Chapter 4

DISCUSSION
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Solid-state fermentation (SSF) is a process whereby an insoluble substrate is fermented with sufficient moisture but without free water (Hesseltine, 1976). SSF has been reported to be a relatively low cost appropriate technology for upgrading of amylaceous materials as a protein source in animal feeding. (Raimbault, 1981; Senez et al., 1983; Hesseltine, 1987 and Pandey, 1992).

SSF offers several advantages over liquid fermentation, mainly in the areas of biomass energy conservation and solid wastes management. It offers excellent promises on pollution control by producing negligible liquid effluents in industrial process involving production of various products. In the SSF process, the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells.

Filamentous fungi have a number of properties, which make them important both scientifically and industrially. The industrial importance of filamentous fungi is illustrated by their applications which ranges from production of organic acids and antibiotics to a number of industrial enzymes. Thus, the selection of an appropriate solid substrate plays an important role in the development of an efficient SSF process. Since there is no free water available in the substrate, the relative humidity play a vital role.
The enzyme amylase is of great significance in biotechnology due to its wide area of industrial applications. Amylase find its application in a number of industrial process such as in food, fermentation, textiles and paper industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields such as clinical and analytical chemistries.

4.1. ISOLATION, PURIFICATION AND MAINTENANCE OF AMYLOLYTIC FUNGI

The present investigation deals with the isolation of amylolytic fungi from various parts of Kerala and their in vitro production of extracellular amylases, which have potential applications in industrial processes. Enumeration of extracellular amylase producing fungi was done by inoculating serial dilutions of soil into yeast extract, malt extract starch medium. Colonies which showed clearing zones around them when flooded with Lugol’s iodine solution were taken as positive for extracellular amylase production (Soni and Sandhu, 1990). The area of clearance was brought about by the utilisation of starch in the media. This is one of the simplest method used for the screening of microorganisms with extracellular amylase activity.

With extracellular microbial products, the usual practice is to grow large number of organisms on agar plate media and to relate each organisms production capability to the radius of the product’s zone of diffusion around the colony which may be inducible or repressible. The agar medium is designed to optimise induction and minimise the chances of repression.
Sixty one strains were isolated from soil samples collected from various parts of Kerala were tested for amylase activity. The strains were identified as *Aspergillus* sp, *Rhizopus* sp, *Penicillium* sp, *Saccharomyces* sp, *Mucor* sp, and *Neurospora* sp. Enzyme secretion by the fungal isolates in mineral salt starch medium and wheat bran were studied after an incubation period of four days. The reason for four day incubation is to ensure maximum enzyme production of extracellular amylase. The amylase activity was assayed by DNS method (Miller, 1959).

The pattern of enzyme production at different hours of incubation (0-120 hours) by the top ten ranked strains were determined. All the strains showed maximum amylase production at 96 hours in SSF on wheat bran and SmF on Mineral salt starch medium—*Penicillium* SBSS 1 (269 ulgds), *Saccharomyces* SBSS 2 (138 ulgds), *Rhizopus* SBSS 3 (150 ulgds), *Mucor* SBSS 4 (240 ulgds), *Neurospora* SBSS 5 (230 ulgds), *Mucor* SBSS 6 (185 ulgds), *Aspergillus* SBSS 7 (198 ulgds), *Aspergillus* SBSS 8 (188 ulgds), *Penicillium* SBSS 9 (220 ulgds) and *Neurospora* SBSS 10 (215 ulgds).

All the strains tested had the ability to degrade starch and to produce extracellular amylase. In all cases, the patterns of enzyme activity produced consisted of peaks followed by sharp declines. Some of these declines may have been caused by the activities of extracellular acid proteases. At any rate, these oscillations must be taken into consideration for maximum enzyme production at minimum incubation period.

The strain *Penicillium* species showed highest amylase activity when subjected to submerged fermentation (SmF) and solid state
fermentation (SSF) was selected for further study. It was identified as *Penicillium janthinellum* by the Institute of Microbial Technology (IMTECH), Chandigarh and deposited in their culture collection (NCIM 4960). This species of *Penicillium* was reported for the first time to produce α-amylase. Amylolytic enzymes are known to be synthesized by a number of fungi. Many *Penicillium* sp are also reported to produce extracellular amylase (Doyle et al., 1988; Hasha and Ohta, 1994).

The extent of amylase production is dependent upon a variety of factors, including the make up of the fermentation medium particularly the composition of the medium and how it was prepared. The variation in the yield of amylase, with changes in fermentation medium have been reported by many workers.

SSF offers the greatest possibility when fungi are used because, unlike other microorganism, which grow in nature on solid substrates. Some of the advantages of SSF over conventional submerged cultures for work involving fungi are simplicity of equipment, low moisture content which prevents bacterial contamination (Hesseltine, 1987) and less requirement of solvent to extract the enzymes, which greatly reduces energy requirements and problems of pollution.

The effect of wheat bran on amylase yield is shown in Table 9. Amylase of *Penicillium* sp are produced by SSF usually on wheat bran moistened to 50% and incubated at room temperature (30 ± 2°C). It is generally accepted that semi solid culture results in higher yields of these enzymes than that are observed in liquid culture. Semi solid culture allows development of spores and fruiting bodies in fungi to a much greater extent than observed in submerged fermentation (Ward, 1985).
The culture of *Penicillium janthinellum* effectively colonised the surfaces of the substrate wheat bran in SSF and exhibited dense growth. The maximum amylase concentration (269 ul/gds) was obtained after 4 days of incubation. The particle size and chemical composition of the substrate are of critical importance (Lonsane *et al.*, 1985). The suitability of wheat bran is apparent from sufficient nutrients that it contains and its ability to remain loose even in the moist state where it provides large surface area and aerobic conditions.

Commercial wheat bran consists of 8.5 % starch and 9.5 % protein in addition to various minerals (Fisher, 1973). The uptake of nutrients from the wheat bran cells by the culture, is due to their ability to penetrate deeply into the wheat bran particles is well established in SSF (Lonsane *et al.*, 1985). Some of the vital nutrients necessary for optimum growth and the formation of product process may also be present in wheat bran at sub optimal levels.

India is an agricultural country where different types of cereal bran are largely available at a very cheap price. So the use of wheat bran as a substrate for α-amylase production by *Penicillium janthinellum* seems quite encouraging from a commercial viewpoint. It may therefore be reasonably stated the data obtained in the present investigation would possibly serve as a useful guide for exploiting amylase activity of *Penicillium janthinellum* in the interest of commercial utilisation.
4.2. \( \alpha \)-AMYLASE PRODUCTION UNDER SOLID STATE FERMENTATION USING WHEAT BRAN

Among the physical parameters, the 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. Most of the Bacillus strains used commercially for the production of bacterial \( \alpha \)-amylases by submerged fermentation have an optimum pH between 6.0 and 7.0 for growth and enzyme production. This is also true of strains used in the production of the enzyme by solid state fermentation.

In fungal processes, the buffering capacity of some media constituents sometimes eliminates the need for pH control (Chahal, 1983). The pH value also serves as a valuable indicator of the initiation and end of enzyme synthesis (Friedrich et al., 1989). It is reported that Aspergillus oryzae 557 accumulated \( \alpha \)-amylases in the mycelia when grown in phosphate or sulphate deficient medium and was released when the mycelia were replaced in a medium with alkaline pH (Yabuki et al., 1977).

Medium pH play an important role in \( \alpha \)-amylase production (Narayanan and Shanmugasundaram, 1967; Kundu and Das, 1970, Michelenia and Castillo, 1984; Gautaum et al., 1991; Joshi and Ball, 1993, Chadha et al., 1995). Initial pH of the medium is an important parameter which affects the growth and product formation by microorganisms under both SSF and SmF. Good buffering capacity of some of the substrates used in SSF helps in eliminating the need
for pH control during fermentation (Lonsane et al., 1985). This advantage is exploited in the initial adjustment of the pH of the solids using the moistening media of the desired pH.

Kundu and Das (1970) reported that Aspergillus oryzae prefers optimum pH of 4.5 - 5.0 while Brettanomyces naardensis preferred a pH of 4.0 - 4.5 for maximum amylase synthesis (Gautam et al., 1991).

Narayanan and Shanmugasundaram (1967) reported that Fusarium vasinfectum exhibited three pH optima between pH 4.4 and 8.0 i.e., pH 4.4 - 5.0, pH 5.8 and pH 7.8 - 8.0. Michelena and Castillo (1984) reported that Aspergillus foetidus exhibited an optimum pH of 6.8 with sharp decline in enzyme yields for initial pH above or below this value. The results indicate clearly the importance of careful control of initial pH for maximum enzyme production. But in the present study Penicillium janthinellum preferred the optimum pH for maximum amylase production was found to be 5.0 (292.75 u/gds), although amylase yield was significant over a range of pH 3.0 - 9.0.

The influence of temperature on amylase production is related to the growth of the organism. Among the 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). α-amylase production has also been reported at 50°C by Thermomyces lanuginosus (Mishra and Maheshwari, 1996) and at 55°C by the thermophilic fungus Thermomonospora fusca (Busch and Stutzenberger, 1997).
Temperature is directly related to the metabolic activities of the microorganism and it affects proper growth and product formation by the organism (Lonsane et al., 1985). Every organism has its optimum temperature at which it grows best resulting in higher yield of the desired product and hence temperature should be maintained at optimum of the microorganism that is used for solid state fermentation (Kundu and Das, 1970; Michlena and Castillo 1984; Gautam et al., 1991; Chadha, 1995; Lin et al., 1998).

Gautam (1991) reported that *Phaffia rhodozyma* exhibited an optimum temperature of 45°C for maximum enzyme production. *Aspergillus foetidus* exhibited an optimum temperature of 45°C for maximum enzyme production (Michelena and Castillo, 1984). In the present study *Penicillium janthinellum* produced significant levels of amylase over a range of temperature between 30 to 55°C with an optimum at 35°C (294.50 u/gds). Higher temperature led to decline in enzyme production.

The critical importance of moisture content on microbial growth and product yield in SSF has been emphasised earlier (Ramesh and Lonsane, 1990). Microbiological activity on a substrate will progressively decrease at low water contents. Moisture is reported to cause swelling of the substrates thereby facilitating better utilisation of substrates by the microorganism (Kim et al., 1995). But high moisture content leads to reduction in yield, during SSF, which in turn is due to reduction in interparticle spaces, decreased substrate degradation and impaired oxygen transfer (Sandhya and Lonsane, 1984). Normally, moisture content of 30 - 80% is required for a significant level of
enzyme production with an optimum between 50 - 60%, depending on the material (Oriol et al., 1988). In the present study, it was observed that the level of initial moisture content required for maximum amylase yield (364.75 u/gds) was 60% for wheat bran. Similar observations were reported earlier by Selvakumar et al., (1996). Pandey (1990) reported that the optimum moisture content of wheat bran medium for amylase production by Aspergillus niger as 50 - 55%.

Effect of particle size on enzyme production during SSF was reported by Pandey (1991) and Selvakumar et al., (1996). Particle size of the substrate is a critical factor for enzyme production by SSF. Pandey (1991) reported that the smaller particle substrate gives high enzyme activity.

Particle size and shape are extremely important in SSF. They affect the surface area to volume ratio of the particle, the packing density within the substrate mass, and the size and shape of the void spaces between the particles. Small particles, or particles with large flat surfaces, tend to pack together closely, making it difficult to aerate the substrate mass. If the microorganism can penetrate into the particle this increases the directly accessible substrate and decreases the distances over which diffusion needs to occur. In this case, the optimal particle size will be influenced by the depth of penetration. The optimal particle size often represents a compromise between the accessibility of nutrients and the availability of oxygen.

Ramesh and Lonsane (1989) used wheat bran of particle size 800 μm inorder to obtain higher yield of α-amylase by Bacillus licheniformis. In the present study, the particle size of 425-500 μm,
favoured maximum enzyme production. Beyond this size, there is a decrease in enzyme activity. Identical observations were reported earlier by Selvakumar et al., (1996). The maximum enzyme activity was obtained with substrate containing particles of 425-500\(\mu\)m (385.75 u/gds) while the lowest enzyme activity with substrate containing particles bigger than 1.4mm. Similar trends was also observed with substrate containing particles of mixed size (Pandey, 1991).

The variation in optimum incubation time required for maximum enzyme yield with the substrates could be attributed to the differences in their physical nature and biochemical status which consequently, influences the aeration rate and nutritional availability for the fungi during SSF. Impact of incubation period on amylase production was reported by Soni and Sandhu (1990) Joshi and Ball (1993) and Chadha et al., (1995).

Soni and Sandhu (1990) reported that in *Trichosporon beigelli* and *Saccharomyces cerevisiae* the production of extracellular amylases which exhibited an initial increase with increase of biomass. The maximum activity was observed at the end of the exponential phase and decreased gradually thereafter. Similar observations have also been reported earlier by Clementi et al., (1980) and Sandhu et al., (1987) reported that the yield of exo and endo amylases increases appreciably, in *Saccharomycopsis fibuligera* during growth and attains its maximum in stationary phase.

Chadha et al., (1995) reported that in *Streptomyces albaduncus*, an initial lag period of 2 hour was observed which was followed by
The maximum \( \alpha \)-amylase activity was observed after 56 hours. Chadha et al., (1995) reported that in alkalophilic bacteria the maximum amylase activity was observed on the second day. The present study shows the fermentation period required for maximum enzyme yield as 96 hours (405 u/gds), the incubation beyond 96 hours led to the decrease in enzyme production. The reduction in the enzyme yield after 96 hours is probably due to enzyme denaturation by protease.

\( \alpha \)-amylases is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose (Tonomura et al., 1961; Yabuki et al., 1977; Lachmund et al., 1993; Morkeberg et al., 1995). Most reports available on the induction of \( \alpha \)-amylase in different strains of Aspergillus oryzae suggest that the general inducer molecule is maltose. Apart from maltose, in some strains, other carbon sources such as lactose, trehalose, \( \alpha \)-methyl-D-glucoside also served as inducers of \( \alpha \)-amylase (Yabuki et al., 1977).

\( \alpha \)-amylases production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes (Bhella and Altosaar, 1985; Morkeberg et al., 1995). However, the role of glucose in the production of \( \alpha \)-amylases in certain cases is controversial. \( \alpha \)-amylases production by Aspergillus oryzae DSM 63303 was not repressed by glucose rather, a minimum level of the enzyme was induced in its presence (Lachmund et al., 1993). However, xylose or fructose have been classified as strongly
repressive although they supported good growth in *Aspergillus nidulans* (Arst and Bailey, 1977).

There was an increase in the product yield after incorporation of additional carbon source during SSF was reported by Upton and Fogarty (1977); Gayal and Khandeparkar (1979); Emayavarambdan and Ramabadran (1979); Srivastava *et al.*, (1981); Gogoi *et al.*, (1987); Melasneimi (1987); Soni *et al.*, (1991); Freer (1993); Lin *et al.*, (1998) and Mamo and Gessesse (1999).

Freer (1993) reported that in *Streptococcus bovis JBl*, starch and maltose induce 12 fold amylase activity than glucose as sole carbon source. The production of amylase appears to be regulated. Gayal *et al.*, (1997) reported that in *Saccharomyces fibuligera* mannose, glucose, fructose, sucrose, maltose, dextrin and starch act as excellent source for both growth and amylase production. Cellobiose also supports the amylase synthesis although the growth was rather low. The ability to synthesize α-amylase even in presence of easily metabolisable monosaccharides and disaccharides indicate the absence of catabolite repression in *Saccharomyces fibuligera*. This is contrary to the reports that glucose acts as a catabolite repressor in the production of amylase in *Saccharomyces fibuligera* (Stepanov *et al.*, 1975; Clementi *et al.*, 1980; Sills and Stewart, 1982).

Emayavarambdan and Ramabadran (1979) reported that in *Bacillus* species addition of glucose resulted in only small increase of amylase. This might be either due to the inhibitory effect of intermediary products of glucose or due to glucose repression (Neidhart and Magasanik, 1956).
In the present study of the carbon source tested maltose (429 u/gds), starch and dextrin could effect an enhancement in the enzyme yield. Identical results were reported earlier by Upton and Fogarty (1977); Melasneimi, (1987); Soni et al., (1991) and Mamo and Gessese (1999).

The role of inorganic nitrogen source on enzyme production was reported by Upton and Fogarty, (1977); Emayavarambdan and Ramabadran, (1979); Michelena and Castillo, (1984); Gogoi et al., (1987); Lin et al., (1998) and Mamo and Gessese (1999).

Michelena and Castillo (1984) reported that the supplementation of nitrogen salts greatly increased the enzyme yields in Aspergillus foetidus. The inhibitory effect of some of the salts may be related to the pH changes associated with their use in the medium. Growth was not markedly affected in any case. (NH₄)₂SO₄, NH₄Cl or NH₄NO₃ reduced the final yields of amylase. While other salts like NaNO₃ and NaNO₂ stimulated enzyme production. Of the different inorganic nitrogen sources when tested for their effect on amylase production it was observed that KNO₃ (442 u/gds) and NaNO₃ (420 u/gds) served as best nitrogen source. Beneficial effect of nitrate has also been observed by other workers for other organisms. (Jonnson, 1967, Karashunov and Kazavalov 1972 and Prudhov et al., 1972).

The impact of organic nitrogen source on amylase production was reported by Upton and Fogarty, (1977); Emayavarambdan and Ramabadran, (1979); Michelena and Castillo, (1984); Gogoi et al., (1987); Lin et al., (1998) and Mamo and Gessese (1999).
Gogoi et al., (1987) reported that in *Saccharomycopsis fibuligera*, yeast extract and corn steep liquor supported both growth and amylase production. Maximum yield of amylase was obtained with yeast extract at a concentration of 0.5%. Corn steep liquor at a concentration higher than 0.2% reduces the final amylase yield although the growth was stimulated at higher concentration. Peptone did not serve as a good promoter of amylase synthesis. Presence of \((\text{NH}_4\text{)}_2\text{SO}_4\) in the medium, either alone or in presence of yeast extract inhibited the amylase synthesis.

Mamo and Gessesse (1999) reported that in *Bacillus* sp proteose peptone and tryptone were used as nitrogen sources. Compared to these two nitrogen source, growth and enzyme production was lower in media containing yeast extract. No growth was detected using \(\text{NO}_3\), \(\text{NH}_4\) or urea as nitrogen source. Growth and amylase production was influenced by the type of nitrogen source used in the medium.

Organic nitrogen sources have been preferred for the production of \(\alpha\)-amylase. Yeast extract have been used in the production of \(\alpha\)-amylase from *Streptomyces* sp (Mc Mohan et al., 1999), *Bacillus* sp-IMD 435 (Hamilton et al., 1999 a) and *Halomonas meridiana* (Coronado et al., 2000). Yeast extract have also been used in conjunction with other nitrogen sources such as bactopeptone in the case of *Bacillus* sp. IMD 434 (Hamilton et al., 1999 a) ammonium sulphate in case of *Bacillus subtilis* (Dercova et al., 1992), ammonium sulphate and casein for *Calvatia gigantea* (Kekos et al., 1987) and soybean flour and meat extract for *Aspergillus oryzae* (Imai et al.,
Yeast extract increased the productivity of α-amylase by 110-156% in *Aspergillus 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 bacterial strains; Krishnan and Chandra (1983) and Yoshigi *et al.*, (1985). Soybean meal and casamino acids by *Aspergillus oryzae* (Ueno *et al.*, 1987). Corn steep liquor has also been used for the economical and efficient production of α-amylase from a mutant of *Bacillus subtilis* (Shah *et al.*, 1990). Apart from this, various inorganic salts such as (NH₄)₂SO₄ for *Aspergillus nidulans* (Lachmund *et al.*, 1993) and *Aspergillus oryzae* (Morkeberg *et al.*, 1995), ammonium nitrate for *Aspergillus oryzae* (Kundu *et al.*, 1973) and Vogel salts for *Aspergillus fumigatus* (Goto *et al.*, 1998) have been reported to support better α-amylase production in fungi.

The present study indicates that the tryptone and peptone enhances the maximum enzyme production followed by beef extract, liver extract and urea. Beef extract and liver extract were only good sources for growth but not for amylase production. In *Penicillium janthinellum* only peptone and tryptone served as good complex nitrogen sources for growth and amylase formation. Compared to inorganic nitrogen sources such as NaNO₃ and KNO₃ growth and enzyme production was lower in media containing complex organic nitrogen source.

K⁺, Na⁺, Fe²⁺, Mn²⁺, Mo²⁺, Cl⁻, SO₄²⁻ had no effect while Ca²⁺ was inhibitory to amylase production by Aspergillus oryzae E1 212 (Kundu et al., 1973). Mg²⁺ plays an important role and production was reduced to 50% when Mg²⁺ was omitted from the medium. Na⁺ and Mg²⁺ were showed a coordinated stimulation of enzyme production by Bacillus sp CRP strain. Addition of zeolites to control ammonium ions in Bacillus amyloliquefaciens resulted an increase in the yield of α-amylase. An inverse relationship between α- amylase production and growth rate was observed for Streptomyces sp in the presence and absence of Co²⁺ (McMohan et al., 1999), the presence of Co²⁺ enhancing the final biomass levels by 13-fold, albeit with a reduction in enzyme yield.

Here in the present study the maximum production of amylase was induced by manganese (458 ul/gds). While all other metal ions showed a negative impact on amylase production. The stimulation of amylase by manganese does not appear to have reported earlier. This is contrary to the report that manganese act as an inhibitor in the production of amylase in Aspergillus oryzae E212 (Kundu et al., 1973).

Amino acids in conjunction with vitamins have also been reported to affect α- amylase production. However, no conclusion can
be drawn about the role of amino acids and vitamins in enhancing the α- amylase production in different microorganisms as the reports are highly variable. α-amylase production by *Bacillus amyloliquefaciens* ATCC 23350 increased by a factor of 300 in presence of glycine (Zhang et al., 1983). The glycine was not only act as a source of nitrogen but rather it affects α- amylase production by controlling pH thereby increasing amylase production. β-alanine, Dl-nor valine and D-methionine were effective for the production of alkaline amylase by *Bacillus* sp A-40-2. However, the role of amino acids was considered neither as a nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion (Ikura and Horikoshi, 1987). It has been reported that only asparagine gave good enzyme yields (Kundu et al., 1973). While the importance of arginine for α- amylase production from *Bacillus subtilis* has also been well documented. (Lee and Parulekar, 1993).

The impact of amino acids on enzyme production was reported by Patil and Shastri (1982). Amino acids viz. serine, threonine, valine, methionine and phenylalanine at a concentration of 0.1 and 0.5% stimulated the growth while amino acids viz. lysine, leucine at high concentration decreased the enzyme production. The result of the present experiment indicate that asparagine increases the enzyme production while all other amino acids tested stimulated the growth but with no effect on α-amylase synthesis. Asparagine enhances the enzyme synthesis confirming the earlier reports Black and Gray, (1953) and Black and Wright, (1953) that various amino acids and intermediates that were formed in the cleavage of asparagine, facilitated maximum synthesis of amylase (468 u/gds).
Lin et al., (1998) reported the impact of various surfactants on amylase production. Triton X-100 and sodium dodecyl sulphate had lethal effect on growth. The enzyme production was slightly affected by Tween-80 at a concentration less than 0.5 %. Chandra et al., (1980) reported that the non ionic surfactants such as Tween-80 and Triton X-100 have an important role in the enhancement of amylase production by *Bacillus licheniformis*. However, non ionic surfactants were inhibitory towards growth and amylase production in *Bacillus* sp. TS-23. In the present study it was observed that among the various surfactants, Tween-80 plays an important role in the enhancement of amylase production (483 ul/gds).

Use of low molecular weight dextran in combination with either Tween-80 or Triton X-100 for α-amylase production in the thermophilic fungus *Thermomyces lanuginosus* has been reported (Arnesen et al., 1998). Triton X-100 had no effect, whereas Tween-80 increases the α-amylose activity 27-fold.

The role of vitamins on enzyme production was reported by Emayavarambdan and Ramabadran (1979). Among the various vitamins riboflavin favoured maximum amylase production followed by ascorbic acid, folic acid and nicotinic acid. Jayachandran, (1969) observed the stimulatory effect of various vitamins on amylase production by *Thermoascus aurantiacus*. It is observed from the present study that among the various vitamins riboflavin (491 ul/gds) favoured maximum enzyme production followed by ascorbic acid (376 ul/gds), folic acid (357 ul/gds) and nicotinic acid (338 ul/gds). Similar observations were also reported earlier by Jayachandran.
(1969) and Emayavaramdan and Ramabadran (1979). Addition of ascorbic acid, folic acid and nicotinic acid inhibits the production of amylase. This might be either due to pH changes associated with their use in the medium or due to inhibitory effect of intermediary products.

The role of sugar alcohols on α-amylase production was reported by Gayal and Khandeparkar (1979) and Patil and Shastri (1982).

In *Bacillus subtilis* glycerol and mannitol suppressed the amylase production although the growth in the presence of mannitol is quite high. Sorbitol stimulates amylase synthesis. Similar effect of mannitol was observed in the case of *Thermactinomyces vulgaris* where better growth with mannitol without any substantial formation of amylase occurred (Kuo and Hartman, 1966). Sorbitol, the stereoisomer of mannitol behaved differently and it stimulates better amylase synthesis. In the present study of the sugar alcohols tested sorbitol could effect an enhancement in the enzyme yield. While glycerol and mannitol suppressed the amylase production. The inhibitory effect of glycerol and mannitol may be related to the pH changes associated with their use in the medium.

Medda and Chandra (1980) reported that the presence of 7.0% NaCl in the medium results in high yield of amylase from *Bacillus licheniformis*. Contrary to this the present study shows the maximum level of amylase in the absence of NaCl (485 u/gds). Further, the enzyme yield decreased progressively with increase in NaCl concentration. Horikoshi (1971) reported that an alkalophilic *Bacillus* sp. A-40-2 did
not show any enhancement of growth and enzyme production after addition of NaCl in the medium.

The impact of sodium salts of organic acids on amylase synthesis was reported by Gayal and Khandeparkar (1979); Patil and Shastri (1982) and Pal and Das (1984).

Gayal and Khandeparkar (1979) reported that in the case of *Bacillus subtilis* the organism did utilise the salts of organic acids to various extents which was revealed by the differential growth of the organism, although no significant production of amylase was observed. In *Polyporus ostreiformis* 5mM concentration of sodium citrate stimulate amylase synthesis. The observations indicate that metabolic activities of *Polyporus ostreiformis* was highly sensitive to even minute traces of sodium citrate leading to over production of amylase.

Patil and Shastri (1982) reported that in *Alternaria alternata*, sodium pyruvate at lower concentration stimulated protease synthesis, while at higher concentrations sodium pyruvate suppressed growth and protease production. Sodium pyruvate increased the pH of the culture medium.

In the present study, of the sodium salts of organic acids tested trisodium citrate could effect an enhancement in enzyme yield while sodium succinate and sodium pyruvate suppressed the amylase production. Addition of sodium citrate resulted in only small increase of enzyme when compared to control. This increase might be due to the presence of sodium in sodium citrate. The sodium salts of organic
acids which were found inhibitory or having no significant effect on amylase production were deleted in subsequent steps.

The impact of organic acids on enzyme production was reported by Patil and Shastri (1982). Supplementation of organic acids decreases the enzyme production and growth of cells. Addition of citric acid, maleic, acid, succinic acid, pyruvic acid and fumaric acid resulted in complete inhibition of growth and enzyme production. In the present study, the various organic acids tested suppresses the amylase production. Similar results with organic acids were also recorded by Prudkov et al., (1972). All the organic acids were found to decrease the pH of the culture medium.

The impact of various inhibitors on amylase production was reported by Narayanan and Shanmugasundaram (1967); Johnson et al., (1968); Vukelic et al., (1992); Fagerstrom and Kalkkinen (1995); and Lin et al., (1998). Johnson et al., (1968) reported that in Aspergillus wentii starch hydrolysis was completely inhibited by 0.001 M EDTA, which reflect the need for calcium, which otherwise shown to be necessary for bacterial α-amylase activity (Nishida et al., 1967). Johnson et al., (1968) reported that 0.001M concentration of sodium arsenate, sodium azide and cyclohexamide were proved to be potent inhibitors of amylase. It was observed from the present study that all inhibit growth and enzyme production.

4.3. Purification and characterisation of α-amylase

Uguru et al., (1997 a) purified a thermostable extracellular α-amylase from Bacillus subtilis having the specific activity of
2200 Units/lmg per litre. Bolton et al., (1992) purified an α-amylase to homogeneity using a combination of ammonium sulphate precipitation, ion exchange chromatography and gel filtration. An extracellular thermostable amylase produced by Bacillus licheniformis was purified by two phase separation in polyethylene glycol/dextran system followed by gel filtration and anion exchange chromatography. (Ivanova et al., 1993). Thermostable α-amylase from yeast Cryptococcus sp was purified in just one step by using cyclodextrin-sepharose 6B column (Iefuji et al., 1996). Priest et al., (1985) purified a novel α-amylase from the yeast Lipomyces kononenkaoe to homogenity by ammonium sulphate treatment, affinity binding on cross-linked starch and DEAE-Biogel A chromatography.

An overall yield of 38.3% was obtained after 216 fold purification of amylase from Filobasidium capsuligenum (De Mot and Verachtert, 1985) while a 118 fold purified amylase with 53% yield was obtained from Saccharomycopsis fibuligera (Gogoi et al., 1987). Amylase from Lactobacillus cellobiosus D-39 was purified with an yield of 1.49% (Sen and Chakraborty, 1986) where as the enzyme from Bacillus subtilis-159 was purified 82 fold with 35% yield (Gayal et al., 1997).

The purification of α-amylase have largely been limited to a few species of mesophilic fungi (Khoo et al., 1994, Planchot and Colonna, 1995, Chang et al.; 1995, Park et al., 1995 and Abouzeid, 1997). In the present study a maximum 66.4 % of yield for α-amylase with 40.7 fold purification and 183.2 U/mg protein
specific activity was obtained after purification. Electrophoretic studies revealed a single band upon polyacrylamide gel electrophoresis both SDS-PAGE and native PAGE.

The pH optima of α-amylases vary from 2.0 to 12.0 (Vihinen and Mantsala, 1989). α-amylases from most bacteria and fungi have pH optima in the acidic to neutral range (Pandey et al., 2000). α-amylases are generally stable over a wide range of pH from 4.0 to 11.0 (Saito, 1973, Fogarty and Kelly, 1979; Vihinen and Mantsala, 1989; Khoo et al., 1994, Hamilton et al, 1999 a and b); however, α-amylases with stability in a narrow range have also been reported (Robyt and Ackerman, 1971, Krishnan and Chandra, 1983, Coronado et al., 2000).

The α-amylase isolated from various microorganisms were reported to be active and stable over different ranges of pH. Amylase produced from Aspergillus awamori exhibited optimum activity at pH 4.8-5.0 (Bhella and Altosaar, 1985) while amylase from Aspergillus flavus showed a pH optima of 7.0 (Abouzeid, 1997). Amylase isolated from Lipomyces starkeyi exhibited optimum activity at pH 3.5-4.0 (Soni et al., 1991). While that from Lipomyces kononenkoae exhibited optimum activity at pH 4.5-5.0 (Prieto et al., 1995). Similarly Penicillium janthinellum shows the activity and stability at a pH range of 4.0-7.0, having an optimum at pH 5.0.

Thermostable α-amylase from Thermomyces lanuginosa (Jensen and Olsen, 1992) shows lower optimum pH than the α-amylase from Aspergillus oryzae but higher than Mucor pusillus.
(Planchot and Colonna, 1995) though it resembles $\alpha$-amylase from *Talaromyces emersonii* (Bhella and Altosaar, 1985). The $\alpha$-amylases isolated from various microorganisms were reported to be active and stable over different ranges of temperatures. Amylase produced from *Aspergillus awamori* showed the optimum activity at $50^0\text{C}$ (Bhella and Altosaar, 1985) while that from *Aspergillus flavus* was $55^0\text{C}$ (Khoo, 1994).

The temperature optimum for the activity of $\alpha$-amylase is related to the growth of the microorganisms (Vihinen and Mantsala, 1989). The lowest temperature optimum is reported to be 25-30$^0\text{C}$ for *Fusarium oxysporum* amylase (Chary and Reddy, 1985) and the highest of 100 and 130$^0\text{C}$ from archaeabacteria, *Pyrococcus furiosus* and *Pyrococcus woesei*, respectively (Giri et al., 1990; Ray, 2004).

The optimum activity of amylase isolated from *Aspergillus flavipes* was found to be 60-80$^0\text{C}$ (Frolova et al., 2002), while amylase produced from *Aspergillus foetidus* was 45$^0\text{C}$ (Michelena and Castillo, 1984). The studies on the temperature tolerance of the purified amylase show that the enzyme has an optimum temperature of 50$^0\text{C}$ for its activity and retained 80% activity at 60$^0\text{C}$.

The substrate specificity of $\alpha$-amylase varies from microorganism to microorganism. In general, $\alpha$-amylases display highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen and maltotriose.
The enzyme obtained in the present study recorded high substrate specificity towards amylose and amylopectin and 35% specificity towards glycogen. High molecular weight substrates, namely amylose and amylopectin were the better substrates for α-amylase. Glycogen was hydrolysed at a lower rate. This result is in agreement with the reports of amylase produced by *Filobasidium capsuligenum* (De Mot and Verachtert, 1985). The relative rates of substrate hydrolysis indicate that amylose was more easily hydrolyzed than amylopectin or glycogen, because α-1,4 linkages were attacked more easily than were α-1,6 linkages. The enzyme from *Bacillus licheniformis* (Krishnan and Chandra, 1983) showed high affinity towards amylose. N-ethylmaleimide had no inhibitory effect on the enzyme, hence it was concluded that, no sulphydryl or carboxyl residues are essential for the catalytic activity of the amylase. This is in agreement with the earlier reports of amylase produced by *Bacillus cereus* NY-141 (Yoshigi *et al*., 1985).

The inhibitory effect of chelating agent EDTA (32%) demonstrated the ion requirements of this α-amylase activity, is consistent with a putative metalloprotein with a bound divalent cation. This inhibition is very common among amylases with ion requirements.

Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Fe^{2+} and Fe^{3+} were potent inhibitors of α-amylase activity in *Penicillium janthinellum*. Most amylases are metallo enzymes containing at least one metal atom per molecule of enzyme. This metal plays an important role in catalytic activity. In the presence of Ca^{2+} α-amylase are more resistant (Moranelli *et al*., 1987). The activity in *Penicillium janthinellum* was slightly higher in
presence of Mg$^{2+}$ and Ca$^{2+}$, and these ions permitted the stabilization and prolongation of the activity. The effect of Zn$^{2+}$ varied markedly among amylases in *Schwanniomyces alluvius* (Moranelli et al., 1987) and *Bacillus cereus* NY-14 which holds a potent inhibitory effect, whereas for *Aspergillus kawachii* (Mikami et al., 1987) has no effect at all. For *Penicillium janthinellum*, Zn$^{2+}$ had a significant inhibitory effect. The inhibition observed may be due to competition between the exogenous cation and the protein associated cation, resulting in reduced metalloenzyme activity.

4.4 Compatibility with various commercial detergents

90% of the original activity of the enzyme was retained in case of Henko, Vim and White Giant. Addition of CaCl$_2$ to the detergent and enzyme mixture increases the stability of enzyme in presence of detergents.

Nehra et al., (2004) reported that alkaline protease isolated from *Aspergillus* sp was 90% stable in Vim, Avis, Nirma and Wheel and the stability was increased in the presence of CaCl$_2$.

Due to high competition in the field of detergent industry, the composition of the detergent always undergoes much change with time to improve its quality and to bring out effective cleaning. Under these conditions it is significant to note that the proposed amylase from *Penicillium janthinellum* is stable and retains its 90% activity even in the presence of new detergent formulas as that of Henko, Vim and White Giant.
Different hydrolytic enzymes being used for laundry formulation, it seems that although it started as early as in 1913, detergent development and process technology have taken more than eight decades to reach the present status. Even today, large scale efforts are on the way to develop detergents which not only clean clothes of dust particles, but are simultaneously efficient in removing stains of oils, proteins, tannins and other problem molecules. Although attempts for the utilisation of proteases, lipases, α-amylases and cellulases in detergents have been made, great scope for further development of a novel detergent exists which will not only be economical but also be “complete” and efficient in all respects. Such a detergent can only be produced by using microbial biocatalysts.

Penicillium janthinellum was observed increased production of α-amylase under SSF in the present study indicates its potential for industrial scale application in the future. In fact this strain is observed for the first time to produce α-amylase in large scale. The observations made with respect to optimization of growth conditions and process parameters that govern maximum production of α-amylase by this strain strengthen the potential of the organism for industrial use.

The results indicate that the composition of the medium is a major factor in regulating the synthesis of extracellular enzyme. Extracellular enzymes have been successfully produced in SSF using Penicillium species. This clearly indicates that Penicillium species are amenable for cultivation in SSF.
Amylases are among the most important enzymes used in industrial processes. Although, the use of amylases, \( \alpha \)-amylases in particular, in starch liquefaction and other starch based industries has been prevalent for many decades and a number of microbial sources exists for the efficient production of this enzyme, the commercial production of this enzyme has been limited to a few selected strains of fungi and bacteria. Moreover, the demand for these enzymes is further limited with specific applications as in the food industry, wherein fungal \( \alpha \)-amylase are preferred over other microbial sources due to their more accepted GRAS status.
4.5. CONCLUSION

The present project suggests *Penicillium janthinellum* as a potential candidate for industrial exploitation in the field of α-amylase production. Perhaps it is the first report that *Penicillium janthinellum* could produce α-amylase under SSF. The optimum conditions for maximal amylase production include – pH (5.0), temperature (35°C), 60% moisture content, particle size (425-500μm), 96 hours of incubation period, 1.0% maltose, 0.5% KNO₃, 0.5% MnSO₄•5H₂O, 0.2% Asparagine, 0.05% Tween-80 and 0.1% riboflavin. The enzyme produced by the organism also has several beneficial properties needed for an industrial enzyme. It shows activity and stability over a wide range of pH and temperature. The enzyme is highly compatible with commercial detergents and retain almost complete activity in their presence. Hence this enzyme may also be used along with the new formulations of detergents to make it more effective and also to make it more eco-friendly. However, scale up studies are needed for the development of an economic bioprocess for commercial exploitation of this organism for α-amylase production.