Journal of Biochemistry, Biotechnology and Biomaterials, 1(1):36-43, 2016

Journal Home page: http://molcare.org/jbcbb

Production and purification of α-Amylase by *Aspergillus sydowii* through liquid state and solid state fermentation

*Ume Salma Liaqat[1], M. Javaid Asad[*2], Raja Tahir Mahmood[3], M. Gulfraz[4] and Sajid Nadeem[1]*

Abstract

In the present study, *Aspergillus sydowii* was used to produce an industrially relevant enzyme α-amylase. The enzyme was then purified and characterizes to increase its applicability. Production was enhanced by optimizing various parameters like pH, temperature, fermentation period and size of inoculum. Maximum production of α-amylase was observed after 72 hrs at 50 °C temperature, at pH 4.5 and in the presence of 3 mL inoculum size. It was then purified through ammonium sulfate precipitation and gel filtration chromatography. It was then characterize to find its optimum pH, temperature, Km and Vmax. The characterization of α-amylase revealed that it has maximum activity at pH 4.5 and 50 °C temperature. The study of kinetic parameters showed that it has Km value 1.40 mM and Vmax value 9.17 μM/mL/min. The results obtained in current study suggested that α-amylase could be very useful for many industrial processes.

Key words: α-*amylase, Aspergillus sydowii, Fermentation, Purification, Characterization*

Full length article: Received: 31 Jan, 2016 Revised: 10 Feb, 2016 Accepted: 10 Feb, 2016 Available online: 15 Feb, 2016

Affiliations of authors: [1] *Department of Biology,* [2] *Department of Biochemistry, PMAS-Arid Agriculture University Rawalpindi*
[3] *Department of Biotechnology, Mirpur University of Science and Technology (MUST), Mirpur AJK*
[4] *Department of Chemistry, COMSATS Institute of Information Technology (CIIT), Abbottabad*
***Corresponding Author**: mjavaidasad@gmail.com

1. Introduction

Amylases are among the most important enzymes and have great significance in many fields especially present day biotechnology (Sundarram and Murthy, 2014). These can be produced from several sources such as plants, animals and micro-organisms like bacteria and fungi (Pandey *et al*., 2000). Fungal sources are not only confined to terrestrial isolates but also on marine isolates mostly to *Aspergillus* and *Penicillium* species (Gouda and Elbahloul, 2008).

The growth of microorganisms on humid solid substrates with minor free water is known as Solid state fermentation (Pandey *et al*., 2001; Raul *et al*., 2014). For cost effective production of α-amylases agro-industrial wastes have been reported as very good substrates for solid state fermentation (Kumar and Duhan, 2011; Kumar *et al*., 2011).

They have been studied extensively due to an important group of hydrolytic enzymes and their latent applications in the biotechnological based products like pharmaceutical industries, food, paper, and detergent (Maarel *et al*., 2002; Krishnan *et al*., 2006). Purpose of this research was to optimize different fermentation parameters for maximum production of α-amylase in solid and liquid state cultures of *A. sydowii* using waste bread as starch containing inducer substrate (Ellaiah *et al*., 2002; Maryam *et al*., 2010). The enzyme produced under optimum conditions was also purified and characterized for its possible industrial applications.

2. Material and Methods

2.1. Substrate

Waste bread was used as a substrate of *A. sydowii* for the production of α-amylase. Waste bread is cheap and easily available in Pakistan and is suitable starch containing substrate for the economic production of amylases.

2.2. Preparation of Substrate

Waste bread was obtained from Fatima Jinnah Women Hostel Arid Agriculture University Rawalpindi. It was air dried for twenty days to remove wetness. It was then oven dried at 50 ^{o}C for 48 hrs and then crushed into powder (40 mm mesh size) from the Department of Soil Sciences PMAS Arid Agricultural University Rawalpindi (Singh and Rani, 2014).

2.3. Fermentative Organism

A. sydowii was used as fermentative organisms for the production of α-amylase. It was obtained from Industrial and Environmental Biotechnology Laboratory, Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi. The fungal culture was raised on PDA media plates having pH 5 at 45 ^{o}C (Mahmood *et al*., 2013).

2.4. Inoculum Preparation:

For fermentation process inoculum of the fungus was prepared in agar less potato dextrose media. pH of the media was maintained with 1 M HCl / NaOH at 5. The autoclaved media was inoculated with fungal culture aseptically and incubated at 45 ^{o}C and 150 rpm for 72-96 hrs. The number of spores (107-109/mL) was calculated with biomass monitor (ABER 220) (Asgher *et al*., 2013).

2.5. Fermentation Process

The α-amylase production was carried out by Solid State Fermentation (SSF) as well as Liquid State Fermentation (LSF) of waste bread. Both processes were compared and the better process was optimized to enhanced α-amylase production. For experiments 250 mL flasks were used and SSF was performed with 5 g substrate having 70% moisture level while LSF was carried out with excess of water in substrate. Each experiment was run in triplicate to avoid any error.

2.6. Sample Harvesting

For the harvesting of extracellular α-amylase from SSF flasks, 50 mL distilled water was added and these were shaked at 150 rpm for 30 min before filtered with filter paper. The filtrate was centrifuged at 10,000 rpm for 10 min to remove impurities and used as crude enzyme. The mixture of LSF was filtered, centrifuged and used as crude enzyme for the enzymatic assay (Kumari *et al*., 2011; Mahmood *et al*., 2013).

2.7- Enzyme assay

The α-amylase activity assay was performed in test tubes by taking 0.5 mL crude enzyme, 1 mL (1%) starch solution and 0.5 mL phosphate buffer (pH=5). The whole mixture was incubated at 30 ^{o}C for 5 min and then added 3 mL DNS solution to stop

reaction. The absorbance was taken at 540 nm to measure the quantity of complex compounds formed with enzymatic products and the enzyme activity was determined by comparing the absorbance with that of standards (Asgher *et al*., 2013).

2.8. Optimization of Enzyme Production

To enhance the production of α-amylase by *Aspergillus sydowii* various parameters were optimized like; temperature, pH, time period and inoculum size. One parameter was optimized at one time keeping other constants and optimized value was used in the next experiment. All the experiments were run in triplicate to avoid any error during experiments.

2.9. Purification of Enzyme

The crude enzyme obtained at optimized conditions was partially purified by ammonium sulfate precipitation. Different concentrations of ammonium sulfate (20-60%) were added in crude enzyme for overnight and enzymatic assay was performed on next day (Nooralabettu, 2014).

2.10. Characterization of Enzyme

The α-amylase was characterized to determine its optimum temperature, optimum pH and values of Km and Vmax. The optimum temperature was determined by performing enzymatic assay in triplicate at different temperature (40-60 ^{o}C) while optimum pH was determined by assay at different pH values (pH 3-7) in triplicate. The kinetic parameters (km and Vmax) were determined by using various conc. of substrates (2-10 mM). Values obtained were used to draw line-weaver Burk double reciprocal plot that was used to calculated Km and Vmax.

3. Results and Discussion

3.1. Comparison of Solid State and Liquid State Fermentation

Initially two fermentation processes were compared for the production of α-amylase by maintaining similar conditions for both. It was observed that SSF gave the better production of α-amylase as compared to LSF process (Fig.1). Based on initial results SSF process was further optimized to enhance production. Due to ease of access to the substrate and static conditions there was more growth and production in SSF compared to LSF process.

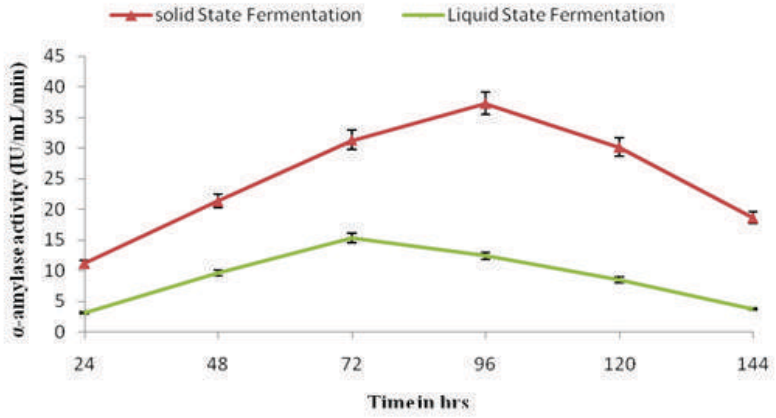

Fig. 1. Comparison of SSF and LSF processes for α-amylase production

3.2. Optimization of α-*amylase Production*

The production of α-amylase through SSF process was enhanced by optimization of various independent parameters. There was maximum activity (17.22±0.831 IU/mL/min) observed after 72 hours of fermentation which decreased as time period increased (Fig. 2). This is possibly due to depletion of nutrients and accumulation of waste products in the media. The production was further

enhanced when pH was optimized. The results showed that there was maximum production and activity (32.18±1.072 IU/mL/min) of α-amylase at pH 4.5 of the fermentation media (Fig. 3). These findings suggested that α-amylase produced by *A. sydowii* was acidic and suitable for industrial processes taking place in acidic conditions.

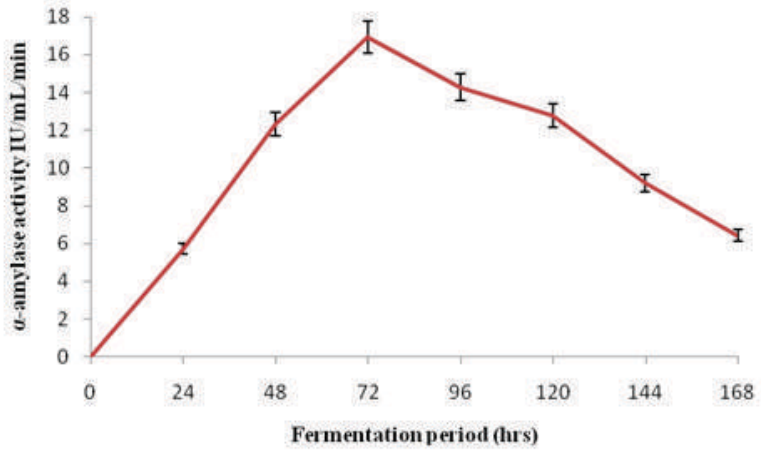

Fig. 2. Effect of fermentation period on α-amylase production by *Aspergillus sydowii*

Temperature played a vital role in growth of fungus and secretion of enzymes. Thermophillic microorganisms and their enzymes are preferred because these catalyzed reactions at higher temperature, suitable for industrial processes. There was maximum production of αamylase (38.39±1.127 IU/mL/min) at 50 ^{o}C temperature (Fig. 4). Further increased in temperature decreased the enzymatic activity because higher temperature effect the structure of enzyme. Any change in the structure of enzymes decreased its affinity towards substrate and its activity. The most suitable inoculum size for the maximum production of α-amylase (37.34±1.004 IU/mL/min) was 3 mL in 5 g of substrate (Fig. 5). The inoculum size associated with the number of spores in the media.

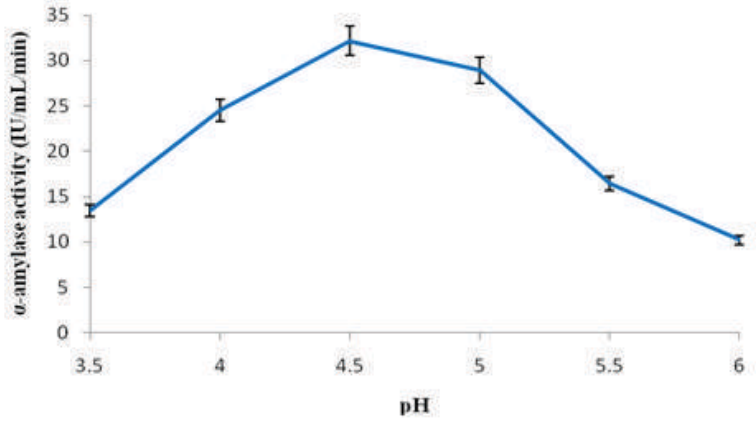

Fig. 3. Effect of pH on α-amylase production by *Aspergillus sydowii*

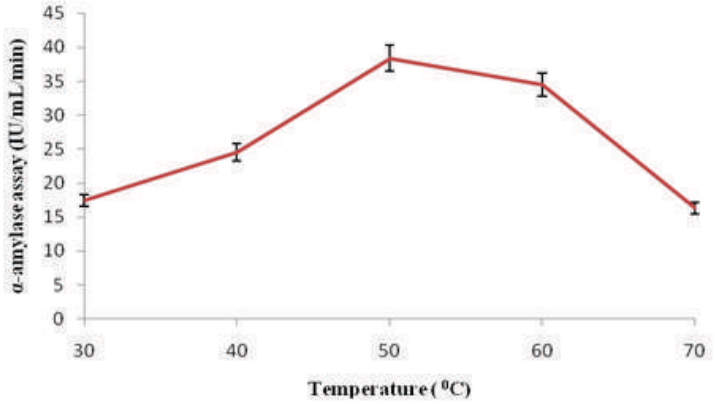

Fig. 4. Effect of temperature on α-amylase production by *Aspergillus sydowii*

There are many studies reported that secretion of fungal enzymes is affected by surrounding conditions. Presence of suitable conditions will enhance while non-suitable conditions definitely decrease enzymes production (Hang *et al*., 2003). So, selection/optimization of fermentation parameters increases the production of extracellular enzymes. The most important parameters that affect enzymes production are; fermentation period, temperature, initial pH and inoculum size (Kumari and Kayastha, 2011; Asgher *et al*., 2013).

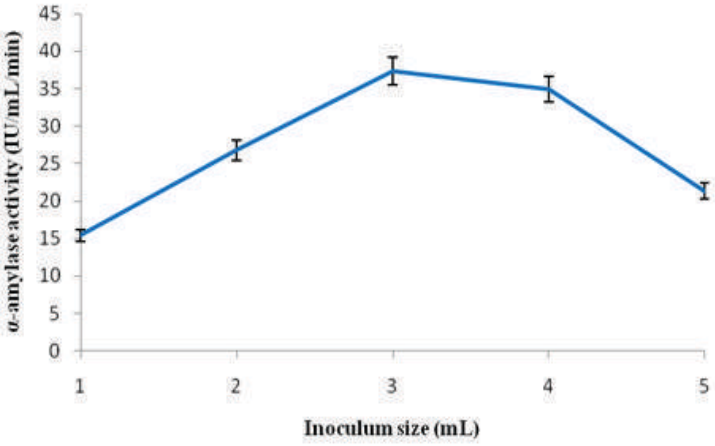

Fig. 5. Effect of inoculums size on production of -amylase by *Aspergillus sydowii*

3.3. Purification of α ***-amylase***

The α-amylase produced at optimized conditions was partially purified by ammonium sulfate precipitation and gel filtration chromatography. The maximum purification was observed at 50% concentration of ammonium sulfate with maximum activity (45.76±1.352 IU/mL/min). When crude enzyme solution was passed through column (Sephadex G-100), the maximum activity (53.71±1.383 IU/mL/min) was observed in elution number VIII. The purification results in decreased in concentration of extra proteins from the mixture. There are certain studies reported the increase in activity of extracellular enzymes after ammonium sulfate precipitation and gel filtration chromatography (Mahmood *et al*., 2013). Purification may enhance the applicability of the industrial enzymes and partial purification is suitable for many industrial processes (Jabbar *et al*., 2008 and Ahmed *et al*., 2008).

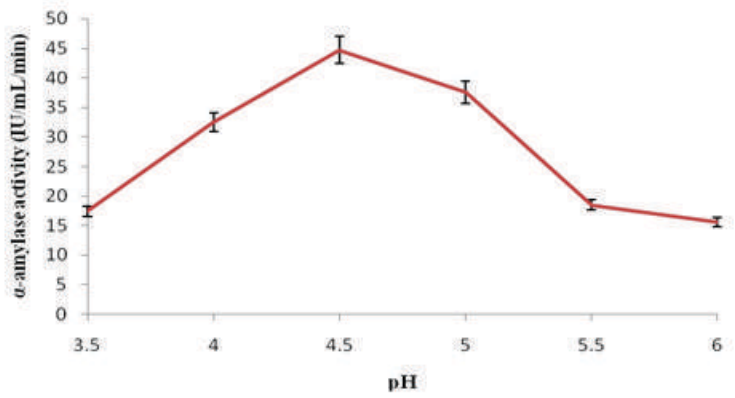

Fig. 6. Effect of varying pH on α-amylase activity

3.4. Characterization of α ***-amylase***

The α-amylase was characterized for optimum pH, optimum temperature and kinetic parameters. To determine optimum pH the activity assay was performed at pH from 3.5 to 6. It was observed that maximum activity (44.72±1.277 IU/mL/min) was obtained at pH 4.5 (Fig.6) which revealed that α-amylase produced by A. sydowii is acidic. To determine optimum temperature of α-amylase produced by A. sydowii activity assay was performed at various temperature 45-65 ^{o}C in duplicate. The results showed that maximum activity (42.48±1.094 IU/mL/min) was observed at 50 ^{o}C (Fig. 7). Further increased in temperature negatively affect the enzyme activity possibly due to de-naturation of enzyme structure. The maximum rate of reaction (Vmax) of α-amylase and substrate concentration that gave half of Vmax (Km) was also determined by catalyzing reaction with various concentrations of substrate.

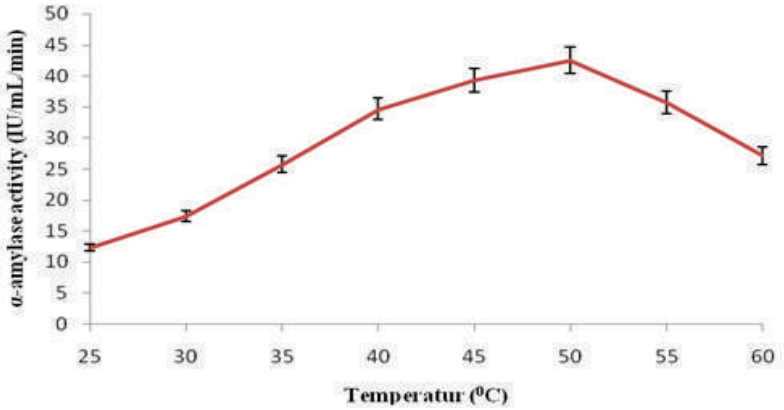

Fig. 7. Effect of varying temperature on α-amylase activity

Activity obtained was used to draw Line-Weaver Burk plot between inverse of substrate conc. and rate of reaction of reaction (Fig. 8). Equation obtained from plot was used to calculated Km and Vmax. The results gave 9.17 µM/mL/min value for Vmax and 1.40 mM value of Km. The results suggested that produced enzyme could be a good industrial enzyme for various applications. Activity at higher temperature by enzymes secreted from thermophillic microorganisms were also reported by other previous studies. Fungal enzymes showed activity at broader pH range which increased the applicability of these enzymes for industrial processes where change in conditions taking place quickly (Wenworth *et al*., 2004; Signh *et al*., 2011; Bukhari and

Rehman, 2015).

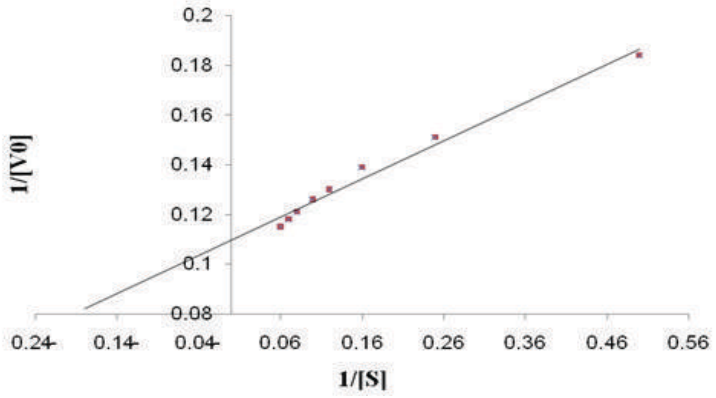

Fig. 8. Line-weaver Burk plot to find out Km and Vmax

4. Conclusion

There was maximum production of α-amylase after 72 hrs at 50 ºC and 5.5 pH. Thermophillic α-amylase isolated from *A. sydowii*, working in acidic conditions shall be very useful for industrial processes like starch cellulose conversion, high fructose corn syrup preparations. Kinetic studies suggested that α-amylase from *A. sydowii* has good affinity for its substrate.

5. References

Ahamed, A. and P. Vermette. 2008. Culture-basedstrategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. Biochem. Eng. J. 40: 399-407.

Asgher, M., F. Bashir and H. M. N. Iqbal. 2013. A Comprehensive Ligninolytic Pre-Treatment Approach From Lignocellulose Green Biotechnology to Produce Bio-Ethanol. Chem. Eng. Res. Des. 92: 1571-1578.

Bukhari, D. A. and A. Rehman. 2015. Purification and characterization of α-amylase from *Bacillus subtilis* isolated from local .nvironment. Pak. J. Zool. 47: 905-911.

Ellaiah, P., B. Srinivasulu and K. Adinarayana. 2002. A review on microbial alkaline proteases. J. Sci. Indus. Res.61: 690-704.

Gouda, M. and Y. Elbahloul. 2008. Statistical optimization and partial characterization of amylases produced by halo tolerant Penicillium Sp. W. J. Agri. Sci. 4 (3): 359-368.

Hung, T.C., R. Giridhar, S. H. Chiou W. T. Wu. 2003. Binary immobilization of *Candida rugosa* lipase on chitosan. J. Mol. Catal. B: Enzym. 26, 69-78.

Jabbar, A., M. H. Rashid, M. R. Javed, R. Perveen and M. Aslam. 2008. Kinetics and thermodynamics of a novel endoglucanase (CMCase) from *Gymnoascella citrine* produced under solid state condition. J. Ind. Microbiol. Biotechnol. 33: 515-524.

Krishnan, S., D. Ganga, K. M. Nampoothiri, C. R. Soccol and A. Pandey. 2006. α-amylases from microbial sources:an overview on recent developments. Food Technol. Biotechnol. 44:173–184.

Kumar, A. and J. S. Duhan. 2011. Production and Characterization of Amylase Enzyme Isolated from *Aspergillus sydowii* MTCC-104 Employing Solid State Fermentation. Int. J. Pharma Biosci. 2(3): 250-258.

Kumar, V., N. R. Sankar. R. Shailaja, K. Saritha, E. Siddhartha, S. Ramya, G. Giridhar and R. V. Sahaja. 2011. Purification and Characterization of α-Amylase Produced by *Aspergillus sydowii* using Banana Peels. J. Cell Tissue Res. 11: 2775-2780.

Kumari, A. and A. M. Kayastha. 2011. Immobilization of soybean (Glycine max) α-amylase onto chitosan and amberlite MB-150 beads: optimization and characterization. J. Mol. Catal. B-Enz. 69: 8-14.

Maarel, V. M. J. E. C., B. Vanderveen, J. C. Uitdehaag, H. Leemhuis and L. Dijkhuizen. 2002. Properties and applications of starch converting enzymes of the α-amylase family. J. Biotechnol. 94:137-155.

Mahmood, R., T. M. J. Asad, N. Mehboob, M. Mushtaq, M. Gulfraz, S. H. Hadri, M. Asgher and N. M. Minhas. 2013. Production, Purification and Characterization of Exoglucanase by *Aspergillus fumigatus*. Appl. Biochem. Biotech. 170: 895-908

Maryam, H., H. R. Seyed, A. S. Seyed, M. M. Seyyed, K. Khosro and S. Mohammad. 2010. Development of a solid state fermentation process for production of an α- amylase with potentially interesting properties. J. Biosci. Bioeng. 110:333-337.

Nooralabettu, K. P. 2014. Optimisation of ammonium sulfate precipitation method to achieve high throughput concentration of crude alkaline phosphatase from Brown shrimp (*Metapenaeus monoceros*) hepatopancreas. Int. J. Anal. Biosci. 2:7-16.

Pandey, A., G. Szakacs, C. R. Soccol, J. A. Rodriguez-Leon and V. T. Soccol, V. T. 2001. Production, purification and properties of microbial phytases. Bioresour. Technol. 77: 203-214.

Pandey, A., P. Nigam. C. R. Soccol, V. T. Soccol and D. Singh. Mohan, R. 2000. Advances in microbial amylases. J. Biotechnol: Appl. Biochem. 31:135-152.

Raul, D., T. Biswas, S. Mukhopadhyay, S. K. Das and S. Gupta. 2014. Production and partial purification of α-amylase from Bacillus subtilis (MTCC 121) using solid state fermentation. Biochem. Res. Int. doi.org/10.1155/2014/568141.

Sundarram, A. and T. P. K. Murthy. 2014. α-Amylase production and applications: a review. J. Appl. Environ. Microbiol. 2: 166-175.

Singh, P. and A. Rani. 2014. Isolation and partial characterization of amylase producing *Bacillus* sp. from Soil. Int. J. Pharm. Technol. Res. 6: 2064–2069.

Singh, A., N. S. Singh, N. Suthar and V. K. Dubey. 2011. Glutaraldehyde-Activated Chitosan Matrix for Immobilization of a Novel Cysteine protease, Procerain B. J. Agric. Food Chem. 59: 6256-6262.

Wentworth, D., S. D. Skonberg. D. W. Donahue and A. Ghanem. 2004. Application of chitosan entrapped β-galactosidase in a packed-bed reactor system. J. Appl. Polym. Sci. 91: 1294-1299.