



**PRODUCTION AND CHARACTERIZATION OF AMYLASE FROM *RHIZOPUS ORYZAE*
USING AGRICULTURAL WASTES AS SUBSTRATE**

Shaheen Sulthana¹, H. Haseena Banu² and S. Suganthi^{1*}

¹Dept. of Biochemistry, Bhaktavatsalam Memorial College for Women, Korattur, Chennai-80.

²Dept. of Pathology, Dr ALM P-G IBMS, University of Madras, Taramani Campus, Chennai - 113.

*Corresponding Author: Dr. S. Suganthi

Asst. Professor, Dept. of Biochemistry, Bhaktavatsalam College.

Article Received on 10/05/2018

Article Revised on 30/05/2018

Article Accepted on 20/06/2018

ABSTRACT

Fungal α -amylase, an enzyme which holds tremendous industrial and pharmaceutical applications is screened in the present study using the zero value material for submerged fermentation using the sugarcane baggage, banana peel and tapioca waste. Amylase was screened using starch agar plate assay and optimization of amylase production was done in fermentation media using different pH, carbon source, nitrogen source and substrate as three different agriculture wastes. The banana peel showed a maximum activity (231U/ml) of amylase. The maximum activity was observed at pH 6.0 (153U/ml), Maltose served as carbon source (174U/ml) while Yeast extract served as nitrogen source (187U/ml). The enzyme was stable at 40°C and the pH was observed to be 7.0. Hence, the present study demonstrates that alpha amylases can be produced from *Rhizopus oryzae* using agricultural wastes and the amylase enzymes is thermostatically very stable.

KEYWORDS: *Rhizopus oryzae*, Fungal α -amylase, Agricultural wastes.

1. INTRODUCTION

Amylases are the enzymes which catalyze the hydrolysis of starch into sugars and have been most commonly used in industry. α -Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. The production of α -amylase is essential for conversion of starch molecules into oligosaccharides. Starch-converting enzymes are used in the production of malto dextrin, modified starches, or glucose and fructose syrups. A large number of microbial α -amylases have applications in different industrial sectors such as food, textile, paper and detergent industries.^[1]

α -Amylases (E.C.3.2.1.1) are enzymes that produce low molecular weight products, such as glucose, maltose and maltotriose units by catalyzing the hydrolysis of internal α -1,4-glycosidic linkages in starch.^[2] The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Industrial sectors have made maximum utilization of enzymes from fungal and bacterial sources.^[2] Submerged fermentation (SmF) has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture.^[3,4] In industry

based fermentation process both fermentation medium and fermentation process condition are known to play an important role in the optimization of the fermentation processes due to their impact on the economy and practicability of the process.^[5]

The present study defines the effects of culture conditions on amylase production in batch experiments in shake flasks and under controlled conditions in a laboratory incubator. The study also explores the role of agro-industrial wastes in the enzyme synthesis.

2. MATERIALS AND METHODS

2.1. Fungal culture collection and maintenance

The fungal culture *Rhizopus oryzae* was procured from CAS in Botany, University of Madras, Chennai 600 025. The fungal culture was maintained in Potato Dextrose Agar Medium (PDA) and also these organisms were maintained at 4°C in slants as a mother culture.

2.2. Media Preparation - Potato Dextrose Agar Media

200g of peeled and sliced potatoes were boiled for one hour in one liter of water. It was filtered and made up the volume to one liter and then 3g of glucose (dextrose) and agar (15g) were added and heated until agar gets dissolved and then the media was sterilized using an autoclave. This media composition was used for the culture *Rhizopus oryzae*. The pH of the medium was maintained at 6.5.

2.3. Amylase screening of *Rhizopus oryzae* by agar plate assay

Rhizopus oryzae culture was grown on PDA and was cut into 5 mm discs with the help of borer. Three discs were placed on Petri plate containing autoclaved 15 mL of glucose, yeast extract and peptone agar (GYP) medium (glucose 1 g, yeast extract 0.1 g, peptone 0.5 g, agar 16 g and distilled water 1000 mL) with 0.2% soluble starch at pH 6.0.^[6] After incubation, the plates were flooded with 1% iodine solution in 2% Potassium iodide. Zone of clearance around the colony was measured.^[7]

2.4. Preparation of agro-industrial residues

Four agriculture by-products namely; Sugarcane baggase, Topica peel, banana peel and starch were washed and then dried at 50°C overnight in order to get constant weight. The dried substrates were grinded in a laboratory grinder and a particle size of 5 mm was selected by sieving to remove the large particles and used for further studies.^[8]

2.5. Submerged fermentation process

It was carried out in 250 ml plugged Erlenmeyer flasks, each containing 100 ml sterile starch broth medium and inoculated with 1% of standard inoculum (2.3·10⁶ CFU ml⁻¹) for the tested bacterial isolate which incubated at 50°C on rotary shaker at 150 rpm for 48 h. The fermented medium was centrifuged at 10,000 rpm for 10 min in order to determine periodically the cell dry weight and amylases activity in the precipitate and supernatant, respectively. All the experiments were carried out at least in triplicate.^[9]

2.6. Optimization of amylase production from *Rhizopus oryzae*

2.6.1. Effect of different pH

Liquid state fermentation was carried out using mineral salts solution with different pH ranging from 6.0 to 10.0 adjusted with 1 N NaOH or 1 N HCl, the flasks were incubated at 30 ± 2°C for 120 h and the enzyme production was measured every 24 h.

2.6.2. Effect of different carbon sources

Different carbon source namely glucose, sucrose, lactose and maltose were amended separately at the concentration of 4% at pH 6.0. Then the experimental flasks were inoculated with *Rhizopus oryzae* and incubation at 30±2°C for 120 h. The culture filtrate was collected by centrifugation at every 24 h interval and the supernatant was used for the estimation Glucose oxidase activity and protein content.

2.6.3. Effect of different Nitrogen sources

Different nitrogen sources namely ammonium sulphate, ammonium nitrate, sodium nitrate, yeast extract and peptone were amended at the concentration of 1% separately in the production medium. The *Rhizopus oryzae* was inoculated and incubated for 120 h. The culture filtrate was collected by centrifugation at every 24 h interval and the supernatant was used for the

estimation of Glucose oxidase activity and protein content.

2.7. Amylase activity

The culture broth was filtered using Whatman filter paper No.1, the filtrate was centrifuged at 3500 g for 10 minutes at 4°C and the supernatant was used for enzyme assay. Amylase activity was determined at room temperature in a reaction mixture containing 1mL of 1mol L⁻¹ sodium acetate buffer (pH 6.0), 0.5 mL 1% starch (w/v) and 0.5 mL of the crude enzyme extract. After 20 minutes of incubation, the liberated maltose was estimated by dinitrosalicylic acid (DNS) method.^[10] One unit of amylase activity (U) is defined as the amount of enzyme releasing one μmol of reducing sugar mL⁻¹ min⁻¹, with maltose as standard under the assay conditions mentioned above. The denatured culture filtrate served as control.

2.8. Purification of Amylase

2.8.1. Ammonium Sulphate Precipitation

The organism was grown for 48 hours as described previously. The cells were separated by centrifugation (10 000 rpm, 15 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4°C. Dialysis was performed with 14 KDa dialysis tubing. The protein was resuspended in 50 mM Phosphate buffer and dialyzed (Memracel MD 44-14X100CLR) against the same buffer.

2.9. Biochemical Characterization of Glucose oxidase

2.9.1. pH optimum and stability of partial purified Amylase

The pH optimum of the enzyme was measured at 37°C by the titrimetric method using olive oil as the substrate. The following buffer (0.1 M) was used: sodium acetate (pH 4.0-6.0), potassium phosphate (pH 7.0) and Tris-HCl (pH 8.0-10.0). To determine the pH stability of Glucose oxidase, the enzyme was pre incubated in different buffers for 6 h at 30°C.

2.9.2. Temperature optimum and stability of partial purified Amylase

The temperature optimum of the enzyme was evaluated by measuring the lipase activity at different temperatures (20–80°C) in 50 mM phosphate buffer pH 7.0 with CMC as substrate. The effect of temperature on glucose oxidase stability was determined by measuring the residual activity after 30 min of pre-incubation in 50mM phosphate buffer (pH 7.0) at various temperatures.

3. RESULTS

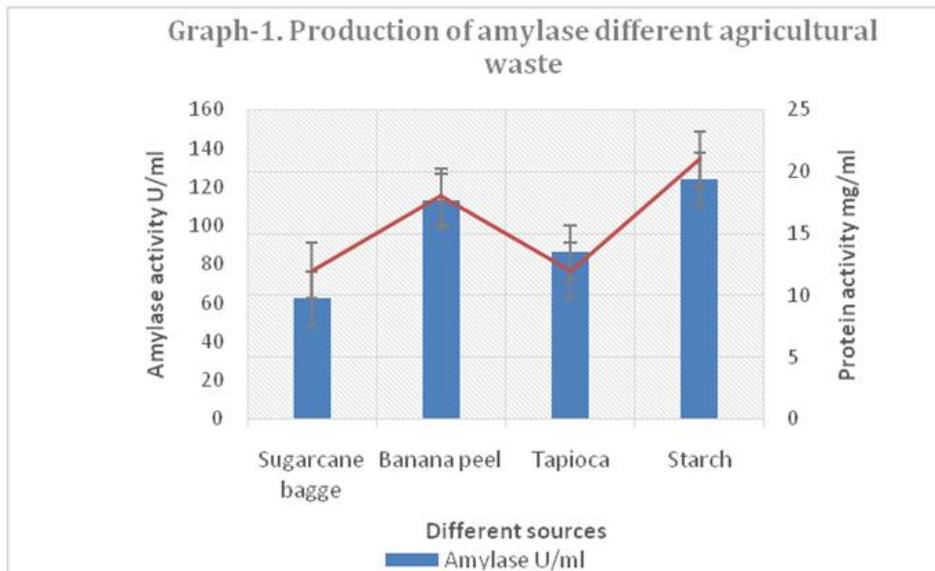
3.1. Amylase screening of *Rhizopus oryzae* by Agar plate assay

Rhizopus Oryzae screened for the amylolytic activity on solid media and the one which showed maximum zone of clearance, was selected for the optimization of amylase activity.

3.2. Production of amylase in different agricultural waste

Four substrates i.e. banana peel, tapioca peel, sugarcane waste and commercial starch were used in the present study as agricultural wastes. The highest enzyme activity

(124 U/ml/min and 113 U/ml/min) was observed from the extract obtained using starch and banana peel in Graph-1.

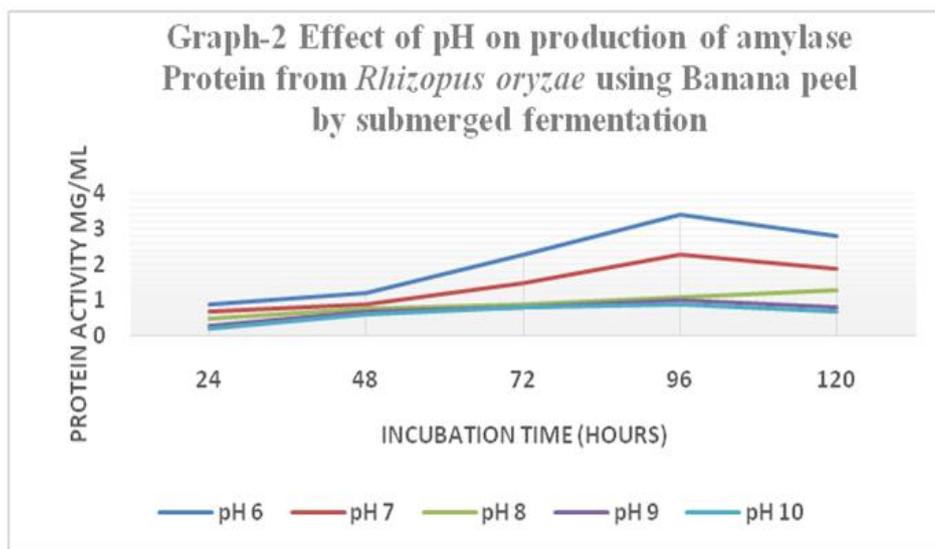


3.3. Optimization of amylase production by *Rhizopus oryzae* using banana peel

3.3.1. Effect of pH

It was evident that the pH significantly influenced the extracellular protein content and amylase activity in

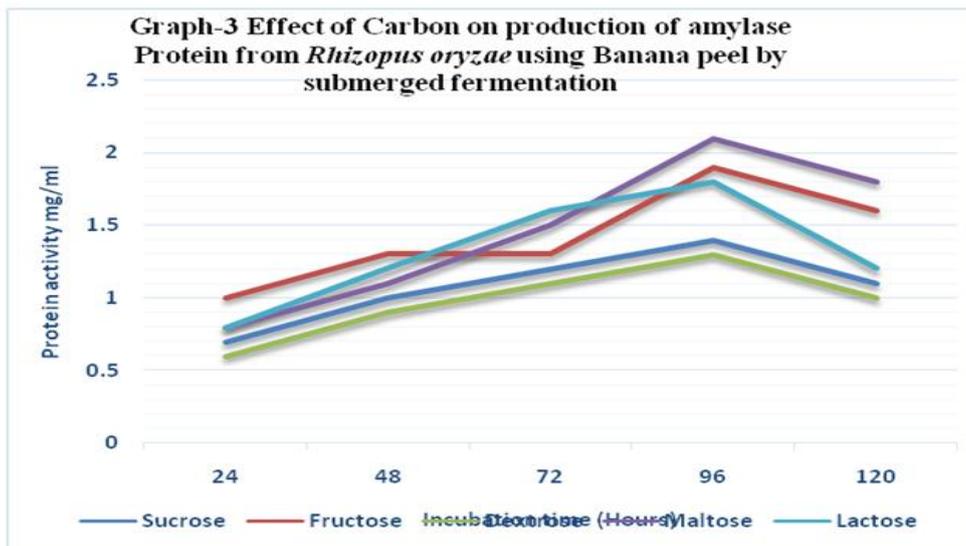
Rhizopus oryzae. The fungi were able to release a maximum protein content of 3.4mg/ml at pH 6.0 after 96 h and amylase of 68 U/ml at pH 6.0 after 96 h (Graph-2).



3.3.2. Effect of different carbon source

Among the different concentrations Maltose, Lactose, Glucose, Fructose and Sucrose tested, *Rhizopus oryzae* produced a maximum extra cellular protein content of

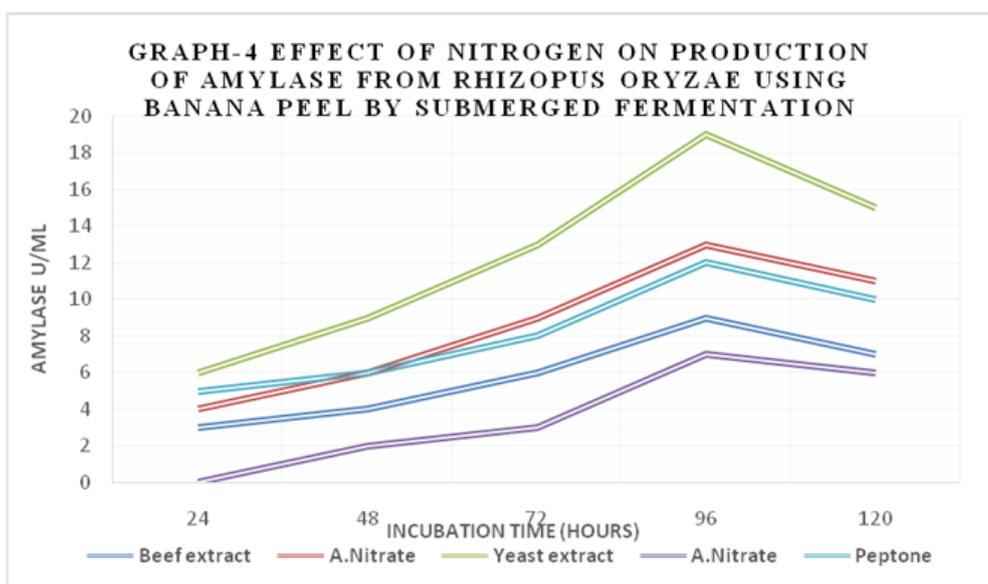
2.1 mg/ml in maltose at 96 h and maximum enzyme activity of 51 U/ml at 96 h compared to the rest of sugars (Graph-3).



3.3.3. Effect of different nitrogen sources

Different nitrogen source such as peptone, ammonium nitrate, ammonium nitrate, beef extract and yeast extract

was tested for extracellular protein and amylase production in *Rhizopus oryzae*.



Among them, yeast extract supported a maximum extracellular protein content of 2.4 mg/ml at 96 h and lipase production of 48 U/ml at 96 h (Graph-4).

3.4. Partial Purification of Amylase

Salting out and dialysis

The cell free culture filtrate of *Rhizopus oryzae* was collected after 96 h of incubation and its proteins were

precipitated by salting out with ammonium sulphate (70%). The crude protein precipitation was dialyzed, concentrated and used for further analysis. The amylase of *Rhizopus oryzae* was purified to homogeneity according to the procedure summarized in Table-1.

Table 1: Purification of amylase from *Rhizopus Oryzae*.

Purification step	Volume (ml)	Total Protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Purification fold	Yield %
Crude extract	100	2190	12300	5.7	1	100
70% (NH ₄) ₂ So ₄	80	1210	10730	9.1	1.63	87.8

3.4. Effect of different pH on partial purified amylase activity

The pH optimum of the partially purified amylase is around 6.0. However, this enzyme retains more than 30% of its activity over a broad pH interval, from 5.0 to 7.0. The amylase activity determined at 30°C and pH 6.0 is 10 U/ml (Table-2).

Table 2: Effect of different pH on partial purified amylase activity.

pH	Relative activity U/ml
pH 3	3
pH 4	4
pH 5	8
pH 6	10
pH 7	9
pH 8	6
pH 9	3
pH 10	1

Table-3 Effect of different Temperature on partial purified amylase activity

The temperature stability profile of amylase activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 40 to 60°C with highest activity (12 U/ml) detected at 40°C incubation temperature for 1 h (Table-3).

Temperature °C	Relative activity U/ml
4°C	10
20°C	11
30°C	12
40°C	11.3
50°C	10.1
60°C	7
70°C	5.2
80°C	3
90°C	2.2
100°C	1.3

4. DISCUSSION

Amylases can be derived from various sources, such as plants, animals and microorganisms, but in spite of these sources, microbial sources are generally the most sort forms in industry^[11] as there is a possibility of increasing the levels of microbial enzyme synthesized by classical genetic techniques, continuous culture selection, induction, or optimization of growth conditions for the enzyme of interest.^[12,13] Even, among the microbial sources, the fungal amylases are preferred over other microbial sources because of their more acceptable GRAS (generally regarded as safe) status, the hyphal mode of growth and good tolerance to low water activity, and high osmotic pressure conditions make fungi most efficient for bioconversion of solid substrates^[14] and thus attracting increasing attention as source of amylolytic enzymes suitable for industrial applications.^[15,16]

The ability to access and rapidly screen discrete biotopes to discover novel α -amylases has proven to be extremely valuable in this study. Although the naturally occurring α -amylases described in this work were similar to each other at the amino acid level, the environmental conditions, *i.e.* high temperature and low pH, prevailing at the sites of sample collection apparently has had a strong influence on their biochemical properties.

The highest production of amylase by the *Rhizopus oryzae* was in a culture medium initially adjusted to pH 6. Considerable amounts were also recorded at pH 5, 7 and 8 but low levels in more acidic or alkaline cultures (Graph-2). The results of our study was in concurrence with the studies carried out by Uguru *et al.* 2011^[17] and Khan and Yadav 2011^[18] since maximum production of α -amylase by *A. niger* was observed in media adjusted to pH 6 and 6.2, respectively.

Carbon sources tested, starch resulted in maximum amylase production by *Rhizopus oryzae* by maltose; glucose and fructose were the least effective carbon sources (Graph-4). Starch was also found to be the optimal carbon source by Balkan and Ertan 2007^[19] for *Penicillium chrysogenum*. The highest yields of amylase by *Rhizopus oryzae* were achieved in cultures supplemented with yeast extract, followed by peptone (Graph-4). Balkan and Ertan 2007^[19] found maximum production of amylase by *Penicillium chrysogenum* with sodium nitrate, while Gupta *et al.* 2008^[20] and Chimata *et al.* 2010^[21] studying *A. niger* and *Aspergillus* MK07 and Erdal and Taskin 2010^[22] studying *Penicillium expansum* found that peptone was the optimum nitrogen source.

Maximum activity of the enzyme was 5% (w/v) in yeast extract (2.4 U/ ml) at 50°C and pH 7.5–8.5. At lower and higher temperatures (10 and 70°C), 47% and 50% reductions of the enzyme activity were observed, respectively. The enzyme showed greater activity in higher pH values (Table-2). In pH 9, only 20% reduction of the enzyme activity was detected. Amylase activity of *Micrococcus sp.* was reported as maximum in pH 7.5, while at pH 9, amylase activity was reduced by more than 50%.^[23] Amylase activity of *M. halobius* and *M. varians* subsp. halophilus was maximum at pH 6–7.

5. CONCLUSION

α -Amylase is one of the most widely used enzymes for the production of fermented foods and starch and the demand for amylase is increasing with a widening spectrum of applications. In our study, *Rhizopus oryzae* were found to be the best local isolates for α -amylase production. Use of the active fungi for the production of α -amylase would allow large-scale production and the development of the food and starch industries.

Declaration of Interest

The authors declare that there are no conflicts of interest among authors. The authors alone are responsible for the preparation of the manuscript.

REFERENCES

- Paula Monteiro de Souza and Pérola de Oliveira Magalhães (2010). Application of microbial α -amylase in industry – A review. *Braz J Microbiol*, 2010; 41(4): 850–861.
- Gupta R., Gigras P., Mohapatra H., Goswami V.K., Chauhan B (2003). Microbial α -amylases: a biotechnological perspective. *Process Biochem*, 2003; 38: 1599-1616.
- Couto, S.R.; Sanromán, M.A. (2006). Application of solid-state fermentation to food industry- A review. *Journal of Food Engineering*, 2006; 76: 291-302.
- Gangadharan, D.; Sivaramakrishnan, S.; Nampoothiri, K.M.; Sukumaran, R.K.; Pandey, A. (2008). Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. *Bioresour Technol*, 2008; 99: 4597-4602.
- Francis, F.; Sabu, A.; Nampoothiri, K.M.; Ramachandran, S.; Ghosh, S.; Szakacs, G.; Pandey, A. (2003). Use of response surface methodology for optimizing process parameters for the production of α -amylase by *Aspergillus oryzae*. *Biochem. Eng. J.*, 2003; 15: 107–115.
- Kathiresan K., Manivannan, S(2006). α -Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. *Afr J Biotechnol*, 2006; 5: 829-832.
- Maria G.L., Sridhar, K.R., Raviraj, N.S.(2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *J Agric Technol*, 2005; 1(XX): 67-80.
- Singh, R.K., Kumar, S., Kumar, S. (2009). Production of α -amylase from agricultural byproduct by *Humicola lanuginosa* in solid state fermentation. *Curr Trends Biotechnol Pharmacol*, 2009; 3(2): 172–180.
- Fossi, B.T., Tavea, F., Jiwoua, C., Ndjouenkeu, R. (2011). Simultaneous production of raw starch degrading highly thermostable α -amylase and lactic acid by *Lactobacillus fermentum* 04BBA19. *Afr J Biotechnol*, 2011; 10: 6564–6574.
- Miller, G.L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal Chem*, 1959; 31(3): 426–428.
- Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R. (2000). Advances in microbial amylases. *Biotechnol Appl Biochem*, 2000; 31: 135-152.
- Ali, S., Mahmood, S., Alam, R., Hossain, Z. (1989). Culture condition for production of glucoamylase from rice bran by *Aspergillus terreus*,” *Mircen. Journal of Applied Microbiology and Biotechnology*, 1989; 5(4): 525–532.
- Konsoula, Z., Liakopoulou-Kyriakides, M (2007). “Co-production of α -amylase and β -galactosidase by *Bacillus subtilis* in complex organic substrates”. *Bioresource Technology*, 2007; 98(1): 150–157.
- Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology*, 1998; 1(3): 114–140.
- Mishra, R.S., Maheshwari, R(1996). Amylases of the thermophilic fungus *Thermomyces lanuginosus*: their purification, properties, action on starch and response to heat. *Journal of Biosciences*, 1996; 21(5): 653–672.
- Hernandez, M. S., Rodríguez, M. R., Guerra, N. P., Rosés, R. P. (2006). “Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries”. *Journal of Food Process Engineering*, 2006; 73: 93–100.
- Uguru, G.C., Akinayanju, J.A., Sani, A.(2011). The use yam peel for growth of locally isolated *Aspergillus niger* and amylase production. *Enzyme Microbiology and Technology*, 2011; 21: 48-51.
- Khan, J.A., Yadav, S.K. (2011). Production of alpha amylase by *Aspergillus niger* using cheaper substrates employing solid state fermentation. *Int J Plant Anim Environ Sci.*, 2011; 1(3): 100-108.
- Balkan B., Ertan F.(2007) Production of α -amylase from *Penicillium chrysogenum* Food Technol. Biotechnol., 2007; 45: 439–442.
- Gupta, A., Gupta, V.K., Modi, D.R., Yadava, L.P. (2008). Production and characterization of α -amylase from *Aspergillus niger*. *Biotechnology*, 2008; 7(3): 551-556.
- Chimata M.K., P. Sasidhar, S. Challa Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation Afr. J. Biotechnol., 2010; 9(32): 5162–5169.
- Erdal S., M. Taskin Production of α -amylase by *Penicillium expansum* MT-1 in solid state fermentation using waste loquat (*Eriobotrya japonica* Lindley) kernels as substrate Rom. Biotechnol. Lett., 2010; 15(30): 5342–5350.
- Khire, J.M.(1994). Production of moderately halophilic amylase by newly isolated *Micrococcus* sp. 4 from a salt-pan. *Lett Appl Microbiol*, 1994; 19: 210– 212.