MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8587
Vol 6, No 2, June 2012, p 83-88

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.6.2.5

SHORT COMMUNICATION

Isolation and Identification of A Thermostable Amylase-Producing Bacterium from Hatuasa Hotspring

DOMINGGUS MALLE[1]*, JUNUS PICARIMA[2], LAURY CHARA HUWAE[2], INDRA RAHMAWATI[3], AND WAHYU PURBOWASITO[3]

[1] *Faculty of Agriculture, Pattimura University, Jalan Ir M Putuhena, Kampus Poka Ambon, Ambon 97233, Indonesia;*
[2] *Faculty of Mathematics and Natural Sciences, Pattimura University, Jalan Ir M Putuhena, Kampus Poka Ambon, Ambon 97233, Indonesia;*
[3] *Center for Biotechnology , BPPT, Puspiptek-Serpong, Tangerang Selatan, Indonesia*

Hot springs are a common source of thermophyles which produce thermostable enzymes. The objective of this study was to isolate and identify thermostable amylase-producing bacteria from a local geothermal spring. An amylase–producing bacterium strain was isolated from this hot spring which excreted amylase after being grown on starch agar screening plates at 37 °C. It was identified as *Bacillus megaterium* using the method of 16S ribosomal DNA. The organism is a rod-shape and is a spore-forming bacterium. Maximum amylase production was achieved after incubation in the production media for 72 h. Preliminary analysis of the secreted amylase showed that the enzyme could bind to DEAE-Sepharose matrix and was discharged by eluting with 0.5 M NaCl. The partially purified enzyme was stable up to 75 °C, showing that this enzyme might have potential application in the starch-processing industry.

Key words: *Bacillus*, hotsprings, isolation, thermostable amylase

Sumber air panas merupakan habitat umum mikroba termofilik yang menghasilkan enzim-enzim termostabil. Tujuan penelitian ini adalah untuk mengisolasi dan mengidentifikasi bakteri penghasil amilase termostabil dari sumber mata air panas lokal. Satu strain bakteri penghasil enzim amilase berhasil diisolasi. Bakteri tersebut menghasilkan enzim amilase setelah ditumbuhkan pada suhu 37 °C pada media agar yang mengandung pati. Hasil analisis gen subunit 16S rRNA menunjukkan bahwa bakteri tersebut adalah *Bacillus megaterium*. Bakteri tersebut berbentuk batang dan menghasilkan spora. Produksi enzim amilase maksimum diperoleh setelah bakteri dikultivasi pada media produksi selama 72 jam. Analisis pendahuluan enzim yang disekresi menunjukkan bahwa enzim tersebut terikat pada matriks DEAE-Sepharose dan terlepas dari matriks setelah dielusi dengan larutan NaCl 0,5 M. Amilase dari fraksi NaCl 0,5 M tetap aktif hingga suhu 75 °C. Hal ini menunjukkan bahwa enzim amilase ini mungkin memiliki potensi untuk digunakan pada industri pengolahan tepung pati.

Kata kunci: amilase termostabil, *Bacillus*, isolasi, sumber air panas

Starch is a common reserve energy store of plants and is one of the most abundant biopolymers on earth. It is a polymer of α-glucose units connected with α-1,4- and α-1,6-glycosidic linkages. Starch consists of amylose, a linear polymer of glucose, (15-25%) and amylopectin, a branched glucose polymer, (75-85%). Due to its complex structures, hydrolysis of starch requires a combination of hydrolytic enzymes to release glucose units and small oligosaccharides (Bertoldo and Antranikian 2001). To hydrolyze starch completely into 6-carbon glucose units, at least 2 different enzymes are needed, namely, (1) an enzyme which breaks down α-1,4 linkages such as α-amylase, α-glycosidase, and β-amylase; and (2) enzyme that hydrolyzes α-1,6 linkages such as pullulanase and isoamylase (Van der Maarel *et al.* 2002). Enzymatic hydrolysis (saccharification) of starch is usually carried out at relatively high temperature conditions between 80-90 °C. Hence, heat-resistant enzymes are required (Burhan *et al.* 2003). Thermostable amylase has various commercial applications related to starch processing including brewing, natural sweetener production, textile and paper production, bioethanol production, and detergent manufacture. This type of enzyme constitutes about 25% of the total enzymes needed in industrial processes (Sidhu *et al*. 1997; Pandey *et al*. 2000). Due to its promising applications, the search for new sources of thermostable amylase continues. Bacteria have become important sources of thermostable amylase for industrial application because they produce amylases with greater

*Corresponding author; Phone: +62-911-322653 ; E-mail: minggus@gmail.com

thermostability properties as compared to fungal amylases. In addition, the genera *Bacillus* is the major source for the production of thermostable amylase and they have been widely used in commercial applications (Prakash and Jaiswal 2010).

Bacillus megaterium has attracted attention from biotechnologists due to the scientific fact that this bacterium is non-pathogenic and found in the soil and hot water springs. This species has become of special interest because of its promising biotechnological applications such as enzyme production (Oyeleke *et al.* 2010; Gurudeeban *et al.* 2011; Sajitha *et al.* 2011), recombinant protein production (Stammen *et al*. 2010) and recombinant antibody fragment productions (Jordan *et al*. 2007). Another advantage of this bacterium is that it does not produce alkaline proteases (Rygus and Hillen 1991). The Province of Maluku possesses a lot of hot springs. Hence, it is possible that bacteria which can produce thermostable amylase exist there. To our knowledge, there is no investigation yet regarding the study of thermophyles isolated from those locations. In this study, we aimed to isolate and identify a bacterium that produced a thermostable amylase from a Maluku hot spring.

Microbes were isolated from Hatuasa hotsprings, Tulehu village, District of Central Maluku. A 50 µL aliquot of sample was spread on Luria-Bertani (LB) agar (Difco) plate sumpelented with 1% w/v of starch (from potato; Sigma, USA) as the sole carbon source and then incubated at 37 °C overnight. A colony that produced the largest clear zone after iodine staining was isolated and grown onto another starch-containing LB agar plate for futher isolation and regeneration. The morphological and biochemical characterizations of the amylase-producing isolate were determined by the electron microscope and enzymatic tests, respectively. The isolated bacterial strain was designated as "HAT1". This bacterium has capability of hydrolyzing starch in LB-agar plate containing 1% w/v soluble starch as indicated by the presence of a clear zone on the plate after iodine staining. The colony forming the largest clear zone was taken for further isolation steps to obtain a single colony. The target colony was assayed for its morphological properties which showed that it is a rod-shape and spore-forming bacterial strain (Fig 1).

For further identification, the 16S rRNA gene of the isolate was amplified using polymerase chain reaction (PCR) method from the genomic DNA. Genomic DNA was isolated using InstaGene kit following the manufacturer's instructions. Two universal primers for bacterial 16S rRNA gene, 8F (forward) 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse) 5'-GGTTACCTTGTTACGACTT-3' were used to amplify the target gene (Gee *et al*. 2003). PCR amplification was carried out using a PCR Thermal Cycler (TAKARA, Japan) with a thermal profile: denaturation of 96 ºC for 3 min, and 30 cycles of 96 ºC for 45 s (denaturation), 56 ºC for 30 s (annealing) and 72 ºC for 2 min (polymerization), followed by the last cycle of polymerization at 72 ºC for 7 min. The PCR product was electrophoresed on 1% w/v agarose gel and visualized after ethidium bromide staining. The PCR band was excised and extracted using a GeneAid Kit following the procedure provided. The purified PCR product was sequenced using ABI PRISM 3130x1Genetic Analyzer (Applied Biosystem, USA). PCR amplification showed that a fragment about 1500 bp long appeared on 1% w/v agarose gels using its genomic DNA as template. Sequencing analysis of this fragment resulted in a partial sequence of about 996 bp long. The partial sequence of the 16S rRNA gene obtained was compared with known bacterial 16S rRNA genes using BLAST at NCBI (www.ncbi.nih.nlm.gov/BLAST/), showing 99% homology with *Bacillus megaterium*. Prior to phylogenetic analysis, the nucleotide sequence was aligned with other related bacteria using ClustalW. An evolutionary distance tree was generated using the Neighbor-joining algorithm as implemented in the MEGA version 5.0 Software (Tamura *et al*. 2011).

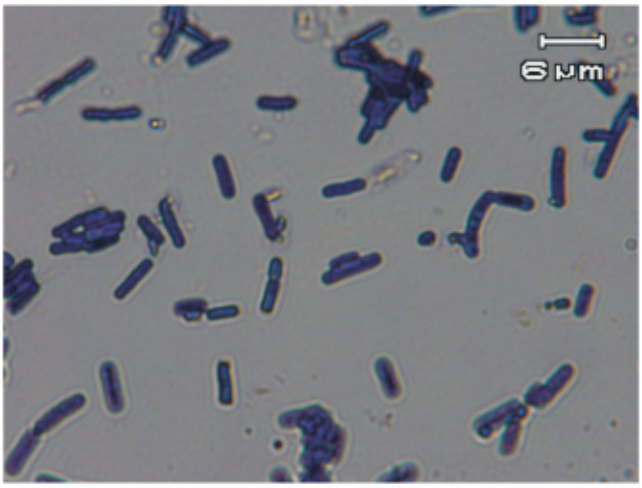

Fig 1 Morphology of the isolate HAT1 showing a rod-shape type under the Scanning Electron Microscope.

To evaluate enzyme production, 5 mL of fresh culture of the isolate which was grown overnight was transferred into 100 mL of sterilized production media containing 6 g Bacto Trypton, 0.5 g $MgSO_4.5H_2O$, 0.5 g KH_2PO_4, 0.5 g NaCl, and 1.0 g starch (Vidyalakshmi *et al.* 2009). The production media was incubated at 30 ºC with orbital shaking at 100 rpm for 6 days. A sample

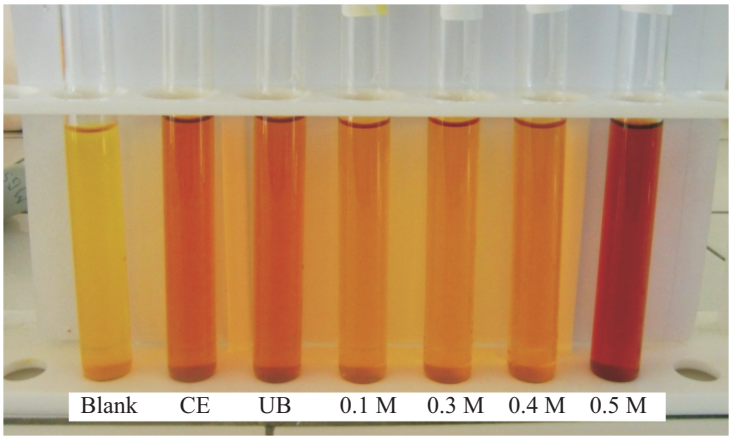

Fig 2 Amylase activity of the DEAE-Sepharose fractions after DNS assay. The 0.5 M NaCl fraction shows the highest amylase activity as compared to other fractions. (CE=crude extract; UB=unbound).

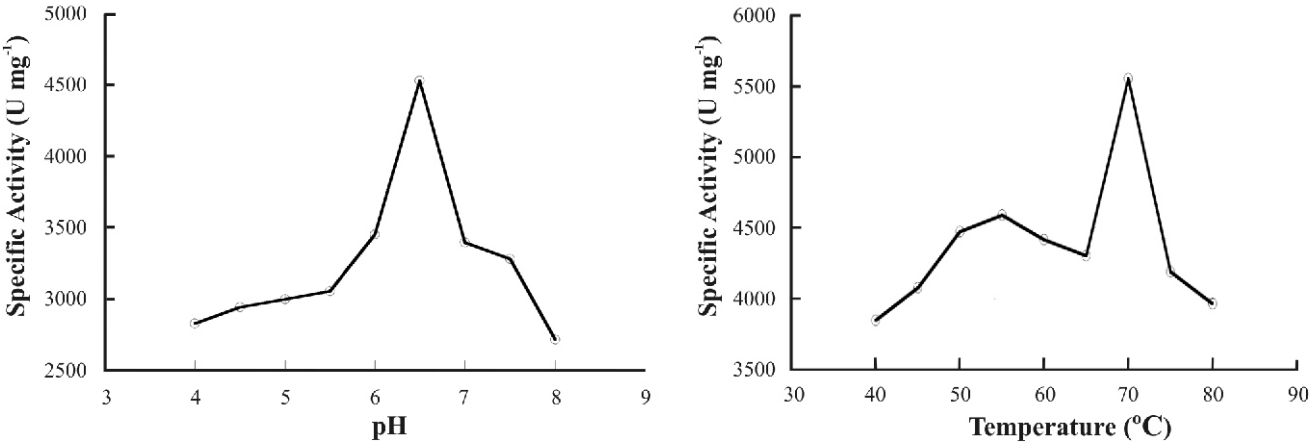

Fig 3 (A) Effect of pH on partially-purified amylase activity; (B) Effect of temperature at the optimum pH on partially-purified amylase activity.

was taken every 24 h for amylase activity evaluation. Amylase activity was determined using the dinitrosalicylic (DNS) acid method according to Miller (1959) with slight modification. Briefly, a reaction mixture containing buffer solution and starch substrate was incubated at 30 ºC for 10 min. Then, a 100 µL aliquot of enzyme solution was added and incubation was continued for additional 3 min. The reaction was stopped by the addition of 1 mL of DNS solution. Subsequently, the mixture was incubated in boiling water for another 10 min. After that, the mixture was put into an iced-water bath. The absorbance of the mixture was measured spectrophotometrically at λ 540 nm. The protein concentration was measured at λ 280 nm and by the method of Bradford (1976). The amylase was partially purified using ion exchange chromatography according to the method of Scopes (1994). The crude extract of amylase was applied to a DEAE-Sepharose Fast Flow column (GE Life Science, England) which had been previously equilibrated with two volumes of 50 mM acetate buffer (pH 5.6). The column was then washed with two volumes of the same buffer to remove all unbound proteins. The bound proteins were eluted fractionally with a linear gradient of NaCl from 0-0.5 M. All fractions were analyzed for their protein content and amylase activity was assayed following the methods as described above. The fraction with highest amylase activity was further analyzed for its optimum pH and thermostability properties according to Takeuchi *et al.* (2006). To determine the optimum pH, a series of pH solutions ranging from 4.0-8.0 were prepared. Then, the amylase activity of the fractions was assayed using these prepared buffer solutions. To determine thermostability, the amylase activity of the fraction was assayed at the optimum pH with different temperatures ranging from 40-80 ºC (with a 5-degree scale interval). The amylase secreted by the isolate bound to DEAE-Sepharose Fast Flow column and almost completely discharged with 0.5 M NaCl (Fig 2). The amylase had an optimum pH at 6.5.

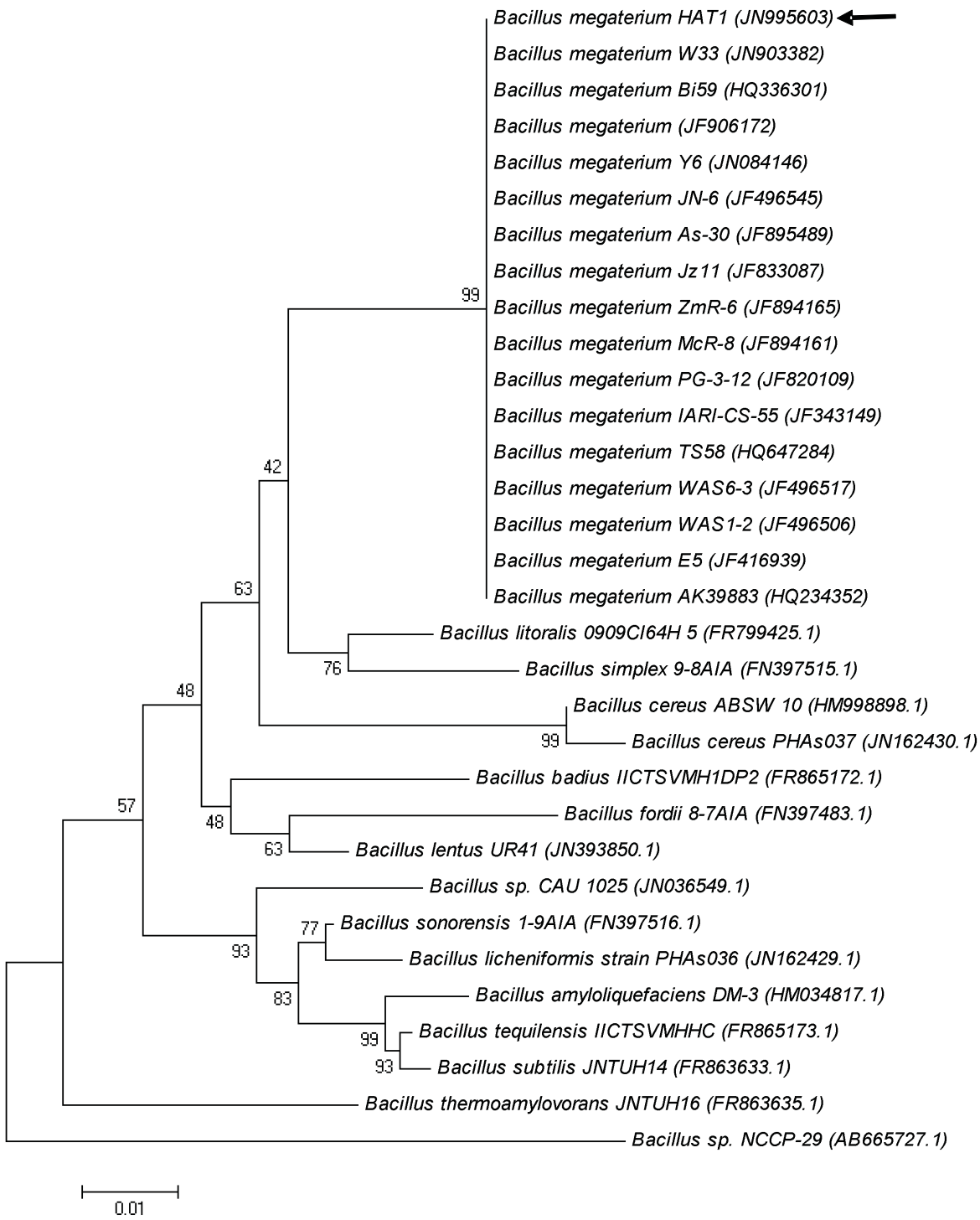

Fig 4 Phylogenetic tree constructed using the *Neighbor-joining Method* between the isolate HAT1 and several related *Bacillus* sp. obtained from GenBank.

At low pH (4.0), the activity of the amylase remained low which was about 2826.47 U mg^{-1} protein, and this increased to a maximum activity of 4529.14 U mg^{-1} protein at pH 6.5. However, the activity suddenly decreased to 2712.88 U mg^{-1} protein at pH 8.0. In terms of temperature, the activity of the amylase which was about 3848.05 U mg^{-1} protein at 40 ºC and increased until the temperature reached 70 ºC (5550.71 U mg^{-1} protein). Beyond this temperature, the activity of the enzyme dropped quickly to 3961.55 U mg^{-1} protein at 80 ºC (Fig 3).

The isolate was capable of degrading soluble starch in the isolation media, as indicated by the formation of a clear zone after the iodine test. The clear zone indicated that the starch had been hydrolyzed into simple sugars, thus losing the capability of starch to bind to iodine leading to the disappearance of blue color. Morphological analysis revealed that the isolate HAT1

is a rod-shaped, gram positive, spore-forming strain. Recently, similar work has been undertaken by Gurudeeban *et al*. (2011), who successfully isolated a rod-shaped, spore-forming and Gram positive amylase producing bacterium *B. megaterium* from white mangrove (*Avicennia marina*) leaves. Also, Sajitha *et al*. (2011) isolated an amylase producing bacterium *B. megaterium* from an estuarine ecosystem. These studies prove that *B. megaterium* is a potential source of bacterial amylase.

The BLAST Analysis of the 16S rRNA gene sequence confirmed that the isolate HAT1 is most closely related to *Bacillus* sp. with 99% of homology. Phylogenetic analysis using Distance-based Method of the known bacterial 16S rRNA genes revealed that the isolate has high similarity with *Bacillus megaterium* (Fig 4). This has been confirmed that the p-distance among the *B. megaterium* strains is zero, indicating that they are all closely related.

Amylase secreted by the isolate was partially purified using ion exchange chromatography on a DEAE-Sepharose Fast-flow column. After loading the crude extract solution, the pass-through fraction (eluant) showed very weak amylase activity, indicating that the amylase was bound to the matrix. The binding of the target protein to the column shows that the enzyme protein has a total negative charge at its surface. The bound amylase was almost completely discharged from the column after elution with 0.5 M NaCl. This fraction showed highest amylase activity after DNS assay among other fractions (Fig 2). Most of the known bacterial amylases were purified using anion exchange chromatography (Shih and Labbe 1995; Hagihara *et al*. 2001; Ballschemiter *et al*. 2006). The pH optimum of the enzyme was almost similar to that of the hotspring water (6.7), while the enzyme could retain its thermal stability above the temperature of the hotspring, i.e. 60 ºC. It is clear that beyond the optimum temperature (70 ºC), the activity of the amylase sharply decreased, indicating that denaturation of the enzyme may have begun to occur. This optimum thermal property of the amylase is higher than those of an estuarine *B. megaterium,* 35 ºC (Sajitha *et al*. 2011) and of *B. megaterium* from White Mangrove leaves which is also 35 ºC (Gurudeeban *et al*. 2011). This thermostability property might be due to fact that the isolated strain inhabits a thermophilic environment as compared to the two former strains.

Based on the 16S rRNA gene sequence analysis, and the multiple sequence alignment with other related *Bacillus* sp, the isolate HAT1 is similar to *B. megaterium*. The partial nucleotide sequence of the 16S rRNA gene of the isolate has been deposited in the GenBank with the accession number: JN995603.

Based on our experimental results, the properties of the partially purified enzyme indicate that the enzyme might have potential application in starch processing. However, for scientific reasons, pure enzyme must first be obtained. Thus, further studies based on enzyme purification and characterization are recommended.

REFERENCES

Ballschemiter M, Futterer O, Liebl W. 2006. Identification and characterization of a novel intracellular alkaline α-amylase from the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl Environ Microbiol. 72(3):2206-2211. doi:10.1128/AEM.72.3.2206-2211.2006.

Bertoldo C, Antranikian G. 2001. Amylolytic enzymes from hyperthermophiles. Methods Enzymol. 330:269-289.

Bradford, MM. 1976. A rapid sensitive method for quantitition of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 72(1-2):248-254.

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(10):1397-1403. doi: 10.1016/S0032-9592(03)00037-2.

Gee JE, Sacchi CT, Glass MB, De BK, Weyant RS, Levett PN, Whitney AM, Hoffmaster AR, Popovic T. 2003. Use of 16S rRNA Gene Sequencing for Rapid Identification and Differentiation of *Burkholderia pseudomallei* and *B. mallei*. J Clin Microbiol. 41(10): 4647-4654. doi:10.1128/JCM.41.10.4647-4654.2003.

Gurudeeban S, Satyavani K, Ramanathan T. 2011. Production of extracellular α-amylase using *Bacillus megaterium* isolated from White Mangrove (*Avicennia marina*). Asian J Biotechnol. 3(3):310-316. doi:10.3923/ajbkr.2011.310.316.

Hagihara H, Igarashi K, Hayashi Y, Endo K, Kitayama KI, Ozaki K, Kawa S, Ito S. 2001. Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM-K38. Appl Environ Microbiol. 67(4):1744-750. doi: 10.1128/AEM.67.4.1744-1750.2001.

Jordan E, Hust M, Roth A, Biedendieck R, Schirrmann T, Jahn D, Dübel S. 2007. Production of recombinant antibody fragments in *Bacillus megaterium*. Microb Cell Fact. 6:2. doi:10.1186/1475-2859-6-2.

Miller GL. 1959. Use of dinitrosalycilic acid reagent for determination of deducing sugars. Anal Chem. 31(3): 426-428. doi: 10.1021/ac60147a030

Oyeleke SB, Auta SH, Egwim EC. 2010. Production and characterization of amylase produced by *Bacillus megaterium* isolated from a local yam peel dumpsite in

Minna, Niger State. J Microbiol Antimicrob. 2(7): 88-92.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. 2000. Advances in microbial amylases. Biotecnol Applied Biochem. 31(2): 135-152.

Prakash O, Jaiswal N. 2010. α-Amylase: an ideal representatives of thermostable enzymes. Appl Biochem Biotechnol. 160(8):2401-14. doi: 10.1007/s12010-009-8735-4.

Rygus T, Hillen W. 1991. Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. Appl Microbiol Biotechnol. 35(5): 594-599. doi: 10.1007/BF00169622.

Sajitha N, Vasanthabharathi V, Lakshminarayanan R, Jayalakshmi S. 2011. Amylase from an estuarine *Bacillus megaterium*. Curr Res J Biol Sci. 3(2): 110-115.

Scopes RK. 1994. Protein purification: principles and practice. 3rd Edition. New York: Springer-Verlag.

Shih NJ, Labbe RG. 1995. Purification and characterization of an extracellular α-amylase from *Clostridium perfringens* type A . Appl Environ Microbiol. 61(5): 1776-1779.

Sidhu GS, Sharma P, Chakrabarti T, Gupta JK. 1997. Strain improvement for the production of a thermostable α-amylase. Enzyme Microb Technol. 21: 525-530.

Stammen S, Muller BK, Korneli C, Biedendieck R, Gamer M, Franco-Lara E, Jahn D. 2010. High-yield intra- and extracellular protein production using *Bacillus megaterium*. Appl Environ Microbiol. 76(12): 4037-4046.

Takeuchi A, Shimizu-Ibuka A, Nishiyama Y, Mura K, Okada S, Tokue C, Arai S. 2006. Purification and characterization of an α-amylase of *Pichia burtonii* isolated from the traditional starter "Murca" in Nepal. Biosci Biotechnol Biochim. 70: 3019-3024.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum persimonymethods. Mol Biol Evol. doi:10.1093/molbev/msr121.

Van der Maarel MJ, Van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L. 2002. Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol. 94(2): 137-155.

Vidyalakshmi R, Paranthaman R, Indhumathi J. 2009. Amylase Production on Submerged Fermentation by *Bacillus* spp. World J Chem. 4(1): 89-91.