

Full Length Research Paper

Characterization of immobilized post-carbohydrate meal salivary α -amylase

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Saliva containing amylase was collected from an individual 5 min after a carbohydrate meal and filtered using a dialysis bag to remove starch particles. The filtered saliva was immobilized on calcium alginate beads. The effect of experimental parameters like pH, temperature and substrate concentration on the activity of the immobilized post-carbohydrate meal salivary α -amylase was determined. The immobilized salivary α -amylase had an optimum activity at temperature 40°C and pH 7.0. The activation energy (E_a) as obtained from the Arrhenius plot was 31.4 kJ/Mol. The kinetic parameters K_m and V_{max} of the immobilized α -amylase were found to be 1.6 mg/ml and 16.4 μ mol/min, respectively; this was compared to that of free salivary α -amylase ($K_m = 0.0048$ mg/ml) and α -amylases from fungi and bacteria sources. Immobilization tends to increase the K_m of the immobilized enzyme, indicating a low affinity for substrate, however the enzymes K_m was lower than that of some microbial α -amylases. The results obtained from the characterization of immobilized post-carbohydrate meal salivary α -amylase in this study show that immobilization had no significant effect on the enzyme and compared to kinetic parameters of microbial α -amylase, immobilized salivary α -amylase may not be of significant benefit as alternative source of α -amylase in the industrial bioprocesses.

Key words: Enzyme activity, carbohydrate, immobilized enzyme, industrial bioprocess, kinetics, salivary α -amylase.

INTRODUCTION

Alpha amylases have been on increasing demand for various industries, due to their applications in the production of wide array of products, ranging from conversion of starch to sugar syrups, textile, paper, brewing, baking, distilling industries, preparation of digestive aids, production of cakes, and the production of cyclodextrins for the pharmaceutical industry

(Sivaramakrishnan et al., 2006; Gupta et al., 2003; Reddy et al., 2003). These enzymes account for about 30% of the world's enzyme production (Chi et al., 2009; Van der Maarel et al., 2002). Hence, there is enormous interest in developing alpha amylases with better properties. Alpha amylases (endo-1, 4- α -D-glucanglucanohydrolase EC 3.2.1.1) are extracellular endo enzymes that randomly

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Abbreviations: E_a , Activation energy.

cleave α -1,4 linkages between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units (Uma and Nasrin, 2013). Most of the α -amylases are metallo-enzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. The α -amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes (Sobukola and Aboderin, 2012). The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana et al., 2005).

The human buccal cavity (mouth) can be likened to a template of industrial reactor whose salivary amylase may possess some industrial applications when characterized. Although, the quantity of salivary amylase may be small in the mouth, it may possess better kinetic parameters which could be genetically engineered to complement microbial sources of the amylase. The human salivary α -amylases initiate the chemical process of digestion in the mouth and like amylases from other sources; break down large insoluble starch molecules into soluble starches producing successively smaller starch molecules and ultimately maltose. They act only on linear α -1,4-glycosidic bonds, and hence depend on other enzymes like β -amylase and glucosidase for complete hydrolysis of branched products (Sivaramakrishnan et al., 2006; Robert et al., 2006; Uma and Nasrin, 2013).

Research has shown that the enzyme is optimally active at temperature of 37°C and pH range of 6.7 to 7.0 (Vijayaraghavan et al., 2011). However, the enzyme is inactivated in the stomach by gastric juice at pH 3.3 (Agamemnon and Stefan, 2003). The enzyme is a metalloenzyme and thus requires the presence of metallic ions like calcium and sodium for effective catalytic activity (Siddhartha et al., 2013). The aim of this study was to examine the kinetic property of the immobilized human salivary α -amylase as a possible alternative source of industrial enzyme.

MATERIALS AND METHODS

Collection of salivary α -amylase

Saliva (5 ml) was collected from ten (10) individuals 5 min after a carbohydrate meal. The saliva samples were then filtered using a porous material to remove starch particles. About 1.5 g of sodium alginate was dissolved in 50 ml of distilled water and autoclaved at 121°C for 15 min after cooling to room temperature. 4.5 ml of the crude enzyme sample was added and mixed. The mixture was then allowed to stand for 10 min. The enzyme-alginate mixture was carefully pumped through sterile syringes drop wise into beaker containing 250 ml of sterile 0.12 g calcium chloride in order for the mixture to form beads. The beads were kept in solution for 1 h at 4°C to ensure complete precipitation according to a modified method of James (1992).

Determination of the number of beads of immobilized salivary α -amylase with highest activity

The number of beads that gave the maximum activity with time was determined. Six (6) test tubes were used. The first test tube contained 1 bead + 5 ml of 1% starch + 1 ml of NaCl. The second test tube contained 2 beads + 5 ml of 1% starch + 1 ml of NaCl until the sixth test tube with 6 beads. It was found that 2 beads of size 2 mm yielded the maximum activity and hence was used to characterize the immobilized enzyme. Activity of the immobilized salivary α -amylase was defined as the amount of glucose produced in mM/min at a given temperature when the substrate is hydrolyzed by the enzyme.

Effect of substrate concentration, temperature and pH on α -amylase activity

Starch concentration for optimum extracellular amylase activity varied from 5.0 to 30 mg/ml in 0.1 M potassium phosphate buffer (pH 7.5). Kinetic data were transformed into Lineweaver–Burk plots with graph pad prism program (version 5.0). The K_m value was calculated by slope of the curve. Extracellular α -amylase activity was also performed at different temperatures ranging from 20 to 70°C. Effect of pH was assayed using 0.1 M pH buffer solutions ranging from pH 4.5 to 9 in increments of one pH unit, (Note that phosphate buffer is only good for pH = 4.5 to 9 due to the dissociation constant). All enzyme assay measure either the consumption of substrate or production of products over time. Initial rate experiments were used to measure the amylase activity. When an enzyme is mixed with a large excess of substrate, the enzyme-substrate intermediates build up in a fast initial transient. Then the reaction achieves a steady-state kinetics in which enzyme substrate intermediates remains approximately constant over time and the reaction rate changes relatively slow. Rates are measured for a short period after the attainment of a quasi-steady state, typically by monitoring the accumulation of products with time. Because the measurements are carried for a short period of time also large excess substrate, the assumption that the amount of free substrate is approximately equal to the amount of initial substrate is usually made. The enzyme activity is the measure of the quantity of active enzyme present. There are two ways to measure enzyme activity; monitoring the disappearance of substrate or the appearance of product. Measuring the appearance of the product is usually accurate because detecting small changes in $[P]$ ($[P]=0$) is easier to measure than detecting small changes in $[S]$.

Amylase activity = moles of substrate per unit time = rate x reaction volume.

RESULTS

The result from the plot of immobilized post carbohydrate meal salivary α -amylase activity against temperatures values ranging from 25 to 70°C showed that temperature effect on the immobilized salivary α -amylase was optima at 40°C (Figure 1). In Table 1, optimal temperatures and activities of immobilized human salivary α -amylase samples were compared with known microbial α -amylases. The result showed that the salivary amylase had significantly ($p<0.05$) lowered optimum temperature than the microbial sources. The pH effect on the immobilized enzyme was also determined within pH values range of 4.5 to 9.0; the enzyme was found to be

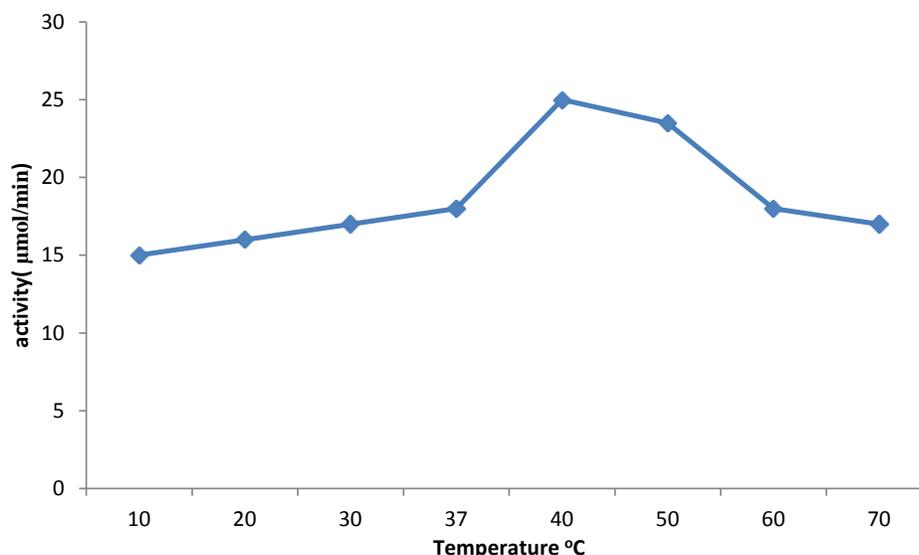


Figure 1. Effect of temperature on human immobilized salivary α -amylase activity showing optimum temperature of 40°C.

Table 1. Comparison between residual activity of human salivary α -amylase and microbial α -amylases at different temperatures.

Organism	Temperature range (°C)	Residual activity (%)	Temperature Optimum (°C)	References
Human salivary (immobilized)	20 - 70	25 (20 min)	40	This study
<i>Bacillus</i> sp. PS1-3	65 - 100	50 (80°C for 2.5 h)	70	Goyal et al. (2005)
<i>Pyrococcus furiosus</i>	80 - 100	50 (98°C for 13 h)	100	Vieille and Zeikus (2001)
<i>Aspergillus tamarii</i>	50 - 60	90 (65°C for 3 h)	55	Moreira et al. (2004)
<i>Lactobacillus manihotivorans</i>	50 - 70	70(80°C for 2.5 h)	55	Aguilar et al. (2000)

optimally active at pH 7.0 (Figure 2); this was compared with optimal pH values of fungal and bacteria α -amylases as shown in Table 2. The activation energy of the immobilized enzyme obtained from an Arrhenius plot was 1.4 kJ/mol (Figure 3). The K_m and V_{max} of the enzyme were 1.6 mg/ml and 16.4 μ mol/min, respectively, as obtained from a Line weaver-Bulk plot (Figure 4).

DISCUSSION

In spite of the wide distribution of α -amylases from fungal and bacterial sources, the diversity of its industrial application creates the need to search for novel α -amylases with novel and improved properties. The rate of hydrolysis of starch by α -amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} and other stabilizing agents. α -amylases with properties suitable for industrial conditions and applications have to be appropriately selected based on their high demand. Results obtained from this

research work showed that the immobilized salivary α -amylase activity increased as temperature was increased from 20°C. This initial rise in temperature could be due to the increase in probability of effective collision between the reactants as a result of an increase in their average kinetic energy (Okoye et al., 2013). The optimal temperature was obtained at 40°C (Figure 1), followed by a sudden decrease in the activity with further increase in temperature. It is desirable that α -amylases be active at high temperatures of gelatinization (100 to 110°C) and liquefaction (80 to 90°C) to economize the industrial bioprocess; α -amylases from microbial sources seems to be more thermostable (Table 1) than the immobilized salivary α -amylase as observed in this research work (Haki and Rakshit, 2003).

Similarly, the results also showed that the activity of the immobilized enzyme was affected by changes in pH. As the pH of the medium was increased, the activity of the enzyme also increased up to an optimum value of 7.0 (Figure 2). Further increase in pH resulted to a fall in activity and this could possibly be due to ionization of amino-acid side chains that are involved in the catalytic

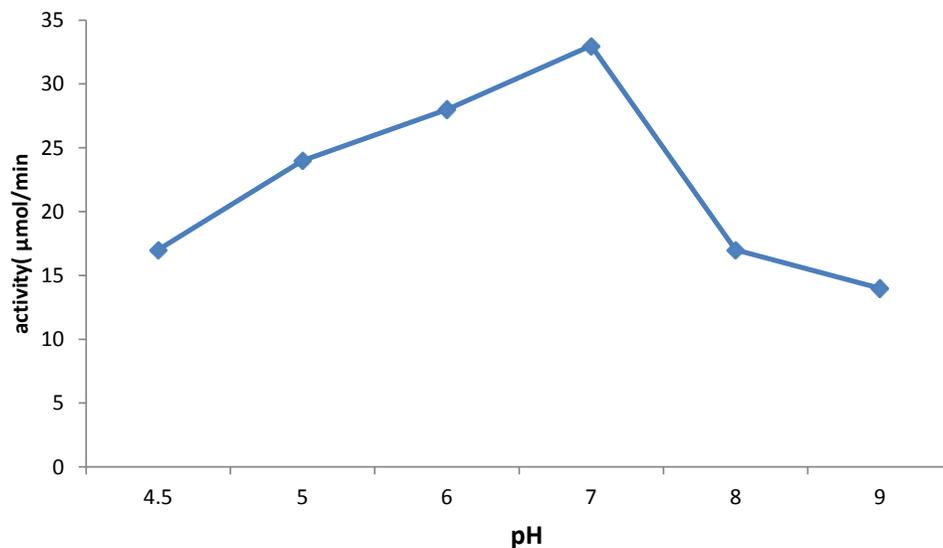


Figure 2. Effect of pH on the activity of human immobilized salivary α -amylase showing optimum pH at 7.

Table 2. Comparison between residual activity of human salivary α -amylase and microbial α -amylases at different pH.

Organism	pH range	Residual activity (%)	pH optimum	Reference
Human saliva (immobilized)	4.5 - 9	32 (20 min)	7.0	This study
<i>Bacillus</i> sp. PS-7	5.0 - 8	96 (pH = 5.0 for 90 min)	6.5	Sodhi et al. (2005)
<i>Bacillus</i> sp. PS1-3	5.0 - 5.5	55 (pH = 10 for 15 h)	5.5	Goyal et al. (2005)
<i>Bacillus</i> sp. ANT-6	9 - 13	90 (pH 2.0 for 30 min)	10.5	Burhan et al. (2004)

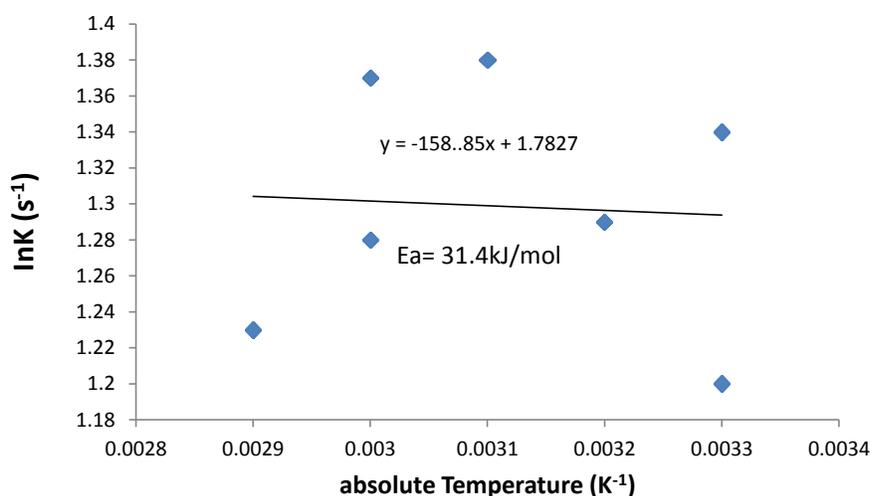


Figure 3. Arrhenius plot of human immobilized salivary α -amylase showing activation energy.

mechanism or may reflect an onset of denaturation of the enzyme (Sundarram and Murthy, 2014). A comparison of

the optimum pH of the immobilized salivary α -amylase to those of microbial α -amylases showed that some

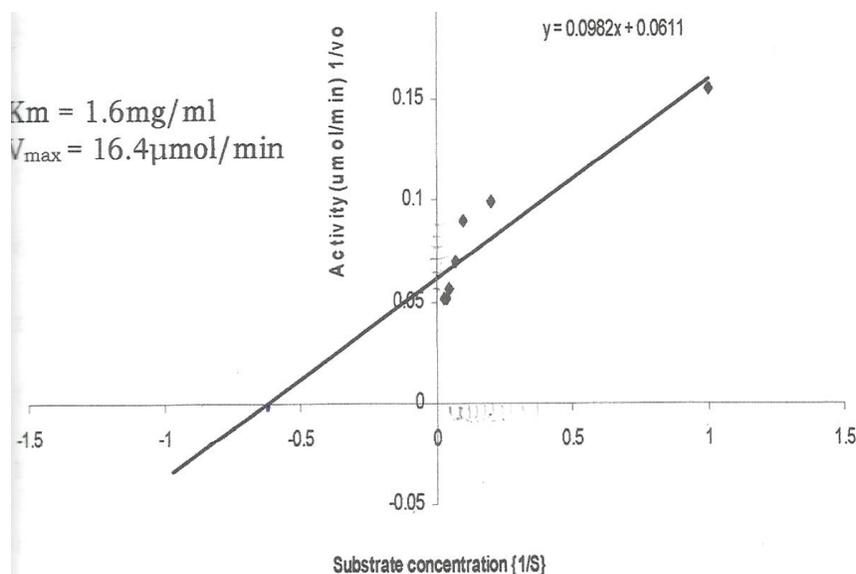


Figure 4. Line Weaver Burk plot of immobilized salivary α -amylase showing K_m and V_{max} values

Table 3. Comparison of K_m values of immobilized salivary α -amylase and α -amylases from microbial sources.

Human/microbial species	K_m (mg/ml)	Reference
Human saliva (immobilized)	1.6	This study
<i>Bacillus</i> sp. PB3224	2.7	Orlando et al. (1983)
<i>B. subtilis</i>	3.8	Ciobanu et al. (1976)
<i>B. subtilis</i> 65	1.2	Hayashida et al. (1988)
<i>Bacillus</i> sp. KCC103	1.9	Nagarajan et al. (2006)
truncated <i>Bacillus subtilis</i> alpha-amylase in <i>E. coli</i>	14	Marco et al. (1996)

microbial amylases are optimally active under acidic conditions, some under basic while others in a neutral medium. The activation energy (E_a), of the reaction was also investigated and found to be 31.4 kJ/mol (Figure 3). This is the minimum energy reactants, must possess to overcome the activation barrier. The role of the enzyme is to reduce this barrier, thereby increasing the rate of reaction, hence the formation of products. From the Arrhenius plot, E_a is best regarded as the experimentally determined parameter that indicates the sensitivity of a reaction rate to temperature. Enzymes facilitate the rate of catalysis by providing an alternative route with less E_a for reacting species. Hence, E_a (31.4 kJ/mol) in this study is the energy required to hydrolyse starch to glucose molecules. More energy will have been required if the reaction was devoid of an enzyme.

The kinetic parameters K_m and V_{max} of the immobilized enzyme were obtained from a Lineweaver-Burk plot using starch as the substrate. The K_m and V_{max} were 1.6 mg/ml and 16.4 $\mu\text{mol}/\text{min}$, respectively. The

value of the immobilized enzyme K_m was lower than K_m values from microbial sources (Table 3). The K_m of an enzyme, relative to the concentration of its substrate under normal conditions permits prediction of whether or not the rate of formation of product, will be affected by the availability of the substrate. Since, K_m is the major factor determining the enzymes' affinity for its substrate, which also affects the rate at which the enzyme is saturated by its substrate (Das et al., 2011) an enzyme with low K_m has high affinity for its substrate and vice versa.

In conclusion, from the results obtained from immobilization of α -amylase from human saliva compared to that from microbial sources, immobilized salivary α -amylase is not thermally stable and may not be active in an acidic or basic medium though it has a low K_m , signifying that the enzyme may have high affinity for its substrate starch which is of significant advantage to the industrial processes. Most industrial processes operate at very high temperature and during bioprocesses lot of acid and basic metabolites are released that alters the pH of

the bioreactor which may result to loss of enzyme activity. However, amylases from some microbial sources can operate in both acidic and basic medium and thus immobilized salivary α -amylase may not be a perfect alternative for industrial purposes.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

- Agamemnon D, Stefan S (2003). Color atlas of Physiology; Nutrition and Digestion. Chap.10, p.255.
- Aguilar G, Morlon-Guyot J, Trejo-Aguilar Guyot JP (2000). Purification and characterization of an extracellular α -amylase produced by *Lactobacillus manihotivorans* LMG 18010T, an amylolytic lactic acid bacterium. *Enzyme Microb. Technol.* 27:406-413.
- Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G(2003). Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process. Biochem.* 38:1397–1403.
- Chi Z, Chi Z, Liu G, Wang F, Ju L, Zhang T(2009). Saccharomycopsis fibuligera and its applications in biotechnology. *Biotechnol. Adv.* 27 (4):423-431.
- Ciobanu A, Lascu G, Berescu V, Nicolescu L (1976). Cooling techniques in food industry. *Abacus*; p. 20.
- Das S, Singh S, Sharma V, Soni M(2011). Biotechnological applications of industrially important amylase enzymes. *Int. J. Pharma BioSci.* 2(1):486-496.
- Goyal N, Gupta JK, Soni SK (2005). A novel raw starch digesting thermostable α -amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme Microb. Technol.* 37:723-734.
- Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B(2003). Microbial α -amylases: a biotechnological perspective. *Process Biochem.* 38 (11):1599-1616.
- Haki GD, Rakshit SK (2003). Developments in industrially important thermostable enzymes: a review. *Bioresour. Technol.* 89(1): 17-34.
- Hayashida S, Teramoto Y, Inoue T(1988). Production and characteristics of raw potato starch digesting α -amylase from *Bacillus subtilis* 65. *Appl. Environ. Microbiol.* 54:1516-1522
- James DW(1992). *Analytical Biochemistry.* 201:331-335
- Marco JL, Bataus LA, Valencia FF, Ulhoa CJ, Astolfi-Filho S, Felix CR (1996). Purification and characterization of a truncated *Bacillus subtilis* α -amylase produced by *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 44:746-752.
- Moreira FG, Lenartovicz VL, Peralta RM(2004). A thermostable maltose-tolerant α -amylase from *Aspergillus tamari*. *J. Basic Microbiol.* 44:29-35.
- Nagarajan DR, Rajagopalan G, Krishnan C (2006). Purification and characterization of a maltooligosaccharide forming α -amylase from a new *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 73:591-597.
- Okoye IG, Ezugwu AL, Udenwobele DI, Eze SOO, Anyawu CU, Chilaka FC (2013). production and partial characterization of cellulases from *Aspergillus fumigatus* using two distinct parts of corn cob as carbon sources. *Niger. J. Biotechnol.* 26:50-59.
- Orlando AR, Ade P, Di Maggio D, Fanelli C, Vittozzi L(1983). The purification of a novel amylase from *Bacillus subtilis* and its inhibition by wheat proteins. *Biochem. J.* 209:561–564.
- Reddy NS, Nimmagada A, Sambasiva Rac RS (2003). An over view of the microbial α amylase mini review. *Afr. J. Biotechnol.* 2:645–648.
- Robert KM, Darl KG, Victor WR (2006). *Harpers illustrated biochemistry* 27th edition. The McGraw Hill Companies Inc. Chap. 6, p.482.
- Satyanarayana T, Rao JLUM, Ezhilvannan M(2005). α -Amylases. In: *Enzyme Technology*, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), Asia Tech Publishers Inc., New Delhi, India. pp. 189–220.
- Siddhartha MK, Chandana MV, Sridevi V (2013). Production and optimization of glucoamylase from wheat bran by *Aspergillus oryzae* NCIM 1212 under solid state fermentation. *Int. J. Appl. Innov. Eng. Manage.* 2:318-323.
- Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A (2006). α -Amylases from Microbial Sources--An Overview on Recent Developments. *Food Technol. Biotechnol.* 44 (2):173–184.
- Sobukola OP, Aboderin AT (2012). Studies on some properties of starches from three *Mucuna* species. *Int. Food Res. J.* 19: 913-921.
- Sodhi HK, Sharma K, Gupta JK, Soni SK (2005). Production of a thermostable α -amylase from *Bacillus* sp. PS-7 by solid-state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochem.* 40:525–534.
- Sundaram A, Murthy TPK (2014). α -Amylase Production and Applications: A Review. *J. Appl. Environ. Microbiol.* 2(4):166-175.
- Uma MN, Nasrin A(2013). production and optimization of glucoamylase from agro waste by using *Aspergillus niger* and *Aspergillus flavus*. *Int. J. Chem. Pharm. Sci.* 4: 34-38.
- Van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002). Properties and applications of starch-converting enzymes of the α -amylase family. *J. Biotechnol.* 94:137–155.
- Vieille K, Zeikus GJ (2001). Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms of thermostability. *Microbiol. Mol. Biol. Rev.* 65:1-43.
- Vijayaraghavan P, Remya CS, Prakash SG (2011). Production of α -amylase by *Rhizopus microsporus* Agricultural By-products in Solid State Fermentation. *Res. J. Microbiol.* 6:366-375.