AMYLASE

Starch is one of the common and useful carbohydrate polymers found in nature. It is the main storage form of carbohydrate in higher plants. It is synthesised by all autotrophic plants using glucose as building block. Next to cellulose, starch is the important carbohydrate polymer in nature. It serves as major source for carbon and energy for most of the organisms, providing up to 80% calories intake by humans. Starch is present as microscopic granules in storage tissues of the plants, which are quite resistant to penetration by water or hydrolytic enzymes. It is mainly stored in seeds, tubers, roots and in parenchyma cells as a long-term reserve. In addition to its importance as a major source of carbon and energy for man and most other organisms, it has many industrial applications in the manufacture of paper, textiles, pharmaceuticals and biodegradable polymers, and its hydrolysates have a wide range of importance (Van der Maarel *et al.,* 2002).

3.1 Structure and properties of starch:

Chemically, starch is a polymer of glucose units linked through α-1, 4 and the α-1, 6 bonds. The presences of two different linkages indicate the possibilities for existence of two types of starch molecules. As a result, starch granule is a heterogeneous structure (Figure 15) consisting of two high molecular weight

polymers, amylose and amylopectin that are joined with intra and inter hydrogen bonding in the granules (Smith *et al.,* 1995; Gessler *et al.,* 1999).

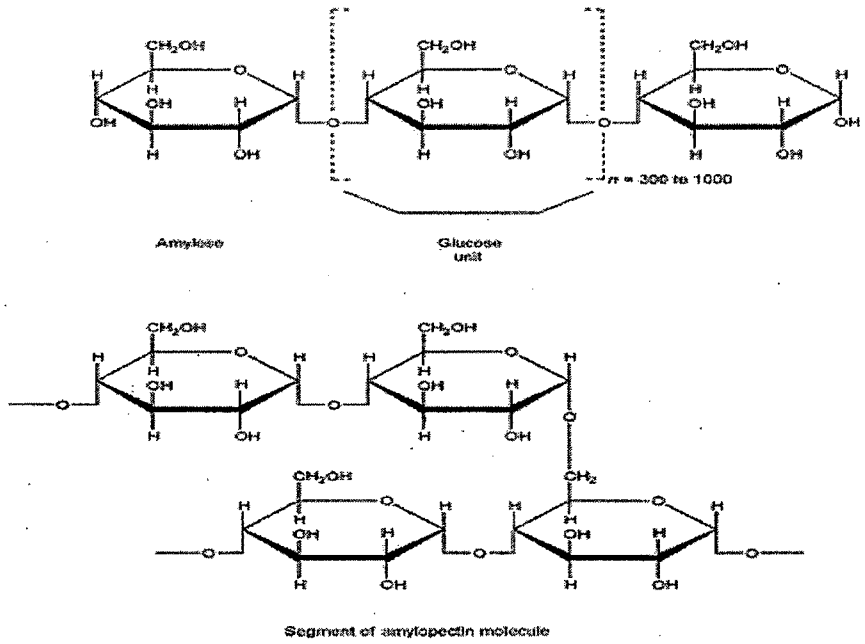

Figure 15: Structure of starch.

The relative proportion of these polymers plays a critical role in physical properties of native starch and its derivatives (Fogarty, 1983; Van der Maarel *et al.,* 2002). Amylose is mainly a linear polysaccharide formed by α-1, 4-linked D-glucose residues, having some α-1, 6-linked branching points. Amylopectin has a highly branched structure. The relative content of amylose and amylopectin varies with the source of starch. The degree of polymerisation (DP) of amylose chains ranges from 350 to 1000 glucose units depending on the source (Whistler and Daniel, 1985). DP of straight chain units in amylopectin ranges from 10-60 but total DP can be several thousand glucose units. Average amylose content in starch can vary between 0 and 75% approximately but a typical value is 20–25%. Amylopectin is formed when the linear polymer is formed by α-1, 4-glucose residues, branched by α-1, 6 linkages. The degree of branching is approximately one per twenty-five glucose units in the unbranched segments. Amylopectin may

accounts for 75-85% of most starches. The complete amylopectin molecule contains on an average about 20,00,000 glucose units (Alavi, 2003). Generally, native starch is found in partly crystalline form. Amylopectin forms the crystalline component whereas; amylose exists mainly in amorphous form (Zobel, 1992).

3.2 Enzyme required for hydrolysis of starch:

Starch is hydrolysed by an enzyme amylase. The amylolytic enzymes form a large group of starch hydrolases and related enzymes hyrolysing starch, glycogen and other related oligo and polysaccharides (Vihinen and Mantsala, 1989; Pandey *et al.,* 2000; Janecek, 2009). It is a common way of binding of a glucose residue of the substrate in the enzyme active centre, termed conventionally as a substrate-binding subsite (Davies *et al.,* 1997) that is responsible for the activity of amylolytic enzymes. As more enzymes have been isolated and characterised, it has become clear that the classifications hitherto used are somewhat problematic. Many hydrolases have overlapping specificities and the use of poorly defined substrates which further hinders proper classification. Gomes *et al.,* (2005) classified amylase into endoamylases, exoamylases and debranching amylases on the basis of their mode of actions and the way in which it hydrolyze starch (Figure 16).

Endoamylases

Endoamylases randomly cleaves α-1, 4-glycosidic bonds in amylose, amylopectin and related polysaccharides in the entire molecule. It results in oligosaccharides of varied chain lengths with alpha configuration on the first carbon of the reducing glucose unit (Guzman and Paredes, 1995; Van der Maarel *et al.,* 2002; Reddy *et al.,* 2003). As reported by Sivaramakrishnan *et al.,* (2006) that α-amylases (EC 3.2.1.1) are the well known endoamylases, which are the most widely, distributed enzymes in nature. Majority of them are extracellular and a few others are found to be intracellular. Such intracellular α-amylases enable organisms either to utilize

maltodextrin or storage polysaccharides during the exponential growth phase (Ballschmiter *et al.*, 2006).

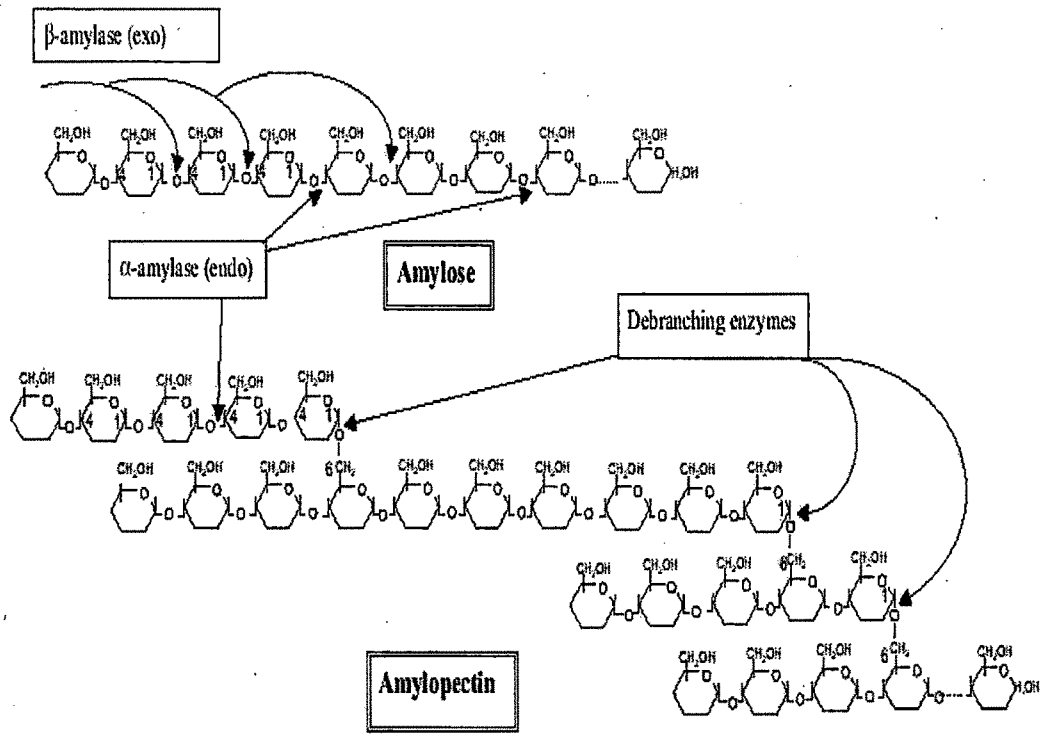

Figure 16: Starch hydrolysing enzymes.

Exoamylases

The exoamylases act preferentially on α-1, 4 linkages from the nonreducing end successively, resulting in low molecular weight products. Exoamylases of microbial origin are various types with respect to bond and substrate preference as well as products formed. According to Sivaramakrishnan *et al.*, (2006), these enzymes either cleave α-1, 4-glycosidic bonds as β-amylase (EC 3.2.1.2) or cleave both α-1, 4 and α-1, 6-glycosidic bonds like glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20).

Fungal glucoamylase is the most important industrial enzyme; which has wide application in the starch processing industry. Fungal glucoamylases are known for their activity usually at acidic pH, and have low thermostability. All the

glucoamylase so far studied are glycoproteins, the carbohydrate groups being necessary for maintaining the structural stability of the enzyme conformation (Shenoy *et al.,* 1985).

Debranching amylases

Debranching amylases include pullulanase (EC 3.2.1.41) and isoamylases (EC 3.2.1.68). Pullulanase specifically acts on α-1, 6 linkages in pullulan, starch, amylopectin and related oligosaccharides while isoamylase (EC 3.2.1.68) hydrolyses α-1, 6 linkages in amylopectin. Debranching enzymes exclusively degrade amylopectin, thus result in long linear polysaccharides (Israilides *et al.,* 1999; Sivaramakrishnan *et al.,* 2006).

3.3 Industrial application of Amylases:

As a raw material, starch can be used directly in textiles, paper industries, in the manufacturing of biodegradable plastic film, pharmaceuticals, adhesives, etc. In addition, starch can be chemically or enzymatically processed into a variety of starch derivatives such as glucose, maltose and syrups of various dextrose levels that are used as sweetener or as chemical feed for further bioconversion into different products. Starch is produced commercially from cereal crops such as corn, wheat, sorghum and rice. It is also produced from the tubers and roots of plants such as cassava, potato, etc (Ramachandran *et al.,* 2004). Important industrial application of amylase has been listed below:

Starch Processing

Conversion of starch into sugar syrups (glucose, maltose, maltotriose, dextrins, sugar, or fructose syrups, etc.) forms the major part of the starch processing industry. The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products (Reddy *et al.,* 2003). In industrial starch processing, the synergistic action of both bacterial α-amylase and fungal glucoamylase is employed. Application of enzymes in the production of

sweeteners from starch has potential to make food processing less dependent on sugarcane in areas which are unsuitable for sugar production or where starch is more cheaply accessible than sugarcane. Furthermore, bioconversion of starch into sweetener offer alternative uses for starchy materials, which are highly perishable and lacks proper preservation techniques particularly in the tropical countries (Hyum and Zeirkus, 1985).

Textile desizing

Prior to weaving of yarn into fabric, the warp yarns are coated with a removable sizing agent to lubricate and protect yarn from abrasion during weaving. Historically, starch has been used as a main sizing agent for cotton fabrics due to its excellent film forming capacity. Prior to dying of the fabrics, the sizing agent applied to these fabrics must be removed. Before the discovery of amylase enzymes, the only alternative to remove the starch based sizing agents was extended treatment with caustic soda at high temperature. However, such chemical treatment was not totally effective in removing the starch. Appropriate desizing by application of α-amylase is the possible alternative against chemical treatment (Kumar *et al.,* 1990; Tsurikova *et al.,* 2002).

Beverage alcohol and fuel ethanol production

In beer industries, microbial amylases are used to aid cereal amylase in the production of fermentable sugar. Over the past decades, there has been an increasing interest in fuel ethanol as a result of increased environmental concern and higher crude oil prices. Ethanol fuels can be derived from renewable resources such as agricultural crops and their by products. Therefore, enzymes such as α-amylase, glucoamylase and cellulases are important in the production of fermentable sugars to produce ethanol (Kirk *et al.,* 2002).

Baking

Food fermentation processes are reliant on both endogenous and microbial enzymatic activities for the degradation of starch and other polymers. In baking industries, α-amylase and glucoamylase are employed to supplement the natural enzyme coming from the grains in producing fermentable glucose by yeast. The microbial amylases are also important to improve the shelf life of the bread (Sahlstrom and Brathen, 1997).

Other applications of amylases have been reported in the field of clinical, medical and analytical chemistry (Pandey *et al.,* 2000; Cherry *et al.,* 2004). To some extent, amylases are also been used to improve digestibility of some of the feed ingredients (Kumar *et al.,* 1990). In spite of all these applications of amylases in various industries, there is little work on the fungal enzymes from the tropical countries. In respect to this, production of amylase in the present investigation has been carried out by using submerged fermentation. The aims of investigation were:

- Screening of wood rot fungi for production of amylase.
- Production of amylase by submerged fermentation.
- To study the effect of various parameters on amylase production.
- Complete/Partial purification of amylase.
- Characterisation of amylase.
- Molecular characterisation of amylase by SDS PAGE and Native PAGE.

MATERIALS AND METHODS

3.4 General procedures:

3.4.1 Chemicals

Potato starch, malt extract, sugars, KH_2PO_4, K_2HPO_4, were procured from SRL (Sisco Research Labs, India), whereas Dinitro salicylic acid was obtained from Himedia (India). Medium range molecular marker was procured from Bangalore Genei (India). All the other chemicals used were of analytical grade.

3.4.2 Sterilisation

Fungal growth media, required solutions and glass wares were sterilized by autoclaving at 121 °C and 15 lbs pressure for 30 minutes. Solutions which are heat sensitive were purified by Whatmann filter paper No.1. Glass wares were baked overnight in an oven for preserving solutions of electrophoresis. For all the experiments, including media preparation, isolation of fungi and enzyme production procedures, distilled water was used. For the purification of enzymes and electrophoresis process, double distilled water was used.

3.4.3 Centrifugation

Crude enzyme and ammonium sulphate precipitation was done in polypropylene centrifuge tubes at 10,000 rpm (rotation per minute), 4 °C temperature in a REMI cooling centrifuge.

3.5 Fungal isolate and growth medium:

3.5.1 Isolation of fungal strains

Small pieces of infected wood along with fungal fruiting bodies were excised with the help of hammer and chisel from different forests of Gujarat state. Immediately after the collection, some of the samples were fixed in Formaldehyde-Acetic acid-Alcohol (Berlyn and Miksche, 1976), while rest of the samples were packed in sterile polyethylene bags for the isolation and identification of the fungi. Small

pieces of fruiting body and suitably trimmed wood samples were surface sterilized with aqueous $HgCl_2$ solution (0.1%) with intermediate washing by sterile distilled water followed by absolute alcohol. Before plating on malt agar plates these samples were washed with sterile distilled water and the plates were incubated in B.O.D. incubator at 28 °C and 75% Rh. Cultures obtained from these samples were then transferred to freshly prepared malt agar plates and pure cultures were obtained by making serial transfers on malt agar medium. After 7 days growth, these cultures were maintained in 4% malt agar media at 4 °C.

3.5.2 Screening of fungal strains for amylase activity

About 30 fungal strains were collected from the decaying wood of the trees growing in different forests (*viz.* Girnar forest-Junagadh, Satpuda forest of Dediapada and Panchmahal, Pavagadh and Shivrajpur) of Gujarat state. All these fungal strains were subjected to activity assay for screening of amylolytic strain. Fungus was inoculated on starch malt agar media (malt + 1% starch). Based on maximum zone of clearance, strain KSR-1 was further used for production of amylase enzyme by submerged fermentation. The strain was identified by morpho-taxonomic features by Agharkar Research Institute (Pune) and it was identified as *Chrysosporium asperatum* Carmichael (Class Euascomycetes; Onygenales; Onygenaceae).

3.5.3 Inoculum Preparation

Inoculum for enzyme production was prepared by inoculating loop full culture of *C. asperatum* in distilled water with Tween 80 (0.1%). Spores having 10^6 spores/ml has been used as inoculum in the production medium. Flasks were kept on rotary shaker (Nova, India) at 150 rpm at 30 °C.

3.5.4 Enzyme production

Production media with composition enlisted in Table 27 has been used. Temperature and pH of production media was adjusted 30 °C and 5.0 respectively.

Chemicals	g/l
Malt extract	10
Peptone	3
K_2HPO_4	1
K_2HPO_4	0.04
Starch potato	30

Table 27: Fungus was grown in EP medium for amylase production.

3.5.5 Enzyme harvestation

Harvestation of enzyme was done after the interval of every 24 hours, and enzyme activity was checked by taking out 2-3 ml of broth from fermentation media till 5 days after the addition of inoculum. Enzyme was harvested using cooling centrifuge at 10,000 rpm at 4 °C. Crude enzyme obtained was further subjected to ammonium sulphate precipitation and purification.

3.6 Optimization of media for enzyme production:

3.6.1 Different substrate and substrate concentration

Different substrates were tested for maximum production of amylase by submerged fermentation. Substrates used were starch potato, starch soluble, corn starch were procured from SRL (India) Ltd. while wheat bran, corn bran and raw starch were procured from the local markets of Vadodara. To check the effect of different concentrations of substrate on enzyme production, various concentration of starch potato were used in production media.

3.6.2 Effect of different media parameters on production of enzyme

Spore suspension of different inoculums sizes such as 5×10^5, 10^6, 5×10^6 and 10^7 spores/ml of mineral solution was prepared in 0.1% Tween 80 from 72 hours grown culture of *Chrysosporium asperatum.*

3.6.3 Effect of pH and temperature on production of enzyme

To study the effect of pH on amylase production, pH of the culture medium was adjusted from 2-9 and enzyme activity was checked individually at all pH levels. Similarly, to check the effect of temperature on amylase production, production media was kept at different temperatures ranging from 20-70 °C.

3.6.4 Effect of media parameters

For carbon sources (2%), glucose, maltose, lactose, sucrose, fructose, xylose, etc. were used to examine the impact of different carbon sources on amylase production. For nitrogen sources (1%), yeast extract, peptone, beef extract, and malt extract were supplemented as individual components in production media. Similarly metal ions, in concentration of 1mM were added to access the effect of metal ions on production of amylase enzyme.

3.7 Parameters for amylase activity:

Activity of amylase was performed as described by Miller (1959).

Na-Acetate buffer pH 5.0 (for 100 ml): Na-Acetate 50 ml of 0.1M and 50 ml of 0.1M acetic acid was mixed and pH 5.0 was adjusted by adding acetic acid in solution.

Substrate: 1% potato starch was prepared by dissolving 1g of starch potato in 100 ml of Na-Acetate buffer.

Enzyme Assay:

Amylase activity was determined by incubating a mixture of 0.1 ml of enzyme and 1% starch dissolved in 0.1M Na-Acetate buffer (pH 5) at 30 °C for 25 minutes. The reducing sugar released after 25 minutes was measured using DNSA reagent as

described by Miller (1959). Composition of DNSA reagent has been given in Table 7. One unit (U) of amylase activity is defined as the amount of enzyme that releases 1μmol of reducing sugar as glucose per minute under defined assay conditions. All the sets have been performed in triplicates and the standard error has been reported.

3.7.1 Ammonium sulphate precipitation

Crude amylase was partially purified by adding ammonium sulphate in crude broth. For different percent saturations, ammonium sulphate was added according to ammonium sulphate precipitation (Table 28). Saturation of crude enzyme was done from 20%-80% at 4 °C in REMI cooling centrifuge. After centrifugation, pellets obtained were dissolved in minimum quantity of Na-Acetate buffer (pH 5.0). All saturated fractions were checked for amylase activity and the fractions having maximum activity were subjected to dialysis. Dialysis of enzyme was carried out by using a membrane filter with cut off value 12000-14000 Da against Na-Acetate buffer with pH 5.0.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

Table 28: Ammonium sulphate precipitation table.

3.7.2 Affinity purification and characterization of enzyme

Dialysed enzyme was mixed with 1.25 ml of alginate solution (2%w/v) and the final volume was made up to 5.0 ml with Na-Acetate buffer (20mM, pH 5.0). The mixture was then incubated for 1 hour at 25 °C with continuous shaking at 60 rpm. The enzyme bound alginate was precipitated by adding 0.350 ml of $CaCl_2$ (1.0M) solution. The final concentration of $CaCl_2$ in the solution was 70mM, which is known to precipitate alginate nearly quantitatively (98%). After 20 minutes of incubation at 25 °C, the precipitate was centrifuged at 10000 rpm for 10 minutes at 25 °C. The supernatant and subsequent washings with buffer containing 70mM $CaCl_2$ were collected until no enzyme activity was detected in the washings. The amount of bound enzyme was calculated by the difference of initial activity before the addition of alginate and the activity of the supernatant and washings.

3.7.3 Effect of substrate concentration on production of enzyme

Six different concentrations as: 0.5%, 1%, 1.5%, 2%, 2.5% and 3% of starch potato were used for checking effect of substrate concentration on enzyme activity. Flasks were kept on rotary shakers and enzyme activity was determined by performing the amylase assay using DNSA method at pH 5.0.

3.7.4 Effect of pH on amylase activity

Amylase activity was measured at different pH from 2.0 to 6.0 with various buffers. Phosphate buffer was used for pH 4.0 to 7.0 and citrate buffer was used for the range of pH 5.0 to 7.0.

3.7.5 Effect of temperature on amylase activity

Similarly, activity of the amylase was also measured for different temperature range starting from 10-60 °C. The enzyme was incubated for 25 minutes with starch potato (in 0.1M Na-Acetate buffer), as a substrate at above mentioned range

of temperatures and the enzyme activity was checked individually for each temperature.

3.7.6 Molecular weight determination

Electrophoresis of the purified enzyme was done using Medox Electrophoresis unit (Bioneeds Instruments Pvt. Ltd., Ahmedabad). Gels of 15×10 cm in size were employed in experiments. In all the experiments, 10% gels were used to examine single band of purified enzyme. Composition resolving gels and staking gels used were as follows (Table 29, 30).

Composition	Resolving (7ml)
30% Polyacrylamide	2.25
1.5 M Tris Buffer (pH 8.8)	1.75
10% SDS	0.07
10 % APS (Ammonium per sulphate)	0.07
TEMED	0.0028
Distilled water	2.75

Table 29: Composition of 10% resolving gel.

Chemicals	Stacking (4ml)
30% Polyacrylamide	0.68
1.5 M Tris Buffer(pH 6.8)	0.5
10% SDS	0.04
10% APS(Ammonium per sulphate)	0.04
TEMED	0.004
Distilled water	2.72

Table 30: Composition of stacking gel.

Running buffer used for electrophoresis is Glycine-SDS buffer. Electrophoresis was done by method described by Laemmli (1970). Protein samples were heated in

boiling water bath with loading dye for 3 minutes. Loading dye was composed of Bromophenol blue, glycerol, Tris buffer (pH 6.4) and distilled water. Voltage for electrophoresis was set 80V. The electrophoresis time was variable from 2 hours to 5 hours, depending on the gel size and the purpose of the experiment.

Native gel Electrophoresis

Native gels

For activity staining, 10% gel was prepared with similar composition without addition of SDS in it.

Staining and photography of gel

CBB (Comassie Brilliant Blue R-250) with 40% methanol and 10% acetic acid was used as staining solution for detection of protein bands. Destaining solution comprised of 40% methanol and 10% acetic acid with no CBB in it. Photographs of stained gel were taken by Sony digital camera DSC T10, (Sony, India).

For native electrophoresis, gel was immersed in 1% starch dissolved in respective optimum pH buffer for 1 hour, followed by 2 minutes washing with distilled water. The gel was stained with 0.0005% iodine in 0.05% potassium iodide for 5 minutes. Excess iodine was washed off with cold distilled water and the gel was soaked in 1% acetic acid after visualization of α-amylase activity bands. The bands were observed and photographs were taken by Sony digital camera DSC T10 with 8 megapixel.

RESULTS AND DISCUSSION

3.8 Selection of strains for Amylolytic activity:

Out of the 30 fungal cultures isolated and purified from the infected wood samples, one of the culture giving promising results was selected. All the cultures were inoculated on the optimised growth media supplemented with 1% Starch and zone of clearance was observed by flooding the plate with 0.1% Iodine solution. Based on maximum zone of clearance, strain KSR-1 was further used for production of amylase enzyme by submerged fermentation. For maximum yield of amylase enzyme, strain was inoculated in liquid medium containing starch as a sole carbon source at 30 °C. Culture that was showing potential results was identified by its morpho-taxonomic features and it was identified as *Chrysosporium asperatum* Carmichael (Class Euascomycetes; Onygenales; Onygenaceae).

Maximum growth of *C. asperatum* at 30 °C indicates that the fungus is mesophilic in nature. The organism is considered to be mesophilic when optimal temperature for growth is between 25 °C and 35 °C. They are aerobic in nature and can grow over a wide range of hydrogen ion concentration (Omemu *et al.,* 2005). In Euascomycetes, *Chrysosporium sp.* has been explored by earlier workers (Kirk *et al.,* 1978; Gold *et al.,* 1982) due to its ability to degrade xenobiotic compounds and production of commercially important enzymes. In fungi, detailed studies on purification of α-amylase have largely been limited to a few species of fungi (Abou-Zeid, 1997) and there is no information available on species investigated in the present study.

3.9 Optimisation of amylase enzyme under submerged fermentation:

3.9.1 Effect of inoculum size and incubation time on enzyme production

Though, inoculum size plays an important role in the production of amylase enzyme, no much appreciable differences were observed when inoculum size was varied much to that of optimum inoculum size. It was found that a high inoculum

level leads to decrease in enzyme production. As reported earlier by Hema *et al.,* (2006) that there was no effect of inoculum size on the amylase production. In the present study also our results are in agreement with Hema *et al.,* (2006) which showed no effect of inoculum size on the production of amylase enzyme.

The incubation time for achieving maximum enzyme level was governed by the characteristics of the culture which is based on growth rate and enzyme production by fungi (Kunamneni, 2005). Production of amylase increases with the increase in the incubation time and it was found maximum after 72 hours of inoculation. Further increase in the incubation period resulted in decrease production of amylase. It might be due to the fact that after 72 hours, production of other by-products by the fungus and depletion of the nutrients might be responsible for it. Duochuan *et al.*, (1997) reported that these by-products produced by the fungus inhibits its growth and thus leads to decrease in enzyme production.

3.9.2 Effect of different substrate and substrate concentration on enzyme production

An ideal substrate to improve fungal amylase production should combine a biomass increasing property with enzyme synthesis induction. Various concentrations of substrate (starch) ranging from 1-6% were checked for optimization of amylase enzyme activity. As shown in Figure 17, maximum activity was observed after 72 hours of incubation in media containing starch potato (3%). Initially, as concentration of starch increases there is significant increase in enzyme activity but as soon as all sites of substrate have been filled there is decline in enzyme activity (John and Charles, 1973).

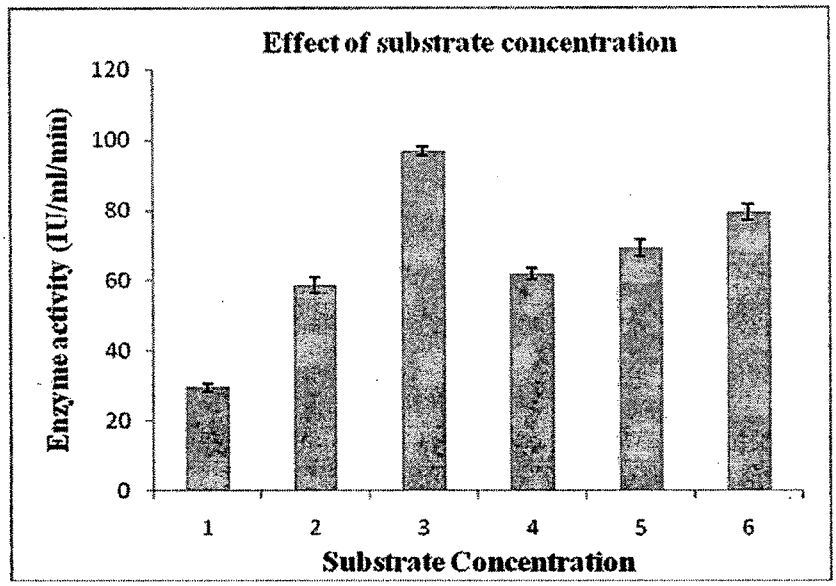

Figure 17: Effect of substrate concentration on production of enzyme.

Effect of different substrates on the amylase production was checked by using starch potato, corn starch, starch soluble, starch maize and raw starch from sweet potato. Among these different substrates, maximum production of an enzyme was seen in starch potato after 72 hours of incubation (96.85 IU/ml/min). On the other hand, corn starch gave maximum enzyme activity (76.45 IU/ml/min) after 24 hours of incubation. However, compared to corn starch, enzyme activity was relatively higher after 72 hours of incubation in starch potato. Depending on the origin of starch, there are often variations in the composition of amylose and amylopectin and the quantity of lipids in starch (Aberle *et al.,* 1994; Hoover, 2001). According to Aberle *et al.,* (1994) and Hoover (2001), cereal starch has approximately 28% amylose, 72% amylopectin and 6.0% lipids, while tuber starch has approximately 20% amylose, 80% amylopectin and 0.1% lipids. Therefore, it is inferred that the composition and variation in the molecular weight of starch could affect the mode of enzyme action (Cruz *et al.,* 1997). In the present study, composition of starch potato and variation in its molecular weight might be responsible for the variations in the production of enzyme. Lower affinity of the enzymes towards low molecular weight substrates than the highly polymerized glucan is consistent with the properties of α-amylase and glucoamylase. When the

amount of starch was increased, production of the enzyme was reduced. It may be due to the fact that increases in the amount of carbon source than the optimum level leads to the reduction of enzyme formation (Asgher *et al.,* 2007).

3.9.3 Effect of carbon sources on production of enzyme

Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product like maltose (Tonomura *et al.,* 1961; Morkeberg *et al.,* 1995; Gupta *et al.,* 2003). Supplementation of carbon source in production media plays an important role in enzyme production. In the present investigation also, supplementation of different carbon sources to the production media showed slightly increase in amylase production. After 72 hours of incubation, maximum activity was found in media supplemented with xylose (99.19 IU/ml/min), while minimum activity was found in media containing sucrose (81.09 IU/ml/min) (Figure 18). There are reports that not only the carbon source, but also the mycelial age affects the synthesis of α-amylase by *Aspergillus oryzae* M-13 whereas, five days starved non growing mycelia were the most appropriate for optimal induction by maltose (Yabuki *et al.,* 1977). In the present study, one week old culture was used for the production of enzyme. Like most other inducible enzymes, production of α-amylases is also subjected to catabolite repression by glucose and other sugars (Bhella and Altosaar, 1985; Morkeberg *et al.,* 1995). Available literature indicates that inducer molecule may vary from species to species and a molecule proved to be good inducer of α-amylase in one strain may not be good inducer for other fungal species. However, in *C. asperatum* media supplemented with xylose as an inducer found to be good inducer of enzyme as compared to other sugars. Most reports available on the induction of α-amylase on different strains of *A. oryzae* suggest that the general inducer molecule is maltose. There is a report of a 20-fold increase in enzyme activity when maltose and starch were used as inducers in *A. oryzae* (Arst and Bailey, 1977; Gupta *et al.,* 2003). Similarly strong α-amylase induction by starch and maltose in the case of *A. oryzae* DSM 63303 has been reported (Lachmund *et al.,* 1993). Apart from maltose,

in some strains, other carbon sources as lactose, trehalose, α-methyl D-glycoside also served as inducers of α-amylase (Yabuki *et al.*, 1977). Addition of supplements in SSF for amylase production has already been studied previously, which indicates that glucose and sucrose are important supplementations for amylase production by *A. niger* which belongs to same class: Ascomycetes (Ariff and Webb, 1998). On the other hand, role of glucose in the production of α-amylase in certain cases is controversial. α-amylase production by *A. oryzae* DSM 63303 was not repressed by glucose rather; a minimal level of the enzyme was induced in its presence (Lachmund *et al.*, 1993). However, xylose or fructose has been classified as strongly repressive although they supported good growth in *Aspergillus nidulans* (Arst and Bailey, 1977).

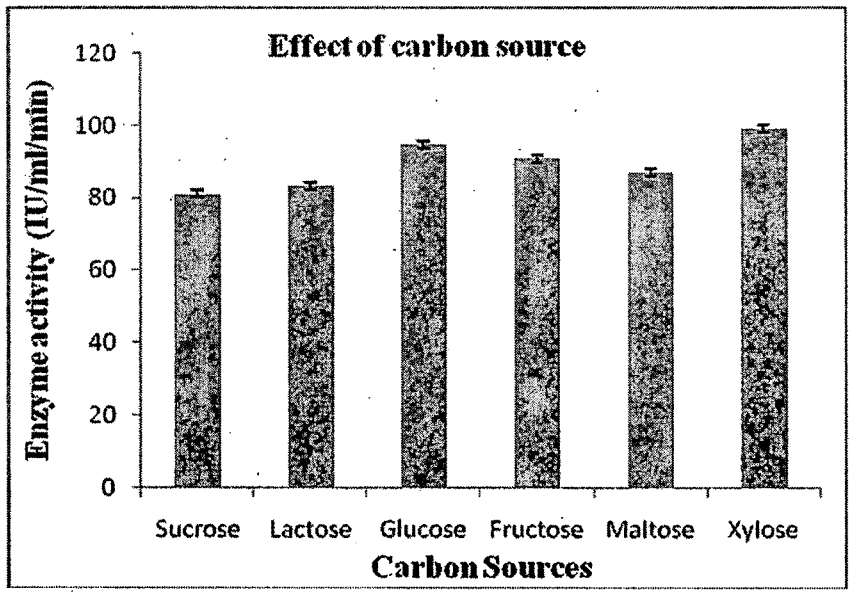

Figure 18: Effect of carbon source on production of enzyme.

Earlier workers dealing with SSF have suggested that soluble starch is the best source for production of amylase enzyme with *A. fumigatus* and *A. niger* (Goto *et al.*, 1998). However, our observation differs from previous reports, which indicates that combination of two different carbon sources increase the production of amylase by submerged fermentation.

3.9.4 Effect of nitrogen sources on production of enzyme

As a nitrogen source, both organic and inorganic nitrogen sources have been added as supplement in the media. After 72 hours of incubation, maximum enzyme production using yeast extract as nitrogen source was recorded (Figure 19). Similar findings were also recorded in SSF, where yeast extract was added as supplement with coarse waste of rice bran (Hema *et al.,* 2006). The inorganic nitrogen sources also have inducing effect on the production of amylase. Among the different nitrogen sources evaluated, the ammonium sulphate was found to be the best inducer for amylase. Lineback *et al.,* (1996) has reported the regulation of amylase formation takes place only after the supplementation of nitrogen source. Among the different nitrogen sources, easily metabolizable nitrogen source like ammonium sulphate is better than other nitrogen sources. In the present study also, ammonium sulphate showed maximum enzyme production after 48 hours of incubation. Yeast extract has been used in the production of α-amylase from *Streptomyces* sp. (McMahon *et al.,* 1999), *Bacillus* sp. IMD 435 (Hamilton *et al.,* 1999a) and *Halomonas meridian* (Coronado *et al.,* 2000). Yeast extract has also been used in conjunction with other nitrogen sources such as bactopeptone in the case of *Bacillus* sp. IMD 434 (Hamilton *et al.,* 1999b), ammonium sulphate for *Bacillus subtilis* (Dercova *et al.,* 1992; Gupta *et al.,* 2003), ammonium sulphate and casein for *C. gigantean* (Kekos *et al.,* 1987) and soybean flour and meat extract for *A. oryzae* (Imai *et al.,* 1993). Yeast extract increased the productivity of α-amylase by 110-156% in *A. oryzae,* when used as an additional nitrogen source than when ammonia was used as a sole source (Pedersen and Nielsen, 2000). Various other organic nitrogen sources have also been reported to support maximum α-amylase production by various bacteria and fungi. However, organic nitrogen sources *viz.* beef extract, peptone and corn steep liquor supported maximum α-amylase production by different bacterial strains (Hayashida *et al.,* 1988; Cheng *et al.,* 1989). Apart from this, various inorganic salts such as ammonium sulphate for *A. oryzae* (Morkeberg *et al.,* 1995) and *A. nidulans* (Lachmund *et al.,* 1993),

ammonium nitrate for *A. oryzae* (Kundu *et al.*, 1973) and vogel salts for *A. fumigatus* (Goto *et al.*, 1998) have been reported to support better α-amylase production in fungi. It has been reported that during the production of amylase by SSF method using wheat bran, urea acts as the best nitrogen source for induction of amylase activity (El-Safey and Ammar, 2004). According to Haq *et al.*, (2002) although, urea releases ammonium ions slowly, it was found not to be a good source of nitrogen. This was attributed to low urease activity of the organism.

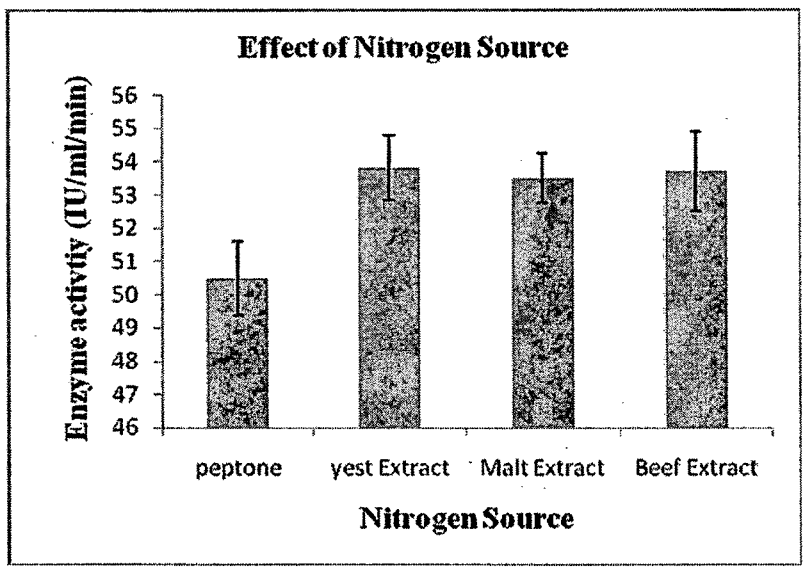

Figure 19: Effect of nitrogen source on production of enzyme.

3.9.5 Effect of pH and temperature on production of enzyme

Among the physical parameters, pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. As the enzymes are very sensitive to pH, selection of optimum pH is very much essential for the production of amylase (McMohan *et al.*, 1999). In the present study, the effect of pH on the enzyme production was studied. The fermentation medium was carried out at different pH range (2.0-8.0). Production of amylase was found to be the highest at pH 5 (Figure 20). There are reports that fungal cultures give optimum enzyme production at pH 5.0 by using

various substrates (Nahas and Waldermarin, 2002; El-Safey and Ammar, 2004). It is evident from the data that amylase production by *C. asperatum* and enzyme activity showed a broad range of pH preference allowing the use of enzyme in both acidic and alkaline conditions.

The influence of temperature on amylase production is related to the growth of the organism. Among fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25-37 °C. Optimum yields of α-amylase were achieved at 30-37 °C for *Aspergillus oryzae* (Kundu *et al.,* 1973; Ueno *et al.,* 1987; Gupta *et al.,* 2003). Production of α-amylase has also been reported at 55 °C by the thermophilic fungus *Thermomonospora fusca* (Busch and Stutzenberger, 1997) and at 50 °C by *Thermomyces lanuginosus* (Mishra and Maheshwari, 1996; Kunamneni, 2005). In the present study, enzyme activity increases with increase in temperature from 10 °C and maximum enzyme production was observed at temperature 30 °C (96.15 IU/ml/min). Optimum temperature for the amylase activity required by various fungi has also been studied by earlier workers and they have noted a wide range of temperature preference depending upon their nature of adaptation (Ellaiah *et al.,* 2002; Kunamneni, 2005; Omemu *et al.,* 2005). Amylase activity was increased progressively with increase in temperature from 20 °C reaching a maximum at 60 °C in *Aspergillus niger* (Omemu *et al.,* 2005), while it was found to be optimum 35 °C for *Aspergillus falvus* var. *columnaris* (Ellaiah *et al.,* 2002). In the present study, being a mesophilic species, *Chrysosporium* showed 30 °C as optimum temperature for maximum enzyme production.

3.10 Effect of Temperature and pH on enzyme activity:

Temperature and pH are the most important factors, which markedly influence enzyme activity. The optimum pH was observed at 5 as indicated in Figure 20.

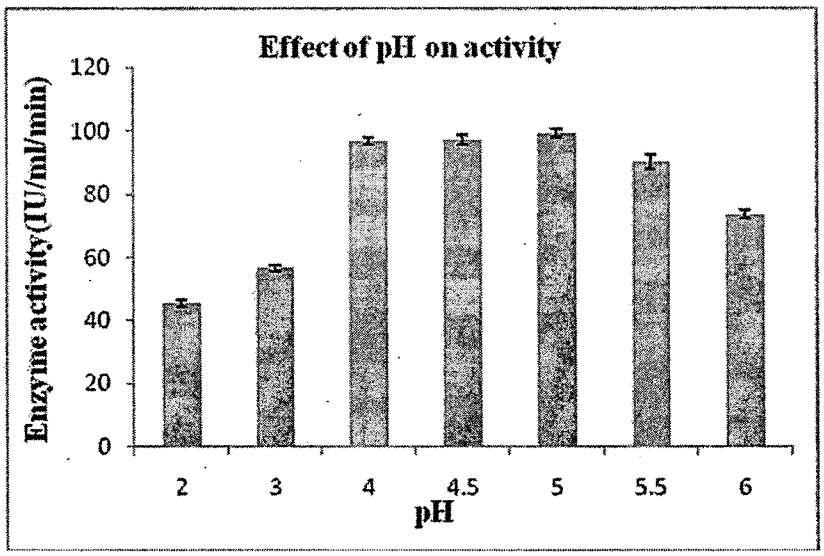

Figure 20: Effect of pH on enzyme activity.

After pH 5.0, there is continuous decline in activity of amylase enzyme. The influence of temperature on the activity of the enzyme showed that it increase with increase in temperature from 10 °C and reach maximum at 30 °C (Figure 21).

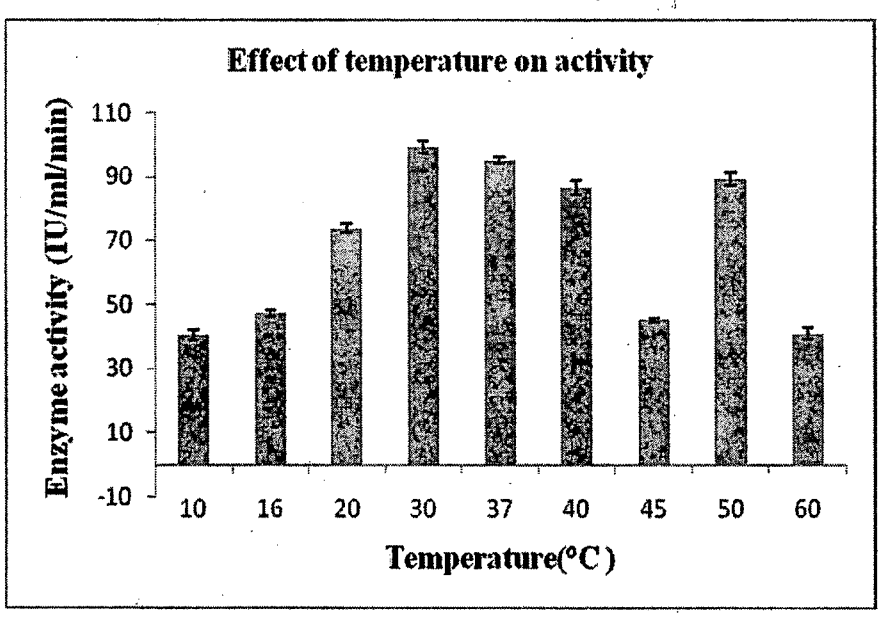

Figure 21: Effect of Temperature on enzyme activity.

However it retains its 80% of activity at even 50 °C for 20 minutes indicating that it is thermostable in nature. It shows the highest activity at 30 °C and least activity

at low temperature at 10 °C. Least activity has also been observed at high temperature 60 °C. The high temperature inactivation may be due to incorrect conformation of proteins due to hydrolysis of the peptide chain, destruction of amino acid, or aggregation (Schokker and Van Boekel, 1999).

3.10.1 Effect of substrate concentration and incubation time on activity

Five concentrations of starch potato (substrate): 0.5%, 1%, 1.5%, 2%, 2.5% were used to study the effect of substrate concentration on enzyme activity. Maximum activity was found after 20 minutes of incubation in 1% substrate concentration (96.35 IU/ml/min). However, amylase activity was increased with increase in amount of starch in the enzyme substrate mixture, while it was declined when the substrate concentration was exceeded more than 1%. Declined enzyme activity may be associated with saturation of catalytic sites of enzymes by 1% substrate concentration. Decreased enzyme activity due to saturation of catalytic sites by substrate concentration is reported in the studies (John and Charles, 1973).

Enzyme activity increases with the increase in incubation time. Activity was measured from the five minutes (23.75 IU/ml/min) of incubation and maximum activity was observed after incubation of 20 minutes (99.19 IU/ml/min) as shown in Table 31. However, it began to decline after 20 minutes of incubation.

Incubation time (minutes)	Enzyme activity (IU/ml/min)
5	23.75±1.54
10	40±1.33
15	65.65±0.98
20	99.19±0.90
25	98.25±1.25
30	99±1.10
40	72±1.45

Table 31: Effect of incubation time on amylase activity.

3.10.2 Effect of metal ions on production of enzyme

Four different metal ions Cu^{2+}, Zn^{2+}, Fe^{3+}, Mg^{2+} in the forms of their salts were supplemented in media to investigate their effect on amylase production. Metal ions at 1mM concentration were added in reaction mixture and activity obtained has been represented in Table 32. Perusal of literature indicates that supplementation of metal ions enhance the enzyme production (Castro *et al.*, 1997; Ghanem *et al.*, 2000; Seyis and Nilufer, 2005). However, role of metal ions appears to be controversial. In some fungal species, addition of metal ions enhanced enzyme production (Castro *et al.*, 1997; Ghanem *et al.*, 2000; Seyis and Nilufer, 2005) and its activity while in other species, metal ions inhibited enzyme production (Laderman *et al.*, 1993; Reyed, 2007). In the present investigation also, addition of metal ions showed no significant increase or decrease in production and activity of enzyme. Effect of Ca^{2+} at two different concentrations (5mM and 10mM) has also been analysed and no inhibitory effect of Ca^{2+} ions was found on enzyme activity. Similar studies have also been carried out by earlier workers and it was found that the amylase did not require any specific ion for its catalytic activity (Reyed, 2007).

Metal ions	Enzyme activity (IU/ml/min)
$CuCl_2$	97.15±0.95
$ZnCl_2$	96.25±1.12
$FeCl_3$	98.32±1.25
$MgSO_4$	98±0.65

Table 32: Effect of metal ions on amylase activity.

Inhibition of amylase activity in presence of Ca^{2+} has been reported (Laderman *et al.*, 1993; Reyed, 2007) due to presence of metallo enzymes containing a metallic ion for catalytic activity. Tanaka *et al.*, (1987) and Sakano *et al.*, (1992) investigated the inhibition of amylase occurs in presence of Ca^{2+} in *Clostridium*

butyricum and *Thermoactinomyces vulgaris* respectively. On the contrary, other reports also indicate no effect of metal ions on the amylase activity (Dutta *et al.,* 2006; Alva *et al.,* 2007; Reyed, 2007).

3.11 Ammonium sulphate precipitation and purification of enzyme:

Crude filterate was subjected to ammonium sulphate precipitation. Different percentage such as 20%, 40%, 60%, 80% saturated fractions were pulled out and maximum activity was observed in 60% fractions. Dialysis of enzyme was done in membrane cut off value 12000-14000 Da against Na-Acetate buffer (pH 5.0). Dialysed enzyme was then subjected to further purification by affinity precipitation using sodium alginate as described by Teotia *et al.,* (2001). SDS-PAGE of purified amylase showed homogenous nature in band pattern (mw 55000 Da), which was further characterized by native gel electrophoresis. Khoo *et al.,* (1994) purified α-amylase enzyme produced by *Aspergillus flavus* using ammonium sulfate precipitation and ion exchange chromatography. Their study reported that enzyme is homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Abou-Zeid (1997) also purified extracellular α-amylase from the same species of *Aspergillus flavus* by a starch adsorption method. Similarly, Chakraborty *et al.,* (2000) investigated thermostable α-amylase enzyme and purified it to homogeneity by ammonium sulfate fractionation and TECC on DEAE-cellulose column.

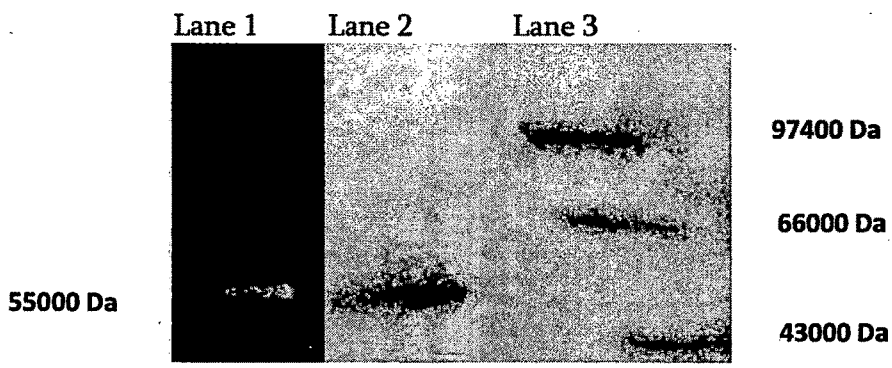

Figure 22: SDS PAGE and Native PAGE of purified enzyme.

Lane 1: Native gel for Amylase activity.
Lane 2: Purified enzyme.
Lane 3: Ladder Medium Range (Bangalore Gene Pvt.Ltd. Cat. No. RPMW-M 106005).

Properties of amylase:

Screening of various fungal strains for production of amylase showed *C. asperatum* as a good producer of amylase. In the present study, production of amylase was carried out by submerged fermentation and it was partially purified and characterized. Purification of amylase was done by affinity precipitation using sodium alginate. Purified enzyme showed maximum activity at pH 5.0, temperature 30 °C. Enzyme showed no inhibitory effect against Ca^{2+} metal ions. Molecular mass (55000 Da) was determined by denaturing gel electrophoresis, and enzyme was further characterized using native gel electrophoresis. Results of this study indicated that *C. asperatum* species is one of the potent producer of amylase in family Onygenaceae other than *P. chrysosporium* of the same family.