Identification a Novel Raw-Starch-Degrading-α-Amylase from a Tropical Marine Bacterium

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Abstract: Problem statement: Bacteria from the surface of the tropical marine hard coral *Acropora* sp. were screened for producing raw-starch-degrading-α-amylase. **Approach:** Based on its 16s rDNA sequence, a bacterium that produced the highest amylolitic activity was identified as *Bacillus amyloliquifaciens* ABBD. The bacterial isolate secreted a α-amylase extracellularly and then the enzyme was partially purified by ammonium sulfate precipitation followed by anion exchange chromatography. **Results:** Electrophoresis results both SDS-PAGE and native PAGE suggested that the enzyme was a heterodimeric protein (97 kDa) consisting of 45 and 55 kDa subunits. The α-amylase had an optimum pH of 7.0 and temperature of 60°C. More than 80% activity of the enzyme was retained under high salt conditions (up to 20% NaCl). The enzyme remained stable at 50°C for 1 h. Starch hydrolysis by the enzyme at 70°C yielded oligosaccharides (G2-G4) and at room temperature yielded glucose/maltose (G1 and G2). **Conclusion:** The *B. amyloliquifaciens* ABBD α-amylase was capable of degrading various raw starch granules from corn, rice, cassava and sago at room temperature.

Key words: α-amylase, amylolytic enzymes, *Bacillus amyloliquifaciens*, C-terminal domain, Glycosyl Hydrolase (GH), hard coral *Acropora* sp., marine bacterium, Marine Broth (MB), Thin Layer Chromatography (TLC), yielded oligosaccharides

INTRODUCTION

α-Amylases (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) hydrolyze starch and related 1,4-linked polysaccharides by cleaving internal α-1,4 glycosidic bonds releasing α-anomer products. α-Amylase is among the most important industrial enzymes as it is used in sugar-, brewing-, textile-, detergent-and bioethanol industries (Reichelt, 1983; Guzmán-Maldonado *et al*., 1995; Pandey *et al*., 2000; Van Der Maarel *et al*., 2000; Gupta *et al*., 2003). Thus, α-amylases with industrially desirable characteristics, such as wide range pH- and salt stability (Al-Quadan *et al.*, 2009) and the capability to digest raw starch at room temperature are of utmost importance. Hence, worldwide efforts for searching microorganisms producing α-amylases with such properties are being undertaken.

According to Henrissat (1991), α-amylases belong to the Glycosyl Hydrolase (GH) family 13 (http://afmb.cnrs-mrs.fr/CAZY/fam/GH13.html). The structural features of the family include the core domain (domain A) consisting of a $(\beta/\alpha)_8$ barrel, the domain B protruding from domains A third β-strand and the C-terminal domain (C) with a Greek key motif. Owing to the incapability to penetrate crystalline structures, only few amylolytic enzymes are capable of attacking raw starch granules. The latter enzymes possess the starch binding domain predominantly at their C-termini (for review see (Machovic and Janecek, 2006).

α-Amylases degrading raw starch granules have been reported to occur in a wide range of organisms (Demirkan *et al*., 2005; Matsubara *et al*., 2004; Iefuji *et al*., 1996; Mehrabadi and Bandani, 2009a,2009b), however, few reports on α-amylases from marine sources are known (Vidilaseris *et al.*, 2009). Here we report a hard coral associated bacterium producing raw-starch- degrading-α-amylase, *Bacillus amyloliquifaciens* ABBD. The enzyme properties such as quaternary structure, biochemical features and its action on raw starch granules as well as its hydrolysis pattern are described.

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MATERIALS AND METHODS

Bacterial isolate and culture condition: Of three isolates obtained from the surface of the hard coral *Acropora* sp. collected from Bandengan Water, Jepara, North Java, Indonesia by scuba diving at depth of approximately 5 m, the bacterial strain ABBD displaying highest starch degrading activity was chosen for this study (Fig. 1). The isolate was routinely maintained on Marine Broth (MB) medium containing 0.25% bacto peptone, 0.05% yeast extract and 0.1% soluble starch (Merck) in sea water. The culture was grown in a shaker incubator (New Brunswick Scientific model C25KC) at 37°C and 150 rpm for 18 h.

Identification of 16S rDNA: Bacterium grown to the stationary phase was harvested by centrifugation at 5000×g for 10 min at 4°C. Genomic DNA was isolated and purified using the Wizard Genomic Purification Kit (Promega). Degenerated forward- and reverse primers for amplification of the 16S DNA were 5'-AGAGTTTGATC (A/C) TGGCTCAG-3' (BactF1) and 5'-GGTTAC(G/C)TTTGTTACGACTT-3' (UniB1), respectively. PCR amplification was performed in a final concentration of 25 µL, containing 5 ng chromosomal DNA, 0.8 µm of each primer, 0.4 mm dNTP, 0.025 U Taq DNA polymerase, 50 mm KCl, 1.5 mm $MgCl_2$ and 10 mm Tris-HCl, pH 8.3. The following profile was applied for the PCR: DNA-denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and elongation at 72°C for 2 min. The initial and final cycle included denaturation at 94°C for 4 min and elongation at 72°C for 7 min to ensure full extension of the product. Amplified PCR products were analyzed in a 1% agarose gel and nucleotide sequences determined by the method of dye-end terminator using BactF1 and UniB1 as forward and reverse primer, respectively, performed by Macrogen Inc., (Seoul, Korea). The 16S rDNA sequence of the isolate was aligned with those in the GenBank database. Multiple alignments of sequences and calculation of the level of sequence similarity were carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The neighbor-joining phylogenetic analysis was performed by applying the MegAlign DNAstar program.

Enzyme preparation: To the supernatant obtained from bacterial cultures, ammonium sulfate was gently added to reach 80% saturation, followed by centrifugation at 22.000×g for 30 min at 4°C. The precipitate was dissolved in a minimum volume of 25 mm Glycine-NaOH buffer, pH 9.0 and dialyzed against the same buffer. The supernatant was loaded onto a Resource-Q column (Pharmacia) equilibrated with 25 mm Glycine-NaOH buffer, pH 9.0. After washing and

Fig. 1: Strain ABBD showing amylolytic activities. The strain cultured in MB agar containing 2% bacto agar at 37°C overnight, was stained by iodine solution containing 2% KI and 0.2% I_2. A clear zone on a blue background showed amylolytic activities

removing unbound proteins, the column was eluted by applying a linear gradient from 0-0.5% NaCl in the same buffer at a flow rate of 6 mL min^{-1}. Active fractions were pooled and concentrated using a filter membrane (GE Healthcare) (30 kDa molecular weight cut off) and used for the characterization.

Enzyme activity assay: Amylolytic activities were measured by the method of Fuwa (1954): 50 µL of soluble starch in 25 mm Glycine-NaOH buffer, pH 9.0 was mixed with 50 µL of enzyme solution. After incubation at 60°C for 10 min, the enzymatic reaction was terminated by the addition of 50 µL 1 M HCl. To the reaction mixture, 50 µL iodine solution containing 2% KI and 0.2% I_2 and 800 µL 25 mm Glycine-NaOH buffer, pH 9.0 was added. Absorption was measured at 600 nm and the activity was calculated using soluble starch as the standard. Hydrolysis of 1 mg mL^{-1} soluble starch in 1 min was defined as 1 U mL^{-1} of enzyme activity. Specific activity is given as U mg^{-1} protein.

Protein determination: Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoresis: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) under non reducing conditions was carried out according to Laemmli (1970) using 10% acrylamide gels. Electrophoresis was run at 150 V for 60 min at room temperature. After electrophoresis, a part of the gel was silver stained. To identify amylase band (clear zone on a blue background), the other part of gel was incubated for 45 min at 70°C in 1% soluble starch

containing 25 mm Glycine-NaOH buffer, pH 9.0 and then stained with iodine solution.

Non-denaturing gel electrophoresis (native-PAGE) was performed similar to the SDS-PAGE procedure but lacking sodium dodecyl sulfate. Zymogram staining was performed by incubating the gels for 45 min at 70°C in 1% soluble starch in 25 mm Glycine-NaOH buffer, pH 9.0 and then stained with iodine solution.

Effect of pH on enzyme activity: The optimal pH of the α-amylase was determined by standard assays at different pH values (4.0-10.0). The pH solution was adjusted using a JBScreen pH-2D buffer system (Jena Bioscience). The buffer contained succinic acid, sodium dihydrogen phosphate, glycine (2:7:7) and was adjusted with 1 m NaOH to reach the appropriate pH.

Effect of salinity on enzyme activity: The effect of NaCl on α-amylase activity was determined by the enzyme at different NaCl concentrations (0-20%) under standard assay conditions at pH 7.0

Effect of temperature on enzyme activity and stability: To determine the optimum temperature for the α-amylase activity, assays were performed at 30, 40, 50, 60, 70 and 80°C. For checking thermal stability, enzymes were heated for 15-60 min at 40, 50, 60, 70 and 80°C. Subsequently, samples were immediately cooled on ice and the residual activities were determined.

Determination of degradation products: Thin Layer Chromatography (TLC) was employed for identification of oligosaccharides in starch hydrolysates (Hansen, 1975). Briefly, each sample containing starch hydrolysates was spotted on a silica gel plate and then developed in a chromatographic jar in a solvent system of butanol, ethanol and water (5:5:3) until the solvent reached the top of the plate. After drying at room temperature to get rid of the solvent, plates were sprayed with 50% concentrated H_2SO_4 in methanol and subsequently dried in an oven at 110°C for 10 min to develop the black color of the spots. Sugars (G1-G7) were used as markers.

Degradation of raw starch granules: Amylolytic activity on raw starch granules from rice, corn, cassava and sago was checked by mixing 10% of the respective raw starch suspension in a volume ratio of 1:1 with the enzyme solution. Reaction mixtures were incubated at 37°C for 4 days, then centrifuged and the pellet was dried in an oven at 50°C. The pellets were photographed using a scanning electron microscopy (SEM Jeol JSM6063lA0).

RESULTS AND DISCUSSION

The bacterium characteristics: During the microbial screening process -of the three culturable isolates obtained from the surface of the hard coral *Acropora* sp.- strain ABBD was found to exhibit the highest amylolytic activities (Fig. 1). Cells of strain ABBD positively reacted in the Gram-staining procedure and had a rod-like shape. Sequences of two PCR products obtained by applying primers BactF1 and UniB1 and chromosomal DNA of the strain ABBD as a template yielded the almost complete nucleotide sequence of the 16S rDNA of 1414 bases. The sequence has been deposited in the GenBank database under accession no FJ654647.

By performing a BLAST search within the GenBank database, the most similar sequences were identified. Subsequently, based on selected sequences of close neighbors, a phylogenetic tree was constructed (Fig. 2). At the close of the analysis *B. amyloliquefaciens* strains GH58 and GH28 (Meerak *et al.*, 2008) were identified as being almost identical, i.e. with 99% identity. Thus, with respect to the phylogenetic tree of the 16S rDNA obtained by applying the clustal method (Higgins and Sharp, 1989), strain ABBD is considered to be a representative of the species *B. amyloliquefaciens*.
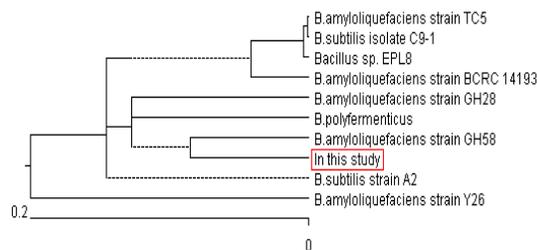

Fig. 2: Phylogenetic tree based on virtually complete 16S rDNA sequence data. The GenBank accession numbers of *B. amyloliquefaciens* strain TC5, *B. subtilis* isolate C9-1, *Bacillus* sp. EPL8, *B. amyloliquefaciens* strain BCRC 14193, *B. amyloliquefaciens* strain GH58 *B. amyloliquefaciens* strain GH28, *B. polyfermenticus*, *B. subtilis* strain A2 and *B. amyloliquefaciens* strain Y26 are AB300808, EU257446, EU359775, EF433408, AB301024, AB301002, AY149473, EU567068 and AB300820, respectively. The scale bar corresponds to 0.2 substitutions per base position
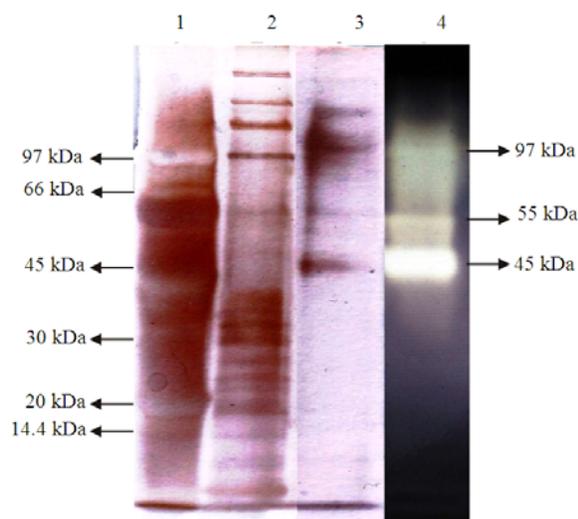
Fig. 3: SDS-PAGE profile and its zymogram of BAA. Lane 1, 2 and 3 represent SDS-PAGE of protein marker, crude enzyme and partially purified enzyme, respectively; whereas lane 4 indicates zymogram of partially purified enzyme

Quaternary structure of the α-amylase: Since the *B. amyloliquefaciens* α-amylases are routinely extracellular enzymes, it was possible to partially purify the enzyme from the supernatant of the isolate; we applied a strong anion exchange column (Resource-Q column, Pharmacia) for such purpose. To determine quaternary structure of α-amylase, SDS-PAGE and Native PAGE were employed, together with their zymogram to observe the α-amylase bands.

SDS-PAGE result under non reducing conditions showed three protein bands with estimated molecular masses of 97, 55 and 45 kDa, respectively. All of the protein bands displaying amylolytic activities were detected from their zymogram (Fig. 3).

However, the zymogram of native-PAGE result identified only a single amylolytic protein with a molecular mass of 97 kDa (Fig. 4). Our findings suggested that the quaternary structure of the α-amylase of the *B. amyloliquefaciens* strain ABBD (hereafter called BAA) was a heterodimeric complex consisting of two different subunits: 55 and 45 kDa. Apparently, BAA molecules under denaturing conditions such as in SDS-PAGE were partially separated into its subunits. Most interestingly, after incubation in the non denaturing buffer, BAA refolded and showed amylolytic activities for both, in each of the subunits and in the complex. In terms of heterodimeric complex formation of a quaternary structure, the BAA was a novel α-amylase that differed from all BAA so far known, as most were monomeric (Table 1).
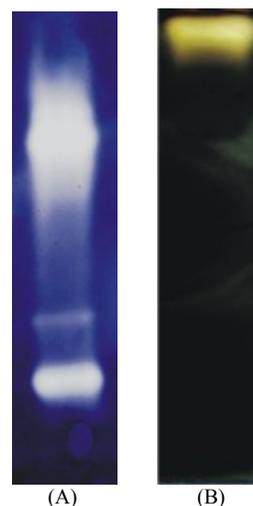

Fig. 4: Zymogram of BAA on SDS-PAGE under non reducing condition (A) and native-PAGE (B)

The α-amylase properties: The BAA exhibited an optimum pH of 7.0 (Fig. 5A). The enzyme was relatively stable in acidic conditions with at least 40% of the activity retained at pH 4.0-5.0; and more than 50% of the activity was observed in the range of pH 6.0-9.0. Such characteristics may be important for industrial processes in which a broad pH-range is needed. It was noteworthy to state that the total loss of activity occurred at pH 10.0.

The effect of NaCl on the BAA is depicted in Fig. 5B. The enzyme retained more than 80% of its activity in NaCl up to 20%. As the bacterium was isolated from the surface of a hard coral from the sea, the halophilic properties of the enzyme matched our expectations. The enzyme activity that could adapt to a wide range of salt concentrations might be important for industrial application.

Optimal temperature of the BAA occurred at 60°C (Fig. 5C). At least 50% of the activity was detectable at temperatures between 40 and 70°C (the enzyme was kept under such temperatures for 10 min). However, more than 80% loss of activity was observed at temperatures above 80°C. Based on temperature characteristic, the BAA can be considered as a mesophilic enzyme.

Thermal stability of the BAA at pH 7.0 is given in Fig. 5D. The enzyme retained more than 60% activity at 50°C for 1 h. Consistent with its mesophilic character, only half of the BAA activity was obtained when the enzyme was incubated at 60°C for 1 h and its activity completely lost at 80°C for 30 min.

Table 1: Comparison of characteristics of some of BAA from different strains or sources

| Strains/ sources	Mr (kDa), subunit(s)	pI	Optimum pH	Optimum temperature (°C)	Temperature stability (°C)	Reference
ABBD	97, heterodimer	NA	7.0	60	50	This study
158	68, monomer	6.25	6.0	65	65	(Kochhar and Dua, 1990)
Onion powder isolate	60, monomer	5.2	5.5	50-70	40	(Granum, 1979)
ATTCC 23842	58, monomer	NA	5.0	50	40	(Gangadharan *et al.*, 2009)
Turkish soil isolate	52, monomer	NA	6.0	55	40	(Demirkan *et al.*, 2008)
NCIM 2829	67.5, monomer	NA	NA	NA	NA	(Mithu *et al.*, 2005)
BAN, Novozyme	55.8, monomer	NA	NA	NA	NA	(Takkinen *et al.*, 1983)

NA = not available
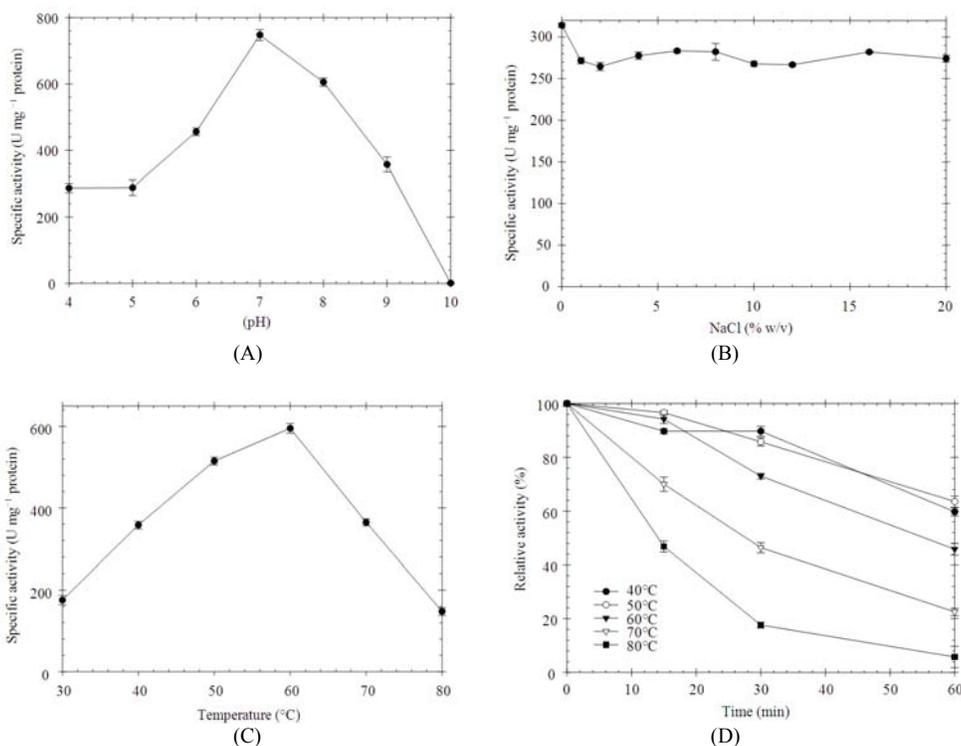
Fig. 5: Effects of pH (A), NaCl concentration (B), temperature (C) and thermal stability (D) on BAA activity. Data were the average of three different experiments

Starch degradation profiles: To understand hydrolysis pattern of soluble starch by BAA, the TLC was performed. An early reaction product of starch digestion at 70°C yielded maltose (G2) to maltoheptaose (G7), whereas small amounts of glucose (G1) were observed (Fig. 6A). The end products of starch digestion at 70°C were predominantly G2, G3 and G4, with minor amount of G1. Interestingly, starch hydrolysis by the enzyme at room temperature yielded predominantly G1 and G2 (Fig. 6B). With respect to the major products of hydrolysis, the BAA action is useful for starch liquefying, short-chain-oligosaccharide forming, or starch saccharifying.

Degradation patterns towards various raw starch granules by BAA at room temperature were observed by the SEM (Fig. 7). BAA degraded corn- and sago granules as digging means (from the surface to the center) producing holes on the granules. The enzyme hydrolyzed rice- and cassava granules as peeling ways (scattered over the surface). Adsorption of enzymes on starch granules remains unclear; however, some studies of amylases showed the important role of the C-terminal domain of α-amylase for starch binding (Machovic and Janecek, 2006). For industrial use, the capability of BAA to degrade raw starch granules at lower temperatures may give advantages owing to it will reduce the cost of energy. The relative activity of the BAA on raw starch granules from corn, rice, cassava and sago was 100, 34, 30 and 18%, respectively.
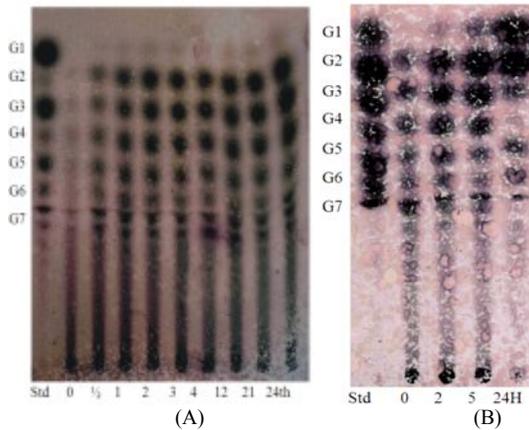
Fig. 6: Time course of digestion of soluble starch by BAA at 70° (A) and room temperature (B). Std: G1, G2, G3, G4, G5, G6 and G7 denote glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose, respectively
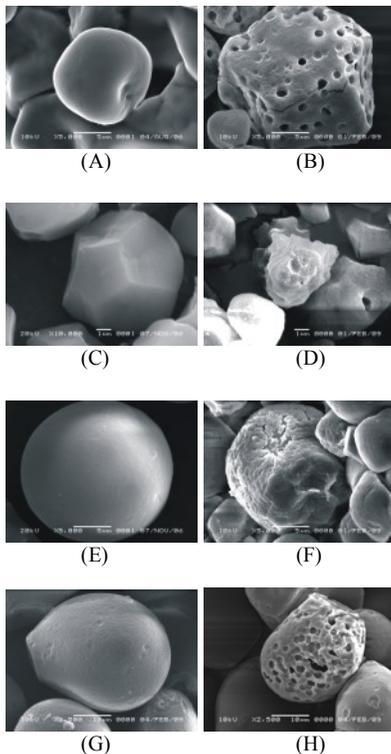

Fig. 7: Scanning electron microscopy of untreated and treated raw starch granules by BAA. A,B: corn; C,D: rice; E,F: cassava; and G,H: sago. Untreated raw starch granules were A, C, E and G; whereas treated raw starch granules by BAA were B, D, F and H

CONCLUSION

The present study has yielded a raw starch degrading α-amylase produced by a hard coral associated bacterium, *B. amyloliquifaciens* strain ABBD. In terms of heterodimeric complex formation, the BAA from strain ABBD is a novel α-amylase differing from the published BAA. The α-amylase of the *B. amyloliquifaciens* strain ABBD showing activities in a broad pH-range, in high salt concentrations, at lower temperatures as well as on raw starch granules may be a promising candidate for starch processing industries. To understand the mechanism of the enzyme activity, further research will focus on cloning and overexpressing gene(s) encoding BAA, as well as on structural studies of the enzyme.

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REFERENCES

Al-Quadan, F., H. Akel and R. Natshi, 2009. Characteristics of a Novel Highly Thermostable and Extremely Thermophilic Alkalitolerant Amylase from Hyperthermophilic Bacillus Strain HUTBS71. OnLine J. Biol. Sci., 9: 67-74. DOI: 10.3844/ojbsci.2009.67.74

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254. DOI: 10.1016/0003-2697(76)90527-3

Mithu, D., P.D. Kali and P.K. Chakrabartty, 2005. Purification and characterization of α-amylase from *Bacillus amyloliquefaciens* NCIM 2829. Indian J. Biochem. Biophys., 42: 287-294.

Demirkan, E.S., B. Mikami, M. Adachi, T. Higasa and S. Utsumi, 2005. α-Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. Process Biochem., 40: 2629-2636. DOI: 10.1016/j.procbio.2004.08.015

Fuwa, H., 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. Tokyo, 41: 583-603.

Gangadharan, D., K.M. Nampoothiri, S. Sivaramakrishnan and A. Pandey, 2009. Biochemical characterization of raw-starch-digesting α-amylase purified form *Bacillus amyloliquefaciens*. Applied Biochem. Biotechnol., 158: 653-662. DOI: 10.1007/s12010-008-8347-4

Granum, P.E., 1979. Purification and physicochemical properties of an extra cellular amylase from a strain of *Bacillus* amyloliquefaciens isolated from dry onion powder. J. Food Biochem., 3: 1-12. DOI: 10.1111/j.1745-4514.1979.tb00631.x

Gupta, R., P. Gigras, H. Mohapatra, V.K. Goswami and B. Chauhan, 2003. Microbial α-amylases: A biotechnological perspective. Process Biochem., 38: 1599-1616. DOI: 10.1016/S0032-9592(03)00053-0

Guzmán-Maldonado. H., O. Paredes-López and C.G. Biliaderis, 1995. Amylolytic enzymes and products derived from starch: a review. Crit. Rev. Food Sci. Nutr., 35: 373-403. DOI: 10.1080/10408399509527706

Hansen, S.A., 1975. Thin-layer chromatographic method for identification of oligosaccharides in starch hydrolyzates. J. Chromatogr., 105: 388-390. DOI: 10.1016/S0021-9673(01)82270-6

Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J., 280: 309-316.

Higgins, D.G. and P.M. Sharp, 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci., 5: 151-153. DOI: 10.1093/bioinformatics/5.2.151

Iefuji, H., M. Chino, M. Kato and Y. Iimura, 1996. Raw-starch-digesting and thermostable α-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. Biochem. J., 318: 989-996. PMCID: PMC1217715

Kochhar, S. and R.D. Dua, 1990 Thermostable liquefying α-amylase from *Bacillus amyloliquefaciens*. Biotechnol. Lett., 12: 393-396. DOI: 10.1007/BF01024438

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685. DOI: 10.1038/227680a0

Machovic, M. and S. Janecek, 2006. Starch-binding domains in the post-genome era. Cell. Mol. Life Sci., 63:2710–2724. DOI: 10.1007/s00018-006-6246-9

Matsubara, T., Y.B. Ammar, T. Anindyawati, S. Yamamoto and K. Ito *et al.*, 2004. Degradation of raw starch granules by α-amylase purified from culture of *Aspergillus awamori* KT-11. J. Biochem. Mol. Biol., 37: 422-428. PMID: 15469729

Meerak, J., P. Yukphan, M. Miyashita, H. Sato and Y Nakagawa *et al.*, 2008. Phylogeny of γ-polyglutamic acid-producing *Bacillus* strains isolated from a fermented locust bean product manufactured in West Africa. J. Gen. Appl. Microbiol., 54: 159-166. PMID: 18654037

Mehrabadi, M. and A.R. Bandani, 2009a. Assessing of α-amylase activity of midgut in wheat bug *Eurygaster maura*. Am. J. Applied Sci., 6: 478-483. DOI: 10.3844/ajassp.2009.478.483

Mehrabadi M. and A.R. Bandani, 2009b. Study on salivary glands α-amylase in wheat bug *Eurygaster maura* (Hemiptera: Scutelleridae). Am. J. Applied Sci., 6: 555-560. DOI: 10.3844/ajassp.2009.555.560

Pandey, A., P. Nigam, C.R. Soccol, V.T. Soccol and D. Singh *et al.*, 2000. Advances in microbial amylases. Biotechnol. Applied Biochem., 31:135-152.

Reichelt, J.R., 1983. Industrial Application. In: Industrial Enzymology, Godfrey, T. and J.R. Reichelt (Eds.). Nature Press, New York, pp: 375-379.

Takkinen, K., R.F. Pettersson, N. Kalkkinen, I. Palva and H. Söderlund *et al.*, 1983. Amino acid sequence of α-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. J. Biol. Chem., 258: 1007-1013.

Van Der Maarel, M.J.E.C., B. van der Veen, J.C.M. Uitdehaag, H. Leemhuis and L. Dijikhuizen, 2002. Properties and applications of starch-converting enzymes of the α-amylase family. J. Biotechnol., 94: 137-155. DOI: 10.1016/S0168-1656(01)00407-2

Vidilaseris, K., K. Hidayat, D.S. Retnoningrum, Z., Nurachman, A.S. Noer and D. Natalia, 2009. Biochemical characterization of a raw starch degrading α-amylase from the Indonesian marine bacterium *Bacillus* sp. ALSHL3. Biologia, 64: 1047-1052. DOI: 10.2478/s11756-009-0190-8