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Characterization and application of alkaline α-amylase from *Bacillus licheniformis* MTCC1483 as a detergent additive

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Abstract

Alpha-amylases (1-4, α D- glucano hydrolase EC 3.2.1.1) are endoenzymes that hydrolyses α–1, 4- glycosidic linkage in starch and related substrate in an endo-fashion producing oligosaccharides, glucose and α–limit dextrin. To cater the constant growing need of this enzyme in detergent industrial purposes, alkaline amylase secreted by *Bacillus licheniformis* MTCC1483 was studied in order to test its suitability as a detergent additive. It was found to produce 21.6 U/ml of alkaline amylase after 24 hours of incubation. The amylase was further characterized with respect to pH and temperature optima and stability, detergent compatibility. The optimum pH and temperature for amylase activity was found to be 8.0 and 40°C. The enzyme was found to retain around 80% residual activity in the pH range of pH 6.0-10.0. The enzyme showed highest stability at 40°C for 1 h of incubation while at 60°C, 70% of the original activity was lost, respectively. Compatibility of enzyme amylase with certain commercial laundry detergents was also shown to be good as enzyme retained up to 100%, 85% and 55% of their activities after 30 min of incubation at 40°C. Supplementation of the enzyme to the detergent Tide® improved the cleansing ability of the detergent with maximum strain removal in terry cloth followed by cotton fabric stained with potato curry sample. All these properties make this amylase an ideal choice for application in detergent formulations.

Introduction

The industrial enzyme producers sell enzymes for a wide variety of applications. The most widely used industrial enzymes include protease, lipase, cellulase and amylase that remove soils based on proteins, lipids, and polysaccharides. The estimated value of world market is presently about US$ 2.7 billion and is estimated to increase by 4% annually through 2012 (Deb *et al*., 2013). About 75% of industrially produced enzymes are utilized for detergent, textile, starch, baking and animal feed industries. Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (Das, 2011). Detergent enzymes account for about 37% of the total world-wide enzyme production and the detergents industry, regulated by the European Commission (EC) 648/2004, Detergent Directive, is considered as the largest enzyme consuming sector (Hasan *et al.*, 2010). Enzyme based detergents have better cleaning properties as compared to synthetic detergents. The enzyme containing detergents are used as they are environment friendly; completely biodegradable and can also improve the fabric quality and keeping color bright. Amylases catalyze the hydrolysis of starch which is a major polysaccharide used as food ingredients in baby food, spaghetti, custards, sauce, chocolate, pasta and gravy.

Alpha-amylases (1-4, α D- glucano hydrolase EC 3.2.1.1) are extracellular endoenzymes that hydrolyze internal α–1-4- glycosidic linkage in starch and related substrate in an endo-fashion producing oligosaccharides, glucose and α–limit dextrin (Fogarty *et al*., 1999). Alpha-amylases find potential applications in pharmaceuticals, baking, brewing, textile (in desizing fabric), paper, syrup industries and detergent manufacturing processes (Bajpai and Bajpai, 1989; Hewitt and Solomons, 1996; Sivaramakrishnan, 2006). Amylases occur widely in nature but only microbial lipases are commercially significant as they can be produced at low cost and are thermostable and alkalistable. They are usually produced by bacteria belonging to the genus *Bacillus* for industrial applications such as *B. amyloliquefaciens, B. stearothermophilus, B. subtilis* and *Bacillus licheniformis* (Sajedi *et al*., 2005). Recently many reports have been made regarding the washing efficiency of amylases from microbial sources (Joshi, 2011; Sindhu *et al.*, 2011). The present paper deals with characterization of alkaline α-amylase from *Bacillus licheniformis* MTCC1483 and its compatability and potential application in

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detergent industry.

Materials and Methods

The alkaliphilic *Bacillus licheniformis* MTCC1483 for amylase production was procured from MTCC, IMTECH, Chandigarh. Amylase production was carried out in a medium containing (w/v) starch (10%), $MgS0_4$ (0.5%), peptone (5%), yeast extract (2%), $CaCl_2$ (0.15%) and $(NH_4)_2PO_4$ (1%). The flasks were incubated at 30°C for 24 h under shaking. Whole broth was centrifuged at 10000 rpm for 15 minutes at 4°C. The clear supernatant (crude enzyme) was used for estimation of amylase activity. The strain was routinely maintained on nutrient agar slants at 4°C.

Amylase assay

The enzyme activity was assayed following the method of Bernfeld (1995) using 3,5-dinitrosalicylic acid. An enzyme blank with 3,5-dinitrosalicylic acid added before the addition of enzyme was used as control. The amount of reducing sugar released was quantified using 3,5- dinitrosalicylic acid with maltose as standard at 540 nm with a spectrophotometer (UV-Vis, Shimazdu). One unit of alpha amylase activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute from soluble starch at pH 7.0 when incubated at 30°C.

Characterization of amylase from Bacillus licheniformis

Evaluation of pH and temperature optima and stability

The pH and temperature activity profiles for amylase from *Bacillus licheniformis* MTCC1483 was studied in the pH range of 6.0 to 11.0 by mixing the enzyme with sodium phosphate buffer (pH 6.0 and 7.0), Tris buffer (pH 8.0), glycine-NaOH (pH 9.0 and 10.0) and $NaHCO_3$-NaOH buffer (pH- 11.0) in the ratio 1:1 at different temperatures (25- 60°C) at pH 8.0 using starch as substrate.

pH stability profile was studied by pre incubating the crude amylase from *Bacillus licheniformis* for 1 hour with various buffers covering a pH range of 6.0 to 11.0. Samples were withdrawn after every 15 minutes and residual activity (%) was assayed. To determine the temperature stability, crude enzyme preparation was pre incubated in the temperature range of 40 to 60°C for 1 hour 15 minutes time intervals and the residual activity (%) was assayed under standard assay conditions.

Effect of detergents on amylase activity

The compatability of amylase from *Bacillus licheniformis* with several commercially available detergents of Indian market was studied. The tested commercial detergents includes Tide® (Procter & Gamble, USA), Surf excel blue® (Hindustan Lever Ltd., Mumbai, India), Fena Detergent bar® (Fena Pvt. Ltd, New Delhi, India) and Rin® (Hindustan Lever Ltd., Mumbai, India), Ariel® (Procter & Gamble, USA), Admiral Plus® (V.R Industries (P) Ltd., India) were purchased from local market. Detergents at a concentration of 7 mg/ml were incubated with 0.5 ml (10 units) of the alkaliphilic *Bacillus licheniformis* amylase at 40°C for 1 hour. Aliquots (0.5 ml) were withdrawn at intervals of 15 minutes and the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of detergents served as controls.

Washing test with amylase preparations

Wash performance of amylase from *Bacillus licheniformis* in the presence of Surf excel blue detergent on potato curry stains was studied. The fabrics selected were cotton and terrycloth (2x2 inches). The stains on the fabrics were made by placing 100 ul of potato curry sample on the fabric. The fabrics were then left at room temperature and then kept in oven at 60°C for 2 hours so that the potato curry stains could bind to the fabric material firmly. The cloth pieces were washed maintaining four different conditions: Set a-Distilled water (50 ml), Set b-Distilled water (50 ml) + 1 ml of commercial detergent (7 mg/ml), Set c-Distilled water (50 ml) + amylase enzyme, Set d-Distilled water (50ml) + 1 ml of detergent (7 mg/ml) + amylase enzyme. Washing of potato curry soiled fabrics maintaining the above four different conditions was performed for 1 hour in water bath at 40°C and 80 rpm of stirring. After incubation the cloth pieces were taken out from each set, rinsed with water, dried and visually examined. Controls consisted of soiled cloth pieces without enzyme treatment. Aliquot (2 ml) from samples and control were collected after every 15 minutes till 1 hour from the respective setups. The treated and untreated samples were compared both visually and spectrophotometrically.

Results and Discussion

Bacillus licheniformis produced clear zone on incubation at 30°C for 24 h on medium containing starch as substrate suggesting the presence of amylolytic activity. The bacterium accumulates amylase in culture fluid when grown aerobically at 30°C for 24 h in a medium composed of 10% starch. The amylolytic activity of the strain was confirmed

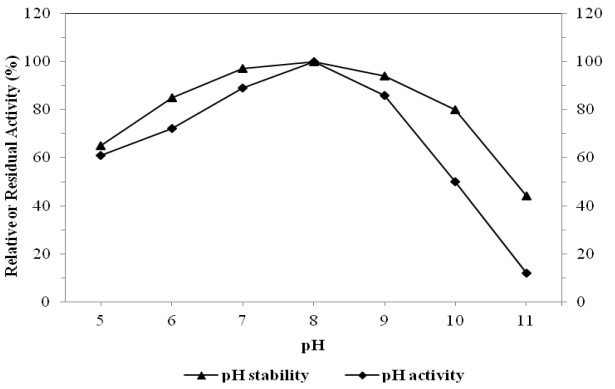

Figure 1. Effect of pH on enzyme activity and stability of MTCC 1483

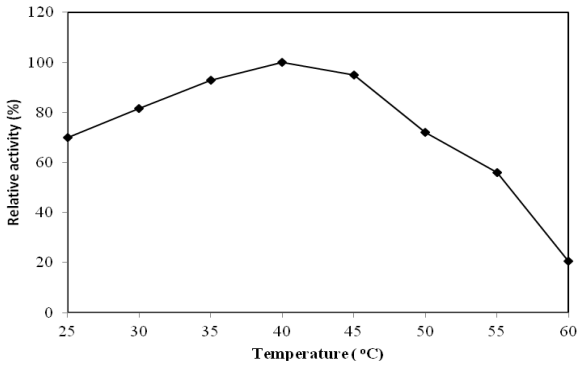

Figure 2. Effect of temperature on the activity of MTCC 1483

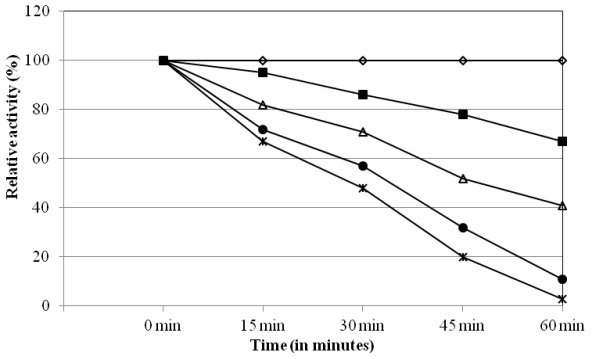

Figure 3. Thermal stability of amylase from *Bacillus licheniformis* MTCC 1483

using soluble starch as an enzyme substrate, which was found to be 21.6 U/ml at 24 h of incubation.

Effect of pH on amylase activity and stability

The amylase from MTCC 1483 was found to be active over pH range of 6.0 to 10.0 with maximum activity at pH 8.0 indicated that the enzyme functioned optimally at pH 8.0. The activity increased linearly from pH 6.0 to 8.0 followed by a drastic decrease in the activity beyond pH 8.0 (Figure 1). Amylases from *Bacillus* species have been reported to show variation in pH optima depending on species and strain. In the present study, the amylase from *Bacillus licheniformis* was found to be relatively stable between pH ranges of 6.0 to 10.0 retaining around 80% activity after 45 min of incubation at 40°C (Figure 1). Comparable high optimum pH and high pH stability for *Bacillus* sp. had been reported by various researchers (Joshi, 2011; Dahiya *et al.*, 2010; Asgher *et al.*, 2007). However, amylase from *Bacillus licheniformis* has an optimal pH value of 8.0 and is stable in an alkaline pH range retaining more than 80% enzyme activity after 45 min of incubation, indicating that the enzyme is an alkalitolerant. Since the amylase was active at alkaline pH, studies on its compatibility and its applications in detergents were undertaken.

Effect of temperature on amylase activity and stability

The amylase from *Bacillus licheniformis* was found to exhibit maximum activity at 40°C and as temperature increased from 40 to 60°C, the enzyme activity showed a declining trend as represented in Figure 2. Thermal stability profile of alpha-amylase from MTCC 1483 was studied in the temperature range of 40 to 60°C at pH 8.0 using starch as substrate. The enzyme showed highest stability at 40°C till 1 hour of incubation, and the least stability at 60°C retaining only 20% of original activity at 60°C after 45 minutes of incubation as observed from Figure 3. Temperature optima of 37 and 40°C were reported from *Bacillus* sp. AB04 (Behel *et al*., 2006) and *Bacillus* sp. (Nusrat and Rahman, 2007) whereas, higher temperature optima of 50 and 55°C was reported by various researchers (Sindhu *et al*., 2011; Dahiya *et al.*, 2010; Suman and Ramesh, 2010). The thermostability of amylase from *Bacillus licheniformis* is comparable to that reported for *Bacillus* sp. AB04 (Behel *et al*., 2006) however, 100% stability has been observed for various other *Bacillus* sp. at higher temperatures (Al-Quadan *et al*., 2009; Das *et al*., 2004; Valaparla, 2010). For the optimal performance of the detergent alpha amylase should have optimal temperature of 40 to 60°C and should have alkaline pH. Thus the enzyme is found to be suitable for application in detergent industry.

Stability of enzyme with commercial detergents

In order to check the compatibility with detergents, the crude amylase from *Bacillus licheniformis* was incubated with detergents at a concentration of 7 mg/ml for 1 h at 40°C. The detergents were previously deactivated for endogenous enzyme. The data presented in Figure 4 showed high stability of enzyme preparation in the presence of all the commercial detergents tested retaining 100 % of its activity in the

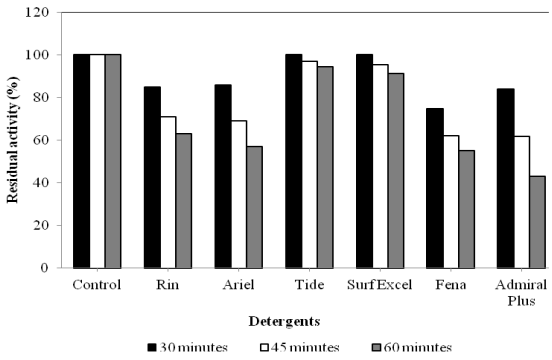

Figure 4. Stability of the amylase from *Bacillus licheniformis* in the presence of various commercial detergents

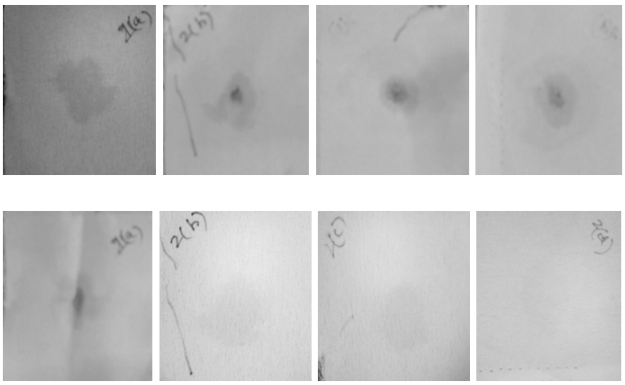

Figure 5. Wash performance of amylase from MTCC 1483 on potato curry stains using terrycloth fabrics at 40°C for 1 h.

A). Untreated (B) Treated. Sample a: Washed with distilled water only, Sample b: detergent with water, Sample c: amylase with water, Sample d: detergent+ water+ amylase.

presence of Tide and Surf excel detergents after 30 minutes of incubation and more than 95 % stability after 45 min of incubation. The crude enzyme retained 85% of its activity in the presence of Rin and Ariel after 30 min of incubation at 40°C. Whereas, Fena and Admiral plus tested retained only 55% and 43% of its activity after 1 h incubation at 40°C. Good numbers of similar reports are available on compatibility and wash performance for amylases. Joshi, (2011) reported *Bacillus circulans* PN5 producing amylase that retains more than 90% activity after exposure for 30 min to all commercial detergents tested. Amylase from *Bacillus licheniformis* NHI (Hmidet *et al.,* 2009) possess excellent stability and compatibility with the various solid and liquid detergents tested. Similarly, Correa *et al*. (2011) reported increase in the activity of α-amylase in the presence of several detergents tested. Strain TSCVKK in the presence of commercial detergents Rin and Surf, whereas Ariel had no effect on activity. High compatability of amylase with commercially available detergents (Henko, Vim and White Giant) where 90% of the original activity of the enzyme was retained was reported by Sindhu *et al.* (2011). Based on the results, amylase from thermostable *Bacillus licheniformis* possessing compatibility with a wide range of commercial detergents can be used for the preparation of various detergent formulations.

Stain removal

In removing potato curry stain from the selected cotton and terrycloth fabrics, it was observed that the amylase possess high capability in removing curry stains from the selected fabrics. Due to its greater efficiency in removing stains from fabric selected it could be used as an alkaline amylase in detergents as powder or solution. As observed from figure, detergent alone or enzyme amylase alone is not effective in removing the potato curry strain from the selected fabrics. It is the combination of amylase to detergent which leads to complete removal of strain from all the fabrics.

Spectrophotometrically it was observed that terrycloth fabric had performed best by removing the stain to the maximum after 45 minutes of incubation at 40°C and 80 rpm of stirring in a water bath. Spectrophotometric comparison compares on the basis of absorbance of sample. Sample showing maximum absorbance signifies that the stain is removed maximally. It was also observed that the removal of stain by Set- A (only distilled water) is very less as observed from figure 5 and is almost equals for selected cotton and terrycloth fabric at all the time interval. In both cotton and terrycloth, maximum strain removal was observed after 45 min of incubation at 40°C. Similar results were noted by Correa *et al.* (2011) who observed that supplementation of Campeiro® with the enzyme preparation improved its cleansing performance for removing egg yolk and tomato sauce stains. *Bacillus subtilis* (MAFE 118079) crude enzyme can effectively remove a variety of strains such as chocolate, bread jam and gravy (Rameshkumar and Sivasudha, 2011).

Conclusion

This work describes the characterization and applicability of amylase preparation as a detergent additive. The amylase preparation showed stability of enzyme at wide temperature range possessing maximum activity at high alkaline pH and at high temperatures. Enzyme stability at wide temperature range indicate its potential to be used in hot as well as cold wash cycles. The crude amylase exhibited high stability in the presence of various commercial

detergents tested. Maximum stain removal was observed for terrycloth fabric where incorporation of enzyme to the detergent improved the cleansing ability of the detergent followed by fabric cotton. Amylase from *Bacillus licheniformis* thus can be considered as a potential candidate to be for application in the detergent industry.

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References

Al-Quadan, F., Akel, H. and Natshi, R. 2009. Characteristics of a novel highly thermostable and extremely thermophilic alkalitolerant amylase from hyperthermophilic *Bacillus* strain HUTBS7J. Online Journal of Biological Sciences 9: 67-74.

Asgher, M., Asad, M. J., Rahmanm, S. U. and Legge, R. L. 2007. A thermostable amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. Journal of Food Engineering 79: 950-955.

Bajpai, P. and Bajpaim, P. 1989. High-temperature alkaline alpha-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnology and Bioengineering 33: 72-78.

Behel, A., Singh, M. K., Sharma, M. K., Puri, P. and Batra, N. 2006. Characterization of alkaline α-amylase from *Bacillus* sp. AB 04. International Journal of Agricultural Biology 8: 80-83.

Bernfeld, P. 1955. Amylases α and β methods. In: Methods in Enzymology, USA, p 149-158. New York: Academic Press.

Correa, T., Moutinho, S. S., Martins, M. and Martins, M. 2011. Simultaneous α-amylase and protease production by the soil bacterium Bacillus sp. SMIA-2 under submerged culture using whey protein concentrate and corn steep liquor: compatibility of enzymes with commercial detergents. Food Science and Technology 31: *http://dx.doi.org/10.1590/S0101-20612011000400003*

Dahiya, P., Arora, P., Chand, S., Chaudhury, A. and Dilbaghi, N. 2010. Characterization of an extracellular alkaline lipase from *Pseudomonas mendocina* M-37. Journal of Basic Microbiology 50: 1-7.

Das, K., Ooley, R. and Mukherjee, A. K., 2004. Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular alpha amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional fermented food of India. Biotechnology and Applied Biochemistry 40: 291-298.

Das, S. 2011. Biotechnological applications of industrially important amylase enzyme. International Journal of Pharma and BioSciences 2: 486-496.

Deb, P., Talukdar, S. A., Mohsina, K., Sarker, P. K. and AbuSayem, S. M. 2013. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. Springer Plus 2: 154-163.

Fogarty, W. M., Dooylc, E. M. and Kelly, C.T. 1999. Comparison of the action pattern of two high maltose forming alpha-amylase on linear malto-oligosaccharides. Enzyme and Microbial Technology 25: 330-335.

Hasan, F., Shah, A., Javed, S. and Hameed, A. 2010. Enzymes used in detergents: Lipases. African Journal of Biotechnology 9: 4836-4844.

Hewitt, C. J. and Solomons, G. L. 1996. The production of alpha-amylase (E.C:3. 2. 1. l.) by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium. Journal of Industrial Microbiology 17: 96-99.

Hmidet, N., El-Hadj, Ali., Haddar, A., Kanoun, S., Alya, S. K. and Nasri, M. 2009. Alkaline proteases and thermostable alpha-amylase coproduced by *Bacillus licheniformis* NHI- Characterization and potential application as detergent additive. Biochemical Engineering Journal 47: 71-79.

Joshi, B. H. 2011. A novel thermostable alkaline α-amylase from Bacillus circulans PN5: Biochemical characterization and production. Asian Journal of Biotechnology 3: 58-67.

Nusrat, A. and Rahman, S. R. 2007. Comparative studies on the production of extracellular amylase by three mesophilic *Bacillus* isolates. Bangladesh Journal of Microbiology 24: 129-132.

Rameshkumar, A. and Sivasudha, T. 2011. Optimization of nutritional constitute for enhanced alpha amylase production using by solid state fermentation technology. International Journal of Microbiology Research 2: 143-148.

Sajedi, R. H., Naderi-Manesh, H., Khajeh, K., Ahmadvand, R., Ranjbar, B. and Asoodeh Moradian, F.2005. A Ca-independent alpha-amylase that is active and stable at low pH from the Bacillus sp. KR-8104. Enzyme and Microbial Technology 36: 666-671.

Sindhu, R., Suprabha, G.N. and Shashidhar, S. 2011. Purification and characterization of alpha -amylase from *Penicillium janthinellum* (NCIM 4960) and its application in detergent industry. Biotechnology Bioinformatics and Bioengineering 1: 25-32.

Sivaramakrishnan, S., Gangadharan, D., Naampoothiri, K. M., Socool, C. R. and Pandey, A. 2006. Alpha amylases from microbial sources- an overview on recent developments. Food Technology and Biotechnology 44: 173-184.

Suman, S. and Ramesh, K. 2010. Production of a thermostable extracellular amylase from thermophilic *Bacillus* species. Journal of Pharmaceutical Sciences and Research 2: 149-154.

Valaparla, V. K. 2010. Purification and properties of a thermostable alpha amylase by *Acremonium sporosulcatum*. International Journal of Biotechnology and Biochemistry 6: 25-34.