CHARACTERISATION OF A MALTOGENIC ALPHA AMYLASE OF *ASPERGILLUS NIGER* AND STUDIES ON THE ENZYME OVERPRODUCING MUTANT

THESIS

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Abstract/Synopsis	Characterisation of a 53 kDa maltogenic α-amylase from the culture filtrate of *A. niger* is new to literature. Production of this enzyme by the fungus could be improved by UV mutation and temperature selection. Studies using the maltogenic α-amylase over producing mutant showed that high molecular mass precursor starch hydrolysing enzyme is degraded to G1 and G2 forms of glucoamylase and maltogenic α-amylase by autocatalysis in order to overcome secretional difficulties associated with reduced cell wall porosity to high molecular mass proteins. Standardization of fermentation conditions to a pilot plant scale described a process for the enzyme production by submerged fermentation. In defining the media components for maltogenic α-amylase production, acarbose as a screening reagent appeared effective since it inhibited all other amylases.
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10th March 2004

CERTIFICATE

I hereby certify that this thesis entitled **'Characterisation of a maltogenic alpha amylase of *Aspergillus niger* and studies on the enzyme overproducing mutant'** submitted by **Mr. Ravi Kumar Kadeppagari** for the award of Doctor of Philosophy in Biotechnology, to the University of Mysore, is the result of Research work carried out by him in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under my guidance during the period 2000 to 2004.

S. Umesh Kumar
Guide

DECLARATION

I hereby declare that this thesis entitled **'Characterisation of a maltogenic alpha amylase of *Aspergillus niger* and studies on the enzyme overproducing mutant'** submitted to the University of Mysore, for the award of Doctor of Philosophy in Biotechnology is the result of research work carried out by me in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. S. Umesh Kumar**, during the period 2000 to 2004.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Place: Mysore
Date: 10th March 2004

Ravi Kumar Kadeppagari
Candidate

To

My Parents, Who motivated me

My Teachers, Who enabled me

My Brothers, Who encouraged me

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CONTENTS

PREFACE

Starch is a primary food reserve available in nature. Processing of starch gains importance due to the potential industrial applications of its derived products. Starch derivatives have major applications in food, pharmaceutical and textile industries. Adopting enzyme technology revolutionized the processing of starch. Hence, there is a continuous search for new starch degrading enzymes (amylases) for use in starch processing to evolve newer food products. Microorganisms have been shown to produce a wide array of amylases and several of these have already found applications in starch processing industries. Among microbes, filamentous fungi specifically *Aspergillus* spp are potential candidates for enzyme production due to their metabolic diversity and since economic yields can be obtained by simple fermentation methods. In all the above studies, strain improvement is an indispensable method for obtaining strains that produce higher levels of amylases.

Though several amylases of the filamentous fungus *Aspergillus niger* have been described in literature, the fundamental question regarding their secretion is still unclear. Basic studies on the enzymes secreted by the fungus revealed that amylase production requires processing. Apparently identification of this event resulted in the characterization of maltogenic α-amylase production by the organism. Details of this study are described in the thesis.

INTRODUCTION

The filamentous fungus *Aspergillus*, is world wide in distribution and it is one of the ubiquitous groups of fungi. Saprophytic nature of *Aspergillu*s was not recognized until nineteenth century, even though *Aspergillus* was described as early as 1729 (Micheli, 1729). *Aspergillus* spp are major contaminants of natural and man made products and cause infections in human and animals. They are also beneficial due to their valuable biochemical products. They are used as fermenting agents, where their metabolic powers are harnessed for commercial production of primary and secondary metabolites.

The review of literature presented deals with only those aspects relevant to the present investigation.

THE FUNGUS *ASPERGILLUS*

The name *Aspergillus* (rough head) was given to the fungus by Micheli (1729) after distinguishing conidiophores from rough heads. Culturing of the mold was introduced in the early 1850's by De Bary's laboratory. In 1860's, Raulin and van Tieghem used molds for fermentation. Thom and Church published the *Aspergilli* as a taxanomic monograph in 1926, where they presented critical opinion on the relationship of described species. In that monograph they discussed the status of species and whether they can be retained in the genus or placed elsewhere. Raper and Fennell (1965a) placed *Aspergilli* under 18 groups based primarily on morphology or colour.

MORPHOLOGY OF *ASPERGILLUS*

Submerged mycelium of the fungus constitute vegetative mass of *Aspergilli* from which conidiophores arise. Many species of *Aspergillus* produce concentric zonate colonies, that result from periodical production of conidia during colony development. However, *A. glaucus* and *A. sulphureus* develop conidial heads in localized areas in contrast to concentric zones. Colour of the aerial parts (conidiophores, heads and conidia) of the colony is universally used in the species characterization. In *A. flavus*

colour is deposited only in the conidia while in *A. niger*, conidial wall, sterigmata, vesicle and upper part of the conidiophore contain deposited colour. Outer layers of conidiophore wall are usually coloured in *A. flavipes* and *A. ochraceus*.

Spore bearing head is an important structure in the characterization of *Aspergilli*. The colour, shape, size and arrangement of heads are characteristics of the species of *Aspergillus*. Wehmer (1901) grouped *Aspergilli* into micro and macro *Aspergilli* based on the sizes of the fruiting parts. *A. fumigatus*, *A. nidulans* and *A. versicolor* were grouped under micro *Aspergilli* due to smaller heads that formed columnar masses. *A. niger*, *A. clavatus*, *A. ochraceus* and *A. wentii* were placed under macro *Aspergilli* due to their globose and large heads. *A. flavus* and *A. candidus* produced heads of different sizes and shapes in the same colony.

In all *Aspergilli,* foot cells bear the conidiophore perpendicular to their long axis. Entire aerial hyphae composed of continuous series of foot cells each bearing a conidiophore is the characteristic feature of *A. oryzae*. Conidiophores enlarge towards the apex where it dilated to form the vesicle. In *A. glaucus,* conidiophore is articulate which is characterized by septate conidiophores with distinct cells. In *A. niger*, the ends of conidiophore splinter like bundles of lath. The vesicle may be globose, hemispherical, elliptical or clavate and it provides surface for the attachment of spore bearing sterigmata. Some species bear a single layer (uniseriate) of sterigmata, while in others, first series of cells that are perpendicular to the surface of the vesicle are primary sterigmata, which bear two or more cells called secondary sterigmata. These in turn bear a chain of conidia. Some species of *A. niger* group show increase in the length of the primary sterigmata after full formation of secondary sterigmata. The primary sterigmata are often called basidia and the secondary sterigmata the phialides.

The conidium or spore is separated from the sterigmata by a septum and conidia are connected in chains. Shape and size of conidia are characteristic of species. In most of the species, the multinucleate vesicles provide the initial complement of one or more nuclei in each sterigmata. In species containing biseriate sterigmata, each

secondary sterigmata receive one or more nuclei from the primary sterigmata. In most of the species, conidia are uninucleate, however in *A. glaucus* group, up to 12 nuclei have been seen. Iizuka (1955) classified species of *Aspergillus* based on conidial walls.

NATURAL VARIATION IN *ASPERGILLUS*

Aspergilli are characterized by vast diversity and variability, since they are not only isolated from nature but also mutated by using different stimuli. Species of *Aspergillus* are cosmopolitan and adopted to wide environmental conditions. Naturally isolated molds show variations that differ in degree rather than in basic characters. Natural variants can be frequently obtained in a laboratory by selective isolation from sectors or areas of atypical growth or by single spore isolations. *A. fumigatus* mut. *helvola* and *A. nidulans* mut. *albus* represent natural mutations that originated in the absence of artificial stimuli (Raper and Fennell, 1965b).

Some species and strains are subject to continuous variation in character and growth rate that appear abruptly as sectors or over growths or gradually develop as an alteration in the whole culture. Strains of *A. alliaceus* and *A. ochraceus* showing significant difference in the production of Sclerotium were obtained after selective and successive sub culturing. Greene (1933) isolated 448 single spore cultures from *A. fischeri*, among which one group produced very large, scattered perithecia against typical many small perithecia and the other groups produced conidial structures in profusion, but forming few perithecia.

In 1933, Buller reasoned variability in molds to the unmeasured possibilities of nuclear and cytoplasmic disturbance due to hyphal anastomosis (quoted from Raper and Fennell, 1965b). Vegetative hyphae derived from same mycelium (single spore) or different mycelia, produce branches which fuse and sexual process occur either before or after fusion. Anastomosis is usually observable in the fast growing area where colony develops by many spores placed as an inoculum. Anastomosis also leads to

heterokaryosis, which is responsible for many apparent changes. Heterokaryons exhibit "hybrid vigour" that express as enhanced growth (Dodge, 1942).

Wide variations among large number of isolates of any particular species can be expected in colour, amount of sporulation, general habit of growth etc, since the strains represent intermediate stages between the extremes. Typically, *A. terreus* colonies are plane, cinnamon in colour and conidial heads directly arise from substratum with heavy sporing. But typical *A. terreus* var. *boedijni* (Blochwitz, 1934) colonies are much brighter, approximating xanthine orange. In *A. terreus* var. *floccosus* (Shih, 1936), form and colour of conidial heads are typical but, their number is greatly reduced and they are born upon aerial hyphae. *A. terreus* var. *aureus* possess abundant close mycelia and bear numerous pale coloured conidial heads with bright yellow vegetative mycelium. However these strains are not sufficiently distinct from the type strain for placement as separate species.

In *A. sydowi* species, variants that exhibited gradations in colony appearance were isolated. *A. fumigatus* type culture showed heavily sporing velvety colonies, whereas other variants isolated from nature produced floccose colonies with comparatively less conidial heads. Hence, variants arise through gradual changes from well identifiable strains of species. Sudden sharp breaks have been reported from parent cultures leading to the occurrence of altered strains called mutants. In 1937 and 1939 Yuill isolated buff coloured mutant of *A. fumigatus* (*A. fumigatus* mut. *helvola*) and white spored mutant of *A. nidulans* (*A. nidulans* mut. *alba*) respectively (quoted from Raper and Fennell, 1965b). They were thought to arise due to natural phenomena with out the application of any artificial stimuli. Tan head mutant was also isolated from typical black head producing parent strain of *A. niger* (*A. niger* mut. *cinnamomeus*).

INDUCED VARIATIONS IN *ASPERGILLUS*

Artificially imposed stimuli lead to striking mutations in several species of *Aspergillus*. Schiemann (1912) produced two striking mutants by treating *A. niger* strain to different concentrations of potassium dichromate. According to colour, she

designated them as *A. fuscus* (= *A. niger* mut. *schiemanni*) and *A. cinnamomeus* (= *A. niger* mut. *cinnamomeus*).

Barnes (1928) produced series of variations by exposing spores of *A. glaucus* to heat. Galloway (1933) induced marked variation in the colonies of *A. terreus* by using 0.003-0.005% salicylanilide. Thom and Steinberg (1939) and Steinberg and Thom (1940a and 1940b) obtained mutants that showed changes in head colour or conidiophore or fruit forming or mycelial colour in *A. niger* by using chemical stimulus like sodium nitrite.

Whelden (1940) obtained mutants in *A. niger* that possessed brown shaded heads rather than black and larger conidial structures by subjecting conidia to low voltage cathode ray bombardment. Raper *et al.* (1945) obtained tan coloured conidial head producing mutants from *A. terreus* by irradiating spores with ultra violet rays. Same researchers isolated physiological mutants which were not able to utilize nitrate nitrogen but able to grow and sporulate on ammonia nitrogen. These mutants were unable to synthesize thiamin. In *A. niger*, extensive heterokaryosis studies by various authors suggested ultra violet radiation induced multinucleate conditions in conidia. The explanation was that due to blockage of different steps of enzymatic reactions by the mutation, nuclei of two mutants enter the same conidiophore to complement the blocked enzymatic step. Parasexual cycle of *Aspergilli* also consist of heterokaryosis as an essential step (Roper*,* 1952). Mutants affected in general secretory pathway were isolated by subjecting spores of *A. niger* to UV mutagenesis and selection against temperature (Gordon *et al.,* 2000).

Physiological mutations have credible importance in strain improvement for various fermentation processes. Hence strain improvement through induced mutation is a regular practice in all industrial processes that use microbes. In all these studies, mutations were induced with ultra violet rays, ionizing radiations and chemicals like lithium chloride, camphor, nitrous oxide etc. *A. niger* and *A. carbonarius* were improved

for amylase and pectinase production in this laboratory by subjecting the spores to UV rays (Suresh, 1999; Dubey, 1999; Kavitha, 2000).

CARBON METABOLISM IN *ASPERGILLUS*

McCullough *et al.* (1977) reviewed carbon metabolism in the filamentous fungi. Blumenthal (1965) documented the occurrence of Embden-Meyerhoff-Parnas and the pentose phosphate pathways in the *Aspergillus* species. Niederpruem (1965) reported the presence of enzymes of the tricarboxylic acid cycle. Kornberg (1966) illustrated the importance of mutants in the study of carbon metabolism mainly in relation to enzymes of physiological importance.

Wide range of organic compounds are utilized as carbon source for growth by *Aspergillus* species (McCullough *et al.,* 1977). Studies on carbon sources indicated adaptive metabolism, since they lead to growth after an obvious lag period. Wild type strains are little affected in the carbon source utilization by the nature of carbon source and some mutants isolated for deficiencies in carbon metabolism showed difference in growth on nitrate or ammonium (Hankinson, 1974). However nature of the carbon source decided nitrogen regulation and some aminoacids served both as nitrogen source and moderate or poor carbon source for growth (McCullough *et al.,* 1977).

Aspergillus possesses both inducible and constitutive uptake systems. The regulation of uptake of hexoses by a metabolic derivative of acetate i.e. acetyl-CoA was demonstrated by Romano and Kornberg (1969). Hynes (1975) described utilization of amides as carbon source. Inhibition and repression between two carbon sources was demonstrated using mutants (Bailey and Arst, 1975). Regulations of main catabolic enzymes or specific uptake systems were suggested as causes for the differences in the growth of *Aspergilli* on different carbon sources (Romano and Kornberg, 1968; Romano, 1973).

Two types of regulations for carbon utilization involving enzymes and permeases were demonstrated. They are induction of enzymes involved in carbon metabolism and

the carbon catabolite repression, where certain carbon sources, generally the better ones prevent the synthesis of enzymes and permeases required for the utilization of poorer carbon sources

NITROGEN METABOLISM IN *ASPERGILLUS*

Nitrogen metabolism in *Aspergillus* was reviewed by Kinghorn and Pateman (1977). Formamidase, which act on formamide, is a non inducible enzyme produced by *Aspergillus*. Production of this enzyme was subject to ammonium repression and weak carbon repression. Acetamidase hydrolyses aliphatic amides including acetamide to utilize them as nitrogen or carbon sources. This enzyme is inducible and is subject to carbon and nitrogen repression. Another amidase with similar substrate specificity as acetamidase, found to be responsible for the utilization of amides as sole nitrogen sources and subject to only ammonium repression has also been reported from the fungus. In *A. nidulans,* activities of the nitrate reductase and nitrite reductase enzymes (necessary for reduction of nitrate) and hydroxyl amine reductase were found to be induced by nitrate and nitrite and repressed by ammonium (Cove and Pateman, 1963; Pateman *et al*., 1964; Pateman and Cove, 1967).

Mutational studies showed a role for eighteen genes in the utilization of nitrate/nitrite in *A. nidulans* (Gross, 1969; Pateman and Kinghorn, 1976). Mutant strains of *niaD* gene showed inability to utilize nitrate as nitrogen source (Cove and Pateman, 1963; Cove, 1966; Pateman and Cove, 1967; Cove and Pateman, 1969) due to lower levels of nitrate reductase activity compared to the wild type. Mutant strains isolated for their inability to utilize nitrate were able to utilize nitrite and also grow on urate, but could not utilize hypoxanthine as nitrogen source. These mutants designated as *cnx* also showed impaired activity of both nitrate reductase and xanthine dehydrogenase. It was proposed that the *cnx* genes determined the production of cofactor that is required for xanthine dehydrogenase and nitrate reductase activity (Pateman *et al*., 1964).

Evidence was presented for chlorate interference, with the break down of organic nitrogenous compounds, as a cause for chlorate toxicity in *A. nidulans* (Cove, 1976). A

class among mutants affected in the utilization of nitrate as sole nitrogen source was unable to grow on nitrite (*niiA*). These *niiA* mutants produced nitrate reductase but not nitrite reductase (Pateman *et al*., 1967). Mutants that lacked nitrate and nitrite reductases were not able to utilize both nitrate and nitrite. *A. clavatus* utilized ammonium in preference to other nitrogen sources (Robinson *et al*., 1974) due to repression of systems that were required for the utilization of other nitrogen sources by ammonium.

Ability of *A. nidulans* to metabolise different amides as sole nitrogen source has been studied extensively (Hynes and Pateman, 1970). Urea is degraded to ammonium by urease in one step reaction. It was found that specific transport system with high affinity helped the fungus in the uptake of urea and it was lost in *ureA* mutants. Mutation at the locus *asnA* lead to the strains that needed asparagine as supplement and they showed extremely poor growth on ammonium, glutamate, glutamine or aspartate but grown like wild type on asparagine.

STARCH HYDROLYSING ENZYMES OF *ASPERGILLUS*

There are several reviews on the starch hydrolysing enzymes (amylases) of *Aspergillus* in the literature (Vihinen and Mantsala, 1989; Saha and Zeikus, 1989; Nigam and Singh, 1995; Reilly, 1999; Pandey *et al.,* 2000; Ravi-Kumar and Umesh-Kumar, 2004). In 1896, Takemine marketed Takadiastase (a mixture of hydrolytic enzymes obtained after growing *A. oryzae* on wheat bran) in the West (Barbesgaard, 1977). It was perhaps the first marketed amylase. *Aspergillus* enzymes still play an important role in industry, even though many other microbial sources for the enzyme were found. Most important of the *Aspergillus* amylases are the α-amylases and glucoamylases, since they are used in hydrolysis of starch in the industry.

The important application of *Aspergillus* amylases in the industry is for the production of sugar syrups and alcohol. Earlier, starch was processed using concentrated acid at high temperature and pressure. The yield of products was not consistent by this method and there was major starch loss due to retrogradation

(spontaneous precipitation of starch). Advent of amylolytic enzymes of microbial origin replaced starch processing by acid hydrolysis since product yield was consistent with defined end points. In alcoholic fermentation, pre-cooked starchy materials are liquefied with α-amylase and they are subjected to fermentation by yeast after saccharifying with glucoamylase.

Glucose syrups (96-97.5%) are produced from starch in two stages. In the first stage, aqueous starch slurry (30-50% DS) gelatinized at temperatures ranging from 105-110^{0}C is liquefied to reduce viscosity using thermostable α-amylase to evolve α-limit dextrins. In the second stage, glucoamylase, β-amylase or Taka-amylase are used to produce glucose and maltose syrups from the dextrins. Glucoamylases and maltogenic amylases act at acidic pH but at lower temperatures. Hence, the dextrin solution obtained from the thinning reaction is acidified to the required pH and cooled prior to enzyme action. *Aspergillus* is the major source for commercial glucoamylase and Taka-amylase. Production of high fructose syrups involves a three stage processing of starch. The first two stages of starch processing to generate glucose is as described above. In the third stage of processing, isomerisation of glucose to fructose is achieved using glucose isomerase.

High maltose syrups containing 45-60% maltose (10-25% maltotriose, 0.5-3% glucose and 35-50% higher saccharides), extremely high maltose syrups with 70-85% maltose (8-21% maltotriose, 1.5-2% glucose and 45-60% higher saccharides) and high conversion syrups containing 30-47% maltose (8-15% maltotriose, 35-43% glucose and 60-70% higher saccharides) are also obtained by using glucoamylase in addition to maltogenic amylases (Saha and Zeikus, 1987). Other than for sweetness, maltose syrups are used for their functional properties like low hygroscopicity, low viscosity in solution, resistance to crystallization, reduced browning capacity, good heat stability etc. in foods. These properties of maltose syrups have applications as moisture conditioners, crystallization inhibitors, stabilizers, carriers and bulking agents (Saha and Zeikus, 1987).

Amylolytic enzymes are widely used in modifying texture in breads. They find an important application in biochemically modifying barley to substitute malt in the brewing process for the manufacture of beer. Staling, that makes bread hard and brittle is due to reversion of starch granules from soluble to insoluble form (retrogradation). In bread making, thermostable α-amylase reduces the retrogradation of starch to improve the shelf life of breads. α-Amylases, due to their stability at high temperatures, are able to work after the baking process, breaking the starch to smaller length dextrins preventing retrogradation. Production of dextrins from starch reduces high dough viscosity in bread and accelerates the fermentation process by yeast thereby, improving the crust formation. Thus fungal amylolytic enzymes play vital role in various industries.

ASPERGILLUS **PROTEASES**

Proteases are enzymes that hydrolyse proteins to yield amino acids and peptides. *A. awamori*, *A. niger* and *A. saitoi* produce extracellular acid proteases. Alkaline proteases have been also reported from *A. niger*, *A. flavus, A. oryzae, A. sojae* and *A. sulphureus* (Bossman, 1973; Bretschneider *et al.,* 1973; Turkova *et al.,* 1972). The synthesis of acid protease by *A. niger* is constitutive and is synthesized *de novo* in response to sulphur limitation in the culture (Tomonaga *et al.,* 1964). Extracellular neutral and alkaline proteases of *A. nidulans* are non inducible and they are repressed by carbon, nitrogen and sulphur metabolites (Cohen, 1973a). In *A. oryzae,* de novo synthesis of extracellular protease occurred in response to carbon catabolite derepression (Klapper *et al.,* 1973). Strains of *A. nidulans, A. flavus, A. fumigatus, A. oryzae, A. tamarii* and *A. terreus* produced extracellular neutral and alkaline protease in response to nitrogen starvation but not in the presence of ammonium (Cohen, 1977). Normal growth and differentiation of extracellular protease defective mutant of *A. nidulans* demonstrated dispensability of the enzyme (Cohen, 1973b) and it was concluded that the region behind the hyphal tip was active in secretion of protease in *A. niger* (Yanagita and Nomachi, 1967).

Three acid carboxypeptidases (I, II and III) were reported from *A. oryzae* (Nakadai *et al.,* 1972a,b,c). Leucine aminopeptidase IV of 130 kDa was also reported

from *A. oryzae* (Nakadai and Nasuno, 1977). An acid carboxy peptidase with molecular weight of 125 kDa by gel filtration and 72 kDa by SDS disc gel electrophoresis and isoelectric point of 4.07 was characterised in *A. saitoi* ATCC14322 (Takeuchi and Ichishima, 1986). Subtilisin like protease and aspartic protease genes *pep* D and *pep* E from *A. niger* have been cloned and characterised (Jarai *et al.,* 1994a,b). Thermostable acid protease with temperature and pH optima of 60^0C and 3.0-4.0 respectively was reported from *A. niger* (Singh *et al.,* 1994). Other reports of carboxypeptidases of *A. niger* are Kumagai *et al.* (1981) and Degan *et al.* (1992). A 95 kDa lysine aminopeptidase that act at pH between 7.5-8.0 was reported from *A. niger* (Basten *et al.,* 2001).

Proteolytic degradation of heterologous proteins expressed in *A. niger* by host proteases reduced the yield of target proteins (Archer *et al.,* 1992). Bovine chymosin yields in *A. awamori* were improved by deleting the *pep* A gene that coded for aspergillopepsin (Berka *et al.,* 1990). UV-derived protease deficient mutants of *A. niger* resulted in reduced degradation of target proteins (van den Hombergh *et al.,* 1995). Acid proteases have been shown to be responsible for the formation of multiple forms of glucoamylase in *A. niger* (Aalbaek *et al.,* 2002).

CULTIVATION OF *ASPERGILLUS*

Growth of *Aspergillus* as a monoculture for the production of metabolites is long known. Cultivation of *A. oryzae* for conversion of cereal preparations prior to their alcoholic fermentation is one of the early mould fermentations used by Japanese. Another early mould fermentation is utilization of *A. niger* to hydrolyse ester form of gallic acid found in gall nuts. Use of *Aspergillus* for enzyme fermentations trace back to 1896, when Takemine marketed Takadiastase after growing *A. oryzae* on wheat bran (Barbesgaard, 1977). *Aspergillus* can be cultivated either by solid state or submerged cultures.

Spore suspensions are commonly used as inocula in fermentations. But there are problems of clumping and dormancy with spores. Hence, adequately grown mycelial

inocula are used in scale-up studies. Growth, colony formation, genotype, hyphal branching and hyphal interactions are important biological factors that influence the productivity in fermentations. In solid state, filamentous fungi develop aerial, surface and submerged hyphae, where as in submerged culture a variety of morphological forms result due to three dimensional growth of hyphae. The aggregation of swollen spores or newly germinated spores lead to colony formation. Fungal morphology during submerged culture was described as flocculent, pelleted, granular and loose pellets (Whitaker and Long, 1973) depending on aggregation of hyphae. The suitability of any strain for fermentation is generally determined by their genotype since range of strains show varied response to a particular environment. It was speculated that features of a colony during submerged fermentation of filamentous fungi are determined by branching characteristics of hyphae (Butler, 1966). In 1949, Foster reported that short highly branched swollen hyphae result under adverse conditions like lack of oxygen, high acidity, high concentrations of ferricyanide and toxic metals (quoted from Cocker and Greenshields, 1977). Any adhesion between hyphae affects the mycelial response to agitation. Machek and Fencl (1973) found that hyphae could elongate according to linear or exponential rules, but there were also many intermediate types of growth. Though their graph showed the doubling time of 1.7 h Trinci (1971) calculated a mean doubling time of 2.54 h. Contradiction may be due to differences in strains or conditions. It was shown that on solid medium, individual hyphae extend according to linear kinetics (Trinci and Banbury, 1967). However, Smith (1924) reported that individual hyphae longer than 200 μm were able to extend exponentially on solid media.

Media composition, metabolite production and nutrient uptake influence the growth of the fungus during fermentation. Trace metals have profound effect on the growth of *Aspergillus* species. Calcium, copper, iron, mercury, zinc, lead, lithium, sodium, rubidium and aluminium will affect growth of *Aspergillus* species. In liquid cultures several of these ions act as flocculating and chelating agents altering the physico-chemical balance of the medium. Deficiency or excess of most of these cations result in morphological aberrations, sub-optimal fermentation and poor product accretion (Cocker and Greenshields, 1977). Presence of individual amino acids in

addition to inorganic nitrogen sources has stimulatory effet on the growth rate (Kasatkina, 1961). Derivatives and analogues of nucleic acid bases like caffeine, theobromine, 5-bromo uracil etc. inhibit growth by preventing nucleotide base synthesis or substituting nucleotide base and leading to consequent mutagenic effects. Short chain fatty acids like formic, acetic, butyric, propionic, capric, valeric, levulenic etc. inhibit growth of *Aspergillus* species by preventing uptake of nutrients, phosphate and sulphate at the cell surface (Cocker and Greenshields, 1977). Higher fatty acids like oleic and linoleic but not palmitic acid are also utilized as sole carbon source by *A. niger*. Hexoses are as good as pentoses for fungal growth with certain exceptions, which depend on the strain. Disaccharides and starch are good sources of carbon for growth except lactose, which gives poor growth. Another important carbohydrate parameter is the level of sugar acids and acids of TCA cycle in the medium. Antifoams and detergents reduce oxygen transfer in submerged fermentors and it was found that 0.05-0.1% non ionic detergents encouraged filamentous growth of *A. niger*. Tween 20 was found to inhibit growth since it lead to large pellet formation (Cocker and Greenshields, 1977).

The simplest fermentor configuration suitable for mould culture is open tray or dish used in Koji process. Various adaptations like flat bottles and flexible pillow tanks have also been used. The stirred tank reactor is most widely used reactor configuration. Other alternate type reactors are pressure cycle fermentor, airlift, bubble column and loop fermentors with mechanical or pneumatic recycling (Cocker and Greenshields, 1977). In stirred fermentors, the problems are compounded by the typical suspension rheology as for filamentous fungi due to high viscosity and non Newtonian rheology. Hence, submerged fermentations with filamentous fungi are subject to poor mixing, stalled impellers, wall growth, dead zones and blockages. Biodisc fermentors are adopted for filamentous fungi like *Aspergillus* species, where fungus grows as a film on the rotating discs submerged partly in the medium. This facilitates gas transfer in a simple fashion. In air lift or bubble column designs, due to comparatively low sheer rates, mycelial fragmentation is lesser than in stirred fermentors (Cocker and Greenshields, 1977). In submerged cultures, concentration of

oxygen and carbon dioxide dissolved in the medium has to be controlled within critical limits to avoid deleterious effect. Heterogeneity occurs even in stirred fermentors at high biomass concentrations due to dead spots. Air lift and bubble column fermentors involve heterogeneity that associated with colony sedimentation rates. More heterogeneity is found in solid state tray cultures and biodisc fermentors where the mycelium forms a pellicle (Cocker and Greenshields, 1977).

It is difficult to compare product titres in solid state and submerged processes due to differences in the physical state of the media. The expenditures in solid state are lower than those in submerged fermentation as shown in case of amyloglucosidase plants (Ghildyal *et al.,* 1985). The energy input in solid state fermentation involving tray and other static bio reactors is lower due to absence of agitation of medium (Lonsane, 1994) and surface aeration is sufficient in tray fermentors (Lonsane *et al.,* 1985). Bacterial contamination was controlled efficiently in solid state fermentation due to lower water activities (Lonsane, 1994). The solid nature of the substrate, limited use of water and static conditions lead to easier induction of spores in solid state fermentation (Laroche and Gros, 1986) and spore formation can be suppressed by using medium at higher depth or by agitating the medium at higher rates (Lonsane, 1994). End product inhibition and catabolite repression are largely absent in solid state cultivation. Proteinases when produced by submerged fermentation, invariably contain carbohydrate moiety (Aunstrup, 1980), but such carbohydrate is absent when produced in solid state (Tsujita and Endo, 1976). Enzyme production phase is longer in solid state fermentation for the bacterial α-amylase production compare to submerged fermentation (Ramesh and Lonsane, 1990). Processes have also been developed for large scale production of pectinases and amyloglucosidases by solid state fermentation (Ghildyal *et al.,* 1981; Kavitha and Umesh-Kumar, 2000; Ravi-Kumar *et al.,* 2002; Eugeneraj *et al.,* 2003). Submerged fermentations for the production of amylases and pectinases have also been reported (Kundu and Das, 1970; Hayashida, 1975; Saha *et al.,* 1979; Morkeberg *et al.,* 1995; Eugeneraj *et al.,* 2002; Venkatesh *et al.,* 2002; Aalbaek *et al.,* 2002). In order to quantitate growth of the fungus during solid state fermentation, this laboratory has developed an ELISA procedure (Dubey *et al.,* 1998).

THE PROBLEM

Food processing industries use amylases of microbial origin for the production of sweeteners and syrups, in the manufacture of modified foods, for the production of bakery products and in brewing. Several industrial applications of these enzymes and the awareness that the amylases produced by microorganisms vary in the properties of end product formation from starch and its derivatives have resulted in the search for newer enzymes of microbial origin. Typically the amylases produced by the fungus *Aspergillus niger* are important because basic studies from this laboratory (Dubey *et al*., 2000) have shown that the several amylases arise by probable proteolytic processing of a precursor enzyme. Since that study made an observation on the presence of Taka-amylase in the fungal culture filtrates and the report on the production of this amylase by *A. niger* was new to literature, studies were undertaken to confirm the earlier observation by purifying and characterizing the enzyme protein. The enzyme had a molecular mass of 53 kDa by SDS-PAGE and reacted with starch to produce α-maltose and α-maltotriose as products. The **first part** of the thesis describes characterization of the enzyme as a maltogenic α-amylase.

Maltogenic α-amylase was shown to be a product of protease processing (Dubey *et al*., 2000) of starch hydrolysing enzyme (Suresh *et al.*, 1999). Since the earlier study did not identify the protease involved, attempts were made to locate the protease activity that caused the processing of amylase in the fungal cell. By selection for effect on secretory pathway, a mutant overproducing maltogenic α-amylase and protease was isolated. Based on the studies using the mutant and the identification of *in situ* protease activity in the precursor amylase it was concluded that amylase processing occurred due to an autocatalytic process. These details are described in the **second part** of the thesis.

Studies using the mutant isolated in this investigation showed that the strain was promising since it could secrete higher concentrations of maltogenic α-amylase when grown in culture. Hence media components were standardized for optimizing the

enzyme yield and using the data obtained, fermentation was also scaled up. **Third part** of the thesis describes these results.

MATERIALS AND METHODS

General methods used for studies involving microorganisms were routinely followed. Culture media were prepared in glass distilled water and then pH adjusted with dilute hydrochloric acid or sodium hydroxide. Cultures used in this study were maintained at 4^0C. Ultra pure water obtained from Milli Q-system was used for enzyme assays, protein estimations and all other analytical procedures. Media, glassware and plastic ware were sterilized at 15 lbs pressure and 121^0C for 20 min.

CHEMICALS

Enzyme substrates like Lintner's soluble starch, casein (Hammarsten grade), gelatin and 4-chloro-1-naphthol were procured from BDH Laboratories, Poole, England and Sigma Chemicals, St. Louis, USA. Standards for enzyme assays and protein estimation, glucose, maltose, tyrosine and bovine serum albumin were obtained from Sigma Chemicals. Detection reagents like 3,5-dinitrosalicylic acid, Folin-Ciocalteu's phenol reagent, silver nitrate, Coomassie Blue G 250, R 250 and Amido black were obtained from Loba Chemie, India and Sigma Chemicals.

Reagents used for electrophoresis, acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate, N, N, N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate, dithiothreitol, and mercaptoethanol were from BDH Laboratories and Sigma Chemicals. Termamyl and glucose oxidase peroxidase were from Novo Nordisk Laboratories, Denmark and J. Mitra and Co., New Delhi, India. Glucosidase inhibitor, acarbose tablets were from Bayer pharmaceuticals, Mumbai, India.

Serological reagents like goat anti-rabbit IgG tagged with biotin and avidin peroxidase were also from Sigma Chemicals. Nitrocellulose membranes were procured from Amersham Pharmacia Biotech, UK. Buffer salts like Tris, sodium acetate and the

detergent Triton X-100 were from Sigma Chemicals. Corn starch, corn flour and corn steep liquor were market samples. Peptone and yeast extract were from Himedia, Mumbai, India.

ORGANISM

Aspergillus niger van Tieghem used in this study was isolated in this laboratory and maintained at culture collection of Central Food Technological Research Institute, Mysore, India under the accession No. CFTRI 1105 (Ramasesh *et al.,* 1982a).

MEDIA

Parent and mutant cultures were maintained on potato starch agar slants at 4^0C. They were sub cultured periodically every 2 months and 4-5 day old slants were used for experiments.

Potato starch agar (PSA)

	g/L
Potato	250
Starch	10
Agar agar	20
pH	5.5

For submerged cultures, the medium contained the following components.

	g/L
Corn starch	100
Peptone	10
Yeast extract	5
pH	5.5

Corn starch was dextrinised with Termamyl at boiling temperature prior to addition of other components. Cultures were grown at 30^0C on rotary shaker (250 rpm)

for 24-120 h. Culture filtrates obtained after filtration through Whatman No.1 paper were used as crude enzyme.

ENZYME ASSAYS

Schimadzu UV-visible recording spectrophotometer (UV-160A) was used for all spectrophotometric analyses.

Amylase activities of culture filtrates and solid-state extracts were assayed using starch and maltose as substrates by determining either reducing sugar equivalents or glucose formed. Reducing sugars that formed due to amylase activity were estimated by dinitrosalicylic acid method and glucose was estimated by glucose oxidase-peroxidase method.

Dinitrosalicylic acid method (Bernfeld, 1955): Dinitrosalicylic acid reagent contained following constituents

	g/L
3,5-dinitrosalicylic acid	10
Sodium hydroxide (anhydrous)	16
Sodium-potassium tartarate	300

Dinitrosalicylic acid and sodium hydroxide were dissolved in 400 to 500 ml water. Then 300 g of sodium-potassium tartarate was added slowly and the volume was made to 1L with water. Reducing sugars were estimated by adding 1 mL of appropriately diluted sample to 1 mL dinitrosalicylic acid reagent taken in test tubes. The mixture was boiled for 5 min, cooled and the volume was made to 7 mL using distilled water. The colour intensity was measured at 540 nm. The difference in optical densities of the blank and the test samples were read against the standard graph prepared by using maltose (1 mg/mL stock).

Glucose oxidase-peroxidase method (Dahlqvist, 1961): Glucose oxidase-peroxidase reagent was prepared by dissolving 125 mg glucose oxidase and 0.5 mg

horse radish peroxidase in 100 mL of sodium phosphate buffer (0.5 M, pH 7.2) that contained 0.5 mL *0*-dianisidine (1% solution in 95% ethanol). The glucose in samples was estimated after adding 10 µL of appropriately diluted sample to 3 mL of the reagent. Following incubation at 37^0C for 1 h, the colour intensity was measured at 420 nm against the blank (that contained no sample). Glucose was quantitated using a standard graph prepared using 'ANALAR' glucose (1 mg/mL stock).

Protease: Protease activities in the culture filtrates were assayed by determining tyrosine equivalents due to protease action on casein. Released tyrosine equivalents were estimated using Folin-Ciocalteau phenol reagent (Ichishima, 1970). The released tyrosine equivalents were measured by adding 2.5 mL Na_2Co_3 (0.4 M) and 0.5 mL Folin-Ciocalteau phenol reagent (1:5 diluted with water) to 0.5 mL of sample and incubating at 30^0C for 30 min. The intensity of the colour produced was measured at 660 nm against the blank. Tyrosine (0.02 mg/mL) was used as standard.

SUBSTRATES

Granular starch (2%), amylose (2%) and amylopectin (2%; Sigma) were used as substrates for estimating amylase activity. Substrates were prepared by making a slurry in cold water and then gelatinized by heating. The volume was made to 100 mL with 1.0 M sodium acetate buffer (pH 4.3) to obtain a substrate solution in 0.1 M buffer. Maltose (2%) was prepared by dissolving the sugar directly in sodium acetate buffer (0.1 M; pH 4.3). All preparations were made in standard flasks.

Casein (2%) was used as substrate for protease assay. Weighed casein (Hammarsten grade) was suspended in 10 mL distilled water for 15 min. To this, 60 mL of water and 1.5 mL of 1.0 N HCl were added and casein was dissolved by stirring the mixture on boiling water bath. The final volume was made to 100 mL with water in a standard flask.

DETERMINATION OF ACTIVITY

The maltogenic α-amylase activity was determined at 40^0C and pH 4.3 by estimating released reducing sugars as maltose equivalents from starch. Enzyme reaction was carried out using 5 mL substrate and 1 mL appropriately diluted enzyme. For some of the reactions, the enzyme was diluted 1:2 with 0.125 % acarbose containing sodium acetate buffer (0.1 M; pH 4.3). Reaction was stopped after 30 min with 1 mL NaOH (4 M). Substrate and enzyme blanks were used as controls. Activity corresponded to μ moles of maltose equivalents produced min^{-1} mL^{-1} of culture filtrate.

Starch hydrolysing enzyme and glucoamylase activities were determined by glucose oxidase-peroxidase method by quantitating glucose formed due to amylase reaction at 25^0C (Suresh, 1999). One mL substrate (2% maltose) was treated with 1 mL of appropriately diluted enzyme for 30 min and the reaction was stopped with 3 mL of Tris buffer (1.66 M, pH 7.6). This sample was used for glucose estimation maintaining enzyme and substrate blanks. Activity corresponded to μ moles of maltose degraded min^{-1} mL^{-1} of culture filtrate.

Protease activity was determined by quantitating released tyrosine equivalents after enzyme action on substrate at 30^0C for 46 h. One mL substrate was treated with appropriately diluted 0.2 mL enzyme and the reaction was stopped with 1.2 mL trichloroacetic acid (0.4 M). The mixture was filtered through Whatman No.1 paper and 0.5 mL of filtrate was used as the sample for quantitating released tyrosine equivalents as described above. Activity corresponded to μ moles of tyrosine released min^{-1} mL^{-1} of culture filtrate (Ravi-Kumar *et al.,* 2004).

PROTEIN ESTIMATION

Protein was quantitated by dye binding method (Spector, 1978). The reagent consisted of Coomassie Blue G 250 prepared in 3 % perchloric acid (optical density at 465 nm between 1.3 and 1.5). Protein was quantitated by adding appropriately diluted sample to 1.5 mL of the reagent and diluting to 3 mL. Colour was measured at 595 nm

against the blank and optical density was read against bovine serum albumin (1 mg/mL) standard.

Specific activity corresponded to activity mg $^{-1}$ protein.

PRODUCT ANALYSIS

Paper Chromatography (Trevalyan *et al.,* 1951): Standards and aliquots of the reaction mixture (substrate treated with amylase) were spotted on Whatman No. 1 chromatography paper and ascending run was carried out in butanol: pyridine: water (6:4:3) solvent system. After 5 repeated runs, the sugars were visualized by staining with alkaline silver nitrate reagent.

High performance liquid chromatography (HPLC): Sugars in the product after starch hydrolysis with enzyme were separated in Supelco LC-NH_2 column (25x4.6 mm, 5 ì m) using acetonitrile: water (80:20) as the solvent system with a flow rate of 1 ml min^{-1} (Suresh *et al*., 1999a) and identified by HPLC (Shimadzu SCL-6A HPLC) equipped with RID-6A detector.

ELECTROPHORESIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed in 10 % gels according to Laemmli (1970). Protein samples were prepared in sample buffer that contained reducing agents (dithiothreitol and mercaptoethanol) under denaturing conditions. The proteins were visualised after staining with silver nitrate (Morrissey, 1981).

Two dimensional SDS-PAGE (Ravi-Kumar *et al.,* 2004): After the first electrophoretic run in 10% polyacrylamide gels at 100 mA using Tris-glycine buffer (0.025 M; pH 8.8), the lane containing the separated proteins was cut and used for the second electrophoretic run. 10% SDS-polyacrylamide gel was cast after placing the lane horizontally and electrophoresis performed as above. The proteins in gels were visualised after silver staining.

Zymograms: Amylase activities were detected as enzyme zymograms after separating the proteins by SDS-PAGE in Lintner's soluble starch (0.1%) incorporated 10% gels. The SDS sample buffer used contained no reducing agents. The samples diluted in the buffer were used directly for electrophoresis without boiling. The amylase proteins were renatured by incubating in sodium acetate buffer (0.1 M, pH 4.3) for 30 min at 30^0C. For studying the effect of acarbose on maltogenic α-amylase, proteins were renatured in 0.125% acarbose containing sodium acetate buffer. Amylase activities were detected as zones of clearance after staining with iodide/iodine solution (0.6 g I_2 and 6 g KI L^{-1}).

Enzyme zymograms for protease activities were performed after separating the proteins by SDS-PAGE in 10% acrylamide gels containing gelatin (0.1%) under non reducing conditions as described above. SDS was removed from the gels by incubating them in Triton-X-100 (2.5%) containing acetate buffer (0.02 M, pH 4.0) for 1 h. The protein was renatured by incubating the gels in buffer for 5 h at 30^0C. Sodium acetate buffers of different pH (3.5-6.0) were used for identifying pH optimum for protease activity. Gels were fixed and stained for 30 min in amido black (0.1%) solution made in methanol: acetic acid: water (30:10:60). Protease activity was identified as zone of clearance after destaining the gels in the same solution without amido black (Christa and Eugene, 1980).

Molecular mass (kDa) determination: Molecular mass of the proteins were determined by running the following standards alongside the protein samples

Molecular weight standards (Sigma)	**kDa**
Bovine milk α-lactalbumin	14.2
Bovine erythrocytes carbonic anhydrase	30.1
Egg albumin	43.0
Bovine serum albumin	67.0
Rabbit muscle phosphorylase B	94.0

Based on the migration of standards, the molecular mass of the sample was determined using Bioprofil Image Analysis System (Vilber Lourmat, France).

PURIFICATION OF PROTEIN

Maltogenic α-amylase protein was purified from 72 h culture filtrate by ammonium sulphate fractionation. The enzyme protein was fractionated at 60% ammonium sulphate saturation and it was dissolved in sodium acetate buffer (0.1 M, pH 4.3). After dialysis against the same buffer at 4^0C to remove ammonium sulphate, the purity of the sample was checked by performing SDS-PAGE and silver staining.

The amylases fractionated in the 60 to 90 % ammonium sulphate fraction were dialysed against the sodium acetate buffer (0.02 M, pH 4.0) at 4^0C to remove ammonium sulphate. The sample was subject to preparative SDS-PAGE under non-reducing conditions and protease protein containing gel portion was cut by referring to zymogram assay. SDS was removed by washing the cut gel with Triton-X-100 (2.5%) and protein was renatured by incubating the gel in sodium acetate buffer (0.02 M, pH 4.0) for 5 h. Then protein was electroeluted (50 V, 30 min) from the gel and its purity was checked by SDS-PAGE followed by silver staining.

IMMUNOLOGICAL METHODS

Antibodies were raised in rabbits by injecting 10 μg of protein (Spector, 1978) via intra-muscular route with Freund's complete adjuvant (Sigma) on days 0, 14, 28 and 42 (Shankar and Umesh-Kumar, 1994). Antiserum was collected by marginal ear vein bleeding after 2 days of last injection. Antiserum was separated from blood clot and stored at -15^0C with sodium azide as preservative.

Ring test: Serially diluted antibody in Tris buffer saline (Tris buffer 0.01 M, pH 8.0; NaCl 0.85 %) was taken in a Durham's tube and an equal volume of antigen was layered on the antibody with out mixing. The tube was incubated at 37^0C and the reaction was visualized as white precipitate at the interface of antigen-antibody. Ring test was regularly used to check the antibody titre of bleeds.

Ouchterlony immuno double diffusion (Nagarajan and Umesh-Kumar, 1990): Two millimeter thick 1% agarose gels were prepared in 0.1% sodium azide containing Tris buffer saline on glass slides and wells were cut using cork borer for loading the reagents. The peripheral wells contained the antigen and central well the antibody. The slides were incubated at 37^0C in the humid chamber for 24 – 48 h for the reaction. Reactions were visualized as white precipitates between the peripheral and central wells. To confirm reactions, slides were stained with Coomassie Blue R 250.

Western blotting: Proteins were separated in 10% SDS-polyacrylamide gels and electroblotted on to nitrocellulose membranes for 16 h at 30 V (Towbin *et al*., 1979). Membranes were blocked with 15% skim milk solution at 37^0C overnight. Proteins were identified by subjecting the membranes to the reactions of antibody (1:500 and 1:2000 dilutions for 71- and 125-kDa antibodies respectively) in TTN buffer (0.01 M Tris, pH 8.0; 1 % Tween 20 and 0.85 % NaCl). After washing the membranes in TTN buffer, antibody reactivity was visualized indirectly using goat anti-rabbit IgG conjugated to biotin (1:1000 dilution in TTN buffer) and avidin-peroxidase conjugate (1:1000 dilution in TTN buffer). Tris-HCl buffer (0.05M, pH 7.6) containing hydrogen peroxide (0.025%) and 4-chloro-1-naphthol (0.04%) was used as substrate for the peroxidase enzyme reaction (Dubey *et al.,* 2000).

Enzyme inhibition assay (Suresh *et al.,* 1998): The antibody to the enzyme was confirmed by performing enzyme inhibition assay. Nitrocellulose membranes containing immobilized antibody were incubated for 30 min at 37^0C in a solution of enzyme containing a defined activity. The residual enzyme activity was estimated in the enzyme solution and decrease in activity assayed as antibody mediated absorption, confirmed reactivity of the antibody to the enzyme protein.

MUTATION EXPERIMENTS

UV mutation: Young spore suspension (2 or 3 day old) prepared from the cultures of the *A. niger* grown on potato dextrose agar slants was used for inducing mutation. 100 µL suspension (OD_{550} = 5.0) was spread on plates containing peptone

(2%), dextrose (2%), yeast extract (1%) and agar-agar (2%) medium. Spores were exposed to UV rays to obtain 40% kill by inverting the plates on a Transilluminator (302 nm) for inducing mutation (Johnston and Dover, 1988). Plates containing mutated colonies were incubated at 4^0C in the dark overnight to prevent photo induced repair of the mutation.

Selection: Temperature selection was carried out by incubating the plates at 42^0C for 24 h. The surviving colonies that emerged after incubating the plates at 30^0C for 24 h were screened on potato starch agar plates for starch clearance. The mutants were purified by picking mycelia from fast growing non sporulant sectors. Final selection of mutants for study was done by assaying the short listed strains for amylase activity. For this, the mutants were grown for 72 h in a medium containing corn starch (10%), peptone (1%) and yeast extract (0.5%).

SCALE UP STUDIES

Submerged fermentation was carried out at 30^0C in 12 L Chemap^R Fermentor for 64 h with aeration (1 vvm) and agitation (200-500 rpm).

Twenty four hour inoculum corresponding to log phase of the culture was raised by growing a loopful of culture from 4 or 5 day old PSA slants in 10 mL corn flour (10%) and corn steep liquor (1%) broth. For fermentor studies, the inoculum was transferred to 90 mL corn flour (6%) and corn steep liquor (6%) broth and grown for 24 h.

The pH of the production medium (12 L) taken in fermentor (6% corn flour and 6% corn steep liquor) was adjusted to 5.5 with sodium hydroxide (4 M). Termamyl (6 mL) was added and the medium sterilized at 121^0C for 20 min. After cooling, the inoculum was added and samples were analysed for enzyme activity at regular intervals of time.

EXPERIMENTAL RESULTS

PART I

CHARACTERISATION OF MALTOGENIC ALPHA AMYLASE

Publication

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INTRODUCTION

Starch is a polymer of glucose produced by the plants as their principle food reserve polysaccharide. Starch is stored in seeds (cereals and millets) and tubers like tapioca (cassava) and potato. Starch is a major source of carbohydrate for human and animal diet. Starch or its liquefied products are found in almost every processed food, and in the U.S.A, fructose syrups are the primary sweeteners.

In its native state, starch is crystalline and the shape of the granules vary according to the source of starch. The property of starch granules to swell reversibly in cold water is used industrially to loosen the starch-protein in grain matrix. Starch irreversibly swells forming a paste, when boiled in water in a process called gelatinisation. This increases viscosity of a food product.

Structurally, starch can be separated into two polymers called amylose and amylopectin. Amylose is a linear polymer of D-glucose units linked together by α-1,4-glucosidic linkages whereas amylopectin has branching at α-1,6 positions of starch. The concentration of amylose and amylopectin in each plant varies. Waxy starches from maize contain 2% amylose and 98% amylopectin whereas most starches contain 15 to 30% amylose. Starch granules exist in a ring structure of amylose and amylopectin extending from the hilum towards the edge of the granule.

Starch hydrolases act either by cleaving α-1,4 and α-1,6 glucosidic bonds at random positions releasing oligosaccharides of different chain lengths called dextrins or from the non reducing ends of the starch molecules releasing D-glucose successively. Smaller chain lengths of starch molecules like disaccharides and oligosaccharides are also substrates for specific enzymes. Hence, enzyme processing of starch define processes for the preparation of food components, substrates for the production of beverages and industrial alcohol using microorganisms and define technologies for modified starch components used in paper, textile, soap, cosmetic and pharmaceutical industries.

ENZYMES INVOLVED IN STARCH DEGRADATION

Microorganisms are the major source for starch hydrolases, generally called amylases (Pandey *et al.,* 2000). The number of amylases produced by microorganisms in culture can be easily identified by performing enzyme zymograms after electrophoresis (Ravi-Kumar and Umesh-Kumar, 2004) in starch incorporated sodium dodecyl sulfate containing polyacrylamide gels (SDS-PAGE) under non reducing condition (Dubey *et al.,* 2000). Amylases are classified according to the specific glucosidic bond it cleaves as α-1,4-glucanases and α-1,6-glucanases. Endo glucanases act on interior bonds of starch while, exo glucanases cleave the bonds successively from non reducing ends of starch. Activities of amylases result in smaller molecules called dextrins, disaccharides and monosaccharides. Glycosyl transferases are enzymes that synthesize cyclic molecules from starch.

α-Amylase (1,4- α-D-glucan glucanohydrolase, EC 3.2.1.1): α-Amylases are α-1,4-endo glucanases that rapidly decrease starch viscosity resulting in oligosaccharides. Some of the α-amylases produce higher concentrations of mono and disaccharides and they are classified as 'saccharifing α-amylases'. α-Amylases that reduce starch viscosity by producing precursor products for mono and disaccharides are called 'liquefying enzymes'. Though bond specificity of this enzyme is for α-1,4 linkages, some enzymes acting on α-1,6 linkages of starch molecule have also been reported (Sakano *et al.,* 1985). End products of the reaction result in oligosaccharides with α-configuration at the first carbon. Several of these enzymes acting at temperatures above 60^0C (normal gelatinisation temperature of starch molecules) have been identified and characterized from bacterial sources. The enzyme most stable to high temperature, acting at temperatures above 100^0C, has been described from *Bacillus licheniformis* by Novo industries (Rosendal *et al.,* 1979).

α-Amylases of bacterial origin are extracellular and act at near neutral pH. However, the pH optimum of an enzyme from *B. stearothermophilus* was reported to be dependent on temperature (Ogasahara *et al.,* 1970). α-Amylases active at pH as high

as 9.0 and 11 have been described from alkalophilic *Bacillus spp* (Kelly and Fogarty, 1976).

α-Amylase stability at high temperatures require calcium ions for maintaining the protein conformation at high temperatures (Klibanov, 1983). However for *B. licheniformis* thermostable α-amylase, the calcium requirement is low (Chiang *et al.,* 1979). Inhibition of the enzyme activity by calcium ions has also been reported (Umesh-Kumar *et al.,* 1990). Modeling of *Bacillus* α-amylase suggested conserved regions for calcium binding and the amino acids between 116 and 127 are involved in calcium binding in *B. subtilis* α-amylase (Rogers, 1985). In *B. amyloliquefaciens,* calcium is bound outside the active center (Hsiu *et al.,* 1964). In *B. subtilis* Taka-amylase, calcium is bound to the SH group of cysteine (Toda *et al.,* 1968). α-Amylases of bacterial origin contain tryptophan, tyrosine and histidine in their active sites (Vihinen and Mantsala, 1989).

Three-dimensional structure of Taka-amylase (α-amylase of *Aspergillus oryzae*) and *Bacillus* α-amylase revealed that seven glucose units of the substrate are bound by atleast two amino acids on the surface of the molecule. In this reaction, calcium binds close to the active center apparently to stabilize the cleft in the active center (Vihinen and Mantsala, 1989). The catalytic site of Taka-amylase contains conserved aspartic acid at positions 206 and 297 of the protein chain. Taka-amylase A was sequenced completely (Toda *et al.,* 1982). There are also reports on the properties of Taka-amylase (Hanrahan and Caldwell, 1953a,b). Protein engineering of *B. stearothermophilus* α-amylase revealed that aspartic acid is in the catalytic site at 331 position (Vihinen and Mantsala, 1989).

Glucoamylase or Amyloglucosidase (1,4- α-D-glucan glucanohydrolase, EC 3.2.1.3): This enzyme catalyses release of glucose from the non-reducing ends of starch, dextrins and maltose. Glucoamylases widely occur in microorganisms and plants with filamentous fungi as the major source of the enzyme (Fogarty and Kelly, 1980). *Rhizopus* glucoamylase hydrolyses both α-1,6 and α-1,4 linkages of starch

molecule with equal efficiency compared to the *A. niger* glucoamylase which has higher affinity for α-1,4 linkages of starch (Dubey, 1999). Glucoamylase hydrolyses raw starch also (Belshaw and Williamson, 1990).

Glucoamylases of fungal origin contain about 28% carbohydrate, which is responsible for the maintenance of the enzyme conformation (Vihinen and Mantsala, 1989). The carbohydrate is *O*-glycosidically linked to serine and threonine of the protein. Glucoamylases have optimum activity at acidic pH and act at temperatures around 60^0 C. Tryptophan residues have been proposed to be essential for enzyme activity (Olsen *et al.,* 1993). Biochemical analysis after site directed mutagenesis of glucoamylase gene indicated that tryptophan 120 is essential for transitional state stabilization (Olsen *et al.,* 1993). Glutamic acid 179 and 400 were identified as general catalytic acid and base catalyst respectively (Ly and Withers, 1999; Chiba, 1997).

β-Amylase (1,4- α-D-glucan maltohydrolase, EC 3.2.1.2): Occurrence of β-amylase is common in plants and perhaps the best characterized enzymes of microbial origin are of *Clostridium thermosulfurogenes* (Hyun and Zeikus, 1985) and *B. polymyxa* (Robyt and French, 1964). β-Amylase hydrolytically cleaves the penultimate α-1,4 bond at the non reducing ends of starch and causes the production of β-maltose. Since the enzyme cannot act on α-1,6 linkages of starch, it also produces β-limit dextrins. Maltogenic amylase of *B. stearothermophilus* was reported to cleave acarbose and carryout transglycosylation reactions (Park *et al.,* 1998).

Plant β-amylases are not thermostable. The enzyme of the thermophilic anaerobe *C. thermosulfurogenes* is thermostable maintaining its activity at 80^0C for two hours in the presence of 5% starch (Vihinen and Mantsala, 1989). Cysteine residues seem to be essential for both activity and conformation of β-amylase.

Isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68): This enzyme predominantly degrades α-1,6-glucosidic linkages of amylopectin, glycogen, dextrins and oligosaccharides. Its low affinity to short chains of pullulan makes this substrate

less susceptible to the enzyme activity. Isoamylases have been characterized from *Bacillus spp*. (Maitin *et al.,* 2001; Urlaub and Wober, 1975). Molecular mass of the enzyme from microbial sources range from 65 to 121 kDa.

Pullulanase (α-dextrin 6-glucanohydrolase, EC 3.2.1.4): Very few organisms produce this enzyme that hydrolyse α-1,6 linkages of pullulan. Their molecular mass range from 80 to 145 kDa. Pullulanase of *C. thermohydrosulfuricum* and *B. acidopullulyticus* are thermostable with optimum activities in acidic pH (4.9 – 6.0).

α-Glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20): α-Glucosidases are exo acting enzymes that catalyze the splitting of α-glucosyl residue from the non reducing terminals of substrates to liberate α-glucose. Typically they are called maltases, since they hydrolyse maltose to glucose.

Fungal α-glucosidase has a wide substrate specificity degrading starch, dextrins and maltose (Kita *et al.,* 1991). The enzyme purified and crystallized from *A. niger* was a glycoprotein of molecular mass of about 125 kDa as estimated by SDS-Polyacrylamide gel electrophoresis. The activities of this enzyme resembled that of glucoamylase.

Glucose isomerase or xylose isomerase (EC 5.3.1.5): A range of microorganisms like *Streptomyces*, *Bacillus* and *Arthrobacter* normally produce glucose isomerase or xylose isomerase intracellularly. Glucose isomerase generally act at 60^0C, isomerising glucose to fructose. Since its affinity for glucose is low, concentrated solution of substrate is used for isomerization reaction. The molecular mass of the enzyme range from 80 kDa in the case of *Actinoplanes missouriensis* to 157 kDa in the case of *Streptomyces spp*. This enzyme is strongly inhibited by Ca^{+2} and Mn^{+2}. However, it requires magnesium for activity and cobalt for maintenance of stability.

STARCH PROCESSING

Starch based derivatives are produced industrially for different applications. In their unmodified form, starch obtained from cassava is used in puddings, fruit fillings and biscuits. Acid modified starches have been used in paper and textile industries. Hydrolyzed starches form substrates for sweeteners and brewing industries.

In the early years, starch was processed using concentrated acid at high temperature and pressure. By this procedure, the yield of products was not consistent. Starch loss due to retrogradation was also a major constraint in the acid hydrolysis process. Advent of enzyme reactions and their suitability for application in starch processing has nearly replaced the processing of starch by acid hydrolysis. Discovery of thermostable amylases and specificity of the enzymatic reactions upgraded industrial processing of starch since, product yields were consistent with defined end points and saved on cost by reducing starch loss due to retrogradation.

The primary step in starch hydrolysis for processing is its gelatinisation at high temperatures followed by a thinning reaction. Discovery of bacterial α-amylases like Termamyl from *B. licheniformis* which was stable even at temperatures as high as 104^0C, completely replaced acid hydrolysis of starch to make enzyme technology a viable process. Use of this enzyme permitted, processing of even 30% starch slurries in steam jet cookers. Being a liquefying enzyme, the dextrins produced due to the activity of thermostable α-amylase constituted nearly 70% of the product. From these dextrin molecules, cyclodextrins can be created with the enzyme cyclodextrin glycosyl transferase. Cyclodextrins find applications as carriers of fragile vitamins and flavours. Only enzymatic synthesis of cyclodextrins is possible since no chemical means exist for making this class of substance.

Two and three stage enzyme processing of starch defined clear protocols for industrial production of glucose, maltose, fructose and limit dextrins.

Production of sweeteners and confectionaries: Enzymatically hydrolysed starch has been used in the manufacture of syrups (glucose, maltose and fructose), baby food formulations (dextrins) and in confectionaries (maltose and β-limit dextrins).

Glucose is produced from starch in two stages. In the first stage, starch is gelatinized at temperatures ranging from 105-110^{0}C and liquefied to reduce viscosity using thermostable α-amylase to evolve α-limit dextrins. The enzyme reaction is performed at pH around 7.0 to maintain the enzyme stability. In the second stage, glucoamylase or β-amylase enzymes are used to produce glucose and maltose syrups from the dextrins respectively. Glucoamylases and β-amylases act at acidic pH (3.5 – 5.0 and 4.8 – 6.5 respectively) and at temperatures around 60^{0}C. Hence, the dextrin solution evolved from the thinning reaction is acidified to the required pH and cooled prior to enzyme action. *Aspergillus* is the major source for commercial glucoamylase. Since the enzyme is slow acting on α-1,6-linkages of dextrins, pullulanase is added along with glucoamylase to improve enzyme efficiency to evolve glucose syrups containing 95-96% glucose in shorter periods of time (Norman, 1982). Glucoamylases are also known to catalyze reversible condensation of glucose into isomaltose at higher concentrations of glucose. Hence, technologies describing glucose syrup production define a very low concentration of glucoamylase to prevent reversible reactions (Norman, 1982).

In the manufacture of maltose syrups, the dextrins obtained from gelatinized starch after α-amylase treatment is further hydrolyzed using thermostable β-amylase. Products of this enzyme activity evolve β-maltose and β-limit dextrins. α-1,6-Glucosidic bonds of dextrins are resistant to β-amylase activity. Simultaneous treatment of the dextrins with β-amylase and pullulanase improves the yield of maltose but the process efficiency is reduced due to condensation reaction of maltose to form branched tetra-, penta- and hexa- saccharides. Maltose syrups are non hygroscopic and are used in hard candies to avoid stickiness.

Production of high fructose syrup involves a three stage processing of starch. The first two stages of starch processing to generate glucose have been described above. In the third stage of processing, isomerization of glucose to fructose is achieved using glucose isomerase. The enzyme obtained from *B. coagulans* is most widely used (Bucke, 1983). Calcium ions, the potent inhibitor of glucose isomerase, are acquired as impurities from raw materials. Though calcium ions are important for the stability of α-amylase used to liquefy starch, they have to be removed completely prior to the isomerization reaction. Hence, the glucose syrup obtained after glucoamylase treatment is passed through ion resin columns to remove calcium. To make the process economical, glucose isomerase immobilized by cross linking with glutaraldehyde is used in column reactors to achieve isomerization. Glucose isomerases are optimally active at pH 7.5 to 7.8. Hence, prior to conversion of glucose to its isomer fructose, the pH of the glucose syrup is adjusted generally with soda ash or sodium hydroxide. Since isomerization reaction does not result in high fructose syrups, industries employ chromatography columns to partially separate glucose to enable flexibility to sell syrups with different levels of fructose. This is important since fructose is sweeter than glucose and syrups carrying a broad range of sweetness help manufacturers of many food products (Boyce, 1986).

***In situ* starch modifications for food and beverage preparations**: In bread making, use of thermostable α-amylase breaks starch to smaller length dextrins that prevent staling. In brewing of beer, use of amylase apart from thinning the mash and accelerating fermentation, also modifies barley biochemically to substitute for malt. In beer, malt has an impact on flavour and is usually used as source of β-amylase enzyme. β-Amylases of microbial origin can modify β-glucans in the barley to lower the wort viscosity for improving flavour. Activity of this enzyme also releases maltose, which serves as nutrient for yeast in the fermentation process.

AMYLASES OF *ASPERGILLUS NIGER*

Though many amylases have been characterized of *A. niger* (Ravi-Kumar and Umesh-Kumar, 2004), the fundamental question on the need for Taka-amylase

secretion by the fungus has not been answered. Production of Taka-amylase by *A. niger* and *A. oryzae* and higher forms (125- and 71-kDa) of glucoamylase by *A. niger* alone suggested gene segregation for the evolution of species that resulted in the occurrence of separate coding sequences for 61 kDa glucoamylase and 53 kDa Taka-amylase in *A. oryzae*, since the gene responsible for Taka-amylase in *A. oryzae* has been cloned and sequenced (Tsukagoshi *et al.,* 1989; Tanaka *et al.,* 2000) and it was shown from this laboratory that Taka-amylase and glucoamylases were products of post translational processing (Dubey *et al.,* 2000) probably coded by a single gene. Though the study identified Taka-amylase production by *A. niger* based on sequence homology of the N-terminal amino acids with the *A. oryzae* enzyme, the enzyme of *A. niger* was not characterized (Dubey *et al.,* 2000). This was carried out and the results are described in this section.

1.1. AMYLASES OF *ASPERGILLUS NIGER* CFTRI (1105)

From this laboratory, several amylases have been characterized from the culture filtrates of *A. niger* CFTRI (1105). The production of two glucoamylases of molecular mass 69.8 kDa and 89.1 kDa were described from this fungus after growing the strain in a medium containing corn meal, ammonium, sodium and potassium salts (Ramasesh *et al.*, 1982a). However, SDS-PAGE analysis showed that these enzymes migrate as 61- and 71-kDa proteins respectively (Dubey *et al.*, 2000). The fungus was also reported to produce a high molecular mass enzyme which corresponded to 125 kDa in SDS-PAGE gels. The enzyme of pI 4.0 on reacting with starch dextrinised the substrate as the first reaction and saccharified the dextrins to release D-glucose (Suresh *et al.*, 1999a). An α-amylase of *A. niger* CFTRI (1105) was also reported and this enzyme showed dextrinising activity on starch at pH 6.0 (Ramasesh *et al.*, 1982b). Though reports showed variation in the reactivity of enzymes to the starch substrate, antibody cross reactivity studies, reactivity on raw starch and N-terminal amino acid sequence homologies suggested proteolytic processing of the 125 kDa starch hydrolyzing precursor amylase to glucoamylases and an yet uncharacterized 53 kDa Taka-amylase like enzyme (Dubey *et al.*, 2000; Suresh *et al.*, 1999b). Since the identification of Taka-amylase secretion by *A. niger* was new to literature, attempts were made to characterize the protein from the fungal culture filtrates.

For this study, the organism was grown in a medium made of corn starch, peptone and yeast extract. After growing the fungus for 3 days, the culture filtrate was separated from the fungal mycelium by filtering through Whatman No.1 filter paper. Amylase activities in the crude culture filtrate were estimated after carrying out reactions with starch. Defining activity estimations in presence of an inhibitor acarbose, showed that some amylases are not inhibited by acarbose. Hence, for some of the assays for amylase activities, acarbose at 0.125 % concentration was used. Individual amylases in the crude culture filtrate were also identified by performing enzyme zymogram reactions after separating the proteins in SDS-PAGE gels under non reducing conditions. The details of the procedures are described in Materials and Methods.

Zymogram analysis of the culture filtrate proteins of *A. niger* CFTRI (1105) for amylase activities at pH 4.3 identified 4 reactive zones corresponding to molecular mass 125-, 71-, 61- and 53-kDa (**Fig. 1A**). Zymogram reactions carried out at pH 6.0 characteristically identified a single reactive zone corresponding to molecular mass 53 kDa (**Fig. 1B**). Assays for the enzyme activities performed with the culture filtrate revealed the presence of glucoamylases as evidenced by the estimation of glucose in the product (**Table 1**). Interestingly, the enzymes that reacted with starch to produce glucose were inhibited in the presence of acarbose in the assay since no glucose was detected in such reactions even though, reducing sugars were estimated in enzyme assays (**Table 1**).

Table 1: Amylase activities at pH 4.3 in the culture broth of *A. niger* (1105)

Activities in the absence of acarbose		Activities in the presence of acarbose	
Glucoamylase[a] (Units/mL)	Total amylase[b] (Units/mL)	Glucoamylase[a] (Units/mL)	Total amylase[b] (Units/mL)
70	148	Nil	67

[a] Unit = μ mole glucose produced/min

[b] Unit = μ mole glucose equivalents produced/min

The two glucoamylases and the precursor starch hydrolyzing enzyme are known to have a pH optima for activity at 4.3. It is also known that the α-amylase produced by the fungus has a pH optima at 6.0 (Ramasesh *et al.*, 1982b). Hence, the results on the zymogram reactions and acarbose inhibiting the amylases producing glucose from starch as product suggested that glucoamylases and the precursor enzyme are inhibited in the presence of the inhibitor. The assays also revealed that an enzyme active at pH 4.3 not inhibited by acarbose was present in the culture filtrates of *A. niger* CFTRI (1105). The enzyme did not hydrolyse starch to glucose since no glucose was

Fig. 1

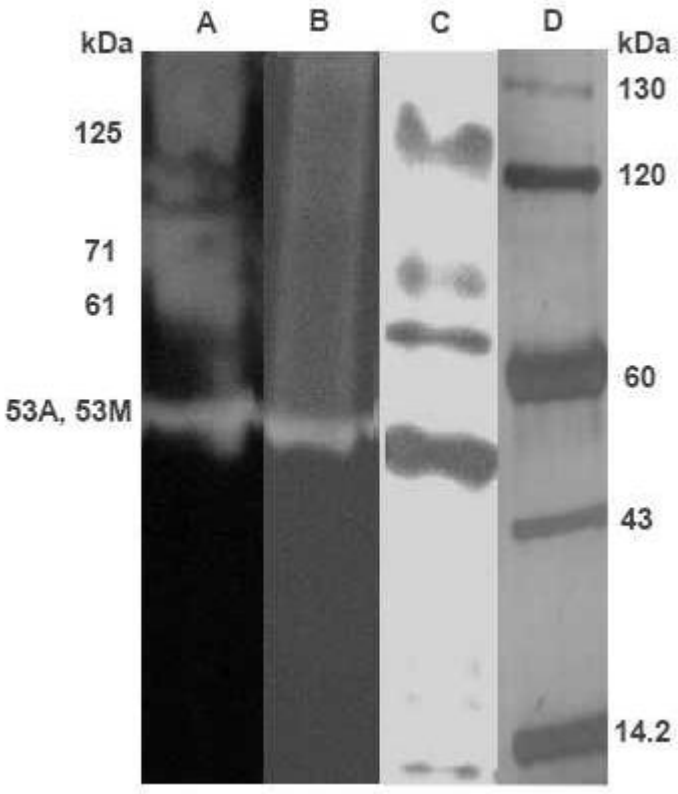


Fig. 1**.** Zymogram reactions of amylases of *A. niger* after SDS-PAGE performed under non reducing conditions in 10% acrylamide gels and corresponding silver stained proteins. 9 μg of protein was loaded in each well.

A. Zymogram reaction at pH 4.3
B. Zymogram reaction at pH 6.0
C. Silver stained proteins
D. Molecular weight markers

125 kDa- Starch hydrolyzing enzyme
71- and 61-kDa- Glucoamylases
53 kDa A- α-Amylase (active at pH 6.0)
53 kDa M- Amylase active at pH 4.3

detected despite the presence of reducing sugars. Zymogram analysis identified this enzyme as a protein of 53 kDa molecular mass.

The above results suggested further studies on the characterization of the 53 kDa amylase active at pH 4.3.

1.2. MUTATION AND SELECTION OF *ASPERGILLUS NIGER* FOR OVERPRODUCTION OF 53 KDA AMYLASE ACTIVE AT pH 4.3

Growth of *A.niger* CFTRI (1105) in a medium containing starch showed the production of 53 kDa amylase that was active at pH 4.3 (**Fig.1A**). Acarbose, a pseudotetrasaccharide has been described as a potent inhibitor of glucoamylases of *A. niger* (Olsen *et al*., 1993). Preliminary studies have shown inhibition of the activity of precursor starch hydrolyzing enzyme and glucoamylases by acarbose (Ex. No. 1.1). Since estimation of amylase activities in presence of acarbose suggested the identification of 53 kDa amylase protein in the culture broth, mutation and selection for cultures that over produced this enzyme was carried out using acarbose in the assays.

Amylase secretion by *A. niger* is apparently affected by secretional difficulties due to reduced porosity of the cell walls of the fungus to high molecular mass proteins (Wosten *et al*, 1991; Money, 1990; Dubey *et al*, 2000). Based on this reasoning, mutants affected in general secretory pathway could be a choice for studies on amylase excretion. Temperature tolerant mutants of *A. niger* were isolated since, Gordon *et al* (2000) had described that such mutants have an impaired secretory pathway.

Mutation was induced in *A. niger* CFTRI (1105) using ultra violet irradiation. Mutants were selected for temperature tolerance by incubating the plates at 42^0C for 24 h. For this study, mutants isolated were grown in corn starch, peptone and yeast extract for 72 h and the culture filtrates obtained after filtering through Whatman No.1

filter paper were assayed for the 53 kDa amylase and other amylases active at pH 4.3. The details are described in Materials and Methods.

In this study, temperature selection resulted in a number of mutants and a best mutant was identified for increased 53 kDa amylase production by assays containing acarbose. A mutant strain (258) was found to produce 30% more 53 kDa enzyme compared to the parent. The production could be improved substantially by selecting fast growing sectors and non sporulant sectors of the mutant colony. The details are described in **Fig. 2, 3** and **4**.

Assay for 53 kDa amylase and other amylases was done using starch and maltose as substrates. In starch assays, acarbose was incorporated at 0.125% levels to inhibit interference by other amylases. Enzyme assays showed (**Table 2**) increased 53 kDa amylase production in the mutant strain selected. It appeared that production of the enzyme was associated with decrease in activities of starch hydrolyzing enzyme and glucoamylases in the culture filtrate (**Table 2**).

Table 2. Relative activities of other amylases and the 53 kDa amylase in the culture filtrates of parent and mutant strains of *A. niger*

Culture	Precursor Starch hydrolysing enzyme and glucoamylases (Units/mL)[a]	53 kDa amylase (Units/mL)[b]
Parent	45	130
Mutant	12	202

[a] Specific activity unit = μ moles of maltose degraded min^{-1}

[b] Specific activity unit = μ moles of maltose produced min^{-1}

While starch was used as a substrate for the identificaton of 53 kDa amylase, maltose was used as a substrate for determining the activities of the precursor enzyme and glucoamylases because they have the ability to degrade α-1,4 linkages of maltose.

Fig. 2: Mutational protocol for strain improvement of *A. niger* (CFTRI 1105) for hyper production of 53 kDa amylase active at pH 4.3

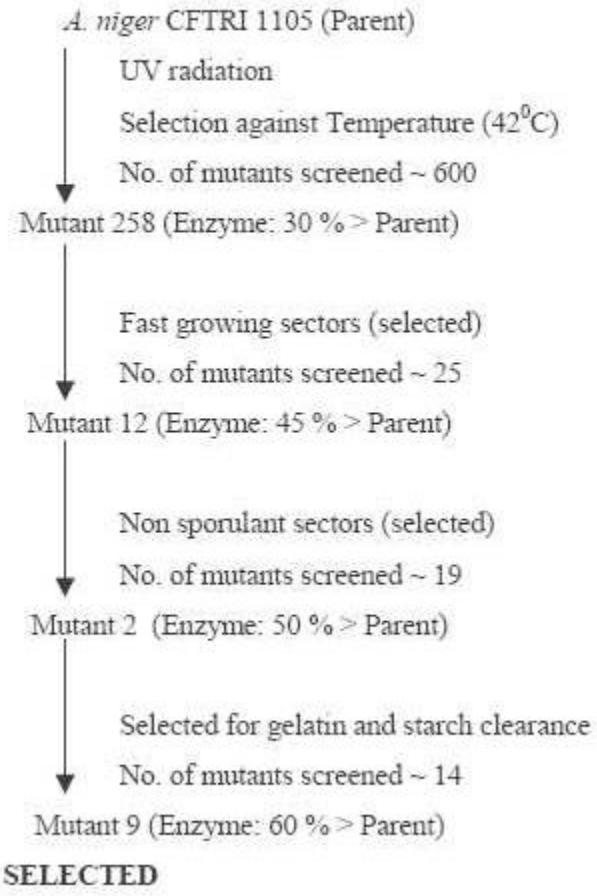


Fig. 3

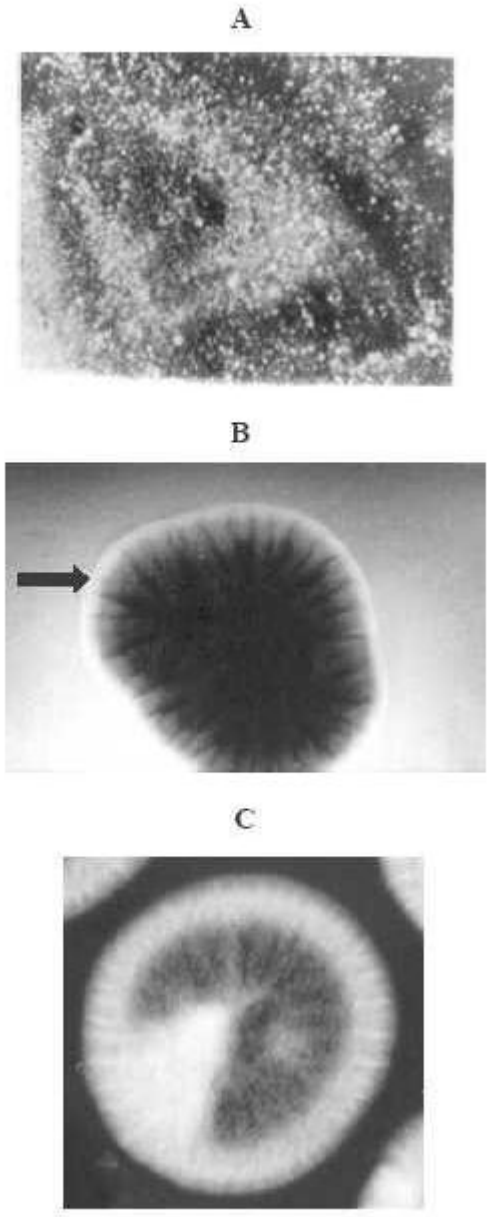


Fig. 3. Mutants of *A. niger*

A. Temperature tolerant mutants of *A. niger*

B. The selected mutant showing fast growing sector (Arrow)

C. The selected mutant showing non sporulant sector

Fig. 4

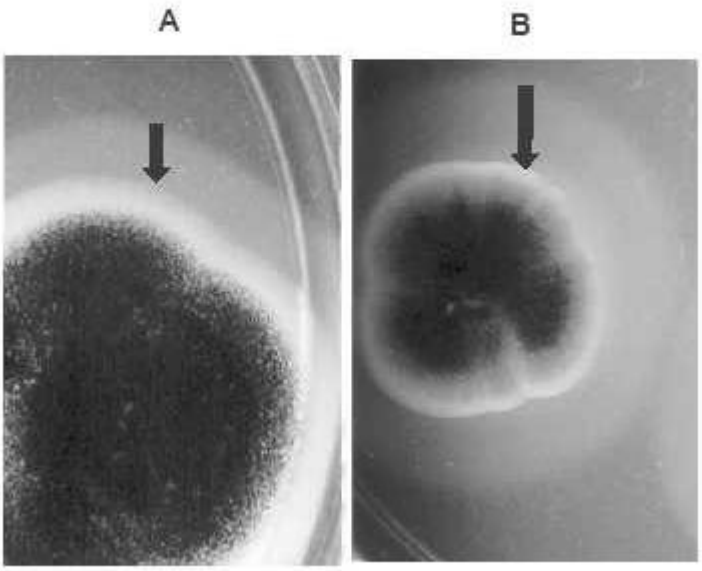


Fig. 4. Plate assay for amylase as indicated by zones of clearance (Arrow)

Mutant (B) and Parent (A) strains of *A. niger* growing on starch containing medium

Since overproduction of 53 kDa amylase was identified in the mutant obtained in this study, the mutant was used in further experiments for characterization of the enzyme. The observation on decrease in the precursor enzyme concomitant with the increase in 53 kDa amylase in the culture filtrates also reasoned proteolysis of the precursor amylase.

1.3. CHARACTERISATION OF THE 53 KDA AMYLASE

In the earlier experiments, identification of a 53 kDa amylase in the culture filtrates of *A. niger* (Ex. No. 1.1) and its overproduction by the mutant (Ex. No. 1.2) were shown. Literature survey suggested that Taka-amylase, a 54 kDa protein produced by *A. oryzae* reacts with starch resulting in the formation of maltose as the product. Apparently this activity identified in the culture filtrates of *A. niger* was a Taka-amylase like enzyme because on reactivity of amylases of culture broth with starch in the presence of acarbose, reducing sugars were identified as product but not glucose. The earlier report from this laboratory also identified a Taka-amylase like enzyme in the culture filtrates of *A. niger* based on N-terminal sequence homology (Dubey *et al.,* 2000). In this experiment the enzyme was purified for further characterization.

Taka-amylase produced by *A. oryzae* is an α-amylase (EC 3.2.1.1). It catalysed the hydrolysis of α-1,4–glucosidic linkages in starch to produce maltose as product. The enzyme characterized of *A. oryzae* was a 54 kDa protein with temperature and pH optima of 40^0C and 5.0 respectively (Matsuura *et al*., 1979; Matsuura *et al*., 1984; Matsubara *et al.*, 1959; Perevozchenko and Tsyperovich, 1972).

The purified 53 kDa amylase was obtained from the culture filtrates of the mutant strain of *A. niger* in a single step by ammonium sulphate fractionation protocol. The purity was established by performing silver staining of SDS-PAGE gels containing the protein. The details are described in Materials and Methods. Temperature optimum for the activity of the purified enzyme was studied by carrying out the reaction at different temperatures. To determine the pH optimum for enzyme activity, the reaction was carried out at 40^0C using different buffers.

The 53 kDa amylase from the culture filtrates of mutant strain of *A. niger* selectively precipitated at 60 % ammonium sulphate saturation. The protein separated in 10 % SDS-PAGE gels resolved as a 53 kDa protein (**Fig. 5B**). Since silver staining of the gels did not identify any contaminating proteins in the purified enzyme preparation,

Fig. 5

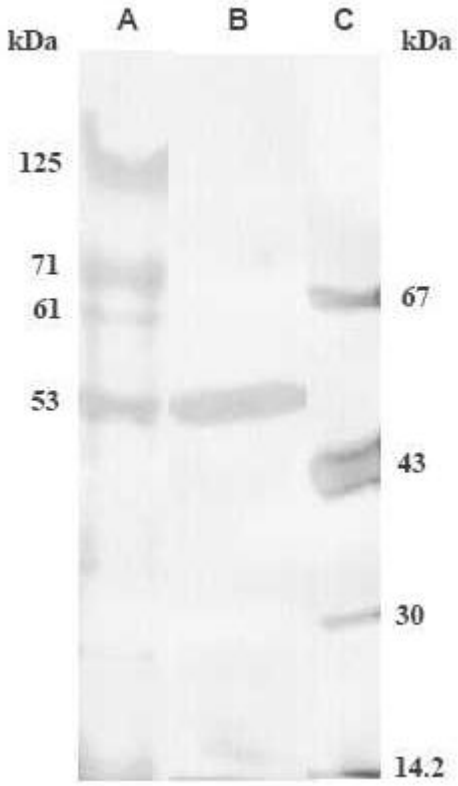


Fig. 5. Purification of the 53 kDa amylase from the culture broth of the mutant strain

Silver stained gels showing:

A. Amylase proteins of crude culture broth
B. Purified protein
C. Molecular mass standards

the enzyme was used as a pure protein for further studies to determine the pH and temperature optima for the activity on starch.

1.3.1. pH OPTIMA OF THE ENZYME

Preliminary experiments showed that the enzyme was optimally active at 40^0C. Hence the pH optima for enzyme activity was determined at 40^0C, using the purified enzyme dissolved in various buffers of 0.1 M concentration. Buffers used for this study were glycine-HCl (2.0-3.4), acetate (3.5-5.6), phosphate (5.7-8.0) and tris buffers (9.0).

The results showed that the enzyme was optimally active at pH 4.3. A small reduction in the activity (2%) occurred when the pH was raised to 5.0. Only 36.42 % of the enzyme activity was measured at pH 3.0 and apparently the enzyme was denatured below pH 3.0 because, no activity could be measured at pH 2.0 (**Fig. 6**). Similarly, at neutral and alkaline pH very little enzyme activity was measured.

The enzyme was found to be active in the acidic region between pH 4.0 and 5.0 (optimum activity at pH 4.3).

1.3.2. TEMPERATURE OPTIMA OF THE ENZYME

At pH 4.3, the enzyme showed an optimum activity at 40^0C. At 50^0C, there was a reduction of 35 % in the enzyme activity and at temperatures above 50^0C, the enzyme was less stable since, activity was drastically lost at these temperatures (**Fig. 7**).

Calcium ions have been shown to impart thermostability to α-amylases of microbial origin (Vihinen and Mantsala, 1989). Hence, thermostability of the *A. niger* enzyme was determined using $CaCl_2$ (0.05 M).

Fig. 6

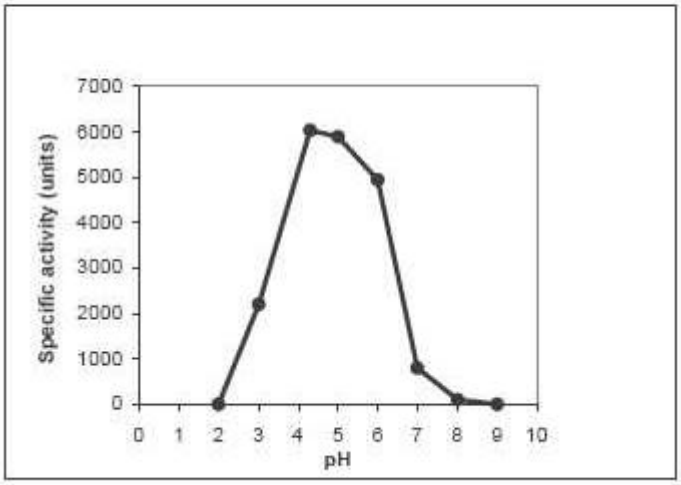


Fig. 7

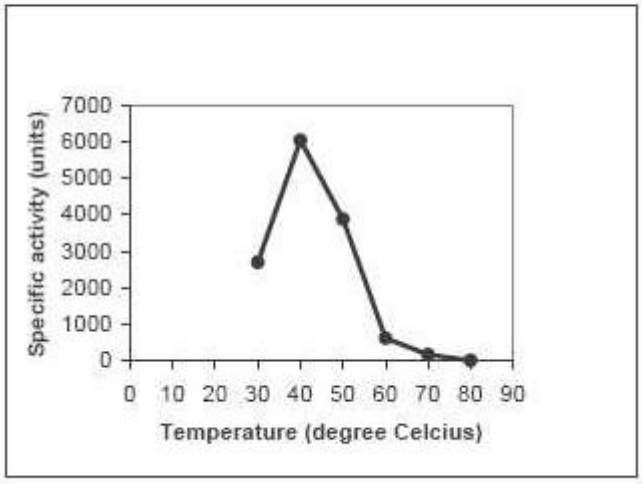


Fig. 6. pH optima of the 53 kDa amylase

Enzyme activity was estimated at 40^0C and different pH (2.0-9.0) by carrying out the reaction with 100 mg starch (in 5 mL) in sodium acetate buffer (0.1 M, pH 4.3). Enzyme units: 603

Fig. 7. Temperature optima of the 53 kDa enzyme

Activity estimation same as in Fig. 6 but at different temperatures (30-80^{0}C). pH of the reaction mixture was 4.3.

Addition of calcium to the enzyme stabilized it for 30 min at 50^{0}C. In the absence of calcium, no significant activity could be determined after 30 min incubation of enzyme at 50^{0}C (**Table 3**).

Table 3: Thermostability of the 53 kDa amylase

Temperature (^{0}C)	Residual activity of raw enzyme (%)	Residual activity of enzyme in the presence of calcium (%)
40	87.53	100
50	2.85	100
60	0.649	4.93
70	0	0
80	0	0

100% enzyme activity = 1237 units

1.4. SUBSTRATE AFFINITY OF THE ENZYME

Starch is a branched molecule made of amylose and amylopectin. Since the enzyme characterized was shown to act on starch, substrate affinity of the enzyme for starch and its individual components (amylose and amylopectin) was determined in this experiment.

The enzyme was not active on pullulan, suggesting that it did not hydrolyse α-1,6-linkages of starch molecule. The K_m estimated of the enzyme for gelatinized starch, amylopectin and amylose were 0.41 %, 0.62 % and 3.5 % respectively (**Fig. 8 A, B** and **C**).

The K_m values of the enzyme for different substrates described above showed highest affinity of the enzyme for branched molecules like starch and amylopectin compared to linear amylose.

Fig. 8

A

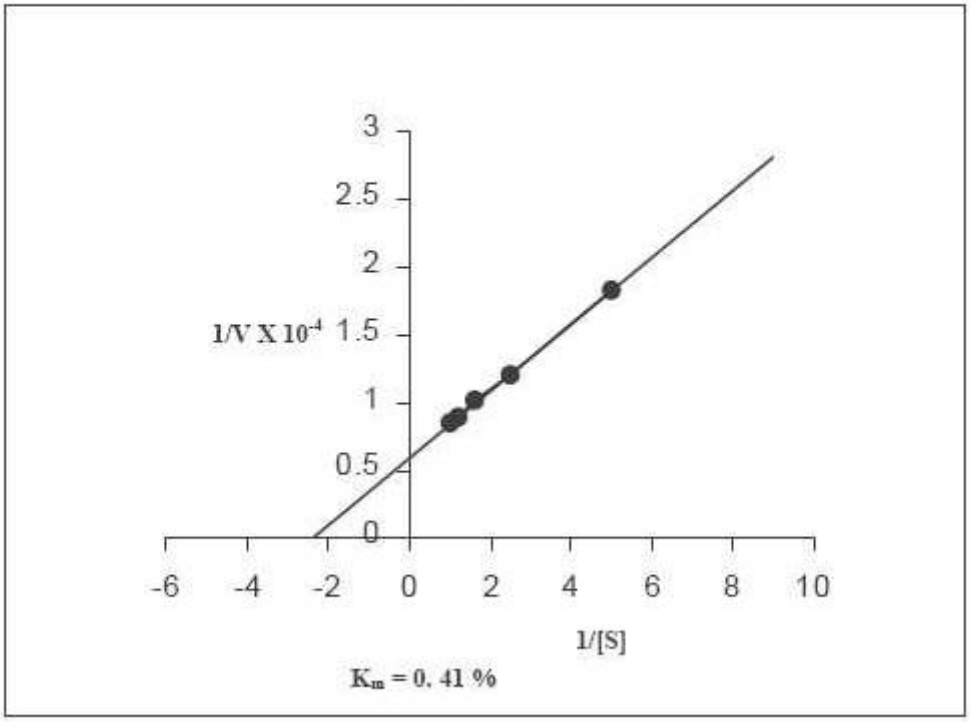


Fig. 8

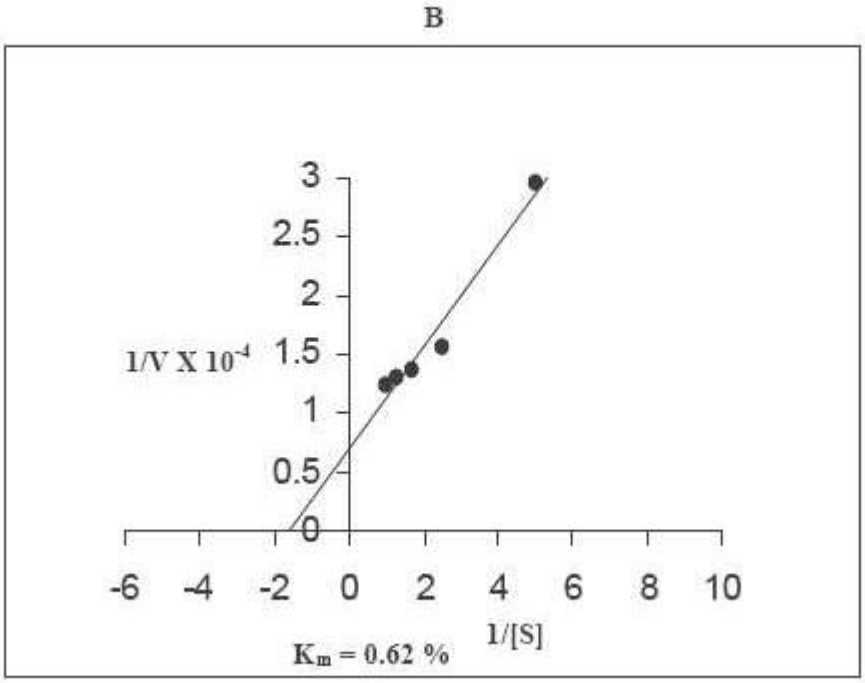


Fig. 8

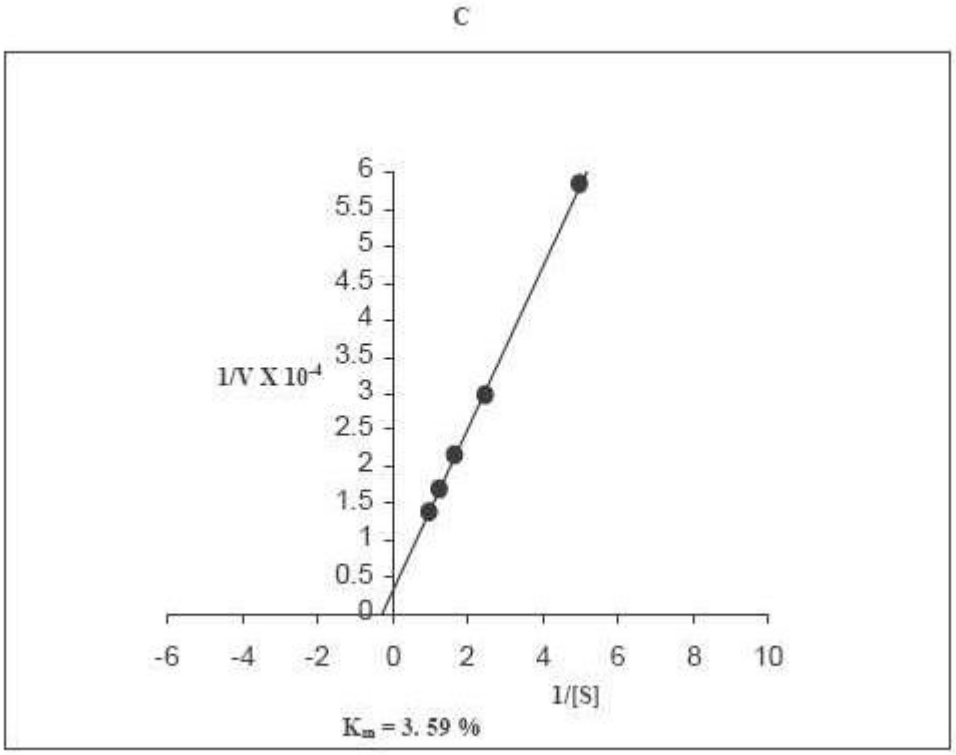


Fig. 8. Substrate affinity of the 53 kDa amylase on starch, amylopectin and amylose

A. Lineweaver-Burk plot for the determination of enzyme K_m to starch. 1.4 μg of enzyme protein was added to the substrate (0.2 to 1 %) and the reaction was carried out at 40^0C. Specific activity corresponded to μ moles of maltose equivalents produced min^{-1}.

   K_m = 0.41% starch

B. Lineweaver-Burk plot for the determination of enzyme K_m to amylopectin

   Conditions were same as described in Fig. 8A.

   K_m = 0.62% amylopectin

C. Lineweaver-Burk plot for the determination of enzyme K_m to amylose

Details were same as described in Fig. 8A.

K_m = 3.59% amylose

1.5. PRODUCTS OF STARCH HYDROLYSIS DUE TO THE ACTIVITY OF 53 KDA AMYLASE

Earlier experiments on the identification and characterization of the 53 kDa amylase obtained from the culture filtrates of the mutant strain of *A. niger* suggested Taka-amylase like enzyme production by the fungus. In order to further consolidate the results on the production of Taka-amylase like enzyme by the fungus, time course study on the products formed from starch by the enzyme activity was analysed to

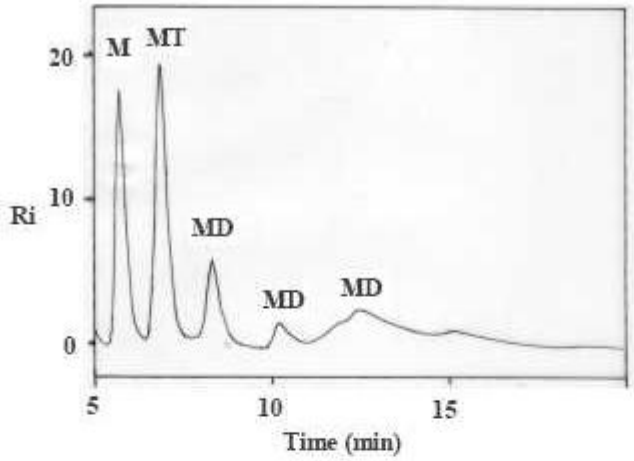
i. differentiate the enzyme from the other 53 kDa α-amylase produced by the fungus (Ramasesh *et al.*, 1982b)
ii. identify the sugars in the product and
iii. understand the nature of enzyme activity

For this study, the 53 kDa amylases (Taka-amylase like enzyme and the α-amylase) were purified from the *A. niger* culture broths. The purified enzymes were treated to gelatinized starch at their optimum temperature and pH of activity for varying periods of time and the products formed were analysed by HPLC and paper chromatography. The details of the experimental procedure are described in Materials and Methods.

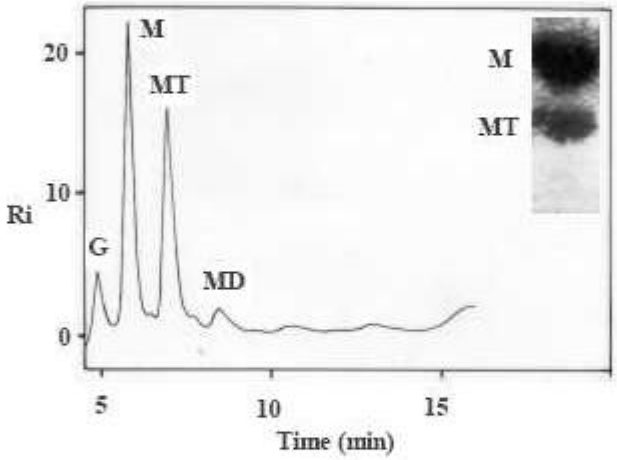
Fig. 9 shows the products of starch formed due to reactivity with the 53 kDa Taka-amylase like enzyme. The enzyme during the first 30 min of reaction, produced maltose, maltotriose and maltodextrins as products. Longer incubation of the substrate with the enzyme resulted in the accumulation of maltose and reduction in maltodextrins. Very little glucose was identified in the reaction. Apparently, the reaction was complete in 60 min because there was no significant variation in the product formed when the incubation was extended to 90 min.

Fig. 9

A


M
MT
MD
MD
MD
20
10
0
Ri
5
10
15
Time (min)

B


M
MT
G
MD
M
MT
20
10
0
Ri
5
10
15
Time (min)

C

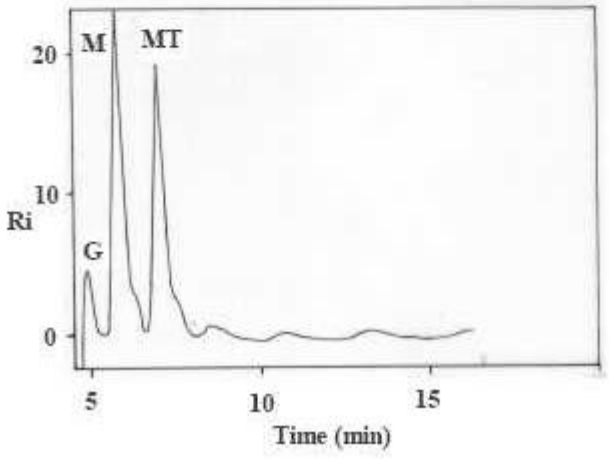

M
MT
G
20
10
0
Ri
5
10
15
Time (min)

Fig. 9. Product analysis by HPLC after the action of purified 53 kDa Taka-amylase like enzyme on Lintner's soluble starch

A. The products after 30 min of enzyme action
B. The products after 60 min of enzyme action
   Inset: Identification of sugars on paper chromatogram
C. The products after 90 min of enzyme action

The reaction was carried out at 40^0C and pH 4.3.
G: Glucose
M: Maltose
MT: Maltotriose
MD: Malto dextrin
Ri: Refractive index

In contrast to this activity, the known α-amylase of *A. niger* CFTRI (1105) rapidly hydrolysed starch at pH 6.0 to result in maltodextrins only (**Fig. 10**).

Thus, the product analysis confirmed that the 53 kDa amylase with optimum activity at pH 4.3 acted on starch like the Taka-amylase of *A. oryzae*.

Fig. 10

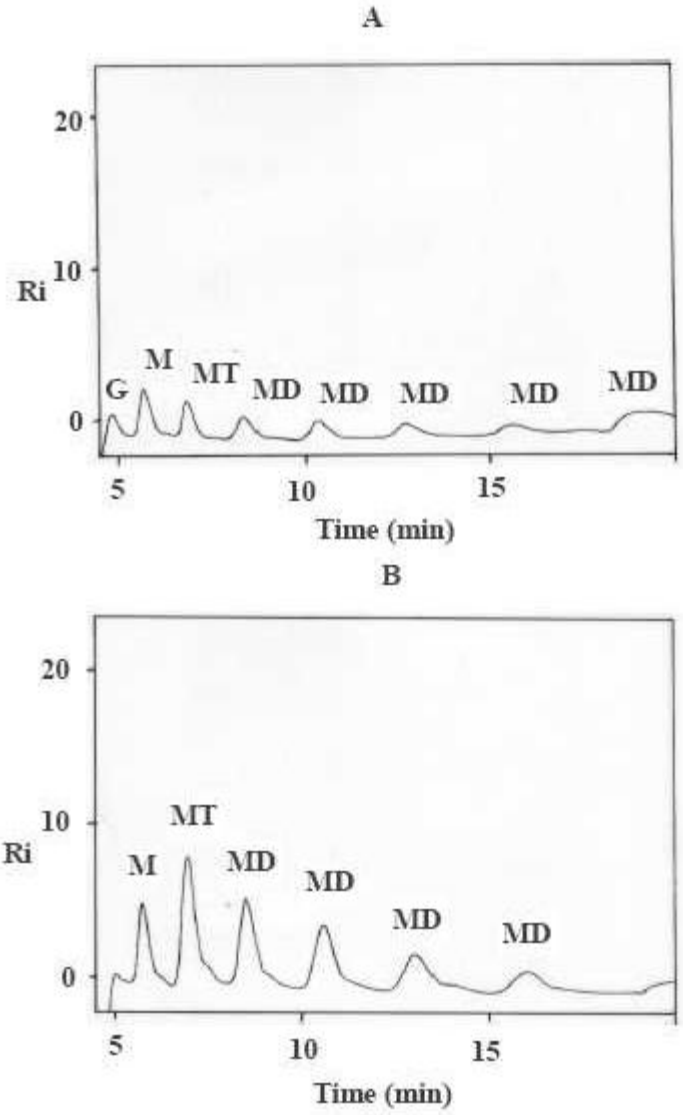


Fig. 10. Product analysis by HPLC after the action of purified α-amylase on Lintner's soluble starch

A. The products after 30 min of enzyme action

B. The products after 60 min of enzyme action

The reaction was carried out at 60^0C and pH 6.0.

G: Glucose

M: Maltose

MT: Maltotriose

MD: Malto dextrin

Ri: Refractive index

1.6. ANOMERIC CONFIGURATION OF THE PRODUCTS OF STARCH HYDROLYSED WITH 53 KDA TAKA-AMYLASE LIKE ENZYME OF *ASPERGILLUS NIGER*

Maltogenic amylases are differentiated from other amylases by the type of maltose formed as the product. The anomeric configuration of the product generally is of the β-type, when β-amylase reacts with starch. This is known of *Streptomyces* spp, *Bacillus* spp and *Clostridium thermosulphurogenes* (Ray and Nanda, 1996; Hyun and Zeikus, 1985). Since the enzyme characterized in the present study was found to produce maltose and maltotriose as products of starch hydrolysis, the configuration of the anomeric carbon was studied to differentiate the enzyme from the β-amylases known of bacterial strains.

The α or β-configuration at the anomeric carbon of the sugars in the product after enzymic reaction was analysed by polarimetry. For this, a 0.6 mL reaction mixture made with Lintner's soluble starch (1%) in sodium acetate buffer (0.1 M; pH 4.3) and purified enzyme were taken in a 10 mm cell and the reaction was allowed to proceed at 30^0C. Optical rotation was measured at 30^0C in a Perkin Elmer (model 243) polarimeter by using the sodium line. When the optical rotation became approximately constant, about 4 mg sodium carbonate was added, and the mutarotation of the mixture was measured as a downward shift for α-configuration and upward shift for β-configuration (Hyun and Zeikus, 1985).

The products in the reaction mixture of starch and the 53 kDa Taka-amylase like enzyme produced a downward shift in optical rotation (**Fig. 11**) on the addition of the base. This showed that the products produced were of a α-anomeric configuration.

Fig. 11

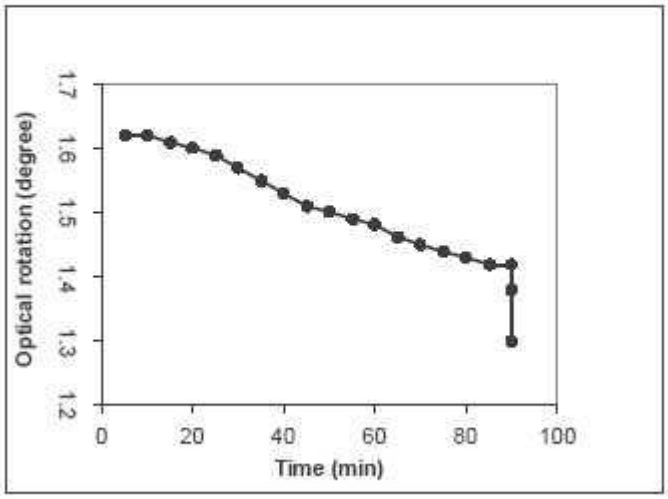


Fig. 11. Analysis of anomeric carbon configuration of products

Products formed due to the action of purified 53 kDa Taka-amylase like enzyme on Lintner's soluble starch, showed down ward shift in optical rotation after the addition of sodium carbonate. Optical rotation of the products was measured at 30^0C in a Perkin Elmer (model 243) polarimeter by using the sodium line.

CONCLUSIONS

The results described in the experiments characterized a Taka-amylase like enzyme produced by *A. niger*. Based on the reactivity of the enzyme to starch, production of maltose and maltotriose as products and since the products formed had an α-anomeric configuration, this enzyme of *A. niger* has been characterized as maltogenic α-amylase. The maltogenic α-amylase of *A. niger* was differentiated from Taka-amylase of *A. oryzae* by their reactivities to maltotriose. The Taka-amylase of *A. oryzae* has been shown to bind to maltotriose and release glucose (Matsuura *et al.*, 1984). However, time course study of products of starch hydrolysis using the enzyme of *A. niger* (Ex.No. 1.5) showed accumulation of maltotriose as one of the major products. This suggested that the enzyme of *A. niger* does not act on maltotriose. Zinc ions not inhibiting the activity of maltogenic α-amylase of *A. niger* (**Table 4**) also differentiated it from the Taka-amylase of *A. oryzae* because zinc has been shown to reduce *A. oryzae* Taka-amylase activity (Toda and Narita, 1967).

Table 4: Effect of zinc and mercuric ions on maltogenic α-amylase

salt	Residual activity (%)
$ZnCl_2$ (0.05 M)	100
$HgCl_2$ (0.05 M)	0

100% activity = 1145 units

Though the above properties differentiated the two amylases of *A. niger* and *A. oryzae*, antibody cross reactivity (**Fig. 12**) showed that the two enzymes shared a number of structural features in addition to the identical N-terminal sequence (Dubey *et al.*, 2000).

Fig. 12

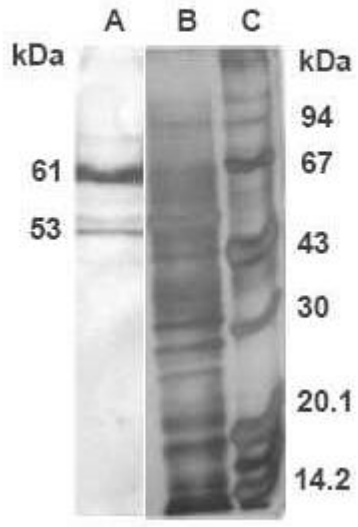


Fig. 12. Amylase Cross reactivity

A. Western blot showing reactivity of antibodies raised against 125 kDa amylase of *A. niger* with amylases of *A. oryzae*
B. Silver stained crude culture filtrate proteins of *A. oryzae* after separation in 10% SDS-polyacrylamide gels
C. Molecular weight markers

However, in the light of the differences in properties, the enzyme of *A. niger* has been referred to as maltogenic α-amylase in the subsequent sections of the thesis.

PART II

STUDIES ON AMYLASE PROCESSING USING THE MALTOGENIC ALPHA AMYLASE OVERPRODUCING MUTANT STRAIN OF *ASPERGILLUS NIGER*

Publication

Ravi–Kumar, K., Venkatesh, K.S. and Umesh-Kumar, S. 2004. Evidence that cleavage of the precursor enzyme by autocatalysis caused secretion of multiple amylases by *A. niger*. **FEBS Lett.** 557: 239-242

INTRODUCTION

Insight into carbohydrate binding properties of amylases and their mechanisms of action acquired through a combination of x-ray crystallography, chemical modification, inhibition studies and site directed mutagenesis, explained functionality of amylases for specific action patterns. Replacement of histidine 238 with aspartic acid in α-amylase showed thermostability reduction but the mutant protein derived, hydrolyzed starch to give rise to a different oligodextrin profile (Svensson and Soggard, 1992). In another approach, replacement of histidine 133 with tyrosine resulted in α-amylase that was more stable (Svensson and Soggard, 1992). The tryptophan 178 to arginine and serine 119 to tyrosine mutations in *A. niger* glucoamylase increased the cleavage of α-1,6 glucosidic bonds of starch without significantly decreasing activity towards α-1,4 bonds (Svensson and Soggard, 1992).

The extreme thermostability of *B. licheniformis* α-amylase was ascribed to electrostatic interactions involving few specific lysines (Tomazic and Klibanov, 1988). Stabilization against irreversible thermal inactivation was achieved in a chimeric gene constructed using *B. amyloliquefaciens* and *B. licheniformis* α-amylase genes and by deleting arginine 176 and glycine 177, substituting lysine 269 with alanine and asparagine 266 with aspartic acid (Suzuki *et al.,* 1989). Truncation of amylolytic enzymes has also led to the understanding of starch granule binding function. Hence, protein engineering of amylases through chemical modifications and site directed mutagenesis described the art of changing enzyme properties without losing catalytic efficiency (Gottschalk *et al.,* 1998). Selectivity of glucoamylase for increasing glucose yields by site directed mutagenesis of the glucoamylase catalytic domain (Fang *et al.,* 1998a; Fang *et al.,* 1998b; Liu *et al.,* 1998; Liu *et al.,* 1999), modified the dimension of the active site just enough to prevent the prevalent byproduct isomaltose, from binding the active site without affecting the binding of maltose. This was achieved by stiffening the α-helices of the protein by mutating glycine to alanine or producing disulphide bonds by substituting cysteine on adjacent loops that restricted the movement of the active site so that it did not accommodate isomaltose.

Thermostability of glucoamylase was also found to increase when amino acid substitutions were made by site directed mutagenesis. Significantly, replacement of glycine 137 with alanine was reported to stiffen an α-helix that improved the enzyme thermostability at temperatures over 70^0C. This was because the mutation retarded general unfolding of the enzyme (Chen *et al.,* 1996). Several other mutants obtained by site directed mutagenesis have also shown substantial improvement in thermostability and activity of glucoamylase (Reilly, 1999).

Most cloned α-amylase genes have been expressed using their own promoters in bacteria and fungi. High level production using cloned genes has been obtained for α-amylase in *B. amyloliquefaciens* and *A. oryzae* and β-amylase in *A. schirousami*. Glucoamylase gene has been characterized and expressed in *Saccharomyces cerevisiae* under the control of enolase (*ENO1*) promoter (Nunberg *et al.,* 1984; Innis *et al.,* 1985). This study described construction of the yeast strain that hydrolysed and simultaneously fermented starch, because of the expression of *A. awamori* glucoamylase gene. Granular starch hydrolysing glucoamylase of *Rhizopus* has been cloned and expressed in yeast (Ashikari *et al.,* 1986). Comparison of amino acid sequences of different amylases showed conserved regions among amylases (Nakajima *et al.,* 1986; Svensson, 1988).

Glucoamylases occur as multiple forms in several fungi. Since all fungal glucoamylases are glycoproteins, the difference in molecular mass was attributed to varying amounts of carbohydrate moieties (Hayashida and Yohino, 1978). The glucoamylases of molecular mass 71-and 61-kDa from *A. niger* referred to as G1 and G2, have been extensively studied (Lineback *et al.,* 1969). The cause for occurrence of the two glucoamylases was initially thought to be due to differential splicing of mRNA encoded by a single gene that contained a number of intervening sequences (Boel *et al.,* 1984a,b). But it was shown that the 61 kDa G2 protein arose by a proteolytic splitting–off of a 10 kDa peptide from the 71 kDa G1 protein C-terminus (Svensson *et al.,* 1986). The activity differences of G1 and G2 on raw starch were reasoned to the C-terminus peptide. Recently it was shown that a 125 kDa precursor starch hydrolysing

enzyme is the major enzyme in *A. niger* and this enzyme is apparently processed by proteases to give rise to 71-, 61-kDa glucoamylases and 53 kDa Taka-amylase enzyme (Dubey *et al.,* 2000). However protease involved in this processing was not identified. This part of the thesis describes experiments on amylase processing in relation to protease.

2.1. THE PROTEASES OF THE MUTANT STRAIN OF *ASPERGILLUS NIGER*

Characterization of maltogenic α-amylase in the culture filtrates of *A. niger* described in the previous section suggested, the production of the enzyme by the fungus due to defects in general secretory pathway. However, concomitant decrease in the activities of starch hydrolysing enzyme and glucoamylases in the culture filtrates and the earlier report from this laboratory (Dubey *et al.,* 2000) that the α-amylase and the glucoamylases secreted by *A. niger* are proteolytically processed products of the starch hydrolysing enzyme hypothesised a role for protease in the maltogenic α-amylase production. It has been shown earlier that aspergillopepsin, an acid protease was responsible for the secretion of multiple forms of glucoamylase (Mackenzie *et al.,* 2000). *A. niger* mutant deficient in extracellular proteases was found to accumulate starch hydrolysing enzyme in the culture filtrate (Suresh, 1999). Based on the above reports, experiments were carried out to identify the role for proteases in maltogenic α-amylase production in *A. niger*.

The mutant strain of *A. niger* described in the previous section was assayed for the production of proteases in culture. Plate assays were carried out in gelatin incorporated medium (gelatin, 2%, dextrose, 2%, yeast extract, 1% and agar-agar, 2%). Cultures were grown in broth made of peptone (1%), starch (10%) and yeast extract (0.5%) for the production of amylases and the culture filtrates were assayed for protease activity using casein as substrate. The details are described in Materials and Methods.

The mutant strain of *A. niger* was found to overproduce extracellular proteases as evidenced by increased zones of clearance in plate assay containing gelatin incorporated medium (**Fig. 13**). Assay of culture filtrates for proteases after 72 h growth showed, five fold increased production of acid proteases in the mutant strain compared to that produced by the parent (**Table 5**).

Fig. 13

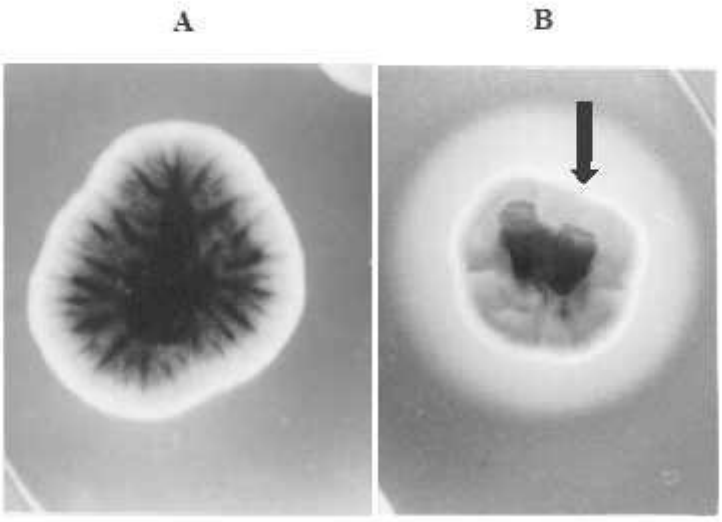


Fig. 13. Protease production as indicated by zones of clearance by mutant (B) and parent (A) strains of *A. niger* on gelatin containing medium. Arrow indicate the zone of clearance due to protease production.

Table 5: Specific activity of total proteases in the culture broths of parent and mutant strains of *A. niger*

Culture	Specific Activity (Units)
Parent	0.066
Mutant	0.33

These results suggested a role for proteases in the maltogenic α-amylase production by the mutant strain of *A. niger*.

2.2. EXTRACELLULAR AMYLASES OF *ASPEGILLUS NIGER*

A suggestive data on protease processing of the precursor starch hydrolysing enzyme causing the accumulation of maltogenic α-amylase and secretion of multiple forms of amylases by the mutant strain of *A. niger* was obtained in the earlier experiment (Ex. No. 2.1). In order to further evidence the above observation, two dimensional electrophoresis studies were performed with the culture filtrate proteins of the mutant and parent strains to identify degradations of amylases. Separation of proteins by two dimensional electrophoresis was important since, preliminary experiments showed that uni-dimensional gels did not clearly identify degradations.

Proteins were separated by two-dimensional SDS-PAGE, since this method improved the resolution for protein separation. Proteins were separated in 10 % SDS-PAGE gels maintaining identical conditions for both the electrophoretic runs. Protein samples for electrophoresis were prepared with reducing agents (dithiothreitol and 2-mercaptoethanol) under denaturing conditions. The proteins were visualized after silver nitrate staining. For the identification of amylases, Western blot reactions were performed with the protein transferred to nitrocellulose membranes after separation by two dimensional electrophoresis. Antibodies raised to the precursor starch hydrolysing enzyme (Dubey *et al.,* 2000) were used to probe the amylase proteins. The details are described in Materials and Methods.

Two-dimensional SDS-PAGE separation of culture filtrate proteins of the parent strain of *A. niger* showed the occurrence of a prominent 125 kDa precursor amylase protein and a low concentration of 71- and 61-kDa glucoamylases. Two proteins were identified at the 53 kDa position (**Fig. 14**). The reactivity of the antibody raised against the starch hydrolysing enzyme with the homologous protein and the 71- and 61-kDa glucoamylases evidenced identification of the enzymes described above (**Fig. 14 inset**). In contrast to this result, two-dimensional SDS-PAGE analysis showed that the culture filtrate of the mutant strain of the fungus contained a degraded precursor amylase (**Fig. 15**). Importantly, Western blot analysis also showed the presence of prominent 71 kDa

glucoamylase and 53 kDa maltogenic α-amylase in the culture filtrates (**Fig. 15 inset**). Interestingly, in the mutant culture filtrates, two minor proteins corresponding to molecular mass 30 and 45 kDa were identified. These proteins were not the amylases since, the antibody did not react in western blot reaction (no reaction).

The results suggested accumulation of 71 kDa glucoamylase and 53 kDa maltogenic α-amylase in the culture filtrates of the mutant due to degradation of the precursor 125 kDa starch hydrolysing enzyme. The occurrence of minor proteins at 30 and 45 kDa positions in the mutant, not observed in the culture filtrates of the parent strain had relevance to proteases, since proteases characterized of *A. niger* were of similar molecular mass.

Fig. 14

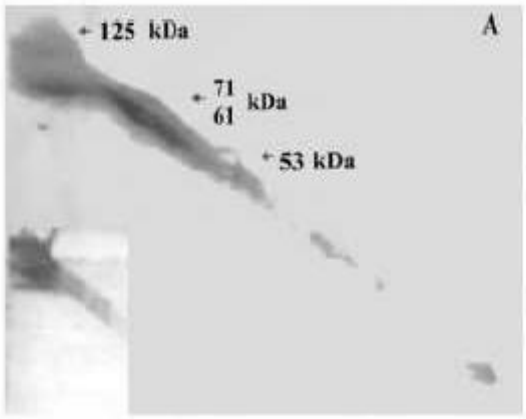

125 kDa
A
71
61
kDa
53 kDa

Fig. 15

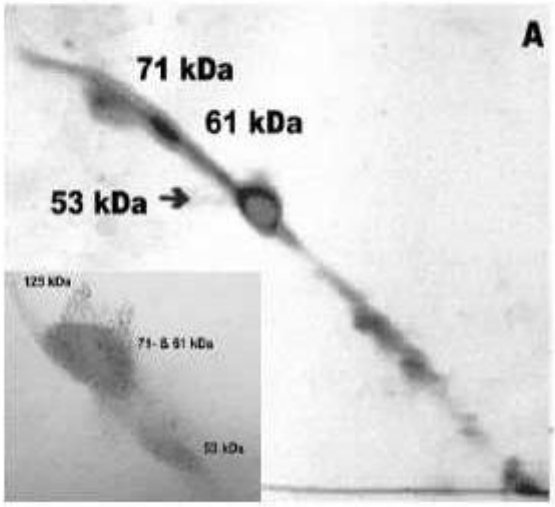

A
71 kDa
61 kDa
53 kDa
125 kDa
71- & 61 kDa
53 kDa

Fig. 14. Silver stained two-dimensional SDS-PAGE of total culture filtrate proteins (15 μg) of parent strain (A). **Inset**: corresponding Western blot reaction. The protein from the culture filtrates of parent (A) strain of *A. niger* after two-dimensional SDS-PAGE was probed with antibody raised against the precursor 125 kDa starch-hydrolysing enzyme in the Western blot reaction.

Fig. 15. Silver stained two-dimensional SDS-PAGE of total culture filtrate proteins (15 μg) of mutant strain (A). **Inset**: corresponding Western blot reaction. The protein from the culture filtrates of mutant (A) strain of *A. niger* after two-dimensional SDS-PAGE was probed with antibody raised against the precursor 125 kDa starch-hydrolysing enzyme in the Western blot reaction.

2.3. THE PROTEASES OF *ASPERGILLUS NIGER*

The observation that the mutant strain of *A. niger* over produced protease (Ex. No. 2.1) that apparently caused the degradation of the starch hydrolysing enzyme (**Fig. 15**) and the identification of 30 and 45 kDa proteins which corresponded to proteases of *A. niger* (NCBI Ac. No: A41025 GI: 101761; Mackenzie *et al.,* 2000) suggested experiments to identify the protease activity involved in amylase processing. Since zymogram assays for visualizing a protease activity after SDS-PAGE has been described in the literature (Christa and Eugene, 1980), this method was used to assay the proteases in the culture filtrates of the fungus.

Enzyme zymograms were performed after separating the culture filtrate proteins under non reducing conditions in 10% SDS polyacrylamide gels containing 0.1% gelatin. The activity was visualized as zones of clearance. The details of the procedure are described in Materials and Methods.

Zymogram analysis of the crude culture filtrate proteins of the parent and mutant strains of *A. niger* separated in gelatin containing polyacrylamide gels showed activity regions for proteases (**Fig. 16**). These activities were visualized at regions corresponding to molecular masses 125-, 71- and 45-kDa (**Fig. 16B**). The protease activity at the region corresponding to the molecular mass 45 kDa was unique to the mutant strain that showed degraded precursor starch hydrolysing enzyme (Ex. No. 2.2). This protease was absent in the parent strain (**Fig. 16A**).

The zymogram results identifying protease activities in the two amylolytic enzymes is new to literature. The occurrence of the 45 kDa protease only in the culture filtrate of the mutant strain of the fungus is probably an aspergillopepsin, since the molecular mass of this enzyme corresponded with that of the aspergillopepsin reported in literature (Mackenzie *et al.,* 2000).

Fig. 16

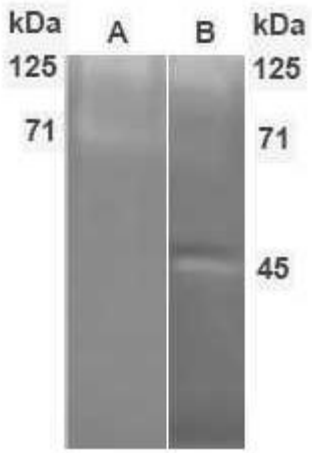


Fig. 16. Zymogram analysis of the crude culture filtrate proteins of the parent (A) and mutant (B) strains of *A. niger* for protease activities after separating the proteins (5 μg) in gelatin containing polyacrylamide gels

2.4. PROTEASE ACTIVITIES OF THE AMYLOLYTIC PROTEINS

Occurrence of protease activities in the amylolytic proteins identified by the zymogram reactions described in the previous experiment suggested a probable role for this activity in amylase protein processing in *A. niger*. To further confirm the above observation on the protease activity associated with amylases and to exclude the role of aspergillopepsin, the amylases were selectively precipitated from the culture filtrate of the mutant and assayed for protease activity by zymogram reaction.

The total culture filtrate proteins of the mutant strain were subject to ammonium sulphate fractionation at two saturations (60% and 90%). The precipitate obtained after centrifugation at 5000 x *g* for 20 min was dissolved in sodium acetate buffer (0.02 M, pH 4.0) and dialysed against same buffer to remove ammonium sulphate. Zymograms to visualize the protease activities were carried out as stated earlier. Zymogram assays were performed at different pH to identify the pH optimum for the enzyme activity. The details are described in Materials and Methods.

Ammonium sulphate fractionation of total culture filtrate proteins showed that maltogenic α-amylase and aspergillopepsin precipitated at 60% ammonium sulphate saturation. At this ammonium sulphate saturation, other amylases were assayed in the supernatant. The enzymes present in the supernatant of 60% ammonium sulphate saturation were precipitated at 90% ammonium sulphate saturation. Assay for protease activity by zymogram reactions of the protein precipitated after 90% ammonium sulphate saturation revealed a prominent protease activity at the region corresponding to the 71 kDa glucoamylase in the culture filtrates of the mutant strain of *A. niger*. This activity was optimum at pH 4.0. At pH 6.0, only very little protease activity could be visualized (**Fig. 17**). Protease activity zymograms performed with the amylases of the parent strain of *A. niger* after ammonium sulphate fractionation identified the activities in the 125 – and 71-kDa amylases (**Fig. 16A**).

These results located a prominent protease activity in the 71 kDa glucoamylase protein of the mutant strain of *A. niger*. The protease was also found to be optimally active at pH 4.0.

Fig. 17

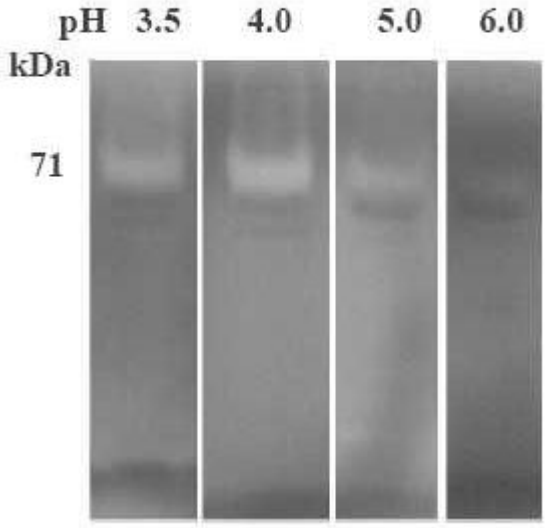


Fig. 17. Effect of pH on protease activity as determined by zymogram analysis

The gels were incubated at different pH to visualise the activity. Identical protein concentrations (16.93 μg) were used in all lanes.

2.5. *IN VITRO* PROTEASE ASSAY

The identification of protease activity by enzyme zymograms in the amylases described in the earlier experiment is new to literature. In order to confirm the zymogram assays, efforts were made to estimate the activity by *in vitro* assays. Since 90% ammonium sulphate fractionation selectively precipitated the amylases (**Fig. 18E**), this fraction was used for protease activity determinations.

Identification of protease activities inherent to the amylolytic enzymes suggested an assay that contains the enzymes from the 90% ammonium sulphate fraction. Hence this fraction was incubated in the buffer at 30^0C and assayed for the release of tyrosine and tryptophan using Folin-Ciocalteau phenol reagent (Ichishima, 1970). Activity for proteases was also done using casein as substrate. After the reaction, protein was precipitated with trichloro acetic acid and tyrosine and tryptophan in the supernatant was estimated using Folin-Ciocalteau phenol reagent. The unit activity corresponded to µ moles of tyrosine equivalents released min^{-1}. The unit activity was determined in terms of specific activity of enzyme after estimating the total protein concentration. The details are described in Materials and Methods.

No protease activity could be determined as tyrosine or tryptophan equivalents when the amylases were alone incubated and assayed for protease activity. However, these enzymes in the presence of casein as substrate, hydrolysed the substrate after 46 h of incubation to reason an activity determination of 4.98 units. Occurrence of protease activity in the amylolytic enzymes identified by the zymogram reactions could not be confirmed by the *in vitro* assays described.

Fig. 18

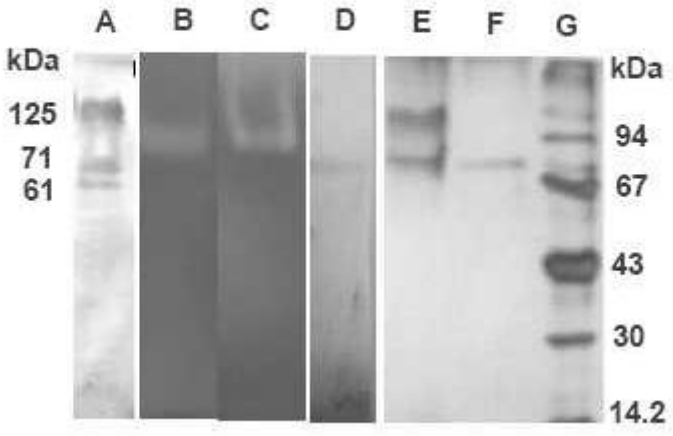


Fig. 18. Reactions of the electroeluted 71 kDa protein

A. Western reactions of the antibody raised to the purified enzyme (F. Silver stained (3 μg); D. Homologous reaction) with amylases in the crude culture filtrate of the parent (6 μg).

B & C. Zymograms of the purified enzyme (3 μg) showing protease (B) and amylase (C) activities. Electrophoresis of the protein in substrate containing gels caused migrational shifts. E: Proteins precipitated at 60-90% ammonium sulphate fractionation G: Molecular mass standards

Since these assays suggested the presence of protease as a contaminating enzyme and was at a variance from the reaction obtained with the zymogram assays, effect of acarbose on the protease activity was studied. This experiment was based on the presumptions that

i. The protease activity of the amylases does not result in the release of tyrosine or tryptophan for estimation by Folin-Ciocalteau phenol reagent.
ii. The inherent protease activity of amylase can hydrolyse casein.

iii. If the protease activity occurred as an inherent property of the amylase, acarbose that inhibit the amylases could possibly cause the inhibition of the protease activity as well and this could be measured with casein substrate.

Amylases taken in the buffer containing 0.125% acarbose, when used for protease activity determination with casein substrate resulted in the determination of 3.62 units of specific activity. This was about 27% reduced activity compared to the activity determined with out acarbose.

The above results suggested that the activity on casein was due to the protease activity inherent to the amylolytic enzymes and that acarbose caused only partial inhibition of this activity. These results also suggested a need for studies on the protease activity using the purified amylase protein.

2.6. PURIFICATION OF THE ENZYME

Though zymogram assays showed the occurrence of a protease activity in the amylolytic enzymes of *A. niger*, *in vitro* assays using casein suggested a possible contaminating protein in the ammonium sulphate fraction which was not identified by the amido black staining of the gels in zymograms. Hence, reactivity in terms of purified protease was found important to confirm that a protease activity indeed occurred as a inherent activity to the amylolytic proteins.

Since the apparent protease activity in the amylolytic enzymes was more prominent in the protein produced by the mutant strain of the fungus, the culture filtrate of the mutant strain grown in corn starch (10%), peptone (1%) and yeast extract (0.5%) containing medium was used for enzyme purification. The enhanced protease of the mutant protein was useful for zymogram assays for purification due to the absence of an *in vitro* assay. The protein was purified by electro elution after preparatory SDS-PAGE. Details of the procedures are described in Materials and Methods.

The protease activity visualised in the protease enzyme zymogram when purified, resolved as a single homogenous band in the silver stained SDS-PAGE gels (**Fig. 18F**). The migration of purified protein as 71 kDa protein in the SDS-PAGE gels suggested that it is the G1 form of glucoamylase. Zymogram reactivity of the protein for protease and amylase after SDS-PAGE in gels containing gelatin or starch confirmed that the protein is an amylolytic enzyme with inherent protease activity. Mobility shifts of the protein was observed in the substrate containing gels (**Fig. 18B** and **C**) which was probably due to the presence of substrates.

Significance of the above results has been discussed in the 'Discussion' section of the thesis.

2.7. SUBSTRATE AFFINITY OF THE ENZYME

The reactivity of the purified G1 form of glucoamylase to gelatin observed as a clearing zone in the zymogram reaction and the effect of glucoamylase inhibitor acarbose on the protease activity (Ex. No. 2.5) suggested that casein hydrolysis observed in the earlier experiment was due to the protease activity inherent to the amylase protein. Hence the affinity of the enzyme for the two substrates, starch and casein was studied.

Figures 19A and **B** describe the K_m determined of the purified G1 form of glucoamylase on starch and casein substrates respectively. The 71 kDa protein showed higher K_m on casein (0.64 %) compared to the starch (0.22 %), indicating its lower affinity to the protein substrate compared to starch.

Higher affinity of the enzyme for starch showed that the enzyme was an amylase with a low protease activity as determined by higher K_m to casein.

Fig. 19A

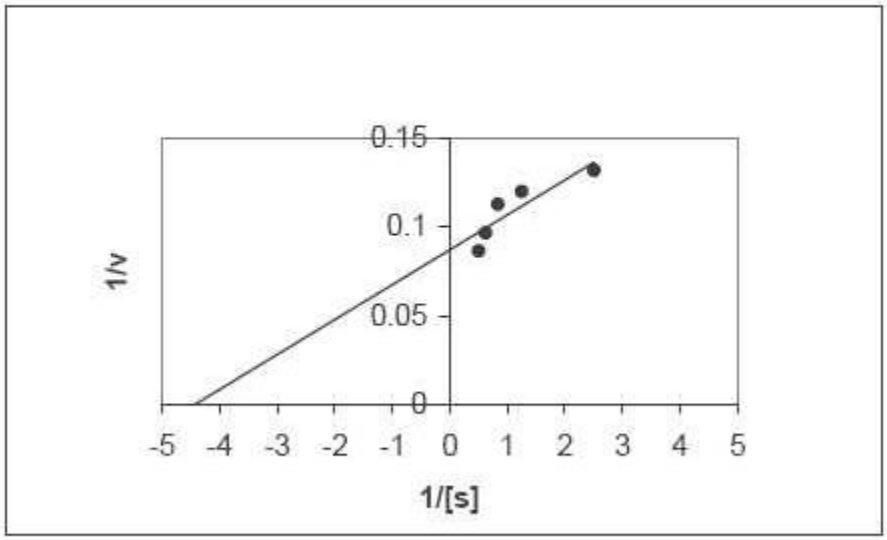


Fig. 19B

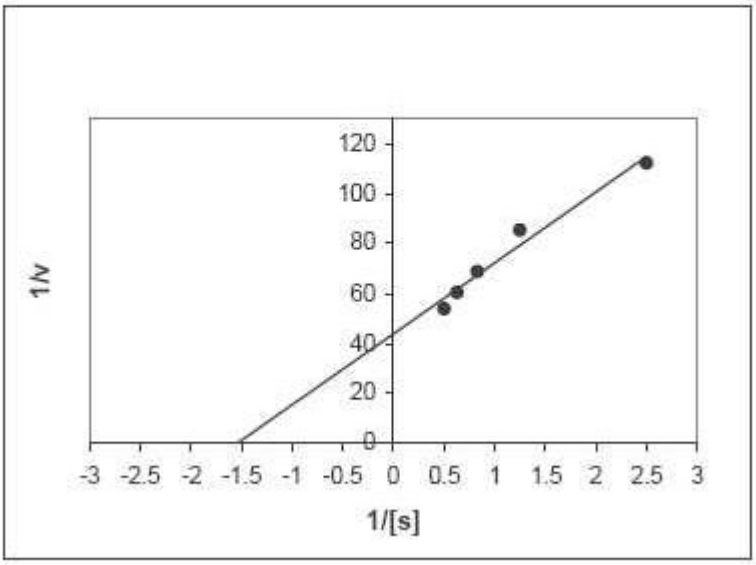


Fig. 19. Substrate affinity of the purified 71 kDa protein on starch and casein

A. Lineweaver-Burk plot for the determination of enzyme K_m to starch. 5.16 μg of enzyme protein was added to the substrate (0.4 to 2 %) and the reaction was carried out at 30^0C.
   K_m = 0.22 % starch
B. Lineweaver-Burk plot for the determination of enzyme K_m to casein. Details as described in Fig. 19A.
   K_m = 0.64 % casein

2.8. ANTIBODY REACTIVITY

Based on antibody cross reactivity it was earlier suggested that 71- and 61-kDa glucoamylases and 53 kDa Taka-amylase (maltogenic α-amylase) were products derived by proteolysis of 125 kDa starch hydrolysing enzyme (Dubey *et al.,* 2000). Enzyme zymograms and assays described in this study localized the amylase and protease activities in the same protein. To further substantiate this observation, reactivity of antibodies raised to the pure protein was studied.

Reactivity of the antibodies raised against the purified enzyme in rabbits was tested by ring test and Ouchterlony immuno double diffusion assay. The reactivity of the antibody to the enzyme protein was also confirmed by performing an antibody mediated enzyme inhibition assay. Cross reactivity of the culture filtrate proteins were visualized by Western blot reactions after separating the crude proteins in SDS-PAGE gels. The procedural details are described in Materials and Methods.

The antibody reacted with the homologous antigen in the ring test. The purified protein (antigen) on reacting with the antibody in the Ouchterlony double immuno diffusion reaction showed a single line of precipitation (**Fig. 20**). The antibody immobilized on the nitro cellulose membrane (Suresh *et al.,* 1998) when treated to the

enzyme present in solution, caused 18% reduction in the enzyme activity. Western blot reactions of the antibody with the crude culture filtrate proteins is shown in **Fig. 18A.**

The antibody cross reacted with 125- and 61-kDa proteins apart from the homologous reaction with the 71 kDa G1 form of glucoamylase.

The results are discussed in the 'Discussion' section of the thesis.

Fig. 20

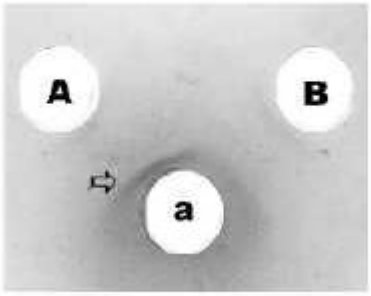


Fig. 20. Ouchterlony immuno double diffusion reaction

Reaction of undiluted (A) and 1:100 diluted (B) protease protein with antibody (a) was visualised as a line of precipitation (Arrow).

2.9. *IN VITRO* AUTO CATALYSIS OF 125 KDA STARCH HYDROLYSING ENZYME

Occurrence of a protease activity inherent to the precursor starch hydrolysing enzyme reasoned auto catalytic cleavage of the protein to form 71 kDa G1 form of glucoamylase and 53 kDa maltogenic α-amylase. To further evidence these observations, proteolysis of the precursor was studied by an *in vitro* experiment.

The purified precursor was stored at 4^0C for 7 days and Western blot analysis was carried out with the homologous antibody to identify the cleaved products.

Results showed degradation of purified 125 kDa protein upon storage at 4^0C. The products of auto catalysis of the precursor were 71- and 61-kDa glucoamylases and 53 kDa maltogenic α-amylase (**Fig. 21**).

These results supported the earlier observation on the occurrence of an inherent protease activity in the 125 kDa precursor amylase.

Fig. 21

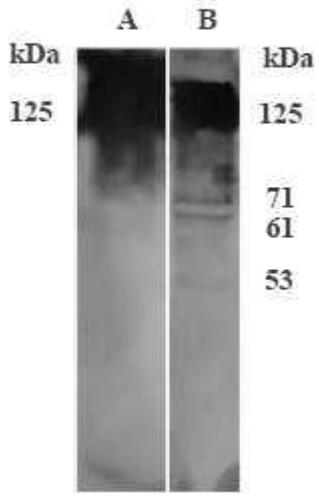


Fig. 21. Autocatalytic degradation of the precursor starch hydrolysing enzyme (10 μg) during storage as identified by Western blot reactions.

The purified precursor starch hydrolysing enzyme (A) was stored at 4^0C for 7 days in sodium acetate buffer (0.02 M) and analysed for degradation (B) using homologous antibody. Overloading of the protein in the wells was necessary to visualize the degraded products. Overloading affected protein transfer for Western blot reaction.

PART III

MEDIA OPTIMISATION AND SCALE UP STUDIES FOR MALTOGENIC ALPHA AMYLASE PRODUCTION

INTRODUCTION

Submerged and solid state fermentations are parts of the processes used for the production of enzymes from *Aspergillus*. Amylases were produced by solid-state fermentation from *A. oryzae* for the first time by Takemine. Solid-state method of fermentation is limited by the lack of control over fermentation conditions and heavy sporulation eventhough it has several economical advantages. Amylolytic enzymes are also produced by submerged fermentation (Saha *et al.,*1979) wherein the conditions are very well controlled for an easy down stream processing of the product. In view of the availability of above methods for the production of enzymes, both the methods were used for optimizing media components for the production of the 53 kDa maltogenic α-amylase using the mutant (9) strain of *A. niger* in this study.

After carrying out strain improvement for maltogenic α-amylase, various media components were screened for the production of maltogenic α-amylase. Based on laboratory experiments, scale up studies were also conducted. Stabilization studies of the enzyme were carried out for storage. Effect of acarbose on the production of maltogenic α-amylase in the submerged fermentation was also investigated. The details are described in this part of the thesis.

3.1. EFFECT OF ACARBOSE ON MALTOGENIC ALPHA AMYLASE PRODUCTION IN SHAKE FLASK CULTURES

Acarbose is a potent α-glucosidase inhibitor and it inhibits glucoamylases of *A. niger* (Olsen *et al*., 1993). In this study, it was shown that acarbose inhibited the activity of 125-, 71- and 61-kDa amylases (**Table 1**) but not the reactivity of the maltogenic amylase. Ex. No. 2.5 described the inhibition of the protease activity inherent to the glucoamylase by acarbose. Based on these observations and in order to use acarbose for media screening for maltogenic α-amylase production, the effect of this inhibitor on the growth of the organism was studied.

The parent and mutant strains of *A. niger* were grown in the medium containing corn flour (4%), peptone (1%) and yeast extract (0.5%) with and with out acarbose at 0.005% concentration. After 24 h growth, the culture filtrates were separated by filtering through Whatman No. 1 filter paper and the enzyme activity was visualised as enzyme zymogram reactions. For this study, after electrophoresis in starch incorporated gels, the zymogram reaction was carried out in acetate buffer (0.1 M, pH 4.3) containing 0.125% acarbose. The details are as described in Materials and Methods.

In the absence of acarbose in the medium, the mutant strain of the fungus produced 2.19 times more maltogenic α-amylase compared to the parent (**Table 6**). Generally strains grown in the medium containing acarbose (0.005%) produced more maltogenic α-amylase. The parent strain showed 113 % increase in the production of maltogenic α-amylase while the mutant showed only 49 % increase (**Table 6**). Higher productivity of maltogenic α-amylase in the culture filtrates of the parent compared to the mutant described above was further visualised in the zymogram reaction (**Fig. 22**).

It was also interesting to see increased maltogenic α-amylase excretion by the fungus in the presence of acarbose.

Fig. 22

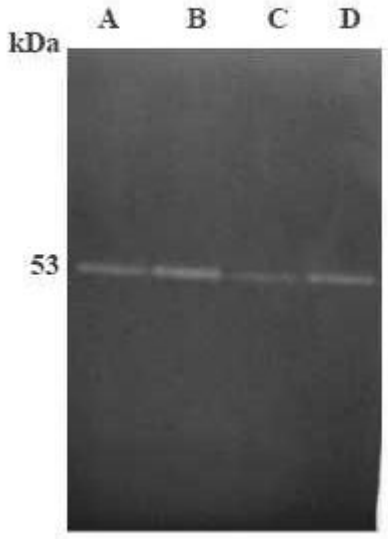


Fig. 22. Effect of acarbose on maltogenic α-amylase production during submerged growth

The culture filtrates of the mutant (A and B) and parent (C and D) strains of *A. niger* were analysed for maltogenic α-amylase in enzyme zymogram reactions. 4 μg of protein was used in all the lanes.

A and C: Medium containing no acarbose
B and D: Acarbose containing medium

Table 6: Relative specific activities of maltogenic α-amylase in the culture filtrates of *A. niger* strains

Culture	Control (Units/mL)[a]	Acarbose containing medium (Units/mL)[a]
mutant	68	102
parent	31	66

[a]Unit = μ moles of reducing sugars as maltose equivalents produced min^{-1}

Higher activities of maltogenic α-amylase estimated in the culture filtrates after growing in the acarbose containing medium apparently suggested the need for more of this enzyme for utilization of starch substrate since acarbose was shown to inhibit the activities of all other amylases produced by this fungus.

3.2. MEDIA STANDARDISATION FOR MALTOGENIC ALPHA AMYLASE PRODUCTION

Report on the production of maltogenic α-amylase by *A. niger* shown in this study is new to literature. Production of the enzyme due to proteolytic degradation of the precursor amylase (Dubey *et al.,* 2000; Ravi-Kumar *et al.,* 2004), has already been discussed. These observations suggested that formulation of a medium for maltogenic α-amylase production by *A. niger* depends on several factors. They are:

i. That the fungus grows and produces high concentrations of the precursor enzyme.
ii. Precursor amylase production and the autocatalytic activity result in the accumulation of maltogenic α-amylase; and
iii. Maltogenic α-amylase produced should not be degraded by the proteases, since a protease, aspergillopepsin, was identified in the present study as a secreted protein of the mutant strain of the fungus (**Fig. 16B**).

In order to identify the factors described above and since general enzyme assays do not reflect the cause for decreased enzyme yields, it was found necessary to standardize a presumptive method to rapidly identify the components needed for defining a medium for maltogenic α-amylase production by the fungus. In an earlier study, it has been shown that use of corn flour/corn starch resulted in increased production of the precursor amylase (Suresh, 1999). Hence, media based on starch (primary carbon source) was used and amendments were carried out to evaluate the enzyme production by the fungus. In some of the assays, acarbose was incorporated in the medium to short list the medium for further studies. Clearance of starch due to amylases was identified by iodide/iodine solution and the comparative zones of starch clearance in the presence of acarbose was used to visualise maltogenic α-amylase production.

In acarbose containing potato starch agar plates, maltogenic α-amylase activity was identified as zones of starch clearance due to the degradation of starch to maltose and maltotrioses (**Fig. 23B**). In the absence of acarbose in the medium, due to the activity of glucoamylases and precursor amylase, larger zones of clearance surrounding the colonies were seen (**Fig. 23A**). Comparison of zones of clearance when the mutant was grown on various media in the presence of acarbose (0.005%) gave a presumptive indication on the quantity of maltogenic α-amylase produced. Larger the zones of clearance, higher were the enzyme productivity. Hence based on this presumption, the components for a medium were approximated.

Identification of maltogenic α-amylase production in plate assays containing acarbose was useful in the presumptive identification of media components for further studies.

Fig. 23

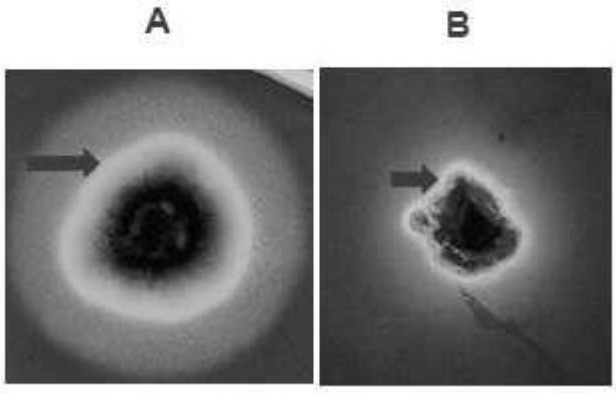


Fig. 23.Amylase production in the presence (B) and absence (A) of acarbose

Arrows indicate zones of starch clearance due to amylase activity after staining with iodide/iodine solution.

3.3. MEDIA COMPONENTS FOR ENZYME PRODUCTION BY SUBMERGED FERMENTATION

In addition to strain improvement for defining a technology, inexpensive medium for fermentation is important for obtaining low value products like amylases. Hence, in this study, media components that showed promise for obtaining increased maltogenic α-amylase yields were tested for maltogenic α-amylase production by the fungus in submerged fermentation. Growth conditions were as already stated in Materials and Methods.

Activity estimations after growing the fungus in different media (**Table 7**) showed economic yields of the enzyme when corn flour was used as a carbon source. Among nitrogen sources, peptone could be replaced with corn steep liquor to increase enzyme production. Use of essential amino acid arginine did not improve the enzyme production to any significant levels.

Based on the above experiment, the medium consisting of corn flour (6%) and corn steep liquor (6%) was defined for the production of maltogenic α-amylase by submerged fermentation since this medium is also economical on cost basis.

Table 7:Optimization of media components for maltogenic α-amylase production by the mutant strain (9) of A. niger in submerged cultures (Initial pH was 5.5)

Media components	Medium No.																						
	Composition (g/L)																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Corn flour	40				40	40	20	60	80	60	60	60	60	60	60	60	60	60	60	60	60	60	60
Corn starch		40																					
Glucose			40																				
Maltose				40																			
Peptone	10	10	10	10			10	10	10	2	4	6	8										
Yeast extract	5	5	5	5			5	5	5	5	5	5	5										
Corn steep liquor					20									30	40	50	60	80	100	40	40	40	40
$(NH_4)_2HPO_4$						15																	
$NH_4H_2PO_4$						15																	
Gelatin																				1			
Casein																					1		
Cysteine																						1	
Arginine																							1
Activity(Units/mL)	42	43	37	39	39	0	35	78	21	29	34	38	54	42	60	33	70	60	50	60	66	15	63

3.4. PILOT PLANT STUDIES

Using the media standardized for maltogenic a-amylase production (corn flour, 6% and corn steep liquor, 6%), the fermentation was scaled to 12 L.

In this study, a chemap ^{R} fermentor was used (Fig. 24). The conditions and other details for fermentation were as described in Materials and Methods. Two fermentor experiments were carried out to check the effect of pH on the enzyme production. Both the trials were carried out keeping the initial pH of the medium as 5.5. Preliminary studies showed increase of pH after the initial drop to 4.5 from 5.5. Hence for the first trial (Fig. 25A), the fungus was grown without adjusting the pH. In the other trial (Fig. 25B) when the pH was dropped to 4.5 it was maintained by the addition of hydrochloric acid (1N).

Assay for the enzyme at regular time intervals showed the production of the enzyme by the fungus in the fermentor after 18-20 h of fermentation (Fig. 25B). This was followed by a gradual increase in enzyme production till 39 h and 77 units of enzyme activity was estimated at this time in both the trials (Fig. 25A and B). This suggested that pH had no effect on enzyme production. However, there was a decrease in amylase activity after 39 h when the pH was maintained at 4.5 after the initial drop during the growth of the fungus (Fig. 25B). In the trial where pH maintenance was not carried out (Fig. 25A), there was a sharp increase in activity (108 units) after the completion of fermentation (39 h).

Decreased specific activity after fermentation when the pH was not maintained (Fig. 25A), suggested cell lysis and protein increase.

Fig. 24

A

B

Fig. 24. A. Chemap [R] Fermentor used for scale-up studies

B. Fermentor (enlarged) showing fungal growth

Fig. 25A

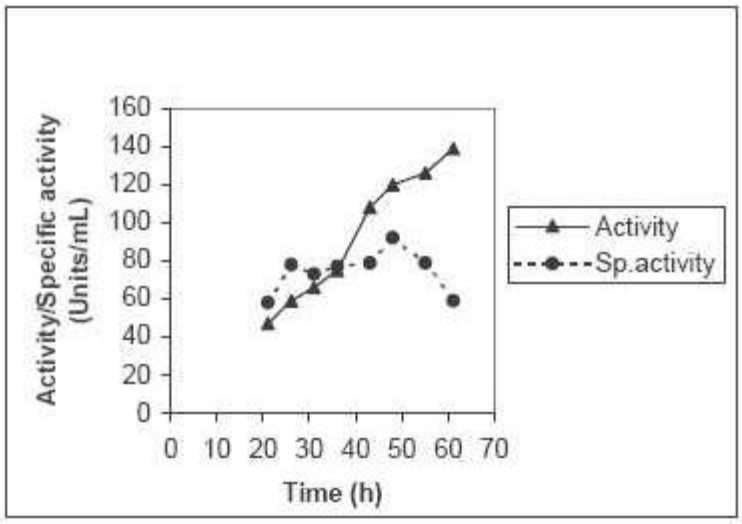


Fig.25B

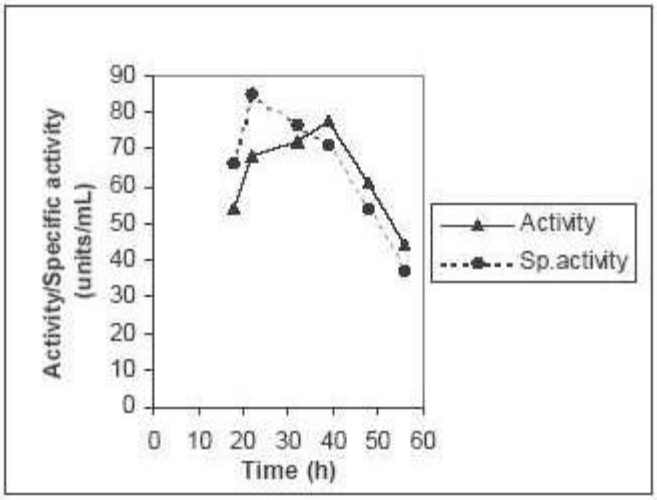


Fig. 25. Scale-up studies (12L) for maltogenic α-amylase production using the mutant strain of A. niger

A. Submerged batch fermentation carried out with out maintaining pH
B. Submerged batch fermentation carried out by maintaining pH at 4.5

3.5. EFFECT OF MEDIUM COMPONENTS ON MALTOGENIC ALPHA AMYLASE PRODUCTION

Maltogenic α-amylase production by the mutant strain occurred due to proteolysis of the precursor starch hydrolysing enzyme (Ex. No. 2.9). Even though plate assay that contained incorporated acarbose in the medium showed maltogenic α-amylase production by the fungus in different media, growth of the fungus in shake flask cultures using some of the media components resulted in no activity determinations of the enzyme in the culture filtrates. This was typical in the case of the medium containing ammonium salts as the nitrogen source (Ex. No. 3.3). To understand the reason for non production of the enzyme in the medium and in order to reason the role of ammonium salts, the culture filtrates of the fungus obtained after growth in the medium made of corn flour (4%), $(NH_4)_2HPO_4$ (1.5%) and $NH_4H_2PO_4$ (1.5%) were analysed for maltogenic α-amylase and protease by performing Western blot and zymogram reactions.

The culture filtrate proteins precipitated with 90% ammonium sulphate saturation were used for SDS-PAGE performed under reducing conditions for two-dimensional Western blot analysis. Protease activity was visualised as zymogram reaction under non reducing conditions. The details of the methodology are described in Materials and Methods.

Western blot analysis of proteins after two-dimensional SDS-PAGE identified only two proteins in the culture filtrates. Based on molecular mass of the protein they were identified as 71-and 61-kDa glucoamylases (**Fig. 26A**). Typically, the maltogenic

α-amylase and the precursor starch hydrolysing enzymes were absent in the culture filtrates.

Zymogram analysis showed the occurrence of a 58 kDa protease protein in the culture filtrate of the fungus grown in the medium containing ammonium salts (**Fig. 26C**).

The results are discussed in the 'Discussion' section of the thesis.

Fig. 26

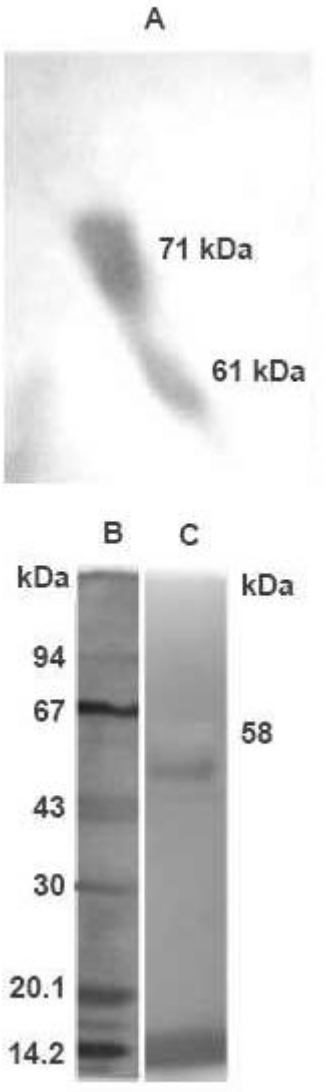


Fig. 26. Extracellular amylase and proteases of the mutant strain grown in the medium containing ammonium salts

A. Western reactions of antibodies raised against 125 kDa amylase with the culture filtrate protein separated in 10% two-dimensional SDS-polyacrylamide gels
B. Molecular weight markers
C. Zymogram reaction showing 58 kDa protease activity in the culture filtrate

3.6. SCREENING OF MEDIA COMPONENTS FOR ENZYME PRODUCTION BY SOLID STATE FERMENTATION

A. niger has been shown to produce amylases when grown in solid state (Selvakumar *et al.,* 1996). Since the mutant isolated in this study produced maltogenic α-amylase in submerged fermentation, effect of solid media on the enzyme production was studied. Ingredients for solid state cultivation (**Table 8**) were mixed in glass petriplates using 25 mL of hydrochloric acid (0.2 N) or water and sterilized for 45 min at 15 lbs pressure. The strain grown for 24 h in liquid medium made of corn flour (10%) and corn steep liquor (2%) was used to inoculate the solid medium and the fungus was allowed to grow for 48-120 h at 30^0C. Crude enzyme was obtained by extracting 10 g of moldy bran in 100 mL sodium acetate buffer (0.1 M, pH 4.3) for 30 min. After filtration through Whatman No.1 paper, maltogenic α-amylase activity was determined using starch as the substrate. Activity corresponded to μ moles of maltose equivalents produced min^{-1} g^{-1} dry moldy bran.

Results of screening experiments are summarized in **Table 8**. Micro nutrients improved the enzyme yields when the fungus was grown in solid substrates (Media 2 and 3). Rice bran substrate containing micro nutrients resulted in the production of 144 units of the enzyme.

Table 8: Standardization of solid state media components for maltogenic a-amylase production by themutant (9) strain of A. niger

Media components	Medium No.						
	1	2	3	4	5	6	7
Wheat bran (g)	25	25	25				
Corn flour (g)	0.5	0.5	0.5	25			
Rice bran (g)					25		
Tippe waste (g)						25	
Bagasse (g)							25
KCl (mg)			25	25	25	25	25
$CuSO_4.5H_2O$ (mg)			0.25	0.25	0.25	0.25	0.25
$ZnSO_4.7H_2O$ (mg)			0.5	0.5	0.5	0.5	0.5
0.2 N HCl (mL)		25	25	25	25	25	25
Water (mL)	25						
Activity (Units)	85	89	117	12	144	37	17

3.7. STABILIZATION STUDIES

Studies on the stability of the enzyme after fermentation are an important requirement and since production of maltogenic a-amylase requires studies on the enzyme storage for defining a process technology, this was under taken to assess the stability of the enzyme in the crude preparation.

Culture broth obtained after growing the mutant strain of A. niger in corn flour (6%) and corn steep liquor (6%) broth was used as enzyme in this study. Studies were carried out using the enzyme stored with and without additives like sodium benzoate and calcium chloride. Corn starch was included for some of the experiments, since substrates have been reported to stabilize enzymes. Stabilization studies were carried out with enzyme stored at 4^0, 28^0 and 35^0C.

Results (Table 9) showed stability of maltogenic a-amylase in the presence of calcium chloride at 4^0C. At 28^0 and 35^0C, there was a significant loss in the activity of the enzyme. Sodium benzoate and corn starch could not stabilize the enzyme. Interestingly an increase in the activity of the enzyme was observed after the storage period of 49 days. This apparently was due to proteolytic degradation of the precursor amylase in the crude enzyme preparation resulting in the formation of maltogenic a-amylase.

Table 9: Stabilization studies of the maltogenic α-amylase for storage

Storage period (Days)	Sample	Storing temperature					
		4^0C		28^0C		35^0C	
		Activity (Units)	%Activity change	Activity (Units)	%Activity change	Activity (Units)	%Activity change
14	1	91	0	0	0	0	0
	2	97	0	0	0	0	0
	3	95	0	94	0	57	-38.7
	4	96	0	0	0	0	0
	5	101	+8.6	71	-23.6	0	0
35	1	61	-34.4	0	0	0	0
	2	60	-35.4	0	0	0	0
	3	81	-12.9	62	-33.3	36	-61.2
	4	63	-32.2	0	0	0	0
	5	81	-12.9	0	0	0	0
49	1	64	-31.1	0	0	0	0
	2	65	-30.1	0	0	0	0
	3	95	0	57	-38.7	45	-51.6
	4	68	-26.8	0	0	0	0
	5	93	0	0	0	0	0
66	1	78	-16.1	0	0	0	0
	2	87	-6.4	0	0	0	0
	3	110	+18.2	43	-53.7	50	-46.2
	4	85	-8.6	0	0	0	0
	5	107	+15	0	0	0	0
103	1	77	-17.2	0	0	0	0
	2	77	-17.2	0	0	0	0
	3	113	+21.5	11	-88.1	44	-52.6
	4	94	0	0	0	0	0
	5	113	+21.5	0	0	0	0

Table 9: Stabilization studies of the maltogenic α-amylase for storage

Original activity of the enzyme sample that used for storage studies was 93 Units mL^{-1}

- and + corresponds to decrease and increase in the activity

± 5 Units of original activity was considered no change in activity

1. Enzyme sample with out stabilizers
2. Enzyme sample with sodium benzoate (1000 ppm)
3. Enzyme sample with $CaCl_2$ (0.03 M)
4. Enzyme sample with raw corn starch (0.2 %)
5. Enzyme sample with $CaCl_2$ (0.03 M) and raw corn starch (0.2 %)

DISCUSSION

AMYLASES OF ASPERGILLUS NIGER

The saprophytic ability of *Aspergillus* has defined species of the fungus as organisms capable of producing several enzymes. The strains of the species *A. niger* being food safe due to its GRAS status, have been recognized for the production of enzymes by fermentation for use in several industrial applications. Of the enzymes produced by the fungus, amylases are industrially important since they have applications in food, pharmaceutical and brewing industries. Even though the fungus has been characterized for several amylases like amyloglucosidase or glucoamylase (Ramasesh *et al.*, 1982a), α-amylase (Ramasesh *et al.*, 1982b), α-glucosidase (Kita *et al.,* 1991) and starch hydrolysing enzyme (Suresh *et al.,* 1999a), the report from this laboratory that the organism also produces a Taka-amylase (Dubey *et al.,* 2000) raised the fundamental question on the need for the organism to secrete so many amylases to degrade a single substrate, the starch, for utilization as a growth substrate. The observation that all the amylases can be identified in the culture broth after growth of the organism in a starch containing medium (**Fig. 1**) suggested that the production of the enzymes is not sequential. Hence, secretion of the enzyme can not be reasoned to regulation by the type of sugar formed upon hydrolysis of starch during growth of the organism by each of the amylase.

Zymogram analysis of culture filtrate proteins of *A. niger* for amylase activities at pH 4.3 identified four reactive zones corresponding to 125-, 71-, 61- and 53-kDa molecular mass (**Fig. 1A**). At pH 6.0, zymogram reactions for amylase activities identified only a single reactive zone corresponding to molecular mass 53 kDa (**Fig. 1B**). The occurrence of two amylases of molecular mass 53 kDa perhaps reasoned the description of one of these which was active at pH 6.0 (Ramasesh *et al.*, 1982b). Only later studies at this laboratory identified the occurrence of another 53 kDa amylase protein (Dubey *et al.,* 2000) and this was studied in this investigation. Amylase assays using culture filtrate at pH 4.3 revealed that glucose was a major product of hydrolysis (**Table 1**). This showed the presence of glucoamylases and the starch hydrolysing

enzyme as the major amylases in the culture filtrate of the parent strain. Activities on maltose further confirmed their presence in the culture filtrate. Activity estimations on starch using the inhibitor acarbose (pseudotetrasaccharide which is a potent inhibitor of glucoamylases) revealed inhibition of glucose formation after starch hydrolysis (**Table 1**). These results suggested that the activity of one of the amylases not the glucoamylases or starch hydrolysing enzyme, was free of acarbose inhibition in the assay. However the enzyme of *A. niger* was not characterized. Since the assay in presence of acarbose described the formation of reducing sugars and maltose as products, the enzyme was probably the Taka-amylase. It has been shown earlier in this laboratory (Dubey *et al.,* 2000) that Taka-amylase is produced by the fungus and it was identified in *A. niger* by N-terminal sequence homology of the pure protein with that produced by *A. oryzae*.

MUTANTS OF *ASPERGILLUS NIGER*

Secretion of high molecular mass proteins, specifically glucoamylases, by *Aspergillus* is known to occur through the hyphal tips (Wosten *et al.,* 1991; Gordon *et al.,* 2000) where cell wall is less developed. Such a protein secretion has been reasoned to reduced porosity of cell walls affecting secretion of high molecular mass proteins. Hence multiple amylase production by *A. niger* was reasoned to post translational processing of precursor high molecular mass starch hydrolysing enzyme by proteases to overcome secretional difficulties associated with cell wall porosity resulting in the formation of 71-, 61-kDa glucoamylases and 53 kDa Taka-amylase (Dubey *et al.,* 2000). In order to consolidate the understanding on the mechanism of amylase processing, mutants affected in general secretory pathway were isolated by temperature selection (Gordon *et al.,* 2000) after subjecting the spores to UV rays (Ex. No. 1.2). The assumption was that the mutants so isolated would have deformed structures thereby secreting all the enzymes. In this isolation protocol for mutants, a screening for hyper protease production was also included since the earlier study predicted a role for proteases in the amylase processing that would allow the isolation of mutants overproducing the low molecular mass 53 kDa amylase active at pH 4.3.

Using the screening procedures described above, a mutant that overproduced the 53 kDa amylase active at pH 4.3 was obtained (**Fig. 4B**) and analysis showed that the enzyme was produced due to proteolytic processing of high molecular mass amylase (described below).

CHARACTERISATION OF THE 53 KDA AMYLASE PRODUCED BY THE MUTANT

The enzyme purified from the culture broth of the mutant strain of *A. niger* had an apparent molecular mass of 53 kDa by SDS-PAGE (**Fig. 5**). It was optimally active at pH 4.3 (**Fig. 6**). Though the enzyme showed optimum activity at 40^0C (**Fig. 7**), it was less stable at this temperature. However, addition of 0.05 M $CaCl_2$ allowed the stability of the enzyme at 50^0C for 30 min (**Table3**). The enzyme showed highest affinity to branched starch and amylopectin molecules compared to linear amylose (**Fig. 8A, B** and **C**). The enzyme was not active on pullulan, suggesting its non-reactivity to α-1,6-linkages because pullulan is a glucose polymer made of this linkage. Polarimetric studies showed that the enzyme produced products of α-anomeric configuration since the products of the enzyme reaction on starch produced a downward shift in optical rotation after the addition of the base (**Fig. 11**).

Time course studies on the analysis of products of starch hydrolysis by purified enzyme (**Fig. 9**) showed maltose, maltotriose and maltodextrins as the first products. This suggested an exo and endo activities for the enzyme. Prolonged reaction showing accumulation of maltose and maltotriose as major products and reduction in maltodextrins suggested that the initial activity of the enzyme released maltose from the non reducing end of starch and inability to hydrolyse the α-1,6-linkages caused the formation of maltotriose. In contrast, the α-amylase of *A. niger* which was active at pH 6.0 (Ramasesh *et al.*, 1982b) rapidly hydrolyzed starch to result in maltodextrins only (**Fig. 10**). The product analysis and characterization studies confirmed that the 53 kDa amylase characterized of *A. niger* in this study acted like a Taka-amylase of *A. oryzae* (EC 3.2.1.1). The Taka-amylase of *A. oryzae* is known to be a 54 kDa protein that hydrolysed α-1,4-glucosidic linkages in starch to evolve maltose as product. Interestingly, the temperature and pH optima of 40^0C and 5.0 respectively reported of

Taka-amylase of *A. oryzae* (Matsuura *et al*., 1979; Matsuura *et al*., 1984; Matsubara *et al.*, 1959; Perevozchenko and Tsyperovich, 1972) also corresponded with the amylase characterized of *A. niger* in this study. Antibody cross reactivity (**Fig. 12**) showed that the two enzymes shared a number of structural features in addition to the identical N-terminal amino acid sequence (Dubey *et al.,* 2000). Polarimetric studies differentiated the 53 kDa amylase of *A. niger* from β-amylase which is also a maltogenic amylase usually reported of *Streptomyces*, *Bacillus* spp. and *C. thermosulphurogenes* (Ray and Nanda, 1996; Hyun and Zeikus, 1985), because starch upon degradation by β-amylase result in the production of maltose with β-anomeric configuration.

Despite the N-terminal **ATPAD** sequence of the enzyme showing homology to that of the *A. oryzae* Taka-amylase (Dubey *et al.,* 2000), the two enzymes differed marginally in their properties. While the hydrolysis of starch by the *A. niger* enzyme accumulated maltotriose as one of the products (**Fig. 9**), Taka-amylase of *A. oryzae* has been shown to bind to maltotriose and release glucose (Matsuura *et al.*, 1984). Like wise, the activity of *A. niger* enzyme was not inhibited by zinc ions (**Table 4**) unlike inhibition of Taka-amylase of *A. oryzae* by zinc (Toda and Narita, 1967). Though small, the differences in the enzyme properties were significant. Hence the enzyme of *A. niger* was more a maltogenic α-amylase rather than the Taka-amylase *per se*.

AMYLASE PROCESSING IN *ASPERGILLUS NIGER*

Amylases reported from bacteria are usually single protein species with typical properties. However, multiple amylases have been described from the species of *Aspergillus* (Vihinen and Mantsala, 1989). As already discussed, *A. niger* has been reported to produce two forms of glucoamylase (Ramasesh *et al.*, 1982a), α-amylase (Ramasesh *et al.*, 1982b), starch hydrolysing enzyme (Suresh *et al.,* 1999a), α-glucosidase (Kita *et al.,* 1991) and the maltogenic α-amylase earlier referred to as Taka-amylase (Dubey *et al.,* 2000). Though, cloning and sequence analysis of the glucoamylase gene of *A. niger* identified differential splicing of the glucoamylase mRNA for the secretion of G1 and G2 forms of the enzyme (Boel *et al.,* 1984a,b), studies using the peptide fragments obtained from G1 and G2 glucoamylases of *A. niger* suggested

post-translational proteolysis in the COOH-terminal region of G1 to result in the G2 enzyme form (Svensson *et al.,* 1986). Since two forms of glucoamylase were also reported of *A. awamori, A. siato* and *Rhizopus* species, such a proteolysis was apparently responsible for the generation of multiple forms of amylases in these fungi as well. Based on N-terminal amino acid sequence homology of the starch hydrolysing enzyme protein of *A. niger* with the G1 and G2 forms of glucoamylase, cross reactivity of the antibody raised to starch hydrolysing enzyme with G1 and G2 glucoamylases and identification of raw starch hydrolysing activities in the starch hydrolysing enzyme and G1 form of glucoamylase it was reasoned that the starch hydrolysing enzyme was the amylase precursor from which G1 and G2 forms of glucoamylase and Taka-amylase arose by proteolytic processing (Dubey *et al.,* 2000). However, a protease involved in such a processing was not characterized.

In this study, a mutant of *A. niger* affected in the general secretory pathway isolated by selection for temperature tolerance (Gordon *et al.,* 2000) also showed five fold increased protease activity compared to the parent when grown in the medium containing starch (0.33 U/mg *vs.* parent 0.066 U/mg). Growth of the mutant in the same medium showed accumulation of maltogenic α-amylase (202 U/mg *vs.* parent 130 U/mg) and concomitant decrease of the other amylases active at pH 4.3 (12 U/mg *vs.* parent 45 U/mg). This favoured the hypothesis that proteases have a role in amylase processing. Two-dimensional SDS-PAGE analysis for the amylases of the mutant and parent strains of the fungus showed degradation of 125 kDa starch hydrolyzing enzyme and accumulation of glucoamylases and the 53 kDa maltogenic α-amylase (**Fig. 14** and **15**). Thus it appeared that for efficient utilization of starch substrate and since the cell walls of *A. niger* were less permeable to high molecular mass proteins, the precursor starch hydrolysing enzyme was proteolytically processed to lower molecular mass 53 kDa maltogenic α-amylase and 71-, 61-kDa glucoamylases (**Fig 14** and **15**).

Aspergillopepsins have been reported to be the major proteases produced by *A. niger* and the mutants affected in aspergillopepsin production were shown to over produce glucoamylase in culture (Mackenzie *et al.,* 2000). In the culture filtrates of the

mutant strain of *A. niger,* zymogram analysis identified a clear 45 kDa protease (**Fig. 16B**) which was apparently an aspergillopepsin due to similarity of the molecular mass reported of the enzyme. However, this protease activity was characteristically absent in the culture filtrate of the parent (**Fig. 16A**). Since the parent strain also produced multiple forms of amylase despite characteristic absence of aspergillopepsin in the culture filtrate, the results suggested that the higher protease activity assayed in the culture filtrate of the mutant strain was due to over production of aspergillopepsin and that this protease had no role in the degradation of starch hydrolysing enzyme obtained in the Western blot assay (**Fig. 15**). Zymogram analysis also showed the occurrence of protease activities in the regions corresponding to 125 kDa starch hydrolysing enzyme and 71 kDa G1 form of glucoamylase (**Fig. 16**). This could not be reasoned to proteases to co-migrate with the two amylolytic enzymes because, in the earlier study, a clear N-terminal amino acid sequence was obtained of the 125 kDa starch hydrolysing enzyme by micro sequencing (Dubey *et al.,* 2000). To reason the inherent protease activity in starch hydrolysing enzyme as a cause for its degradation, the purified enzyme of the parent strain was incubated at 4^0C and analyzed for the degraded products by performing Western blot reactions using homologous antibody. After seven days incubation, degradation of protein and formation of the glucoamylase and maltogenic α-amylase was observed (**Fig. 21**).

Ammonium sulphate precipitation of the culture filtrate proteins of the mutant strain of *A. niger* separated aspergillopepsin from the amylases. While, aspergillopepsin and maltogenic α-amylase precipitated at 60% ammonium sulphate concentration, increasing the concentration to 90% precipitated other amylases in the supernatant. The precipitated amylases when assayed for protease by zymogram analysis showed a prominent activity at pH 4.0 in the 71 kDa glucoamylase protein (**Fig. 17**). Since this activity could reason the degradation of the 71 kDa glucoamylase to 61 kDa enzyme, *in vitro* assay for protease was carried out by incubating the 60-90 % ammonium sulphate fraction (**Fig. 18E**) dissolved in sodium acetate buffer. Despite 46 h incubation, no protease activity was detected as tyrosine or tryptophan equivalents. However, the enzyme in the presence of casein as substrate, hydrolyzed the substrate

after 46 h of incubation to account for an activity determination of 4.98 units in terms of tyrosine equivalents. The typical hydrolysis patterns observed showed that the enzyme has an amylase activity and a non specific protease activity releasing tyrosine/tryptophan from casein. This was further substantiated by K_m determination using the purified 71 kDa G1 form of glucoamylase (**Fig. 18F**). These studies showed higher affinity of the enzyme to starch (**Fig. 19A**) compared to casein (**Fig. 19B**). Since protease assay was possible with casein as substrate, effect of acarbose on this activity was studied to study effect of inhibition of amylase on protease activity. In the presence of acarbose (0.125%) reduction in 27% protease activity (3.62 U) was determined compared to control (4.98 U).

Even though the enzyme showed an activity with casein, assays required 46 h incubation. Occurrence of other proteases in the culture broth that interfered with the assay described the need for an easy assay to determine protease activity within the amylase. Hence the zymogram analysis described above was used as a means to purify the proteolytic enzyme by electro elution from the gels after preparatory SDS-PAGE. Antibodies raised to the purified enzyme (**Fig. 18F**) reacted with 125-, 71- and 61- kDa proteins in a Western blot analysis (**Fig. 18A**). The purified protein also showed both protease and amylase activities in zymogram reactions (**Fig. 18B** and **C**). Antibody cross reactivity (**Fig. 18A**) and detection of protease activities in the starch hydrolysing enzyme and the 71 kDa glucoamylase (**Fig. 16A** and **B**) described above, evidenced processing of the precursor amylase by the inherent protease to give rise to the 71 kDa G1 form of glucoamylase and the 53 kDa maltogenic α-amylase. Further processing of the G1 form by the inherent protease apparently caused the formation of the 61 kDa G2 form of the enzyme. Absence of protease activity in the G2 form and maltogenic α-amylase, made these enzymes stable products of amylase protein processing in *A. niger*. The observation that the mutant strain of the fungus had a more pronounced protease activity in the G1 form compared to the parent strain reasoned more degradation of the starch hydrolysing enzyme for the accumulation of maltogenic α-amylase. Thus autocatalysis by an inherent protease activity appeared to be the cause for multiple amylase secretion by *A. niger* (Ravi-Kumar *et al.,* 2004).

ANALYSIS OF GLUCOAMYLASE FOR PROTEASE MOTIFS

Though the identification of protease activities in the two amylolytic proteins explained autocatalysis as the cause for multiple amylase secretion by the fungus, it has also to reason the evolution of higher protease activity in the mutant strain that caused accumulation of maltogenic α-amylase. Since the mutant strain of the fungus was isolated by treatment with ultra violet radiation, the cause for enhanced protease activity in the amylases could only be due to point mutations in the amylase gene. An alignment of the G1 glucoamylase with the following protease sequences available at the NCBI was carriedout.

Protease	**NCBI Accession No.**
Aspergillopepsin I	Q12567 GI:2499820
Acid protease A	1817166A GI:229046
Microbial aspartic proteinase	JN0630 GI:484394
Serine-type carboxypeptidase I	S78072 GI:7428206
Carboxypeptidase D	S57907 GI:1363898
Serine-type carboxypeptidase	S55328 GI:1362511
Serine-type D-Ala-D-Ala carboxypeptidase	S48220 GI:1084263
Serine-type carboxypeptidase sxa2	B42249 GI:539077
Serine-type D-Ala-D-Ala carboxypeptidase	S17674 GI:80853

The Multalin version 5.4.1 (Corpet, 1988) sequence alignment of the above sequences showed no meaningful homology. However specific consensus regions within the G1 enzyme sequence was possible with a serine type carboxypeptidase I (E.C. 3.4.16.-) of *A. niger* (NCBI Accession No. S78072 GI:7428206). This is shown in **Fig. 27**.

Gl 188 SYVAQYWNQTG 198
Sp 156 **S**YAGMYVPYIA 166

Gl 389 SSSSTYSSIVDAVKTFADGFVSIVETHAASNGSMSEQYDK 428
Sp 337 SSWGPLPSVIERTNNTIIGH-GWL**D**YLLFLNGSLATIQNM 375

Fig. 27. Multalin alignment of serine type carboxy peptidase I sequence (Sp) of *A. niger* with glucoamylase G1 protein sequence (Gl). Letters in bold indicate the catalytic amino acids of serine type carboxy peptidase I. Consensus regions are underlined.

Since the consensus regions included catalytic aminoacid residues (S and D) of serine type carboxypeptidase I it was inferred that protease motifs occur within the glucoamylase G1 and that G1 enzyme can have a proteolytic activity (Ravi-Kumar *et al.,* 2004).

PRODUCTION OF MALTOGENIC ALPHA AMYLASE BY *ASPERGILLUS NIGER*

Unlike other amylases of the fungus, maltogenic α-amylase was not inhibited by the sugar analogue, acarbose. Estimation of higher activities of maltogenic α-amylase in the culture filtrates of the mutant strain of *A. niger* after growing in the medium containing acarbose (**Table 6**) suggested that the fungus produced more quantities of the precursor which was proteolytically processed to evolve maltogenic α-amylase. Since this enzyme was not inhibited by acarbose in the culture broth, such a processing was necessary to support the growth of the fungus on the starch containing medium. Further evidence to the statement above is on the estimation of higher activities of the maltogenic α-amylase when the parent strain was grown in the medium containing acarbose (**Table 6**).

In this study, apart from defining maltogenic α-amylase production, acarbose was also useful as an inhibitor for specifically monitoring enzyme production. By assays using acarbose it was found that in certain culture broths, specifically when nitrogen source as ammonium salts were provided, maltogenic α-amylase production could not be detected (**Table 7**). Though, proteolytic degradation of the precursor and the occurrence of G1 form of glucoamylase in such culture broth were observed by Western blot reactions (**Fig. 26A**), the absence of maltogenic α-amylase could be associated only to its degradation by certain proteases. Analysis of the culture broth for protease activity by zymogram reaction identified the secretion of a 58 kDa protease by the

fungus when the culture was grown in a medium containing ammonium salts (**Fig. 26C**). The occurrence of this protease which was unique in culture broths of the fungus grown in the medium containing ammonium salts suggested probable degradation of the maltogenic α-amylase by this protease. There are several media optimization studies on amylase production by *A. niger* (Suresh, 1999; Dubey, 1999; Cocker and Greenshields, 1977). These studies relate amylase production by the fungus either to growth of the fungus in the given medium or to the importance of C/N ratio for enzyme production. Studies have also shown that higher growth rates of the fungus in certain media did not necessarily support amylase production. Based on above reports described in the literature, the finding of this study that specific proteases are produced in certain media which probably affect the stability of enzymes of interest in culture suggested a need for monitoring proteases when cultivation conditions and media components are optimised for amylase production.

MEDIA OPTIMISATION AND SCALE-UP STUDIES FOR MALTOGENIC ALPHA AMYLASE PRODUCTION

In addition to improvement of strain, economically viable medium is an important requirement for the production of a metabolite using microorganisms. This is more important when considering individual production of low value products like amylases because cost towards media components should be inexpensive. Earlier it was shown from this laboratory that corn flour/corn starch are better carbon sources for amylase production (Suresh, 1999; Dubey, 1999). In this study, it was found that corn flour and peptone resulted in the highest yields of maltogenic α-amylase (78 U/mL). Replacing peptone with corn steep liquor reduced the enzyme yields only by 8 units/mL. Thus corn steep liquor being a much cheaper substrate, it was used to define the process for enzyme production. Higher enzyme yields also suggested that proteases did not affect the stability of maltogenic α-amylase when the fungus was grown in the medium containing corn steep liquor and corn flour.

Based on the laboratory experiments that optimized the medium components as corn flour (6%) and corn steep liquor (6%), the process was scaled-up to 12 L by

cultivating in Chemap ^{R} Fermentor (**Fig. 24**). Estimation of highest enzyme activity of 77 units/mL after 39 h fermentation (**Fig. 25A** and **B**), indicated the reproducibility of the results of the lab scale experiment. Pilot plant trial was carried out with (**Fig. 25B**) and without (**Fig. 25A**) pH maintenance. These experiments were necessary since fermentation trials showed an increase of pH after the initial drop to 4.5. Enzyme assays carried out at regular intervals of time showed gradual increase in enzyme production until 39 h in both the trials. This suggested that acidic pH had no effect on the enzyme production. However, there was a decrease in activity after 39 h when the pH was maintained at 4.5 (**Fig. 25B**). In contrast to this observation, when the pH of the fermentation medium was not maintained there was a sharp increase in activity (108 U/mL) after the completion of fermentation (39 h). Decreased specific activity after 39 h observed in this experiment (**Fig. 25A**) suggested that the increased production of maltogenic α-amylase estimated could be due to autolysis of the cell resulting in the release of the intracellular precursor enzyme and its subsequent degradation in the culture broth. This observation supports the earlier statement that much of the precursor enzyme is retained in the cell due to reduced porosity of the cell wall and its release occurred due to cell lysis.

Since *A. niger* was shown to produce amylases when grown by solid state (Ghildyal *et al.,* 1985; Selvakumar *et al.,* 1996; Eugeneraj *et al.,* 2003) effect of this cultivation condition for the fungus on the production of maltogenic α-amylase was studied. Results (**Table 8**) showed that addition of micro nutrients to the bran improved the enzyme yields and rice bran along with micro nutrients resulted in the highest production (144 U/g dry moldy bran) of the enzyme. In solid state unlike submerged cultures, the growth of the fungus is attributed to the metabolic activity of the hyphal tips where the cell wall is less formed. Since much of the enzyme secretion occurred at the hyphal tips it was presumed that secretion was unaffected by the cell wall porosity resulting in higher quantities of enzyme production.

Stability is an important feature in all technological studies involving enzymes. Storage studies carried out at 4^0, 28^0 and 35^0C to assess the stability of the crude

enzyme (obtained after growing the fungus on corn flour (6%) and corn steep liquor (6%) containing medium) by using stabilizers (sodium benzoate, calcium chloride and corn starch) showed that maltogenic α-amylase was stable only at 4^0C in the presence of calcium chloride (**Table 9**). Sodium benzoate and corn starch could not stabilize the enzyme at 4^0, 28^0 or 35^0C. Interestingly there was an increase in the enzyme activity after storage for 49 days. This can be attributed to the degradation of the precursor amylase in the crude enzyme preparation resulting in the formation of maltogenic α-amylase due to autocatalysis by the inherent protease.

In conclusion it can be stated that characterization of a 53 kDa maltogenic α-amylase from the culture filtrate of *A. niger* is new to literature. Production of this enzyme by the fungus could be improved by UV mutation and temperature selection. Studies using the maltogenic α-amylase over producing mutant showed that high molecular mass precursor starch hydrolysing enzyme is degraded to G1 and G2 forms of glucoamylase and maltogenic α-amylase by autocatalysis in order to overcome secretional difficulties associated with reduced cell wall porosity to high molecular mass proteins. Standardization of fermentation conditions to a pilot plant scale described a process for the enzyme production by submerged fermentation. In defining the media components for maltogenic α-amylase production, acarbose as a screening reagent appeared effective since it inhibited all other amylases.

SUMMARY

This thesis describes characterization of a maltogenic α-amylase of *A. niger* and studies on the mutant overproducing the enzyme in relation to amylase processing.

The enzyme was differentiated from the other amylases in the culture filtrates of the fungus by using zymogram reactions and acarbose in the amylase assays. Zymogram reactions and SDS-PAGE identified maltogenic α-amylase as a 53 kDa protein. The enzyme was interesting since it had properties not reported of starch hydrolysing enzyme, glucoamylases and α-amylase. However, its production by the parent culture of *A. niger* was not significant.

The strain of *A. niger* was improved for higher enzyme production using a mutation protocol that involved UV treatment, temperature selection and screening for overproduction for protease and amylase. The enzyme purified from the culture filtrates of the mutant strain showed optimum activity at pH 4.3 and 40^0C. But the enzyme was stable in the presence of $CaCl_2$ at 50^0C. The enzyme showed higher affinity to starch compared to amylopectin and amylose. The enzyme produced maltose as major product along with maltotriose after reacting with starch. This was differentiated from the α-amylase active at pH 6.0 due to the identification of only maltodextrins as products after reaction with starch. The polarimetric study showed that the product of maltogenic α-amylase activity on starch was sugar with α-anomeric configuration. Though the enzyme behaved like Taka-amylase of *A. oryzae* it differed from Taka-amylase of *A. oryzae* in its reactivity to maltotriose and effect of zinc ions on its activity.

Mutant strain that showed five fold increase in protease activity compared to the parent also overproduced the maltogenic α-amylase. The enzyme production was usually associated with a decrease of other amylases active at pH 4.3. This suggested a role of protease in maltogenic α-amylase overproduction. Two-dimensional SDS-PAGE analysis of amylase proteins of the mutant and parent strains of the fungus showed degradation of 125 kDa starch hydrolysing enzyme and accumulation of 71-

and 61-kDa glucoamylases and 53 kDa maltogenic α-amylase. This suggested proteolytic processing of high molecular mass 125 kDa amylase to lower molecular mass 53 kDa maltogenic α-amylase and 71- and 61-kDa glucoamylases in the fungus in order to overcome secretional difficulties associated with less permeability of cell walls of *A. niger* to high molecular mass proteins.

Identification of Aspergillopepsin (45 kDa protease) in the culture filtrate of mutant strain by zymogram analysis and characteristic absence of it in the parent strain suggested that higher protease activity in the culture filtrate of mutant strain was due to the aspergillopepsin. The role of aspergillopepsin in amylase processing was ruled out since the parent strain that lacked aspergillopepsin also produced multiple forms of amylases. Zymogram analysis also showed the occurrence of protease activity in the regions corresponding to 125 kDa starch hydrolysing enzyme and 71 kDa G1 form of glucoamylase. Degradation of purified 125 kDa starch hydrolysing enzyme and formation of maltogenic α-amylase and glucoamylases after storage reasoned the inherent protease activity of starch hydrolysing enzyme. When selectively precipitated amylases were assayed for protease by zymogram analysis, they showed a prominent activity at pH 4.0 in the 71 kDa glucoamylase protein. The purified 71 kDa protein showed both protease and amylase activities in zymogram reactions. 71 kDa enzyme showed higher activity to starch compared to casein suggesting the enzyme was an amylase with a low protease activity. The antibody raised against this protein reacted with 125-, 71- and 61-kDa proteins.

Antibody cross reactivity, self degradation of 125 kDa starch hydrolysing enzyme and identification of protease activity in 125- and 71-kDa amylases evidenced that the processing of the 125 kDa starch hydrolysing enzyme was due to autocatalysis by an inherent protease activity to give rise to 71 kDa glucoamylase and 53 kDa maltogenic α-amylase. Further processing of the 71 kDa glucoamylase by an inherent protease apparently caused the formation of the 61 kDa glucoamylase since 71 kDa enzyme showed inherent protease activity and protease motifs. Alignment of the 71 kDa glucoamylase sequence with that of protease sequences showed regions of consensus

with a serine type carboxypeptidase I of *A. niger*. These consensus regions included catalytic residues of serine type carboxypeptidase I suggesting protease motifs within the 71 kDa glucoamylase. Thus point mutations in these regions due to UV radiation probably caused the mutant to evolve with enhanced protease activity that degraded the 125 kDa starch hydrolysing enzyme and accumulated 53 kDa maltogenic α-amylase.

Optimization of media components using mutant strain at laboratory level resulted in an inexpensive media for maltogenic α-amylase production by submerged and solid state fermentation. Based on laboratory experiments, the process for enzyme production was scaled to 12L. Acarbose in the growth medium increased enzyme yields. Stabilization studies showed that the enzyme requires $CaCl_2$ and a temperature of 4^0C for its storage.

The results are discussed in relevance to the literature pertinent to this investigation.

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