1988
Bacitracin	*Bacillus Sps.*	Polyacrylamide	Morikawa et. al., 1980
Candicin	*S. griseus*	κ- Carrageenan	Constantinides and Mehta, 1991
Cyclosporin A	*Tolypociadium inflatum*	κ- Carrageenan, Celite	Foster et.al., 1983; Chun and Agathos, 1993
Hybrid Antibiotic	*S.llvidans*	Self Aggregate pellets	Sarra et.al., 1997
Mithramycin	*S. iverini*	Agar	Egresi et.al., 1992
Neomycin	*S.fradiae*	Cellulose beads	Park et.al., 1994
Penicillin G	*P.chrysogenum*	Polyacrylamide celite, κ-carrageenan	(Jones et.al., 1986; Kalogerakis, et.al, 1986; Deo and Gaucher, 1984; Mahmoud et.al., 1987 a, 1987b), Keshavarz et.al., 1990.

Rifamycin B	*S. rimosus Pf12ER 182.34-2*	Glass wool	Farid et.al., 1994.
B) Organic acids			
Citric Acid	*Aspergillus niger, Yarrowia lipolitica*	Na-Alginate, Agar, Hollow Fiber, Polyacrylamide, Polyurethane foam, Na-cellulose sulphate	Khare et. al., 1994; Chung and Chang, 1988; Mittal et.al., 1993; Sanroman et.al., 1994; Ostr, et.al., 1994; Mansfeld et.al., 1995
Lactic Acid	*Lactobacillus delbreackii, L.cursei, L.lactis sps lactis, L.helveticus*	Resin, Ca-alginate, Locust beand gel	Jianlong et.al., 1994; Kaufman et.al., 1994; Norton et.al., 1994; Cachon et.al., 1995.
Acetic acid	*Acetobacter Sp. K-1024*	κ-carraggenan	Mori et.al., 1989
Gluconic	*Aspergillus niger*	Ca-alginate	Rao and Panda, 1994
Kojic acid	*A. oryzae*	Alginate	Kqak and Rhee, 1992
Fumaric acid	*Rhizopus arrhizus*	Cork, clay	Buzzini et.al., 1993
Gibberilic acid	*Gibberilla fujikuroi*	Ca-alginate	Saucedo et.al., 1989
Vanillic acid	*Pseudomonas fluorescens*	Na-alginate	Bare et.al., 1994
C) Enzymes			
Peptidase	*Yeast*	ENT-2000	Paz et.al., 1993
β-amylase	*Bacillus megaterium*	Acrylamide	Ray et.al., 1995
Glucoamylase	*Aureobasidium pullulans*	Ca-alginate	Federici et.al., 1990 a, 1990b
Ribonucelase	*Aspergillus clavatis*	PVA	Abraham et.al., 1991
Protease	*Humicola lutea*	Wort agar	Grozeva et.al., 1993
Alkaline Phosphatase	*E.coli*	κ-carrageenan	Mannin et.al., 1989
Glucose oxidase	*A.niger*	Wheat, rye	Federici et.al., 1994
Hydantinase	*Pseudomonas sp.*	Polyacrylamide	Kim et.al., 1994
Lignin peroxidase	*Phanerochaete chysogenum*	Polystyrene-divinyl benzene	Ruckenstein and Wang, 1994
D)Bio-transformations			
Ethanol	*Zymomonas sp., Yeast, Z.mobilis, Kluyveromyces marxianus, S.cerevisiae*	Resin gel, Polymers, Ca-alginate, Fibrous matrix, PVA,	Ida et. al., 1993; Lu and Fujimura, 1993; Kazuaki et.al., 1994; Castellar et.al., 1994; Vorlop et.al., 1993;

		Microcarriers	Gelson and Thomas 1993; Nolan and McHale, 1994; Mebin and Shich,1992; Cheong et.al., 1993
Acetone, butanol, ethanol	*Clostridium acetobutylicum*	Bonechar, Carrageenan	Qureshi and Maddox, 1995; Davison and Thompson, 1993
Xylitol	*Candida pelliculosa,* *Methanobacterium sps.*	Photocrosslinkable Resin ENT 4000	Nishio et.al., 1989

Though initially our knowledge on physiology of immobilized cells was limited and hypothetical, the use of microelectrodes and development of noninvasive techniques to study the immobilized cells under microenvironment have revealed significant information pertaining to metabolic structural alterations occurring in the cell under immobilized phase. Though a variety of carrier materials have been tried, there are very few reports comparing these in terms of their performance, long-term stability, and cost. The observations made with immobilized cells and altered morphology indicate the influence of anchorage on cell metabolism. An important area of research requiring greater focus is the bioreactor design and its long-term operation. Except for a couple of experimental ventures, most of the experiments have been carried out on a very small scale, and hence very difficult to scale up. The future research should centre around not only for developing feasible microbiological processes with immobilized cells but also for carrying out extensive research in bioreacter design to solve some of the engineering problems, specially the ones that are connected with diffusional limitations.

3.2.6. Application of immobilized cells for biodecaffeination:

Caffeine is an alkaloid naturally occurring in coffee, cocoa beans, cola nuts and tea leaves, and is a central nervous system stimulant. It is known to show toxicity when fed in excess and is even mutagenic in-vitro (Friedman and Waller, 1983a and b). Excessive consumption of caffeine through beverages is associated with a number of health problems (Friedman and Waller, 1983a and b, Srisuphan and Bracken, 1986, Dlugosz et.al., 1996). Increasing knowledge of the effects of caffeine on human health led to the development of processes for decaffeination using solvents which are considered unsafe for humans. Biotechnological means of decaffeination have been considered as safe alternatives for the conventional decaffeination processes. Since 1970's several studies have been conducted by several groups in the world on the identification of caffeine degrading organisms for possible use in the development of biodecaffeination technologies.

Caffeine degrading bacteria and fungi have immense potential in the decaffeination processes for utilization of coffee, tea and other caffeine containing wastes which are otherwise unusable and pose sever health and environmental problems (Roussos, et.al., 1995). Although several reports on the use of free cells of bacteria and fungi for the degradation of caffeine are available, they are limited to solid state fermentation of caffeine containing agro wastes (Jarquín, 1987). Caffeine is now being determined as a marker for contamination of water and processes involving decontamination of caffeine laden waste waters have high environmental significance. Literature in this area is scanty. Middelhoven and Beckker- (1982) report the immobilization of a caffeine-resistant strain of *Pseudomonas putida*

isolated from soil in agar gel particles which were continuously supplied with a caffeine solution in a homogeneously mixed aerated reaction vessel. The caffeine degradation was monitored in this reactor system. No other reports are available on the immobilization of microbial cells for the degradation of caffeine. Caffeine degrading microorganisms utilizing caffeine as the sole source of carbon and nitrogen have been isolated and characterized which have enzymes that bring about the actual degradation of the substrate. In this communication, we report the isolation of an effective caffeine-degrading microbe (*P. alcaligenes* MTCC 5264) from soil, its growth and decaffeination studies.

3.3. MATERIALS AND METHODS:

Sodium alginate, κ- carrageenan, agar, potassium chloride, peptone and yeast extract were procured from M/s HiMedia Labs, Mumbai, India. Sucrose, potassium dihydrogen orthro phosphate, ammonium sulphate and toluene were from Qualigenes Fine Chemicals, Mumbai, India. Gelatin (Porcine Skin), γ-aminopropyl trimethoxy silane, sodium cyanoborohydride, polyethyleneimine and glutaraldehyde were procured from Sigma-Aldrich, St. Louis, USA. All other chemicals and reagents were procured from standard sources and were of highest purity. Double distilled water was used in all the experiments.

3.3.1. Studies on biodecaffeination by *P. alcaligenes* MTCC 5264:

Pseudomonas alcaligenes MTCC 5264 which was isolated in our lab from soils of coffee plantations and maintained by sub-culturing on caffeine containing nutrient agar slants was used for the present study. Studies on the degradation of caffeine by this strain, immobilization of whole cells in different matrices and biodecaffeination of pure caffeine solutions and caffeine containing real samples were carried out.

3.3.2. Growth of cells and induction for biodecaffeination:

A loop full of actively growing culture of *P. alcaligenes* MTCC 5264 was transferred to 100 ml of nutrient broth containing 0.3 g/l caffeine and incubated at 30^{o}C in an orbital shaker set at 150rpm for 24 hrs. A 5% v/v of the 24 hrs grown pre inoculum was transferred to 100 ml of nutrient broth containing 0.3g.L^{-1} caffeine and grown under the same conditions.

Aliquots of samples (10 ml) were drawn from each flask at known intervals of time for the measurement of cell growth. Biomass accumulated in the flasks after 24 hours was harvested by centrifugation in a bench top centrifuge (Kubota, Japan) at 16,000g for 20 min at 0-4^0 C to form a pellet. The biomass pellet was aseptically transferred into a 500ml flask containing 100ml of caffeine liquid medium (CLM) containing 1g/l caffeine and incubated at 30^{o}C on the orbital shaker for a period of 48hrs for inducing the cells to degrade caffeine. These induced cells (incubated in CLM for 48 hrs) were harvested by centrifugation as before. The cells were washed several times to remove caffeine. 10 gms of these induced cells were suspended in 100 ml of phosphate buffer, which were used for caffeine degradation experiments.

3.3.3. Caffeine degradation experiments:

Induced cell suspension (10 ml) was aseptically transferred to 90ml of CLM containing 1g/l caffeine and incubated on a rotary shaker at 30^{o}C set at 150 rpm for 24 hours. 0.5 ml aliquot samples were drawn at 2, 4, 6, 12 and 24 hours, centrifuged and the supernatants were analyzed for residual caffeine content.

Studies on the effect of pH and temperature on biodecaffeination by the isolate were carried out by incubating the induced cells in CLM containing 1g/l caffeine, which was adjusted to different pH and temperature.

For studying the effect of inoculum on caffeine degradation, induced cell suspension was added to CLM containing 1g/l caffeine so as to obtain inoculum levels of 1.2, 2.5, 5.0 and 7.5%w/v (based on wet weight). Studies on the effect of caffeine concentration on the biodecaffeination efficiency of the isolate were carried out by incubating the induced cells in CLM containing caffeine in the concentration

range of 1-5 g/l. Samples were drawn at known intervals of time and analyzed for caffeine content.

3.3.4. Studies on biodecaffeination by immobilized *P. alcaligenes* MTCC 5264:

3.3.4.1. Immobilization of cells:

Entrapment method of immobilization of the cell was carried out using different matrices. A scheme of preparation of immobilized cells of *P. alcaligenes* MTCC 5264 is given in Scheme A below:

Scheme A: Preparation of immobilized cells of *P. alcaligenes* MTCC 5264 for biodecaffeination.

Slant (*P. alcaligenes* MTCC 5264)

↓ (Loop full of culture)

10 ml Nutrient Broth (0.3g/l caffeine)

↓ **12 hrs**

100 ml Nutrient Broth (0.3g/l caffeine)

↓ **24 hrs**

Harvesting Biomass

↓ **Washing**

CLM (1 g/l Caffeine)

↓ **48 hrs**

Harvesting Induced Biomass

↓ **Washing**

Cell pellet (resuspended in Buffer)

↓

Mixing with Matrix

↓

Curing

↓

Packed Bed reactor studies

a) Gelatin:

Gelatin (porcine skin) was used as the matrix for the immobilization of cells of *P. alcaligenes.* Solutions of gelatin in the concentration range of 3-20% was prepared by dissolving required weight of solid gelatin powder in 100 ml water maintained at 60^{O}C. Cells of *P. alcaligenes* (Ten grams by wet weight) were mixed with 100 ml of gelatin maintained at 30^{O}C and mixed thoroughly to obtain a homogeneous suspension. This suspension was then dropped slowly into an aqueous solution of 1.5% v/v glutaraldehyde at 4°C using a surgical syringe. Another approach for achieving good bead characteristics was tried in which the modified curing solution consisted of toluene and chloroform in the ratio of 3:1 with 1.5% glutaraldehyde. Another modification of the curing solution using a mixture of paraffin oil and chloroform in the ratio of 3:1 with 1.5% glutaraldehyde.

b) κ–Carrageenan:

A 3% w/v solution of κ–Carrageenan was prepared by dissolving 3 grams of κ-Carrageenan in 100ml of distilled water maintained at $60^{o^{\circ}}$C. The solution was then cooled to 40-$45^{o^{\circ}}$C and 10 grams wet weight of cells previously equilibrated to the same temperature was suspended in the solution. The mixture was maintained at a temperature of 40-45^{o}C and mixed thoroughly to obtain a homogenous suspension. This suspension was then dropped slowly into an aqueous solution of 2% KCl at 4°C using a surgical syringe. Different concentrations of κ–Carrageenan and potassium chloride in the range of 3.5 – 15%, 2-20% respectively were tried and the curing time was also optimized.

c) Agar:

Immobilisation of the cell of *P. alcaligenes* MTCC 5264 in agar was done by dissolving 3 gm of agar in 100ml of distilled water 80^{O}C and then cooling it to 40^{O} C with subsequent mixing of 10 gm of cell equilibrated to the same temperature. After thorough mixing, the suspension was dropped through a syringe into water maintained at 4 ^{O}C.

d) Sodium alginate:

Sodium alginate solution (3% w/v) and was prepared and sterilized. 10 grams cell by wet weight was mixed thoroughly in the alginate solution. The cell suspension was dropped slowly into a solution of 0.22 M Calcium chloride at 4^{o}C. The beads were kept for primary curing at 4^{o}C for 24 hours and secondary curing was done in 0.02 M calcium chloride for 48 hours at 4^{o}C. Traces of calcium chloride were removed by through washing with distilled water and used for further studies.

3.3.4.2. Biodecaffeination by immobilized cells:

a) Shake flask experiments:

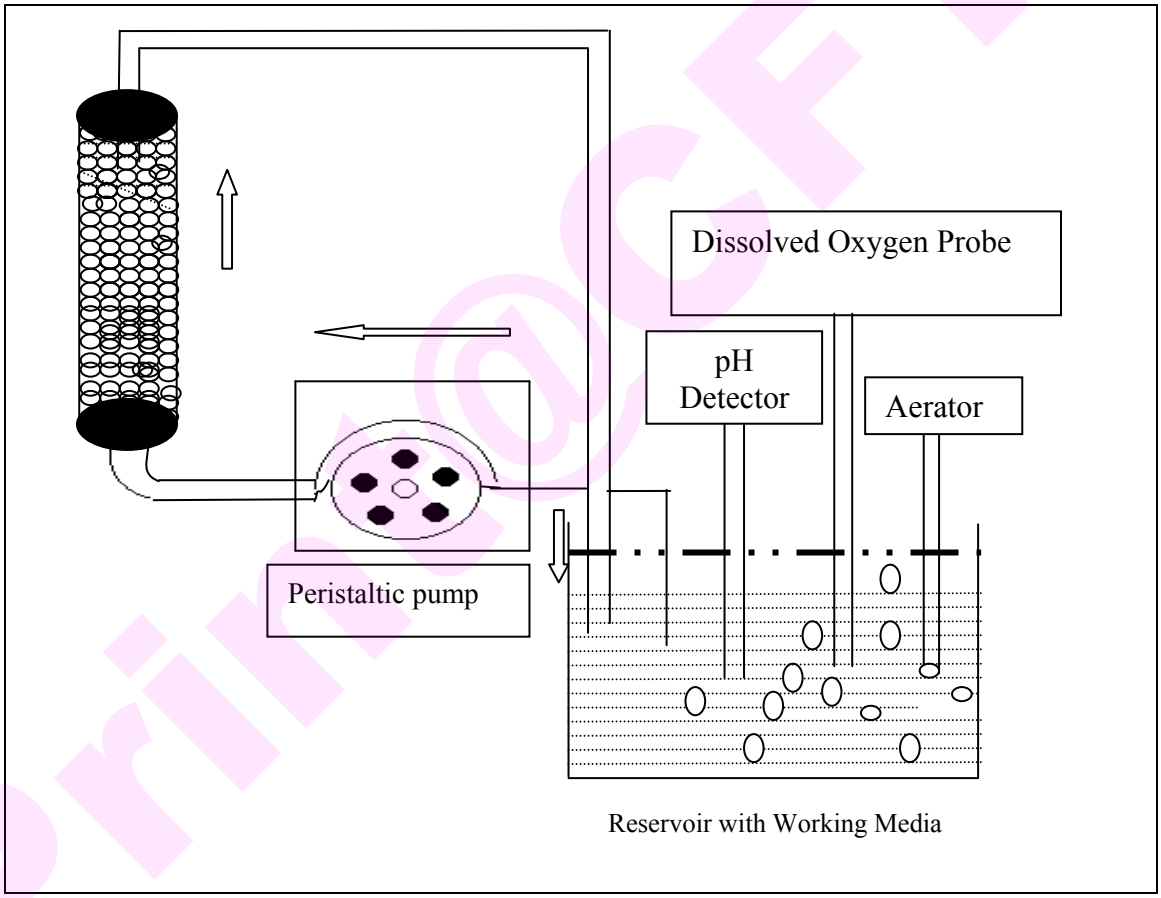
Immobilized cells of *P. alcaligenes* MTCC 5264 were tested for the ability to degrade caffeine in shake flask. 10 gm of the immobilized beads were incubated with 100 ml of the working medium under agitation on a rotary shaker set at 200 rpm at 30±2^{o}C.

b) Packed column reactor studies:

Cells of *P. alcaligenes* MTCC 5264 immobilized in different matrices was packed into glass columns of 2 cm internal diameter and 20 cm length. The total bed volume was 34.54 ml. Working medium, i.e. PO_4 Buffer, 5µM, and pH 7.2 containing

1g.L^{-1} caffeine was then passed through the column at a flow rate of 5 ml/minute. The medium was continuously saturated with oxygen by bubbling air into the reservoir (Figure 3.3.4.2.). Samples were drawn at regular intervals of time and were analyzed for the residual caffeine content by HPLC. Caffeine degradation (%) was calculated using equation 1.

Figure 3.3.4.2. Schematic of immobilized cell based reactor for biodecaffeination studies.



3.3.5. Optimization of parameters for biodecaffeination using immobilized cells of *P. alcaligenes* MTCC 5264:

Following parameters were optimized for biodecaffeination using immobilized cells of *P. alcaligenes* MTCC 5264.

3.3.5.1. Effect of Buffer composition and phosphate concentration:

Various buffers like Tris-Cl, acetate, carbonate and phosphate buffer were tested for degradation of caffeine by *P. alcaligenes* MTCC 5264 by incubating the immobilized cells with different buffers containing caffeine. The effect of phosphate concentration on stability of calcium alginate was determined by incubating the immobilized cells with phosphate buffer containing phosphates in the range of 5µM to 100mM.

3.3.5.2. Effect of pH:

For maximal caffeine degradation it is important that the working medium should be at an optimal pH, which would maintain the cells in their viable state, and the enzymes responsible for decaffeination are highly active. Phosphate buffer at different pH was used for biodecaffeination of caffeine solution and the residual caffeine at different pH was recorded.

3.3.5.3. Temperature:

Immobilized cells of *P. alcaligenes* MTCC 5264 were incubated with phosphate buffer containing 1g.L^{-1} of caffeine which was adjusted to 18, 28, 32, 48 and 60^0C in a water bath and incubated for 96 hours and checked for biodecaffeination ability.

3.3.5.4. Effect of cell loading on the immobilization of whole cell for decaffeination:

Cell suspensions containing w/v of cells (wet weight) in 3% sodium alginate solution were prepared and dropped slowly into a 2% calcium chloride solution and cured in the same solution. The immobilized cells were then incubated with caffeine and their decaffeination ability was checked.

3.3.5.5. Effect of flow rate:

To enable a proper retention time of the sample in the column for increased contact an optimum flow rate is necessary. For this, flow rates of 1, 2, 3, 5, 7 and 10 ml/min were tested.

3.3.5.6. Oxygen availability:

The caffeine-degrading organism being an aerobe needs oxygen for its viability. Further, some of the enzymes involved in caffeine degradation pathway are also oxygenases necessitating the presence of molecular oxygen in the system. Therefore to check the requirement of oxygen for efficient degradation of caffeine, experiment was conducted without external oxygen supply.

3.3.5.7. Biodecaffeination of tea extract using immobilized cells:

Extract of tea sample was prepared by boiling 10 grams of tea powder in 100ml of distilled water then filtering it. The caffeine content of the extract was checked by HPLC analysis (Martin et al., 2000). The extract was then recirculated through the column at a flow rate of 5ml/min for 5 hours. Aliquots of samples (1ml) were collected at regular intervals and analyzed for residual caffeine through HPLC analysis.

% Caffeine degradation = (a- b) x 100/a ---------- Eqn.1

where**, a** = initial caffeine concentration, and

b = residual caffeine concentration

3.4. RESULTS AND DISCUSSION:

3.4.1. Studies on biodecaffeination by immobilized cells of *P. alcaligenes* MTCC 5264:

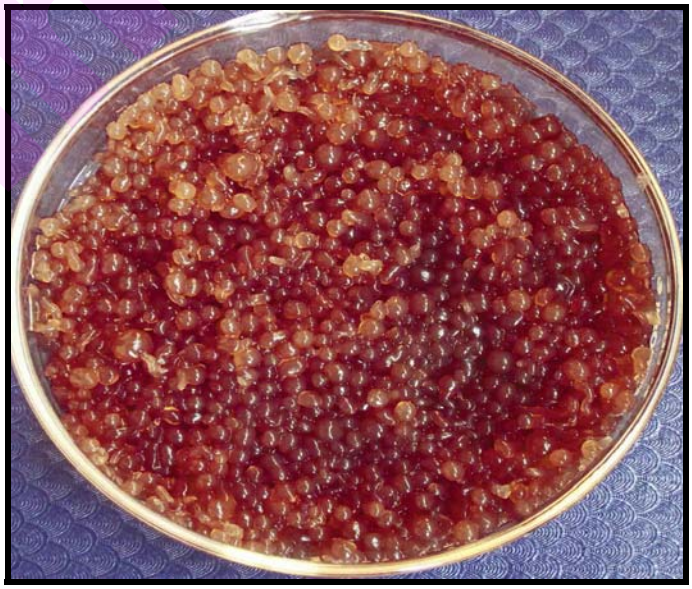
Biomass production and induction of the cells for Biodecaffeination was carried out as reported in Chapter 2, Section 2.3.2.8.

3.4.1.1. Immobilization of *P. alcaligenes* MTCC 5264 in different matrices:

a) Gelatin:

Gel formation was achieved by dropping the gelatin- cell suspension into cold water. Bead formation was not uniform and tailing of beads was observed. The beads were also not physically stable when packed into a column. Bead formation by cross-linking with glutaraldehyde was tried. During bead formation in the curing solution tailing of the beads was observed. The tailing of beads persisted even when the glutaraldehyde concentration was increased to 5% (Fig. 3.4.1.1).

Figure 3.4.1.1. Induced cells of *P. alcaligenes* MTCC5264, immobilized in gelatin.



Changing the curing condition by using toluene and chloroform in the ratio of 3:1 with 1.5% glutaraldehyde led to formation of beads with good strength and shape. But the use of toluene is toxic to cells. Another modification of the curing solution using a mixture of paraffin oil and chloroform in the ratio of 3:1 with 1.5% glutaraldehyde gave beads with good stability. However this approach necessitates extensive washing to remove the paraffin oil on the surface. Despite giving several washes a coat of oil persisted. When *P. alcaligenes* MTCC 5264 immobilized using this method was used for decaffeination, it was found that caffeine degradation was not considerable for further work to be carried out. Also because of the toxicity of the solvents used it's not a viable approach for use in case of real samples. Only 3% caffeine degradation was observed in 80 hours using gelatin as the matrix for bead formation (Figure. 3.4.1.2.).

Under Scanning electron microscope (SEM) of the beads appeared to be compact (Figure 3.4.1.3) probably having low porosity. The low porosity of the beads offers diffusional resistance to caffeine and oxygen entering the beads leading to a low efficiency of the beads. Also the high concentration of glutaraldehyde, used for cross linking may be toxic to the cell probably leading to loss of viability of many cells in the matrix, hence the low rate of caffeine degradation.

The beads obtained were not of uniform size and also lacked bead strength and hindered the flow, when packed into a column. Hence this matrix was not used for further studies.

Figure 3.4.1.2. Biodecaffeination of pure caffeine solution (1g.L^{-1}) by *P. alcaligenes* MTCC 5264 immobilized in different matrices.

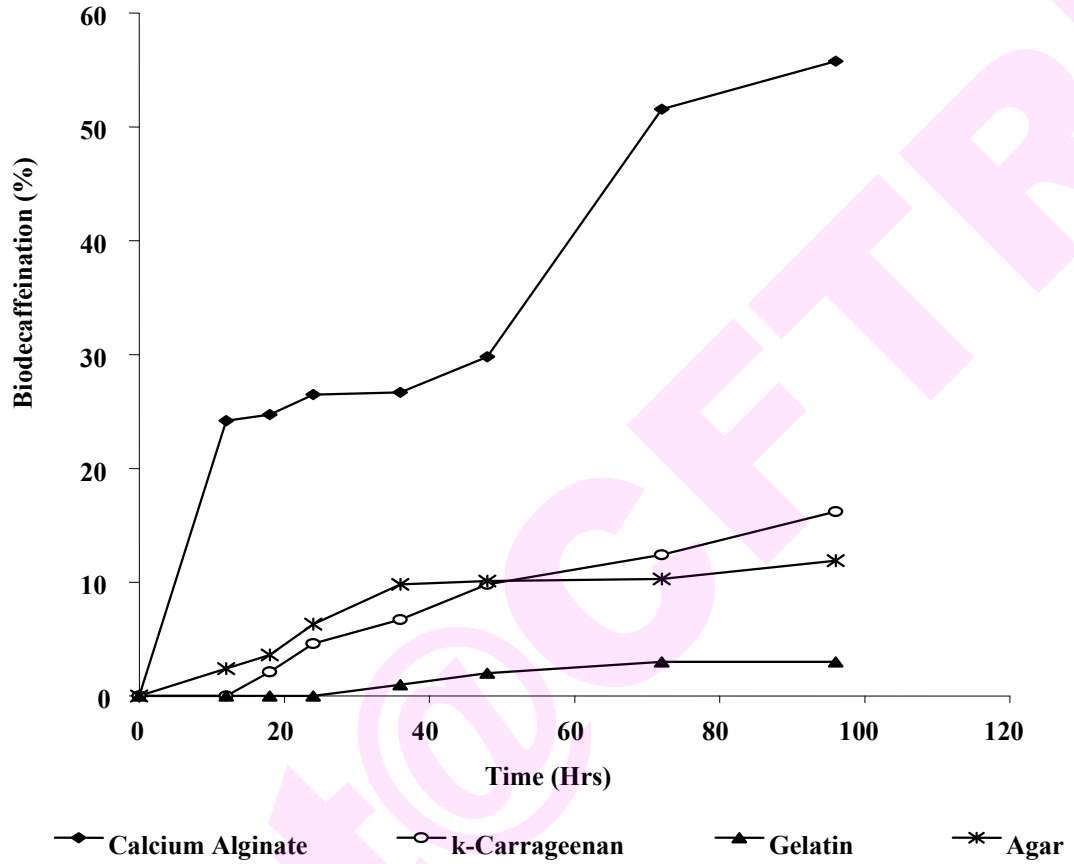
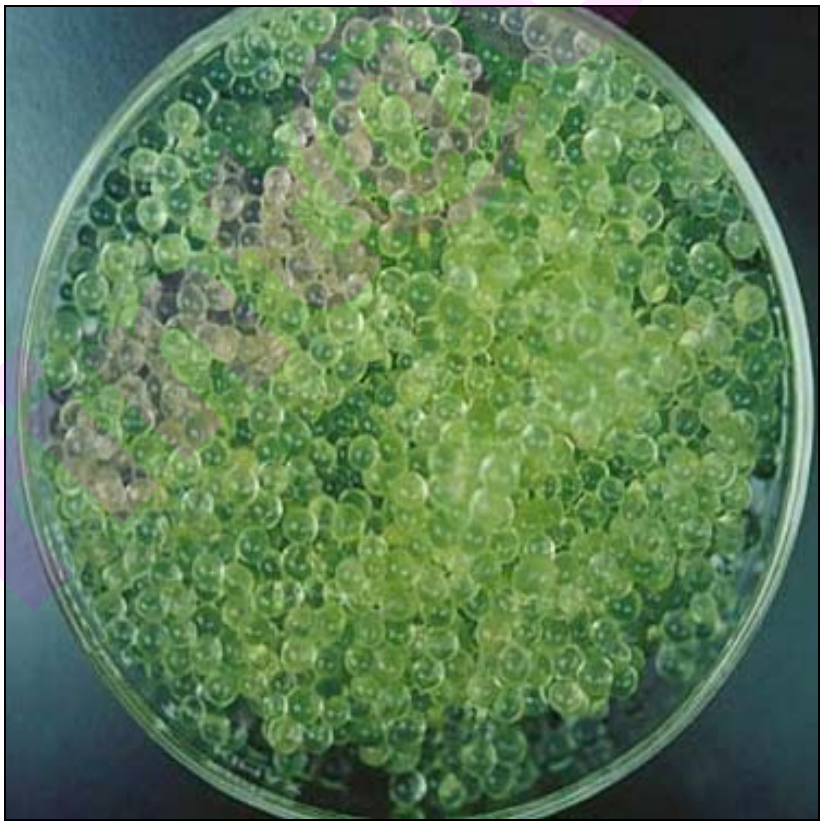


Figure 3.4.1.3: Scanning electron microscope photograph of *P. alcaligenes* MTCC 5264 immobilized in gelatin (2000 X).

b) κ–Carrageenan:

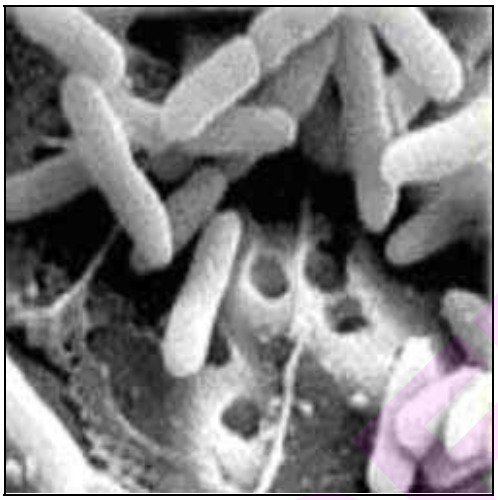
Induced cells of *P. alcaligenes* MTCC 5264 were immobilized in κ-carrageenan (Fig. 3.4.1.4). The beads formed by immobilization in κ–carrageenan were fragile. An initial curing in 20% KCl for 30 minutes at 4^{o}C and subsequent curing at the same temperature in a solution of 2% KCl for 24 hours was found satisfactory. But after the completion of curing and incubation of the beads with caffeine solution under agitation or packing in a column led to the rapid disintegration of the beads.

Figure 3.4.1.4. Induced cells of *P. alcaligenes* MTCC5264, immobilized in κ-carrageenan.



Only 16% biodecaffeination could be achieved by this matrix (Fig. 3.4.1.2).

Figure 3.4.1.5: Scanning electron microscope photograph of *P. alcaligenes* MTCC 5264, immobilized in κ-carrageenan (7000X).



Although the porosity of this matrix was good (Fig. 3.4.1.5), the low mechanical strength of the beads and low biodecaffeination efficiency of this matrix, its use was discontinued.

c) Agar:

The beads obtained using agar as matrix were soft and not able to withstand pressure when they were packed in column (Fig. 3.4.1.6). Only 12% decaffeination was possible after 96 hrs. (Fig. 3.4.1.2).

Figure 3.4.1.6. Induced cells of *P. alcaligenes* MTCC 5264, immobilized in agar.

d) Sodium alginate:

Of all the matrices tried sodium alginate was found to be the best in terms of bead uniformity, bead strength, handling conditions, porosity and caffeine degradation results. Figure 3.4.1.7., represents the cells of *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.

Figure 3.4.1.7. Induced cells of *P. alcaligenes* MTCC 5264, immobilized in calcium alginate.

Maximum caffeine degradation (55.67%) was observed with *P. alcaligenes* immobilized in calcium alginate beads (Fig. 3.4.1.2).

Figure 3.4.1.8: Scanning electron microscope photograph of *P. alcaligenes* MTCC 5264 immobilized in sodium alginate (5000 X).

From the electron microscopic picture of the beads (Fig. 3.4.1.6) indicates that the beads have high porosity and enable uniform distribution of the cells. The mild and non toxic nature of the gel also enables higher biodecaffeination rates. Hence it was the matrix of choice for further studies.

3.4.2. Optimization of parameters for decaffeination using immobilized cells of *P. alcaligenes* MTCC 5264 in calcium alginate beads:

The effect of buffer composition, phosphate concentration, pH, temperature, aeration, packed bed volume, cell loading and flow rate on the biodecaffeination of caffeine solution by *P. alcaligenes* MTCC 5264 was studied and the parameters for optimum biodecaffeination were optimized.

3.4.2.1. Phosphate concentration:

Figure 3.4.2.1., represents the efficiency of biodecaffeination by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate incubated in different buffers. No biodecaffeination was found in Tris-Cl, acetate and carbonate buffers, but complete decaffeination could be achieved with phosphate buffer. Therefore biodecaffeination studies using immobilized cells were carried out in phosphate buffer.

Initially a phosphate ion concentration of 100 mM was used for the decaffeination experiments and at this concentration of phosphate ions a rapid disintegration of the beads was observed. Salts like phosphates, potassium and EDTA are known to destabilize the calcium alginate beads by displacing the calcium ions from the alginate hydrogels (Kierstan and Bucke, 1977; Mattiasson, 1983) resulting in disintegration of the beads. Therefore experiments were conducted to see the effect of different concentrations of phosphate ions on the strength of the beads. Various

concentrations of phosphate ranging from 100 mM to 5 µM were tried and it was found that 5µM phosphate concentration was optimum for degradation of caffeine and maintaining the bead strength (Table 3.3.2.1).

Further studies on decaffeination were carried out at this phosphate concentration.

Figure 3.4.2.1.Effect of buffer composition on biodecaffeination by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.

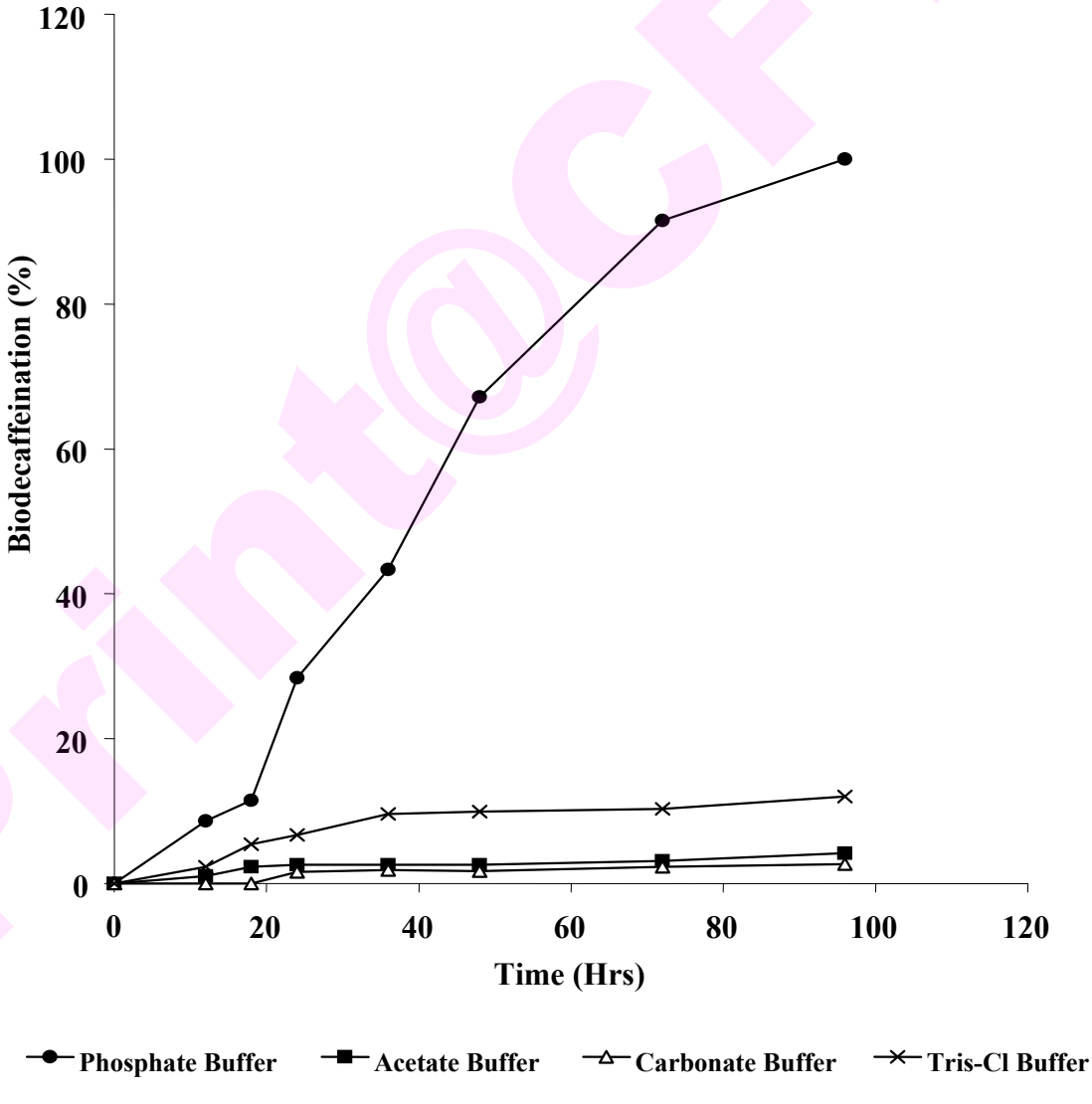


Table 3.4.2.1: Effect of phosphate concentration on strength and stability of calcium alginate beads with immobilized *P. alcaligenes* MTCC 5264.

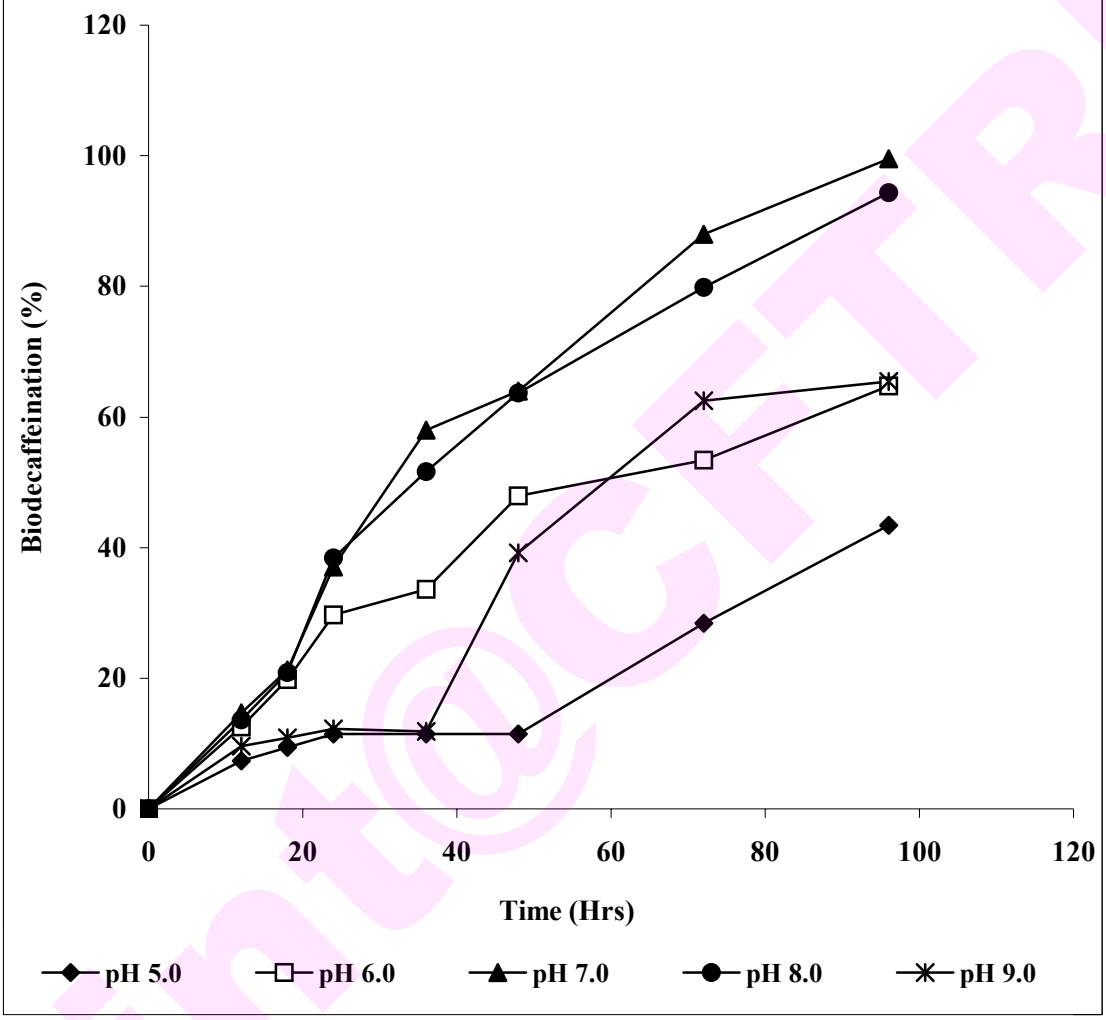
S.No	Phosphate Conc.	Time taken for bead disintegration in hrs
1.	100mM	10
2.	50mM	12
3.	100µM	35
4.	5µM	No disintegration

3.4.2.2. pH:

For maximal caffeine degradation it is important that the working medium should be at an optimal pH, which would maintain the cells in their viable state, and the enzymes responsible for biodecaffeination are highly active. Figure 3.4.2.2., shows the effect of pH on biodecaffeination by immobilized cells of *P. alcaligenes* MTCC 5264. Biodecaffeination at lower pH is very low due to the low activity of the enzymes in the cells. Only 43% decaffeination was possible at pH 5.0 and increased with increase in pH up to 8.0 (Fig. 3.4.2.2).

Maximum biodecaffeination (99.5) was observed at pH 7.0. Biodecaffeination was more than 94% even at pH 8.0, indicating that the cells are capable of biodecaffeination at neutral and slightly alkaline conditions. The cells however could biodecaffeinated only 65% caffeine at pH 9.0. This is due to the higher alkalinity of the medium where the enzymes are not active.

Figure 3.4.2.2. Effect of pH on biodecaffeination of cafeine solution (1g.L^{-1}) by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.

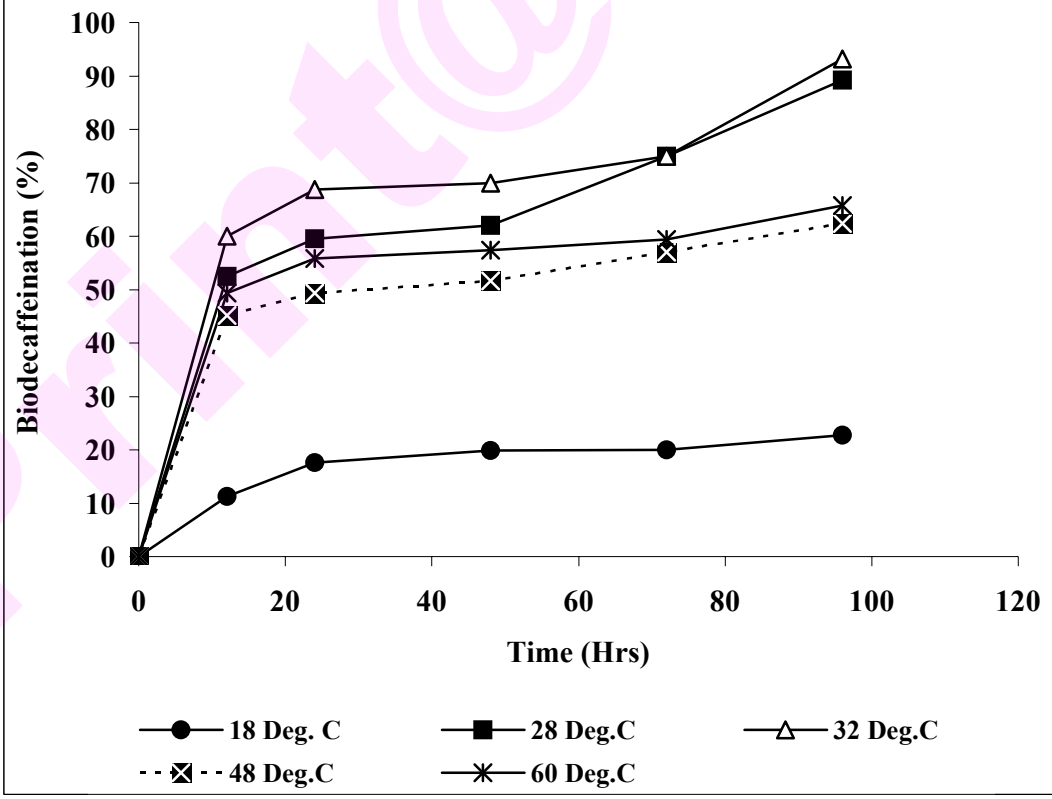


Studies on biodecaffeination by free cells of *P. alcaligenes* MTCC 5264 also show that the organism has the maximum biodecaffeination ability at pH 6.8-8.0 (Sarath et.al., 2005). Decaffeination was still possible under acidic (pH 5.0, 43% and basic, pH 9.0, 65%) conditions by the organism probably due to the protective effect of the calcium alginate matrix conferred on the cells. Further it is known that immobilization protects the biocatalysts from extremes of pH and temperature.

3.4.2.3. Temperature:

P. alcaligenes MTCC 5264 is a mesophilic organism and grows best at 30±2^{0}C (Sarath et.al, 2005). From figure 3.4.2.3., it can be observed that biodecaffeination is achieved at 32^{0}C. At lower temperature 18^{0}C the biodecaffeination is only 22%. Maximum biodecaffeination was observed at 28 and 32^{0}C (89.2 and 93.6% respectively). At these temperatures the cells are metabolically active and degrade maximum caffeine. Temperatures above this are detrimental to the cells leading to a loss of activity of the enzymes involved in the biodecaffeination. The organism however degrades 65% caffeine even at 60^{0}C and has the possibility of using the organism at higher temperatures also.

Figure 3.4.2.3. Effect of temperature on biodecaffeination by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.



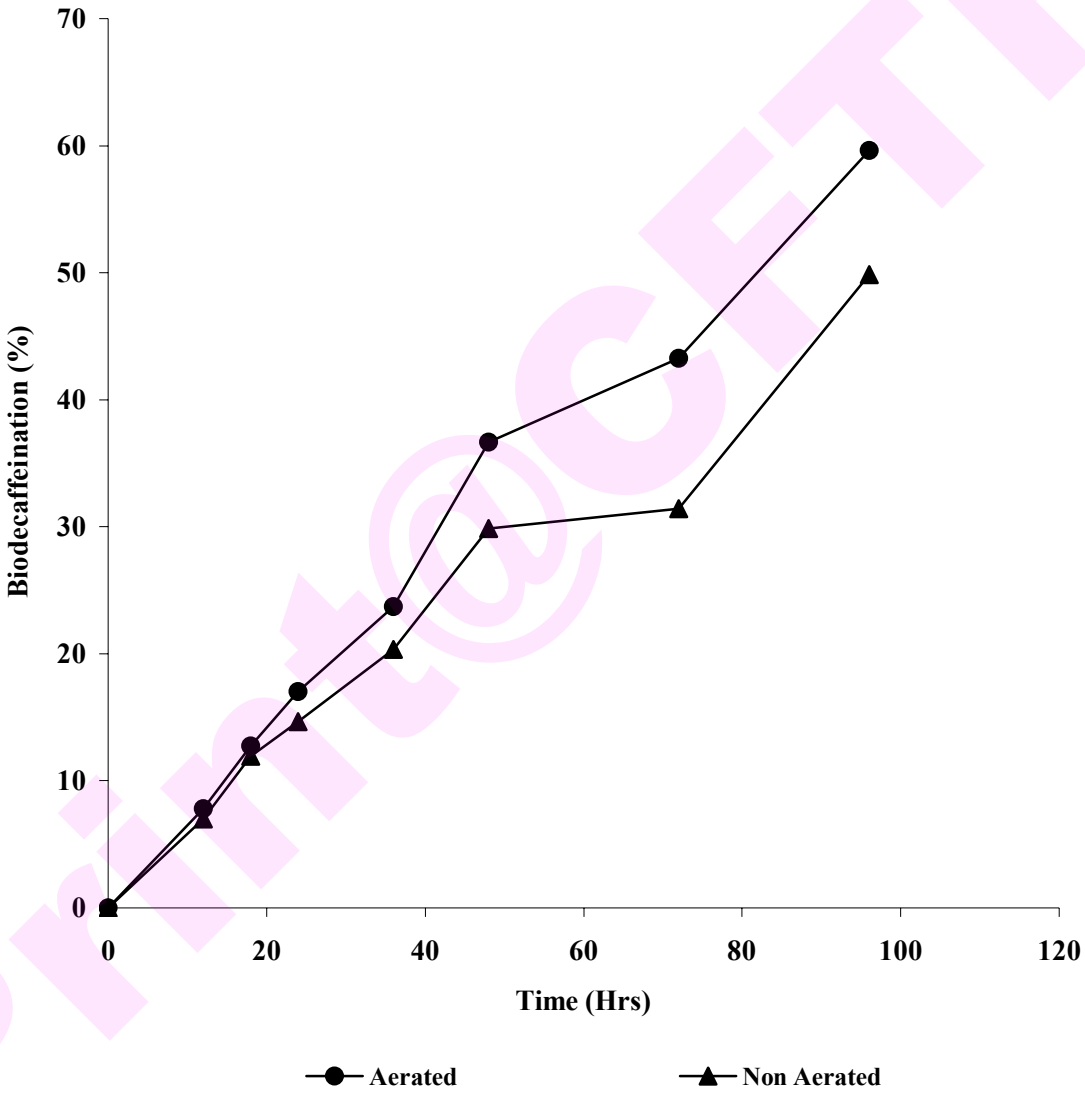
3.4.2.4. Oxygen availability:

The caffeine-degrading organism being an aerobe needs oxygen for its viability. Further, some of the enzymes involved in caffeine degradation pathway are also oxygenases necessitating the presence of molecular oxygen in the system. Therefore to check the requirement of oxygen for efficient degradation of caffeine, experiment was conducted without external oxygen supply. It was found that the degradation of caffeine was 45.9% at the end of 96 hours in comparison to the 55.9% caffeine degradation in 96 hours in the system with oxygen supply (Fig. 3.4.2.4).

This suggests that there is a requirement of external oxygen for more efficient caffeine degradation. In the experiments involving more than two columns most of the dissolved oxygen in the working medium is expected to be completely used up in the first part of the column itself, which may lead to a decrease in the efficiency of the system. In our experiments, aeration of the liquid medium in the reservoir was accomplished by using a simple aquarium pump. The experimental set up consisted of an extra reservoir after each column, which was aerated with a laboratory aerator. There was no significant improvement or decrease in the efficiency of decaffeination by the immobilized cell system indicating that oxygen consumption was not very rapid as was expected earlier. The low biodecaffeination levels in the packed bed reactor compared to shake flask studies indicate that the oxygen availability in the packed bed reactor is low leading to low rates of degradation of caffeine by the organisms. It might also be possible that most of the dissolved oxygen in the caffeine solution will have been consumed by the cells present in the beads at the inlet of the column leading to oxygen deprivation to the cells at the top leading to oxygen stress

and this might be accounting for the low caffeine degradation rate in the packed bed column.

Figure 3.4.2.4. Effect of aeration on biodecaffeination by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.

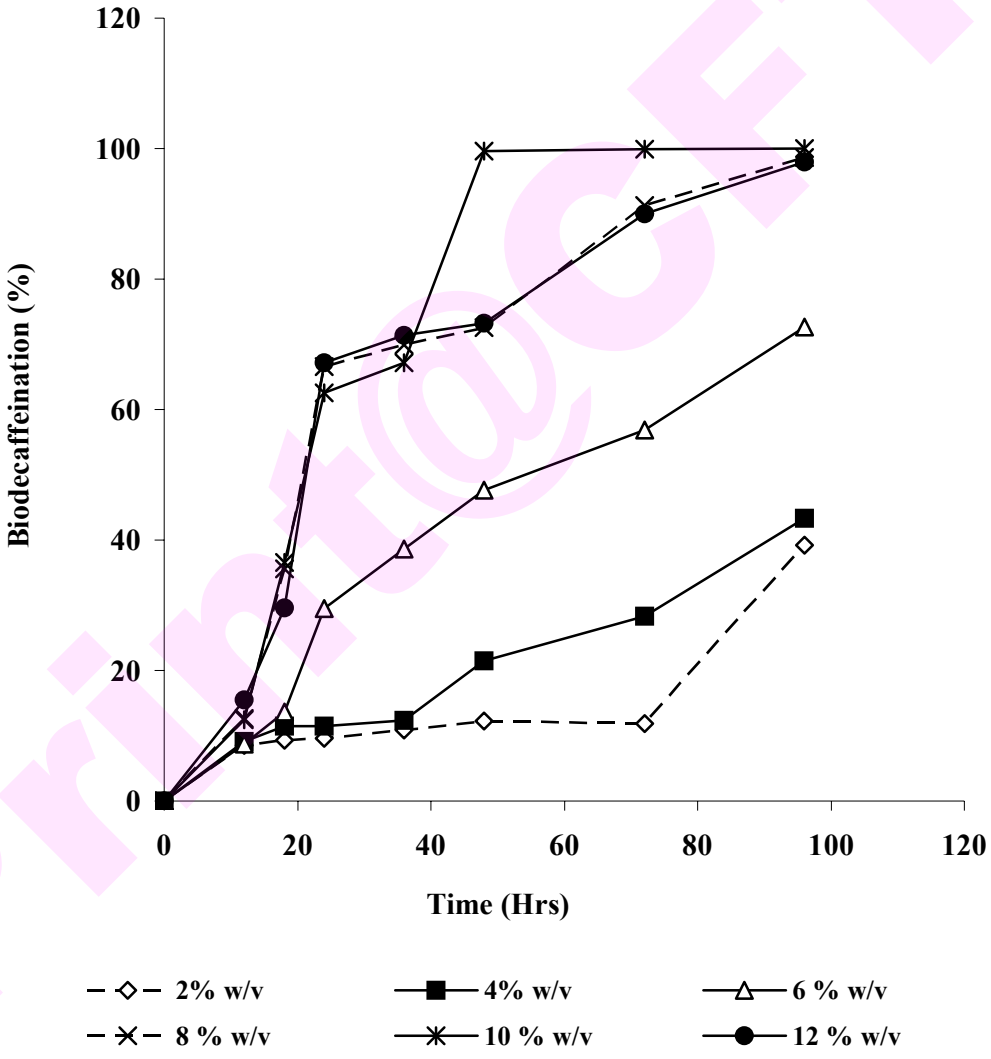


3.4.2.4.Effect of cell loading on the immobilization of whole cell for decaffeination:

Figure 3.4.2.5., represents the effect of cell loading on the biodecaffeination of caffeine solution by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate

beads. At low cell concentrations (2 and 4% w/w of cells) only 40-43 % decaffeination was achieved. This is supposed to be due to the low number of bacterial cells present for degrading caffeine in the solution. Caffeine being inhibitory to the cells might also be involved in the lower biodecaffeination levels.

Figure 3.4.2.5. Effect of cell loading on biodecaffeination by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate.



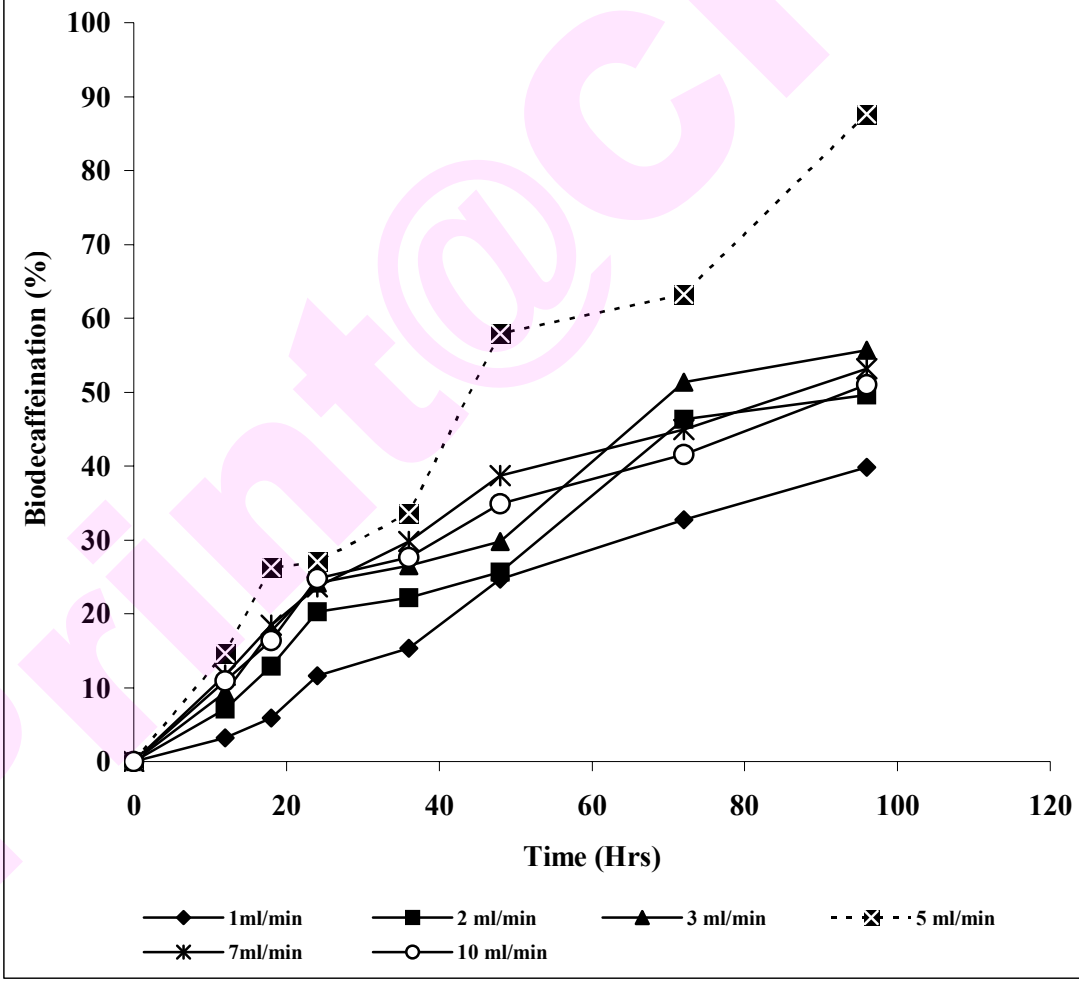
Biodecaffeination increased with an increase in the cell loading up to 10% w/w in the gel. It was found that 10 % w/v of the cells was the best for decaffeination in the packed bed reactor system and caffeine was almost completely degraded within 48 hrs of incubation. Cell concentrations above 10% showed a slightly lower efficiency of biodecaffeination, probably owing to the high cell density and competition for oxygen and other nutrients. Also the byproducts of caffeine metabolism might exert an inhibitory effect on the cells leading to lower level of biodecaffeination. So 10% cell loading was found to be optimum and was used for further studies.

3.4.2.6. Flow rate:

The utilization of a substrate by an immobilized biocatalyst depends on the retention time of the sample. Higher retention rimes enable better bioconversion and lower times lead to low substrate bioconversion. However too high retention times lead to accumulation of reaction products in the vicinity of biocatalysts leading to product inhibition, decreasing the efficiency of the immobilized bioreactor system. The retention times of any substrate in a flow through reactor are determined by the flow rate of the carrier buffer. In the studies on the optimization of flow rate for efficient biodecaffeination by immobilized cells of *P. alcaligenes* MTCC 5264, it was found that a flow rate of 5ml/min was optimum. 87.6 % biodecaffeination was achieved at a flow rate of 5ml/min (Fig. 3.4.2.6). At flow rates of 1, 2 and 3 ml/min, the biodecaffeination efficiency was only 39, 49 and 51% respectively. The lower flow rates lead to high retention times in the reactor and the time of contact of caffeine with the beads is high. However this will lead to accumulation of caffeine

degradation products like ammonia and carbon dioxide which limit the oxygen availability to the cells leading to low biodecaffeination levels. At higher flow rates, the retention times are too low leading to low contact times with caffeine and the biodecaffeination decreases. It was found that 5 ml/ min was optimum for efficient caffeine degradation in the system (Fig.3.4.2.6.).

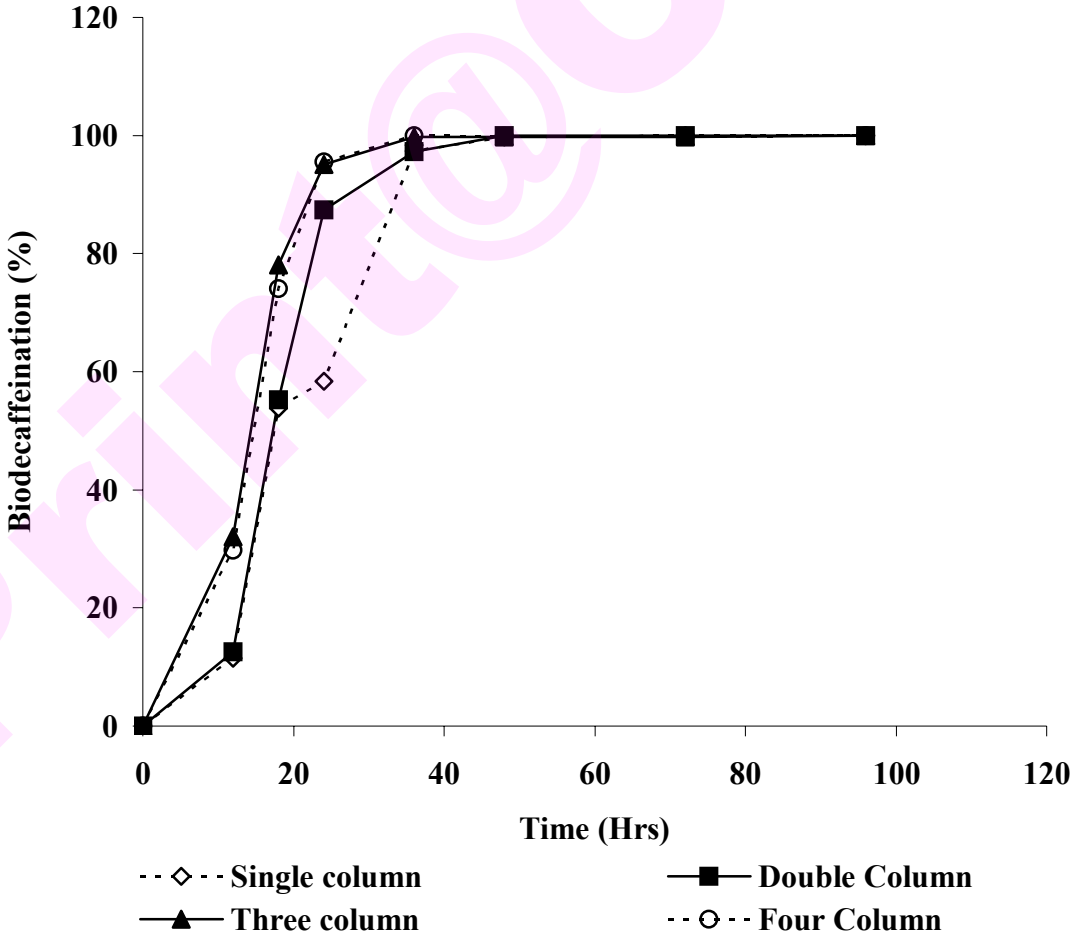
Figure 3.4.2.6. Effect of flow rate on biodecaffeination of caffeine solution by immobilized cells of *P. alcaligenes* MTCC 5264.



3.4.2.7. Bed volume:

It is known that as the height of the packed bed column increases the pressure on the beads increases. It has been reported that strength of alginate beads is not enough to withstand pressure in long columns. However, longer columns become necessary when larger contact areas are needed. In order to get better decaffeination by increasing the bed volume, studies were carried out in 3 and 4 column reactors. *P. alcaligenes* MTCC 5264 immobilized in Calcium alginate was packed into single and two columns of 2 cm internal diameter and 20 cm length.

Figure 3.4.2.7. Effect of packed bed volume on biodecaffeination by P. alcaligenes MTCC 5264 immobilized in calcium alginate.



In the single column reactor 97.3% of the initial caffeine was degraded within 36 hrs of incubation (Fig. 3.4.2.7), whereas 87.6% degradation of caffeine occurred within 24 hours of incubation in the two-column reactor. Faster degradation in the two-column reactor compared to the single column reactor is due to the increase in the amount of the immobilized catalyst. Further increase in the number of columns and thereby the bed volume did not lead to better biodecaffeination. Even in the case of a two column system, the biodecaffeination capacity between the single and two columns did not differ much. Therefore further studies were carried out using a single column.

3.4.3. Studies on biodecaffeination by immobilized cells under optimized conditions:

Studies were carried on caffeine degradation using immobilized whole cells of *P. alcaligenes* MTCC 5264 under optimized conditions. Calcium alginate was found to be the best matrix and it was found to degrade caffeine efficiently in solution containing pure caffeine at $1g.L^{-1}$ at pH 6.8, in 5μM Phosphate buffer, at $30^{o}C$, under a flow rate of 5ml/minute. The optimum cell loading was 10% w/w, and complete degradation of pure caffeine by immobilized cells of *P. alcaligenes* MTCC 5264 was possible in a single column reactor. Under these conditions complete biodecaffeination could be achieved within 36 hours of incubation (Fig. 3.4.3). The immobilized cells of *P. alcaligenes* MTCC 5264, were used for the biodecaffeination of tea extracts.

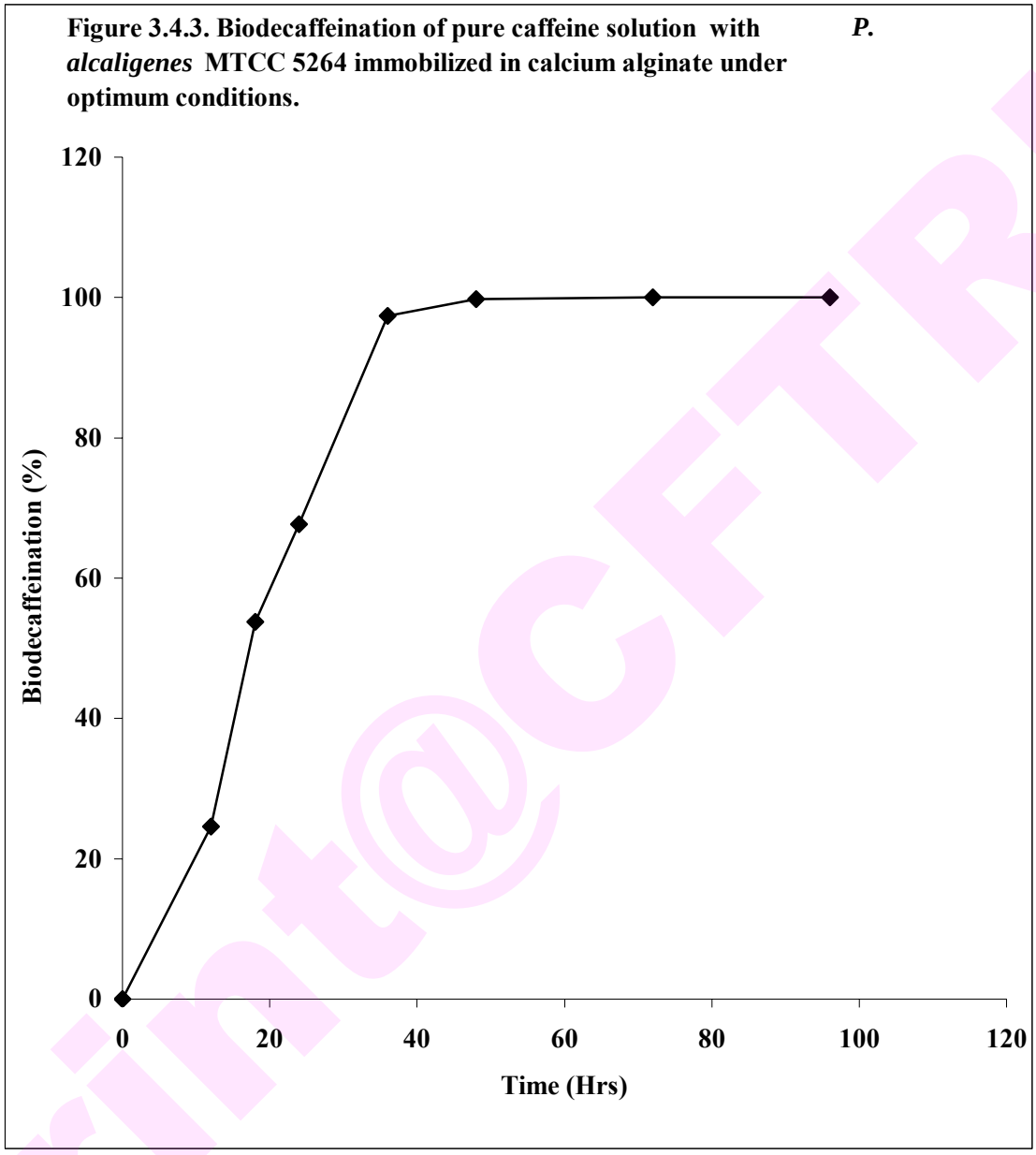


Figure 3.4.3. Biodecaffeination of pure caffeine solution with *P. alcaligenes* MTCC 5264 immobilized in calcium alginate under optimum conditions.

3.5.1. Biodecaffeination of tea extract by *P. alcaligenes* MTCC 5264 immobilized in calcium alginate:

The caffeine content in the sample as analyzed by HPLC was found to be 0.6g/L. Only 7.3% of the initial caffeine was degraded after 96 hrs of incubation (Fig. 3.5.1). Fermentation of sugars occurred in the tea extract. One probable reason for the low biodecaffeination ability of the immobilized cells is the inactivation of the

enzyme due to the formation of polyphenol-protein complexes. The cells also were utilizing the sugars present in the sample which was evident by the emanating fermented odor from the extract after 24 hrs of incubation. The presence of easily assimilable organic compounds in the medium would lead to the utilization of these compounds in preference to caffeine. Leaching of bacterial cells into the extract was observed due to the destabilization of alginate due to the components in the tea extract. Also presence of live bacterial cells is undesirable in food processing if the process of decaffeination is to be used for decaffeination of commercial beverages like coffee, tea, instant tea, chocolates etc.

3.6. CONCLUSIONS:

The isolate (*Pseudomonas alcaligenes* MTCC 5264*)* used in this study is an efficient caffeine degrader, which may be useful in the development of an environment friendly biodecaffeination process. The potential use of this organism for the development of an immobilized cell based bio-decaffeination process has been tried and the results were promising in the case of pure caffeine solutions. Experiments on real samples lead to a loss in the quality of the tea and coffee samples. Though the application of this system is limited in the area of tea and coffee, the immobilized cell based system can be used for the treatment of waste waters from coffee and tea processing units where the effluents are rich in caffeine and are responsible for pollution of the water bodies.

3.7. REFERENCES:

1. Abraham, T. E., Jamuna, R., Bansilal, C.V., Ramakrishna, S. V., *Starch/Starke*, (1991), **43:** 113–116.
2. Axelsson, A., Sisak, C., Westrin, B. A., Szajani, B., (1994), Diffusional characteristics of a swelling gel and its consequences for bioreactor performance. *Chem. Eng. J*. **55:** B35–39.
3. Bare, G., Delaunois, V., Rikir, R., Thonart, P. H., (1994), Bioconversion of Vanillin into Vanillic Acid by Pseudomonas fluorescens Strain BTP9 - Reactor Design and Parameters Optimization. *Appl. Biochem. Biotech*. **45/46:** 599–610.
4. Braham, J.E., (1987), Coffee pulp in other species. In: Brahan, J.E., Bressani, R. (Ed.) Coffee pulp**:** composition, technology, and utilization. Guatemala City: Institute of Nutrition of Central America and Panama, 39-49.
5. Brinker, C.J., Scherrer, G., (1990), The physics and chemistry of sol-gel processing. *Academic, Boston*.
6. Bryant, S.J., Nuttleman, C.R., Anseth, K.S., (2000), Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 Fibroblasts in vitro. *Journal of Biomaterial Science Polymer Edition.* **11:** 439-457.
7. Buzzini, P., Gobbetti, M., Rossi, J., *Ann. Microbiol. Enzymol*., (1993), **43:** 53–60.
8. Cachon, R., Molin, P., Divies, C., (1995), Modeling of continuous Ph-stat stirred tank reactor with Lactococcus lactis ssp. lactis bv. diacetylactis immobilized in calcium alginate gel beads. *Biotechnol. Bioeng.*, **47:** 567–574.
9. Castellar, M. R., Borrego, F., Canovas, M., Iborra, J. L., (1994), Optimization of the start-up of a passively immobilized Zymomonas mobilis system for continuous ethanol production. *Process Biochem*., **29:** 569–574.
10. Cheong, S. H., Park, J. K., Kun, B. S., Chang, H. N., (1993), Microencapsulation of yeast cells in the calcium alginate membrane. *Biotechnol. Tech*., **12**: 879–884.
11. Chibata, I., Tosa, T., Sato, T., (1974 a), Immobilized Aspartase-Containing Microbial Cells: Preparation and Enzymatic Properties, *Appl. Environ. Microbiol*. **27:** 878-885.

12. Chibata, I., Tosa, T., Sato,T., Mori,T., (1974b), Basic Studies for Continuous Production of L-Aspartic Acid by Immobilized *Escherichia coli* Cells *Appl. Environ. Microbiol*. **27:** 886-889.
13. Chu, C. H., Sakiyama, T., Yano, T., (1995), *Biosci*. *Biotech*. *Biochem*., **59:** 717.
14. Chun, G. T., Agathos, S. N., (1993), Dynamic response of immobilized cells to pulse addition of L-valine in cyclosporin A biosynthesis. *J*. *Biotechnol*., **27:** 283–294.
15. Chung, B. H., Chang, H. N., (1988), Aerobic fungal cell immobilization in a dual hollow-fiber bioreactor: Continuous production of a citric acid. *Biotechnol*. *Bioeng.* **32**: 205–212.
16. Constantinides, A., Mehta, N., (1991), Periodic operation of immobilized live cell bioreactor for the production of candicidin. *Biotechnol*. *Bioeng.*, **37:** 1010–1020.
17. Dalili, M., Chau, P. C., (1988), Production of actinomycin D with immobilized *Streptomyces parvullus* under nitrogen and carbon starvation conditions. B*iotechnol*. *Lett*., **10(5):** 331–336.
18. Davison, B. H., Thompson, J. E., (1993), *Appl*. *Biochem*. *Biotehcnol*., **39/40:** 415-426.
19. Deo, Y. M., Gaucher, G. M., (1984), Semicontinuous and continuous production of penicillin-G by Penicillium chrysogenum cells immobilized in κ-carrageenan beads. *Biotechnol*. *Bioeng.*, **26:** 285–295.
20. Dixit, V., Piskin, E., Arthur, M., Denizli, A., Tuncel, S.A., Denkbas, E., Gitnick, G., (1992), Hepatocyte immobilization on PHEMA microcarriers and its biologically modified forms, *Cell Transplant***.1(6):**391-9.
21. Dlugosz, L., Belanger, K., Hellenbrand, K., (1996), Maternal caffeine consumption and spontaneous abortion: a prospective cohort study. *Epidemiology* **7**:250–5.
22. Egresi, A., Sisak, G. S., Harasanyi, I., Szajani, B., (1992), An improved method for the entrapment of microbial cells into a thermogel. *Biotechnol*. *Tech*. **6:** 261–264.

23. Farid, M. A., El Diwany, A. I., El, Enshasy, H. A., (1994), Production of oxytetracycline and rifamycins B and SV with cells immobilized on glass wool**.** *Acta Biotechnol*., **14**: 67–74.

24. Federici, R. G., Federici, F., Petruccioli, M., (1990a), Continuous production of glucoamylase by immobilized growing cells of *Aureobasidium pullulans Biotechnol*. *Lett*., **12:** 661–666.

25. Federici, F., Petruccioli, M., Miller, M. W., (1990b), Enhancement and stabilization of the production of glucoamylase by immobilized cells of *Aureobasidium pullulans* in a fluidized-bed reactor. *Appl*. *Microbiol*. *Biotechnol*., **33(4):** 407–409.

26. Federici, J., Gromada, A., Szczodrak, J., (1994), *Microbiol*. *Res*. **149:** 425–428.

27. Forstr, M., Mansfeld, J., Schellenberger, A., Dautzenberg, H., (1994), Immobilization of citrate-producing Yarrowia lipolytica cells in polyelectrolyte complex capsules. *Enz. Microb. Technol*., **16:** 777–784.

28. Foster, B. C., Coutts, R. T., Pasutto, F. M., Dossetor, J. B., (1983), Production of cyclosporin a by carrageenan-immobilized tolypocladium inflatum in an airlift reactor with external loop. *Biotechnol*. *Lett*., **5:** 693-696.

29. Friedman, J., Waller, G.R., (1983a), Caffeine hazards and their prevention in germinating seeds of coffee (*Coffea arabica* L.). *J. Chem. Ecol*. **9**:1099-1106.

30. Friedman, J., and Waller, G.R. (1983b), Seeds as allelopathic agents. *J. Chem. Ecol.* **9:**1107-1115.

31. Gelson, C. D., Thomas, A., (1993), A novel fluidised bed bioreactor for fermentation of glucose to ethanol using alginate immobilised yeast. *Biotechnol*. *Tech*., **7(5):** 397–400.

32. Groboillot, A., Boadi, D. K., Poncelet, D. and Neufeld, R., (1994), Immobilization of cells for. application in the food industry. *Critical Rev. Biotechnol*., **14**: 75–107.

33. Grozeva, L., Tchorbanov, B., Aleksieva, P., (1993), Production of casein hydrolysates by extracellular acid proteinases of immobilized *Humicola lutea* cells. *Appl*. *Microbiol*. *Biotechnol*., **39:** 512–514.

34. Guoqiang, D., Kaul R.H., Mattiasson, B., (1992), Immobilization of *Lactobacillus casei* cells to ceramic material pretreated with polyethylenimine *Appl. microbiol. Biotechnol*, **37(3)**: 305-310.

35. Ida, T., Hitoshi, I., Yoshimi, A., Masahiro, S., (1993), Continuous ethanol fermentation in molasses medium using Zymomonas mobilis immobilized in photo-crosslinkable resin gels. *J. Ferment. Bioeng.*, **75:** 32–35.

36. Jamuna, R., Vora, S., Sai, P. S. T., Ramakrishna, S. V., (1992), Optimization of critical parameters for immobilization of yeast cells to alginate gel matrix. *J. Ferment. Bioeng.*, **73**: 319–322.

37. Jianlong, W., Ping, L., Ding, Z., (1994), Extractive fermentation of lactic acid by immobilized*Lactobacillus casei* using ion—exchange resin. *Biotechnol. Tech.*, **8:** 905–908.

38. Jones, A., Wood, D. N., Razniewska, T., Gaucher, G. M., (1986), *Can. J. Chem. Eng.*, **64:** 547–552.

39. Kalogerakis, N., Linarda, T., Behie, L. A., Svrcek, W. Y., Gaucher, G. M., (1986), *Can. J. Chem. Eng.*, **64:** 581–587.

40. Kanasawud, Hjorleifsdottir, Holst, Mattiasson, B., (1989), "Studies on immobilization of the thermophilic bacterium Thermus aquaticus YT-1 by entrapment in various matrices," *Appl. Microbiol. Biotech.* **31**:228-233.

41. Kaufman, E. N., Cooper, S. P., Davison, B. H., (1994), *Appl. Microbiol. Biotechnol.*, **45/46:** 545–554.

42. Kazuaki, Y., Yukan, S., Taro, K., Masayuki, O., Akira, O., (1994), Ethanol production by encapsulated and immobilized yeast. *Biotechnol. Tech.*, **8:** 271–274.

43. Kennedy, J. F., Cabral, J. M. S., (1985), in *Immobilized Cells and Enzymes* (ed. Woodward, J.), IRL Press. 19–37.

44. Keshavarz,T., Eglin, R., Walker, E., Bucke, C., Holt, G., Bull, A. T., Lilly, M. D., (1990), The large-scale immobilization of Penicillium chrysogenum: Batch and continuous operation in an air-lift reactor. *Biotechnol. Bioeng.*, **36:** 763–770.

45. Khare, S. K., Jha, K., Gandhi, A. P., (1994), Use of agarose-entrapped *Aspergillus niger* cells for the production of citric acid from soy whey. *Appl. Microbiol. Biotechnol.*, **41:** 571–573.

46. Kierstan M., Bucke C., (1977), The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnol Bioeng*. **19(3):**387-97.

47. Kim, D. M., Kim, G. J., Kim, S. K., (1994), Enhancement of operational stability of immobilized whole cell D-hydantoinase. *Biotechnol. Lett.*, **16**: 11–16.

48. Kqak, M. Y., Rhee, J. S., (1992), Controlled mycelial growth for kojic acid production using Ca-alginate-immobilized fungal cells. *Appl. Microbiol. Biotechnol.*, **36:** 578–583.

49. Kurosawa, H., Matsumura, M., Tanaka, H., (1989), Oxygen diffusivity in gel beads containing viable cells. *Biotechnol. Bioeng.*, **34**, 926–932.

50. Lee, G. K., Long, M. E., (1974), U.S. Patent. No. 3,821,086,

51. Livage, J., (1997), Sol gel processes. *Current Opinion in Solid State and Materials Science.* **2:** 132-138.

52. Lu, M.Z., Lan, H.L., Wang, F.F., Chang, S.J., Wang, Y.J., (2000), Cell encapsulation with alginate and 2-phenoxycinnamylidene-acetylated poly(allylamine). *Biotechnol.Bioeng.*. **70:** 479-483.

53. Lu, Z., Fujimura, T., (1993), *Radiat. Phys. Chem.*, **42:** 923–926.

54. Mahmoud, A. H., El Sayed, M., Rehm, H. J., (1987a), Semicontinuous penicillin production by two *Penicillium chrysogenum* strains immobilized in calcium alginate beads *Appl. Microbiol. Biotechnol.*, **26:** 211–214.

55. Mahmoud, A. H., El Sayed, M., Rehm, H. J., (1987b), Continuous penicillin production by *Penicillium chrysogenum* immobilized in calcium alginate beads. *Appl. Microbiol. Biotechnol.*, **26:** 215–218.

56. Mannin, C., Barbotin, J. N., Thomas, D., Lazzaroni, J. C., Portaier, R., (1989), Production of alkaline phosphatase by immobilized growing cells of *Escherichia coli* excretory mutants. *Appl. Microbiol. Biotechnol.*, **32**:143–147.

57. Mansfeld, J., Forster, M., Hoffmann, T., Schellenberg, H., (1995), Coimmobilization of Yarrowia lipolytica cells and invertase in polyelectrolyte complex microcapsules. *Enzyme Microbiol*. *Technol*., **17:** 11–17.

58. Marek, M., Kas, J., Valentová, O., Demnerová, K., and Vodrá zka, Z., (1986), Immobilization of cells via activated cell walls. *Biotech. Letts.* **8(10):** 721-724.

59. Martin, M. J., Fernandez, P. L., Gonzalez, A. G., Pablos, F., (2000), HPLC determination of catechins and caffeine in tea. Differentiation of green, black and instant teas. Analyst, **125(3):** 421-425.

60. Martinsen, A., Skjak-Break, G, Smidsrod, O., (1989), Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads**.** *Biotechnol*. *Bioeng.*, **33:** 79–89.

61. Mattiasson, B. (1983), Immobilization methods, p. 4–19. *In: Immobilized Cells and Organelles*, Vol. **1**. Mattiasson, B. (Ed.) CRC Press, Inc., Boca Raton, Florida.

62. Mcginis, R., (1985) *Sugar J*., **38**, 8.

63. Mebin, E., Shich, W. K., (1992), *Chem*. *Eng. J*., **50**: B17–B22.

64. Middelhoven, W. J., Bakker, C. M., (1982) Degradation of caffeine by immobilized cells of *Pseudomonas putida* strain C 3024. *European. J. Appl. Microbial Biotechnol*. **15;** 214-217.

65. Mignot, L., Junter, G. A., (1990), Diffusion in immobilized-cell agar layers: influence of bacterial growth on the diffusivity of potassium chloride. *Appl*. *Microbiol*. *Biotechnol*., **33**: 167–171.

66. Mittal, Y., Mishra, I. M., Varshney, B. S., (1993), *Biotechnol*. *Tech*., **15:** 41–46.

67. Mori, A., Matsumoto, N., Imai, C., (1989), *Biotechnol*. *Lett*., **11:** 183–188.

68. Morikawa, Y., Karube, I., Suzukis, S., (1980), Continuous production of bacitracin by immobilized living whole cells of *Bacillus* sp. *Biotechnol*. *Bioeng*., **22**: 1015.

69. Nassif, N., Bouvet, O., Rager, M.N., Roux, C., Coradin, T., Livage, J., (2002), Living bacteria in silica gels. *Nature Materials.* **1:** 42-44.

70. Nassif, N., Roux, C., Coradin, T., Rager, M.N., Bouvet, O.M.M., Livage, J., (2003), A sol-gel matrix to preserve the viability of encapsulated bacteria. *Journal of Materials Chemistry.* **13:** 203-208.

71. Nguyen, A. L., Luong, J. H. T., (1986), Diffusion in κ-carrageenan gel beads. *Biotechnol*. *Bioeng.* **28:** 1261–1267.

72. Nishida, Y., Sato, T., Tosa, T., Chibata, I., (1977), Immobilization of Escherichia coli cells having aspartase activity with carrageenan and locust bean gum. *Enzyme Microb*. *Technol*., **1**: 95–99.

73. Nishio, N., Sugawa, K., Hayase, N., Nagai, S., (1989), Conversion of D-xylose into xylitol by immobilized cells of Candida pelliculosa and Methanobacterium sp. HU. *J*. *Ferment. Bioeng.*, **67:** 356–360.

74. Nolan, A. M., McHale, A. P., (1994), Ethanol production at 45° by an alginate-immobilized, thermo-tolerant strain of *Kluyveromyces marxianus* following growth on glucose-containing media. *Biotechnol*. *Lett*. **16**: 849–852.

75. Norton, S., Lacroix, C., Vuillemard, J. C., (1994), Kinetic study of continuous whey permeate fermentation by immobilized Lactobacillus helveticus for lactic acid production. *Enz. Microb*. *Technol*., **16:** 457–466.

76. Novarro, J. M. and Durand, G., (1977) *Eur*. *J*. *Appl*. *Microbiol*., **4**: 243–254.

77. Ogbonna, J. C., Amuno, Y. and Nakamura, K., (1989), Elucidation of optimum conditions for immobilization of viable cells by using calcium alginate. *J*. *Ferment. Bioeng.*, **67:** 92–96.

78. Pandya, Y., Knorr, D., (1991), Diffusion characteristics and properties of chitosan coacervate capsules. *Process Biochem*., **26:** 75–81.

79. Park, S. O., Ohta, N., Okabe, M., (1994), Neomycin production by partial immobilization Streptomyces fradiae on cellulose beads in an air-lift bioreactor. *J*. *Ferment*. *Bioeng.*, **78:** 265–268.

80. Paz, D. E. D., Aantana, M. H. A., Eguchi, S. Y., (1993), *Appl*. *Biochem*. *Biotechnol*., **39/40:** 455–466.

81. Qureshi, N., Maddox, I. S., (1995), Continuous production of acetone-butanol-ethanol using immobilized cells of *Clostridium acetobutylicum* and integration with product removal by liquid-liquid extraction. *J. Ferment. Bioeng.* **80:** 185–189.

82. Rao, D. S., Panda, T., (1994), *Bioprocess Eng.*, **11:** 209–212.

83. Ray, R. R., Jana, S. C., Nanda, G., (1995), *J. Basic Microbiol*., **2:** 113–116.

84. Roussos, S., Angeles-Aquiahuatl, M.D.L., Trejo-Hernandez, M.D.R., Gaime-Perraud, I,, Favela, E., Ramakrishna, M., (1995), Biotechnological management of coffee pulp-isolation, screening, characterization, selection of caffeine degrading fungi and natural microflora present in coffee pulp and husk. *Appl Microbiol Biotechnol;* **42**:756–62.

85. Ruckenstein, E., Wang, X. B., (1994), Production of lignin peroxidase by Phanerochaete chrysosporium immobilized on porous poly(styrene-divinylbenzene) carrier and its application to the degrading of 2-chlorophenol. *Biotechnol*. *Bioeng.* **44:** 79–86.

86. Ruggeri, B., Sassi, G., Specchia, V., Bosco, F., Marzona, M., Alginate beads coated with polyacrylamide resin: potential as a biocatalyst. (1991), *Process Biochem*., **26**: 331–335.

87. Sakiyama, T., Chu, C.H., Fujii, T., Yano, T., (1993), *J. Appl*. *Polymer Sci*., **59**:

88. Sanroman, A., Pintado, J., Lema, J. M., (1994), A comparison of two techniques (adsorption and entrapment) for the immobilization of *Aspergillus niger* in polyurethane foam. *Biotechnol*. *Tech*. **8:** 389–394.

89. Sarath Babu, V.R., Patra, S., Karanth, N.G., Varadaraj M.C., Thakur M.S., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708, *Enz, Microb. Tech*. **37:** 617-624.

90. Sarra, M., Casas, C., Godia, F., (1997), Continuous production of a hybrid antibiotic by *Streptomyces lividans* TK21 pellets in a three-phase fluidized-bed bioreactor. *Biotechnol*. *Bioeng.*, **53:** 601–610.

91. Saucedo, J. E. N., Barbotin, J. N., Thomas, D., (1989), Continuous production of gibberellic acid in a fixed-bed reactor by immobilized mycelia of *Gibberella fujikuroi* in calcium alginate beads. *Appl*. *Microbiol Biotechnol*., **30:** 226–233.

92. Smeds, K.A., Grinstaff, M.W., (2001), Photocrosslinkable polysaccharides for *in situ* hydrogel formation. *J. Biomed. Mat. Res.* **54:** 115-121.

93. Smidsrod, O., Skjak-Braek, G., (1990), Alginate as immobilization matrix for cells. *Trends in Biotechnology.* **8:** 71-78.

94. Srisuphan W, Bracken MB.(1986), Caffeine consumption during pregnancy and association with late spontaneous abortion. *Am J Obstet Gynecol* **154**:14–20.

95. Sun, P., Li, B., Wang, Y., Ma, J., Din, D., He, B., (2003), 1H NMR studies of poly (Nisopropylacrylamide) gels near the phase transition. *European Polymer Journal.* **39:** 1045-1050.

96. Takata, I., Tosa, T., Chibata, I., (1978), *J. Solid. Phase Biochem.*, **2:** 255–366.

97. Teixeira, J. A., Mota, M., Venancio, A., (1994), Model identification and diffusion coefficients determination of glucose and malic acid in calcium alginate membranes. *Chem. Eng. J.*, **56:** B9–B14.

98. Van Wezel, A.L., (1967), Growth of Cell Strains and Primary Cells on Microcarriers in Homogeneous Culture. *Nature*. **216:** 64-65.

99. Vorlop, K. D., Klein, J., (1981), Formation of spherical chitosan biocatalysts by ionotropic gelation. *Biotechnol. Lett.*, **3:** 9–14.

100. Vorlop, K., Di, E., David, G. F., (1993), Design of Ca-alginate immobilized yeast cell beads with controlled low density to enhance their fluidization behaviour in bioreactors. *Biotechnol. Tech.*, **7:** 287–292.

101. Webb, C., (1989), The role of cell immobilization in fermentation technology. *Aust. J. Biotechnol.* **3(1):**50-5.

102. West, T. P., Strohfus, B., (1996), Fungal cell immobilization on ion exchange resins for pullulan production, *Microbios*, **88(356):** 177-187.

103. Westrin, I. A., (1990), Diffusion in immobilized-cell gels. *Appl. Microbiol. Biotechnol.*, **34:** 189–190.

104. Willaert, R. G. and Baron, G. V., (1994), Effectiveness factor calculation for immobilised growing cell systems. *Biotechnol. Tech.*, **8:** 695–700.

105. Yamagiwa, K., Shimizu, Y., Kozawa, T., Onodera, M., Ohkawa, A., (1993), *J. Chem. Eng. Japan*. **26:** 449–450.

106. Yaskovich G. A., (1998), the role of cell surface hydrophobicity in adsorption immobilization of bacterial strains. *Appl. Biochem. Microbiol*. **34(4):** 373-376.

107. Yu, D., Voloponi, J., Chhabra, S., Brinker, C.J., Mulchandani, A., Singh, A., (2005), Aqueous sol–gel encapsulation of genetically engineered *Moraxella* spp. cells for the detection of organophosphates. *Biosens. Bioelec.* **20(7):** 1433-1437.

CHAPTER 4

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF CAFFEINE DEMETHYLASE

4.1. SCOPE OF THE WORK:

The xanthine alkaloid, caffeine is present in several food materials, beverages and pharmaceutical preparations. The excessive consumption of caffeine containing beverages like coffee and tea has been reported to be the cause for several ill effects on human health, which paved way for research into the development of processes for decaffeination. Commonly decaffeination processes employ organic solvents which are reported to be unsafe, thus leading to research in the area of biotechnology for development of processes for biodecaffeination.

A basic requirement in development of biodecaffeination processes is the isolation and identification of enzymes involved in the biodegradation of caffeine. This chapter deals with the isolation, identification and characterization of caffeine demethylase from *Pseudomonas alcaligenes* MTCC 5264. The first section of the chapter deals with a review of the literature on research carried out by different groups in the world on biodegradation of caffeine. The second section of chapter deals with methodologies used in the work.

The third section of the chapter deals with the results and discussion. The enzymes involved in biodecaffeination were isolated and identified. The pathway for caffeine degradation has been elucidated in this work. Caffeine demethylase, a rate limiting enzyme which is highly labile even under sub zero temperatures has been isolated, characterized and stabilized and the results are detailed in the third section of the chapter. The chapter ends with the conclusions and future perspectives of research on caffeine demethylase.

4.2. INTRODUCTION:

Caffeine has deleterious effects on cardiac patients and women (James, 1997; Leviton and Cowan, 2002; Waring et. al., 2003). Reports are also available on the effects of caffeine on health and of its toxic effects to animals and plants (Pincheira et.al., 2003; Meyer et.al., 2004). Decaffeination is being carried out widely in beverages because of the growing belief that the chronic ingestion of caffeine can have adverse effects on health. The biological decaffeination of coffee pulp not only decreases the caffeine content in it but also improves the nutritional value of the coffee pulp (Rojas et. al., 2003). Hence, caffeine degradation is essential from both health and environmental point of view.

In plants, degradation of caffeine occurs through sequential demethylation that finally results in the formation of xanthine. The demethylation reactions have been found to be catalyzed by demethylase enzymes, viz., *N*-1 demethylase, *N*-7 demethylase and *N*-3 demethylase. Xanthine is then converted into CO_2 and ammonia by purine catabolism (Mazzaffera, 1993; Ashihara et.al., 1996; Ashihara et.al., 1997; Vittoria and Mazzaffera, 1998; Koyama et.al., 2003). In contrast to plants, the degradation pathway in mammals results in the formation of methyl xanthines and methyl uric acids by cytochrome P450 enzymes CYP1A2, CYP3A4, CYP2E1 xanthine oxidase and *N*-acetyl transferase (Cornish and Christman, 1957; Khanna et.al., 1972; Wreck and Fevereisen, 2000; Caubet et.al., 2004). In fungi, the initial degradation product has been found to be theophylline. Other dimethyl and monomethyl xanthines were also detected as products of caffeine degradation. However, the catabolic pathway is not clearly known (Hakil et.al., 1998). In

bacteria (*Pseudomonas*), caffeine is initially converted into theobromine and paraxanthine parallely by demethylases.

Studies on caffeine degradation by microorganisms were not reported till 1970 probably because caffeine was regarded as toxic to bacteria (Sundarraj and Dhala, 1965; Putrament et.al., 1972; Kihlman, 1974). Bacterial strains capable of degrading caffeine belong to *Pseudomonas* and *Serratia* genus (Kurtzman and Schwimmer, 1971; Woolfolk ,1975; Vogels and Drift, 1976; Blecher and Lingens, 1977; Asano et.al., 1993; Porres et.al., 1993; Mazzaffera et.al., 1994; Roussos et.al., 1994; Hakil et.al., 1998; Hakil et.al., 1999; Brand et.al., 2000; Tagliari et.al., 2003; Ramarethinam and Rajalakshmi, 2004). The enzymes involved in the degradation of caffeine in microorganisms are demethylases and oxidases (Asano et.al., 1993; Asano et.al., 1994; Hohnloser et.al., 1980; Yano and Mazzaffera, 1999; Sideso et.al., 2001; Madyastha and Sridhar, 1998; Madyastha et.al., 1999). Yano and Mazzafera, (1999) attempted to purify caffeine demethylase, but found that the purified enzyme was labile and it rapidly lost its activity. It has been observed that the use of cryoprotectants and freeze drying to low moisture contents improved the stability of the enzyme (Sideso et.al., 2001). In general, the caffeine degrading enzymes are very labile and more studies are required to improve the stability of the enzymes, which will help in developing a specific process for caffeine degradation. In mixed culture consortium belonging to *Klebsiella* sp. and *Rhodococcus* sp., caffeine was directly oxidized by the enzyme caffeine oxidase at the C-8 position leading to the formation of 1,3,7-trimethyluric acid and this process did not have demethylation steps (Madhyasta et.al., 1999). Only partial characterization of this

enzyme is available (Madyastha et.al., 1999, Sideso et.al., 2001). Though enzymes involved in degradation of caffeine are known, in vitro enzymatic studies for caffeine degradation are not yet reported. Since demethylase enzymes are not very stable more studies on enzyme stability and biochemical characterization is required.

Caffeine degrading bacteria, belonging to the *Pseudomonas sps*, demethylate caffeine to yield xanthine, which is further catabolised to NH_3 and CO_2. The pathways via which this is achieved were elucidated. It has been found that caffeine-degrading microorganisms utilizing caffeine as the sole source of carbon and nitrogen have enzymes that bring about the actual degradation of the substrate (Sarath et.al., 2005).

A few reports in the literature have already described the isolation of bacteria strains from soil with the ability to degrade caffeine (Woolfolk, 1975; Blecher and Lingens, 1977; Gluck and Lingens, 1987; Mazzaffera et.al., 1994).

Only a few reports are available on the partial purification and characterization of caffeine demethylase complex (Sideso et.al., 2001) but the complete purification and characterization of this enzyme has not been done till now. In lieu of the need for the development of an enzymatic decaffeination process, and in view of the instability of the enzyme, an in-depth knowledge of the biochemical and biophysical characteristics of the enzyme is essential. This chapter deals with the studies on isolation, identification and characterization of caffeine demethylase enzyme from *P. alcaligenes* MTCC 5264 which was isolated and characterized at our laboratory previously (Sarath et.al., 2005).

4.3. MATERIALS AND METHODS:

Caffeine (99.9%), lysozyme, Tris, sodium cyano borohydride, phenazine methosulphate (PMS), methyl xanthine standards, xanthine oxidase, uricase, urease, trifluoro acetic acid (TFA), acrylamide, silver nitrate, DEAE-Sephadex A-50 and trypsin (MB Grade) were procured from M/s Sigma-Aldrich, St. Louis, USA.

Dichlorophenol indo phenol (DCPIP), nicotinamide adenosine dinucleotide phosphate, reduced tetra sodium salt (NADPH sodium salt), nicotinamide adenosine dinucleotide (NAD), dithiothreitol, phenyl methyl sulfonyl fluoride (PMSF), NNN-bisacrylamide, TEMED, ammonium persulphate (APS), sodium dodecyl suplhate (SDS, MB Grade), caffeine (LR Grade), uric acid standard, urea standard and methylene blue were purchased from Sisco Research laboratories, Mumbai, India.

Peptone and yeast extract were from HiMedia labs, Mumbai, India. All other reagents were of the highest purity and were procured from standard sources.

4.3.1. Extraction of enzymes:

Induced cells of *P. alcaligenes* MTCC 5264 were harvested by centrifugation at 12000 g for 30 minutes at 4^{o}C. The biomass pellet was washed several times with ice cold buffer (Tris-Cl, 50 mM; pH 6.8) and frozen at –20^{o}C. 10 grams of the frozen pellet was thawed into 100 ml of Lysis buffers containing 1-5mg/ml of lysozyme, 1mM PMSF, 0.1mM DTT and 15 %v/v of glycerol at 37^{o}C for 1 hour. The lysate was then centrifuged at 12000 g for 30 minutes to separate the cell debris. The supernatant obtained was designated as crude enzyme and used for further purification. Different methods of lysis of cells were tried which include freezing and thawing, mechanical lysis on a bead mill (dynomill), ultrasonication,

combination of lysozyme and freeze thawing. Glass beads (0.25-0.75 mm dia) were used in the dynomill. 10 gms of glass beads were added to 10gms (wet weight) of cell pellet suspended in 100 ml of Tris-Cl buffer (100mM, pH 6.8) containing 4% w/v sorbose as the stabilizer and maintained at 5-10^{o}C. The dynomill was set at 2000 rpm and cell lysis was brought about in 5 cycles of 5 minutes each with intermittent cooling for 5 minutes. Cold water was circulated throughout the lysis period to avoid heating of the contents. The protein content of the cell free extract (CFE) was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein. All the experiments involving enzyme purification and characterization were carried out at 4^{o}C unless otherwise specified.

4.3.2. Identification of enzymes involved in degradation of caffeine:

The enzyme involved in the N-demethylation of caffeine to its respective dimethyl xanthines has not been reported conclusively. Although few reports indicate the involvement of a demethylase with monooxygenase activity (Asano et.al., 1994) and the involvement of a caffeine oxidase (Madhyasta et.al., 1999), the reports have been inconclusive. The involvement of an oxidase in the degradation pathway does not fit into the existing biochemical pathway elucidated for the degradation of caffeine as it involves no demethylation steps. Therefore, a demethylase model is much valid. In lieu of the inconclusivity of the nature of the enzyme involved in the N-demethylation of caffeine, extensive studies on the nature of this reaction were required. Thus experiments were conducted to identify the enzymes responsible for caffeine degradation. The following enzymes were assayed. The enzyme involved in the first step of caffeine degradation is believed to

be a demethylase. The exact nature of this enzyme is not known and studies were carried out on the extraction, purification and identification of the demethylase and the experimental details are described in section 4.4.3.8.

4.3.2.1. Caffeine oxidase:

a) Spectrophotometric assay:

Caffeine oxidase activity in the cell free extract was checked according to the method of Madhyastha et al, 1999. In brief, the reaction mixture consisted of 10 mM Caffeine, 10 mM DCPIP, 1mM PES in 1 ml of 100 mM phosphate buffer at pH 7.2. The reaction was initiated by the addition of 100 µl of crude enzyme. Enzyme activity was measured as a decrease in the absorbance of the reaction mixture due to the reduction of DCPIP.

b) Incubation Test:

100µl of crude enzyme was incubated with 10 mM caffeine in 1 ml of 100 mM phosphate buffer pH 7.2, and incubated at 30± 2^{o}C for 1 hour. The reaction was terminated by addition of 10µl of glacial acetic acid. The reaction mixture was analyzed by HPLC for the presence of 1,3,7-trimethyluric acid. HPLC analysis was carried out in a Shimadzu LC 10A system with a diode array detector and connected to a octadecyl silica column (Luna C18, Phenomenex, 4.2 x 250 mm) under isocratic conditions with water and acetonitrile (85:15) as the mobile phase.

4.3.2.2. Xanthine oxidase:

Xanthine oxidase is known to accept theobromine and theophylline as the substrates and convert them into their respective uric acids. The presence of this enzyme was tested by assayed by methylene blue reduction test (Bray, 1963). The

reaction mixture contained 10 mM hypoxanthine, 25mM methylene blue and 100 µl of crude enzyme in 1 ml of reaction Buffer (100 mM, PO_4; pH 7.2). The enzyme activity was recorded as a decrease in the absorbance of the reaction mixture at 600 nm. One unit of xanthine oxidase activity is defined as the conversion of 1 µM of methylene blue reduced per minute, which is equal to 1µM of uric acid formed from the substrate.

4.3.2.3. Heteroxanthine demethylase:

Heteroxanthine demethylase activity in the crude enzyme extract was determined by the method of Gluck and Lingens (1988). The reaction mixture consisted of paraxanthine (100µl), crude enzyme extract (100µl), and NADPH (20µl) in 1 ml of phosphate buffer (50mM, pH 6.8). This reaction mixture was incubated for three hours at 30^0C. Samples were drawn at 0, 30, 60, 120 and 180 minutes and analyzed for their caffeine content by HPLC and product formation. One unit of activity is defined as the formation of 1µM of 7-methyluric acid formed per minute.

4.3.2.4. Xanthine dehydrogenase:

Xanthine dehydrogenase activity in the crude enzyme extract was determined by the method of Gluck and Lingens (1987). The reaction mixture consisted of theobromine (100µl), crude enzyme extract (100µl), and NAD (20µl) in 1 ml of phosphate buffer (50mM, pH 7.0). This reaction mixture was incubated for three minutes at 30^0C. Enzyme activity was recorded as an increase in the absorbance of the reaction mixture at 340 nm. One unit of xanthine dehydrogenase activity is defined as the formation of 1µM of NADH from NAD per minute.

4.3.2.5. Uricase:

The uric acid formed by the oxidation of theobromine and other dimethyl xanthines are converted to allantoin by the enzyme uricase. Uricase activity was determined by incubating uric acid with crude extract and the removal of uric acid was monitored spectrophotometrically (Mahler et.al., 1955). The reaction mixture consisted of 10mM uric acid and 100µl of crude enzyme in 1 ml of phosphate buffer (100mM, pH 6.5). The activity was recorded as the decrease in the absorbance at 290nm. One unit of uricase activity is defined as the formation of 1µM of allantoin from uric acid per minute.

4.3.2.6. Allantoinase:

Allantoinase activity in the crude enzyme was detected by incubating the crude enzyme extract (100µl) with allantoin (100µl) in Tris-Cl buffer (100mM; pH. 6.8) for 1 hr and analyzing the reaction mixture for the production of allantoic acid by HPLC.

4.3.2.7. Allantoicase:

Allantoicase activity in the crude enzyme extract was determined by incubating the crude enzyme extract (100µl) with allantoic acid (100µl) in Tris-Cl buffer (100mM; pH.7.0) for 1 hr and analyzing the reaction mixture for the reduction in the allantoic acid content in the reaction mixture by HPLC.

4.3.2.8. Urease:

Urea formed by the action of allantoicase is now converted to ammonia and carbon dioxide by the enzyme Urease that marks the final step in the degradation of caffeine. Urease activity was determined by estimation of the amount of ammonia

released by using Nessler's reagent (Jayaraman, 1988). The reaction mixture consisted of 1 ml urea and 1 ml of crude enzyme. The reaction mixture was incubated at 30^0C for 1 hour and samples drawn at 0, 5, 15, 30 and 60 minutes. 100µ.l of Nessler's reagent was added and absorbance recorded at 490 nm. One unit of urase activity is defined as the formation of 1µM of ammonia per minute.

4.3.2.9. Caffeine demethylase:

Initially assay protocol for this enzyme was not available. Therefore, to confirm the presence of this enzyme in the crude extract, a HPLC based method for detection of enzymatic product was used. The reaction mixture consisted of caffeine (100µl), crude enzyme extract (100µl), and NADPH (20µl) in 1 ml of phosphate buffer (50mM, pH 7.2). This reaction mixture was incubated for three hours at 30^0C. Samples were drawn at 0, 30, 60, 120 and 180 minutes and analyzed for their caffeine content by HPLC and product formation. Enzyme activity was recorded as the number of micromoles of caffeine consumed per minute.

a) Oxygenase activity of caffeine demethylase:

The enzyme assay was done by using a dissolved oxygen meter (EDT Instruments, UK). The reaction mixture contained 100µl of enzyme suspended in 1 ml of buffer and continuously agitated. When the dissolved oxygen probe showed a stable response, 0.1% w/v caffeine (100µl) was injected and the response was recorded as a drop in the dissolved oxygen content (Gouda et.al., 2000). The reaction was stopped by addition of 5µl of Glacial acetic acid and the reaction mixture was analyzed for the theobromine content by HPLC. The oxygen (mM) consumed in this reaction was calculated determined from the value obtained from

the oximeter and the amount of oxygen consumed per mM of theobromine produced was determined.

4.3.3. Purification of caffeine demethylase:

Purification of caffeine demethylase was carried out and the following steps were involved. All the experiments were carried out below 5^{o}C in a cold room unless otherwise specified. All the buffers and reagents used were prepared in triple distilled water and were of the highest quality. The enzyme preparations were stored at 4^{o} C in a refrigerator till further use. All the buffers contained protease inhibitors and thiol reducing agents.

4.3.3.1. Salting out:

Salting out of the enzyme was carried out with ammonium sulphate at different concentrations (Jakoby, 1971). Solid ammonium sulphate was added to the crude enzyme at 4^{o}C slowly and then centrifuged at 6000 rpm for 10 minutes. The supernatant was decanted slowly without disturbing the pellet. The pellet was dissolved in minimal amount of buffer and checked for activity. Based on the presence of the enzyme, either the pellet or supernatant were selected for further processing. The pellet containing enzyme activity was desalted by passing through a Sephadex G-10 column. Active fractions were collected, pooled and subjected to further purification.

4.3.3.2. Ion exchange chromatography:

Ion exchange chromatography was carried out on a DEAE-Sephadex A-50 column (Yamamoto and Ishihara, 1999). The matrix was first swelled by boiling 10 grams of the gel with 200 ml of distilled water. The gel was then equilibrated with

Tris-Cl buffer and packed into a column of 2 cm X 20 cm. The column was further equilibrated and used for purification of the enzyme. The packed bed volume was 20 ml. 5 ml of the crude enzyme (0.64 mg/ml of protein) was loaded on the column and the column was washed with equilibration buffer to remove the unbound fractions. Bound proteins were eluted by an increasing salt gradient. The unbound and bound fractions were checked for enzyme activity by incubating the fraction with pure caffeine (2mM) and NADPH (1 mM) at 30^{o}C for 3 hours. The reaction was stopped by addition of 50 µl of glacial acetic acid and analyzed for the presence of products by HPLC. Active fractions were pooled and the purity of enzyme was checked by SDS-PAGE.

4.3.3.3. Gel permeation chromatography:

Purification of the enzyme by gel permeation chromatography (Ward and Arnott, 1965), was carried out in a Sephadex G -100 column of 1cm X 120 cm dimensions (bed Volume= 144 cc), equilibrated with Tris-Cl buffer and 1.5 ml of the sample (1.19 mg /ml protein) was applied to the top of the column. Elution was carried out in the same buffer containing 100 mM NaCl to avoid non specific binding to the matrix. 2 ml fractions were collected and active fractions were pooled and used for further purification.

4.3.3.4. Hydrophobic interaction chromatography:

Hydrophobic interaction chromatography (HIC) was carried out on a phenyl sepharose column (Machold, et.al., 2002). The matrix was washed off all the remaining preservative, equilibrated with Tris-Cl buffer containing 1.0 M ammonium sulphate and packed into a column of 2 cm X 20 cm. The packed bed

volume was 10 ml. 2 ml of the crude enzyme (0.64 mg/ml of protein) was loaded on the column and the column was washed with equilibration buffer to remove the unbound fractions. Bound proteins were eluted by a negative ammonium sulphate gradient. The unbound and bound fractions were checked for enzyme activity by incubating the fraction with pure caffeine (2 mM) and NADPH (1 mM) at 30^{o}C for 3 hours. The reaction was stopped by addition of 50 µl of glacial acetic acid and analyzed for the presence of products by HPLC. Active fractions were pooled and the purity was checked by SDS-PAGE.

4.3.3.5. Affinity chromatography:

The biogel blue sepharose (Biorad labs, UK) was packed to a column (15mm X 100 mm) and used for purification of caffeine demethylase by a modification of the method of Johan and Mooibroek, (1998). The column was equilibrated with 50 mM NaCl and 2 ml of the enzyme (0.26 mg/ml of protein) from HIC fractions was loaded on the column and washed with equilibration buffer to remove the unbound fractions. Bound proteins were eluted by increasing sodium chloride gradient (50-500 mM). The unbound and bound fractions were checked for enzyme activity by incubating the fraction with pure caffeine (2 mM) and NADPH (1 mM) at 30^{o}C for 1 hour. The reaction was stopped by addition of 50 µl of glacial acetic acid and analyzed for the presence of products by HPLC. Active fractions were pooled and the purity was checked by SD- PAGE according to the method of Laemmli (1970).

4.3.4. Characterization of caffeine demethylase:

4.3.4.1. Trypsin digestion for LC-MS analysis:

Active fractions were pooled and concentrated. The protein content was checked and to 750µg of the protein, 75 µg of trypsin was added in Tris-Cl buffer pH 8.0 and incubated at 38^{o}C for 1 hour. The reaction mixture was further incubated for 12 hours at room temperature to complete the digestion (Stone and Williams, 1996). The tryptic digest thus obtained was used for LC MS analysis.

4.3.4.2. Liquid chromatography with mass spectroscopic (LC-MS) analysis of caffeine demethylase:

Active fractions of caffeine demethylase enzyme obtained from affinity chromatography were injected into a Q-Tof Ultima mass spectrometer equipped with a liquid chromatograph connected to a C8 column (Shevchenko et.al., 1996). The chromatographic separation of the protein was done under an increasing gradient of 70% acetonitrile containing 0.1% TFA and water containing 0.1% TFA (Papayannopoulos, 1995). The peaks were directly injected into the mass spectrophotometer attached with electro spray ionization injector in the positive mode. The collision energy was 10.0 kV. The mass peaks obtained were analyzed by mass lynx software and the masses of the proteins were calculated using the mass finder option. The peptides obtained by tryptic digestion were also analysed in the ESI positive mode and the sequencing of the mass fragments was done by using MASCOT software (Matrix Science Inc. Boston, USA; www.matrixsciences.com).

4.3.4.3. Determination of the metal in the enzyme atomic absorption spectroscopy:

The metal present in the enzyme was also determined by complete digestion of the protein in nitric acid followed by atomic absorption spectroscopy (Shimadzu-

AA-6800 Atomic Absorption Spectrophotometer), with iron standards (Freedman and Peisach, 1984).

4.3.4.4. Determining the co-factors for the enzyme:

The enzyme was dialyzed against buffer for 18 hrs with three changes in buffer and then incubated with the co factors FAD, FADH, NAD, NADH, NADP and NADPH and the preference to these co-factors was studied.

4.3.4.5. Stabilization of caffeine demethylase:

The enzymes extracted from cells are known to be unstable in vitro and caffeine demethylase enzyme activity is lost rapidly in solution. Therefore, stabilization of caffeine demethylase a key enzyme in the biodecaffeination process is very important. Several stabilizing agents were used for the stabilization of caffeine demethylase enzyme. Lysozyme, bovine serum albumin (BSA) and gelatin were used as protein based stabilizing agents and sorbose, mannose, and raffinose were used as the polyols for the stabilization of the enzymes. Gelatin, BSA and lysozyme were added to the enzyme preparation at 2 mg/ml to 10 ml of crude enzyme and incubated at room temperature for 3 hrs. All the polyols were incubated at concentrations of 2% w/v. The residual enzyme activity was determined by spectrophotometric assay of the caffeine demethylase activity at different intervals of time. The enzyme preparation incubated with stabilizing agents was then stored at 4^{o}C in a refrigerator. The residual activity of the demethylase was checked every 10 days over a period of 90 days.

4.4. RESULTS AND DISCUSSION:

4.4.1. Extraction of enzymes:

Several methods were employed in the present study for the extraction of the enzymes involved in caffeine degradation in cells of *P. alcaligenes*. The cells were harvested from shake flask and caffeine demethylase enzyme activity in the cell free extract (CFE) was used as a measure of the enzyme activity in the study and represented in the study. Table 4.4.1.1., represents the effect of different lysis methods on the extraction of the enzymes.

Among the physical methods of lysis sonication for 10 minutes with 1 minute cycles and 5 minutes intermittent cooling was found to be efficient with 44.9 U/ml of caffeine demethylase activity and a protein content of 0.388mg/ml. The protein content in the CFE increased with increasing the time of sonication, but there was a drastic loss in the enzyme activity which is attributed to the mechanical stress effected on the enzymes. Cell lysis by freezing and thawing was not efficient in extraction of the enzyme and the protein content was also very low (0.112mg/ml) compared to other methods. The enzyme activity was also low (2.8 U/ml) by using this method. However, a lysis by freezing and thawing of the cells followed by sonication was found to improve the extraction of the enzymes (0.554 mg/ml protein and 12.6 U/ml) of activity.

Dynomill treatment was found to be more efficient among the physical methods of cell lysis with 0.964mg/ml protein and 78.54U/ml of enzyme activity. This method is useful in lysis of cells at a large scale. Enzymatic method of lysis was found to be the best in terms of yields and activity of caffeine demethylase in

CFE. Maximum caffeine demethylase activity and protein content (was found in CFE obtained by lysis using lysis buffer containing 4 and 5mg/ml of lysozyme (327.2 & 329.1 U/ml respectively) and are represented in Table 4.4.1.1., below. Lysozyme is a cell wall degrading enzyme and only acts on the cell wall of the bacteria. This method of lysis is mild and does not affect the enzyme activities in the CFE and it accounts for the high caffeine demethylase activity. The mechanical methods of lysis have several disadvantages of being harsh on the cells leading to loss of enzyme activities. It was concluded that lysozyme treatment was the best method of cell lysis and was used for further experiments.

Table 4.4.1.1. Effect of cell lysis method on extraction of caffeine demethylase from *P. alcaligenes* MTCC 5264.

S. No.	Lysis Method	Protein content (mg/ml)	Activity of caffeine demethylase (U/ml)
1.	Sonication (5 Min)	0.144	13.2
2.	Sonication (10 Min)	0.388	44.9
3.	Sonication (15 Min)	0.397	26.7
4.	Sonication (20 Min)	0.402	5.76
5.	Freezing and Thawing (1 Cycle)	0.023	-
6.	Freezing and Thawing (2 Cycles)	0.048	-
7.	Freezing and Thawing (3 Cycles)	0.112	2.8
8.	Freezing and Thawing + Sonication.	0.554	12.6
9.	Lysozyme (1mg/ml)	0.64	150.7
10.	Lysozyme (2 mg/ml)	1.047	184.0
11.	Lysozyme (3 mg/ml)	1.112	198.8
12.	Lysozyme (4 mg/ml)	1.127	327.2
13.	Lysozyme (5 mg/ml)	1.134	329.1
14.	Dynomill	0.964	78.54

4.4.2. Product formation using crude enzyme extract:

This crude enzyme extract incubated with caffeine showed the presence of theobromine, paraxanthine, allantoin which was confirmed by TLC and HPLC (Table 4.4.2.1). This observation confirms that the *P. alcaligenes* produced the required enzymes for degrading caffeine. The metabolites produced by the organism indicate that caffeine undergoes the same pathway indicated by Blecher and Lingens (1977).

Table 4.4.2.1. HPLC analysis of reaction mixture of crude enzyme.

S.No	Sample ID	RT of Peaks detected	Peak Area	Product
1	Zero Hr. Reaction Mixture	2.955	885	Allantoin
		3.725	2406	Theobromine
		5.828	926892	Caffeine
2.	One Hr. Reaction Mixture	2.487	1306	Unknown
		2.652	2010	Unknown
		2.732	3630	Unknown
		3.028	40155	Allantoin
		3.783	**5121**	**Theobromine**
		4.05	**1028**	**Paraxanthine**
		5.893	836735	Caffeine
3.	Two Hr. Reaction Mixture	2.707	1582	Unknown
		3.068	44780	Allantoin
		3.735	**8356**	**Theobromine**
		4.052	**2646**	**Paraxanthine**
		5.828	762510	Caffeine
4.	Three Hr. Reaction Mixture	2.745	3652	Unknown
		3.108	**40471**	**Allantoin**
		3.752	**11438**	**Theobromine**
		4.055	**9698**	**Paraxanthine**
		5.948	659348	Caffeine

4.4.3. Identification of enzymes in crude extract responsible for degradation of caffeine:

4.4.3.1. Caffeine oxidase:

Initial experiments on the identification of the enzymes responsible for caffeine degradation by *P. alcaligenes* MTCC 5264 showed an oxygenase activity. Madhyasta et.al., (1999), using a consortium of *Klebsiella* sp. and *Rhodococcus* sp. reported the presence of a caffeine oxidase which oxidizes caffeine at the C-8 position producing 1,3,7-trimethyl uric acid. However, when purification of caffeine degrading enzymes from crude extract of *P. alcaligenes* MTCC 5264 was attempted, there was a rapid loss in the activity. The reasons for this loss in the activity were not known. When the dialyzed enzyme was assayed for activity in the presence of NADPH, there was a regain in the activity. The reason for the loss and regain of activity was also found to be due to cofactor leaching during the dialysis step (Table 4.4.3.1).

This made us rethink whether the enzyme was an oxidase or it belongs to other classes of enzymes. The loss of activity after dialysis and partial regain in the activity of the enzyme by addition of NADPH lead to confirm whether the enzyme is a oxygenase or a dehydrogenase. So the crude enzyme extract was assayed for its specificity towards caffeine, theobromine and hypoxanthine. We have confirmed that that the partially purified enzyme does not accept theobromine and hypoxanthine as the substrates but accepts caffeine as substrate. This proves that the enzyme was not a dehydrogenase as reported by Blecher and Lingens (1988). The attempts were now made to identify the actual nature of the enzyme involved in demethylation of caffeine with oxygenase activity but not producing the C-8

oxidation product (1,3,7 trimethyl uric acid as reported by Madhyasta et.al., 1999) confirmed that the enzyme is not an oxidase. Further studies were done to identify the characteristics of the demethylating enzyme.

Table 4.4.3.1. Observations on experiments conducted on assay of enzyme (oxygenase) for caffeine.

S. No.	Reaction mixture	Activity (Δ Abs/min)	Result
1.	DCPIP + PES + Caffeine	0.000	No activity
3.	DCPIP + PES + CFE + Caffeine	-0.271	Activity
4.	Dialyzed CFE + PES + DCPIP + Caffeine	-0.000	No activity
5.	Dialyzed CFE + NADPH + DCPIP + PES + Caffeine	-0.028	Activity regained
6.	15 hours dialyzed + NADPH + caffeine + DCPIP + PES	-0.258	Increased activity
7.	Cyt c reductase + caffeine + NADPH + DCPIP + PES	-0.002	No response

4.2.3.2. Xanthine dehydrogenase (XDH) :

Dehydrogenase enzymes are known to accept NAD as co-factors and are involved in the second step of degradation (Fig. 4.4.2.2) of caffeine in bacteria (Blecher and Lingens, 1988). Incubation of crude enzyme extract after dialysis with NAD as cofactor showed that the enzyme catalyzed the conversion of hypoxanthine and theobromine to their respective uric acids. However this enzyme does not accept caffeine as the substrate. (Table 4.4.3.2 & 4.4.3.3). This confirms the presence of xanthine dehydrogenase (XDH) enzyme in the crude enzyme extracts of *P. alcaligenes* MTCC 5264 which is similar to that reported by Blecher and Lingens (1988) and this enzyme is involved in a degradation step after the 1N-demethylation of caffeine. The activity of XDH in CFE was found to be 196.6 U/ml

when theobromine was used as substrate (Table 4.2.2.2.) and 87.5 U/ml when paraxanthine was used as substrate (Table 4.2.2.3).

Table 4.4.3.2: Activity of xanthine dehydrogenase in crude enzyme extract incubated with theobromine as substrate.

Time (Sec)	Absorbance (340nm)	Δ ABS
0	0.924	00
30	0.978	0.054
60	1.074	0.087
90	1.148	0.096
120	1.232	0.158
150	1.363	0.215
180	1.476	0.113
210	1.598	0.122
240	1.676	0.078
270	1.743	0.067
Rate=0.661 abs/min; Activity = 196.6 U/ml		

Table 4.4.3.3: Activity of xanthine dehydrogenase in crude enzyme extract incubated with paraxanthine as substrate.

Time (Sec)	Absorbance (340nm)	Δ ABS
0	0.919	00
30	0.936	0.017
60	0.969	0.033
90	0.998	0.029
120	1.032	0.034
150	1.076	0.044
180	1.119	0.043
210	1.152	0.053
240	1.216	0.064
270	1.283	0.067
Rate=0.231 abs/min Activity = 87.5 U/ml		

These observations indicate that the enzyme involved in the first step of degradation of caffeine is not a dehydrogenase and the actual enzyme involved in demethylation of caffeine was identified as caffeine demethylase which is discussed in detail in the following sections (4.4.3.8).

4.4.3.3. Heteroxanthine demethylase:

Heteroxanthine demethylase activity in the crude extract was determined and it was found that the enzyme acts upon the dimethyl xanthines Viz., theobromine and paraxanthine and converts them to monomethyl xanthine (7-methyl xanthine) (Fig. 4.4.2.2). This enzyme is also involved in the demethylation of 3,7- dimethyl uric acid and 1,7- dimethyl uric acid formed by the action of xanthine oxidase on theobromine and paraxanthine converting them to 7-methyl uric acid. This enzyme however does not accept caffeine as substrate. The heteroxanthine demethylase activity in CFE was found to be 154.36U/ml.

4.4.3.4. Xanthine oxidase:

Xanthine oxidase is a ubiquitous enzyme involved in the degradation of purines and is expressed in high levels in almost all the living systems, which along with other enzymes acts as a recycling system of old and degraded genetic material for the cells through the salvage pathways (Vitória and Mazzafera, 1999; Moriwaki, et.al., 1999). Xanthine oxidase was found to be the enzyme involved in the oxidation of the dimethyl and monomethyl xanthines formed during the degradation of caffeine (Fig. 4.4.2.2., Table. 4.4.3.4.).

From our experiments it was found that xanthine oxidase was responsible for the formation of uric acid in the caffeine degradation pathway of *P. alcaligenes* MTCC 5264. The xanthine oxidase activity in the enzyme extract was found to be 522 U/ml. It accepts theobromine and paraxanthine as substrates to form the respective methyl uric acids, which were detected by HPLC analysis of the reaction mixtures of enzyme and caffeine.

Table 4.4.3.4. Xanthine oxidase activity in cell free extract of *P. alcaligenes* MTCC 5264.

Time (Sec)	Absorbance (600nm)	Δ ABS
0	1.921	0
30	1.456	-0.465
60	1.117	-0.339
90	0.894	-0.223
120	0.656	-0.258
150	0.421	-0.235
180	0.373	-0.048
Rate= -0.522 abs/min (which is equivalent to 522 U/ml in enzyme extract)		

4.4.3.5. Uricase:

Uric acid formed by the activity of xanthine oxidase on methyl xanthines is converted to allantoin by the enzyme uricase (urate oxidase), which is the ring hydroxylating enzyme (Fig.4.4.2.2.). This enzyme is involved in the opening of the purine ring of methyl xanthines (degraded products of caffeine) (Vogels and Van der Drift, 1976; Bergmann et.al., 1962; Burg and Stein, 1972; Campbell, Jr., L. L, 1954; Trijbels and Vogels, 1967).

Table 4.4.3.5. Uricase activity in cell free extract of *P. alcaligenes* MTCC 5264.

Time (Sec)	Absorbance (340nm)	Δ ABS
0	0.985	0
30	0.761	-0.224
60	0.652	-0.109
90	0.601	-0.051
120	0.534	-0.067
150	0.501	-0.033
180	0.478	-0.023
Rate= -0.169 abs/min (Activity = 98.7 U/ml) 1 Unit = 1μM of uric acid converted to allantoin/minute = 0.0017 Abs/min.		

The uricase activity in the extract was found to be 98.7 U/ml (Table 4.4.3.5). Uricase enzyme is involved in the 5th step of the caffeine degradation pathway in *P.*

alcaligenes MTCC 5264. The results of this study are also similar to that reported by Vogels and Van der Drift, (1976); Blecher and Lingens, (1988) and several others. Further studies were conducted on the identification of other enzyme produced by *P. alcaligenes* MTCC 5264.

4.4.3.6. Allantoinase and Allantoicase:

Allantoin formed during the oxidation of uric acid is degraded to allantoic acid by allantoinase. Allantoic acid is further degraded to urea and glyoxylic acid by allantoicase. Glyoxylic acid formed is further converted to Urea by glyoxalate dehydrogenase. Uricase activity was determined in the CFE and allantoin produced by the above reaction was monitored by HPLC analysis of the reaction mixture (Table 4.4.3.5, Table 4.4.2.1) and allantoin and allantoic acid were detected in the reaction mixture. Allantoic acid produced during the degradation of allantoin is further converted to ureidoglycolate which is believed to be brought about by allantoicase. Ureidoglycolate is further degraded to urea and glyoxylic acid by the action of glyoxylate dehydrogenase which was observed in the reaction mixture.

4.4.3.7. Urease:

Urea formed in the above reactions is further converted to ammonia and carbon dioxide by the enzyme urease present in the enzyme extract from *P. alcaligenes* MTCC 5264. The production of ammonia which was the end product of caffeine degradation was monitored by Nessler's reagent (Table 4.4.3.6). It was also observed that the pH of the reaction mixture increased from 6.8 to 8.7. The urease activity in CFE was found to be 48.4 U/ml.

Table 4.4.3.6. Assay for urease enzyme in cell free extract.

Time (Min)	set 1 (Abs 490 nm)	Set 2 (Abs 490 nm)
0	0.201	0.204
5	0.399	0.412
15	0.49	0.492
30	0.533	0.539
60	0.76	0.7772
(Activity = 48.4 U/ml)		

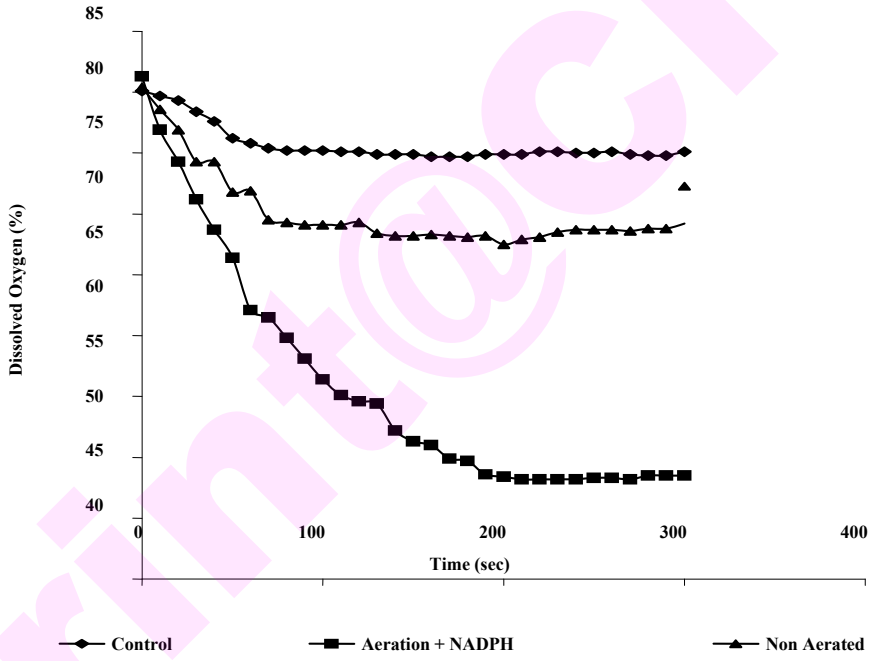
4.4.3.8. Caffeine demethylase:

The first step of caffeine (containing 3 methyl groups) degradation includes a demethylation reaction, which was confirmed by the product formation i.e., theobromine having 2 methyl groups. The enzyme activity *in vitro* has not been conclusively reported earlier. It is also interesting to know that this enzyme is a mono-oxygenase requiring NADPH as a cofactor and the characteristics of this demethylase are described below. The oxygenase nature of this enzyme is responsible for the positive result in the DCPIP assay in our earlier experiments. However the HPLC analysis of the enzyme reaction mixture showed the formation of theobromine which is a N-Demethylation product in contrast to the C-8 oxidation product reported by Madhyasta et.al., (1998, 1999), and a demethylase fits more into the reaction scheme than an oxidase as found in our observations. The oxidase model does not fit into the caffeine degradation pathway of *P. alcaligenes* MTCC 5264 (Fig. 4.4.2.2.). The monooxygenase activity was observed along with the demethylating activity in the presence of NADPH as a cofactor confirming our hypothesis.

a) Monooxygenase activity of caffeine demethylase:

The monooxygenase nature of the enzyme was determined by oximetry (Fig. 4.4.3.1.). It was observed that the enzyme requires one mole of oxygen per mole of caffeine converted. It was also found that the enzyme is oxygen dependent and has a monooxygenase activity not an oxidase activity, indicating that the enzyme is also a monooxygenase as described in literature (Asano et. al.. 1994).

Figure 4.4.3.1. Determination of caffeine demethylase activity in CFE from *P . alcaligenes* MTCC 5264 by oximetry.



From the results obtained in all the experiments so far we could conclude that the enzyme involved in the first step of the caffeine degradation pathway is a NADPH dependent monooxygenase (Fig. 4.4.2.2). There are a few reports available

in literature indicating the presence of a monooxygenase in the caffeine degradation pathway (Middelhoven and Lommen, 1984).

Reports on the enzyme involved in caffeine degradation (Blecher and Lingens, 1977; Asano et.al., 1994) indicate that a demethylating enzyme in the caffeine degradation pathway which is a mixed function oxygenase, which requires the presence of a cofactor (NAD(P)H).

The reaction scheme is as follows:

$$\text{Caffeine} + \text{NAD(P)H} + O_2 \xrightarrow{\text{CDM Enzyme}} \text{Theobromine} + CH_3OH + \text{NAD(P)}^+$$

From the above experiments the enzymes involved in the caffeine degradation pathway of *P. alcaligenes* MTCC 5264 have been identified and Figure 4.4.2.2 represents the caffeine degradation pathway in *P. alcaligenes* MTCC 5264. The activity of caffeine demethylase enzyme from *P. alcaligenes* MTCC 5264 is similar to the cytochrome P450 found in eukaryotes. The bacterial caffeine demethylase differs from the cytochrome P450 as it is not membrane bound and enzyme preparations from *P. alcaligenes* MTCC 5264 do not show the carbon monoxide spectrum characteristic to cytochrome P450. Moreover, the enzyme does not require a cytochrome c reductase as a co factor which is very essential for Cytochrome P 450 and occurs as a single protein (Fig. 4.4.4.4, Section 4.4.4.) in the cell free extracts of *P alcaligenes* MTCC 5264. Thus this enzyme is a novel protein and has clear distinction from the cytochrome P450, caffeine oxidase (Madhyasta et. al., 1999) and caffeine demethylase complex described by Sideso et.al., (2001).

Figure 4.4.3.2. Caffeine degradation pathway in *P. alcaligenes* MTCC 5264.

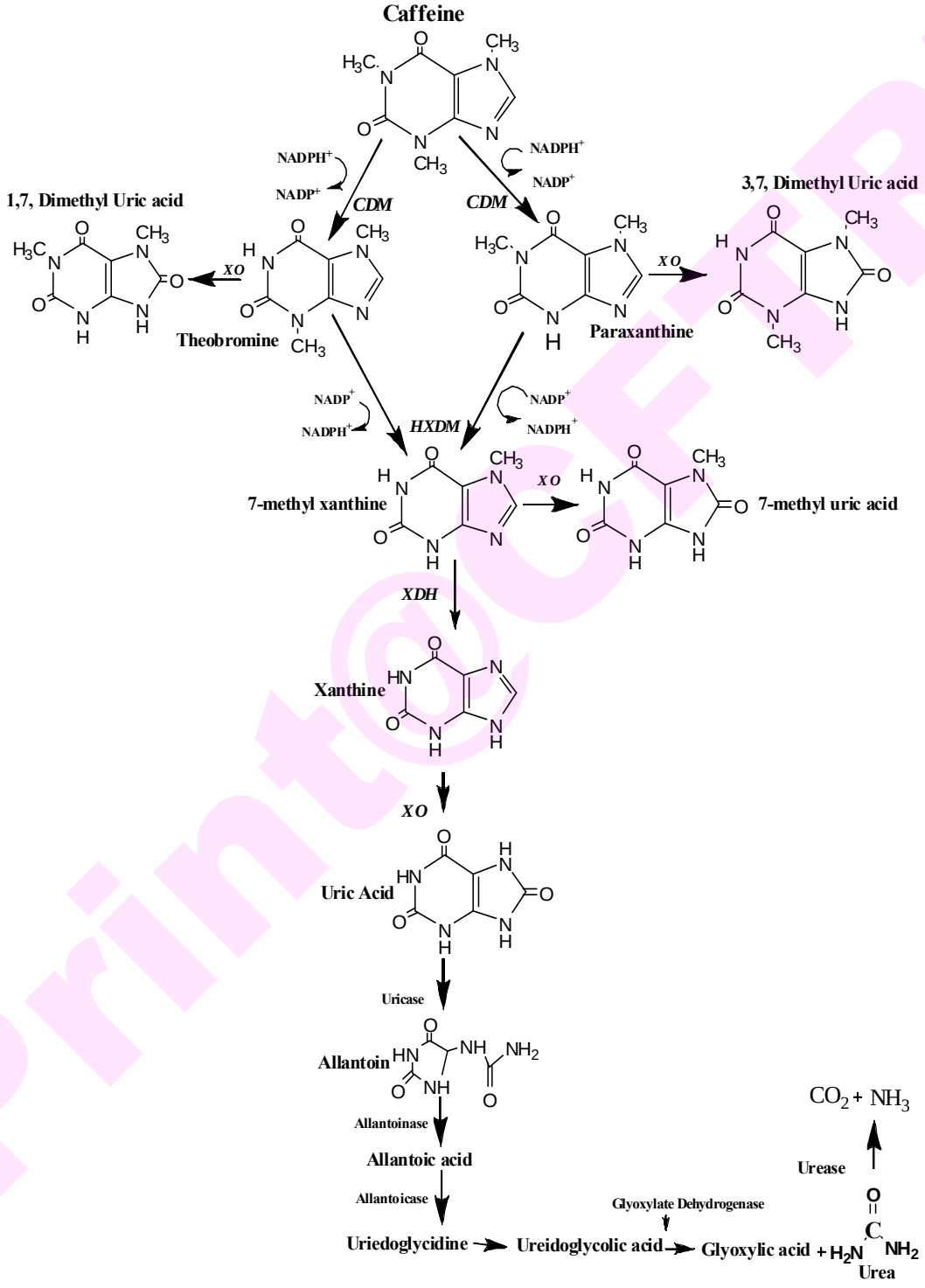
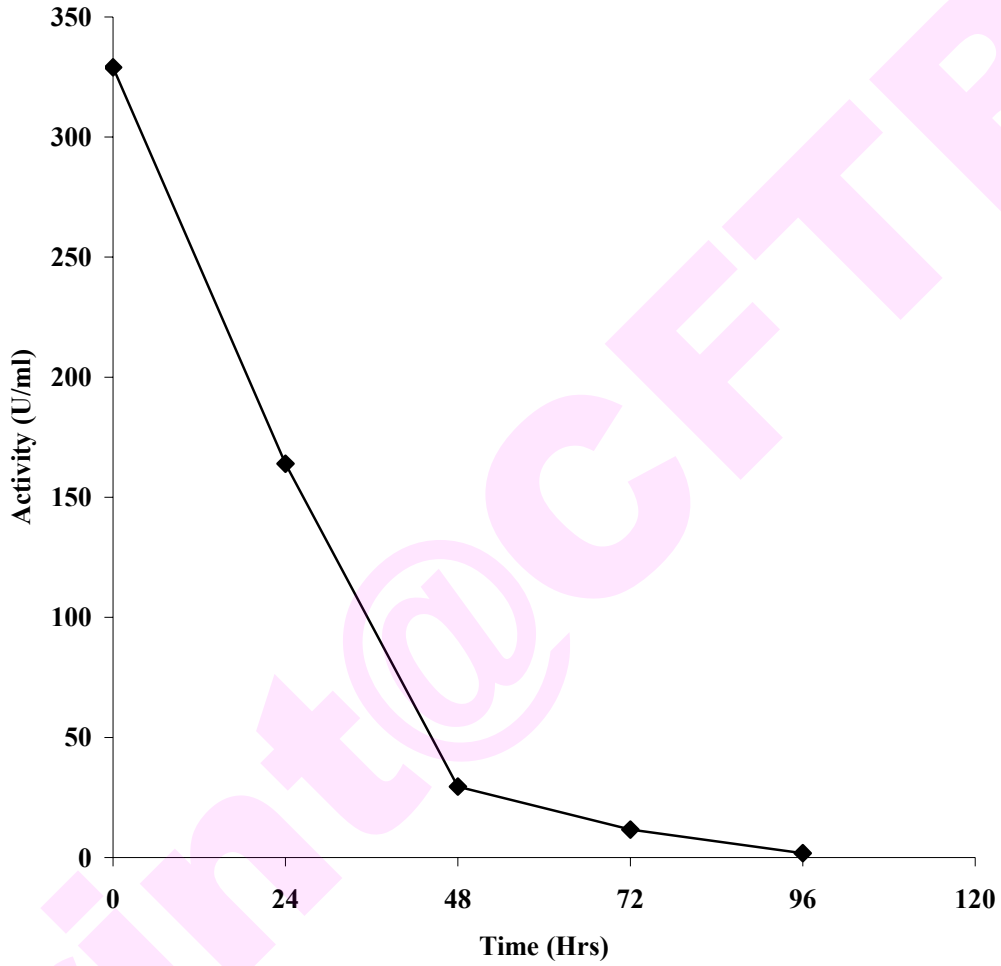


Figure 4.4.3.2., represents the caffeine degradation pathway in *P. alcaligenes* MTCC 5264. Caffeine is converted to theobromine and paraxanthine by a 1N and 3N-demethylase respectively. The dimethyl xanthines are then oxidized to their respective uric acids by the action of xanthine oxidase. The dimethyl uric acids undergo further demethylation by heteroxanthine demethylase to produce 7-methyl xanthine. The 7-methyl xanthine is demethylated to xanthine dehydrogenase and by the action of xanthine oxidase is converted to uric acid. Uricase further converts the uric acid to allantoin which is degraded to allantoic acid, uriedoglycolate and urea by the action of allantoinase, allantoicase and glyoxylate dehydrogenase respectively. Urea is further converted to ammonia and CO_2 by the action of urease. The methyl groups removed by demethylases and the dehydrogenase are oxidized to methanol which is further oxidized to formaldehyde. Formaldehyde is degraded to CO_2 and water by the action of formaldehyde dehydrogenase. The formaldehyde oxidation was confirmed by monitoring the formation of formaldehyde in the reaction mixture followed by its disappearance. Thus the products of caffeine degradation are degraded to safe and simpler molecules like NH_3 and CO_2 (Figure 4.4.3.2).

4.4.4. Storage stability of caffeine demethylase:

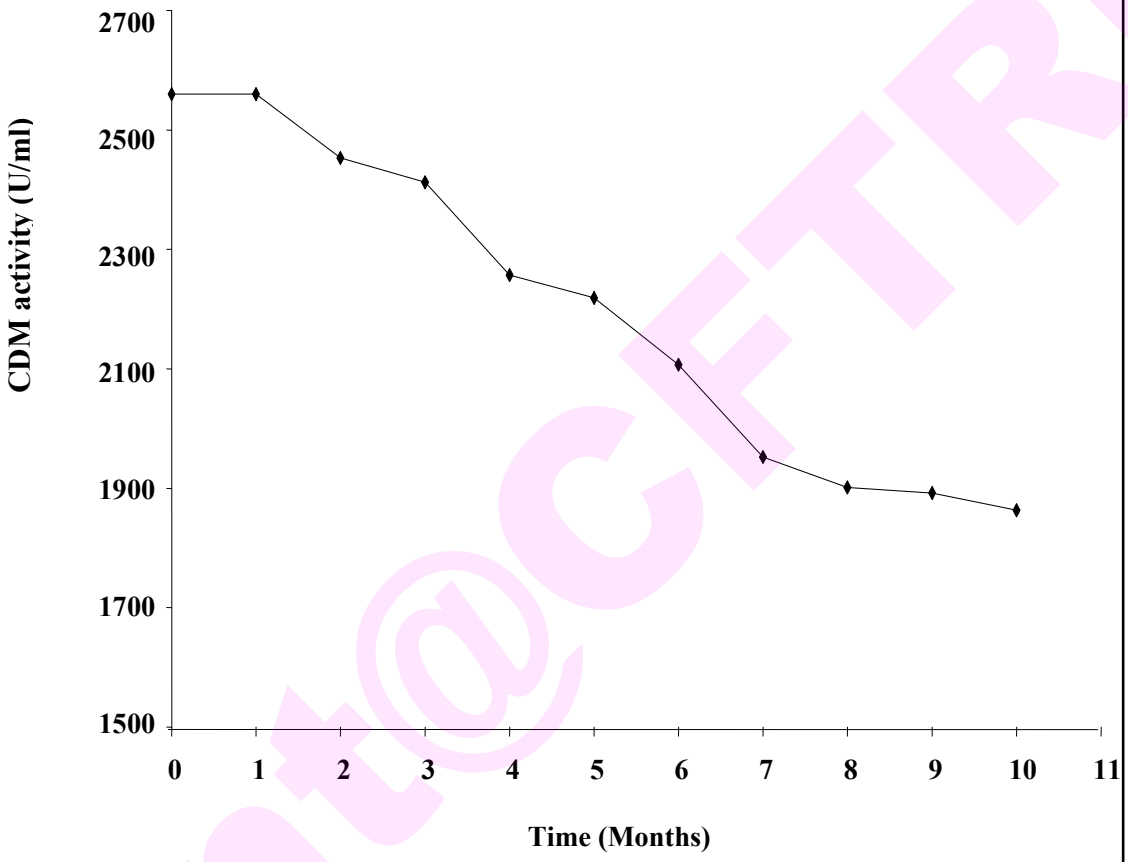
It is generally known that the demethylases are unstable after extraction from the cells and rapidly lose activity even after preservation at 4^0C (Gluck and Lingens, 1988; Sideso et. al., 2001). Experiments conducted on the purification of the enzyme showed a loss in the activity on storage at 4^0C over a period of 96 hours (Fig. 4.4.4.1a) and thus the enzyme was not stable in pure form.

Figure 4.4.4.1 a. Storage stability of caffeine demethylase stored at 4°C.



It was interesting to note that frozen cell free extracts were found to retain 73% of the initial caffeine demethylase activity after 10 months of storage (Fig. 4.4.4.1b.). The frozen cell free extracts could be used for the biodecaffeination of caffeine solutions and materials.

Figure 4.4.4.1.b. Storage stability of caffeine demethylase in CFE frozen and stored at -20°C.



Purification of the demethylase was attempted and it was found that the activity loss increased with each step of purification. During the ammonium sulphate precipitation step about 50% of the initial activity was lost. Dialysis step also lead to complete loss of activity of the enzyme which was partially restored by the addition of the cofactors. Therefore studies were conducted on the stabilization of caffeine demethylase enzyme.

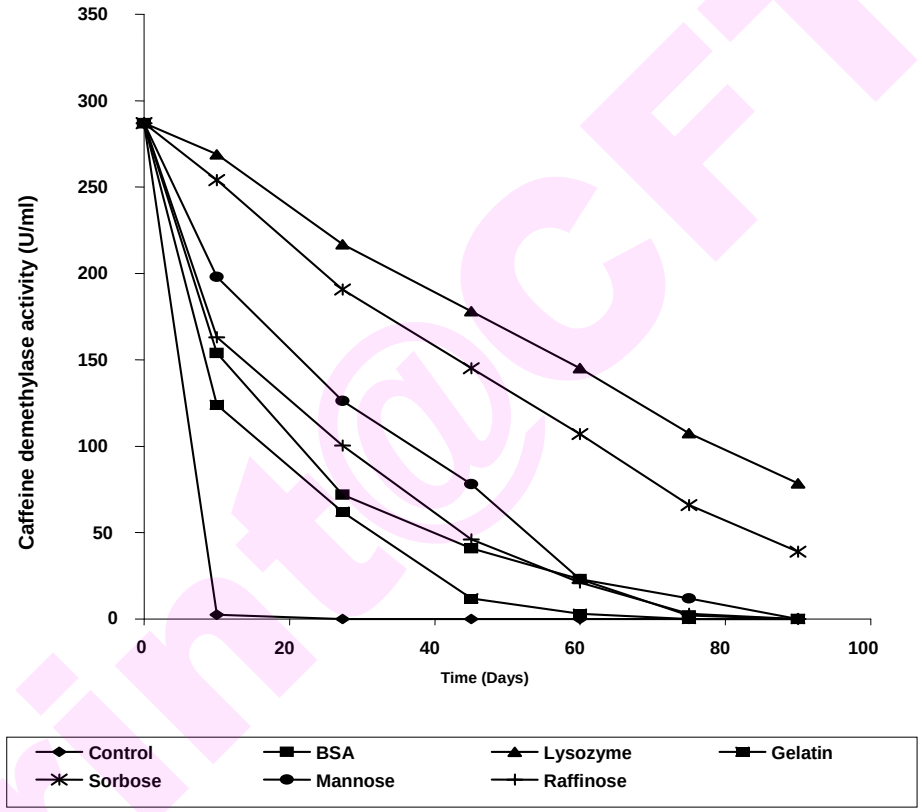
4.4.4.1. Stabilization of caffeine demethylase:

It is generally known that the demethylases are unstable after extraction form the cells and rapidly lose activity even after preservation at 4^{o}C (Gluck and Lingens, 1987; Asano et. al., 1993, Sideso et. al., 2001). Initial experiments conducted in our laboratory on the purification of the enzyme showed a loss in the activity on storage at 4^{o}C over a period of 96 hours. The enzymes extracted from cells are known to be unstable in vitro and caffeine demethylase enzyme activity is lost rapidly in solution. Therefore stabilization of caffeine demethylase a key enzyme in the biodecaffeination process is very important. Several stabilizing agents were used for the stabilization of caffeine demethylase enzyme.

Lysozyme, bovine serum albumin (BSA) and gelatin are known to stabilize enzymes and are known as protein based stabilizing agents (PBSA). In our laboratory at CFTRI, Gouda et. al., (2002, 2003) have reported the stabilization of glucose oxidase, mutarotase and invertase by the use of these PBSAs for biosensor application up to 1000 analyses. The effectiveness of these PBSAs along with sorbose, mannose, and raffinose on the stabilization of the caffeine demethylase enzyme in solution was tested. The stability of caffeine demethylase in solution in the presence of PBSAs and Polyols improved. In the absence of stabilizing agents the half life (T ½) of the enzyme was less than 24 hrs (Fig. 4.4.4.2.). Lysozyme at 2 mg/ml in the enzyme preparation was found to confer maximum stability to the enzyme with a T ½ of 60 days, where as BSA and gelatin could stabilize the enzyme for less than 20 days only (Fig. 4.4.4.2.). Lysozyme is a basic protein (pI~11) may be interacting with the caffeine demethylase enzyme through ionic

interactions and protecting the enzyme against loss of activity. In case of polyols, sorbose was found to be the best stabilizing agent (Fig. 4.4.4.2.) and the stabilization is brought about by increase in the hydrophobicity of the medium offered by the polyol.

Figure 4.4.4.2. Stabilization of caffeine demethylase by PBSAs and polyols.



These polyols are known to form a hydrophobic cage around the enzyme molecule protecting it from the destabilizing agents. A similar effect of protection through increased hydrophobic interactions was observed in our previous studies on stabilization of glucose oxidase (GOD) using silanization, which could stabilize GOD at higher temperatures upto 75^{o}C (Sarath et.al., 2004). The stabilized enzyme preparation was very essential for biodecaffeination of tea and coffee and was

found useful in the development of biodecaffeination process which is reported in the next chapter of the thesis.

4.4.5. Purification of caffeine demethylase:

The protein content and caffeine demethylase activity in the crude CFE were found to be 1.104 mg/ml and 329.1 U/ml respectively. Purification of caffeine demethylase was carried out using different procedures and is described below.

4.4.5.1. Salting out:

The enzyme was salted out at an ammonium sulphate concentration of 30-60 % w/v. The activity however dropped by around 50 % (Fig.4.4.5.4). There was a slight increase in the protein content after salting out may be due to the concentrating effect. The protein content in the enzyme preparation after salting out was 1.19mg/ml and caffeine demethylase activity was 163.26 U/ml.

4.4.5.2. Gel permeation chromatography:

Gel permeation chromatography on Sephadex G-100 column lead to a further purification of caffeine demethylase. The activity was 156 U/ml and the protein content was 0.56 mg/ml (Fig.4.4.5.1. and Fig.4.4.5.4.). The specific activity of caffeine demethylase was 278.57 U/mg protein. From Fig. 4.4.5.1., it was observed that the enzyme eluted from the gel in the fractions from 40-45 and a 2 fold increase in the purity was observed.

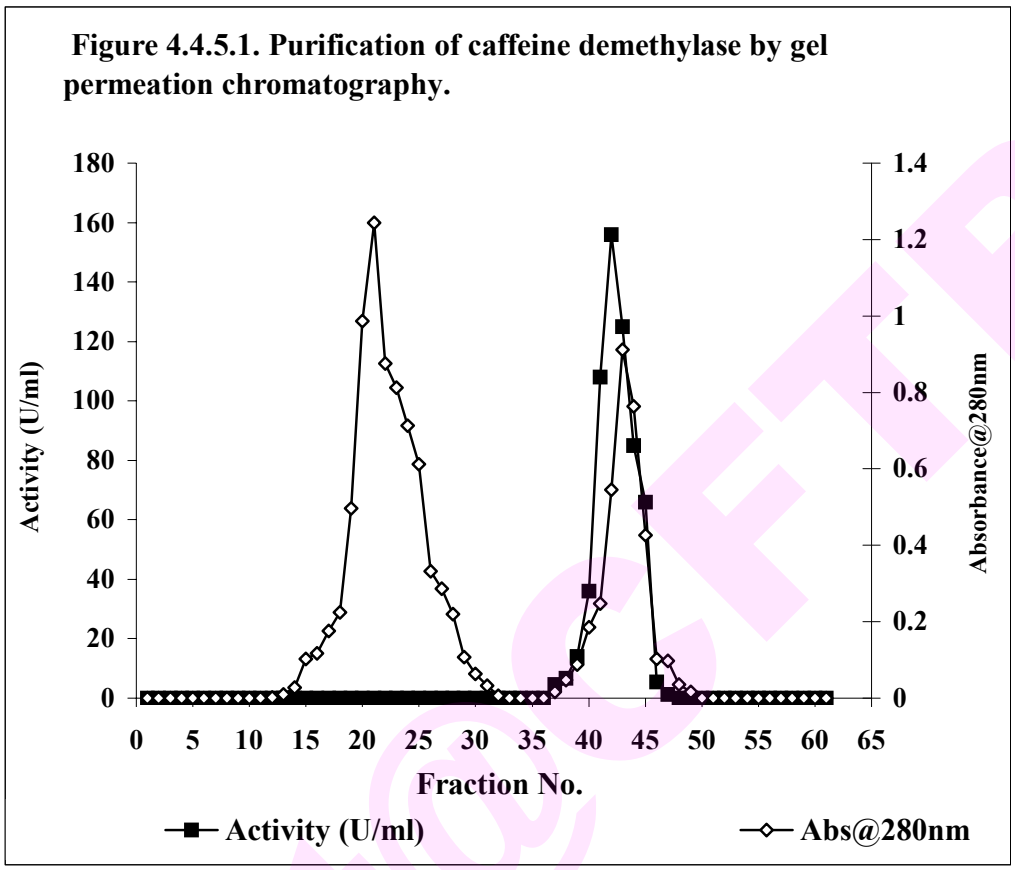


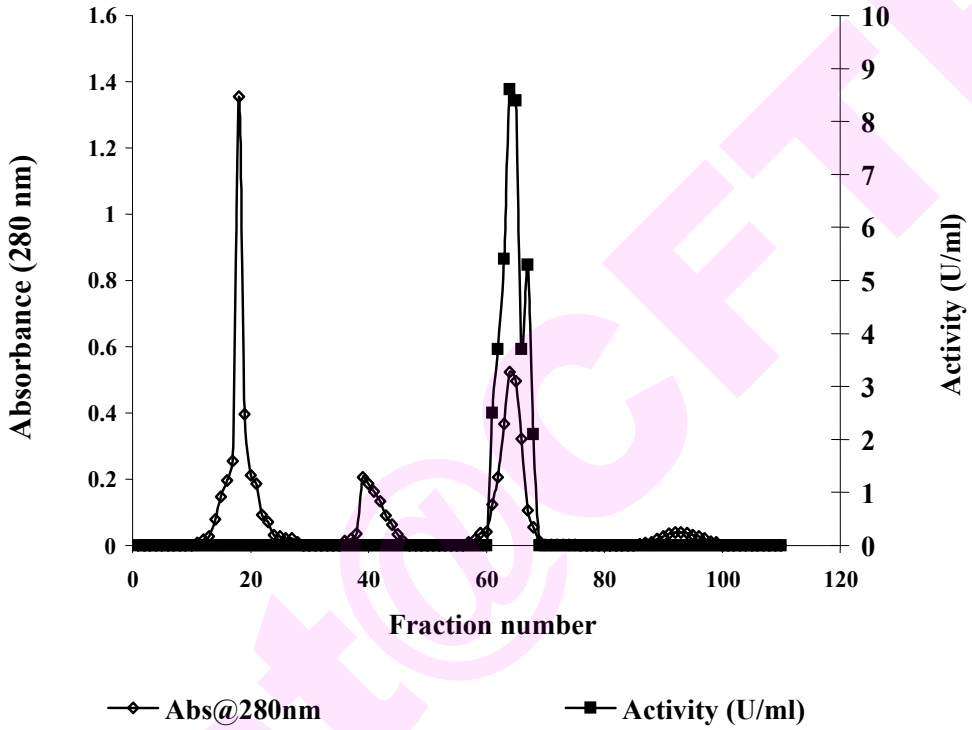
Figure 4.4.5.1. Purification of caffeine demethylase by gel permeation chromatography.

4.4.5.3. Ion exchange chromatography:

Purification of the enzyme was further tried by ion exchange chromatography (Fig. 4.4.5.2.). The enzyme was bound to the column and eluted with 0.25 M NaCl. However the yield was very less with only 0.19 mg/ml of protein and 8.7 U/ml of activity. The protein content in the purified enzyme preparation was found to decrease from 0.56 mg/ml to 0.19mg/ml and the specific activity was 45.18 U/mg protein. It was observed that the protein binds to the gel and complete elution of the enzyme was not occurring leading to a very high reduction of enzyme activity and yield. This method of purification was thus

discontinued due to the loss of enzyme activity as well as yield. Further purification was tried by hydrophobic interaction chromatography.

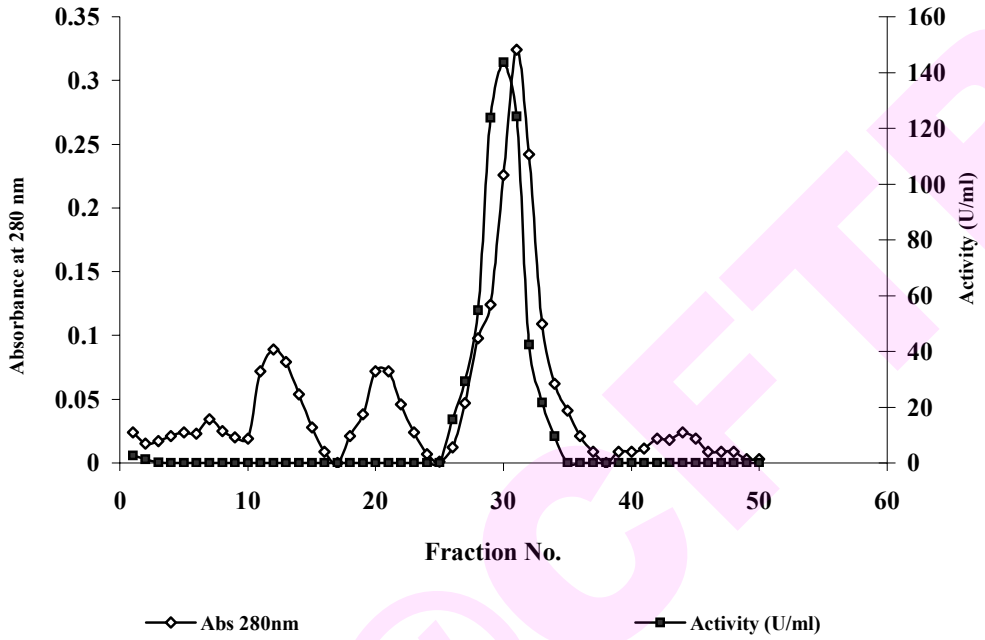
Figure. 4.4.5.2. Purification of caffeine demethylase by ion exchange chromatography on DEAE-Sephadex G-75.



4.4.5.4. Hydrophobic interaction chromatography (HIC):

Fig.4.4.5.3. represents the purification of caffeine demethylase by HIC. The enzyme was found to bind to phenyl sepharose matrix and was eluted with 0.5 M ammonium sulphate in the buffer, indicating the enzyme to be more hydrophobic in nature. The activity and yield were also better compared to ion exchange chromatography with 143.7 U/ml of activity and 0.36 mg/ml. The specific activity of the enzyme also increased from 278.57 U/mg protein in case of gel permeation chromatography to 399.16U/mg protein.

Figure 4.4.5.3. Purification of caffeine demethylase by hydrophobic interaction chromatography.



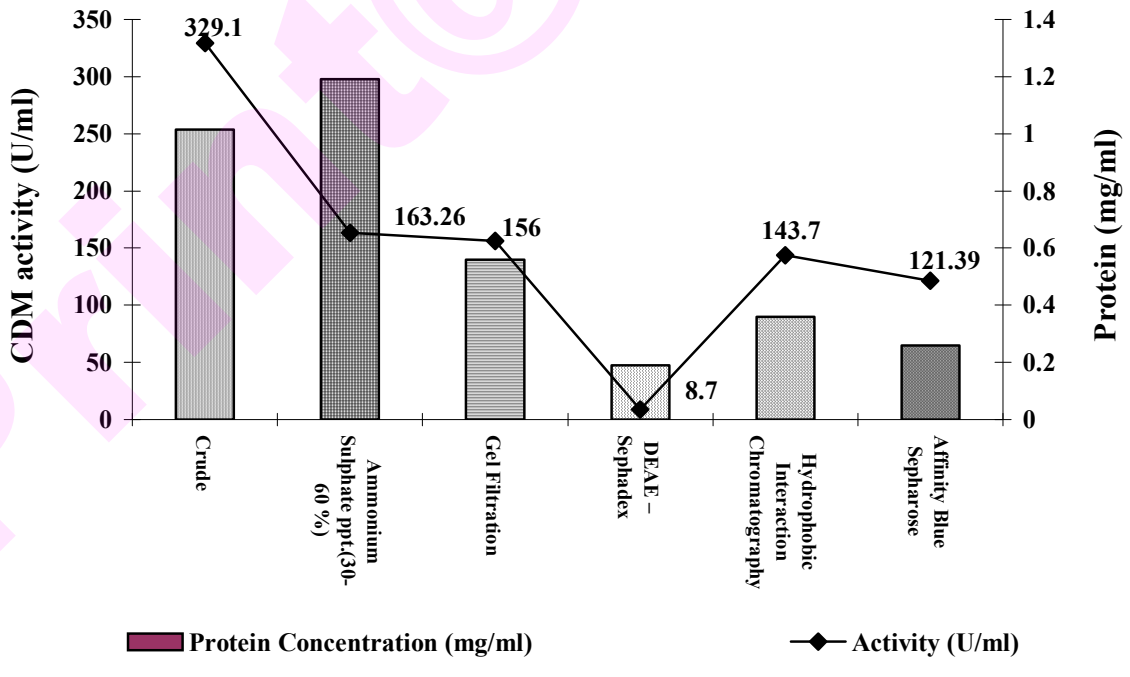
4.4.5.5. Purification of caffeine demethylase by affinity chromatography:

Active fractions obtained from hydrophobic interaction chromatography were purified on a blue sepharose column; the active fractions were pooled, concentrated and stored at 4^0C until further use. Cibacron Blue (Blue Sepharose) matrix is used for the purification of enzymes containing a NAD/NADPH binding region. Johan and Mooibroek (1998) have reported the purification of NADP-mannitol dehydrogenase from the button mushroom by affinity chromatography on a Blue sepharose column. The enzyme is a NADP dependent enzyme and was purified to homogeneity by the use of blue sepharose. Caffeine demethylase is a NADPH dependent enzyme and the enzyme binds to the blue sepharose column through the NADPH fold leading to purification of the enzyme under a gradient of

sodium chloride. The purified enzyme preparation on analysis by SDS-PAGE was found to contain two bands which were identified as 1N and 3N-demethylases producing theobromine and paraxanthine respectively and the results are discussed in section 4.4.6.1. below. The activity of caffeine demethylase was found to be 121.3U/ml and the protein content was 0.26mg/ml. The specific activity was found to increase to 468.84 U/mg protein after purification by affinity chromatography on blue sepharose.

Figure 4.4.5.4., represents the total activity profile of caffeine demethylase during purification by different methods. There is a noted decrease both in the protein content (1.014-0.39mg/ml) as well as the activity (329.1-29 U/ml).

Figure 4.4.5.4. Activity profile of caffeine demethylase during purification steps.



This enzyme is highly labile to many factors as atmospheric air, temperature etc, which is reported in literature (Sideso et.al., 2001).

4.4.6. Biochemical characterization of caffeine demethylase:

4.4.6.1. Assay of pure caffeine demethylase:

Confirmation of activity of the enzyme in gel is usually done by activity staining. As very little information is known about this enzyme, and due to the high lability activity staining in gels was not possible. Therefore, the presence of this enzyme in gel was confirmed by cutting the bands and incubating each of the two bands separately with caffeine. The reaction was stopped after 3 hours and the reaction mixture was analyzed by HPLC and TLC (Table 4.4.6.1., Fig. 4.4.6.1. & 4.4.6.2.).

Table 4.4.6.1. HPLC analysis of the reaction mixture of purified caffeine demethylase obtained from SDS PAGE gel.

S.No	Sample ID	Peaks detected	Peak Area	Comments
1	Zero Hour Reaction Mixture	2.583	31561	Unknown
		3.073	10271	Unknown
		5.827	435129	Caffeine
2.	Three Hour Reaction Mixture	2.642	16584	Unknown
		3.103	9524	Unknown
		3.932	**626**	**Theobromine**
		5.845	404326	Caffeine

From Table 4.4.6.1., it is evident that theobromine was formed after incubating the uppermost band (Fig. 4.4.6.1.) with caffeine, thus establishing the presence of the enzyme and the lower (second) band contained the 3N-demethylase producing paraxanthine. Further work on caffeine demethylase was carried out on

the characterization of 1N-demethylase responsible for producing theobromine only and the following sections describe the characterization of 1N-demethylase.

Figure 4.4.6.1. HPLC analysis of the incubation mixture of caffeine demethylase I purified by HIC.

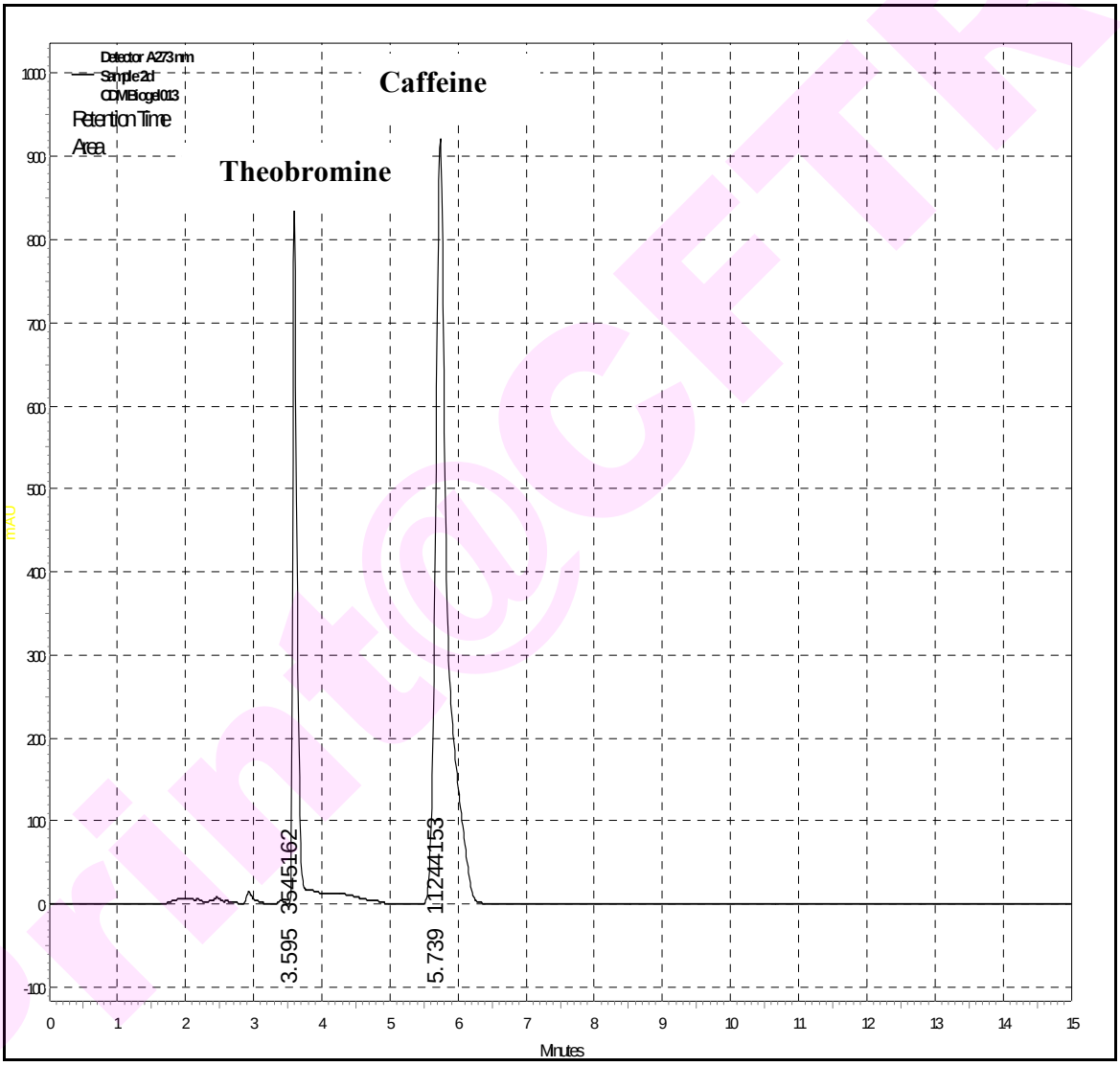
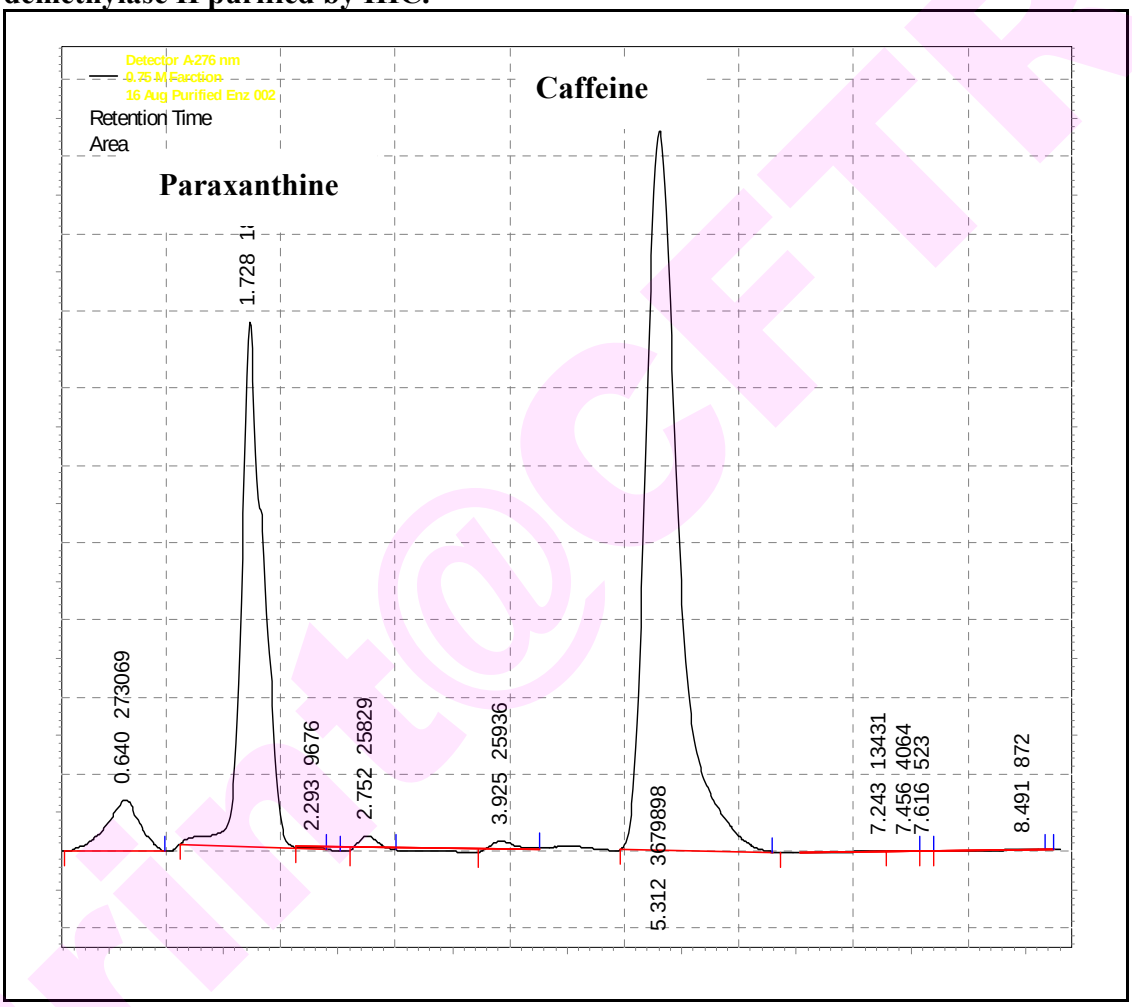


Figure 4.4.6.2., represents the HPLC analysis of reaction mixture containing caffeine incubated with caffeine demethylase obtained from the lower band (CDM2) in the SDS PAGE. Paraxanthine was produced by the action of this

enzyme on caffeine and it proves that the second isoform of caffeine demethylase (CDM 2) is a caffeine 3N-demethylase.

Figure 4.4.6.2. HPLC analysis of the incubation mixture of caffeine demethylase II purified by HIC.



Enzyme Kinetics:

Figure 4.4.6.3 represents the substrate saturation kinetics of caffeine demethylase. The enzyme kinetics of caffeine 1N-demethylase were plotted using easyplot software (www.jlc.com/Perell.htm). From the plot, the Vmax of caffeine1N-demethylase was found to be 0.3531 mM.min^{-1}.mg^{-1} protein.

Figure 4.4.6.3. Substrate saturation plot of caffeine demethylase from *P. alcaligenes* MTCC 5264.

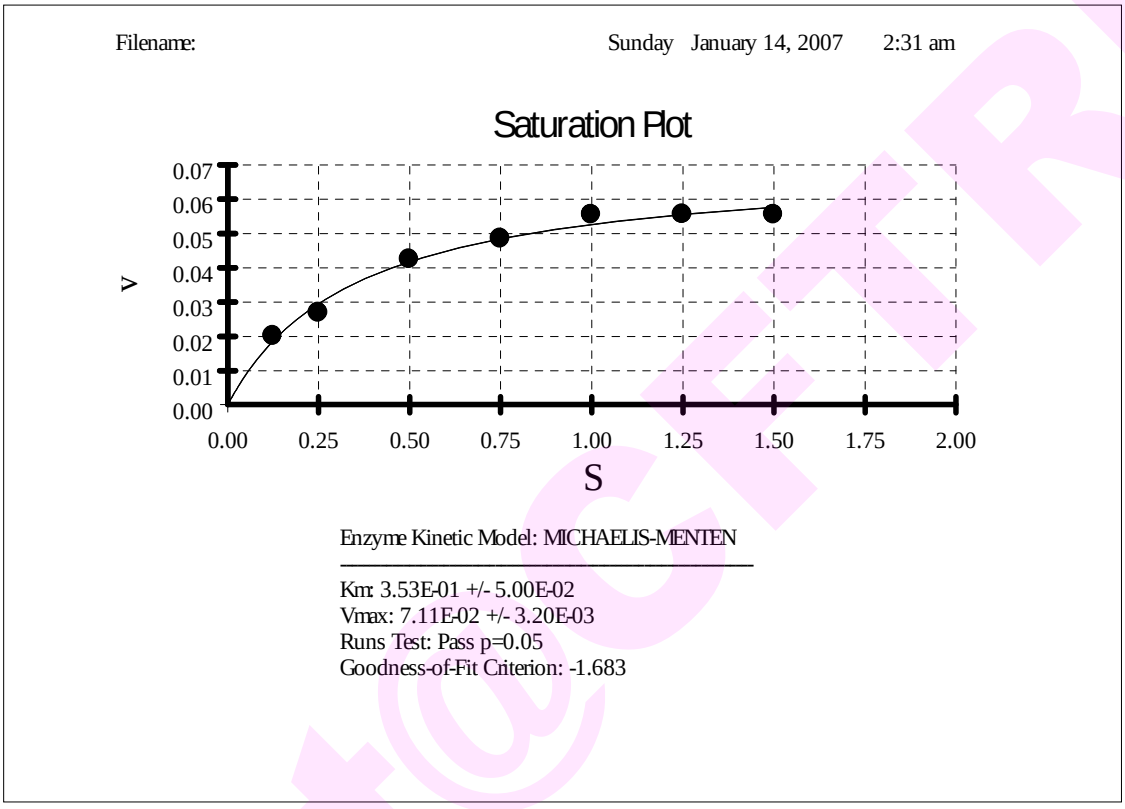
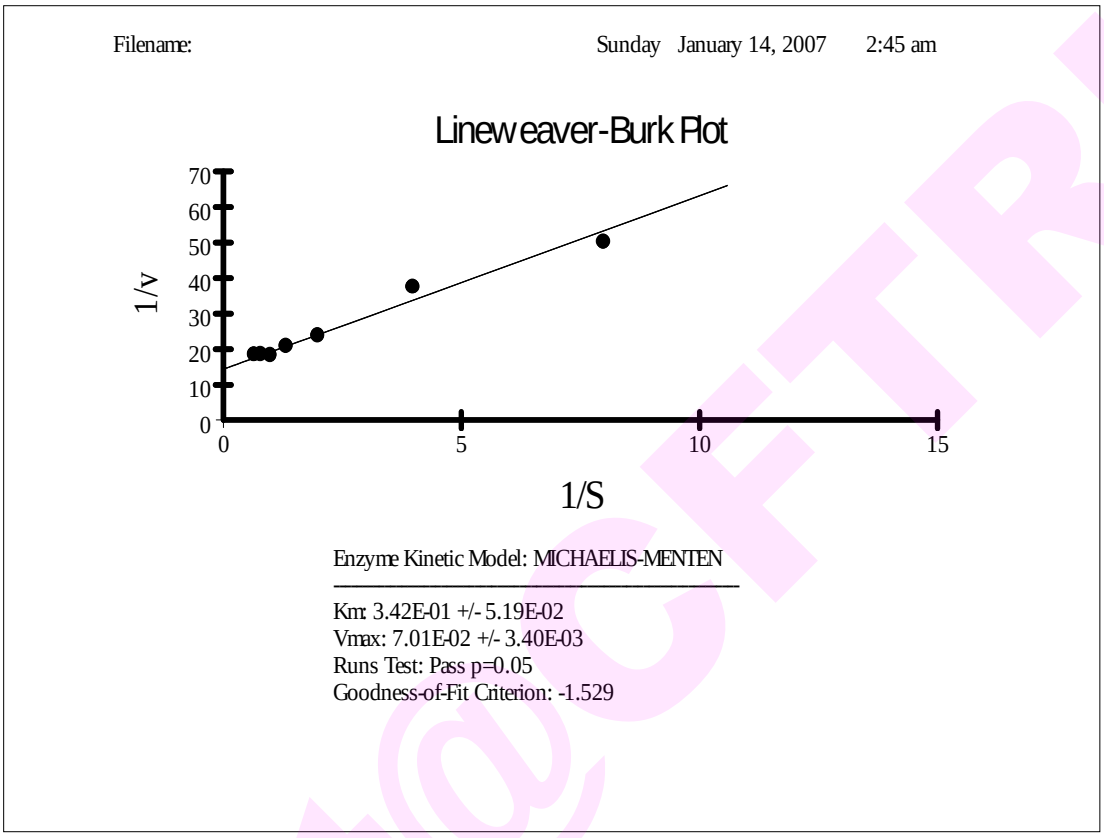


Figure 4.4.6.4., represents the double reciprocal plot (Lineweaver-Burk plot) of caffeine 1N-demethylase activity with different concentrations of caffeine. From the slope of the equation, the **Km** value of the enzyme was found to be 0.0711mM.

Figure 4.4.6.4. Double reciprocal plot of caffeine demethylase activity.



4.4.6.2. Sodium dodecyl sulphate poly acrylamide gel electrophoresis: (SDS PAGE):

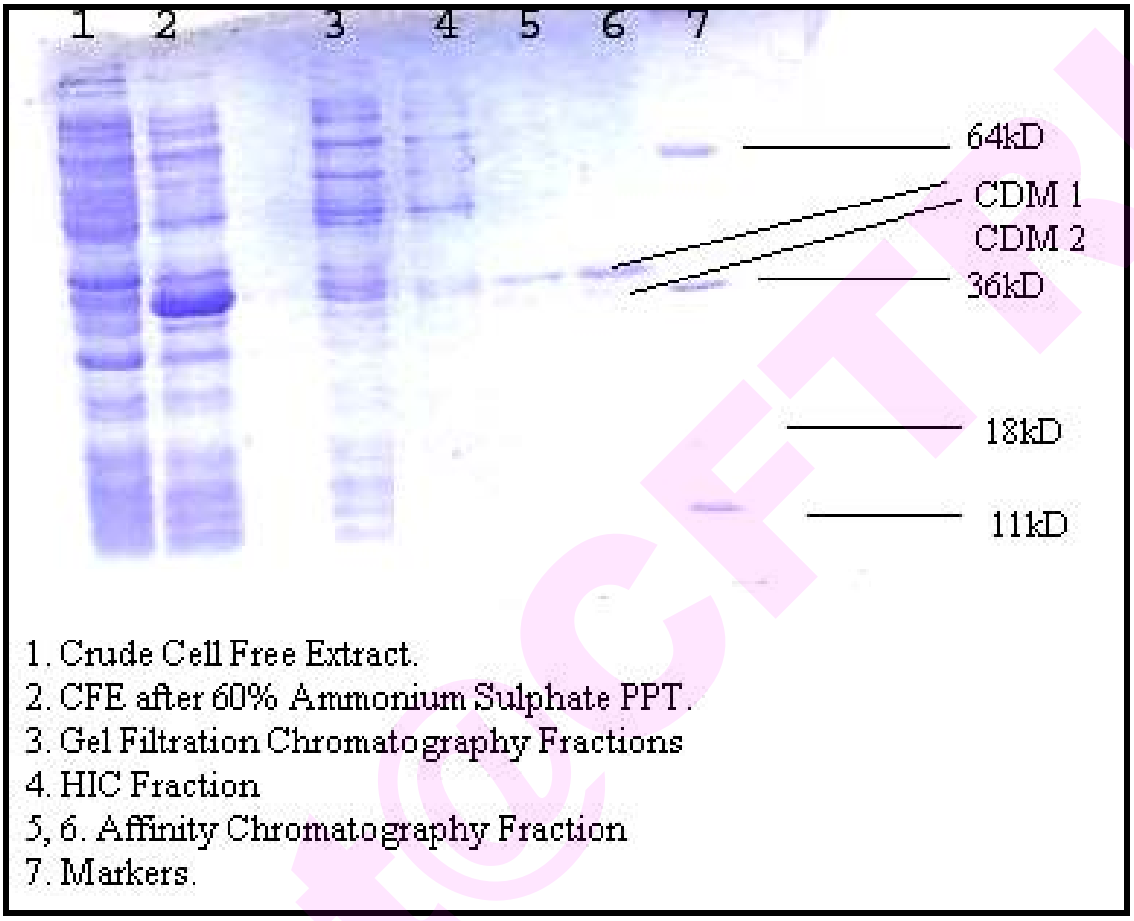
SDS-PAGE was carried out according to the method of Laemmli (1970). The protein could be purified to homogeneity (Fig. 4.4.6.5.). From Fig. 4.4.6.5., it can be observed that the purity of caffeine demethylase increased with each step of purification. Studies were conducted on the purification of caffeine demethylase from bacteria. The enzyme was highly unstable and lost activity very rapidly even at low temperatures. The enzyme required cofactors and oxygen for activity. It is also reported in literature that demethylases are highly unstable (Asano et al., 1993, Sideso et., al., 2001). Further the expression levels of this protein are also too low,

which is another constraint in working with this enzyme added to scanty literature is available on the purification of demethylases.

Caffeine demethylase purified by affinity chromatography on blue sepharose showed two bands CDM1 and CDM2 (lane 5 and 6), which were found to be two isoforms of the enzyme catalyzing the production of theobromine (CDM1) and paraxanthine (CDM2). Lane 1 represents the crude CFE from induced cells of *P. alcaligenes* MTCC 5264. Lane 2 represents the active fractions after ammonium sulphate fraction of the CFE. Lanes 3 and 4 represent the active fractions obtained from gel filtration chromatography and hydrophobic interaction chromatography respectively.

Although several groups have tried to purify and stabilize this enzyme, they have met with very little success. One probable reason for the highly unstable nature of the enzyme may be due to the presence of three aspartyl-prolyl (D-P) bonds in the enzyme which results in autolysis of the protein under acidic conditions. Landon, (1977) has also reported that proteins with aspartyl-prolyl bonds undergo autolysis under acidic conditions. The amino acid sequence of the 1N-demethylase was found to contain 3 D-P bonds at Asp143-Pro144, Asp183-Pro184 and Pro214-Asp215 in the enzyme (Sequence in Page- 240) and studies showed that the enzyme undergoes autolysis at pH below 7.0.

Figure 4.4.6.5 SDS-PAGE of crude and purified caffeine demethylase.



4.4.6.3. Determination of metal in the enzyme:

The metal content in the enzyme was determined by atomic absorption spectroscopy (AAS). It was found that the enzyme contained iron in the active site. Table 4.4.6.2., represents the iron content of the enzyme determined by AAS. It was found that 6µg of iron was present in every milligram of purified caffeine demethylase. The iron atom in the caffeine demethylase is present bound to the porphyrin ring of the rieske- iron sulphur protein of the enzyme. Rieske iron sulphur proteins are present in cytochrome P450 among eukaryotes and in demethylases (Gassner et.al., 1995; Kauppi et.al., 1998; Parales et.al., 1999 among

bacteria. The Fe-S center is involved in the catalysis of planar molecules (Asano et. al., 1994) and is believed to be involved in the catalysis of caffeine also.

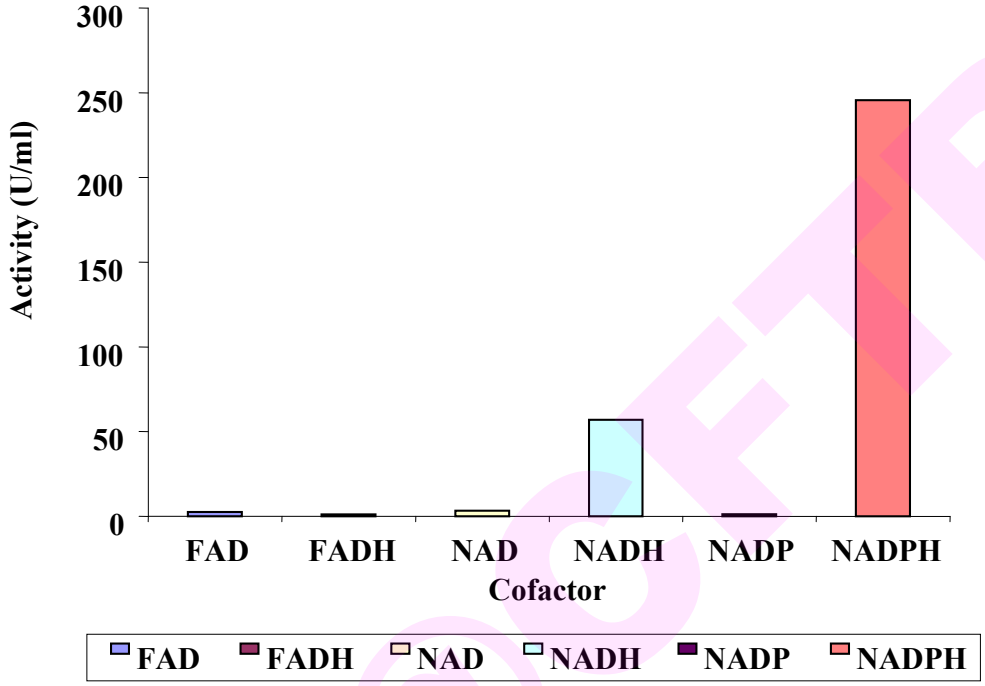
Table 4.4.6.2. Determination of metal content in caffeine demethylase by AAS.

Type	Sample ID	Abs.	BG	Final Conc.
BLK		0.0017	-0.0033	0
STD	Fe	0.0397	-0.0059	0.5
STD	Fe	0.0748	-0.0114	1
STD	Fe	0.1467	-0.0108	2
UNK	Blank	-0.0063	-0.0169	-0.1405
UNK	CDM	0.4352	-0.0219	6.0387
UNK	CDM duplicate	0.438	-0.0226	6.0769

4.4.6.4. Determination of co-factor for caffeine demethylase.

Figure 4.4.6.6., represents the effect of different cofactors on the activity of caffeine demethylase. It was found that NADPH was the best co-factor for caffeine demethylase. The oxidized form of FAD, NAD and NADP were not accepted as cofactors by the enzyme, whereas NADH and NADPH have given high activity of the caffeine demethylase. Addition of FADH did not improve the enzyme activity. The results indicate the enzyme is highly specific to reduced forms of NAD and NADP.

Figure 4.4.6.6. Determination of the cofactor requirement of caffeine demethylase.



4.4.7. Characterization of caffeine demethylase:

4.4.7.1. Characterization of caffeine demethylase by LC-MS analysis:

The purity of the protein was tested by HPLC and SDS-PAGE. Two proteins were found in the fraction from affinity chromatography on blue sepharose. Further it was confirmed that both the bands correspond to two isoforms of caffeine demethylase and differ in molecular weight. The 1N-demethylase (CDM1, Figure 4.4.6.5) was further characterized by separation on a C-8 column and the peak eluted from the column was injected to the MS directly, and analyzed in the ESI positive mode. The peaks corresponding to the enzyme were identified (Fig 4.4.7.1.a,b).

Figure 4.4.7.1a: LC MS analysis of pure caffeine demethylase.

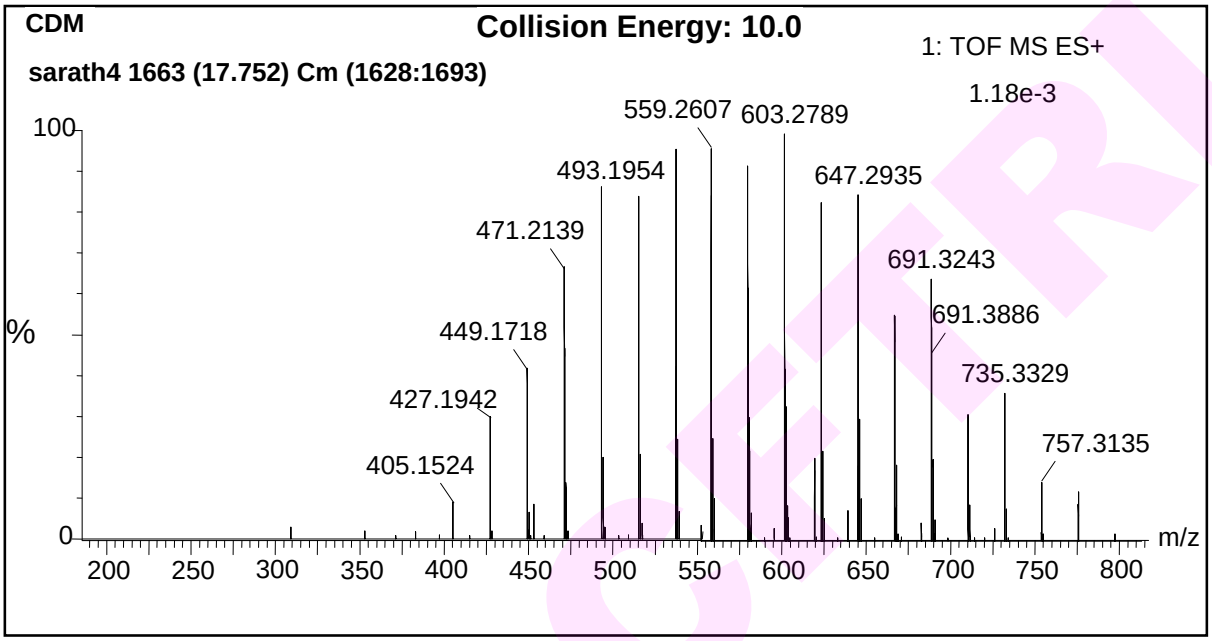
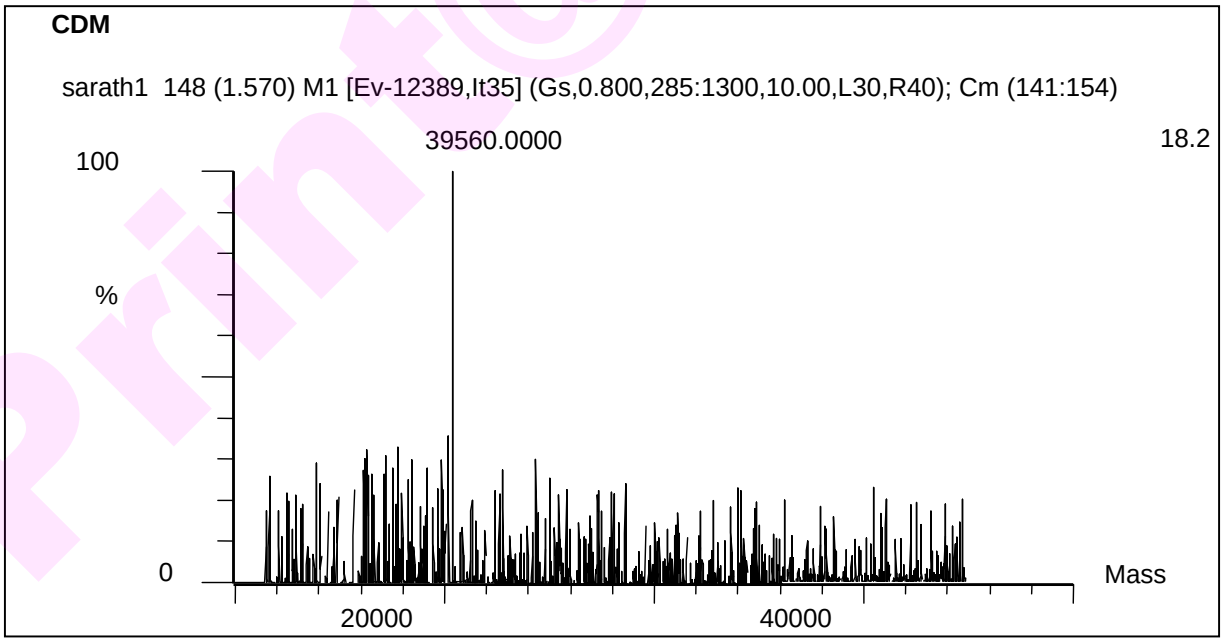


Figure 4.4.7.1b: Molecular mass graph of caffeine demethylase analyzed by LC MS.



The LC MS analysis revealed the molecular mass of the protein was 39,560 daltons (39.56 KDa) (Fig. 4.4.7.1b).

4.4.7.2. Sequencing of the protein:

Amino acid sequencing of caffeine demethylase was done by LC MS analysis of the protein in ESI positive mode in a Waters Q-Tof LC MS System. The method employed was top down, by trypsin digestion followed by sequencing of the mass fragments according to the method described by Papayannopoulos, 1995. The protein was digested by trypsin and the tryptic digest was subjected to HPLC on a C8 column (2.1 mm ID, 12 Cm Length) under an increasing gradient of acetonitrile containing TFA. The eluted fractions were directly injected to the MS system with the ESI Probe set in positive mode.

Several mass fragments were obtained (Figure 4.4.7.2a, b and c). These peaks show matches to, rieske Fe-S protein, cytochrome oxidase and a hypothetical protein.

Figure 4.4.7.2a. Peptide mass finger prints of tryptic digest of caffeine demethylase.

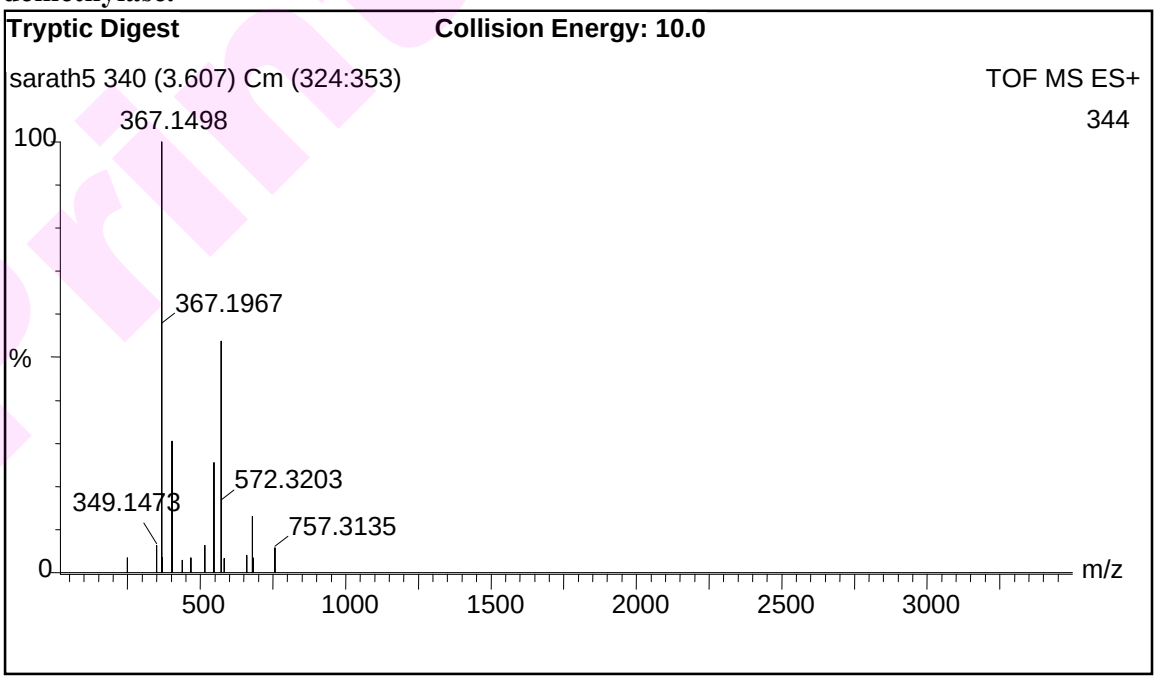


Figure 4.4.7.2b. Peptide mass finger prints of tryptic digest of caffeine demethylase.

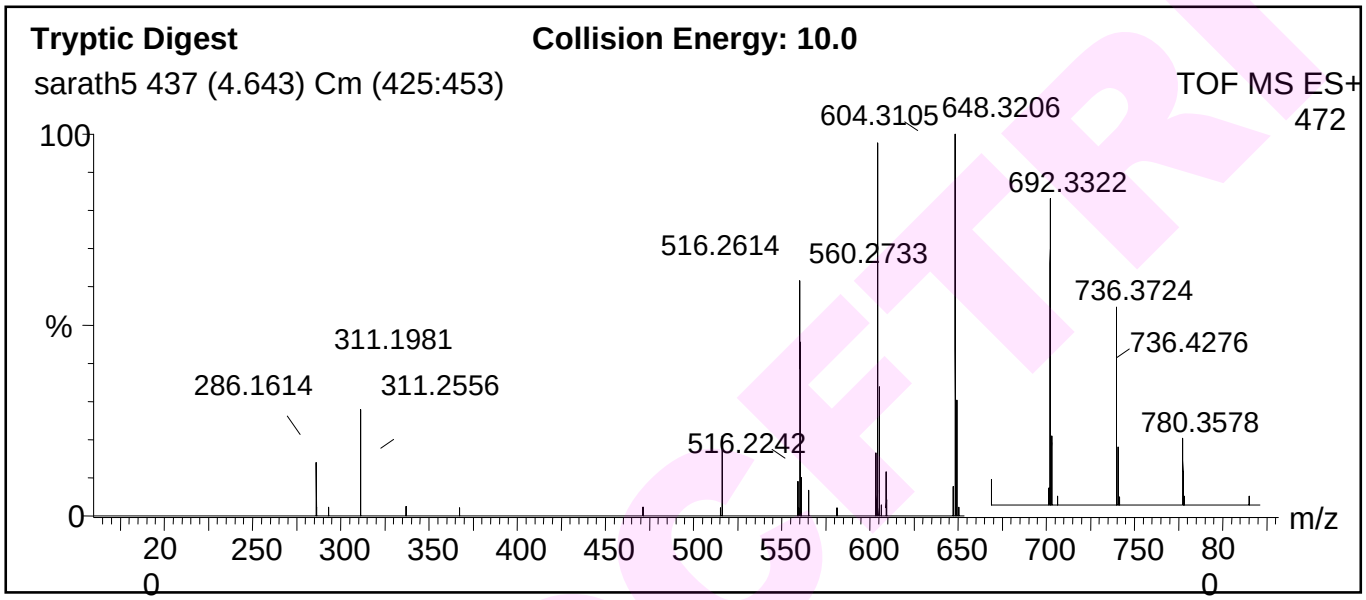
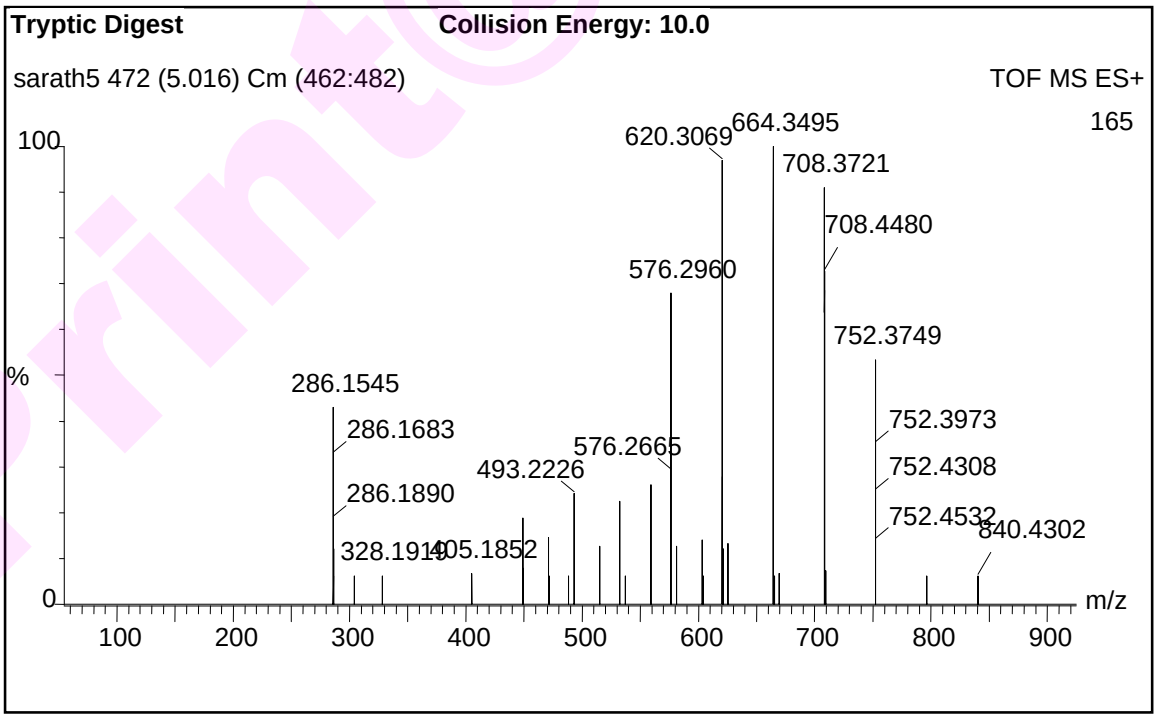


Figure 4.4.7.2c. Peptide mass finger prints of tryptic digest of caffeine demethylase.



The peptide mass finger prints were analyzed using MASCOT peptide mass analysis software developed by Matrix Life Sciences Ltd (www.matrixsciences.com). The peptide sequences thus obtained were used for deriving the amino acid sequence of the protein using bioinformatics and method developed by Shevchenko, et. al., (1996).

No information is available for the amino acid sequence of the enzyme. The probable genomic DNA for the protein was available from a Japanese patent (Imai et.al., 1996) which was obtained by deletion mutation of a caffeine degrading strain of *Pseudomonas putida* and cloning the gene into a strain without caffeine degrading capability. The gDNA sequence is as follows:

>gi|2175607|dbj|e07469.1| gdna encoding caffeine demethylase

gtagactgtctttacgccccgcacattctctatcgtaaaaatttccacgctgagcccacgctaatttaatcaagtctcattca
actgagccgcacagccgcgtgtcattgagtgatcaccggcagtaacattttacggctgagcggcttgcagcaatgtttctt
ttatgcaccaaactcgcccacctaatgcactaaatcgaaccattaaaaacttagctaatactctaaaatctattggatttgac
cacttacatttttaaccgtccagagcctatccgaagttgacacgcgggtcagaaatggcctatttttttctttgtgtaccagat
gataaatctgatgtaccagccgtatgattggcgtaaatacaccattaccaagtgtttacccttttctatgagtagatgtaggag
acagggcactcagctgagttgagtgttcatacataacaacgtcatccacaaaggctacatacatggaacagacaatcaat
aacaacgatcgcgagtaccttcggcacttttggcatcccgtctgtacagtgacagaactggaaaaggcccacccctcca
gcctaggcccaataggggtgaagcttctaaatgagcaattggttgttgctaaacttagtggccaatacgtcgcaatgcatg
atcgctgcgcacatcggtcggcaaagctctccctgggcaccatcgctaatgatcgactgcaatgcccttatcatgggtgg
cagtacgacacggaaggtgcatgtaaactagtgccggcgtgccccaacagccccattcctaatcgagctaaagttcag
cgattcgattgtgaagagcggtacggtctgatttgggtaaggctggactcaagttatgcttgcactgagatcccatacttca
gtgcagcaagcgatccgaaacttcgagtcgtgatccaagaaccctattggtggaacgcaacagcagagcgacgttgg

gaaaactttacagacttttcccattttgcgtttatccaccctggcacgctgtttgatcctaacaacgcggaaccgccgatcgt
accgatggatcgttttaatggccaattccgtttcgtttacgataccccggaagatatggccgttccagatcaagccccaatt
gggtcgttctcttatacctgcagcatgcccttcgctatcaatctggaagtcgctaagtactcaagcaattcattgcatgtgct
tttcaacgtgtcatgcccagttgacgatagcactaccaagaacttcttgctgttcgcaagggagcaggctgacgattcag
attatcttcacattgcatttaatgatttagtctttgctgaagataagcctgtgatcgagtctcaatggccgaaggatgctccgg
ctgatgaagtttcggttgtcgcggataaagtctcgatccagtatagaaaatggctgcgggaactgaaagaggcccatca
agacggtgctcaggctttccgtagtgcgttgctggactccgtgatcgagagcgatcgaagctacacctaacatttgcgta
tgagggtggcgcactgcgccttttttttagggtcaaaaaaagacggcctcctaggaggccgtaaactcgctacgtccaa
ctcgtattagggcttcttgaatgaatagacagccaatttgttcccgtcgagatcgcgcacatatg

The coding region of this protein was deduced by using the 6 frame SOPMA analysis (www.ncbi.nlm.nih.gov/SOPMA).

The deduced Sequence of the protein is:

MEQTINNNDRKYLRHFWHPVCTVTELEKAHPSSLVPIGVKLLNEQLVVAK
LSGQYVAMHDRCAHRSAKLSLGTIANDRLQCPYHGWQYDTEGACKLVP
ACPNSPIPNRAKVQRFDCEERYGLIWVRLDSSYACTEIPYFSAASDPKLR
VVIQEPYWWNATAERRWENFTDFSHFAFIHPGTLFDPNNAEPPIVPMDRF
NGQFRFVYDTPEDMAVPDQAPIGSFSYTCSMPFAINLEVAKYSSNSLHVL
FNVSCPVDDSTTKNFLLFAREQADDSDYLHIAFNDLVFAEDKPVIESQWP
KMLRLMKFRLSRIKSRSSIENGCGN

Using peptide cutter software, the probable cleavage sites and resultant fragments were obtained.

The peptide sequences derived from LC MS analysis of the protein were compared by sequence alignment tools (Clustal w 1.8.1). Peptides with matching score over 95% were only selected for the sequencing. The total score for the over all peptides with the derived sequence was more than 96%. Therefore, it was confirmed that the protein was indeed a caffeine demethylase. The sequence of caffeine demethylase deduced by us has more similarity to the one reported in the Japanese patent, which is concluded as a caffeine 1N-demethylase producing theobromine.

4.4.7.3. Prediction of chemical composition and properties CDM:

The protein is 323 AA long and the molecular weight is 39,560Da, assuming that there are no phosphorylations or glycosylations of the residues like proline. The theoretical pI is 6.07, which shows that the protein is acidic in nature. Experimental results also show that the optimum pH for catalysis is 6.8.

Total number of negatively charged residues (Asp + Glu) is 38 and total number of positively charged residues (Arg + Lys) is 33.

Atomic composition:

Carbon	C	1658
Hydrogen	H	2511
Nitrogen	N	451
Oxygen	O	480
Sulfur	S	17

The predicted molecular formula of the protein is $C_{1658}H_{2511}N_{451}O_{480}S_{17}$ and total number of atoms is 5117.

Estimated half-life of the enzyme considering N-terminal of the sequence considered as M (Met) <10 hours in bacterial cell, in vivo and <30 minutes invitro.

The instability index (II) is computed to be 51.94 and classifies the protein as unstable. And the protein appears to be compact, as a globular domain.

The enzyme does not share similarity in sequence or structure to any other proteins except in the rieske region, which makes it a novel enzyme and hitherto unknown. Several attempts to purify and characterize this enzyme have been unsuccessful, due to its high instability and autolytic nature. The protein has 3 aspartyl prolyl bonds, which are unstable at acidic conditions. Therefore, the protein undergoes autolysis and loses activity rapidly.

4.4.7.4. Structure prediction of the enzyme:

a) Secondary structure:

The secondary structure of caffeine demethylase was predicted using Hierarchical Neural Network Software (Bairoch, et. al., 1997).

Figure 4.4.7.3. Hierarchical neural network result for caffeine demethylase for prediction of secondary structure.

```
   10        20        30        40        50        60        70
    |         |         |         |         |         |         |
MEQTINNNDRKYLRHFWHPVCTVTELEKAHPSSLGPIGVKLLNEQLVVAKLSGQYVAMHDRCAHRSAKLS
Ccccccchhhhhhhhccccceeeeccccccccchhhhhhhhhhhhhhhhcccchhhhhhhhhhchcccc
LGTIANDRLQCPYHGWQYDTEGACKLVPACPNSPIPNRAKVQRFDCEERYGLIWVRLDSSYACTEIPYFS
Cchecccccccccccccccccccceecccccccccccccchhhhcchccccceeeeeecccccccccccee
AASDPKLRVVIQEPYWWNATAERRWENFTDFSHFAFIHPGTLFDPNNAEPPIVPMDRFNGQFRFVYDTPE
Cccccceeeeeecccccchhhhhhhhhcccceeeeecccccccccccccccccccccccceeeeecccc
DMAVPDQAPIGSFSYTCSMPFAINLEVAKYSSNSLHVLFNVSCPVDDSTTKNFLLFAREQADDSDYLHIA
Ccccccccccccccccccchchhhhhhhhccccceeeeeeecccccccchhhhhhhhhcccccchheee
FNDLVFAEDKPVIESQWPKMLRLMKFRLSRIKSRSSIENGCGNHchheecccccchhhhhhhhhhhhhh
hhhecccccccccccc
```

C= Random coil
H= Helix
E= Beta pleated Sheet

Results show that 13.93 % of the protein is beta pleated in nature, 27.55% alpha helical and 58.51 % of the protein occurs as random coils (Fig. 4.4.7.3, 4.4.7.4 a & b).

Figure 4.4.7.4 a. Graphical representation of the predicted secondary structure of caffeine demethylase.

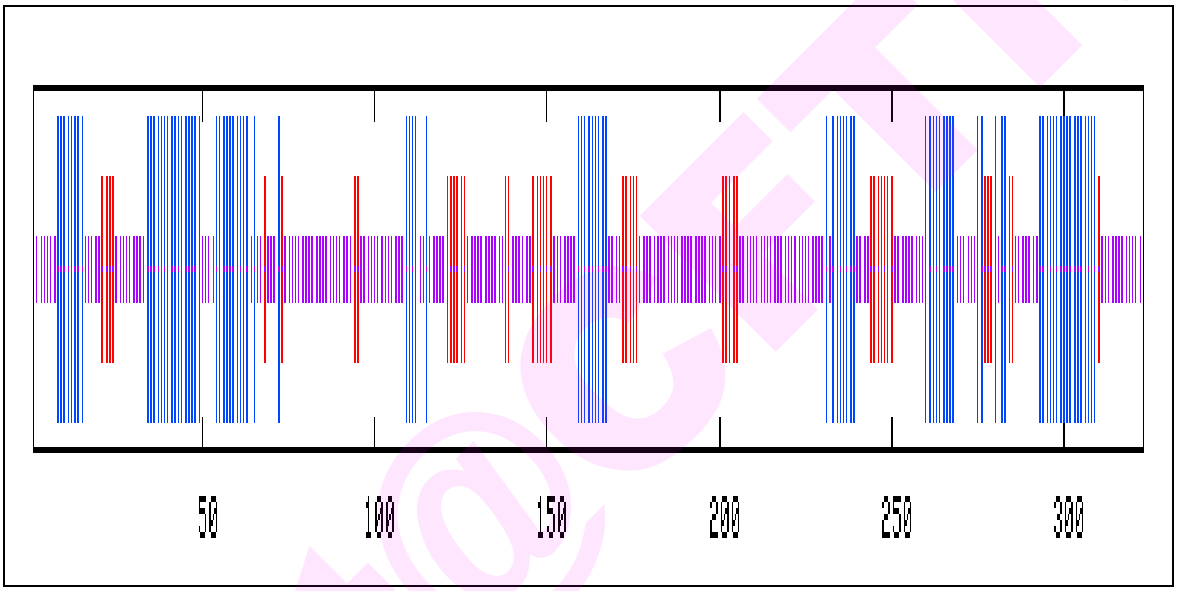


Figure 4.4.7.4b. Graphical representation of secondary structure of caffeine demethylase (HNN).

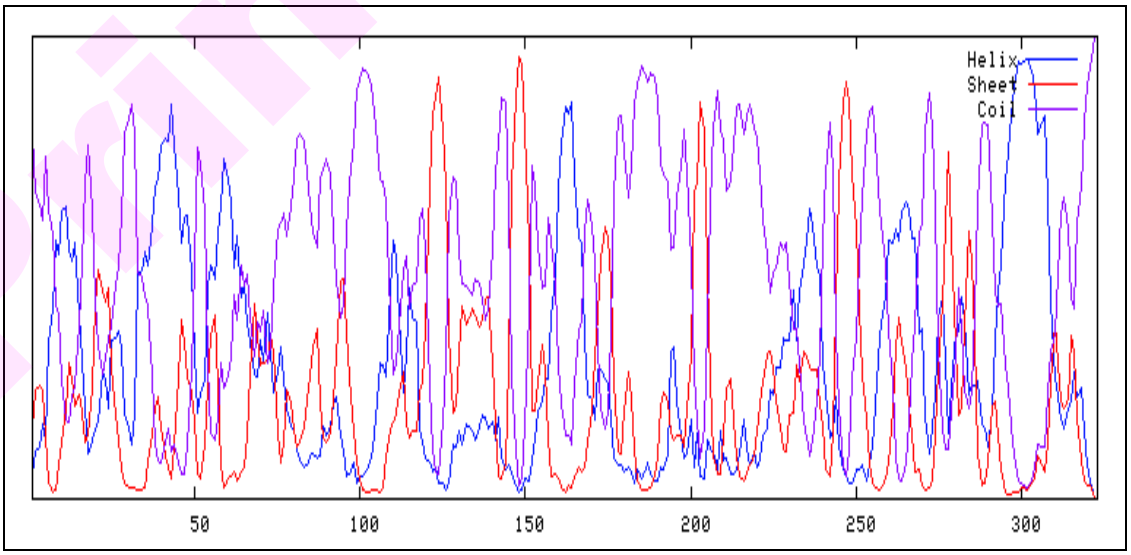
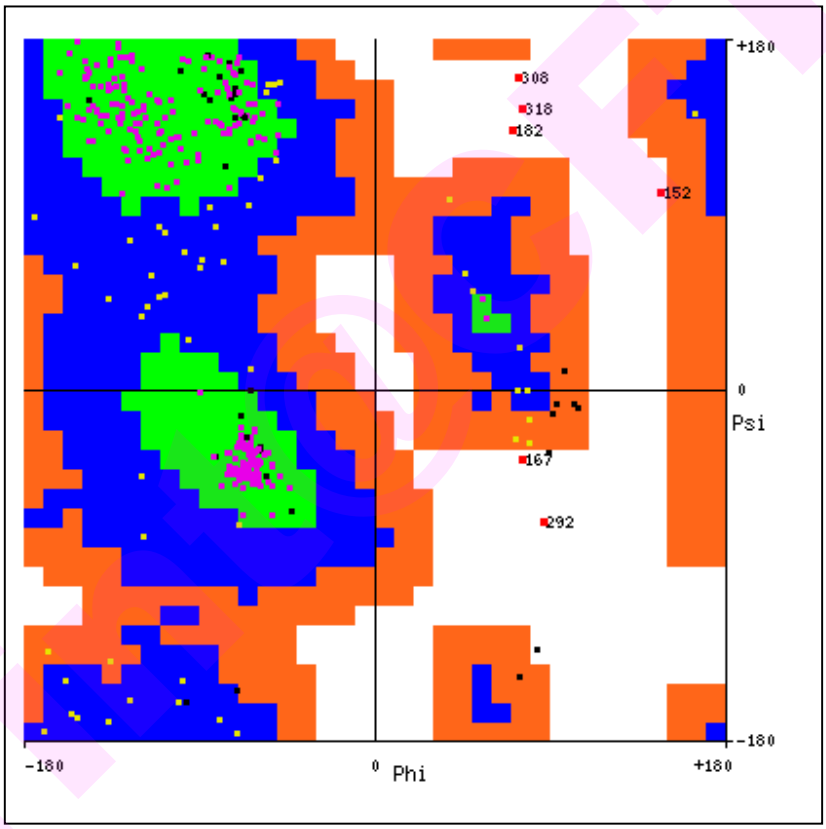


Figure 4.4.7.5. represents the Ramachandran plot of the caffeine 1N-demethylase. From the plot it can be observed that 79.7% of the residues lie in the core region of the protein and 16.1% lie in the allowed region. The number of glycines is 12 which accounts for 3.7% of the protein and 23 prolines are present in the sequence accounting for 7.2% of the enzyme.

Figure 4.4.7.5. Ramachandran plot of caffeine demethylase enzyme.



CORE ALLOWED GENEROUS DISALLOWED PRO GLY

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.

residues in CORE:	79.7 % (228)
residues in ALLOWED:	16.1 % (46)
residues in CORE+ALLOWED:	95.8 %
residues in GENEROUS:	2.1 % (6)
residues in DISALLOWED:	2.1 % (6)
number of GLY:	12 (3.7 %)
number of PRO:	23 (7.2 %)

DISALLOWED region
GENEROUS region
ALLOWED region
CORE region

4.4.7.5. Sequence homology studies:

Sequence homology of the protein was determined using clustal w bioinformatics software. The following proteins were selected for homology modeling of caffeine demethylase.

Sequence homology of the protein determined using Clustal w bioinformatics software.

MSF: 2288

tr|Q40000000 ....
.....
tr|Q8NN29|Q8 ....
.....
tr|Q4KAS7|Q4 .... .LLQPYRLKH
LTLRNRIITT……..
tr|O54036|O5 ....
.....
sp|P12609|VA ....
.....
sp|O87278|ST .... .LLQPYQLKH
LTLRNRIIVT……
sp|P38364|PI EIVMDLHRc. .....HGPI VRIGPNRYDF DTMEALKIIY
RIGNALPKAD……
sp|Q9UBB5| MB ....
.....
sp|O60341|LS ....
.....
sp|P93846|CP KFMRGPIPMI REQYAALGSV FTVPIITRRI TFLIGPE...
.....
sp|Q4PJW3|CP AFGKSPIEFL EDAYEKYGPV FSFTMVGKTF TYLLGSE...
.....
sp|Q9Y8G7|C5 EFTSNPLSDL NRLADTYGPI FRLRLGAKAP IFVSSNSLIN
EVCDEKrf……..
sp|P08686|CP LLQPDLPIYL LGLTQKFGPI YRLHLGLQDV VVLNSKRTIE
EAMVKKwadf…..
sp|P37118|C7 QLGSLPHRSL HKLSQKYGPV MLLHFGSKPV IVASSVDAAR
\DIMKTHDVVW
sp|Q9T0K2|C7 QLSLHTHRSL RSLSLRYGPL MLLHFGRTPV LIVSSADVAH
DVMKTHDLVC

tr|Q40000000 ....
tr|Q8NN29|Q8 ....

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tr|Q4KAS7|Q4    SHEPAYPEEG MPKKLYRAYH VERAKAGVAM TMTAGSAAI. .....SKDS
tr|O54036|O5    ......... ......... ......... ......... .........
sp|P12609|VA    ......... ......... ......... ......... .........
sp|O87278|ST    SHEPAYPEEG MPKKLYRAYT VERARGGVAM TMTAGSAAV. .....SKDS
sp|P38364|PI    yyi..... ......... ..PFGlpssp nlfdvqnpar hsAMKK.QVA
sp|Q9UBB5|      MB  ......... ......... ......... ......... .........
sp|O60341|LS    ......... ......... ......... ......... .........
sp|P93846|CP    ......... .....VSAHF FKGNEAEMSQ ......... ..QEVY.RFN
sp|Q4PJW3|CP    ......... .....AAALL FNSKNEDLNA ......... ..EEVYSRLT
sp|Q9Y8G7|C5    KKTLKSVLS QVREGVHDGL FTAFEDEPNW ......... ..GKAH.RIL
sp|P08686|CP     AGRPEPLTYK LVSKNYPDLS LGDYSLLWKA ......... ..HKKL.TRS
sp|P37118|C7     ASRPKSSIVD RLSYGSKDVG FSPFGEYWRR ......... ..AKSI.TVL
sp|Q9T0K2|C7    ANRPKTKVVD KILSGGRDVA FAPYGEYWRQ ......... ..MKSI.CIQ

tr|Q40000000    ......... ......... ......... ......... .........
tr|Q8NN29|Q8    ......... ......... ......... ......... .........
tr|Q4KAS7|Q4    PPVFNNILA. ......... ......... ......... .........
tr|O54036|O5    ......... ......... ......... ......... .........
sp|P12609|VA    ......... ......... ......... ......... .........
sp|O87278|ST    PPVFNNLLA. ......... ......... ......... .........
sp|P38364|PI    SLYTMTALLS YEAGVDGQTI ILKEQLQRFC DQ.....KQV IDLPQFLQYY
sp|Q9UBB5|MB    ......... ......... ......... ......... .........
sp|O60341|LS    ......... ......... ......... ......... .........
sp|P93846|CP     VPTFGPGVV. ......... ......... ......... .........
sp|Q4PJW3|CP     TPVFGKGVA. ......... ......... ......... .........
sp|Q9Y8G7|C5     VPAFGPLSIR .....GMFPE MHDIATQLCM KFARHGPRTP IDTSDNFTRL
sp|P08686|CP      ALL...LGIR .....DSMEP VVEQLTQEFC ERMRAQPGTP VAIEEEFSLL
sp|P37118|C7      HLLSNTRVQS YRNVRAEETA NMIGKIRQGC D......SSV INLGEHLCSL
sp|Q9T0K2|C7     NLLNNKMVRS YEKIREEEIK RMIEKLEKas csssp...SP VNLSQILMTL
tr|Q40000000     LEKAHPSSLG PIGVKLLNEQ LVVAKLSGQY VA.MHDRCAH RSAKLSLG..
tr|Q8NN29|Q8    ......... ......... ......... ......... .........
tr|Q4KAS7|Q4    ......... ......... ....EVVGWM KD.LTDECHE HGAAVMIQLT
tr|O54036|O5      Iadk..... PLGRQICGEK IVFYRGHENR VAAVEDFCrt r.APLSLG..
sp|P12609|VA    ......... .....ICNER MVIYRGAGQR VAALEDFCPH RGAPLSLG..
sp|O87278|ST    ......... ......... ....EIVPWI RE.MTDAVHE EGAVIMIQLT
sp|P38364|PI    ......... ......... ......... ......... .........
sp|Q9UBB5|MB ......... PGGGRCCPEQ ......... ......... .........
sp|O60341|LS    ......... ......... ......... ......... .........
sp|P93846|CP     EAEEYFSKWG ESGTVDLKYE L......... ......... .........
sp|Q4PJW3|CP     ETKEYFKSWG ESGEKNLFEA L......... ......... .........
sp|Q9Y8G7|C5    ......... ......... ....ELHPFI EA.MGDFLTE SGnrnrrppf
sp|P08686|CP    ......... ......... ......... ......... .........
sp|P37118|C7    ......... ......... ......... ......... .........
sp|Q9T0K2|C7    ......... ......... ......... ......... .........

tr|Q40000000    AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
tr|Q8NN29|Q8     LRVGApeyvl vaggigitai rsmasllkkl GANYRIHFAA RSLDAMAYKD
tr|Q4KAS7|Q4    ......... ......... ......... GANYCLDRIY NAGAAYCIHN
tr|O54036|O5    ......... ......... ......... ......... .........
sp|P12609|VA    ......... ......... ......... ......... .........
sp|O87278|ST    ......... ......... ......... GANYCLDRIY QGGAAYCIHN
sp|P38364|PI      AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
sp|Q9UBB5|MB    ......... ......... ......... ......... .........
sp|O60341|LS    ......... ......... ......... ......... .........
sp|P93846|CP    AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
sp|Q4PJW3|CP    AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
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sp|Q9Y8G7|C5   AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
sp|P08686|CP   AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
sp|P37118|C7   AALFAGQHTS SITSTWTGA. ......... ...YMLRFKQ YFAEAVEEQK
sp|Q9T0K2|C7  DMFLAGTATT LSFLEWAMT. ......... ...ELMRNPK VMKKLQEEIR

tr|Q40000000   PRGPRAT.. ESGKRMDCPA LPPGWKKEEV IRKSGLSAGK SDVyyfsp
tr|Q8NN29|Q8    ELVAEH..GD KLhlhldseg t......... ......... .........
tr|Q4KAS7|Q4   .AATGR ETSMPHEVPK AAHPR..... ......... .........
tr|O54036|O5   .HRHRREV.. LSARHMENIM APPFWRMAL. ......... .........
sp|P12609|VA    ......... .....MEGIL APPFWRAAL. ......... .........
sp|O87278|ST    .....AATGR ELTMPHSIAK AhcrR..... ......... .........
sp|P38364|PI    THVKED.... PIRFQQS... ......... ......... .........
sp|Q9UBB5|MB PRGPRAT.. ESGKRMDCPA LPPGWKKEEV IRKSGLSAGK SDVyyfsp..
sp|O60341|LS   .PRGPRAT.. ESGKRMDCPA LPPGWKKEEV IRKSGLSAGK SDVyyfsp
sp|P93846|CP   DVMKRH..GD KIDHDIL... ......... ......... .........
sp|Q4PJW3|CP   QKTVCGENLP PLTYDQL... ......... ......... .........
sp|Q9Y8G7|C5   EVVGRG.... PVLVEHL... ......... ......... .........
sp|P08686|CP   HELGPGASSS RVPYKDR... ......... ......... .........
sp|P37118|C7   ...GLAQGKS EITEDDL... ......... ......... .........
sp|Q9T0K2|C7    SSSPQDlf.. .VTEKEA... ......... ......... .........

tr|Q40000000   N.....FLLF A......... ......... ......... REQADDSDYL
tr|Q8NN29|Q8   FSP.EVF... ......... ......... ......... ....HI....
tr|Q4KAS7|Q4   GRNVLVYDDA GD...HAGLQ AAE.F.IARS GARTEIMTPD RSFAPE....
tr|O54036|O5   Y.....FWVL A......... ......... ......... CNFAAQDQAL
sp|P12609|VA   Y.....FWGM A......... ......... ......... RSFRPEDNEL
sp|O87278|ST   GTNVLIFDDA GD...HAALQ AAE.F.LATA GARVEIMTPD RSFAPE....
sp|P38364|PI   GNDASVFRPE RWLETKGNLN igg....SFA FGAGSRSCIG KNISIL....
sp|Q9UBB5|MB  LNT.SQPLCK AFIVTDEDIR k......... ......... .........
sp|O60341|LS   GAp....... ......... ......... ......... .........
sp|P93846|CP   KNP.DSYDPD RFgpgreedk aagaF.SYIS FGGGRHGCLG EPFAYL....
sp|Q4PJW3|CP    VER.LDFNPD RYLEDSpas. GEK.F.AYVP FGAGRHRCIG ENFAYV....
sp|Q9Y8G7|C5   GNDADKFIPE RMLDDEfarl nkeypnCWKP FGNGKRACIG RPFAWQ....
sp|P08686|CP   ERP.HEFWPD RFLEp..... GKN.S.RALA FGCGARVCLG EPLARL....
sp|P37118|C7   ENP.EEYQPE RFLNSDADVK GLN.F.KLLP FGAGRRGCPG SSFAIA....
sp|Q9T0K2|C7   GTDAEEFKPE RHLDTNLDFQ GQD.F.KFIP FGSGKRICPG IGFTSA....

tr|Q40000000   TANIREGQGK IFSEDLEMLE RQQQNLLAYP......... .........
tr|Q8NN29|Q8    ......... .......QVP ELGLHATVNK ......... .........
tr|Q4KAS7|Q4    ......... .......VMG MNLVPYMRSL QQLDTTFTVT YRLKAVERQG
tr|O54036|O5   TANIREGQGK IFSEDLEMLE RQQQNLLAYP ......... .........
sp|P12609|VA   TARIREGQGT IFAEDLEMLE QQQRNLLAWP ......... .........
sp|O87278|ST    ......... .......VMA MNLVPYMRCL QKLDVTFTVT YRLEAVEKSG
sp|P38364|PI   ......... .......EMS KAIPQIVRNF ......... .........
sp|Q9UBB5|MB    ......... ......... ......... ......... .........
sp|O60341|LS    ......... ......... ......... ......... .........
sp|P93846|CP    ......... .......QIK AIWTHLLRNF ......... .........
sp|Q4PJW3|CP    ......... .......QIK TIWSTMLRLY ......... .........
sp|Q9Y8G7|C5    ......... .......ESL LAMVVLFQNF ......... .........
sp|P08686|CP    ......... .......ELF VVLTRLLQAF ......... .........
sp|P37118|C7    ......... .......VIE LALARLVHKF ......... .........
sp|Q9T0K2|C7    ......... .......LIG VTLANIVKRF ......... .........
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tr|Q40000000     KFRLSRIKsr ssiengcgn. ..... ..... .....
tr|Q8NN29|Q8      DESMLEAL.. ..... ..... ..... .....
tr|Q4KAS7|Q4      EQLLATIGTD YSDLEK.... ..... ..... .....
tr|O54036|O5     QRNLLKLNID AGGVQS.... ..... ..... .....
sp|P12609|VA      ERPLLKLNID AGGVQS.... ..... ..... .....
sp|O87278|ST      NELVAHVGSD YGGISK.... ..... ..... .....
sp|P38364|PI     DIEINHGdmt wknecwwfvk pe..... ..... .....
sp|Q9UBB5|MB      ..... ....QE.... ..... ..... .....
sp|O60341|LS      ..... ..... ..... ..... .....
sp|P93846|CP      EFELVSPF.. ..... ..... ..... .....
sp|Q4PJW3|CP      EFDLIDGYFP tvnyttmiht pekp..... ..... .....
sp|Q9Y8G7|C5      NFTMTDPnya leikqtltik pdhfyinatl rhgmtptele hvlagngats
sp|P08686|CP     TLlpsgdalp slqp..... ..... ..... .....
sp|P37118|C7     DFALPEGIKP EDLDMTETIG ..... ..... .....
sp|Q9T0K2|C7      NWRMDVEPQR VQHDLTEATG ..... ..... .....
```

Legend :

tr|Q40000000	Caffeine demethylase *Pseudomonas alcaligenes* CFTRI
tr|Q8NN29|Q8	Ferredoxin-NADPH reductases) family 1 (Vanillate O-Demethylase)
tr|Q4KAS7|Q4	N-methylproline demethylase, putative - *Pseudomonas fluorescens*
tr|O54036|O5	Vanillate demethylase A - *Pseudomonas putida*
sp|P12609|VA	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82) (4-hydroxy- 3-methoxybenzoate demethylase) - *Pseudomonas* sp.
sp|P38364|PI	Pisatin demethylase (EC 1.14.-.-) (Cytochrome P450 57A2) - *Fusarium solani*
sp|Q9UBB5|MB	HUMAN Methyl-CpG-binding domain protein 2
sp|O60341|LS	HUMAN Lysine-specific histone demethylase
sp|P93846|CP	Cytochrome P450 51 (EC 1.14.13.70) (CYPLI) (P450-LIA1) (Obtusifoliol 14-alpha demethylase)
sp|Q4PJW3|CP	BOVIN Cytochrome P450 51A1 (EC 1.14.13.70) (CYPLI) (P450LI) (Sterol 14-alpha demethylase
sp|Q9Y8G7|C5	Bifunctional P-450:NADPH-P450 reductase (Fatty acid omega-hydroxylase) (P450foxy) [Includes: Cytochrome P450 505 (EC 1.14.14.1); NADPH—cytochrome P450 reductase (EC 1.6.2.4)
sp|P08686|CP	HUMAN Cytochrome P450 XXI (EC 1.14.99.10) (Steroid 21-hydroxylase) (21- OH) ase
sp|P37118|C7	Cytochrome P450 71A2 (EC 1.14.-.-) (CYPLXXIA2) (P-450EG4) - *Solanum melongena*
sp|Q9T0K2|C7	Cytochrome P450 71A20 (EC 1.14.-.-) - *Arabidopsis thaliana*

The protein was found to have less than 31% similarity with existing proteins, which indicates that the protein is novel. The region of similarity is in the Rieske Fe-S cluster, which is a dioxygenase subunit protein belonging to iron-sulfur oxidoreductase ferredoxin electron transport protein family, and has a length of 116 AA.

4.4.7.6. Tertiary structure prediction:

The tertiary structure of caffeine 1N-demethylase was predicted using fold recognition server and the template used was **1ndo** (pdbid). Figure 4.4.6.6 represents the predicted tertiary structure of caffeine demethylase. Based on the predicted structure, the protein is organized into a domain containing a six membered β-pleated sheet barrel. β-sheet barrels in enzymes are usually involved in the channeling of the substrate to the active site and in the solvent accessibility. These are present in the core of the enzyme, concealed in the hydrophobic pockets of the enzyme. A 3 membered β -sheet saddle is also present in the enzyme. Two histidines and one isoleucine residue appear to be involved in the binding of caffeine to the enzyme.

The function of 2 extended sheets towards the surface of the protein is not known. The alpha helices of the protein reside on the surface of the protein indicating they are composed mostly of hydrophilic residues. It is a common feature of the NADPH binding sites and Fe-S centers of the protein are present in the periphery and mostly comprise of α-helices as in the case of other demethylases like vanillate demethylase, lanosterol demethylase etc., which also have rieke Fe-S centers, involved in NADPH/NADH oxidation (Bernhardt, 1975; Buswell and Ribbons, 1988; Cartwright and Smith, 1967). NADPH binding in these proteins occurs at the surface of the protein in α- helices of the surface domains of the protein. The Fe-S center (Figure 4.4.7.6.) with a prophyrin ring is involved in the binding of oxygen to the enzyme. The rest of the protein is organized as random

coils. The random coils are mostly involved in the stabilization of the back bone of the protein.

Figure 4.4.7.6: Predicted 3D structure of caffeine demethylase enzyme based on PSSM output.

They are mostly comprised of proline residues, which are responsible for bend in the coils.

Figure 4.4.7.7. 3D Structure Fe-S center of rieske iron sulphur protein of vanillate demethylase.

Literature shows that vanillate demethylase is a two-component enzyme classified as a IA oxygenase (Buswell and Ribbons, 1988). It comprises a reductase containing both a flavin and a [2Fe-2S] redox center and an oxygenase containing, in addition to a substrate-binding site, an iron binding site and a Rieske-type [2Fe-2S] cluster. Little is known about how structure influences function in vanillate demethylase. Demethylases involved in the metabolism of *p*-anisate in *Pseudomonas putida* (Bernhardt, 1975) and vanillate in *P. testosteroni* (Buswell and Ribbons, 1988) and *P. fluorescens* (Cartwright and Smith, 1967) are air sensitive and unstable. The vanillate demethylases from *P. testosteroni* and *P. fluorescens* are mixed-function oxygenases and have a wide substrate specificity: *m*-anisate, *p*-anisate, *m*-toluate, 3,4,5-trimethoxybenzoate, and 3,4-dimethoxybenzoate were oxidized by vanillate-induced cells (Cartwright and Smith, 1967). As described here, the *Acinetobacter* vanillate demethylase also possesses a

broad substrate range. Inferences can be drawn about the mechanism of vanillate demethylase from results obtained with the evolutionarily related enzyme phthalate dioxygenase (Gassner et.al., 1994). In this enzyme, electrons for hydroxylation flow from NADH to flavin mononucleotide to [2Fe-2S] in the reductase and from the Rieske-type [2Fe-2S] center to the Fe21 site in the oxygenase, where oxygen binding and hydroxylation occur (Gassner et.al., 1995; Kauppi et.al., 1998; Parales et.al., 1999). As recently shown for the naphthalene dioxygenase, another member of this group of aromatic dioxygenases, Fe1 of the Rieske [2Fe-2S] center is coordinated by two cysteinyl residues and Fe2 is coordinated by two histidyl residues (Pavel et.al., 1994). The iron atom at the active site is coordinated by two histidyl residues and one aspartyl residue. Aspartate 205 in the catalytic domain of this enzyme has been shown to be essential for activity. The C-terminal regions of the a subunit of the oxygenase component of 2-nitrotoluene 2,3-dioxygenase and biphenyl dioxygenase were shown to be responsible for substrate specificity. Very scanty literature is available on the exact docking of caffeine to the active site of the caffeine demethylase enzyme. However, from the sequence similarity in the Fe-S region of most of the demethylases with caffeine demethylase, it can be inferred that the oxygen binding and NADPH oxidation is common to all the demethylases. A report on caffeine demethylase (Sideso et.al., 2001) indicates a flexible active site of the enzyme and the enzyme also accepts theobromine as a substrate. However our experiments show that, the demethylase is specific for caffeine only and two demethylases are involved in the degradation of caffeine in *Pseudomonas alcaligenes* MTCC 5264. Further these enzymes are present as separate proteins in

contrast to what has been reported in literature (Sideso et.al., 2001). The docking of caffeine to human cytochrome p450 (CYP 1A2) shows that the active site is present in the same plane as the heme group and caffeine is bound parallel to the iron in the heme group (Regal and Nelson, 2000). The 1,3 and 7th methyl groups are oriented 6.7 Å, 6.5 Å and 6.7 Å apart from the Fe center in the heme and therefore a preference to 3 N demethylation is shown by the enzyme followed by 1 N demethylation and the least preference to 7 N demethylation. The caffeine demethylases in the bacterial system also produce theobromine (3 N demethylation) and 1 N demethylation (paraxanthine). The active site in CYP1A2 can fit two caffeine molecules, one parallel to the heme and the other perpendicular to the heme leading to the formation of theobromine and paraxanthine. This does not fit to the bacterial demethylase because both the products are produced by different enzymes. One probable explanation can be derived from the CYP 1A2 model, where in the 1 N demethylase the caffeine molecule is oriented perpendicular to the iron favouring the formation of paraxanthine and parallel in the 3 N demethylase favouring theobromine production. This same model seems to apply to caffeine demethylase from *P. alcaligenes* MTCC 5264.

Further, the exact docking of caffeine to the active site of the enzyme is being studied and will be undertaken in future.

4.5. CONCLUSIONS:

The enzymes involved in caffeine degradation by *P. alcaligenes* MTCC 5264 were identified as caffeine 1N and 3N demethylases, xanthine oxidase, heteroxanthine demethylase, xanthine denhydrogenase, uricase, allantoinase, allantoicase and urease. Caffeine demethylase enzyme is found to be the key

enzyme in the pathway and it has not been purified and characterized earlier due to its high instability. The use of protein based stabilizing agents and polyols conferred stability to the enzyme and the enzyme was purified and characterized to homogeneity. It was found that two demethylases (caffeine 1N and 3N demethylase) are involved in the first step of caffeine degradation in *P. alcaligenes* MTCC 5264. The enzyme involved in 1N-demethylation of caffeine was characterized by LC MS analysis and bioinformatics tools. The tertiary structure of the enzyme has been predicted using bioinformatics approach. The mode of binding of caffeine to the active site of the enzyme has been proposed to be parallel to the heme group similar to CYP 1A2 activity in humans. The enzyme is highly specific to caffeine unlike a few models proposed in literature.

Further studies will be carried out in our laboratory on the cloning and hyper expression of the gene coding for caffeine demethylase in a suitable expression vector and the hyper production of caffeine demethylase enzyme in a suitable expression system. Further work is required to be carried out on the stabilization of the caffeine demethylase enzyme which is a very important aspect in the development of biotechnological processes for decaffeination.

4.6. REFERENCES:

1. Asano, Y., Toshihiro, K. and Yamada, H. (1993). Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.***57**:1286-1289.
2. Asano, Y., Toshihiro, K and Yamada, H. (1994). Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No.352**.** *Biosci.Biotech.and Biochem*. **58(12)** : 2303-2304.
3. Ashihara, H., Monteiro, A.M., Moritz, T., Gillies, F.M., Crozier, A., (1996), Catabolism of caffeine and related purine alkaloids in leafs in *Coffee arabica* L. *Planta*. **198:**334–39.
4. Ashihara, H., Gillies, F.M., Crozier, A., (1997), Metabolism of caffeine and related purine alkaloids in leafs of tea (*Camellia Sinensis*). *Plant Cell Physiol*. **38:**413–9.
5. Bairoch, A., Bucher, Hofmann, K., (1997), The PROSITE database, its status in 1997. *Nucl. Acids Res*., **25:** 217-221.
6. Bergmann, F., Waron, U. H., Govrin, K.H., Goldberg, H., Leon, S., (1962), Some specific reactions of the purine-oxidizing system of *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, **55**: 512-22.
7. Bernhardt, F. H. P. H. S. H., (1975), A 4-methoxybenzoate O-demethylase from *Pseudomonas putida*: a new type of monooxygenase system. *Eur. J. Biochem*. **57:**241–256.
8. Blecher, R., Lingens, F., (1977), Metabolism of caffeine by *Pseudomonas putida*. *Hoppe Seyler's Z. Physiol. Chem.* **358:**807–17.
9. Bradford, M. M., (1976), A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. **72**:248–254.
10. Brand, D., Pandey, A., Roussos, S., Soccol, C.R., (2000), Biological detoxification of coffee husk by filamentous fungi using a solid-state fermentation system. *Enzyme Microb. Technol.* **27:**127–33.

11. Bray, R.C., (1963), Xanthine oxidase. In : Boyer, P.D., Lardy, H. and Myrbäck, K. (Eds.), The Enzymes, 2nd ed., vol. 7, Academic Press, New York, 533-556.

12. Burg, A.W., Stein, M.E., (1972), Urinary excretion of caffeine and its metabolites in the mouse. *Biochem Pharmacol.* **21(7):**909-922.

13. Buswell, J. A., Ribbons, D. W., (1988), Vanillate O-demethylase from *Pseudomonas* spp. *Methods. Enzymol.* **161:**294–301.

14. Campbell, Jr., L. L., (1954), The mechanism of allantoin degradation by a *Pseudomonas, J Bacteriol.;* **68(5**): 598–603.

15. Cartwright, N. J., Smith, A.R.W. (1967), Bacterial attack on phenolic esters: an enzyme demethylating vanillic acid. *Biochem. J*. **102:**826–841.

16. Caubet, M.S., Comte. B., Brazier. J.L., (2004), Determination of urinary 13Ccaffeine metabolites by liquid chromatography–mass spectrometry: the use of metabolic ratios to assess CYP1A2 activity. *J. Pharm. Biomed. Anal.* **34:**379–89.

17. Cornish, H.H., Christman, A.A., (1957), A study of the metabolism of theobromine, theophylline and caffeine in man. *J. Biol. Chem.* **228:**315–23.

18. Freedman J.H., Peisach, J., (1984), Determination of copper in biological materials by atomic absorption spectroscopy: a reevaluation of the extinction coefficients for azurin and stellacyanin. *Anal. Biochem.* **141(2):**301-10.

19. Gassner, G. T., Ludwig, M. L., Gatti, D.L., Correll, C.C., Ballou, D.P., (1995), Magnetic circular dichroism studies of exogenous ligand and substrate binding to the non-heme ferrous active site in phthalate dioxygenase. *Chem. Bio*l. **1:**173–183.

20. Gassner, G., Wang, L., Batie, C., Ballou, D.P., (1994), Reaction of phthalate dioxygenase reductase with NADH and NAD: kinetic and spectral characterization of intermediates. *Biochemistry* **33:**12184–12193.

21. Gluck, M., Lingens, F., (1987), Studies on the microbial production of thebromine and heteroxanthine from caffeine. *Appl. Microbiol. Biotech.* **25**: 334-340.

22. Gluck, M., Lingens, F., (1988), Heteroxanthinedemethylase, a new enzyme in the degradation of caffeine by *Pseudomonas putida. Appl. Microbiol. Biotechnol.* **28**: 59-62.

23. Gouda, M. D., Thakur, M. S., Karanth, N. G., (2001), Stability Studies on Immobilized Glucose Oxidase Using an Amperometric Biosensor - Effect of Protein Based Stabilizing Agents, *Electroanalysis,* **13 (10):** 849-855.

24. Gouda M.D., Thakur M.S., Karanth N.G., (2002), Reversible Denaturation Behavior of Immobilized Glucose Oxidase, *Appl. Biochem. Biotechnol*. **103(1-3):** 471-480.

25. Gouda, M.D., Singh, S.A., Rao, A.G.A., Thakur, M.S., Karanth, N.G., (2003), Thermal Inactivation of Glucose-Oxidase - Mechanism and Stabilization Using Additives, *J. Biol. Chem*. **278 (27):** 24324-24333.

26. Hakil, M., Denis, S., Gonz´alez, G.V., Augur, C., (1998), Degradation and product analysis of caffeine and related dimethyl xanthines by filamentous fungi. *Enzyme Microb. Technol.* **22:**355–9.

27. Hakil, M., Voisinet, F., Gonz´alez, G.V., Augur, C., (1999), Caffeine degradation in solid-state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources. *Process. Biochem*. **35:**103–109.

28. Hohnloser, W., Osswalt, B., Lingens, F., (1980), Enzymological aspects of caffeine demethylation and formaldehyde oxidation by *Pseudomonas putida* C1. *Hoppe seyler's Z. Physiol. Chem.* **361:**1763– 1766.

29. Imai, Y., Nakane, S., Koide, Y., (1996), Caffeine demethylase gene-containing DNA fragment and microbial process for producing 3-methyl-7-alkylxanthine, United States Patent 5550041.

30. Jakoby, W.B., (1971), Crystallization as a purification technique, *Enzyme Purification and Related Techniques,* in *Methods in Enzymology,* Vol. 22, Jakoby, W.B., Ed., Academic Press, London.

31. James, J.E., (1997), Is habitual caffeine use a preventable cardiovascular risk factor? *Lancet*. **349:**279–281.

32. Jayaraman, (1988), *Laboratory manual in Biochemistry*. 3rd edition. New Delhi. Wiley Eastern Limited. pp. 114 - 115, 130 -131.

33. Johan M. H. S., Mooibroek, H., (1998), Cloning and Characterization of NADP-Mannitol Dehydrogenase cDNA from the Button Mushroom, *Agaricus bisporus*, and Its Expression in Response to NaCl Stress. *Appl Environ Microbiol.* **64(12):** 4689–4696.

34. Kauppi, B., Lee, K., Carredano, E., Parales, R. E., Gibson, D.T., Eklund, H., Ramaswamy, S., (1998), Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. *Structure* **6:**571–586.

35. Khanna, K.L., Rao, G.S., Cornish, H.H., (1972), Metabolism of caffeine-3H in the rat. *Toxicol*. *Appl*. *Pharmacol.* **23:**720–730.

36. Khilman, B.A., (1974), Effects of caffeine on the genetic material. *Mutat. Res.* **26:**53–71.

37. Koyama. Y., Tomoda, Y., Kato, M., Ashihara, H., (2003), Metabolism of purine bases, nucleosides and alkaloids in theobromine-forming *Theobroma cacao* leaves. *Plant Physiol. Biochem.* **41:**977–984.

38. Kurtzman, Jr. R.H., Schwimmer, S., (1971), Caffeine removal from growth media by microorganism. *Experientia* **27:**481–482.

39. Laemmli, U.K**.,** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

40. Landon., (1977), Cleavage at aspartyl-prolyl bonds, *Methods Enzymol.* **47:**145-149.

41. Leviton, A., Cowan, L., (2002), A review of the literature relating caffeine consumption by women to their risk of reproductive hazards. *Food. Chem. Toxicol.* **40:**1271–1310.

42. Machold, C., Deinhofer, K., Hahn, R., Jungbauer, A., (2002), Hydrophobic interaction chromatography of proteins - I. Comparison of selectivity, J. *Chromatog. A*, **972(1):** 3-19.

43. Madyastha, K.M., Sridhar, G.R., (1998), A Novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem. Biophys. Res. Commun.* **249:**178–181.

44. Madyastha, K.M., Sridhar, G.R., Vadiraja, B.B., Madhavi, Y.S., (1999), Purification and partial characterization of caffeine oxidase-A novel enzyme from a mixed culture consortium. *Biochem. Biophys. Res. Commun.***263:**460–464.

45. Mahler, H.R., Hübscher, G., Baum, H., (1955), *J. Biol. Chem.* **216:** 625-641.

46. Mazzaffera, P., (1993), 7-Methyl xanthine is not involved in caffeine catabolism in *Coffea dewevrei*. *J. Agric. Food Chem.* **41:**1541–1543.

47. Mazzaffera, P., Olsson, O., Sandberg, G., (1994), Degradation of caffeine and related methyl xanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microb. Ecol.* **31:**199–207.

48. Meyer, L., Caston, J., Lieberman, H.R., (2004), Stress alters caffeine action on investigatory behaviour and behavioural inhibition in the mouse. *Behav. Brain. Res.* **149:**87–93.

49. Middelhoven, W.J., Lommen, A., (1984), Degradation of caffeine by *Pseudomonas putida* C3024. *Anton. van Leeuw.*. **50:**298-30.

50. Moriwaki, Y., Yamamoto, T., Higashino, K., (1999), Enzymes involved in purine metabolism-a review of histochemical localization and functional implications. Histol Histopathol. **14(4):** 1321-1340.

51. Papayannopoulos, I. A., (1995), The interpretation of collision-induced dissociation tandem mass Spectra peptides. Mass Spectromet. Rev. **14:** 49-73.

52. Parales, R. E., Parales, J. V., Gibson, D.T., (1999), Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity. *J. Bacteriol.*, **181:**1831–1837.

53. Pavel, E. G., Martins, L. J., Ellis, Jr. W.R. Solomon, E.I., (1994), Magnetic circular dichroism studies of exogenous ligand and substrate binding to the non-heme ferrous active site in phthalate dioxygenase. *Chem. Biol.* **1:**173–183.

54. Pincheira, J., L´opez-S´aez, J.F., Carrera, P., Navarreteb, M.H., Torre, C.D.L., (2003), Effect of caffeine on in vivo processing of alkylated bases in proliferating plant cells. *Cell. Biol. Int.* **27**:837–843.

55. Porres, C., Alvarez, D., Calzada, J., (1993), Caffeine reduction in coffee pulp through silage. *Biotechnol Adv* . **11(3):**519–523.

56. Putrament, A., Baranowska, H., Bilinsky, T., Prazmo, W., (1972), On the specificity of caffeine effects. *Mol. Gen. Genet.* **118:**373–379.

57. Ramarethinam, S., Rajalakshmi, N., (2004), Caffeine in tea plants [*Camellia sinensis* (L.) O. Kuntze]: in situ lowering by *Bacillus licheniformis* (Weigmann) Chester. *Indian. J. Exp. Biol.* **42(6):**575–580.

58. Regal K. A., Nelson, S.D., (2000), Orientation of caffeine within the active site of Human Cytochrome P450 1A2 based on NMR longitudinal (T1) relaxation measurements, *Arch. Biochim. Biophy.* **384:** 47-58.

59. Rojas, J.B.U., Verreth, J.A.J., Amato, S., Huisman, E.S., (2003), Biological treatments affect the chemical composition of coffee pulp. *Bioresour. Technol.* **89:**267–274.

60. Roussos, S., Hannibal, L., Aquiahuatl, M.A., Hernandes, M.R.T., Marakis S., (1994), Caffeine degradation by *Penicillium verrucosum* in solid-state fermentation of coffee pulp: critical effects of additional inorganic and organic nitrogen sources. *J. Food Sci. Technol.* **31:**316–319.

61. Sarath Babu V.R., Kumar M.A., Karanth, N.G., Thakur, M.S., (2004), Stabilization of immobilized glucose oxidase against thermal inactivation by silanization for biosensor applications. *Biosen. Bioelect*. **19:** 1337-1341.

62. Sarath Babu, V.R., Patra, S., Karanth, N.G., Varadaraj, M.C., Thakur, M.S., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. Enz. *Microb. Technol*. **27:**617-624.

63. Shevchenko, A., Wilm, M., Vorm, O., Mann, M., (1996), Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels**.** *Anal. Chem*. **68:** 850-858.

64. Sideso, O.F.P., Marvier, A.C., Katerelos, N.A., Goodenough, P.W., (2001), The characteristics and Stabilization of a caffeine demethylase enzyme complex. *Int. J. Food Sci. Technol*. **36:**693–698.

65. Silvarolla, M.B., Mazzafera, P., de Lima, M.M.A., (2000), Caffeine content of ethiopian *Coffea arabica* beans. *Genet. Mol. Biol.* **23**:213–215.

66. Stone, K.L., Williams, K.L., (1996), Enzymatic Digestion of Proteins in Solution and in SDS Polyacrylamide Gels, in *The Protein Protocols Handbook*, eds. J.M. Walker, Humana Press Inc., Totowa, NJ, pp.415 ff.

67. Sundarraj, C.V., Dhala, S., (1965), Effect of naturally occurring xanthines on bacteria (I). Antimicrobial action and potentiating effect on antibiotic spectra. *Appl Microbiol.* **13:**432–436.

68. Tagliari, C.V., Sanson, R.K., Zanette, A., Franco, T.T., Soccol, C.R., (2003), Caffeine degradation by *Rhizopus delemar* in packed bed column bioreactor using coffee husk as substrate. *Braz. J. Microbiol*. **34:**102–104.

69. Trijbels, F., Vogels, G.D., (1967), Allantoate and ureidoglycolate degradation by *Pseudomonas aeruginosa*. *Biochim Biophys Acta.* **132(1):**115–126.

70. Vit´oria, A.P., Mazzafera, P., (1998), Caffeine degradation in fruits and leaves of *Coffea arabica* and *Coffeea dewevrei*. *Pesq Agropec. Bras.* **33:**1957–1961.

71. Vitória, A.P., Mazzafera, P., (1999), Xanthine Degradation and Related Enzyme Activities in Leaves and Fruits of Two *Coffea* Species Differing in Caffeine Catabolism, *J. Agric. Food Chem.* **47(5)**: 1851 – 1855.

72. Vogels, G. D., Van der Drift, C., (1976) Degradation of purines and pyrimidines by microorganisms, *Bacteriol Rev.* **40(2):** 403–468.

73. Ward, D.N., Arnott, M.S., (1965), Gel filtration of proteins, with particular reference to the glycoprotein, luteinizing hormone. *Anal. Biochem*. **12:**296-302.

74. Waring, W.S., Goudsmit, J., Marwick, J., Webb, D.J., Maxwell, S.R.J., (2003), Acute caffeine intake influences central more than peripheral blood pressure in young adults. *Am. J. Hypertens.* **16:**919–924.

75. Woolfolk, C.A., (1975), Metabolism of *M*-methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. *J. Bacteriol.* **123:**1088–1106.

76. Wreck-Reichhart, D., Feyereisen R., (2000), Cytochrome P-450 a success story. *Genome Biol.* **6:3003**. 1–9.

77. Yamamoto, S., Ishihara, T., (1999), Ion-exchange chromatography of proteins near the isoelectric points. *J. Chromatog*. A. **(852)**, 31-36.

78. Yano-Y.D.M., Mazzafera, P. (1999), Catabolism of caffeine and purification of a xanthine oxidase responsible for methyluric acids Production in *Pseudomonas Putida* L. *Revista Microbiol*. **30:**62– 70.

79. Yano-Y.D.M., Mazzafera, P., (1998), Degradation of caffeine by *Pseudomonoas putida* isolated from soil. *Allel. J.* **5:**23–34.

CHAPTER 5

BIODECAFFEINATION

OF COFFEE & TEA

5.1. SCOPE OF THE WORK:

This chapter deals with the development of a biodecaffeination process for coffee and tea using enzymes isolated from *Pseudomonas alcaligenes* MTCC 5264. The first section of this chapter deals with an introduction to biodecaffeination, a survey of literature on studies carried out by different groups around the world and studies carried out in our laboratory. The later sections of the chapter deal with the methods, and results of the studies on biodecaffeination of coffee and tea.

Biodecaffeination of green coffee beans was carried out using the multienzyme system immobilized in calcium alginate beads and 98% of the caffeine could be completely degraded in the beans. Cell free extract was used for the biodecaffeination of tea during fermentation and 80% of the caffeine in the tea dhool was degraded within 90 minutes of incubation. Inhibitory factors like caffeine polyphenol complexes and polyphenol protein complexes which were hindering the biodecaffeination were prevented by the use of glycine in the dhool.

The chapter ends with suggestions on future work to be carried out on biodecaffeination and future prospects of biodecaffeination.

5.2. INTRODUCTION:

Biodecaffeination is defined as the complete removal of caffeine and related methyl xanthines form caffeine containing materials like coffee, tea, cocoa etc., by the use of enzymes/cells capable of degrading caffeine. It is a process in which caffeine and its metabolites are removed from caffeine containing materials without altering the taste, flavor and quality of the materials like tea and coffee.

Coffee, tea, chocolates and cola beverages are caffeine containing food stuffs and beverages discovered by serendipity, have become a part of customs and traditions in several countries. In course of time they have created history of their own to become one of the largest consumed beverages only second to water. Caffeine is a methylated xanthine alkaloid derivative (1,3,7-trimethylxanthine), structurally related to purine and contains an imidazole and a purine ring. The presence of three methyl groups imparts the stimulatory effects to caffeine. Apart from the stimulatory effects, caffeine when consumed in excess is associated with several ill effects on human health. Caffeine has deleterious effects on cardiac patients and women (Camargo and Toledo, 1998; Krestschmar and Baumann, 1999; Caudle and Bell, 2000). Reports are also available on the effects of caffeine on health and of its toxic effects to animals and plants (Landolt et.al., 1995; Shilo et.al., 2000). Therefore the demand for decaffeinated products is increasing at a rate of 3% per annum, and decaffeinated coffee and tea share 15% of the world market. Since the 1940's several processes for decaffeination have been developed using solvents such as methylene chloride. These processes suffer from the disadvantages of stripping off the flavor and aroma of the beverages, being expensive and use of solvents which are not safe to the

environment. Moreover, the presence of these solvents in traces also has been a subject of concern to human health. Therefore the approach is towards a natural and safe method of decaffeination.

Biotechnological routes of decaffeination are considered to be safe and green alternatives for the chemical decaffeination processes. Literature survey indicates the capability of several strains of bacteria and fungi for degrading caffeine and strains of *Pseudomonas* are known to be the most potent in terms of caffeine degradation. Woolfolk (1975) was the first to investigate caffeine degradation by *Pseudomonas putida*. Since then, several caffeine degrading bacteria, such as *Pseudomonas cepacia*, were isolated which demethylate caffeine to yield 3,7-dimethylxanthine, which is further catabolised to NH_3 and CO_2. Biotechnological route of decaffeination serves a better alternative both in terms of consumer health and sensory properties for which these beverages are consumed. Studies in this area have since large been concentrating on the production of caffeine free plants through genetic engineering techniques like gene knock out, gene silencing etc (Keya et.al., 2003, Ogita et.al., 2004). However these techniques have not reached a stage where they can be applied at a massive scale. Even then a major question lies in the application of these technologies on a scale of replacing at least a part of the 2.34 million hectares of tea plantations around the world. Decaffeinated products have a niche market and complete decaffeination is not revered by al the consumers. A low caffeine product may be preferred to a no caffeine product for enjoying health benefits as well as the refreshing effects of the caffeine in the beverages and food products. Enzymatic biodecaffeination offers a control over the extent and scale of decaffeination.

Moreover this process does not include genetic modification of the caffeine containing plants leaving the defense system of the plant intact.

Studies on the development of a biodecaffeination process using immobilized cells of *Pseudomonas alcaligenes* MTCC 5264 isolated in our laboratory (Sarath et. al., 2005) showed promising results. However, there was a detrimental effect of the organism on the final product quality. Similarly Schwimmer and Kurtzman (1972) have reported the decaffeination of coffee infusion by *Penicillium roquefortii.* Haas and Stieglitz (1980) have reported the decaffeination of aqueous caffeine-containing liquids, such as coffee extracts, with Pseudomonad microorganisms.

All these processes have been limited to laboratory scale studies and their commercial exploitation is not possible as the live microbial cells would utilize simple substrates present in the coffee or tea extracts leading to degradation of the quality of the products. Therefore, enzymatic processes for decaffeination are advantageous over whole cell based processes due to the specificity of the enzymes only to caffeine and other methyl xanthines.

In bacteria (*Pseudomonas*), caffeine is initially converted into theobromine and paraxanthine parallely by demethylases. Although the enzymes involved in the degradation of caffeine by microorganisms have been reported in literature (Blecher and Lingens, 1977), no enzymatic process has been successful as yet, owing to the high instability of the caffeine demethylase, a rate limiting enzyme in the pathway of caffeine degradation. The enzyme involved in the N-demethylation of caffeine, a demethylase has not been characterized till date due to its very high lability even under storage at 4-8^{o}C. Yano and Mazzafera (1999), attempted to purify caffeine

demethylase but found that the purified enzyme was labile and it rapidly lost its activity. It has been observed that the use of cryoprotectants and freeze drying to low moisture contents improved the stability of the enzyme (Sideso et. al., 2001). In general, the caffeine degrading enzymes are very labile and more studies are required to improve the stability of the enzymes, which will help in developing a specific process for caffeine degradation. In mixed culture consortium belonging to *Klebsiella* sp. and *Rhodococcus* sp., caffeine was directly oxidized by the enzyme caffeine oxidase at the C-8 position leading to the formation of 1,3,7-trimethyluric acid and this process did not have demethylation steps. Only partial characterization of this enzyme is available (Madhyasta and Sridhar 1998, Madhyasta et.al., 1999).

Though enzymes involved in degradation of caffeine are known, in vitro enzymatic studies for caffeine degradation are not yet reported. Since demethylase enzymes are not very stable more studies on enzyme stability and biochemical characterization are to be carried out. The development of an enzymatic Bio-decaffeination process for tea and coffee requires many inhibitory factors to be overcome like protein-polyphenol complexes (Karl et. al., 1996; Elisabeth et. al., 2004) and caffeine–polyphenol interactions (Nicola, et.al., 1996; Charlton et.al., 2000) which tend to drastically limit the success of this process by inhibiting the enzyme activities and the availability of caffeine to the enzymes. Therefore this area of coffee biotechnology, towards the development of an enzymatic decaffeination process thus still lies under explored.

During our studies on biodecaffeination, we have found that the demethylase activities could be maintained in the cell free extracts (CFE) of *P. alcaligenes* MTCC

5264 by addition of stabilizing agents to the CFE. The successful isolation, identification and stabilization of caffeine demethylase and the other enzymes involved in caffeine degradation by *P. alcaligenes* MTCC 5264 is described in detail in chapter 4 of this thesis resulted in the development of biodecaffeination processes for coffee and tea during fermentation which are reported in this chapter.

5.3. MATERIALS AND METHODS:

Caffeine, theobromine, lysozyme, Tris, methyl xanthine standards and (+) catechin were procured from M/s Sigma-Aldrich, St. Louis, USA. Caffeine (LR Grade), uric acid standard, urea standard were purchased from Sisco Research laboratories, Mumbai, India. Peptone and yeast extract were from HiMedia labs, Mumbai, India. All other reagents were of the highest purity and were procured from standard sources. Analytical grade glycine, glycerol, HPLC grade methanol, magnesium oxide, ammonium sulphate, potassium dihydrogen orthophosphate and other chemicals were procured from Qualigenes Fine Chemicals, Mumbai, India. Coffee beans were procured from the local market. Black tea dhools were kind gifts from Tantea Industries, Gudalur and AVT Natural Products, Chennai, India.

5.3.1. Biomass production:

A loop full of an actively growing culture of *P. alcaligenes* MTCC 5264 was inoculated into sucrose growth medium (containing in g/L of the following components: sucrose, 20.0; ammonium aulphate, 3.0; potassium dihydrogen ortho phosphate, 1.5; yeast extract, 3.0 and peptone, 1.5 at a pH of 6.8) and incubated at 30± 2^{o} C on a rotary shaker at 150 rpm. Caffeine was dosed after 48 hours of growth and further incubated till 96 hours. Samples were drawn at every 12 hours and the growth and enzyme production were checked. Growth was determined by recording the absorbance of the culture broth against a sterile broth blank in a spectrophotometer set at 600 nm. The induction of the biodecaffeinating enzymes in the cells was determined by analysis of residual caffeine in the broth by HPLC. After

the caffeine was completely degraded in the medium, the biomass was harvested by centrifugation at 12000 g for 30 minutes at 4^{o}C to obtain a pellet of the biomass. The biomass was washed in PBS to rid it off the media components.

5.3.2. Isolation of biodecaffeinating enzymes:

Induced cells of *P. alcaligenes* MTCC 5264 were harvested by centrifugation at 12,000 g for 30 minutes at 4^{o}C. The biomass pellet was washed several times with ice cold buffer (Tris-Cl, 50 mM; pH 6.8) and frozen at –20^{o}C. 10 grams of the frozen pellet was thawed into 100 ml of Lysis buffer containing 2 mg/ml of lysozyme, 1mM PMSF, 0.1mM DTT and 15%v/v of glycerol at 37^{o}C in a water bath (Julabo, Germany) for 1 hour. The lysate was then centrifuged at 12,000 g for 30 minutes at 4^{o}C in a refrigerated centrifuge (Kubota, Japan) to separate the cell debris. The supernatant obtained was designated as the cell free extract (CFE) was stored frozen at -20^{0}C in 10ml aliquots till further use. Required amounts of the CFE was thawed on ice before use and used for the experiments.

5.3.3. Biodecaffeination studies:

5.3.3.1. Biodecaffeination of pure caffeine solutions:

Biodecaffeination of pure caffeine solution by the enzymes was carried out by incubating 1ml of the CFE with 1ml of caffeine (10mM) and 300µl of NADPH (1mM) in 7.7ml of Tris-Cl Buffer (100mM, pH 6.8) and incubation at 30±2^{o}C for 2 hours. Samples were drawn at every 30 minutes and analyzed for the metabolites by HPLC.

5.3.3.2. Biodecaffeination with immobilized enzymes:

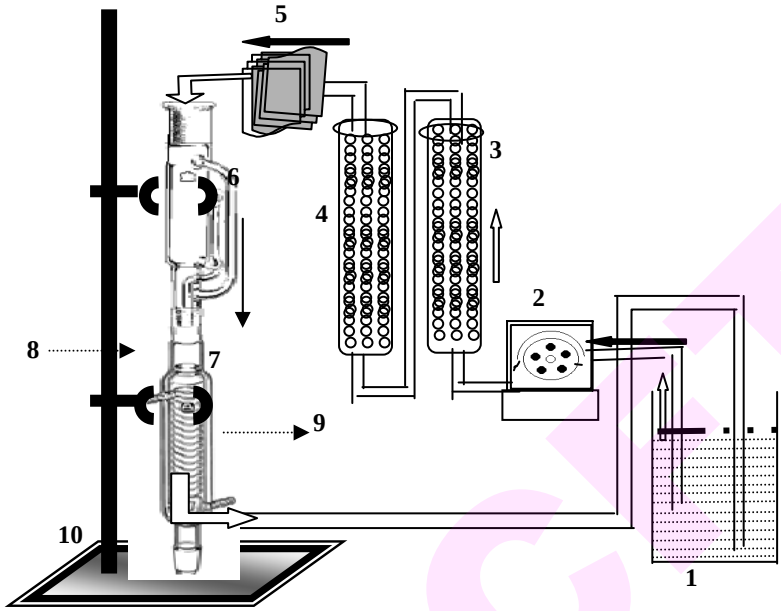
a) Immobilization of enzymes:

Sodium alginate was used for the immobilization of the enzymes. 10 ml of the enzyme extract was mixed with 90 ml of the matrix prepared at 3% w/v concentration and dropped into curing solutions consisting of calcium chloride (200mM). The beads were washed off the curing solutions with buffer and packed into glass columns of 25 mm x 500 mm dimensions. The efficiency of biodecaffeination by the immobilized enzymes was tested by passing pure caffeine solution (1g/L) and determining the caffeine degradation at different time intervals. Several parameters for decaffeination like flow rate, retention time, packed bed volume, extract to packed bed volume ratio, temperature, pH and aeration which influence the caffeine degradation were optimized.

b) Biodecaffeination of green coffee beans:

Green coffee beans were packed into an extractor and water heated to 80^0C was dripped over the beans. The extract was collected after cooling over a cooling coil and was passed through a bioreactor containing immobilized enzymes (Fig.5.3.3.2). The caffeine content in the extract was checked at regular intervals by HPLC analysis of the sample and the degradation products were also identified by comparing with standards. The decaffeination process was terminated after 99% of the initial caffeine was degraded. The extract was then concentrated and reabsorbed into the beans after which the beans were dried and roasted.

Figure 5.3.3.2. Schematic of the process for continuous bio-decaffeination of coffee beans/Solids.



1. Reservoir **2. Pump**
3. Column 1 **4. Column 2**
5. Heating Unit **6. Extractor**
7. Cooling Unit **8. Water Inlet**
9. Water Outlet **10. Stand**

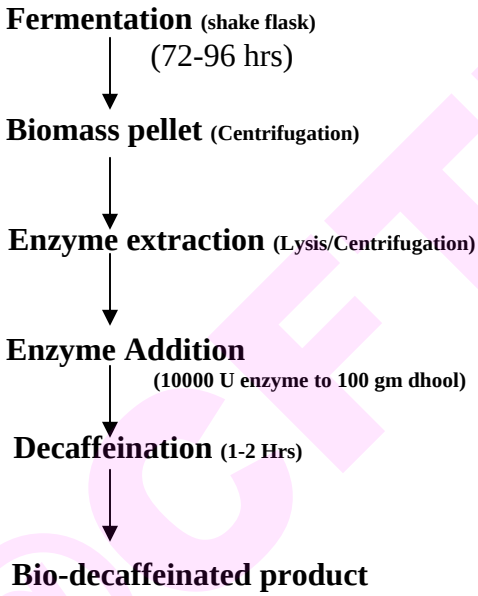
c) Biodecaffeination of black tea dhool:

Biodecaffeination of black tea dhool was carried out as follows:

100 gms of black tea dhool was carefully weighed and the moisture content was adjusted accordingly. To the moist dhool 10 ml of CFE containing required enzyme activity was added and mixed uniformly. The dhool-CFE mixture was then spread over a perforated tray (30 x 20 x 5 cm) and incubated for 90 minutes at 28±2^{o}C. Aeration was facilitated by blowing air from the bottom of the perforated tray through a blower. The dhool-CFE mixture was mixed intermittently and samples were drawn at 30-minute intervals and the residual caffeine content was determined by HPLC.

A flow chart of the Biodecaffeination of tea is given below.

Flow sheet for bio-decaffeination of tea during fermentation:



5.3.4. Optimization of parameters for biodecaffeination of tea:

Tea dhool is rich in polyphenols, proteins and caffeine apart from a variety of biochemicals. We target the biodecaffeination of tea during the fermentation process to synchronize tea fermentation with biodecaffeination to make the process natural. Moreover, biodecaffeination during fermentation adds to the preservation of quality of tea as well as the process economics to the tea manufacturers. Several factors which tend to inhibit the biodecaffeination during this stage need to be overcome. Therefore, parameters like moisture, aeration, intermittent enzyme addition and mixing were optimized and inhibitory interactions of proteins with polyphenols, caffeine-polyphenol interactions which directly influence the biodecaffeination process were minimized using different approaches.

5.3.5. Analytical:

Sample preparation for caffeine analysis in the dhool was done according to the ISO method of caffeine analysis. In brief, 10 grams of the moist dhool was weighed carefully and dried in a hot air oven till the moisture content of the dhool is reduced to below 5% w/w. The sample was then ground to a fine powder using a mortar and pestle, sieved through a 100-mesh sieve and the finely powdered tea was carefully weighed to the nearest 1 gram. This sample was placed in a sample bottle and 300 ml of distilled water was added to the sample. 4.5-5.0 grams of magnesium oxide was added to the bottle and the sample was heated at 90^{o} C for 20 minutes. The sample was allowed to cool and filtered through a 0.22µm filter. 20µl of the sample was injected to HPLC and the peak area was recorded. Two caffeine standards (0.1mg/ml and 1mg/ml) were also prepared and injected prior to analysis of the sample. 20µl of the standard caffeine was injected to HPLC connected to a Luna C18 column (4.6 x 250 mm) set at a flow rate of 1ml/min with water and methanol in the ratio of 70:30 as the mobile phase. The peak area of the caffeine was recorded with a photo diode array detector set at 280 nm. Caffeine concentration in the sample was calculated using the equation 1:

$$C_s = \frac{W_s \times \text{dilution factor} \times P_s \times C_{cf}}{P_{cf}} \quad \text{…….. Eqn:1.}$$

Where
C_s= Concentration of caffeine in the sample
W_s= Weight of sample
P_s = Peak area of sample
C_{cf} = Concentration of Standard Caffeine
P_{cf} = Peak area of standard caffeine

5.4. RESULTS AND DISCUSSION:

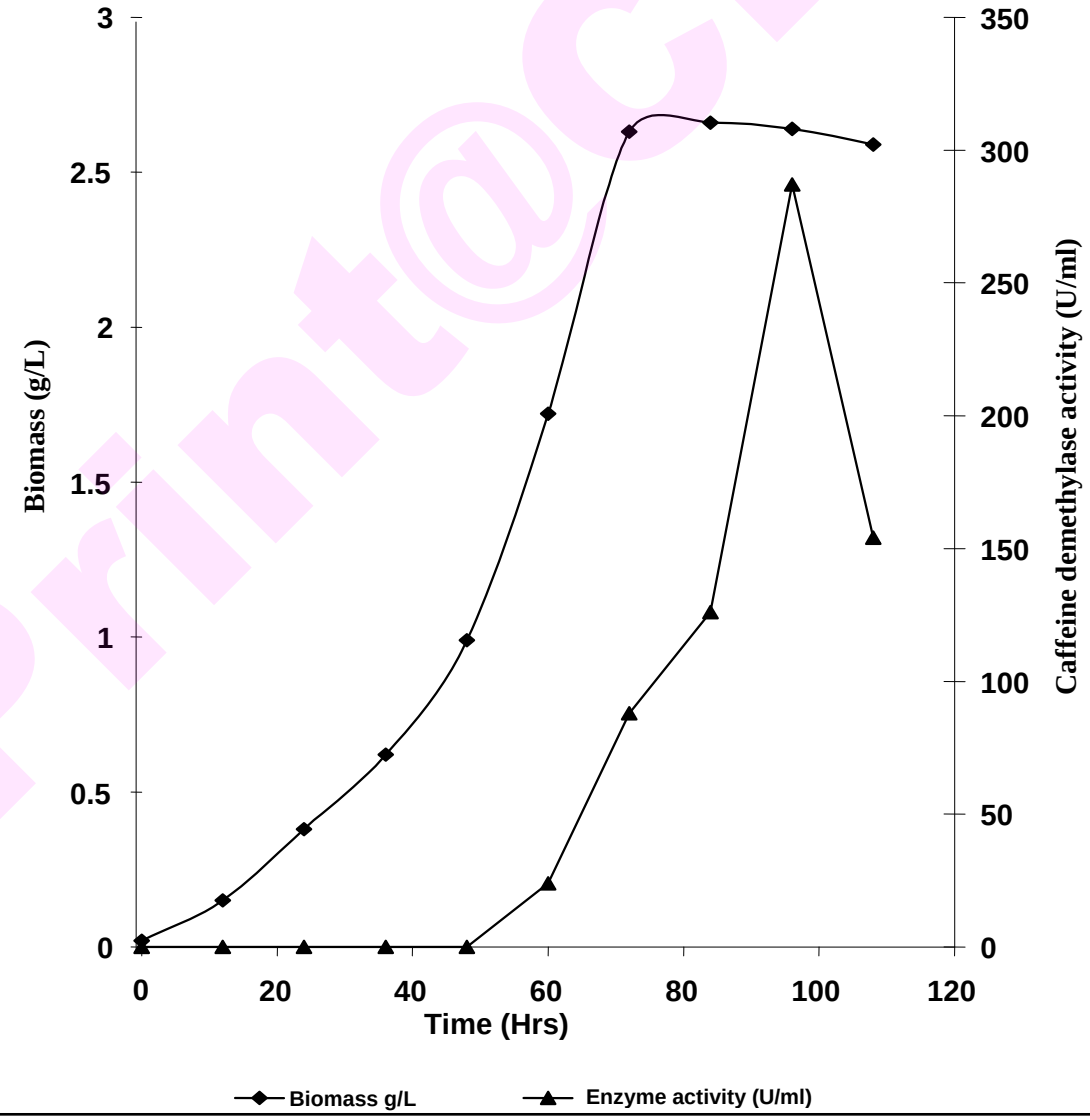
5.4.1. Biomass production:

Figure 5.4.1.1., represents the growth and enzyme production by *P. alcaligenes* MTCC 5264 in sucrose growth medium. Maximum growth was achieved after 72 hours of growth. Caffeine was dosed after maximum biomass was accumulated so as to achieve a high biomass as well as high amount of enzyme expressed. This will in effect enable the maximum utilization of caffeine by the bacterial cells due to competition for nutrients. During the late logarithmic phase of growth under batch culture, the availability of simple nutrients like sugars in the medium is low. Therefore the microorganism will utilize caffeine as the sole source of carbon and nitrogen adapting itself to utilize the otherwise toxic substrate. Moreover, high cell density results in a low amount of caffeine available per cell reducing the net concentration of caffeine in the vicinity of the cells. It is also known that metabolism or accumulation of secondary metabolites occurs in the stationary phase of an organism (Kellerhals et. al., 1999). Highest biomass production (2.64 $g.L^{-1}$) and caffeine degradation rate of 0.053 $g.L^{-1}.h^{-1}$, was achieved at 48 hrs of caffeine dosing i.e., 96 hrs of growth (Fig.5.4.1.1). A decrease in the biomass was observed at 108 hrs of incubation indicating the cells entered a death phase. At this stage the caffeine degradation rate also dropped to 0.044 $g.L^{-1}.h^{-1}$. Therefore the cells were harvested at 96 hours of growth, lysed and the CFE was used for further experiments.

5.4.2. Isolation of enzymes for biodecaffeination of tea and coffee:

Induced cells of *P. alcaligenes* MTCC 5264 were harvested and enzymes were extracted by lysis using lysozyme as a lysis agent and protein based stabilizing agent. The protein content in the CFE was determined by the method of Bradford (1976) and was found to be 0.64 mg/ml. The caffeine demethylase activity which is rate limiting in the biodecaffeination is considered in the expression of enzyme activities for biodecaffeination and is mentioned in the following sections. The caffeine demethylase activity in the CFE was 329.1 U/ml (Fig.5.4.1.1.).

Figure 5.4.1.1. Growth and caffeine demethylase production by *P. alcaligenes* MTCC 5264.



5.4.3. Biodecaffeination studies:

The enzymes involved in biodecaffeination are inducible in nature and are expressed in the presence of caffeine. Studies in our laboratory showed that the enzymes extracted from cells of *P. alcaligenes* MTCC 5264 were found to degrade caffeine invitro (Chapter 4). The ability of the extracted enzymes to degrade pure solutions of caffeine was checked in order to confirm the activities of the enzymes in the CFE and is reported below.

5.4.3.1. Biodecaffeination of pure caffeine solutions:

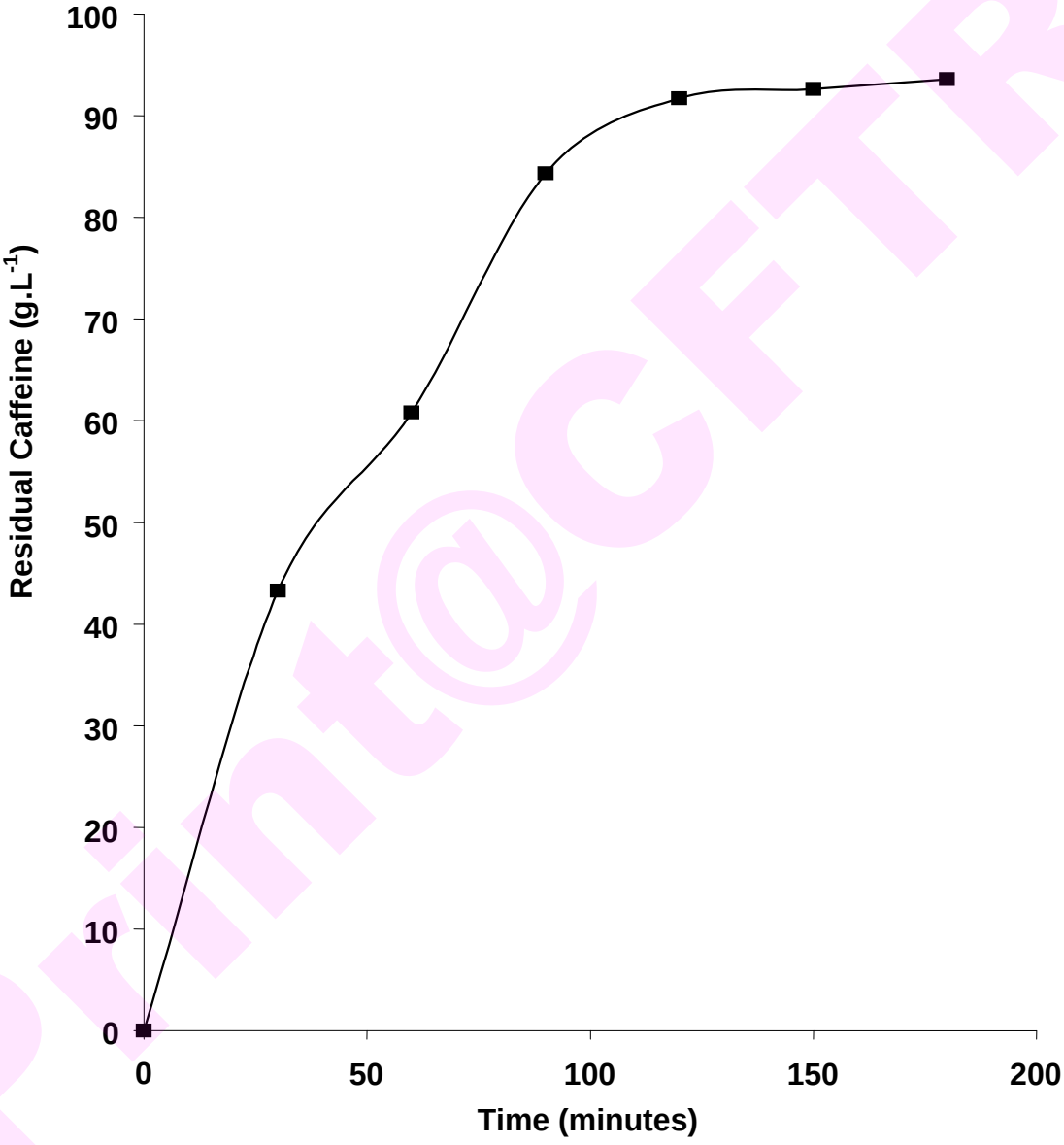
The CFE was incubated with caffeine and the products of the reaction were analyzed by HPLC. Theobromine, paraxanthine, allantoin; other degradation products such as uric acid, allantoin, allantoic acid, urea and ammonia were identified in the reaction mixture (Table 4.4.2.1; Chapter 4). The theobromine content in the sample increased with time of incubation and after 1 hr of incubation, paraxanthine, allantoin and xanthine were detected in the reaction mixture. The concentrations of these products were found to increase with the time of incubation, whereas the concentration of caffeine was reduced in the reaction mixture proving that biodecaffeination was occurring in the presence of CFE.

5.4.3.2. Biodecaffeination with immobilized enzymes:

CFE was immobilized in calcium alginate gel by entrapment and packed into a column (20 x 2.5 cm). Pure caffeine solution ($1g.L^{-1}$) was then passed through the column at a flow rate of 5ml/min. The caffeine content in the solution was determined at regular intervals. Figure 5.4.3.2, represents the biodecaffeination of pure caffeine solution in packed bed reactor containing immobilized CFE. It was found that 91.7%

biodecaffeination was achieved within 2 hours of incubation and 93.6% biodecaffeination was achieved after 3 hours of incubation.

Figure 5.4.3.2. Biodecaffeination of pure caffeine by immobilized CFE of *P. alcaligenes* MTCC 5264.

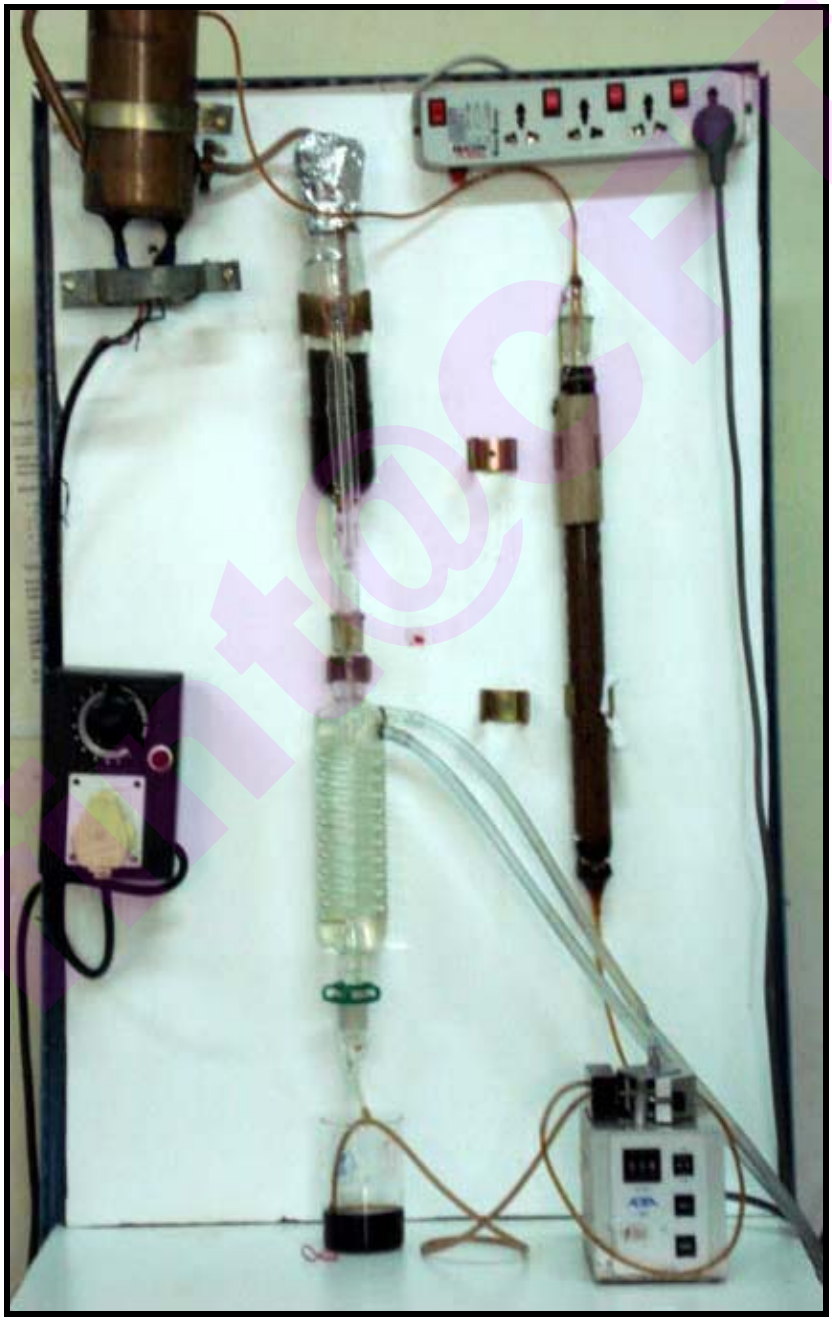


5.4.3.3. Biodecaffeination of green coffee beans:

Biodecaffeination of green coffee beans was carried out in an extraction and biodecaffeination system shown in Figure 5.4.3.3. With the optimized conditions of

5ml/min flow rate, a temperature of 30^{o}C and pH 6.8 the caffeine content in the coffee beans was reduced by 98%. The extraction water used was concentrated by heating and reabsorbed by the beans. The coffee beans were dried and roasted.

Figure 5.4.3.3. Laboratory scale apparatus for biodecaffeination of coffee beans.



5.4.4. Biodecaffeination of black tea dhool:

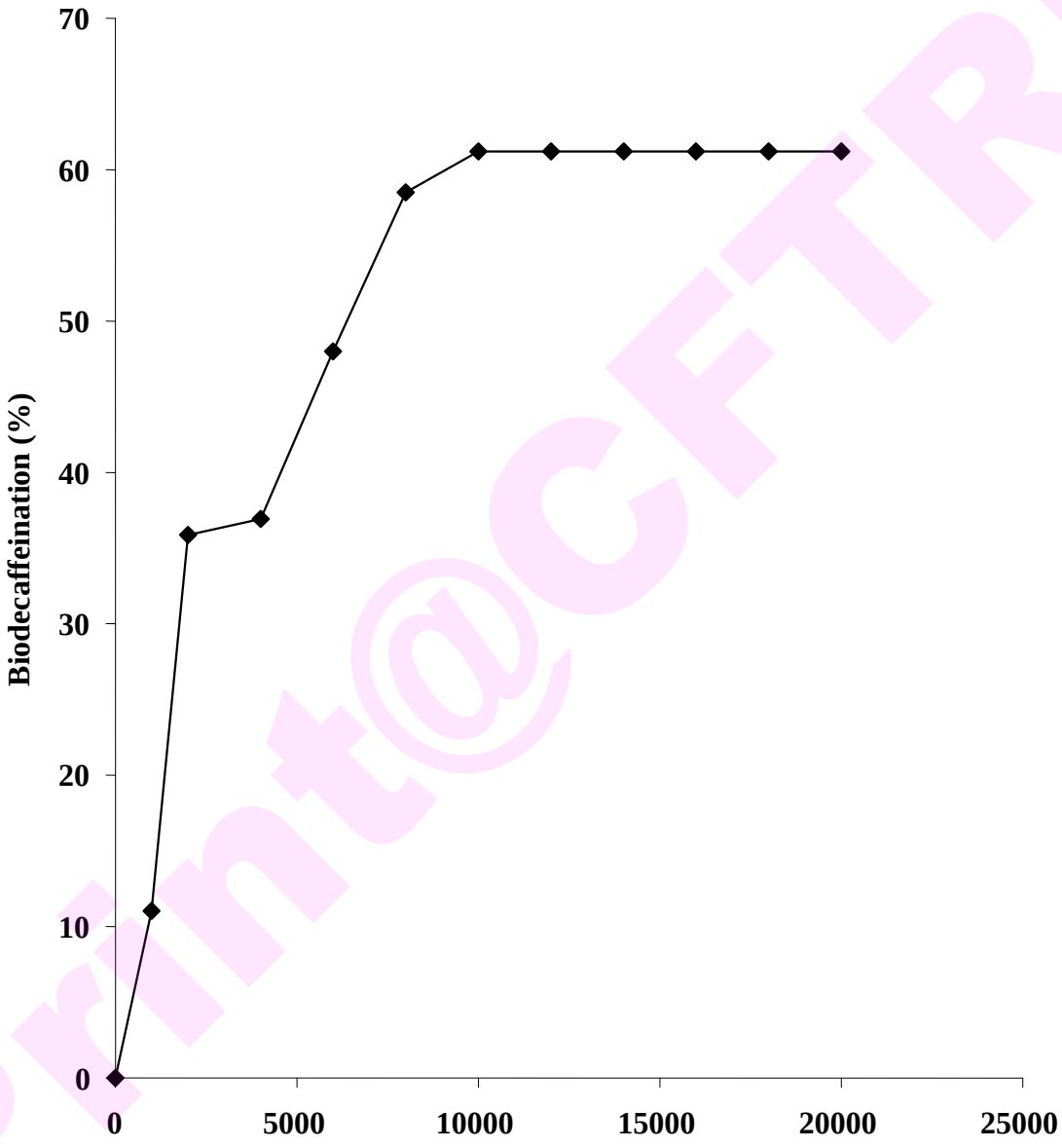
Tea dhool is a complex mixture of several components like polyphenols, caffeine, fiber, condensed tannins, etc. which influence the enzyme activity, caffeine availability to the enzymes and the degradation of caffeine in tea dhool. The color, flavor and aroma of made tea are due to the formation of several complex mixtures like theaflavins, thearubigins and caffeine-theaflavin complexes. Therefore, several parameters need to be optimized for efficient biodecaffeination of tea dhool. Parameters, which were considered crucial for caffeine removal by the enzymes, were optimized for the development of the biodecaffeination process. Aeration, enzyme concentration, caffeine availability in the dhool and prevention of the formation of inhibitory factors like caffeine-polyphenol and protein polyphenol complexes in the tea dhool during biodecaffeination were found to be important factors and were optimized.

5.4.4.1. Optimization of parameters for biodecaffeination:

a) Effect of initial enzyme activity on biodecaffeination of tea dhool:

The activity of caffeine degrading enzymes is critical for achieving required degree of biodecaffeination. It is known that caffeine demethylase is the rate-limiting enzyme in the degradation of caffeine by bacteria (Asano et. al., 1993) and the activity of caffeine demethylase in the CFE plays an important role in the biodecaffeination. Therefore, caffeine demethylase enzyme activities in the CFE largely control the whole process of decaffeination.

Figure 5.4.4.1. Effect of enzyme activity on biodecaffeination of tea dhool.



It was found that maximum decaffeination could be achieved by adding caffeine demethylase at 10000 U/100 gms of dhool (Fig. 5.4.4.1.). The caffeine content in the test dhool was found to be 2.986% w/w corresponding to 153.76mM of caffeine.

With an enzyme activity of 1000 U/100 gms only 11.04% of the initial caffeine was degraded in 90 minutes (Fig. 5.4.4.1.). Biodecaffeination increased to 35.86% as the concentration of the enzyme was increased to 2000 $U.100g^{-1}$ dhool. The corresponding values for 4000, 6000 and 8000 $U.100g^{-1}$ dhool were 36.92, 47.99 and 58.49% respectively.

A maximum of 61.21% biodecaffeination (corresponding to 76.78mM of caffeine degraded) was observed within 90 minutes using an enzyme activity of 10000 $U.100g^{-1}$ dhool. The reason for very low caffeine degradation in the dhool at low enzyme concentrations might be attributed to the high caffeine concentration in the sample requiring high enzyme activities. At enzyme activities above 10000 Units, no further caffeine degradation was observed probably owing to the non availability of caffeine to the enzyme.

By the end of 90 minutes most of the moisture in the dhool would be lost due to aeration leading to a drought effect in the dhool. This usually reduces the activity of polyphenol oxidase activity in the dhool in normal fermentation processes (Ritcher, 1934; Lipmann, 1943) and a similar effect is observed on caffeine demethylase activity in the dhool.

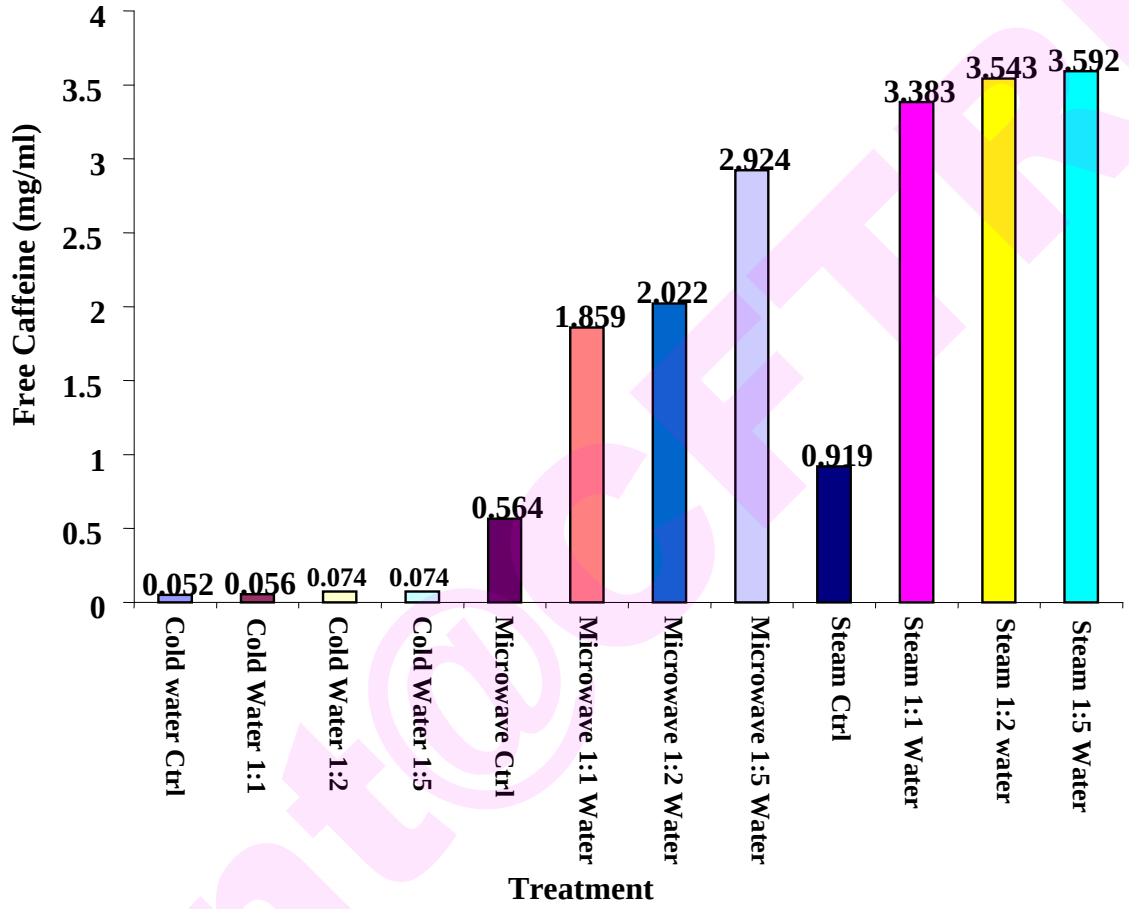
b) Effect of different treatments of dhool on caffeine availability:

Caffeine is present in the vacuoles of the tea leaf and is extracted along with the polyphenols and other leaf components into the sap during the process of cutting, tearing and curling of the leaves. The extent of extraction of caffeine into the sap is a critical factor determining the success of a biodecaffeination process. It is known that caffeine extraction from the dhool can be enhanced by an increase in the temperature (Liang, & Xu, 2003). Figure 5.4.4.2., represents the effect of different physical treatments such as steaming, microwave heating and cold water extraction on the availability of caffeine to the enzymes for biodecaffeination.

Extraction with water in the ratio of 1:5 with the dhool at room temperature resulted in the extraction of 0.076 mg/ml of caffeine (Fig. 5.4.4.2.) (Actual caffeine content in dhool = 2.98% w/w). The extractability of caffeine increased with the heat treatments with a maximum of 2.924 mg/ml of caffeine with microwave treatment at 1:5 ratios of dhool to water.

Maximum extraction of caffeine was observed when the dhool was steamed for 10 minutes with 1:5 ratio of dhool to water (3.54mg/ml). As the latent heat of steam is very high, it allows for the better diffusion of the water into the fibers and interiors of the tea dhool particles enabling efficient extraction of caffeine from the tea dhool.

Figure 5.4.4.2. Effect of different treatments of dhool on availability of caffeine for biodecaffeination of tea.



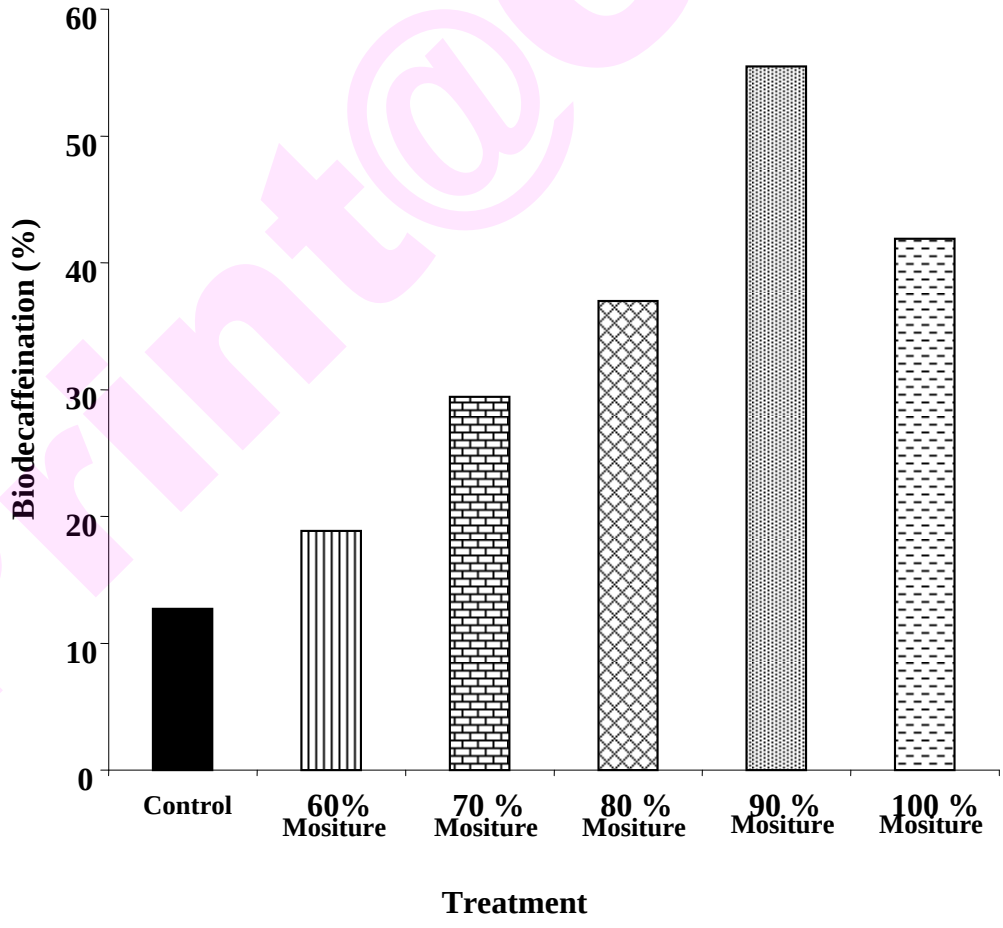
c) Moisture content of dhool:

Most of the enzymatic reactions occur in aqueous environments and water activity plays a major role in the activity of the enzymes (Skujins and. McLaren, 1967). Even enzymes catalyzing reactions in organic media are known to have a single layer of water in the active site (Yang and Russell, 1996). The moisture content of tea dhool ranges from 68-72% on a dry weight basis. The availability of caffeine to the active site of the enzyme depends on the moisture content in the dhool. Moisture content therefore, plays an important role in determining the diffusion of caffeine into

the vicinity of the enzymes to enable decaffeination and it is also known to regulate activities of several enzymes (Shoshana and Richmond, 1976).

From Figure 5.4.4.3., it is evident that the biodecaffeination increases with the increasing moisture content of the dhool. At 60% moisture the decaffeination was only 18.86% which increased to 55.47% at moisture content of 90%. At 100% there was a slight decrease in the decaffeination, probably owing to the draining of juices from the dhool and improper aeration and contact of the enzyme with caffeine in the dhool. A moisture content of 90% was found to be optimum for biodecaffeination of the dhool.

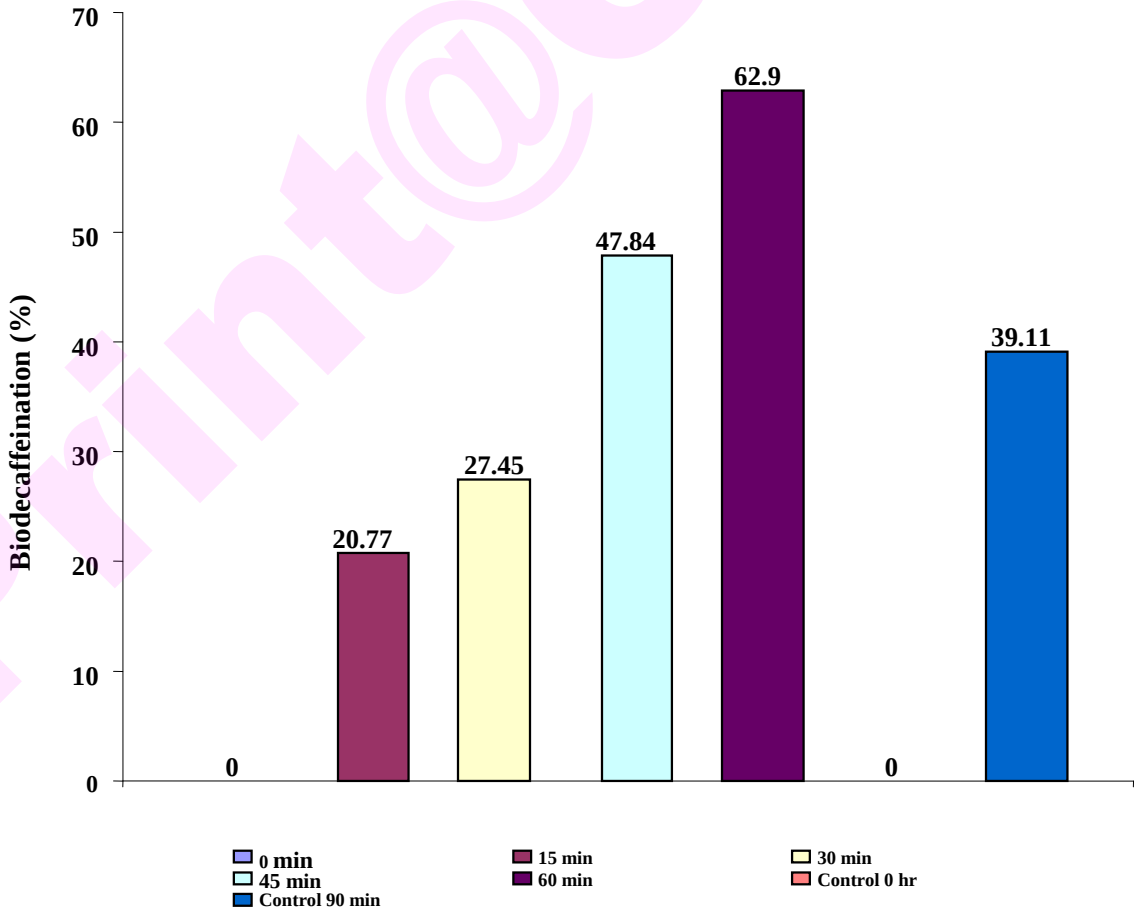
Figure 5.4.4.3. Effect of moisture content on biodecaffeination of tea dhool.



d) Effect of intermittent enzyme mixing on biodecaffeination:

The polyphenols in tea are known to interact with proteins and affect their functional properties. If the protein is an enzyme the activity of the enzyme is reduced over time (Haslam, 1974). The activity of caffeine demethylase in the CFE added to the dhool at the start of the reaction was found to decrease with time. A similar phenomenon was observed in the activities of polyphenol oxidase and other oxidative enzymes in tea by Ravichandran and Parthiban (1998). Intermittent addition of enzyme during biodecaffeination improved the caffeine degradation rate from the dhool (Fig. 5.4.4.4.).

Figure 5.4.4.4. Effect of intermittent enzyme addition on biodecaffeination of tea dhool.



In the control experiment where CFE was added only at the start of the biodecaffeination only 39.11% caffeine was degraded in 90 minutes. Addition of enzyme at different time intervals showed an improved in the total caffeine removal from the dhool with 47.9 % caffeine degraded within 60 minutes and 62.9 % caffeine degraded in 90 minutes (Fig.5.4.4.4.). From the above results, it is evident that enzyme addition at 30 and 60 minutes of incubation will enhance the biodecaffeination of tea dhool.

e) Oxygen requirement:

The tea fermentation process is highly oxygen intensive and the enzymes involved in the decaffeination are also monooxygenases and oxidases involving high oxygen expenditure. Roberts and Sharma, 1940 and Sreerangachar 1943 also reported that the activity of polyphenol oxidase in tea dhool demands high oxygen levels in the dhool during fermentation. Aeration plays a major role in the biodecaffeination of tea dhool. The enzymes involved in decaffeination viz. caffeine demethylase, heteroxanthine demethylase and xanthine oxidase are oxygenases and oxidase respectively. Caffeine degradation by the enzymes from *P. putida* requires molecular oxygen: 3 moles for the demethylation reaction, ½ mole for the urate oxidase reaction and 2 moles for electron transport (Middelhoven and Lommen, 1984). Studies on the development of a biosensor for caffeine at our laboratory have also indicated the presence of oxygenases in the system and a high oxygen requirement for good activity by the cells (Sarath et.al., 2006). Table 5.4.4.1., represents the effect of oxygenation on the biodecaffeination of dhool by the enzymes in the CFE. In the control experiment only 27.24% of the initial caffeine was degraded within 90 minutes of incubation, whereas under agitation 54% of the initial caffeine was degraded. When the sample was aerated there was a slight increase in the caffeine degradation in the dhool (59.6%) thus it proves the importance of aeration in the biodecaffeination of tea.

Table 5.4.4.1. Effect of aeration and agitation on the biodecaffeination of dhool.

Sample	Residual Caffeine (%w/w)	Biodecaffeination (%)
Control Dhool	2.948	0

Static Dhool	2.958	0
Static Enzyme	2.152	24.24
Agitated Control	2.812	0
Agitated Enzyme	1.294	53.98
Aerated Control	2.923	0
Aerated Enzyme	1.181	59.59

f) Caffeine polyphenol interactions:

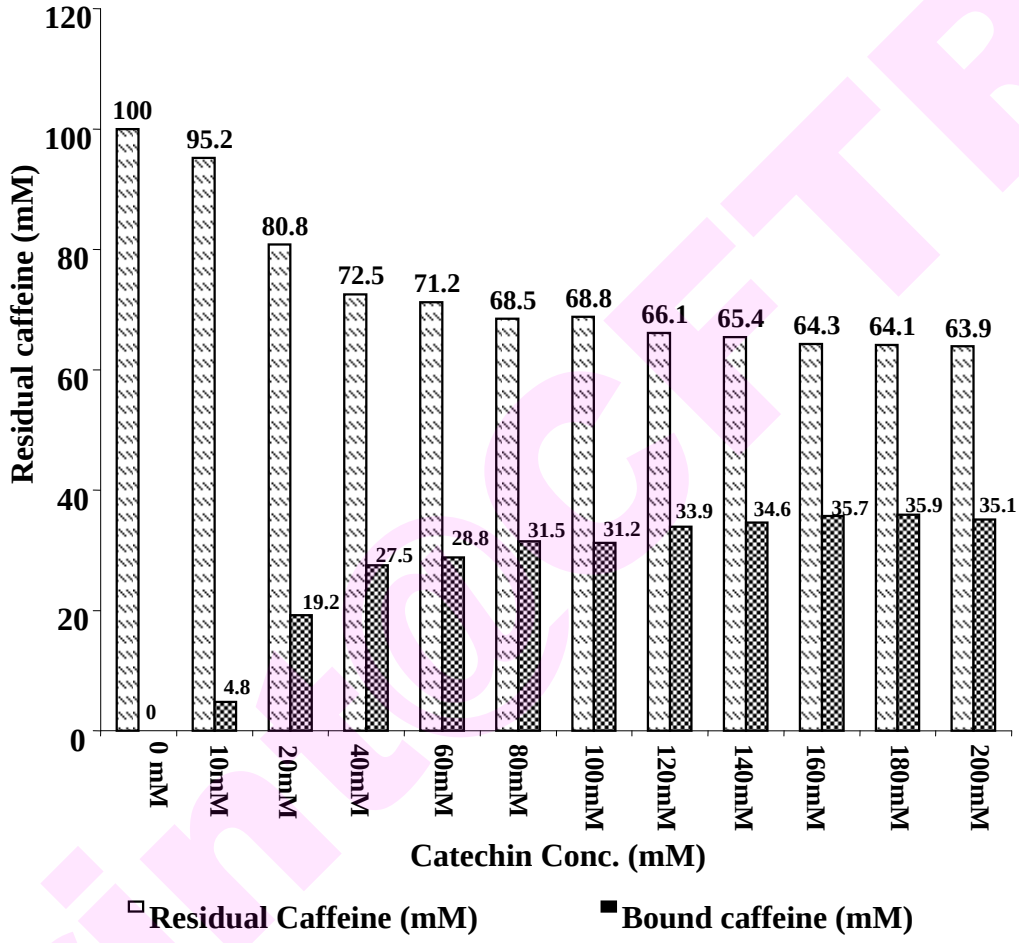
Caffeine is known to bind to polyphenols during the fermentation process leading to formation of caffeine polyphenol complexes. Caffeine-polyphenol complexes play an important role in imparting the creaming property of black tea. Two molecules of caffeine are known to bind to a molecule of theaflavin or catechin through hydrophobic interaction and hydrogen bonding (Collier et.al., 1972; Arnone and Marchessault, 1968).

The complexation of caffeine with polyphenols in tea would limit the availability of caffeine to the enzymes which will lead to a low degree of decaffeination. It was found that the availability of free caffeine decreased with an increase in the concentration of catechins in solution (Fig. 5.4.4.5).

Formation of condensed tannins would further limit the caffeine availability which is evident from figure 5.4.4.5. Initially 100mM caffeine was incubated with different concentrations of catechin and the recovery of caffeine in solution was found to decrease from 86.2-68.8 mM when the concentration of catechins was increased from 10-80mM (Fig. 5.4.4.5). It was observed that only 60mM caffeine was freely available in solution at a concentration of 80mM catechin. This proves that the

availability of caffeine for decaffeination was limited by the formation of caffeine-polyphenol complexes which affects the biodecaffeination.

Figure 5.4.4.5. Interactions of caffeine with polyphenols.



This phenomenon was not observed in the case of coffee. Inhibition of caffeine-polyphenol interactions is required for the efficient degradation of caffeine in dhool by the enzymes. We have observed a decreased decaffeination as the time of decaffeination progressed from 0-90 minutes (Figure 5.4.5.1). This is in part contributed by the caffeine-polyphenol complexes formed during fermentation of tea dhool.

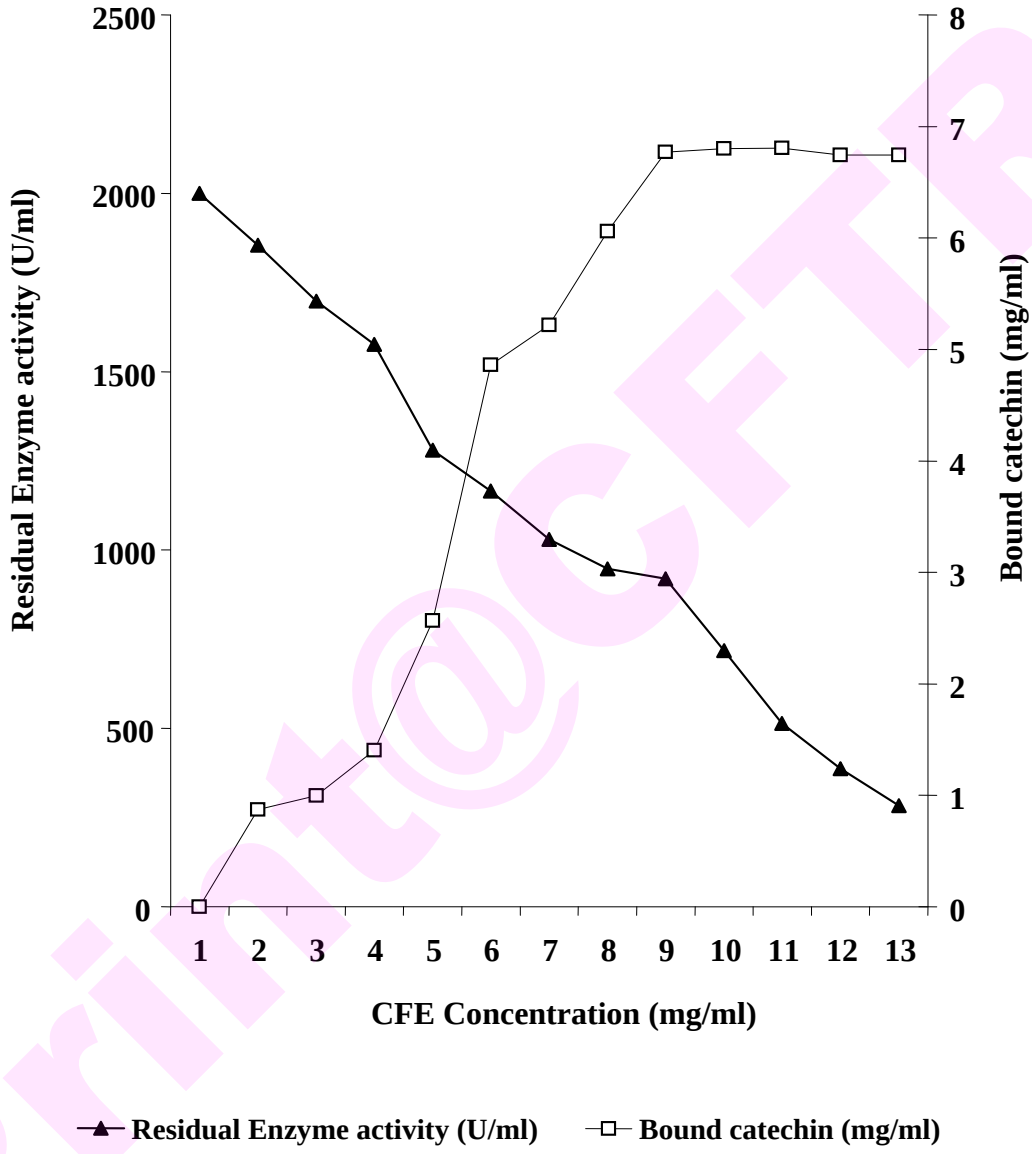
g) Protein polyphenol interactions

Proteins interact with tannins and polyphenols present in foods leading to the formation of complexes, which inhibit the enzyme activities (Haslam, 1974). The effect of catechin concentration and the rate of fermentation of tea govern the activities of externally added enzymes.

Fast fermenting clones of tea have a high rate of inhibition of the enzymes whereas slow fermentors show a lower inhibition of the enzymes. Biodecaffeination of tea dhool obtained from two different clones of tea (obtained from Tamilnadu and Kerala) varying in their fermentation ability showed that biodecaffeination was higher in the slow fermenting tea whereas the fast fermentor showed a lower biodecaffeination (Data not shown).

Studies also indicate that the formation of protein-polyphenol complexes is dependent on the size of the protein, the residues on the protein and also the size of the condensation products. Haslam (1974) showed that the enzyme α-glucosidase was inhibited by polyphenols due to complexation and that the inhibition increased as a function of the size of the condensation products. Small polyphenols like catechins had little effect compared to larger polyphenols like tannins and theaflavin gallates. Figure 5.4.4.6., represents the effect of protein polyphenol interactions on the activity of caffeine degrading enzymes. It was found that as the enzymatic oxidation of the tea progressed, the caffeine degradation rate slowed down (Fig. 5.4.4.6.). As the time of incubation progressed the amount of free catechin decreased from 10 mg/ml to 3.23 mg/ml.

Figure 5.4.4.6. Interaction of catechin (10mg/ml) with CFE.



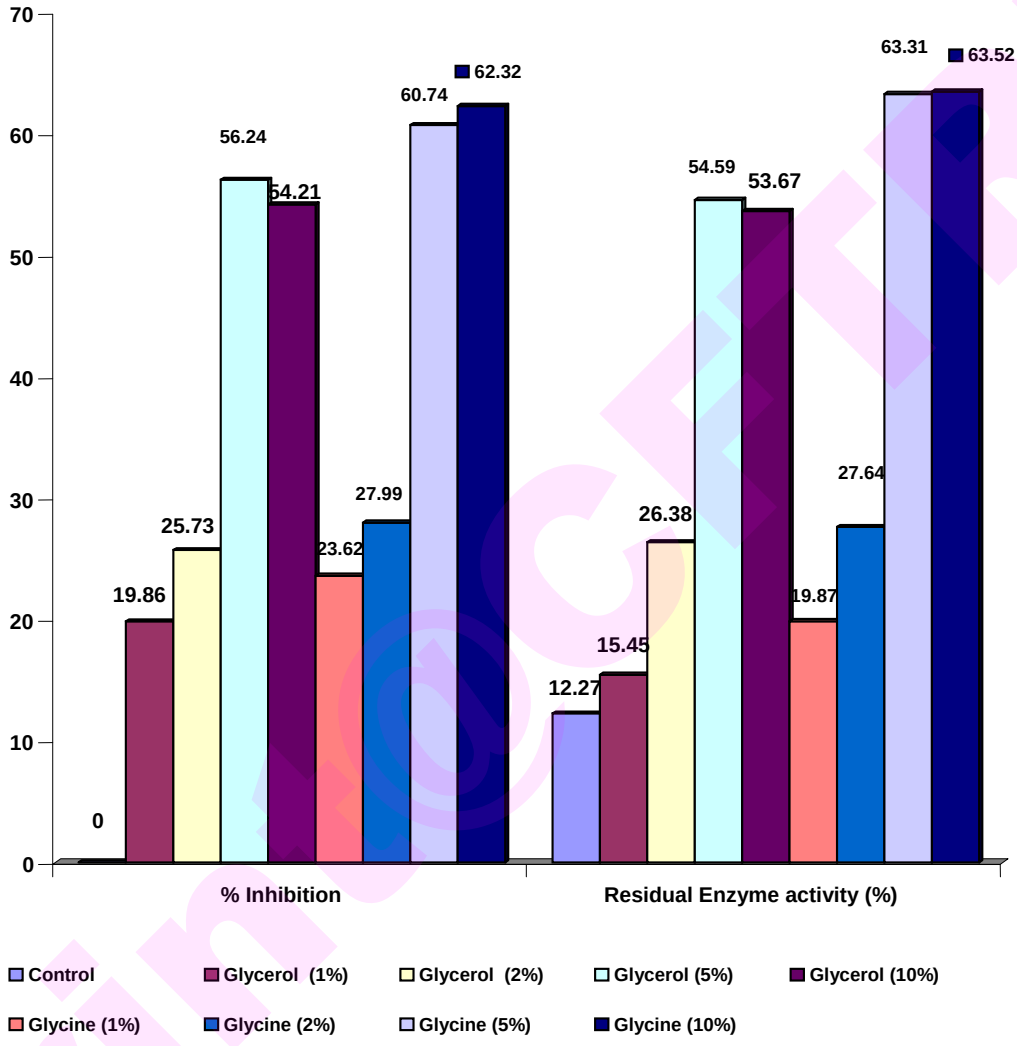
The enzyme activity also decreased from 2000 Units to 283 U in 90 minutes of incubation (Fig 5.4.4.6).

h) Prevention of protein-polyphenol interactions:

Protein polyphenols interactions are mostly hydrophobic in nature and several organic reagents can inhibit their formation. Several agents like methanol (Craft et al.,

2000), acetone (Haslam, 1974), tetrahydrofuran (THF), dioxin (Siebert et.al., 1996), glycine (Kotaro et.al., 1998), glycerol etc. are reported to be used for the removal of protein polyphenol interactions. Application of organic solvents like acetone, methanol, THF, dioxins etc. have detrimental effects on the health of the consumer. Moreover these solvents tend to denature the enzymes leading to a loss of the enzyme activities. Alternative molecules which are safe should be used for the development of biodecaffeination processes. Glycine, an amino acid and glycerol were checked for their efficiency of inhibiting the protein polyphenol interactions. It was observed that glycerine at a concentration of 5% w/w could inhibit 56 % of the protein polyphenol interactions, whereas glycine could inhibit 60.74% of the interactions leading to the retention of 63% Enzyme activity (Figure 5.4.4.7). Several studies also have indicated that glycine would reverse the protein polyphenol interactions in model systems. Glycine at 5% w/v was found to be the best in terms of inhibition of the complex formation as well as the retention of enzyme activity. Several studies on protein polyphenol interactions have shown that proline rich proteins (PRPs) have much higher affinity for the polyphenols and play a major role in the perception of the astringency of the tea. It is known that proline, glycine, and glutamine together account for 70% to 88% of all amino acids in the PRPs (Wong et.al., 1979) and is responsible for the high affinity of these proteins to polyphenols and tannins present in foods. In the tea dhool externally added glycine competitively binds to the polyphenols and tannins in the dhool rendering a protective effect on the enzymes in the CFE. This explains the higher decaffeination ability in the sample containing externally added glycine.

Figure 5.4.4.7. Effect of glycine and glycerol on inhibition of protein-polyphenol interactions in tea dhool.



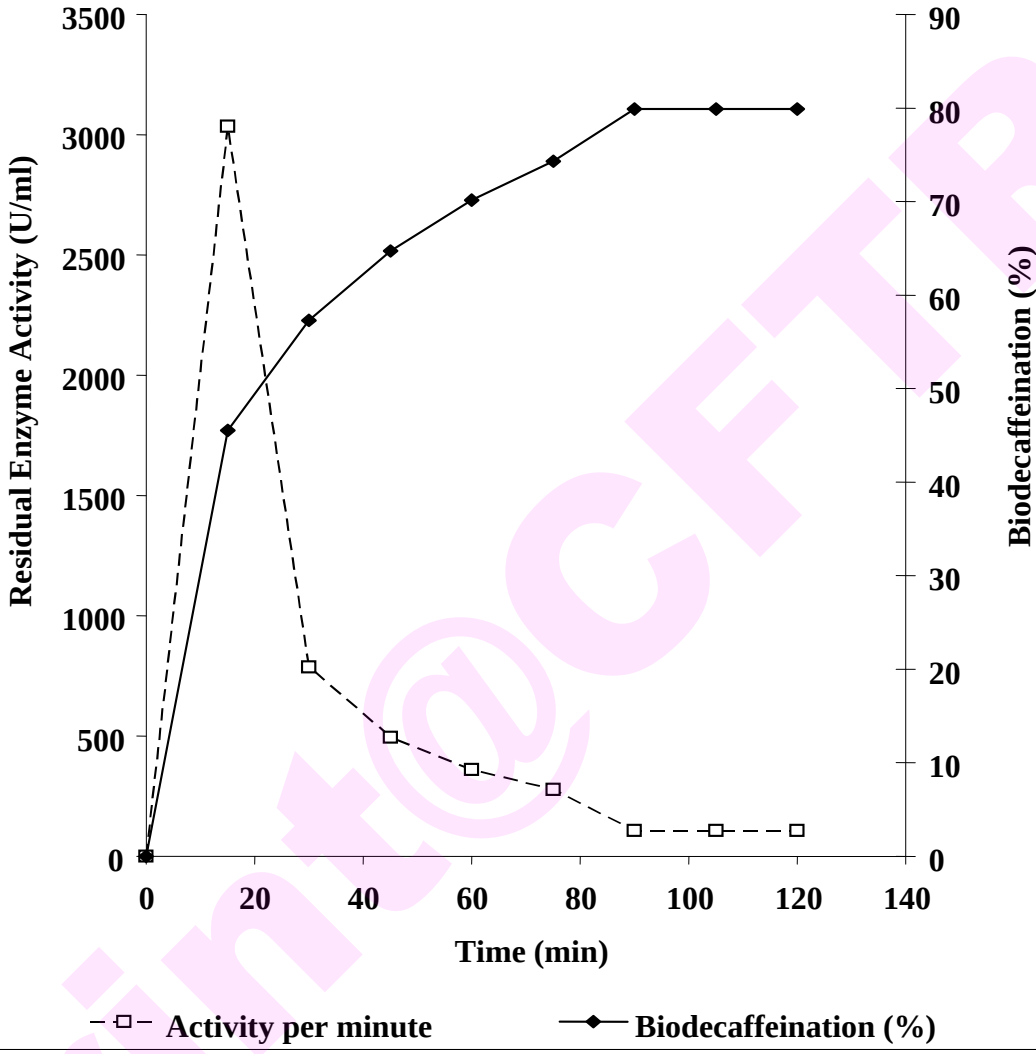
5.4.5. Biodecaffeination of black tea under optimum conditions:

With the optimized conditions (as above) 80% of the initial caffeine could be degraded from the tea dhool within 90 minutes (Fig. 5.4.5.1.). The residual caffeine in the dhool was found to be 0.633% w/w. Maximum enzyme activities and biodecaffeination (45.52 %) occurred within the first 15 minutes of addition of the

enzymes. The rate of removal of caffeine decreased as the reaction progressed with 57.31% caffeine degraded at the end of 30 minutes. The reaction slowed down further and reached a plateau at 90 minutes of incubation and the biodecaffeination was 80%.

This decrease in activity after 90 minutes of incubation is due to the formation of caffeine-polyphenol complexes and protein polyphenol complexes in the dhool. As tea fermentation progresses theaflavins and thearubigins are formed which have higher affinity of binding to caffeine and proteins present in the dhool (Brown, 1966; Chao and Chiang, 1999, Haslam, 2003). This would limit the efficiency of biodecaffeination through two major phenomena, one limiting the availability of caffeine and the other limiting the enzyme activity. These two phenomena were also observed during the biodecaffeination of black tea and account for the decreasing rate of biodecaffeination with time.

Figure 5.4.5.1. Kinetics of caffeine degradation during biodecaffeination of tea dhool.



5. 5. CONCLUSIONS:

You can't teach old dog new tricks (so the adage goes). But it is not always true in the case of microorganisms. They appear to be constantly on a learning spree adapting themselves to utilize different substrates and adapting to new environments. Enzymes isolated from the induced cells of *P. alcaligenes* MTCC 5264 were found to decaffeinate a solution of caffeine completely. This discovery led to an in-depth quest into the possibility of developing a biodecaffeination process. Interest in the production of decaffeinated tea and coffee arose more than 50 years ago, stimulated by research on the ill effects of caffeine when these beverages were consumed in excess. Since then several processes on decaffeination using solvents and other chemicals were developed. Each of these processes has limitations such as loss of flavor, adverse effects of the solvents on health etc.

In the present work, we succeeded in decaffeinating tea dhool by using enzymes isolated from *P. alcaligenes* MTCC 5264 and defined this process as a **"Biodecaffeination Process"** for the first time. This work builds on previous studies by us in which *P. alcaligenes* MTCC 5264 was induced to produce the enzymes involved in degradation of caffeine. A major breakthrough is the ability of these enzymes to decaffeinate tea invitro. A major bottle neck in this process is that the enzymes themselves are highly unstable invitro. Application of these enzymes in the biodecaffeination process was limited by many factors such as protein polyphenol interactions, caffeine-polyphenol complexes and the inactivation of the enzymes. An integrated approach of controlling the adverse reactions on the enzymes and the

availability of caffeine to the enzymes was effective in achieving 80% biodecaffeination.

In sum, this work represents an in-depth study on the development of new routes for the decaffeination of caffeine containing materials like tea and coffee through the biotechnological route. This process is safe both to the consumer as well as the environment. Hayashibara International Inc.'s GRAS panel considers the enzyme from *P. amylodermosa*, a soil isolate can be safely used for the production of isoamylase enzyme for food applications (www.FDA.org/CFSAN: Agency response letter: GRAS Notice No. GRN 000085) and also isoamylase from the fore said isolate is approved by the Japanese Food Additive Regulations, 1998. *P. alcaligenes* is a soil isolate from coffee and tea garden soils. It is not a known opportunistic pathogen and the enzymes produced by *P. alcaligenes* may be considered safe inline with Hayashibara International Inc.'s isoamylase for food applications.

Achieving 80% decaffeination by using enzymes under so many adverse conditions was not easy. A few challenges still lie ahead. Achieving 90% or more decaffeination is required and an in-depth study into this is being carried out.

5.6. REFERENCES:

1. Arima, K., Nose, K., (1968), Studies on Bacterial Urate-Oxygen Oxidoreductase. I. Purification and Properties of the Enzyme, *Biochim Biophys Acta.* **151**: 54.
2. Asano, Y., Komeda. T., Yamada. H., (1993). Microbial production of theobromine from caffeine. *Biosci. Biotechnol. Biochem.* **57:**1286-1289.
3. Asano, Y., Komeda T., Yamada, H., (1994), Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No. 352, *Biosci. Biotechnol. Biochem*., **58(12):** 2303-2304.
4. Baxter N.J., Michael P. W., Terence H. L., Haslam, E., **(**1996), Stacking interactions between caffeine and methyl gallate, *J. Chem. Soc., Faraday Trans.***, 92:** 231 – 234.
5. Bergmeyer, H.U., Gawehn, K., Grassl, M., (1974), in *Methods of Enzymatic Analysis* (Bergmeyer, H.U. ed.) Second Edition, Volume I, 521-522, Academic Press Inc., New York.
6. Blecher, R.; Lingens, F., (1977), The metabolism of caffeine by a *Pseudomonas putida* strain. *Hoppe-Seyler's Zeitschrift fuer Physiologische*, **358**: 807-817.
7. Bradford, M. (1976), A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding" *Anal. Biochem*. **72:** 248-254.
8. Camargo, M.C.R., Toledo, M.C.F., (1998), Caffeine content of commercial Brazilian coffee. *Cienc Technol Aliment*. **18:** 421–424.
9. Caudle, A.G., Bell, L.N., (2000), Caffeine and theobromine content of ready to eat chocolate cereals. *J Am Diet Assoc.* **100:** 690–692.
10. Charlton, A. J., Davis, A. L., Jones, D. P., Lewis, J. R., Davies, A. P., Haslam, E., Williamson, M. P., (2000), The self-association of the black tea polyphenol theaflavin and its complexation with caffeine. *J. Chem. Soc., Perkin Trans.* **2:** 317-322.
11. Elisabeth, J., John, O'C.J., Patrick, A. F., Mike, P.W., (2004), Molecular Model for Astringency Produced by Polyphenol/Protein Interactions, *Biomacromolecules*, **5 (3):** 942 -949.
12. Gluck, M.; Lingens, F. (1987), Studies on the microbial production of theobromine and heteroxanthine from caffeine. *Appl. Microbiol. Biotechnol*., **25:** 25334-253340.
13. Haas, G., Stieglitz, B., (1980), Microbiological decaffeination of aqueous solutions. Canadian Patent, 1086553.
14. Haslam E., (1974), Polyphenol–protein interactions. *Biochem. J*., **139(1):** 285–288.
15. Kaplan, A. V., (1960), Deterrmination of Urease Activity, *Methods Biochem Anal.* **17:** 320.
16. Karl J. S., Nataliia V.T., Penelope, Y. L., (1996), Nature of Polyphenol-Protein Interactions, *J. Agric. Food Chem.* **44 (1):** 80 -85.
17. Kellerhals, M., Hazenberg, W., Witholt, B., (1999), High Cell Density Fermentations of Pseudomonas oleovorans for the Production of mcl-PHAs in Two Liquid-Phase Media, *Enz. Microb. Technol.* **24**: 111-116.

18. Keya, C.A., Crozier, A., Ashihara, H., (2003), Inhibition of caffeine biosynthesis in tea (*Camellia sinensis*) and coffee (*Coffea arabica*) plants by ribavirin. *FEBS Lett.* **554:** 473-477.
19. Kretschmar JA, Baumann TW. (1999), Caffeine in citrus flowers. *Phytochem.*, **52:** 19–23.
20. Kusuda, M., Hatano, T., Yoshida,T., (2006), Water-Soluble Complexes Formed by Natural Polyphenols and Bovine Serum Albumin: Evidence from Gel Electrophoresis**,** *Biosci. Biotechnol. Biochem.,* **70(1):** 152-160.
21. Landolt HP, Dijk D-J, Gauss SE, Borb´ely AA. (1995), Caffeine reduces, low-frequency delta activity in the human sleep EEG. *Neuropsychopharmacol*. **12**: 229–238.
22. Madyastha, K.M., Sridhar, G.R., (1998), A Novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem. Biophys. Res. Commun*. **249**:178–81.
23. Madyastha, K, M., Sridhar, G.R., Vadiraja, B.B., Madhavi, Y.S., (1999), Purification and partial characterization of caffeine oxidase-A novel enzyme from a mixed culture consortium. *Biochem Biophys Res Commun*. **263**:460–464.
24. Middelhoven, W.J., Lommen, A., (1984), Degradation of caffeine by *Pseudomonas putida* C3024; the effect of oxygen concentration. *Antonie van Leeuwenhoek* **50:**298-300.
25. Ogita, S., Uefuj, H., Yamaguchi, Y., Koizumi, N., Sano, H., (2003), Producing decaffeinated coffee plants. *Nature* **423:** 823.
26. Sarath Babu V.R., Patra, S., Thakur, M.S., Karanth, N.G., Varadaraj, M.C., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708, *Enz. Microb. Technol*. **37(6):** 617-624.
27. Schwimmer, S., Kurtzman, R., (1972), Fungal decaffeination of roasted coffee infusions. *J. Food Sci*. **37:** 921-924.
28. Shilo, L., Sabbah, H., Hadari, R., Kovatz, S,, Weinberg, U., Dolevm S., (2002), The effects of coffee consumption on sleep and melatonin sectretion. *Sleep Med*. **3:** 271–273.
29. Sideso, O. F. P., Marvier, A. C., Katerelos, N. A., Goodenough, P.W., (2001), The characteristics and stabilization of a caffeine demethylase enzyme complex. *Int. J. Food Sci. Tech* **36(6):** 693-698.
30. Trijbels, F., Vogels, G.D., (1966a), Degradation of allantoin by *Pseudomonas acidovorans*, *Biochim Biophys Acta*. **113(2):** 292-301.
31. Trijbels, F., Vogels, G.D., (1966b) Allantoinase and uriedoglycolase in *Pseudomonas* and *Penicillium* Species. *Biochim Biophys. Acta.* **118 (2):** 387-95.
32. Woolfolk, C.A., (1975), Metabolism of N-methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. *J. Bacteriol.* **123**: 1088-1106.
33. Woolfolk, C.A., (1985), Purification and properties of a novel ferricyanidelinked xanthine dehydrogenase from *Pseudomonas putida* 40. *J. Bacteriol*. **163:** 600-609.
34. Yano, Y.D.M., Mazzafera, P., (1999), Catabolism of caffeine and purification of a xanthine oxidase responsible for methyluric acids production in *Pseudomonas Putida* L. *Rev Microbiol*. **30**: 62–70.

CHAPTER 6

DEVELOPMENT OF A

BIOSENSOR FOR CAFFEINE.

PREAMBLE TO CHAPTER 6:

Caffeine determination in tea, coffee, cola drinks, pharmaceutical preparations and other caffeine containing materials is highly important in determining the quality of the beverages and in determining the caffeine intakes. In our laboratory we have isolated microorganisms capable of degrading caffeine and the enzymes involved in biodecaffeination were identified and characterized. With the leads obtained in our research on enzymes involved in caffeine degradation and with the expertise available on the development of biosensors in our laboratory, added with the lack of existing biosensors for caffeine, we had an inquisition into the development of a biosensing method for determination of caffeine and have developed a whole cell based biosensor which is described in this chapter.

6.1. SCOPE OF THE WORK:

Coffee and tea are the most popular beverages across the world and their caffeine content has an important role of the caffeine in determining the quality of coffee and tea beverages. Further, on account of the harmful effects of caffeine its efficient measurement is relevant. In this context, the development of a sensitive, rapid and cost effective method for monitoring caffeine is greatly needed. Although a few biosensing methods for caffeine are reported, they have limitations in application for commercial samples. The development and application of new caffeine detection methods remains an active area of investigation, particularly in food and clinical chemistry. Caffeine biosensor has wide applications in determination of caffeine in tea, coffee, chocolates, cola beverages, sports drinks, energy drinks, pharmaceutical preparations and in determining the health of the liver (where caffeine is used as a

marker for Cytochrome P450 activity in humans) and in the determination of caffeine in waste waters as an indicator of pollution.

An amperometric biosensor based on immobilized cells of *P. alcaligenes* MTCC 5264 was developed and is described in detail in this chapter. The physicochemical parameters for efficient operation of the biosensor were optimized and are described in detail in this chapter. Studies on interferences due to other metabolites like sugars were also carried out and the efficiency of the biosensor in analysis of real samples like coffee and tea was checked by analysis by HPLC and are described in detail in this chapter. The chapter ends with recommendations and future perspectives of the caffeine biosensor.

6.2. INTRODUCTION:

Demand for biosensors has increased markedly in recent years, driven by needs in many commercial and research sectors for specific sensors that are capable of rapid, reliable measurements (Keusgen, 2002). Development of biosensors is of interest for diverse applications ranging from biochemical profiling of normal and diseased cells (metabolomics), clinical diagnostics, drug discovery and biodefense, to more straightforward analyses such as fermentation, process monitoring, environmental testing, and quality control of foods and beverages.

Coffee and tea are the most popular beverages across the world and their caffeine content has an important role of the caffeine in determining the quality of coffee and tea beverages. Further, on account of the harmful effects of caffeine (described in chapter 1 of this thesis) its efficient measurement is relevant. In this context, the development of a sensitive, rapid and cost effective method for monitoring caffeine is greatly needed.

Conventionally, high-performance liquid chromatography (HPLC) separation (Moriyasu et al., 1996; Kunagi and Tabei 1997) and UV-spectrophotometric detection (Turk and Guzin, 2002), methods are applied to coffee beans, tea and other caffeine containing materials for caffeine content determinations. Other methods such as capillary electrophoresis, (Zhao and Lunte, 1997), thin layer chromatography (TLC) (Kunagi and Tabei, 1997) and gas chromatography (GC) (Statheropoulos et.al., 1996), are used for separation of caffeine in the analysis of mixtures, combined with detection methods such as mass spectroscopy (Statheropoulos et. al., 1996) and FTIR spectrophotometry (Norton and Griffith, 1995). However, expensive instrumentation,

highly skilled technicians and complicated and time-consuming procedures are required for such methods.

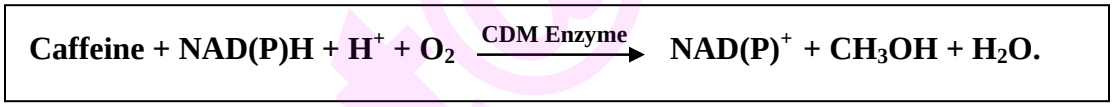
Another possible technique is flow injection immunoassay using a solid phase reactor, which makes the assay faster because no separation step is needed (Rico et al., 1991). However the time and cost difficulty in raising monoclonal antibody for low molecular weight compound such as caffeine, antibody purification, and the need for their manipulation with extreme care, are the disadvantages of this approach. A biosensor based on inhibition of 3,5-cyclic phosphodiesterase (CPDE) from bovine heart in combination with a pH electrode for the detection of caffeine in coffee was reported by Pizzariello et. al., (1999). This work is limited to academic interest due to the costs and difficulty in obtaining the enzyme. Moreover this biosensor also suffers from the disadvantage of interference due to other compounds in real samples.

The development and application of new caffeine detection methods remains an active area of investigation, particularly in food and clinical chemistry. Significant research and development activity has been devoted to preparing compact analytical devices comprising a bioactive sensing element integrated with a suitable transducing system, known as biosensors, for determination of various inorganic, organic and biological substances in our laboratory at CFTRI. The main advantages of these devices are their specificity, sensitivity and ease of sample preparation, and the fact that no other reagents besides a buffer and a standard are usually required (Thakur and Karanth, 2003). With these advantages in view, investigations have been

carried out in this work to develop a microbial biosensor for the estimation of caffeine in food and beverage samples.

6.2.1. Principle of microbial based biosensor for the detection of caffeine:

As it is well known that oxidation of the substrate using oxidase enzyme involves oxygen uptake, which can be monitored when these enzymatic reactions are brought about in the vicinity of a dissolved oxygen electrode. In microorganisms, the enzymatic degradation of caffeine is brought about by sequential demethylation, by an oxygenase, which by the utilization of oxygen degrades it into theobromine or paraxanthine. A stoichiometric relation exists between the amount of caffeine in the sample and the amount of oxygen consumed based on which, the amount of caffeine in the sample can be determined.



6.3. MATERIALS AND METHODS:

Caffeine, theophylline, theobromine, gelatin, glutaraldehyde and para chloro mercuri benzoic acid (Ultra Pure) were obtained from Sigma Chemicals, St. Louis, U.S.A. Analytical grade, di-nitro phenyl hydrazine, polyvinyl alcohol, and polyvinyl pyrrollidone were procured from Sisco Research Laboratory Chemicals, Mumbai, India. κ-carrageenan, dehydrated nutrient agar and nutrient broth (AR) were obtained from M/s HiMedia Labs, Mumbai, India. All other chemicals were of high purity and were procured from standard sources.

A microbe previously isolated and characterized in our laboratory as *Pseudomonas alcaligenes* MTCC 5264 (Sarath et.al., 2005), which was found to have potent caffeine degradation capability was used for the studies. The isolate was cultivated in a modified nutrient broth containing caffeine (0.03% w/v) to obtain biomass. The microbial cells were induced according to the method described in Chapter 2.

6.3.1. Construction of the caffeine biosensor:

The biosensor comprises a biological sensing element (immobilized cells of *P. alcaligenes* MTCC 5264), transducer (amperometric elctrode), amplification and detector systems (dissolved oxygen probe, EDT instruments, UK) as shown in Figure 6.3.1.1, which was earlier reported (Gouda et. al. 2000) and is described as follows.

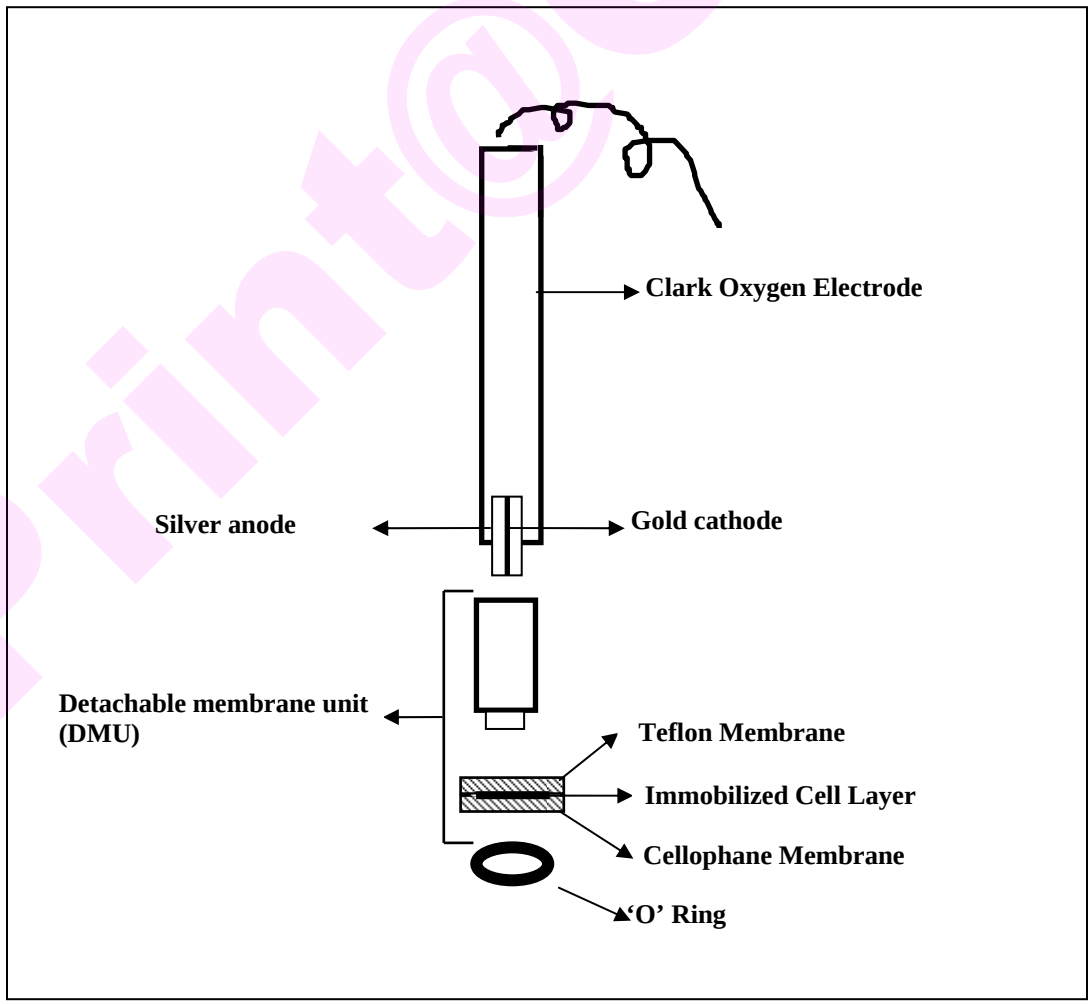
6.3.1.1. Immobilization of whole cells:

Immobilization of microbial cells was done according to the method of Gouda et. al., (1997, 2001, 2002) with slight modifications. Three immobilizing agents viz.,

gelatin, polyvinyl alcohol, and polyvinyl pyrrollidone were used. In brief, the following steps were involved in the immobilization of cells.

The harvested microbial cells were washed 4-5 times. A 10% w/v suspension of the induced cells was prepared in phosphate buffer (pH 7.2). 50µl of cell suspension was carefully pipetted onto a cellophane membrane (2 cm x 2 cm) and 100µl of a 10% w/v of the immobilizing agent was added on the membrane and mixed with a glass rod to form a fine layer of the cells. The membrane was kept at room temperature for one hour to allow crosslinking. These membranes were preserved in buffer at 4 ^{o}C for further use.

Fig. 6.3.1.1: Schematic diagram of the whole cell electrode used for the biosensor.



6.3.1.2. The biological sensing element:

In order to construct the biosensor for caffeine, immobilized cells having caffeine degrading activity immobilized on semi permeable membrane were fixed on to the electrode surface by using an 'O' ring shown in Figure 6.3.2.1. The schematic diagram of the complete biosensor setup is shown in Figure 6.3.1.2. Gas permeable teflon membrane (WTW, Germany) was used as the inner membrane in the sensor element and cellophane membrane (3000-6000 MWCO; Gambro, Lund, Sweden) was used as the outer membrane. Electrode poisoning due to electrochemically interfering compounds like metal ions and ascorbic acid and metal chelating agents like citric acid, is avoided during real sample analysis due to the selective permeability of the teflon membrane to gases only. A 10 ml glass container with 2.5 ml phosphate buffer was used as the sample cell. Air was continuously bubbled using a simple aquarium pump to keep the contents mixed as well as oxygen supplied continuously.

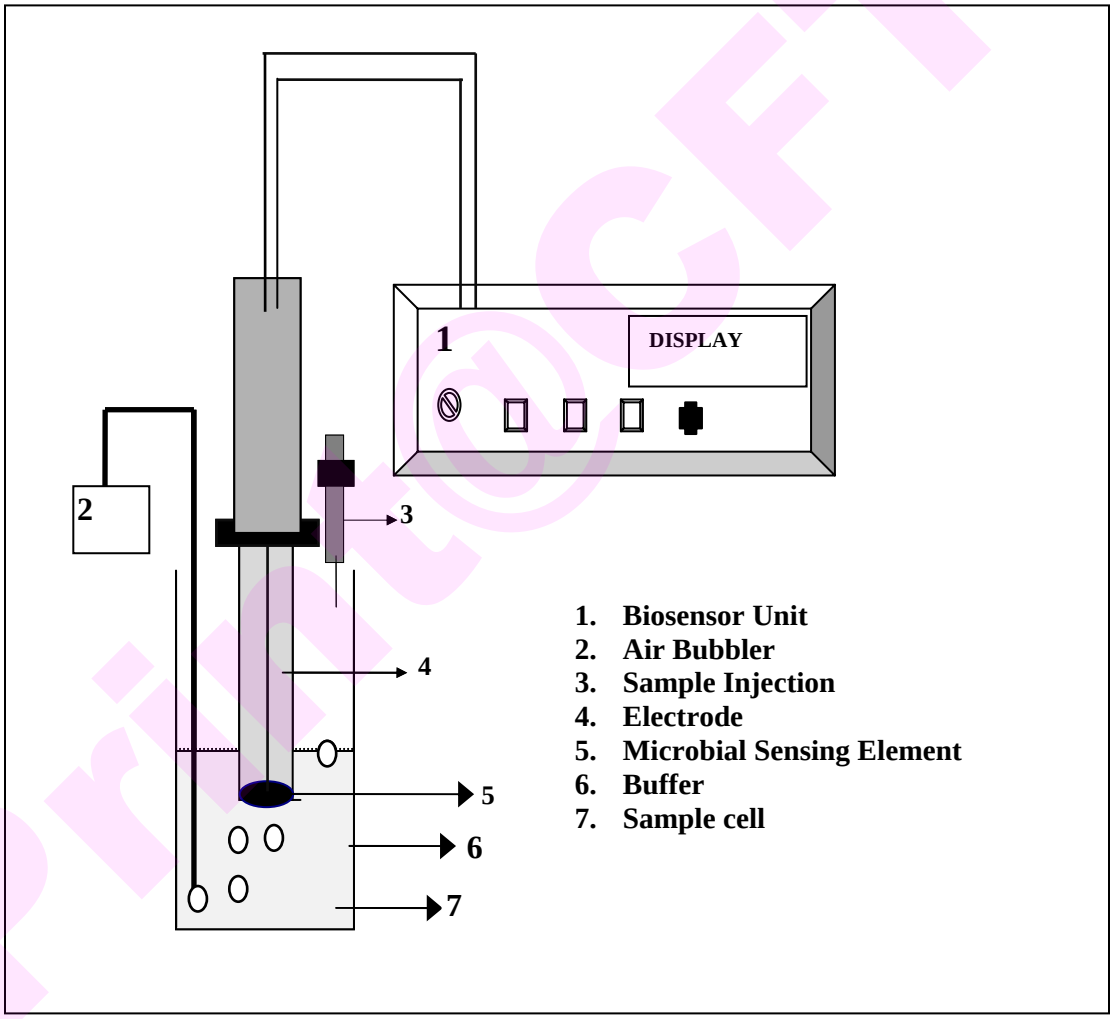
6.3.1.3. Construction of the transducer-amplifier-detector system:

In order to apply the potential to the electrode and to process the signal from the electrode, a clarke type dissolved oxygen (DO) sensor (EDT Instruments, UK) was used. The reduction of oxygen at the cathode due to the biochemical reaction gives an output voltage/current, which is proportional to the oxygen concentration in solution, which can be correlated with the analyte concentration.

6.3.1.4. Measurement of response by immobilized microbial cells for caffeine:

Initially, the electrode kept in the sample-cell containing buffer was polarized for one hour. Then 100µl of the sample containing caffeine was injected and the decrease in DO with time was recorded.

Figure 6.3.1.2: Schematic diagram of the biosensor for the estimation of caffeine.



The response of the DO electrode as decrease of DO at steady state was plotted against the caffeine concentration (% w/v) on X-axis and electrode response (Δ % DO) on Y- axis.

6.3.1.5. Response of xanthine oxidase to caffeine:

The enzyme involved in the first step of degradation of caffeine has not been characterized, but reports are available on the further degradation of caffeine to xanthine, by xanthine oxidase, uricase, allantiocase, allantoinase, glyoxylate dehydrogenase and urease. To check for the interference of xanthine oxidase activity (XOD) in the determination of caffeine, XOD was immobilized and its response to caffeine, theobromine, and paraxanthine (0.1% w/v) was recorded.

6.3.1.6. Preparation of real samples for biosensor analysis:

Instant coffee and tea were prepared by dissolving 0.5 g of each in 125 ml boiling water (Blauch and Stanley, 1983) and were kept for 30 minutes on a magnetic stirrer and filtered using a Whatman filter Paper No.1. 25 µl of the sample was then injected and the response was recorded.

6.4. RESULTS AND DISCUSSION:

6.4.1. Construction of caffeine biosensor:

6.4.1.1. Immobilization of whole cells:

For immobilization of cells, different agents viz. gelatin, polyvinyl pyrrollidone and polyvinyl alcohol were tried. The biosensor gave good response for caffeine (1% w/v) showing a steady state depletion of 53.2 % DO concentration as shown in Table 6.4.1.1.

It was observed that polyvinyl alcohol is a better immobilizing agent in terms of a higher response and reusability, which may be due to a higher oxygen permeability and diffusivity (Reidel et.al, 1988). With PVA as stabilizing agent, about 15-25 analyses could be done with a single membrane.

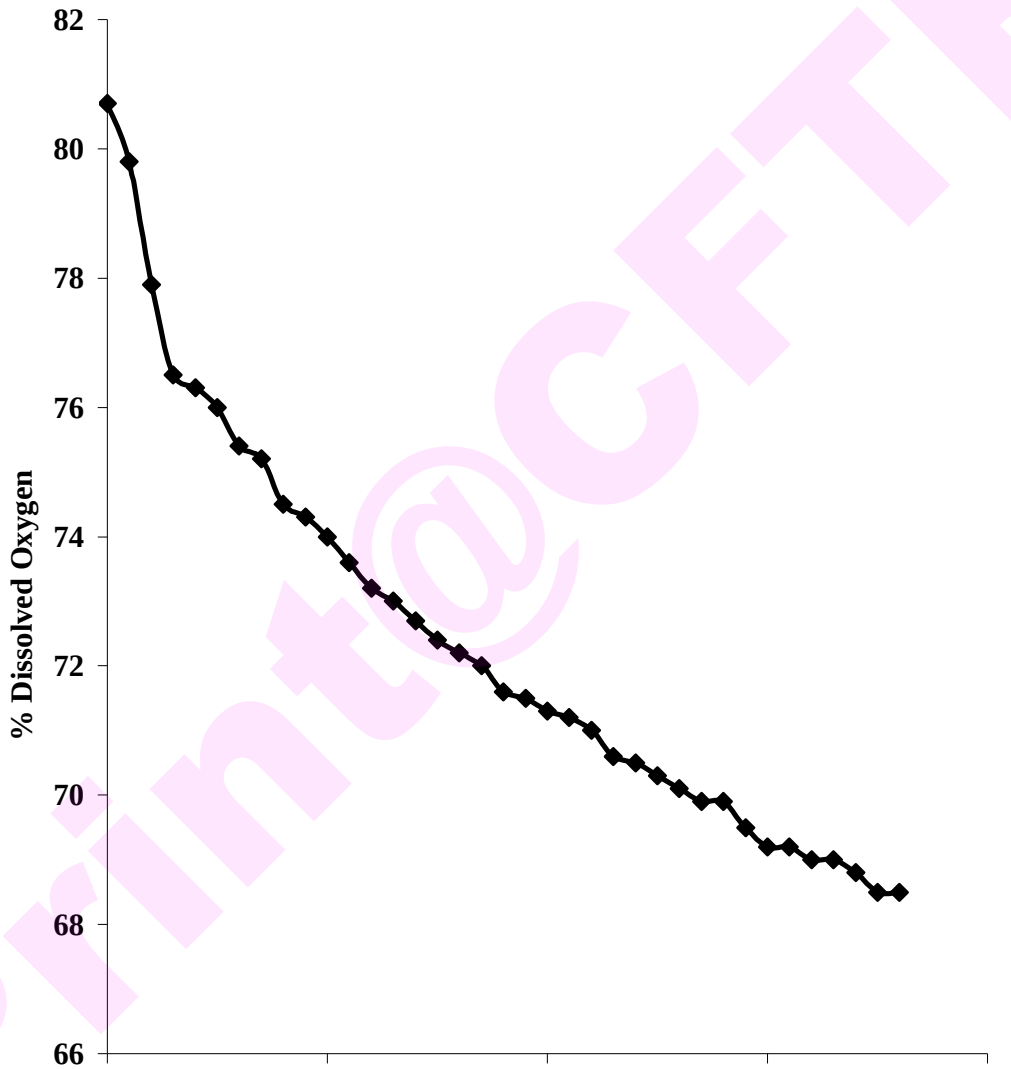
Table 6.4.1.1: Response of the Biosensor using different immobilizing agents.

Stabilizing agent	% DO Depletion shown by the biosensor after 3 minutes	No. of analyses with one sensor element
Gelatin	1.7	1
Polyvinyl pyrrollidone	1.6	2
Polyvinyl alcohol	**53.2**	**15**

6.4.1.2. Response of biosensor to caffeine:

The response of the biosensor to 1% w/v of caffeine is shown in Figure 6.4.1.1.

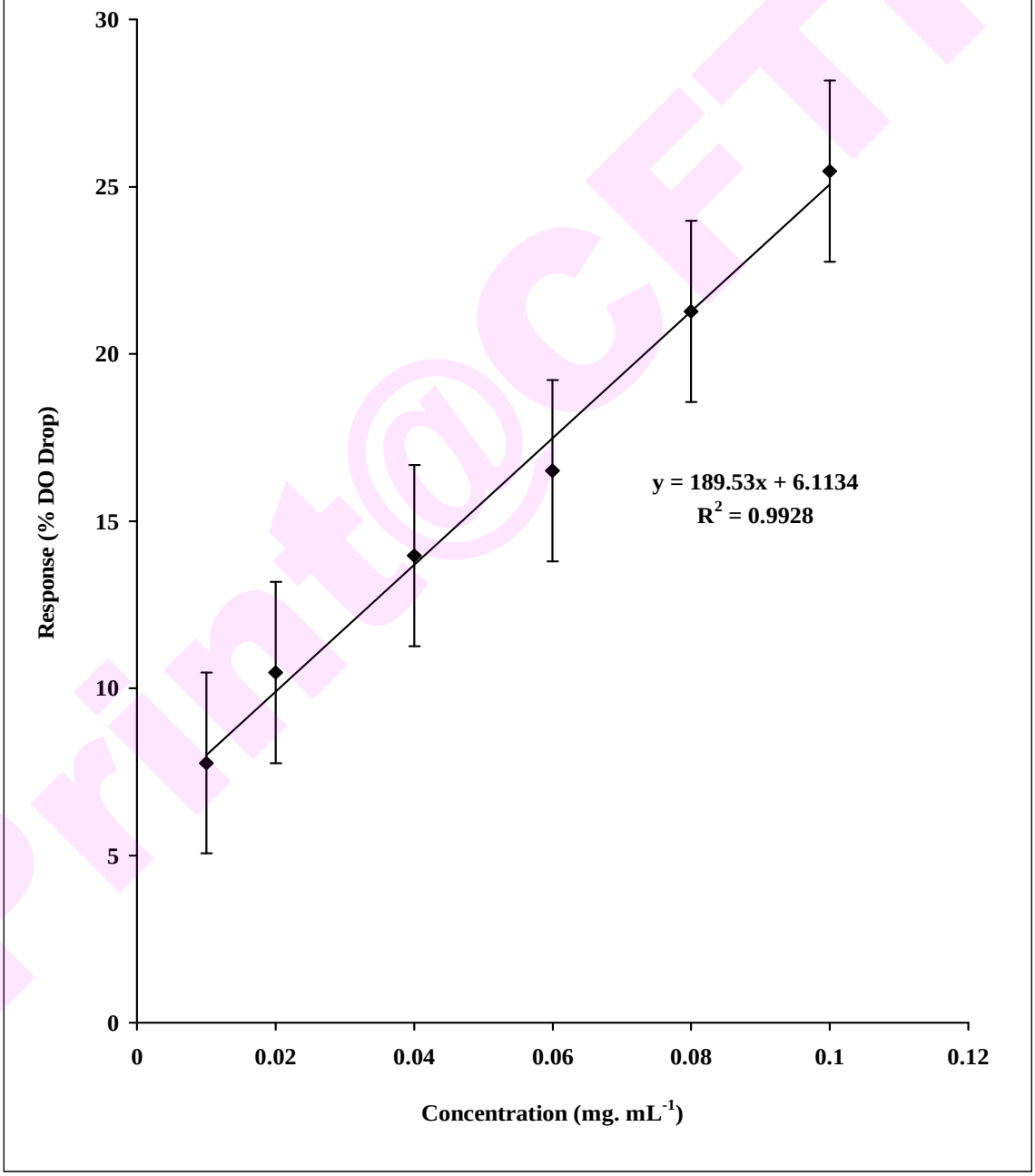
Figure 6.4.1.1. Response of the biosensor to 1% w/v of pure caffeine.



6.4.1.3. Calibration curve of caffeine using biosensor:

A calibration graph for caffeine in the concentration range of 0.01 to 0.1% w/v of caffeine was plotted with a regression value of 0.9928 (Fig. 6.4.1.2)

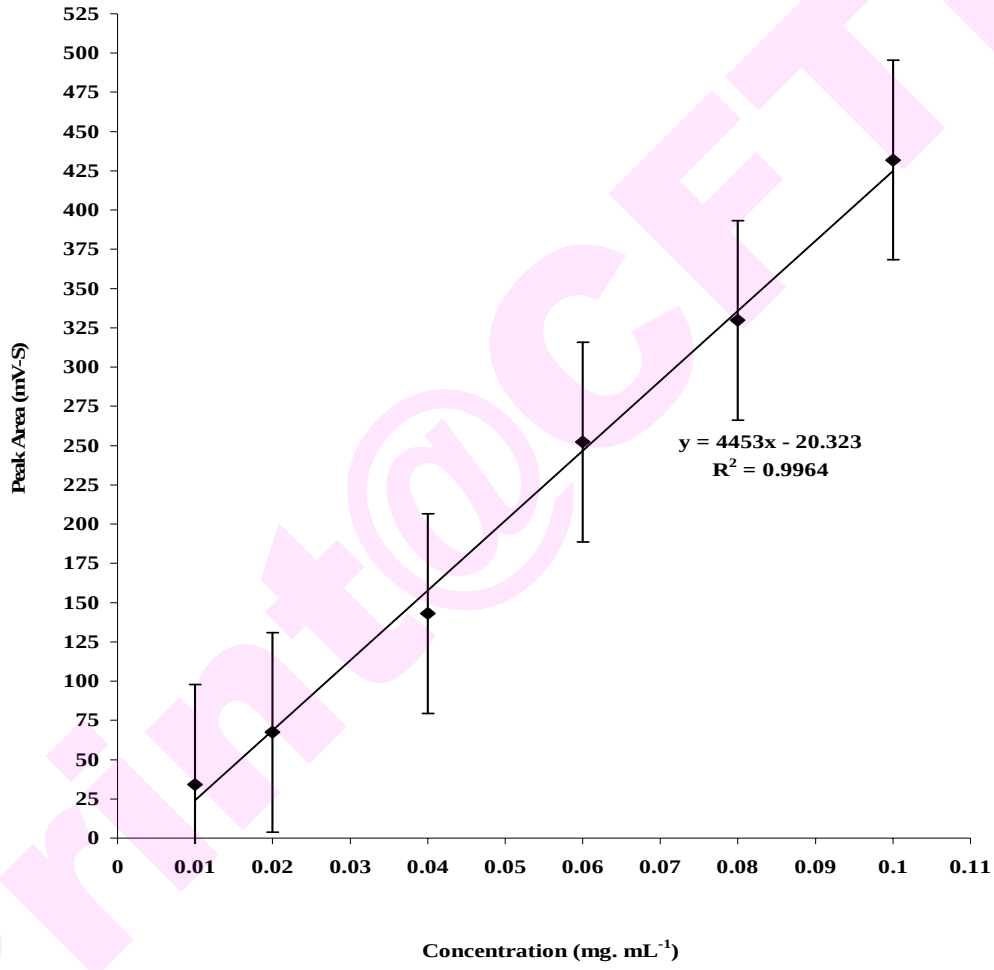
Figuire 6.4.1.2. Calibration of caffeine using immobilized cell based biosensor.



6.4.2. Calibration for caffeine using HPLC:

A linear calibration curve for caffeine was prepared in the range of 0.01- 0.1mg mL^{-1} with a regression value of 0.9964. (Fig. 6.4.2.1.)

Figure 6.4.2.1. Calibration for caffeine using HPLC.



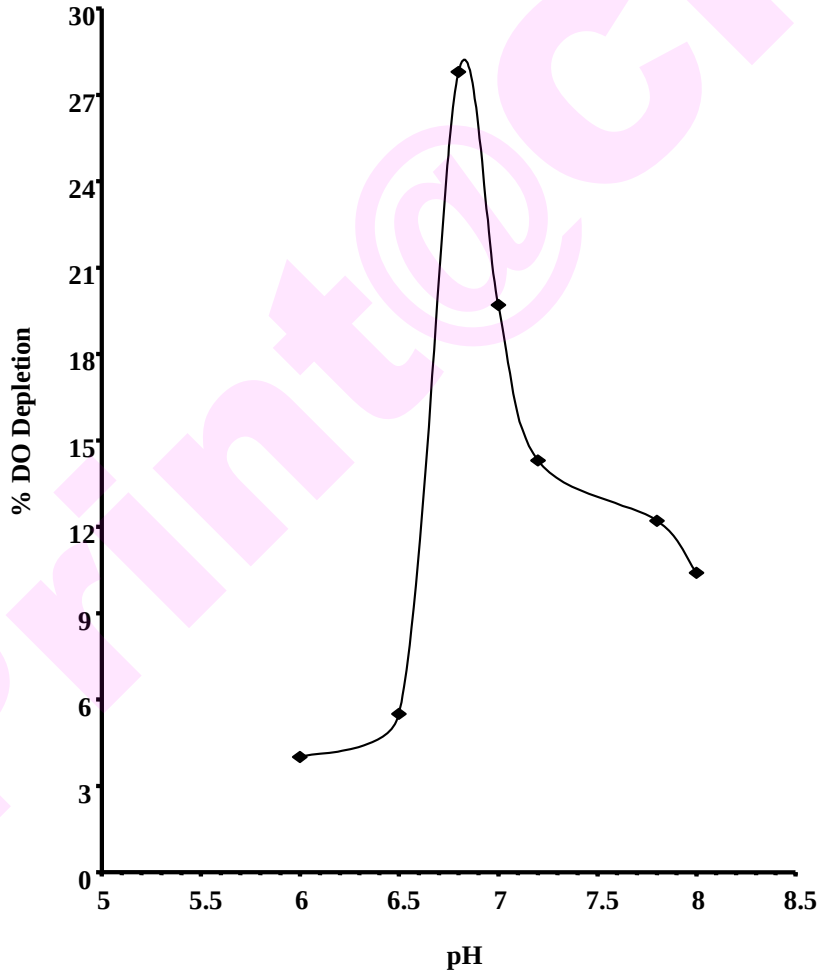
6.4.3. Optimization of parameters for the biosensor:

The following parameters were studied to develop the biosensor for caffeine estimation.

6.4.3.1. pH:

The immobilized cell had a maximum activity at pH 6.8 (Figure 6.4.3.1). Therefore, phosphate buffer (100 mM) at pH 6.8 was used for further studies on the biosensor for caffeine. Studies on the caffeine degradation by *P. alcaligenes* MTCC 5264 also showed a pH optimum of 6.8 for efficient caffeine degradation (Sarath et.al., 2005) and also the optimum pH for caffeine demethylase is 6.8. This explains the reason for the optimum operation of caffeine biosensor at pH of 6.8.

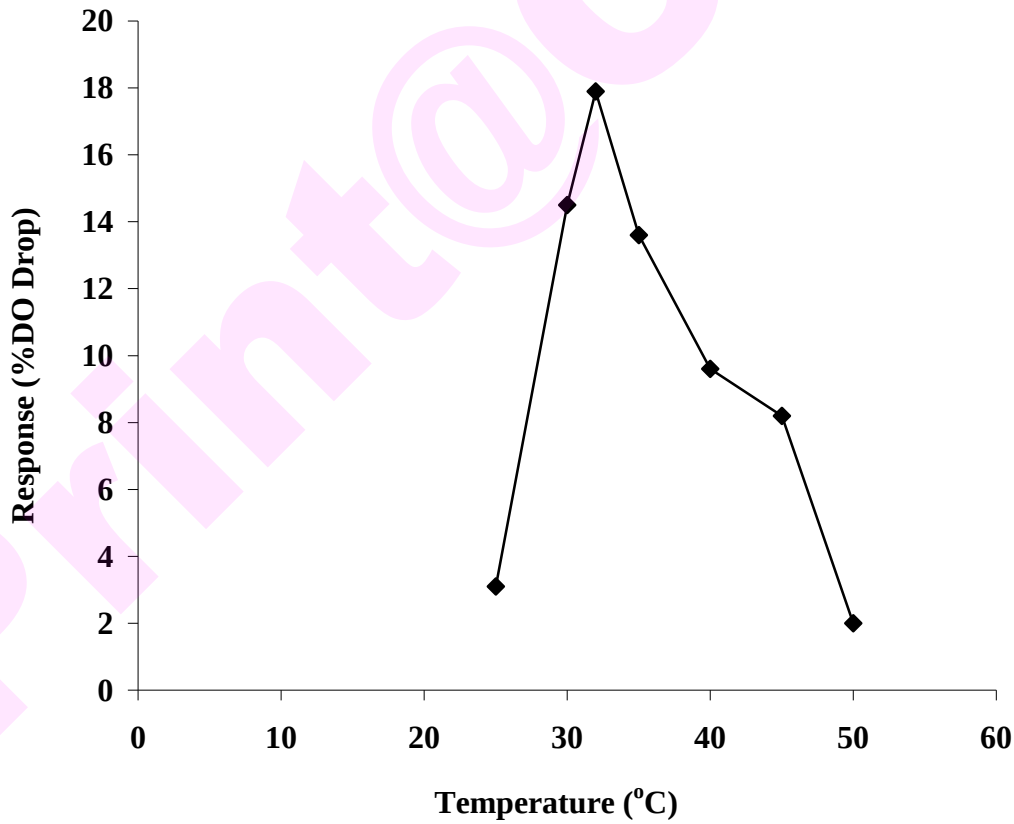
Figure 6.4.3.1. Effect of pH on response of biosensor to caffeine.



6.4.3.2. Temperature:

The optimum temperature for the caffeine response was found to be 32°C. The response of the immobilized cells based biosensor gradually increased upto a temperature of 35^{0}C, but above this temperature the activity gradually decreased and the cell membrane was inactivated at 50^{0}C (Fig. 6.4.3.2). *P. alcaligenes* MTCC 5264 is a mesophile and has a optimum temperature of degradation of caffeine at 30°C (Sarath et.al., 2005). The results on biosensor also are concurrent with those obtained in our earlier studies.

Figure 6.4.3.2.: Effect of temperature on the response of biosensor to caffeine



6.4.3.3. Interference of different compounds in caffeine estimation:

Glucose was found to interfere with the estimation of caffeine, whereas the other sugars sucrose and fructose did not show any interference (Table 6.4.3.1.). The response of the immobilized cells to glucose was considerably higher. So elimination of glucose in real samples is needed for the efficient estimation of caffeine. Introduction of an immobilized glucose oxidase membrane can be a good method for the elimination of interference due to glucose.

Table 6.4.3.1. Effect of interfering agents on caffeine biosensor response.

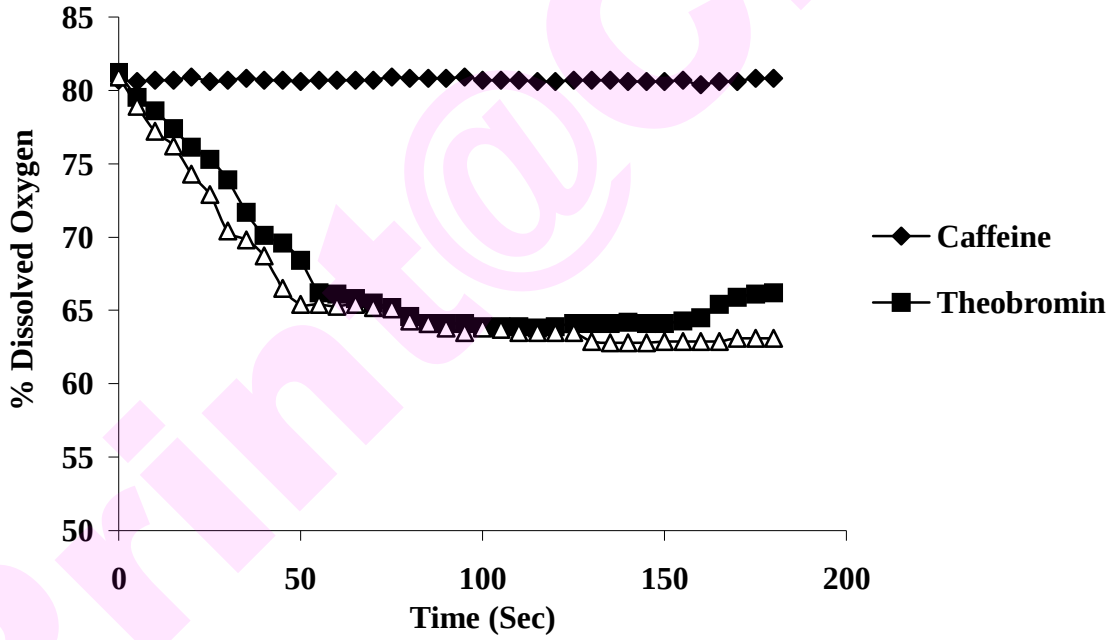
S.No	Interfering agent (1 % w/v)	Response (% DO drop)
1	Theophylline	2.00
2	Theobromine	3.50
3	Sucrose	4.00
4	Glucose	5.00
5	Fructose	0.20
6	Glycine	1.20
7	Tyrosine	0.60
8	Histidine	1.10
9	Ascorbic acid	1.50
10	Catechin	1.20

- **Response to 1.0 % w/v caffeine = 20.6**

6.4.3.4. Response of xanthine oxidase to caffeine:

Immobilized xanthine oxidase did not respond when caffeine was injected, but there was a notable response when theobromine and theophylline were injected (Figure 6.4.3.3). This proved that the degradation of caffeine was not brought about by xanthine oxidase as reported by (Ohe et.al., 1979) but another oxygenase enzyme, which is supposedly caffeine demethylase, is responsible for the degradation of caffeine.

Figure 6.4.3.3. Response of immobilized xanthine oxidase to caffeine, theobromine and theophylline



6.4.3.5. Analysis of caffeine in real samples by biosensor and by HPLC:

Analysis of caffeine contents of real samples was done by using HPLC and microbial biosensor and presented in Table 6.4.3.2.

The biosensor results were in correlation with HPLC results. The spectrophotometric results show a very high error % indicating matrix interferences. The samples need prior treatment for analysis by HPLC and Spectrophotometric methods. The error % was calculated taking HPLC as standard.

Table 6.4.3.2. Analysis of caffeine in real samples by biosensor and by HPLC.

Sample	Caffeine Concentration (% w/v)		%Error
	Biosensor	HPLC	
Instant Coffee	0.056	0.052	(+) 07.69
Commercial Coffee Sample 1	0.030	0.033	(-) 09.09
Commercial Coffee Sample 2	0.035	0.034	(+) 02.94
Commercial tea Sample 1	0.036	0.038	(-) 05.26
Commercial tea Sample 2	0.076	0.072	(+) 05.55
Cola Drink 1	0.042	0.047	(-) 10.63
Cola Drink 2	0.046	0.050	(-) 08.00

6.4. CONCLUSIONS:

An amperometry based microbial biosensor for the analysis of caffeine in beverages and fermentation samples was developed using immobilized whole cells of *P. alcaligenes* MTCC 5264. The biosensor was found to have maximum efficiency at a pH of 6.8 and a temperature of 32°C.

Sugars like glucose and sucrose were tested for interference in the results and were found to interfere in the analysis. An approach for the removal of these sugars prior to injection in the biosensor was tested by passing the sample through a packed column of immobilized glucose oxidase enzyme. Tests on the use of glucose uptake inhibitors were carried out and it was found that the inhibitors also inhibited the analysis of caffeine. Further work is to be carried out on increasing the selectivity of the immobilized system to caffeine.

6.5. PERSPECTIVES:

Caffeine biosensor has lot of potential applications in tea, coffee and other beverages along with pharmaceutical caffeine based products and in clinical applications for determining the health status of humans (as a marker for Cytochromoe P450 activity in Liver). The biosensor devised for the analysis of caffeine with a working range of 0.01% -0.1 % w/v of caffeine is found to have large number of commercial applications. Industries such as coffee, tea and soft drinks where the concentration of the caffeine is to be monitored for safety of human health and the level of caffeine is of critical importance. In view of these facts caffeine biosensor will have potential market world over.

This device helps overcome the problem of time consuming and tedious sample preparation and analytical procedures commonly involved in conventional analysis methods. The main advantage of this method is the fast response time, which enables the quick estimation of caffeine in real samples. However, the whole cell system may respond to substrates such as Theophylline, Theobromine and other methyl xanthines which usually are present in real samples. This tends to give rise to

some inaccuracy in the analysis of real samples. Although this effect was minimum in the present biosensor, this problem can be avoided by the use of pure caffeine demethylase enzyme for highly selective analysis of caffeine.

Future work would be carried out on the development of an enzyme based biosensor for the determination of caffeine. The proposed enzyme biosensor is anticipated to be free of interferences and enable more analyses in a shorter time.

6.6. REFERENCES:

1. Alpdogan, G., Karabina, K. Sungur, S. (2002), Derivative spectrophotometric determination of caffeine in some beverages. *Turk. J. Chem.* **26(2):** 295-302.
2. Asano, Y., Komeda, T., Yamada, H., (1993), Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem*. **57**: 1286–1289.
3. Blauch, J.L., Tarka J.R., Stanley, M., (1983), HPLC determination of caffeine and theobromine in coffee, tea and instant hot cocoa mixes. *J. Food Sci.* **48**: 745-748.
4. Blecher, R,, Lingens, F., (1977), Metabolism of caffeine by *Pseudomonas putida*. *Hoppe Seyler's Z Physiol Chem*. **358:**807–817.
5. Europaisches, A., *Coffeinum*, (1978) p 670: *Theophyllinum*, (1978) p 1213; Deutscher Apotheker Verlag Stuttgart.
6. Gouda, M.D., Thakur, M.S., Karanth, N.G., (1997), A dual enzyme amperometric biosensor for monitoring organophosphorous pesticides. *Biotechnol. Tech*. **11(9):** 653-655.
7. Gouda, M.D., Thakur, M.S., Karanth, N.G., (2001), Optimization of the multienzyme system for sucrose biosensor by response surface methodology. *World J. Microbiol. Biotech.* **.17(6):** 595-600'
8. Gouda, M.D., Thakur, M.S., Karanth, N.G., (2001), Stability studies on immobilized glucose oxidase Using an amperometric biosensor - Effect of protein based Stabilizing agents. *Electroanalysis***. 13(10):** 849-855.
9. Kalmar, J. M. and Cafarelli, E., (1999), Effects of **caffeine** on neuromuscular function. *J Appl Physiol.* **, 87:** 801-808**.**
10. Keusgen, M., (2002), Biosensors: new approaches in drug discovery. *Naturwissenschaften*, **89**: 433–444.
11. Kunugi A and Tabei K, (1997), Simultaneous determination of purine alkaloids in daily foods by high performance thin layer chromatography. *HCR J High Resol Chromatogr* **20:** 456-458.
12. Moriyasu, Saito, K., Nakazato,M., Ishikawa,F., Fujinuma,K., Nishima,T., Kunagi, A. Norton, K.L. and Griffith, P.R., (1995), simultaneous determination of caffeine, theobromine and theophylline in foods by HPLC. *J. Chromatog. A*, **703:** 503-522.
13. Ohe, T., Watanabe, Y., (1979), Purification and properties of xanthine dehydrogenase from *Streptomyces cyanogenus* . *J. Biochem (Tokyo***); 86:** 45-53.
14. Pizzariello, A., Svorc, J., Stredansky, M., Miertus, S., (1999) A biosensing method for detection of **caffeine** in coffee. *J. Sci. Food Agri.*, **79 (8):** 1136 – 1140.
15. Pons, F.W., and Muller, P., **(**1990), Induction of frameshift mutations by caffeine in *Escherichia coli* K12, *Mutagenesis*, **5:** 173-177**.**
16. Rico, C.M., Fenandez, M.D., Gutirrez, A.M., Golovela, L.A., Stein, H.J and Scheller, F., (1995), Development of a flow fluoroimmunosensor for determination of theophylline. *Analyst* , **120:** 2589-2591.

17. Ritchie, J.M.. (1975) The xanthines. *The pharmacological Basis of therapeutics*, 5th Edn. Mac Millan, New York. pp 367-368.
18. Riedel, K., M. Lehmann, K. Tag, R. Renneberg, and G. Kunze, (1998), *Arxula adeninivorans* based sensor for the estimation of BOD. *Anal. Lett.* **31:** 1-12.
19. Srisuphan, W., Bracken, M.B., (1986), Caffeine consumption during pregnancy and association with late spontaneous abortion. *Am J Obstet Gynecol* **154**:14–20.
20. Statheropoulos, M., Samargadi, E., Tzamtzis, N and Geogakopoulous, C., (1996), Principal component analysis for resolving coeluting substances in gas chromatography mass spectrometry doping control analysis. *Anal. Chim. Acta*, **331**: 53-61.
21. Sambrook, J., Fritsch, E .F. and Maniatis, T., (1989), Bacterial Cell Maintenance. In Molecular Cloning: A laboratory manual, Vol.1, Cold Spring Harbor laboratory Press, New York.
22. Thakur, M.S. and Karanth, N.G., (2003) Research and development of Biosensors for food analysis in India, in Advances in Biosensors, Vol. 5, Perspectives in Biosensors. Ed. Malhotra, B.D. and Turner, A.P.F, Elsevier science, Amsterdam, ISBN: 0-444-51337-X, 131-160.
23. Zhao, Y.P. and Lunte, C.E., (1997) Detemination of caffeine and its metabolites by micellar electrokinetic capillary electrophoresis. *J. Chromatog.B*, **628:** 265-274.

List of Publications:

Patents:

1. A process for the bio- decaffeination of solutions containing caffeine (428/Del/03, Columbia Patent applied, US 4171411).
2. A biosensor device for the determination of caffeine (Patent Applied).
3. A process for the production of caffeine demethylase enzyme for use in biodecaffeination and biotransformation of Caffeine to methyl xanthines. (Patent Applied).
4. An improved enzymatic process for the Decaffeination of caffeine containing liquid systems (Patent Applied).
5. A Process for the biodecaffeination of tea during fermentation (Patent Applied).
6. A Bioreactor Design for Enzymatic Decaffeination (Patent Applied).

Publications:

1) Sarath Babu, V.R., Patra, S., Karanth, N.G., Thakur, M.S., Varadaraj, M.C., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. *Enzyme and Microbial Technology*. **27**, 617-624).

2) Sarath Babu, V.R., Patra, S., Karanth, N.G., Thakur, M.S., Kumar, M.A., (2007), Development of a biosensor for Caffeine. *Analytica Chimica Acta.* **582(2):** 329-334.

3) Sarath Babu, V.R., Thakur, M.S., Enzymatic biodecaffeination of tea: Effects of different physicochemical parameters. (MS under preparation)

4) Sarath Babu, V.R., Thakur, M.S., Biodecaffeination of tea during fermentation: Technological challenges and opportunities. (MS under preparation)

5) Sarath Babu, V.R., Thakur, M.S., Studies on effect of physicochemical parameters on biodecaffeination by *Pseudomonas alcaligenes* MTCC 5264. (MS under preparation)

6) Sarath Babu, V.R., Thakur, M.S., Biodecaffeination of coffee beans: Development of an immobilized enzyme based bioreactor system. (MS under Preparation)

7) Sarath Babu, V.R., Thakur, M.S., Studies on Stabilization of caffeine demethylase. (MS under preparation).

8) Sarath Babu, V.R., Thakur, M.S., Studies on quality improvement of tea by external addition of enzymes. (MS under preparation).