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Full Length Research Paper

Streamlining of procedure parameters for the generation of α- amylase from Penicillium janthinellum (NCIM 4960) under solid state fermentation

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The production of extracellular amylase was studied in solid state fermentation by *Penicillium janthinellum* (NCIM 4960) using wheat bran as a solid substrate. The physical and chemical parameters influencing SSF were optimized. The maximum enzyme activity obtained was 300 U/gds under optimum conditions of pH 5.0, an incubation temperature of 35°C, maltose (1% w/w) as carbon source, $(NH_4)_2SO_4$ - (0.2% w/w) as nitrogen source, particle size (425 - 500 µ), incubation time (96 h), moisture content (60%), and surfactant (Tween-80, 0.05% w/w).

Key words: Extracellular, amylase, *Penicillium janthinellum*, optimization, soil, fermentation.

INTRODUCTION

Amylases are one of the most important enzymes used in industrial processes. Although, the use of amylases, α-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 is limited to only 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 α-amylase are preferred over other microbial sources due to their more accepted GRAS status.

Two major classes of starch degrading enzymes are identified in microorganisms - α-amylases and glucoamylases. α-amylases (E.C-3.2.1.1) are extracellular enzymes that randomly cleave α- 1,4 glucosidic linkages between adjacent glucose units in the linear amylase chain and glucoamylase (E.C-3.2.1.3) hydrolyses single glucose units from the non-reducing ends of amylase and amylopectin in a stepwise manner and also able to hydrolyse the α- 1,6 linkages at the branching points of amylopectin at a slower rate than α-1,4 linkages. α-Amylases is secreted as a primary metabolite and its secretion is growth associated (Sudo et al., 1994 and Spohr et al., 1998). α-Amylases are universally distributed throughout the animal, plant and microbial kingdoms. α-Amylases find application in baking, brewing, detergent, textile, paper and distilling industry (Pandey et al., 2000).

The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are achieved. The cost of enzyme production in submerged fermentation is high; this leads to search of alternative methods. The use of agroindustrial residues make solid state fermentation more economic (Ellaiah et al., 2002). Baysal et al., 2003 reported amylase production using wheat bran as substrate. The effects of the starch, protein and soluble oligosaccharides contents in wheat bran on the production of extracellular amylase were reported in *Penicillium decumbens* by Sun et al., 2007.

The present investigation deals with the isolation of amylolytic fungi from soil samples collected from South Kerala, India and optimization of process parameters for maximal production of amylase under SSF. The strain was identified as *Penicillium janthinellum* by 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. In this paper we report a number of factors that influence amylase production by *P. janthinellum* (NCIM 4960) under SSF.

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MATERIALS AND METHODS

Microorganism

P. janthinellum (NCIM 4960) used in the present study was isolated from soil samples collected from southern part of Kerala, India. Screening was carried on starch agar plate (Soni et al., 1996). It was maintained on potato dextrose agar slants at 4°C.

Amylase production under solid state fermentation

Wheat bran procured from local market was used as solid substrate. Solid state fermentation was carried out in 250 ml Erlenmeyer flask containing 20 g of wheat bran moistened to 60% with mineral salt starch solution containing (g/l) Na_2HPO_4-6.0, NaH_2PO_4 - 3.0, KCl - 1.0, $MgSO_4.7H_2O$ - 0.1 (pH- 7.0) (Ramesh and Lonsane, 1989).

Culture conditions

Spores of the selected fungus were harvested from seven day old slant cultures by suspension in sterile distilled water containing 0.01% Tween-80.The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution was used as inoculum (Ramachandran et al., 2004; Agrawal et al., 2005). The spore suspension diluted to desired count (5×10^7 spores/ml) served as an inoculum. Inoculum was added to 20 g wheat bran in a 250 ml conical flask moistened to 60% water content with salt solution. The flasks were incubated at 35°C for 96 h under stationary conditions in a BOD incubator. After incubation, amylase was extracted by shaking for 30 min with 50 ml distilled water. Solids were separated by squeezing through two fold cheese cloth and then filtering through Whatman No: 1 filter paper. The filtrate was used as source of amylase.

α-Amylase assay

α-Amylase was assayed by incubating 0.5% of soluble starch solution (Prepared in 0.1 M phosphate buffer) at 55°C for 15 min (Bernfield, 1955). The reaction was terminated by adding 1 ml of dinitrosalicylic acid reagent followed by incubating in a boiling water bath for 10 min and the final volume was made up to 12 ml with distilled water and optical density was taken at 540 nm (Miller, 1959). One unit of - amylase activity was defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under assay conditions and expressed as units per gram dry substrate.

Impact of process parameters on α-amylase production during solid state fermentation

The impact of various process parameters influencing α- amylase synthesis by *P. janthinellum* (NCIM 4960) under solid state fermentation was studied. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments (Uyar and Bysal, 2004). The effect of process parameters on enzyme production was determined by incubating at different pH (3 - 11), temperature (30 - 55°C), additional carbon source, nitrogen source, moisture content (20 - 80%), particle size, incubation time (24 - 168 h) and surfactant.

Effect of initial pH of the medium

The effect of initial pH on enzyme yield by fungus during solid state fermentation was studied by adjusting the pH of the mineral salt solution used to moisten the substrate to various pH levels (pH 3 – 11) using 1 N NaOH and 1 N HCl. The other conditions were kept constant.

Effect of incubation temperature

The effect of incubation temperature on enzyme production by fungi during SSF was determined by incubating the flasks at different temperature (30 - 55°C) keeping other conditions constant.

Effect of incubation time

The effect of incubation time on enzyme production by the fungi was studied by incubating the inoculated flask for a total period of 168 h and estimating the enzyme production at regular intervals of 24 h.

Effect of nitrogen source

The effect of nitrogen source on enzyme production by the fungi was studied by incorporating 0.2% (w/w) level of nitrogen in the SSF medium. The nitrogen sources tested include ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, sodium nitrite and potassium nitrate.

Effect of initial moisture content

The effect of initial moisture content of the solid medium on enzyme production was determined by preparing the solid substrates with varying initial moisture contents in the range of 20 - 80%. This was achieved by altering the amount of salt solution used to moisten the substrates. The optimum moisture content achieved by this step was used for subsequent experiments.

Effect of particle size of the substrate

The effect of particle size on enzyme production by the fungi was evaluated by using substrates of different particle size. Commercial wheat bran contained particles of different sizes; hence they were sieved by using standard sieves (Secor, India) of known mesh sizes ranging from 300 - 1400 µ.

Effect of surfactants

The effect of surfactants on enzyme synthesis by fungi during SSF was evaluated by incorporating 0.05% (w/w) level of surfactants in the medium. The surfactants tested include tween-80, triton X-100 and SDS.

Effect of additional carbon source

The effect of carbon source on enzyme production by the fungi during solid state fermentation was determined by incorporating at 1% (w/w) level in the medium. The carbon sources tested include dextrin white, sucrose, maltose, glucose, galactose, starch, xylose, arabinose, fructose and lactose. The other conditions were kept constant.

RESULTS AND DISCUSSION

Effect of initial pH of the medium

The results presented in Figure 1 showed that maximum amylase and was produced at pH 5.0 (275 U/gds). Amylase yield was significant over a range of pH 3 - 9, with

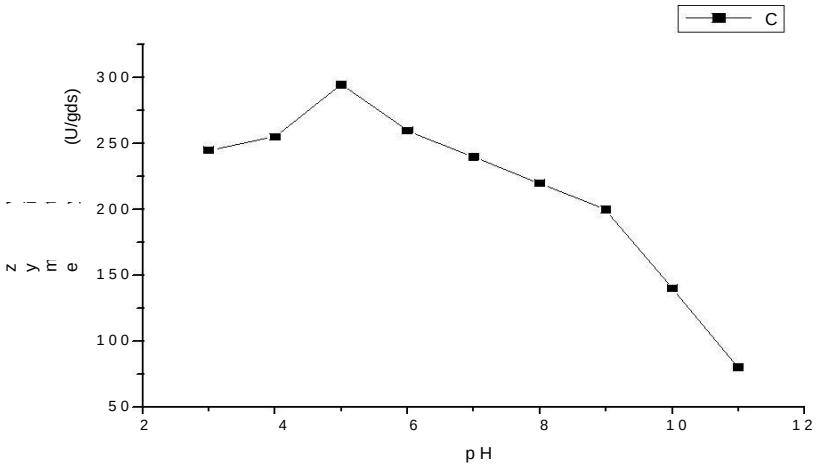

Figure1. Effect of p^H on α- amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

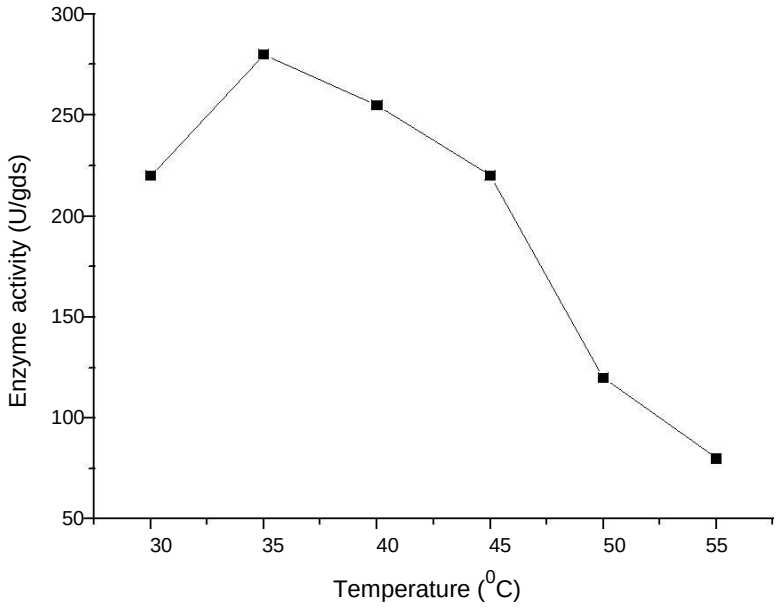

Figure 2. Effect of temperature on α-amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

optimum at pH 5.0. Initial pH of the medium plays an important role in growth and product formation.

Effect of incubation temperature

Solid state fermentation is usually carried out in the temperature range of 25 - 35°C (Krishna and Chandrasekharan, 1996).

A significant level of enzyme was produced by the fungi over a range of temperature 30°C to 45°C, with an optimum at 35°C (Figure 2). It was noted that higher temperature led to decline in the enzyme production while the temperature above 45°C the enzyme yield was decreased. Temperature above 45°C results in moisture loss of the substrate, which will affect metabolic activities of the microorganism, which results in reduced growth and enzyme production.

Effect of incubation time

The results presented in Figure 3 indicate that amylase production was 220 U/gds up to 20 h and increased to 278 U/gds after 96 h of incubation. After 96 h there was a sharp decrease in enzyme production which may be due to denaturation of the enzyme.

Effect of nitrogen source

The results presented in Figure 4 indicate that $(NH4)2SO4$

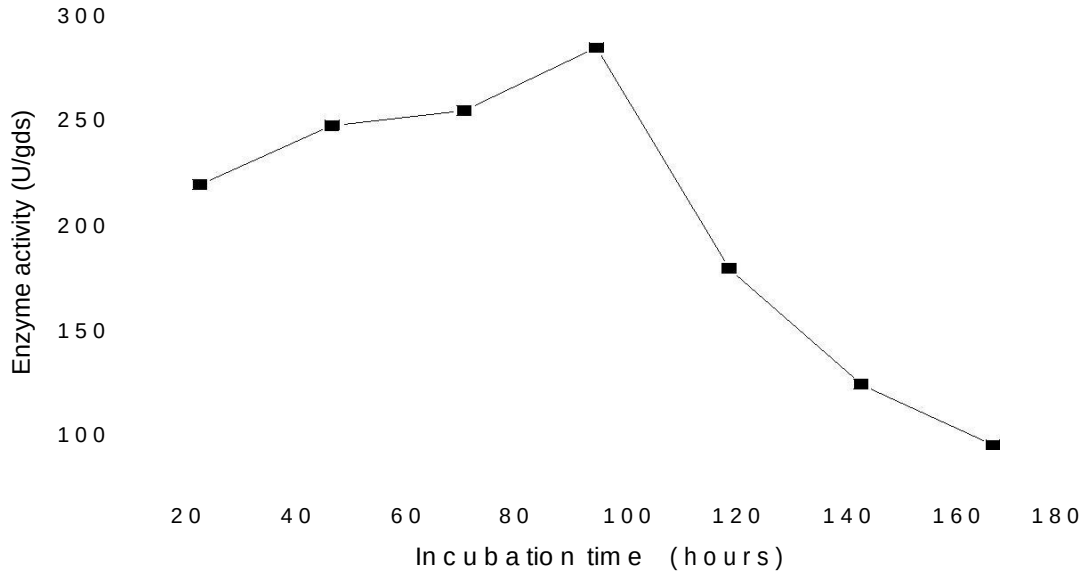

Figure 3. Effect of incubation time on α-amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

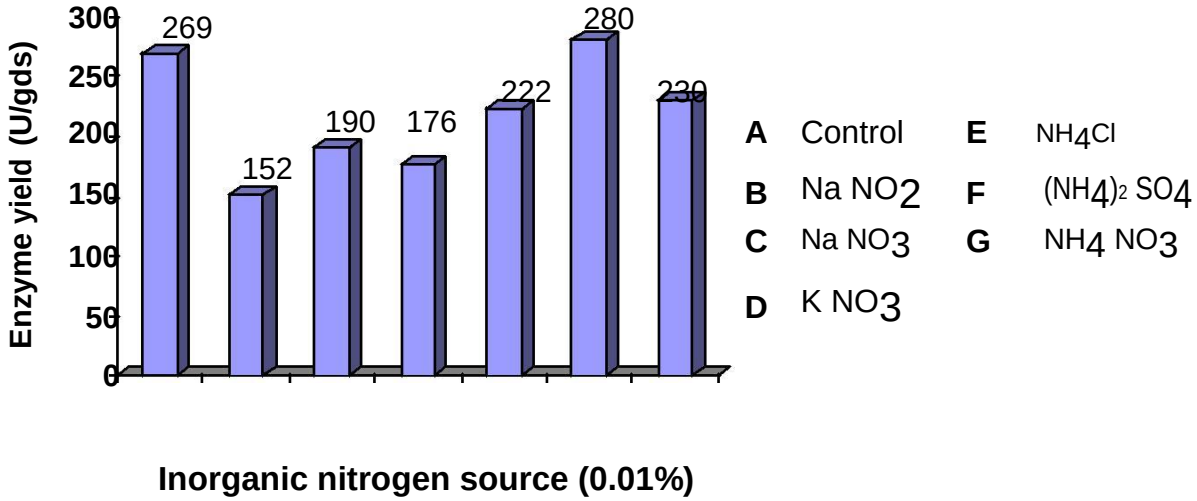

Figure 4. Effect of nitrogen source on α-amylase production *Penicillium janthinellum* (NCIM 4960) on SSF

enhanced maximal amylase production (280 U/gds). NH_4NO_3, NH_4Cl, $NaNO_3$, KNO_3 and $NaNO_2$ showed inhibitory effect on enzyme production. The inhibitory effects of some of the salts may be related to the pH changes associated with their use in the medium. Michelena and Castillo (1984) reported that the supplementation of nitrogen salts greatly increased the enzyme yields in *Aspergillus foetidus.*

Effect of moisture content

The results presented in Figure 5 indicate that an initial moisture content of 60% was optimum for maximum enzyme yield with wheat bran. Increase in moisture content resulted in clumping of the solid particles and consequent reduction in enzyme yield. Microbiological activity on a substrate will progressively decrease at lower water contents. Optimum yield was observed as 295 U/gds at 60% (w/w) moisture content which decreased to 220 U/gds at 80% (w/w) moisture content. Moisture causes swelling of substrate facilitating better utilization of the substrate. Increase in moisture content leads to reduction in product yield, during SSF, is due to reduction in interparticle spaces, decreased substrate degradation and impaired oxygen transfer (Ramesh and Lonsane, 1990 and Sandhya and Lonsane, 1994).

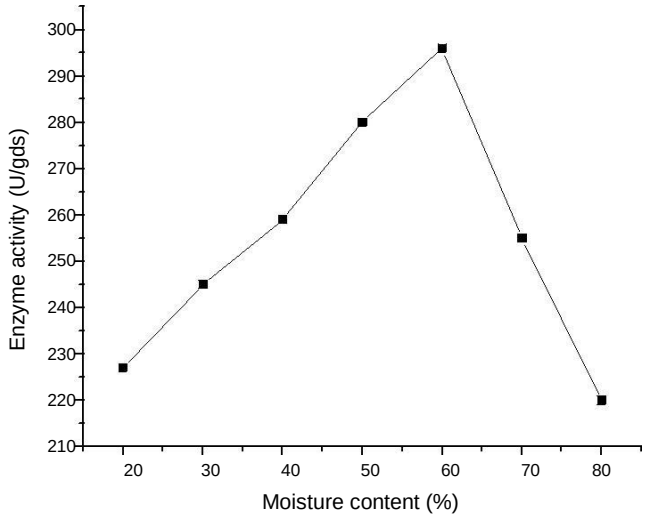

Figure 5. Effect of moisture content on α-amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

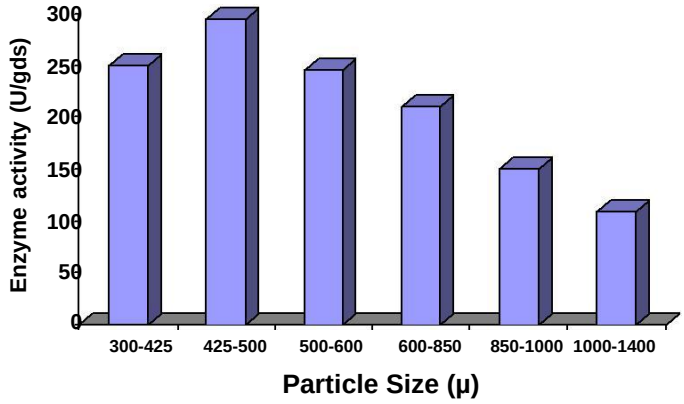

Figure 6. Effect of particle size on α-amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

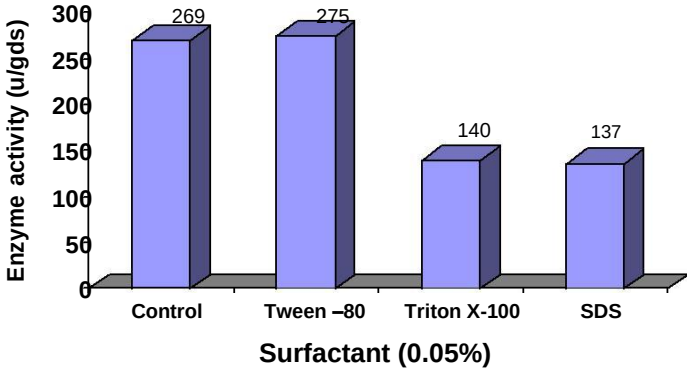

Figure 7. Effect of various surfactants on α-amylase production by *Penicillium janthinellum* (NCIM 4960) on SSF.

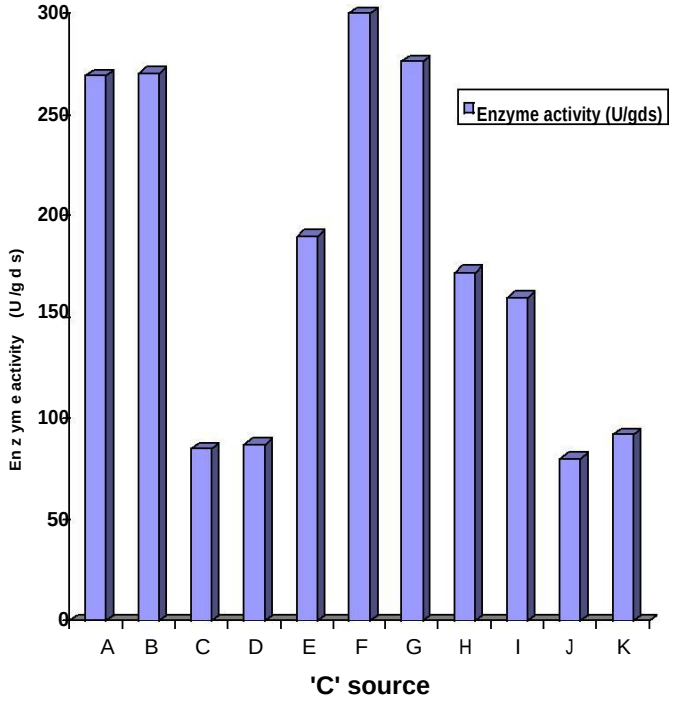

Figure 8. Effect of additional carbon source on α-amylase production by *Penicillium janthinellum* (NCIM 4960).

A; Control, B; Dextrin white, C; Sucrose, D; Glucose, E; Arabinose F; Maltose, G; Starch H; Galactose I; Xylose J; Frustose and K; Lactose.

Effect of particle size

The results presented in Figure 6 suggest that particle size of 450 - 500 µ favored maximal enzyme production. However, further reduction in size, there is a decrease in the enzyme yield and activity. Lowest enzyme activity is attained with the substrates containing particles bigger than 850 µ. Particle size of the substrate is a critical factor for enzyme production by SSF. For smaller particles the surface area for growth is more, interparticle space is less while for larger particles the surface area for growth is less and the interparticle space is more.

Effect of surfactants

The results presented in Figure 7 indicate that among the various surfactants tested Tween- 80 plays an important role in amylase production. Triton X- 100 and sodium do decyl sulphate inhibited growth which in turn affected enzyme synthesis.

Effect of additional carbon source

The results presented in Figure 8 indicate that maltose enhanced amylase production (300 U/gds) when compared to other carbon sources. Identical observations were earlier reported by Lachmund et al., 1993 and Morkeberg et al., 1995. α-Amylase is an inducible enzyme and is generally induced in the presence of starch

or its hydrolytic product, maltose. Starch and dextrin were comparable. Fructose, sucrose and glucose inhibited growth and amylase production. Identical observations were earlier recorded in *A. oryzae* (Yabuki et al., 1997). α-Amylases production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes (Morkeberg et al., 1995; Bhella and Altosaar, 2004).

Conclusion

Penicillium janthinellum (NCIM 4960) was observed to hyper produce α-amylase under SSF in the present study indicating 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 maximal 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.

In the light of modern biotechnology, α-amylases are now gaining importance in biopharmaceutical applications. Still, their application in food and starch based industries is the major market and thus the demand of α-amylases would always be high in these sectors.

REFERENCES

Agrawal D, Patidar P, Banerjee T, Patil S (2005). Alkaline protease production by a soil isolate of *Beauveria feline* under SSF condition: parameter optimization and application to soy protein hydrolysis. Proc. Biochem. 40: 1131–1136.

Baysal Z, Uyar F, Aytekin C (2003). Solid-state fermentation for production of -amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Process Biochem. 38: 1665–1668.

Bernfield P (1955). Amylases, - and In: Methods in Enzymology, Academic Press, New York, USA. 1: 149–158.

Bhella RS, Altosaar I (2004). Role of CAMP in the mediation of glucose catabolite repression of glucoamylase synthesis in *Aspergillus awamori* . Curr. Genet. 14(3): 247-252.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergilus* species, Process Biochem. 38: 615–620.

Krishna C, Chandrasekaran M (1996). Banana waste as substrate for - amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation. Appl. Microbiol. Biotechnol. 46(2): 106-111.

Lachmund A, Urmann U, Minol K, Wirsel S, Ruttkowski E (1993). Regulation of -Amylase formation in *Aspergillus oryzae* and *Aspergillus nidulans* transformants. Curr. Microbiol. 26: 47-51.

Michelena VV, Castillo FJ (1984).Production of amylase from *Aspergillus foetidus* on rice flour medium and characterization of the enzyme. J. Appl. Bacteriol. 56: 395-407.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426- 428.

Morkeberg R, Carlsen M, Nielsen J (1995). Induction and repression of -amylase production in batch and continuous cultures of *Aspergillus oryzae*. Microbiol. 141: 2449-2454.

Pandey A, Soccol CR, Nigam P, Brand D, Mohan R, Roussos S, (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochem. Eng. J. 6: 153–162.

Ramesh MV, Lonsane BK (1989). Solid state fermentation for production of higher titres of thermostable alpha-amylase with two peaks for pH optima by *Bacillus licheniformis* M27. Biotechnol. Lett. 1: 49–62.

Ramesh MV, Lonsane BK (1990).Critical importance of moisture content of the medium in alpha amylase production by *Bacillus licheniformis* M 27 in a solid state fermentation system. Appl. Microbiol. Biotechnol. 33: 501-505.

Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, Pandey A (2004). Coconut oil cake: A potential raw material for the production of -amylase. Biores. Technol. 93: 169–174.

Sandhya X, Lonsane BK (1994). Factors influencing fungal degradation of total soluble carbohydrates in sugar cane pressmud under solid state fermentation. Proc. Biochem. 29: 295-301.

Soni SK, Bath KS, Soni R (1996). Production of amylase by *Saccharomycopsis capsularis* in solid state fermentation. I. J. Microbiol. 36: 157-159.

Spohr A, Carlsen M, Nielsen J, Villadsen J (1998). -Amylase produc-tion in recombinant *Aspergillus oryzae* during Fed-batch and continuous cultivation. J. Ferment. Bioeng. 86(1): 49–56.

Sudo S, Ishikawa T, Sato K, Oba T (1994). Comparison of acid stable -amylase production by *Aspergillus kawachii* in solid-state and submerged cultures, J. Ferment. Bioeng. 77(5): 483–489.

Sun X, Liu Z, Qu Y, Li X (2007).The Effects of Wheat Bran Composition on the Production of Biomass-Hydrolyzing Enzymes by *Penicillium decumbens*. Appl. Biochem. Biotech. 146(1-3): 119-128.

Uyar F, Baysal Z (2004).Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Proc. Biochem. 39: 1893–1898.

Yabuki M, Ono N, Hoshino K, Fukui S (1997). Rapid induction of alpha-amylase by non-growing mycelia of *Aspergillus oryzae*. Appl. Environ. Microbiol. 34(1): 1-6