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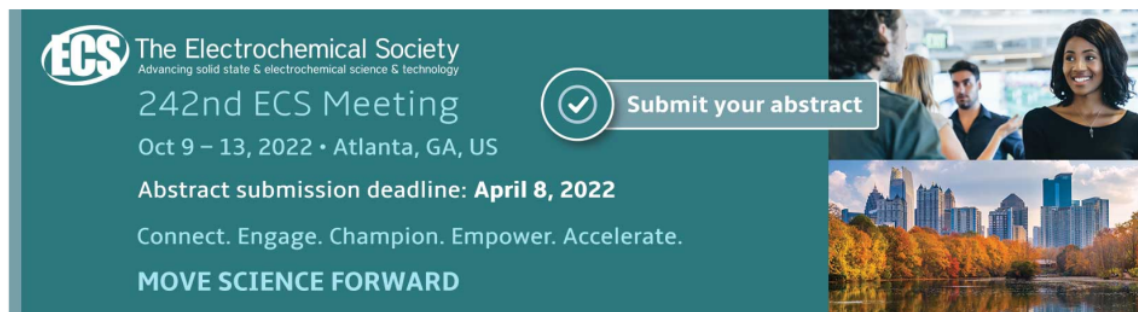
Isolation and selection of amylase-producing microbes isolated from *ragi tape* and cassava *tape* available on the markets

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Isolation and selection of amylase-producing microbes isolated from *ragi tape* and cassava *tape* available on the markets

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Abstract. Amylase has an important role in biotechnology development and occupies an important position in the world enzyme market, as a biocatalyst in various industrial fields. This study has the goal to find microbial isolates that have the ability to produce amylase enzymes. The study was conducted in two stages, namely: 1) Isolation and selection of microbes that can produce amylase enzymes using starch as substrate, was incubated for 4-7 days at 30°C. Microbial isolates that can produce amylase enzymes are characterized by the presence of clear zones around the colony after the addition of an iodine solution of 1% in the overgrown media of microbes, 2) Test the activity of amylase enzymes using a dinitrosalicylic acid reagent test. The activity of the amylase enzyme is determined by measurement using a spectrophotometer at a wavelength of 540 nm. The sample used comprised of 7 types of *ragi tape* and 2 samples from cassava *tape* that has been fermented for 5-7 days. The results obtained in the first stage were 65 microbial isolates, 16 of which had clear zones, consisting of 7 isolates from *ragi tape* samples and 9 isolates from cassava *tape* samples. In the enzyme activity test, there are several isolates that have the potential to produce amylase enzymes, these include R514 (0.897 ± 0.018 U/mL), R215.1 (0.814 ± 0.011 U/mL), R513 (0.727 ± 0.042 U/mL) (derived from cassava *ragi tape* samples) and T212.2 (0.812 ± 0.013 U/mL), T216.1 (0.817 ± 0.010 U/mL), T212.1 (0.735 ± 0.023 U/mL), T114 (0.755 ± 0.020 U/mL) (derived from cassava *tape* samples). The isolate with the highest enzyme activity is the R514 which has the value enzyme activity of 0.897 ± 0.018 U/mL and with a fairly high or moderate category, while the lowest enzyme activity is the T111.1 isolate of 0.284 ± 0.020 U/mL.

1. Introduction

Enzymes have an important role as biocatalysts in various industrial fields. One of the enzymes that has this important role is the amylase enzyme. Amylase enzyme is an enzyme that can be used to hydrolyze polysaccharides such as starch into simple sugar [1]. Amylase occupies an important position in the world enzyme market by 25% to 33% [2], [3]. While the consumption of amylase enzymes in the country reached 2,500 tons with an import value of 187.5 billion in 2015 [4]. Amylase enzymes are commonly used in the bread making, textile, detergent and pharmaceutical industries [5], [6]. The most common amylase used in the food industry are the α -amylase (EC.3.2.1.1) and β -amylase (EC 3.2.1.2) [6], [7].



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Amylase is produced from all sources of life (plants, animals, and microorganisms) and its demand continues to increase due to various industrial applications [8]. One source of amylase enzymes is a fermented product of cassava, namely cassava *tape*. Apart from cassava *tape*, of course, the enzyme can also be obtained from the production of microbes used for the production of *tape* itself. Amylase enzymes can be produced by microorganisms especially amylolytic microbes [9]. However, amylase enzymes for industrial purposes are mostly isolated from microbes. The choice of microbes as a source of enzymes because it has several advantages when compared to plants and animals, such as easy and rapid to grow, easier in the production scale-up, cheaper in the production costs, and easy to control in many production parameters:

Ragi tape is an inoculum containing microbial cultures which have amylolytic and fermentative activities. The same types of inoculums as *ragi* in Indonesia are *bubod* from the Philippines, *luk pang* from Thailand, *peh chu* from China, *bakhar*, *mucha*, *ranu*, *u-y-iat* from India, *juipaing* from Malaysia and *nooruk* from Korea. Amylolytic and fermentative microbes that have been successfully isolated by the researchers from various brands of *ragi tape* from several places and markets in Indonesia are a combination of *Amylomyces rouxii*, *Rhizopus oryzae*, *Endomycopsis burtonii*, *Mucor* sp., *Candida utilis*, *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, and some lactic acid bacteria (LAB) *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *L. fermentum* [10], [11], [12]. The researchers from Philippines, Malaysia, Thailand and Vietnam also found similar microbial species in the inoculums used in their regions.

Amylolytic microbes in traditional fermentation have good uses, which will accelerate the process of starch degradation into simple sugars. For example, in the fermentation of cassava *tape* the resulting simple sugar will be converted into organic acids and alcohols which make a distinctive aroma of *tape*. Therefore, the amylolytic ability of the enzyme is interesting because it has potential for many practical purposes. This research is a first step in obtaining amylase enzymes produced by amylolytic microbial isolates from *ragi* and cassava *tape* so that it can be a source of alternative for amylase enzymes. The purpose of the study was to isolate and select amylolytic microbes found in *ragi* and cassava *tape*.

2. Materials and methods

2.1. Materials

Ragi tape samples (*ragi 99*, *ragi Sari Madu*, *ragi NKL*, *ragi Gedang*, *ragi Matahari Cakra*, *ragi Berlian*, and *ragi LBC*), cassava *tape* samples (Jember honey cassava *tape* "Special" and sweet cassava *tape* "Sari Madu"), starch, peptone, beef extract, yeast extract, NaCl, agar powder, iodine, potassium iodine, CH₃COOH, C₂H₃NaO₂, 3,5-dinitrosalicylic acid (DNS), distilled water, and alcohol.

2.2. Isolation and selection of microbes

Microbe isolation was done by spread plate method and isolate purification. The insulation media used starch agar medium (peptone 0.5 g; beef extract 0.15 g; yeast extract 0.15 g; NaCl 0.5 g; starch, 1 g; agar 2 g; distilled water 100 mL) [13], [14]. Before isolation, the sample was first diluted to 10⁻⁷ in NaCl solution, then the spread plate stage was carried out and incubated for 5–7 days at a temperature of 30°C. Then the purification of isolate was conducted using starch agar medium (SAM).

The pure isolate was then tested for a clear zone test by adding 1% iodine solution (iodine 0.2 mL; potassium iodide 0.4 mL; distilled water 100 mL) [13], [14]. The isolate showed a clear zone with the addition of iodine solution, then the isolate can be confirmed to be able to produce amylase enzymes. The data obtained were then calculated the diameter of clear zone and the amylolytic index value. Amylolytic microbial isolates were tested on the amylolytic index based on the formula [15]:

$$IA = \frac{\text{Clear Zone Diameter (mm)}}{\text{Colony diameter (mm)}}$$

2.3. Test of amylase enzymes activity

The activity of amylase enzymes was measured using a combined method from Naguib and Qureshi [16], [17]. Substrates containing 1 mL of amylase enzymes were mixed into a reaction containing 1 mL of 0.5% soluble starch in an acetate buffer (pH 5.6) then incubated at 30°C for 30 minutes [16], [14]. Then the reaction was stopped by adding 2 mL of reagent DNS solution, then heated for 5 minutes. The tubes were cooled to room temperature and absorbance was measured at 540 nm against empty substrates and enzymes [17]. Furthermore, the obtained data calculated the value of enzyme activity and glucose levels produced by comparing with the standard glucose curve.

2.4. Morphological test of amylase enzyme-producing microbe

Selected yeast cultures were inoculated on the medium yeast extract peptone glucose agar (YEPGA) in Petri dish by a scratch method, incubated at room temperature for 3–7 days. Colonies that grow separately (single colonies) were observed morphologically include form, elevation, margin, and color. Microscopic observations were made of the shape of the cell. Cell were observed by staining using methylene blue [18].

2.5. High glucose tolerance test

The isolate was added into the medium yeast extract peptone glucose broth (YEPGB). Each tube contains 5 mL of medium with glucose concentrations of 10% (m/v), 20% (m/v), 30% (m/v), 40% (m/v), and 50% (m/v). The density value of the cell was calculated by absorbance at a wavelength of 600 nm using the UV-Vis Spectrophotometer at an incubation of 30°C for 2 – 5 days [19], [20], [21].

2.6. Effect of incubation temperature on amylase enzyme activity

Substrates containing the enzyme amylase as much as 1 mL were mixed into a reaction containing 1 mL of soluble starch 0.5% in an acetate buffer (pH 5.6) and incubated at 30°C, 35°C, and 40°C for 30 minutes [16], [14]. Then the reaction was stopped by adding 2 mL of reagent DNS solution, subsequently it was heated for 5 minutes. The tubes are cooled to room temperature and the absorbance was measured at 540 nm against empty substrates and enzymes [17].

2.7. Effect of pH on amylase enzyme activity

Substrates containing the enzyme amylase as much as 1 mL were mixed into a reaction containing 1 mL of soluble starch 0.5% in acetate buffers (pH 4, 5, and 6) and incubated at 30°C for 30 minutes [16], [14]. The reaction was stopped by adding 2 mL of reagent DNS solution, after that it was heated for 5 minutes. The tubes were cooled to room temperature and the absorbance was measured at 540 nm against empty substrates and enzymes [17].

3. Results and discussions

3.1 Microbial isolation and selection

A total of 65 isolates were isolated from *ragi* and cassava *tape*, only 16 isolates showed a clear zone after the addition of iodine solution. Table 1 shows the result of isolate selection that has a clear zone diameter of ≥ 1 mm and a list of amyolytic index values.

Table 1. List of isolates capable of producing clear zones

Isolates	Clear zone diameter (mm)	Amyolytic Index
R2I5.1	10.25 \pm 2.54	2.69 \pm 0.84
R6I4	6.25 \pm 3.00	1.38 \pm 0.35
R5I3	15.08 \pm 0.83	1.78 \pm 0.34
R6I1	7.42 \pm 0.83	1.32 \pm 0.05
R1I2	3.92 \pm 0.69	1.42 \pm 0.15
R5I4	12.33 \pm 3.14	3.36 \pm 1.01
R3I9	4.67 \pm 1.91	1.23 \pm 0.14
T1I4	13.25 \pm 2.08	2.18 \pm 0.38
T2I2.1	16.75 \pm 1.48	2.18 \pm 0.23
T1I3.1	9.83 \pm 2.15	1.45 \pm 0.21
T1I1.1	8.25 \pm 1.75	1.31 \pm 0.09
T1I5	8.92 \pm 0.42	1.38 \pm 0.05
T2I6.2	6.92 \pm 1.93	1.36 \pm 0.12
T2I6.1	18.42 \pm 3.49	2.22 \pm 0.39
T2I2.2	18.17 \pm 1.69	2.25 \pm 0.23
T2I5	4.92 \pm 0.57	1.15 \pm 0.02

Note: The number after \pm indicates the deviation value of a treatment

Table 1 shows that the resulting clear zone is 3.92 – 18.42 mm and has an amyolytic index value of 1.15 – 3.36. Here are some examples of clear zone images produced when the addition of iodine solution is done.

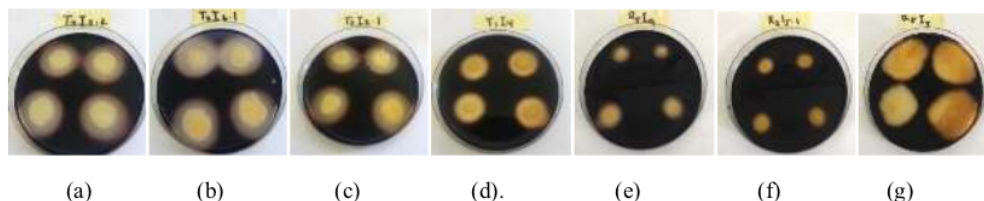


Figure 1. Isolates that produce the highest clear zone: (a) T2I2.2, (b) T2I6.1, (c) T2I2.1, (d) T1I4, (e) R5I4, (f) R2I5.1, (g) R5I3. The clear zone was tested by adding 1% iodine solution

Figure 1 shows that the clear zone formed around the colony indicates that the isolate is capable of hydrolyzing the starch. The starch will form a deep blue complex with iodine reagents. The iodine-starch reaction is caused by the presence of helical amylose and iodine in forming^{I₃⁻} which fills the helical nucleus. Active hydrolysis of starch by amylase enzymes will cause the starch-iodine complex to decompose thus forming a clear zone. The absence of a clear zone around the colony indicates a reaction between iodine reagents and non-hydrolyzed starches in starch-containing mediums [22]. Gana *et al.* [23], reported that the ability or the power to produce the enzyme amylase of a microbe is characterized by the formation of clear zones in the medium containing starch.

Sixteen isolates of microbes that have the ability to produce the enzyme amylase were tested for their ability based on the amylolytic index (AI). Based on the amylolytic index (Table 1), various AI values were obtained. Total of 3 isolates from *ragi* samples and 4 isolates from *tape* samples were potential or capable of producing AI ≥ 1.5 or moderate to high category in producing amylase compared to other isolates, namely isolates R2I5.1, R5I4, R5I3, T1I4, T2I2.1, T2I6.1, and T2I2.2 with AI values of 2.69 ± 0.84 , 3.36 ± 1.01 , 1.78 ± 0.34 , 2.18 ± 0.38 , 2.18 ± 0.23 , 2.22 ± 0.29 , and 2.25 ± 0.23 . Meanwhile isolates R3I9 and T2I5 had the lowest AI values, namely 1.23 ± 0.14 and 1.15 ± 0.02 . This is in accordance with the research of Bansal *et al.* [24] screened fungi capable of producing amylase with an AI of 2.95. Ouedraogo *et al.* [25], who succeeded in isolating yeast and screening potential amylase-producing yeasts from potatoes with an AI value of 2.35. Desai *et al.* [26] succeeded in isolating yeast and obtained an AI value of 2.60.

3.2 Test of amylase enzymes activity

Amylase enzyme activity testing was conducted to determine the activity of amylase enzymes against 16 potential isolates in previous tests. The measurement of amylase enzyme activity was done using microbial isolate culture in suspense form in a 40% glycerol solution. The isolate suspension was grown in starch medium broth with a peptone composition, yeast extract, and starch. Peptone, and yeast extract are sources of organic nitrogen that serve as important macronutrients in cell growth. In addition, the starch being an important macronutrient also acts as a source of carbon inducing amylase enzymes [27]. Here are the results of the amylase enzyme activity test as well as the amount of glucose content produced.

Table 2. Amylase enzyme activity value and amount of glucose produced

Isolates	Absorbance value	Total glucose (mg/mL)	Enzyme activity (U/mL)
R2I5.1	1.327 ± 0.017	1.466 ± 0.019	0.814 ± 0.011
R1I2	0.857 ± 0.035	0.935 ± 0.039	0.520 ± 0.022
R5I4	1.458 ± 0.028	1.614 ± 0.032	0.897 ± 0.018
R3I9	1.095 ± 0.045	1.204 ± 0.050	0.670 ± 0.028
R5I3	1.187 ± 0.066	1.308 ± 0.075	0.727 ± 0.042
R6I1	0.822 ± 0.051	0.897 ± 0.057	0.498 ± 0.032
R6I4	0.960 ± 0.050	1.052 ± 0.056	0.585 ± 0.031
T2I2.1	1.199 ± 0.036	1.322 ± 0.041	0.735 ± 0.023
T1I1.1	0.481 ± 0.032	0.511 ± 0.036	0.284 ± 0.020
T1I5	1.156 ± 0.024	1.274 ± 0.027	0.708 ± 0.015
T1I4	1.231 ± 0.031	1.358 ± 0.035	0.755 ± 0.020
T2I2.2	1.322 ± 0.021	1.461 ± 0.024	0.812 ± 0.013
T2I5	1.036 ± 0.034	1.138 ± 0.038	0.632 ± 0.021
T2I6.1	1.329 ± 0.017	1.47 ± 0.019	0.817 ± 0.010
T2I6.2	1.154 ± 0.050	1.271 ± 0.056	0.706 ± 0.031
T1I3.1	1.081 ± 0.138	1.189 ± 0.156	0.660 ± 0.086

Note: The number after \pm indicates the deviation value of a treatment

Based on the measurements of amylase enzyme activity (Table 2), it can be seen that some potential isolates produce the highest amylase enzyme activity. These include R5I4 (0.897 ± 0.018 U/mL), R2I5.1 (0.814 ± 0.011 U/mL), R5I3 (0.727 ± 0.042 U/mL) (derived from cassava *ragi tape* samples) and T2I2.2 (0.812 ± 0.013 U/mL), T2I6.1 (0.817 ± 0.010 U/mL), T2I2.1 (0.735 ± 0.023 U/mL), T1I4 (0.755 ± 0.020 U/mL) (derived from cassava *tape* samples). The isolate that has the highest enzyme activity is the R5I4

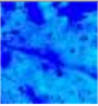
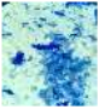
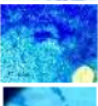
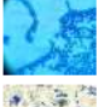
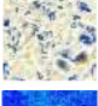
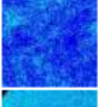

isolate with enzyme activity of 0.897 ± 0.018 U/mL with a fairly high or moderate category, while the lowest enzyme activity in T111.1 isolate is 0.284 ± 0.020 U/mL.

Some studies that are able to produce enzyme activity are quite high, including, Ouédraogo *et al.* [25], successfully measured the activity of amylase enzymes in the potential yeast of amylase-producing results from potatoes of 0.774 U/mL. Oliveira *et al.* [28] successfully measured the activity of amylase enzyme in yeast *Saccharomyces cerevisiae* enzyme activity of 0.734 U/mL.

3.3. Morphological test of amylase-producing microbes

After testing the activity of enzymes, 7 suspected isolates were found to be potential in the production of amylase enzymes. Based on the morphological character of yeast isolates that have been done, the following results are obtained:

Table 3. Morphological observations amylase-producing microbes

Isolates	Image	Morphology				Observation on a microscope
		Form	Elevation	Margin	Color	Form a colony
T212.1		Irregular	Raised	Undulate	Snuff-coloured	Oval
T216.1		Irregular	Umbo	Undulate	Reddish-yellow	Tapering
T212.2		Irregular	Convex	Erose	Cream	Oval
T114		Circular	Convex	Entire	Cream	Oval
R514		Irregular	Raised	Undulate	Cream	Oval
R215.1		Irregular	Convex	Lobate	Cream	Oval
R513		Irregular	Flat	Lobate	Cream	Round

Potential yeast isolates are microscopically round to oval and the presence of buds as a form of breeding. According to Buckle [29] yeast can grow in liquid and solid media in the same way as bacteria. Cell division occurs asexually with the formation of buds, a process that is a typical property of yeast.

Among the yeast isolates observed, some had capsules in their cells. According to Limoli *et al.* [30] some yeasts are covered by slimy extracellular components and are called capsules. The capsule covers the outside of the cell wall and consists mainly of polysaccharides, a starch-like polymer, and heteropolysaccharides that contain more than one type of sugar unit such as pentose, hexose, and glucuronic acid.

3.4. High glucose tolerance test

The test of microbial growth at high glucose concentrations was to determine whether microbial isolates could grow at high glucose concentrations. The value of isolate density at different glucose concentrations can be seen in Figure 2.

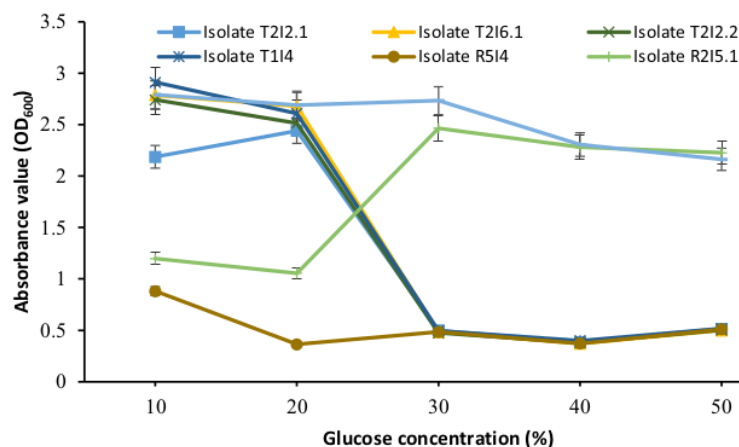


Figure 2. The absorbance isolate values at some glucose concentrations

The high glucose tolerance level (high osmotic pressure) possessed by isolated yeast isolates is at a glucose concentration of 50%. At that concentration, the optical density value measured by the UV-Vis spectrophotometer (λ 600 nm) has the smallest value compared to other glucose concentrations. The isolates of R215.1 and R513 were able to survive and thrive at high glucose concentrations because they could still grow well at a concentration of 50%, while the largest optical density values R513 isolates are produced at a concentration of 30% which indicates their optimum glucose concentration at 30%. The same thing happened in a study conducted by Nasir [21]. R514 isolates cannot tolerate high glucose concentrations because R514 isolates is an isolate that produces amylase enzyme.

3.5. Effect of incubation temperature on amylase enzyme activity

After obtaining microbes that have the potential to produce amylase enzymes, the influence of temperature on the activity of the enzyme produced. Here are the values of enzyme activity at temperatures of 30°C, 35°C, and 40°C, can be seen in Figure 3.

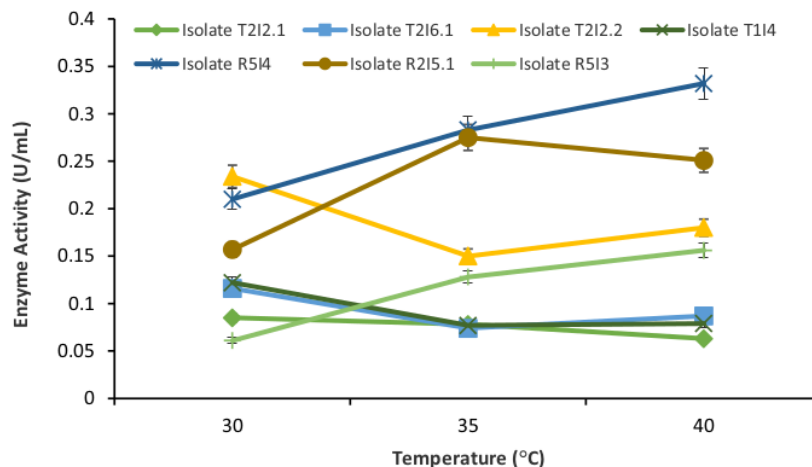


Figure 3. Amylase enzyme activity at different temperatures

In Figure 3 it can be seen that all enzymes are able to survive up to 40°C. However, some enzymes decrease the value of activity at 35°C to 40°C. While isolated R514 and isolated R513 tend to increase to a temperature of 40°C and isolate R514 has the highest amylase enzyme activity compared to other isolates. So, it can be interpreted that each enzyme obtained from a different isolate has different enzyme activity. The best isolate that has resistance to temperature and enzyme activity is isolate R514. Then the research by Vidyalakshmi *et al.* [31] reported that, amylase activity decreased significantly with an increase in temperature beyond 37°C. Divakaran *et al.* [32] also reported that amylase enzyme activity decreased after incubation above 40°C.

3.6. Effect of pH on amylase enzyme activity

After obtaining microbes that have the potential to produce amylase enzymes, then experiments on the effect of pH on the activity of the enzyme amylase produced. Here are the values of enzyme activity at pH 4, 5, and 6, can be seen in Figure 4.

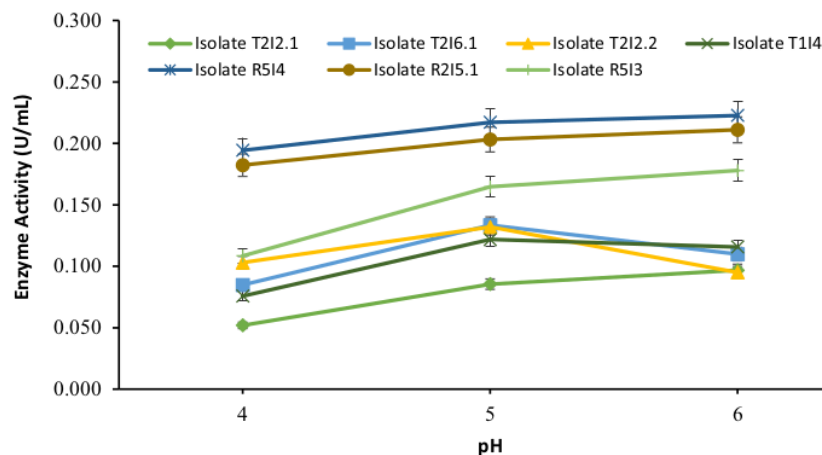


Figure 4. Enzyme activity at different initial pH

Figure 4 indicates that it is still against the activity of enzymes at pH 4, 5, and 6, which indicates that the enzyme can survive at that pH. However, most enzymes of some isolates decrease activity at pH 6 and most have optimum pH conditions at pH 5. In testing the effect of pH on enzyme activity produces the best enzyme obtained from R514 isolates. This is in line with the results of the study of Todoró *et al.* [33] which states that a further increase in pH results in a decrease in the activity of the enzyme amylase. Divakaran *et al.* [32] also stated that the higher the pH, the value of amylase enzyme activity decreased.

4. Conclusion

From the results of this study, we can conclude that there are 16 isolates from 65 isolates that have clear zones after a clear zone test with the addition of iodine solution. While the isolate that has the highest enzyme activity is R514 isolates with enzyme activity of 0.897 ± 0.018 U/mL with a fairly high or moderate category. Furthermore, there are 7 isolates that have the potential to produce amylase enzymes which are able to survive at varying incubation temperatures and pH. The most potential to produce amylase enzymes is R514 isolates.

Acknowledgments

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12
