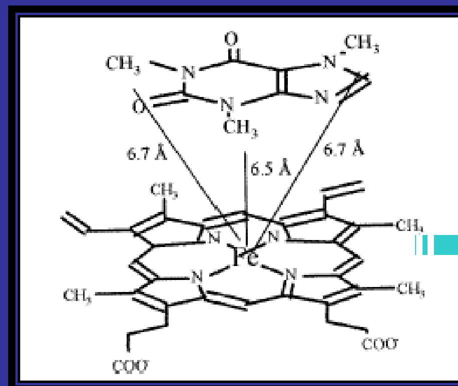
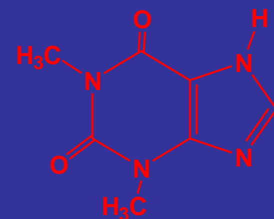
BIOTRANSFORMATION OF CAFFEINE TO VALUE ADDED PRODUCTS

A Thesis Submitted to the
University of Mysore for the award of the degree of





By

Sanjukta Patra, *M.Sc.*,

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

February 2007

BIOTRANSFORMATION OF CAFFEINE TO VALUE ADDED PRODUCTS

A thesis submitted to the

University of Mysore for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

By

SANJUKTA PATRA

Fermentation Technology and Bioengineering Department

Central Food Technological Research Institute

Mysore-570020, India.

February 2007

ACKNOWLEDGEMENT

I avail this opportunity to express my heartfelt thankfulness to my research guide Dr. M.S. Thakur. His inquisitive and visionary nature owes to the genesis of my research topic. His invaluable painstaking guidance, precious suggestions and critical assessment throughout my work were crucial in the completion of my work. His human values and goodness is a lesson to be practiced.

I am immensely grateful to the Director CFTRI, Dr. V. Prakash for allowing me to take up my Ph.D at CFTRI and utilize the facilities to carry out my research work.

My indebtedness to Council of Scientific and Industrial Research for direct financial assistance through CSIR fellowship.

My sincere gratitude to Dr. N.G. Karanth for his valuable guidance in improving my scientific writing skills and analytical thinking. Being associated with him was a privileged honour.

My deep sense of gratitude and indebtedness to Dr. K.N. Gurudutt – an unforgettable teacher for his imprinted lessons. His involvement and guidance, was instrumental in the completion of my work.

My thankfulness to Dr. Bhogesha and Dr. P.K Dash, Scientist at Central Sericulture Research Institute for their guidance and help during the course of my work.

The completion of my work would not have been possible without the help and guidance of Dr. S. Rajaratnam, and Dr. M.N. Shashirekha. The invaluable suggestions of Dr. S.G. Bhatt were crucial for the progress of my work in the right direction.

I would like to thank Dr. M.C. Misra, Dr. S. Divakar, Dr. S.G. Prapulla, Dr. A. P. Sattur, Mr. Eugene Raj, Dr. Yogesh, Mr. M.A. Kumar and Dr. Somashekhar for their support and help in carrying out my work. My heartfelt thankfulness to staff CIFS and all other departments of CFTRI who have rendered their help on professional and personal basis during the course of my work and stay at CFTRI, Mysore.

My sincere thanks to my friends Sujith, Abhijith, Raghuraj, Chidananda, Manash and Mamatha Veeresh with whom I shared my time and experiences during my stay at CFTRI. It was a pleasure to be with all of them. I would also like to thank my senior Naveen Babu for his support. My colleagues at FTBE, Praveen, Lohith, Vijay, Shivakumar, Somshekhar, Jolly, Mabel, Renuka, Ayyappan and Nandini have always been there with me. My sincere thanks to all of them. My thankfulness to Mr. Manjunath, project assistant PPSFT department for his help in carrying out my NMR work. Thanks to Ms. Mamatha for all the help during these years.

Thanks to my senior colleague Dr. Shabana, her presence and interaction was an experience in itself.

My heartfelt thankfulness to Mr. Somanna for rendering me all possible help.

The comforting presence of my senior Sarath, his involvement in all stages of my Ph.D work and completion of my thesis is a pleasure remembrance. I could not have travelled all this way in my work without him.

Thanks to my friend Bindu and little Sonu for all their love and support.

I could not have imagined my stay in Mysore without Ashwin who had been with me in all my crests and troughs on a constant basis. Thanks for understanding me the way I am, I could be 'myself' in his presence.

My thankfulness to Sarath and Pratap annayya for being with me during the most crucial stages of my life and all the moral support and strength.

I owe my immense thankfulness to all my family members. The aspiration of my younger sister, love and encouragement of my mother, patience of my father and all time support of my younger brother have helped me in culminating my work. The liberty given to me by my parents and the faith, which they have in me, deserves a special mention. Without which, I could never realize my dreams. The mortal and immortal presence of my grandmother has always strengthened me.

Thanks to the Almighty for everything I have been endowed with.

Sanjukta Patra

SANJUKTA PATRA,
Senior research Fellow,
Fermentation Technology and
Bioengineering Department,
Central Food Technological Research Institute,
Mysore-570020.

Date: 28-02-2007

DECLARATION

I hereby declare that the thesis entitled **"BIOTRANSFORMATION OF CAFFEINE TO VALUE ADDED PRODUCTS"** 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 2003 – 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.

(SANJUKTA PATRA)

List of Abbreviations:

Abbreviation	Full form
ADP	Adenosine Diphosphate
Al-P	Alkaline phosphatase
AMP	Adenosine Monophosphate
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
BCE	Bioconversion efficiency
BOD	Biological oxygen demand
cAMP	Cyclic Adenosine monophosphate
COD	Caffeine oxidase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CPR	Cytochrome P450 reductase
CEE	Crude Enzyme Extract
CO	Carbon Monoxide
DCPIP	Dichlorophenol indophenol
DNA	Deoxyribo nucleic acid
DNS	Dinitrosalicylic Acid
DMSO	Dimethyl sulfoxide
DTT	Dithio thretol
FTIR	Fourier transform Infra red spectroscopy
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
HXDM	Heteroxanthine demethylase
FAD	Flavin adenine dinucleotide
FMN	Flavin Adenine mononucleotide
FTC	2’, 3’-Dideoxy-5-fluoro-3’-thiacytidine
FTIR	Fourier transform infrared spectroscopy
GMP	guanosine monophosphate
HDAC	histone deacetylase
HDAC2	histone deacetylase-2
HIV	Human immunodeficiency Virus
HSV	Herpes simplex virus
kDa	Kilo dalton
L-DOPA	L-amino acid dihydroxy-L-phenylalanine
L-PAC	L- phenylacetylcarbinol
MEA	Malt extract agar
MWCO	Molecular weight cutoff
MTCC	Microbial type culture collection
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
NADH	Nicotinamide adenine dinucleotide (reduced)
PDA	Potato dextrose agar
PDEs	Phosphodiesterases
PEG	Polyethylene glycol
PMSF	Phenyl methyl sulfonyl fluoride
Ppm	part per million
PKA	phosphokinase
PDE	cAMP-phosphodiesterase
Px	paraxanthine
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
SDS PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
SEM	Scanning electron microscopy
SSF	Solid state fermentation
Tb	Theobromine
Tp	Theophylline
TEMED	NNN-bisacrylamide, tetramethylethylene

diamine

TLC Thin layer chromatography

TOC Total organic carbon

TGF-β Tissue growth factor β

TNF-α Tumor necrosis factor

UV Ultraviolet

VZV *Varicella zoster*

XDH= Xanthine dehydrogenase

XO= Xanthine oxidase

List of Schemes and Figures:

Figure no.	Title of Scheme and Figure	P. No.
Scheme 1.	Structure of purine.	31
Scheme 2.	Structure of xanthine.	31
Scheme 3.	Structure of caffeine.	33
Scheme 4.	Structure of caffeine and adenosine.	34
Scheme 5.	Biotransformation of caffeine in mammalian system.	35
Scheme 6.	Structure of theophylline.	37
Scheme 7.	Structure of theobromine.	40
Scheme 8.	Structure of paraxanthine.	42
Scheme 9.	Structure of 8-methoxymethyl-1-methyl-3- (2-methylpropyl) xanthine.	43
Scheme 10.	Structure of 1-methyl-3-isobutylxanthine.	43
Scheme 11.	Structure of acyclovir.	44
Scheme 12.	Structure of pentoxyfylline.	45
Figure 2.3.1a.	Caffeine biotransforming fungal cultures isolated from coffee and tea plantation soil.	73
Figure 2.3.1b.	Caffeine biotransforming fungal cultures isolated from coffee and tea plantation soil.	73
Figure 2.3.2.	Caffeine degradation by various isolates grown in screening media.	76
Figure 2.3.3.	Biotransformation efficiency of the fungal isolates from caffeine to theophylline.	77
Figure 2.3.5.1.	Pure culture of *P. citrinum* MTCC 5215 growing on potato dextrose agar showing yellow pigmentation at the reverse of colony.	80
Figure. 2.3.5.2.	*P. citrinum* MTCC 5215 growing on potato dextrose agar containing 0.03% caffeine showing green colony colour and yellow exudates.	81
Figure 2.3.5.3.	Light microscopic photograph of *P. citrinum* MTCC 5215 stained with cotton blue showing branched, vegetative and septate hyphae.	81
Figure 2.3.5.4.	Dark field micrograph of *P. citrinum* MTCC 5215 showing conidiophore arising directly from the substratum.	82
Figure. 2.3.5.5.	Scanning electron micrograph of *P. citrinum* MTCC 5215.	82
Scheme: 3.1.	Structure of caffeine showing positions of methyl group.	90
Figure 3.3.2.1a.	FTIR of biotransformed theophylline.	111
Figure 3.3.2.1b.	NMR of biotransformed theophylline.	112

Figure 3.3.2.2a.	FTIR of biotransformed 1-methylxanthine.	113
Figure 3.3.2.2b.	NMR of biotransformed 1 methylxanthine.	114
Figure 3.3.2.3a.	FTIR of biotransformed xanthine.	115
Figure 3.3.2.3b.	NMR of biotransformed xanthine.	116
Figure 3.3.2.4a.	FTIR of biotransformed uric acid.	117
Figure 3.3.2.4b.	NMR of biotransformed uric acid.	118
Figure 3.3.2.5a.	FTIR of biotransformed allantoin.	119
Figure 3.3.2.5b.	NMR of biotransformed allantoin.	120
Figure 3.3.2.6a.	FTIR of biotransformed allantoic acid.	121
Figure 3.3.2.6b.	NMR of biotransformed allantoic acid.	122
Figure 3.3.2.7.	FTIR of biotransformed urea.	123
Scheme: 3.3.6.	7-*N* demethylation in *P.citrinum* mediated by cytochrome P450.	128
Figure 3.3.6.1.	Heme group of cytochrome P450 showing the position of caffeine in the active site in human cytochrome P450 system.	129
Figure 3.3.6.2.	CO absorption spectra of cytochrome P450 isolated from *P. citrinum* MTCC 5215.	129
Figure 3.3.6.3.	Gel electrophoresis of enzyme extract from induced and uninduced biomass to determine the inducible nature of cytochrome P450.	131
Figure 3.3.6.4.	Purification of Cyt P450 by gel permeation chromatography.	132
Figure 3.3.6.5.	HPLC chromatogram showing biotransformation of caffeine to theophylline in reconstituted enzyme system.	133
Scheme: .3.7.1.	Biotransformation of theophylline to 1 methyl xanthine by heteroxanthine demethylase in *P. citrinum*.	135
Scheme: 3.3.7.2.	Biotransformation of 1 methyl xanthine to xanthine by xanthine dehydrogenase in *P. citrinum*.	136
Scheme: 3.3.7.3.	Biotransformation of xanthine to uric acid by xanthine oxidase in *P. citrinum*.	137
Scheme: 3.3.7.4.	Biotransformation of uric acid to allantoin by uricase in *P. citrinum*.	138
Scheme: 3.3.7.5.	Biotransformation of allantoin to allantoic acidby allantoinase in *P. citrinum*.	139
Scheme: 3.3.7.6.	Biotransformation of allantoic acid to urea by allantoicase in *P. citrinum* MTCC 5215.	140
Scheme: 3.3.7.7.	Biotransformation of urea to carbon dioxide and ammonia by urease in *P. citrinum*. MTCC 5215.	141

Figure 3.3.8.	Caffeine biotransformation pathway in *P. citrinum* MTCC 5215.	146
Figure 4.2.3.12.	Production of theophylline in 10L laboratory fermentor.	172
Figure 4.2.3.2a.	Effect of carbon source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	179
Figure 4.2.3.2b.	Effect of glucose concentration on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	180
Figure 4.3.1.3.	Effect of corn steep liquor on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	182
Figure 4.3.1.4a.	Effect of caffeine concentration on biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	184
Figure 4.3.1.4b.	Effect of caffeine concentration on growth of *P. citrinum* MTCC 5215.	185
Figure 4.3.1.5.	Effect of pH on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	186
Figure 4.3.1.6a.	Effect of temperature on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	188
Figure 4.3.1.6b.	Effect of temperature on growth of *P. citrinum* MTCC 5215.	188
Figure 4.3.1.7.	Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	189
Figure 4.3.1.8.	Effect of inoculum load on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	190
Figure 4.3.1.9.	Effect of concentration of $FeSO_4$ on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	192
Figure 4.3.1.10.	Effect of allopurinol concentration on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	193
Figure 4.3.1.11.	Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimized condition in shake flask.	196
Figure 4.3.1.12.	Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimized condition in 10L fermentor.	197
Figure 4.3.2.1a.	Response curve for biotransformation of caffeine to theophylline at 120hour.	202
Figure 4.3.2.1b.	Response curve for biotransformation of caffeine to theophylline at 72 hour.	202
Figure 4.3.2.1c.	Response curve for biotransformation of caffeine to theophylline at 96hour.	203
Figure 4.3.2.1d.	Response curve for biotransformation of caffeine to	203

	theophylline at 144hour.	
Figure 4.3.2.2a.	Response curve for caffeine degradation at 144 hour.	207
Figure 4.3.2.2b.	Response curve for caffeine degradation at 72 hour.	208
Figure 4.3.2.2c.	Response curve for caffeine degradation at 96 hour.	208
Figure 4.3.2.2d.	Response curve for caffeine degradation at 120 hour.	209
Figure 4.3.2.3.	Response curve for maximum biomass accumulation	212
Figure 4.3.2.4a.	Crude extract and purified theophylline.	215
Figure 4.3.2.4b.	FTIR spectra of standard theophylline and purified biotransformed theophylline.	216
Figure 5.1.1.1.	Section of coffee fruit showing various anatomical sections.	225
Figure 5.1.1.2.	Disposed coffee pulp after coffee processing by wet method at a coffee-processing unit in Mercara.	227
Figure 5.1.1.3.	Coffee pulp and coffee hull by generated by dry method of coffee processing disposed at coffee processing unit in Mysore.	228
Figure 5.3.1.	Caffeine extraction profile using soxhlet method.	257
Figure: 5.3.4.1.	*P. florida* growing on coffee pulp.	269
Figure: 5.3.4.2.	*P. florida* growing on coffee hull.	269
Figure. 5.3.4.3.	*P. florida* growing on spent coffee.	270

Table No.	Title of Table	P. No.
1.1.6.	Fungal cytochrome P450 with known gene sequences.	15
1.1.7.1.	Biotransformation reactions in human/animal systems – an over view.	21
1.1.9.3.	Biotransformation for value addition to food products.	28
1.2.1.	Applications of methyl xanthines.	33
2.3.2.	Source of isolation and caffeine biotransformation ability of the fungal isolates.	75
2.3.4.	Identification of caffeine biotransforming isolates.	78
2.3.5.	Characterization of the isolate capable of transforming caffeine to theophylline.	79
3.1.5.	Caffeine biotransformation in different living systems.	97
3.3.1.	Biotransformed products formed by *P. citrinum* MTCC 5215.	110
3.3.5.	Isolation of microsomes by different methods.	125
3.3.6.	Biotransformation of caffeine to theophylline by incubating with microsomes.	125
3.3.7.1.	HXDM activity in crude enzyme extract of *P. citrinum* MTCC 5215.	135
3.3.7.2.	XDH activity in crude enzyme extract of *P. citrinum* MTCC 5215.	136
3.3.7.3.	XO activity in crude enzyme extract of *P. citrinum* MTCC 5215.	138
3.3.7.4.	Uricase activity in crude enzyme extract of *P. citrinum* MTCC 5215.	139
3.3.7.5.	Allantoinase activity in crude enzyme extract of *P. citrinum* MTCC 5215.	140
3.3.7.6	Allantoicase activity in crude enzyme extract of *P. citrinum* MTCC 5215.	141
3.3.7.7.	Urease activity in crude enzyme extract of *P. citrinum* MTCC 5215.	142
4.2.3.1.	Media Screening of for biotransformation of caffeine to theophyllineby *P. citrinum* MTCC 5215.	164
4.2.4.	Coding and assigned concentrations of variables of different levels of the central composite rotatible design.	174

4.3.1.1	Biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 in screening media.	177
4.3.1.3.	Effect of nitrogen source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	182
4.3.1.5.	Optimization of pH for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	186
4.3.1.11.	Comparison of growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 before and after optimization of conditions in shake flask.	195
4.3.1.12.	Comparison between shake flask and fermentor studies for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimized conditions.	197
4.3.2.1a.	Experimental design and results of the central composite rotatible design.	199
4.3.2.1b	Analysis of variance for the experimental results of the CCRD for biotransformation of caffeine to theophylline.	200
4.3.2.1c.	Regression coefficient of variables for the experimental results of the CCRD for biotransformation of caffeine to theophylline.	200
4.3.2.1d.	Optimized medium composition for biotransformation of caffeine to theophylline.	201
4.3.2.1e.	Validation of experimental results for biotransformation of caffeine to theophylline.	201
4.3.2.2a.	Analysis of variance (ANOVA) for the experimental results of the CCRD for maximum caffeine degradation in *P. citrinum* MTCC 5215.	205
4.3.2.2b.	Regression coefficient of variables for the experimental results of the CCRD for maximum caffeine degradation in *P. citrinum* MTCC 5215.	206
4.3.2.2c.	Optimized medium composition for maximum caffeine degradation in *P. citrinum* MTCC 5215.	206
4.3.2.2d.	Validation of experimental results for maximum caffeine degradation in *P. citrinum* MTCC 5215.	206
4.3.2.3a.	Analysis of variance (ANOVA) for the experimental results of CCRD for maximum biomass accumulation.	210
4.3.2.3b.	Regression coefficient of variables for the experimental results of CCRD for maximum biomass accumulation.	211
4.3.2.3c.	Optimized medium composition for maximum biomass accumulation.	211
4.3.2.3d.	Validation of experimental results for maximum biomass accumulation.	211
4.3.2.4a.	Choice of extraction of solvent for downstream processing of theophylline.	214

4.3.2.4b.	Purification of theophylline from fermented broth.	215
5.1.3.1.	Composition of coffee pulp.	235
5.1.7.1.	Advantages of vermicompost over chemical fertilizer.	241
5.1.7.2.	Advantages of vermicomposting over general composting.	242
5.1.7.3.	Nutrient profile of vermicompost and farm yard manure.	242
5.3.1.	Extraction of caffeine using sodium carbonate and bicarbonate.	256
5.3.2.	Decaffeination of coffee processing wastes by *P. citrinum* MTCC 5215.	259
5.3.3.	Production of enzymes by the fungal isolates in SSF using coffee pulp and wheat bran as substrates.	263
5.3.4.1.	Production of mushroom using coffee processing wastes.	267
5.3.4.2.	Chemical analysis of mushroom produced.	268
5.3.4.3.	Caffeine concentration in substrates and mushroom fruiting bodies.	268
5.3.5.1.	Production of vermicompost using coffee processing wastes.	275
5.3.5.2.	Population profile during vermicompost using coffee processing wastes.	275

BIOTRANSFORMATION OF CAFFEINE TO VALUE ADDED PRODUCTS

Synopsis submitted to the

University of Mysore for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

By

SANJUKTA PATRA

Department of Fermentation Technology and Bioengineering

Central Food Technological Research Institute

Mysore-570020, India.

February 2007

Title: BIOTRANSFORMATION OF CAFFEINE TO VALUE ADDED PRODUCTS

Synopsis

Caffeine is a naturally occurring molecule belonging to the xanthine alkaloid family and is present in tea, coffee over 60 other plant species. It is also available as a by-product of decaffeination process, coffee and tea processing wastes and by chemical synthesis. The world production of coffee is 6.795 million tons and the world decaffeinated coffee market is about 15% of the total coffee market. About 2 lakh tons of caffeine is generated per year from decaffeination of tea and coffee. Besides this, lots of caffeine is available from unutilized coffee processing wastes such as coffee pulp and hull. Every ton of coffee, when processed, generates 40% processing wastes in the form of coffee pulp and coffee hull. These processing wastes contain caffeine ranging from 1.1-2.2%, which can be extracted efficiently and used as substrates for useful biotransformation where caffeine is converted into potent therapeutic molecules leading to value addition. Biotransformation of caffeine and bioconversion of the wastes to safe products is an effective solution to the above problems. Caffeine extracted from these wastes can be biotransformed by using suitable caffeine biotransforming microbial cultures to valuable methyl xanthines such as theophylline, theobromine and paraxanthine, which are potent therapeutic molecules. Production of theophylline by biotransformation of caffeine will obviate the disadvantages of chemically synthesized theophylline and lead to utilization of abundantly available caffeine and value addition.

The processing wastes of caffeine such as coffee pulp and coffee hull are considered as major agro-industrial wastes, as they are associated with several anti-physiological and inhibitory factors like high content of caffeine, tannins and polyphenols. Efficient methods of utilization or disposal of these wastes are still not available and are therefore disposed by the processing units and left unused, causing pollution of the nearby water bodies and soil. Effective utilization of these processing wastes is required not only to save the environment, but also utilize it for the benefit of the producers, which would generate revenues in the lean season and generate extra employment. Further, microbial cultures can also be used for the decaffeination of the

processing wastes and their efficient utilization. The removal or minimization of the anti nutritional factors in these processing wastes makes them suitable to have alternative applications in animal feed, organic fertilizer, as a substrate in solid state fermentation, biogas production, edible mushroom production and vermiculture.

Work reported in the thesis involves the isolation, screening and characterization of caffeine utilizing fungal cultures. The most potent fungal culture, which could biotransform caffeine to theophylline, was characterized as *Penicillium citrinum* and used for the production of theophylline. The caffeine biotransformation pathway of *P. citrinum* was elucidated and the enzymes responsible for biotransformation of caffeine were identified. Optimization of parameters for caffeine biotransformation by the selected microorganism was done. Work was also carried out towards the efficient utilization of coffee processing wastes and spent coffee.

Proposed objectives of the thesis:

1. Isolation, screening and characterization of microbial cultures for biotransformation.
2. Identification and characterization of biotransformed products.
3. Studies on the enzymes involved in the biotransformation of caffeine.
4. Optimization of parameters for growth and caffeine biotransformation by selected microorganism.
5. Utilization of coffee processing wastes and spent coffee.

Thesis organization:

The thesis entitled "Biotransformation of caffeine to value added products" consists of five chapters.

Chapter-1:

The first section of chapter 1 is a review of literature on biotransformation. It presents an overview of general biotransformation, its significance, the major developmental stages, advantages and applications of the process in various industrial sectors with a brief account on a few commercial successes. Enzymes are the key

elements of biotransformation and their role in the development of industrially successful processes have been discussed. Besides this, major biotransforming systems such as human/animal system, plant and microbial systems have also been discussed in this section. The second section of this chapter brings out the importance of various methyl xanthine molecules, their availability in nature, the disadvantages of chemical method of synthesis and the need for the production of methyl xanthines from caffeine by green route.

Chapter-2:

Chapter 2 is on screening of caffeine biotransforming fungi. The first section of this chapter focuses on the survey of various groups of caffeine biotransforming microbial cultures. Further, the work carried out on the isolation of potent fungal cultures capable for biotransforming caffeine is discussed. 34 caffeine biotransforming fungal cultures were isolated which were further screened for their biotransformation efficiency by growing them in liquid culture media containing caffeine. The most potent caffeine biotransforming strain was identified and characterized as *P. citrinum* MTCC 5215. The biotransformation product from caffeine by this strain was identified as theophylline.

Chapter-3:

The third chapter focuses on biotransformation of caffeine by *P. citrinum* MTCC 5215. The first section of the chapter consists of information on biotransformation of caffeine in various biological systems such as human, fungi, yeast, and bacteria. The second section of this chapter deals with the identification of all the biotransformation products and their respective enzymes involved in biotransformation of caffeine and elucidation of the biotransformation pathway of caffeine in *P. citrinum* MTCC 5215. This chapter also details the studies on the identification of cytochrome P450, involved in the biotransformation of caffeine to theophylline. This is the first conclusive report confirming the enzyme involved in the biotransfromation of caffeine to theophylline in fungi. The other enzymes involved in the caffeine degradation pathway were identified as heteroxanthine demethylase, xanthine dehydrogenase, xanthine oxidase, uricase, allantoinase, allantoicase, and urease and the details are described in the third section of

this thesis. Products formed during the biotransformation of caffeine were isolated, purified and identified by techniques such as thin layer chromatography, high performance liquid chromatography, fourier transformation infrared spectroscopy and nuclear magnetic resonance spectroscopy. A comprehensive study of the biotransformed products and their respective enzymes led to the elucidation of the complete pathway of caffeine biotransformation in *P. citrinum* MTCC 5215. The chapter ends with conclusions and future perspectives in the development of a bioprocess for the production of theophylline from caffeine through biotransformation.

Chapter-4:

Chapter 4 deals with studies on the optimization of parameters for production of theophylline. The first section of the chapter discusses the therapeutic importance of theophylline. The crucial parameters which affect biotransformation of caffeine to theophylline have been discussed. The second section of the chapter is on optimization of physico-chemical parameters for efficient biotransformation of caffeine to theophylline. The results of the experiments are described in the third section of the chapter. The results obtained show that glucose was the best carbon source, corn steep liquor the best nitrogen source, $FeSO_4$ when incorporated into the media enhances the process of biotransformation and allopurinol inhibits the further degradation of the biotransformed theophylline. The physical parameters, which were optimized, were pH 5.6 and temperature 28°C. Additional studies were also carried out using response surface methodology for optimizing the media conditions for theophylline production using higher concentration of caffeine, which will help towards process development and scale up studies. Study has been carried out for the downstream processing of the biotransformed theophylline. The biotransformed theophylline was isolated and purified and purity of the biotransformed theophylline was confirmed by various analytical techniques such as mixed melting point analysis, HPLC, FTIR and NMR. The chapter ends with recommendations on the development of processes for theophylline production at a larger scale.

Chapter-5:

Chapter 5 is on the utilization of caffeine containing coffee processing wastes. The first section discusses about the various coffee processing wastes and the several limitations, which renders them, unfit for any use. The various measures, which can be taken, to remove these inhibitory factors and their further use has been detailed. The second section of this chapter deals with various approaches for the efficient utilization of coffee processing wastes namely coffee pulp, coffee hull and spent coffee.

The third section of the chapter details the results obtained in this study. Coffee pulp and hull was used for the extraction of caffeine and used for biotransformation to produce theophylline. Biodecaffeination of coffee pulp and coffee hull was carried out using *P. citrinum* MTCC 5215. Complete decaffeination of coffee pulp was observed in a period of 72 hours, whereas coffee hull could not be decaffeinated. Coffee pulp was also used for the production of various commercially important enzymes such as amylase, amyloglucosidase, protease, caffeine oxidase and pectinase by solid-state fermentation. Coffee pulp proved to be a better substrate for the production of enzymes such as caffeine oxidase, protease and pectinase compared with wheat bran as substrate. Coffee pulp, coffee hull and spent coffee were used successfully for mushroom production. Coffee pulp showed 99% bioconversion efficiency (BCE) as compared to rice straw which showed 80% BCE. Successful production of mushroom was possible on coffee hull for the first time with spawn run of 10 days. Besides this the mushroom produced on coffee hull showed highest protein content of 25.83% w/w. Coffee pulp and spent coffee were also used for vermicomposting using *Eisenia foetida.* The vermicompost produced using coffee pulp and spent coffee were analyzed and found to have high NPK content which has been discussed in detail in this chapter.

Conclusions and future recommendation:

The work reported in this thesis resulted in the isolation of a potent fungal culture, for the biotransformation of caffeine to theophylline, which was identified as *P. citrinum* MTCC 5215. The enzyme for biotransformation of caffeine to theophylline was identified as cytochrome P450. The pathway for the biotransformation of caffeine by this strain was elucidated; all the biotransformed products were isolated, purified and

identified. A medium was designed with optimum physical and chemical parameters for efficient biotransformation of caffeine to theophylline. Isolation and purification of theophylline from the fermented broth was carried out. In the last chapter of the thesis, efficient utilization of coffee processing wastes such as coffee pulp, coffee hull and spent coffee have been carried out successfully, for extraction of caffeine and utilizing it for biotransformation, for mushroom cultivation, vermiculture and for enzyme production in SSF.

This work has further prospects in the development of a process for commercial production of theophylline and it's utilization as a therapeutic compound. Further studies on purification of cytochrome P450, cloning and hyper expression of the enzyme, and its molecular characterization is recommended.

Dr. M.S. Thakur **Sanjukta Patra**
Guide.

CHAPTER -1

REVIEW OF LITERATURE

1.0. Scope of the review:

Biotransformation is gaining prominence in the changing world scenario of transforming the chemical industries from conventional chemical catalysis towards applications of biocatalysts for achieving better yields through environmentally safe and simpler bioprocesses.

The first part of this chapter presents an overview of general biotransformation, its significance, the major developmental stages, advantages and applications of the process in various industrial sectors with a brief account on a few commercial successes. Involvement of enzymes in biotransformation and the role of different classes of enzymes in the development of industrially successful biotransformation processes have been discussed in general. Besides this major biotransforming systems such as human, animal, plant and microbial systems have also been described in brief. The role of biotransformation in bioremediation of environmental pollutants has also been brought about in the thesis. Second part of this chapter focused the therapeutic importance of various methyl xanthine molecules including caffeine, their availability in nature, the disadvantages of chemical synthesis methods and the need for the production of methyl xanthines by biotransformation of caffeine. The last part of the chapter deals with conclusions and possibilities of development of processes for biotransformation of caffeine to methyl xanthines of therapeutic interest keeping in view the availability of caffeine as a by-product of decaffeination process, coffee and tea processing wastes and by chemical methods of synthesis.

1.1. Introduction:

The conversion of a compound from one form to another by biological system is known as biotransformation. It is the molecular or atomic structure alteration of a compound due to enzymatic reactions. They are organic reactions utilizing biological catalysts, which may be whole cells or enzymes and may be used as free or immobilized, in aqueous or two phase systems or as cross linked enzyme crystals. Enzymes as biocatalysts can be used for regio-and stereoselective reactions and to introduce chirality in ways that would be very difficult or impossible for classical synthetic processes. A series of biotransformations make a biochemical pathway. Hence, either a single biotransformation or few biotransformation steps in continuation leads to formation of product/s of interest, detoxification or metabolism of the compound by the living system. Biotransformation products can be intermediate metabolites or terminal products of a process in the organisms.

Biotransformation is a growing field of biotechnology, which encompasses both enzymatic and microbial biocatalysis. Sufficient progress has been made in research, on the key drivers of biotransformations, including isolation, screening and characterization of microbes and their enzymes, their utilization in extreme environments, the manipulation, alteration, augmentation of metabolic pathways and the use of combinational biosynthesis and biocatalyst methodologies for the development of new compounds. The convergence of biology and chemistry has enabled a plethora of industrial opportunities to be targeted, while discoveries in biodiversity and the impact of molecular biology and computational science are

extending the range of natural and engineered biocatalysts that can be customized for clean industrial processes.

Biotransformation has the potential to produce existing products more cost-effectively by efficient utilization of raw materials, lower investment costs, lower energy demand, and lower disposal costs due to less hazardous emissions. It can also provide a basis for completely new products and system/s solutions and has the potential to replace classical chemical production processes, for instance in the sectors of fine chemicals, bulk chemicals, and energy sources. Some of the important products through biotransformation are ethanol, glutamic acid, antibiotics, vitamins, acrylamide and iso-glucose syrup. Several studies estimate the share of biotransformation processes in the production of various chemical products to be around 5 %, by 2010 however, they postulate that this figure will soar to about 20 % (Pacl *et al*, 2004). Alone for the chemical industry, the potential value addition of biotransformation product is predicted to reach € 11–22 billion worldwide annually by 2010 (Pacl *et al*, 2004).

In the present study, emphasis will be given to microbial biotransformation and some transformations will be dealt in short to understand the wider prospectives of biotransformation and how they have been instrumental in the production of various compounds with ease, which were not possible by other routes. The advantages of the biotransformation methods because of which they are gaining prominence in the field of synthesis have also been discussed. The classification of biotechnological generated compounds as “natural” has increased the attention to biotransformation systems.

1.1.1. History of biotransformation:

Biotransformation processes have been used by mankind for several thousand years. For example, the biotransformation of ethanol to acetic acid (vinegar) by *Acetobacter* was developed concomitantly with ethanol production from fermentable sugars in Babylon (Mesopotamia), Egypt, Mexico, and Sudan as early as 5000 B.C. It was the first reported biotransformation process applied on industrial scale. Biotransformations were observed by humans long before they were appreciated as having an underlying microbial cause. In 1858, Louis Pasteur provided evidence for the role of specific microorganisms conducting favorable fermentations of fruit juice (Pasteur, 1858). The properties of enzymes, the principle biocatalysts, became generally appreciated from kinetic studies conducted in the early 1900s (Michaelis and Menten, 1913). An important industrial-scale fermentation to produce acetone to meet the wartime needs of Great Britain was developed in 1916 (Glazer and Nikaido, 1995). Conversion of glucose from starchy materials into high fructose syrup, which has enhanced sweetening properties, by glucose isomerase is an example of a biotransformation operated at the giant scale of 15 million tonnes per year and has been in use since 1974.

1.1.2. Industry sectors involving biotransformation:

Biotransformation reactions are ubiquitous in nature and the industrial sectors which have exploited the ability of biotransformations to commercially and economically successful technologies are:

- Pharmaceuticals
- Fine and Bulk Chemicals
- Food
- Cosmetics

Production of antibiotics, insulin, vitamins, steroids, prostaglandins, and alcohol are some of the commercially successful biotransformation reactions.

1.1.2.1. Pharmaceuticals:

Exciting and innovative developments in biotechnology have opened a plethora of opportunities for the pharmaceutical industries. More than 20 percent of new medicines launched in 2003 were produced using biotechnology, and nearly 80 percent of those under development either use biotechnology or are derived from it (Frost and Sullivan, 2003).

1.1.2.2. Bulk products and polymers:

'Bulk products' represent products exceeding production of 10,000 tons annually. According to a study by McKinsey & Company, biotransformation will significantly affect bulk products and polymers. It is expected that, by the year 2010, 6-12 % of bulk products and polymers produced by chemical means will be replaced by biotransformation processes (Alchema, 2006).

1.1.2.3. Fine and specialty chemicals:

The term 'fine chemical products' refers to substances that are highly functional and for which world demand is typically below 10,000 tons per year. The current world market of biotransformation processes in this area is estimated to be US-$ 50 billion, the potential volume within the next 10-20 years will be around US-$ 250 billion. A study by McKinsey & Company (2003) anticipates that 30-60 % of all fine chemicals produced will involve biocatalytic steps by 2010 (Alchema, 2006).

1.1.2.4. Food:

Value addition to food products has gained vital importance in our country due to diversity in socio-economic conditions, industrial growth, urbanization and globalization. It is not merely to satisfy producers and processors by way of higher monitory return but also with better taste and nutrition. Value is added by changing their form, colour and other such methods to increase the shelf life of perishables. Hence, there is a great scope to extend requisite technical know how to agro based industrial units for the production of value added food products. Today, the world's neutraceutical market is more than $100 million. Health foods contribute another $250 million (Singh *et al*, 2004). Development of biotransformed flavour, color, low calorie sweetener etc. are some of the demanding projects in the food sector, besides the already existing traditional ones (Longo and Sanroman, 2006; Gatfield, 1988).

1.1.2.5. Cosmetics:

Increasing consumer concerns about health have triggered growing demand for cosmeceutical products. Cosmeceuticals now represent up to 50 per cent of supplement sales in some countries. The category is estimated to be worth €3.5 billion globally and is one of the biggest areas of innovation (Lonza, 2004). In order to meet consumer needs and to capitalize on this expanding market, the ingredients used must be chosen with safety, efficacy and quality in mind. Biotransformation can quench all the above worries.

Biotransformation has been made to produce not only new ingredients, but also improved processes to make ingredients previously produced by chemical

synthesis or extraction from natural sources. Production of aromatic oil, tea tree oil, anti oxidative compounds, allantoin are some of the examples.

1.1.3. Potential benefits of biotransformations:

Biotransformation processes are beneficial over other methods of synthesis and are listed below.

- Environment friendly (Green technology)
- Improved reaction efficiencies
- Improved enantioselectivities
- Process economy
- Improved total conversions
- Reduced by-products
- Cleaner effluent streams
- Shorter manufacturing routes
- Control/selection of required enantiomers by asymmetric route development
- Chiral intermediates
- Resolution of racemic mixtures
- Eliminate need for extreme conditions as heavy metals, high temperature, high pressure and extreme pH
- Neutral reaction conditions
- Natural processing

1.1.4. Systems of Biotransformation:

Enzymes, the key elements of biotransformation can be used in various forms depending on the requirement. A few biotransformation systems are described below.

1. Free cells
2. Free enzyme
3. Immobilized cells
4. Immobilized enzymes
5. Cross linked enzyme systems
6. Tissue culture
7. Biotransformation in organic phase

1.1.4.1. Biotransformations by free cells:

a) Taxol: Taxol is used in the treatment of various cancers including breast cancer. Cells of *Taxus brevifolia* have been used economically for the production of taxol (Harshad and Heble, 2003). Production of taxol by free cell culture technique is of great commercial value owing to the enormous market potential of taxol, the scarcity of the *Taxus* tree, and the costly synthetic process (Cragg *et al*, 1993).

b) Biotransformation of citronellol by whole cells of *Rhodotorula minuta* is one more commercial success in the field of biotransformation. Citronellal (3,7-Dimethyl-6-octanal), a monoterpene which occurs in the L or D form, bears distinct odor characteristics and also occurs as a constituent of essential oils in *Eucalyptus citriodora* (Betts, 2000). Citronellal can be further hydrogenated to produce citronellol (3, 7-dimethyl-6-octanol), which is a commercially important product due to the peculiar rose like characteristics odor of the product (Guenther, 1950).

c) Biotransformation of caffeine to theophylline by free cells of *Penicillium citrinum* MTCC 5215will be discussed in the subsequent chapters of this thesis.

1.1.4.2. Biotransformation by immobilized cells:

a) Cells of *Candida utilis* immobilized in calcium alginate have been used for the production of L- phenylacetylcarbinol (L-PAC) from benzaldehyde (Shin and Rogers, 1995), which is precursor of ephedrine. L-ephedrine is widely used in pharmaœutical preparations as a decongestant and anti-asthmatic compound. It can be produced by biotransformation of benzaldehyde using various yeasts.

b) Immobilized cells of *Papaver somniferum* has been used for the biotransformation of codeinone to codeine and the conversion yield was 70.4% and about 88% of codeine produced was secreted into the medium (Furuya, 1984).

1.1.4.3. Immobilized enzymes:

a) Immobilization is carried out to increase concentration, stability and reusability of enzymes. It is significantly advantageous over free enzymes when the biocatalyst is of increased operational (or storage) stability, desired activity per unit volume, and easily recovered to facilitate biocatalyst recycle, continuous process development, simplification of the work-up procedure and overall process cost improvements. Different carriers (inorganic and organic from natural or synthetic materials) can support enzymes through processes of adsorption, covalent binding, entrapment or cross linking (Buchholz, 2005; Kallenberg, 2005; Lalonde and Margolin, 2002).

b) Immobilization of cholesterol esterase to prepare 2', 3'-Dideoxy-5-fluoro-3'-thiacytidine (-)-FTC: 2', 3'-Dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC, (-)-1] which is the active ingredient in the antiviral drug Emtriva (Liotta and Choi, 1991; Liotta, 1992; Painter, 2000). Precipitation of cholesterol esterase onto solid support was done using acetone, followed by cross-linking with glutaraldehyde (0.25 percent v/v). This was then used for the continuous production of (-)-FTC allowing up to 15 times reusability leading to an economic process development.

1.1.4.4. Cross-linked enzyme crystals:

Cross-linked enzyme crystals (CLECs) are a novel form of immobilized biocatalyst designed for application in large-scale biotransformation processes. They

are found to be more resistant to harsh environmental conditions, such as extremes of temperature and pH and the presence of solvents or proteases, than the free enzymes. Cross-linking of the crystals with glutaraldehyde also yields mechanically robust catalysts that can withstand various forces associated with shear in agitated vessels and particle compression in repeated dead-end filtration cycles. The technique is also applicable to the preparation of combination - CLECs, containing two or more enzymes, for use in one-reaction vessel, for multi step continuous reaction. For example, an oxynitrilase/nitrilase combi-CLEC can be used for the synthesis of (*S*)-mandelic acid from benzaldehyde in high yield and enantiomeric purity (Sheldon *et al*, 2005; Roy and Abraham, 2006).

1.1.4.5. Tissue culture:

a) Digitoxin: Biotransformation is carried out in the form of tissue culture where the reaction requirement is for a particular differentiated tissue. Plant tissue culture for production of cardiac glycoside digitoxin, a cardiotonic has been used by Hildebrandt and Riker (1953). *Digitalis lanata* and *D. purpurea* are the two species commonly used for the production of digitoxin with good biotransformation efficiency.

b) Vinblastine and Vincristine: Biotransformation using plant tissue culture has been used successfully as an alternative route by Misawa *et al* (1988) for the efficient production of anticancer drugs, vinblastine and vincristine. They are dimeric indole alkaloids and have become highly valued drugs in cancer chemotherapy due to their potent antitumor activity against various leukemias, Hodgkin's disease and solid tumors. They are currently produced commercially by extraction from *Catharanthus*

roseus (Apocyanaceae) plants, but the process is not efficient because of very low concentrations of the alkaloids in the plant.

1.1.4.6. Biotransformation in organic phase:

Biotransformation in organic phase is a solution for those biotransformation reactions where there is a problem of (*i*) instability and low solubility of substrates and/or products in water and (*ii*) substrate and/or product inhibition. They are instrumental for reactions involving reactants of low aqueous solubility, such as steroid compounds (Buckland et al., 1975; Fukai and Tanaka, 1981). The main advantages of an aqueous/organic two-phase biotransformation system over a single aqueous phase system are that, the organic phase serves as reservoir for reactants that are soluble to a very small extent in the aqueous phase and that the differing solubilities of the reactant and the product in the aqueous or organic phase may be exploited to achieve product separation. The presence of an organic phase offers certain other advantages, including a reduced incidence of microbial contamination.

a) Steroid biotransformation: Organic phase biotransformation using octane as solvent for the ll-β hydroxylation of the steroid precursor Reichstein's substance S (cortexolone) to hydrocortisone by *Curvularia lunata* cells is a model system for organic phase biotransformation (Harish and Gina, 1994).

b) Terpenoid synthesis: Terpenoid biotransformation is one of the examples of organic phase biotransformation where cells of *Rhodococcus erythropolis* can biotransform turpenes such as limonene and pinene into their oxygenated derivatives (terpenoids) such as limonene 1,2 diol, which are widely used as flavours and fragrances (Carla *et al*, 2000).

Biotransformation in organic media using immobilized enzyme and whole cells of *Mycobacterium* sp. have been used successfully for the production of active sterols by sitosterol side chain cleavage. The increase in the volumetric productivity of the reaction system and the shift of the reaction equilibrium in favour of product synthesis leads to high product yield as there is reduction of substrate/product inhibition (Cabral *et al*, 1997).

1.1.5. Characteristic features of biotransformation:

Biotransformations have been found to be advantageous over chemical reactions because of the following characteristic features:

1) Regiospecificity: The substrate molecule is usually attacked at a particular site, even if several groups or equivalents are present. For example: In the second step of commercially synthesized ascorbic acid, D-sorbitol is converted to L-sorbose by highly selective dehydrogenation by *Acetobactor soboxydans.* Among the six hydroxyl groups of D-sorbitol, microbial oxidation takes place exclusively at position 2, producing L-sorbose as the only product with higher yields.

2) Reaction specificity: The catalytic activity is usually restricted to a single reaction type. In other words side reactions are not expected as long as one enzyme is involved in a particular biotransformation.

3) Stereo specificity: Since the reactive centre of an enzyme provides an asymmetrical environment, it can easily make differentiation between enantiomers of a racemic mixture. Therefore, only one or preferentially one of the enantiomers is attacked. Similarly, if an enzyme reaction produces a new asymmetric centre, usually only one of the possible enantiomers is formed resulting into optically pure compound.

4) Functionalization at non-activated carbon: Biotransformations can selectively introduce functional groups at certain non-activated positions in a molecule, which cannot be attacked by chemical reagents. For example, cortisone acetate was prepared by this method**.**

5) Mild reaction conditions: Activation energy of chemical reaction is distinctly lowered by interaction of substrate and enzyme and thus biotransformation takes place under mild conditions such as neutral pH, room temperature and normal pressure. Even a labile compound may be converted into product using low energy consumption, without undesired decomposition or isomerisation**.**

1.1.6. Enzymes - the key elements of biotransformation:

Enzymes are the key elements for biocatalysis in all types of Biotransformations. They catalyze an enormous number of reactions that are necessary for the synthesis, modification and degradation of the organic molecules that make up living organisms, or that are produced by these organisms for their existence, protection and communication. Metabolic pathways and cell growth entail highly diverse reactions such as the formation of carbon–carbon, peptide, or ester bonds, saturation/unsaturation of carbon–carbon bonds, and oxidation. The corresponding enzymes have been subdivided in six different classes according to their catalytic properties:

1) Oxidoreductases - oxidation/reduction
2) Transferases - transfer of functional groups
3) Hydrolases – hydrolysis reactions
4) Lyases - addition/elimination of small molecules
5) Isomerases – isomerization reactions
6) Ligases - formation/cleavage reactions

1.1.6.1. Oxidoreductases:

Cytochrome P450 monooxygenase system, flavin-containing monooxygenase system, alcohol dehydrogenase, aldehyde dehydrogenase, monoamine oxidase, peroxidases and NADPH-cytochrome P450 reductase are some of the important enzymes involved in biotransformation of oxidative nature. They are all cofactor dependent enzymes. The reducing or oxidizing equivalent is either supplied or taken by the cofactor. The most commonly needed cofactors are $NADH/NAD^+$, $NADPH/NADP^+$, $FADH/FAD^+$, ATP/ADP (Chenault and Whitesides, 1987; Wong and Whitesides, 1994).

Fungal cytochrome P450s are considered to be responsible for the catalysis of several interesting biotransformations. They are categorized from 51 to 64 (Nebert *et al*, 1987). Table 1.1.6. shows an overview of all fungal cytochrome P450s categorized on the basis of their amino acid sequence. Prospects of application of fungal cytochrome P450s exists in the production of pharmaceuticals and fine chemicals by biotransformation and in bioremediation. They are considered to be interesting enzymes to be used as biotransforming catalysts, as some of them are capable of introducing a hydroxyl group regio- and stereospecifically into their respective substrate (Deutsch *et al*, 1978; Kreiner *et al*, 1996; Breslow *et al*, 1997; Boucher *et al*, 1994; Trager, 1989). Progesterone 11 α-hydroxylase from *Rhizopous nigricans*, a filamentous fungus, has been used in the production of glucocorticosteroids for two decades (Hanisch *et al,* 1980; Aharonowitz and Cohen, 1981). A hydroxyl group is introduced at carbon 11 of progesterone stereospecifically, shortens the chemical synthesis of these steroids from 37 to 11 steps. The application of cytochrome P450s

is hampered for several reasons. They have, in general, low specific activites resulting in low production rates of the compounds of interest. Besides this, these enzymes can only be used in whole cell systems, as the enzymes are relatively labile outside the cell, need electron donating enzymes, and need NADPH for the reaction, which is expensive. The use of whole cell suspension solves these problems but is disadvantageous as the metabolization of the product of interest may occur, the mycelia may be depleted of reducing equivalents and contaminating substances may be excreted into the medium, resulting in more difficult downstream processing. These problems can be overcome by introducing multiple copies of the cytochrome P450 and NADPH cytochrome P450 reductase into the organism, which results in better specific activities (Truan *et al*, 1993; Panzhou *et al*, 1998). Another way of improving the specific activity is by the covalent attachment of the reductase to the cytochrome P450 (Hara *et al*, 1999).

Table 1.1.6. Fungal cytochrome P450 with known gene sequences.

Family	Substrate	Product	Organism	Remarks	Reference
CYP51	lanosterol/ ebirucol	14-demethyl lanosterol	Probably in all Fungi and yeast	Target for antifungal drugs	Vandenb-ossche and Koymans, 1998
CYP52	Alkane(s)	1-OH-alkane	*C. maltose* *C. tropicalis*	Inducible by substrate	Schunck *et al*,1989; Sanglard, and Loper, 1989
CYP53A1 CYP53B1	Benzoate and Derivatives Benzoate/ isovalerate	4-OH-benzoate 4-OH-benzoate/ Isobutene+ CO_2	*A. niger* *R. minuta*	Inducible by benzoate and p-aminobenzoate Inducible by phenyl-alanine and benzoate	Vaan *et al*, 1990; Fukuda, 1993

CYP54	n.i	n.i	*N. crassa*	Inducible by cycloheximide	Attar, 1990
CYP55	NO	N_2O	*F. oxysporum* *C. tonkinese*	Soluble; no O_2 used;	Toritsuka *et al,* 1997; Shoun *et al,* 1989
CYP56	Tyrosine	Dityrosine	*S. cerevisiae*	Product is a cell wall component	Briza et al,1989; Briza *et al*, 1994
CYP57	Pisatin	14α-demethyl pisatin	*N. haematococca (F. oxysporum)*	Pisatin is a phytoalexin	Weltring *et al*, 1988; Maloney and VanEtten, 1994
CYP58	n.i	n.i	*F.sporotrichiodes*	Trichothecene metabolism	Hohn *et al*, 1995
CYP59	n.i	n.i	*A. nidulans*	Involved in aflatoxin biosynthesis	Brown *et al*, 1996
CYP60	Averantin	Averufin	*A. parasiticus*	Involved in aflatoxin biosynthesis	Yu *et al*, 1997
CYP61	Lanosterol	Lanosterol-22-desaturase	*S. cerevisiae*	Ergosterol biosynthesis	Kelly *et al,* 1997
CYP62	n.i	n.i	*A. nidulans*	Involved in aflatoxin biosynthesis	Brown *et al,* 1996
CYP63	n.i	n.i	*P. chrysogenum*	unknown	Brown *et al*, 1996
CYP64	O-methyl-sterigmato-cystin	Aflatoxin(s)	*A. parasiticus*	Involved in aflatoxin biosynthesis	Yu *et al*, 1998

*** n.i. = not - identified**

Cytochrome P450 monooxygenase system plays a vital role in the metabolism of methyl xanthines, particularly caffeine in humans, animals and fungi. Caffeine metabolism is carried out by cytochrome P450 monooxygenase system along with

NADPH-cytochrome P450 reductase in animal systems. The role of this particular enzyme in caffeine metabolism and the subsequent product formation will be discussed in detail in the following chapters.

1.1.6.2. Transferases:

Of all biological reactions this class of biocatalysts is one of the most common enzymes (Ager, 1999). Glutathione S-transferases, UDP-glucoron(os)-yltransferases, N-acetyltransferases are some of the important enzymes involved in biotransformation reactions.

Transketolase is an example of transferse enzyme and catalyses the transfer of a two-carbon ketol unit from xylulose 5-phosphate to an aldehyde acceptor such as erythrose 4-phosphate, producing a new C-C bond and chiral centre with high enantioselectivity. An alternative ketol donor, beta-hydroxypyruvate, can be used to ensure that the reaction is irreversible, producing CO_2 as a byproduct, and hence cost effective at large scale.

1.1.6.3. Hydrolases:

Of the various enzyme classes, hydrolases, which catalyze the cleavage or synthesis of ester and amide-bonds, have found the widest range of applications. It is estimated that approximately 80% of all industrial biotransformation enzymes are members of the hydrolase class (Wrotnowski, 1997). A large number of biotransformation processes use hydrolytic reactions, including lipases, proteases, esterases, nitrilases, etc. An interesting group of hydrolases, the hydantoinases, can be used to produce chiral amino acids and their derivatives, as chiral starting materials in the production of pharmaceuticals such as antibiotics. The reactions are

stereoselective, giving D- or L-products (Burton *et al*, 1998). These compounds can be efficiently synthesized by the biocatalytic conversion of substituted hydantoins, in reactions catalyzed by microbial isolated enzymes or resting cells, in solution or immobilized in bioreactors.

Hydrolases are used in laundry detergents (lipases, proteases) and in the food industry (amylases, proteases, lipases, esterases), and also as biocatalysts for the synthesis of chemicals. Hydrolases have found major uses because it is relatively easy to develop practical applications for these enzymes. They can be formulated as immobilized cell preparations or as partially purified enzyme preparations. In addition, as these enzymes catalyze slightly exergonic equilibrium reactions, they do not require cofactors or complex reaction conditions. Given sufficient enzyme stability, the application of hydrolases *in vitro* is straightforward. Additionally, the direction of the reaction can be reversed by using appropriate reaction conditions as product removal, low water concentration, etc.

1.1.6.4. Lysases:

Lysases catalyse reactions involving C-C, C-O, and C-N bonds. In industrial processes these enzymes are most commonly used for synthesis. The addition of a molecule to an unsaturated substrate is of interest in synthetic processes. For example; phenylalanine ammonia lyase catalyses the formation of an asymmetric C-N bond yielding the L-amino acid dihydroxy-L-phenylalanine (L-DOPA). The product is applied for the treatment of Parkinsonism, which is caused by a lack of L-dopamine and its receptors in the brain (Tsuchida *et al*, 1993; Yamada, 1998).

1.1.6.5. Isomerases:

Depending on the type of isomerism, isomerases may be called as epimerases, racemases, cis-trans-isomerases, tautomerases or mutases. Isomerases are easy to utilize, but have found few applications, the most important of which is the production of high-fructose corn syrup from glucose using glucose isomerase. Isomerases are also used to racemize unwanted stereoisomers in amino acid resolutions.

1.1.6.6. Ligases:

Ligases join two molecules with covalent bonds as C-O, C-S and C-N bonds. These biocatalysts play a major role in genetic diagnostics. Specific enzymes called DNA ligases catalyse the formation of C-O bonds in DNA synthesis.

1.1.7. Categorization of biotransformation:

The tools of biotransformations are principally mammalian, plant or microbial cells. Hence, biotransformation can be categorized into three different classes depending on their occurrence in a particular living system. They are:

a) Biotransformation in animal system/human system
b) Biotransformation in plants
c) Biotransformation in microbes

1.1.7.1. Biotransformation in human/animal system:

Biotransformation in human body plays a central role in the elimination of drugs and other foreign compounds (xenobiotics) from the body. Most organic compounds entering the body are relatively lipid-soluble (lipophilic). Once in the bloodstream, these molecules can diffuse passively through other membranes to reach various target organs to effect their pharmacological actions. But when they are

needed to be excreted out of the body, they need to be converted to water-soluble products. The formation of water-soluble metabolite not only eliminates drugs, but also leads to compounds that are generally pharmacologically inactive and non-toxic.

a) Biotransformation of xenobiotics in human/animal systems:

Biotransformations of xenobiotics are crucial reactions for the detoxification mechanism of the body. It involves the Phase I or functionalisation reaction where active functional groups are generated on the drug moiety. The Phase II or conjugation reaction involves the addition of small molecules or functional group to make the drug water soluble in nature so that it can be extracted out easily. Table 1.1.7.1., describes the phase I and phase II reactions, the types of reaction covered under each class and the enzymes involved in these reactions.

Table 1.1.7.1. Biotransformation reactions in human/animal systems – an over view.

S.No	Reaction type	Nature of reaction	Enzymes involved	Reference
1.	Phase I or functionalization reactions a) Oxidative reactions	1) Oxidation of aromatic moieties 2) Oxidation of olefins. 3) Oxidation at benzylic, allylic carbon atoms and carbon atoms α to carbonyl and imines. 4) Oxidation at aliphatic and alicyclic carbon atoms. 5) Oxidation involving carbon heteroatom. 6) Carbon-nitrogen systems (aliphatic and aromatic amines; includes N-dealkylation, oxidative	Cytochrome P450 monooxygenase system, Flavin-containing monooxygenase system, Alcohol dehydrogenase and Aldehyde dehydrogenase, Monoamine oxidase, co-oxidation by Peroxidases	Rendic and Dicarlo, 1997

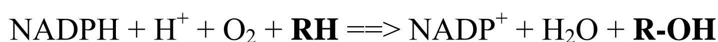
		deamination, N-oxide formation, N-hydroxylation). 7) Carbon-Oxygen systems (O-dealkylation). 8) Carbon-sulfur systems (S-dealkylation, S-oxidation and desulfuration). 9) Oxidation of alcohols and aldehydes.		
	b) Reductive reactions	1) Reduction of aldehydes and ketones. 2) Reduction of nitro and azo compounds.	NADPH-cytochrome P450 reductase, reduced (ferrous) cytochrome P450.	Rendic and Dicarlo, 1997
	c) Hydrolytic reactions	1) Hydrolysis of esters and amides. 2) Hydration of epoxides and arene oxides by epoxide hydrase.	Esterases and amidases, epoxide hydrolase	Rendic and Dicarlo, 1997
2.	Phase II or conjugation reactions	1) Glucoronic acid conjugation. 2) Sulfate conjugation. 3) Conjugation with glycine, glutamine and other amino acids. 4) Organic phase biotransformation using solvents as octane 5) Acetylation. 6) Methylation.	Glutathione S-transferases , mercapturic acid biosynthesis, UDP-glucoron(os)yltransferases, N-acetyltransferases, amino acid N-acyl transferases, sulfotransferases	Rendic and Dicarlo, 1997

b) Enzymes involved in biotransformation in human/animal systems:

Cytochrome P450 family is known as the body's detoxifying enzymes. They are a large group of monooxygenase enzymes responsible for the metabolism of drugs/toxins and require NADPH and O_2 for their activity (Garrett and Grisham, 1995). They also require NADPH-cytochrome P450 reductase (CPR) as the electron

donor protein. Over 60 key forms of this enzyme are known, with hundreds of genetic variations possible, producing a wide variety of susceptibility to specific drugs and toxins. These enzymes are located in the endoplasmic reticulum and are highly concentrated in the liver and small intestine. Additionally, P450 enzymes are also found in the mitochondrial membrane (Modi *et al*, 1995). P450s encompass a highly diverse "superfamily" of hemoproteins and one of their most relevant functions is that of metabolizing drugs in humans.

A typical cytochrome P450 catalysed reaction is:



NADPH + H^+ + O_2 + **RH** ==> $NADP^+$ + H_2O + **R-OH**

Where **RH** is the substrate and **R-OH** is the product.

1.1.8. Biotransformation in plants:

Many important pharmaceuticals are produced as secondary metabolites by the plants. Secondary metabolites are used as food, flavours, colour, dyes, perfumes, scented oils in aromatherapy, as industrial products such as rubber and oils. To large extent secondary metabolites in plants determine the host range of herbivorous insects. As caffeine produced in *Coffea arabica* helps the plant in protecting itself from insect attack (Frischknecht *et al*, 1986).

Commercially important plant biotransformation products:

Taxol (plaxitaxol), a complex diterpene alkaloid found in the bark of the *Taxus* tree, is one of the most promising anticancer agents known due to its unique mode of action on the micro tubular cell system (Jordon and Wilson, 1995, Cragg *et al*, 1993). The dimeric indole alkaloids, vincristine and vinblastine produced in *Catharanthus roseus* are valuable drugs in cancer chemotherapy due to their potent

antitumor activity against various leukemias and solid tumors. Some other therauptic compounds of plant biotransformation origin are alkaloid compounds such as hyoscyamine, atropine, cocaine, codeine, morphine and terpenoids like azadirachtin, artemisinin and plant steroids like saponin. Glycosides as nojirimycin and glucosinolates, phenols as resveratrol, phenazines as pyocyanin and phenazine-1-carboxylic acid, polyketides as erythromycin and discodermolide, fatty acid synthase products as phoroglucinol, nonribosomal peptides as vancomycin, thiostrepton, gramicidin, bacitracin, ribosomal peptides as microcin-J25.

1.1.9. Biotransformation in microbes:

Microbial transformations refer to reactions catalyzed by microbial enzymes, leading to specific and useful metabolite accumulation in the reaction media. Micro-organisms have a unique ability to produce a wide variety of different natural products, many of them with valuable biological activities (e.g. antibiotics, anti-virals and anti-fungals). Many enzymes involved in the biosynthesis of these metabolites catalyze reactions that are difficult to perform using conventional synthetic approaches in the laboratory. As such, they constitute a class of catalysts with great potential for the various biotransformations and remain the preferred method for biotransformations as they offer a broad field of inexhaustible possibilities for the future. Microbes can act as biocatalysts to carry out complex sequences of reactions very specifically which offer cost-effectiveness over the costly chemical transformation alternatives. Micro-organisms can be used to catalyze chemical reactions which otherwise are very difficult to occur and 11-hydroxylaion in steroids is an example. Microbes can be manipulated to overproduce the products by

eliminating feedback inhibition and repression mechanisms as well as inducing secretion of product. Microbial enzymes are highly versatile in nature, more adoptogenic than higher cells and are thus widely exploited as they decompose or metabolize the substrate at a faster rate. Enzymes are available for catalyzing various reactions leading to desired modifications/conversion in the structure of a compound. Success depends on skillful selection of microorganism for particular conversion. Many different groups of microorganisms like bacteria, yeast, fungi, algae etc. have been used efficiently for various purposes such as:

1) Metabolite or product formation.
2) Bioremediation
3) Biotransformation for value addition to food products.

1.1.9.1. Metabolite or product formation:

Microorganisms produce various metabolites/molecules during their life cycle, which can be categorized as primary and secondary metabolites. These metabolites/molecules are not only important to the microorganisms but they can be used beneficially for various human requirements also. The microbial milieu can be manipulated to make the microorganism over-produce its metabolites, which can be further used by humans. Some examples of important microbial biotransformation are as follows:

Pharmaceutical compounds:

Microbially biotransformed medicines – both natural compounds and their semi-synthetic derivatives are being used in almost everything from anticancer drugs, antibiotics to anticholesterolemics and immunosuppressants. Production of such compounds is eco-friendly, high yielding and economical. Microbial products have a

long history in medicinal treatment, dating back to the discovery of penicillin in the 1920s.

1) Theophylline:

Pharmaceutically important compounds such as theophylline can be produced by biotransformation using microbial cultures as *P. citrinum* MTCC 5215 which will be discussed in detail in the following chapters.

2) Antibiotics:

Most of the antibiotics such as penicillin, tetracycline, streptomycin, aureomycin ciprofloxacin, cephalosporin are produced by microbial fermentation. Penicillin is produced by microbial fermentation using *P. notatum.* Penicillin acylase produced by *Saccharomyces cerevisiae* and *Kluyvera citrophila* is used in biotransformation of penicillin G to semi-synthetic penicillins (Beers *et al*, 2004 and Robert, 2004). The production of tetracycline by fermentation was described by Minieri *et al* (1954). It is a broad-spectrum antibiotic produced by the *Streptomyces*, and is used against many bacterial infections.

3) Steroids:

Recent development in the area of pharmaceutical biotransformation has been in the use of microbial biotechnology in steroid transformations. World Market for steroid biotransformation is estimated to be £500million. Pharmaceutical industry has great interest in the biotransformation of steroids for production of steroid hormones. Delta-1- dehydrogenation, 11β and 20β-reduction are some important steps because they introduce changes in steroid structure that influence biological activity. Steroids provide sex hormones and adrenal cortex hormones, e.g. progesterone, and cortisone

that have extensive application in birth control pills and anti-inflammation respectively. Cortisone and its analogue; prednisolone have a very large market in alleviation of arthritis and other inflammation problems e.g. sports injury and diseases such as asthma.

On C-17 side chain cleavage, steroid precursors yield steroid drugs, which can be done by any chemical means, but the yield is very poor. However microorganisms as *Rhizopous arrhizus* and *R. nigricans* cleave this side-chain with high efficiency and increased yield. Some other important biotransformations include introduction of 16 α-hydroxyl group into 18-hydroxy cortisone and 18-hydroxyl group for preparation of aldosterone (Iizuke and Natio, 1967)

4) Prostaglandins:

Structurally prostaglandins are derivatives of prostanoic acid and have a cyclopentane ring with two side-chains attached to adjacent carbon atoms. The commercial source of availability of prostaglandins is chemical synthesis and extraction from living sources, which has very low yield and is time consuming. Microbial biotransformation of prostaglandins is gaining importance. Recently, they are also being synthesized enzymatically from certain open chain C_{20}–unsaturated fatty acids that include

a) 8,11,14 eicosatrienoic acid
b) 5,8,11,14—eicosatetraenoic acid or arachidonic acid.
c) 5,8,11,14,17-eicosapentaenoic acid.

5) Vitamins:

Vitamins are nutrients required in very small amounts for essential metabolic reactions in the body. Until the 1900's, vitamins were obtained solely through food

intake. But the present scenario has shifted towards microorganisms for their production. Presently, vitamin B12 or cyanocobalamin is obtained by combination of cobalamin with cobalt salt catalysed by microorganisms such as *Streptomyces olivaceous, S. griseous, Bacillus megaterium, Pseudomonas denitrificans, Propionibacterium shermani, P. freudenreichii.* Vitamin B12 is also recovered as by product of streptomycin and aureomycin fermentations. Vitamin B2 or riboflavin is a parent compound of flavins–FAD and FMN co-enzymes and the fungus *Ashbya gossypii* is a prodigious producer of vitamin B_2 (Michael and John, 2006)

6) Commodity chemicals:

The various commodity chemicals produced by biotransformation are acetic acid, and other organic acids as citric acid, lactic acid, acetone, butanol, ethanol etc.

1.1.9.2. Bioremediation:

Bioremediation is also known as biodegradation enhancement and includes any purposeful use of microbes to degrade/biotransform unwanted substances in the environment to ecofriendly compounds. It is the application of biotransformation to alleviate environmental problems. It can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the environment altered by contaminants to its original condition. The goal of all bioremediation efforts is to reduce the potential toxicity of chemical contaminants in the field by using microorganisms, plants, and animals to transform, degrade, or immobilize toxicants. There is already a significant base of knowledge about many pathways for organic degradation, and several important contaminant degradation mechanisms are presently under detailed investigation. Fundamental knowledge of biodegradation and

biotransformation mechanisms has contributed significantly in improving the performance of bioremediation in the field. Recently discovered biotransformation processes for metal biotransformations and biosequestration, coupled aerobic and anaerobic processes, co-metabolism, biotransformations in the presence of alternative electron donors/acceptors, and biotransformations catalyzed by consortia (Ehrlich, 1990; Francis, 1990; Lovley, 1993; Macaskie, 1991) are under investigation by several groups. Bioremediation can be employed to attack specific soil, water, natural body contaminants such as chlorinated hydrocarbons that are degraded by microbes. Using genetic engineering to create organisms specifically designed for bioremediation has great potential (Lovely, 2003).

1.1.9.3. Biotransformation for value addition to food products:

Biotransformation reactions have been used successfully for value addition to food products (Nickerson and Sinskey, 1972). Table 1.1.9.3 shows some examples of microbes, which act upon food materials to enhance nutrition and flavour.

Table 1.1.9.3. Biotransformation for value addition to food products

S. No	Food	Raw material	Biotransforming microorganism
1.	Pickle	Cucumber	*Leuconostoc mesenteroides, Lactobacillus*
2.	Chocolate	Cocao beans	*Saccharomyces cerevisae, Candia rugosa, Kluyveromyces marxianus*
3.	Bread	Flour	*Saccharomyces cerevisae*
4.	Sauerkraut	Cabbage	*Leuconostoc plantarum*
5.	Soy sauce	Soybean	*Aspergillus oryzae*
6.	Curd	Milk	*Lactobacillus bulgaris*
7.	Cheese	Milk	*Lactobacillus lacti, Lactophilus acidophilus*
8.	Decaffeinated coffee	Coffee beans	*Pseudomonas alcaligenes* MTCC 5264
9.	Decaffeinated tea	Tea leaves	*P. alcaligenes* MTCC 5264

1) Biodecaffeination of tea and coffee by microbial enzymes:

Excessive consumption of caffeine through beverages is associated with a number of health problems like adrenal stimulation, irregular muscular activity (Essig *et al*, 1980; Spriet *et al*, 1992), cardiac arrhythmias (Kalmar and Cafarelli, 1999) and increased heart output. Excess caffeine is reported to cause mutation (Pons and Muller, 1990), inhibition of DNA repairs and inhibition of adenosine monophosphodiesterase (Blecher and Lingens, 1977) and during pregnancy causes malformation of fetus and may reduce fertility rates (Srisuphan and Bracken, 1986). It also causes osteoporosis, i.e. decrease in mineral density (Eskenazi, 1993). Conventional methods of decaffeination usually involve the use of solution containing aqueous extract of coffee/tea and decaffeinating agents such as methylene chloride, ethyl acetate, charcoal or carbon, triglycerides and supercritical CO_2 (Katz, 1987). However, these methods suffer from drawbacks like harmful effects due to solvent residues, loss of essential flavours and high cost. Use of microorganisms is being researched into, in the recent years as a potential method for decaffeination (Sarath Babu *et al*, 2005). Many caffeine biotransforming microorganisms have been reported (Blecher and Lingens, 1977; Asano *et al*, 1993; Asano *et al,* 1994; Woolfolk, 1975; Gluck and Lingens, 1987; Gluck and Lingens, 1988; Mazzafera *et al*, 1996; Trijebels and Vogels, 1966; Middlehoven and Bakker, 1982; Vogels and Van der, 1982; Woolfolk and Downward, 1977; Middlehoven and Lommen, 1984; Madyastha *et al*, 1999). Work was carried out in our laboratory on the isolation and characterization of microorganisms capable of biotransforming caffeine to methyl xanthines. A potent caffeine degrading strain of *P. alcaligenes* MTCC 5264 was

isolated at our lab (Sarath *et al.,* 2005). The enzymes involved in the caffeine degradation pathway have been identified and a rate limiting enzyme viz., caffeine demethylase which has potential of application in the biotransformation of caffeine to theobromine and paraxanthine has been identified and used for biodecaffeination which is eco friendly and safer as compared to the conventional decaffeination methods.

2) Biotransformation of vanillin:

Vanillin is frequently used as aromatic flavor compound in food and cosmetics industries. The most intensively studied process for producing natural vanillin by biotransformation is based on the use of ferulic acid as a substrate. The gram-positive microorganisms *Amycolatopsis* sp. strain HR167 and *Streptomyces setonii* have been used to convert ferulic acid to vanillin (Achterholt *et al*, 2000; Muheim and Lerch, 1999).

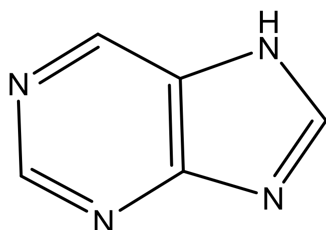
3) Amino acid production:

Amino acids are used as starting materials in the chemical industry for the production of various neutraceuticals and food additives. Some examples of microbially biotransformed amino acids are glutamic acid, phenylalanine, aspartic acid (aspartame = Nutrasweet), lysine (an essential amino acid) and tryptophan. Production volumes of L-leucine range from 10 tons per year, 10,000 tons per year for phenylalanine, 700,000 tons per year for L-lysine and 1.5 million tons per year for glutamic acid (Alchema, 2006).

1.2. Introduction to methyl xanthines:

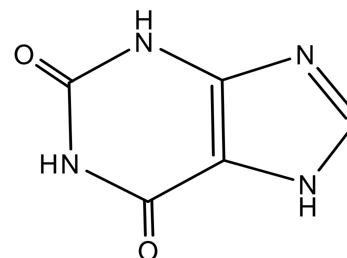
Caffeine, theophylline, theobromine, paraxanthine, and 7-methylxanthine are some of the naturally occurring methyl xanthines of interest (Bresler, 1904; Willaman and Schubert, 1961; Kretschmar and Baumann, 1999). They bear structural resemblance to the purine base guanine, which is a DNA/RNA nucleoside (Scheme 1 and 2). The other methyl xanthines are pentoxyfylline, 8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine, 1-methyl-3-isobutylxanthine etc. Some of the methyl xanthines such as theophylline is therapeutically important molecule and is produced as one of the products of the caffeine biotransformation pathway of fungi. However, the commercial availability of theophylline is by chemical synthesis, which uses various toxic chemicals and solvents, the remnants of which are not desirable. Hence, the need for the production of these therapeutically important molecules by safer ecofriendly methods as biotransformation. Theophylline can be produced by the method of biotransformation using potent microbial cultures and caffeine as the substrate.

Scheme 1. Structure of purine.



Purine

Scheme 2. Structure of xanthine.



Xanthine

Purine (Scheme 1) is the parent molecule for methyl xanthines and are present in nature as the parent compound for adenine and guanine, two of the four

basic constituents of the nucleotides that form DNA and RNA, the compounds of life. Every living organism contains DNA and RNA. Purine is also the basis of ATP, nature's "energy currency". Thus studies on these methyl xanthines and purine molecules gain prominence.

The oxidation of purine by adding two oxygen atoms forms xanthine (Scheme 2). This forms the parent molecule for the methylxanthines. There are three pyrrole type nitrogens, which can be N-methylated to form methylxanthines. Methyl xanthines are an important group of compounds because of their structural similarity to the nucleotide base adenine. Table 1.2.1., shows the therapeutic importance of most of the methyl xanthines and their derivatives as theophylline (Tp) (Bradley and Lichtenstein, 2001; Kathryn and Kamada, 2000; Kurosawa, 2002), pentoxyphylline (Gude *et al*, 1996), acyclovir (Vajpayee and Malhotra, 2000). Different pharmacological studies have revealed that methyl xanthines possess various desirable pharmacological properties such as their ability to inhibit cyclic nucleotide phosphodiesterases (PDEs) (Beavo and Reifsnyder, 1990) and as adenosine receptor antagonist (Daly, 1982; Daly *et al*, 1991). In general, the above mentioned pharmacological activities are reduced in derivatives that lack substituents at position 1 or contain substituents at position 7, as compared with the corresponding 1,3-dialkyl xanthines. The order of potency of the naturally occurring methyl xanthines is theophylline>caffeine>theobromine (Bradley and Lichtenstein, 2001).

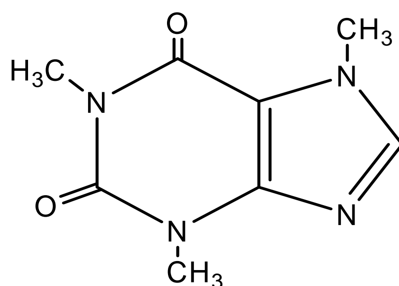
Table 1.2.1. Applications of methyl xanthines.

S.No	Products	Therauptic uses
1.	Theophylline	Used for asthma, apnea, diuretic, stimulates CNS, inhibitor of δ-P110 enzyme (antileukemia property), antimetastatic
2.	Theobromine	Diuretic, enhancer in cancer chemotherapy
3.	Pentoxyphylline	Inhibits TNF-α by decreasing gene transcription, antimetastatic activity, cytoskeleton depolymeriser
4.	Acyclovir	Antivirus
5.	3-Iso butyl-1 methyl xanthine	Adenosine receptor antagonist
6.	1-Isobutyl-3 methyl xanthine	Adenosine receptor antagonist
7.	Diethyl -8 phenyl theophylline	Adenosine receptor antagonist
8.	8-phenyl theophylline	Adenosine receptor antagonist
9.	8-phenyl sulphophenyl theophylline	Adenosine receptor antagonist
10.	Verophylline	Adenosine receptor antagonist

1.2.1. Caffeine:

If all three available pyrrole type nitrogen atoms are methylated, the product is caffeine.

Scheme 3. Structure of caffeine.



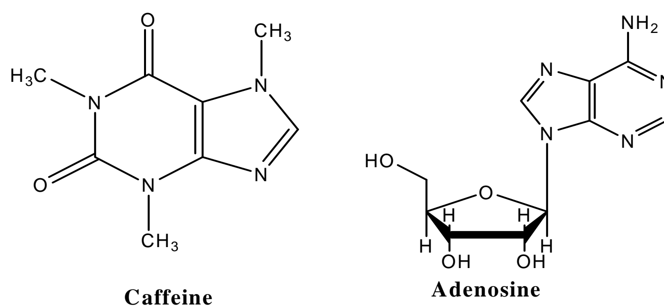
IUPAC name 1,3,7-trimethyl-1*H*-purine-2, 6 (3*H*,7*H*)-dione
Molecular formula $C_8H_{10}N_4O_2$
Molar mass 194.19 g/mol

Caffeine is a xanthine alkaloid compound that acts as a stimulant in humans. It is found in the beans, leaves, and fruit of over 60 plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding upon them (Frischknecht *et al*, 1986; Nathanson, 1984). Caffeine is a central nervous system (CNS) stimulant, having the effect of warding off drowsiness and restoring alertness. Beverages containing caffeine, such as coffee, tea, soft drinks and energy drinks, enjoy popularity great enough to make caffeine the world's most widely consumed psychoactive substance.

Mechanism of action:

Caffeine's principal mode of action is as an antagonist of adenosine receptors in the brain (Fisone *et al*, 2004). As caffeine molecule is structurally similar to adenosine (scheme 4), it binds to adenosine receptors on the surface of cells without activating them (a "false transmitter" method of antagonism). The reduction in adenosine activity results in increased activity of the neurotransmitter dopamine, largely accounting for the stimulatory effects of caffeine.

Scheme 4. Structure of caffeine and adenosine.



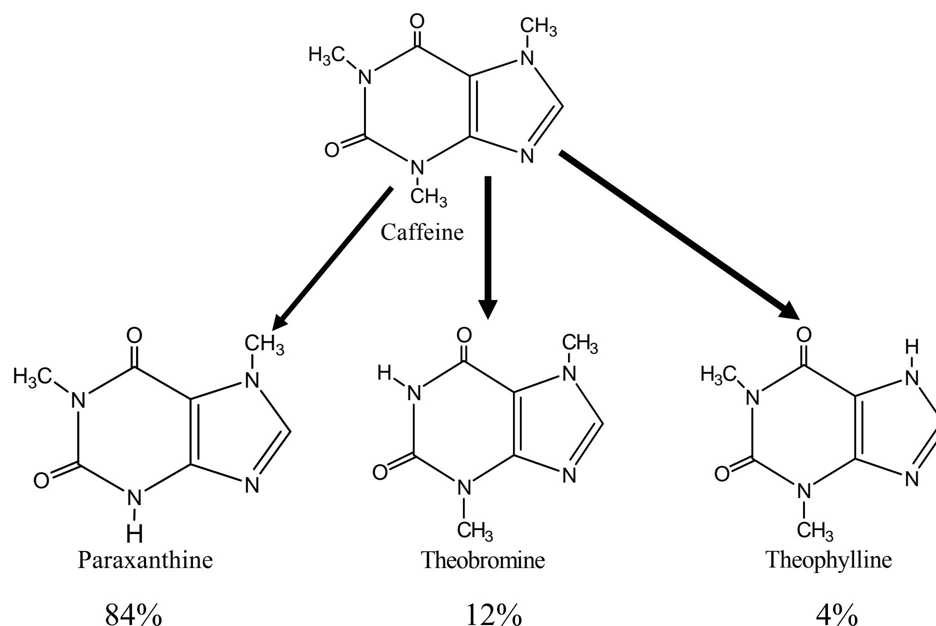
Caffeine can also increase levels of epinephrine (Graham *et al*, 1994). Acute usage of caffeine also increases levels of serotonin, causing positive changes in mood. It is also known as a competitive inhibitor of cAMP-phosphodiesterase (cAMP-PDE),

an enzyme which converts cyclic AMP (cAMP) in cells to its noncyclic form, thus allowing cAMP to build up in cells. Cyclic AMP participates in the messaging cascade produced by cells in response to stimulation by epinephrine. By blocking its metabolism, caffeine intensifies and prolongs the effects of epinephrine and epinephrine-like drugs such as amphetamine, methamphetamine, or methylphenidate.

Biotransformation of caffeine:

In mammalian system, caffeine is biotransformed in the liver by the cytochrome P450 enzyme system (specifically, the 1A2 isozyme) into three primary metabolites: paraxanthine (84%), theobromine (12%), and theophylline (4%) as shown in scheme 5 (Warrens, 1969; Woon-Gye and Young-Nam, 1997). The caffeine biotransformation in plant parts like coffee fruits and leaves proceeds via theobromine, theophylline, N3-methylxanthine, N7-methylxanthine, xanthine, allantoin, allantoic acid and urea (Kalberer, 1965; Suzuki and Waller, 1984a; Suzuki and Waller, 1984b, Ashihara and Crozier., 1999).

Scheme 5. Biotransformation of caffeine in mammalian system.



Reports on caffeine biotransformation pathway show that theobromine and paraxanthine (Asano *et al*, 1993, Sarath Babu *et al*, 2005) are produced by bacteria and theophylline is produced by fungi (Schwimmer *et al*, 1971). Caffeine biotransformation in yeast occurs via formation of theophylline (Birkett *et al*, 1981; Sauer *et al*, 1982).

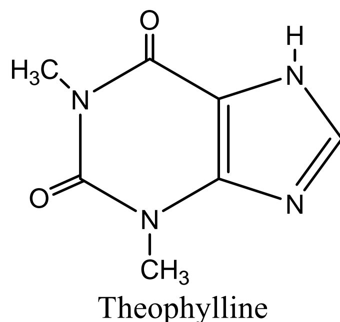
Analogs of caffeine:

Caffeine and analogs that contain ethyl, propyl, allyl, propargyl and other substituents in place of methyl at 1-, 3- and 7-positions are antagonists to the two major classes (A1 and A2) adenosine receptors. Potency at both receptors increased as methyl group is replaced with larger substituents (Daly, 1991). Certain analogs with only one of the three-methyl groups of caffeine replaced by larger substituents are somewhat selective for A2 receptors. Nearly all the 22 analogues of caffeine are available and are more potent than caffeine. The most potent caffeine analogue is 1,3-di-n-propyl-7-propargylxanthine, which is about 100-fold more potent than caffeine at both A1 and A2 receptors.

1.2.2. Theophylline:

It is naturally found in black tea and green tea and in tea leaves (0.3% by mass). It was first extracted from tea leaves around 1888 by the German biologist Albrecht Kossel. The drug was chemically identified in 1896 and eventually it was synthesized by German scientist, Wilhelm Traube (Finar, 1975). Theophylline's first clinical use in asthma came in the 1950s.

Scheme 6. Structure of theophylline.



Theophylline

IUPAC name: 1,3-dimethyl-7H-purine-2,6-dione
Formula - $C_7H_8N_4O_2$
Mol. weight 180.164 g/mol

The conventional therapeutic uses of theophylline are:

- Chronic obstructive diseases of the airways
- Chronic obstructive pulmonary disease (COPD)
- Bronchial asthma.

Theophylline possesses therapeutic properties such as diuretic, cardiac stimulation, smooth muscle relaxation and has been successfully used for the treatment of asthma (Bradley and Leichtenstein, 2001; Kathryn and Alan, 2000), apnea, diuretic (Homer, 2001) and as a central nervous system stimulator. Theophylline shows bronchodilator effects, arising out of its ability for inhibition of phosphodiesterase (PDEs), which catalyze the breakdown of cyclic AMP and GMP to 5' AMP and 5'GMP respectively. Inhibition of PDEs leads to an accumulation of cyclic AMP and GMP thereby increasing the signal transduction through these pathways and leading to increased levels of cyclic AMP, promoting smooth muscle relaxation. The ability of theophylline to stabilize mast cells and other anti-inflammatory and immunomodulatory effects of theophylline has also been observed. Adenosine-stimulated release of mediators from mast cells,

neutrophil activation, induction of 1L-1β and 1L-1α, synthesis and release of tumour necrosis factor (TNF-α) and cytokine release from T-lymphocytes are inhibited by theophylline (Kathryn and Alan, 2000).

Theophylline has been shown to inhibit tissue growth factor β (TGF-β) mediated conversion of pulmonary fibroblasts into myofibroblasts in COPD and asthma patients via cAMP-PKA pathway and suppresses COL1 mRNA, which codes for the protein Collagen (Yano *et al*, 2006). Theophylline can restore the reduced HDAC (histone deacetylase) activity that is induced by oxidative stress (i.e. in smokers), returning steroid responsiveness toward normal, *in vitro* (Ito *et al*, 2002). Corticosteroids switch off the inflammatory response by blocking the expression of inflammatory mediators through deacetylation of histones, an effect mediated via histone deacetylase-2 (HDAC2) and theophylline has been shown to directly activate HDAC2 (Ito *et al*, 2001). Thus theophylline could prove to be a novel form of adjunct therapy in improving the clinical response to steroids in smoking asthmatics.

Histochemical and biochemical studies were carried out on the inhibition of alkaline phosphatase (Al-P) activity in rat cerebral cortex with various methylxanthine derivatives and the results show that theophylline is a potent inhibitor of alkaline phosphatase (Al-P) activity (Sugimura and Muzitani, 1979).

The binding of naturally occurring methylxanthines such as theophylline, theobromine and caffeine to nucleic acids are reckoned to be pivotal as they are able to modulate the cellular activities. The interaction of yeast RNA binding efficacy of theophylline, theobromine and caffeine was studied by Johnson *et al* (2003) using UV absorption differential spectroscopy and Fourier Transform Infrared (FTIR)

spectroscopy. The differential UV-spectrum reveals the greater RNA binding activity for theophylline (85 ± 5%), whereas moderate and comparatively less binding activity for theobromine (45 ± 5%) and caffeine (30 ± 5%). Thus, the discrimination in the binding affinity of methylxanthines with RNA molecule shows that strong RNA binding drugs like theophylline can selectively be delivered to RNA targets of microbial pathogens having the mechanism of RNA catalysis (Johnson *et al*, 2003).

Recent therapeutic uses of theophylline:

The structural resemblance of theophylline to adenine is being exploited to venture into the possibilities of its use as antiviral, anticancerous, antitumourous activity. Theophylline induces growth inhibition and apoptosis in tumorous cells (Lazaros *et al,* 2002). It has been reported to have antileukemia property as it inhibits δ-P110 enzyme (Lazaros *et al,* 2002). Theophylline has been shown to block the transcription of the HIV viral gene in infected CD4 T cells by 94% (Kurosawa, 2002).

Theophylline analogs:

The theophylline analogues are relatively nonselective except for the 1-ethyl analogue and the 1, 3-diallyl analogue, which are selective for the A2 receptor, and the 1, 3-di-n-propyl, 1, 3-diisobutyl, and 1,3-dibenzyl analogues, which are selective for the A1 receptor. 1,3-di-n-propylxanthine is 20-fold more potent than theophylline at the A1 receptor and 5-fold more potent at the A2 receptor (Bruns *et al*, 1983). 1,3-dipropyl substitutions enhance potency compared to theophylline. An 8-phenyl substituent produces a considerable increase in potency, which is augmented by certain para substitutions on the 8-phenyl ring. Combining an ortho amino with a para-chloro substituent on the 8-phenyl ring affords further increases in potency.

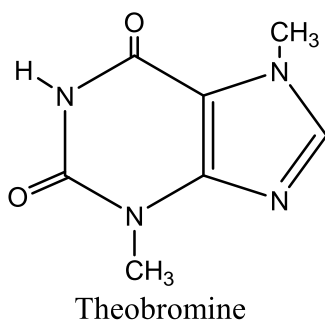
Combining all of these substituents results in 1,3-dipropyl-8-(2-amino-4-chlorophenyl xanthine, a compound of extraordinary receptor affinity, with a K_i for adenosine A1 receptors of 22 pM. It is 4,000,000 times more potent than xanthine itself and 70,000 times more potent than theophylline.

Theophylline is produced as a microbial biotransformation product of caffeine in *Penicillium roqueforti* (Schwimmer *et al*, 1971) and is also produced by biotransformation of caffeine by *P. citrinum* MTCC 5215 as will be discussed in the subsequent chapters.

1.2.3. Theobromine:

Theobromine (Scheme 7) is derived from *Theobroma*, the genus of the cacao tree, which is composed of the Greek roots theo ("God") and broma ("food"), meaning "food of the gods", with the suffix -ine given to alkaloids and other basic nitrogen-containing compounds. It is the primary alkaloid found in cocoa and chocolate, and is one of the causes for mood-elevating effects of chocolate.

Scheme 7. Structure of theobromine.



Theobromine

IUPAC name: 3,7-dihydro-3,7-dimethyl-1H-purine-2,6-dione
Formula - $C_7H_8N_4O_2$
Mol wt - 180.16

Theobromine is found in cacao (1.50% by mass), lesser extent in tea and coffee, and as a human metabolite of caffeine.

Uses of theobromine:

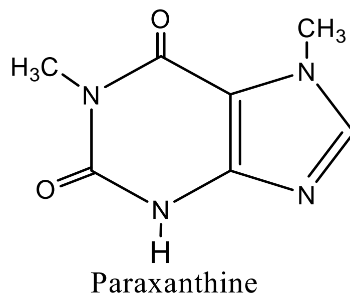
In medicine, it is used as a diuretic, vasodilator, and a myocardial stimulant. It has been used for the treatment of prostate cancer (Slattery and West, 1993). Scientists have recently discovered that theobromine has an antitussive effect superior to codeine by suppressing vagus nerve activity (Usmani *et al*, 2005). Theobromine is known to induce gene mutations in lower eukaryotes and bacteria. It has been used as an experimental teratogen in mice and rabbits (Rambali *et al*, 2002). It is also a contributing factor in acid reflux because it relaxes the esophageal sphincter muscle, allowing stomach acid access to the esophagus. Theobromine is an adenosine receptor antagonist and exerts antiangiogenic properties in many types of tumors in Balb/c mice cutaneous angiogenesis assay. Theobromine inhibits angiogenic activity of ovarian cancer cells as well as CD45 positive lymphocytes isolated from peritoneal ascitic fluid of ovarian cancer patients (Barcz *et al*, 2000). Theobromine inhibits the doxorubicin efflux from tumor cells, increasing the doxorubicin concentration in a tumor, enhancing antitumor effect of doxorubicin (Kakuyama and Sadzuka, 2001). Theobromine is produced by biotransformation of caffeine in bacterial species such as *P. putida* (Blecher and Lingenes, 1977), *P. alcaligenes* MTCC 5264 (Sarath *et al*, 2005).

1.2.4. Paraxanthine:

Paraxanthine (Scheme 8) is a major metabolite of caffeine in humans (>70% of caffeine that has been consumed ends up as this compound). After intake, roughly

80% of caffeine is demethylated at the 3-position to yield paraxanthine. Paraxanthine has a number of physiological effects on animals. In humans, the compound acts as a nonselective, competitive inhibitor of adenosine receptors. As a result, paraxanthine triggers an elevated diastolic blood pressure and an increase in plasma epinephrine.

Scheme 8. Structure of paraxanthine.



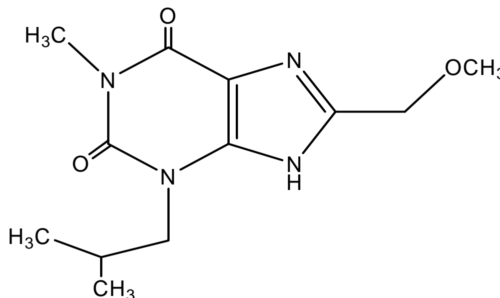
Molecular formula: $C_7H_8N_4O_2$
Molar mass: 180.16 g/mol

Furthermore, the compound has lipolytic properties and its presence in the blood causes an increase in serum free fatty acid concentration. Paraxanthine, unlike caffeine, acts as an enzymatic effector of Na^+/K^+ATPase. As a result, it is responsible for increased transport of potassium ions into skeletal muscle tissue. Similarly, the compound also stimulates increases in calcium ion concentration in muscle. Paraxanthine is also produced as one of the caffeine biotransformation products in bacterial species *P. alcaligenes* MTCC 5264. The demethylation of caffeine to paraxanthine is by demethylation at 3N position by demethylase enzyme *P. alcaligenes* MTCC 5264.

1.2.5. 8-Methoxymethyl-1-methyl-3- (2-methylpropyl) xanthine:

This molecule (Scheme 9) specifically inhibits calmodulin-sensitive cGMP phosphodiesterase and it selectively inhibits Ca^{2+}/calmodulin-dependent phosphodiesterase (Wells, 1988; Ahn., 1989).

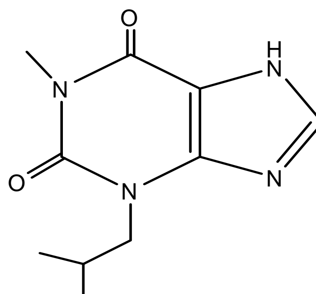
Scheme 9. Structure of 8-methoxymethyl-1-methyl-3- (2-methylpropyl) xanthine.



1.2.6. 1-Methyl-3-isobutylxanthine:

1-Methyl-3-isobutylxanthine is a potent cyclic nucleotide phosphodiesterase inhibitor that increases cyclic AMP and cyclic GMP in tissue and thereby activates cyclic nucleotide-regulated protein kinases.

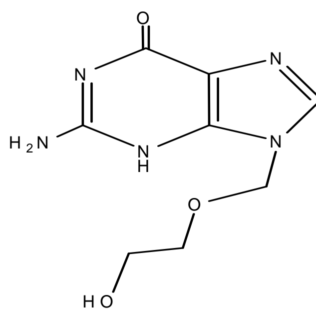
Scheme 10. Structure of 1-methyl-3-isobutylxanthine.



1.2.7. Acyclovir:

Acyclovir (scheme 11) has structurally evolved from basic xanthine molecule and has proved to be a promising antiviral drug to control infections caused by herpes virus. Vidarabine has been the earliest available drug against *Herpes simplex* (HSV) and *Varicella zoster* (VZV), but is an agent that is rarely used at present. Acyclovir has replaced vidarabine in treating herpes infections in immunoincompetent and immuno compromised patients.

Scheme 11. Structure of acyclovir.



IUPAC name: 2-amino-9-(2-hydroxyethoxymethyl)- 3*H*-purin-6-one
Formula: $C_8H_{11}N_5O_3$
Mol. weight: 225.21

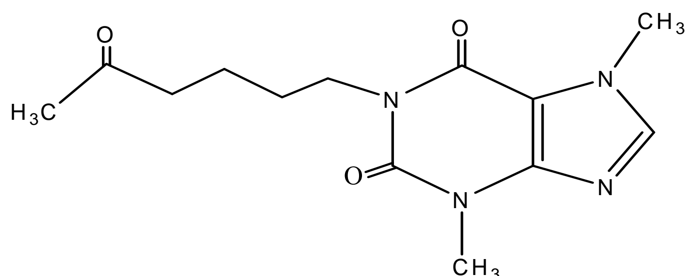
The low oral bioavailability of acyclovir, as well as emergence of drug resistant strains have stimulated efforts towards development of newer compounds for treatment of herpes infection. Newer drugs as sorivudine which is a nucleoside analogue has been pursued in treating herpes infections. Acyclovir and its related compounds are methyl xanthine derivative, which have been used as potent therauptic molecules.

1.2.8. Pentoxyfylline:

Pentoxifylline (Scheme 12), is a methyl xanthine derivative, improves blood flow by decreasing its viscosity and also increases fibrinolytic activity in plasma. Pentoxifylline administration either through IV or IP reduces peritoneal adhesion formation by altering peritoneal fibrinolytic activity (Tohoku, 2006). Pentoxifylline has been found to be a potent inhibitor of primary post-traumatic adhesion formation in a rodent model (Steinleitner *et a*l, 1990).

A few methyl xanthines occur naturally in plant parts whereas the rest are available by chemical synthesis. The extraction of naturally occurring methyl xanthines from plant sources is uneconomical. The commercial availability of all methyl xanthines including theophylline is only through chemical synthesis.

Scheme 12. Structure of pentoxyfylline.



The commercial production of theophylline by chemical synthesis (Finar, 1975) which uses materials such as dimethyl urea and ethyl cyanoacetate. Besides using various toxic chemicals and solvents the chemical method of synthesis results in low yields, is time consuming, involves high cost and is not eco friendly. The traces of chemicals and solvents in the product are not acceptable when used for sensitive therapeutic applications. Besides this the methyl xanthines and their derivatives such as acyclovir, pentoxyphylline etc. are synthesized by chemical means via group transfer mechanism which uses heavy metal compounds such as cyanoiodates and organomercurials (Lech and Piotr, 2001). Production of these methyl xanthines by biotransformation will obviate all the disadvantages of the present methods besides being ecofriendly, giving high yields, being economical. Keeping in view the scope and requirement for the production of methyl xanthines and their derivatives by biotransformation, the present work aims at the production of methyl xanthines as theophylline by biotransformation.

Conclusion:

The process of biotransformation is ubiquitous in nature and has been instrumental in the development of various commercial processes, which are not possible otherwise. It has the potential to produce existing products more cost-

effectively by lower consumption of raw materials, lower investment costs, lower energy demand, and lower disposal costs due to less hazardous emissions making it highly acceptable. It can also provide a basis for completely new products and system solutions and has the potential to replace classical chemical production processes. The high specificity of biotransformation processes attributes to its uniqueness and gives it an edge over other methods of synthesis. Biotransformation systems are operational in various life forms such as humans, animals, plants and microbes. Microbial systems are the most easily manipulative in nature and have been used for the development of many successful commercial processes. There have been reports of fungi, which have the capability of biotransforming caffeine into valuable methyl xanthine as theophylline. These naturally occurring fungal cultures can be utilized for the production of therapeutically important methyl xanthines as theophylline and further worked upon towards the development of successful commercial processes. The production of theophylline by biotransformation will obviate the disadvantages of the chemical synthesis method of theophylline production. Further work in subsequent chapters will be dealt with theophylline production by fungal cultures from caffeine.

The isolation, identification and characterization of caffeine degrading fungi, selection of a potent fungal strain with the capability to biotransform caffeine to theophylline, and studies on the optimization of physicochemical parameters for the development of a biotransformation process for theophylline production are reported in the following chapters of this thesis.

References:

Achterholt, S., Priefert, H., and Steinbüchel, A. (2000), Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol*. **54:**799-807.

Ager, D. J. (1999), Handbook of chiral chemicals, Marcel Dikker Inc, New York/Basel

Aharonowitz, Y., and Cohen, G. (1981), The microbial production of pharmaceuticals. *Sci. Am*. **245**: 140-152.

Ahn, H. S. (1989), Effects of selective inhibitors on cyclic nucleotide phosphodiesterases of rabbit aorta*: Biochem. Pharmacol*. **38**: 3331.

Alchema (2006), 28th International Exhibition-Congress on Chemical Engineering, Environmental Protection and Biotechnology. Frankfurt am Main, Germany May 15 – 19.

Andersson, H. C., Hallström, H. and Kihlman, B. A. (2004), Intake of caffeine and other methylxanthines during pregnancy and risk for adverse effects in pregnant women and their foetuses. Ekspressen Tryk and Kopicenter, www.norden.org

Asano, Y., Toshihiro, K., and Yamada, H. (1993), Microbial production of theobromine from caffeine. *Biosci. Biotechnol. Biochem*. **57**:1286–1289.

Asano, Y., Toshihiro, K, and Yamada, H. (1994), Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No. 352. *Biosci. Biotechnol. Biochem.* **58**:2303–2304.

Ashihara, H. and Crozier, A. (1999), Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Advances in Botanical. Research.* **30**:117- 205.

Attar, R. M., Grotewold, E., Taccioli, G. E., Aisenberg, G. D., Torres, H. N., and Judewicz, N. D. (1990), A cycloheximide-inducible gene of *Neurospora crassa* belongs to the cytochrome P-450 superfamily. *Nucl. Acid. Res*. **17**: 7535-7536.

Barcz, E., Sommer, E., Janik, P., Marianowski, L., and Skopinska-Rozewska, E., (2000), Adenosine receptor antagonism causes inhibition of angiogenic activity of human ovarian cancer cells. *Oncol. Rep.* **7**(6):1285-1291.

Beavo, J. A., and Reifsnyder, D. H., (1990), Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors; *Trends in Pharmacol. Sc.* **11**:150-155.

Beers, M. H., and Robert B, eds. (2004), *The Merck Manual*, 2nd home ed. West Point, PA: Merck and Co.

Betts, T. J. (2000), Solid phase microextraction of volatile constituents from individual fresh Eucalyptus leaves of three species. *Planta Medica*. **66**(2): 193-195.

Birkett, D. J, Grygiel, J. J, and Mines, J. O. (1981), Metabolic deposition of methyl xanthines in man. In: Theophylline and other methylxanthines, N. Rietenbrock, Woodcock B.G, Staib A.H (eds). Vieweg Verlag, The Proceedings of an International Symposium, Frankfurt:29th april and 3rd May 1981, 149-158.

Blecher, R., and Lingens, F. (1977) The metabolism of caffeine by a *Pseudomonas putida* strain. *Hoppe-Seyler's Z Physiol Chem*. **358**:807–817.

Boucher, J. L., Delaforgh, M., and Mansuy, D. (1994), Dehydration of alkyl- and arylaldoximes as a new cytochrome P450-catalyzed reaction: mechanism and stereochemical characteristics. *Biochemistry*. **33**:7811-7818.

Bradley, J. W., and Lichtenstein, L. M., (2001), Drugs used in the treatment of asthma, *In* Alfred Goodman Gilman (ed.), Pharmaceutical basis of therapeutics, 10th ed. p733-754 Mc Graw-Hill Medical Publication division.

Bresler, H. W. (1904), Über die Bestimmung der Nucleinbasen in Safte von Beta vulgaris. *Hoppe-Seyler´s Z. Physicol. Chem*. **41**:535-541.

Breslow, R., Huang, Y., Zhang, X. J., and Yang, J. (1997), An artificial cytochrome P450 that hydroxylates unactivated carbons with regio- and stereoselectivity and useful catalytic turnovers. *Proc. Natl. Acad, Sci.* U. S. A. **94** (2): 11156-11158.

Briza, P., Breitenbach, M., Ellinger, A., and Segall, J. (1990), Isolation of two developmentally regulated genes involved in spore wall maturation in Saccharomyces cerevisiae. *Genes Dev*. **4** (10): 1775- 1789.

Briza, P., Eckerstorfer, M., and Breitenbach, M., (1994), The Sporulation-Specific Enzymes Encoded by the DIT1 and DIT2 Genes Catalyze a Two-Step Reaction Leading to a Soluble LL-Dityrosine-Containing Precursor of the Yeast Spore Wall. *Proc. Natl. Acad. Sci.* U. S. A. **91**(10), 4524-4528.

Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., Adms, T. H., and Leonard, T. J. (1996), Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc. Natl. Acad, Sci*. U. S. A. **93**(4): 1418-1422.

Bruns, R. F., Daly, J. W, and Snyde,r S. H. (1983), Adenosine receptor binding: structure-activity analysis generates extremely potent xanthine antagonists. *Proc Natl Acad Sci U S A*. **80** (7): 2077–2080.

Buchholz, K. (2005), Immobilisation of enzymes (Including applications), *Biocatalyst and enzyme technology*, Wiley-VCH Verlag GmbH and Co., pp. 243-282

Buckland, B. C., Dunnill, P., Lilly, M. D. (1975), The Enzymatic Transportation of water insoluble reactants in non aqueous solvents. Conversion of Cholesterol to Cholest-4-ene-3-one by a *Nocardia* sp. *Biotechnol. Bioeng*. **17**:815-826.

Burton**,** S. G. , Dorrington, R. A. Hartley, C. Kirchmann, S., Matcher, G. and Phehane, V. (1998). Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoinase-producing bacteria. *J. Mol. Catal B: Enzymatic*, **5** (1-4), 301-305.

Cabral, J. M. S., Aires, M. R., Pinheiro, H. and Prazeres. D. M. F. (1997), Biotransformation in organic media by enzymes and whole cells. *J. of Biotechnol* **59** (1-2):133-143.

Carla, C. C., Carvalho, R., Frederik, K. and Manuela, R. M. (2000), Botransformation of Limonene-1,2-epoxide to Limonene-1,2-diol, *Food.Technol. Biotechnol.* **38** (3): 181–185.

Chenault, H. K, and Whitesides, G. M. (1987), Regeneration of nicotinamide cofactors for use in organic synthesis. *Appl.Biochem. Biotechnol*. **14**:147-197.

Clarke, R. J. and Macrae, R. (eds) (1988), *Coffee.* **Vol. 1-3**, Elsevier, New York.

Cragg, G. M., Schepartz, S. A., Suffuess, M., and Grever, M. R. (1993), The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *J. Nat. Prod*. **56**: 1657-1668.

Daly, J. W. (1982), Adenosine receptors: targets for future drugs. *J.Medical Chemistry*. **25**:197-207.

Daly, J. W., Hide, I., Muller, C. E. and Shamim, M. B. (1991), *Medical Pharmacol.* **42**: 309-321

Deutsch, J., Leutz, J. C., Yang, Gelboin, H. V., Chiang, Vatsis, and Coon, M. J. (1978), Regio- and Stereoselectivity of Various Forms of Purified Cytochrome P-450 in the Metabolism of Benzo[a]pyrene and (-) trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene as shown by Product Formation and Binding to DNA. *Proc. Natl. Acad, Sci.* U. S. A. **75**:3123-3127.

Ehrlich, H. L. (1990), *In:* Geomicrobiology. Marcel Dekker, Inc., New York.

Eskenazi. (1993), Caffeine during pregnancy: grounds for concern? *J Am Med Assoc* **270**:2973–2974.

Essig, D., Costill, D. L., and Handel, P. J. (1980), Effects of caffeine ingestion on utilization of muscle glycogen and lipid during leg ergometer cycling. *Int. J Sports. Med.* **1**:86–90.

FAO (1996), Year book. Production 1996. FAO, Rome, Table 78, 172.

Finar, I. L Organic Chemistry (1975), 5th Edition, vol-2; (Reprint 1996), Stereochemistry and the chemistry of natural products; Chapter-16, Purines and Nucleic acids, P-809.

Fisone, G., Borgkvist, A., and Usiello, A. (2004), Caffeine as a psychomotor. stimulant: mechanism of action. *Cell. Mol. Life. Sci*. **61**: 857–872.

Frischknecht, P.M., Ulmer, D.J., and 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.

Frost and Sullivan Research Service, (2003), Advances in Biotechnology for Chemical Manufacture - Part 2 (Technical Insights).

Fukai, S. and Tanaka, A. (1981), Bioconversion of Lipophilic Compounds by Immobilised Microbial Cells in Organic Solvents. *Acta Biotechnol*., **1** (4), 339-477.

Fukuda, H., Fujii, T., Daimon, H., Iwata, M., Ogawa, T., Tanase. S., and Morino, Y. (1993) Purification and characterization of cytochrome P450 from an isobutene-forming microorganism, Rhodotorula minuta *Biosci. Biotech. Biochem*. **57**(9): 1599-1601.

Furuya, T.**,** (1984), *Japanese Patent*, Kokai Sho-59-159790.

Garrett, R. H., and Grisham, C. M. (1995), *Biochemistry*. Florida: Saunders College Publishing; 753, 799.

Gatfield, I. L., (1988), Production of flavour and aroma compounds by biotechnology, *Food Technol.* **10**:110–122.

Glazer, A. N. and Nikaido, H. (1995), *In:* Microbial Biotechnology: Fundamentals of Applied Microbiology. Freeman, New York.

Gluck, M., and Lingens, F. (1987) Studies on the microbial production of the bromine and heteroxanthine from caffeine. *Appl. Microbiol. Biotechnol.* **25**:334–340.

Gluck, M., and Lingens, F. (1988), Heteroxanthine demethylase, a new enzyme in the degradation of caffeine by *Pseudomonas putida*. *Appl Microbiol Biotechnol* **28**:59–62.

Graham, T., Rush, J., and Van, S. M. (1994), Caffeine and exercise: metabolism and performance. *Can. J Appl. Physiol.* **19** (2): 111-138.

Gude, R. P, Ingle, A. D., and Rao, S. G. A. (1996), Inhibition of lung homing of B16F10 by Pentoxifylline, a microfilament depolyerizing agent. *Cancer letters*. **106**:171-176.

Guenther, E. (1950), Essential oils of the plant family Gramineae. In: *The Essential Oils*, D. Van Nostrand Co, Inc., Canada. **4**:20-155.

Hanisch, W. H., Dunhill, P., and Lilly, D. (1980), Optimization of the production of progesterone 11**α**-hydroxylase by *Rhizopus nigricans. Biotech. Bioeng*. **22**(3): 555-570.

Hara, M., Miyake, J., Asada, Y., and Ohkawa, H. (1999), Purified Fusion Enzyme between Rat Cytochrome P4501A1 and Yeast NADPH-Cytochrome P450 Oxidoreductase. *Biosci. Biotechnol. Biochem*. **63**: 21-28.

Harish, K. S and Gina, S. S. (1994), Solvent Selection and Productivity in Multiphase Biotransformation Systems. *Biotechnol. Prog.* **10**:*187*-192.

Hildebrandt, A. L. and Riker, A. J., (1953), Culture des tissus vegetaux 42.

Hohn, T. M., Desjardins, A. E., and Mccormick, S. P. (1995), The Tri4 gene of Fusarium sporotrichioides encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *Mol. Gen. Genet*. **248** (1): 95-102.

Homer, A. B, (2001), Bronchodilator and other agents used in asthma chapter 19, p 305-321 *In* Basic and clinical pharmacology, 6th ed., Bertrom G. Katzung(ed.) Lange Medical Book.

Iizuke, H. and Natio, A. (1967), Microbial transformation of steroids. University of Tokyo Press, Tokyo.

Ito, K., Lim, S., and Caramori, G. (2001), Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *FASEB J*, **15**:1110-1112.

Ito, K., Caramori, G., and Lim, S. (2002), Expression and activity of histone deacetylases in human asthmatic airways. *Am. J Respir. Crit. Care. Med.* **166**:392-396.

Johnson, I. M., Kumar, S. G, and Malathi, R. (2003), RNA binding efficacy of theophylline, theobromine and caffeine. *J Biomol. Struct. Dyn*. **20**(5): 687-692.

Jordon, M. A. and Wilson, L. (1995), Microtuble polymerization dynamics, mitotic, and cell death by paclitaxel at low concentration, *American Chemical Society* Symposium Series, **Vol. 583**, Chapter X, pp. 138-153.

Kakuyama, A., and Sadzuka, Y., (2001), Effect of methylxanthine derivatives on doxorubicin transport and antitumor activity. *Curr. Drug. Metab*. **2**(4):379-395.

Kalberer, P. (1965), Breakdown of caffeine in the leaves of *Coffea arabica* L. *Nature* **205**: 597-598.

*Kallenberg, A. I. (2005), *Adv. Synth. Cat.* **347**: 905-926.

Kalmar, J. M., and Cafarelli, E. (1999), Effects of caffeine on neuromuscular function. *J Appl. Physiol.* **87**:801–808.

Kathryn, B. and Alan, K. K. (2000) Ch-35 Asthma p-651 *In* Eric T.Herfindal,Dick R.G.(ed.), Text book of therapeutics-Drug and disease management, 6th ed, Williams and Wilkins.

Katz, S. N. (1987), Decaffeination of coffee. In: Clarke RJ, Macrae R, editors. *Coffee technology*. New York: Elsevier Applied Science.

Kelly, S. L., Lamb, D. C., Baldwan, B. C., Corran, A. J., and Kelly, D. E. (1997), Characterization of *Saccharomyces cerevisiae* CYP61, Sterol Δ^{22}-Desaturase, and Inhibition by Azole Antifungal Agents. *J. Biol. Chem*. **272**(15): 9986-9988.

Kreiner, M., Braunegg, G., Raady, A., Griengl, H., Kopper, I., Petsch, M., Plachota, P., Schoo, N., Weber, H., and Zeiser, A. (1996), Stereospecific Biohydroxylations of Protected Carboxylic Acids with Cunninghamella blakesleeana. *Appl, Environ. Microbiol.* **62**(7): 2603-2609.

Kretschmar, J. A. and Baumann, T. W. (1999), Caffeine in Citrus flowers. *Phytochemistry*. **52** (1):19-23.

Kurosawa, (2002), Caffeine inhibits the synthesis of the HIV Virus in T cells by 94%, http://Grouppekurosawa.com.

Lalonde. J., Margolin A., (2002), Immobilisation of enzymes, *Enzyme Catalysis in Organic Synthesis*, 2nd ed. K. Drauz, H. Waldmann, Eds. Wiley-VCH Verlag GmbH, (Ch 6), pp. 163-184

Lazaros, C. F, Nathalie, D., Chariklia, K., Karen, E. A, Jorgen, J., and Peter, R. S. (2002), Direct Effects of Caffeine and Theophylline on p110 and Other

Phosphoinositide 3-Kinases. Differential effects on lipid kinase and protein kinase activities, *J. Biol. Chem.* **277** (40): 37124–37130.

Lech, S. and Piotr, W. (2001,1-30 September) Synthesis – Some heteroaromatic organomercurials, their synthesis and reaction:A review, http:// www.mdpi.org/ecsoc-5.htm.

Liotta, D. C., Choi, W. B. PCT Int. Appl. WO 91252418 (1991).

Liotta, D. C., PCT Int. Appl. WO 9214743 (1992).

Longo, M. A. and Sanroman, M. A. (2006), Production of Food Aroma Compounds, *Food. Technol. Biotechnol.* **44** (3): 335–353.

Lovley, D. R. (1993), Dissimilatory metal reduction. *Annual Review of Microbiology* **47**:263- 290.

Lovley, D. R (2003), Cleaning up with genomics: applying molecular biology to bioremediation. *Nature reviews. Microbiology.* **1** (1): 35–44.

Lonza AG (2004), L-Carnitine for your skin. Cosmoceuticals**.**

Macaskie, L. E. (1991), The application of biotechnology to the treatment of waste products produced from the nuclear fuel cycle: Biodegradation and bioaccumulation as a means of treating radionuclide-containing streams. *Critical Reviews in Biotechnol.* **11**:41-112.

Madyastha, K. M, Sridhar, G. R, Bhat, B. V, and Madhavi, S. Y. (1999), Purification and partial characterization of caffeine oxidase. *Biochem. Biophys. Res. Commun.* **263**:460–464.

Maloney, A. P., and VanEtten, H. D. (1994), A gene from the fungal plant pathogen Nectria haematococca that encodes the phytoalexin-detoxifying enzyme pisatin demethylase defines a new cytochrome P450 family. *Mol. Gen. Genet*. **243** (5): 506-514.

Mazzafera, P., Olsson, O., and Sandberg, G. (1996), Degradation of caffeine and related mehtylxanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microb. Ecol*. **31**:199–207.

Michaelis, L. and Menten, M. L. (1913), Die Kinetik der Invertasewirkung. *Biochem J.* **49**:333–369.

Michael, T. M., and John, M. M. (2006), Industrial microbiology, Chapter 30 in Biology of Microorganisms, Eleventh edition. Pearson Prentice Hall, Inc.

Middlehoven, W. J, and Bakker, C. M. (1982), Degradation of caffeine by immobilized cells of *Pseudomonas putida* strain C3024. *Eur J Appl Microbiol Biotechnol* **15**:214–217.

Middlehoven, W. J, and Lommen, A. (1984), Degradation of caffeine by *Pseudomonas putida* C3024 the effect of oxygen concentration. *Antonie Van Leeuwenhock*. **50**:298–300.

Minieri, P. P., Firman, M. C,. Mistretta, A. G,. Abbey, A,. Bricker, C. E,. Rigler, N. E and Sokol, H. (1954), A new broad spectrum antibiotic product of tetracycline group. In *Antibiotics annual* 1953-1954. *Medical Encyclopaedia,* Inc., New York, pp 81-87.

Misawa, M., Endo, T., Goodbody, A., Vukovic, J., Chapplet, C., Choi, L., and Kutney. J. P. (1988), Synthesis of dimeric indole alkaloids by cell free extracts from cell suspension cultures of *Catharanthus roseus*. *Phytochem*. **27** (5): 1355-1359.

Modi, S. P, Boyle, W. U., Gibson, M. B., Lian , L. L., and Roberts, G. C. K. (1995), NMR studies of substrate binding to cytochrome P450 bm3: comparison to cytochrome P450cam. *Biochemistry*. **34**: 8982-8988.

Muheim, A., and Lerch, K. (1999). Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.* **51**:456-461.

Nathanson, J. A., (1984), Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science.* **226** (4671): 184-187.

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., and Waterman, M. R. (1987), The P450 gene superfamily: recommended nomenclature. *DNA*. **6**: 1-11.

Nickerson, J. T. and Sinskey, A. J. (1972), Microbiology of Food and Food Processing. American Elsevier, New York.

Pacl, H., Festel, G. Wess G. (2004) The Future of Pharma R&D : Challenges and trends. P 27-38, Festel Capital (Huenenberg)

Painter, G. R., PCT Int. Appl. WO 2000009494 (2000).

Panzhou, X. R., Crettonscott, E., Zhou, X. J., Yang, M. X., Lasker, J. M., and Sommadossi, J. P. (1998), Role of human liver P450s and cytochrome b5 in the reductive metabolism of 3'-azido-3'-deoxythymidine (AZT) to 3'-amino-3'-deoxythymidine. *Biochem. Pharmacol*. **55** (6): 757-766.

*Pasteur, L. (1858), Me´moire sur la fermentation appele´e lactique. *Ann. Chim. Phys.* **52**: 404–418.

Pons, F. W, and Muller, P. (1990), Induction of frameshift mutations by caffeine in *Escherichia coli* K12. *Mutagenesis.* **5**:173–177.

Rambali, B., Van A, Schenk, I. E., Wolterink, G. G., Van, W., Stevenson, H., and Vleeming, W., (2002), The contribution of cocoa additive to cigarette smoking addiction, RIVM report 650270002/2002)

Rendic, S., and Dicarlo, F. J. (1997), Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab. Rev.* **29** (1-2), 413-580.

Roy, J. J., and Emilia, T. A. (2006), Continuous biotransformation of pyrogallol to purpurogallin using cross-linked enzyme crystals of laccase as catalyst in a packed-bed reactor. *J. Chem. Technol. and Biotechnol.* **81**(11): 1836-1839.

Sanglard, D., and Loper, J. C. (1989), Characterization of the alkane-inducible cytochrome P450 (P450alk) gene from the yeast Candida tropicalis: identification of a new P450 gene family. *Gene*. **76**: 121-136.

Sarath Babu, V. R, Patra, S., Thakur, M. S., Karanth, N. G, and Varadaraj, M. C., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. *Enz. Microb. Technol.* **37**: 617-622.

Sauer, M., Kappeli, O., and Fiechter, A. (1982), Comparison of the cytochrome P-450 containing monooxygenases originating from two different yeasts. *Deveop. in Biochem.* **23**:452-457.

Schunck, W. H., Kargel, E., Gross, B., Mauersberger, S., Kopke, K., Kiessling, U., Strauss, M., Gaestel, M., and Muller, H. G. (1989), *Biochem. Biophys. Res. Commun*. **161**: 843-850.

Schwimmer, S., Khurtzman, R. H., Heftmann, E. (1971), Caffeine metabolism by *Penicillium roqueforti*. *Arch. Biochem. Biophys.* **147**:109–113.

Sheldon, R. A., Schoevaart, R., and Van, L. L. M. (2005), Cross-linked enzyme aggregates (CLEAs): A novel and versatile method for enzyme immobilization**.** *Biocatal. and Biotrans.* **23**(3-4): 141 – 147

Shin, H. S, and Rogers, P. L (1995), Biotransformation of benzeldehyde to L-phenylacetylcarbinol, an intermediate in L-ephedrine production, by immobilized *Candida utilis*. *Appl. Microbiol. Biotechnol.* **44**: 7-12.

Shoun, H., Suyama, W., and Yausi, T. (1989), Soluble, nitrate/nitrite-inducible cytochrome P-450 of the fungus, Fusarium oxysporum. *FEBS Lett*. 244 (1): 11-14.

Singh, P., Mathur, A. N., and Verma, S. K. (2004), Value Addition of Agricultural Produce : For Export Promotion. Vedams eBooks (P) Ltd.

Slattery. M, and West, D. (1993). Smoking, alcohol, coffee, tea, caffeine, and theobromine: risk of prostate cancer in Utah (United States). *Cancer Causes Control.* **4** (6): 559-563.

Spriet, L. L, MacLean, D. A, Dyck, D. J, Hultman, E., Cederblad, G., and Graham, T. E. (1992), Caffeine ingestion and muscle metabolism during prolonged exercise in humans. *Am J Physiol Endocrinol Metab*. **262**:891–898.

Srisuphan, W, and Bracken, M. B. (1986), Caffeine consumption during pregnancy and association with late spontaneous abortion. *Am J Obstet Gynecol*, **155**:14–20.

Steinleitner, A., Lambert, H., Kazensky, C., Danks, P., and Roy, S. (1990), Pentoxifylline, a methylxanthine derivative, prevents postsurgical adhesion reformation in rabbits. Data demonstrate a marked inhibition of adhesion reformation after lysis of pelvic adhesions under the influence of pentoxifylline in rabbits. *Obstetrics & Gynecology*. **75**:926-928.

Suffness, M., and Boca, R. F. L. (1995), Taxol: Science and Applications. *CRC Press.*

Sugimura, K. and Mizutani, A., (January 1979) The inhibitory effect of xanthine derivatives on alkaline phosphatase in the rat brain. *Histochemistry and Cell Biology*. **61**(2): 115-120.

Suzuki, T. and Waller, G. R. (1984a), Biosynthesis and biodegradation of caffeine, theobromine, and theophylline in *Coffea arabica* L. Fruits. *J. Agric. Food Chem*. **32**:845-848.

Suzuki, T. and Waller, G. R. (1984b), Biodegradation of caffeine: formation of theophylline and theobromine from caffeine in mature *Coffea arabica* fruits. *J. Sci. Food Agric*. **35**:66-70.

Tohoku. (2006), Pentoxifylline, a Methyl Xanthine Derivative, Reduces Peritoneal Adhesions and Increases Peritoneal Fibrinolysis in Rats *J. Exp. Med*. **209**(3): 312-316.

Toritsuka, N., Shoun, H., Singh, U. P., Park, S. Y., Iizka, T., and Shiro, Y. (1997), Functional and structural comparison of nitric oxide reductases from denitrifying

fungi Cylindrocarpon tonkinense and Fusarium oxysporum. *Biochem. Biophys. Acta*, **1338**(1): 93-99.

Trager, W. F. (1989), Stereochemistry of cytochrome P-450 reactions. *Drug Met. Rev*. **20**: 1989-1994.

Trijebels, F, and Vogels, G. D (1966), Degradation of allantoin by *Pseudomonas acidovorans*. *Biochim. Biophys. Acta.* **113**:292–301.

Truan, G., Cullin, C., Reisdorf, P., Urban, P., and Pompon, D. (1993), Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5.*Gene*. **125** (1): 49-55.

Tsuchida, T., Nishimoto, Y., Kotani, T., and Liizumi, K. (1993), Production of L-3,4-dihydoxyphenylalanine, Ajinomoto Co.Ltd., JP 5123/77A.

Usmani, O, Belvisi, M., Patel, H., Crispino, N., Birrell, M., Korbonits, M., Korbonits, D., and Barnes, P. (2005), Theobromine inhibits sensory nerve activation and cough. *FASEB J.* **19** (2): 231-233.

Vaan, G. R. F. M., Boschloo, J. G., Kuijvenhoven, A., Lange, J., van Vark, A. J., Bos, C. J., van Balken, J. A. M., Pouwels, P. H., and van den Hondel, C. A. M. J. J. (1990), Isolation and molecular characterisation of the benzoate-para-hydroxylase gene (bphA) of Aspergillus niger: a member of a new gene family of the cytochrome P450 superfamily.*Mol. Gen. Genet*. **223** (2): 192-197.

Vajpayee, M., and Malhotra, N., (2000), Antiviral drugs against Herpes infection. *Indian Journal of Pharmacology.* **32**: 330-338.

Vandenbossche, H., and Koymans, L. (1998), Cytochromes P450 in fungi. *Mycoses*. **41**(Suppl. 1): 32-38.

Vogels, G. D., and Van der D. C. M. (1982), Degradation of purines and pyrimidines by microorganisms. *Bacteriol Rev*. **40**:403–468.

Warrens R.N., Metabolism of xanthine alkaloids in man, *Journal of Chromatography* **40**: 468-469, 1969.

Wells, J., N. and Miller, J., R. (1988), Methylxanthine inhibitors of phosphodiesterases. *Meth. Enzymol.* **159**: 489.

Weltring, K. M., Turgeon, B. G., Yoder, O. C., and VanEtten, H. D. (1988), Isolation of a phytoalexin-detoxification gene from the plant pathogenic fungus Nectria haematacocca by expression in Aspergillus nidulans. *Gene*. **68**: 335-344.

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

Wong, C. H, and Whitesides, G. M. (1994), Enzymes in synthetic Organic Chemistry, Elsevier Science Ltd., Oxford.

Woolfolk C. A. (1975), Metabolism of *N*-methylxanthines by *Pseudomonas putida* strain isolated by enrichment in caffeine as sole source of carbon and nitrogen. *J Bacteriol*. **123**:1088–1096.

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

Woon-Gye, C. and Young-Nam, C. (1997), Oxidation of caffeine to theobromine and theophylline is catalysed primarily by Flavin containing Monoxygenases in liver microsomes. *Biochem. Biophys. Res. Comm*. **235**: 685-688.

Wrotnowski, C. (1997), Unexpected niche applications for industrial enzymes drives market growth, *Gen. Eng. News.* **1**: 14-30.

Yamada, H. (1998) Screening of novel enzymes for the production of useful compounds, in: New frontiers in screening for Microbial Biocatalysis(Kieslich.,K.; Van der Bleck; C.P., de Bont,J.A.M., Van der Tweel, W.J.J., eds) pp13-17, Studies in Organic Chemistry 53, Elsevier, Amsterdam.

Yano, Y., Yoshida, M., Hoshino, S., Inoue, K., Kida, H., Yanagita, M., Takimoto, T., Hirata, H., Kijima, T., Kumagai, T., Osaki, T., Tachibana, I., and Kawase, I. (2006), Anti-fibrotic effects of theophylline on lung fibroblasts. *Biochem. Biophys. Res. Comm*. 341: 684-690.

Yu, J., Chang, P. K., Cary, J. W., Bhatnagar, D., and Cleveland, T. E. (1997), avnA, a gene encoding a cytochrome P-450 monooxygenase, is involved in the conversion of averantin to averufin in aflatoxin biosynthesis in Aspergillus parasiticus. *Appl. Environ. Microbiol*. **63**(4): 1349-1356.

Yu, J., Chang, P. K., Ehrlich, K. C., Cary, J. W., Montalbano, B., Dyer, J. M., Bhatnagar, D., and Cleveland, T. E. (1998), Characterization of the Critical Amino Acids of an *Aspergillus parasiticus* Cytochrome P-450 Monooxygenase Encoded by *ordA* That Is Involved in the Biosynthesis of Aflatoxins B_1, G_1, B_2, and G_2. *Appl. Environ. Microbiol*. 64 (12): 4834-4841.

*** Original reference not seen.**

CHAPTER -2

SCREENING OF CAFFEINE BIOTRANSFORMING FUNGI

2.0. Scope of the work:

Microorganisms are major elements effecting biotransformation and has been used successfully for the production of many commercial processes. Selection of appropriate microbial culture is the most crucial step for the development of efficient microbial processes. Targeted isolation programme is the first and foremost step in this direction. Although caffeine is known to inhibit the growth of several microorganisms, certain microbial species are known to utilize caffeine and convert it into valuable compounds of therapeutic importance.

The first section of this chapter focuses on the survey of literature on various groups of caffeine biotransforming microbial cultures. Further, the work carried out on the isolation of potent fungal cultures capable for biotransformation of caffeine is discussed. 34 caffeine biotransforming fungal cultures were isolated which were further screened in liquid culture media containing caffeine to check their biotransformation efficiency. The most potent caffeine biotransforming strain was identified and characterized as *P. citrinum* MTCC 5215. The biotransformation product of caffeine by this chosen strain was identified as theophylline. This strain was further used for the development of process for biotransformation of caffeine to theophylline.

2.1. Introduction:

Microorganisms play a key role in biotransformation during the process of metabolism, to convert abundantly available raw materials into valuable products. The biotransformation of caffeine by microorganisms leads to the formation of various valuable compounds such as theophylline, theobromine, paraxanthine, uric acid, allantoin etc. which can be exploited for the development of biotransformation processes. Targeted isolation of potent caffeine biotransforming microorganisms is the first and foremost step in the production of biotransformed compounds from caffeine, using microorganisms.

Caffeine is a xanthine alkaloid and is produced in *Coffea arabica* and sixty other plant species, many of them belonging to *Camellia*, *Theobroma* and *Cocoa* genera (Suzuki and Waller, 1988). The production of caffeine is a potent survival strategy for the plant and a method to compete for essential nutrients in a stressful environment. Caffeine found in plants at doses, as in *C. arabica* is toxic to a variety of insects and fungi and inhibits the growth of other plants and bacteria near the germinating seeds of tea and coffee (Frischknecht *et al*, 1986; Nathanson, 1984). Friedman and Waller (1983a, b) noted that caffeine readily leaches out of the seeds into aqueous media, and is stored in the surrounding soil in soluble form and further shows its allelopathic action. They also observed that caffeine present in the soil inhibits mitosis in the roots of many plants and thereby, reduces their 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. Caffeine's solubility in water provides for simple transport

into the nearby soil, inhibiting microbial growth. However, certain species of microorganisms are known to develop mechanisms of tolerating caffeine on exposure to increasing concentration for a longer period of time and further biotransform the caffeine. Hence, enrichment of soil/media with increasing concentrations of caffeine has been used for the selective isolation of caffeine biotransforming microorganisms.

Studies on caffeine biotransformation by microorganisms were not reported till 1970 because caffeine was regarded as toxic to bacteria (Sundarraj and Dhala, 1965; Putrament *et al*, 1972; Kihlman, 1974). Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. First report on caffeine degradation by microorganisms was in early 1970 (Kurtzman and Schwimmer, 1971). Since then, progress has been achieved on using caffeine as sole nutrient source for microbial growth (Woolfolk, 1975; Vogels and Drift, 1976; Schwimmer *et al*, 1971; Roussos *et al*, 1995). Microbial systems initially adapt to caffeine containing media/environment and then biotransform it into various metabolites. Various caffeine biotransforming microorganisms belonging to *Aspergillus, Penicillium, Trichoderma, Fusarium, Humicola, Stemphylium, Pseudomonas, Serratia, Klebsiella, Rhodococcus, Acinetobacter*, *Bacillus coagulans* etc. are reported in literature. These caffeine utilizing microorganisms were isolated from soils of coffee and tea plantations, leaves of coffee and tea plants, infected coffee cherries and other infected tea and coffee plant parts. Gaime *et al* (1993) have accomplished a study to know the different groups of caffeine biotransforming microorganisms naturally occurring in coffee pulp. 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 and bacteria were found to be the predominant microorganisms. The authors 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. A comprehensive knowledge of the microorganisms able to thrive on coffee processing wastes, and caffeine tolerance of these microorganisms will help in the further development of processes towards the utilization of these wastes.

2.1.1. Biotransformation of caffeine by bacteria:

Several studies were carried out to investigate the use of caffeine, as a source of energy for growth of microorganism (Mazzafera *et al*., 1994; 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), comparing its resemblance with the caffeine metabolism pathway. Most of the studies of caffeine biotransformation pathway were carried out with bacteria isolated from soil, mainly those belonging to the *Pseudomonads* group, with particular attention to *P. putida* (Burr and Caesar, 1985). Some reports in the literature have described the isolation of bacterial strains from soil with an ability to biotransform caffeine (Asano *et al*, 1993; Asano *et al*, 1994; Woolfolk, 1975; Gluck and Lingens, 1987; Gluck and Lingens, 1988; Trijebels and Vogels, 1966; Middlehoven and Lommen, 1984; Vogels and Van der Drift, 1976;

Woolfolk and Downward, 1977; Middlehoven and Bakker, 1982; Madyastha *et al*, 1999; Gluck and Lingens, 1987; Mazzafera *et al*., 1996). Bacterial strains belonging to *Pseudomonas* and *Serratia* genus capable of degrading caffeine have also been reported by Blecher and Lingens (1977) and Mazzafera *et al* (1994). Yamaoka-Yano and Mazzafera (1998) isolated more than 20 bacterial strains from soil collected under coffee plants, observing predominance of *Pseudomonas* sp., which was also the most efficient caffeine degrader.

Asano *et al* (1993), isolated caffeine-degrading *P. putida* from soil samples by an enrichment culture technique using medium containing caffeine which was further used for the biotransformation of caffeine to theobromine. Attempts were made for biological production of caffeine biotransforming intermediates with the help of inhibitors. Asano *et al* (1993) reported the production of theobromine formed by 1*N* demethylation of caffeine in *Pseudomonas* strain. Sarath *et al* (2005) isolated a strain of *P. alcaligenes* MTCC 5264, from coffee plantation soil by enrichment technique and showed that caffeine is biotransformed to theobromine and paraxanthine and finally to other products of the caffeine metabolism pathway.

Bacteria have been 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). Ramarethinam and Rajalakshmi found in situ lowering of caffeine in tea leaves without affecting the quality of the other tea components by spraying tea plants with a suspension of *Bacillus licheniformis*. The caffeine was biotransformed to further metabolites, hence, lowering the caffeine content of the tea leaves (Ramarethinam and Rajalakshmi, 2004).

2.1.2. Biotransformation of caffeine by fungi:

Various caffeine-utilizing fungi have been reported which belong to *Aspergillus, Penicillium, Trichoderma, Fusarium, Humicola, and Stemphylium* species. Schwimmer *et al* (1971) isolated caffeine degrading fungal strains of *Penicillium roqueforti* from agar containing trace elements and caffeine, which metabolized caffeine to theophylline and further methyl xanthines. Caffeine biotransformation has been observed in fungal species like *Stemphyllium* (Kurtzman and Schwimmer, 1971), *Penicillium* sp. (Schwimmer *et al*, 1971) and *Aspergillus* sp. (Roussos *et al*, 1995). Schwimmer *et al* (1971) have identified theophylline as the first product of the caffeine biotransformation pathway in fungi. Roussos *et al* (1995) have reported the isolation of 272 strains of filamentous fungi and several bacterial and yeast species from soil, leaves of coffee plants and coffee cherries. The isolation of microorganisms was carried out in three semi synthetic culture media containing sucrose and coffee pulp extract, purified by conventional techniques and selected on the basis of their caffeine degrading ability in well defined liquid medium containing caffeine. The study gives a comparative evaluation of natural micro flora in coffee pulp and coffee husk. They found that the percentage distribution of fungi, bacteria and yeast was almost similar in all the samples except in coffee husk where the fungal population was slightly higher than in the other samples. This particular work also reveals the wide diversity in the microbial population with respect to selective media containing functional nutritional groups like cellulose, starch and pectin. Such target oriented isolation programmes opens ventures on how these isolated microorganisms can be utilized further for various purposes. From the studies conducted by Hakil *et al*

(1998) it was observed that, *Aspergillus tamari*, *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium commune* showed appreciable growth when caffeine was used as the sole source of nitrogen. *Aspergillus tamari* and *Penicillium commune* showed good caffeine degrading ability (about 60%) whereas others showed less than 20% caffeine degradation. Bioremediation of coffee pulp to reduce the caffeine content has been studied mainly through fungal systems. Among the microbial community present in coffee pulp, only a few species like *Aspergillus*, *Penicillium* and *Rhizopus* could biotransform caffeine (Roussos *et al*, 1995). *Rhizopus* sp. produced a higher quantity of biomass, whereas *Aspergillus* sp. showed more efficient caffeine biotransformation (92%). The biotransformation 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 *Aspergillus niger* the critical parameters affecting caffeine degradation were temperature and pH and the optimal values were 28°C and pH 4, 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* and it was accumulated in the fruiting bodies of *Pleurotus* sp. It has been reported that there was a shorter lag phase when the inoculum was grown in caffeine (Blecher and Lingens, 1977) suggesting the growth of inoculum in caffeine before fermentation to attain higher biotransformation rates.

Packed bed fermentation of *Rhizopus delemar* with coffee pulp as a substrate has been reported to produce theophylline and 3-methyl xanthine as the major metabolites formed by degradation of caffeine in the pulp (Tagliari *et al*, 2003; Hakil *et al*, 1998).

All the earlier work on the isolation of caffeine metabolizing microorganisms is based on enrichment techniques. The strategy of these microbes is acclimatization in the caffeine containing media and then its utilization. Besides using these isolated microbes for caffeine degradation, one more aspect, which can be ventured, is production of biotransformed compounds using these microbes and caffeine as the substrate. This is because caffeine is metabolized by subsequent demethylation of the three different methyl groups. The first step of demethylation of caffeine leads to the formation of theophylline (Tp), theobromine (Tb) or paraxanthine (Px), depending on the preference of methyl group attacked by the microorganism. These dimethyl xanthines (Tp, Tb and Px) are further transformed to mono methyl xanthines which are metabolized to form uric acid. Uric acid is then metabolized to allantoin, allantoic acid, uric acid, urea and finally to carbon dioxide and ammonia. Some of the products of caffeine biotransformation such as theophylline, theobromine, uric acid and allantoin are therauptically important compounds. Hence, the biotransformation of caffeine to these compounds using suitable caffeine metabolizing microorganisms has great scope of application. The present chapter aims at the isolation of such microorganisms, which can be used efficiently for the biotransformation of caffeine to value added products.

2.2. Materials and Methods:

Theophylline, caffeine, theobromine, 1-methyl xanthine, xanthine, uric acid, urea (99.9%) was procured from Sigma chemicals, St. Louis, USA. Caffeine (LR) used for biotransformation was purchased from Loba Chemicals, Mumbai, India. Potassium hydrogen orthophosphate, disodium hydrogen orthophosphate, calcium chloride, magnesium sulphate were of analytical grade and procured from Qualigenes Fine Chemicals, Mumbai. Ethyl acetate, butanol and acetic acid were obtained from Qualigenes Fine Chemicals, Mumbai. HPLC Grade acetonitrile and methanol were procured from Merck, Germany. Potato dextrose agar and agar agar were obtained from Himedia. All other chemicals used were of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India. Soil samples for isolation of caffeine utilizing microorganisms were collected from tea and coffee plantations.

Analytical methods:

1) Thin layer chromatography (TLC) for the identification of methyl xanthines:

TLC for the identification of biotransformed methyl xanthines was carried out using precoated TLC plates from Merck (Germany). Samples were loaded on the plates as spots and developed with a solvent system containing butanol, acetic acid, water (4:1:1). After the mobile phase was run till 1 cm below the top of the plate, the plate was removed, air dried and viewed under a UV transilluminator (CAMAG, Germany) set at 254 nm.

2) High Performance Liquid Chromatography (HPLC) for the identification of methyl xanthines:

Detection of biotransformation products of caffeine was done by HPLC on a RP C-18 column [5µm, 250mmx4.6mm, Phoenomenex] using LC-10A system

(Shimadzu, Japan) with a mobile phase containing water and acetonitrile in the ratio of 85:15, run under isocratic condition at a flow rate of 1ml/min and connected to a UV detector set at 273nm.

3) Microscopy:

Phase contrast microscopy of the fungal samples was done using, Olympus, Model BX40F4 (Japan). Scanning electron microscopy was carried out using Lieo Electron Microscope (Cambridge, England).

2.2.1. Isolation and purification of caffeine biotransforming culture:

Isolation of caffeine biotransforming microorganisms was done using the enrichment technique. Soil samples were collected from coffee and tea plantations of Mercara, Karnataka and Gudalur, Tamilnadu. Coffee husk, hull and spoiled coffee seeds were collected from Chamundi coffee curing works, Mysore. Following growth media were used for the isolation of caffeine biotransforming fungi.

a) Modified M9 media:

A medium designated as M9 (Sambrook *et al*, 1989) containing the following constituents in gm/L: $Na_2HPO_4.7H_2O$ - 1.5; KH_2PO_4 - 2.0, NaCl - 0.25, Caffeine - 0.1, pH - 5.6, fortified with 20 gm/L of agar. The soil samples were serially diluted in sterile distilled water, poured in the isolation media mentioned above and incubated at 28±2°C and allowed to grow for 7 days. Well-isolated colonies were picked and subjected to further purification. Pure cultures of fungi were obtained after successive transfers of individual colonies and incubation for 72 hours at 28±2°C. The purified strains were further stored in PDA slants containing 0.03% caffeine.

b) Caffeine agar media:

Soil sample (1 gm) and coffee pulp (1 gm) were taken individually and mixed well with 10 ml each of sterile distilled water. Ten-fold serial dilutions (10^{-1} to 10^{-9}) of the above suspension were made and 0.1 ml of the dilutions was inoculated on agar plates fortified with 1gm/l of caffeine, incubated at 28±2°C and allowed to grow for 7 days. Isolated colonies growing on the above plates were selected and purified further to obtain pure cultures. The isolated fungi were then maintained on PDA slants containing 0.03% caffeine.

2.2.2. Screening of isolated cultures for caffeine biotransformation:

The selection of fungi for biotransformation was done in the screening media. 72-hour-old slant of the isolated cultures were prepared and inoculated (spore suspension) into 100ml of screening media [having composition in (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; caffeine – 1; sucrose – 1] in 500ml Erlenmeyer flask and incubated in a rotary shaker at 150 rpm and (28±2°C) for 240 hours. Samples were collected at regular intervals and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was then subjected to TLC and HPLC analysis to check for caffeine biotransformation.

Identification of biotransformed methyl xanthine:

After fermentation, the biomass was separated from the fermented broth by filtration followed by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was then subjected to TLC and HPLC for identification of biotransformed methyl xanthines formed during the biotransformation of caffeine by

the isolated fungal cultures. The biotransformed methyl xanthines were compared with methyl xanthine standard using TLC and HPLC methods.

2.2.2.1. Screening of isolated cultures for biotransformation of caffeine to theophylline:

Spore suspension of 72 hour old slants of the isolated strain were inoculated into biotransformation media [having composition in (g/L) Na_2HPO_4- 0.12; KH_2PO_4 -1.3; $MgSO_4$ - 0. 3; $CaCl_2$ - 0.3; Sucrose -5.0; caffeine - 1.0] into 500ml conical flask containing 100 ml media. The flasks were then incubated in a rotary shaker at 150 rpm at 28±2°C. Samples were collected at regular intervals and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was then subjected to TLC and HPLC analysis to check for biotransformation of caffeine to theophylline.

2.2.3. Characterization and identification of the isolates:

The culture, which gave maximum biotransformation of caffeine to theophylline, was characterized and identified. To obtain data on the description and identification of the strain, the culture was grown on potato dextrose agar medium, observed under microscope for morphological characteristics and compared by reference classical keys reported in the literature (Bernet and Hunter, 1972; Ainsworth 1973; Riddel, 1950). Morphological and microscopical characters of the isolated culture were studied and recorded in Table 2.3.5.

2.2.4. Microscopy studies:

1) Phase contrast microscopy:

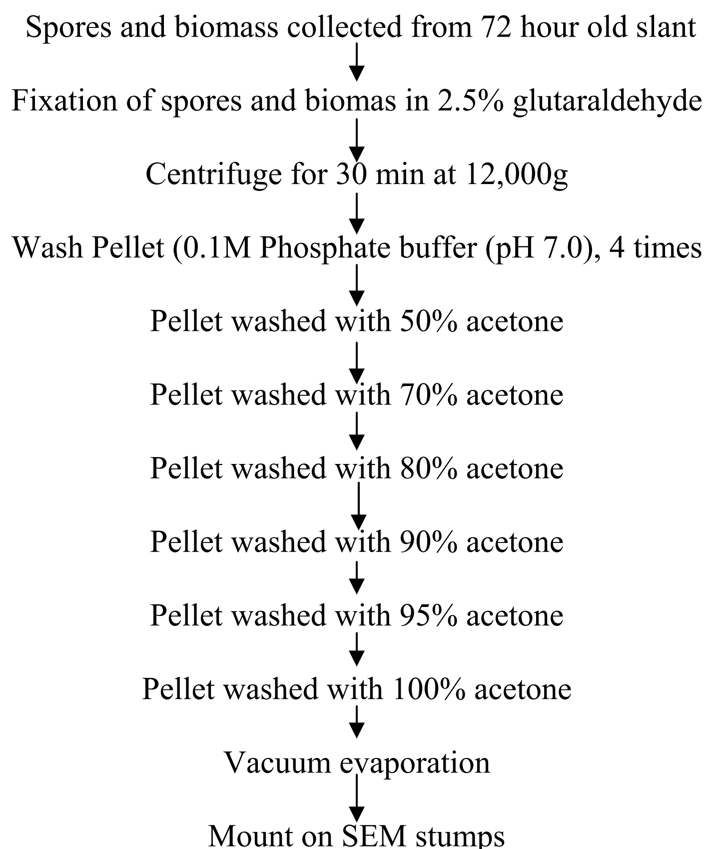
72 hour old slant of the chosen culture was inoculated into the biotransformation media and grown for a period of 48 hours. The suspended biomass from the liquid culture was taken and washed thoroughly with phosphate buffer (pH

7.2. 0.1 M). The biomass was further incubated with cotton blue stain and used for microscopy studies using phase contrast microscope, Olympus, Model BX40F4 (Japan).

2) Scanning electron microscopy (SEM):

The most potent culture was grown on PDA containing 0.03% caffeine and incubated for 72 hour. Fully grown fungal mycelia of the 72 hour old slant was used for scanning electron microscopy studies. SEM was done using LV 435 VP (Lieo Electron Microscope, England). The protocol for SEM studies as described by Samson and Staplers (1977) was followed and is given below.

Sample preparation for scanning electron microscopy.



2.3. Results and Discussion:

A selective and directed screening method based on enrichment technique was adopted for the selection of microorganisms capable of biotransforming caffeine and several fungal strains were isolated from coffee and tea plantation soils.

2.3.1. Isolation and purification of caffeine biotransforming culture:

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 as they have been acclimatized to the caffeine leached into the soil. In the present study, 34 fungal cultures were isolated from coffee and tea plantation soil capable of biotransforming caffeine. They were designated F1 to F34 and have been represented in figure 2.3.1a and figure 2.3.1b. This is because caffeine leaches out from the fallen plant parts of coffee and tea plant and is accumulated in the soil in soluble form (Friedman and Waller, 1983a). Caffeine is inhibitory to most microorganisms and only those microorganisms which can metabolise caffeine could grow in its presence in the soil (Frischknecht *et al*, 1985). Besides this, the media used for isolation of fungal cultures contained caffeine, which helps in elimination of microorganisms, not having caffeine tolerance (Sunderraj and Dhala, 1965; Putrament *et al*, 1972; Kihlman 1974). In the caffeine agar media only those microorganisms could grow which could utilize caffeine as the sole source of carbon and nitrogen. Hence, only the caffeine metabolizing fungal cultures were isolated.

The isolated cultures were further screened for their caffeine biotransformation ability in caffeine containing liquid media.

Figure 2.3.1a. Caffeine biotransforming fungal cultures isolated from coffee and tea plantation soil.

Figure 2.3.1b. Caffeine biotransforming fungal cultures isolated from coffee and tea plantation soil.

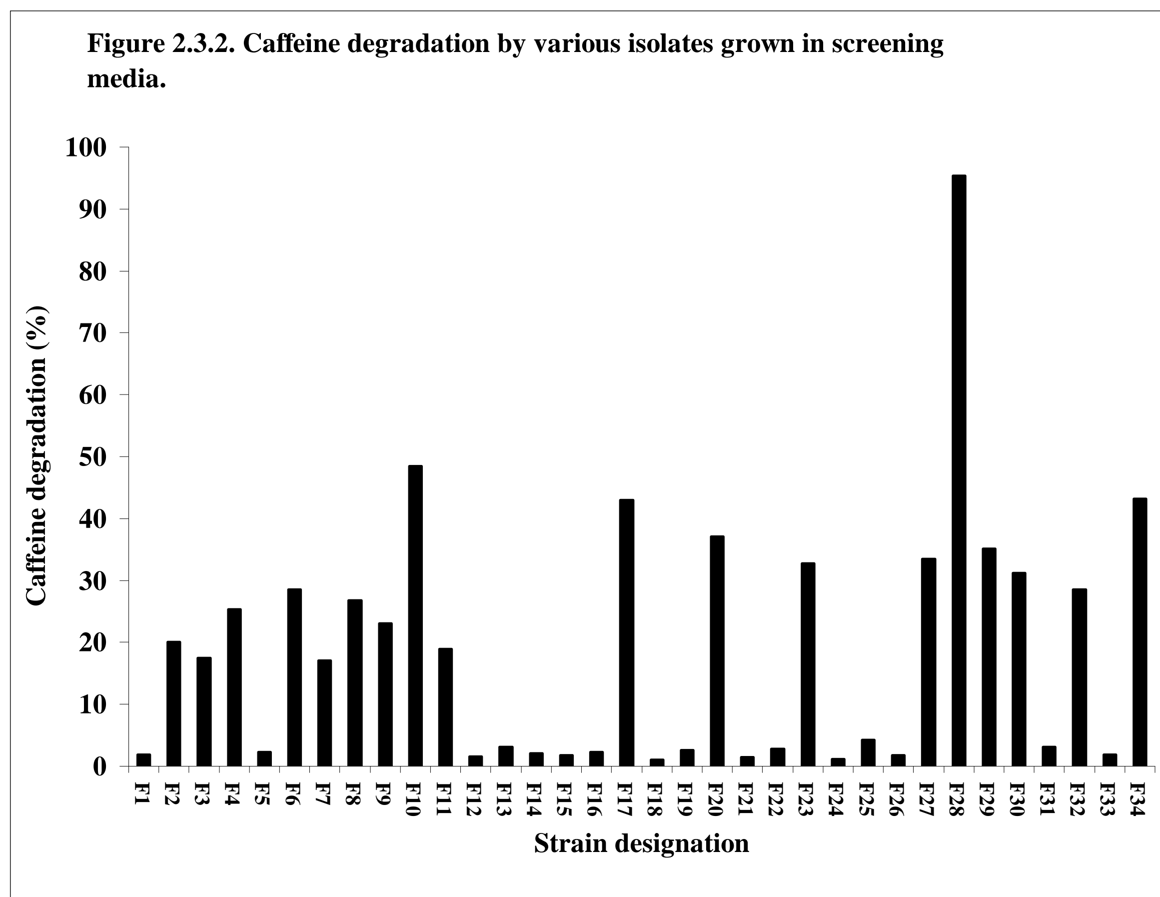
2.3.2. Screening of isolated cultures for caffeine biotransformation:

It was observed that all 34 isolated fungal cultures could grow in the screening media and metabolize caffeine. Of the 34 isolated strains, 18 strains could biotransform caffeine to theophylline (Table 2.3.3). Table 2.3.3. also shows the isolation source of the caffeine biotransforming strains. It was observed that besides theophylline, 1-methyl xanthine and xanthine were also produced by the isolate F28. The isolates which could biotransform caffeine to theophylline can be used for the production of theophylline by biotransformation of caffeine and will be discussed in the upcoming chapters of this thesis.

Figure 2.3.2. represents the efficiency of caffeine metabolism by the isolated strains in the screening media. F28 was the most efficient strain metabolizing 95.4% of caffeine. The other strains utilizing caffeine reasonably efficiently were F10 (50%), F17 (44.2%), F20 (38%), F23 (34%) and F34 (43%). These caffeine metabolizing cultures have the potential of utilization in the decaffeination of coffee processing wastes such as coffee pulp and coffee hull leading to detoxification of these processing wastes. Besides, these caffeine tolerant fungal isolates can also be used for the production of industrially important enzymes in solid state fermentation (SSF) using coffee processing waste as substrate.

Table 2.3.2. Source of isolation and caffeine biotransformation ability of the fungal isolates.

S. No.	Strain designation	Source of isolation	Biotransformed compounds
1.	F2	Tea plantation soil	Theophylline
2.	F3	Spoiled coffee seeds	Theophylline
3.	F4	Spoiled coffee seeds	Theophylline, unidentified product
4.	F5	Coffee plantation soil	Theophylline
5.	F6	Tea plantation soil	Theophylline
6.	F7	Coffee plantation soil	Theophylline, unidentified product
7.	F8	Dumped coffee pulp	Theophylline
8.	F9	Spoiled coffee seeds	Unidentified product
9.	F10	Coffee plantation soil	Theophylline
10.	F11	Coffee plantation soil	Unidentified product
11.	F17	Tea plantation soil	Theophylline
12.	F20	Tea plantation soil	Theophylline
13.	F23	Dumped coffee pulp	Theophylline
14.	F27	Dumped coffee pulp	Theophylline
15.	F28	Coffee plantation soil	Theophylline, 1-methyl xanthine, xanthine, uric acid, allantoin, allantoic acid
16.	F29	Dumped coffee pulp	Theophylline
17.	F30	Dumped coffee hull	Theophylline
18.	F32	Tea plantation soil	Theophylline
19.	F34	Coffee plantation soil	Theophylline



2.3.3. Screening of isolated cultures with the capability of biotransformation of caffeine to theophylline:

While all the isolated strains were able to utilize caffeine during growth, theophylline was produced only by 18 strains (Table 2.3.3 and Fig: 2.3.3). Among these, the most potent strain was F28, showing 80% conversion of caffeine to theophylline (Fig 2.3.4). Other strains, which could biotransform caffeine to theophylline, were F7 (35%), F20 (25%), F27 (23%), F29 (22%) and F34 (20%). It has been reported that the biotransformation of caffeine occurs via theophylline in fungi (Schwimmer *et al*, 1971; Kurtzman and Schwimmer, 1971). Schwimmer *et al* (1971) reported the biotransformation of caffeine to theophylline by *Penicillium roqueforti* grown in a media containing sucrose and caffeine. However, the study

was confined to the identification of the biotransformed products of caffeine by the fungal strain and no work was carried out towards the production of theophylline. The isolated potent caffeine metabolizing strains can be used for the commercial production of theophylline because of the demand for biotransformed theophylline as compound of therapeutic importance. The present work of production of theophylline by biotransformation is the first ever report.

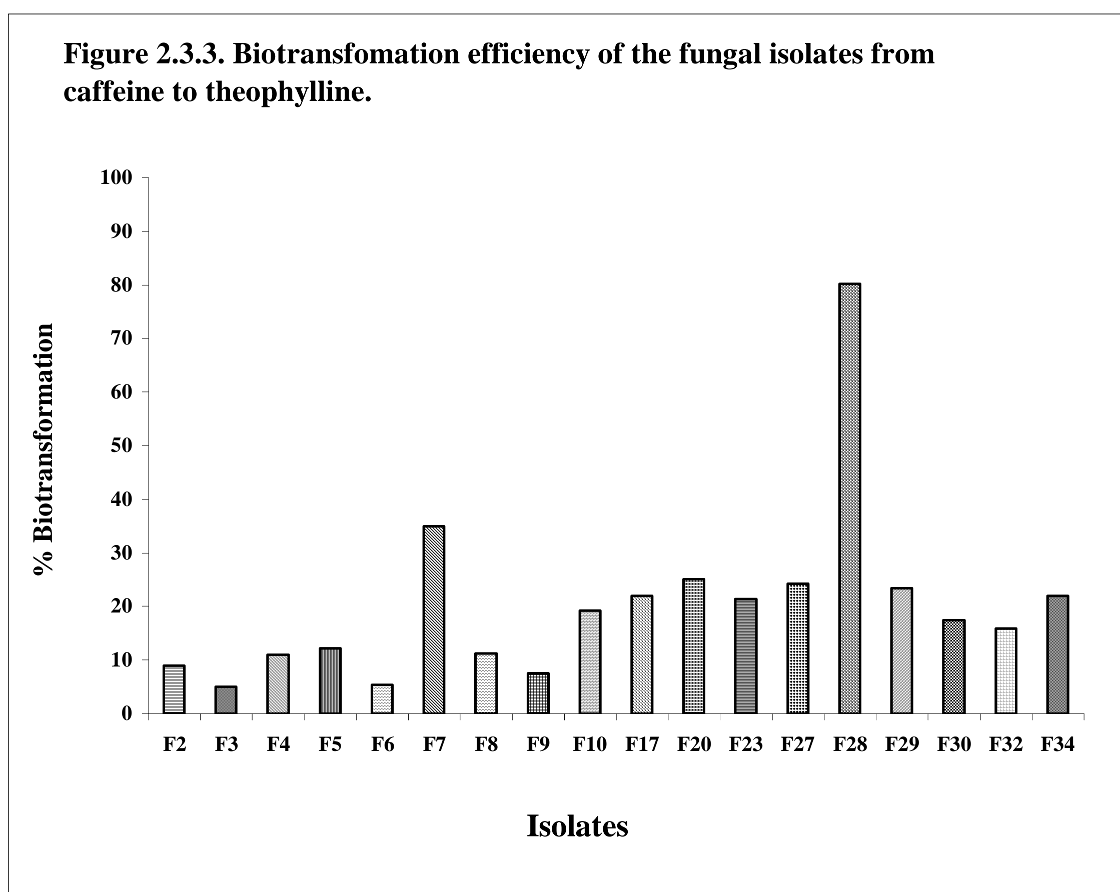


Figure 2.3.3. Biotransfomation efficiency of the fungal isolates from caffeine to theophylline.

2.3.4. Characterization and identification of the isolates:

The isolated caffeine biotransforming fungal strains were identified to the genus level. Table 2.3.4. shows the total number of strains belonging to different genus. Maximum number of strains (10) belonged to genus *Aspergillus*. The next

abundant genus was *Rhizopous* and *Penicillium* having six and five strains respectively. The most potent biotransforming strain F28 was identified as *Penicillium citrinum*.

Table: 2.3.4. Identification of caffeine biotransforming isolates.

Sl.No	Genus	Total No. of strains	Isolate designation.
1.	*Penicillium*	**5**	**F3, F6, F15, F28, F34**
2.	*Aspergillus*	**10**	**F7, F8, F9, F11, F23, F26, F27, F30, F31F33**
3.	*Fusarium*	**1**	**F16**
4.	*Cladosporium*	**1**	**F25**
5.	*Rhizopous*	**6**	**F2, F4, F20, F24, F25, F32**
6.	*Neurospora*	**1**	**F29**
7.	*Trichoderma*	**1**	**F19**
8.	*Basidiomycetes*	**2**	**F13, F14**
9.	Yeast	**2**	**F1, F17**
10.	Unidentified	**5**	

2.3.5. Cultural and morphological characteristics of the isolate F28:

The most potent isolate F28 showing 80% conversion of caffeine to theophylline was characterized. Different cultural and microscopic studies of the selected strain were carried out and the fungal strain was identified to the species level (Bernet and Hunter, 1972; Raper *et al*, 1949, Ainsworth, 1973). The details of characterization of the strain are given in Table 2.3.5. It was identified to be *Penicillium citrinum*. The culture was deposited in MTCC patent culture deposit under the Budapest treaty and assigned the accession number MTCC 5215. Yellow pigmentation was observed at the reverse of the culture slants, a typical feature of *Penicillium* sp. Figure 2.3.5.1. represents the culture of *P. citrinum* showing yellow pigmentation at the reverse of the colony. The mycelium is green in colour and has velvety texture (Fig. 2.3.5.2). The colony was initially white in colour and turned

greenish after 48 hours of growth. The vegetative hypha was observed to be creeping, septate and branched in nature (Fig. 2.3.5.3). Conidiophore was found to arise directly from the substratum, it was observed to be erect, septate and comparatively short in length as seen from Figure 2.3.5.3. The conidiophore was unbranched and terminated by clusters of flask-shaped phialides five in number as shown in figure 2.3.5.4 and figure 2.3.5.5. The spores (conidia) were produced in chains from the tips of the phialides, with the youngest spore at the base of the chain, and were green in colour, globose and granular in appearance as shown in figure 2.3.5.5.

Table 2.3.5. Characterization of the isolate capable of transforming caffeine to theophylline.

Observation	Morphological, cultural characterisitcs	Observation
Colony characteristics	Growth	Restricted
	Character of growth	Velvety
	Character of margin	Undulate
	Amount of sporulation	Heavy
	Colony colour	Green
	Colour changes during growth period	Light to dark green
	Transpired drops (exudates), abundance and color	Low, yellow
	Odour	Moldy
	Colony reverse, colour and colour changes	Yellow, Yellow to brown
Microscopic characteristics	Vegetative hyphae	Creeping, septate, branched
Conidial stage	Penicilli	Consisting of terminal cluster of conidiophores
	Colour	Green

Conidiophore	Origin and character	Conidiophore arising directly from the substratum Erect, Septate, Comparatively short, 150μ in length
	Branches	None
Sterigmata	Number	Five
Identification	*Penicillium citrinum*	

Figure 2.3.5.1. Pure culture of *P. citrinum* MTCC 5215 growing on potato dextrose agar showing yellow pigmentation at the reverse of colony.

Reverse of colony of *P. citrinum* MTCC 5215 showing yellow pigmentation.

Figure. 2.3.5.2. *P. citrinum* MTCC 5215 growing on potato dextrose agar containing 0.03% caffeine showing green colony colour and yellow exudates.

Figure 2.3.5.3. Light microscopic photograph of *P. citrinum* MTCC 5215 stained with cotton blue showing branched, vegetative and septate hyphae.

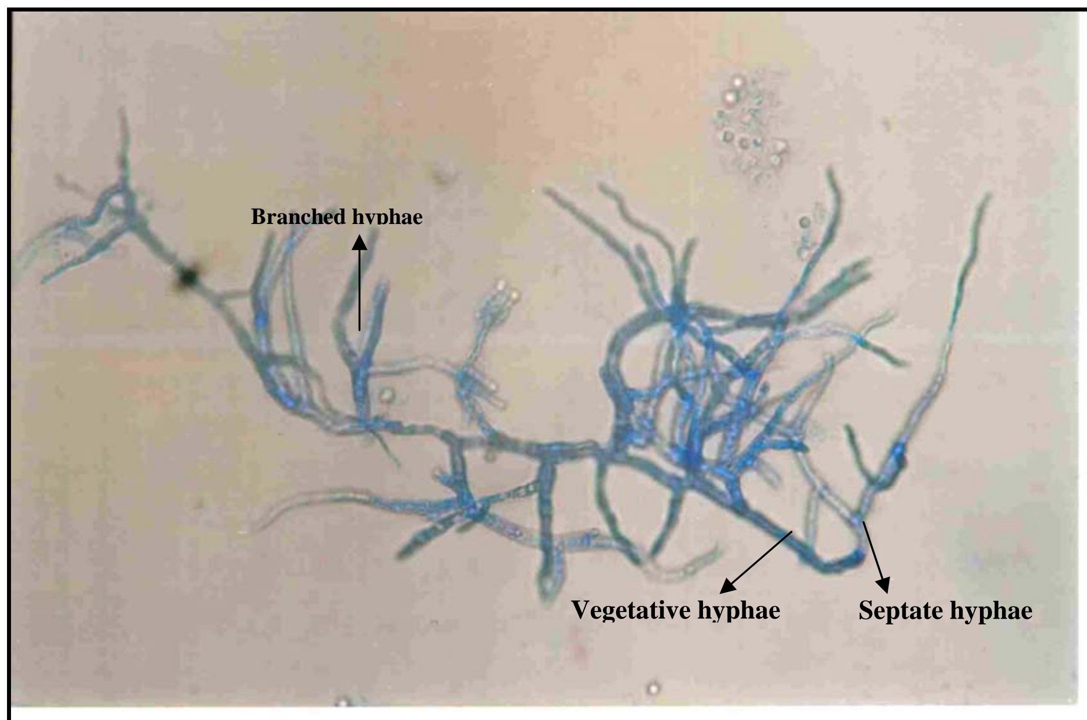


Figure 2.3.5.4. Dark field micrograph of *P. citrinum* MTCC 5215 showing conidiophore arising directly from the substratum.

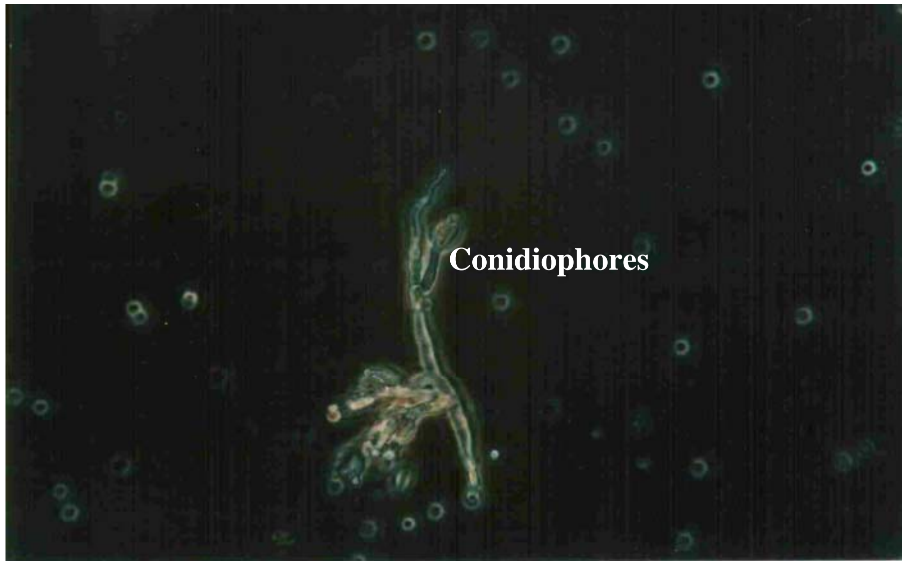


Figure. 2.3.5.5. Scanning electron micrograph of *P. citrinum* MTCC 5215.

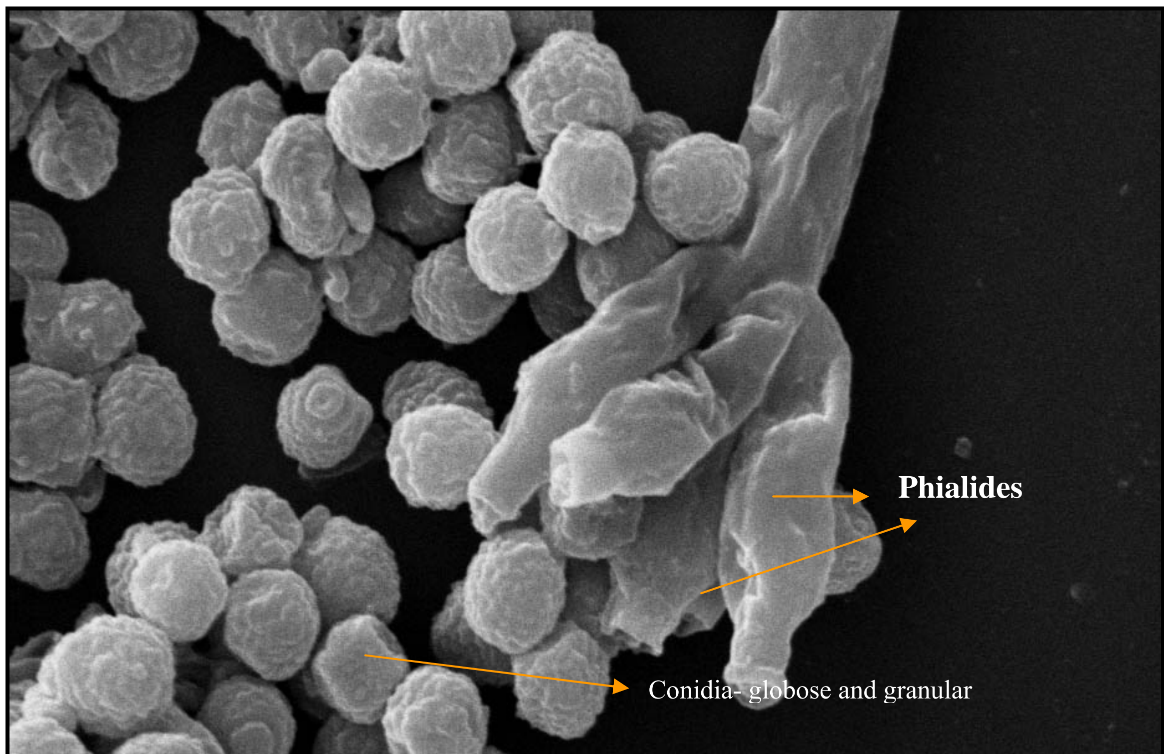


Figure 2.3.5.5. shows scanning electron microscopic pictures of *P. citrinum* MTCC 5215 with unbranched conidiophore arising from submerged hyphae with compact verticil of five phialides – an identifying feature of the citrinum species. Further it shows penicilli consisting of one series of metulae containing phialides without branches - an identifying feature of sub section Asymmetrica.

Taxonomic Classification of *Penicillium citrinum*.

Kingdom : Fungi
Phylum : Ascomycota
Class : Euascomycetes
Order : Eurotiales
Family :Trichomaceae
Genus : *Penicillium*
Species : *Citrinum*

Identification features of the species observed from morphological studies:

Colony on PDA, showed bluish green colour, becoming brownish-olive when old, with sterile white margin (Fig 2.3.5.2.). The reverse of colony developed yellow pigmentation after 72 hours of growth (Fig 2.3.5.1.). Aerial part of colony consisted of densely standing conidiophores, where tufts of aerial hyphae arise (Fig 2.3.5.3.). Conidiophores were observed to arise separately from submerged hyphae or from mycelium on the surface, producing a compact verticil of phialides, 5 in number (Figure. 2.3.5.5.). The conidial chains were formed in columns, a separate column arising from each verticil of cells. They were 3 µ in size, green in colour, globose and granular in nature (Fig: 2.3.5.5).

Conclusion:

34 fungal cultures were isolated from soil samples of tea and coffee plantations, which were capable of metabolizing caffeine and they were designated as isolates F1-F34. The most efficient caffeine utilizing strain (F28) metabolised 95.4% caffeine. The other strains utilizing caffeine reasonably efficiently were F10 (50%), F17 (44.2%), F20 (38%), F23 (34%) and F34 (43%). The isolated caffeine metabolizing strains were further used for the production of enzymes in solid-state fermentation using coffee pulp as substrate as will be discussed in Chapter-5. Out of these 34 strains, 18 strains could biotransform caffeine to theophylline in the screening media. F28 was found to be the most potent strain for the biotransformation of caffeine to theophylline. It showed biotransformation efficiency of (80%). This isolate was further characterized and was identified as *P. citrinum* MTCC 5215 based on various cultural and microscopic characteristics. This was found to be an efficient biotransforming strain and further work on the production of theophylline was carried out with this strain. This particular caffeine metabolizing strain was also used for biodecaffeination of coffee processing wastes as coffee pulp and coffee hull and will be discussed in subsequent chapters.

References:

Ainsworth, G. C., (1973), Introduction and keys to the higher taxa. In: The Fungi: An advanced treatise**. 4a**: A taxonomic review keys: Academic Press, New York.

Asano, Y., Komeda, T., and Yamada, H., (1993), Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.* **57**:1286–1289.

Asano, Y., Toshihiro, K., and Yamada, H., (1994), Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No. 352. *Biosci. Biotechnol. Biochem.* **58**:2303–2304.

Bernet, H. L., and Hunter, B. B., (1972), Illustrated general of imperfect fungi, 3rd edn, Burgels, Minneapolis.

Blecher, R., and Lingens, F., (1977), The metabolism of caffeine by a *Pseudomonas putida* strain. *Hoppe-Seyler's Z. Physiol. Chem.* **358**:807–817.

Brand, D., Pandey, A., Roussos, S., and Soccol, C. R., (2000), Biological detoxification of coffee husk by filamentous fungi using a solid-state fermentation system. *Enz. Microb. Technol.* **27**:127–133.

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

Escohotado, A., and Ken, S., (1999), A *Brief History of Drugs: From the Stone Age to the Stoned Age*. Park Street Press.

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

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

Frischknecht, P. M., Ulmer, D. J., and 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.

Gaime-P., Roussos, S., and Martinez-Carrera, D., (1993), Natural microorganisms of the fresh coffee pulp. *Micol. Neotrop. Apl*. **6**: 95-103.

Gluck, M., and Lingens, F., (1987), Studies on the microbial production of the theobromine and heteroxanthine from caffeine. *Appl. Microbiol. Biotechnol.* **25**:334–340.

Gluck, M., and Lingens, F., (1988), Heteroxanthine demethylase, a new enzyme in the degradation of caffeine by *Pseudomonas putida*. *Appl. Microbiol. Biotechnol.*

28: 59–62.

Hakil, M., Denis, S., Gonzalez, G.V., and Augur, C., (1998), Degradation and product analysis of caffeine and related dimethyl xanthines by filamentous fungi. *Enz. Microb. Technol* . **22**:355–359.

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

Kumar, N., Pandey, S., Bhattacharya, A., and Ahuja, P. S. (2004) Do leaf surface characteristics affect *Agrobacterium* infection in tea [*Camellia sinensis* (L.) O. Kuntze]? *J. Biosci*. **29** (3): 309–317.

Kurtzman, R.H., and Schwimmer, S., (1971), Caffeine removal from growth media by microorganism. *Experientia.* **27**:481–482.

Leifa, F., Pandey, A., and Soccol, C. R. (2000), Solid-state cultivation—an efficient method to use toxic agro-industrial residues. *J. Basic Microbiol.* **40**(3):187–197.

Madyastha, K.M., Sridhar, G.R., Bhat, B.V., and Madhavi, S.Y., (1999), Purification and partial characterization of caffeine oxidase. *Biochem. Biophys. Res. Commun*. **263**:460–464.

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

Middlehoven, W.J., and Bakker C. M., (1982), Degradation of caffeine by immobilized cells of *Pseudomonas putida* strain C3024. *Eur. J. Appl. Microbiol. Biotechnol.* **15**:214–217.

Middlehoven, W. J., and Lommen, A., (1984), Degradation of caffeine by *Pseudomonasputida* C3024 the effect of oxygen concentration. *Antonie Van Leeuwenhock.* **50**:298–300.

Nathanson, J. A., (1984), Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science.* **226** (4671): 184-187.

Putrament, A., Baranowska, H., Bilinsky, T., and Prazmo, W., (1972), On the specificity of caffeine effects. *Mol. Gen. Genet.* **118**:373–379.

Ramarethinam, S., and 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.

Raper, K. B., Thomas, C., and Fennel, D. I., (1949), Chapter-IX Asymmetrica-Velutina In: Manual of Penicillia. The Williams and Wilkins Company, Baltimore, U.S.A.

Riddel, R.W., (1950), Permanent stained mycological preparations obtained by slide culture. *Mycologia*. **42**:265-270.

Roussos, S., Angeles-A. M. D. L., Trejo-Hernandez, M. D. R., Gaime-Perraud, I., Favela, E., and 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**:756–762.

Sarath Babu, V. R., Patra, S., Thakur, M.S., Karanth, N.G., and Varadaraj, M. C., (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708, *Enz. Microb. Technol*. **37**: 617-622

Salmones, D., Mata, G., and Waliszewski, K. N., (2005), Comparative culturing of *Pleurotus* sp. on coffee pulp and wheat straw: biomass production and substrate biodegradation. *Bioresour. Technol*. **93**:537–544.

Sambrook, J., Fritsch, E. F., and Maniatis, T., (1989), Bacterial cell maintenance. Molecular cloning: a laboratory manual, **Vol: 1**. New York: Cold Spring Harbor laboratory Press.

Samson, R. A., and Staplers, J. A., (1977), Preparation techniques of fungal specimens for scanning electron microscopy. Abstracts Annual Congress of the Netherlands Society for Electron Microscopy. November-p 140

Schwimmer, S., Khurtzman, R. H., and Heftmann, E., (1971), Caffeine metabolism by *Penicillium roqueforti*. *Arch. Biochem. Biophys*. **147**:109–113.

Silva, C. F., Scwan, R. F., Dias, E. S., and 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.

Sundarraj, C.V., and Dhala, S., (1965), Effect of naturally occurring xanthines on bacteria (I). Antimicrobial action and potentiating effect on antibiotic spectra. *Appl. Microbiol.* **13**:432–436.

Suzuki, T., and Waller, G. R., (1988), Metabolism and analysis of caffeine and other methyl xanthines in coffee, tea, cola, guarana and cacao. In: *Modern Methods of Plant analysis.New Series,* H. F. Linsken and J. F. Jackson, eds **8**: 184-220, Berlin, Heidelberg: Springer-Verlag.

Tagliari, C.V., Sanson, R. K., Zanette, A., Franco, T.T., and 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.

Trijebels, F., and Vogels, G. D., (1966), Degradation of allantoin by *Pseudomonas acidovorans*. *Biochim. Biophys. Acta*. **113**:292–301.

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

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.

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

Yamaoka-yano, D. M., and Mazzafera, P. (1998), Degradation of caffeine by *Pseudomonas putida* isolated from soil. *Allelopathy Journal*. **5**: 23-34.

CHAPTER -3

BIOTRANSFORMATION OF CAFFEINE BY Penicillium citrinum MTCC 5215

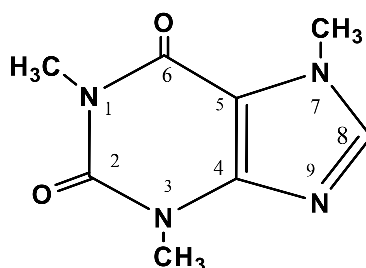
3.0. Scope of the work:

Caffeine is naturally present in a variety of plants such as *Coffea arabica* and sixty other plant species. The abundance of caffeine in several plants, food products and beverages leaves a wide scope for value addition to the molecule through biotransformation into therapeutically and commercially valuable products. Caffeine is biotransformed in different living systems by different enzyme systems leading to the formation of various methyl xanthine products such as theophylline, theobromine, paraxanthine, 1-methyl xanthine, uric acid, allantoin etc. The caffeine biotransformation pathway in bacterial system has been elucidated and extensively studied. However, the caffeine biotransformation pathway of fungi has not been fully worked out yet. The present chapter aims at the identification of all the products of caffeine biotransformation pathway, their respective enzymes and the complete pathway elucidation in *Penicillium citrinum* MTCC 5215. A comprehensive knowledge of the biotransformation products and their respective enzymes will help in the development of processes for the production of biotransformed products having therapeutic importance. In the present study the enzyme responsible for biotransformation of caffeine to theophylline was identified as cytochrome P450 and the complete pathway of caffeine biotransformation in fungi has been elucidated for the first time.

3.1. Introduction:

Caffeine has been consumed and enjoyed by humans throughout the world for centuries. It is the world's most widely consumed psychoactive substance taken in the form of beverages such as coffee, tea, soft drinks and energy drinks. The widespread natural occurrence of caffeine in a variety of plants undoubtedly played a major role in the long-standing popularity of caffeine-containing products, especially beverages. It was during the last century that consumers became exposed to caffeine as a food additive (cola drinks) as well. It is biotransformed by various living species such as bacteria, fungi, plants, earthworms and other animal species by the sequential demethylation of the methyl groups in varying order. The position of various methyl groups in the caffeine molecule is shown in scheme 3.1. The biotransformed products vary from one living system to another as the enzymes involved in the different biotransformation steps vary. The biotransformation pathway of caffeine leads to the formation of many methyl xanthines and other compounds of therapeutic importance; hence the biotransformation of caffeine can be exploited for the production of such compounds.

Scheme: 3.1. Structure of caffeine showing positions of methyl group.



Caffeine

3.1.1. Biotransformation of caffeine in plants:

Caffeine is the end product of a long biosynthetic pathway and can be catabolized by both tea and coffee plants. It has been reported that caffeine has a metabolic half-life of only a few months in tea plants. Caffeine is degraded at a variety of rates in different species of higher plants via a series of demethylation steps. Ashihara *et al* (1996) demonstrated that catabolism of caffeine in leaves of the coffee plant, *C. arabica*, involves the following steps: caffeine →theophylline → 3-methylxanthine→xanthine pathway. Xanthine is further degraded in the conventional purine catabolism pathway (via uric acid, allantoin, and allantoate) to CO_2 and NH_3. The demethylation reactions are 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 (Ashihara *et al*, 1997; Koyama *et al*, 2003; Ashihara *et al*, 1996; Mazzafera, 1993, Vitoria and Mazzafera, 1998). The slow degradation of caffeine compared with that of theophylline indicates that the conversion of caffeine to theophylline is a major rate limiting step in the catabolism of caffeine, and provides a ready explanation for the high endogenous caffeine content of *C. arabica* leaves (Ashihara *et al*., 1996). It is now known that theophylline is associated primarily with caffeine biodegradation, whereas theobromine is involved in both biosynthesis and biodegradation of caffeine (Ashihara *et al*., 1999). Both immature and mature coffee fruits and leaves degrade caffeine to theobromine, theophylline, *N*-3 methylxanthine, *N*-7 methylxanthine, xanthine, allantoin, allantoic acid, and urea (Kalberer, 1965; Suzuki and Waller, 1984a; Ashihara *et al*., 1996). Suzuki and Waller (1984b) studied the pattern of

formation of theobromine, theophylline, and caffeine in coffee berries during the growth stages of the bean, and showed that theophylline arises from the breakdown of caffeine in excised, mature coffee berries.

3.1.2. Biotransformation of caffeine in mammals:

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; Caubet *et al*, 2004; Wreck-Reichhart and Feyereisen, 2000). Methylated xanthines and their respective uric acids formed due to degradation are excreted from the body through urine (Rao *et al*, 1973). In the case of human beings, 1-*N* and 3-*N* demethylation occurs and leads to the formation of theophylline, theobromine and paraxanthine, which is excreted in the urine (Warrens, 1969; Woon-Gye and Young-Nam, 1997). The metabolism of methylxanthines primarily occurs in the liver. In adults, nearly 98% of an oral dose is found to be excreted in the urine and 1-3% in the faeces (Arnaud, 1985). Less than 2 % of the ingested caffeine is excreted unchanged in the urine. At least 98 % is transformed in the liver by demethylation, oxidation at the 8-position in the purine ring, or ring opening between positions 8 and 9 to yield di-and monomethylxanthines, tri-, di-, and monomethyluric acids, and various methylated uracil derivatives (Arnaud and Welsch, 1980; Arnaud, 1984, Kalow, 1985). Most of these metabolites are further metabolized to secondary and tertiary metabolites. The most important step in the biotransformation of caffeine in man is the removal of the 3-methyl group to yield 1,7-dimethylxanthine or paraxanthine. Metabolism of caffeine to paraxanthine usually represents almost 80 %

of the primary caffeine metabolism (Callahan *et al*., 1982; Callahan *et al*., 1983), but different factors affecting this reaction may change the kinetics of caffeine metabolism. Paraxanthine is further oxidized to 1-methylxanthine and 1,7-dimethyluric acid, or is acetylated to 5-acetyl-6-formylamino-3-methyluracil (Callahan *et al*., 1982, Branfman *et al*., 1983; Tang *et al*., 1983; Kalow, 1985). A substantial part of the 1-methylxanthine formed is hydroxylated to 1-methyluric acid. In human urine, paraxanthine represents about 11 % of the total metabolites of caffeine, 1,7-dimethylxanthine 14%, 1-methylxanthine 24%, 5-acetyl-6-formylamino-3-methyluracil 10%, and 1-methyluric acid 28% (Cornish and Chistman, 1957; Branfman *et al*., 1983; Tang *et al*., 1983; Callahan *et al*., 1982; Callahan *et al*., 1983). Minor primary metabolites of caffeine are theobromine, theophylline, and 1, 3, 7-trimethyluric acid, which are formed in reactions of 1-*N*-demethylation, 7-*N* demethylation, and 8-hydroxylation, respectively. Knowledge about the enzymology of caffeine metabolism in human has increased during recent years. CYP1A2 is the most essential enzyme in the metabolism of caffeine, although other CYP enzymes, flavin monooxygenase, and *N*-acetyltransferase are also involved in its metabolism (Kalow, 1985; Butler *et al*., 1989; Berthou *et al*., 1992; Fuh *et al*., 1992; Tassaneeyakul *et al*., 1994; Chung and Cha, 1997). CYP2E1, CYP3A4 and mutant CYP2D6 are three other CYP enzymes able to metabolise caffeine, but with lower affinity for the compound, and, therefore, have roles at physiological relevant caffeine concentrations in human beings. The fifth enzyme in humans that can oxidise caffeine is CYP1A1. However, this enzyme is nearly absent in the liver, and may be induced by compounds acting via the Ah-receptor, as for example compounds

occurring in tobacco (Parkinson, 1996). Flavin monoxygenase is able to catalyse *N*-1 and *N*-7demethylations of caffeine, but not the other primary reactions, whereas only *N* acetyltransferase 2 are able to conjugate acetyl groups to caffeine (Kalow, 1985; Hardy *et al*., 1988; Chung and Cha, 1997). *N*-acetyltransferase 2, on the other hand, forms 5-acetylamino-6-formylamino-3-methyluracil by acetylating paraxanthine (Kalow 1985; Ohsako and Deguchi, 1990; Grant *et al*., 1991). Thus, it can be concluded that several enzymes are involved in the metabolism of caffeine, but quantitatively CYP1A2 is the key and most important enzyme in the metabolism and kinetics of caffeine. It is noteworthy that CYP1A1, CYP1A2, CYP2D and CYP2E1 are able to catalyze several different types of primary metabolic reactions of caffeine. The product pattern produced by CYP1A2 from rats, mice and humans acting on caffeine varies (Fuh *et al*., 1992). Human CYP1A2 predominantly *N*-3 demethylates caffeine to paraxanthine and produces less than 15 % other products. Paraxanthine is also the major product in the catalytic reaction of mouse CYP1A2 but here, theobromine represents about 25 %, and theophylline and 1,3,7-trimethyluric acid together about 10 % of the total metabolism. The rat CYP1A2, however, metabolises caffeine to equal parts of paraxanthine and theobromine (about 40 % each), less than 20 % theophylline, and and only a little 1,3,7-trimethyluric acid.

3.1.3. Biotransformation of caffeine in bacteria:

The enzymes involved in the degradation of caffeine in microorganisms are demethylases and oxidases (Asano *et al*, 1994; Hohnloser *et al*, 1980; Yamoka and Mazzafera, 1999; Yamoka and Mazzafera, 1998). In bacteria (*Pseudomonas*), caffeine is initially converted into theobromine and paraxanthine parallely, by

demethylases. Further demethylation forms xanthine with 7-methyl xanthine as the intermediates. There is also an evidence of oxidation of xanthine, monomethyl and dimethyl xanthines to their respective uric acids, 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 (Mazzafera *et al*, 1994). In case of bacteria, 1-*N* demethylation occurs leading to the formation of theobromine (Asano *et al,* 1993, Sarath Babu *et al*, 2005). 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 was possible (Madyasta and Sridhar, 1998; Madyasta *et al*, 1999).

The pathway of caffeine biotransformation in bacteria has been elucidated as:

Caffeine → theobromine/paraxanthine/1,3,7 trimethyl xanthine → 7 methyl xanthine/3 methyl xanthine → xanthine → uric acid → allantoin → allantoic acid → urea → CO_2 + NH_3.

3.1.4. Biotransformation of caffeine in yeast:

Birkett (1981) has worked out caffeine metabolism in yeast, which is similar to that in humans. Cyt P450 was detected in enzyme extracts of caffeine degrading yeast by Sauer *et al* (1982).

3.1.5. Biotransformation of caffeine in fungi:

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). More studies are required to identify the enzymes involved in the biotransformation of caffeine in fungal systems. Though the product of demethylation of caffeine has been reported as theophylline by Schwimmer *et al* (1971), the enzyme responsible for biotransformation of caffeine to theophylline has not been identified yet. Table 3.1.5 represents the various enzyme systems involved in caffeine biotransformation. Further no reports are available elucidating the complete pathway of caffeine biotransformation in fungi and the enzymes responsible for the formation of these biotransformation products.

The biotransformation pathway of caffeine in *Penicillium citrinum MTCC* 5215 is described in detail in the present work. The biotransformation of the initial tri methyl xanthine to various products, the identification of the products using various analytical techniques and the enzymes involved in the formation of biotransformed products is described. In bacteria, caffeine is biotransformed into two products viz. theobromine and paraxanthine by 1-*N* demethylation and 3-N demethylation respectively (Asano *et al,* 1993, Sarath Babu *et al*, 2005), and is brought about by caffeine demethylase. Madyastha *et al* (1999) reported the biotransformation of caffeine at the C-8 position leading to the formation of 1,3,7-trimethyluric acid by *Klebsiella* sp. and *Rhodococcus* sp, the enzyme for biotransformation was identified as caffeine oxidase. In *P. citrinum* MTCC 5215, the enzyme involved in the biotransformation of caffeine to theophylline was neither caffeine demethylase nor caffeine oxidase. Work was carried out in our laboratory on the identification of the

caffeine demethylase enzyme in view of the earlier work on the identification of the biotransforming enzyme in different systems and in the fungal system reported here. .

Table 3.1.5. Caffeine biotransformation in different living systems.

S.No	Biological system	Reaction type	Enzyme responsible	Product formed	Reference
1.	Humans	3-N, 1-N, 7-N demethylation, N-8 oxidation	CYP1A1, CYP1A2, CYP2D and CYP2E1	Paraxanthine, theobromine, theophylline, 1,3,7 trimethyl uric acid	Kalow, 1985; Butler *et al*., 1989; Berthou *et al*., 1991; Fuh *et al*., 1992; Tassaneeyakul *et al*., 1994; Chung and Cha, 1997
2.	*Pseudomonas putida* 352	1-N demethylation	Caffeine demethylase	Theobromine	Asano *et al*, 1993
3.	*Pseudomonas alcaligenes* MTCC 5264	1-N and 3-N demethylation	Caffeine demethylase	Theobromine, paraxanthine	Sarath *et al*, 2005, Sarath *et al*, 2006
4.	Mixed culture consortium of *Klebsiella* and *Rhodococcus*	N-8 oxidation	Caffeine oxidase	1,3,7 trimethyl uric acid	Madyasta *et al*, 1999
5.	*Serratia marcescens*	N-8 oxidation	Caffeine demethylase	1,3,7 trimethyl uric acid	Mazzafera *et al*, 1994
6.	Yeast	3-N, 1-N, 7-N demethylation	Cyt P450	Paraxanthine, theobromine, theophylline	Birkett, 1981; Sauer *et al*, 1982
7.	*Penicillium roqueforti*	7-N demethylation	Not identified	Theophylline	Schwimmer *et al*, 1971
8.	*Aspergillus niger*	7-N demethylation	Not identified	Theophylline	Ina, 1971
9.	*Stemphylium sp.*	7-N demethylation	Not identified	Theophylline	Kurtzman and Schwimmer, 1971
10.	*Rhizopus delemar*	7-N demethylation	Not identified	Theophylline	Brand *et.al*, 2000

3.2. Materials and Methods:

3.2.1. Materials:

Caffeine, theophylline, 1 methyl xanthine, 3 methyl xanthine, xanthine, uric acid, allantoin, allantoic acid, urea (99.9%) were procured from Sigma chemicals, St. Louis, USA. Cytochrome P450 reductase and dilauryl phosphatidyl choline were also obtained from Sigma chemicals, St. Louis, USA. Caffeine for biotransformation was purchased from Loba chemicals, Mumbai, India. Polyethylene glycol (PEG), N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer was obtained from Sisco Research Laboratories. dithio thretol (DTT), nicotinamide adenosine dinucleotide phosphate, reduced tetra sodium salt (NADPH sodium salt), nicotinamide adenosine dinucleotide (NAD), phenyl methyl sulfonyl fluoride (PMSF), NNN-bisacrylamide, tetramethylethylenediamine (TEMED), ammonium persulphate (APS), sodium dodecyl suplhate (SDS, MB Grade), methylene blue and sodium cholate were purchased from Sisco Research laboratories, Mumbai, India. Potassium hydrogen orthophosphate, di sodium hydrogen ortho phosphate, calcium chloride, magnesium sulphate, glucose were of analytical grade and procured from Qualigenes Fine Chemicals, Mumbai. Ethyl acetate, butanol and acetic acid were obtained from Qualigenes Fine Chemicals, Mumbai. HPLC Grade acetonitrile and methanol were procured from Merck, Germany. Potato dextrose agar and agar agar was obtained from Himedia, Mumbai. Corn steep liquor was procured from Anil Starch and Chemicals, Mumbai. All other chemical used were of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India.

3.2.2. Methods:

1) Thin layer chromatography (TLC) for identification of biotransformed products:

TLC for identification of biotransformed methyl xanthines was run using precoated TLC plates from Merck (Germany) with a solvent system containing butanol, acetic acid, water (4:1:1) and was visualized in a CAMAG UV illuminator.

2) High Performance Liquid Chromatography (HPLC) for identification of biotransformed products:

HPLC was carried out using RP C-18 column [5µm, 250mmx4.6mm, Phoenomenix column] using LC-10A (Shimadzu, Japan) system with a isocratic mobile phase of water and acetonitrile (85:15) at a flow rate of 1ml/min and wavelength of 273nm.

3) Fourier Transform Infrared (FTIR) spectroscopy for identification of biotransformed products:

IR absorption spectra were recorded using a Perkin Elmer Model 2000 Infrared Fourier-transform spectrophotometer, with samples prepared in KBr.

4) Nuclear magnetic resonance (NMR) spectroscopy for identification of biotransformed products:

NMR spectra were recorded at 500 MHz, using 500MHz Bruker Avance instrument. ^{1}H and ^{13}C NMR was carried out by dissolving about 20 mg of purified theophylline in DMSO.

3.2.2.1. Product profiling of caffeine biotransformation by *P. citrinum* MTCC 5215:

Isolated strain *P. citrinum* MTCC 5215 was maintained on potato dextrose agar slants containing 0.03% caffeine. Spores of 72 hour old cultures were inoculated at approximate concentration of $5x10^{5}$ spores/ml into media composed of the

following components in (g/L): Na_2HPO_4- 0.12, KH_2PO_4-1.3, $MgSO_4$-0.3, $CaCl_2$-0.3, glucose-20.0, caffeine-1.0. Corn steep liquor was added at 5.0ml/L. 100 ml of the biotransformation media was taken in 500ml Erlenmeyer flasks, and inoculated with *P. citrinum* MTCC 5215. Biotransformation was carried out in a rotary shaker at 150 rpm and 28°C. Samples were drawn at regular intervals of 6 hours and checked for biotransformed product formed. Estimation of biomass was done by separating it from the broth by filtration and drying at 80°C till constant weight was obtained.

3.2.2.2. Isolation, purification and identification of biotransformed products:

After fermentation, biomass was separated from the broth by filtration. The broth was then extracted with ethyl acetate (1:1) for a period of 2 hours on a rotary shaker. The ethyl acetate layer was vacuum concentrated to get the crude extract. The crude extract obtained was dissolved in methanol and run along with standard methyl xanthines in TLC and HPLC to check for the biotransformed products formed. After identification of the biotransformed products by comparing with standard methyl xanthine samples, each individual product was purified by preparative TLC and subjected to FTIR and NMR for chemical identification.

3.2.2.3. Biomass production for enzyme identification:

Biomass production for enzyme identification was carried out as explained in section 3.2.2.1. The induced biomass of *P. citrinum* MTCC 5215 was harvested by filtration of the culture fluid through double layer muslin cloth. The filtered biomass was washed extensively with buffer (Phosphate buffer, 0.1 M, pH 7.2, prechilled at 4°C) to remove media components. After washing, the biomass was stored at -20°C with 0.01M caffeine. For further use, the frozen biomass was thawed, washed

extensively with buffer to remove all caffeine content and used for further work.

3.2.2.4. Preparation of crude enzyme extract (CEE):

Earlier experiments conducted show that the caffeine biotransforming enzyme is intracellular in nature. Hence, attempts were made to extract the enzyme by cell lysis using dynomill. After fermentation, biomass was washed with buffer to remove the media components and used for enzyme extraction. Washed biomass (100gm) was transferred to the chamber of dynomill and 200gm of pre cooled glass beads and 300ml of isolation buffer (50mM HEPES buffer, pH 8.0, containing 15% (v/v) glycerol, 1mM DTT, 0.25mM PMSF) was added. Disruption was carried out for 10 mins and the cycle was repeated 4 times with 10 mins interval. The homogenate was filtered through muslin cloth and centrifuged for 30 mins at 12,000g at 4°C in a cooling centrifuge (Kubota, Japan). The supernatant contained glassy glycogen layer which was separated by filtration through muslin cloth. The filtrate obtained was termed as crude enzyme extract (CEE) and used for further studies. All the experiments involving enzyme purification and characterization were carried out at 4°C unless otherwise specified.

3.2.2.5. Isolation of microsomes:

Isolation of microsomes for the identification of cytochrome P450 was carried out using various techniques such as calcium chloride precipitation, polyethylene glycol (PEG) precipitation and ultracentrifugation (Estabrook and Werringler, 1978; Mitoma *et al*, 1956). The following methods of microsome isolation were used in the present study.

a) Calcium chloride precipitation:

In recent years, one of the procedures for microsomal isolation which has been extensively studied and gaining acceptance is a method involving aggregration of microsomes with calcium ions (Kamath *et al*, 1971; Schenkman and Cinti, 1972; Kamath and Ananth Narayan, 1972; Cinti *et al*, 1972; Kupfer and Levin, 1972). To the crude extract, Ca^{2+} ions (calcium chloride) at a concentration of 8mM was added to the enzyme extract with stirring and allowed to settle for two hours followed by centrifugation in a refrigerated centrifuge for 30 mins at 25,000g for 15 min. The microsomal pellet obtained was then resuspended and washed with phosphate buffer, (pH 7.2, 0.05M). Washed microsomal pellet was centrifuged at 25,000g for 15 min and used for further studies.

b) Polyethylene glycol precipitation:

To the crude extract obtained polyethylene glycol (PEG 8000) was added at concentration of 8% by slow stirring. The solution was kept at 4°C for 2 hours and then centrifuged at 20,000g for 30 min. The pellet obtained was resuspended in phosphate buffer, pH 7.2, 0.05M and centrifuged at 20,000g for 30 min. The microsomal pellet obtained was used for further studies.

c) Ultracentrifugation:

The crude enzyme extract was used for the isolation of microsomes. It was subjected to ultracentrifugation at 100,000g for 1 hour at 4°C using L-7 65, Sorvall centrifuge. The microsomal pellet was carefully removed and resuspended in phosphate buffer (0.05M) using homogeniser with a teflon pestle, with cycles of 30 secs each with intermittent cooling for 30 secs. The homogenized microsomes were

again subjected to ultracentrifugation at 100,000g for 1 hour at 4°C.The supernatant obtained was used for assay of cytochrome P450 (Ballard *et al*, 1990).

3.2.2.6. Identification of cytochrome P450:

Cytochrome P450 are mixed-function oxidases**,** located in microsomes. Cytochrome-P450 enzyme complex has four required components in order to be functional, which are listed below (Chenault and Whitesides, 1987):

- Cytochrome-P450 Enzyme
- Cytochrome-P450 Reductase
- O_2
- NADPH

The identification of cytochrome P450 for the biotransformation of caffeine to theophylline in *P. citrinum* MTCC 5215 was done by incubation test, carbon monoxide (CO) reduction test and reconstitution of purified cytochrome P450.

(1) Incubation test:

The microsomal pellet obtained by ultracentrifugation was homogenized with teflon pestle homogeniser, with cycles of 30 secs and solubilised with sodium cholate. The solubilised microsome was incubated with buffer (phosphate buffer 0.1M, pH 7.2) containing NADPH (10mM) and caffeine (1mM). Samples were collected at intervals of 30 mins and analyzed for the formation of theophylline using TLC and HPLC.

(2) CO reduction spectra of cytochrome P450:

CO was generated by dropping formic acid (HCOOH) over concentrated sulphuric acid (H_2SO_4). The CO generated was passed through aqueous KOH (10%) to trap any CO_2 liberated during the reaction. Differential spectra of microsomal

preparation were measured in a Shimadzu UV 1601 spectrophotometer with quartz cuvettes of 1 cm optical path. Microsomal preparations containing, 1.71 mg/ml of protein in 0.1M, phosphate buffer, (pH 7.2), were placed in both the reference and sample samples. The baseline was recorded and the sample was saturated by passing CO for about 60 seconds. Reduction of the sample was done by adding about 50mg of sodium dithionite ($Na_2S_2O_4$). The reduced enzyme when reacted with CO gave absorbance at 450 nm, which is the characteristic spectrum for cytochrome P450 enzyme (Estabrook *et al*, 1972; Omura and Sato, 1964).

3.2.2.7 Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) to determine the inducible nature of enzyme:

To determine the inducible nature of the caffeine biotransforming enzyme, *P. citrinum* MTCC 5215 was cultivated in biotransformation media (composition in section 3.2.2.1). The media for control experiment contained no caffeine. Fermentation was terminated after 48 hours and biotransformation of caffeine to theophylline was checked by TLC and HPLC. The harvested biomass was filtered, washed and used for microsome isolation. The induced and uninduced microsomal preparations were solubilised and then loaded into SDS PAGE. SDS-PAGE was carried out according to the method of Laemmli (1970). The bands were visualized after staining with coomassie blue and silver staining.

3.2.2.8 Gel filtration chromatography for purification of cytochrome P450:

Purification of the crude enzyme was carried out in a Sephadex G-75 column according to the method of Ward and Arnott (1965). The column dimensions were 1cm x 120 cm and the bed volume of 144 cm^3. The column was equilibrated with Tris–HCl buffer, pH 8.0 and 1.5 ml of sample (0.4068mg/ml protein) was applied to

the top of the column. Elution was carried out using the same buffer containing 100mM NaCl to avoid non-specific binding to the matrix. 15ml fractions were collected and active fractions were pooled concentrated on Amicon filters (10kDa, MWCO) and used for further work.

3.2.2.9 Reconstitution of cytochrome P450 enzyme:

300µl of active fractions of cytochrome P450 containing 0.2 mg/ml of protein collected from gel filtration chromatography were reconstituted with 100µl of cytochrome P450 reductase (0.5mg/ml protein concentration), 100 µl of dilauryl phosphatidyl choline, 10µl NADPH (10mM), 50µl caffeine (1mM), in 400µl phosphate buffer (pH 7.2, 0.1M). The reaction mixture was incubated at 30°C for two hours and checked for the biotransformation of caffeine to theophylline using HPLC analytical technique.

3.2.2.10. Identification of enzymes involved in caffeine degradation pathway:

For the assay of rest of the enzymes of the pathway, CEE was prepared as explained in section 3.2.3.4. Incubation test as well as spectrophotometric assays was performed for the identification of the enzymes.

(1) Heteroxanthine demethylase (HXDM):

Heteroxanthine demethylase activity in the CEE was determined according to the method of Gluck and Lingens, (1988). Activity of HXDM was recorded as a decrease in absorbance at 340nm due to the oxidation of NADPH during the reaction. The reaction mixture contained 790µl of phosphate buffer (50mM, pH 7.3) 10µl NADPH (10mM), and 100µl of substrate (1mM). The different substrates used were heteroxanthine, theophylline and theobromine and caffeine. One unit of enzyme

activity is defined as the decrease in absorbance of 0.001 at 340nm. Further, incubation test was also carried out, and the incubation mixture contained the above mentioned components in the same proportion. Theophylline was used as the substrate in the incubation mixture and at 30°C, the product formed was analyzed by HPLC.

(2) Xanthine dehydrogenase (XDH):

Xanthine dehydrogenase assay was done according to the method of Tatsuhiko and Yasuto, (1978). Enzyme activity was recorded as the increase in absorbance at 340nm due to the reduction of NAD to NADH. Reaction mixture consisted of 700µl Tris HCl buffer (100mM, pH 7.5), 100µl of substrate (2mM), 10µl NAD (2mM), 90µl KCl (100mM). Substrates used were hypoxanthine, 1 methyl xanthine, theophylline, theobromine and caffeine. One unit of enzyme activity is defined as the increase in absorbance of 1at 340 nm. Incubation test was carried out at 30°C using the above mentioned reaction mixture and 1 Methyl xanthine as the substrate, product formed was analyzed by HPLC.

(3) Xanthine oxidase (XO):

Xanthine oxidase is known to convert xanthine and methyl xanthines into their respective uric acids. Xanthine oxidase activity in the CEE was determined according to the method of Bray (1963). The presence of xanthine oxidase in the CEE was assayed by methylene blue reduction at 600nm. 1ml of reaction mixture contained 770µl phosphate buffer (pH 7.2, 100mM), 100µl of crude enzyme extract, 100µl substrate (10mM), and 30µl methylene blue (25mM). The different substrates used were xanthine, caffeine, theophylline, theobromine and. One unit of enzyme

activity was defined as the amount of enzyme, which caused decrease in absorbance of 1 unit. The incubation mixture contained above mentioned reaction components and xanthine as the substrate. Product formed was analyzed by HPLC.

(4) Uricase:

Enzyme assay for uricase in the CEE was analyzed by incubating crude enzyme extract with uric acid and monitoring the removal of uric acid by decrease in absorbance at 290nm spectrophotometrically according the method of Mahler *et al*, (1955). The reaction mixture contained 890µl of phosphate buffer, (pH 6.5, 100mM), 100µl uric acid (10mM) and 100µl of crude enzyme extract.

(5) Allantoinase:

Allantoinase activity in the crude enzyme extract was confirmed by incubation test at 30°C. The incubation mixture contained CEE 500µl, 1.4ml Tris buffer (pH 8.0, 100mM) and 100µl allantoin (10mM). Samples were collected every 2 hours and analysed by HPLC for the utilization of allantoin and its conversion to allantoic acid.

(6) Allantoicase:

Allantoicase activity in the crude enzyme extract was confirmed by incubation test at 30°C. The incubation mixture contained CEE 500µl, Tris buffer (pH 7.2, 100mM), 1.4ml and 100µl allantoic acid (10mM). Samples were collected at regular interval and analyzed by HPLC for the utilization of allantoic acid and its conversion to urea.

(7) Urease:

Urease activity in the crude enzyme extract was determined by the estimation of the amount of ammonia released by Nessler's reagent according to the method of Jayaraman, (1988). The reaction mixture contained 1 ml of urea (10mM) and 1 ml of crude enzyme extract. The reaction mixture was incubated at 30°C for 1 hour. Samples were collected at regular intervals of 15 mins each. 100µl of Nesslers reagent was added to the samples collected and absorbance was recorded at 490nm.

3.3. Results and Discussion:

3.3.1 Cultivation of *P. citrinum* MTCC 5215 for product profiling during biotransformation of caffeine:

The various products formed during biotransformation of caffeine in *P. citrinum* MTCC 5215, were identified by HPLC analysis and reported in Table 3.3.1. The first demethylation product from caffeine biotransformation in *P. citrinum* MTCC 5215, was theophylline and it appeared in the broth after 30 hours of fermentation. The result obtained is in accordance with the results obtained by Schwimmer *et al*, 1971, where the first product of caffeine biotransformation in *Penicillium roqueforti* was theophylline. In the present studies it was observed that further demethylation of theophylline by *P. citrinum* MTCC 5215 led to the formation of 1-methyl xanthine and it appeared in the broth after 48 hours of fermentation. Removal of a methyl group from 1-methyl xanthine, led to the formation of xanthine, which was then oxidized to uric acid and it, appeared in the media after 60 hours of biotransformation. Uric acid was biotransformed to allantoin, which further gets converted to allantoic acid. Allantoic acid is converted to urea, which is finally broken down to ammonia and CO_2. Hence, the order in which the various products of caffeine biotransformation appear in the fungal strain *P. citrinum* MTCC 5215 is **caffeine → theophylline → 1-methyl xanthine → xanthine → uric acid → allantoin → allantoic acid → urea → $CO_2 + NH_3$.**

Biotransformation of caffeine has been studied in *Penicillium roqueforti* (Kurtzman and Schwimmer 1971; Schwimmer *et al*, 1971) and *P. chrysogenum* (Allam and Eazainy, 1969). These studies showed that both the fungal strains could utilize caffeine as a source of nitrogen. Schwimmer *et al* (1971) identified the first

intermediatary metabolite of caffeine biotransformation as theophylline but further biotransformation products of caffeine metabolism were not identified by them. In the present work, a complete study has been carried and all the biotransformation products of the pathway have been identified till the formation of CO_2 and NH_3.

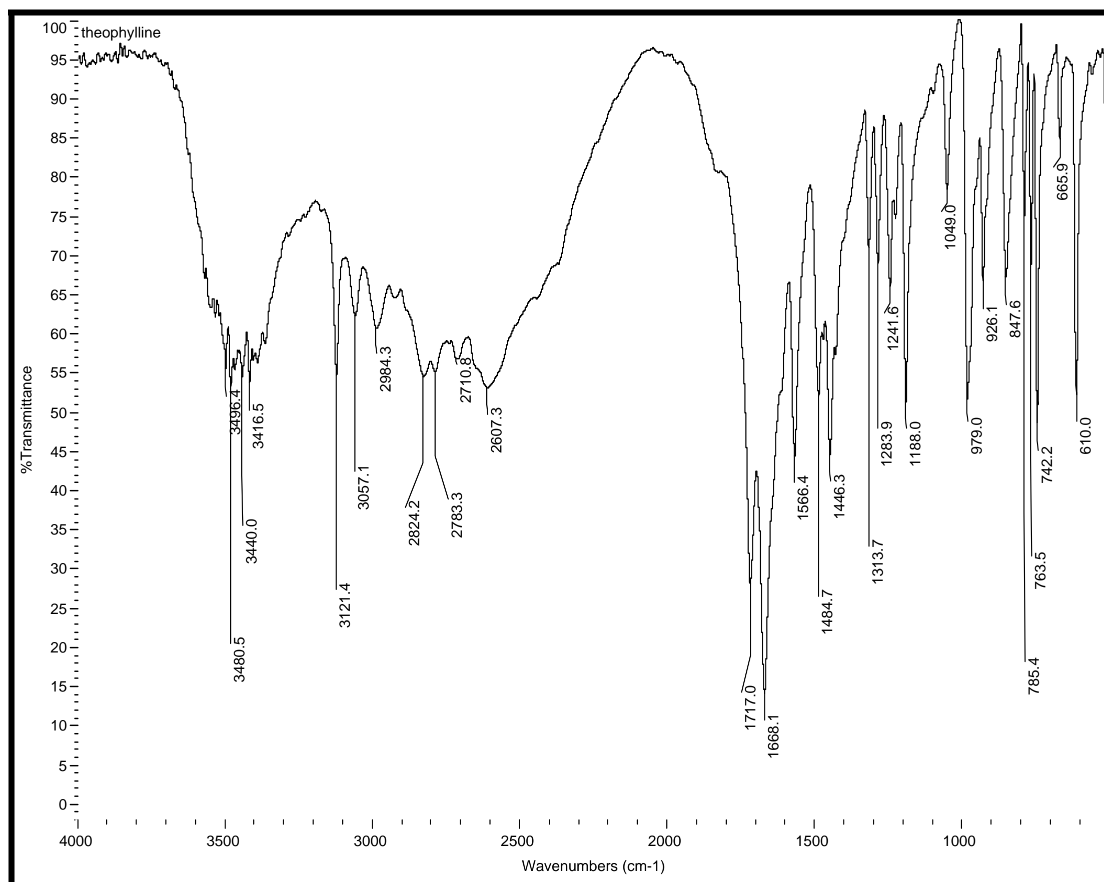
Table 3.3.1. Biotransformed products formed by *P. citrinum* MTCC 5215.

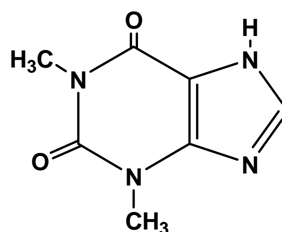
S.No	Time (Hrs)	Biotransformed products formed in *P. citrinum* MTCC 5215
1.	0	Caffeine
2.	6	Caffeine
3.	12	Caffeine
4.	18	Caffeine
5.	24	Caffeine
6.	30	Caffeine, Theophylline
7.	36	Caffeine, Theophylline
8.	42	Caffeine, Theophylline
9.	48	Caffeine, Theophylline, 1 Methyl xanthine
10.	54	Caffeine, Theophylline, 1 Methyl xanthine, Xanthine
11.	60	Caffeine, Theophylline, 1 Methyl xanthine, Xanthine, Uric acid
12.	66	Theophylline, 1 Methyl xanthine, Xanthine, Uric acid
13.	72	Theophylline, 1 Methyl xanthine, Xanthine, Uric acid, Allantoin, Allantoic acid
14.	78	1 Methyl xanthine, Xanthine, Uric acid, Allantoin, Allantoic acid, Urea
15.	84	1 Methyl xanthine, Xanthine, Uric acid, Allantoin, Allantoic acid, Urea, Ammonia
16.	90	Uric acid, Allantoin, Allantoic acid, Urea, Ammonia
17.	96	Uric acid, Allantoin, Allantoic acid, Urea, Ammonia
18.	102	Allantoin, Allantoic acid, Urea, Ammonia
19.	108	Urea, Ammonia

3.3.2. Isolation, purification and identification of biotransformed products:

The biotransformed products of caffeine were identified by HPLC and have been represented in Table 3.3.1. They were purified by preparative TLC. The purified products obtained were confirmed by FTIR and NMR and the chromatograms are presented in figure 3.3.2.1a to figure 3.3.2.7.

Figure 3.3.2.1a. FTIR of biotransformed theophylline.

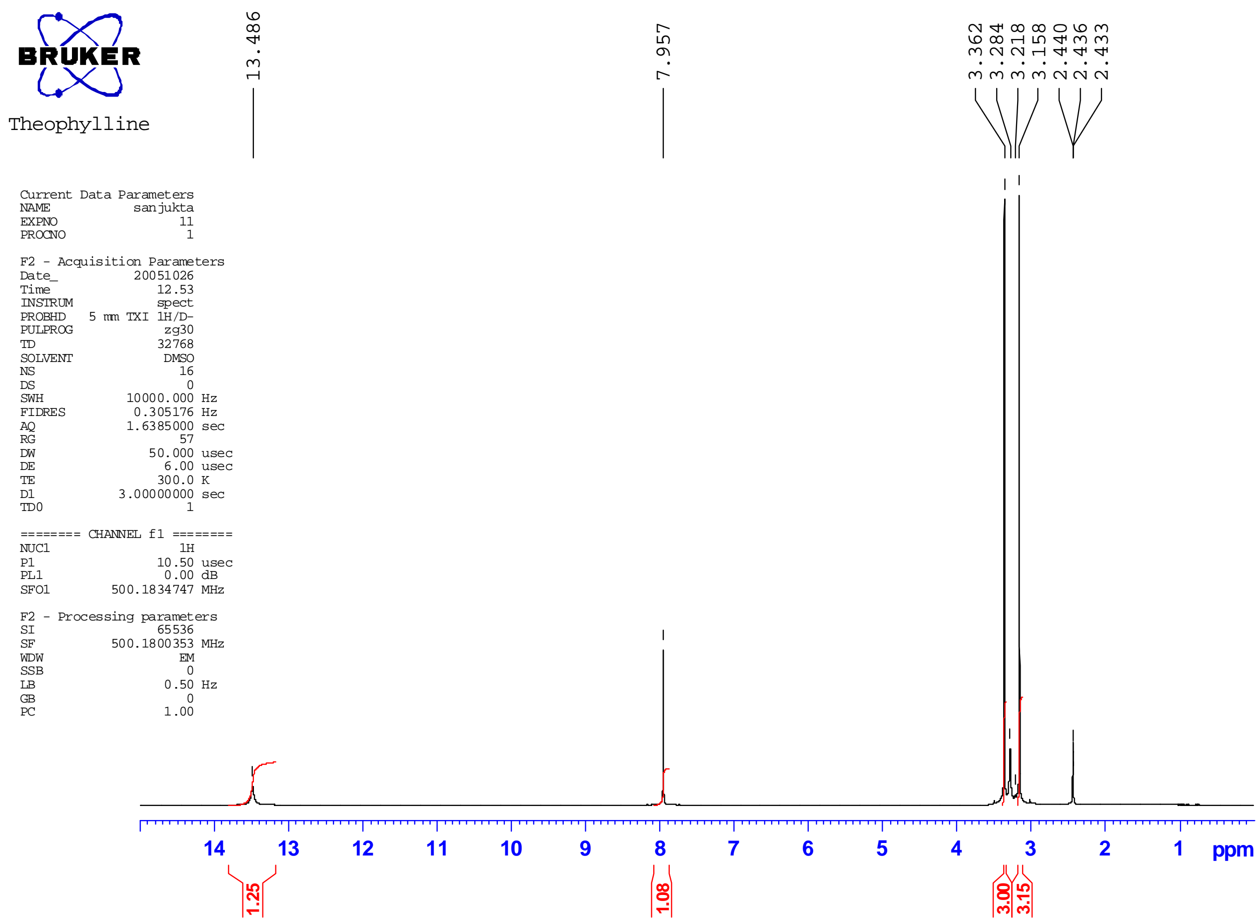




Theophylline

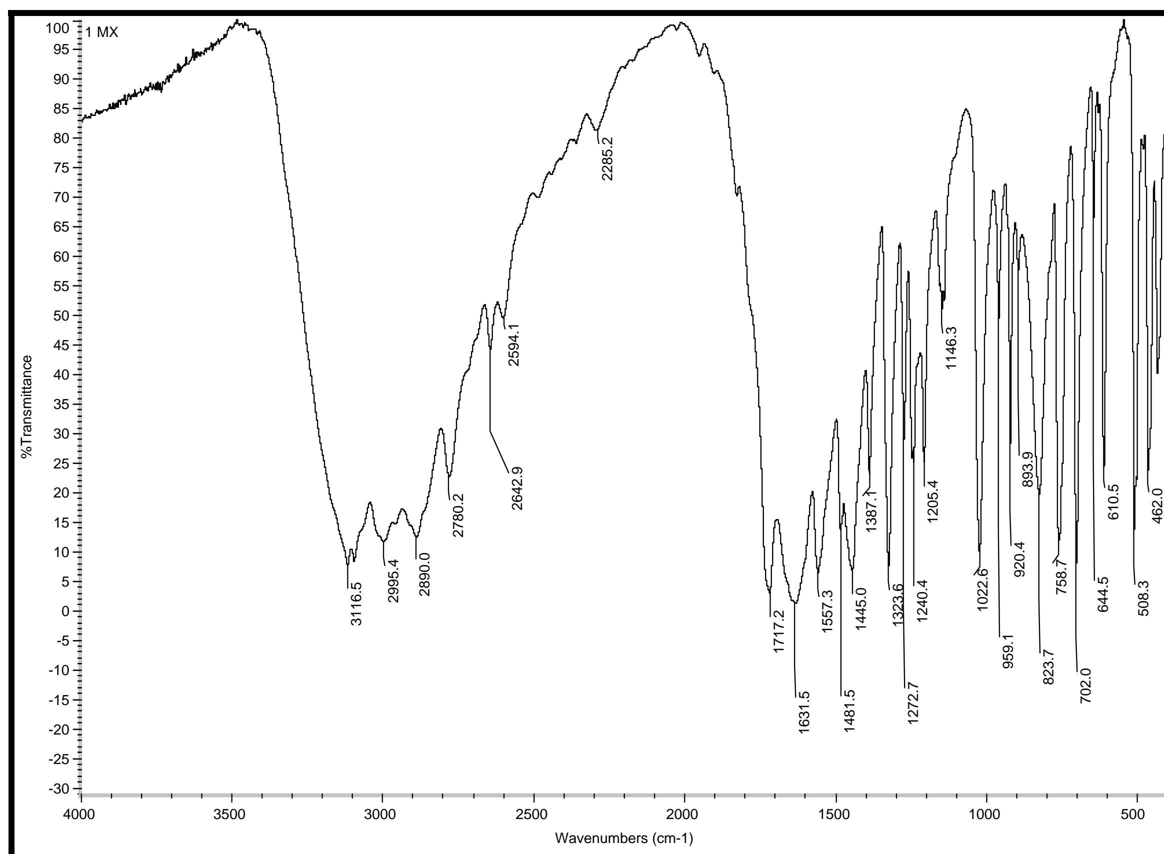
IR max (KBr) 1668 (C=O), 1556 (C=C), 1313 (Ar. ter. amine), 1283 (Ar. sec. amine) cm^{-1}.

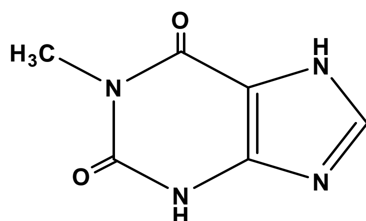
Figure 3.3.2.1b. NMR of biotransformed theophylline.



[1] H NMR: (DMSO in δ units): 3.15 (1N CH_3), 3.36 (3NCH_3), 3.28 (=C-H), 7.96 (NH).

Figure 3.3.2.2a. FTIR of biotransformed 1-methylxanthine.

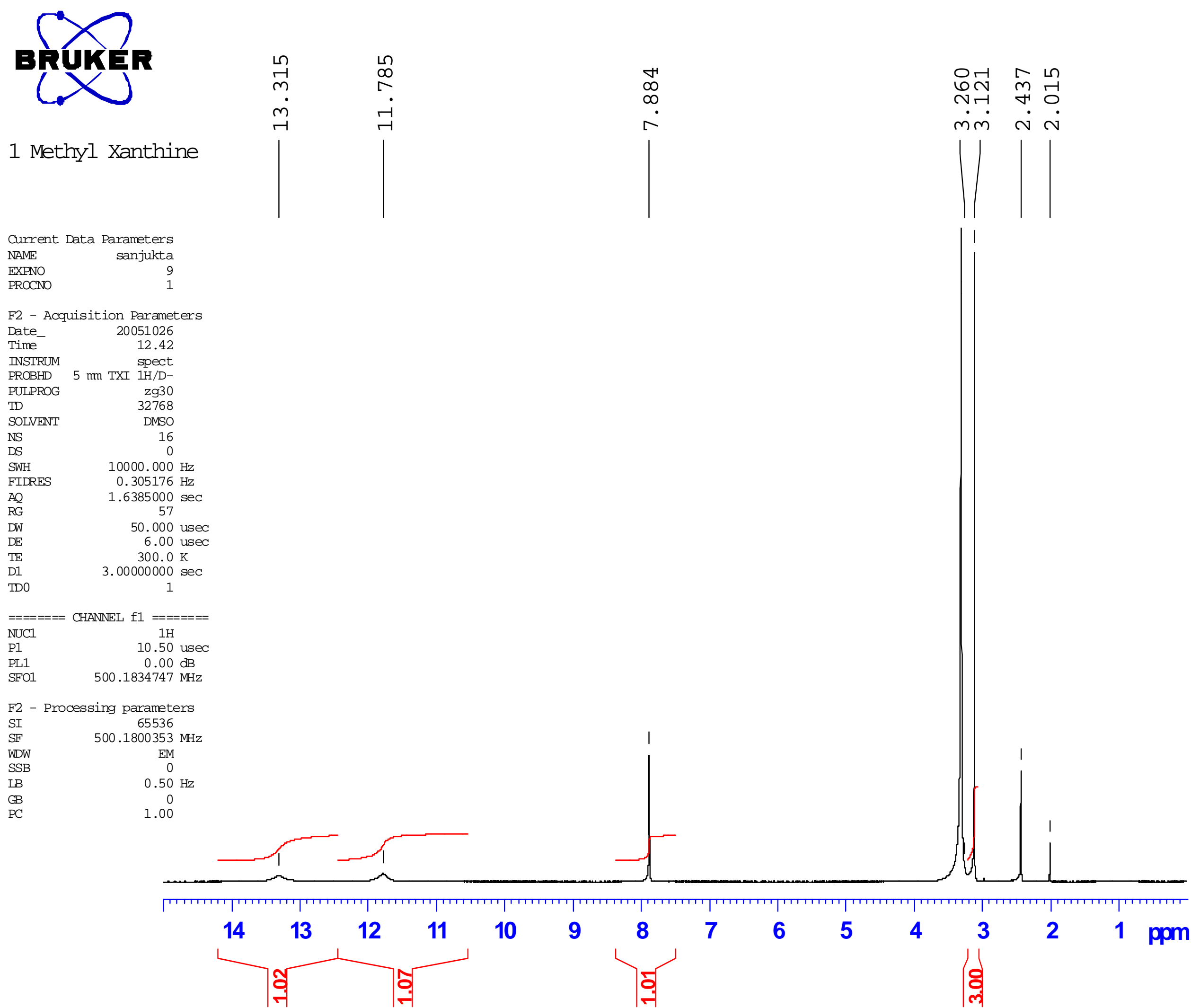




1-Methyl Xanthine

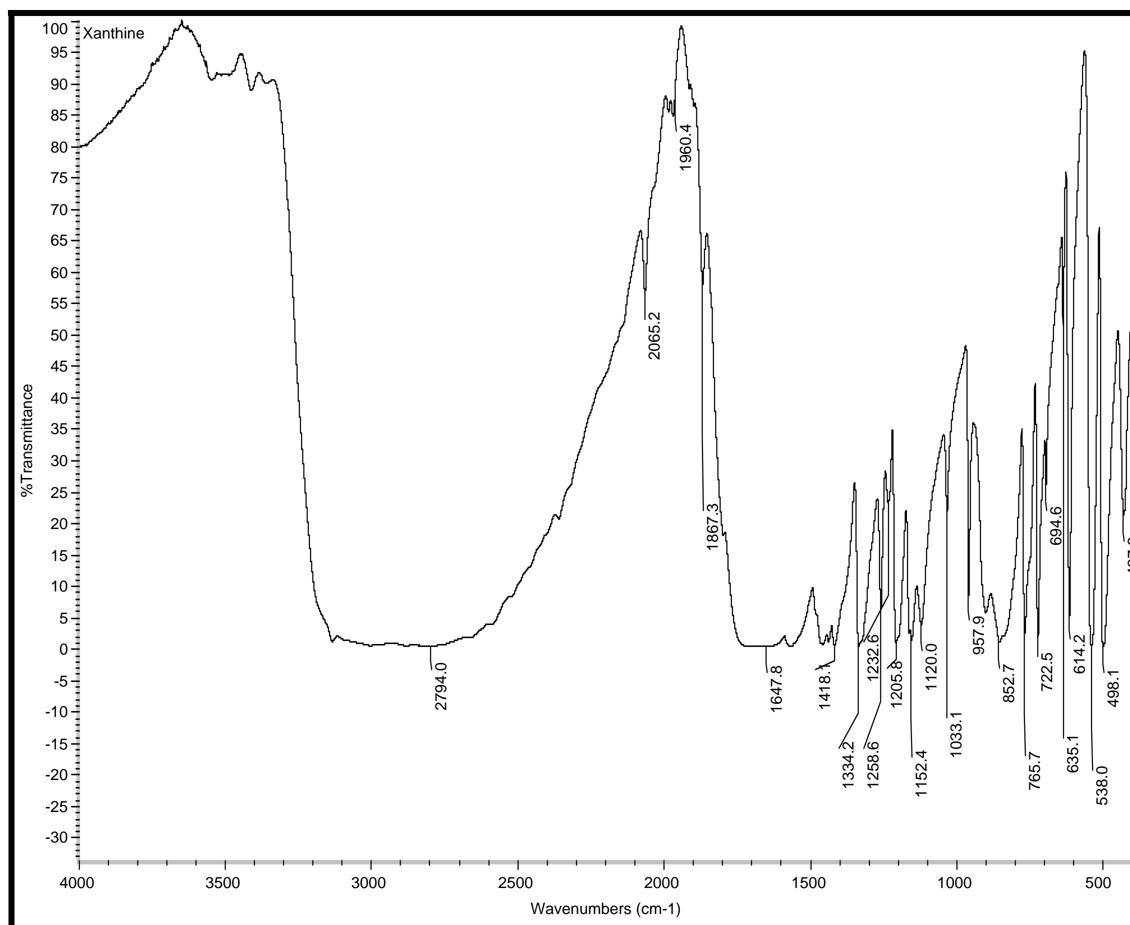
IR max (KBr): 1631 (C=O), 1557 (C=C), 1323 (Ar. ter. amine), 1272 (Ar. sec. amine) cm^{-1}

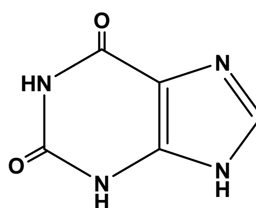
Figure 3.3.2.2b. NMR of biotransformed 1 methylxanthine.



[1] H NMR: (DMSO in δ units): 3.121 (1N-CH_3), 13.315 (3N-H), 7.884 (8), 11.785 (7 or 9).

Figure 3.3.2.3a. FTIR of biotransformed xanthine.

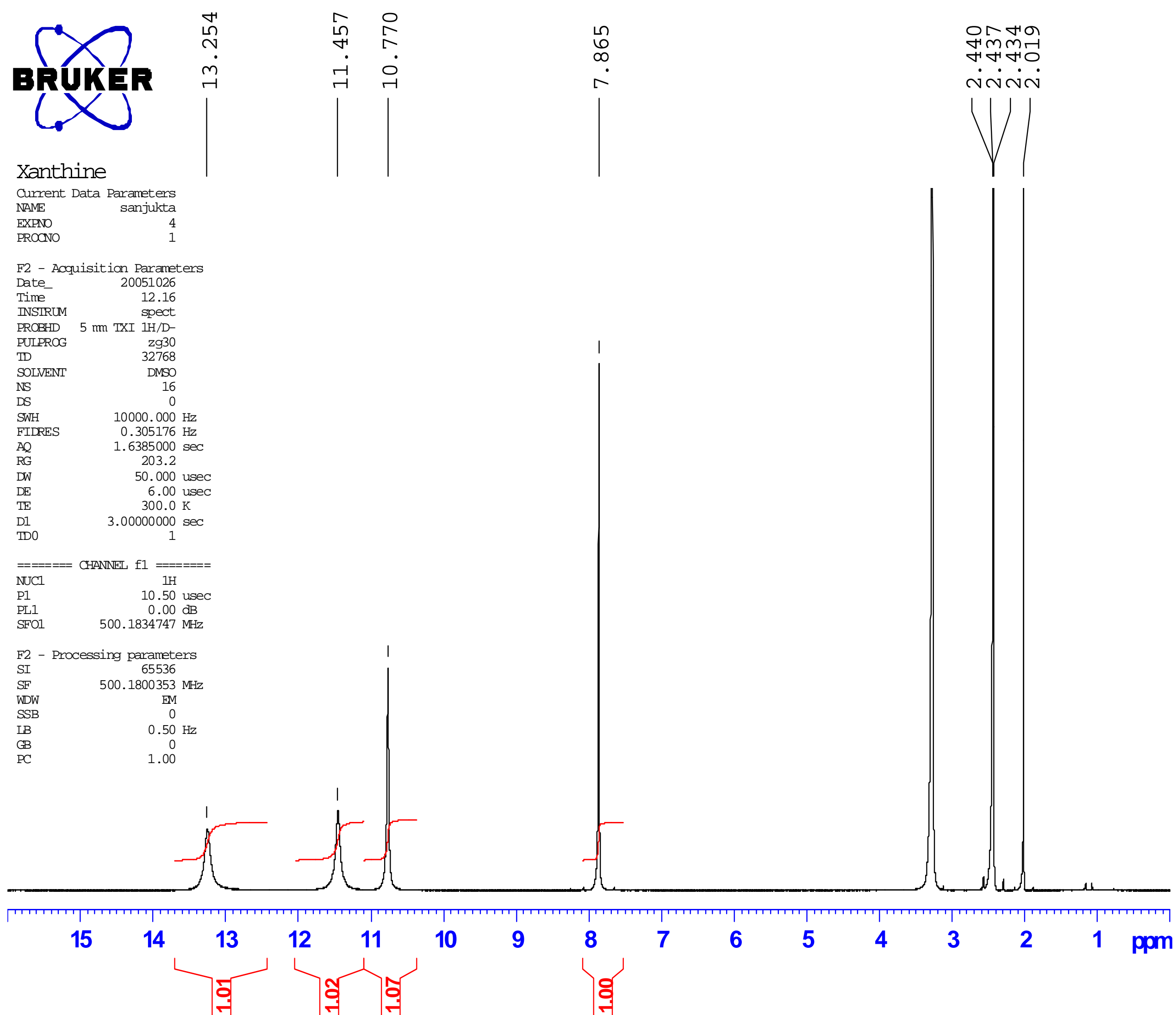




Xanthine

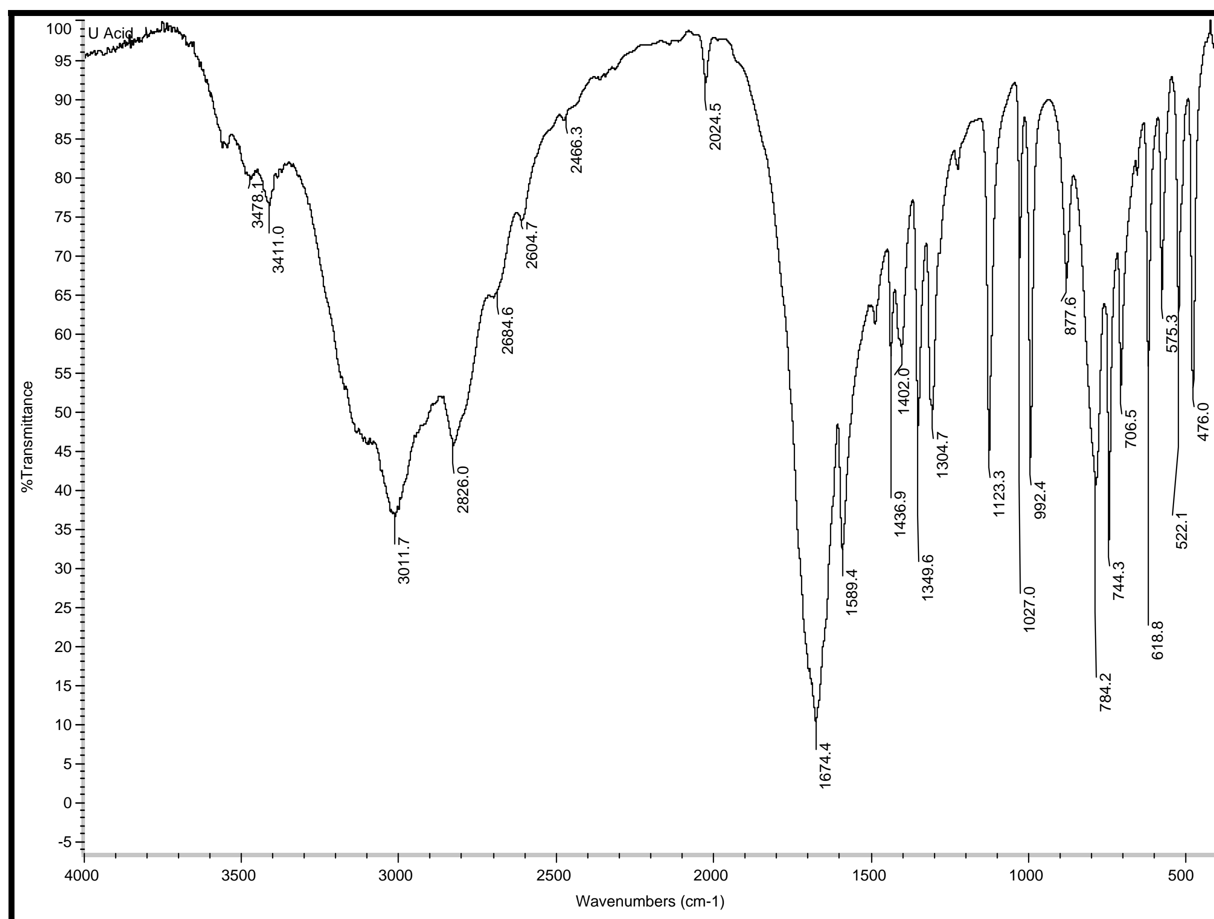
IR max (KBr): 1647 (C=O), 1566 (C=C), 1334 (Ar. ter. amine), 1258 (Ar. sec. amine) cm^{-1}

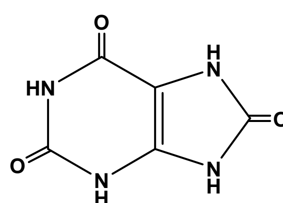
Figure 3.3.2.3b. NMR of biotransformed xanthine.



[1] H NMR: (DMSO in δ units): 13.254 (1N-H), 11.457 (3N-H), 7.865 (8), 10.770 (7 or 9).

Figure 3.3.2.4a. FTIR of biotransformed uric acid.

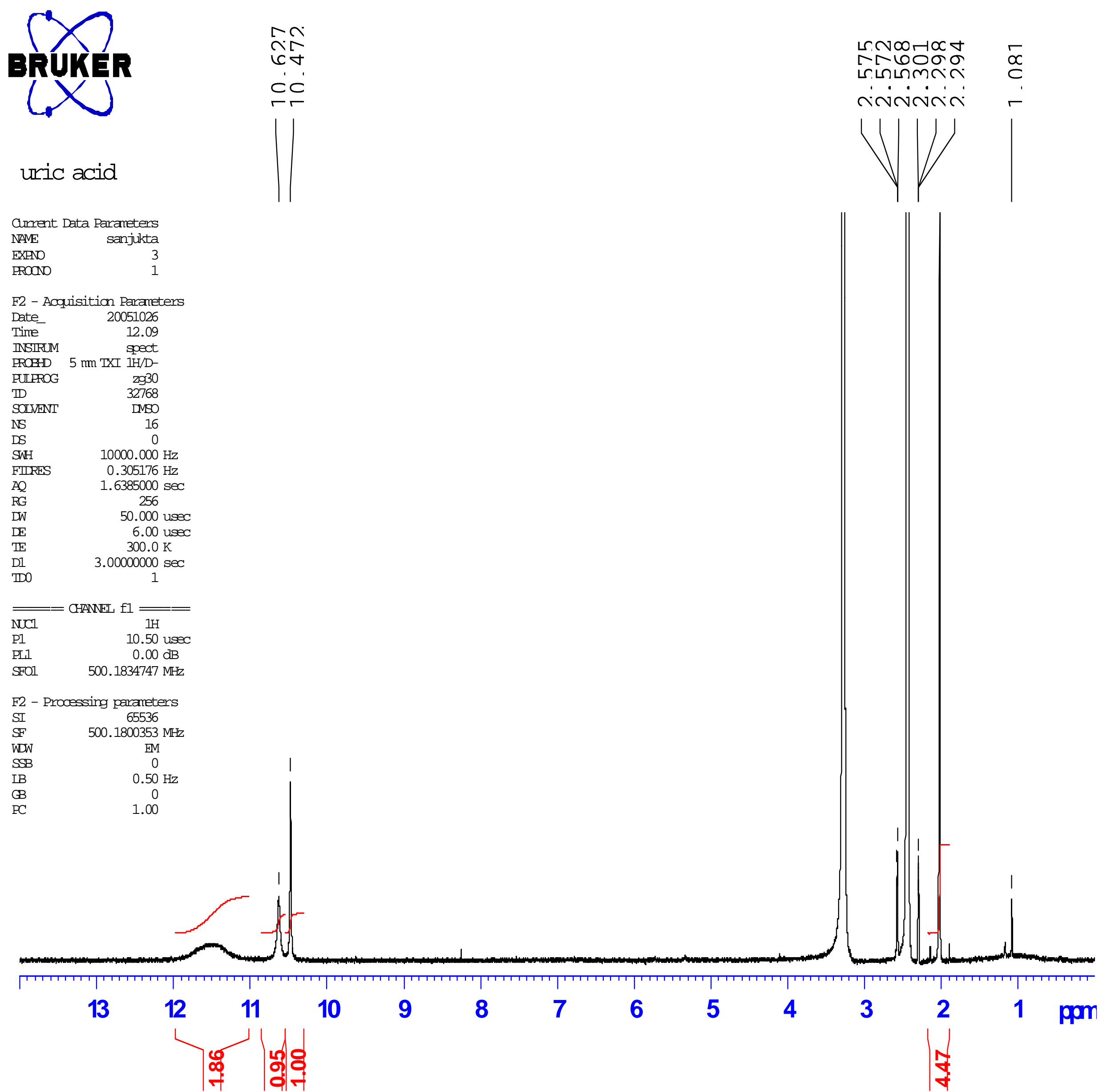




Uric Acid

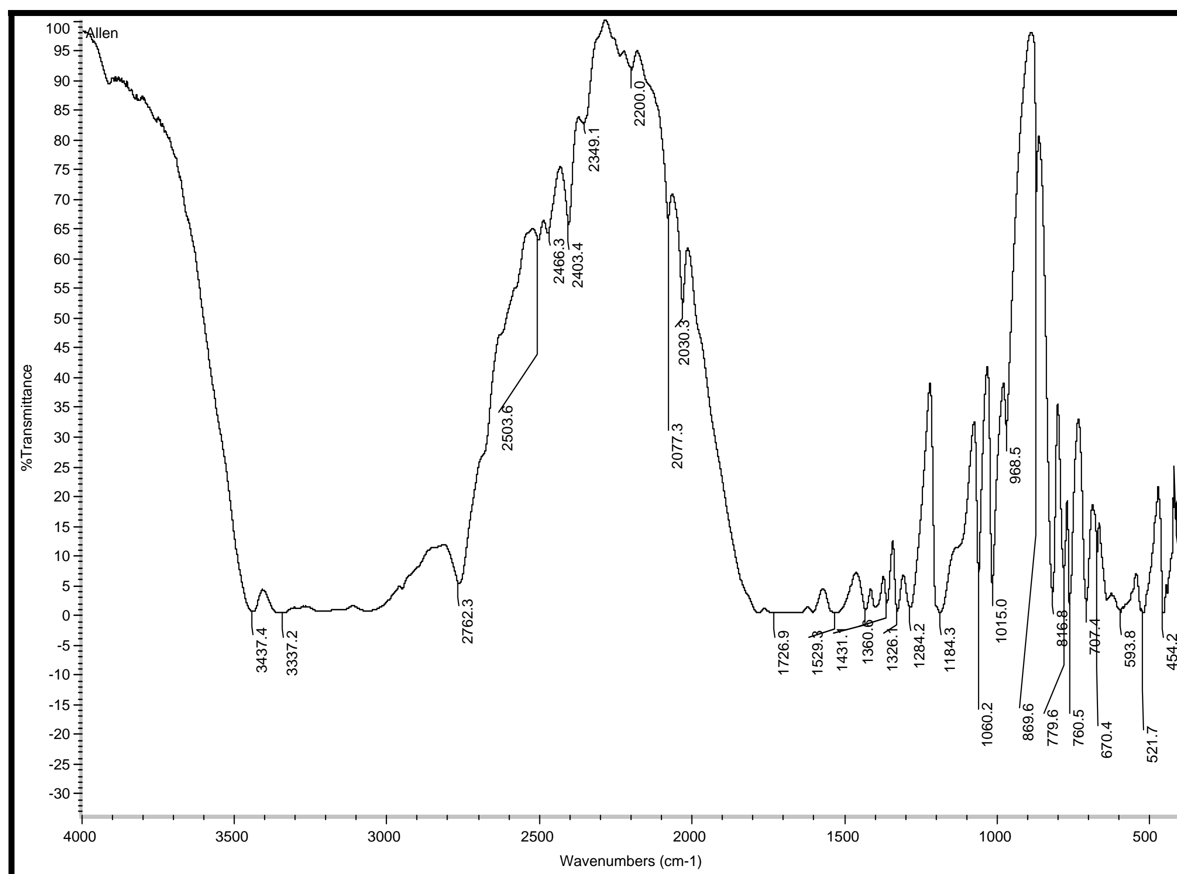
IR max (KBr): 1674 (C=O), 1589 (C=C), 1349 (Ar. sec. amine) cm^{-1}

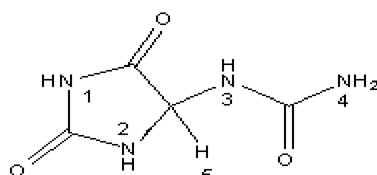
Figure 3.3.2.4b. NMR of biotransformed uric acid.



[1] H NMR: (DMSO in δ units): Broadening (1N-H, 3-NH), 10.472 (9 N-H), 10.627 (7N-H)

Figure 3.3.2.5a. FTIR of biotransformed allantoin.

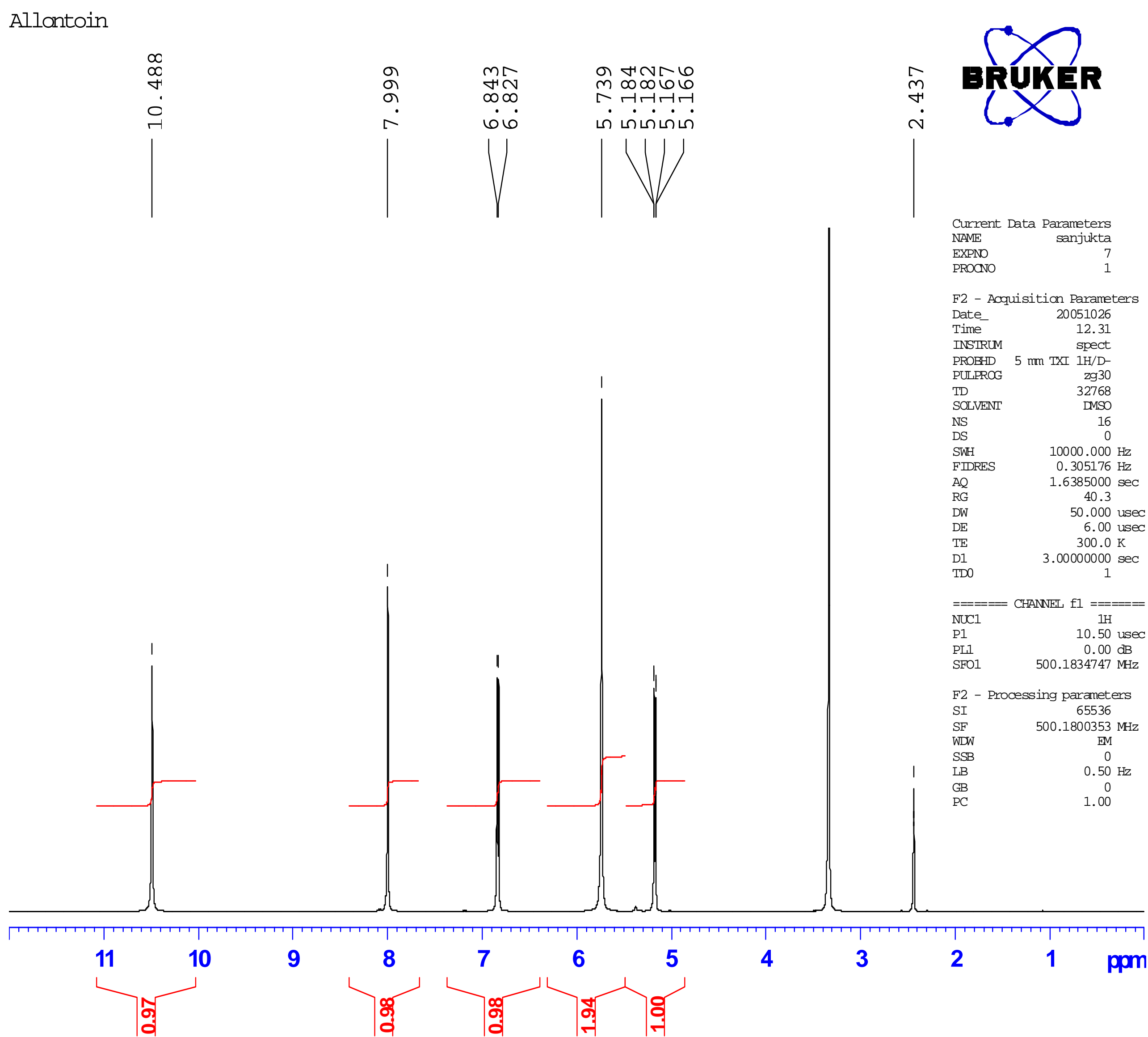




Allantoin

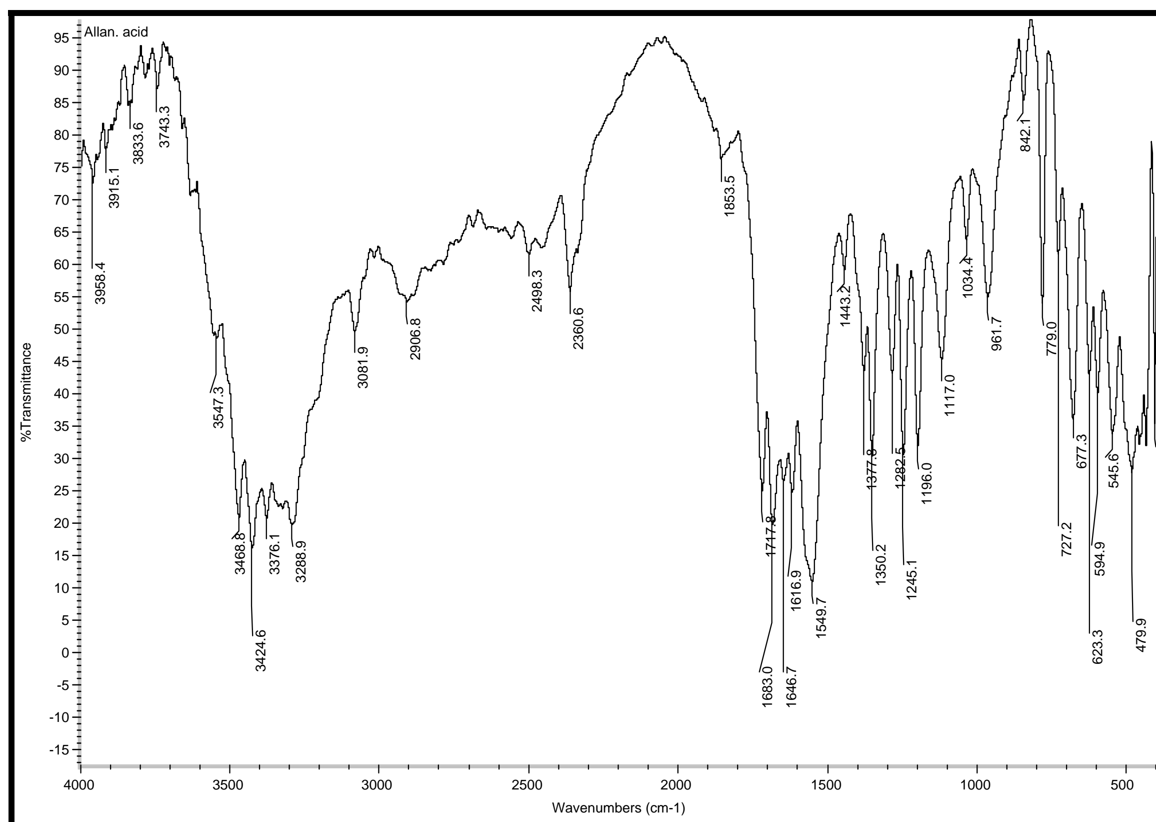
IR max (KBr): 1726 ^{1} (C=O), 3437 (Pr. amine), 3337 (Sec. amine), 1284 (Ar. sec. amine) cm^{-1}

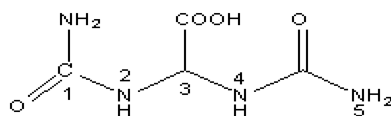
Figure 3.3.2.5b. NMR of biotransformed allantoin.



[1] H NMR: (DMSO in δ units): 10.4883 (1), 7.999 (2), 6.835 (3), 5.7388 (4), 5.175ppm.

Figure 3.3.2.6a. FTIR of biotransformed allantoic acid.

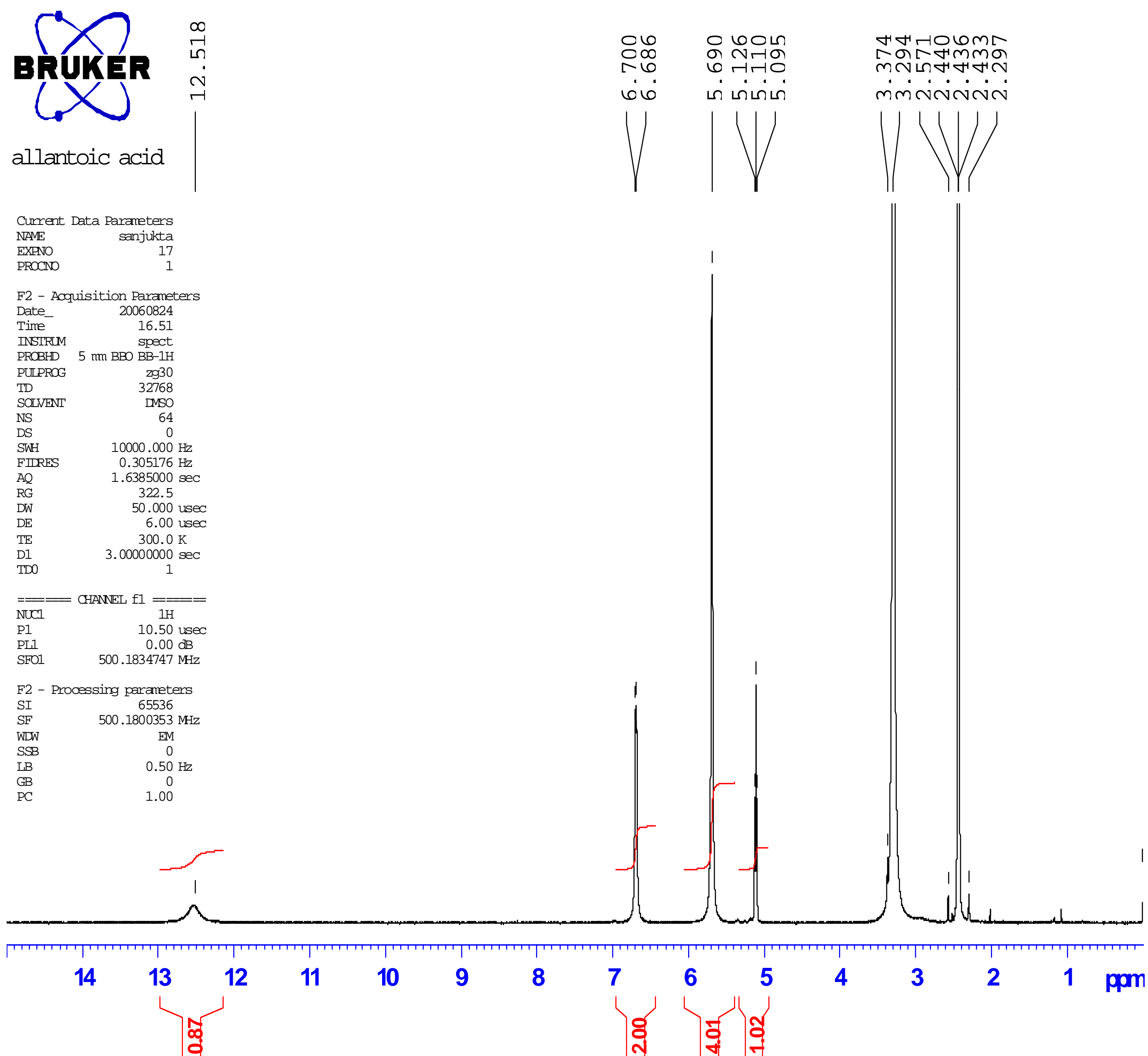




Allantoic acid

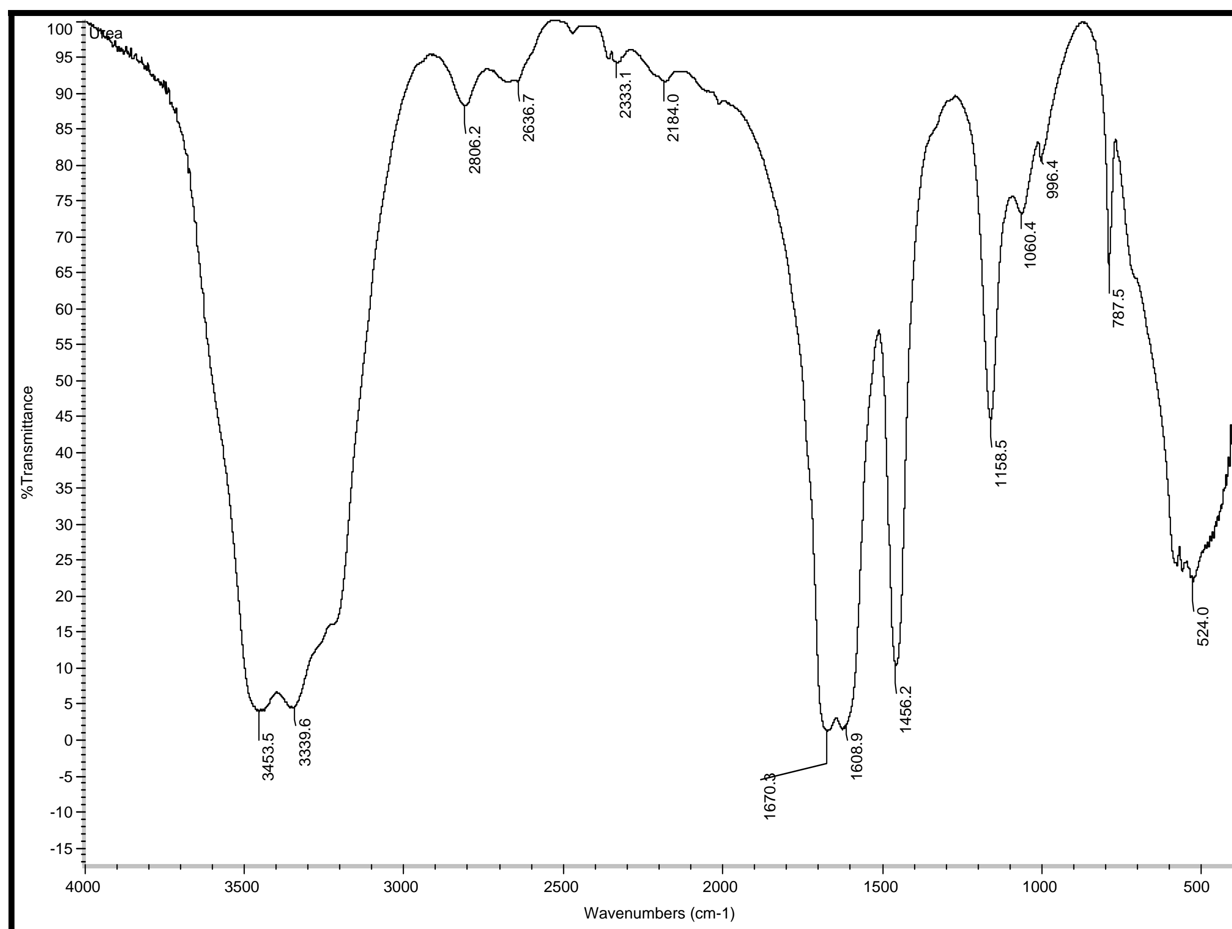
IR max (KBr): 1683 (C=O), 3468 (Pr. Amine), 3376 (Sec. amine), 1646 (Carboxylate) cm^{-1}

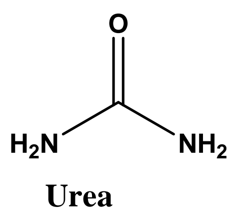
Figure 3.3.2.6b. NMR of biotransformed allantoic acid.



[1] H NMR: (DMSO in δ units): 5.690 (1), 6.693 (2), 5.110 (3), 6.693 (4), 5.690(5), 12.518(6).

Figure 3.3.2.7. FTIR of biotransformed urea.





Urea

IR max (KBr): 1670 (C=O), 3453 (Pr. Amine) cm^{-1}

3.3.3. Cultivation of *P. citrinum* MTCC 5215 for identification of enzyme:

The various products formed during fermentation were monitored and reported in Table 3.3.1. For the identification of each individual enzyme of the pathway, fermentation was terminated depending on the time at which the biotransformation product accumulation was maximum in the growth media, as there will be maximum activity of enzyme expressed for formation of product. After termination of fermentation, the biomass was used for enzyme extraction and further studies for the identification of respective enzymes.

3.3.4. Preparation of crude enzyme extract:

Extraction of enzyme was carried out using dynomill and the protein concentration of crude enzyme extract was found to be 520μg/ml.

3.3.5. Preparation of microsomes:

The protein content of the microsome preparations obtained by calcium chloride precipitation and PEG precipitation was 0.12mg/ml and 0.16mg/ml respectively (Table 3.3.5). The microsomes obtained by these method have to be washed thoroughly as the presence of calcium chloride and PEG makes the isolated microsomes slimy in nature (Schenkman and Cinti, 1972; Kamath and Ananthnarayan, 1972). Repeated washing leads to loss in yield, is time consuming and tedious. Hence, these two methods of microsome isolation were discontinued. Ultracentrifugation was found to be the best method for the preparation of microsomes as this method of isolation gave maximum yield of 0.3mg from 1 ml of crude enzyme extract as shown in Table 4.3.2. The microsomes obtained by

ultracentrifugation are free from any cell component contamination (Mitoma *et al*, 1956).

Table 3.3.5. Isolation of microsomes by different methods.

S.No	Method of isolation	Yield of microsomes
1.	Ultracentrifugation	0.3mg/ml
2.	Precipitation with $CaCl_2$	0.12mg/ml
3.	Precipitation with PEG	0.16mg/ml

3.3.6. Enzyme assay and characterization of cytochrome P450:

1) Incubation test:

The incubation of solubilised microsomes with caffeine and NADPH led to the formation of theophylline as analyzed by TLC and HPLC. Table 3.3.6. shows the results obtained by incubation test of microsomes. At the end of six hours 52.8% of caffeine was biotransformed to theophylline. This proves that the enzyme responsible for biotransformation of caffeine to theophylline is present in the isolated microsomes.

Table 3.3.6. Biotransformation of caffeine to theophylline by incubating with microsomes.

S.No	Time (Hours)	Biotransformation of caffeine to theophylline (%)
1.	1	15.6
2.	2	34.8
3.	4	46.5
4.	6	52.8

2) Enzyme assay for cytochrome P450:

CO reduction spectra were obtained by reduced cytochrome P450 as explained in section 3.2.2.6. The reduced CO spectrum obtained shows a distinct peak at 450nm and is presented in figure 3.3.6.2. cytochrome P450 is best

characterized by the maximum absorbance at 450 nm for the CO adduct of the reduced hemoprotein and is caused by a thiolate anion at the fifth coordination of the heme-group present in these enzymes (Bart, 1999). Heme group of cytochrome P450 as shown in figure 3.3.6.1. is the active site of the cytochrome P450 enzyme.

The CO spectra was obtained with caffeine induced mycelia whereas in the non induced mycelium, no peak was obtained at 450 nm, suggesting the inducible nature of the enzyme. The results obtained from incubation test and from the CO spectra obtained, it can be concluded that cytochrome P450 is the enzyme responsible for the biotransformation of caffeine to theophylline and is inducible in nature. This reaction involves demethylation at 7-*N* position of the caffeine molecule. The results obtained vary from human cytochrome P450 1A2 (Cyp 1A2), as in case of *P. citrinum* MTCC 5215 only one product (theophylline) is formed. The major enzyme responsible for the biotransformation of caffeine in humans is Cyp 1A2 and the products of biotransformation in humans are paraxanthine (80%), theobromine (12%), and theophylline (4%) (Cornish and Kristman, 1957). 1, 3, 7-trimethyluric acid is also formed in the human liver, but theophylline is the only product of biotransformation in fungi distinguishing it from the human Cyp 1A2 system. It has been reported that the putative active site of cytochrome P450 1A2 consists of a rectangular slot surrounded by several aromatic side chains, all of which are positioned perpendicular to the heme (Fig. 3.3.6.1.) (Kelly and Sidney, 2000). These aromatic residues restrict the size of the cavity such that, only planar structures of particular dimensions will be able to occupy it. Caffeine sits within this active site in three different orientations, which would lead to three different *N*-demethylation

products (Kelly and Sidney, 2000) as shown in figure 3.3.6.1. The human P450 1A2 is based on the crystal structure of P450 BM3 (Lozano *et al*, 1997). Analysis of the resultant active site containing caffeine show that *N*-3 methyl group is closest to the heme, and the demethylation at this position accounts for approximately 80% of caffeine metabolism in humans, the rest of demethylation leads to 12% theobromine, 4% theophylline and 1% 1,3,7 tri methyl uric acid (Lelo *et al*, 1986; Gu *et al*, 1992). There are two resultant orientations of caffeine, one with and one without a water molecule between the *N*-3 methyl group and the iron (Lozano *et al*, 1997). In both the orientations caffeine is perpendicular to the heme. The later is in agreement with the studies of Sanz *et al.,* (1994), wherein the authors proposed that areas of minimum molecular electrostatic potential (MEP) minima serve to align the caffeine molecule, via hydrogen bonding, within the active site of human P450 1A2. The favored site of oxidation is supposed to be at a distance of 3Å from the MEP minimum and the deepest minimum on caffeine is 3 Å from the *N*-3 methyl group in case of Cyp 1A2.

In *P. citrinum* MTCC 5215, the only product of biotransformation is theophylline (Scheme 3.3.6). The probable reason may be that the orientation of caffeine in the heme *N*-7 methyl group is closest to the active site maintaining a minimum distance of 3 Å. Hence, it is the favored site of oxidative demethylation leading to the formation of theophylline. This explanation can be considered keeping in view the fact, that the active site in cytochrome P450 is conserved in all cytochrome P450s irrespective of the source and their substrate specificity. Hasemann and co workers (1995) have concluded that all cytochrome P450s have conserved secondary and tertiary structure. The core structure of cytochrome P450s shows

conservation of all α-helices and β sheets, with considerable variation in those structure elements known to be involved in substrate binding (Hasemann *et al*, 1995). This can be compared with demethylation of caffeine in *Pseudomonas alcaligenes* MTCC 5264 where the biotransformation products formed are theobromine (major product) and paraxanthine. Biotransformation of caffeine to theophylline occurs via 7-*N* demethylation in *P.citrinum* by cytochrome P450. The enzyme also requires NADPH and oxygen as shown in the scheme (scheme 3.3.6.).

Scheme: 3.3.6. 7-*N* demethylation in *P.citrinum* mediated by cytochrome P450.

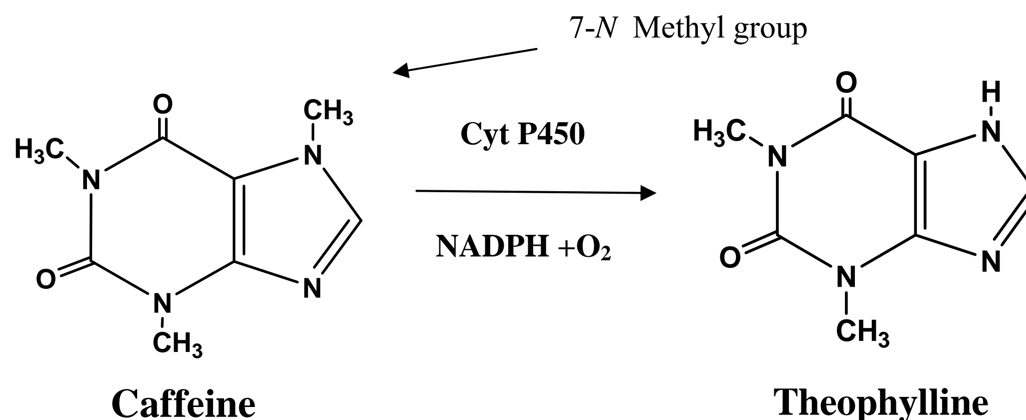


Figure 3.3.6.1. shows 3-*N* methyl group of caffeine nearest to the Fe, resulting in 3-*N* demethylation and formation of paraxanthine which is the major product (80%) of first step of caffeine demethylation in humans.

Figure 3.3.6.1. Heme group of cytochrome P450 showing the position of caffeine in the active site in human cytochrome P450 system (Adapted from Regal and Nelson, 2000).

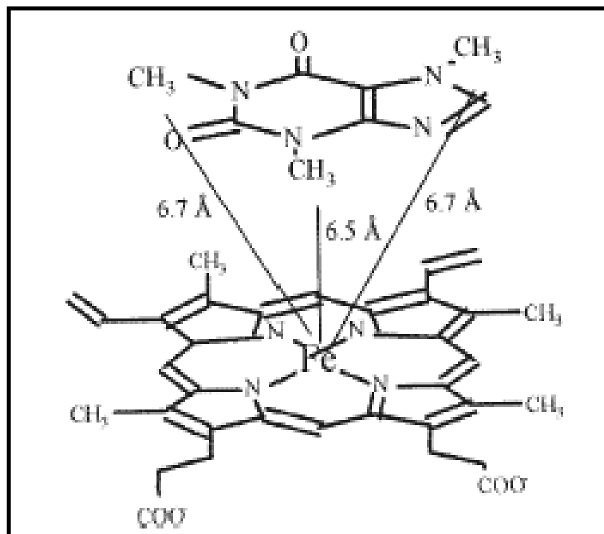
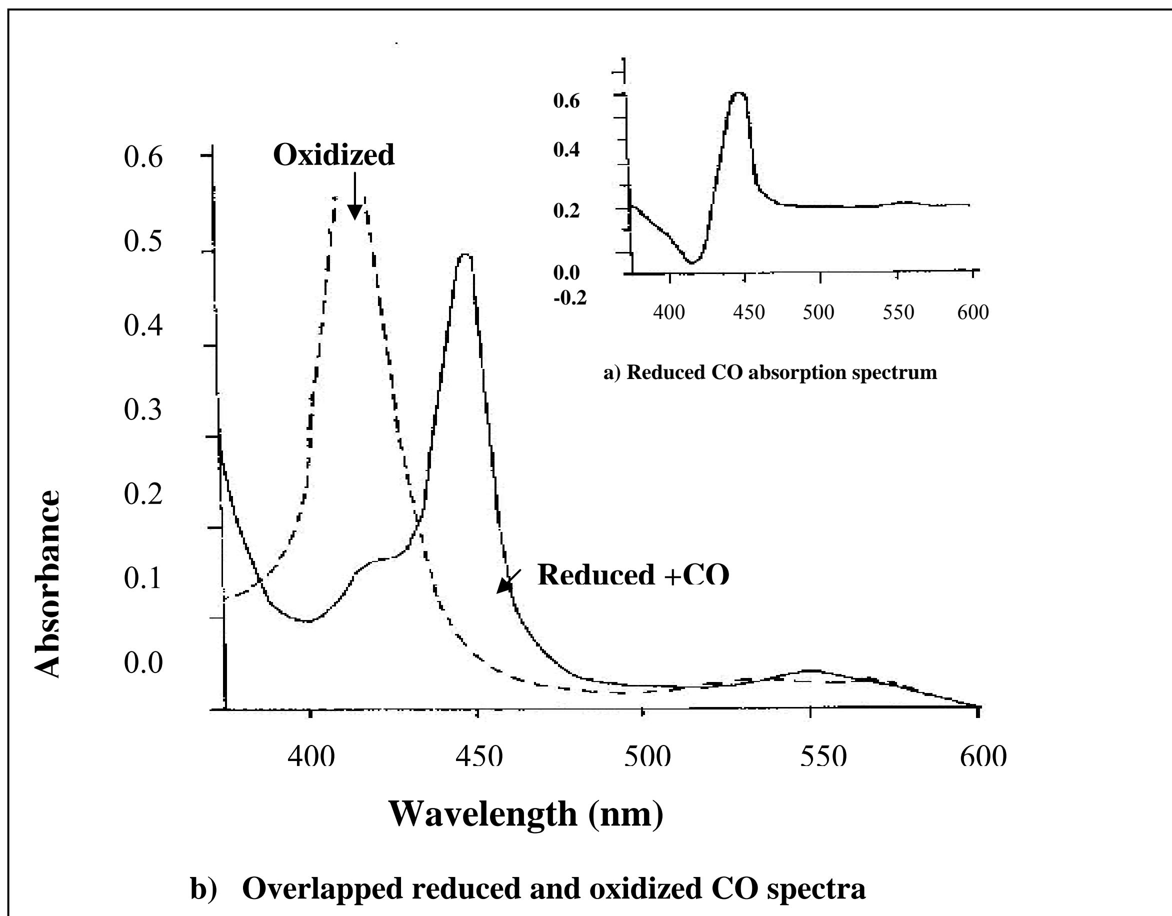


Figure 3.3.6.2. CO absorption spectra of cytochrome P450 isolated from *P. citrinum* MTCC 5215.



b) Overlapped reduced and oxidized CO spectra

The enzyme extract was reduced by adding sodium dithionite (-----) and subsequently CO was added (——) to obtain the reduced CO absorption spectra which shows maximum absorption at 450nm.

3) Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) of induced cytochrome P450:

SDS-PAGE was carried out according to the method of Laemmli (1970) figure 3.3.6.3. represents the SDS-PAGE carried out with the induced and uninduced enzyme. The CEE of the induced biomass shows a protein band, which corresponds to the enzyme responsible for the biotransformation of caffeine to theophylline as shown in lane 3 and 4 of figure 3.3.6.3. The corresponding band is not found in the uninduced biomass enzyme preparation as is evident from the results of Lane 1 and 2. The microsomes obtained from the induced biomass showed the characteristic CO spectrum (Fig. 3.3.6.2). These results confirm that the cytochrome P450 enzyme responsible for biotransformation of caffeine to theophylline is inducible in nature.

Figure 3.3.6.3 Gel electrophoresis of enzyme extract from induced and uninduced biomass to determine the inducible nature of cytochrome P450.

- **Lane 1 and 2 -** Enzyme extract from uninduced biomass
- **Lane 3 and 4 –** Enzyme extract from induced biomass

4) Gel filtration chromatography for purification of cytochrome P450:

Gel filtration chromatography was carried out for the purification of cytochrome P450 and the results are represented below in figure 3.3.6.4. Fraction number 30 to 35 showed maximum protein content as is evident from figure 3.3.6.4. These fractions were further used to check the activity of the cytochrome P450 enzyme by reconstitution studies.

Figure 3.3.6.4. Purification of Cyt P450 by gel permeation chromatography.

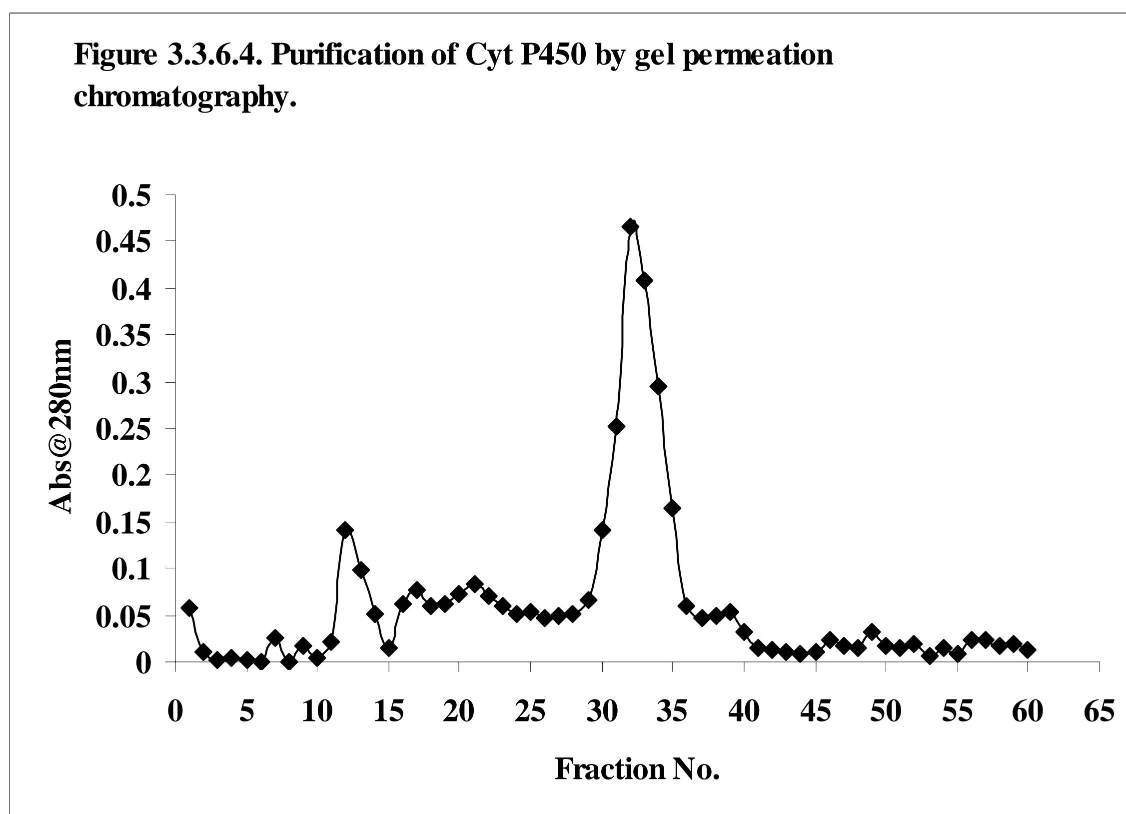
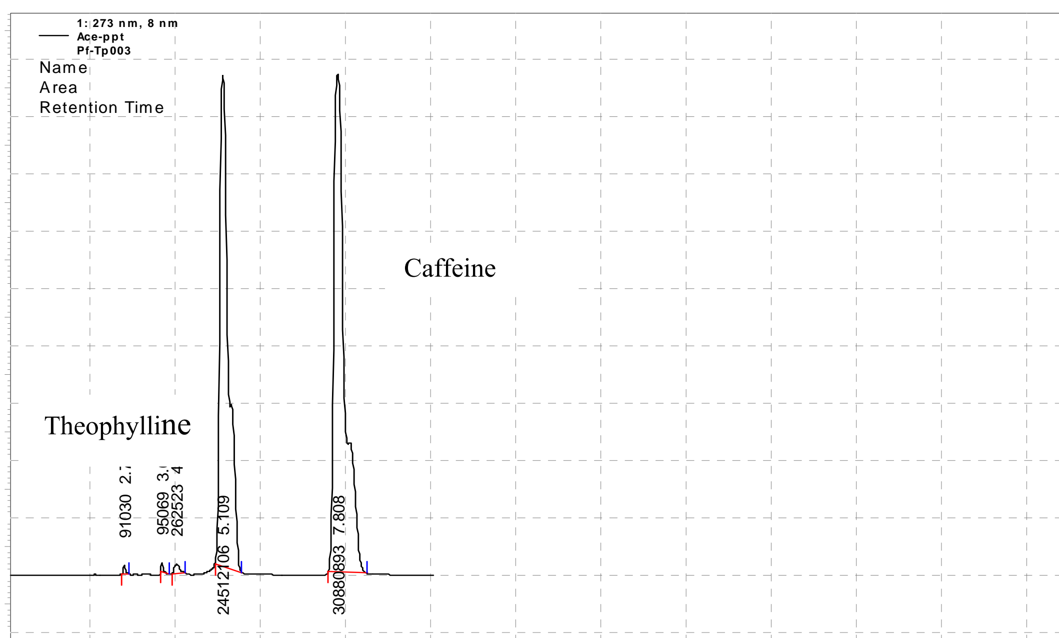


Figure 3.3.6.4. represents the partial purification of Cytochrome P450 by gel filtration chromatography on a Sephadex-G75 column. The fractions with high absorbance at 280nm were reconstituted and the activity was checked. The active fractions were pooled, concentrated and stored at $4^{o}C$ until further use.

5) Reconstitution of cytochrome P450:

The reconstituted enzyme system consisted of fractions 30-35 obtained by gel filtration chromatography. Caffeine was biotransformed to theophylline in the reconstituted enzyme system as shown in figure 3.3.6.5. The results showed 34.2% of biotransformation of caffeine to theophylline after two hours of incubation. The purified fraction when reconstituted with cytochrome P450 reductase, dilauryl phosphatidyl choline, NADPH and caffeine converted caffeine to theophylline confirming that cytochrome P450 is the enzyme responsible for the biotransformation of caffeine to theophylline and it requires a reductase system for its function. Omission of cytochrome P450 reductase or dilauryl phosphatidyl choline or NADPH from the system did not show conversion of caffeine to theophylline, confirming their requirement for the functioning of cytochrome P450 enzyme.

Figure 3.3.6.5. HPLC chromatogram showing biotransformation of caffeine to theophylline in reconstituted enzyme system.



No reports are available on the identification of enzyme responsible for biotransformation of caffeine to theophylline in fungal systems. The enzyme responsible for biotransformation of caffeine to theophylline was identified as cytochrome P450 which removes the 7-*N* methyl group of caffeine by oxidative demethylation. The enzyme was found to be microsomal bound and inducible.

3.3.7. Enzyme assay for other enzymes involved in caffeine biotransformation pathway of *P. citrinum* MTCC 5215:

3.3.7.1. Heteroxanthine demethylase (HXDM):

The enzyme responsible for the biotransformation of theophylline to 1-methyl xanthine by 3-*N* demethylation was identified as HXDM. The spectrophotometric assay results have been shown in Table 3.3.7.1. The enzyme activity observed was 24.48U/mg of crude protein extract as shown in Table 3.3.7.1. Scheme 3.3.7.1. shows the formation of 1-methyl xanthine from theophylline by 3-*N* demethylation. The incubation test confirms the conversion of theophylline to 1-methyl xanthine. Heteroxanthine demethylase also accepted heteroxanthine and theobromine as substrates but not caffeine. The order of substrate preference was heteroxanthine>theobromine>theophylline. Gluck and Lingens (1988) have reported that, heteroxanthine demethylase was the enzyme responsible for the demethylation of heteroxanthine to xanthine by 7-*N* demethylation using NADPH as the cofactor in *Pseudomonas putida*.

Scheme: 3.3.7.1. Biotransformation of theophylline to 1 methyl xanthine by heteroxanthine demethylase in *P. citrinum*.

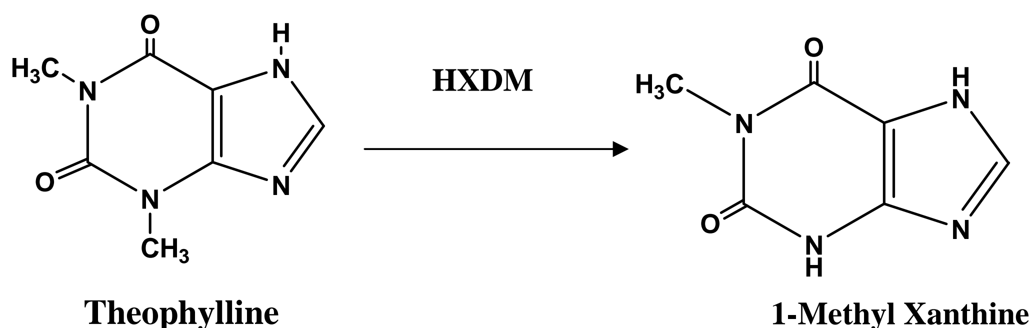


Table 3.3.7.1. HXDM activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Absorbance @340nm	ΔAbs/min
1.	1.286	0.249
2.	1.261	0.234
3.	1.281	0.249
4.	1.279	0.248
5.	1.289	0.250
Rate=0.246abs/min	**Enzyme activity: 24.48U/mg of crude protein**	

3.3.7.2. Xanthine dehydrogenase (XDH):

Xanthine dehydrogense was identified as the enzyme for the conversion of 1-methyl xanthine to xanthine by spectrophotometric assay and the results have been presented in Table 3.3.7.2. The enzyme activity observed was 30.45 U/mg of protein in the CEE. It does 1-*N* demethylation leading to the formation of xanthine (scheme 3.3.7.2). Results of incubation test confirmed the conversion of 1-methyl xanthine to xanthine by XDH. The enzyme also accepted 1-methyl xanthine and hypoxanthine as substrate but did not accept theobromine and caffeine. This enzyme has been reported to be present in certain microorganisms such as *Streptomyces*, *Aspergillus nidulans*,

Neurospora crassa, and *Pseudomonas aeruginosa*, carrying out *N* demethylation (Vogels and Van der Drift, 1976; Bradshow and Barker, 1960; Smith et al, 1976; Sin, 1975; Woolfolk and Downward, 1977; Lyon and Garrett, 1978).

Scheme: 3.3.7.2. Biotransformation of 1 methyl xanthine to xanthine by xanthine dehydrogenase in *P. citrinum*.

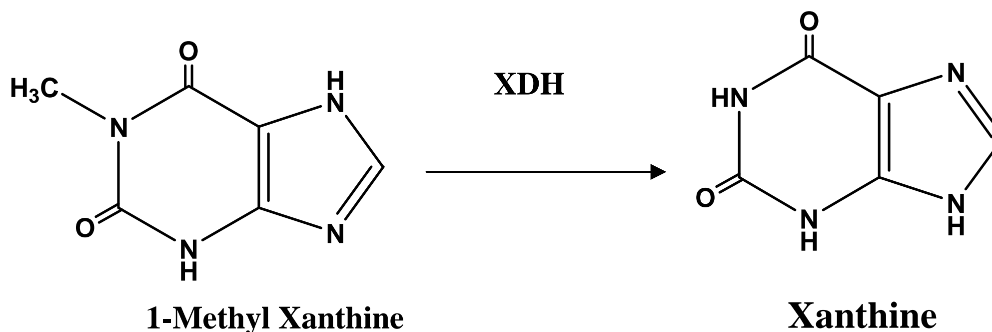


Table 3.3.7.2. XDH activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Absorbance @340nm	ΔAbs/min
1.	0.936	0.312
2.	0.897	0.301
3.	0.931	0.307
4.	0.909	0.315
5.	0.889	0.297
Rate=0.306abs/min, Enzyme activity : 30.45 U/mg of protein		

3.3.7.3. Xanthine oxidase (XO):

Xanthine oxidase was identified as the enzyme responsible for the conversion of xanthine to uric acid in *P. citrinum* MTCC 5215. The results of spectrophotometric assay have been presented in Table 3.3.7.3. It introduces one oxygen atom at C-8 position as shown in scheme 3.3.7.3. The enzyme activity observed was 9.33U/mg of protein in CEE as shown in Table 3.3.7.3. Xanthine oxidase have been reported from

various microbial sources where they perform oxidation of xathine and methyl xanthines to their respective uric acids. Incubation test confirmed the conversion of xanthine to uric acid by xanthine oxidase. However, the enzyme also accepted caffeine and theobromine as substrate but not theophylline. Woolfolk and Downward (1978) reported xanthine oxidase from *Arthrobacter* sp. Yozo and Toru (1981) have reported the purification and properties of xanthine oxidase from *Enterobacter cloacae* KY3034, converting xanthine to uric acid. The enzyme was found to have molecular weight of 128kd and showed similarity in the prosthetic group of the enzyme isolated from other microbial sources. Xanthine oxidase contains nonheme iron, sulfide, flavin, and molybdenum in a molar ratio of 8:8:2:1.5 (Smith *et al*, 1967). Xanthine oxidase uses various electron acceptors as ferricyanide, 2,6 dichlorophenolindophenol, dyes, ferrodoxin and NAD. However, NAD has been reported to be the most effective electron acceptor in most microbial xanthine oxidases (Sin, 1975; Lyon and Garrett, 1978; Sakai and Jun, 1979; Ohe and Watanabe, 1979)

Scheme: 3.3.7.3. Biotransformation of xanthine to uric acid by xanthine oxidase in *P. citrinum*.

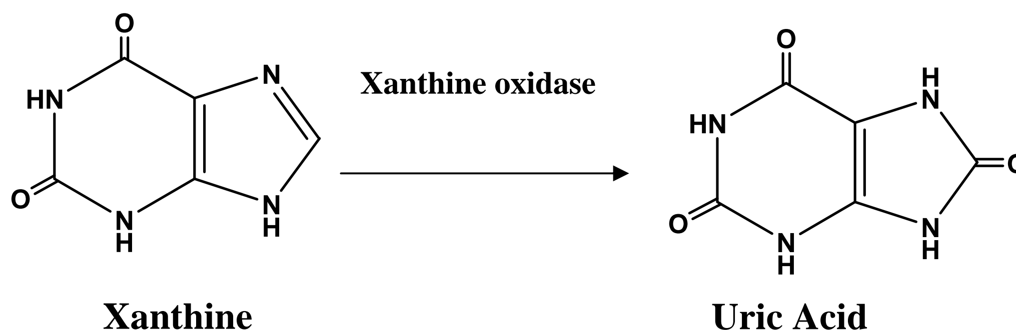


Table 3.3.7.3. XO activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Absorbance @600nm	ΔAbs/min
1.	1.546	0.0977
2.	1.503	0.0879
3.	1.517	0.0912
4.	1.533	0.0959
5.	1.537	0.0962
Rate=0.0938abs/min	**Enzyme activity : 9.33U/mg of protein**	

3.3.7.4. Uricase:

The enzyme for the biotransformation of uric acid to allantoin was identified as uricase by spectrophotometric assay and the results have been presented in Table 3.3.7.4. The enzyme activity observed was 73.37 U/mg of protein in CEE. Uricase cleaves the purine ring and leads to formation of allantoin in fungal and bacterial species as shown in scheme 3.3.7.4. (Blecher and Lingens, 1977). The enzyme is also responsible for the conversion of uric acid to allantoin in plants and animals (Franke *et al*, 1965).

Scheme: 3.3.7.4. Biotransformation of uric acid to allantoin by uricase in *P. citrinum*.

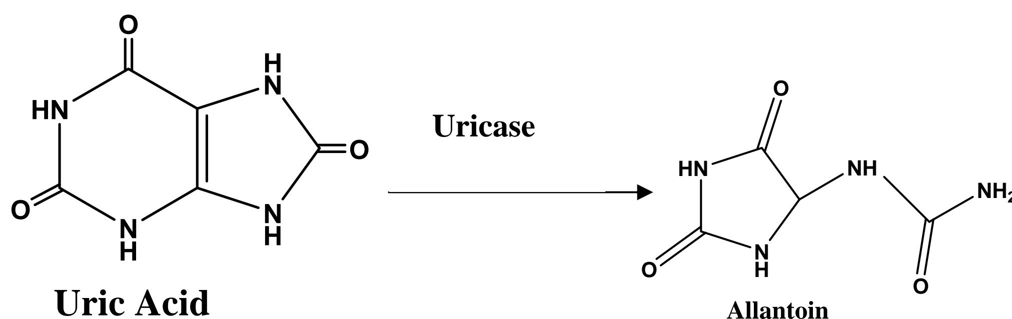


Table 3.3.7.4. Uricase activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Absorbance @290nm	ΔAbs/min
1.	1.368	0.741
2.	1.327	0.729
3.	1.370	0.744
4.	1.362	0.735
5.	1.365	0.738
Rate=0.737abs/min	**Enzyme activity : 73.37 U/mg of protein.**	

3.3.7.5. Allantoinase:

Allantoinase was identified as the enzyme responsible for biotransformation of allantoin to allantoic acid in *P. citrinum* MTCC 5215. The activity of allantoin was confirmed by incubation test and the results have been presented in Table 3.3.7.5. 64.81% conversion of allantoin to allantoic acid occurred in 6 hours. Scheme 3.3.7.5. shows the imidazole ring opening by allantoinase leading to formation of allantoic acid. Allantoinase has also been reported as the enzyme for conversion of allantoin to allantoic acid in *Pseudomonas putida* by Van der Drift *et al* (1975).

Scheme: 3.3.7.5. Biotransformation of allantoin to allantoic acidby allantoinase in *P. citrinum*.

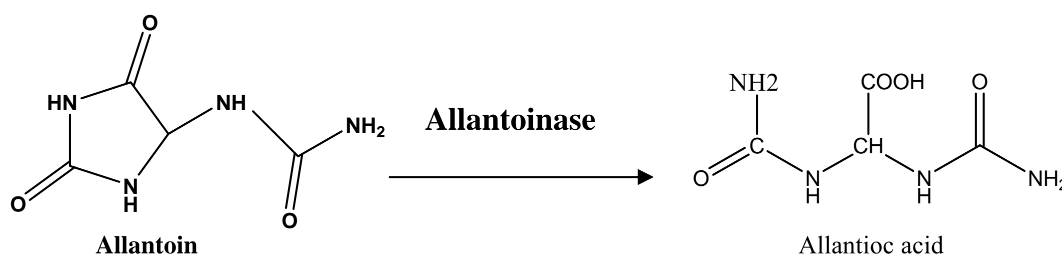


Table 3.3.7.5 Allantoinase activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S. No	Time (Hours)	% Conversion of allantoin to allantoic acid.
1.	0	0
2.	2	22.10
3.	4	31.74
4.	6	64.81

3.3.7.6. Allantoicase:

Allantoicase converted allantoic acid to urea in *P. citrinum* confirmed by incubation test and have been presented in Table 3.3.7.6. Scheme 3.3.7.6. shows the conversion of allantoic acid to urea by allantoicase. No direct spectrophotometric assay methods are available for the detection of allantoicse. Therefore, confirmation of the presence of enzyme was carried out by product formation during incubation test and analyzing the product by HPLC. 59.27% of conversion of allantoic acid to urea was observed in 6 hours. Blecher and Lingens (1977) identified allantoicase as the enzyme for the conversion of allantoic acid to urea in *P. putida* using the analytical methods of Young and Conway (1942) modified by Trijbels and Vogels (1966).

Scheme: 3.3.7.6. Biotransformation of allantoic acid to urea by allantoicase in *P. citrinum* MTCC 5215.

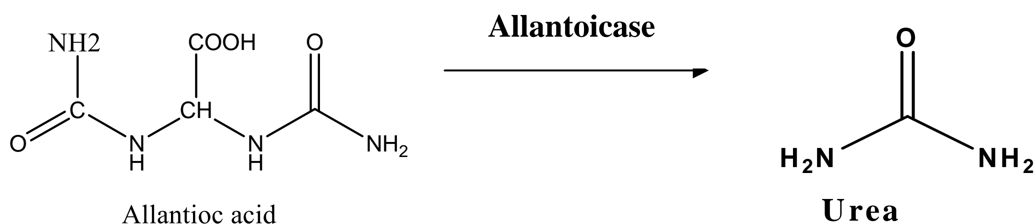


Table 3.3.7.6 Allantoicase activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Time (in Hours)	% Conversion of allantoic acid to urea determined by HPLC
1.	0	0
2.	2	13.25
3.	4	37.19
4.	6	59.27

3.3.7.7. Urease:

Urease was identified as the enzyme for the conversion of urea to ammonia and carbon dioxide. The activity of urease was monitored by Nessler's reagent and the results have been presented in Table 3.3.7.7. Scheme 3.3.7.7. shows conversion of urea to carbon dioxide and ammonia. Urease has been reported as the enzyme responsible for the conversion of urea to carbon dioxide and ammonia in bacterial species *P. putida* (Blecher and Lingens, 1977). It also degrades urea in the purine metabolism pathway of various organisms (Stewart, 1965).

Scheme: 3.3.7.7. Biotransformation of urea to carbon dioxide and ammonia by urease in *P. citrinum*. MTCC 5215.

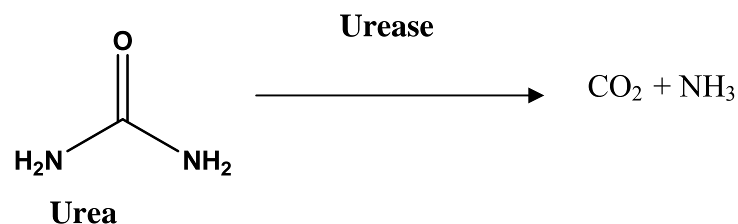


Table 3.3.7.7. Urease activity in crude enzyme extract of *P. citrinum* MTCC 5215.

S.No	Time (min)	Absorbance @490nm
1.	0	0.175
2.	15	0.310
3.	30	0.402
4.	45	0.517
5.	60	0.625

3.3.8. Caffeine degradation pathway in *P. citrinum* MTCC 5215:

The results obtained for the identification of the metabolites as shown in section 3.3.1, 3.3.2 and the identification of the enzymes responsible for the biotransformation of these metabolites have been explained in section 3.3.6. Compilation of both the above results summarizes the complete pathway of caffeine biotransformation in *P. citrinum* MTCC 5215 and have been represented in figure 3.3.8. The pathway reports all the biotransformed metabolites and their respective enzymes. The caffeine biotransformation pathway in a fungal strain has been elucidated for the first time in the present work. However, the caffeine biotransformation pathway in *P. putida* has been worked out by Blecher and Lingens (1977). Blecher and Lingens (1977) studied degradation of caffeine by *P. putida* strains isolated from soil and identified 14 catabolites: Theobromine, paraxanthine, 7monomethylxanthine, xanthine, 3,7-dimethyluric acid, 1,7-dimethyluric acid, 7methyluric acid, uric acid, allantoin, allantoic acid, ureidoglicolic acid, glioxilic acid, urea and formaldehyde. Yamaoka-Yano and Mazzafera (1999) studied the caffeine degradation pathway in *P. putida* strain, and the results were in agreement with results previously obtained by Blecher and Lingens (1977). The caffeine

degradation pathway in *P. alcaligenes* MTCC 5264 has been elucidated in our lab. In fungal strain caffeine is biotransformed to theophylline by cytochrome P450 as explained in section 3.3.6. Sauer *et al* (1982) obtained indications that caffeine in yeast was also biotransformed by cytochrome P450, suggesting that the catabolic pathway might be similar to that of animals. In humans, several cytochrome P-450 isoforms are responsible for caffeine biotransformation (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 cyt P450 in caffeine biotransformation mechanism. In bacterial species it has been identified as caffeine demethylase in *P. alcaligenes* MTCC 5264, further it was isolated and purified in our lab. The products formed by bitransformation of caffeine in this particular strain were theobromine and paraxanthine. Gluck and Lingens (1987) reported caffeine biotransformation 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 made by Mazzafera *et al*. (1994) with *S. marcescens.* Madyastha *et al* (1999) reported caffeine oxidase as the enzyme for the biotransformation of caffeine to 1,3,7 trimethyl uric acid by mixed culture consortium of *Klebsiella* and *Rhodococcus*. The enzymes responsible for the further biotransformation of dimethyl xanthines and other metabolites of the pathway havebeen identified by Blecher and Lingens 1977 and Yamaoka-Yano and Mazzafera 1999 in bacteria.

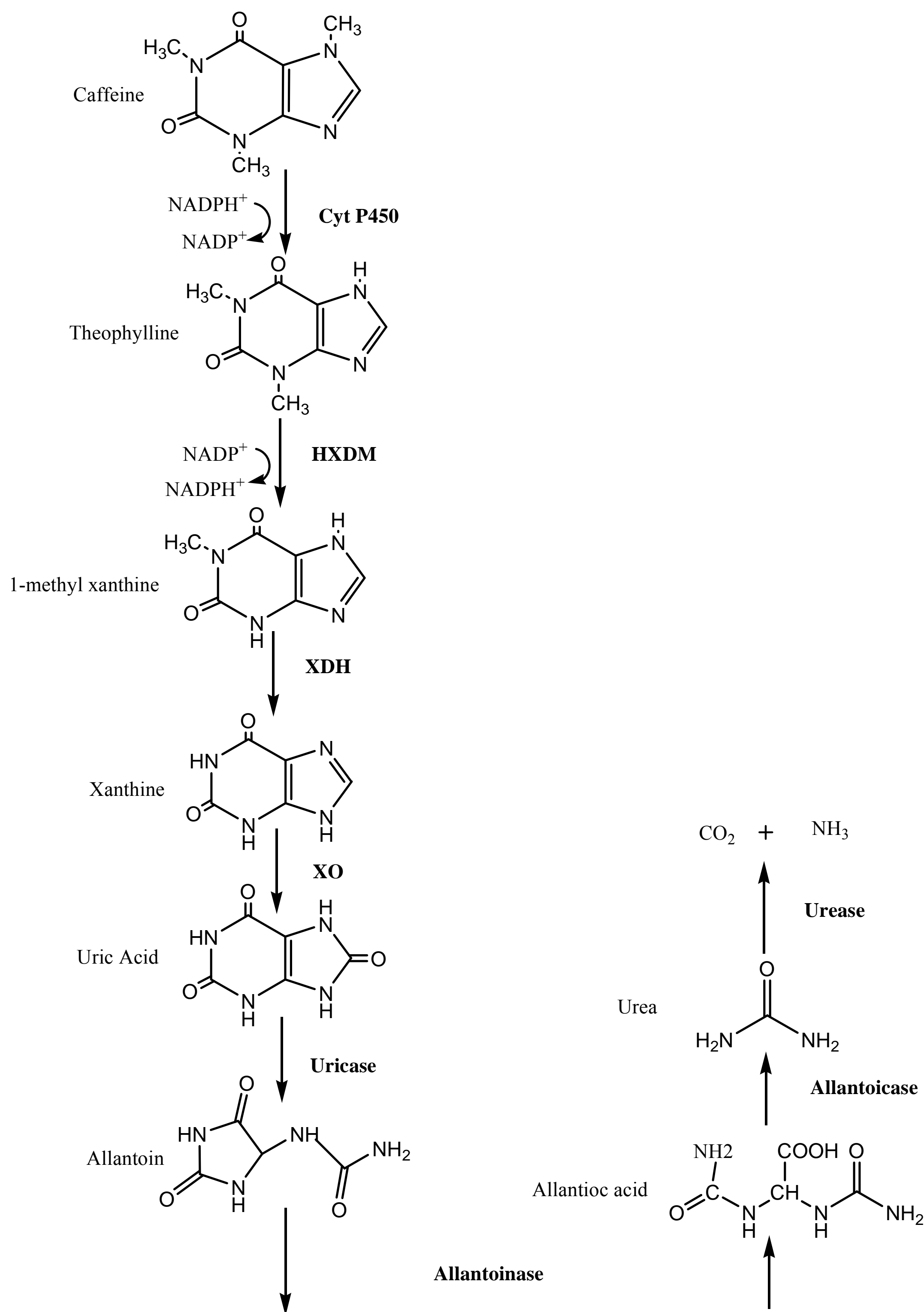
In the present study the demethylation of caffeine at 7-*N* position occurs by cytochrome P450. *N*-demethylation is an oxidative reaction and proceeds – especially in the case of *N*-alkyalated compounds – through the formation of an *N*-hydroxymethyl intermediate (Keberle *et al*, 1963). This compound is unstable and breaks down to form a demethylated product and formaldehyde. Hohnloser *et al* (1980) reported the demethylation of caffeine to dimethyl xanthines by the formation of formaldehyde in the strain of *P. putida* C1. The above mechanism stands in agreement with the demethylation of caffeine to theophylline by cytochrome P450 in *P. citrinum* MTCC 5215. The same mechanism can also be applied to the cleavage of O-alkyl bonds. The enzymes involved in these O and *N*-dealkylation reactions were first investigated in microsomal enzyme systems (Brodie *et al*, 1958). In 1971, the properties of microbial enzymes which attack alky-nitrogen bonds were reviewed by Large, who discussed three classes of these enzymes (dehydrogenases, oxidases and mono-oxygenases). In case of *P. citrinum* MTCC 5215, 7-*N* demethylation occurs and it is by a Cyt P450 and the reaction is monooxygenase in nature.

The dimethyl xanthine obtained after the first demethylation reaction was carried out by heteroxanthine demethylase, which demethylated theophylline to the next metabolite 1 methyl xanthine and the 1-methyl xanthine obtained was further demthylated to xanthine by xanthine dehydrogenase. Both the above demethylations can be classified under the dehydrogenase class.

Further reaction in the biotransformation pathway occurs by the breakdown of purine structure. The enzymes of the pathway and the metabolites during the further biotransformation of xanthine to further biotransformed products is common to both

the biotransformation pathway of fungi and bacteria and bears resemblance to the purine degradation pathway. Xanthine oxidase is the enzyme which cleaves the purine ring structure leading to formation of uric acid. The further degradation of uric acid was carried out by uricase forming allantoin. The imidazole ring of allantoin was broken down by allantoinase, forming allantoic acid. The allantoic acid was broken down by allantoicase to form urea, which was finally metabolized to carbon dioxide and ammonia.

Figure 3.3.8. Caffeine biotransformation pathway in *P. citrinum* MTCC 5215.



3.4 Conclusion:

The enzymes involved in the caffeine biotransformation pathway in *P. citrinum* MTCC 5215 were isolated and identified. Using the information of the enzyme assays, the caffeine biotransformation pathway in *P. citrinum* MTCC 5215 was elucidated. This is the first comprehensive report on the enzymes involved in biotransformation of caffeine in fungi. It has been conclusively reported for the first time that the biotransformation of caffeine to theophylline is by the enzyme cytochrome P450 which is inducible in nature. Theophylline is the only biotransformation product formed by the demethylation of caffeine unlike in bacterial system *P. alacaligenes* where the products are theobromine and paraxanthine. This is also in disagreement with the animal system where the products are paraxanthine, theophylline, theobromine and 1,3,7 trimethyl uric acid. The complete elucidation of the biotransformation pathway shows that theophylline, and 1 methyl xanthine are formed during biotransformation of caffeine. These methyl xanthines have pharmaceutical significance. Besides this, uric acid and allantoin are also formed as biotransformed products after the opening of the purine ring. Uric acid has been used as an antioxidant and allantoin has been used in various cosmetic preparations. The complete knowledge about the enzymes of each step of biotransformation would help in designing processes for the biotransformation of the important metabolites of the pathway. The knowledge gained in the present study sheds light on the further development of biotechnological processes for the production of pharmaceutically important methyl xanthines and further work is underway in our laboratory.

References:

Allam, A. M., and Eazainy, T. A. (1969), Degradation of xanthine by Penicillium chrysogenum. *J. Gen. Microbiol*. **56**: 293-300.

Arnaud, M. .J. and Welsch, C. (1980), Caffeine metabolism in human subjects. 9th Symposium of the Association Scientifique Internationale du Cafe, ASIC Colloq., London. 385-396.

Arnaud, M. J. (1984), Products of metabolism of caffeine. In: P.B. Dews (Ed.), Caffeine. Perspectives from Recent Research, Springer-Verlag, Berlin, 3-38.

Arnaud, M. .J. (1985), Comparative metabolic disposition of [1-Me^{14}C] caffeine in rats, mice and chinese hamsters. *Drug Met. Dispos*. **13**: 471-478.

Asano, Y., Komeda, T. and Yamada, H. (1993), Microbial production of theobromine from caffeine. *Biosc, Biotechnol and Biochem*. **57**:1286-1289.

Asano, Y., Toshihiro, K, and Yamada, H. (1994), Enzymes involved in theobromine production from caffeine by *Pseudomonas putida* No. 352. *Biosci. Biotechnol. Biochem.* **58**:2303–2304.

Ashihara, H., Monteiro, A. M, Moritz, T., Gillies, F. M, and Crozier, A. (1996), Catabolism of caffeine and related purine alkaloids in leafs of *Coffeea arabica* L. *Planta* **198**:334–339.

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**:413–419.

Ashihara, H. and Crozier, A. (1999), Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Adv. in Bot. Res.* **30**:117-205.

Ballard, S. A., Kelly, S. L., Ellis, S.W., and Troke, P.F. (1990), Interaction of microsomal cytochrome P-450 isolated from *Aspergillus fumigatus* with fluconazole and itraconazole. *J. Med. Vet. Mycol.* **28** (4): 327-334.

Bart, F. (1999), Fungal cytochrome P450 involved in hydroxylations of aromatic compounds. PhD Thesis submitted to Delft University of Technology, Netherland.

Berthou, F., Ratanasavanh, D., Alix, D., Carlhant, D., Riche, C. and Guillouzo, A. (1988), Caffeine and theophylline metabolism in newborn and adult human hepatocytes: A comparison with adult rat hepatocytes. *Biochem. Pharmacol*., **37**: 3691-3700.

Berthou, F.; Guillois, B.; Riche, C.; Dreano, Y.; Jacqzaigrain, E.; and Beaunes, P.H. (1992), Interspecies variation in caffeine metabolism related to cytochrome P4501A enzymes. *Xenobiotica*. **22**: 671-680.

Birkett, D. J, Grygiel, J. J, Mines, J. O. (1981), Metabolic deposition of methyl xanthines in man. *In*: Theophylline and other methylxanthines, N. Rietenbrock, Woodcock B.G, Staib A. H (eds). Vieweg Verlag. The Proceedings of an International Symposium, Frankfurt: 29th april and 3rd May 149-158.

Blecher, R., Lingens, F. (1977), Metabolism of caffeine by *Pseudomonas putida*. *Hoppe Seyler's Z Physiol Chem.* **358**: 807–817.

Bradford, M. 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.

Bradshow, W. H and Barker, H. A. Purification and Properties of Xanthine Dehydrogenase from *Clostridium cylindrosporum*. (1960), *J. Biol. Chem*. **235**: 3620-3629.

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.

Branfman, A. R., McComish, M. F., Bruni, R. J., Callahan, M. M., Robertson, R. and Yesair, D.W. (1983), Characterization of diaminouracil metabolites of caffeine in human urine. *Drug Metabol. Disp*. **11**: 206-210.

Bray, R. C. (1963), Xanthine oxidase. *In*: Boyer, P. D., Lardy, H. and Myrback, K. (Eds.), The Enzymes, 2nd ed., vol. 7, Academic Press, New York, 533-556.

Brodie, B. B, Gillette, J. R and La Du, B. N. (1958), Enzymatic Metabolism of Drugs and other Foreign Compounds. *Ann. Rev .Biochem*. **27**: 427-454.

Butler, M. A., Iwasaki., M., Guengerich, F. P., and Kadlubar, F. (1989), Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*- oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci*.. **86** : 7696-7700.

Callahan, M. M., Robertson, R. S., Arnaud, M. J., Branfman, A. R., McComish, M. F.,and Yesair D. W. (1982), Human metabolism of [1-methyl-^{14}C]- and [2-^{14}C]caffeine after oral administration. *Drug Met. Disp*. **10**: 417-423.

Callahan, M. M., Robertson, R. S., Branfman, A. ., McComish, M. F. and Yesair, D. W. (1983), Comparison of caffeine metabolism in three nonsmoking populations

after oral administration of radiolabeled caffeine. *Drug Metabol. Disp*. **11**:211-217.

Caubet, M. S, Comte, B, and Brazier, J. L. (2004), Determination of urinary ^{13}C caffeine metabolites by liquid chromatography–mass spectrometry: the use of metabolic ratios to assess CYP1A2 activity. *J Pharm. Biomed. Anal*. **34**: 379–389.

Chenault, H. K, Whitesides, G. M. (1987), Regeneration of nicotinamide cofactors for use in organic synthesis. *Appl.Biochem. Biotechnol*. **14**:147-197.

Chung, W. G., and Cha, Y. N. (1997), Oxidation of caffeine to theobromine and theophylline is catalyzed primarily by flavin-containing monooxygenase in liver microsomes. *Biochem. Biophys. Res. Commun*. **235**: 685-688.

Cinti, D. L., Moldevs, P., and Schenkmar (1972), Kinetic parameters of drug-metabolizing enzymes in Ca 2+ -sedimented microsomes from rat liver. *Biochem. Pharmacol*. **21**: 3249.

Cornish, H. H, Christman, A. A. (1957), A study of the metabolism of theobromine, theophylline and caffeine in man. *J Biol Chem.* **228**: 315–323.

Dixon, G.H., and Kornberg, H. L., Assay methods for key enzymes of the glyoxylate cycle (1959), *Biochem.J.* **72**: 3-9.

Erickson, S. K., and Bosterling, B. (1981), Cholesterol 7a-hydroxylase from human liver: partial purification and reconstitution into defined phospholipid-cholesterol vesic1es. j. *Lipid* ***Res.*** **22:** 872-876.

Estabrook, R. W., Peterson. J., Baron, J., and Hildebrandt, A. The spectroscopic measurement of cytochromes associated with drug metabolism. (1972), *Methods Pharamacol.* **2**: 303-350.

Estabrook, R.W. and Werringler, J. (1978), The measurement of difference spectra:Application to the cytochromes of microsomes. *Methods Enzymol*. **52**: 212-220.

Franke, W. Thiemann, A., Remily, C., and Mockheye, K. (1965), On knowing ureide-splitting enzymes. I. Soy bean allantoinase. *Enzymologia.* **29**: 251-271.

Fuh, U., Doehmer, J., Battula, N., Wölfel, C., Kudla, C., Keita, Y., and Staib, A. H (1992), Biotranformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of Fredericks, A. B., Benowitz, N. L and single cytochome P450 isoforms. *Biochem. Pharm.,* **43**: 225-235.

Gluck, M., and Lingens, F. (1987), Studies on the microbial production of theobromine and heteroxanthine from caffeine. *Appl. Microbiol. Biotechnol.* **25**:334-340.

Gluck, M., and Lingens F (1988), Heteroxanthine Demethylase, a new enzyme in the degradation of caffeine by *Pseudomonas putida*. *Appl. Microbiol. Biotechnol.* **28**:59-62.

Grant, D. M., Blum, M., Beer, M., and Meyer, U. A. (1991), Monomorphic and polymorphic human arylamine *N*-acetyltransferases: a comparison of liver isozymes and expressed products of two cloned genes. *Mol. Pharmac*. **39**: 184-191.

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

Hakil, M., Denis, S., Gonz´alez, G. V., and Augur, C. (1998), Degradation and product analysis of caffeine and related dimethyl xanthines by filamentous fungi. *Enzyme Microb Technol* **22**:355–359.

Hardy, B. G., Lemieux, C., Walker, S. E. and Bartle, W. R. (1988), Inter individual and intra individual variability in acetylation: Characterization with caffeine. *Clin. Pharmacol. Ther*. **44**: 152-157.

Hasemann, C.A., Kurumbil, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995), Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure*. **3** (1): 41-62.

Hohnloser, W., Osswalt, B., Lingens, F. V (1980), Enzymological aspects of caffeine demethylation and formaldehyde oxidation by *Pseudomonas putida* C1. *Hoppe seyler's Z Physiol Chem* **361**:1763–1766.

Ina, K., Biochemical studies of caffeine. (1971), Degradation of caffeine by mold. *Nippon Nogeikagaku Kaishi*. **45** (8): 378-380.

Jayaraman, (1988), *Laboratory manual in Biochemistry*. 3rd edition. New Delhi. Wiley Eastern Limited. pp. 114 - 115, 130 -131.

Kalberer, P. (1965), Breakdown of caffeine in the leaves of *Coffea arabica* L. *Nature* **205**: 597-598.

Kalow, W. (1985), Variablity of Caffeine Metabolism in Humans. *Drug Res*. **35**:319-324.

Kamath, S. A., Kummerow, F. A., and Ananth, N. K (1971), A simple procedure for the isolation of rat liver microsomes.*FEBS Lett.* **17**: 90-92.

Kamath, S. A. and Ananth, N. K (1972), Interaction of Ca 2+ with endoplasmic reticulum of rat liver: a standardized procedure for the isolation of rat liver microsomes. *Anal. Biochem*. **48** (1): 53-58.

Keberle, H., Riess, W., Schimd, K. and Hoffman (1963), On the stereospecific metabolism of the optical antipodes of alpha-phenyl-alpha-ethyl-glutarimide (Doriden). *Arch. Int. Pharmadyn*. **142**: 117-124.

Kelly, A. R and Sidney, D. N (2000) Orientation of caffeine within the active site of human cytochrome P450 1A2 based on NMR longitudinal (T_1) relaxation measurements. *Arch. Biochem. Biophy.* **384** (1): 47-58.

Khanna, K. L, Rao, G. S., and Cornish, H. H. (1972), Metabolism of caffeine-3H in the rat. *Toxicol Appl Pharmacol*. **23**: 720–730.

Koyama, Y., Tomoda, Y., Kato, M., and Ashihara, H. (2003), Metabolism of purine bases, nucleosides and alkaloids in theobromine-forming *Theobroma cacao* leaves. *Plant Physiol Biochem* **41**: 977–984.

Kupfer, D and Levin, E. (1972), Monooxygenase drug metabolizing activity in $CaCl_2$ -aggregated hepatic microsomes from rat liver. *Bichem. Biophys. Res. Commun.* **47** (3): 611-618.

Kurtzman, R. H., Jr., and Schwimmer, .S. (1971), Caffeine removal from growth media by microorganisms. *Experimentia*. **27**(4): 481-482.

Laemmli, U.K**.,** (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Large, P. J (1971), The oxidative cleavage of alkyl-nitrogen bonds in micro-organisms. Xenobiotica. *Xenobiotica*. **1**(4): 457-467.

Lelo, A., Miners, J. O., Robson, R. A., and Birkett, D. J. (1986), Quantitative assessment of caffeine partial clearances in man. *Br.J.Clin.Pharm*. 22: 183-186.

Lozano, J. J., Lopez-de-Brinas, E., Centeno, N. B., Guizo, R., and Sanz, F. (1997), Three-dimensional modelling of human cytochrome P450 1A2 and its interaction with caffeine and MeIQ. *J.Comput. Aided. Mol.Design.* **11**(4): 395-408.

Lyon, E. S and Garrett, R. H. (1978), Regulation, purification, and properties of xanthine dehydrogenase in Neurospora crassa. *J.Biol.Chem.* **253 (8)**: 2604-2614.

Machida, Y., and Nakanishi. T. (1981), Purification and properties of xanthine Oxidase from *Enterobacter cloacae*. *Agric. Biol. Chem.* **45** (2): 425-432.

Madyastha, K. M, and Sridhar, G. R. (1998), A Novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem Biophys Res Commun.* **249**: 178–181.

Madyastha, K. M., Sridhar, G. R., Vadiraja, B. B., and 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.

Mahler, H. R., Hubscher, G., and Baum, H. (1955), Studies on uricase. I. Preparation, purification, and properties of a cupro protein. *J. Biol. Chem.* **216** (2): 625-641.

Mazzafera, P. (1993), 7-Methyl xanthine is not involved in caffeine catabolism in *Coffea dewevrei*. *J Agric Food Chem.* **41**:1541–1543.

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

Mazzafera, P., Crozier, A. and Magalhães, A. C. (1991), Caffeine metabolism in *Coffea arabica* and other species of Coffee. *Phytochem.* **30**: 3913-3916.

Mitoma, C., Posner, H. S., Retz, H. C., and Udenfriend, S., (1956), *Arch .Biochem. Biophys*. **61**: 431.

Ohe,.T and Watanabe, Y. (1979), Purification and properties of xanthine dehydrogenase from *Streptomyces cyanogenus. J. Biochem.* **86** (1): 45-51.

Omura, T., and Sato, R. (1964), The carbon monoxide-binding pigment of liver microsomes. Ii. Solubilization, purification, and properties. *J. Biol.Chem.* **239**: 2379-2385.

Ohsako, S. and Deguchi, T. (1990), Cloning and expresion of cDNAs for polymorphic and monomorphic arylamine Nacetyltransferases from human liver. *J.Biol. Chem*. **265**: 4630-4634.

Parkinson, A. (1996), Biotransformation of xenobiotics. In Casarett and Doull's Toxicology: The Basic Science of Poisons.ed. Klaassen, C.D., MacMillan, 5th edition, New York, 113-186.

Rao K. L, Khanna H. H. and Cornish. (1973), Identification of two new metabolites in rat urine. *Experientia*. **29**: 953–955.

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.

Roussos. S, M. M. Refugio, Gaime P., E. F., Ramakrishna, M., Raimbault, M. Viniegra-Gonzalez, G. (1995), Biotechnological management of coffee pulp-isolation, screening characterization, selection of caffeine degradaing fungi and natural microflora; *Appl. Microb. Biotechnol*. **42**: 756-762.

Sakai, T and Jun, H. K. (1979), Purification, crystallization, and some properties of xanthine dehydrogenase from Pseudomonas synxantha A3. *Agric.Biol.Chem.* **43**: 753-760.

Sanz, F., Lopez-de-Brinas, E., Rodriguez, J., and Manaut, F. Theoretical Study on the Metabolism of Caffeine by Cytochrome P-450 1A2 and its Inhibition (1994), *Quant. Struct. Act. Relat.* **13** (3): 281–284.

Sarath Babu, V. R, Patra S., Thakur, M. S, Karanth, N. G., and Varadaraj, M. C. (2005), Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708,.*Enzyme Microbial Technology*. **37**: 617-622.

Sarath Babu, V. R., Patra, S., Kumar, M. A., Karanth, N. G., Thakur, M. S. (2007), Development of a Biosensor for caffeine. *Analytica Chimica Acta*, **582** (2): 329-334.

Sauer M, Kappeli, O, and Fiechter, A. (1982), Comparison of the cytochrome P-450 containing monooxygenases originating from two different yeasts. *Dev. in Biochem.* **23**: 452-457.

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.

*Schenkman, J. B. and Cinti, D. L (1972), *Life. Sci.* **11**: 247-253.

Schwimmer, S; Ralph, H; Kurtzman, J. R; and Erich, H (1971), Caffeine metabolism by *Penicillium roqueforti*. *Arch. of Biochem and Bioph*. **147**: 109-113.

Sin, I. L. (1975), Purification and properties of xanthine dehydrogenase from Pseudomonas acidovorans. *Biochim. Biophys. Acta*, **410**: 12-16.

Smith, S. T., Rajagopalan, K. V. and Handler. P. (1967), Purification and properties of xanthine dehydrogenase from *Micrococcus lactilyticus*. *J. Biol. Chem*. **242**: 4108-4117.

Stewart, D. J. (1965), The urease activity of fluorescent Pseudomonads. *J. Gen. Microbiol.***41**: 169-174.

Suzuki, T. and Waller, G.R. (1984a), Biosynthesis and biodegradation of caffeine, theobromine, and theophylline in Coffea arabica L. Fruits. *J. Agric. Food Chem*. **32**:845-848.

Suzuki, T. and Waller, G. R. (1984b), Biodegradation of caffeine: formation of theophylline and theobromine from caffeine in mature *Coffea arabica* fruits. *J. Sci. Food Agric*. **35**: 66-70.

Tang, B. K., Grant, D. M., and Kalow, W. (1983), Isolation and identification of 5-acetylamino-6-formylamino-3-methyluracil as a major metabolite of caffeine in man. *Drug Metabolism and Disposition.* **11**: 218-220.

Tassaneeyakul, W., Birkett, D. J., McManus, M. E., Tassaneeyakul, W., Veronese, M. E., Anderson, T., Tukey, R. H. and Miners, J. O. (1994), Caffeine metabolism by human hepatic cytochomes P450:contributions of 1A2, 2E1 and 3A isoforms. *Biochem. Pharm*., **47**: 1767-1776.

Tatsuhiko, O., and Yasuto, W. (1979), Purification and properties of xanthine dehydrogenase, *J. Biochem.* **86**: 45-53.

Trijbels, F. and Vogels, G. D. (1966), *Biochimica. Acta.* **113**: 292-301. Van der D. L., Vogels, G. D and Van der D, C (1975) *Biochimi. Biophys. Acta*. **391**: 240-246.

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.

Vogels, G. D. and Van der D. C. (1976), Degradation of purines and pyrimidines by microorganisms. *Bacteriol. Rev*. **40**(2): 403-468.

Ward, D. N., and Arnott, M. S., (1965), Gel filtration of proteins, with particular reference to the glycoprotein, luteinizing hormone. *Anal. Biochem*. **12:**296-302.

Warrens, R. N. (1969), Metabolism of xanthine alkaloids in man, *Journal of Chromatography.* **40**: 468-469.

Woolfolk, C. A and Downward, J. S (1977), Distribution of xanthine oxidase and xanthine dehydrogenase specificity types among bacteria. *J. Bacteriol.* **130** (3): 1175-1191.

Woolfolk, C. A and Downward, J. S (1978), Bacterial xanthine oxidase from Arthrobacter S-2. *J. Bacteriol.* **135** (2): 422-428.

Woon-Gye, C. and Young-Nam, C. (1997), Oxidation of caffeine to theobromine and theophylline is catalysed primarily by Flavin containing Monoxygenases in liver microsomes. *Biochem. Biophys. Res. Comm*. **235**: 685-688.

Wreck-Reichhart, D. and Feyereisen, R. (2000), Cytochrome P-450 a success story. *Genome Biol*. **6**: 3003–3009.

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

Yamoka-Yano, 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.

Young, E. G and Conway, C. F. (1942), On the estimation of allantoin by the Rimini-schryver reaction. *J. Biol. Chem*. **142**(2): 839.

Yozo, M. and Toru, N. (1981), Purification and Properties of Xanthine Oxidase From Enterobacter cloacae . *Agric. Biol. Chem*., **45** (2): 425-432.

*** Original reference not seen.**

CHAPTER -4

OPTIMIZATION OF PARAMETERS FOR THEOPHYLLINE PRODUCTION

4.0. Scope of the work:

Theophylline is a xanthine alkaloid and a therapeutically important molecule and has been conventionally for the treatment of several diseases. It has also been reported to posses anti leukemia property. It blocks the transcription of the HIV viral gene in infected CD4 T cells by 94%. However the commercial availability of theophylline is by chemical synthesis using Traube's method, which involves various toxic chemicals and solvents the remnants of which are not desirable. Biotransformation is a viable alternative for the production of theophylline, which has been discussed in this chapter. The crucial parameters, which affect biotransformation of caffeine to theophylline, have been discussed. Optimization of physico-chemical parameters for efficient biotransformation of caffeine to theophylline was carried out. Additional studies using statistical design based on response surface methodology were also carried out for optimizing the media conditions for theophylline production using higher concentration of caffeine, which will help towards process development and scale up studies. Study was also carried out towards the downstream processing of the biotransformed theophylline. The biotransformed theophylline was extracted from the broth and purified and the purity confirmed by various analytical techniques as melting point analysis, HPLC, FTIR and NMR and the results are discussed in detail in the last sections of the chapter.

4.1. Introduction:

Theophylline is an important therapeutic compound because of its structural similarity to purine. It is classified as one of the potent methyl xanthines of therapeutic importance. Conventionally, it has been successfully used for the treatment of asthma (Bradley and Leichtenstein, 2001, Kathryn and Kamada, 2000), cardiac stimulation,

smooth muscle relaxation, apnea, diuretic (Homer, 2001) and as a central nervous system stimulator. The ability of theophylline to stabilize mast cells and other anti-inflammatory and immunomodulatory effects of theophylline has also been observed. Adenosine-stimulated release of mediators from mast cells, neutrophil activation, induction of 1L-1β and 1L-1α, synthesis and release of tumor necrosis factor-α (TNF-α) and cytokine release from T-lymphocytes are inhibited by theophylline. It has been reported to have antileukemia property as it inhibits δ-P110 enzyme (Lazaros *et al,* 2002). It is a potent inhibitor of alkaline phosphatase (Al-P) activity. It has been reported that theophylline show RNA binding efficiency. Hence, it can be used as a RNA binding drug and can be selectively delivered to RNA targets of microbial pathogens having the mechanism of RNA catalysis (Johnson *et al*, 2003). Theophylline acts on B16F10 melanoma, tumor induced angiogenesis as well as tumor regression and has reported to posses anti leukemia property and blocks the transcription of the HIV viral gene in infected CD4 T cells by 94% (Kurosawa, 2002).

Theophylline is found naturally in tea leaves and guarana beans. But extraction of theophylline from natural sources has not been commercialized because of the very low yield of the extraction process. Commercially theophylline is produced by chemical synthesis, and has several disadvantages such as low yield, is time consuming, involves high cost and is not eco friendly. Thus biotransformation is a viable alternative for the production of theophylline from caffeine, which has been discussed in this chapter.

4.1.1. Microbial biotransformation:

Microbial transformations refer to reactions catalyzed by microbial enzymes, leading to specific and useful metabolite accumulation in the reaction media. They are instrumental in biotransformation of valuable molecules. Microorganisms, during their metabolic processes produce a variety of products through stereoselective, regiospecific and selective conversion of substrates to their derivatives. This ability of the microorganisms can be exploited for the efficient production of valuable biotransformed compounds. The present chapter describes the use of a fungal strain of *Penicillium citrinum* MTCC 5215, for the production of theophylline by biotransformation of caffeine. Understanding the microorganism and the biotransformation pathway occurring in the microbe is the first and foremost step for a successful biotransformation process. It is also necessary to understand the particular biotransformation step, which leads to the required product formation, the enzyme involved in the biotransformation step, the nature of the enzyme and its requirements for maximum activity. A complete knowledge of the above factors would help in manipulation of the reaction conditions to favor maximum biotransformation.

Advantages of microbial biotransformation:

Microbial biotransformation is advantageous over other types of biotransformation as many measures can be taken to enhance the biotransformation reaction, such as:

- Manipulation of media
- Strain improvement
- Use of inhibitors

The caffeine biotransformation pathway in *P. citrinum* MTCC 5215 was elucidated (described in chapter 3). The enzymes involved in the biotransformation pathway have also been identified. This knowledge about the pathway and the enzymes

helps in understanding the organism and its nutritional requirements, to help design an efficient biotransformation process for the production of theophylline. Attempt has been made to study the nutritional requirements of *P. citrinum* MTCC 5215, optimization of parameters for maximum biotransformation of caffeine to theophylline.

4.1.2. Media optimization:

Microbial growth requires suitable environmental conditions, a source of energy, and nourishment. These requirements can be divided into two categories, physical and chemical. Physical requirements include: temperature, pH and osmotic pressure. Temperature plays an important role in the growth and metabolic activity of microorganism, as each individual enzyme of the microorganism has its optimum temperature for maximum activity. pH of the media offers suitable environment to the microorganism for growth and metabolic activity. Chemical growth requirements include: sources of carbon (C), nitrogen (N), oxygen (O), hydrogen (H), sulfur (S), phosphorous (P) and trace elements. C, N, P and S, are classified as macronutrients. Trace elements, also categorized, as micronutrients are essential for the growth of microorganisms, but needed in very small amounts. Iron is required for the synthesis of heme-containing compounds (such as cytochromes of the electron transport system) and for certain other enzymes (Tortora *et al*, 1995).

Besides their growth requirements, microorganisms when endowed/supplied with specific requirements, produce targeted products. Hence, media optimization is a essential requirement for the production of targeted compounds. Besides this, improved production can be achieved by incorporation of specific inhibitors into the media.

4.1.3. Response surface methodology (RSM):

Response surface methodology (RSM) explores the relationships between several explanatory variables and response variables to obtain maximum response. The method was introduced by Box and Wilson in 1951. The main idea of RSM is to use a sequential experimental procedure to obtain an optimal response using a first-degree polynomial model. RSM is easy to estimate and apply, even when little is known about the process and is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time (Ravi and Susheelamma, 2005). Interactions between variables can be identified and quantified by such a technique. In the present chapter RSM, with experimental central composite rotatible design (CCRD) has been applied to optimize biotransformation of caffeine to theophylline. RSM and CCRD have been used to demonstrate the effects of three media components (caffeine, glucose as carbon source and corn steep liquor as nitrogen source) on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.2. Materials and Methods:

4.2.1. Materials:

Standard theophylline and caffeine were procured from Sigma Chemicals, St. Louis, USA. Caffeine (LR grade), used for biotransformation was purchased from Loba Chemicals, Mumbai, India. Potassium 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. Corn steep liquor was procured from Anil Starch and Chemicals, Mumbai. All other chemicals were of the highest purity and were procured from standard sources.

4.2.2. Methods:

Analytical methods:

1) Thin layer chromatography (TLC) for identification of biotransformation products:

TLC for identification of biotransformed methyl xanthines was run using precoated TLC plates from Merck (Germany) with a solvent system containing butanol, acetic acid, water (4:1:1) and was visualized in a CAMAG UV illuminator.

2) High Performance Liquid Chromatography (HPLC) for identification of biotransformation theophylline:

HPLC was carried out using RP C-18 column [5µm, 250mmx4.6mm, Phoenomenix column] using LC-10A (Shimadzu, Japan) system with a isocratic mobile phase of water and acetonitrile (85:15) at a flow rate of 1ml/min and wavelength of 273nm.

3) Fourier Transform Infrared (FTIR) spectroscopy for identification of biotransformation theophylline:

IR absorption spectra were recorded using a Perkin Elmer Model 2000 Infrared Fourier-transform spectrophotometer, with samples prepared in KBr.

4) Nuclear magnetic resonance (NMR) spectroscopy for identification of biotransformation theophylline:

NMR spectra were recorded at 500 MHz, using 500MHz Bruker Avance instrument. ^{1}H and ^{13}C NMR was carried out by dissolving about 20 mg of purified theophylline in DMSO.

4.2.3.1. Screening of media for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Theophylline production by *P. citrinum* MTCC 5215 was carried out in twenty different standard media and the designed biotransformation media. The composition of various media are given in Table 4.2.3.1. All the media contained caffeine at concentration of 1g/L and the pH of the media was adjusted to 5.6±0.2. The prepared media was autoclaved at 121°C for 20 min at 15 psi. The autoclaved media was cooled, inoculated with spore suspension of *P. citrinum* MTCC 5215 and incubated on rotary shaker at 150 rpm at 28±2°C. Samples were collected at regular intervals and analyzed by HPLC to check for biotransformed theophylline.

Table 4.2.3.1. Media Screening of for biotransformation of caffeine to theophyllineby *P. citrinum* MTCC 5215.

Media Composition (g/L), pH 5.6			
M.1. Lindenberg synthetic media		**M.2. Hobb's medium**	
Glycerol	30.0	Glucose	20.0
$NaNO_3$	2.0	NaCl	5.0
K_2HPO_4	1.0	Na_2SO_4	5.0
$MgSO_4.7H_2O$	0.5	$NaNO_3$	4.5
$FeSO_4.7H_2O$	0.4	K_2HPO_4	1.2
		Tris	1.2
		$MgSO_4.7H_2O$	1.0
		$ZnSO_4$	0.01
M.3. Czepek-Dox broth		**M.4. O-Brien synthetic media**	
Sucrose	30.0	Glucose	20.0
$NaNO_3$	3.0	Glycine	2.6
K_2HPO_4	1.0	Sodium acetate	1.36
$MgSO_4.7H_2O$	0.5	$(NH_4)_2SO_4$	0.54
KCl	0.5	$K_2HPO_4.3H_2O$	0.05
$FeSO_4.7H_2O$	0.01	$ZnSO_4.7H_2O$	0.03
		$FeSO_4.7H_2O$	0.025
		$CuSO_4.5H_2O$	0.016
		$MnSO_4.4H_2O$	0.012
		$CaCl_2.2H_2O$	0.05
		$MgSO_4.7H_2O$	0.5
M.5. Dulaney's medium		**M.6. Thornberry's medium**	
Glucose	10.0	Glucose	10.0
NaCl	5.0	KH_2PO_4	2.38
K_2HPO_4	2.0	K_2HPO_4	5.65
$MgSO_4.7H_2O$	0.4	NH_4NO_3	4.0
$CaCl_2$	0.4	$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	0.02	Sodium lactate	11.2
$ZnSO_4.7H_2O$	0.01	$ZnSO_4.7H_2O$	0.14
$(NH_4)_2HPO_4$	4.0	$FeSO_4.7H_2O$	0.014
		$MnSO_4.4H_2O$	0.084
		$CuSO_4.5H_2O$	0.0016

M.7.Baron'smedium

Glucose	15.0
NH4NO3	4.0
MgSO4.7H2O	0.25
NaCl	5.0
Sodium citrate	1.0
KH2PO4	0.1
K2HPO4	0.1
CaCO3	3.0

M.8. Numerof's medium

Glucose	20.0
Glycine	2.6
Sodium acetate	1.36
(NH4)2SO4	0.54
FeSO4.7H2O	0.03
CuSO4.5H2O	0.5
K2HPO4	0.5
CaCl2	0.05

M.9. Complex organic media

Glucose	25.0
Soya bean flour	25.0
Yeast extract	3.0
$(NH_4)_2SO_4$	2.0
$CaCO_3$	2.0
NaCl	2.0
KH_2PO_4	0.15

M.10. Lumb's medium

Glucose	20.0
$MgSO_4.7H_2O$	10.0
Sodium citrate	1.0
NaCl	2.5
$CaCl_2$	0.87
KH_2PO_4	0.5
Glycine	5.0
$FeSO_4.7H_2O$	0.0075
$MnSO_4.4H_2O$	0.008
$CuSO_4.5H_2O$	0.001
$ZnSO_4.7H_2O$	0.0014
$(NH_4)_2 MO_4.4H_2O$	0.0018

M.11. Corn meal salt medium

Cornmeal	50.0
Na_2HPO_4	1.15
KH_2PO_4	0.25
KCl	0.2
$MgSO_4.7H_2O$	0.2

M.12.Carbohydrate carcode medium

Potato starch	5.0
Glucose	5.0
Ribose	5.0
Glycerol	5.0
Soyaflour	20.0
$(NH_4)_2SO_4$	0.2
Yeast extract	2.0
Bactopeptone	2.0

M.13. ISP production media I

Soyabean Meal	25.0
Glucose	25.0
$NaNO_3$	4.0
K_2HPO_4	0.05
NaCl	2.5
$CaCO_3$	0.4

M.14. ISP production media II

Soluble Starch	25.0
Corn Steep Liquor	10.0
$(NH_4)_2 SO_4$	5.0
$CaCO_3$	5.0
$ZnSO_4$	0.04

M.15. ISP production media III		**M.16. ISP production media IV**	
Glycerol	20.0	Glucose	10.0
Peptone	5.0	Soluble Starch	10.0
Yeast Extract	3.0	Peptone	7.5
Meat Extract	3.0	Meat Extract	7.5
$CaCO_3$	2.5	NaCl	3.0
M.17. ISP production media V		**M.18. ISP production media VI**	
Soyabean Meal	15.0	Glucose	33.0
Glucose	15.0	Soluble Starch	33.0
Glycerol	2.5	Soyabean Meal	34.0
Sodium Chloride	5.0	$(NH_4)_2SO_4$	13.0
$CaCO_3$	3.0	K_2HPO_4	13.0
		NaCl	2.5
		$CaCO_3$	12.5
M.19. ISP production media VII		**M.20. ISP production media VIII**	
Soyabean Meal	20.0	Soyabean Meal	10.0
$(NH_4)_2SO_4$	5.0	Dextrose	5.0
Meat Extract	4.0	Corn Steep Liquor	5.0
Yeast Extract	2.5	Soluble Starch	1.0
Glucose	6.0	$CaCO_3$	7.0
KCl	4.0		
$CaCO_3$	0.1		
K_2HPO_4	0.1		

M.21.Biotransformation media	
Na_2HPO_4	0.12
KH_2PO_4	1.3
$CaCl_2$	0.3
$MgSO_4$	0.3
Sucrose	20

4.2.3.2. Optimization of carbon source for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Carbon is a requirement for the growth of *P. citrinum* MTCC 5215 as well as for biotransformation to be carried out. The different carbon sources used were glucose, maltose, sucrose, fructose, cellulose, pectin and starch. Biotransformation was carried out in the designed media consisting of (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3;

$CaCl_2$ - 0.3; caffeine – 1. The different carbon sources mentioned above were added individually to the media at 20g/L concentration. The control experiment was carried in media without the addition of any carbon source. After initial optimization studies, glucose was found to be the best carbon source for biotransformation of caffeine to theophylline and was chosen for further optimization studies. Glucose was used in the concentration range of 5 to 50 g/L. 100ml of media was prepared with above mentioned constituents in 500ml Erlenmeyer flask and autoclaved at 121°C for 20 min at 15 psi. The autoclaved media was cooled and inoculated with 5 ml spore suspension of *P. citrinum* MTCC 5215 and incubated on rotary shaker at 150 rpm at 28±2°C. Samples were collected at regular intervals and analyzed by HPLC to check for the biotransformation of caffeine to theophylline. Accumulation of biomass was measured by drying the biomass to constant weight at 80°C.

4.2.3.3. Optimization of nitrogen source for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Nitrogen source is a requirement for the growth of *P. citrinum* MTCC 5215 and biotransformation of caffeine to theophylline by the fungal strain. The various nitrogen sources used for biotransformation of caffeine to theophylline are yeast extract, peptone, beef extract, casein, soybean meal, corn steep liquor, urea, $NaNO_3$ NH_4NO_3, NH_4Cl, $NaNO_3$ and $(NH_4)_2SO_4$. Biotransformation was carried out in designed biotransformation media having the following composition (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; caffeine – 1, glucose-20. The above mentioned nitrogen source was added to the media individually at concentration of 3g/L and the control experiment was carried in media containing no additional nitrogen source. Corn steep liquor (30% protein) was found to be the best nitrogen source and chosen for further optimization. The concentration

of corn steep liquor used was in the range of 5 to 20 ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.4. Optimization of caffeine concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Caffeine is the substrate for biotransformation to be carried out. However, higher concentrations of caffeine are inhibitory to the growth of microorganism, (Frischknecht, 1985; Nathanson, 1984; Fries and Kihlman, 1948; Waller et al. 1986). Hence, optimization of caffeine concentration is an important parameter to obtain maximum biotransformation and growth. Biotransformation was carried out in designed biotransformation media having the following media composition (in g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; glucose-20 and corn steep liquor at concentration of 5ml/L. Caffeine was used in the concentration range of from 1g/L to 10g/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.5. Optimization of pH for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

pH plays a crucial role during the growth and metabolism of an organism. Most of the molds grow between pH 5 and 6. Hence optimization of pH is a requirement for any process optimization. Biotransformation of caffeine to theophylline was carried out in media of varying pH ranging from 3-8 having the following composition (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; glucose-20, caffeine – 1. Corn steep liquor was used at concentration of 5ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.6. Optimization of temperature for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Temperature plays a major role in the biotransformation of caffeine to theophylline. Optimization of temperature was carried out by incubating the fungal cultures at different temperatures between 20°C to 40°C in media having following composition (g/L) Na_2HPO_4 - 0.12; KH_2PO4 - 1.3; $MgSO_4$ - 0.3; CaCl2 - 0.3; glucose-20, caffeine – 1. Corn steep liquor was used at concentration of 5ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.7. Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Biotransformation was carried out with different inoculum ages ranging from 24 hours to 96hours. PDA slants containing 0.03% caffeine were inoculated with spores of *P. citrinum* MTCC 5215 and incubated at 28±2°C in incubator for 24, 48, 72 and 96h. These slants were then used as inoculum for biotransformation of caffeine to theophylline in media having following composition (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; CaCl2 - 0.3; glucose-20, caffeine -1, Corn steep liquor-5ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.8. Optimization of inoculum load for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Optimization of inoculum load is an important parameter for biotransformation of caffeine to theophylline. 72 hour old inoculum slants of *P. citrinum* MTCC 5215 was used at various inoculum loads varying from $1x10^5$ to $7x10^5$ spores/ml and 5ml spore suspension was inoculated into 100ml media. Biotransformation of caffeine to theophylline was carried out in media having following components (in g/L) Na_2HPO_4 - 0.12; KH_2PO4 - 1.3;

$MgSO_4$ - 0.3; $CaCl_2$ - 0.3; glucose-20, caffeine – 1, Corn steep liquor- 5ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.9. Optimization of $FeSO_4$ concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Metal ions influence the activity of enzymes by either enhancing or inhibiting their activity (Elvina *et al*, 1998). The caffeine biotransforming enzymes are also influenced highly by the presence of certain metal ions in the media. The enzyme for biotransformation of caffeine to theophylline was identified as cytochrome P450 as discussed in chapter-3. It is an iron containing enzyme hence, the addition of $FeSO_4$ in the media will lead to the enhancement of enzyme production. Different concentrations of $FeSO_4$ (1 to 5 mM) were used in the biotransformation media having following composition (in g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; glucose-20, caffeine – 1. Corn steep liquor was used at concentration of 5ml/L. All other conditions were similar as described in section 4.2.3.2.

4.2.3.10. Optimization of allopurinol concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Allopurinol is an analog of natural purines, and is quickly metabolised to oxipurinol, which is a xanthine oxidase inhibitor (Kelley and Beardmore, 1970). Xanthine oxidase converts purines and methyl xanthines into their respective uric acids (Blecher and Lingens, 1977). Allopurinol was used in the media in concentrations ranging from 1mM to 5mM to inhibit the further utilization of theophylline. The biotransformation media composed of (g/L) Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; glucose-20, caffeine – 1. Corn steep liquor at concentration of 5ml/L and FeSO4 at 3mM concentration was added to the media.

4.2.3.11. Production of theophylline with optimized conditions in shake flask:

The results obtained from various optimization experiments led to the designing of optimized media and was used for further studies.

Optimized media composition (g/L) Caffeine - 1.0, Glucose - 20.0, Na_2HPO_4 -0.12, K_2HPO_4 - 1.3, $MgSO_4$ - 0.3, $CaCl_2$- 0.3. Corn steep liquor - 5ml/L. $FeS0_4$ was added at 3mM and allopurinol at 4mM concentration. The optimized pH was 5.6, optimum temperature 28°C, optimized inoculum age was 72 hours and optimized inoculum load was 5x 10^5 spores/ml. All the components were dissolved in water; the pH was adjusted to 5.6 and sterilized at 121°C for 20 min. Spore suspension of *P. citrinum* MTCC 5215 at concentration of 5x10^5 spores/ml was inoculated into the medium and incubated under agitation on a rotary shaker set at 150 rpm at 28°C. Samples were collected at regular intervals and checked for biotransformation of caffeine to theophylline. Accumulation of biomass was measured by drying the biomass to constant weight at 80°C.

4.2.3.12. Production of theophylline with optimized conditions in 10L fermentor:

The optimized media composition (mentioned above) was dissolved in water, the pH adjusted to 5.6 and the media was sterilized at 121°C for 20 minutes. 40 ml of spore suspension of *P. citrinum* MTCC 5215 containing 5x10^5 spores/ml was inoculated into 800ml of the medium and incubated under agitation on a rotary shaker set at 150rpm for 24 hours at 28°C and used as inoculum. 7.2 liters of the optimized biotransformation medium detailed above was prepared and charged into a fermentor (Murhopye, India) and sterilized (Figure 4.2.3.12.). The media was cooled and inoculated with *P. citrinum* grown broth (10% v/v). The impeller speed was set at 200rpm, sterile air was passed at 1v/v/min and the fermentation was terminated after 72 hours of growth. Samples were collected at regular

intervals and checked for biotransformation of caffeine to theophylline. Accumulation of biomass was measured by drying the biomass to constant weight at 80°C.

Figure 4.2.3.12. Production of theophylline in 10L laboratory fermentor.

4.2.4. Response surface methodology:

Additional experiments were carried out using statistical analysis and response surface methodology to predict the maximum biotransformation of caffeine to theophylline, caffeine degradation and biomass accumulation by *P. citrinum* MTCC 5215 with three crucial parameters effecting biotransformation. These three crucial parameters are effect of glucose concentration, corn steep liquor concentration and caffeine concentration.

RSM experimental design:

Concentrations of three media ingredients (glucose, corn steep liquor, caffeine) were optimized through RSM to predict the maximum production of theophylline by biotransformation of caffeine. Further, optimization studies for decaffeination was also carried out along with CCRD to locate the true optimum concentrations of glucose, corn

steep liquor and caffeine for biotransformation to theophylline. To describe the nature of the response surface in the optimum region, a 2^3 factorial CCRD was used at 5 levels as shown in Table 4.2.4. (resulting in total of 21 experiments). The experimental results of the CCRD were fitted with a second order polynomial equation by a multiple regression technique.

$$Y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i x_j + \Sigma\Sigma \beta_{ij} x_i x_j$$

Y is the predicted response, β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables or factors. The quality of fit of the second order model equation was expressed by the coefficient of determination R^2 , and its statistical significance determined by a t-test. The computer software used was statistica, version 5.5 by Statsoft, Inc. (Tulsa, OK USA).

Preliminary studies for media optimization was carried out, glucose was found to be the best carbon source and corn steep liquor as the best nitrogen source. The workable range for glucose was chosen to be 10–50g/L, for corn steep liquor it was 5 – 20ml/L and caffeine in the range of 1-10g/L. 21 different media were prepared as per the experimental design which have been presented in Table 4.3.2.1a The prepared media was autoclaved at 121°C for 20 mins at 15 psi, was cooled and inoculated with 1 x 10^5 spores/ml and incubated on rotary shaker at 150 rpm, 28°C. Samples were collected at regular intervals and analyzed by HPLC to check for the biotransformation of caffeine to theophylline. Accumulation of biomass was measured by drying the biomass to constant weight at 80 °C.

Table 4.2.4. Coding and assigned concentrations of variables of different levels of the central composite rotatible design.

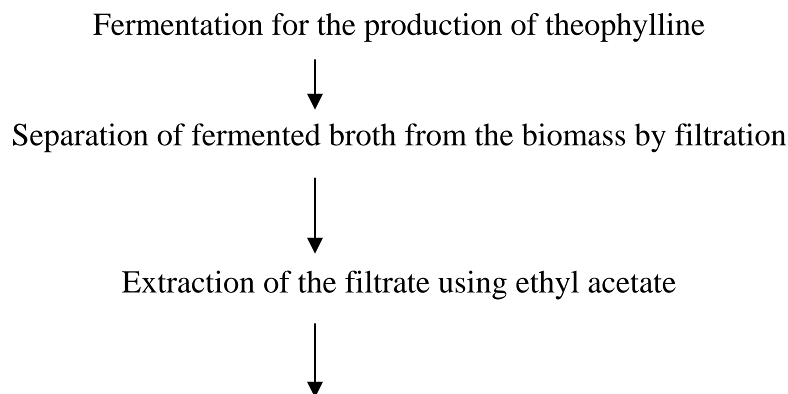
	Symbol	Code levels

Independent variables		-1.682	-1	0	+1	+1.682
Carbon	X_1	0	10.2	25	40.0	50
Nitrogen	X_2	0	4.0	10	15.9	20
Caffeine	X_3	0	2.0	5.0	8.0	10

4.2.5. Downstream processing of biotransformed theophylline:

Methyl xanthines are available either by chemical synthesis or by extraction from natural sources (Finar, 1959; Birdwhistell and Connor, 1971; Helmkamp and Johnson, 1968; Pavia *et al*, 1976). Extraction of methyl xanthines have been carried out by using solvents as chloroform, dichloromethane, ethyl acetate, propanol etc. (Landgrebe, 1993; Mayo *et al*, 1989; Nimitz, 1991; Williamson, 1989, Murray and Hansen, 1995) After fermentation, the broth was separated from the biomass by filtering through muslin cloth and extracted using various solvents. The solvent layer was separated and vacuum concentrated. The concentrate obtained was termed as the crude extract to which acetone was added (8ml/gm of crude extract) to precipitate the theophylline from the rest of the components present. This precipitate was air-dried and crystallized from ethyl alcohol. Chemical identity of theophylline was confirmed by melting point analysis, co-TLC, co-FTIR, NMR with an authentic sample and it’s purity determined by mixed melting point analysis.

Scheme for isolation and purification of theophylline from fermented broth.



Vacuum concentration of the ethyl acetate extract to get the crude extract

Precipitation of theophylline from the crude extract using acetone

Crystallization of precipitated theophylline using ethyl alcohol

Analysis of purified theophylline using TLC, HPLC, FTIR, NMR

4.3. Results and Discussion:

4.3.1.1. Media screening for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Biotransformation of caffeine to theophylline was carried out in 20 different standard media, and the results have been presented in Table 4.3.1.1. However, none of the media could efficiently biotransform caffeine to theophylline. Hence a biotransformation media was designed using various media components and designated as M. 21(Table 4.2.3.1). 31.46% and 17.85% biotransformation was observed in ISP production media VIII (M. 20) and complex organic media (M. 9) respectively. 12.31% biotransformation was observed in corn meal salt media (M.11), whereas, 9.35% and 7.91% of biotransformation was observed in ISP production media V (M. 17) and ISP production media III (M. 15) respectively. Lumb's medium (M. 10) and carbohydrate carcode medium (M. 12) exhibited 5.75% and 4.71% biotransformation respectively. In the rest of the media, biotransformation of caffeine to theophylline was not observed. All the media in which biotransformation was observed contained negligible concentration of inorganic nitrogen suggesting that the presence of externally added inorganic nitrogen source does not allow the organism to take up caffeine from the medium. The media in which biotransformation of caffeine to theophylline was observed, contained organic nitrogen source such as corn steep liquor, soybean meal or yeast extract as nitrogen source. ISP production media VIII (M. 20) contained both soybean meal and corn steep liquor as nitrogen source and showed biotransformation 31.46%. The fungal strain *P. citrinum* MTCC 5215 could take up caffeine from the media even in the presence of external organic nitrogen source such as, soybean meal and corn steep liquor and further biotransform it to theophylline though the efficiency of biotransformation was only 31.46%. Complex organic media (M. 9) contained

soybean flour and yeast extract at concentrations of 25 and 3g/L respectively and showed biotransformation of 17.85%. This suggests that the presence of these nitrogen sources do not inhibit the uptake of caffeine and its biotransformation by the fungal strain *P. citrinum* MTCC 5215. It was observed that only seven out of the twenty standard media could biotransform caffeine to theophylline, but the efficiency of biotransformation was very low in these media. Hence, a biotransformation media was designed and designated M. 21 (composition in Table 4.2.3.1). 79% biotransformation was observed with this media and further work was carried out using this media.

Table 4.3.1.1. Biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 in screening media.

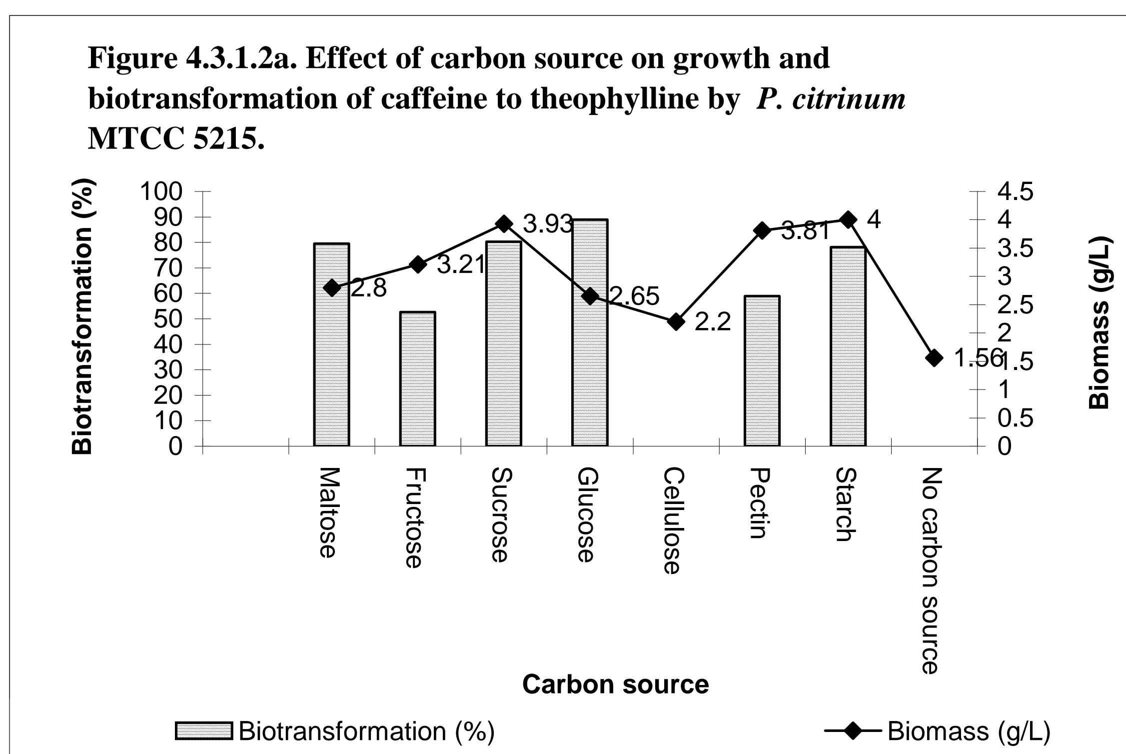
Media No.	Media	Biotransformation of caffeine to theophylline (%)
1.	Lindenberg Synthetic Media	0.0
2.	Hobbs medium	0.0
3.	Czepek-Dox broth	0.0
4.	O-Brien Synthetic media	0.0
5.	Dulaneys medium	0.0
6.	Thornberry’s medium	0.0
7.	Baron’smedium	0.0
8.	Numerofs medium	0.0
9.	Complex organic media	17.9
10.	Lumb’s medium	5.8
11.	Corn meal salt medium	12.3
12.	Carbohydrate carcode medium	4.7
13.	ISP Production media I	0.0
14.	ISP Production media II	0.0
15.	ISP Production media III	7.9
16.	ISP Production media IV	0.0
17.	ISP Production media V	9.4
18.	ISP Production media VI	0.0
19.	ISP Production media VII	0.0
20.	ISP Production media VIII	31.5
21.	Biotransformation media	79.9

4.3.1.2. Effect of carbon source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Carbon sources in the media are required by *P. citrinum* MTCC 5215, for synthesis of proteins, sugars and lipids, to make up cell structures and for biotransformation to take place. No biotransformation took place in the absence of any carbon source in the media, (Fig 4.3.1.2a). Hence, carbon source is a mandatory requirement for biotransformation to occur. Of the various carbon sources used, glucose proved to be the best carbon source, leading to 89% conversion of caffeine to theophylline in a period of 144 hours (Fig 4.3.1.2a). Sucrose, maltose and starch could also biotransform caffeine to theophylline efficiently, showing 80.3%, 79.5% and 78.2% of biotransformation respectively. When fructose was used in the media, the biotransformation observed was only 52.7% whereas with cellulose as carbon source in the media, no biotransformation was observed. The probable reason for this could be the absence of cellulose degrading enzyme in the fungal strain *P. citrinum* MTCC 5215. Further optimization of glucose concentration was carried out in the range of 5 -50g/L. It was observed that the biotransformation of caffeine to theophylline increased with increasing glucose concentration from 5-20g/L (Fig 4.3.1.2b). With further increase in initial glucose concentration beyond 20g/L, there was no considerable increase in biotransformation. Hence, 20g/L of glucose was chosen as the optimum concentration of carbon source to be used in the media for biotransformation. Use of sucrose as carbon source in biotransformation of caffeine to theophylline has been reported by Schwimmer *et al* (1971). Pectin when used as carbon source could biotransform caffeine to theophylline with 58.9% efficiency. This suggests that the chosen fungal strain *P. citrinum* MTCC 5215 can be used for the decaffeination of coffee pulp which contains pectin at a concentration of 6.5% (Penaloza *et al*, 1985; Zuluaga, 1989). The maximum biomass growth (4g/L) was observed with starch as the carbon source (Fig

4.3.1.2a). With sucrose as the carbon source the biomass obtained after 144 hours of growth was 3.93g/L whereas with pectin the biomass obtained after 144 hours of growth was 3.81g/L. With glucose as carbon source, only 2.65g/L of biomass was obtained after 144 hours of growth. However, the time taken for biotransformation was 144hours even after addition of external carbon source, which is not desirable for process development economics. Hence, further modifications were made in the media composition, which is described below.

Figure 4.3.1.2a. Effect of carbon source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.



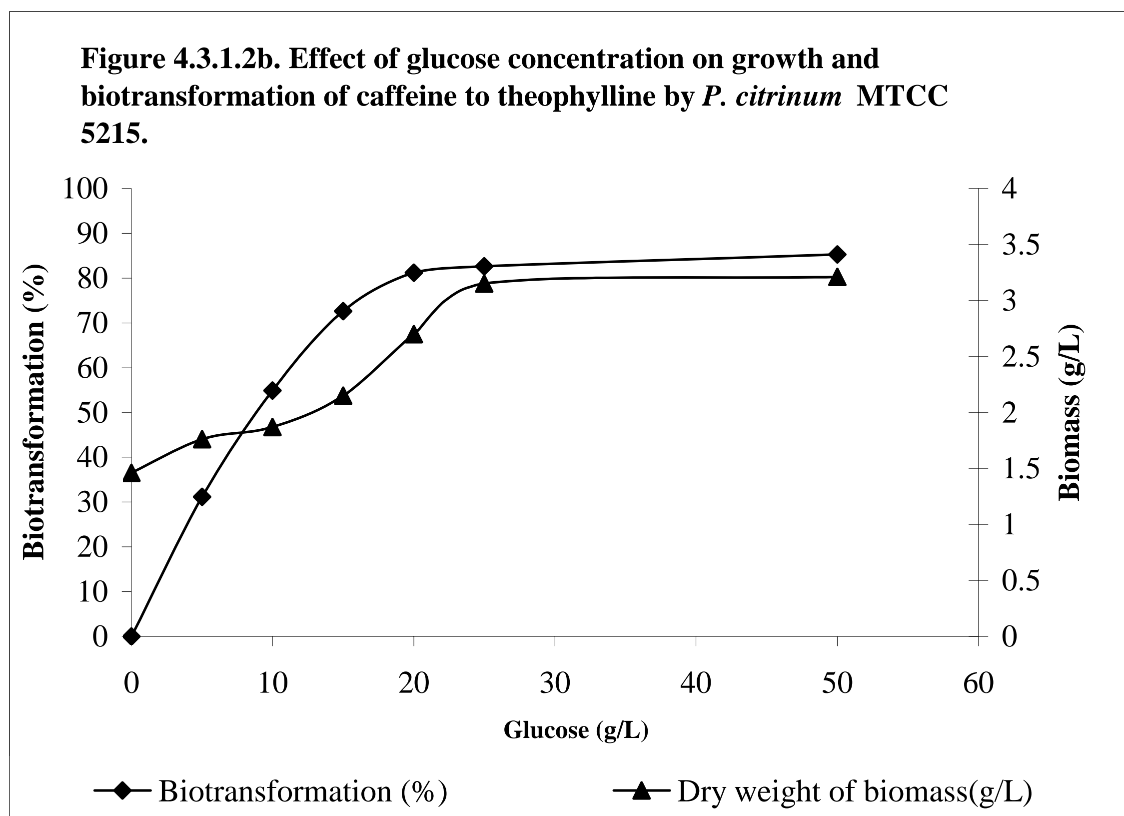


Figure 4.3.1.2b. Effect of glucose concentration on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.3.1.3. Effect of nitrogen source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Nitrogen is a basic requirement for the growth and metabolism of any organism and is required for synthesis of proteins, DNA and RNA. Presence of nitrogen in the media influences the caffeine biotransformation of microorganisms (Penaloza *et al* 1985; Aguilar *et al*, 1983; Hakil, *et.al.*, 1999; Sánchez *et.al* 2004; Blecher, 1976). In the absence of any external nitrogen source, the biotransformation of caffeine to theophylline was only 15%. Biotransformation to theophylline was not observed when peptone, beef extract and casein were used as nitrogen source (Table 4.3.1.3). Similar results were reported by Penaloza *et al* (1985) and Aguilar *et al* (1983) where no caffeine biotransformation was observed in *Aspergillus niger* in the presence of the fore mentioned organic nitrogen sources. However, 5.88% biotransformation was observed when soybean meal was used as an organic

nitrogen source. Biotransformation was enhanced to 64.06% when yeast extract was used as nitrogen source. The enhancement in biotransformation may be attributed to the presence of vitamins, free amino acids and short peptides, which can be utilized efficiently and easily by the fungal strain for growth and further for biotransformation of caffeine. Corn steep liquor as an external nitrogen source showed maximum biotransformation of caffeine to theophylline (80.25%). The enhancement in biotransformation with corn steep liquor in the media may be attributed to the high protein content and presence of essential amino acids (Masood *et al*, 1997). Further optimization of the proportion of corn steep liquor was carried out using different concentrations of corn steep liquor ranging from 5ml/L to 20ml/L. With 5ml/L of corn steep liquor 80.2% biotransformation was observed. Further increase in the concentration of corn steep liquor showed no considerable increase in biotransformation (Fig 4.3.1.3). With the incorporation of corn steep liquor in the media the biotransformation period was reduced from 144hours to 48hours, which is a considerable improvement towards process development, but the biotransformation was reduced from 89.7 to 80.25%.

Maximum biomass growth of 11.2g/L was observed using $(NH4)_2SO_4$ as an external inorganic nitrogen source, but no biotransformation of caffeine to theophylline was observed, whereas the biomass accumulated in the absence of an external nitrogen source was only 2.74g/L(Table 4.3.1.3). From the results obtained it can be concluded that corn steep liquor at a concentration of 0.5% enables higher biotransformation (80.25%) in a period of 48hours and accumulation of a biomass of 7.10g/L on dry weight basis.

Table 4.3.1.3. Effect of nitrogen source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

S.No	Nitrogen source	Biotransformation (%)	Biomass (g/L)

1.	Caffeine	15.00	2.74
2.	Yeast extract	64.06	7.01
3.	Peptone	0.00	9.22
4.	Beef extract	0.00	7.73
5.	Casein	0.00	6.60
6.	Soybean meal	5.88	6.91
7.	Corn steep liquor	80.25	7.10
8.	Urea	0.00	7.15
9.	$NaNO_3$	0.00	5.82
10.	NH_4NO_3	0.00	5.60
11.	NH_4Cl	0.00	6.20
12.	$(NH4)_2SO_4$	0.00	11.20

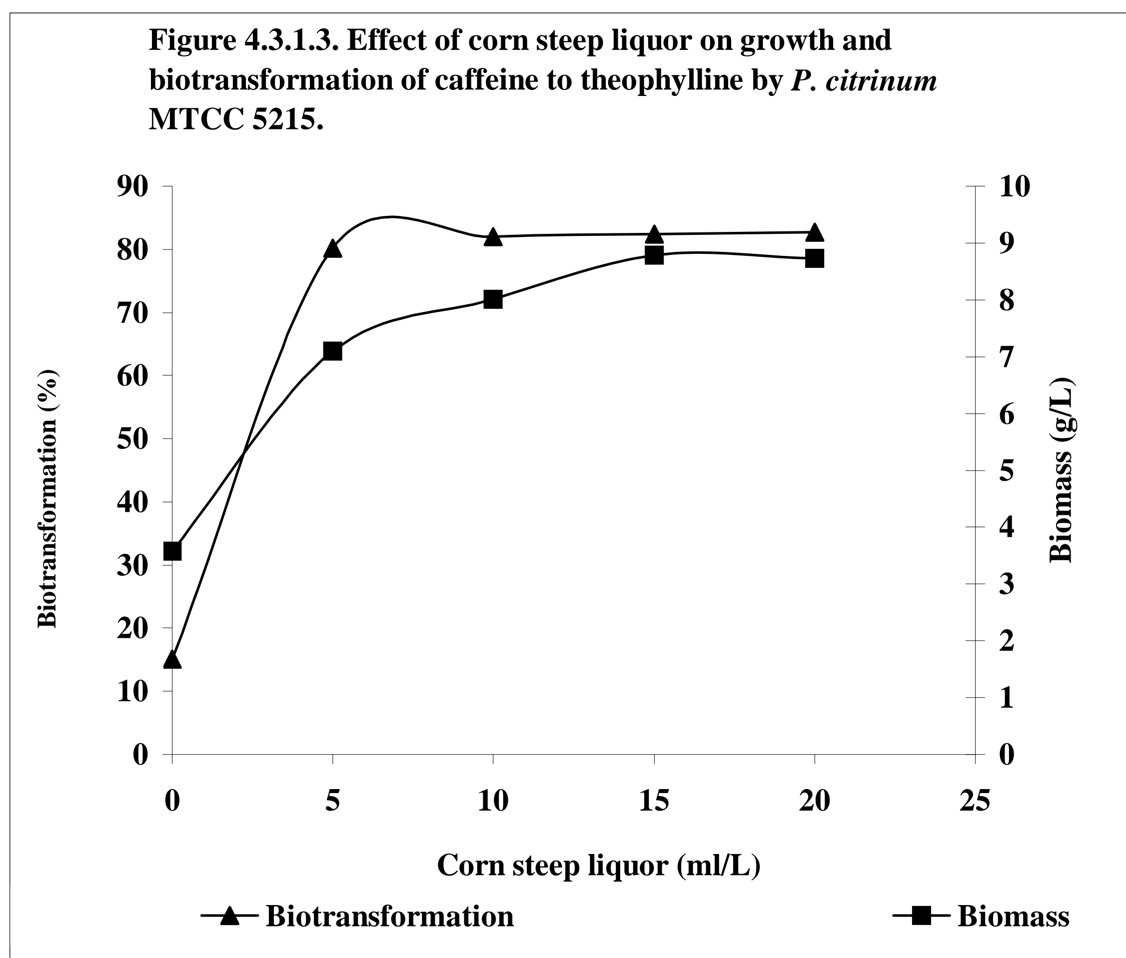


Figure 4.3.1.3. Effect of corn steep liquor on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.3.1.4. Optimization of caffeine concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Biotransformation of caffeine to theophylline decreased with increasing concentration of caffeine. Maximum biotransformation of 82.4% was observed with 1g/L of caffeine in 48hrs (Fig 4.3.1.4b). 75.2% of biotransformation was observed with 2g/L of caffeine at 84hrs, 70.5% of biotransformation was observed at 96hrs with 3g/L of caffeine concentration, 51.2% at 120hours with 4g/L of caffeine. 38.5% of biotransformation was observed at 120 hours with 5g/L of caffeine concentration (Fig 4.3.1.4a). *P. citrinum* MTCC 5215 showed maximum growth of 7.12g/L at 1g/L of caffeine concentration (Fig 4.3.1.4b). The fungal strain showed poor growth beyond 5g/L of caffeine concentration and at 10 g/L of caffeine concentration the growth observed was 0.06g/L (Fig 4.3.1.4b). With 1g/L of caffeine concentration the lag phase observed was 10 hours (Fig 4.3.1.4a). The lag phase increased with increasing concentration of caffeine (Fig 4.3.1.4a). With 5g/L of caffeine the lag phase increased to 38 hours. It was observed that beyond 5g/L of caffeine concentration, *P. citrinum* MTCC 5215 could not biotransform caffeine to theophylline as the fungal strain showed negligible growth above this concentration of caffeine (Fig 4.3.1.4b).

Schwimmer *et al* (1971) had carried out biotransformation of caffeine to theophylline by *P. roqueforti* in sucrose and trace element containing media using caffeine concentration of 1.928g/L and no biotransformation was observed beyond this concentration. This is in accordance with the results obtained by Asano *et al* (1993) who reported the increase in lag phase of growth in *P. putida* with increasing concentrations of caffeine. Higher concentration of caffeine has been reported to be inhibitory to the growth of microorganisms (Frischknecht *et al*, 1986; Nathanson, 1984). Schwimmer *et al* (1971) reported the inhibition of growth of *P. roqueforti* beyond 3.856 g/L. With 1g/L of caffeine

maximum biotransformation was observed with 82.4% of conversion at 48hours and maximum biomass growth of 7.12g/L, hence it was chosen as the concentration for further biotransformation work.

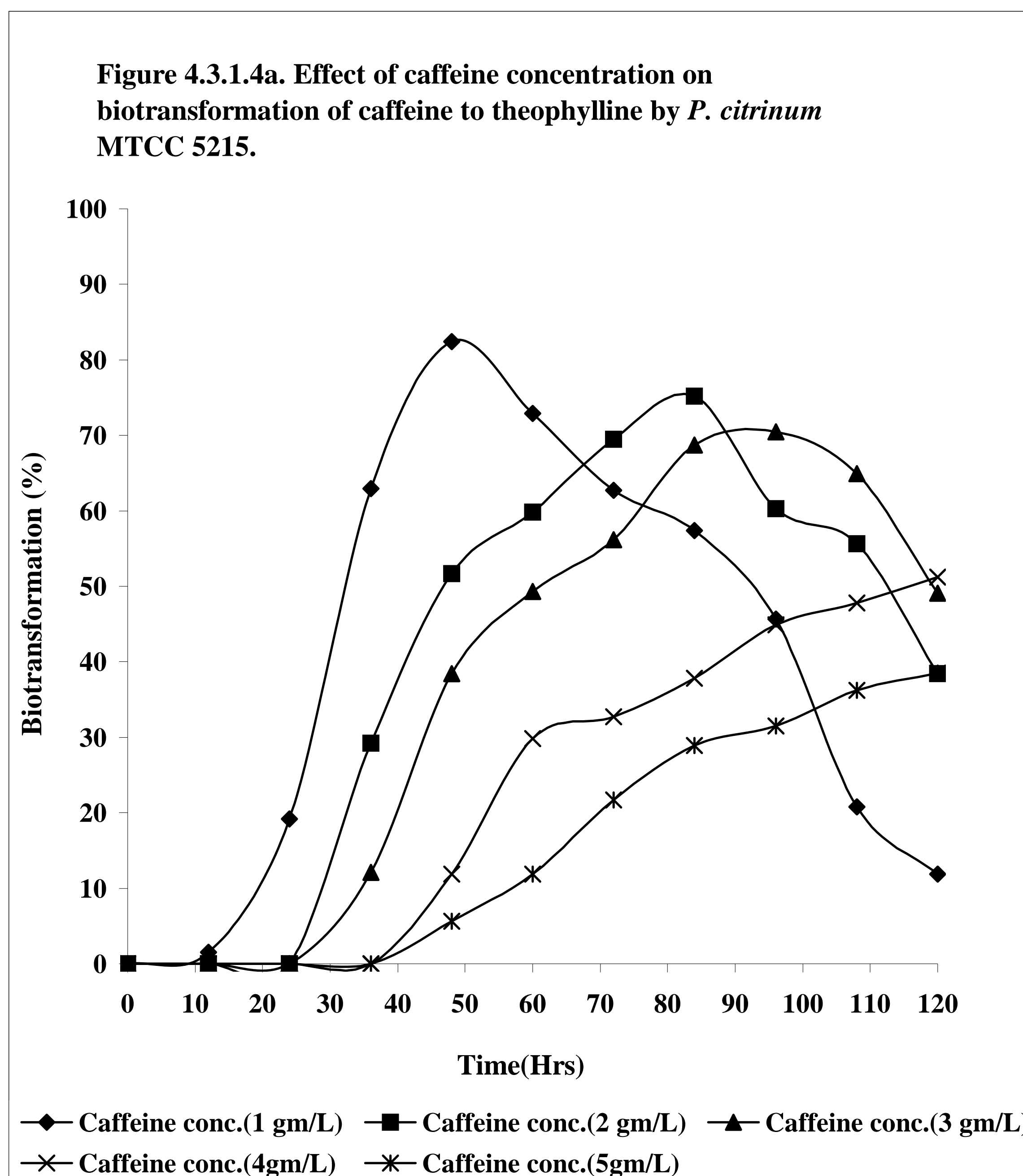


Figure 4.3.1.4a. Effect of caffeine concentration on biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

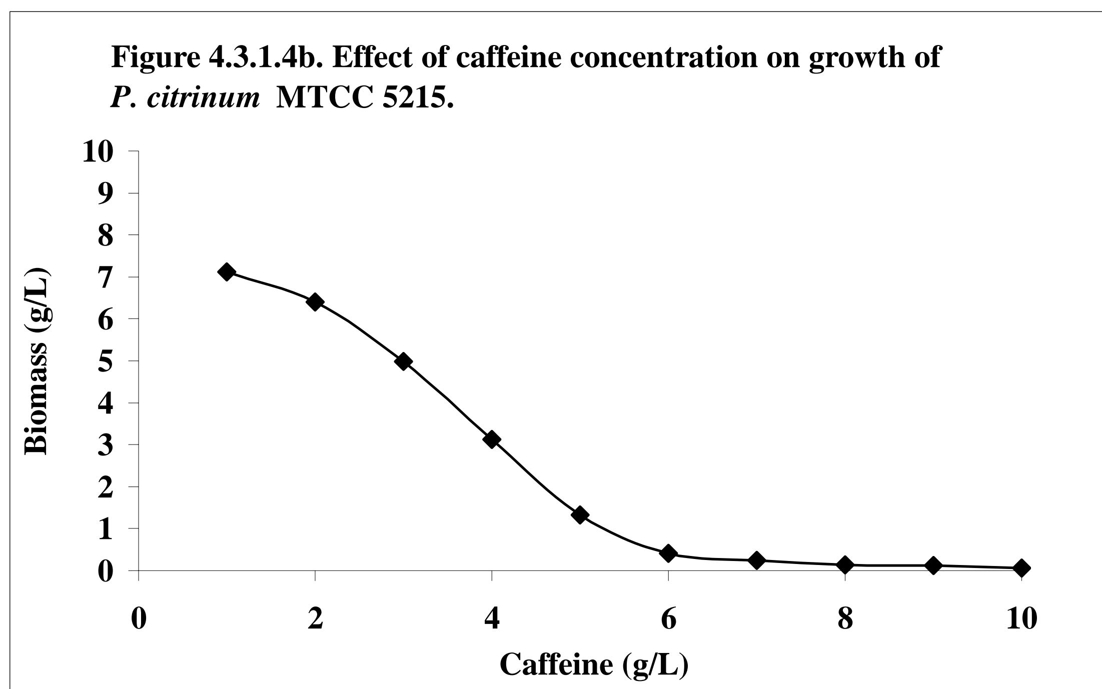


Figure 4.3.1.4b. Effect of caffeine concentration on growth of *P. citrinum* MTCC 5215.

4.3.1.5. Optimization of pH for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

pH is an important physical parameter which effects the growth and metabolism of all microorganisms. Biotransformation and growth of caffeine to theophylline by *P. citrinum* MTCC 5215 was monitored in the pH range of 3-8. Both biotransformation and biomass growth increased with increasing pH (Table 4.3.1.5, Figure 4.3.1.5). At pH 3 the biotransfromation was 9.1% and the biomass accumulated was 2.19g/L. The biotransformation increased from 35.7 % at pH4 to 65.4% at pH 5. At pH 6, 75.5% biotransformation was observed and above this pH the biotransformation decreased. Maximum biotransformation (80.3%) was observed at pH 5.6 (Fig 4.3.1.5). The results obtained are in accordance with the results obtained by Roussos *et. al.*, (1995) who reported maximum biotransformation of caffeine to theophylline by fungi at pH of 5.6. This is because fungal systems require slightly acidic pH for their optimum growth and metabolism (Roussos, *et. al.*, 1995).

Table 4.3.1.5. Optimization of pH for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

S.No	pH	Biotransformation (%)	Biomass (g/L)
1.	3	9.1	2.19
2.	4	35.7	3.56
3.	5	65.4	6.59
4.	6	75.5	7.12
5.	7	61.7	7.36
6.	8	59.7	6.29

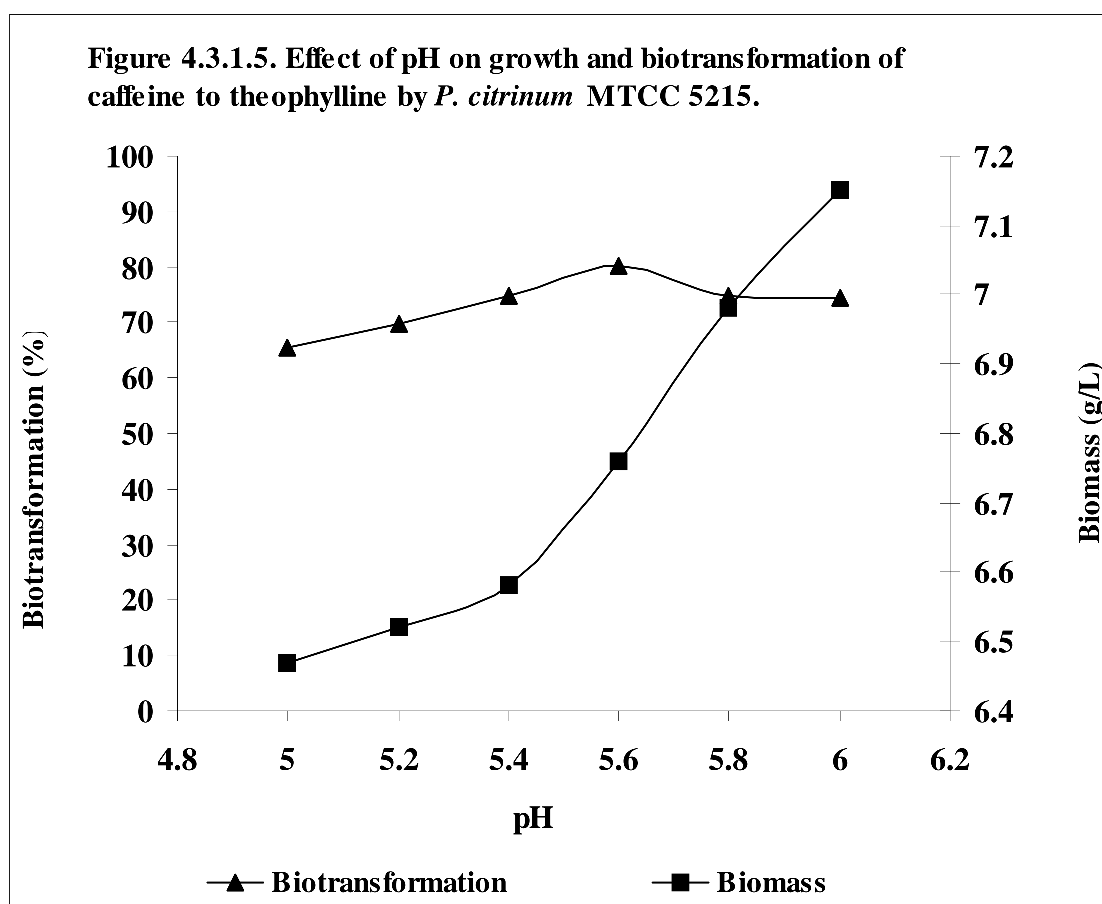


Figure 4.3.1.5. Effect of pH on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.3.1.6. Optimization of temperature for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

It was observed that biotransformation of caffeine to theophylline increased with increasing temperature from 20 to 28°C (Fig 4.3.1.6a). Besides this, the time taken for biotransformation also decreased with increasing temperature. At 20°C, 61.8% of biotransformation was observed at 72hrs and the biotransformation was 71.7% at 24°C. Maximum biotransformation of 82.7% was observed at 28°C in a period of 48hrs (Fig 4.3.1.6a). No biotransformation of caffeine to theophylline occurred beyond 32°C indicating the inability of the biotransforming enzyme system (cyt P450) in the fungal strain to function beyond this temperature. The increase in biotransformation percentage and rate of biotransformation can be correlated, with increasing biomass growth in the temperature range of 20 to 28°C indicating the growth dependent nature of biotransformation. Biomass accumulation was highest at 28°C (7.10g/L) and it decreased beyond this temperature. The biomass accumulation decreased considerably with increasing temperature and the lowest biomass accumulation of 2.12g/L was observed at 40°C (Fig 4.3.1.6b). From figure 4.3.1.6b it can be concluded that the fungal strain *P. citrinum* MTCC 5215 is a mesophile with optimum growth temperature of 28°C. This is in contrast to the reports of Schwimmer *et al* (1971) who have reported the optimum temperature of growth and biotransformation of *Penicillium roqueforti* to be 20°C.

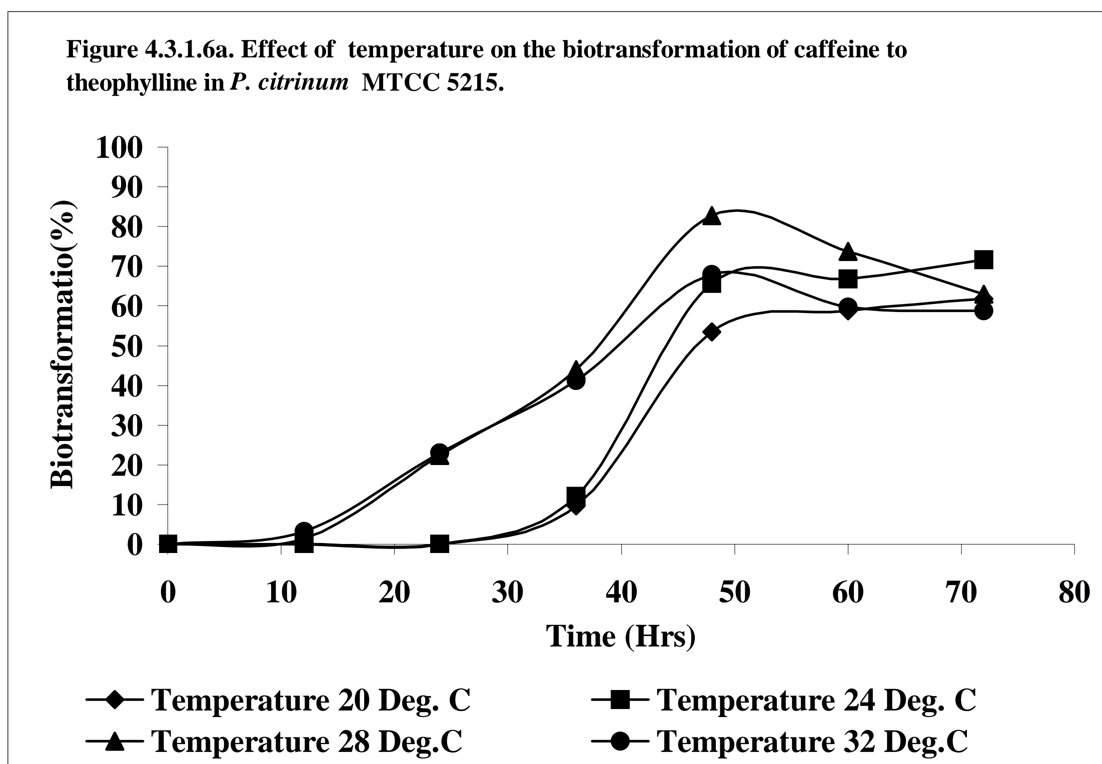


Figure 4.3.1.6a. Effect of temperature on the biotransformation of caffeine to theophylline in *P. citrinum* MTCC 5215.

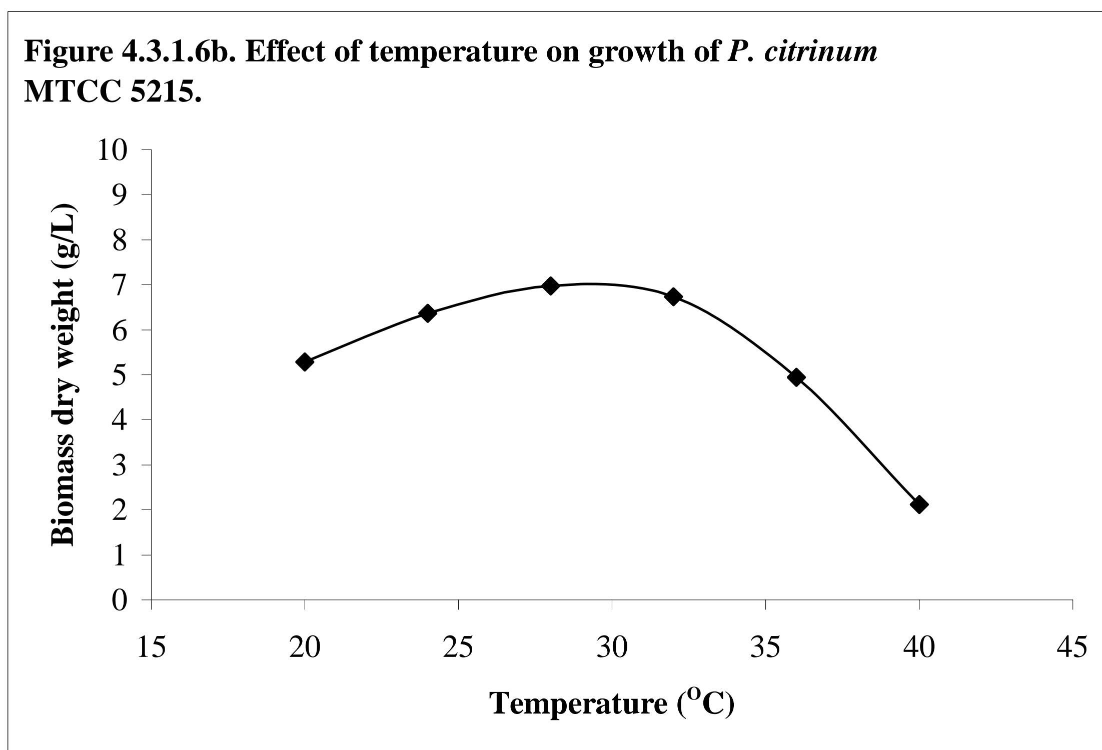


Figure 4.3.1.6b. Effect of temperature on growth of *P. citrinum* MTCC 5215.

4.3.1.7. Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Optimum inoculum age is an important factor, which helps the inoculated organism to acclimatize to the new environment, grow and metabolize there after. It was found that 72 hour inoculum age was the optimum for biotransformation, giving 79.5% conversion of caffeine to theophylline and biomass accumulation of 6.58g/L (Fig 4.3.1.7). Further increase in inoculum age to 96 hours increased the biotransformation to 81.4% but the increase was very marginal over the 72hour old inoculum hence for further experiments were carried out with 72hour old inoculum.

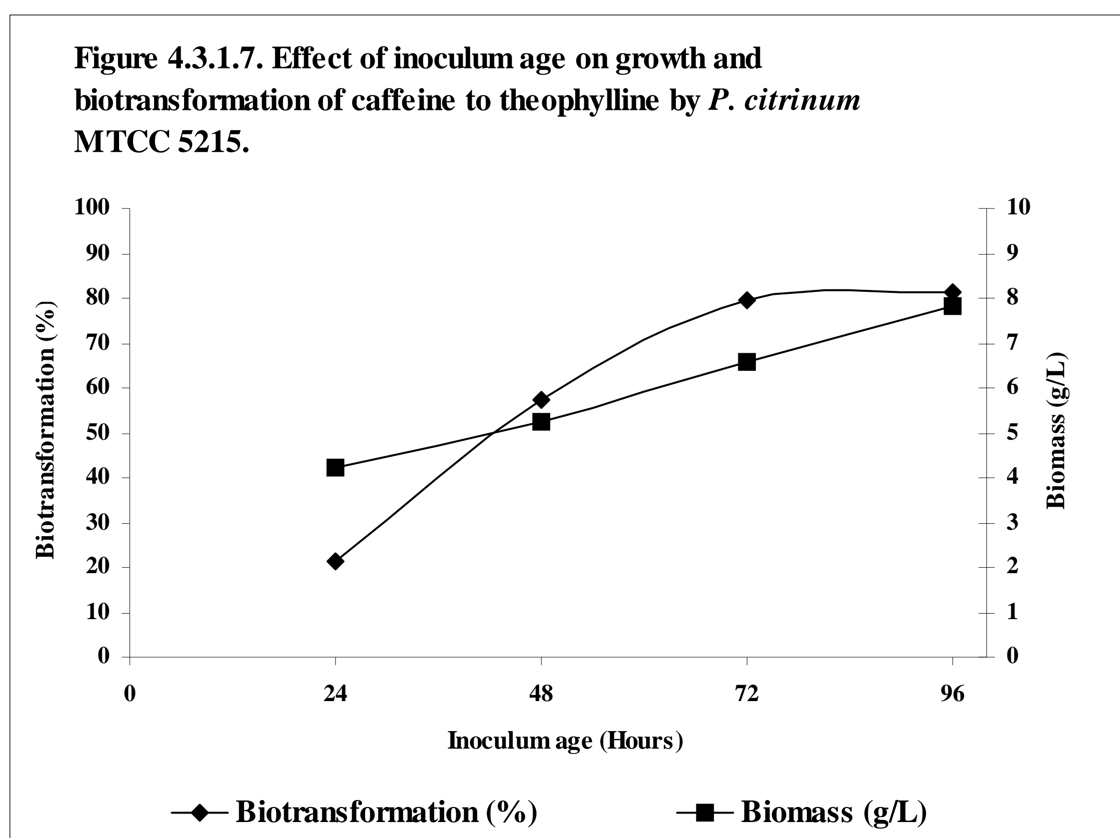
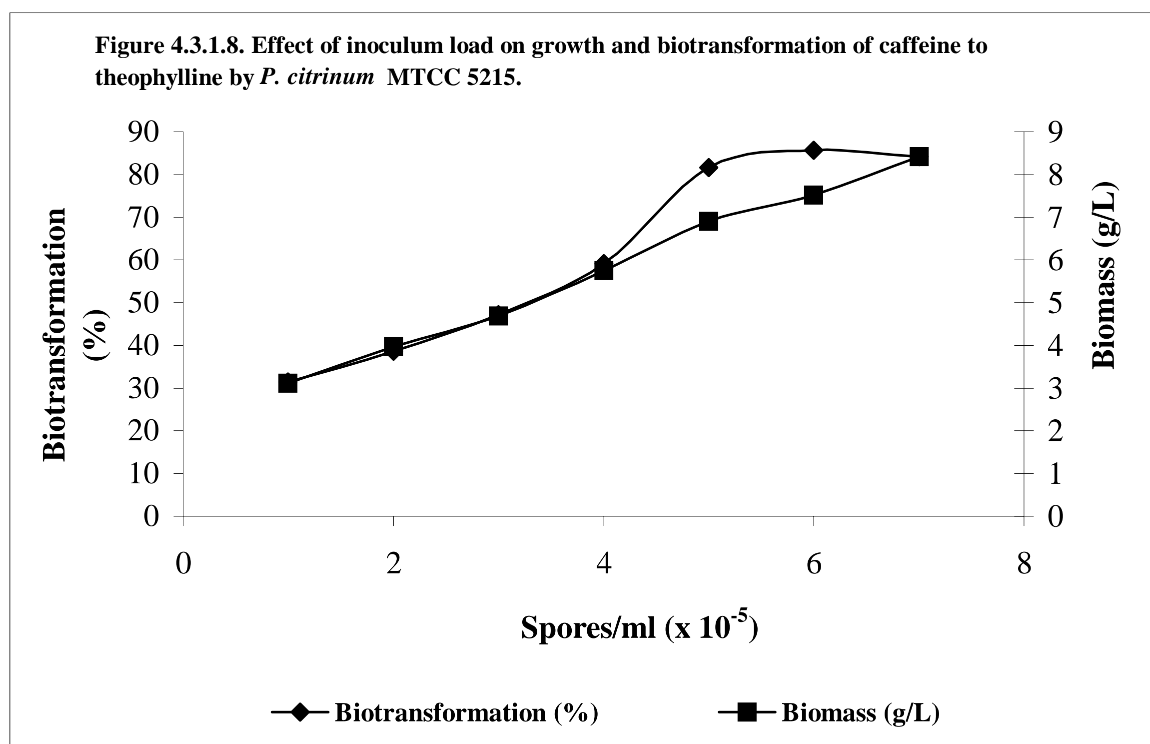


Figure 4.3.1.7. Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.3.1.8. Optimization of inoculum load for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

Based on the previous experiment a 72 hour old inoculum was used and the effect of inoculum load on caffeine biotransformation was studied. The optimum inoculum load was found to be 5x10^5 spores/ml (Fig 4.3.1.8). Lower inoculum load gives slow rate of biomass growth and biotransformation. With increase in inoculum load, a continuous increase in biotransformation and biomass growth was obtained as seen in figure 4.3.1.6. Biotransformation reaches maximum of 83.6% with an inoculum load of 5x10^5 spores/ml, 5ml of spore suspension was inoculated into 100ml of media. With further increase in spore concentration there was no increase in biotransformation and a steady state was observed. This is because with further increase in spore concentration there will be competition for media components leading to lesser growth and biotransformation.

Figure 4.3.1.8. Effect of inoculum load on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.



4.3.1.9. Optimization of $FeSO_4$ concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

The influence of $FeSO_4$ concentration on the biotransformation of caffeine to theophylline has been presented in figure 4.3.1.9. In the absence of $FeSO_4$ in the media maximum biotransformation of 79.7% was observed after 48 hours of fermentation. Biotransformation of caffeine to theophylline increased with increasing concentration of $FeSO_4$ in the growth media (1 to 3mM) (Fig 4.3.1.9). With 1mM of $FeSO_4$ in the growth media the biotransformation % was 82.6 at 48hours. With 2 and 3mM of $FeSO_4$ the biotransformation was 84.6% (42hours) and 86.8% (42hours) respectively. The increase in rate and biotransformation% with addition of $FeSO_4$ is because of the increase in enzyme activity of cytochrome P450 an iron containing enzyme which is the biotransforming enzyme for conversion of caffeine to theophylline. The addition of $FeSO_4$ to the media facilitates the production of iron containing enzymes of the caffeine biotransformation pathway as cytochrome P450, xanthine dehydrogenase, xanthine oxidase etc. (Cornish and Kristman, 1957; Ohe and Watanabe, 1979) enhancing the biotransformation of caffeine. With further increase in concentration of $FeSO_4$ beyond 3mM deviation from the earlier trend was observed. At 4mM of $FeSO_4$ concentration, maximum biotransformation of 83.1% was observed at 42hours and at 5mM $FeSO_4$ concentration the biotransformation % was 77.8 at 42hours (Fig 4.3.1.9). Besides this, at these concentrations of FeSO4, the theophylline accumulated in the media was also degraded faster (Fig 4.3.1.9) indicating the enhanced activity of the xanthine dehydrogenase and xanthine oxidase enzymes. Hence, 3mM $FeSO_4$ concentration was chosen as the optimized concentration for further studies.

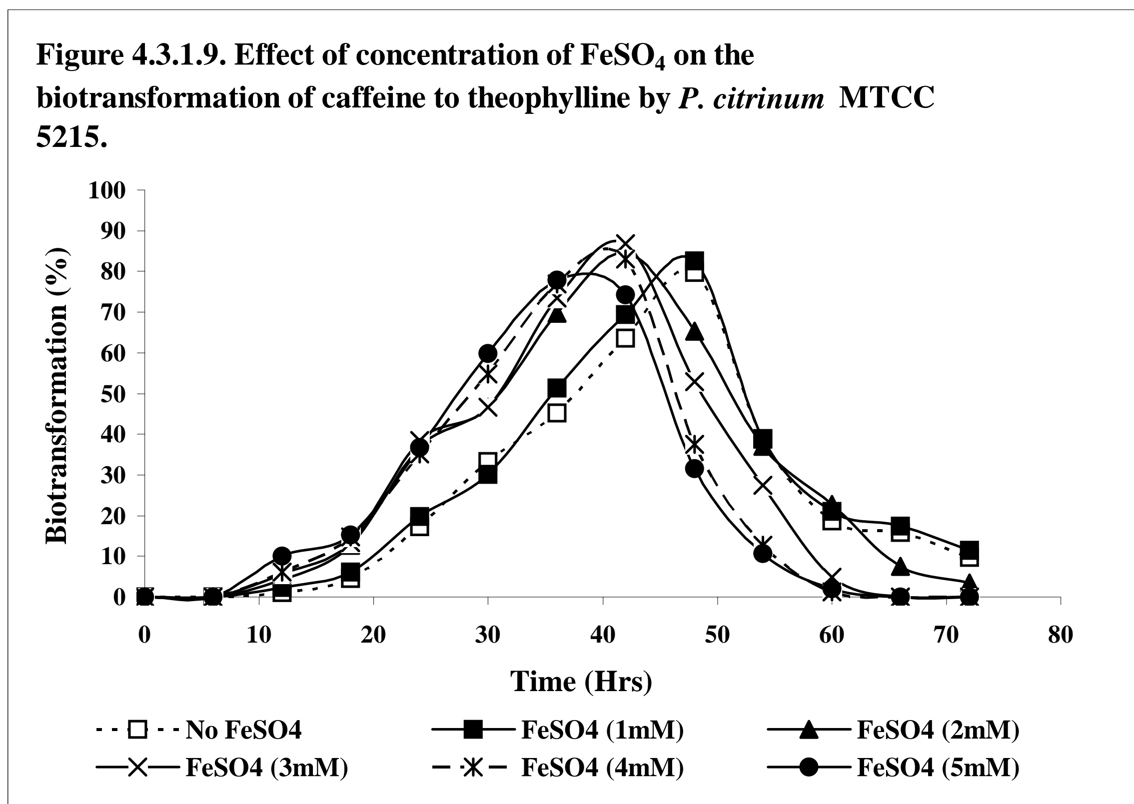


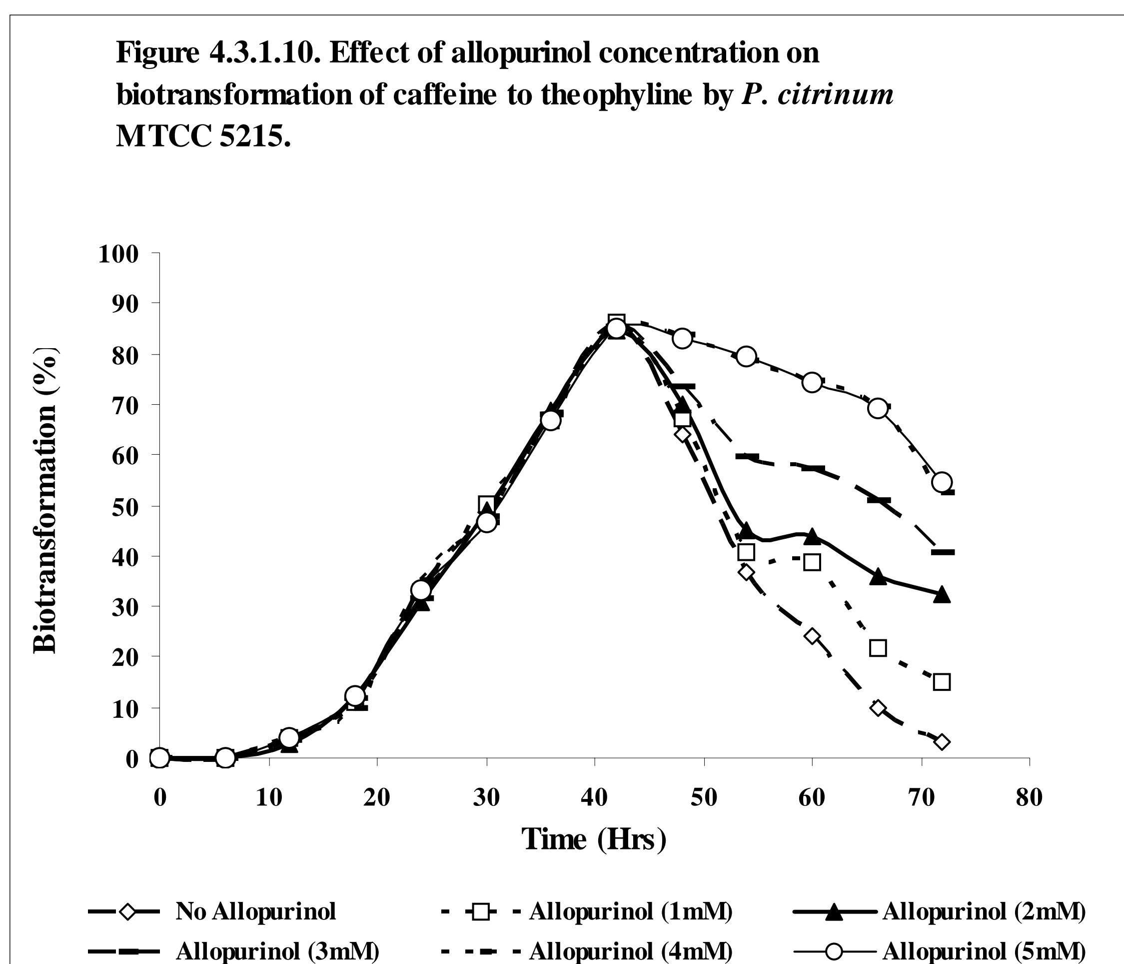
Figure 4.3.1.9. Effect of concentration of $FeSO_4$ on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.

4.3.1.10. Effect of allopurinol concentration on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215:

The effect of allopurinol concentration on the biotransformation of caffeine to theophylline has been presented in figure 4.3.1.10. The degradation of theophylline slows down with increasing allopurinol concentration (Fig 4.3.1.10). In the absence of allopurinol in the media maximum biotransformation of 85.9% was observed at 42hours, which was degraded to 3.1% by the end of 72hours (Fig 4.3.1.10). With 1mM of allopurinol in the media, 86.1% of biotransformation of caffeine to theophylline was observed at 42hours, which was degraded to 15.2% at the end of 72hours. Using 2mM of allopurinol the theophylline degradation was reduced to 32.4%, with 3mM allopurinol it was reduced to 40.9%. 4mM allopurinol could reduce the theophylline degradation to 52.4%, however with further increase in allopurinol concentration to 5mM the theophylline concentration was reduced to 54.5% at the end of 72 hours. There was no considerable difference in the

results obtained with allopurinol concentration of 4mM and 5mM. Hence, 4mM allopurinol was chosen as the optimum concentration and used for further studies. Xanthine oxidase oxidizes xanthine to uric acid in *P. citrinum* MTCC 5215. Allopurinol inhibits xanthine oxidase leading to accumulation of xanthine and the earlier products of the pathway because of feedback inhibition. Similar experiments have been carried out by Schwimmer *et al* (1971) using inhibitors of purine metabolism pathway such as o-phenathroline and allopurinol to enhance the production of theophylline.

Figure 4.3.1.10. Effect of allopurinol concentration on biotransformation of caffeine to theophyline by *P. citrinum* MTCC 5215.



4.3.1.11. Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 under optimized conditions in shake flask:

Optimized growth and biotransformation parameters for *P. citrinum* MTCC 5215 are as follows: media composition (g/L)-Caffeine - 1.0, Glucose - 20.0, Na_2HPO_4 -0.12,

K_2HPO_4 - 1.3, $MgSO_4$ - 0.3, $CaCl_2$- 0.3. $FeS0_4$ - 3mM. Corn steep liquor –5ml/L, allopurinol - 4mM. Optimum pH - 5.6, optimum temperature 28^oC, optimum inoculum age was 72 hours and optimum inoculum load was 5x 10^5 spores/ml.

With the above optimized parameters fermentation was carried out in shake flask and growth and biotransformation was monitored and the results have been compared with the results of growth and biotransformation before optimization of parameters and have been presented in Table 4.3.1.11. Under the optimized conditions, the biotransformation of caffeine to theophylline increased from 79.0% to 85.0% and the time taken for biotransformation reduced from 144hours to 42hours (Table 4.3.1.11 and Fig 4.3.1.11). The reduction in fermentation time from 144hours to 42hours is substantial improvement in terms of process development for production of theophylline. Besides this with optimized parameters the biomass growth increased from 3.93g/L to 15.5g/L and the time taken to attain maximum biomass growth also reduced from 144hours to 48hours (Table 4.3.1.11 and Fig 4.3.1.11). The major contribution for increase in biotransformation from 79.0 to 85.0% and the reduction in fermentation time is the addition of corn steep liquor (5 ml/L) and $FeSO_4$ (3mM) into the media. After addition of corn steep liquor the fermentation time reduced from 144hours to 48hours, which was further reduced to 42hours after the addition of $FeSO_4$ into the media. However, with the addition of $FeSO_4$ the production of other iron containing enzymes of the pathway was also enhanced which led to the breakdown of accumulated theophylline. The breakdown of theophylline was prevented by the addition of allopurinol (4mM) to the media. The increase in biomass growth was contributed by the addition of corn steep liquor and $FeSO_4$ and the optimized temperature and pH.

Table 4.3.1.11. Comparison of growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 before and after optimization of conditions in shake flask.

Observed parameter	Unoptimized condition	Optimized condition
Maximum biotransformation (%)	79.0	85.0
Time taken to attain maximum biotransformation (Hrs)	144	42
Maximum biomass (g/L)	3.93	15.5
Time taken to attain maximum biomass growth (Hrs)	144	48

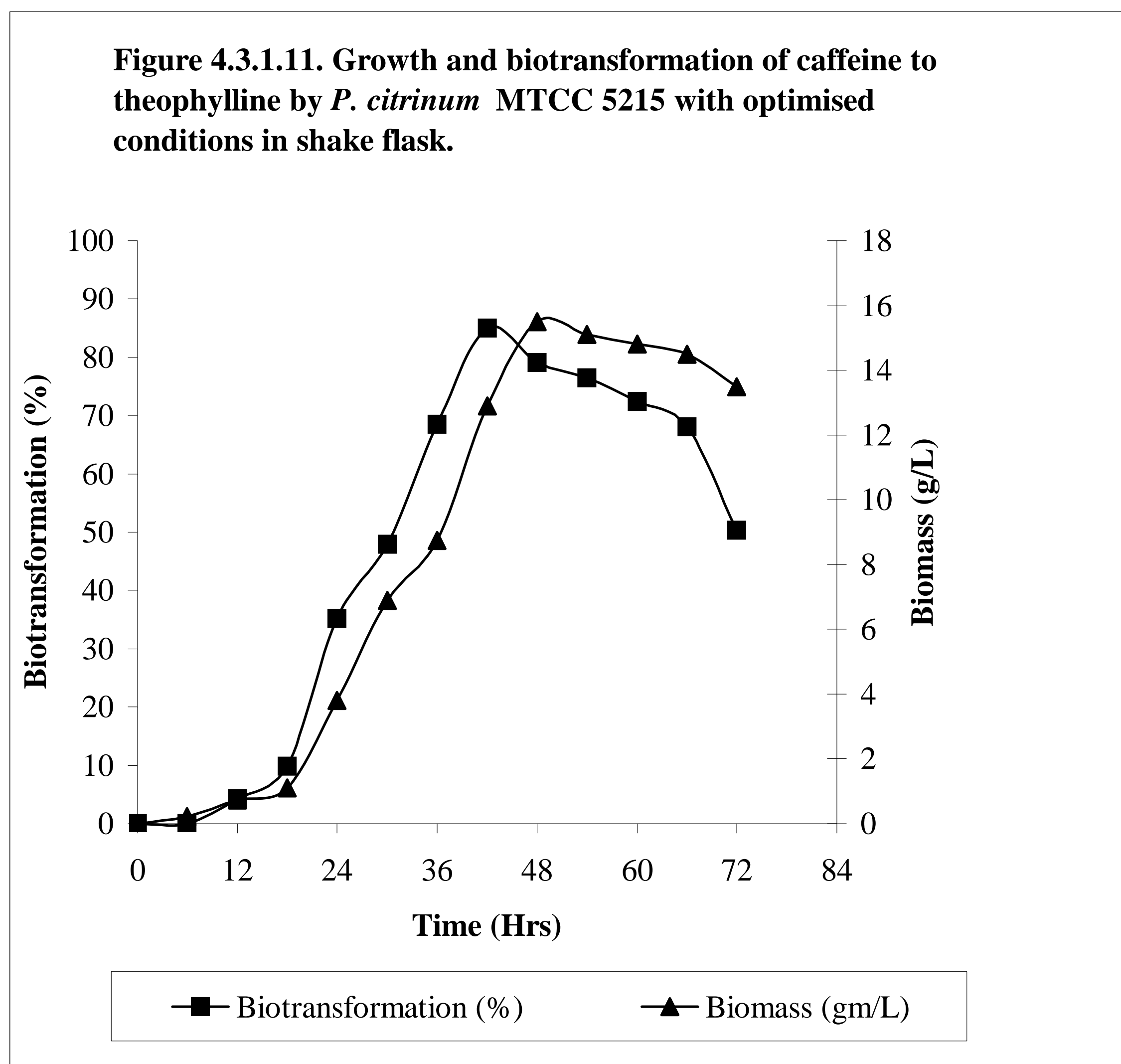


Figure 4.3.1.11. Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimised conditions in shake flask.

4.3.1.12. Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 under optimized conditions in 10L fermentor:

With optimized fermentation parameters, scale up studies were carried out in a 10L lab scale fermentor and the results have been presented in figure 4.3.1.12. The results obtained from optimized shake flask and fermentor studies have been compared in Table 4.3.1.12. The maximum biotransformation was reduced from 85.0% to 83.1% but the time taken to attain maximum biotransformation % remained the same. The maximum biomass growth increased from 15.5 g/L to 21.75 g/L with the time period remaining same in both

the cases. The increased biomass growth in fermentor was because of the controlled conditions of temperature, pH and aeration.

Table 4.3.1.12. Comparison between shake flask and fermentor studies for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimized conditions.

Observed parameter	Production of theophylline in Shake flask	Production of theophylline in Fermentor
Maximum biotransformation (%)	85.0	83.1
Time taken to attain maximum biotransformation (Hrs)	42	42
Maximum biomass (g/L)	15.5	21.75
Time taken to attain maximum biomass growth (Hrs)	48	48

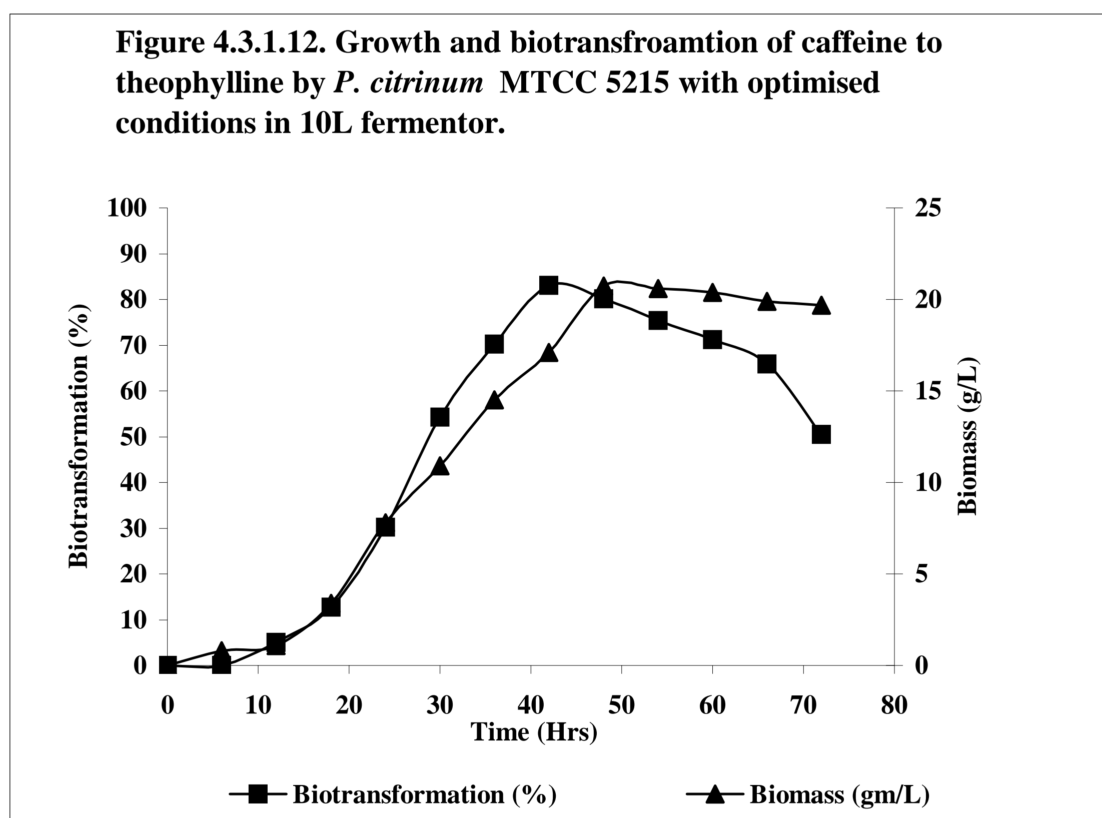


Figure 4.3.1.12. Growth and biotransfroamtion of caffeine to theophylline by *P. citrinum* MTCC 5215 with optimised conditions in 10L fermentor.

4.3.2. Response surface methodology:

4.3.2.1. Biotransformation of caffeine to theophylline:

The levels of the variables for the CCRD experiments were selected according to the 2^3 – factorial design. The point representing high theophylline biotransformation was used as the center point in the next phase of CCRD. The center point of the corresponding composition was selected (25g/L glucose, 10ml/L corn steep liquor and 5g/L caffeine). Further evaluation was conducted with a CCRD experiment. The CCRD design and the corresponding experimental data are shown in Table 4.2.4 and Table 4.3.2.1a respectively. From the experimental results obtained maximum biotransformation of caffeine to theophylline was observed in the 120hour sample. Hence, further discussion of results is done pertaining to the values of 120hour sample. After applying multiple-regression analysis on the experimental values, the results of ANOVA for the 21 trials performed by the experimental design were obtained as shown in Table 4.3.2.1b. The corresponding second-order polynomial response model Equation, that was found after Statistical analysis for the regression is presented below:

$$Y_{(g/L)} = -53.95 + 20.01\,X_1 - 4.12\,X_1^2 + 47.42\,X_2 - 23.66\,X_2^2 + 154.75\,X_3 - 121.11\,X_3^2 + 4.70\,X_1X_2 - 3.36X_1X_3 - 28.77\,X_2X_3$$

The statistical significance of the second-order model equation was checked by the F-test and the fit of the model was also expressed by the coefficient of determination R^2 (as shown in Table 4.3.2.1c), which was found to be 0.9132, indicating that 91.32% of the variability in the response could be explained in the model. This indicated that above represented Equation is a suitable model to describe the response of the experiment pertaining to biotransformation of caffeine to theophylline. The response taken from Table 4.3.2.1b revealed that the linear square coefficients of all the three variables had highly

significant effect on the biotransformation of caffeine to theophylline. Since all eigan values are negative, the response surface is indicated as a maximum point (Fig 4.3.2.1a). Accordingly, three-dimensional graphs were generated for the pair wise combination of the three factors, while keeping third variable at its optimum level for biotransformation of caffeine to theophylline (Fig 4.3.2.1a to Fig 4.3.2.1d) to emphasize the roles played by various factors. The optimal concentrations for the three components as obtained from the maximum point of the model were calculated to be 27.96 g/L for glucose, 9.86 ml/L for corn steep liquor, 4.83 g/L for caffeine and the response graph is represented in Fig 4.3.2.1a. Table 4.3.2.1d shows the optimized medium composition for maximum biotransformation of caffeine to theophylline. Validation of experimental results for biotransformation of caffeine to theophylline was done and the results have been presented in Table 4.3.2.1e. The results indicate that the difference between the observed and predicted values was non significant ($p<0.05$).

Table 4.3.2.1a. Experimental design and results of the central composite rotatible design.

Exp.No	Glucose (g/L)	Corn steep liquor (ml/L)	Caffeine (g/L)	Biotransformation of caffeine to Theophylline (%)	Caffeine degradation (%)	Biomass Dry wt. (g/L)
1.	10.20	4.00	2.00	8.38	91.78	3.65
2.	10.20	4.00	8.00	8.05	16.75	2.08
3.	10.20	15.90	2.00	1.73	47.86	4.25
4.	10.20	15.90	8.00	0.00	17.51	3.78
5.	40.00	4.00	2.00	1.17	98.14	12.47
6.	40.00	4.00	8.00	14.04	40.09	7.84
7.	40.00	15.90	2.00	30.33	92.97	14.11
8.	40.00	15.90	8.00	3.44	22.02	9.05
9.	0.00	10.00	5.00	0.00	0.00	0.33
10.	50.00	10.00	5.00	10.37	85.88	5.84
11.	25.00	0.00	5.00	14.15	48.75	11.78
12.	25.00	20.00	5.00	0.48	8.51	7.81
13.	25.00	10.00	0.00	0.00	0.00	7.99

14.	25.00	10.00	10.00	1.19	21.17	5.52
15.	25.00	10.00	5.00	33.60	80.46	7.30
16.	25.00	10.00	5.00	36.58	81.00	7.50
17.	25.00	10.00	5.00	34.45	81.38	7.47
18.	25.00	10.00	5.00	34.73	81.26	7.59
19.	25.00	10.00	5.00	34.34	80.62	7.39
20.	25.00	10.00	5.00	32.34	81.18	7.47
21.	25.00	10.00	5.00	35.72	82.18	7.31

Table 4.3.2.1b. Analysis of variance for the experimental results of the CCRD for biotransformation of caffeine to theophylline.

Regression = 0.91424					
Variables	**SS**	**df**	**MS**	**F**	**p**
(1) Glucose (L)	170.44	1	170.44	4.76	0.05
Glucose (Q)	1257.09	1	1257.09	35.11	0.00
(2) Corn steep liquor (L)	26.79	1	26.79	0.75	0.41
Corn steep liquor (Q)	1058.85	1	1058.85	29.58	0.00
(3) Caffeine (L)	14.47	1	14.47	0.40	0.54
Caffeine (Q)	1741.93	1	1741.93	48.65	0.00
1L by 2L	138.35	1	138.35	3.86	0.08
1L by 3L	17.88	1	17.88	0.49	0.49
2L by 3L	211.68	1	211.67	5.91	0.03
Error	393.81	11	35.80		
Total SS	4591.98	20			

Table 4.3.2.1c. Regression coefficient of variables for the experimental results of the CCRD for biotransformation of caffeine to theophylline.

Regression = 0.91328				
Variables	**Regression Coefficient**	**Std.Err.**	**t**	**p**
Mean/Interaction.	-53.95	12.99	-4.15	0.00
(1) Glucose (L)	20.00	4.99	4.00	0.00
Glucose (Q)	-4.12	0.70	-5.86	0.00
(2) Corn steep liquor (L)	47.42	12.47	3.80	0.00
Corn steep liquor (Q)	-23.66	4.38	-5.39	0.00
(3) Caffeine (L)	154.76	24.88	6.22	0.00
Caffeine (Q)	-121.11	17.50	-6.92	0.00
1L by 2L	4.70	2.39	1.96	0.08
1L by 3L	-3.36	4.76	-0.70	0.49
2L by 3L	-28.77	11.92	-2.41	0.03

Table 4.3.2.1d. Optimized medium composition for biotransformation of caffeine to theophylline.

S. No	Media component	Concentration	Biotransformation of caffeine to theophylline (%)
1.	Glucose (g/L)	27.96	34.76
2.	Corn steep liquor (ml/L)	9.86	
3.	Caffeine (g/L)	4.83	

Table 4.3.2.1e. Validation of experimental results for biotransformation of caffeine to theophylline.

S. No.	Media components (g/L)		Biotransformation Predicted value (%)	Biotransformation Experimental value (%)	Deviation (P<0.5)
1.	Glucose (g/L)	27.96	34.76	33.94	NS
	Corn steep liquor (ml/L)	9.86			
	Caffeine (g/L)	4.83			
2.	Glucose (g/L)	50.00	10.36	10.27	NS
	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5.00			
3.	Glucose (g/L)	25.00	33.60	34.10	NS
	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5			

Figure 4.3.2.1a. Response curve for biotransformation of caffeine to theophylline at 120hour.

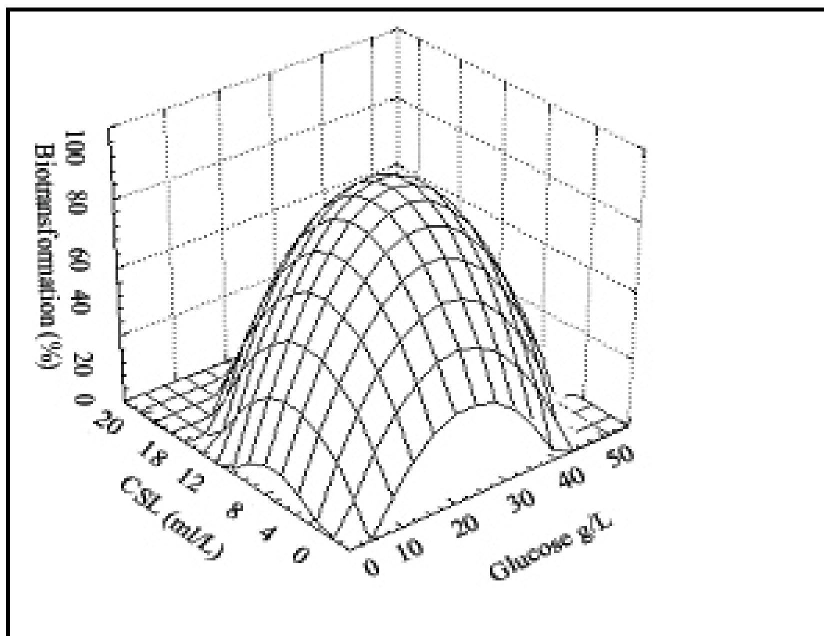


Figure 4.3.2.1b. Response curve for biotransformation of caffeine to theophylline at 72 hour.

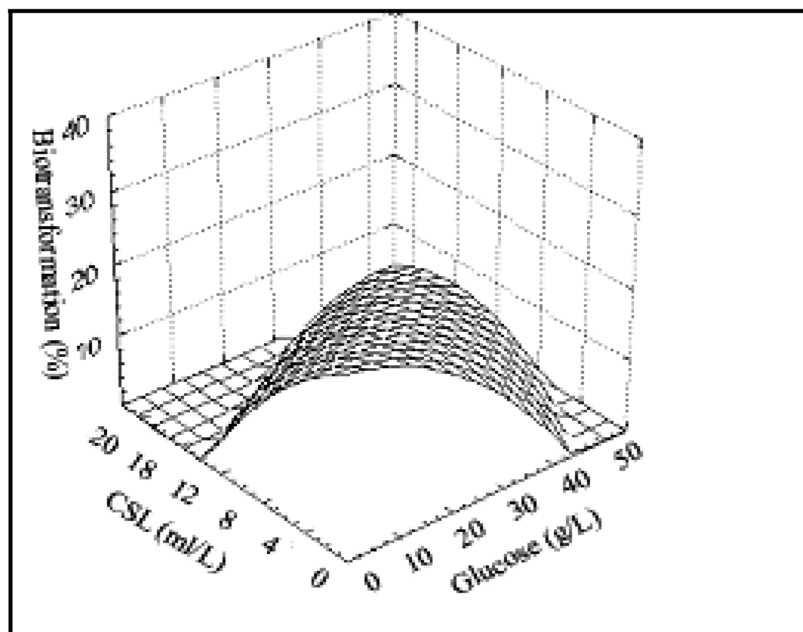


Figure 4.3.2.1c. Response curve for biotransformation of caffeine to theophylline at 96hour.

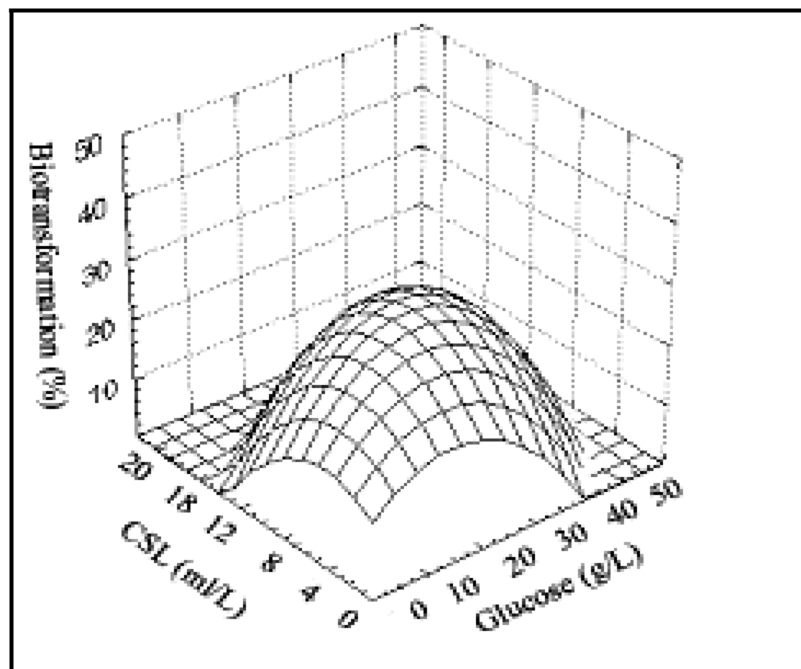
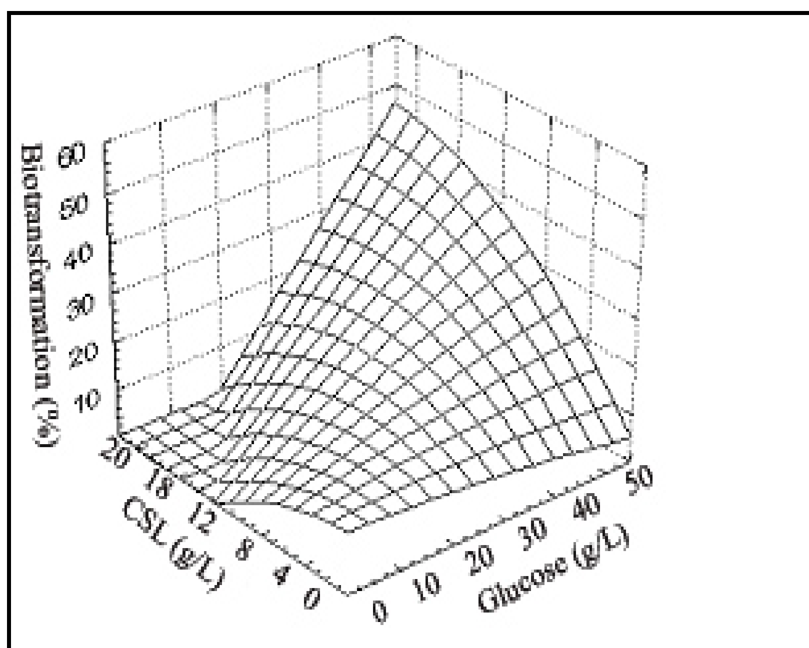


Figure 4.3.2.1d. Response curve for biotransformation of caffeine to theophylline at 144hour.



4.3.2.2. Caffeine degradation:

The CCRD design and the corresponding experimental data for caffeine degradation in *P. citrinum* MTCC 5215 are shown in Table 4.2.4 and Table 4.3.2.1a respectively. From the experimental results obtained maximum caffeine degradation was observed in the 144hour sample, hence, further discussion of results is done pertaining to the values of 144hour sample. After applying multiple-regression analysis on the experimental values, the results of ANOVA for the 21 trials performed by the experimental design were obtained and is shown in Table 4.3.2.2a. The corresponding second-order polynomial response model Equation that was found after Statistical analysis for the regression is represented below:

$$Y_{(g/L)} = -4.68 + 28.24\ X_1 - 3.32\ X_1^2 + 34.63\ X_2 - 35.06\ X_2^2 + 156.45\ X_3 - 211.18\ X_3^2 + 2.88\ X_1 X_2 - 6.82 X_1 X_3 + 22.94\ X_2 X_3$$

The statistical significance of the second-order model equation was checked by the F-test and the fit of the model was also expressed by the coefficient of determination R^2 (as shown in Table 4.3.2.2b), which was found to be 0.6593, indicating that 65.93% of the variability in the response could be explained in the model. This indicates that the above presented Equation is a suitable model to describe the response of the experiment pertaining to caffeine degradation in *P. citrinum* MTCC 5215. The response taken from Table 4.3.2.2b revealed that the linear square coefficients of all the three variables had highly significant effect on the caffeine degradation. Since all eigan values are negative, the response surface is indicated as maximum point. Accordingly, three-dimensional graphs were generated for the pair wise combination of the three factors, while keeping third variable at its optimum levels for caffeine degradation (Fig 4.3.2.2a to Fig 4.3.2.2d), to emphasize the roles played by various factors. The optimal concentrations for the three

components as obtained from the maximum point of the model were calculated to be 42.30 g/L for glucose, 7.80 ml/L for corn steep liquor, 3.44 g/L for caffeine and the response graph is represented in Fig 4.3.2.2a. Table 4.3.2.2c shows the optimized medium composition for maximum caffeine degradation in *P. citrinum* MTCC 5215. Validation of experimental results for caffeine degradation was done and the results are presented in Table 4.3.2.2d. The results indicate that the difference between the observed and predicted values were non significant ($p<0.05$).

Table 4.3.2.2a. Analysis of variance (ANOVA) for the experimental results of the CCRD for maximum caffeine degradation in *P. citrinum* MTCC 5215.

Regression = 0.65854					
Variables	**SS**	**df**	**MS**	**F**	**p**
(1) Glucose (L)	3665.93	1	3665.93	4.91	0.05
Glucose (Q)	817.63	1	817.63	1.09	0.32
(2) Corn steep liquor (L)	1316.27	1	1316.28	1.76	0.21
Corn steep liquor (Q)	2322.78	1	2322.78	3.12	0.11
(3) Caffeine (L)	2893.40	1	2893.40	3.88	0.07
Caffeine (Q)	5316.58	1	5316.58	7.13	0.02
1L by 2L	49.60	1	49.60	0.07	0.80
1L by 3L	69.74	1	69.74	0.09	0.76
2L by 3L	126.24	1	126.25	0.17	0.68
Error	8202.82	11	745.71		
Total SS	24023.04	20			

Table 4.3.2.2b. Regression coefficient of variables for the experimental results of the CCRD for maximum caffeine degradation in *P. citrinum* MTCC 5215.

Regression = 0.65937				
Variables	**Regression Coefficient**	**Std.Err.**	**t**	**p**
Mean/Interaction.	-4.68	58.91	-0.08	0.94
(1) Glucose (L)	28.24	22.65	1.25	0.24
Glucose (Q)	-3.33	3.18	-1.05	0.32
(2) Corn steep liquor (L)	34.63	56.56	0.61	0.55
Corn steep liquor (Q)	-35.06	19.90	-1.76	0.11
(3) Caffeine (L)	156.45	112.81	1.39	0.19
Caffeine (Q)	-211.19	79.33	-2.66	0.02
1L by 2L	2.880476	10.87	0.26	0.79
1L by 3L	-6.82416	21.57	-0.32	0.75
2L by 3L	22.94163	54.02	0.42	0.68

Table 4.3.2.2c. Optimized medium composition for maximum caffeine degradation in *P. citrinum* MTCC 5215.

S.No	**Media component**	**Concentration**	**Caffeine degradation (%)**
1.	Glucose (g/L)	42.30	95.50
2.	Corn steep liquor (ml/L)	7.80	
3.	Caffeine (g/L)	3.44	

Table 4.3.2.2d. Validation of experimental results for maximum caffeine degradation in *P. citrinum* MTCC 5215.

S.No	**Media components (g/L)**		**Biotransformation Predicted value (%)**	**Biotransformation Experimental value (%)**	**Deviation ($P<0.05$)**
1.	Glucose (g/L)	42.30	95.50	94.79	N.S
	Corn steep liquor (ml/L)	7.80			
	Caffeine (g/L)	3.44			
2.	Glucose (g/L)	50.00	86.54	85.88	N.S

	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5.00			
3.	Glucose (g/L)	25.00	79.79	80.46	N.S
	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5.00			

Figure 4.3.2.2a. Response curve for caffeine degradation at 144 hour.

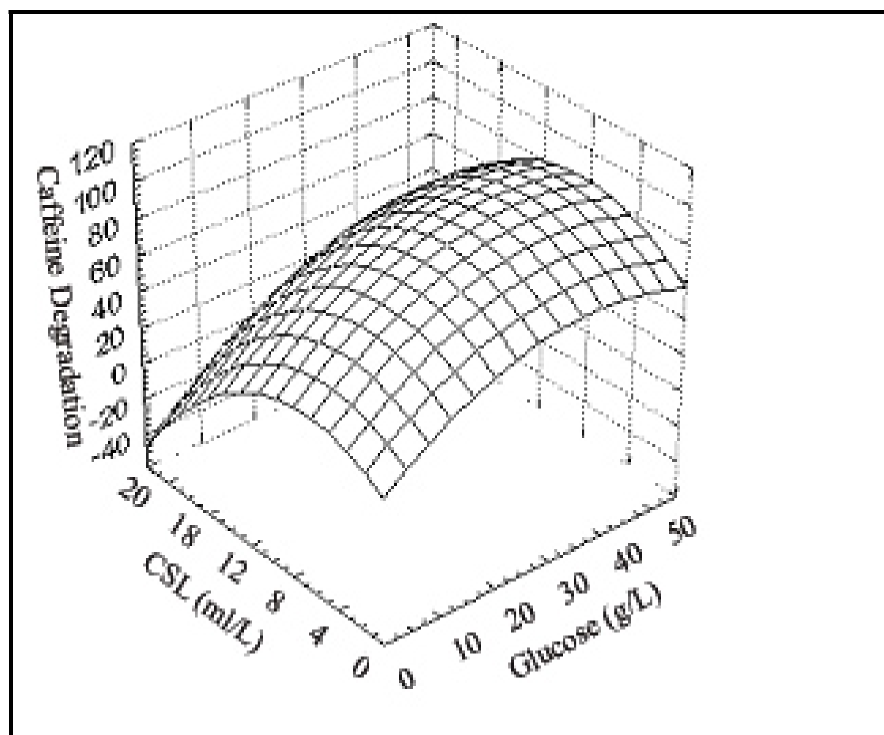


Figure 4.3.2.2b. Response curve for caffeine degradation at 72 hour.

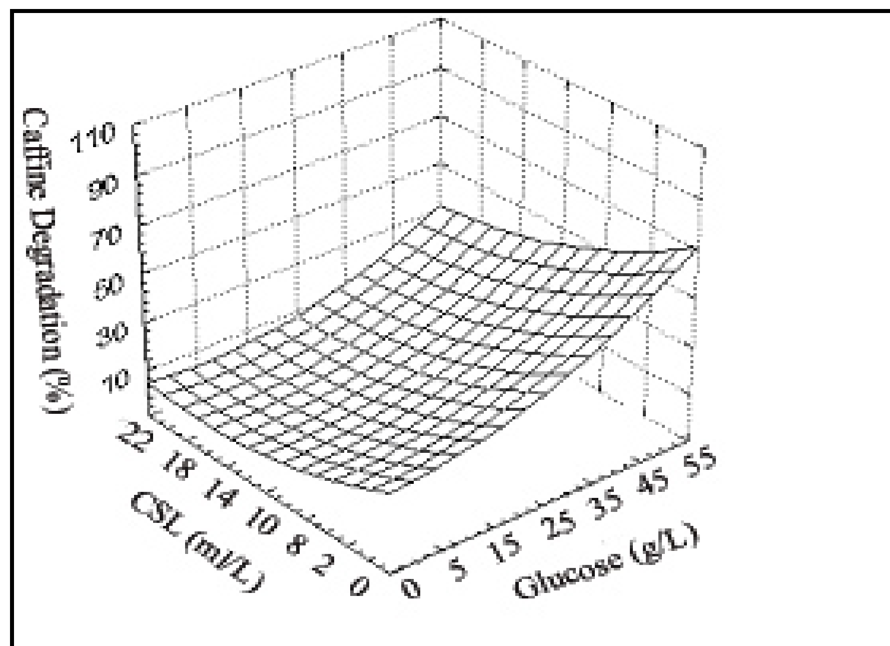


Figure 4.3.2.2c. Response curve for caffeine degradation at 96 hour.

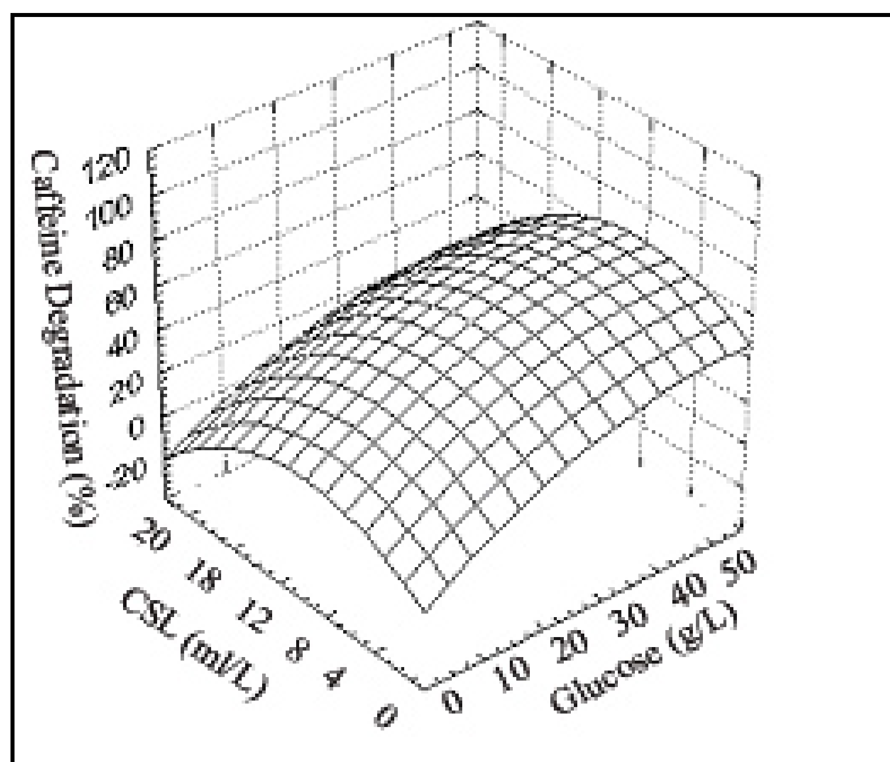
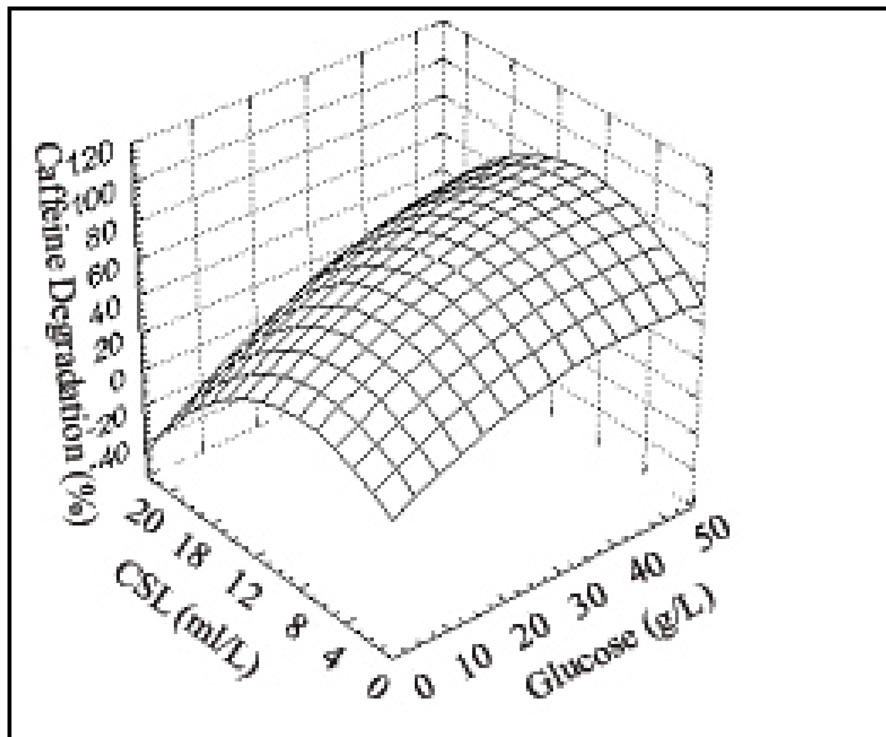


Figure 4.3.2.2d. Response curve for caffeine degradation at 120 hour.



4.3.2.3. Biomass production:

The CCRD design and the corresponding experimental data are shown in Table 4.2.4 and Table 4.3.2.1a respectively. From the experimental results obtained maximum biomass growth was observed at 144hour. Multiple-regression analysis was applied on the experimental values, and the results of ANOVA for the 21 trials performed by the experimental design were obtained as shown in Table 4.3.2.3a. The corresponding second-order polynomial response model Equation that was found after Statistical analysis for the regression is represented below:

$$Y_{(g/L}= 0.97+ 6.07\ X_1 -0.63\ X_1^2\ -6.06\ X_2 -2.73\ X_2^2 + 2.12\ X_3 -1.13\ X_3^2 +0.07\ X_1\ X_2 -2.14X_1X_3 +0.48\ X_2\ X_3$$

The statistical significance of the second-order model equation was checked by the F-test. The fit of the model was also expressed by the coefficient of determination R^2 (as shown in Table 4.3.2.3b), which was found to be 0.8631, indicating that 86.31% of the

variability in the response could be explained in the model. This indicated that above represented Equation is a suitable model to describe the response of the experiment pertaining to biomass growth. The response taken from Table 4.3.2.3b revealed that the linear square coefficients of all the three variables had highly significant effect on the biomass growth. Since all eigan values are negative, the response surface indicated a maximum point (Fig 4.3.2.3). Accordingly, three-dimensional graphs were generated for the pair wise combination of the three factors, while keeping third variable at its optimum levels for biomass growth (Fig 4.3.2.3). The optimal concentrations for the three components as obtained from the maximum point of the model were calculated to be 4.98 g/L for glucose, 2.1 ml/L for corn steep liquor, 1.5 g/L for caffeine and the response graph is represented in figure 4.3.2.3. Table 4.3.2.3c shows the medium composition optimized for maximum biomass growth. Validation of experimental results for biomass growth was done and the results are presented in Table 4.3.2.3d. The results indicate that the difference between the observed and predicted values were non significant ($p<0.05$).

Table 4.3.2.3a. Analysis of variance (ANOVA) for the experimental results of CCRD for maximum biomass accumulation.

Regression = 0.86426					
Variables	**SS**	**df**	**MS**	**F**	**P<0.05**
(1) Glucose (L)	111.22	1	111.22	42.39	0.00
Glucose (Q)	29.37	1	29.37	11.19	0.00
(2) Corn steep liquor (L)	0.16	1	0.166	0.06	0.80
Corn steep liquor (Q)	14.25	1	14.25	5.43	0.03
(3) Caffeine (L)	18.53	1	18.53	7.06	0.02
Caffeine (Q)	0.15	1	0.15	0.05	0.81
1L by 2L	0.03	1	0.03	0.01	0.90
1L by 3L	7.30	1	7.30	2.78	0.12
2L by 3L	0.05	1	0.05	0.02	0.88
Error	28.85	11	2.62		
Total SS	212.61	20			

Table 4.3.2.3b. Regression coefficient of variables for the experimental results of CCRD for maximum biomass accumulation.

Regression = 0.86318				
Variables	Regression Coefficient	Std. Err.	t	P<0.05
Mean/Interaction.	0.96	3.51	0.27	0.78
(1) Glucose (L)	6.07	1.35	4.50	0.00
Glucose (Q)	-0.63	0.18	-3.32	0.00
(2) Corn steep liquor (L)	-6.06	3.37	-1.79	0.09
Corn steep liquor (Q)	2.73	1.19	2.30	0.04
(3) Caffeine (L)	2.12	6.73	0.31	0.75
Caffeine (Q)	-1.14	4.73	-0.24	0.81
1L by 2L	0.075	0.65	0.12	0.91
1L by 3L	-2.15	1.29	-1.66	0.12
2L by 3L	0.48	3.22	0.15	0.88

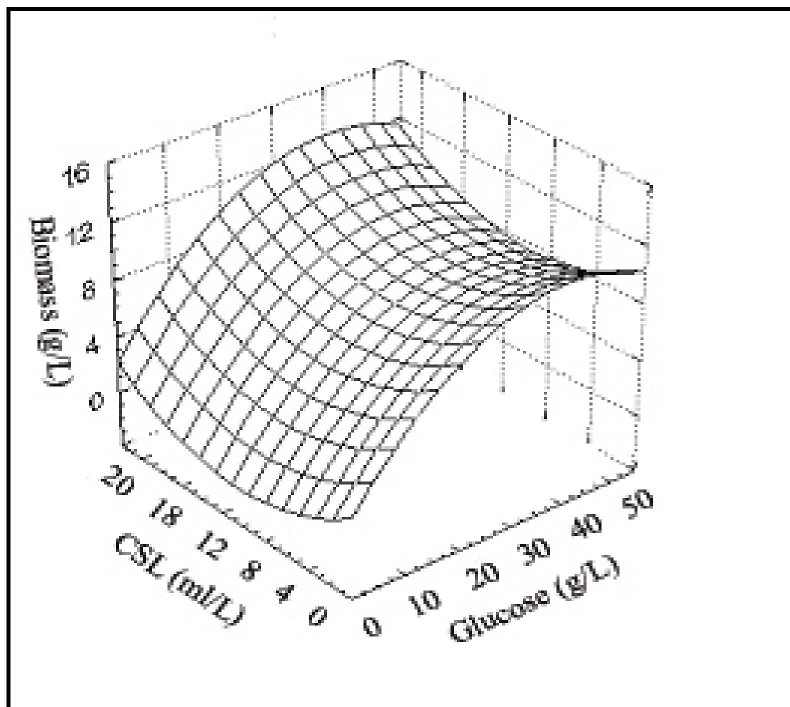
Table 4.3.2.3c. Optimized medium composition for maximum biomass accumulation.

S.No	Media component	Concentration	Dry weight of biomass (g/L)
1.	Glucose (g/L)	49.80	14.26
2.	Corn steep liquor (ml/L)	2.10	
3.	Caffeine (g/L)	1.50	

Table 4.3.2.3d. Validation of experimental results for maximum biomass accumulation.

S.No	Concentration of media components		Predicted value of biomass growth (g/L)	Experimental value of biomass growth (g/L)	Deviation (P<0.05)
1.	Glucose (g/L)	49.80	14.52	14.26	N.S
	Corn steep liquor (ml/L)	2.10			
	Caffeine (g/L)	1.50			
2.	Glucose (g/L)	50.00	5.97	5.84	N.S
	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5.00			
3.	Glucose (g/L)	25.00	7.47	7.30	N.S
	Corn steep liquor (ml/L)	10.00			
	Caffeine (g/L)	5.00			

Figure 4.3.2.3. Response curve for maximum biomass accumulation.



Discussion:

Table 4.3.2.1a represents the experimental design and results of the CCRD. Experimental design 9 of Table 4.3.2.1a shows that the presence of glucose is mandatory for biotransformation and caffeine degradation to take place. It was observed that the biomass growth is almost negligible (0.33g/L) in the absence of glucose. Corn steep liquor as nitrogen source in the media plays crucial role in the biotransformation of caffeine to theophylline and caffeine degradation. Experimental design 11 is devoid of corn steep liquor and the results obtained with this particular media composition, shows that maximum biotransformation observed is only 14.15%, and the caffeine degradation is only 48.75%. Hence, corn steep liquor as nitrogen source enhances biotransformation of caffeine to theophylline as well as caffeine degradation. The enhancement in biotransformation and caffeine degradation can be attributed to the composition of corn steep liquor, which consists of vital amino acids and growth factors, hence, helps in the synthesis of various enzymes for biotransformation of caffeine to theophylline and caffeine

degradation. However, the absence of corn steep liquor did not have much effect on the growth of biomass (11.78gm/L). In the presence of high concentrations of corn steep liquor (20gm/L), as seen from experimental design 12, both caffeine degradation (8.51%) and biotransformation of caffeine to theophylline (0.48%) is almost negligible. This is because higher concentration of additional nitrogen source inhibits the fungal strain from utilizing caffeine. This is in accordance with the results obtained by Roussos *et al*, (1994) who studied the effect of organic and inorganic nitrogen sources on the caffeine biotransformation ability in coffee pulp by *Penicillium verrucosum* and showed that the biotransformation of caffeine was completely inhibited by added nitrogen source both organic and inorganic, in combination or when used individually. Penaloza *et al* (1985) and Aguilar (1983) also reported the inhibition of the degradation of caffeine by *Aspergillus niger* in solid state fermentation when inorganic nitrogen sources were added to the moist coffee pulp media.

No biotransformation was observed in the absence of caffeine as seen from results of experimental design 13. High concentrations of caffeine (10gm/L), lowers the biomass growth (5.52gm/L), as higher concentrations of caffeine is known to inhibit the growth of fungi (Frischknecht *et al*, 1986; Nathanson, 1984). The reduction in biomass growth leads to the reduction in biotransformation rate to negligible values (1.19%) as seen from the results of experimental design 14. With lower concentration of caffeine vigorous growth of biomass (12.47%) was observed, with higher caffeine degradation (98.14%) but the amount of theophylline produced was very less (1.17%) as it was degraded further.

4.3.2.4. Downstream processing of biotransformed theophylline:

The biotransformed theophylline was extracted from the broth by using various solvents and the results have been presented in Table 4.3.2.4a. Using ethyl acetate as the extraction solvent 83mg of crude extract was obtained from 100ml of broth whereas with dichloromethane 60mg of crude extract was obtained, chloroform gave 57.6mg and methanol yielded 47mg of crude extract. Schwimmer *et al* (1971) have worked on the biotransformation of caffeine to theophylline by *P. roquefortii* but the work was confined to the identification of theophylline formed by the biotransformation of caffeine. No work was carried out towards the downstream processing of the biotransformed theophylline. In this particular section theophylline was purified from the crude extract by using two simple steps. It was precipitated from the crude extract using acetone, 560mg of theophylline was obtained from 1gm of crude extract (Fig. 4.3.2.4a). Further purification of theophylline was carried out by crystallizing the theophylline from ethyl alcohol. 92.8mg of pure theophylline was obtained from 100mg of acetone precipitated theophylline (Fig. 4.3.2.4a, Table 4.3.2.4b.)

Table 4.3.2.4a. Choice of extraction of solvent for downstream processing of theophylline:

S. No	Solvent	Weight of crude extract (mg/100ml of broth)
1.	Ethyl acetate	83.0
2.	Dichloromethane	60.0
3.	Chloroform	57.6
4.	Methanol	47.0

Table 4.3.2.4b. Purification of theophylline from fermented broth.

S. No	Treatment	Yield (w/w)
1.	Ethyl acetate extraction of broth	0.83%
2.	Acetone precipitation of crude extract with broth	56%

3.	Crystallization of precipitated theophylline with ethyl alcohol	92.8%

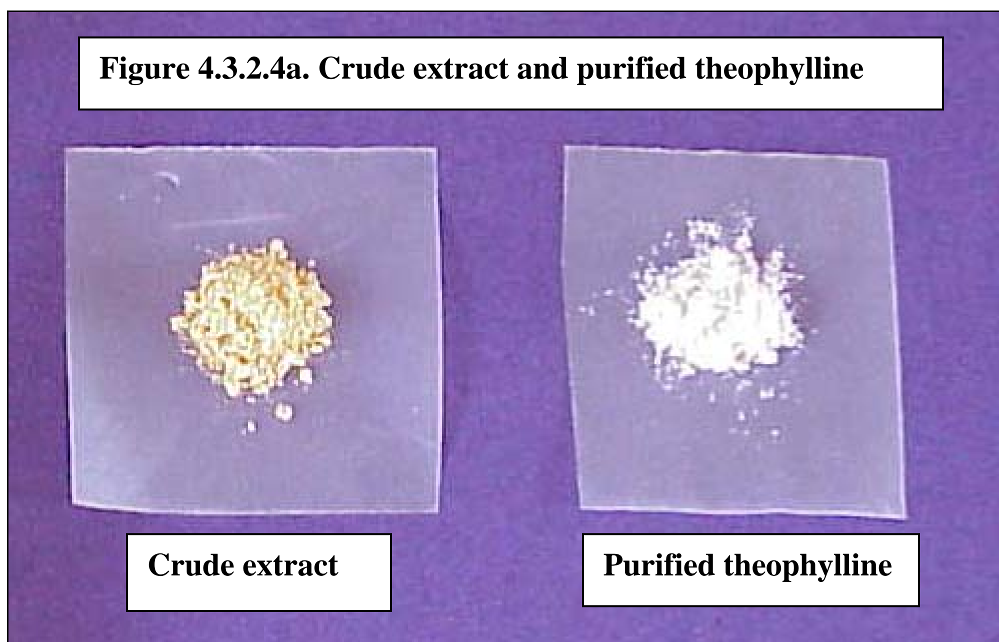


Figure 4.3.2.4a. Crude extract and purified theophylline

Identification of purified theophylline:

1) Melting point determination:

The observed melting point was 272°C (Literature cited 270-274 °C). The purity of biotransformed theophylline was determined by mixed melting point and there was no depression in melting point, suggesting the purity of the compound.

2) HPLC analysis:

HPLC chromatogram of purified biotransformed theophylline was compared with that of standard theophylline and the results showed good agreement in terms of retention time. The peak purity observed was 99.4%.

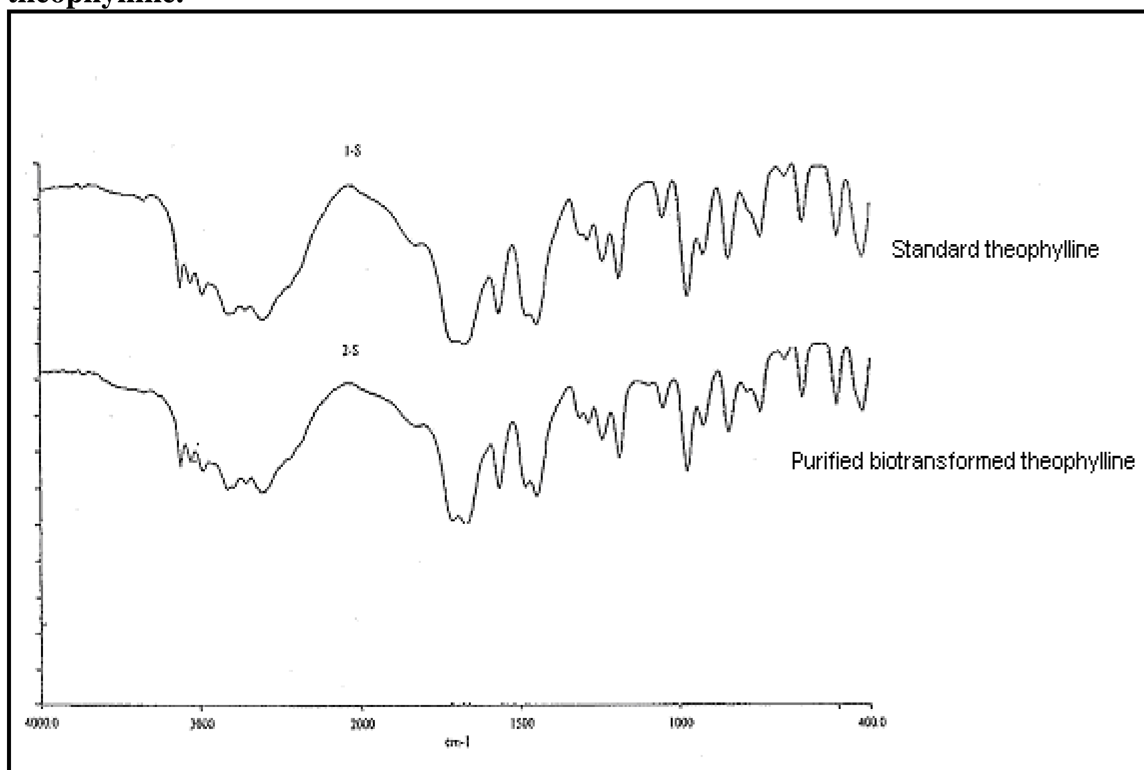
3) FTIR spectroscopy:

FTIR spectra of purified biotransformed theophylline and standard theophylline have been presented in figure 4.3.2.4b, which shows a very good agreement.

The IR data of purified biotransformed theophylline is presented below:

IR Max (KBr) 1706(C=O), 1557(C=C), 1660(C=N), 1436(C-N) cm^{-1}

Figure 4.3.2.4b. FTIR spectra of standard theophylline and purified biotransformed theophylline.



4) NMR spectroscopy:

The purified biotransformed theophylline was subjected to ^{1}H NMR and ^{13}C NMR, and the results have been presented below.

^{1}H NMR: (500MHz): 3.15 ppm (1N-CH_3), 3.36 (3N-CH_3), 3.28(C-H), 7.96 (NH).

^{13}C NMR: (125.7MHz): 27.8(1N-CH_3), 29.8(3N-CH_3), 106.5(C-8), 140.6(C-5), 148.0(C-4), 151.3(C=O), 154.6 (C=O).

Conclusion:

The caffeine metabolizing fungal strain *P. citrinum* MTCC 5215 was used for the production of theophylline in a designed biotransformation media. Optimized biotransformation media composed of (g/L) - Caffeine - 1.0, Glucose - 20.0, Na_2HPO_4 -

0.12, K_2HPO_4 - 1.3, $MgSO_4$ - 0.3, $CaCl_2$- 0.3. $FeS0_4$ - 3mM. Optimized corn steep liquor concentration was 5ml/L and optimum allopurinol concentrationwas 4mM/L. pH 5.6 and 28^oC temperature were the optimum physical parameters. Optimum inoculum age for biotransformation of caffeine to theophylline was 72 hours and inoculum load was optimised to be 5x 10^5 spores/ml. The designed media contained simple ingredients/components and could yield high biotransformation of 85% at reasonable less time period of 42hours. Scale up studies were carried out at 10L laboratory scale fermentor and 83% biotransformation of caffeine to theophylline was obtained in 42 hours.

The results obtained from the RSM studies, showed that maximum biotransformation of caffeine to theophylline occured at 120hour and the optimized media composition for maximum biotransformation of caffeine to theophylline was 27.96 g/L of glucose, 9.86 ml/L of corn steep liquor and 4.83 g/L of caffeine. Maximum caffeine degradation in the fungal strain *P. citrinum* MTCC 5215 occured at 144hour and the optimized media composition was 42.30 g/L of glucose, 7.80 ml/L of corn steep liquor, 3.44 g/L of caffeine. For maximum biomass growth the optimized media composition was 4.98 g/L of glucose, 2.1 ml/L of corn steep liquor and 1.5 g/L of caffeine.

Down stream processing for the recovery of biotransformed theophylline consisted of three simple steps. The chemical identification of the biotransformed theophylline by FTIR and NMR analysis showed good agreement with standard theophylline. The biotransformed theophylline was subjected to mixed melting point analysis with purified standard samples but no depression in melting point was observed indicating the high purity of the biotransformed theophylline and efficiency of the purification method.

The results obtained from the above study, gives a complete knowledge of the behavior of the fungal strain *P. citrinum* MTCC 5215 in increasing concentration of caffeine, glucose and corn steep liquor in varying concentrations and different combinations. The above knowledge will prove to be instrumental in using *P. citrinum* as a strain towards process development for the production of theophylline. Further it will also help towards using this particular strain in decaffeination of coffee processing wastes as coffee pulp, coffee hull and also use of these caffeine containing wastes as SSF substrate for the production of theophylline. Further work on the production of theophylline by biotransformation using immobilized whole cells and immobilized enzyme are in progress.

References:

Aguilar M. D. P (1983), Esudois sobre la utilizacion de carbohidratos, por Aspergillus niger en sistemas de fermentacion solida, usando pulpa de café como sustrato y differentes niveles de nitrogeno. Ph.D Thesis, Universidad de San Carlos de Guatemela, Guatemela.

Alexander, M. (1994), Biodegradation and bioremediation. Academic Press,Inc., San Diego, Calif.

Asano, Y., Komeda, T., and Yamada, H. (1993), Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.* **57**:1286–1289.

Birdwhistell, R. K., and Connor, O. R. R. (1971), The Freeman library of laboratory separates in chemistry (**Vol. 2**). San Francisco, USA:Freeman.

Blecher, R., (1976), Microbial breakdown of caffeine (author's transl), Zentralbl *Bakteriol [Orig B].* **162**(1-2): 180-183. [Article in German]

Blecher, R., and Lingens, F. (1977), Metabolism of caffeine by *Pseudomonas putida*. *Hoppe Seyler's Z Physiol Chem* **358**: 807–817.

Box, G. E. P. and Wilson, K. B. (1951), On the Experimental Attainment of Optimum Conditions (with discussion). *Journal of the Royal Statistical Society* Series B **13**(1): 1-45.

Bradley, J. W., and Lichtenstein, L. M., (2001), Drugs used in the treatment of asthma, *In* Alfred Goodman Gilman (ed.), Pharmaceutical basis of therapeutics, 10th ed. p733-754 Mc Graw-Hill Medical Publication division.

Cornish H. H, and Christman, A. A. (1957), A study of the metabolism of theobromine, theophylline and caffeine in man. *J Biol Chem.* **228**: 315–323.

El'vina. P. M., Margarita, G. V., and German, K. B. (1998), Metal ions as enzyme effectors, *Russ Chem Rev*. **67** (3): 225-232.

Finar, I. L Organic Chemistry (1959), Stereochemistry and the chemistry of natural products. Vol-2 Chapter-16, Purines and Nucleic acids. P-809.

Fries, N., Kihlman, B., Fungal mutations obtained with methyl xanthines. *Nature*, **162:** 573.

Frischknecht, P.M., Ulmer, D.J., and Baumann, T.W., (1986), Purine alkaloid 1948), formation in buds and developing leaflets of *Coffea arabica*: expression of an optimal defense strategy. *Phytochemistry.* **25:**613-616.

Hakil, M., Voisinet, F., Gonz´alez, G.V., and Augur, C., (1999), Caffeine degradation in solid-state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources. *Process Biochem*. **35**:103–109.

Helmkamp, G. K., and Johnson, H. W. (1968), Selected experiments in organic chemistry (2nd ed.) San Francisco, USA: Freeman, pp. 157–158.

Homer, A. B, (2001), Bronchodilator and other agents used in asthma chapter 19, p 305-321 *In* Basic and clinical pharmacology, 6th ed., Bertrom G. Katzung(ed.) Lange Medical Book.

Johnson, I. M., Kumar, S. G, and Malathi, R. (2003), RNA binding efficacy of theophylline, theobromine and caffeine. *J Biomol. Struct. Dyn*. 20(5): 687-692.

Kathryn, B. and Alan, K. K. (2000) Ch-35 Asthma p-651 *In* Eric T.Herfindal,Dick R.G.(ed.), Text book of therapeutics-Drug and disease management, 6th ed, Williams and Wilkins.

Kelley, B.N. and Beardmore, T. D. (1970), Allopurinol: Alteration in pyrimidine metabolism in man. *Science*. **169**: 388-390.

Kurosawa, (2002), Caffeine inhibits the synthesis of the HIV Virus in T cells by 94%, http://Grouppekurosawa.com.

Landgrebe, J. A. (1993), Theory and practice in the organic laboratory (4th ed.). Paci.c Grove, CA: Brooks-Cole, pp. 381–383. Analysis (**Vol 5**: p. 55). New York, USA: Wiley and Sons.

Lazaros, C. F, Nathalie, D., Chariklia, K., Karen, E. A, Jorgen, J., and Peter, R. S. (2002), Direct Effects of Caffeine and Theophylline on p110 and Other Phosphoinositide 3-Kinases. Differential effects on lipid kinase and protein kinase activities, *J. Biol. Chem.* **277** (40): 37124–37130.

Masood, A., Michael, J. L, Robert, A., and Kent, K., T. (1997), Corn steep liquor lowers the amount of inoculum for biopulping, *Tappi journal*. 161-164.

Mayo, D. W., Pike, R . M., and Butcher, S. S. (1989), Microscale organic laboratory (2nd ed.). New York, USA: Wiley, pp. 162–164.

Murray, S. D., and Hansen, P. J. (1995), The extraction of caffeine from tea: An old undergraduate experiment revisited. *Journal of Chemical Education*. **72**: 851–852.

Nathanson, J. A. (1984), Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science.* **226** (4671): 184 -187.

Nimitz, J. S. (1991), Experiments in organic chemistry. Englewood Cli.s, NJ: Prentice-Hall, pp. 61–62.

Ohe,.T and Watanabe, Y. (1979), Purification and properties of xanthine dehydrogenase from Streptomyces cyanogenus. *J. Biochem.* **86** (1): 45-53.

Pavia, D. L., Lampman, G. M., and Kriz, G. S. (1976), Introduction to organic laboratory techniques. Philadelphia, USA: Saunders, pp.58–62.

Penaloza, W., Molina, M. R., Gomez, B. R. and Bressani, R. (1985), Solid-State Fermentation: an Alternative to Improve the Nutritive Value of Coffee Pulp. *Appl. Environ. Microbiol*. **49** (3): 388-393.

Ravi. R. and Susheelamma. N. S. (2005), Simultaneous optimization of a multi-response system by desirability function analysis of '*Boondi'* making - a case study. *J. Food Science,* **70** (8): 539-547.

Roussos. S (1982), Mise au point d'une methode pour l'etude des caracteres morphologiques, biochimiques et nutritionnels des champignons imparfaits. Cah. ORSTOM ser Biol **45**: 25-34.

Roussos, S., Hannibal, L., Aquiahuatl, M. A., Trejo, H., M. R., and 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.

Roussos, S., Angeles, A., M. D. L., Trejo, H., M. D. R., Gaime, P. I., Favela, E., and 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.

Sánchez, G. G., Roussos, S., and Augur, C., (2004), Effect of the nitrogen source on caffeine degradation by *Aspergillus tamarii*. *Letts. Appl. Microbiol.* **38 (1)**: 50-55.

Schwimmer, S., Kurtzman, R. H. and Heftman, E. (1971), Caffeine metabolism by *Penicillium roqueforti*. *Archives of Biochemistry and Biophysics*. **147**: 109-113.

Sugimura, K. and Mizutani, A., (January 1979) The inhibitory effect of xanthine derivatives on alkaline phosphatase in the rat brain. *Histochemistry and Cell Biology.* **61**(2): 115-120.

Tortora, G. J., Funke, B. R., and Case, C. L. (1995), Microbiology. *An Introduction.* Fifth Edition. The Benjamin/Cummings Publishing, Co., Inc., Redwood City, CA, pp. 129-132, 145, 146, 150.

Waller, G. R., Kumari D., Friedman, J., Friedman, N., and 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.

Williamson, K. L. (1989), Macroscale and microscale organic laboratory (2nd ed.). Toronto: Heath, pp. 130–133.

*Zuluaga, V. J. (1989), Utilizacion integral de los subproducion del café. In : Roussos S, Licona FR, Guiterrez RM(eds), Proceedings of I *Seminario International sobre Biotechnologia en la Agroindustria cafetalara* (I SIBAC), Jalapa, Mexico. 63-76.

* **Original reference not seen.**

CHAPTER -5

UTILIZATION OF CAFFEINE- CONTAINING COFFEE PROCESSING WASTES
For value addition

5.0. Scope of the work:

Coffee and tea processing wastes generated during the processing of the coffee berries and tea leaves generate huge amounts of wastes in the form of coffee pulp, hulls, spent coffee, spent tea etc. These wastes are not only rich in nutrients like sugars, protein, and minerals, but are also associated with several anti-physiological factors such as caffeine, tannins, phenolics and indigestible fiber in high concentrations which limit their utilization. The disposed coffee processing wastes leads to pollution of lakes and water bodies around the coffee processing units and also causes soil pollution. In view of the problems mentioned above, alternative ecofriendly methods for coffee pulp utilization have been explored in the present chapter. These processing wastes have been used for the extraction of caffeine for production of theophylline through biotransformation, production of mushrooms, vermicomposting and production of enzymes by solid state fermentation, using coffee pulp as the substrate and are described in detail in this chapter. Caffeine extracted from the coffee wastes was used for biotransformation to theophylline, which was described in Chapter 4. Biodecaffeination of coffee pulp was carried out using *Penicillium citrinum* MTCC 5215. Further these processing wastes were used for the production of mushroom using *Pleurotus* and for vermicomposting using *Eisena fetida*. The coffee processing wastes have been utilized efficiently, which will lead to value addition, generate income in rural areas and prevent environmental pollution.

5.1. Introduction:

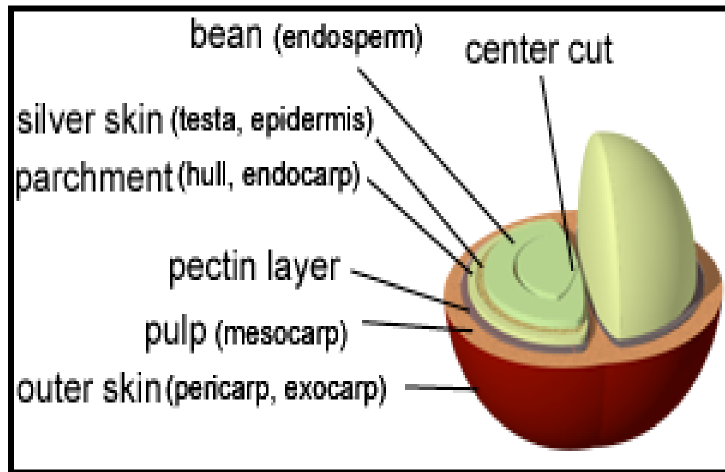
Coffee is one of the most popular beverages consumed world over. The world production of coffee is 6.795 million tons (Upasi, 2006). The processing of coffee is through dry and wet process. In its sojourn from the pod to the cup, 40% of the total coffee cherries produced, are processing waste consisting mainly of coffee pulp and hull. (Rolz *et al*, 1988a; Philip *et al*, 1993). Spent coffee is another from of coffee processing waste rich in caffeine and is generated in the form of spent coffee during the manufacture of instant coffee. These processing wastes consist of several anti-physiological factors such as caffeine, tannins, phenolics and indigestible fiber in high concentrations (Adams and Dougan, 1981; Bressani, 1979a; Bercelos *et al*, 2002; Ricardo, 1980). Caffeine at higher concentrations is known to be antimicrobial and allelopathic, inhibiting germinating seeds (Frischknecht *et al*, 1986; Nathanson, 1984). Due to high caffeine and tannin content, the processing wastes are inhibitory to the growth of microorganisms leading to improper utilization and pollution of lakes around the coffee processing units (Ledger and Tilman, 1972; Christensen, 1981; Martinez-Carrera, 1987).

5.1.1. Coffee processing:

The fruit or "coffee berry" is a two-seeded unit deep crimson when fully mature, about 1 cm in diameter. A cross section of the coffee fruit is shown in figure 5.1.1.1. The coffee berry shows four anatomical fractions: the coffee bean proper or endosperm; the hull or endocarp; a layer of mucilage or mesocarp, and the pulp or mesocarp, the outermost layer is pericarp. Because of the viscous nature of the mucilage when the berry is mature, a slight pressure applied to the berry is

enough to expel the two beans from the fruit. This characteristic has been used to advantage in the process that has been practiced for a long time to separate the beans from the rest of the fruit's structural component.

Figure 5.1.1.1. Section of coffee fruit showing various anatomical sections.



Processing of coffee is the method of converting the raw fruit of the coffee plant (cherry) into the commodity coffee powder. The cherry has the fruit or pulp removed leaving the seed or bean, which is then dried. Two basic methods namely dry (natural) and wet (washing) together with ancillary process, including grading, cleaning and polishing are employed in the processing of coffee.

Wet method:

In the wet method of coffee processing, most of the covering is removed from the beans before they are dried. A mechanical pulping machine removes the pulp after which the seeds are dumped into fermentation tanks where natural enzyme acts upon it to loosen the mucilage so that it can be easily washed from the seeds. Once washed, the beans are left to dry in the sun for 12 - 15 days with the parchment still attached during which time they are raked and turned several times a

day to ensure even drying. Once the beans are dry the parchment is removed. *Coffea* a*rabica* beans are generally wet processed and coffee produced with the wet method is considered superior. The wet method generates coffee pulp and is the most abundant waste produced during the pulping operation of the coffee cherry (Aguirre *et.al*., 1976; Braham, 1979; Bressani, 1979 a,b,c; Cabezas et.al., 1979; Edwards, 1979). It is then transported by water and allowed to ferment naturally. The water is rich in sugars and other nutrient and is drained into the water bodies. This waste water supports a rich microbial growth posing threat to the environment, and its disposal in an efficient manner is a problem. The fermented pulp when dried is used some times as an organic fertilizer and applied to the coffee trees. It is usually dumped in open piles and left there for several months after which it is mainly used as an organic fertilizer or soil conditioner in the coffee fields. Figure 5.1.1.2 represents coffee processing wastes generated by the wet method, dumped into open pits and allowed to ferment naturally.

Wet coffee processing method in a coffee processing unit of Mercara, Karnataka, India is shown in Figure 5.1.1.2. In most of the piles anaerobic conditions are rapidly established and putrefactive fermentation sets in attracting lots of insects. Moreover, constant flow of the pulp liquor out of the pile is usually channeled directly to the rivers or into open ponds contributing to serious water pollution (Hakil *et al*, 1998; Zuluaga *et al*, 1975, Edwards, 1979). Wet coffee processing also results in discharge of high strength, saccharide-rich waste water (Elias, 1979) which is a cause for high BOD levels in water bodies around coffee processing units. Coffee processing in India is carried out by small and medium

sized plantations for whom there are inadequate facilities to carry out efficient resource recovery and effluent treatment. Coffee processing waste water is treated in a system of anaerobic-aerobic lagoons. These anaerobic-aerobic lagoons are operated only during the short coffee pulping season, which is during winter. These lagoons are operated in low winter temperatures, they get overloaded, become malodorous, pose potential hazards to ground and surface water resources (Chanakya and Alwis, 2004) leading to soil and water pollution.

Figure 5.1.1.2. Disposed coffee pulp after coffee processing by wet method at a coffee-processing unit in Mercara.

Dry Process:

The dry process (also known as the natural method) of coffee processing consists of either allowing the fruit to dry in the trees, or harvesting fresh fruits and drying by solar dehydration and then dehulling it. The oldest and simplest method is the dry method in which the coffee cherries are left to dry in the sun for up to four weeks and then hulled to reveal the beans. The dry-process is often used in

countries where rainfall is scarce and long periods of sunshine are available to dry the coffee properly. Coffee pulp and hull are generated during the dry processing method. These processing wastes are disposed by dumping near the processing industries, allowing it to decompose partially and then used in the coffee plantations as fertilizer. Figure 5.1.1.3 shows photographs of coffee pulp, coffee hull and decomposed coffee pulp dumped at a coffee curing station in Mysore, India.

Figure 5.1.1.3. Coffee pulp and coffee hull by generated by dry method of coffee processing disposed at coffee processing unit in Mysore.

In view of the problems mentioned above, alternative ecofriendly methods for coffee processing waste utilization should be explored. Coffee pulp can be used as a substrate for solid-state fermentation for the production of various enzymes, composting, mixing it directly with animal feed ingredients, for production of biogas and organic fertilizer through controlled anaerobic fermentation, for edible mushroom production and vermiculture. Attempts have been made in the present

chapter to explore the possibility of utilizing the various coffee processing wastes for the extraction of caffeine for production of theophylline through biotransformation, production of mushrooms and vermicomposting.

The coffee processing wastes can be utilized in processes, which lead to value addition, generate income in rural areas and prevent environmental pollution. The proposed uses of coffee processing wastes are as follows:

1) Extraction of caffeine for biotransformations.
2) Biodecaffeination of coffee pulp.
3) Use of coffee pulp as substrate for solid-state fermentation for enzyme production.
4) Use of coffee processing wastes for mushroom production.
5) Use of coffee processing wastes for vermiculture for biofertiliser.

5.1.2. Uses of coffee processing wastes:

The utilization of coffee pulp as an animal feed has been mentioned as an attractive possibility; however, such utilization is limited by anti-physiological factors naturally occurring in the material (Bressani, 1979a). It was found that the use of coffee pulps as animal feeds is associated with several anti-physiological effects due to its relatively high caffeine, tannins, polyphenols, high contents of potassium, and fiber (Bercelos *et al*, 2002; Ricardo, 1980). Several technologies to detoxify coffee pulp have been investigated by several authors, but have met with little success (Bressani, 1979b). Decaffeination of coffee pulp 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. Ensiling of coffee

pulp, as well as treatments of the material with calcium hydroxide or potassium bisulfite, has proved to be inefficient in reducing its toxicity (Elias, 1979; Roussos *et.al*, 1995). Strains of *Aspergillus* species have been used in a solid-state fermentation technique for protein enrichment of coffee pulp, for use in animal feed stock (Penaloza *et al*, 1985). From all the studies it was proved that the biological decaffeination of coffee pulp not only decreases the caffeine content in it but also improves the nutritional quality of the coffee pulp (Rojas *et. al*., 2003). Rolz *et al*, (1988b) have reported production of edible mushroom on a commercial scale from coffee pulp after decaffeinating the same by SSF. However, there have been no reports on the use of coffee hull for the production of mushroom. This chapter reports on the successful use of coffee hull for the production of mushroom and will be discussed in detail in the forthcoming sections.

Silage method has been used to stabilize coffee pulp/coffee husk and conserve its nutritional characteristics for better utilization of this major agro industrial waste (Murillo, 1979). Coffee pulp has been used for the production of biogas with yield of $10m^3$/ton (TERI, 1997). A number of studies performed during the last 2 decades revealed the potential of biogas production from coffee pulp and showed the technological feasibility of anaerobic digestion (Boopathy, 1988; Chacon and Fernandz, 1984; Lane, 1983; Wintrebert, 1980). Inhibition of anaerobic degradation by pulp ingredients (tannins, terpenes, terpenoides) have been reported by Field and Lettinga, (1987). The ability of the earthworm *E. foetida* to transform coffee pulp into valuable compost was evaluated by transforming these wastes into a useful organic fertilizer by Orozco *et al* (1996).

5.1.3. Occurrence and extraction of caffeine for biotransformation:

Caffeine is widely distributed within the plant kingdom and is found in about 60 sp. of plants. It is found in higher concentrations in coffee beans (*Coffea arabica*), tea leaves (*Camellia sinensis*), leaves of *Ilex paraguariensis* (mate), guaraná paste (*Paullinia sorbilis*), and kola nuts (*Cola acuminata*). It is also found in many other plants, mainly members of the *Dicotyledoneae*. Altogether it is produced by a large number of species belonging to at least twenty-eight genera over seventeen families in thirteen orders of plants. The highest number (twenty) of caffeine-containing species is found within the family *Sterculiaceae*, where the most important plants are *Cola acuminata* and *Theobroma cacao*.

Usually, caffeine is found only in certain parts of the plant, primarily in leaves, fruits, seeds, and bark. Caffeine is not found in xylem or, with certain exceptions (roots of seedlings of *Cola* and *Paullinia*), in subterranean parts. The caffeine in plants is found in seeds from *Paullinia cupana* and *Paullinia sorbilis*, which may contain as much as 6% caffeine, guarana (4-7%), tea leaves contain approximately 3-5% caffeine, coffee beans 1.1-2.2%, cola nuts 1.5%, and cacao beans 0.03% (Anderson, 2004). Mazzafera and Carvalho (1992) studied the caffeine content of seeds of different *Coffea* species and found that the content varied from 0.4 to 2.4%. The caffeine content in the natural plant products is influenced by the geographical location of its growth, the variety of plant, the climate, and cultural practices (Graham, 1978). The caffeine present in various plant parts and wastes can be extracted efficiently and used further. Caffeine extraction is an important industrial process and can be performed using a number of different solvents.

Benzene, chloroform, trichloroethylene and dichloromethane have all been used over years but for reasons of safety, environmental impact, they have been superseded by two other methods discussed below.

a) Water extraction:

Coffee wastes are soaked in water, which solubilises not only caffeine but also many other compounds, like tannins, phenolics and polyphenols. It is then passed through activated charcoal, which removes the caffeine. The caffeine rich water is then concentrated by evaporation to precipitate the caffeine. The crude caffeine obtained in this manner is then purified by crystallization in cold water or solvents like methylene chloride. Caffeine is also obtained as a by-product of solvent decaffeination of coffee and tea. Coffee manufacturers recover the caffeine and resell it for use in various purposes such as soft drinks and medicines.

b) Supercritical carbon dioxide extraction:

Supercritical carbon dioxide is an excellent nonpolar solvent for caffeine, is safer than the organic solvents that are used for caffeine extraction from coffee beans and tea. The extraction process is simple where CO_2 is forced through the green coffee beans at temperatures above 31.1°C and pressures above 73 atm. Under these conditions, CO_2 is in a "supercritical" state: it has gas like properties which allow it to penetrate deep into the beans but also liquid-like properties which dissolve 97-99% of the caffeine. The caffeine-laden CO_2 is then sprayed with high pressure water to remove the caffeine. The caffeine can then be isolated by charcoal adsorption (as above) or by distillation, recrystallization, or reverse osmosis.

However, the application of supercritical extraction of caffeine has limitations in terms of cost and handling the large volumes of wastes generated and is not considered feasible for detoxification of coffee wastes.

c) Solvent extraction:

Various solvents as chloroform, dichloromethane, and ethyl acetate have been used for the extraction of caffeine. The extraction of caffeine in aqueous solution was generally done using chloroform (Birdwhistell and O' Connor, 1971; Helmkamp and Johnson, 1968; Pavia *et al*, 1976) or methylene chloride (Landgrebe, 1993; Mayo *et al*, 1989; Nimitz, 1991; Williamson, 1989). Murray and Hansen (1995) suggested a less toxic alternative for treatment of tea leaves through the use of water/1-propanol/sodium chloride ternary system, being a suitable replacement for the more traditional water/organochlorine solvent systems.

However, it has been found that the crude caffeine obtained through the Murray–Hansen method after evaporation of 1-propanol was highly contaminated with tannins and sodium chloride. Hampp (1996) introduced an additional cleaning/extraction step, which is commonly performed in organic synthesis. It consists of a wash with a 10% aqueous NaOH solution to extract tannins and sodium chloride from 1-propanol and subsequent drying with anhydrous sodium sulfate prior to evaporation.

In lieu of the shortcomings of the above methods of extraction, a better efficient method of extraction has been attempted, using water extractactable nature of caffeine and its solubility in solvent and will be discussed in detail in the upcoming sections of this chapter. This method will facilitate the efficient

extraction of caffeine from various coffee processing wastes in efficient manner and used as starting material for biotransformation of caffeine to valuable compounds of therapeutic importance.

5.1.3.1. Caffeine containing wastes:

Caffeine is available in plenty in the various coffee processing wastes. These caffeine containing wastes are in various forms as discussed below:

a) Coffee pulp
b) Coffee hull
c) Spent coffee

a) Coffee pulp:

Coffee fruit on a dry weight basis contains coffee pulp 26-30% and mucilage about 5-14% (Ricardo, 1980). The mucilage is made up of pectin materials including protopectin (33%), reducing sugars including glucose and fructose (30%), non-reducing sugars such as sucrose (20%), and cellulose and ash (17%) (Wrigley, 1988). Coffee pulp contains fermentable sugars-23-27% on dry weight basis, which consists mainly of fructose 10-15%, sucrose 2.8-3.2%, galactose 1.9-2.4%, pectin-6.5% (Penaloza *et al*, 1985; Zuluaga *et al*, 1989). The protein content is around (10-13%) and it also has various mineral content as potassium, magnesium, zinc, copper, manganese (Bercelos *et al*, 2002; Elias, 1978; Zuluaga, 1975). The various inhibitory factors present are caffeine (0.7-2.2%), tannins 2.18% and chlorogenic acid (2.6%) (Bressani, 1979a). These inhibitory factors limit the utilization of the coffee pulp, as they are inhibitory to the growth of microorganisms, germination of plants, animal feed. The detailed composition of coffee pulp as shown in Table 5.1.3.1. can be used selectively and suitably as media

for growth, of those microorganisms, which have tolerance towards the inhibitory factors as caffeine and can metabolize the same leading to decaffeination of the coffee pulp. This particular attribute has been made use of, as will be discussed in the forthcoming sections: for the growth of decaffeinating fungal strains, coffee pulp as substrate for SSF substrate for the production of industrially important enzymes, for the production of mushroom using *Pleurotus*, and production of vermicompost using *Eisenia foetida*.

Table 5.1.3.1. Composition of coffee pulp.

S.No	Composition	Percentage (%) dry wt basis
1.	Fermentable sugars	23-27%
2.	Protein content	10-13%
3.	Mineral content	Potassium, magnesium, zinc, copper, manganese
4.	Caffeine	0.7-2%
5.	Tannins	2.18%
6.	Chlorogenic acid	2.6%

b) Coffee hull:

Coffee fruit contains 10-12% coffee hull on a dry weight basis and is the parchment of the coffee fruit (Ricardo, 1980). Caffeine is present in coffee hull in varying percentage of 1.1-2% on dry weight basis. However, coffee hull has high concentration of lignin, and the sugar and protein content is almost negligible (Ricardo, 1980) which restricts its use and degradation by microorganisms making it a disposed, unused agro industrial waste. In the present chapter attempts have been made towards the use of coffee hull in the production of mushroom. Besides this it has also been used for the efficient extraction of caffeine, and further using it for the biotransformation of caffeine to theophylline.

c) Spent coffee:

Spent coffee is generated during the process of instant coffee production. Production of instant coffee generates about 70% of spent coffee waste on wet basis. Ravindranath *et al* (1971) reported the composition of spent coffee as follows: carbon - 33.6 %, hydrogen - 4.2 %, nitrogen - 1.14 %, sulfur - 0.03 %, oxygen - 20.37 %. The determined ash content of spent coffee is 0.66 % and the moisture content is around 40%. The generated spent coffee is disposed by the producing industries by dumping them in the nearby agricultural fields, which is not desirable in the long run as they increase the alkalinity of the soil. In the present chapter attempt has been made towards efficient and economical use of spent coffee. They have been used as substrates for mushroom production and for the production of vermicompost as will be discussed in the upcoming sections.

5.1.4. Decaffeination of coffee processing wastes:

The coffee processing wastes contain 1-2% of caffeine which is the major inhibitory factor in these processing residues limiting the utilization of the coffee pulp and coffee hull, as they are inhibitory to the growth of microorganisms, germination of plants, unsuitable for use as animal feed and as organic fertilizer. Hence, there is a necessity to remove the anti nutritional factors from these coffee processing wastes and make them suitable for various uses. Attempt has been made in this chapter towards the complete removal of caffeine from these processing wastes, which is one of the major inhibitory factors.

5.1.5. Utilization of coffee processing wastes as substrate for solid-state fermentation (SSF):

SSF involves the growth and metabolism of microorganisms on moist, solid substrate medium in which there is no free flowing water (Mitchell and Lonsane, 1990). While the presence of moisture is necessary in SSF, it exists in an absorbed or complex form within the solid matrix. Many microorganisms are capable of growing on solid substrates but only filamentous fungi can grow to a significant extent in the absence of free water. Different agro wastes have been used successfully as SSF substrate. Coffee pulp is one of the major agro industrial wastes, which can be used efficiently as SSF substrate. The chemical composition of coffee pulp (Table 5.1.3.1.) indicates that it is an excellent media for the growth of microorganisms, which have tolerance towards the antinutritional factors as caffeine, tannin and chlorogenic acid. The abundantly available wastes can be used as SSF substrates for the growth of microorganisms producing commercially important enzymes as caffeine oxidase, pectinase, alpha amylase etc.

5.1.6 Mushroom production using coffee processing wastes:

Mushrooms are fruiting bodies of some members of fungi. The fruiting bodies, mushrooms, are fleshy spore bearing structures of the fungi. The "true" mushrooms are classified as *Basidiomycota* (also known as "club fungi"). A few mushrooms are classified by mycologists as *Ascomycota* (or "sac fungi"), the morel and truffle being good examples. There are approximately 14,000 described species of mushrooms. Mushroom cultivation presents an economically important bio-industry that has expanded all over the world in the past few decades.

Various agricultural wastes can be used as substrates for the production of mushroom (Royse, 1996; Royse and Lee, 1980; Stamets, 2000). The economic importance of mushroom lies primarily in their use as food for human consumption and the utilization of agro wastes. The exotic flavour, taste and fleshiness of mushroom have made it an important delicacy in human diet. They also serve as nutriceuticals, as "food that also cures". Nevertheless, the biological potential of mushrooms is probably far from exploited. They are high in fiber and protein, and provide vitamins such as thiamine (B_1), riboflavin (B_2), niacin (B_3), biotin (B_7), cobalamins (B_{12}) and ascorbic acid (C); minerals, including iron, selenium, potassium and phosphorus (Park, 2001). Besides this, the cholesterol and sodium content of mushroom is low, it is also known to have medicinal values and certain varieties of mushrooms can inhibit growth of cancerous tumour. The productivity of mushroom is higher than any crop.

Food, nutritional and medicinal values apart, mushroom growing can be efficient means of waste disposal (agricultural, industrial and family wastes), since it can use these wastes as medium of growth, hence, it is eco-friendly. There has been a recent upsurge of interest in mushrooms as a source of biologically active compounds of medicinal value including anti-cancer, anti-viral, immunopotentiating, and hypocholesterolaemic and hepatoprotective agents. This new class of compounds, termed 'mushroom nutraceutical', are extractable from either the fungal mycelium or fruiting body and represent an important component of the expanding mushroom biotechnology industry (Chang and Buswell, 1996). Of the various types of mushrooms presently cultivated in the world, eight are

important. These are: button, oyster, straw, shikate, woody ear, winter, silver ear and nameko. These account for 99 per cent of the total world production of mushrooms. In India only three types, namely, button, oyster and straw mushrooms are commercially cultivated. Button mushroom (*Agaricus bisporus*) accounts for 90 percent of India's production of mushrooms. About 38 percent of the total world production of mushrooms is button mushroom.

5.1.6.1. Substrates for mushroom production:

Many low-grade agricultural waste products can be used for mushroom cultivation, including the stalks of agricultural produce, corncobs, cotton shells, sugar cane segments, sugar beets, methane, industry waste from cotton mills, slaughterhouses, meat processing plants, paper factories and sewage from livestock feed lots. Cellulose and some nutrients may be added to the waste materials to foster rapid growth. In the present work coffee processing wastes as coffee pulp, coffee hull and spent coffee have been used as substrates for mushroom production which are rendered unused and disposed off because of the presence of various inhibitory factors in them.

5.1.6.2. Mushroom spawn:

The propagating material used by the mushroom growers for planting beds is called spawn. The quality of spawn is crucial for the successful mushroom cultivation. Grain spawn, manure spawn and perlite spawn are the different types of spawn preparation in use.

5.1.7. Utilization of coffee processing wastes for vermiculture production:

Vermicompost (also called Worm Compost, Vermicast, or Worm Manure) is end product of the breakdown of organic matter by special varieties of

earthworms. Chiefly, vermicomposting is a mesophilic process, utilizing microorganisms and earthworms that are active in a temperature range of 50-90° F. The earthworm species (or composting worms) most often used are Brandling worms (*Eisenia foetida*) or Redworms (*Lumbricus rubellus*). Limiting factors for vermicomposting include insufficient water supply, extremely cold weather conditions, poor quality of feedstocks, high salinity in feedstocks, poor management of worm beds, limited surface area and lack of suitable species and ready supply of earthworms to begin and continue the task. Vermicomposting in developing countries could prove to be useful in many instances. In areas where creation of low or semi-skilled jobs is considered advantageous, vermicomposting may supply an opportunity for employment. In addition to worms, a healthy vermicomposting system hosts many other organisms such as insects, molds, and bacteria. Vermiculture can greatly improve soil productivity as the earthworms process soil and organic residues through their gut - clay and organic matter are intimately mixed and coated with organic stabilizing gums and lime secreted from a special gland within the digestive tract of earthworm. The result is that the worm cast consists of just the type and size of water stable soil aggregate, which is needed to hold water while allowing the crop root hairs, to obtain sufficient air and fully exploit the nutrient and moisture reserves contained within. Vermicomposting is akin to composting in that similar feedstocks/organic residuals are used.

Both systems utilize microbial activity to break down organic matter in a moist, aerobic environment. Vermicomposting differs from thermophilic composting in several ways. It is faster, produces no odor, produces a superior

product and minimizes nutrient loss from the soil. Tables 5.1.7.1, 5.1.7.2 and 5.1.7.3. show, how vermicomposting is advantageous over the existing composting methods and the chemical composition of vermicompost (Edwards and Hohlen. 1996; Edwards, 1990; Zorba, 2001). Vermicomposting is considered faster than composting and, because the material passes through the earthworm gut, a significant but not-yet-fully-understood transformation takes place, whereby the resulting earthworm castings (worm manure) are abundant in microbial activity and plant growth regulators, and fortified with pest repellency attributes as well (Edwards, 1990). In short, earthworms, through a type of biological alchemy, are capable of transforming garbage into gold. The present chapter reports the efficient utilization of coffee processing wastes for vermicompost production leading to utilization of these wastes, value addition to the agro wastes and enhancing rural employment.

Table 5.1.7.1. Advantages of vermicompost over chemical fertilizer (Edwards and Hohlen. 1996; Edwards, 1990; Zorba, 2001).

S.No	Chemical Fertilizer	Vermicompost
1.	Expensive	Very Cheap
2.	Continuous use depletes the fertility of the soil	Increases the soil fertility
3.	Chemicals pollute the environment	Environmental friendly
4.	More water required for irrigation	Not much water required
5.	Pesticides required after us	Comparatively much less use of pesticide
6.	Taste difference noticed in crops	The natural taste is preserved

Table 5.1.7.2. Advantages of vermicomposting over general composting.

S.No	Composting	Vermicomposting
1.	Microorganisms decompose substrate	Microorganisms and earthworms combine their activities to transform the substrates.
2.	Takes a longer period to mature	Matures relatively faster than compost
3.	Thermophilic stage must be attained	No thermophilic stage is required
4.	Compost is coarser textured	Vermicompost is finer textured
5.	Risk of heavy metals in the compost	Heavy metals are removed and accumulated within worm bodies

Table 5.1.7.3. Nutrient profile of vermicompost and farm yard manure.

Nutrient	Vermicompost	Farm yard manure
N(%)	1.6	0.5
P(%)	0.7	0.2
K(%)	0.8	0.5
Ca(%)	0.5	0.9
Mg(%)	0.2	0.2
Fe(ppm)	175.0	146.5
Mn(ppm)	96.5	69.0
Zn(ppm)	24.5	14.5
Cu(ppm)	5.0	2.8
C:N ratio	15.5	31.3

5.1.7.1. Properties of vermicompost:

1. Vermicompost is rich in all essential plant nutrients.
2. It provides excellent effect on overall plant growth, encourages the growth of new shoots / leaves and improves the quality and shelf life of the produce.
3. Vermicompost is free flowing, easy to apply, handle and store and does not have bad odour.
4. It improves soil structure, texture, aeration, and water holding capacity.
5. Vermicompost is rich in beneficial micro flora such as nitrogen fixers, phosphate solubilizers, cellulose decomposing micro-flora etc.
6. Vermicompost contains earthworm cocoons and increases the population and activity of existing earthworm in the soil.
7. It prevents nutrient losses and increases the use efficiency of chemical fertilizers.
8. Vermicompost is free from pathogens, toxic elements, weed seeds etc. hence, minimizes the incidence of pest and diseases.
10. It enhances the decomposition of organic matter in soil.

11. It contains valuable vitamins, enzymes and hormones like auxins, gibberellins etc.

5.1.7.2. Advantages of vermicomposting:

- Vermicompost is an ecofriendly natural fertilizer prepared from biodegradable organic wastes and is free from chemical inputs.
- It does not have any adverse effect on soil, plant and environment.
- It improves soil aeration, texture and tilth thereby reducing soil compaction.
- It improves water retention capacity of soil because of its high organic matter content.
- It promotes better root growth and nutrient absorption.
- It improves nutrient status of soil-both macronutrients and micronutrients.

5.1.7.3. Precautions to be taken during vermicomposting:

- Vermicompost pit should be protected from direct sun light.
- To maintain moisture level, spray water on the pit as and when required.
- Protect the worms from ants, rats and birds.

5.1.7.4. Earthworm species used for vermicomposting:

1) The tiger worm *(Eisenia foetida)*:

This is the most commonly used species in commercial vermiculture and waste reduction (Haimi and Huhta, 1990). The species colonizes many organic wastes and is active in a wide temperature and moisture ranges. The worms are tough, readily handled, and survive in mixed species cultures. It is closely related to *Eisenia andrii*, another useful vermicomposting species. The species is commonly used in the U.S., Europe and Australia under the name *Lumbricus rubellus.*

2) ***Eudrilus eugeniae***:

This is a large prolific African worm that is cultured in the U.S. and elsewhere. When large worms are produced under optimum conditions, they are ideal for use as fish bait and in protein processing. It is somewhat difficult to raise

because of its intolerance to low temperature and handling. The use of *E. eugeniae* in outdoor vermiculture is limited to tropical and sub-tropical regions because it prefers warmer temperatures and cannot tolerate extended periods below 16^0C.

3) ***Perionyx excavatus***:

This is a species well adapted to vermicomposting in the tropics. The earthworm is extremely prolific and easy to handle and harvest but it cannot tolerate temperatures below 5^0C, making it more suited to the tropics.

4) ***Dendrobaena venata***:

A large worm with potential to be used in vermiculture and that can also inhabit soils. It has a slow growth rate and the least suitable species for rapid organic matter breakdown.

5) ***Polypheretima elongata***:

The species is suited for use in reduction of organic solids, municipal and slaughterhouse waste, human waste and poultry and dairy manure but it is not widely available. It is restricted to tropical regions, and may not survive temperate winters.

5.2. Materials and methods:

Materials:

Theophylline and caffeine (99.9%) were procured from Sigma chemicals, St. Louis, USA. Caffeine (LR) used for biotransformation was purchased from Loba chemicals, Mumbai, India. Solvents for extraction of caffeine were obtained from Merck, Germany. Polyvinyl alcohol was obtained from Sigma chemicals, St. Louis, USA. All other chemicals used were of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India. Coffee pulp, coffee hulls for mushroom production and vermicomposting were procured from Chamundi coffee curing works, Mysore, India. Spent coffee was obtained from Hindustan Lever Ltd., Mysore, India. Earthworm species of *Eisenia foetida* for vermicompost was obtained from Central sericulture research Institute, Mysore.

Analytical methods:

1) Thin layer chromatography (TLC) for identification of biotransformed products:

TLC was done for the identification of biotransformed caffeine using pre coated TLC plates from Merck (Germany) with a solvent system using butanol, acetic acid, water (4:1:1) and was visualized in a CAMAG UV illuminator.

2) High Performance Liquid Chromatography (HPLC) for identification of biotransformed products:

The caffeine content in the samples was determined by HPLC analysis on a RP C-18 column [5µm, 250mmx4.6mm, Phoenomenex] using LC-10A (Shimadzu, Japan) system with an isocratic mobile phase containing water and acetonitrile in

the ratio of 85:15, set at a flow rate of 1ml/min and connected to a UV detector set at 273nm.

5.2.1. Extraction of caffeine:

Caffeine was extracted from the coffee pulp and coffee hulls by different methods and the extraction efficiency of both the processes were analyzed.

1) Aqueous and solvent phase extraction:

Extraction was done using sodium carbonate and sodium bicarbonate in water as basic pH enhances the caffeine extractability. 2 gm each of sodium carbonate and sodium bicarbonate were added individually to 100 ml distilled water. 5gm each of coffee pulp, coffee hull and waste tea leaves were suspended in the above solutions and boiled for 10 min. The solid mass was removed by filtration and the liquid made up to 100ml. Extraction of caffeine was affected by addition of 100ml of chloroform to the aqueous extract (100ml) and vortexing on a rotary shaker at 200rpm for 1 hr. The chloroform layer was separated and passed through a charcoal bed to rid off the colour and contaminating compounds and vacuum concentrated to get caffeine.

2) Extraction of caffeine using soxhlet:

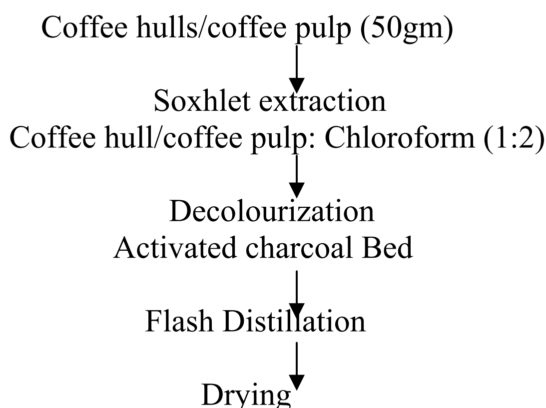
Extraction of caffeine from the coffee wastes was carried out in a soxhlet extractor by two different methods i.e the direct extraction method and pretreatment method of coffee pulp and hull.

a) Direct extraction method:

In the direct extraction method 50 gm coffee pulp/coffee hull was packed into soxhlet extractor and extracted using chloroform. The extract collected

in the solvent reservoir of the soxhlet were collected every 15 min up to 2hrs and the caffeine content of the extract were analyzed by HPLC. The chloroform layer obtained at the end of the process was vacuum concentrated.

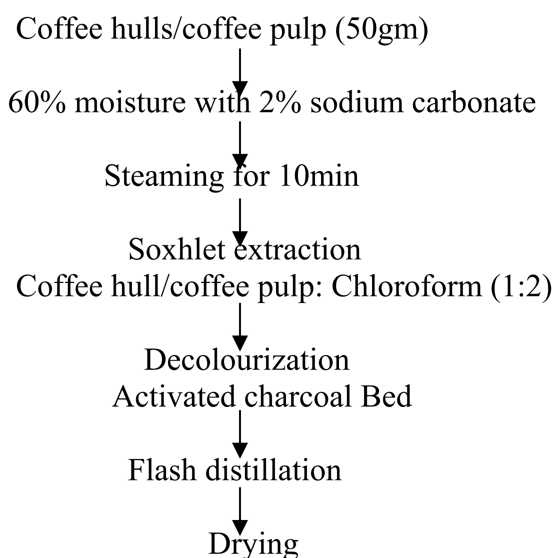
Flow chart of direct soxhlet extraction method



b) Pretreatment method:

Coffee pulp/coffee hull was treated with 2% sodium carbonate with 60% moisture and steamed for 10 mins. The steamed mass was extracted with chloroform in soxhlet as described in above method. The chloroform layer was vacuum concentrated to get the caffeine.

Flow chart of pretreatment extraction method:



5.2.2. Decaffeination of coffee processing wastes:

Coffee pulp and coffee hull were decaffeinated using *Penicillium citrinum* MTCC 5215. 20gm of the substrate was taken and to this mineral salt media was added having following composition (g\L) 0.2N HCl: $ZnSO_4$, 0.79; $FeSO_4$, 0.07; $CuSO_4$, 0.07. The inbuilt moisture content of coffee pulp and coffee hull was determined to be 9.068% and 7.4% respectively. The final moisture content was made up to 65%. It was sterilized for 1hr at 121^oC, 15 psi pressure, inoculated with spores of *P. citrinum* and incubated at 28^oC. After fermentation the samples were autoclaved and extracted with 100ml of ethyl acetate. The ethyl acetate extract was vacuum concentrated and the crude extract obtained was checked for decaffeination using TLC and HPLC.

5.2.3. Production of enzymes by solid-state fermentation (SSF) using coffee pulp as substrate:

a) Isolation of caffeine tolerant microorganisms:

Isolation of caffeine metabolizing fungal strains were carried out as explained in section 2.2.1 of Chapter-2.

b) Screening of isolated cultures for caffeine biotransformation:

The isolated fungal strains were screened for their caffeine biotransformation ability in liquid culture media containing caffeine (discussed in section 2.2.2 of Chapter-2).

c) Enzyme production through Solid-state fermentation:

Coffee pulp was used as substrates for solid-state fermentation. 30 grams each of coffee pulp and wheat bran were taken individually and mixed with 30 ml of mineral salt media containing the following components in 0.2N HCl (g/L): $ZnSO_4$, 0.79; $FeSO_4$, 0.07; $CuSO_4$, 0.07. A spore suspension ($5x10^{-5}$ spores/ml)

was prepared by addition of 5 ml of 0.1% v/v of Tween 20. This spore suspension was then inoculated in the substrate, which was previously sterilized for 1 hour at 121^0C and 15 psi pressure. The inoculated flasks were incubated at 28^OC for 96 hours. After fermentation, extraction of enzyme was done in 100ml of phosphate buffer (0.1M), pH-7.2, at 150 rpm for 2 hours. Biomass was filtered from the enzyme extract using muslin cloth and the filtrate centrifuged at 6000g for 10 min. The supernatant was used for the assay of various enzymes.

d) Enzyme assay:

1. Amyloglucosidase:

Amyloglucosidase activity in the extract was determined by the method reported by Bergmeyer and Veinheim (1984). The enzyme extract was added to test tubes at desired level (0.1 to 0.5ml) and allowed to react with 5ml of 4% starch solution for 60 min at 60° C. For blank, 0.2 ml-distilled water was used. The reaction was stopped by addition of 0.8 ml of 4 N NaOH. Dextrose released was estimated by Dinitrosalicylic Acid Method (DNS) (Miller, 1959). One unit of enzyme is defined as the amount of enzyme which reduces reducing carbohydrate equivalent to 1μM glucose from soluble starch in 1min at 60^oC at pH 4.5.

2. α-Amylase:

α-Amylase activity in the extract was determined by the method of Smith and Roe (1949). In brief, starch solution was incubated with the enzyme extract and then incubated at 60^oC for 10 minutes to hydrolyse the starch. A substrate blank was also prepared by incubating distilled water with enzyme. The reaction was then arrested by adding 2 ml of 1N HCl to each tube. A reagent blank

was prepared with 0.5ml 1N HCl with 50ml-distilled water in a volumetric flask. 0.1ml iodine solution was then added to reagent blank, substrate blank and the sample flask. The volume was made up to 50ml and mixed. The absorbance of substrate blank and sample was recorded at 620 nm against reagent blank. One unit of amylase activity is defined as the amount of enzyme required to hydrolyze one milligram of starch per minute under the experimental conditions mentioned above.

$$\text{Activity} = \frac{Ab_r - Ab_s \text{ x Dilution factor}}{Ab_r \text{ x } 10}$$

Ab_r = Absorbance of substrate blank against reagent blank

Ab_s = Absorbance of hydrolyzed starch sample against reagent blank.

50 = Milligram starch present initially in the substrate used for assay

10 = Time of hydrolysis in min.

3. Protease:

Protease activity in the extract was determined by Turbidometric method of Ennis (1957). Casein (0.1% w/v) was prepared in distilled water. Aliquots of 1.0 ml of this solution were taken and to this 1 ml each of the enzyme extract was added and incubated at 40^0C for 1 hour and the reaction was arrested by addition of a drop of glacial acetic acid. A control was also prepared by adding 1 ml of buffer instead of the enzyme extract. Potassium ferrocynanide solution (0.75%, 4.0 ml) was added to each tube, mixed well and a drop of glacial acetic acid was added to each test tube and mixed vigorously to arrest the reaction. The solutions were then incubated for 10 min and the absorbance was recorded at 600 nm. One unit of protease activity is defined as the conversion of 1μgm of protein to hydrolystae per minute.

4. Caffeine oxidase :

Caffeine oxidase (1,3,7 trimethyl xanthine oxidase) assay was done according to the method of Madhyastha *et al*., (1999). The enzyme activity was monitored at room temperature using phenazine ethosulphate (PES) to couple electron transport from the reduced enzyme to dichlorophenol indophenol (DCPIP). The reaction was initiated by the addition of caffeine. The reduction of DCPIP was followed spectrophotometrically at 600nm, which is the measure of the enzyme activity. One unit of enzyme is defined as the amount of enzyme which forms 1μM of 1,3,7 trimethyl uric acid from caffeine in 1min at 30°C at pH 7.2.

5. Pectinase (polygalacturonase):

Pectinase assay was carried out according to the method of Peter and Ursula (1962). 2ml of polygalacturonic acid substrate (pectin) along with 100 μl enzyme extract was equilibrated to 50°C for 10 min, 100 μl of the reaction mixture was drawn and mixed with freshly prepared 1 ml alkaline copper sulphate solution and 900 μl of water and incubated in a boiling water bath or 20 min. Galacturonic acid formed was estimated by recording the absorbance at 540nm against enzyme and substrate blanks after developing the reaction with 1 ml of arsenal molybdate reagent. One unit of activity is defined as the amount of enzyme which will release 1μM of galactouronic acid from polygalactouronic acid per min at pH 4 and 50°C.

5.2.4. Production of mushroom using coffee processing wastes:

Coffee pulp, coffee hull and spent coffee were used as substrates for mushroom production and the results were compared with rice straw as the control substrate.

a) Culture maintenance:

Monocultures of *Pleurotus florida* were maintained on malt extract (3%) agar (2%) medium (MEA).

b) Production of oyster mushroom spawn:

Pure culture of *P. florida* was maintained on maltose agar media and a 7 day old culture and was used for spawn preparation. Jowar grains (*Sorghum vulgare*) were boiled for 15 min in water and then allowed to soak for another 15 min without heating. The excess water was drained off and the grains are collected in sieves and cooled. The cooled grains were mixed with 2% calcium carbonate. 200gms of prepared grains were filled into polypropylene bags and autoclaved for 2 hr at 121°C. The pH of the material after sterilization should be in the range of 6.5 to 6.7. The bags were inoculated with grain spawn or with bits of agar medium colonized with mycelium and incubated at 22-24°C in a dark place. The mycelium is allowed to completely permeate the grains. 20 day old grain spawn was used for inoculating the mushroom bed substrate (Rajrathnam and Zakia, 1987).

c) Production of oyster mushroom:

Rice straw, coffee pulp, coffee hull and spent coffee were used as substrates, individually and after amendment with 25% rice straw. The substrates were pasteurized for 20 mins at 80°C to ensure that it was free from contamination (Rajrathnam, *et al*, 1979). Water was drained out of the substrate and was cooled to room temperature. Wet straw containing 75% water was spawned (10% spawn on weight basis) along with 1% coarse horsegram powder (mill size ca. 0.5-1.0mm). The mushroom beds were prepared in perforated polyethylene (50µm) bags (25x40

cm). For each treatment fixed weight of substrate was filled into the bags in replicates of 4 and allotted in randomized blocks (Zakia, *et al*, 1993).

d) Mushroom growth and yield:

The polyethylene bags of the mushroom beds were cut open when the fruiting primordial started forming in the perforations of the polyethylene. The exposed substrate blocks were watered to maintain required moisture content. The mushrooms were harvested on the third day of formation of the fruiting primordia. The yield was expressed as % bioconversion efficiency (BCE), grams fresh mushrooms obtained from per 100g initial dry substrate.

e) Mushroom powder preparation:

100gm of mushroom was weighed, sliced into thin pieces and dried in a hot air oven maintained at 45°C till constant weight was obtained. The dried mushroom slices were ground in a mixer cum grinder to a fine powder.

f) Preparation of mushroom powder extract:

1 gm of powdered mushroom was soaked in 5ml of 70% ethanol and incubated on a rotary shaker overnight. The supernatant was collected after centrifugation at 5000 rpm and the extraction process was repeated. The supernatants were pooled together and vacuum concentrated till the final volume was 5ml. This extract was used for further analysis for protein content, free amino acid and total phenolics.

g) Estimation of protein content in the mushroom fruiting bodies:

Total nitrogen content of the mushroom was estimated in the mushroom powder extract by the micro-Kjeldahl method (AOAC 1975). A conversion factor of 4.38 was used for the protein content estimation.

h) Estimation of free amino acid:

Free amino acid in the mushroom powder extract was estimated by ninhydrin method (Yemm *et al*, 1955).

i) Estimation of total phenolics:

The total phenolics estimation in the mushroom powder extract was carried out by Folin-Ciocalteau method (Singleton and Rossi, 1965; Singleton *et al*, 1999)

j) Estimation of caffeine content in fruiting bodies:

Caffeine content in the fruiting bodies was checked by extracting 10gm (wet weight) of mushroom with 10% polyvinyl alcohol (PVA) in 100ml phosphate buffer, (0.1M, pH 7.2). The ground mass was centrifuged at 6,000 g for 20 mins. The caffeine content in supernatant was estimated by HPLC (Sarath *et al*, 2005).

k) Caffeine content in the substrate:

Caffeine content in the matrix was checked by boiling 10gm substrate (at 0hr and at end of the experiment) in 100ml water for 10 mins and then filtering through muslin cloth to separate the solid mass. The filtered extract was centrifuged at 6,000g for 10 min and the supernatant was checked for caffeine content using TLC and HPLC (Sarath *et al*, 2005).

l) Caffeine degradation by *Pleurotus* in shake flask:

Caffeine degradation by *Pleurotus* was checked by inoculating 7 day old cultures in a medium containing the following constituents (g/L) **-** Na_2HPO_4 - 0.12; KH_2PO_4 - 1.3; $MgSO_4$ - 0.3; $CaCl_2$ - 0.3; caffeine – 1; sucrose – 20. The inoculated media was incubated on rotary shaker at 150 rpm and at 28°C. Samples were collected every 24 hours and analyzed for caffeine degradation by HPLC.

5.2.5. Use of coffee processing wastes for vermiculture:

3 kg each of cow dung, coffee pulp and spent coffee individually and combinations of the substrates were prepared and beds of these substrates were spread homogeneously in cardboard boxes measuring 30x20x20 cm with polythene sheets spread at the bottom. 50 worms (*E. foetida*) were introduced into each box and the bed was covered with coarsely cut grass to avoid penetration of light and loss of moisture. Temperature was maintained at 30°C and moisture was maintained by regular spraying of water. The experiment was terminated after 30 days and the worms were harvested. Final count of the worms was taken and the vermicompost was analyzed further. The vermicompost produced was analyzed for NPK content, organic carbon, pH and electrical conductivity.

Estimation of caffeine content in vermicompost:

Caffeine content in the vermicompost was checked by boiling 10gm of substrate (initial substrate and spent substrate) in 100ml water for 10 mins and then filtering through muslin cloth. The filtered liquid was centrifuged at 6,000g for 10 min and the supernatant was checked for caffeine content by HPLC (Sarath *et al*, 2005).

5.3. Results and Discussion:

5.3.1. Extraction of caffeine:

Aqueous and solvent phase extraction:

Extraction of caffeine was done from coffee pulp, coffee hull and waste tea leaves using sodium carbonate and the yield of caffeine was 1.512, 1.228 and 1.231 % w/w respectively (Table 5.3.1). The corresponding experiment was carried out with sodium bicarbonate and the extraction of caffeine was 1.020, 0.898 and 1.312% respectively in coffee pulp, coffee hull and waste tea leaves. Aqueous solution of sodium carbonate effects higher alkalinity leading to a better extraction of caffeine as seen from the results obtained (Table 5.3.1). It is also known that caffeine has the highest solubility in chloroform (12.5% w/v) and caffeine was more selectively separated from the rest of the components present in the aqueous extract when chloroform was used (Radionov, 1926).

Table 5.3.1. Extraction of caffeine using sodium carbonate and bicarbonate.

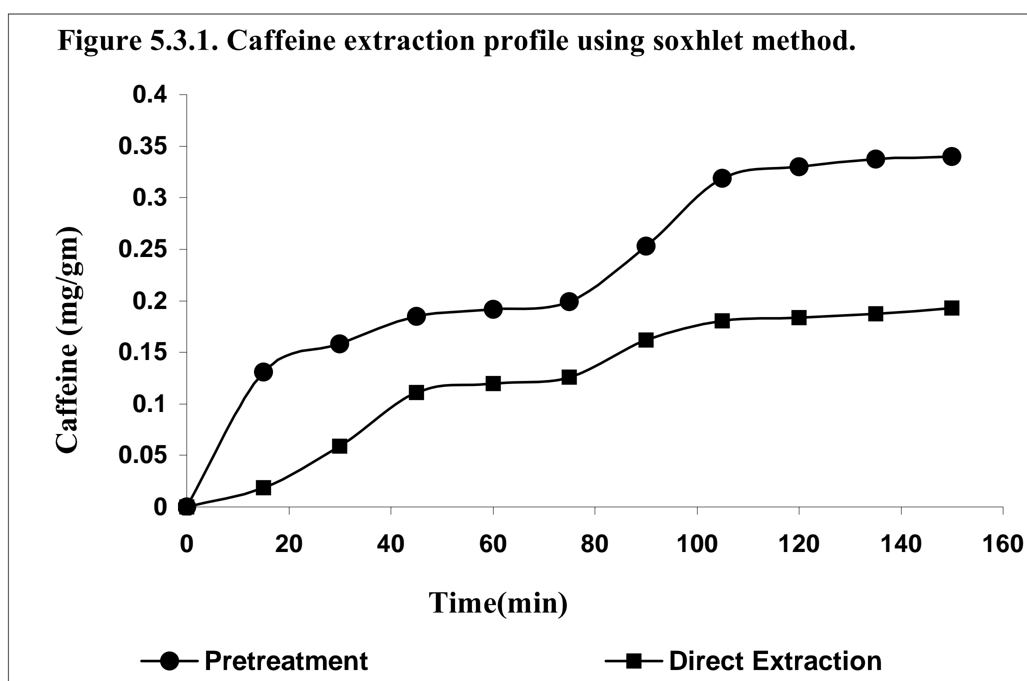
S.No	Salt used	Substrate used for extraction	Yield (% w/w)	% Caffeine in substrate
1.	Sodium carbonate	Coffee pulp	1.512	1-2
2.		Coffee hull	1.228	1.2-2.4
3.		Waste tea leaves	1.231	2-3.5
4.	Sodium bicarbonate	Coffee pulp	1.020	1-2
5.		Coffee hull	0.898	1.2-2.4
6.		Waste tea leaves	1.382	2-3.5

Extraction of caffeine by soxhlet apparatus:

Figure 5.3.1. represents the effect of pretreatment on extraction of caffeine. The extraction rate of the caffeine from coffee pulp was almost twice when the matrices were pretreated with sodium carbonate rather than extracted

directly. It is because of the fact that caffeine dissociates from the complexes such as tannin-caffeine complexes in the alkaline medium, facilitating efficient extraction of the caffeine from the material into the solvent. However, the extraction of caffeine is only 0.34% of the substrate using this method.

Figure 5.3.1. Caffeine extraction profile using soxhlet method.



The results obtained from both the methods of extraction suggest that aqueous and solvent phase extraction is a better extraction method as compared to the soxhlet extraction method where the yield is comparatively low. This can be attributed to the fact that most of the caffeine leaches out into the hot water during boiling. Further, when it is extracted with chloroform, most of the caffeine is extracted into the solvent, as caffeine is highly soluble in chloroform (12.5% solubility) (Radionov, 1926). The plethora of coffee processing wastes can be used for efficient extraction of caffeine using the aqueous and solvent phase sequential

extraction method. The extracted caffeine can further be used for biotransformation of caffeine to valuable therauptic methylxanthies as theophylline.

5.3.2. Decaffeination of coffee processing wastes:

Caffeine is one of the active antiphysiological factors in coffee pulp (Molina *et al*, 1974). Most of the methods for the decaffeination of coffee processing wastes involve either physical or chemical treatments and are characterized either as inefficient in eliminating the toxicity or too expensive in nature (Roussos *et al*, 1994). Few preliminary reports are available on the detoxification of coffee pulp by SSF process (Aquiahuatl *et al*, 1988; Gaime *et al*, 1993; Penaloza *et al*, 1985). Nippon, (1971) studied the decaffeination of coffee pulp by *Aspergillus* but the strain could not decaffeinate coffee pulp completely. Yano and Mazzafera (1996) used a *P. putida* strain to study decaffeination of coffee pulp.

Different proportions of inoculum and pulp were incubated during 30 days, resulting in a reduction of caffeine up to 80%. At shorter incubation periods (9 days) a 40% reduction in caffeine content was observed and time taken for decaffeinating the coffee pulp was too long. Besides, complete decaffeination was not observed using the microbial strains. Hakil *et al*, (1999) studied caffeine degradation in SSF by *Aspergillus tamarii* and concluded that there was a need of external nitrogen source to enhance caffeine degradation in coffee pulp.

Results obtained from present studies and shown in Table 5.3.2. shows that *P. citrinum* MTCC 5215 could grow on coffee pulp and decaffeinate it completely within 72 hours. Decaffeination of coffee pulp using *P. citrinum* MTCC 5215 is advantageous over other methods of physical and chemical decaffeination, as it

decaffeinates coffee pulp completely in 72 hrs, is economical and environment friendly. However *P. citrinum* MTCC 5215 could not decaffeinate coffee hull. The probable reason for this could be that high lignocellulose content, negligible sugar and protein content did not allow the fungal strain to grow on the substrate and further detoxify it. The decaffeinated coffee wastes can further be utilized for the production of valuable enzymes, and nutraceuticals like polyphenols, or for cattle feed as the substrates get enriched with protein and free amino acids due to the growth and metabolism of the microorganisms on the substrate.

Table 5.3.2. Decaffeination of coffee processing wastes by *P. citrinum* MTCC 5215.

S. No	Time (Hrs)	Decaffeination of coffee pulp (%)	Decaffeination of coffee hull (%)
1.	0	0.0	No decaffeination
2.	24	10.7	No decaffeination
3.	48	61.2	No decaffeination
4.	72	99.7	No decaffeination

5.3.3. Production of enzyme using coffee processing wastes:

Production of the enzyme was carried out as described in section 5.2.3. The extract of the fungi grown on coffee pulp and wheat bran was checked for the production of different enzymes viz; amyloglucosidase, αamylase, protease, caffeine oxidase and pectinase and the results have been presented in Table 5.3.3.

1. Amyloglucosidase:

A strain of *Aspergillus niger* was used for the production of amyloglucosidase in SSF and the study included screening of a number of agro-industrial residues including wheat bran, rice bran, rice husk, gram flour, wheat flour, corn flour, tea waste, copra waste, etc., individually and in various

combinations (Pandey, 1990; Pandey *et al*, 1994). However, coffee pulp has not been tried as SSF substrate for the production of amyloglucosidase enzyme. In the present work two of the isolates F8 (*Aspergillus*) and F10 (Yeast) could produce amyloglucosidase in considerably good amount using coffee pulp as substrate for SSF. Isolates F8 (*Aspergillus*) produced 4236.6 U/gm susbstrate and F10 (unidentified) produced 8166.6 U/gm substrate of amyloglucosidase using coffee pulp as substrate (Table 5.3.3). Isolate F10 (unidentified) produced highest amount of amyloglucosidase (8166.6 U/gm substrate) only in coffee pulp but not in wheat bran. Hence, this strain F10 can prove as a promising strain for the production of amyloglucosidase using coffee pulp as the substrate for SSF. The ability of strain F8 and F10 to grow in coffee pulp is because of their ability to detoxify the caffeine content in coffee pulp as these two strains could degrade caffeine in shake flask as shown in section 2.3.2 of chapter-2 (F8 showed 28% caffeine degradation and F10 showed 50% caffeine degradation). After detoxification of the caffeine in coffee pulp these two strains were able to utilize the high amount of fermentable sugars present in coffee pulp (Table 5.1.3.1). Similar results were also reported by Penaloza (1985) and Zuluaga (1989). F8 also proved to be an efficient strain using wheat bran as substrate for SSF and produced 9133.3 U/gm substrate of enzyme activity. Most of the isolates produced the enzyme in larger quantities using wheat bran as substrate as shown in Table 5.3.3. Isolates F2 (*Rhizopous*), F5 (Unidentified), F8 (*Aspergillus*), F9 (*Aspergillus*) and F11 (*Aspergillus*) produced more enzyme in wheat bran as the substrate. F9, a strain of *Aspergillus* produced

the highest amount of enzyme (21500.0 u/gm substrate) with wheat bran as substrate.

2. α-Amylase:

Comparative studies on α-amylase production using different substrates have been carried out (Shah *et al*, 1991; Shankaranand *et al*, 1992; Sudo, 1994) but coffee pulp as substrate of SSF for production of α-amylase has not been reported. In the present work α-amylase could be produced successfully by using coffee pulp as SSF substrate. All the isolates exhibited α-Amylase activity in coffee pulp. However, all the isolates could produce the enzyme in wheat bran also. Amylase activity in the extracts of isolates F4 (*Rhizopous*), F5 (Unidentified fungal strain), F6 (*Penicillium*), F7 (*Aspergillus*), F8(*Aspergillus*), and F9 (*Aspergillus*), was slightly higher in coffee pulp (F4 - 1489.6 U/gm substrate , F5 - 1488.6 U/gm substrate, F6 - 1554. U/gm substrate, F7 - 1412.7 U/gm substrate, F8 -1534.7 U/gm substrate, F9 - 1500.0 U/gm substrate) than in wheat bran, as shown in Table 5.3.3, whereas F11 a strain of *Aspergillus* produced more enzyme with wheat bran as substrate. These results indicate that coffee pulp can also be used as an efficient solid substrate for the production of α-amylase with caffeine metabolizing isolates.

3. Protease:

Interestingly it was observed that the protease production was much higher using coffee pulp as substrate than with wheat bran as substrate (results shown in Table 5.3.3). The enzyme activity of isolate F3 (*Penicillium*) showed highest protease activity 85.3U/gm susstrate in coffee pulp, whereas the highest amount of protease produced with wheat bran as substrate was 61.3 U/gm substrate by F6

(*Penicillium*). Coffee pulp has high protein content of 10-13% (Bercelos *et al*, 2002; Elias, 1978; Zuluaga, 1975). The high protein content in coffee pulp helps in the growth of the fungal strains and further attributes towards the production of protease efficiently than the wheat bran.

4. Caffeine oxidase (COD):

Caffeine oxidase is reported to be produced by a consortium of bacteria of the sps. *Klebsiella* and *Rhodococcus* (Madhyastha *et.al*., 1999). This enzyme was found to be highly inducible requiring the presence of caffeine in the medium for its expression. The production of this enzyme by fungi is hitherto unknown. The fungal isolates F7 (*Aspergillus*), F9 (*Aspergillus*), F10 (unidentified) and F11 (*Aspergillus*) isolated in our laboratory produced caffeine oxidase when grown on coffee pulp as substrate (Table 5.3.3.). Among the caffeine oxidase producers, the enzyme extract from the isolate F9 a strain of *Aspergillus* showed the highest activity (377.6U/gm substrate). None of the isolates produced caffeine oxidase when they were grown in wheat bran as a substrate (Table 5.3.3.) exhibiting the highly inducible nature of the enzyme. Production of caffeine oxidase is a viable alternative from the viewpoint of the commercial production of caffeine oxidase, which has potential application in the biotechnological decaffeination processes.

5. Pectinase (Polygalacturonase):

Production of pectinase using coffee pulp as substrate for SSF has been reported by Boccas *et al*, 1994; Antier *et al*, 1993a; Antier *et al*, 1993b. All the fungi produced good amounts of pectinase enzyme (Table 5.3.3). Isolates F2 (*Rhizopous*), F6 *Penicillium*, F7 (*Aspergillus*), F9 (*Aspergillus*) and F10

(Unidentified), produced more units of pectinase in coffee pulp compared to wheat bran as substrate. Highest activity of 9266.6 U/gm substrate was produced by F10 an unidentified fungal isolate. Coffee pulp contains 6.5% of pectin (Favela, 1989; Penaloza, 1985; Philip *et al*, 1993) and the high pectin content explains the high pectinase activities of the fungal isolates compared to wheat bran as substrate. The fungal strains F2, F6, F7, F9 and F10 also have exhibited high caffeine degradation capability (as shown is Chapter - 2) and it appears that pectinase production by fungal strains is dependent on their ability to degrade the caffeine. The results obtained in this study suggest that strains having caffeine tolerance can be utilized for the efficient production of pectinase using coffee pulp as substrate.

Table 5.3.3 Production of enzymes by the fungal isolates in SSF using coffee pulp and wheat bran as substrates.

Fungal Strain	Enzyme Activity (U/gm solid substrate)									
	Amylo glucosidase		α-Amylase		Protease		Caffeine oxidase		Pectinase	
	Coffee pulp	Wheat bran	Coffee pulp	Wheat bran	Coffee pulp	Wheat bran	Coffee pulp	Wheat bran	Coffee pulp	Wheat bran
F1	0	0	167.7	1089.3	0	7.0	0	0	520.0	2263.3
F2	220.0	8766.7	1427.0	1455.0	70.7	4.0	0	0	8233.3	3966.6
F3	346.6	3200.0	1416.3	1419.3	**85.3**	23	0	0	6266.6	7566.6
F4	220.0	0	1489.6	1265.3	72.3	3.7	0	0	5833.3	3433.3
F5	126.6	10933.3	1488.6	1434.0	59.6	11.0	0	0	7566.6	**13333.3**
F6	347.0	0	**1554.3**	1284.0	61.7	37.0	0	0	8000	4533.3
F7	340.0	12333.3	1412.7	1392.0	75.3	12.6	344.3	0	6233.3	4900.0
F8	4236.6	9133.3	1534.7	1429.6	6.0	0.0	0	0	6933.3	7266.6
F9	220.0	**21500.0**	1500.0	1428.0	31.7	11.6	**377.6**	0	9066.6	7500.0
F10	**8166.6**	0	1314.6	1317.7	1.7	3.6	289	0	**9266.6**	5100.0
F11	63.3	7943.3	1085.3	1323.0	6.3	5.6	200	0	600.0	7366.6
F12	6.7	1403.3	70.0	523.0	0	7.0	0	0	410.0	3026.6
F13	36.7	1040.0	56.0	670.0	0	6.3	0	0	326.6	4123.3
F14	0	1703.3	250	412.0	2.3	4.6	0	0	156.6	593.3
F15	50.0	1410.0	212.7	129.7	3.0	3.0	0	0	520.0	4736.6
F16	106.7	0	159.6	254.3	3.7	2.3	0	0	253.3	1793.3
F17	40.0	0	365.6	375.3	9.0	0	82	0	230.0	1253.3

F18	150.0	770.0	508.0	158.0	12.0	7.6	39.6	0	456.6	2026.6
F19	130.0	1003.3	145.7	188.7	32.6	5.0	0	0	670.0	893.3
F20	260.0	430.0	196.3	261.3	9.0	0	0	0	314.0	1153.3
F21	186.7	1080.0	258	491.3	23.6	0	0	0	450.0	2093.3
F22	100.0	1806.6	708.3	418.7	6.3	5.3	19.6	0	650.0	910.0
F23	76.7	0	219.3	712.0	9.2	5.6	0	0	490.0	2786.6
F24	46.7	780.0	152.3	126.3	5.0	6.3	0	0	196.6	886.6
F25	23.3	0	437.3	202.0	18.0	7.6	16	0	580.0	2620.0
F26	40.0	0	129.7	259.6	21.0	4.6	0	0	226.6	820.0
F27	103.3	2180.0	89.7	132.7	9.7	0	0	0	140.0	2123.3
F28	210.0	1930.0	486.3	424.7	37.3	12.6	30.6	0	380.0	5360.0
F29	133.3	200.0	183.0	156.0	2.0	1.3	0	0	153.3	780.0
F30	190.0	660.0	391.3	91.3	26.3	2.0	0	0	123.3	2080.0
F31	56.6	0	79.0	704.3	15.6	0	0	0	93.3	806.6
F32	103.3	0	421.0	169.7	12.3	1.3	22.3	0	323.3	1040.0
F33	173.3	1506.6	157.3	296.7	19.6	5.3	0	0	136.6	1383.3
F34	66.7	0	226.3	184.0	5.3	9.0	0	0	420.0	586.6

5.3.4. Production of mushroom using coffee processing wastes:

Table 5.3.4.1 represents the yields of mushroom produced by using different coffee processing wastes as substrates. It was observed that coffee pulp gives maximum yield of mushroom and maximum bioconversion efficiency (BCE) of 99.54 whereas *Pleurotus* when grown on coffee pulp amended with 25% rice straw shows 92.54 BCE, this is much higher as compared to BCE obtained from rice straw which is 80.46. Hence, proving that coffee pulp in an unamended form is a better substrate for the production of *Pleurotus*. In both the cases the BCE is better as compared to rice straw substrate. This may be attributed to the rich chemical composition of coffee pulp as (Table 5.1.3). The high concentration of sugar, crude protein and minerals in the coffee pulp helps in the mycelial ramification of *Pleurotus* and in further growth and development of fruiting bodies. Besides this coffee pulp is a good substrate in terms of water holding capacity as evident from the swelling ratio of 1:4. Figure 5.3.4.1 shows photograph of *Pleurotus* growing on

coffee pulp as substrate. Martinez (1987) used coffee pulp for the production of mushroom but observed low BCE. Dulce *et al* (2005) studied the comparative culturing of six strains of *Pleurotus* on coffee pulp and wheat straw to compare the biomass production and substrate degradation. However he reported that the strains of *Pleurotus* used gave better BCE in rice straw as compared to coffee pulp and also accumulation of caffeine was observed in the fruiting bodies. Hence the mushroom produced by this method is not desirable for consumption.

Using coffee hull as substrate *Pleurotus* could be grown successfully with BCE of 35.65 and spawn run of 10 days. Figure 5.3.4.2. shows *Pleurotus* growing on coffee hull as substrate. In both the cases the BCE is less than that obtained in rice straw. The results in Table 5.3.4.1 show that with coffee hull as the substrate the yield of mushroom was obtained in very short period of time. When the coffee hull was amended with 25% rice straw the BCE increased to 52.17 and spawn run of 11 days. This result suggests that coffee hull amended with 25% rice straw shows good conversion efficiency with short spawn run. However since the water holding capacity of coffee hull is very less (swelling ratio of 1:1), it gets dried up fast and has to be watered frequently to maintain the desired moisture level.

Spent coffee used as substrate was given two types of pretreatment i.e. autoclaving and pasteurization. However the pasteurization method leads to water logging in the substrate. Autoclaved spent coffee substrate showed BCE of 69.17 whereas by the same pretreatment method spent coffee amended with 25% rice straw gave BCE of 86.087, which is better than rice straw. Pasteurized spent coffee

gave BCE of 81.50. Figure 5.3.4.3 shows photograph of *Pleurotus* growing on spent coffee as substrate.

Table 5.3.4.2 shows that mushroom produced in coffee hull substrate has maximum protein content of 25.84% and also maximum free amino acid content of 0.46%. The protein and amino acid content is higher than the mushroom produced in rice straw. Mushrrom produced in unamended coffee pulp showed protein content of 15.778% and free amino acid content of 0.318%. Mushroom produced from pasteurized spent coffee showed protein content of 19.145% and free amino acid content of 0.333%. Autoclaved spent coffee showed protein content of 18.314% and free amino acid content of 0.317%. The protein content and the amino acid content in all the coffee processing waste substrates were higher as compared to rice straw substrate (Table 5.3.4.2). Hence, the qualities of mushroom produced in coffee processing wastes have better nutritive value.

Table 5.3.4.2 shows that the total phenolics content of mushroom produced in coffee pulp and amended coffee pulp is 0.584 and 0.592% w/w respectively. The total phenolics content of coffee pulp is 8% on a dry weight basis. However the total phenolics content in the fruiting bodies was recorded to be low suggesting that there is no accumulation of the phenolics components in the fruiting bodies. High concentration of phenolics is not desirable as they would lead to unavailability of protein due to formation of protein-polyphenol complexes. Besides this there was no caffeine accumulation in the fruiting bodies as shown in Table 5.3.4.3. Dulce *et al* (2005) had studied the comparative culturing of *Pleurotus* sp. on coffee pulp and

wheat straw but their results show that there was caffeine accumulation in the fruiting bodies hence not advisable for consumption.

It was also found from the results that the substrates were decaffeinated during the course of mushroom production. Besides this experiments carried out also shows that *Pleurotus* is able to degrade caffeine in liquid culture at the end of 20 days proving the caffeine degrading ability of *Pleurotus*.

Table 5.3.4.1.Production of mushroom using coffee processing wastes.

S.No	Type of coffee waste used and amendmends	Pretreatment	Spawn run (Days)	Yield (in gms) fresh weight	Bioconversion efficiency (BCE)
1.	Coffee Pulp	Pasteurized	15	657	99.545
2.	Coffee pulp (75%) + Rice Straw (25%)	Pasteurized	15	649	92.540
3.	Coffee Hull	Pasteurized	10	410	35.650
4.	Coffee hull (75%) + Rice Straw (25%)	Pasteurized	11	525	52.174
5.	Spent Coffee	Sterilization (Autoclaved)	15	415	69.167
6.	Spent Coffee (75%) + Rice Straw (25%)	Sterilization (Autoclaved)	15	495	86.087
7.	Spent Coffee	Pasteurized	17	326	81.50
8.	Rice Straw	Pasteurized	14	490	80.46

Table 5.3.4.2. Chemical analysis of mushroom produced.

S.No	Type of coffee waste used and amendmends	Pre-Treatment	Total protein content	Free amino acid content (% dry wt)	Total Phenolics content (% dry wt)
1.	Coffee Pulp	Pasteurized	15.778	0.318	0.584
2.	Coffee pulp (75%) + Rice Straw (25%)	Pasteurized	16.821	0.295	0.592
3.	Coffee Hull	Pasteurized	25.838	0.457	0.858
4.	Coffee hull (75%) + Rice Straw (25%)	Pasteurized	22.201	0.423	0.719
5.	Spent Coffee	Sterilization (Autoclaved)	18.314	0.317	0.581
6.	Spent Coffee (75%) + Rice Straw (25%)	Sterilization (Autoclaved)	19.041	0.405	0.624
7.	Spent Coffee	Pasteurized	19.145	0.333	0.591
8.	Rice Straw	Pasteurized	17.357	0.260	0.581

Table 5.3.4.3. Caffeine concentration in substrates and mushroom fruiting bodies.

S.No	Type of coffee waste used and amendmends	Initial caffeine content in substrate (mg/ml)	Final caffeine content in substrate (mg/ml)	Caffeine content in fruiting bodies (mg/ml)
1.	Coffee Pulp	11.2	0.31	-
2.	Coffee pulp (75%) + Rice Straw (25%)	9.7	0.26	-
3.	Coffee Hull	14.5	5.2	-
4.	Coffee hull (75%) + Rice Straw (25%)	12.6	3.7	-
5.	Spent Coffee	7.9	0.22	-
6.	Spent Coffee (75%) + Rice Straw (25%)	7.0	0.31	-

Figure: 5.3.4.1. ***P. florida*** **growing on coffee pulp.**

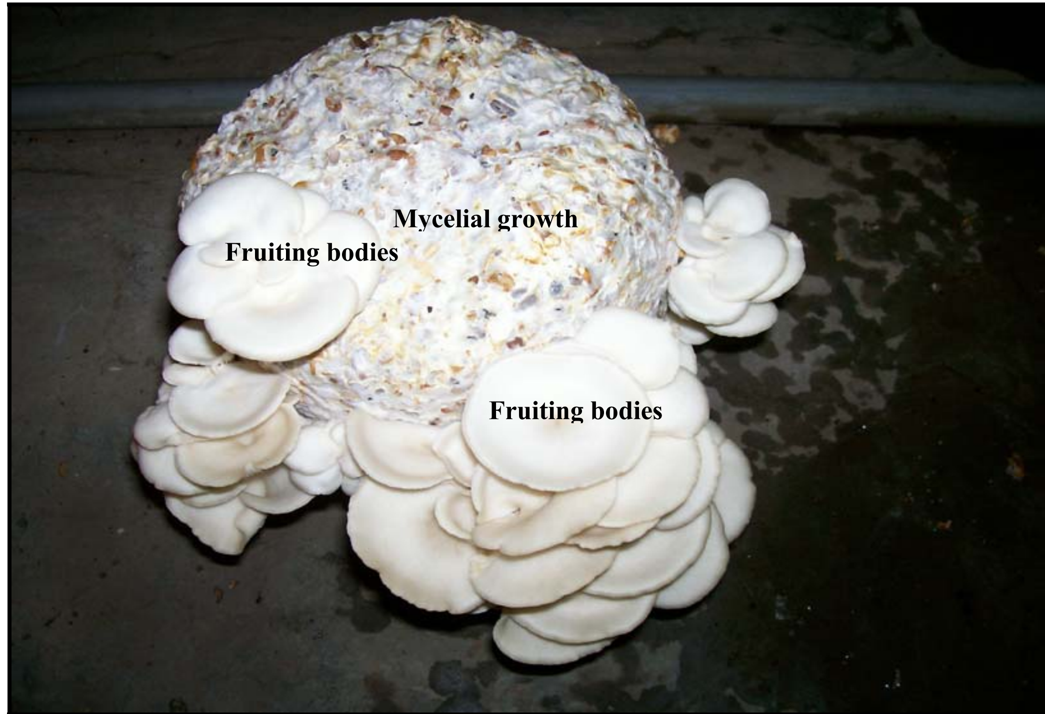


Figure: 5.3.4.2. ***P. florida*** **growing on coffee hull.**

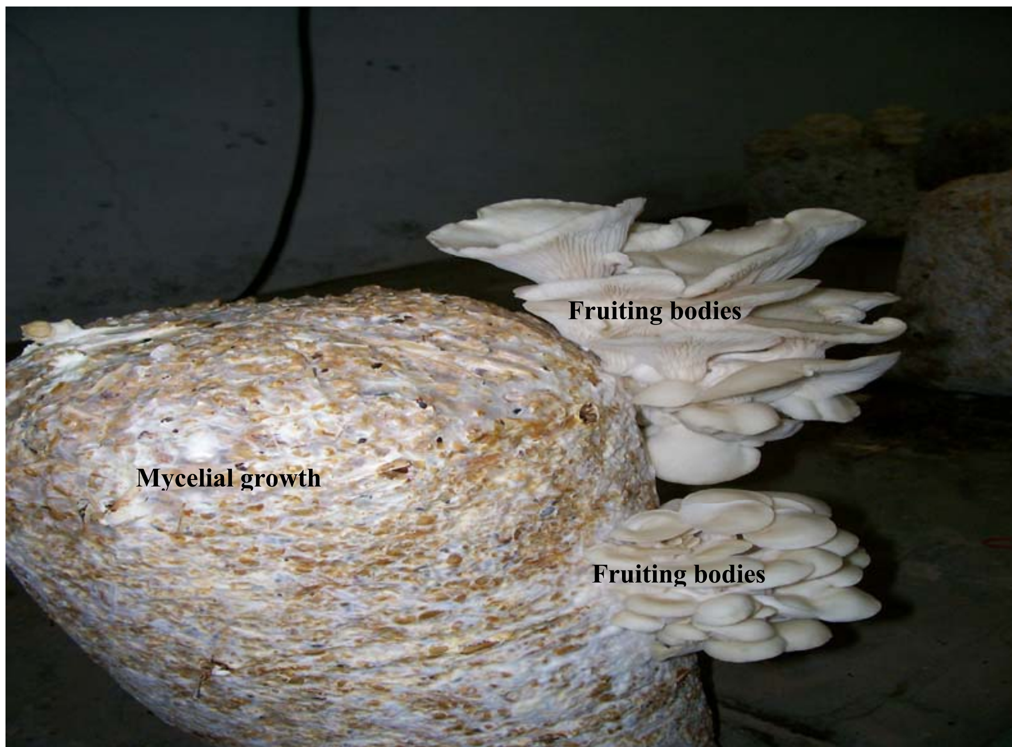
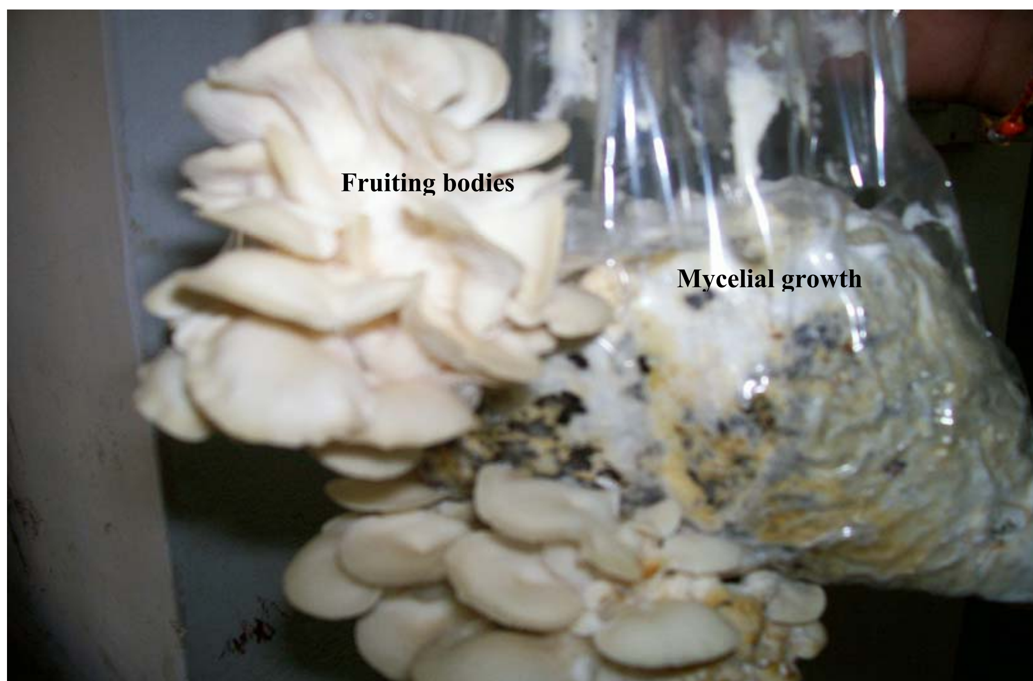


Figure. 5.3.4.3. ***P. florida*** **growing on spent coffee.**



5.3.5. Production of vermicompost using coffee processing wastes:

Coffee pulp is rich in nutrients (Table 5.1.3.1), but contains inhibitory factors like phenolics, tannins and caffeine making it unsuitable for use as fertilizer and feed. Conventionally the pulp is left in piles, and after 3 to 12 months can be used for composting. Another way of composting is to mix coffee husk with cattle manure, and leave the mixture in pits or heaps. However both the above methods take very long time for the conversion of coffee pulp into organic manure, the final product obtained has poor physical and chemical characteristics, besides this, it has been found that the organic manure obtained by this method is not completely devoid of caffeine, hence it can have deleterious effects on plant growth. Vermicomposting obviates both the disadvantages related to the above methods.

Coffee processing wastes, viz., coffee pulp and spent coffee were used for the production of vermicompost using *E. foetida.* It was observed that the time taken for vermicomposting was only 30 days in contrast to 90 days reported by Orozoco (1996). This vermicompost had high NPK content and improvement in the organic carbon content of the wastes was observed (Table 5.3.5.1).

The coffee processing wastes were amended with cow dung in different combinations and the results are presented in Table 5.3.5.1. The total nitrogen content of coffee pulp increased from 2.05% to 3.35% in a period of 30 days whereas the nitrogen content decreased to 1.95% when pulp was amended with dung in the ratio of 1:1. Further the nitrogen content dropped to 1.68% when coffee pulp was amended with dung in the ratio of 2:1. From these experiments it was found that unamended coffee pulp produces better quality of vermicompost in terms of nitrogen content as compared to amended coffee pulp with cow dung. The initial phosphorus content in coffee pulp was found to be 0.14%. The phosphorus content increased to 0.17% in case of unamended coffee pulp vermicompost, whereas there was a considerable increase in phosphorus content in case of amended coffee pulp. It increased to 0.36% both in the case of coffee pulp and cow dung in the ratio of 1:1 and coffee pulp and cow dung amended in the ratio of 2:1. The initial potassium content in coffee pulp was 2.1% and it decreased to 1.23% in after vermicomposting. There was a considerable decrease in potassium content in amended coffee pulp and an amendment of coffee pulp with cow dung in the ratio of 1:1 showed a decrease to 0.58% and in combination of coffee pulp and dung in the ratio of 2:1, it decreased to 0.65% from 2.1%. The results obtained are in

accordance with the results obtained by Orozoco (1996), where after ingestion of the pulp by the earthworms, an increase in available P, Ca, and Mg was observed but a decrease in K was detected.

The total organic carbon (TOC) content decreased from 28.5 to 7.0, the pH increased from 4.78 to 7.54 and the electrical conductivity decreased from 6.96 to 2.63 in case of unamended coffee pulp. The organic carbon content decreased to 11.0 in both the ratios of amended coffee pulp with cow dung. The electrical conductivity decreased in in a combination of coffee pulp and dung in ratio of 1:1, to 2.32 and to 2.34 in combination of coffee pulp and cow dung in the ratio of 2:1. Increase in pH was observed in most of the combinations.

The initial nitrogen content in spent coffee was 1.86%, which decreased to 1.75% after vermicomposting. The nitrogen content increased to 2.74% in case of amendment of spent coffee with cow dung in the ratio of 1:1, and when spent coffee was amended with cow dung in a ratio of 2:1, it increased to 2.17%. In a combination of spent coffee and coffee pulp in the ratio of 1:1 the nitrogen content decreased to 1.75%, and in a combination of spent coffee and coffee pulp in the ratio of 1:2, it increased to 3.00%.

From Table 5.3.5.1, it can be observed that the initial phosphorus content of spent coffee was 0.05%, which increased to 0.11% after vermicomposting with cow dung as amendment in the ratio of 1:1. A further increase in the phosphorus content to 0.80% was observed when spent coffee was amended with cow dung in the ratio of 2:1. In a combination of spent coffee and coffee pulp in the ratio of 1:1, the

phosphorus content increased to 0.40%, and in a combination of spent coffee and coffee pulp in the ratio of 1:2 it increased to 0.18.

Decrease in K content was observed in all the samples, which is a regular observation with vermicompost samples (Orozoco, 1996). The initial organic carbon content of spent coffee is 29.5 and there was a very slight decrease to 29.0% after vermicomposting. There was considerable decrease in TOC in all the combinations of spent coffee except in combination of spent coffee and coffee pulp in the ratio of 1:2, where the decrease was only to 26.0%. The initial electrical conductivity of spent coffee was 0.68 and after vermicomposting the electrical conductivity increased in all the different combinations of spent coffee. Similar was the case with pH, it increased in all the combinations of spent coffee.

As per the results in Table 5.3.5.1 some of the good combinations for vermicompost production are spent coffee and cow dung in the ratio of 1:1, unamended coffee pulp, coffee pulp and cow dung in the ratio of 1:1, spent coffee and coffee pulp in the ratio of 1:2, spent coffee and coffee pulp in the ratio of 1:1, spent coffee: cow dung in the ratio of 2:1.

Nutrient value of vermicompost as reported by Coffee board, Bangalore in the coffee news magazine “Indian coffee” (2005, December Issue) is sited below. The amount of major nutrients in vermicompost varies depending on the raw material: total N (0.4-1.8 %), available phosphorus (0.11 to 0.31 %), available potassium (0.15 to 0.60 %).

During the vermicomposting process a record of the population count of the inoculated earthworm species was maintained and the results are presented below in

Table 5.3.5.2. The results show an increase in population of earthworm during the vermicomposting process and the maximum increase in population was observed in a combination of spent coffee and dung in the ratio of 1:1, the number of earthworms increased from 50 to 135 by the end of the 30-day observation period. The second highest increase in population was observed in spent coffee: coffee pulp (1:1), from 50 to 93, followed by spent coffee unamended fraction, the increase in number was from 50 to 70. It was observed that there is a direct correct correlation between the increase in the total earthworm population an improvement in the quality of vermicompost (Table. 5.3.5.1). The low quality of vermicompost in some combinations like coffee pulp without amendments, in spent coffee alone, and a correlation with the low earth worm counts is due to the initial inhibitory effect of the tannins and caffeine present in the processing wastes. During the 30-day vermicomposting period, the organism first undergoes acclimatization and the initial 50 worms introduced into the matrices utilize a part of the processing wastes removing the inhibitory factors. All the worms introduced were found to have eggs when introduced. After 30 days it was observed that in the case of amendments and in unamended wastes there was a decrease in the number of adult worms in unamended coffee pulp and in combinations of coffee pulp and cow dung (Table 5.3.5.2) indicating the death of the worms and the population of juvenile worms in the partially digested wastes. The juvenile worms then were found to be capable of completely degrading the wastes indicating that the worms have induced the necessary enzymes required to degrade the tannins, phenolics and caffeine in the coffee pulp and spent coffee.

Besides this, the HPLC results of caffeine estimation of the vermicompost produced showed that complete decaffeination occurred during the process of vermicomposting. This proves that the earthworms were capable of detoxifying the caffeine containing vermicompost material.

Table 5.3.5.1. Production of vermicompost using coffee processing wastes.

S. No	Substrate	pH	EC* (m.mohs/ cm	OC** (%)	N (%)	P (%)	K (%)
1.	Coffee pulp (0hr)	4.78	6.96	28.5	2.05	0.14	2.1
2.	Spent coffee (0hr)	5.17	0.68	29.5	1.86	0.05	0.11
3.	Dung control (0hr)	7.46	1.81	7.0	1.41	0.59	0.40
4.	Spent coffee: cow dung (1:1)	7.50	1.78	14.5	2.74	0.80	0.33
5.	Spent coffee control	6.20	1.41	29.0	1.75	0.11	0.33
6.	Coffee pulp: dung (2:1)	7.88	2.34	11.0	1.68	0.36	0.65
7.	Coffee pulp control	7.54	2.63	7.0	3.35	0.17	1.23
8.	Coffee pulp: dung (1:1)	7.80	2.32	11.0	1.95	0.48	0.58
9.	Spent coffee: coffee pulp (1:2)	8.04	3.47	26.0	3.00	0.18	2.5
10.	Spent coffee: coffee pulp (1:1)	8.28	3.64	12.0	1.79	0.36	1.92
11.	Spent coffee: cow dung (2:1)	7.42	2.16	16.5	2.17	0.40	0.36
12.	Dung control	7.52	2.27	7.5	1.37	0.42	0.34

***EC = electrical conductivity is a measure (millimhos per centimeter) of the relative salinity of soil or the amount of soluble salts it contains.**
****OC= Organic carbon content**

Table 5.3.5.2. Population profile during vermicompost using coffee processing wastes.

S. No	Substrate	Worm count (1st day)	Wormcount (30th day)
1.	Spent coffee control	50	70
2.	Coffee pulp control	50	37
3.	Cow dung control	50	44
4.	Spent coffee: cow dung (1:1)	50	135
5.	Spent coffee: cow dung (2:1)	50	42
6.	Coffee pulp: cow dung (2:1)	50	36
7.	Coffee pulp: cow dung (1:1)	50	48
8.	Spent coffee: coffee pulp (1:2)	50	65
9.	Spent coffee: coffee pulp (1:1)	50	93

Conclusion:

In the present work, coffee processing wastes such as coffee pulp, coffee hull and spent coffee have been used efficiently for the extraction of caffeine, production of enzymes, mushrooms and vermicompost. Extraction of caffeine was done efficiently from coffee pulp by aqueous and solvent phase extraction. 1.512% of caffeine could be extracted from coffee pulp using sodium carbonate to facilitate the extraction method. The caffeine extracted from the coffee processing wastes was further used for biotransformation to theophylline using screened fungal culture *P. citrinum* MTCC 5215. *P. citrinum* MTCC 5215, the most potent decaffeinating fungal strain was used for the biodecaffeination of coffee pulp and coffee hull using solid state fermentation. The fungal strain could decaffeinate coffee pulp completely in a period of 72 hours, but could not decaffeinate coffee hull. The decaffeinated coffee pulp can have further uses as animal feed and as organic fertilizer. Coffee pulp proved to be a good SSF substrate for the production of enzymes such as caffeine oxidase, protease and pectinase, α-amylase and amyloglucosidase. F9 a strain of *Aspergillus* produced the highest activity (377.6U/gm substrate). Production of caffeine oxidase by the isolates has potential application in biotechnological process of decaffeination. Protease production was much higher using coffee pulp as substrate than with wheat bran as substrate. The enzyme activity of isolate F3 (*Penicillium*) showed highest protease activity 85.3U/gm substrate in coffee pulp. All the fungal isolates produced good amounts of pectinase enzyme. Highest pectinase activity of 9266.6 U/gm substrate was produced by F10 an unidentified fungal isolate. These isolates can be used for the

commercial production of pectinase using coffee pulp as substrate. Using coffee pulp as substrate, α-amylase and amyloglucosidase were produced and the yields obtained were comparable with that of wheat bran substrate.

It was observed that the processing wastes could be used successfully for the production of mushroom. Coffee pulp gave maximum BCE of 99.545 whereas coffee hull and spent coffee gave better BCE when amended with 25% rice straw than in unamended form. Coffee hull proved to be the best substrate in terms of lesser spawn run period of 10 days, maximum protein content of 25.84% and maximum free amino acid content of 0.46%. Hence, unamended coffee hull which is one of the under utilized processing wastes can be used for the production of mushroom with less spawn run period, high protein and free amino acid content. The results suggest that coffee pulp in unamended form and coffee hull and spent coffee amended with 25% rice straw can be used as successful substrates for mushroom production. It was observed that mushroom produced on coffee processing wastes as substrates ad better nutritive value in terms of high protein content and free amino acid content. However no accumulation of caffeine was observed in the fruiting bodies suggesting the efficiency of *Pleurotus* in degrading caffeine in these processing wastes during its growth. Hence, coffee processing wastes can be used successfully for the production of mushroom using *Pleurotus*.

Vermicomposting was done using coffee pulp and spent coffee in unamended and amended in different ratio with cow dung using *E. foetida.* The analysis of the vermicompost showed increased NPK content. The best combination for vermicompost was spent coffee and cow dung in the ratio of 1:1. Besides this,

unamended coffee pulp also produced vermicompost with high NPK values. From the results obtained it can be concluded that earthworms accelerated the mineralization rate, converted the manures into castings with a higher nutritional value and degrees of humification, and could decaffeinate the substrates used.

The coffee processing wastes were decaffeinated by cultivating fungi, mushrooms and earthworms. Work carried out in the present chapter shows that coffee processing wastes can be used efficiently for the extraction of caffeine, efficient substrate for SSF for enzyme production, production of mushroom, vermicomposting etc.

References:

Adams, M. R., and Dougan, J., (1981), Biological management of coffee processing. *Trop Sci*. **123**: 178-196.

Aguirre, F., Maldonao, O., Rolz, C., Menchu., J. F., Espinosa, R., and Cabrera, S. (1976), Protein from waste. Growing fungi on coffee waste. *Chem.Technol*. **6**: 636-640.

Andersson, H. C., Hallström, H. and Kihlman*, B.A*. (2004), Intake of caffeine and other methylxanthines during pregnancy and risk for adverse effects in pregnant women and their foetuses. Ekspressen Tryk & Kopicenter, www.norden.org

Antier, P., Minjares, A., Roussos, S., Raimbault, M., and Gonzalez, V.G., (1993a), Pectinase hyperproducing mutants of Aspergillus niger C28B25 for solid-state fermentationof coffee pulp. *Enz.Microb.Technol*. **15**:254-260.

Antier, P., Minijares, A., Roussos, S., and Gonzalez, V. G., (1993b), New approach for selecting pectinase producing mutants of Aspergillus niger well adapted to solid state fermentation. *Biotechnol. Tech.* **11** (3): 429–440.

AOAC (1975), Official methods of analysis. 12th edition, Washington DC Association of official analytical chemists. ISBN 0-935584-14-5.

Asano, Y., and Yamada, H. (1994), Enzyme involved in theobromine production from caffeine in *Pseudomonas putida* no. 352. *Biosci. Biotechnol. Biochem*. **58**: 2303-2304.

Aquiahuatl, M. A., Raimbault, M., Roussos, S and Trejo, M. R. (1988), Coffee pulp detoxificationby solid state fermentation, Proceedings of the seminar on solid state fermentation, version of agroindustrial raw materials. ORSTOM, Montpellier, France, 13-26.

Bercelos, A. F., Paiv, P. C., Pereza, J.R.O., Dos, S. V. B., and Cardodo, R. M. (2002), Bromatological parameters of the hulls and dehydrated pulp of coffee (Coffea arabica L.) stored in differentperiods. *Editorial UFLA – Revista*. 26-04-02.

Bergmeyer, H. U. (Ed.), Weinheim V. C. H (1984), *Methods in Enzymatic Analysis*, 3rd edn. **4**: 154.

Birdwhistell, R. K., and Connor, R. R., (1971), The Freeman library of laboratory separates in chemistry **Vol. 2**. San Francisco, USA:Freeman.

Boccas, F., Roussos, S., Gutierrez, M., Serrano, L., and 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.

Boopathy, R. (1988), Dry anaerobic methane fermentation of coffee pulp. *J. Coffee Research.* **18** (2) 59.

Braham, J. E. (1979), Coffee pulp in other species. P 51-54. *In* J.E. Braham and R. Bressani. (ed.). Coffee pulp: composition, technology, and utilization. IDRC Publ. **108e**: International Development Research Centre, Ottawa.

Bressani, R. (1979a), Antiphysiological factors in coffee pulp. *In* J.E. Braham and R. Bressani. (ed.). Coffee pulp: composition technology and utilization. *Int Dev Res Cent Publ* **108e**: 83-88.

Bressani, R. (1979b), Potential uses of coffee berry by products. P 17-24. *In* J.E. Braham and R. Bressani. (ed.). Coffee pulp: composition, technology, and utilization. IDRC Publ. **108e**. International Development Research Centre, Ottawa.

Bressani, R. (1979c), The by products of coffee berries. P 5-10. *In* J.E. Braham and R. Bressani. (ed.). Coffee pulp: composition, technology, and utilization. IDRC Publ. **108e**. International Development Research Centre, Ottawa.

Cabezas. M.T., Flores., A. and Egana. J. I. (1979), Use of coffee pulp in ruminant feeding, P25-38. *In* J.E. Braham and R. Bressani. (ed.). Coffee pulp: composition, technology, and utilization. IDRC Publ. **108e**. International Development Research Centre, Ottawa.

Chacon, G., and Fernandez, J. L. (1984), Capacidad de la pulpa del cafe para la produccion de biogas. Turrialba (IICA). **34** (2) 143-147.

Chanakya, H. N. | Alwis, A. A. P. (2004), Environmental Issues and Management in Primary Coffee Processing. 82 (4): 291-300.

Chang, S. T. and Buswell, J. A. (1996), Mushroom neutraceuticals. *World journal of microbiology and biotechnology*. **12**: (5) 473-476.

Christensen, M. S (1981), Preliminary tests on the suitability of coffee pulp in the dies of common carp (*Cyprinus carpio*) and catfish (*Clarias mossambicus*). *Aquaculture*. **25**: 235-242.

Coffee board (2005 December Issue), Possibility of Vermicomposting in Coffee Plantations. *Indian coffee*.

Dulce. S., Gerardo. M., and Krzysztof, N. W. (2005), Comparative culturing of *Pleurotus* sp. on coffee pulp and wheat straw: biomass production and substrate biodegradation. *Bioresource Technology*. **96**: (5) 537-544.

Edwards, S. S (1979), Central America: fungal fermentation of coffee waste. *In* D. Evans and L. Adler (ed.)., P329-342. Appropriate technology for development: a discussion and case histories. Westview Press, Boulder, Colo.

Edwards, C. A. (Ed.). (1990), *Earthworm Ecology*. Soil and Water Conservation Society,Ankeny, Iowa, USA.

Edwards, C. A. and Hohlen, P. J. (1996), *Biology and Ecology of Earthworms*. 3 rd Edition, Chapman and Hall, United Kingdom. P 230-238.

Elias, L. G. (1978), Composcion quimica de la pulpa de café y otros subproducts. In: pulpade café: Composicion Technologia Y Utilization (Braham, J. R. and Bressani, R. Eds.) INCAP Guatemela city; 19-29.

Elias, L. G. (1979), Chemical composition of coffee berry by products. P 11-16, *In* J.E. Braham and R. Bressani (ed.), Coffee pulp: composition, technology, and utilization. IDRC Publ. **108e**. International Development Research Centre, Ottawa.

Ennis, L. (1957), Turbidometric analysis of proteins, *Methods in Enzymology*. **3**: Academic Press, Inc., 447-445.

Favela, T. E., Huerta, S., Roussos, S., and Gutierrez, M (1989), in I Seminario Internationacional Sobre Biotechnologia en la Agroindustria Cafetalera (Roussos, S., Licona, F. R. and Gutierrez, R. M., eds) Jalapa, Mexico, 145-151.

Field, J. A., and Lettinga, G. (1987), The methanogenic toxicity and anaerobic degradability of hydrolysable tannin. *Water Research*. **21** (3): 367-370.

Frischknecht, P.M., Ulmer, D.J., and 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.

Gaime, P. I., Roussos, S., and Martinez, C. D. (1993), Natural microorganisms of the fresh 39. coffee pulp. *Micol Neotrop Apl*. **6**:95-103.

Graham, D. G. (1978), Caffeine – its identity, dietary sources, intake and biological effects. *Nutrit. Rev*. **36**: 97-102.

Haas, G. and Stieglitz, B. (1980), Microbiological decaffeination of aqueous liquids. US patent US, 4,228,191.

Haimi, J. and Huhta, V. (1990), Effect of earthworms on decomposition processes in the raw humus forest soil: A microcosm study. *Biology and Fertility of Soils*. **10**:78-183.

Hakil, M., Denis. S., Viniegra, G. G. and Augur, C. (1998), Degradation and analysis of caffeine and related dimethyl xanthines by filamentous Fungi. *Enz. Microb. Technol.* **22**: 355-359.

Hakil, M., Voisinet, F., Viniegra, G. G., and Augur, C. (1999), Caffeine degradation in solid state fermentation by *Aspergillus tamarii* : effects of additional nitrogen sources, *Proc. Biochem.* **35**: 103-109.

Hampp, A. (1996), The extraction of caffeine from tea: A modification of the procedure of Murray and Hansen. *Journal of Chemical Education*. **73** (12): 1172-1173.

Helmkamp, G. K., and Johnson, H. W. (1968), Selected experiments in organic chemistry (2nd ed.). San Francisco, USA: Freeman, P157–158.

Landgrebe, J. A. (1993), Theory and practice in the organic laboratory (4th ed.). Paci Grove, C. A: Brooks-Cole, **5**:381–383 New York, USA: Wiley and Sons.

Lane, A.G. (1983), Anaerobic digestion of spent coffee grounds. *Biomass*. **3**: (4) 247-252.

Ledger, H. P., and Tilman, A. D. (1972), Utilization of coffee hulls in cattle fattening rations. *East. Afr. Agric. J.* **37**:234-236.

Madyastha, K. M., Sridhar, G. R, Bhat, B. V., and Sudha, M.Y. (1999), Purification and partial characterization of caffeine oxidase. *Biochem. Biophys. Res. Comm*. **263**: 460-464.

Martinez, C. D. (1987), Design of a mushroom farm for growing *Pleurotus* on coffee pulp. *Mushroom J Trop*. **7**: 13-23.

Mayo, D. W., Pike, R. M., and Butcher, S. S. (1989), Microscale organic laboratory (2nd ed.). New York, USA: Wiley, P 162–164.

Mazzafera, P. and Carvalho, A. (1992), Breeding for low seed caffeine content of coffee (*Coffea arabica* L.) by interspecific hybridization. *Euphytica*. **59**:55-60.

Miller, G. L. (1959), Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* **31**: 426-428.

Mitchell, D. A. and Lonsane, B. K. (1990), Definition, characterization and economic evaluation. In general principles of solid substrate fermentation.

Monograph 1, by H.W.Doelle and C.Rolz (Eds), Rapid publications of Oxford Ltd., UK.

Molina, M., Fuente, D. G, Batten, M. A., and Bressani, R. (1974), Decaffeination – A process to detoxify coffee pulp. *J.Agr.Food.Chem.* **22**: 1055-1059.

Murillo, B. (1979), Coffee pulp silage. Coffee pulp: composition, technology and utilization. *Int Dev Res Cent Publ*. **108e**: 63-70.

Murray, S. D., and Hansen, P. J. (1995), The extraction of caffeine from tea: An old undergraduate experiment revisited. *J. Chem. Edu*. **72**: 851–852.

Nathanson, J. A (1984 October 12), Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science* **226** (4671): 184-187.

Nimitz, J. S. (1991), Experiments in organic chemistry. Englewood Cli.s, N. J: Prentice-Hall, P 61–62.

Nippon N. K. (1971), Degradation of caffeine by mold. *Aspergillus niger* Ina,K. *Biochemical studies of caffeine*. **45** (8): 378-380.

Orozco, F. H., Cegarra J., Trujillo L. M. and Roig, A. (1996), Vermicomposting of coffee pulp using the earthworm *Eisenia fetida*: Effects on C and N contents and the availability of nutrients. **22**: 1-2.

Orue, C, and Bahar, S. (1985), Utilization of solid coffee waste as a substrate for microbial protein production. *J Food Sci Technol*. **22**:10-16.

Pandey, A., (1990) Improvements in solid-state fermentation for glucoamylase production. *Biol. Wastes*. **34** (1): 11–19.

Pandey, A., Ashakumary, L. and Selvakumar, P., Glucoamylase production by *Aspergillus niger* on rice bran is improved by adding nitrogen sources. (1994), *World J. Microbiol. Biotechnol.* **10** (3): 348–349.

Park, K. (2001), Nutritional Value of a Variety of Mushrooms. www.MushWorld.com/sub_en.html. January 5.

Pavia, D. L., Lampman, G. M., and Kriz, G. S. (1976), Introduction to organic laboratory techniques. Philadelphia, USA: Saunders, P 58–62.

Penaloza, W., Molina, M. R., Gomez, B. R. and Bressani, R. (1985), Solid-State Fermentation: an Alternative to Improve the Nutritive Value of Coffee Pulp. *Appl. Environ. Microbiol.* **49** (3): 388-393.

Peter. A. and Ursula. K, (1962), Studies relating to the purification and properties of pectin transeliminase. *Arch. Biochem. Biophys.* **97**(1): 107-115.

Philip, A. M., Roussos, S. Raimbault, M., and Viniegra-Gonzalez, G. (1993), Pectinase - hyperproducing mutants of Aspergillus niger C28B25 for solid state fermentation of coffee pulp., *Enz. Microb. Technol.* **15**: 254-260.

Pulgarin.C., Schwitzguebel,J.P. and Tabacchi,R. (1991), *Biofutur*. **6**:43-50.

Radionov, B (1926), *Bull. Soc. Chem*. **39**: 305-306.

Rajrathnam, S., Singh, N. S and Zakia, B. (1979), Efficiency of carboxin and heat treatment for controlling the growth of *Sclerotium rolfsii* during the culture of the mushroom *Pleurotus flabellatus*. *Ann.f Appl. Biol*. **92**: 323-328.

Rajrathnam, S. and Zakia, B. (1987), *Pleurotus* mushrooms. Part IA. Morphology,life cycle, taxonomy, breeding and cultivation CRC *Critical reviews in Food Science and Nutrition* **26**: 157-223.

Ravindranath, R. Yousuf, A. K., Obi, T. R. Thirumala R, Reddy, S. D. (1971), Composition and characteristics of Indian coffee bean, spent grounds and oil. *J.Sci. and Food Agr*. **23** (3): 307-310.

Ricardo, B. (1980), *Coffea arabica*,. Animal feed resources information system. *Agri.Sci.Nutr*. 573-575.

Rojas. U., Verreth J.A.J, Amato, S., and Huisman, E.A. (2003), Biological treatments affect the chemical composition of coffee pulp. *Bioresource Technol*. **89**:267-274.

Rolz, C., Leon, R., and Arriola, M. C., (1988a), Biological pretreatment of coffee pulp. *Biol. Wastes* **26**:97-114.

Rolz,C., Leon R, Arriola, M.C (1988b), Solid substrate growth of white rot fungi on coffee pulp. *Acta Biotechnol.* **13**:211-223.

Roussos, S., Hannibal, L., Aquiahuatl, M. A., Trejo, H., and Marakis, M. R. (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.

Roussos S., Refugio, M. M., Gaime, P. I., Favela, E,. Ramakrishna, M., Raimbault, M., and Viniegra, G. G. (1995), Biotechnological management of coffee pulp-isolation, screening characterization, selection of caffeine degradaing fungi and natural microflora present in coffee pulp and husk. *Appl. Microbiol. Biotechnol.* **42**: 756-762.

Royse, D. J., and Lee. C. S. (1980) Mushrooms: Their Consumption, Production, and Culture Development. *Interdisciplinary Science Reviews*. 5 (4): 324–331.

Royse, D. J. (ed.). (1996), Mushroom Biology and Mushroom Products: Proceedings of the Second International Conference, University Park, PA, June 9-12, Penn State, State Park, P. A. P581.

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

Shah, N. K., Ramamurthy, V., Kothari, R. M. (1991), Comparative profiles of fungal alpha amylase production by submerged and surface fermentation *Biotechnol. Lett.* **13** (5): 361–364.

Shankaranand, V. S., Ramesh, M. V. and Lonsane, B. K., (1992), Idiosyncrasies of solid-state fermentation systems in the biosynthesis of metabolites by some bacterial and fungal cultures *Process. Biochem.* **27** (1):33–36.

Stamets, P. (2000–2001), A novel approach to farm waste management. Mushroom the Journal. Winter. P 22. www.fungi.com/mycotech/farmwaste.html.

Singleton, V.L, Rossi, J. A. (1965), Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* . **16**:144-158.

Singleton, V.L, Orthofer, R., Lamuela. R. R.. M. (1999), Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* **299**:152-178.

Smith, B.W. and Roe, J. H., (1949), α Amylase activity in fermentation samples. *J.Biol.Chem*. **179**: 53-61.

Sudo, S., Ishikawa, T., Sato, T. and Oba, T. (1994), Comparison of acid-stable α-amylase production by *Aspergillus kawachii* in solid-state and submerged cultures *J. Ferment. Bioeng.* **77**(5): 483–489.

TERI. (1997), Development of reactor design for biomethanation of solid waste. New Delhi: Tata energy research institute.

Upasi (2006)**, www.thehindubusinessline.com/2006/05/26/stories/2006052602311700.htm**

Williamson, K. L. (1989), Macroscale and microscale organic laboratory (2nd ed.). Toronto: Heath, P130–133.

Wintrebert, C. (1980), Augmentation du rendement de la methanisation des pulpes de. cafe. Dissertation. Ecole Nationale Superieure Agronomique de Montpellier (France).

Wrigley, G. (1988), *Coffee*. New York: John Wiley and Sons.

Yano, Y., D. M.; and Mazzafera, P. (1996), Descafeinação da palha de café por bactérias. In: congresso brasileiro de pesquisas cafeeiras, 22. Águas de Lindóia,. Resumos. Rio de Janeiro: Ministério da Agricultura e Reforma Agrária, P 36-39.

Yano, Y., D. M., and Mazzafera, P. (1998) Degradation of caffeine by *Pseudomonas putida* isolated from soil. *Allelopathy Journal*. **5**: 23-34.

Yemm, E. W., Cocking. E. C. and Ricketts. R. E. (1955), The determination of amino acids with ninhydrin. *Analyst*. **80**: 209 – 214.

Zakia, B., Shashirekha, M. N and Rajarathnam, S. (1993), Improvement of the bioconversion and biotransformation efficiencies of the Oyster mushroom (*Pleurotus sajor-caju*) by supplementation of the rice straw substrate with oil seed cakes. *Enzyme and Microbial Technology*. **15**: 985-989.

Zorba, F. (Ed.). (2001), *Worm Digest: The Art of Small Scale Vermicomposting*. Worm Digest, Eugene, Oregon. P 24.

*Zuluaga, J., Bonilla, C., and Quijano, R. M. (1975), Contribucion al studio Y utilizacion de la pulpa de café, In: *Zeme CoL Intern Quim. Cafe*, Hamburg (ASIC costes, A Ed) Paris, 233-242.

*Zuluaga, V. J. (1989), Utilizacion integral de los subproducion del café. In : Roussos S, Licona FR, Guiterrez RM(eds), Proceedings of I *Seminario International sobre Biotechnologia en la Agroindustria cafetalara* (ISIBAC), Jalapa, Mexico. 63-76.

*** Original reference not seen.**

Bibliography:

Section	Title	P. No
	List of abbreviations.	vii
	List of tables.	ix
	List of schemes and figures.	xii
	Chapter 1 – Review of Literature	**1-58**
1.0.	Scope of the review.	1
1.1.	Introduction.	2
1.1.1.	History of biotransformation.	4
1.1.2.	Industry sectors involving biotransformation.	4
1.1.2.1.	Pharmaceuticals.	5
1.1.2.2.	Bulk products and polymers.	5
1.1.2.3.	Fine and specialty chemicals.	5
1.1.2.4.	Food.	6
1.1.2.5.	Cosmetics.	6
1.1.3.	Potential benefits of biotransformation.	7
1.1.4.	Systems of Biotransformation	7
1.1.4.1.	Biotransformations by free cells.	8
1.1.4.2.	Biotransformation by immobilized cells.	8
1.1.4.3.	Immobilized enzymes.	9
1.1.4.4.	Cross-linked enzyme crystals.	9
1.1.4.5.	Tissue culture.	10
1.1.4.6.	Biotransformation in organic phase.	11
1.1.5.	Characteristic features of biotransformation.	12
1.1.6.	Enzymes - the key elements of biotransformation.	13
1.1.6.1.	Oxidoreductases.	14
1.1.6.2.	Transferases.	17
1.1.6.3.	Hydrolases.	17
1.1.6.4.	Lysases.	18
1.1.6.5.	Isomerases.	19
1.1.6.6.	Ligases.	19
1.1.7.	Categorization of biotransformation	19
1.1.7.1.	Biotransformation in human/animal system.	19
1.1.8.	Biotransformation in plants.	22
1.1.9.	Biotransformation in microbes.	23
1.1.9.1.	Metabolite or product formation.	24
1.1.9.2.	Bioremediation.	27
1.1.9.3.	Biotransformation for value addition to food products.	28
1.2.	Introduction to methyl xanthines.	31
1.2.1.	Caffeine.	33
1.2.2.	Theophylline.	36
1.2.3.	Theobromine.	40
1.2.4.	Paraxanthine.	41
1.2.5.	8-Methoxymethyl-1-methyl-3- (2-methylpropyl)	42

	xanthine	
1.2.6.	1-Methyl-3-isobutylxanthine.	43
1.2.7.	Acyclovir.	43
1.2.8.	Pentoxyfyline.	44
	Conclusion.	45
	References.	47
	Chapter 2 – Screening of caffeine biotransforming fungi.	**59-88**
2.0.	Scope of the work.	59
2.1.	Introduction.	60
2.1.1.	Biotransformation of caffeine by bacteria.	62
2.1.2.	Biotransformation of caffeine by fungi.	64
2.2.	Materials and Methods.	67
2.2.1.	Isolation and purification of caffeine biotransforming culture.	68
2.2.2.	Screening of isolated cultures for caffeine biotransformation.	69
2.2.2.1.	Screening of isolated cultures for biotransformation of caffeine to theophylline.	70
2.2.3.	Characterization and identification of the isolates.	70
2.2.4.	Microscopy studies.	70
2.3.	Results and Discussion.	72
2.3.1.	Isolation and purification of caffeine biotransforming culture.	72
2.3.2.	Screening of isolated cultures for caffeine biotransformation.	74
2.3.3.	Screening of isolated cultures with the capability of biotransformation of caffeine to theophylline.	76
2.3.4.	Characterization and identification of the isolates.	77
2.3.5.	Cultural and morphological characteristics of the isolate F28.	78
	Conclusion.	84
	References.	85
	Chapter 3 – Biotransformation of caffeine by *Penicillium citrinum* MTCC 5215.	**89-156**
3.0.	Scope of the work.	89
3.1.	Introduction.	90
3.1.1.	Biotransformation of caffeine in plants.	91
3.1.2.	Biotransformation of caffeine in mammals.	92
3.1.3.	Biotransformation of caffeine in bacteria.	94
3.1.4.	Biotransformation of caffeine in yeast.	95
3.1.5.	Biotransformation of caffeine in fungi	95
3.2.	Materials and Methods.	98
3.2.1.	Materials.	98
3.2.2.	Methods.	

3.2.2.1.	Product profiling of caffeine biotransformation by *P. citrinum* MTCC 5215.	99
3.2.2.2.	Isolation, purification and identification of biotransformed products.	100
3.2.2.3.	Biomass production for enzyme identification.	100
3.2.2.4.	Preparation of crude enzyme extract (CEE).	101
3.2.2.5.	Isolation of microsomes.	101
3.2.2.6.	Identification of cytochrome P450.	103
3.2.2.7	Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) to determine the inducible nature of enzyme.	104
3.2.2.8	Gel filtration chromatography for purification of cytochrome P450.	104
3.2.2.9	Reconstitution of cytochrome P450 enzyme.	105
3.2.2.10.	Identification of enzymes involved in caffeine degradation pathway.	105
3.3.	Results and Discussion.	109
3.3.1	Cultivation of *P. citrinum* MTCC 5215 for product profiling during biotransformation of caffeine.	109
3.3.2.	Isolation, purification and identification of biotransformed products.	110
3.3.3.	Cultivation of *P. citrinum* MTCC 5215 for identification of enzyme.	124
3.3.4.	Preparation of crude enzyme extract.	124
3.3.5.	Preparation of microsomes.	124
3.3.6.	Enzyme assay and characterization of cytochrome P450.	125
3.3.7.	Enzyme assay for other enzymes involved in caffeine biotransformation pathway of *P. citrinum* MTCC 5215.	134
3.3.7.1.	Heteroxanthine demethylase (HXDM).	134
3.3.7.2.	Xanthine dehydrogenase (XDH).	132
3.3.7.3.	Xanthine oxidase (XO).	136
3.3.7.4.	Uricase.	138
3.3.7.5.	Allantoinase.	139
3.3.7.6.	Allantoicase.	140
3.3.7.7.	Urease.	141
3.3.8.	Caffeine degradation pathway in *P. citrinum* MTCC 5215.	142
3.4.	Conclusion.	147
	References.	148
	Chapter 4 – Optimisation of parameters for theophylline production.	**157-222**
4.0.	Scope of the work.	157
4.1.	Introduction.	157
4.1.1.	Microbial biotransformation.	159
4.1.2.	Media optimization.	160

4.1.3.	Response surface methodology (RSM).	161
4.2.	Materials and Methods.	162
4.2.1.	Materials.	162
4.2.2.	Methods.	162
4.2.3.1.	Screening of media for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	163
4.2.3.2.	Optimization of carbon source for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	166
4.2.3.3.	Optimization of nitrogen source for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	167
4.2.3.4.	Optimization of caffeine concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	168
4.2.3.5.	Optimization of pH for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	168
4.2.3.6.	Optimization of temperature for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	169
4.2.3.7.	Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	169
4.2.3.8.	Optimization of inoculum load for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	169
4.2.3.9.	Optimization of $FeSO_4$ concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	170
4.2.3.10.	Optimization of allopurinol concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	170
4.2.3.11.	Production of theophylline with optimized conditions in shake flask.	171
4.2.3.12.	Production of theophylline with optimized conditions in 10L fermentor.	171
4.2.4.	Response surface methodology.	172
4.2.5.	Downstream processing of biotransformed theophylline.	174
4.3.	Results and Discussion.	176
4.3.1.1.	Media screening for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	176
4.3.1.2.	Effect of carbon source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	178
4.3.1.3.	Effect of nitrogen source on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	180

4.3.1.4.	Optimization of caffeine concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	183
4.3.1.5.	Optimization of pH for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	185
4.3.1.6.	Optimization of temperature for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	187
4.3.1.7.	Effect of inoculum age on growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	189
4.3.1.8.	Optimization of inoculum load for growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	190
4.3.1.9.	Optimization of $FeSO_4$ concentration for biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	191
4.3.1.10.	Effect of allopurinol concentration on the biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215.	192
4.3.1.11.	Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 under optimized conditions in shake flask.	194
4.3.1.12.	Growth and biotransformation of caffeine to theophylline by *P. citrinum* MTCC 5215 under optimized conditions in 10L fermentor.	196
4.3.2.	Response surface methodology.	198
4.3.2.1.	Biotransformation of caffeine to theophylline.	198
4.3.2.2.	Caffeine degradation.	204
4.3.2.3.	Biomass production.	209
4.3.2.4.	Downstream processing of biotransformed theophylline.	214
	Conclusion.	217
	References.	218
	Chapter 5 – Utilization of caffeine containing coffee processing wastes for value addition.	**223-286**
5.0.	Scope of the work.	223
5.1.	Introduction.	224
5.1.1.	Coffee processing.	224
5.1.2.	Uses of coffee processing wastes.	229
5.1.3.	Occurrence and extraction of caffeine for biotransformation.	231
5.1.3.1.	Caffeine containing wastes.	234
5.1.4.	Decaffeination of coffee processing wastes.	236
5.1.5.	Utilization of coffee processing wastes as substrate for solid-state fermentation (SSF).	237
5.1.6.	Mushroom production using coffee processing wastes.	237
5.1.6.1.	Substrates for mushroom production.	239

5.1.6.2.	Mushroom spawn.	239
5.1.7.	Utilization of coffee processing wastes for vermiculture production.	239
5.1.7.2.	Advantages of vermicomposting.	243
5.1.7.3.	Precautions to be taken during vermicomposting.	243
5.1.7.4.	Earthworm species used for vermicomposting.	243
5.2.	Materials and methods.	245
5.2.1.	Extraction of caffeine.	246
5.2.2.	Decaffeination of coffee processing wastes.	248
5.2.3.	Production of enzymes by solid-state fermentation (SSF) using coffee pulp as substrate.	248
5.2.4.	Production of mushroom using coffee processing wastes.	251
5.2.5.	Use of coffee processing wastes for vermiculture.	255
5.3.	Results and Discussion.	256
5.3.1.	Extraction of caffeine.	256
5.3.2.	Decaffeination of coffee processing wastes.	258
5.3.3.	Production of enzyme using coffee processing wastes.	259
5.3.4.	Production of mushroom using coffee processing wastes.	264
5.3.5.	Production of vermicompost using coffee processing wastes.	270
	Conclusion.	276
	References.	279

List of publications:

Patents:

1. A Process for the microbial biotransformation of caffeine to theophylline. (329/Del 2006). Inventors - Sanjukta Patra, M.S. Thakur, N.G. Karanth.

2. A Process for Purification of theophylline from fermented broth. Patent filed. Inventors - M.S. Thakur, Sanjukta Patra, V.R. Sarath Babu, K.N Gurudutt, N.G. Karanth.

3. A Process for decaffeination of tea using enzyme. Patent filed Inventors - V.R. Sarath Babu, Sanjukta Patra, M.S. Thakur, N.G. Karanth.

4. A process for the production of vermicompost using coffee processing wastes. Inventors - V.R. Sarath Babu, Sanjukta Patra, M.S. Thakur.

5. A process for the production of edible biomass suing coffee processing wastes. Inventors - Sanjukta Patra, V.R. Sarath Babu, M.S. Thakur, M.N. Shashirekha, M. Rajaratnam.

Publications:

1. Sarath Babu, V.R., Patra, S., Karanth, N. G., Thakur, M.S. and 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. and Kumar, M.A. (2007) Development of a Biosensor for caffeine. *Analytica chimica Acta*. **582(2)**: 329-334.

3. Biotransformation of caffeine to theophylline, Sanjukta Patra, K.N.Gurudutt N. G. Karanth, M.S. Thakur (MS under preparation).

4. Efficient management of coffee pulp as substrate for production of enzymes. Patra, S., Sarath Babu, V.R., Karanth, N. G. and Thakur, (MS under preparation).

5. Patra, S., Sarath Babu, V.R., and Thakur, M.S. Enzymatic pathway of caffeine biotransformation in *Penicillium citrinum* (MS under preparation)

6. Patra, S., Ravi, R. and Thakur, M.S. Media optimization for the production of theophylline using RSM (MS under preparation)

7. Patra, S., Sarath Babu, V.R., Thakur, M.S. M.N. Shashirekha, M. and Rajaratnam Utilization of coffee processing wastes for the production of mushroom (MS under preparation).

8. Patra, S., Sarath Babu, V.R., Thakur, M.S. Detoxification of coffee processing wastes by vermiculture (MS under preparation).