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Full Length Research Paper

Physical and nutritional factors affecting the production of amylase from species of bacillus isolated from spoiled food waste

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Amylase producing *Bacillus* sp. was isolated from spoiled food waste, which yielded 30 U ml^{-1} of amylase in medium containing 4% starch and 2% yeast extract at 37°C, pH 7.0 after 20 h of incubation. Maximum amylase activity was at pH 7.0 and 37°C. The enzyme retained 70% activity at pH 9.0. It exhibited 77% activity at 42°C and decreased to a low value of 25% at 52°C proving it as sensitive for temperature. In addition, the identified enzyme showed a maximum activity (32 U/ml) with sodium dodecyl sulphate compared to other additives. Wheat was found to be a suitable natural source for maximum production of amylase activity. These properties indicated possible use of this amylase in starch saccharification and detergent formulation.

Key words: *Bacillus*, amylase, physical factors, Spoiled Food waste.

INTRODUCTION

Amylases constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Mamo et.al., 1999; Oudjeriouat et al., 2003; Pandey et al., 2000). Bacteria and fungi secrete amylases to the outside of the cells to carry out extra cellular digestion. When they have broken down the soluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cells. Ellaiah et al. (2003) identified amylolytic activity from several fungal species isolated from Indian soils and *Aspergillus* sp. was found to possess the highest amylase activity (73 Uml^{-1}). Ugru et al. (1997) produced extracellular amylase using yam peel as carbon source in shake flask cultures of a hemophilic strain of *Aspergillus niger.* The optimum temperature and pH of the enzyme was found to be 70ºC and 5.5, respectively, with more than 50% activity retained at 80ºC. Hamilton et al. (1999), produced a raw starch-digesting but non-raw starch-adsorbing α-amylase from *Bacillus* sp. Maximum amylase yield was achieved in a medium containing lactose (4%, w/v) as the carbon source and yeast extract (2%, w/v) as the nitrogen source. Zheng et al. (2000) produced α-amylase by fermentation employing *Bacillus subtilis* in an external-loop airlift bioreactor, with a low ratio of height-to-diameter of the riser. The effects of gas flow rate and liquid volume on α-amylase production were investigated. After a 36-h fermentation time, an average of 432.3 U/ml α-amylase activity was obtained under the conditions of liquid and gas flow for the first 12 h of fermentation. The activity was higher than that obtained in shaking flasks and in a mechanically stirred tank bioreactor under optimized operating conditions. Coronado et al. (2000) achieved highest amylase production by growing a moderate halophile *Halomonas meridiana* culture in a growth media comprising 5% NaCl and starch. The amylase exhibited maximal activity at pH 7.0 and found relatively stable in alkaline conditions. Ramesh et al. (2001) studied the effects of various flours on production of thermos table β-amylase and pullulanase using *Clostridium thermosulfurogenes* on submerged fermentation. Among the flours added to PYE basal medium, potato flour was found as

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best substrate for enzyme production under optimal conditions**.** Huang et al. (2003) proposed a segregated model to investigate the inherent relationships between substrate consumption, cell differentiation and product formation for *B. subtilis* strain producing amylase in 22-1 fermentor. The model was able to predict the transient nature of *B. subtilis* in both batch and fed-batch operations. Calderon et al. (2003) investigated fermentation kinetics, growth energetics and α-amylase production by *Lactobacillus fermentum* cultivated in a mixed carbohydrate media in a fermentor. In the present investigation, a *Bacillus sp.* isolated and identified from spoiled food waste was optimized for maximum amylase production.

MATERIALS AND METHODS

Isolation and identification

The contaminated spoiled food waste was collected from a hotel dumping was serially diluted up to 10^{-7} dilution using sterile saline as a blank and the diluted samples were plated into the sterile nutrient agar plates using spread plate technique. The plates were incubated at 37°C° for 24 h. The isolated colonies were further purified by streak plate method using sterile nutrient agar medium. The isolated pure strains were screened for the production of extracellular amylase production using screening medium (starch agar). The pure cultures were streaked at the center of the Sterile Starch agar plates and the plates were incubated at 37°C for 24 h. After incubation, 1% iodine solution was over layered on the agar plates and the observation was made to note the substrate utilized zone around the colony (Figure 1). Only the positive and the better zone formed strain was taken for the further study. Based on the key provided by the Bergey's manual of Systematic bacteriology (Garrity et al., 2001), the isolated strain was identified as *Bacillus* sp.

Inoculum preparation

Media developed by Hamilton et al. (1999) with following composition (in g l^{-1}): Starch 10, Peptone 10, Yeast extract 20, KH_2PO_4 0.05**,** $MnCl_2.4H_2O$ 0.015, $MgSO_4.7H_2O$ 0.25, $CaCl_2.2H_2O$ 0.05 and $FeSO_4.7H_2O$ 0.01 served as inoculation media for all experiments. The pure culture was inoculated into sterile inoculation medium and was incubated at 37°C in a rotary shaker for over night. The fresh over night culture was used as an inoculum for the growth study and the production of enzyme.

Growth studies

250 ml of sterile production broth (Lactose 40 gl^{-1}, Yeast extract 20 gl^{-1}, KH_2PO_4 0.05 gl^{-1}**,** $MnCl_2.4H_2O$ 0.015 gl^{-1}, $MgSO_4.7H_2O$ 0.25 gl^{-1}, $CaCl_2.2H_2O$ 0.05 gl^{-1}, $FeSO_4.7H_2O$ 0.01 gl^{-1}) was prepared and 2% of inoculum was aseptically added in to the medium and incubated at 37°C in shaking incubator. Culture growth was monitored by UV-VIS spectrophotometer at 650 nm.

Enzyme production

Amylase production was carried out in shake flask fermentation using production media (see 'growth studies') comprising of lactose as a carbon source. 500 ml of sterile production broth (pH 7) was prepared in 1 l conical flask and 2% inoculum was transferred aseptically into the production medium and incubated at 37°C for 48 h in an orbitary shaker at 200 rpm. Samples were withdrawn at regular time intervals and analyzed for amylase activity.

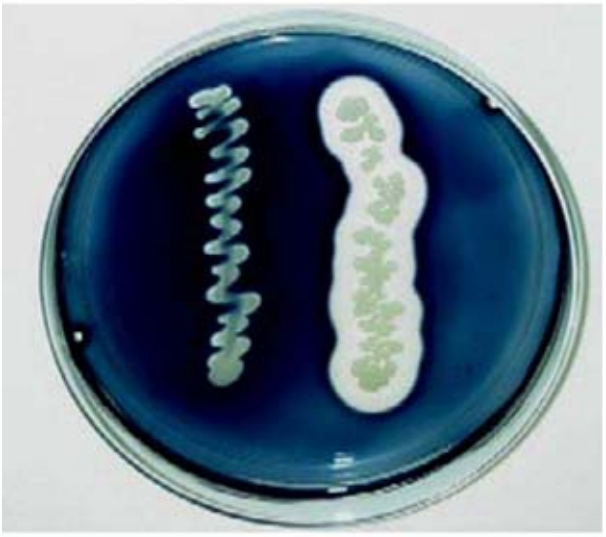

Figure 1. Screening of the isolate for amylase production in starch agar plate.

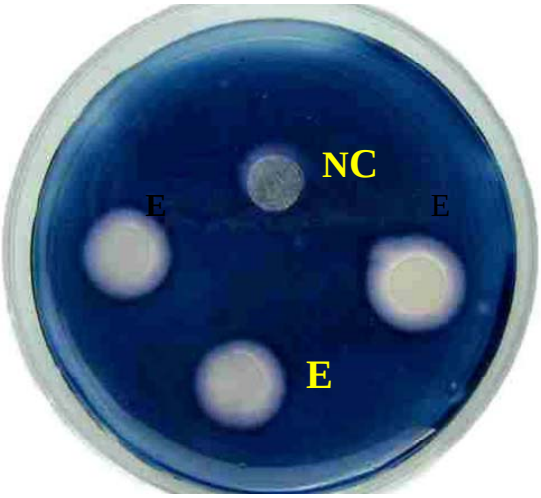

Figure 2. Plate assay for the amylase activity. E = Enzyme and NC = negative control.

Enzyme assay

Plate assay: The plate assay was performed using agar plates amended with starch. The agar plates were prepared amended 2% of starch with 1.5% of agar. After agar solidification, around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37°C for overnight. 1% of iodine solution was over layered on the agar and the observation was made to see the hydrolytic zone around the well (shown in Figure 2). The negative control was maintained by adding sterile water in the separate well.

Chemical assay

Amylase activity was determined by DNS (3,5-dinitro salicylic acid) method using starch as a substrate. Crude culture filtrate was used as enzyme sample. About 0.5 ml of culture filtrate was boiled in a water bath (100ºC) for 20 min and then cooled suddenly in an ice bath for 5 min in order to kill the enzyme. Both killed and active samples were taken for the assay. 1% starch substrate was prepared freshly in 0.1 M phosphate buffer (pH.6.0). The reaction mixture containing 500 µl of substrate (starch) and 500 µl of enzyme solution was incubated at 37°C for 15 min for enzymatic reaction. After incubation, 1 ml of DNS (1%) was added and heated for 15 min in a boiling water to obtain a colored reacted mixture. Absorbency of the solution was measured at 550 nm using UV–VIS spectrophotometer (Beckman DU 40). Killed enzyme mixture served as a blank. One unit of amylase enzyme activity was defined as the amount of starch hydrolyzed during 15 min incubation at 37°C for 1 ml of solution of extract.

Enzyme purification

Amylase produced was partially purified by precipitation with ammonium sulphate and followed by dialysis. Ammonium sulphate precipitation technique was performed by mixing culture filtrate and ammonium sulphate (75%, w/v) solution at 1:3 ratio (Ganesh et al., 2004). The mixture was then stored in cold room for 24 h to precipitate all the proteins present in the sample. Precipitate was removed by centrifuging sample in an ultra centrifuge at 10000 rpm for 10 min. The supernatant was discarded and precipitate obtained was dissolved in 5 ml of 1 M-citrate phosphate buffer (pH.5) (Stamford et al., 2004). Then the mixture was subjected to dialysis.

Dialysis

The pre treatment of the dialysis membrane (Sigma) was done by immersing the membrane into the warm 1 M-citrate phosphate buffer (pH 5) for 10 min. The precipitated protein was then transferred in the dialysis tube and suspended in a beaker containing 500 ml of 1 M-citrate phosphate buffer (pH.5). The buffer was stirred slowly using magnetic stirrer and the entire setup was placed in the cold room for 48 h. Every 12 h buffer was changed periodically to ensure better dialysis. After dialysis, the sample was lyophilized in a lyophiliser (Virtis) and finally stored at 4°C.

Protein content

Total protein content of the sample was determined using Bradford method (Bradford, 1976). 100 mg of Coomassie brilliant blue dye G-250 was dissolved in 50 ml of 95% ethanol. 100 ml of 85% (w/v) phosphoric acid was added and the mixture was made up to 1l. The dye was filtered through Whatman No.1 filter paper and stored in dark bottles refrigeration. 1 ml of culture filtrate was taken and 5 ml of Bradford reagent was added. The contents were mixed well and the absorbency was taken at 595 nm in UV-VIS spectrometer. The blank was prepared by mixing 1 ml of distilled water with 5 ml of reagent. The protein concentration was determined by comparing the value with standard graph prepared using bovine serum albumin.

Effect of temperature on amylase production

The effect of temperature was evaluated by incubating the reaction mixtures (100 ml of sterile production medium was prepared and inoculated with 2% pure culture) at different temperatures (28, 32, 37, 42, 47, and 52ºC) for 48 h. Samples were taken at regular time intervals and analyzed for protein content and amylase activity.

Effect of pH on amylase production

pH in the range of 4.0–9.0 were examined for their effect on amylase production by the selected isolate grown in production media. The pH of the medium was adjusted using 1 N HCl or 1 N NaOH. The flasks were incubated at 37ºC for 48 h. Samples were taken at regular time intervals for protein estimation and amylase activity.

Effect of carbon sources on amylase production

Various carbon sources such as monosaccharides (xylose, galactose and fructose) and disaccharides (lactose, sucrose and maltose) evaluated for their effect on amylase production by replacing starch in the production medium. The flasks were inoculated with 2% inoculum and incubated at 37ºC for 48 h. The optimum carbon source was found by analyzing the results of amylase production.

Effect of additives on amylase production

Amylase activity was measured by incubating production media seeded with 2% inoculum (pH 7.0) at 37ºC for 48 h for different additives. 0.5% of different filter sterilized additives viz. SDS, Triton-X 100 and Tween 20 were added separately and enzyme activity was estimated for all the samples.

Effect of various flours for amylase production

Various natural products like corn, molasses, barley, and wheat were used as substrates for effective enzyme production. Molasses collected from sugar industry and other substrates were prepared by boiling and crushing of respective seeds. The substrates were sterilized and seeded with 2% inoculum. They were incubated at 37ºC for 48 h. The amount of total protein and enzyme produced from different substrates were estimated.

RESULTS AND DISCUSSION

On the basis of level of productivity of the amylase, an isolate producing a maximum of amylase activity was screened from spoiled food waste and used for detailed investigation (data not shown). This isolate was gram positive, rod shaped aerobic, catalase positive and spore forming. On the basis of various morphological and biochemical characteristics, it was identified as *Bacillus* sp. following the criteria laid down in Bergey's Manual of Systematic Bacteriology. Growth study showed that up to 4 h duration the culture was in the lag phase and log phase was observed for 12 h duration (Figure 3). The isolate has shown very short lag phase and moderate log phase which is in agreement with literature values. Amylase production usually initiated during the log phase of the growth and reaches maximum levels during the initial stationary phase. Even though the extra cellular

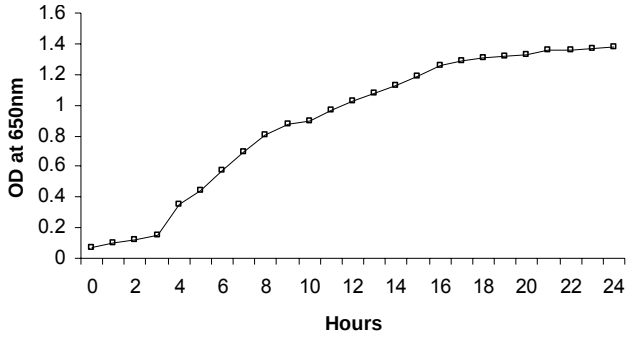

Figure 3. Time course of growth of *Bacillus* sp. in production medium comprising of lactose and yeast extract.

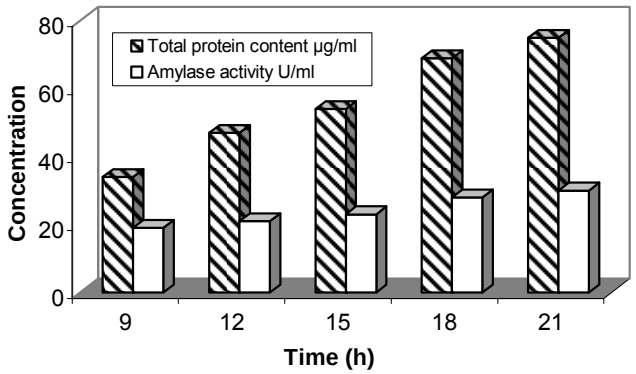

Figure 4. Amylase activity and its total protein content produced by *Bacillus sp.* grown in production media.

enzymes are produced from log phase to initial stationary phase, within the phase the production may be vary. During growth study, samples were taken at regular time intervals for estimation of amylase activity and total protein content. The results revealed that the higher enzyme production was occurred at 21st h (30 U/ml, Figure 4).

Effect of temperature

The environmental parameters are showing great influence in the growth of the organisms and the production of enzymes. The main parameters like temperature, pH are very essential parameters of the production. *Bacillus* sp. was capable of producing amylase in the range of 28–52°C with maximum production at 37°C (22 Uml^{-1}). However, increase in temperature beyond 37°C led to a decline in production of the enzyme (Figure 5). Nearly four fold decrease in amylase yield was observed at 52°C, proving that temperature plays a major role in amylase production from this *Bacillus* strain. The optimal temperatures for amylase production and growth of the organism were found to be same as the organism has growth optima at 37°C and production optima at 37°C. This greatly favors that fermentation can be processed at a constant temperature to increase both biomass and enzyme yield.

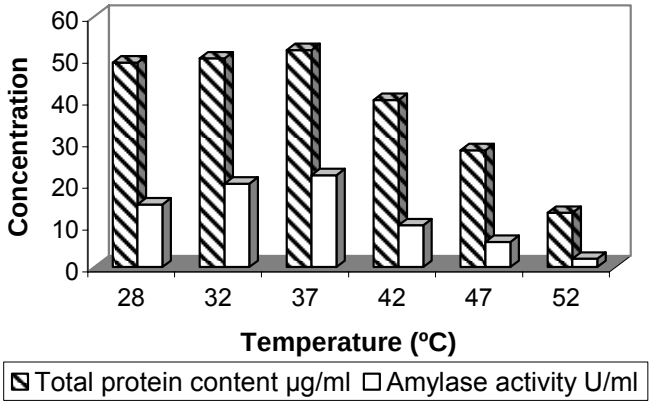

Figure 5. Effect of temperature on amylase activity of *Bacillus* sp. grown in production media.

Effect of pH

Maximum amylase production (24 Uml^{-1}) was achieved at neutral pH 7 by *Bacillus* sp. although pH 4.0–9.0 supported amylase production (Figure 6). The organism did not grow in production medium adjusted to pH 4.0. Results suggest that there is a stimulation of enzyme at neutral pH. Even though max.enzyme activity was observed at neutral pH but considerable amount of activity (15-19 Uml^{-1}) was obtained at alkaline pH showing the wide application nature of identified enzyme.

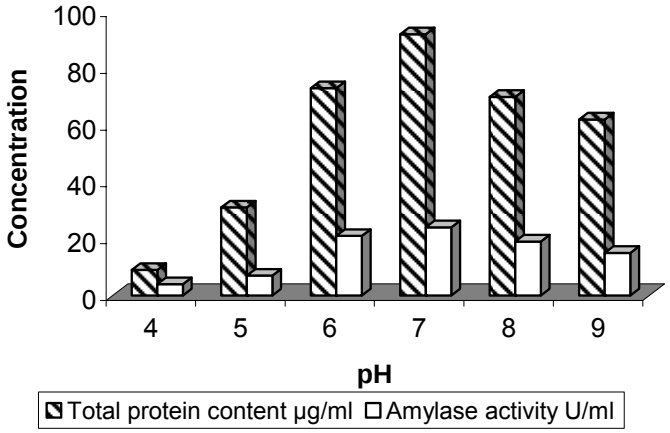

Figure 6. Effect of pH on amylase activity of *Bacillus* sp. grown in production media.

Effect of carbon sources

The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzymes. The isolated strain showed high enzyme yield (31 Uml^{-1}) in lactose-based production media (Figure 7). Glucose and fructose showed a repres-

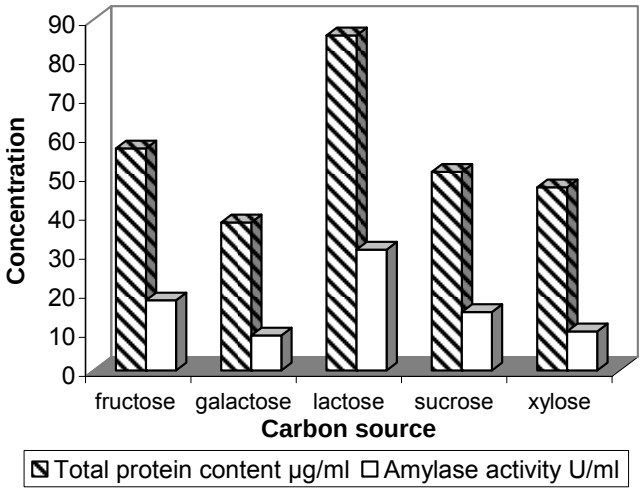

Figure 7. Effect of carbon sources on amylase activity of *Bacillus* sp. grown in production media.

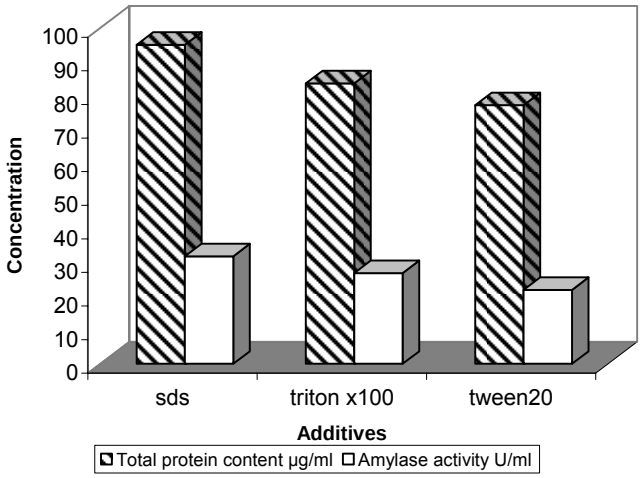

Figure 8. Effect of additives on amylase activity of *Bacillus* sp. grown in production media.

sion effect on amylase production. Similarly, it has been reported by Teodoro and Martins (2000) that synthesis of carbohydrate degrading enzyme in most species of genus *Bacillus* leads to catabolic repression by readily metabolizable substrates such as glucose and fructose. Haseltine et al. (1996) observed that glucose represses the production of amylase in the hyperthermophilic archeon *Sulfolobus solfataricus*. Our results also support these findings.

Effect of additives

The addition of additives some times either increase or decrease in enzyme production. Figure 8 shows clearly that the addition of detergent sodium dodecyl sulfonate (SDS) causes higher production of amylase (32 Uml^{-1}).

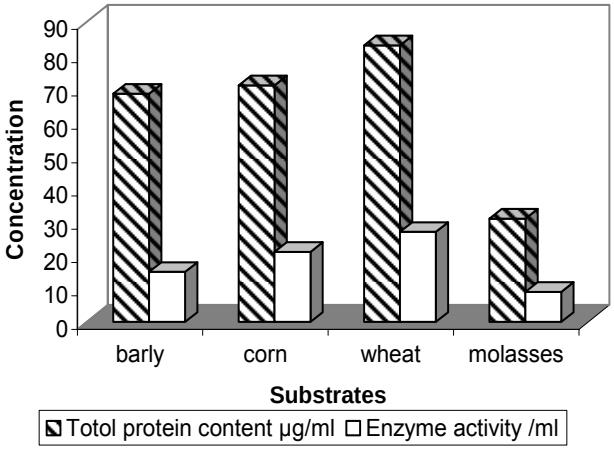

Figure 9. Effect of natural substrates on amylase activity of *Bacillus* sp. grown in production media.

The other additives like Triton X100 and Tween 20 were found to decrease the secretion of amylase. Therefore SDS can be used as a suitable additive for biochemical analysis of identified amylase.

Production from the crude substrates

Natural sources could serve as economical and readily available raw material for production of valuable enzymes. In this study, several natural and industrial wastes were used as substrates (corn, molasses, barley, and wheat). The results from Figure 9 revealed that the maximum production was observed in wheat (27 Uml^{-1}). The reason may be its higher starch content and also the strain was basically isolated from the contaminated starch food. Not only the wheat but also the crude medium prepared using corn also shown high amylase yield (21 Uml^{-1}).

Conclusion

Extra cellular amylase was extracted from *Bacillus* sp. isolated from spoiled food waste. The various factors affecting amylase production was assayed which include pH, different substrate, temperature, natural source and additives. Results showed that pH 7.0 and 37ºC are found to be optimum values for both the growth of the isolate and max. enzyme production. In addition, lactose was found to be a best carbon source and wheat as a suitable natural source for higher amylase production. Further experiments will be done to purify the secreted amylase and stability studies will be performed to enhance the application of enzyme to commercial level.

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REFERENCES

Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72:248-54.

Calderon M, Loiseau G, Guyot JP (2003). Fermentation by Lactobacillus Fermentum Ogi E1 of different Combinations of carbohydrates occurring naturally in cereals: consequence on growth energetic and α-amylase Production. Int. J. Food Microbiol. 80:161-169.

Coronado MJ, Vargas C, Hofemeister J, Ventosa A, Nieto J (2000). Production and Biochemical Characterization of an α-amylase from the moderate halophile Halomonas meridiana. FEMS Microbiol. Lett. 183: 67-71.

Garrity GM, Brenner DJ, Krieg NR, Staley JT (2001). Bergey's Manual of Systematic Bacteriology, second edition, Springer, Newyork.

Ganesh C, Seung Joo H, Koo YM, Paik SR, Chang C (2004). Thermostable Alkaline Protease from a Novel Marine Haloalkalophilic *Bacillus Clausii* Isolate, World J. Microbiol. Biotechnol. 20:351-357

Hamilton M, Catherine K, William Fogarty M (1999). Production and Properties of the raw starch-digesting α-amylase of Bacillus sp. IMD435. Proc. Biochem. 35:27-31.

Haseltine C, Rolfsmeier M, Blum P (1996). The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J. Bacteriol. 178:945-950

Huang H, Ridgwan D, Tingyue GU, MurrayMoo Y (2003). A Segregated modal for hetrologus amylase production by Bacillus subtilis. Enz. Microbial technol. 32:407-413.

Mamo G, Gashe BA, Gessesse A (1999). A highly thermostable amylase from a newly isolated thermophilic *Bacillus* sp. WN11. J. Appl. Microbiol. 86:557–560.

Oudjeriouat N, Moreau Y, Santimone M, Svensson B, Marchis-Mouren G, Desseaux V (2003). On the mechanism of _-amylase: Acarbose and cyclodextrin inhibition of barley amylase isozymes. Eur. J. Biochem. FEBS 270:3871–3879.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem. 31:135–152.

Ramesh B, Reddy PRM, Seenayya G, Reddy G (2001). Effect of various flours on the production of thermos table β-amylase and pullulanase by clostridium thermo sulfurogenes SV2. Biores. Technol. 76: 169-171.

Teodoro S, Martins L (2000). Culture conditions for the production of thermostable amylase by *Bacillus* sp. Brazilian J. Microbiol. 31:298-302.

Uguru GC, Akinayanju JA, Sani A (1997). The use of yam peel for growth of locally isolated *Aspergillus niger* and amylase production. Enz. Microbial Technol. 21:48-51.

Zheng Y, Wang Z, Xiaolong C (2000). α-Amylase productions by *Bacillus subtilis* with drugs in external loop airlift bioreactor. J. Biochem. Engr. 5:115-121.