

Screening, isolation, and characterization of amylase-producing bacteria from Poring Hot Spring Sabah, Malaysia

BAK ZAIBAH FAZAL, CAHYO BUDIMAN^{*}, ZARINA AMIN, CLEMENTE MICHAEL WONG VUI LING
Biotechnology Research Institute, Universiti Malaysia Sabah, Jl. UMS, 88400 Kota Kinabalu, Sabah, Malaysia. Tel./fax.: +60-8832000 ext 5350,
^{*}email: cahyo@ums.edu.my

Manuscript received: 27 April 2022. Revision accepted: 14 May 2022.

Abstract. Fazal BZ, Budiman C, Amin Z, Ling CMW. 2022. Screening, isolation, and characterization of amylase-producing bacteria from Poring Hot Spring Sabah, Malaysia. *Biodiversitas* 23: 2807-2815. Thermostable α -amylases are being used in a wide range of industries, including food, textiles, detergents, pharmaceuticals, and fine chemicals. A good source of thermostable α -amylases in thermophilic bacteria is found in high-temperature habitats like hot springs. Hence, this study aimed to screen, isolate, and characterize amylase-producing bacteria (APB) from the Poring hot spring in Sabah. Sediment and water samples were collected from the hot springs, serially diluted, plated onto the Luria Bertani agar medium containing starch, and incubated at 60°C for 48 hours. The amylase-producing bacterium was identified by the halo formation around the colony after the agar medium was stained with Lugol's solution. Nine colonies were found to be able to form halo zones, with a creamy colony (A7 strain) producing the highest amylolytic index (4.24). Further characterization of the A7 strain showed that the isolate was a Gram-positive, rod-shaped bacterium, with a positive reaction upon oxidase and catalase tests. The 16S rRNA sequence showed that the A7 strain had 99.81% similarity with the *Anoxybacillus flavithermus*, and therefore identified as *A. flavithermus* A7 strain. Further, the growth curve analysis indicated that the A7 strain grew well at 60°C. The 3, 5-dinitrosalicylic acid (DNS) assay showed the crude enzyme secreted by the A7 strain exhibited optimum amylase activity at 60°C with 8.6×10^{-2} U/ml. This is the first APB obtained from hot springs in Sabah and promising for further studies and applications.

Keywords: Amylase producing bacteria, *Anoxybacillus flavithermus*, hot springs, thermostable amylase

Abbreviations: APB: Amylase-producing bacteria; DNS: 3, 5-dinitrosalicylic acid; DNA: Deoxyribonucleic acid; SEM: Scanning Electron Microscope; PCR: Polymerase chain reaction; AI: Amylolytic index

INTRODUCTION

Amylases (E.C. 3.2.1.1) are among the highest demanded biocatalyst that comprises 25-30 % of the global enzyme market (Vaseekaran et al. 2011; Gazali and Suwastika 2018). These enzymes are often used for biotechnology and the food industry (Dawood et al. 2015). Amylases catalyze the hydrolysis of breaking of the bonds in starches, polysaccharides, and complex carbohydrates into easier to absorb simple sugars (Taniguchi and Honnda 2009; Sundarram and Murthy 2014). To date, three groups of amylases have been identified, namely α -, β -, and γ -amylases, which degrade the α -1,4 glycosidic bonds at different positions (Sundarram and Murthy 2014). Unfortunately, the availability of thermostable amylases that are compatible with industrial applications requiring high-temperature treatments remains limited (Kiran et al. 2018). Thermostability is a desired property, especially in high-temperature processes such as gelatinization and liquefaction in starch processing that involve temperatures ranging from 100-110°C and 80-90°C, respectively (Mehta and Satyanarayana 2016). In addition, the application of thermostable amylases in potential industries such as food, pharmaceutical, detergent, textile, and fine-chemical industries can be more economical and advantageous (Teodoro and Martins 2000; Satrimafitrah et al. 2020). The

advantages include decreasing the risk of contamination, cost of external cooling, and increased diffusion rate (Saxena et al. 2007). Therefore, there has been a need and continual search for more novel thermostable amylases.

Amylases are produced by various organisms, including plants, animals, and microbes (Gazali and Suwastika 2018; Saryono et al. 2018; Ardhi et al. 2020). Among these organisms, microorganisms, particularly bacteria, offer advantages for enzyme production, including rapid growth characteristics with simple and economic growth medium and conditions, easy for genetic modification, if necessary, and scale-up production using a bioreactor (Deljou and Arezi 2016). Accordingly, thermostable amylases from the bacteria are also preferable for further studies and applications. These thermostable amylases can be isolated from thermophilic bacteria that live and thrive in high-temperature environments.

Hot springs, where geothermally heated water emerges from the earth's crust, are a great source of thermophilic bacteria with potential thermostable amylase activity (Albejo and Hamza 2017; Gazali and Suwastika 2018; Kiran et al. 2018; Satrimafitrah et al. 2020). Poring hot spring is associated with the latent activity of mount Kinabalu, the highest mountain in Southeast Asia. This hot spring is located at an elevation of about 490 m above sea level and is managed by Sabah Parks as one of the tourist

attractions in Sabah (Sadikun et al. 1992). Hot water of the Poring hot spring can reach up to 70°C on a hot day while 40-60°C on a regular day (Hua 2016), which makes it a potential source for screening of thermophilic bacteria that produce amylases. To note, no prior studies ever reported on the isolation of amylase-producing bacteria (APB) from the Poring hot spring. On the other sides, many other reports revealed the success of thermophilic APB from hot springs in various countries (Sen et al. 2014; Fooladi and Sajjadian 2010; Kumar et al. 2018; Ardhi et al. 2020).

This study describes the isolation and characterization of a moderate thermophilic amylase-producing bacterium from the Poring hot spring. While, the isolated strain was not considered an extreme thermophile, this finding should serve as a proof of concept for the versatility of Poring hot spring as a source of thermophilic enzyme. To our knowledge, this is the first study on amylase-producing bacteria from Sabah hot spring.

MATERIALS AND METHODS

Sample collection

The water and sediment samples were collected from Poring hot spring, Ranau, Sabah, Malaysia (Coordinate: 6.176810876539832, 116.70885297436628; Figure 1). Samples were collected from three different points and kept in sterilized containers, stored in iceboxes during transportation to the laboratory, and stored in a deep freezer (-80°C) until used. The temperature and pH were recorded at 45°C and 6.8 during sampling, respectively.

Screening and isolation

Screening of APB was done by following method as per Kiran et al. (2018), with some modifications. A gram of sediment was weighed and serially diluted with sterile distilled water and spread onto Luria-Bertani (LB) agar

plate containing 1% soluble starch as substrate. Plates were then incubated at 60°C for 48 hours. After incubation, plates were flooded with Lugol's solution for 1 minute, then drained off, and plates were observed for a clear halo zone around the colony against a blue-black background. The colonies showing a clear halo zone were further purified using the streak plate method. The clear zones formed around the pure colony indicate amylase activity, and the amylolytic index (AI) value of each isolate was determined by measuring the ratio of clear zone diameter to its colony diameter (Satrimafitrah et al. 2020). These were performed in triplicate, and the bacterial isolates with the highest AI value were further identified and characterized.

Growth curve

Bacterial cultures were all performed under aseptic conditions. Cultures were incubated in duplicates at six different temperatures (37°C, 50°C, 55°C, 60°C, 65°C, and 70°C) at 180 rpm, and sampled at different time intervals (0-, 2-, 4-, 8-, 16-, 18, 20-, 22-, 24-, 40- and 42-h).

Morphological and biochemical characterization

The selected isolate was observed morphologically under Gram staining and a microscope. The Gram staining was conducted using a Gram staining reagent kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. In addition, further morphological characterization was also done using scanning electron microscopy (SEM). For this purpose, the gold-coated specimen was prepared and further observed under an electron microscope Hitachi S-3400 (Hitachi Ltd, Chiyoda, Tokyo, Japan) at an accelerating voltage of 10 kV. Biochemical characterization was performed based on catalase and oxidase tests. According to the manufacturer's protocol, the tests were done using the Bactident™ kit (Sigma-Aldrich, Germany).

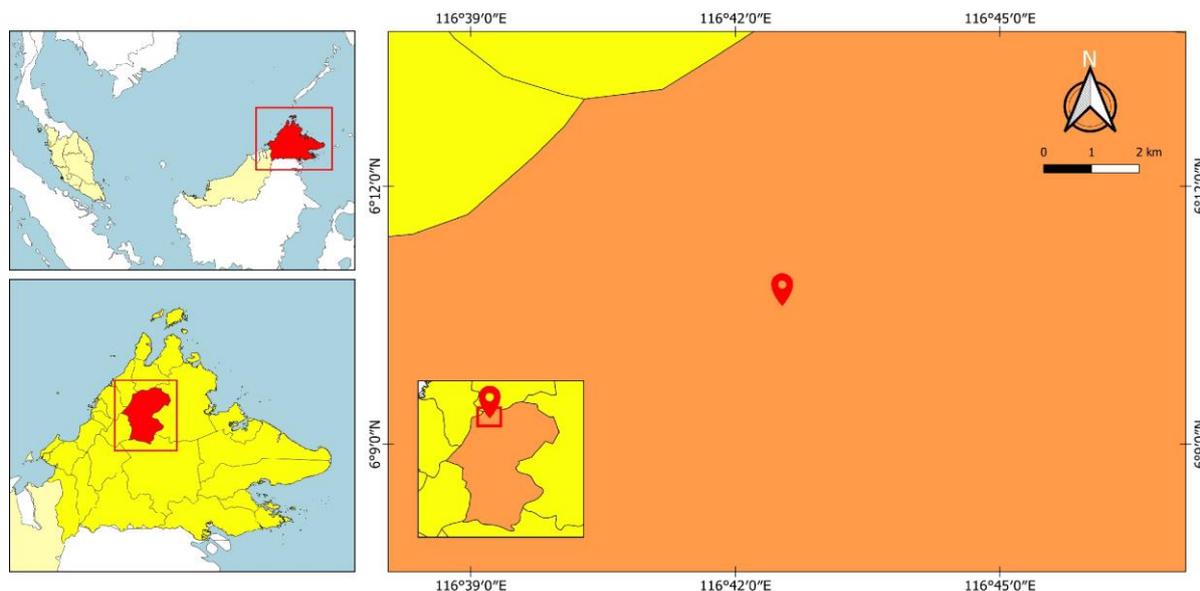


Figure 1. Sampling location of Poring hot spring, Ranau, Sabah, Malaysia. The Map is modified from Google Maps Platform. The scale is shown for distance reference.

Molecular characterization

The selected colony was molecularly characterized based on its 16S rRNA gene sequence. To address this, the selected isolate was cultured overnight in LB broth at 60°C with 180 rpm. Then, the genomic DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen, USA). Isolated DNA was amplified with 16S rRNA universal primer pair BSF8/27 (5'-AGAGTTTGATCTGGCTCAG-3') and BSR1541/20 (5'-AAGGAGGTGATCCAGCCGCA-3') as described previously (Benga et al. 2014). The PCR product was purified under 1% agarose gel electrophoresis and a QIAquick gel extraction kit (Qiagen, Germany) before being subjected to Sanger sequencing. An evolutionary distance tree was generated using MEGA X's Neighbor-joining algorithm (Kumar et al. 2018).

Amylase activity of the crude enzyme

To obtain the crude enzyme, the cells were firstly cultivated at 60°C for 24 hours. The supernatant was separated from the cells by centrifugation at 8,000 g for 10 minutes, washed, and resuspended in 0.9% w/v of NaCl. This supernatant was then designated as crude amylase enzyme (CAE) secreted by the isolated bacterium. The enzymatic activity of the crude enzyme was determined by measuring the increase of reducing sugar as maltose formed by the hydrolysis of starch (Gazali and Suwastika 2018). 3,5-dinitro salicylic acid (DNS) method was used according to Bernfeld (1955). A 1% solution of soluble starch in 50 mM Phosphate Buffer (pH 7.0) was used as a substrate. A reaction mixture (100 µl) containing 50 µl crude enzyme and 50 µl substrate was incubated at 50°C for 15 minutes. After incubation, 50 µl of DNS was added to stop the reaction and then boiled for 15 minutes and diluted 4 times in distilled water. The absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol reducing sugar equivalent to maltose per minute in 50 mM phosphate buffer (pH 7.0) with 1% soluble starch at 50°C. The effect of temperature on amylase activity was determined by performing assays at different temperatures ranging from 37°C to 80°C.

Data analysis

All the data, including AI value and assay activities, were shown as a mean ± standard of deviation from three independent replications and descriptively analyzed (Razali et al. 2020).

RESULTS AND DISCUSSION

Sixty-three colonies from water and sediment samples were observed formed in the screening plate (LB agar with 1% soluble starch). Nevertheless, only 9 colonies from the sediment sample were found to have clear zones surrounding their colony. The AI of these colonies ranged from 1.25 to 4.24, where the creamy white colony with a

round-mouth margin (namely, the A7 colony) was found to have the highest index of 4.24 among other isolates. This indicated that the A7 colony might secrete amylase-degrading enzyme with the highest activity. The clear zone formed by the A7 colony due to the degradation of soluble starch is shown in Figure 2. To note, the observation of starch degradation was done using Lugol's solution, which contains iodine. Lugol's iodine, also known as Lugol's solution, is often used as a reagent for starch detection in routine laboratory and medical tests (Valls et al. 2012). This solution is used to indicate the presence of starches in organic compounds. Lugol's iodine reacts with starch to give a dark blue-black color which is formed due to the interaction between iodine and the coil structure of the polysaccharide polymer (Malle et al. 2012). The ability of A7 colony to degrade the starch leads to the reduction of the polysaccharide polymer structure. Figure 2.B clearly showed that the dark blue-black color of the area around the A7 colony disappeared, which confirmed the starch polymer was degraded. Notably, the screening of bacteria producing amylases was conducted at a high temperature of 60°C, indicating that all isolated bacteria and their amylases were likely to be thermophiles.

The variation of AI among bacteria is a common phenomenon. Gazali and Suwastika (2018) reported that the AI of bacteria is modulated by the growth rate and amount of secreted amylase. According to Gopinath et al. (2017), even within the same genus, species and strain, the level of amylase production varies amongst isolates. Additionally, the amount of amylase generated is dependent on the isolates' source, with those isolated from starch- or amylose-rich sources producing more amylase. In addition to the amylase production level, the specific activity of amylase among bacteria was also different. Nevertheless, observation of AI from agar plate does not necessarily correspond to the specific enzyme activity (Gazali and Suwastika 2018). Rather, AI is convincing enough and widely used to indicate the presence of its enzyme activity.

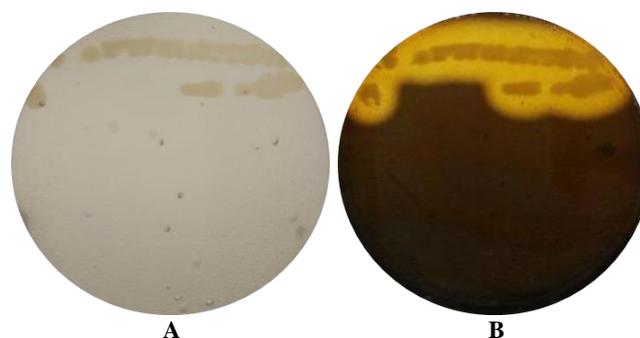


Figure 2. The colony A7 (A) Before flooded with Lugol's solution; (B) After flooded with Lugol's solution

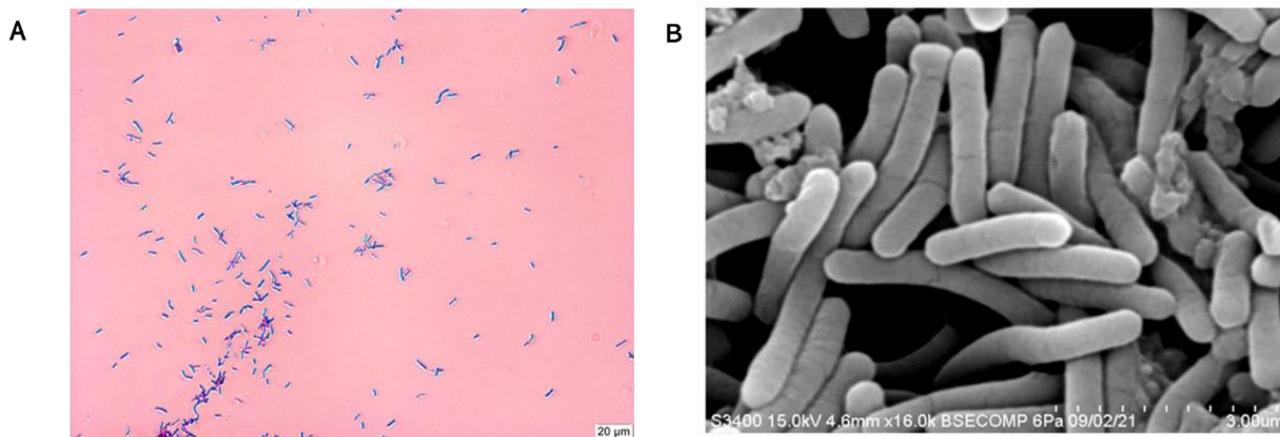


Figure 3. The structural characteristic of A7 strain (A) Gram stain photo (B) SEM electron micrograph

Notably, the AI of the A7 colony was the highest among the other colony, which implied that this colony expressed the amylase at the highest level or best activity. Accordingly, this colony was further isolated and purified to have a single bacterium strain. The isolated A7 strain was then subjected to further morphological and biochemical characterizations. Figure 3.A showed the Gram staining of the A7 strain, which indicated that this bacterium belongs to the Gram-positive group, as shown by its purple color. The Gram staining revealed that the A7 strain forms a rod shape, which was confirmed by observation under SEM (Figure 3.B). The estimated dimension of the A7 strain from the SEM micrograph was about $2.1 \times 0.49 \times 0.39 \mu\text{m}$ for length, width, and diameter, respectively. Some APB isolated from hot springs were also reported as rod shape Gram-positive. To name a few, *Bacillus* sp. from Iranian hot spring (Fooladi and Sajjadian 2010); *Aeromonas veronii* SS2 and *Stenotrophomonas maltophilia* SS3 from Taptapani hot spring, Odisha, India (Sen et al. 2014) and *Bacillus licheniformis* from Bukit Gadang Hot Spring, West Sumatra, Indonesia (Ardhi et al. 2020).

Biochemically, the colony was reactive under catalase and oxidase tests. A positive result on the catalase test indicated that this strain produced enzyme catalase that facilitates cellular detoxification. Catalases, according to Yuan et al. (2021), play a critical role in bacteria's defenses against oxidative stress by accelerating the breakdown of H_2O_2 . Catalases are also engaged in a variety of biological activities, including cell growth and differentiation, and the generation of metabolites. Earlier, Hassan et al. (2020) reported that the bacteria exposed to heat stress led to oxidative damage in their cells, including high lipid peroxidation, lipoxxygenase, and xanthine oxidase level. In addition, heat stress generates high reactive levels of oxygen species (ROS) are generating, namely, superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl free radical ($\cdot\text{OH}$), which ultimately leads to oxidative damage. Accordingly, as the A7 strain was isolated from the thermal environment, it is plausible for this isolate to display a positive catalase phenotype as a defensive mechanism

against oxidative damage due to heat stress of the hot spring temperature. In addition, the positive oxidative result of the A7 strain suggested that it is an aerobic bacterium that uses oxygen as the terminal electron acceptor during respiration.

Nevertheless, the result is unable to confirm of this isolate is a strict aerobe without further investigation. Notably, as the screening method in this study is feasible for isolating only the aerobic group; therefore, it is acceptable to have the A7 strain as an aerobic isolate. In general, there are many Gram-positive APB with rod-shaped and, positively reacted to catalase and oxidase tests were reported previously, including *Bacillus* sp. (Malle et al. 2012; Albejo and Hamza 2017) and *Brevibacillus* sp. (Norashirene et al. 2012).

Further, the growth curve of the A7 strain indicated that this isolate is able to survive at a temperature range between 37°C to 65°C (Figure 4). This growth temperature range fits the range for moderate thermophiles ($45\text{--}85^\circ\text{C}$), as Khalil et al. (2017) reported. Meanwhile, hyperthermophiles and extreme thermophiles grew at $85\text{--}100^\circ\text{C}$ and $>100^\circ\text{C}$, respectively. In addition, thermophilic organisms are generally referred to as the group that thrives at temperatures between 45°C and 122°C (Takai et al. 2008). Figure 4 showed that rapid growth of the A7 strain was observed at 60°C , followed by 55°C and 50°C . Nevertheless, this bacterium reached maximum density at 37°C , with poor growth performance at 65°C and 70°C . While the survival temperature is not extremely high, the performance of the A7 strain at moderately high temperatures convincingly supported that this bacterium is a moderate thermophilic. Rasooli et al. (2008) proposed that the ability of bacteria to proliferate at high temperatures is associated with the production of growth-supporting metabolite components, including enzymes, which are capable of surviving at higher temperatures. Accordingly, the amylase secreted by the A7 strain is therefore predicted as thermostable. This is also supported by the finding that the amylase degrading activity of this bacterium is observable at 60°C .

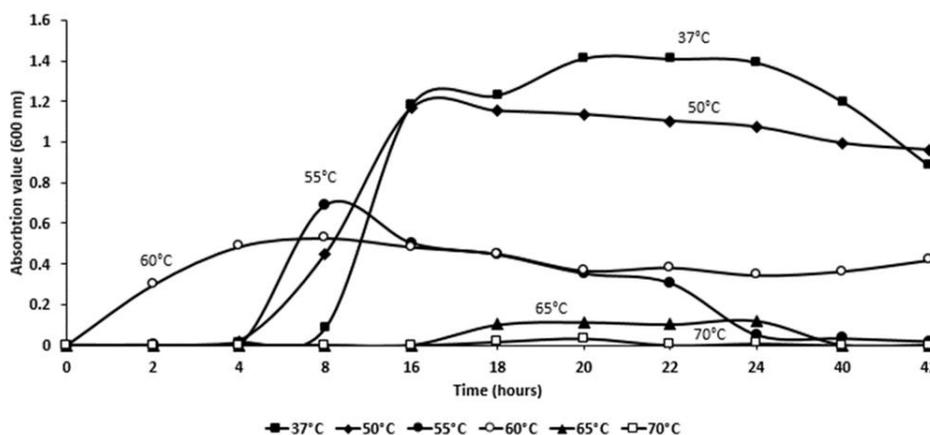


Figure 4 Growth curve of isolate A7

To note, it sounds acceptable to have the A7 strain as a moderate thermophilic bacterium due to the temperature range of the Poring hot spring, as a source of the bacterial screening and isolation, is between 43°C to 59°C at the time of sampling. Similarly, Mohammad et al. (2017) and Lefevre et al. (2010) also isolated several moderate thermostable bacteria from Jordanian and Nevada hot springs, respectively, where both hot springs have moderate temperatures. Nevertheless, some reports also indicated that hyperthermophilic microorganisms were isolated from hot springs. While most hyperthermophilic organisms are archaea, some bacteria are also able to tolerate extreme temperatures (Scott et al. 2021). The temperature of a hot spring is one of the important factors in determining the type of thermophilic organisms. Hot spring with extremely high temperature is likely to provide more hyper or extreme thermophilic organisms. Temperature variations of hot springs are widely reported, ranging from 20°C to more than 75°C (Renaut and Jones 2000). Hot springs located in the active volcanic zones often produce superheated water. While Poring hot spring is often associated with the Kinabalu Mount, this mountain is reported as not an active volcano. Rather, it is essentially a massive pluton formed from granodiorite. Accordingly, it is acceptable that the Poring hot spring is not having an extremely high temperature as the other hot springs from active volcano zones. In addition to temperature, pH and chemical composition of the hot springs were also reported to modulate the bacterial community in the ecosystem (Skirnisdottir et al. 2010; Uribe-Lorio et al. 2019).

Further, molecular characterization was performed for the identification of the A7 strain by sequencing the 16S rRNA gene. This technique has been used extensively in classifying and identifying bacteria and archaea (Kim and Chun 2014). Johnson et al. (2019) reported that 16S sequences are able to distinguish strains (sometimes called subspecies) based on polymorphisms within the gene. Accordingly, the full-length 16S rRNA gene of the A7 strain was amplified using universal primers, which yielded an obvious single amplicon at the expected size of 1500 bp (Figure 5). Further sequence analysis using The Basic Local Alignment Search Tool (BLAST) revealed that the

A7 strain showed 99.81% identity to the 16S rRNA gene of the thermophilic *Anoxybacillus flavithermus*, therefore designated as *A. flavithermus* A7. Other bacteria showing the closest similarity to the A7 strain are shown in Table 1. Notably, some *Anoxybacillus* genus were also isolated from hot springs. Previously, the *A. flavithermus* was reported to be able to survive in temperatures ranging from 55°C to 60°C (Khalil et al. 2017). *Anoxybacillus* sp. was also isolated from Gazlıgöl hot spring in Turkey, and they produced some hydrolytic enzymes, including amylases, specifically α -amylase (Özdemir et al. 2012; Bekler et al. 2021), along with xylanase and pullulanase (Chai et al. 2012; Ellis and Magnuson 2012; Goh et al. 2013). The amylases produced by *Anoxybacillus* sp. were reported to have a different optimum temperature for their activity. For example, amylase of *Anoxybacillus gonensis* AT23 and *Anoxybacillus ayderensis* FMB1 exhibited optimum activity at 50°C and 55°C, respectively (Afrisham et al. 2017; Bekler et al. 2021). Nevertheless, crude amylase of the A7 strain was found to be active at temperatures between 50°C and 65°C, with an optimum activity around 60°C. This temperature was relatively higher than the other *Anoxybacillus* sp. reported earlier (Afrisham et al. 2017; Bekler et al. 2021).

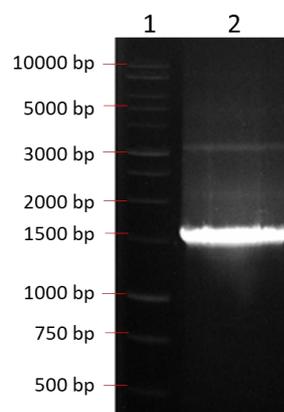


Figure 5. 16S rRNA gene of the isolated A7 on a 1% Agarose Gel; Lane 1: 1Kb DNA Ladder, Lane 2: A7

Table 1. Top BLAST result of the closest species to the A7 strain

Species	Percent identity	E-value	Accession number
<i>Anoxybacillus flavithermus</i> strain K-83	99.81	0.0	MK418411.1
Uncultured bacterium clone TGFC127S5	99.81	0.0	KY355721.1
<i>Anoxybacillus flavithermus</i> WK1	99.81	0.0	CP000922.1
<i>Anoxybacillus flavithermus</i> strain K-1	99.81	0.0	MK418420.1
<i>Anoxybacillus flavithermus</i> strain K-97	99.81	0.0	KC503890.1
<i>Anoxybacillus flavithermus</i> strain AK1	99.81	0.0	KC503890.1
<i>Anoxybacillus</i> sp. JS40	99.72	0.0	KF856614.1
<i>Anoxybacillus suryakundensis</i> strain JS5	99.71	0.0	KF772607.1
<i>Anoxybacillus pushchinoensis</i> strain AE5	99.62	0.0	FN666244.1
<i>Anoxybacillus flavithermus</i> strain K-33	99.52	0.0	MK418427.1

The phylogenetic tree was then constructed between the known bacterial 16S rRNA genes obtained from GenBank (Figure 5). Figure 5 showed that most *Anoxybacillus* were grouped in the same clades as the A7 strain, except *Anoxybacillus pushchinensis* AE5, which was located as an outgroup. While some Bacilli are located at different clades from the A7 strain, the bacillus and A7 are considerably monophyletic groups that share a most common ancestor at a certain point (bootstrap value of 99). Bacillus group, particularly *B. subtilis*, is a well-known amylase producer that dominates the current commercial amylase market (Yan and Wu 2017). Among the Bacillus species, A7 was shown to have the closest genetic distance to *B. megaterium* HAT1, isolated from the Hatuasa hot spring of Maluku, Indonesia. HAT1 was reported to be a thermophilic bacterium that secreted thermostable amylase (Malle et al. 2012).

Amylase activity in the CAE of the A7 strain was tested at different temperatures to see if it behaved like a thermophilic enzyme. Figure 7 shows that the amylase activity of the A7 strain increased when the temperature was raised from 50 to 60°C peaking at 60°C. Hence, the optimum temperature for the amylase activity of the A7 strain was 60°C. Underexposure to temperatures higher than 65°C, the activity was found to drop up to 20% or less. The optimum temperature of the A7 strain (60°C) is under the range of thermostable enzymes and comparable to the optimum temperature of some thermophilic APB, including *B. licheniformis* from hot springs of Himachal Pradesh (50°C), and *B. megaterium* HAT1 (Hiteshi et al. 2016; Malle et al. 2012). Nevertheless, some thermostable APB were shown to have amylase activity at higher optimum temperatures than the A7 strain, including *Bacillus licheniformis* from Iran soil (70°C) and *B. amyloliquefaciens* KCP2 (65°C) (Prajapati et al. 2015; Rasooli et al. 2008). Interestingly, Kiran et al. (2018) isolated 7 APB strains from some hot springs in Bihar, India.

All these strains have an optimum temperature of 50°C. This indicated that the optimum temperatures among thermophilic APB are varied depending on the source of organisms. The discrepancy in the optimum temperature is also related to the structural uniqueness of amylase produced by each APB, further regulating thermal activity

at high temperatures. The remarkable thermostability of this amylase, according to Chang et al. (1997), maybe due to increased ionic contacts, narrower surface area, and higher packing interactions in the interior. Budiman et al. (2012) also implied that polarity of the substrate cavity modulates the sensitivity of enzymes against the temperature. These factors might vary among thermostable amylase, which further differentiates their behaviours under high-temperature exposure. Notably, the assay was done using a crude enzyme (without purification), which might not reflect the real activity of amylase due to the presence of other contaminant proteins which might interfere with the activity. In addition, the real amount of amylase in crude enzyme might also be different among thermostable APB, which differentiate their activities. There is variation in amylase production among species because of the compositions of the medium and other physical properties (Pranay et al. 2019). Obeng et al. (2018) further suggested that similar enzymes produced by different bacteria can behave differently. Budiman et al. (2018) also proposed that the similar enzymes from different organisms might be unique in their activity and also substrate specificity.

As the amylase of the A7 strain has optimum activity at 60°C, this enzyme is therefore considered a promising enzyme for industrial applications. Goyal et al. (2005) suggested that enzymes with optimum temperature exceeding 50°C confirm the suitable character needed by most industrial sectors. The CAE secreted by the A7 strain exhibited optimum temperature at around 60°C with enzyme activity of 8.6×10^{-2} U/ml. This activity is considerably low; nevertheless, it is acceptable since the current study is limited to the crude extract fraction (unpurified one). The contaminants in the extract might interfere with the real activity of the enzyme. Purification of this enzyme will almost certainly improve its specific activity, according to Razali et al. (2021), who found that purification of recombinant protein increased its specific activity. As for comparisons, the amylase activity of *Bacillus* sp. is previously reported to be 57 U/ml (Cordeiro et al. 2002), and *Bacillus subtilis* used in industry can reach 100 U/ml (Asgher et al. 2007). These enzymes, however, are in their pure form and so had higher activity than those from the A7 strain.

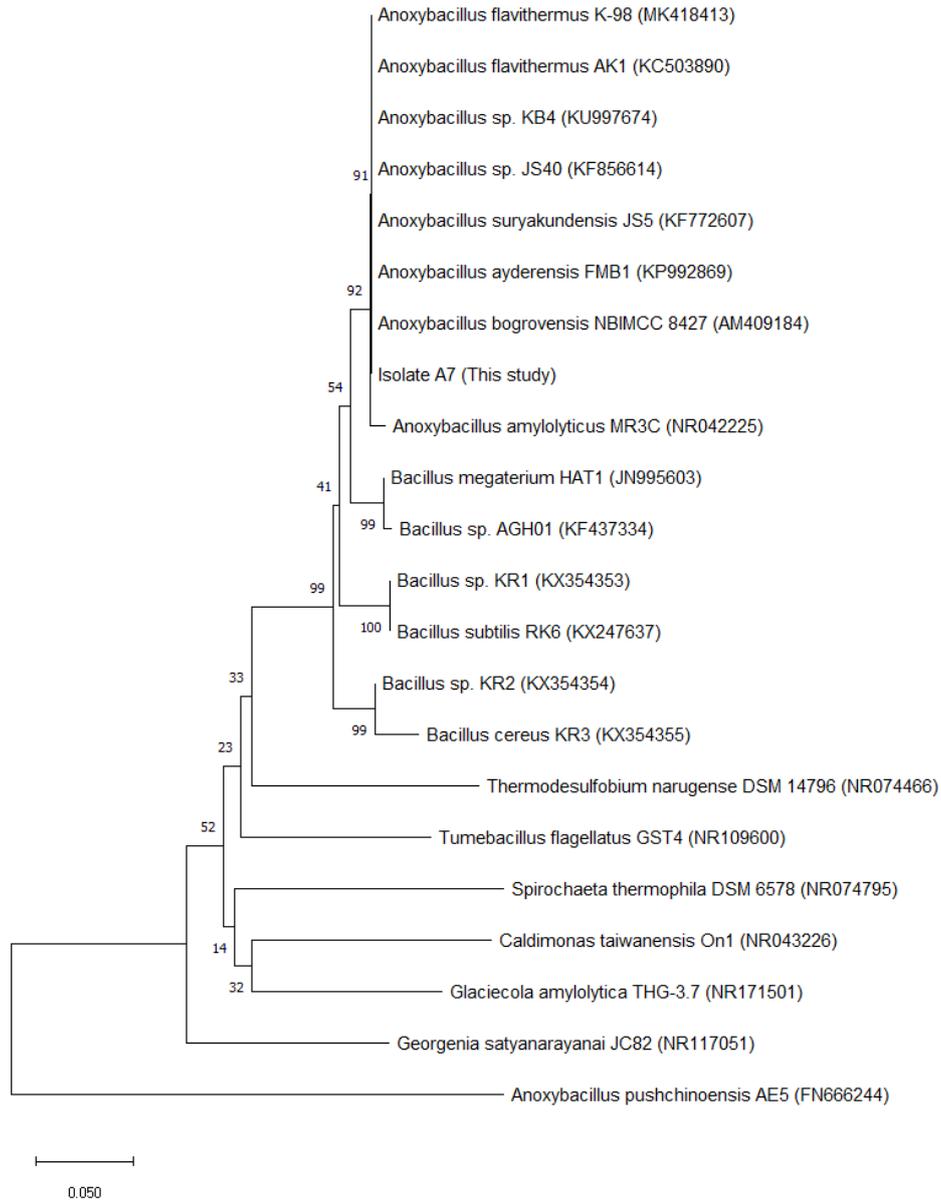


Figure 6. Phylogenetic tree constructed using the neighbor-joining method of strain A7. All bootstrap values (expressed as a percentage of 1,000 replications) were shown at the branch point.

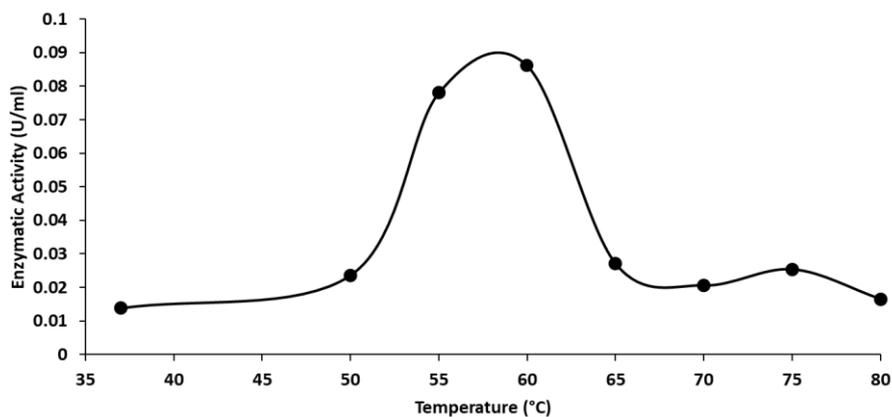


Figure 7. The temperature effect on the amylase activity at pH 7.0.

ACKNOWLEDGMENTS

This study is under GUG0407-2/2019. We thank the Sabah Biodiversity Center and the Sabah Park management for supporting this study (No License: JKM/MBS.1000-2/2 JLD.9(70)).

REFERENCES

- Afrisham S, Badoei-dalfard A, Namaki-Shoushtari A, Karami Z. 2017. Production and characterization of a thermophilic and extremely halotolerant alpha-amylase isolated from *Anoxybacillus gonensis* AT23. *J Microb World* 10 (2): 176-191.
- Albejo AL, Hamza TA. 2017. Isolation and characterization of thermostable amylase producing bacteria from hot spring at isolation and characterization of thermostable amylase producing bacteria from hot spring at Arba Minch Nech Sar National Park, Southern Ethiopia. *Intl J Novel Res Interdiscip Stud* 4 (5): 9-16.
- Ardhi A, Sidauruk AN, Suraya N, Pratiwi NW, Pato U. 2020. Molecular identification of amylase-producing thermophilic bacteria isolated from Bukit Gadang Hot Spring, West Sumatra, Indonesia. *Biodiversitas* 21 (3): 2085-4722. DOI: DOI: 10.13057/biodiv/d210319.
- Asgher M, Asad MJ, Rahman SU, Legge RL. 2007. A thermostable α -amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Eng* 79 (3): 950-955. DOI: 10.1016/j.jfoodeng.2005.12.053.
- Bekler FM, Güven K, Gül Güven R. 2021. Purification and characterization of novel α -amylase from *Anoxybacillus ayderensis* FMB1. *Biocatal Biotransformation* 39 (4): 322-332. DOI: 10.1080/10242422.2020.1856097.
- Benga L, Bente WPM, Engelhardt E, Köhrer K, Gougoula C, Sager M. 2014. 16s ribosomal DNA sequence-based identification of bacteria in laboratory rodents: A practical approach in laboratory animal bacteriology diagnostics. *Lab Anim* 48 (4): 305-312. DOI: 10.1177/0023677214538240.
- Bernfeld P. 1955. Assay Method. In: *Enzyme of Carbohydrate Metabolism* I: 149-158. DOI: 10.1016/0076-6879(55)01021-5.
- Budiman C, Lindang HU, Cheong BE, Rodrigues KF. 2018. Inhibition and substrate specificity properties of FKBP22 from a psychrotrophic bacterium, *Shewanella* sp. SIB1. *Protein J* 37 (3): 270-279. DOI: 10.1007/s10930-018-9772-z.
- Budiman C, Tadokoro T, Angkawidjaja C, Koga Y, Kanaya S. 2012. Role of polar and nonpolar residues at the active site for PPIase activity of FKBP22 from *Shewanella* sp. SIB1. *The FEBS J* 279 (6): 976-986. DOI: 10.1111/j.1742-4658.2012.08483.x.
- Chai YY, Kahar UM, Salleh MM, Illias RM, Mau Goh K. 2012. Isolation and characterization of pullulan degrading *Anoxybacillus* species isolated from Malaysian hot springs. *Environ Technol (United Kingdom)* 33 (11): 1231-1238. DOI: 10.1080/09593330.2011.618935.
- Chang C, Lee J, Choe S, Sweet RM. 1997. Crystal Structure of Thermostable α -Amylase from *Bacillus licheniformis* Refined at 1.7 Resolution. *Mol Cells* 7: 251-258.
- Cordeiro CAM, Martins MLL, Luciano AB. 2002. Production and properties of α -amylase from thermophilic *Bacillus* sp. *Braz J Microbiol* 33: 57-61. DOI: 10.1590/S1517-83822002000100012.
- Dawood ES, Ibrahim SA, El-Nagerabi SAF. 2015. Some properties of thermostable α -amylase of four isolates of *Bacillus licheniformis*. *Nusantara Bioscience* 7: 90-95. DOI: 10.13057/nusbiosci/n070205.
- Deljou A, Arezi I. 2016. Production of thermostable extracellular α -amylase by a moderate thermophilic *Bacillus licheniformis* isolated from Qinarje Hot Spring (Ardebil prov. of Iran). *Periodicum Biologorum* 118 (4): 405-416. DOI: 10.18054/pb.v118i4.3737.
- Ellis JT, Magnuson TS. 2012. Thermostable and alkalistable xylanases produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3. *ISRN Microbiol* 2012: 517524. DOI: 10.5402/2012/517524.
- Fooladi J, Sajjadian A. 2010. Screening the thermophilic and hyperthermophilic bacterial population of three Iranian hot-springs to detect the thermostable α -amylase producing strain. *Iran J Microbiol* 2 (1): 46.
- Gazali FM, Suwastika IN. 2018. Thermostable α -amylase activity from thermophilic bacteria isolated from Bora Hot Spring, Central Sulawesi. *J Phys: Conf Ser* 979: 012001.
- Goh KM, Kahar UM, Chai YY, Chong CS, Chai KP, Ranjani V, Illias RM, Chan KG. 2013. Recent discoveries and applications of *Anoxybacillus*. *Appl Microbiol Biotechnol* 97 (4): 1475-1488. DOI: 10.1007/s00253-012-4663-2.
- Gopinath SC, Anbu P, Arshad MM, Lakshmpriya T, Voon CH, Hashim U, Chinni SV. 2017. Biotechnological processes in microbial amylase production. *BioMed Res Intl* 2017: 1272193. DOI: 10.1155/2017/1272193.
- 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 (7): 723-734. DOI: 10.1016/j.enzymtec.2005.04.017.
- Hassan AH, Hozzein WN, Mousa AS, Rabie W, Alkhalifah DH, Selim S, AbdElgawad H. 2020. Heat stress as an innovative approach to enhance the antioxidant production in *Pseudoaerobicola* and *Bacillus* isolates. *Sci Rep* 10 (1): 15076. DOI: 10.1038/s41598-020-72054-y.
- Hiteshi K, Didwal G, Gupta R. 2016. Production optimization of α -amylase from *Bacillus licheniformis*. *Intl J Adv Pharm Biol Sci* 1: 1-4. DOI: 10.53555/nmbma.v2i5.497.
- Hua AK. 2016. Sustainable tourism in Poring Hot Spring Sabah: An experience. *Intl J Acad Res Environ Geogr* 3 (1): 24-28. DOI: 10.6084/m9.figshare.3362638.v1.
- Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E. 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun* 10 (1): 1-1. DOI: 10.1038/s41467-019-13036-1.
- Khalil A, Sivakumar N, Arslan M, Qarawi S. 2017. Novel *Anoxybacillus flavithermus* AK1: A thermophile isolated from a hot spring in Saudi Arabia. *Arab J Sci Eng* 43 (1): 73-81. DOI: 10.1007/s13369-017-2622-z.
- Kim M, Chun J. 2014. 16S rRNA gene-based identification of bacteria and archaea using the EzTaxon server. *Methods Microbiol* 41: 61-74. DOI: 10.1016/bs.mim.2014.08.001.
- Kiran S, Singh A, Prabha C, Kumari Sushma, Kumari Sonali. 2018. Isolation and characterization of thermostable amylase producing bacteria from hot springs of Bihar, India. *Intl J Pharm Med Biol Sci* 7 (2): 28-34. DOI: 10.18178/ijpmb.7.2.28-34.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35 (6): 1547-1549. DOI: 10.1093/molbev/msy096.
- Lefèvre CT, Abreu F, Schmidt ML, Lins U, Frankel RB, Hedlund BP, Bazylinski DA. 2010. Moderately thermophilic magnetotactic bacteria from hot springs in Nevada. *Appl Environ Microbiol* 76 (11): 3740-3743. DOI: 10.1128/AEM.03018-09.
- Malle D, Picarima J, Chara Huwae L, Rahmawati I, Purbowasito W. 2012. Isolation and identification of a thermostable amylase-producing bacterium from Hatuasa Hotspring. *Microbiol Indones* 6 (2): 83-88. DOI: 10.5454/mi.6.2.5.
- Mehta D, Satyanarayana T. 2016. Bacterial and archaeal α -amylases: Diversity and amelioration of the desirable characteristics for industrial applications. *Front Microbiol* 7 (JUL): 1-21. DOI: 10.3389/fmicb.2016.01129.
- Mohammad BT, Al Daghistani HI, Jaouani A, Abdel-Latif S, Kennes C. 2017. Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. *Intl J Microbiol* 6943952. DOI: 10.1155/2017/6943952.
- Norashirene MJ, Amin AA, Sharina MRN, Fithriah MAN. 2012. Screening and isolation of amyolytic thermophiles from Hulu Langat Hot Spring, Selangor. In 2012 IEEE Symposium on Business, Engineering and Industrial Applications. DOI: 10.1109/BEIAC.2012.6226053.
- Özdemir S, Matpan F, Okumus V, Dündar A, Ulutas MS, Kumru M. 2012. Isolation of a thermophilic *Anoxybacillus flavithermus* sp. nov. and production of thermostable α -amylase under solid-state fermentation (SSF). *Ann Microbiol* 62 (4): 1367-1375. DOI: 10.1007/s13213-011-0385-4.
- Obeng EM, Brossette T, Ongkudon CM, Budiman C, Maas R, Jose J. 2018. The workability of *Escherichia coli* BL21 (DE3) and *Pseudomonas putida* KT2440 expression platforms with auto

- displayed cellulases: a comparison. *Appl Microbiol Biotechnol* 102 (11): 4829-4841. DOI: 10.1007/s00253-018-8987-4.
- Prajapati VS, Trivedi UB, Patel KC. 2015. A statistical approach for the production of thermostable and alklophilic alpha-amylase from *Bacillus amyloliquefaciens* KCP2 under solid-state fermentation. *3 Biotech* 5 (2): 211-220. DOI: 10.1007/s13205-014-0213-1.
- Pranay K, Padmadeo SR, Jha V, Prasad B. 2019. Screening and identification of amylase producing strains of *Bacillus*. *J Appl Biol Biotechnol* 7 (4): 5-2. DOI: 10.7324/JABB.2019.70409.
- Rasooli I, Astaneh SDA, Borna H, Barchini KA. 2008. A thermostable α -amylase producing natural variant of *Bacillus* spp. isolated from soil in Iran. *Am J Agric Biol Sci* 3 (3): 591-596. DOI: 10.3844/ajabssp.2008.591.596.
- Razali R, Kumar V, Budiman C. 2020. Structural insights into the enzymatic activity of protease bromelain of MD2 pineapple. *Pak J Biol Sci* 23 (6): 829-38. DOI: 10.3923/pjbs.2020.829.838.
- Razali R, Subbiah VK, Budiman C. 2021. Technical data of heterologous expression and purification of sars-cov-2 proteases using *Escherichia coli* system. *Data* 6 (9): 99. DOI: 10.3390/data6090099.
- Renaut RW, Jones B. 2000. Microbial precipitates around continental hot springs and geysers. In *Microbial sediments*. Springer, Berlin, Heidelberg. DOI: 10.1007/978-3-662-04036-2_21.
- Sadikun S, Gasim MuhdB, Hamzah U. 1992. Reconnaissance electrical survey for geothermal exploration in the Poring Hot Spring, Ranau, Sabah. *Bull Geol Soc Malays* 31 (July): 145-156. DOI: 10.7186/bgsm31199210.
- Saryono PF, Sari N, Pratiwi NW, Ardhi A. 2018. Morphological identification and hydrolytic enzyme-producing abilities of fungi associated with wilting banana plants (*Musa* sp.). *Res J Chem Environ* 22: 79-86.
- Satrimafitrah P, Razak AR, Hardi J, Puspitasari DJ, Yelenggete I, Rafik. 2020. Thermostable amylase activity produced by thermophilic bacteria isolated from Pulu Hotspring, Central Sulawesi. *J Phys: Conf Ser* 1434: 012034. DOI: 10.1088/1742-6596/1434/1/012034.
- Saxena RK, Dutt K, Agarwal L, Nayyar P. 2007. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Bioresour Technol* 98 (2): 260-265. DOI: 10.1016/j.biortech.2006.01.016.
- Scott KA, Williams SA, Santangelo TJ. 2021. *Thermococcus kodakarensis* provides a versatile hyperthermophilic archaeal platform for protein expression. *Methods Enzymol* 659: 243-273. DOI: 10.1016/bs.mie.2021.06.014.
- Sen SK, Raut S, Satpathy S, Rout PR, Bandyopadhyay B, Mohapatra PK. 2014. Characterizing novel thermophilic amylase producing bacteria from Taptapani hot spring, Odisha, India. *Jundishapur J Microbiol* 7 (12): e11800. DOI: 10.5812/jjm.11800.
- Skirnisdottir S, Hreggvidsson GO, Hjörleifsdottir S, Marteinsson VT, Petursdottir SK, Holst O, Kristjansson JK. 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Appl Environ Microbiol* 66 (7): 2835-2841. DOI: 10.1128/AEM.66.7.2835-2841.2000.
- Sundarram A, Murthy TPK. 2014. α -Amylase production and applications: A Review. *Appl Environ Microbiol* 2 (4): 166-175. DOI: 10.12691/jaem-2-4-10.
- Takai K, Nakamura K, Toki T, Tsunogai U, Miyazaki M, Miyazaki J, Hirayama H, Nakagawa S, Nunoura T, Horikoshi K. 2008. Cell proliferation at 122°C and isotopically heavy CH₄ production by a hyperthermophilic methanogen under high-pressure cultivation. *Proc Natl Acad Sci USA* 105 (31): 10949-10954. DOI: 10.1073/pnas.0712334105.
- Taniguchi H, Honnda Y. 2009. Amylases. *Encyclopedia of Microbiology*. Third Edition:159-173. DOI: 10.1016/B978-012373944-5.00130-9.
- Teodoro CE, Martins ML. 2000. Culture conditions for the production of thermostable amylase by *Bacillus* sp. *Braz J Microbiol* 31 (4): 298-302. DOI: 10.1590/S1517-83822000000400011.
- Uribe-Lorio L, Brenes-Guillén L, Hernández-Ascencio W, Mora-Amador R, González G, Ramírez-Umaña CJ, Diez B, Pedrós-Alió C. 2019. The influence of temperature and pH on bacterial community composition of microbial mats in hot springs from Costa Rica. *Microbiol Open* 8 (10): e893. DOI: 10.1002/mbo3.893.
- Vaseekaran S, Balakumar S, Arasaratnam V. 2011. Isolation and identification of a bacterial strain producing thermostable α -Amylase. *Trop Agric Res* 22 (1): 1-11. DOI: 10.4038/tar.v22i1.2603.
- Valls C, Rojas C, Pujadas G, Garcia Vallve S, Mulero M. 2012. Characterization of the activity and stability of amylase from saliva and detergent: Laboratory practicals for studying the activity and stability of amylase from saliva and various commercial detergents. *Biochem Mol Biol Educ* 40 (4): 254-265. DOI: 10.1002/bmb.20612.
- Yan S, Wu G. 2017. Bottleneck in secretion of α -amylase in *Bacillus subtilis*. *Microb Cell Factories* 16 (1): 1-8. DOI: 10.1186/s12934-017-0738-1.
- Yuan F, Yin S, Xu Y, Xiang L, Wang H, Li Z, Fan K, Pan G. 2021. The richness and diversity of catalases in Bacteria. *Front Microbiol* 12: 645477. DOI: 10.3389/fmicb.2021.645477.