Malaysian Journal of Chemistry, 2025, Vol. 27(1), 200-210
(Special Issue: *International Conference in Organic Synthesis, ICOS 2024*)

Isolation and Characterization of Indigenic Microbes Producing Amylase Enzyme from Paper Industry Liquid Waste

Siti Nafisa[1], Eli Hendrik Sanjaya[1,2]*, Suharti[1], Mieke Alvionita[1], Hafiz Clevanota[1], Hong Chen[3] and Ayom Kusuma[4]

[1]Department of Chemistry, State University of Malang (Universitas Negeri Malang), Jl. Semarang No. 5, Malang, East Java, 65145, Indonesia

[2]Department of Biotechnology, State University of Malang (Universitas Negeri Malang), Jl. Semarang No. 5, Malang, East Java, 65145, Indonesia

[3]Key Laboratory of Water-Sediment Sciences and Water Disaster Prevention of Hunan Province, School of Hydraulic Engineering, Changsha University of Science & Technology, Changsha 410004, China

[4]PT. Tjiwi Kimia Paper Factory Tbk., Indonesia

*Corresponding author (e-mail: eli.hendrik.fmipa@um.ac.id)

Amylase is one of the most widely used enzymes in various industries. However, in Indonesia, its supply largely relies on imports, highlighting the need for domestic production using indigenous Indonesian microbes. This study aimed to isolate and characterize indigenous microbes capable of producing amylase from wastewater of the paper industry in Mojokerto, East Java. The research stages included sample preparation, isolation of amylase-producing microbes, crude amylase extract production, enzyme activity testing, optimization of amylase production, determination of specific activity, and bacterial identification by genotype. The study successfully identified two bacterial isolates, A7 and A8, that produce amylase enzymes, with enzyme activity values of 1.084 ± 0.026 U/mL and 0.784 ± 0.012 U/mL, respectively. Isolate A7 exhibited optimal amylase production at pH 3 and 37°C, with a specific activity of 1.092 ± 0.027 U/mg. In contrast, isolate A8 reached its optimal production at pH 7 and 37°C, with a specific activity of 0.805 ± 0.028 U/mg. Genotypic identification using 16S rRNA revealed that isolate A7 was classified as *Metabacillus niabensis* with a 100% similarity, while isolate A8 was identified as *Fictibacillus nanhaiensis* with a similarity of 99.79%.

Keywords: Isolation and characterization; microbial indigen; paper industry liquid waste; amylase enzyme

Received: September 2024; Accepted: January 2025

Enzymes are biomolecules that function as biocatalysts in a chemical reaction that can accelerate the rate of reaction with very low concentration requirements, and able to accelerate a reaction without participating in the reaction process [1]. The demand for enzymes is substantial and has grown significantly, increasing by 33 thousand tons between 2019 and 2023. [2]. One of the enzymes that is widely used in the industry is the amylase enzyme which is commercially one of the most important enzymes, accounting for around 30% of the global enzyme market and continues to increase [3]. The demand for amylase enzyme in Indonesia is very high and still depends on imported products especially for applications in industries such as food, textiles, starch conversion for syrup sugar, beer fermentation, detergents, and the paper industry.

The need for amylase enzyme is high, the source of amylase enzyme manufacturers is constantly soughtone of the sources is easy to find in microorganisms. The advantages of using microbes as amylase producers include higher production rates, stability, fast growth rates, easy availability of a large number of microbial strains and can produce commercial enzymes that have added value and value compared to amylase from plants and animals [3]. Amylase-producing bacteria are mostly found in waste containing organic matter [4] in the form of carbo-hydrates, proteins and fats [5].

Research on the isolation of amylase-producing microbes from waste has been carried out by several researchers, such as the liquid waste of the sugar industry succeeded in identifying amylase enzyme-producing microbes, namely *Bacillus infantis*, *Bacillus flexus*, and *Pseudomonas nitroreducens* with amylase enzyme activity of 0.108 U/mL, 0.056 U/mL, 0.107 U/mL, respectively [6]. In another study originating from dairy industry waste [7], the species *Bacillus stratosphericus* and *Aspergillus welwitschiae* were obtained with amylase enzyme activity of 24.4 U/mL and 12.58 U/mL. However, until now there has been no research using samples of liquid waste from the paper industry, so in this study the sample was used as an effort to find a source of microbial indigen that produces amylase enzymes.

†Paper presented at the International Conference in Organic Synthesis 2024 (ICOS 2024)

Amylase is an enzyme that catalyzes the hydrolysis of alpha-1,4-glycosidic polysaccharides to produce dextrin, oligosaccharides, maltose, and D-glucose [8], [9]. The enzymatic hydrolysis process is more effective because the enzymes break down the glycosidic bonds specifically without leaving significant residues or color damage [10]. The starch hydrolysis process using amylase enzyme can also be carried out at low temperatures, namely at 30°C so that it can replace the conventional starch hydrolysis process using acid catalysts that require high temperatures (90-125°) [11]. Amylase enzymes in the paper industry are applied as ink removal, cleaning, drainage improvement, and surface coating that can improve the smoothness, strength, and quality of writing [12]. One of them is the paper industry in Mojokerto, East Java. The pulp and paper production process produces waste that contains inorganic and organic compounds. Organic compounds such as carbohydrates or polysaccharides consisting of cellulose, hemicellulose, pectin and lignin derived from wood raw materials as well as the use of amylase enzymes.

This study aims to isolate the indigen microbes that produce amylase enzymes which are characterized based on the optimization of incubation time, pH and temperature, as well as genotype identification of amylase enzyme producing indigen microbial isolates derived from liquid waste from the paper industry in Mojokerto, East Java.

EXPERIMENTAL

Isolation of Amylase Enzyme Producing Microbes

Isolation of amylase enzyme-producing microbes was perfomed by spread plate technique on Starch Agar selective media containing 1% starch and 2.8 grams of Nutrients Agar (Himedia) in 100 mL of aquades. Dilution was carried out using a 0.85% NaCl solution in a ratio of 1:9, inserted into each test tube that had been labeled from a dilution level of 10^{-1} to a dilution level of 10^{-12}. From the dilution results, 100 µL was taken to be spread on the selective medium of Starch Agar, incubated at 37°C for 24 hours in an incubator [13]. Next, the bacteria that grow are selected to be purified using the strike plate technique, incubated at a temperature of 37°C for 24 hours in an incubator.

Then bacterial isolates that have been purified with the strike plate technique, an iodine test is carried out to confirm the existence of a starch hydrolysis reaction on the Starch Agar selective medium [14]. Bacteria that produce clear zones indicate the activity of the amylase enzyme [15]. Next, it is measured by the caliper to determine the magnitude of the amylolitic index. The amylolitic index can be calculated using the following formula (Eq. 1).

$$\text{Amylolytic index} = \frac{\text{Clear zone diameter (mm)} - \text{Colony diameter (mm)}}{\text{Colony diameter (mm)}} \quad \text{(Eq.1)}$$

Production of Crude Amylase Extract

Bacterial isolate was put into 20 mL of liquid medium containing Nutrient Broth (Himedia) and 1% starch then incubated at 37°C 100 rpm. After 24 hours, 10% of the bacterial culture was taken, put into 30 mL of production medium containing (g/L) NB 13, starch 10, $MgSO_4.7H_2O$ 0.25, KH_2PO_4 0.05, $CaCl_2.2H_2O$ 0.05, $MnCl2.4H_2O$ 0.015, and $FeSO_4.7H_2O$ 0.01. Optical Density (OD) was measured at a wavelength of 600 nm which is used to measure the growth curve of bacteria [16] from days 1-10 or until the growth curve decreases. The culture is centrifuged for 15 minutes at 10,000 rpm at 4°C to produce crude extract. Furthermore, the activity of the amylase enzyme was measured by the DNS method and the protein content was measured by the Lowry method.

Amylase Enzyme Activity Test

The DNS method is used to measure the concentration of reducing sugars contained in crude extracts using a glucose solution as the standard solution. Glucose solutions with concentration variations of 0, 50, 100, 150, 200, 250, 300, 350, 400 and 450 µg/mL were used to create the standard glucose curve. The concentration of reducing sugar in the DNS method supernatant was carried out by reacting one mL of supernatant with one mL of 1% starch solution incubated at 37°C for 10 minutes. Then two point five mL of DNS reagent was added. The mixture is reacted at 80-100°C for five minutes. After that, the test tube containing the mixture is cooled to stop the reaction by rinsing it with running water, then ice water so that the reaski tube does not break. Absorbance was measured at a wavelength of 540 nm [17]. The absorbance value is then incorporated into the linear regression equation on the standard curve of glucose to determine the concentration of reducing sugars in supernatants. Amylase Enzyme (AE) activity can be calculated by the following formula (Eq. 2) [18].

$$\text{Enzyme activity} = \frac{\text{Glucose concentration (µg/mL) x Total volume (mL)}}{\text{Mr glucose (µg/µmol) x Incubation time (minutes) x Amylase volume (mL)}} \quad \text{(Eq. 2)}$$

Optimization of Amylase Enzyme Production

In the optimization of amylase enzyme production, it is carried out with variations in incubation time, pH of the production medium and incubation temperature. The incubation time optimization was carried out at 37ºC [19] and at pH seven [7, 20] for one until ten days. The pH optimization of the production media is carried out at pH 3, 4, 5, 6, 7, 8, and 9 by adding HCl and NaOH to the desired pH [7], this pH optimization is carried out at the optimal incubation time that has been obtained previously. Incubation temperature optimization was performed at 27°C, 37°C, 45°C and 55°C with the optimal incubation time and optimal pH previously obtained. Then the bacterial culture that has been obtained from the results of production optimization is centrifuged for 15 minutes at 10.000 rpm at a temperature of 4°C to produce a crude extract. Furthermore, the activity of the amylase enzyme was measured by the DNS method and the protein content was measured by the Lowry method.

Determination of Specific Activity of Amylase Enzyme

Protein concentrations were determined by Lowry's method using Bovine Serum Albumin (BSA) solution as a standard solution. BSA solutions with concentration variations of 20, 40, 60, 80, 100, 120, 140 and 160 µg/mL were used to create the standard curve of the protein. Supernatants of zero point five mL were reacted with two point five mL of biurete reagent, shaken with vortex and incubated at room temperature for 10 minutes. Next, zero point two five mL of Folin-Ciocalteu reagent was added, re-vortified and re-incubated at room temperature for 30 minutes. Absorbance was measured using a UV-Vis Spectrophotometer at 750 nm [21]. The absorbance value is then incorporated into the linear regression equation on the protein standard curve to determine the concentration of protein concentration in the supernatant. Specific Activity of amylase can be calculated by the following formula (Eq. 3).

Species Identification by Genotype

The 16S rRNA gene sequencing method is used to determine the species of bacteria produced. The ZymoBIOMICSTM DNA Miniprep Kit is used to isolate DNA measured using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer at wavelengths of 260/280 nm. Furthermore, the amplification of the 16S rRNA gene by the Polymerase Chain Reaction method using a 16s primer (27F-1492R), and then the DNA size of the PCR results was examined using agarose gel electrophoresis. The appropriate size of DNA is then sequenced to obtain the 16S rRNA gene. These results were then analyzed to confirm similarity with other bacteria in the gene database contained in NCBI using BLAST [22]. Then phylogenetic analysis is carried out to find out its similarity with other species.

RESULTS AND DISCUSSION

Isolation of Amylase-Producing Microbes

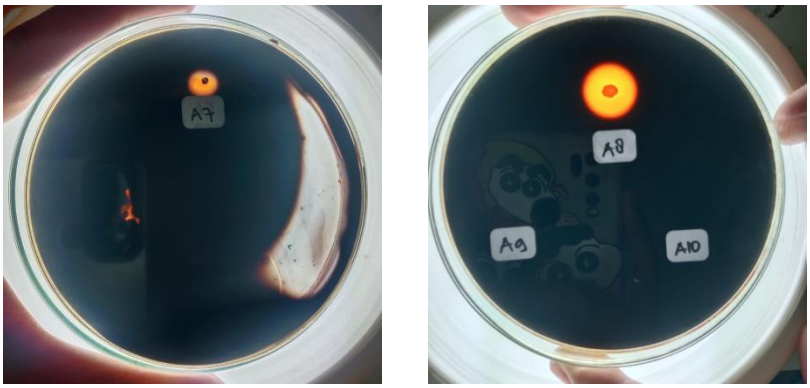
Based on the spread plate method using serial dilutions of paper industry liquid waste samples on Starch Agar selective media, incubated for 24 hours at 37°C, 10 isolates with amylase-producing potential were identified. The results of the spread plate at dilution levels of 10^{-1} to 10^{-12} are summarized in Table 1.

The largest number of colonies was found at 10^{-1} dilution, namely six bacterial isolates. The number of colonies growing in this study is not linear with the level of dilution carried out. The lower the sample concentration, the lower the number of colonies that grow. At dilutions 10^{-4} to 10^{-8} there are no growing colonies, the same is true for dilutions 10^{-10} and 10^{-12}. This is due to the concentration of the sample being too small and not enough to grow. However, at dilution 10^{-9} and 10^{-11} some cells still survive in harsh conditions and grow back.

$$\text{Specific Activities} = \frac{\text{Amylase enzyme activity (U/mL)}}{\text{Protein content (mg/mL)}} \qquad \text{(Eq. 3)}$$

Table 1. The plate spread results were obtained at dilution levels ranging from 10^{-1} to 10^{-12}.

No	Dilution Rate	Number of Bacterial Isolates Collected
1	10^{-1}	6
2	10^{-2}	1
3	10^{-3}	1
4	10^{-4}	-
5	10^{-5}	-
6	10^{-6}	-
7	10^{-7}	-
8	10^{-8}	-
9	10^{-9}	1
10	10^{-10}	-
11	10^{-11}	1
12	10^{-12}	-



(a) (b)

Figure 1. Iodine Test Results (a) A7 isolate and (b) A8 isolate.

Of the 10 bacterial isolates selected, there are two isolates that are positive for producing amylase enzymes, namely bacterial isolates A7 and A8 shown in Figure 1. The formation of a clear zone around the bacterial colony indicates that the starch has been hydrolyzed into simpler compounds (glucose) by bacterial isolates. Meanwhile, the blackish-blue medium indicates that the starch has not been hydrolyzed or called the iodine-amylum complex [13]. The difference in the diameter of clear zones such as A7 and A8 depends on the ability of bacteria to produce the enzyme amylase. This ability (amylolytic index) can be measured by comparing the diameter of the clear zone with the diameter of the colony [23]. The magnitude of the amylolytic index in both isolates can be seen in Table 2.

Table 2. Amylolytic index of amylase-producing bacteria.

Bacterial Isolates	Clear Zone Diameter (mm)	Bacterial Colony Diameter (mm)	Amylolytic Index
A7	7.60	5.65	0.345
A8	14.20	3.45	3.116

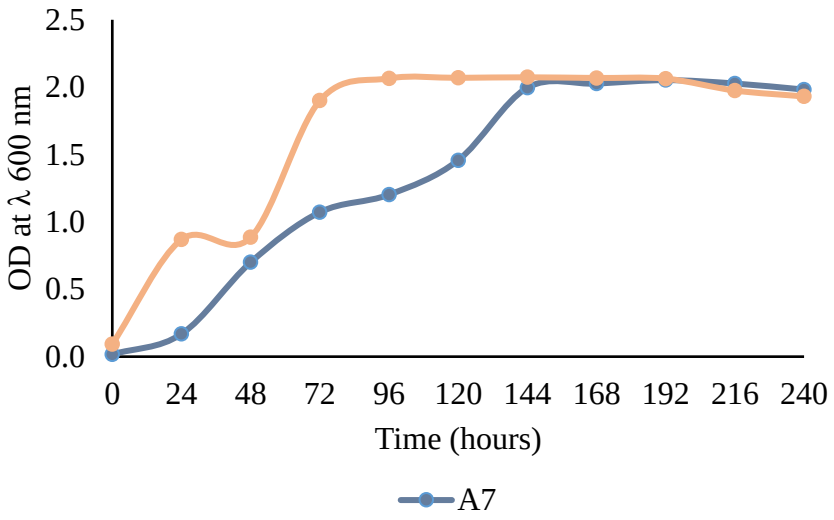


Figure 2. Growth curve of amylase-producing bacteria.

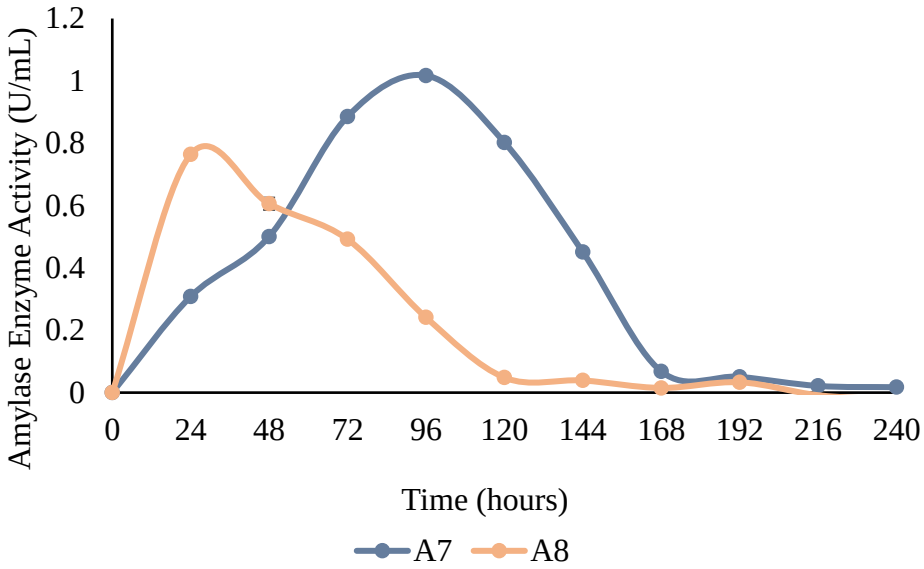


Figure 3. Optimization of incubation time of amylase-producing bacteria.

Based on the results shown in Figure 2, it can be seen that as time goes on, the production media becomes more cloudy, which indicates the growth of bacteria. Figure 2 shows that the exponential phase in isolate A7 occurs at hours 0 to 144, while in isolation A8 occurs at hours 0 to 96. In this phase, the two bacterial isolates increase because bacterial cells undergo rapid dividing. Furthermore, the bacterial isolate underwent a stationary phase and a death phase that occurred at the 216 hours which was shown to decrease the growth curve. In this phase of death, the amount of nutrients decreases and is depleted so that the number of dead cells is more than the living cells.

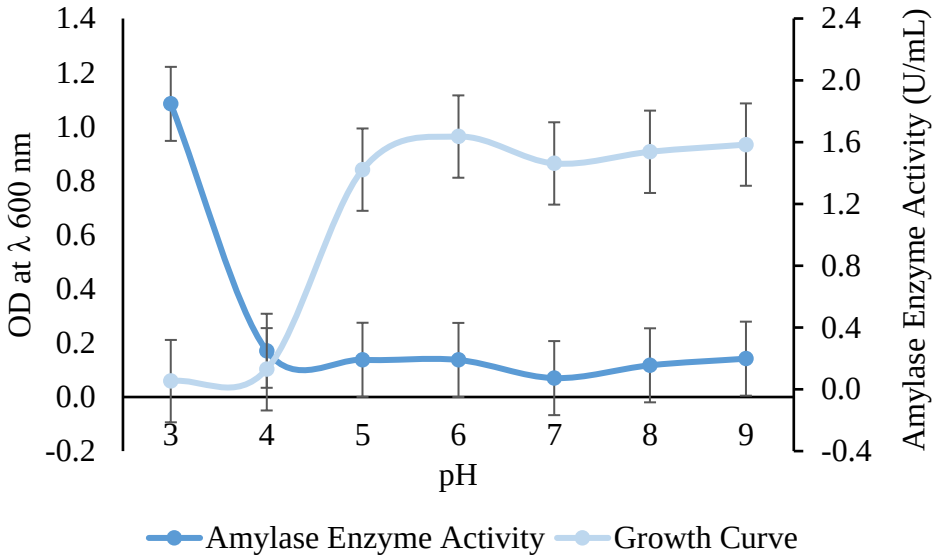
Incubation Time Optimization

Based on Figure 3, the activity value of A8 bacterial isolate enzyme was obtained at 0.764 U/mL, showing a lower result with a difference of 24.88% compared to A7 isolate of 1.017. The two isolates showed different optimal conditions for incubation time, A7 occurred at 96 hours while A8 occurred at 24 hours, because the exponential phase of the A7 growth curve was slower so that it had a slower optimal incubation time as well. Despite having different incubation time conditions, the two isolates showed the highest amylase enzyme activity in the exponential phase (Figure 2). This is supported by research conducted (Oktavia et al., 2018) which also stated that the optimal incubation time condition occurs in the exponential phase, so it is the most effective time to produce high amylase enzyme activity because in this phase bacterial cells undergo rapid dividing.

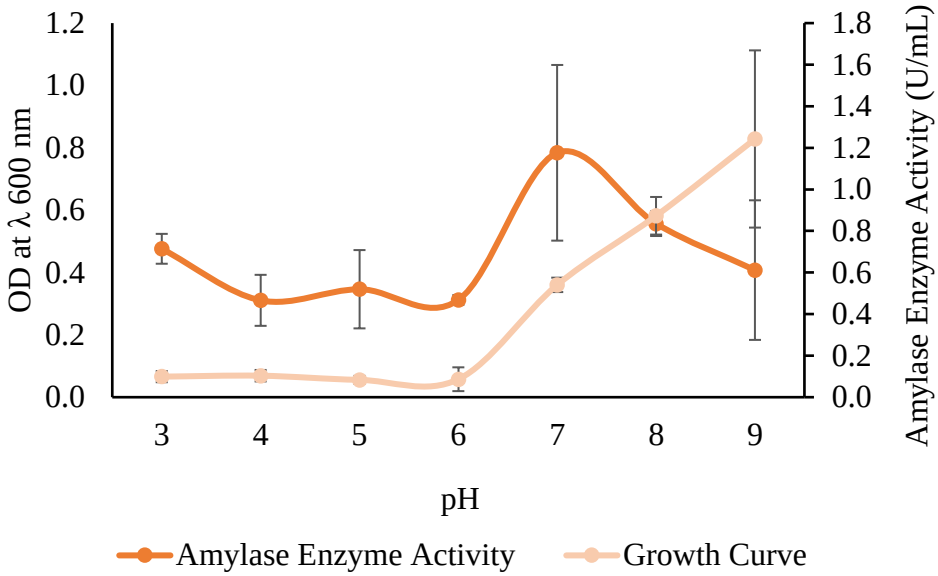
pH Optimization

The production pH optimization in this study was carried out at a temperature of 37ºC [25] with the optimal incubation time obtained in the previous stage, namely A7 optimum at 96 hours and A8 optimum at 24 hours (Figure 2) and in the range of 3 – 9. Based on Figure 4, the optimum pH obtained in A7 isolate is at pH 3 with an enzyme activity of 1.084 U/mL, the optimum pH obtained in this isolate is close to the sample pH of 3.65. Meanwhile, A8 isolate is optimal at pH 7 with enzyme activity of 0.784 U/mL. The result obtained by A7 is 27.68% higher when compared to A8 isolate. If it is associated with Figure 3, it is known that the A7 isolate is slower because this isolate does not work at the optimum pH conditions of the production medium. At the optimum pH of the bacterial production medium, the enzyme protein will form a very precise 3D structure so that the enzyme can bind and process the substrate at a high speed.

In the optimum A7 bacterial isolate at pH 3, this result has not been found in previous studies for the optimal pH condition of amylase enzyme-producing bacteria. pH optimization with the lowest acidic conditions was found at a pH value of 5 in the study [25] and a pH value of 6 in the study [26]. A7 bacterial isolate is a type of acidophilic bacteria that grows in the pH range of 1.0-5.5. These bacteria can survive in environments with acidic pH because they can excrete protons from the intracellular space to maintain the cytoplasm until the pH atmosphere of the environment is close to neutral [27]. The A8 bacterial isolate is optimal at pH 7 so it is included in the neutrophil bacteria type. Because this type of bacteria can live in the pH range of 5.5 to 8.5. The same pH value was also obtained in the study conducted [7] in the isolate of *Bacillus stratosphericus*, other studies also obtained the same pH value in the species *Bacillus subtilis* [28].



(a)



(b)

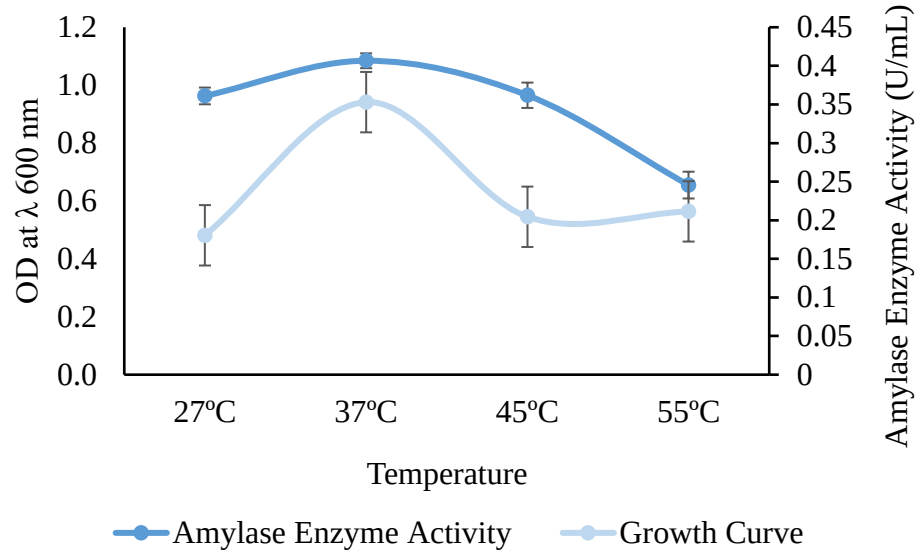
Figure 4. pH optimization of amylase enzyme production media (a) isolate A7 and (b) isolate A8.

Temperature Optimization

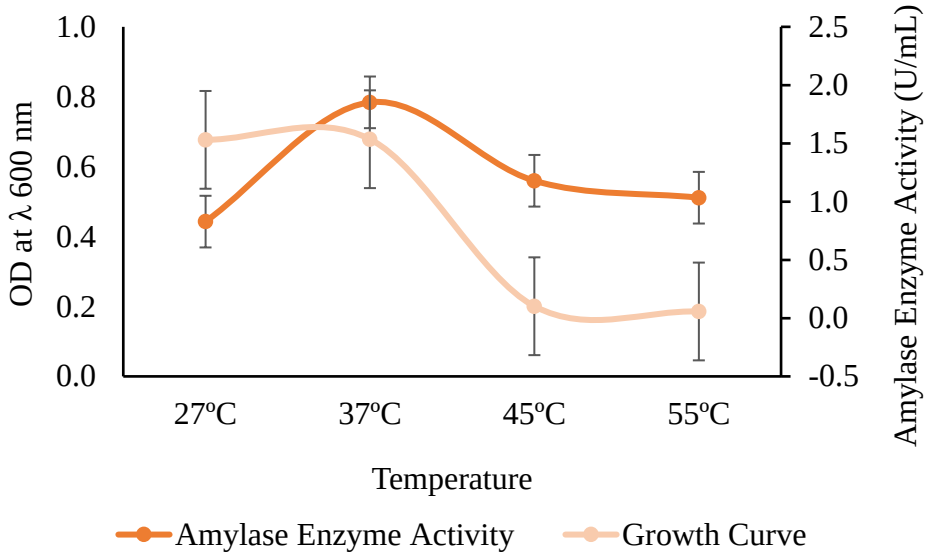
Temperature optimization in this study was carried out at the incubation time and pH that had been obtained previously. The two isolates have the same optimum temperature, which is optimal at 37ºC with an enzyme activity value in A7 isolate of 1.084 U/mL and A8 isolate of 0.784 U/mL (Figure 5). The activity value of the amylase A7 enzyme difference is 27.68% higher when compared to A8 isolate. These isolates include mesophilic bacteria in the range of 15ºC to 45ºC [29] and are a type of bacteria that are often found because they have an optimal temperature of their living environment that is not much different from most living things in general. In the study [30] it succeeded in obtaining the same temperature optimization production of 37ºC with the *Bacillus megaterium* species, the same results were also obtained in the study [19] with the *Bacillus halotolerant* species.

At optimal temperatures, the collision between enzymes and substrates is very effective, so the formation of enzyme-substrate complexes is easier and can increase the resulting product [30]. At 37ºC, the structure of the amylase protein is generally stable so that the activity of the enzyme can be maintained for a longer period of time. At a temperature that is too high, the interaction between molecules weakens so that the fluidity of the cell increases.

This can also damage cell metabolism because there are molecules that should not enter the cell, and can damage proteins and components that make up cells due to denaturation. Meanwhile, at a temperature that is too low from the optimum temperature, it will cause cell metabolism to slow down because the chemical reactions and diffusion that occur in the cell also run slowly [6].



(a)



(b)

Figure 5. Optimization of the production temperature of amylase enzymes (a) isolate A7 and (b) isolate A8.

Table 3. Results of enzyme activity, protein concentration and specific activity of amylase enzyme

Bacterial Isolate	A7	A8
	Average	Average
Amylase Activity (U/mL)	1.084±0.026	0.784±0.012
ProteinConcentration (mg/mL)	0,993	0,971
Amylase Specific Activities (U/mg)	1.092±0.027	0.805±0.028

Determination of Specific Amylase Activity

In this study, the results of the optimum A7 isolate were obtained at the 96 hours incubation time with a pH value of 3 and a temperature of 37ºC, this isolate produced an amylase enzyme activity of 1,084 U/mL. while in the optimum A8 isolate at 24 hours with a pH value of 7 and a temperature of 37ºC, this isolate produces an amylase enzyme activity of 0.784 U/mL. Based on the results of the enzyme activity obtained, the protein concentration and specific activity of the amylase enzyme are obtained in Table 3. A7 bacterial isolate with a value of 1.092 U/mg had a specific activity 26.28% higher than A8 bacterial isolate with a specific activity of 0.805 U/mg. The protein content obtained can be used to determine the purity level of the amylase enzyme, namely by calculating the specific activity of the amylase enzyme. The specific activity of the enzyme indicates the number of units of product produced per mg of the enzyme, where the higher the value, the higher the purity.

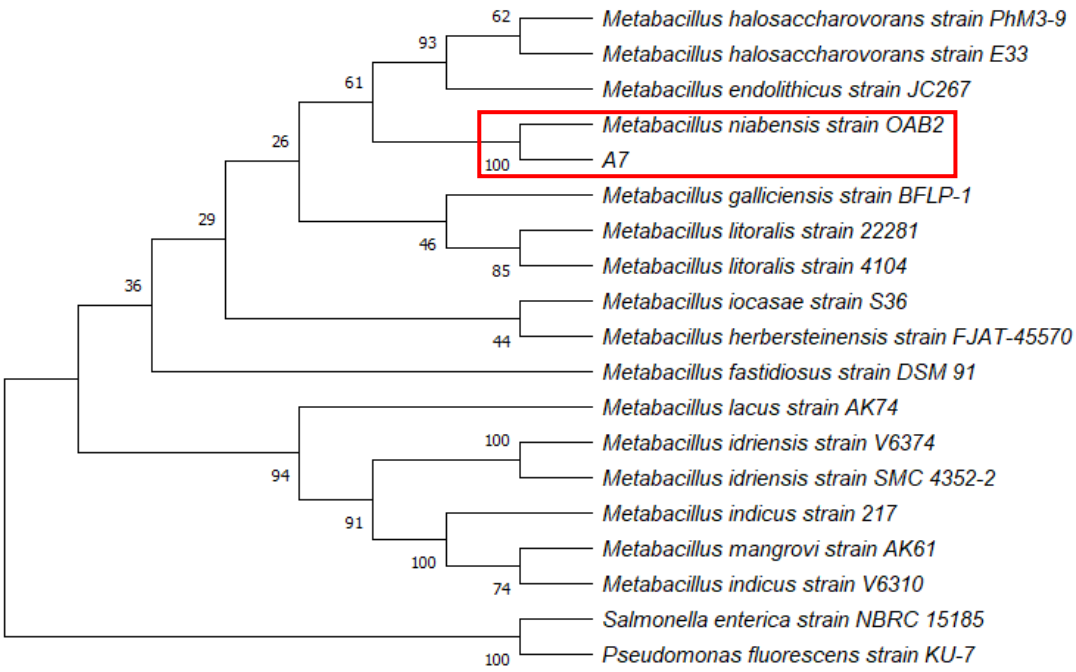
The specific activity obtained was much higher when compared to the study [6], the results of the specific activity of the three bacterial isolates were 0.108 U/mg, 0.198 and 0.208, respectively, in *Bacillus infantis*, *Bacillus flexus*, and *Pseudomonas nitroreducens* isolated from the liquid waste of the sugar industry. This is caused by different sources of bacteria. The concentration of the enzyme correlates with the speed of the reaction, so the amount of product (reducing sugar) produced correlates with the amount of amylase enzyme produced, and the high specific activity of the amylase enzyme indicates that the bacterial isolate can produce more amylase enzymes.

Identification of Bacteria by Genotype

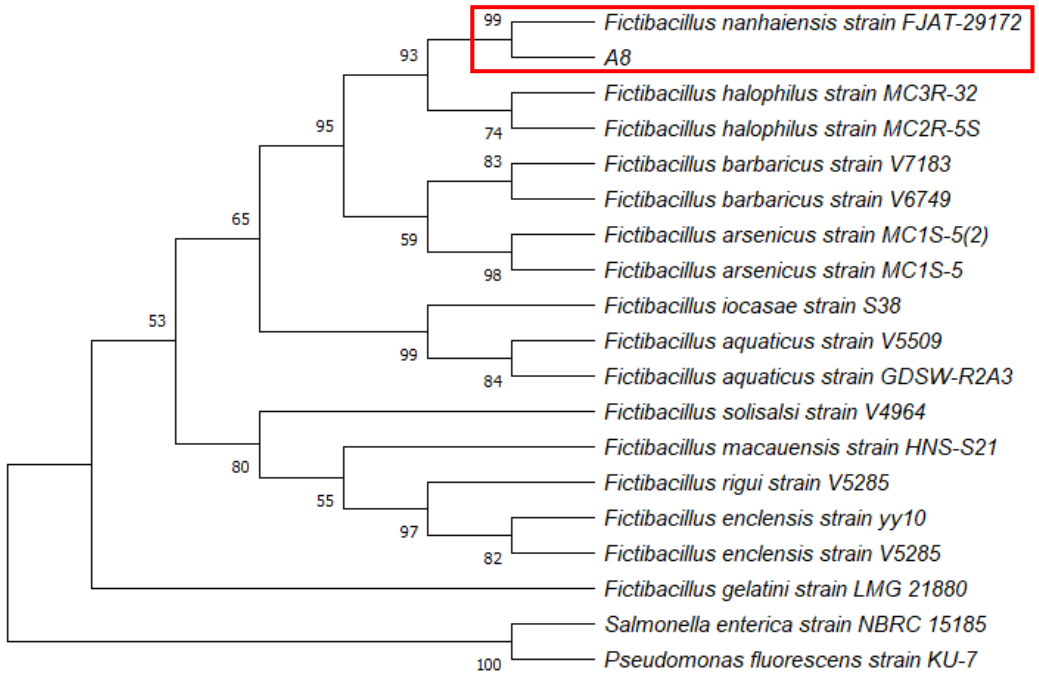
Genotype sequencing of 16S rRNA in isolates A7 and A8 was obtained in sizes of 1416 bp and 1415 bp, respectively. Next, phylogenic analysis was carried out to determine the similarity with species from other bacteria in the gene database contained in NCBI using BLAST. According to Stackebrandt & Goebel (1994), if the similarity of the 16S rRNA gene has a "percentage identity" value above 95%, then the similarity is at the genus level, and if the "percentage identity" value is above 97.5%, the similarity is at the species level [31]. So it is known (Figure 6) that A7 bacterial isolate belongs to the genus *Metabacillus* closely related to *Metabacillus niabensis strain OAB2* with a percentage identity (similarity) of 100%, while A8 isolate belongs to the genus *Fictibacillus* and is closely related to *Fictibacillus nanhaiensis strain FJAT-29172* has a 99.79% similarity. It can be concluded from the statement of Stackebrandt & Goebel (1994), the two bacterial isolates both A7 and A8 have a similarity level at the species level with a value above 97.5% [31]. The results obtained are in line with previous research conducted by [6], reporting that the amylase-producing bacterial isolates obtained have a percentage similarity with *Bacillus infantis*, *Bacillus flexus*, and *Pseudomonas nitroreducens* bacteria of 99.8%, 99.9%, and 99.7%, respectively.

A7 isolate, which has similarities to *Metabacillus niabensis*, is Gram-positive and bar-shaped [32]. The same characteristics are also shared by A8 isolate which bears a resemblance to *Fictibacillus nahaiensis*. The optimum pH condition of *Metabacillus niabensis* bacteria including acidophilic bacteria is optimal at acidic pH, while previous studies [33] on the same species showed optimum at pH 7 (neutrophil bacteria), and in the same genus as *Metabacillus sediminilitoris* also reported optimum at neutral pH [34]. However, not all genera of *Metabacillus* have an optimal neutral pH. While some species can thrive in a neutral pH environment, others can survive in a variety of pH conditions. For example, *Metabacillus dongyingensis*, a plant growth-promoting bacterium, is isolated from alkaline salt-containing soils and can tolerate high salt concentrations [35]. This suggests that some *Metabacillus* species can adapt to environments with varying pH levels. So that the difference in the optimal pH of *Metabacillus niabensis* obtained in this study is because different bacterial sources can show different bacterial optimum conditions as well.

Isolate A7 which is *Metabacillus niabensis* and isolate A8 which is *Fictibacillus nanhaiensis*, both show bacteria that have amylase enzyme activity. However, until now there has been no other study that has done the same stating that the bacteria *Metabacillus niabensis* and *Fictibacillus nanhaiensis* have amylae enzyme activity. Several previous studies have shown that the genus *Bacillus* is an amylase-producing bacterium such as *Bacillus tequilensis* [36], *Bacillus licheniformis* [37], *Bacillus atrophaeus* [9], *Bacillus stratosphericus* [7], *Bacillus halotolerans* [19].



(a)



(b)

Figure 6. Phylogenic dendrograms (a) isolate A7 and (b) isolate A8.

CONCLUSION

This study identified two bacterial isolates producing amylase enzymes: A7 and A8. Isolate A7 showed optimal enzyme activity (1.084±0.026 U/mL) at 96 hours of incubation, while A8 reached its peak activity (0.784±0.012 U/mL) at 24 hours. A7 was most active at pH 3 and 37°C, with a specific activity of 1.092± 0.027 U/mg. In contrast, A8 was optimal at pH 7 and 37°C, with a specific activity of 0.805±0.028 U/mg. Genotype identification using 16S rRNA revealed that isolate A7 had 100% similarity to *Metabacillus niabensis*, and isolate A8 had 99.79% similarity to *Fictibacillus nanhaiensis*.

ACKNOWLEDGEMENTS

The author would like to thank all fellow researchers, especially colleagues at Universitas Negeri Malang who have helped with this research.

REFERENCES

1. Kiti, A. A., Jamilah, I. and Rusmarilin, H. (2020) Studi Kualitatif Aktivitas Amilolitik Bakteri Asam Laktat Yang Diisololasi Dari Pangan Tradisional Aceh Pliek U. *Health and Contemporary Technology Journal*, **1(1)**, 5–9.

2. Badan Pusat Statistik (BPS) (2024).

3. Yassin, S. N., Jiru, T. M. and Indracanti, M. (2021) Screening and Characterization of Thermostable Amylase-Producing Bacteria Isolated from Soil Samples of Afdera, Afar Region, and Molecular Detection of Amylase-Coding Gene. *Int. J. Microbiol*. doi: 10.1155/2021/5592885.

4. Pranay, K., Padmadeo, S. R., Jha, V. and Prasad, B. (2019) Screening and identification of amylase producing strains of bacillus. *J. Appl. Biol. Biotechnol.*, **7(4)**, 57–62. doi: 10.7324/JABB. 2019.70409.

5. Rahayu, I., Masturi, A. & Yulianti, M. (2015) Pengaruh Perubahan Massa Zeolit Terhadap Kadar Ph Limbah Pabrik Gula Melalui Media Filtrasi. *Jurnal Fisika*, **5(2)**.

6. Sanjaya, E. H., Suharti, S., Alvionita, M., Telussa, I., Febriana, S. and Clevanota, H. (2024) Isolation and Characterization of Amylase Enzyme Produced by Indigenous Bacteria from Sugar Factory Waste. *Open Biotechnol. J.*, **18(1)**, 1–13. doi: 10.2174/ 0118740707296261240418114958.

7. Osho, M. B., Awe-Mathias, C. A. K. and Onajobi, I. B. (2021) Biosynthesis and characterizations of extracellular enzymes of microbial isolates from dairy industrial effluent. *Ife Journal of Science*, **23(2)**, 121–134. doi: 10.4314/ijs.v23i2.13.

8. Dinamika, J. (2016) Pengenalan Enzim Amilase (Alpha-Amylase) Dan Reaksi Enzimatisnya Menghidrolisis Amilosa Pati Menjadi Glukosa, **7(1)**, 74–82.

9. Abd-Elaziz, A. L., Karam, A. M., Ghanem, E. A., Moharam, M. M. & Kansoh, M. E. (2020) Production of a novel α-amylase by *Bacillus atrophaeus* NRC1 isolated from honey: Purification and characterization. *Int. J. Biol. Macromol.*, **148**, 292–301.

10. Wahyuningsih, S. (2019) Pengaruh Konsentrasi Enzim α – Amilase pada Hidrolisis Pati Labu Jepang (Kabocha). *CHEESA: Chemical Engineering Research Articles*, **2(1)**, 26. doi: 10.25273/cheesa. v2i1.4433.

11. Hargono, H. (2019) Kinetika Hidrolisis Pati Singkong Manis (Manihot Esculenta) Pada Suhu Rendah. *Jurnal Inovasi Teknik Kimia*, **4(1)**, 11–15. doi: 10.31942/inteka.v4i1.2680.

12. E. Consultancy (2022) Exactitude Consultancy.

13. Rahayu, M. A., Sulistyaningtyas, A. R. and Darmawati, S. (2019) Isolasi Bakteri Hidrolitik Penghasil Enzim Amilase dari Limbah Industri Tapioka. *Prosiding Seminar Nasional*, **II**, 147–155.

14. Jati, O. E., Diponegoro, U., Ayuningrum, D., Diponegoro, U., Sabdaningsih, A. and Diponegoro, U. (2021) Skrining Bakteri Penghasil Enzim Amilase dari Sedimen Tambak Udang Vannamei (*Litopenaeus vannamei*). *JFMR-Journal of Fisheries and Marine Research*, **5(2)**. doi: 10.21776/ ub.jfmr.2021.005.02.15.

15. Ginting, S. S. B., Suryanto, D. and Desrita, D. (2018) Isolasi dan Karakterisasi Bakteri Potensial Probiotik Pada Saluran Pencernaan Ikan Bandeng (*Chanos chanos*). *Acta Aquatica: Aquatic Sciences Journal*, **5(1)**, 23–29. doi: 10.29103/aa.v5i1.390.

16. Prihatini, I. and Dewi, R. K. (2021) Kandungan Enzim Papain pada Pepaya (*Carica papaya L*) Terhadap Metabolisme Tubuh. *Jurnal Tadris IPA Indonesia*, **1(3)**, 449–458. doi: 10.21154/jtii. v1i3.312.

17. Supriyatna, A., Amalia, D., Jauhari, A. A. and Holydaziah, D. (2015) Aktivitas Enzim Amilase, Lipase, dan Protease dari Larva Hermetia Illucens yang Diberi Pakan Jerami Padi. *Jurnal Istek*, **9(2)**, 18–32, Jul, 2015.

18. Karimi-Avargani, M. (2023) Re: How to Calculate Enzyme Activity (for Alpha Amylase using DNS Method) in Excel Sheet? Accessed: Jul. 08. [Online]. Available: https://www.researchgate. net/post/how_to_calculate_enzyme_activity_for _alpha_amylase_using_DNS_method_in_excel _sheet/5efed6e62c4ab673044bd3fa/citation/ download

19. Rafanomezantsoa, P., Gharbi, S., Karkachi, N. and Kihal, M. (2023) Optimization of amylase production by the biological control agent Bacillus halotolerans RFP74 using response surface methodology. *Journal of Genetic Engineering and Biotechnology*, **21(1)**, 63. doi: 10.1186/s43141-023-00519-4.

20. Wang, Y., Tian, X., Zhang, Z., Tian, M. and Zhang, F. (2024) Investigation of the potential mechanisms of α -amylase and glucoamylase through ultrasound intensification, **198(2)**.

21. Martina, V. and Vojtech, K. (2015) A Comparison of Biuret, Lowry and Bradford Methods for Measuring the Egg'S Proteins. *Mendelnet*, **1(1)**, 394–398.

22. Noer, S. (2021) Identifikasi Bakteri secara Molekular Menggunakan 16S rRNA. *EduBiologia: Biological Science and Education Journal*, **1(1)**, 1. doi: 10.30998/edubiologia.v1i1.8596.

23. Sundari, A. S., Purwani, N. N. and Kurniati, A. (2019) Isolasi dan Penentuan Indeks Amilolitik Bakteri dari Sediment Mangrove di Wonorejo, Surabaya. *QUANTUM: Jurnal Inovasi Pendidikan Sains*, **10(1)**, 38–44, May, 2019.

24. Oktavia, Y., Lestari, S. D., Lestari, S., Herpandi and Jannah, M. (2018) Optimasi Waktu Inkubasi Produksi Protease Dan Amilase Isolat Bakteri Asal Terasi Ikan Teri *Stolephorus SP*. *Jurnal Ilmu dan Teknologi Kelautan Tropis*, **10(3)**, 719–725. doi: 10.29244/jitkt.v10i3.18840.

25. Liaqat, U. E. S., Naz, S. and Hussain, H. I. (2024) Optimization and production of highly stable amylase isolated from *Citrobacter portucalensis* using low-cost agro-industrial wastes as substrates: Prospective therapeutics. *Kuwait Journal of Science*, **51(1)**. doi: 10.1016/j.kjs.2023.11.005.

26. Philip, M., Kizhakedathil, J. and S. D. C. (2021) Acid stable α -amylase from *Pseudomonas balearica* VITPS19 — Production, purification and characterization, **30**, 0–7. doi: 10.1016/j.btre.2021.e00603.

27. Amico, S. D., Collins, T., Marx, J., Feller, G. and Gerday, C. (2006) Psychrophilic microorganisms: challenges for life, **7(4)**, 5–9. doi: 10.1038/sj.embor.7400662.

28. Sahu, P. K., *et al.* (2024) Microbial production of α-amylase from agro-waste: An approach towards biorefinery and bio-economy. *Energy Nexus*, **14**, April, 2024. doi: 10.1016/j.nexus.2024.100293.

29. Parker, B. M., Schneegurt, N., Tu, M., Lister, A. -H. T. & Forster, P. (2016) Microbiology. *OpenStax*.

30. Istia'nah, D., Utami, U. and Barizi, A. (2020) Karakterisasi Enzim Amilase dari Bakteri *Bacillus megaterium* pada Variasi Suhu, pH dan Konsentrasi Substrat. *Jurnal Riset Biologi dan Aplikasinya*, **2(1)**, 11. doi: 10.26740/jrba.v2n1.p11–17.

31. Stackebrandt, E. and Goebel, B. M. (1994) Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *Int. J. Syst. Bacteriol.*, **44(4)**, 846–849. doi: https://doi.org/10.1099/00207713-44-4-846.

32. Ilse, S., *et al.* (2023) Metabolomic Insights of Biosurfactant Activity from *Bacillus niabensis* against Planktonic Cells and Biofilm of Pseudomonas stutzeri Involved in Marine Biofouling.

33. Kwon, S., *et al.* (2007) *Bacillus niabensis sp.* nov., isolated from cotton-waste composts for mushroom cultivation, 1909–1913. doi: 10.1099/ijs.0.64178-0.

34. Mao, H., Wei, Y., Gao, Y., Pei, J., Zhang, Y. and Fang, J. (2020) *Metabacillus sediminilitoris sp.* nov., a marine bacterium isolated from a tidal sediment. *Int. J. Syst. Evol. Microbiol.*, **70(10)**, 5211–5216. doi: 10.1099/ijsem.0.004392.

35. Yin, Z., *et al.* (2022) *Metabacillus dongyingensis sp.* nov. Is Represented by the Plant Growth-Promoting Bacterium BY2G20 Isolated from Saline-Alkaline Soil and Enhances the Growth of Zea mays L. under Salt Stress. *mSystems*, **7(2)**, 1–16. doi: 10.1128/msystems.01426-21.

36. Paul, J. S., et al. (2020) Production of biocatalyst α-amylase from agro-waste 'rice bran'by using *Bacillus tequilensis TB5* and standardizing its production process. *Biocatal. Agric. Biotechnol.*, **26(101648)**.

37. Saleh, F., *et al.* (2020) Comparative growth potential of thermophilic amylolytic *Bacillus sp*. on unconventional media food wastes and its industrial application. *Saudi J. Biol. Sci.*, **27(12)**, 3499–3504. doi: 10.1016/j.sjbs.2020.09.045.