IJABR

International Journal of Advanced Biotechnology and Research (IJABR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-10, Issue-2, 2019, pp627-636
http://www.bipublication.com

Research Article

Improvement Amylase Production from Moderately Thermo-Alkaliphile Bacteria

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[Received: 25/05/2019; Accepted: 15/06/2019; Published: 17/06/2019]

ABSTRACT:
Amylase thermo-alkaline is one of the three largest industrial groups enzymes and accounts for approximately 65% of the world enzyme. Amylase thermo-alkaline produced by bacteria or bacteria amylase is an enzyme that can hydrolyze starch to sugar. In the field of the food industry, amylase thermo-alkaline play a role in the manufacture of glucose syrup, bread making, and baby food. In the field of non-food industry, amylase thermo-alkaline play a role in the paper industry, leather tanning, pharmaceuticals, textiles and as detergent additives. Exploration of sources of thermo-alkaline amylase-producing bacteria continues to be carried out to meet industrial needs. The approach to finding new enzyme sources from bacteria isolated from unique environments is the most feasible step. The aim of the study was to determine the biochemical properties of thermo-alkaline production bacteria originating from Pariangan hot spring, West Sumatra, Indonesia and increase amylase production. The method carried out in this study is a screening of thermo-alkaline bacteria, characterization and biochemical assay of bacteria, and optimization medium of amylase production. The results showed that P2.3 bacterial isolates originating from Pariangan hot springs, West Sumatra, Indonesia belong to Bacillus sp. Amylase produced by P2.3 bacterial isolates increases with the concentration of inoculum 10%, urea as nitrogen sources, and wheat as a substrate.

Keywords: diversity, amylase thermo-alkaline, hot spring, 16S rRNA gene

[I] INTRODUCTION

Enzymes can be obtained from renewable resources, can be degraded naturally, require easily achievable temperature and pH conditions, and are both reactant and product-specific [1]. Enzymes (proteases, lipases, and amylase) can be obtained from plants, animals, fungi, and bacteria. Amylase produced by bacteria originating from hot springs with a high temperature and pH is known as thermo-alkaline amylase. Amylase thermo-alkaline is one of the three largest industrial groups enzymes and accounts for approximately 65% of the world enzyme [2]. Amylase thermo-alkaline produced by bacteria or bacteria amylase is an enzyme that can hydrolyze starch to sugar [3].

In the field of the food industry, amylase thermo-alkaline play a role in the manufacture of glucose syrup, bread making, and baby food. In the field of non-food industry, amylase thermo-alkaline play a role in the paper industry, leather tanning, pharmaceuticals, textiles and as detergent additives [4]. Amylase thermo-alkaline is composed in around 90% of all liquid detergents and amylase thermo-alkaline in detergents used for automatic dishwashing is increased [5]. The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry [6]. Amylase thermo-alkaline is needed as a detergent additive because the detergent industry requires enzymes that are stable at high temperatures and pH [7].

Thermo-alkaline amylase produced by thermophilic bacteria is an interesting phenomenon to study because it is stable against extreme temperature and pH environment. Exploration of sources of thermo-alkaline amylase-producing bacteria continues to be carried out to meet industrial needs. The approach to finding new enzyme sources from bacteria isolated from unique environments is the most feasible step. Hot spring is one source of thermo-alkaline amylase bacteria. The aim of the study was to determine the biochemical properties of thermo-alkaline production bacteria originating from Pariangan hot spring, West Sumatra, Indonesia and increase amylase production. Pariangan hot spring is one of the hot springs found in West Sumatra, Indonesia. Pariangan hot spring has a temperature of 51°C and pH 9.0 this causes the amylase producing bacteria to grow well and the high diversity of bacteria. An environment with a high pH will have varying mineral content [8].

[II] MATERIALS AND METHODS

2.1. Screening of thermophile bacteria

The hot water samples inside the bottle are shaken to homogeneous. Hot water samples are poured into a liquid NA medium and leave to freeze. Subsequently incubated at 50°C for 24 to 48 hours. Characteristics of the colony morphology observed include shape, edges, and elevation. Growing bacteria colonies were inoculated into NA medium that had been densely packed with quadrant methods. Subsequently incubated for 24 to 48 hours at a temperature of 50°C and then seen single growing colonies. Any pure isolate that can be grown is assumed to produce amylase on the starch-containing medium and each sample is repeated twice. Bacteria that have been successfully purified, then grown on a selective medium or medium for starch. Each petri dish was placed in a different bacterial colony with one source of the same isolate then incubated for 24 hours at 50°C. Bacteria that successfully grow on selective medium then drop with iodine solution and waited a few minutes until really fused between iodine solution with starch so clear zone clear.

2.2. Amylase activity test

The amylase-producing isolates were grown into 25 ml basal medium (3 g/L K_2HPO_4, 3 g/L KH_2PO_4, 3 g/L $MgSO_4$, 5 g/NaCl, and 10 g/L starch) with pH 7.5 and shaken at 150 rpm at 50°C for 24 hours. When the bacterial growth is subsequently removed 5 ml bacterial cultures into 100 ml basal medium and centrifuged at 150 rpm for 24 hours at 50°C. Bacteria culture formed centrifuged at 5000 rpm for 10 minutes. The supernatant containing the thermostable amylase extract was taken by micropipette and inserted into the microcentrifuge tube for the activity test. The amylase activity test was performed by incubating 0.5 ml of starch 1% for 5 min at 50°C and then adding 0.5 ml of amylase then incubated again for 1 hour at 50°C. To stop the hydrolysis process heating the boiling water (100°C) for 20 minutes. Then added 1 ml of Samogy Nelson solution then vortex and reheated to boiling water for 20 minutes. Further cooled in ice water and added 1 ml of arsenomolibdad solution then shaken then sufficient volume to 10 ml by adding

7 ml aquadest. Measure absorbance at wavelength 540 nm [9].

2.3 Characterization and biochemical assay of bacteria

Characterization of bacteria is done through macroscopic observation, i.e observation of colony form from bacteria, colony edge, elevation, surface, and colony pigmentation. Microscopic observation was done through observation of bacterial cell form, spore staining and reaction to Gram staining [10]. Biochemical properties of bacteria were performed by biochemical tests, such as TSIA, catalase, oxidase, indole, urea citrate, lactose, glucose, sucrose, mannitol, MR, VP, OF, arabinose, nitrate, and gelatin.

2.4.Optimization medium of amylase production:

Optimization medium of amylase production by isolate bacteria such as inoculum size, nitrogen sources, and substrates. Isolate bacteria originated Pariangan hot spring grown in 50 ml of basal medium pH 8.5 (3 g/L KH_2PO_4; 3g/L g K_2HPO_4; 5 g/L $MgSO_4$; 5 g/L NaCl; 10 g/L starch) and incubated for 24 hours at 70°C. Each inoculum with different concentration (4, 6, 8, 10 and 12% v/w) moved into a 100 ml new basal medium pH 8.5 and agitated at a speed of 150 rpm for 24 hours at 70°C. Bacteria cultures were centrifuged at 5.000 rpm and then the supernatant was transferred to a new microcentrifuge tube and assayed for amylase activity. 1% source of nitrogen (urea, yeast extract, peptone, beef extract, and tryptone) added to each production medium. Production medium was cultured aerobically at a rate of 150 rpm with a temperature of 70°C for 24 hours. The inoculum with optimum concentration was transferred into a new production medium and cultured aerobically at 150 rpm with a temperature of 70°C for 24 hours. Bacterial cultures were centrifuged at 5.000 rpm for 5 min. The resulting supernatant was transferred into a new microcentrifuge tube for the amylase test.

Various substrates (rice, wheat, potatoes, and sago) were added to the production medium and cultured aerobically at 150 rpm with a temperature of 70°C for 24 hours. The inoculum with optimum concentration was transferred into a new production medium and cultured aerobically at 150 rpm with a temperature of 70°C for 24 hours. Bacterial cultures were centrifuged at 5.000 rpm for 5 min. The resulting supernatant was transferred into a new microcentrifuge tube for the amylase test.

[III] RESULTS

Pariangan hot water has a pH of 8-9 while the other hot water in West Sumatra generally has a pH of 7 even below 7. This causes Pariangan hot water to be unique compared to other hot springs in West Sumatra, Indonesia.

3.1. Screening of thermophile bacteria

The morphological characterization of thermophilic bacteria showed that the bacteria originating from Pariangan hot spring were cream colored, smooth shallows, and flat elevation (Table-1).

[Table-1]. Morphology characterization of thermophile bacteria.

No	Location	Isolates code	Colour	Shape	Shallows	Elevation
1	P1 (Temperature 50°C, pH 8)	P1.1	Yellowness	Round	Smooth	Arise
		P1.2	Crem	Round	Smooth	Flat
		P1.4	Crem	Round	Smooth	Arise
2	P2 (Temperature 48°C, pH 9)	P2.1	Yellowness	Round	Smooth	Arise
		P2.2	Crem	Round	Smooth	Arise
		P2.3	Crem	Round	Smooth	Flat
		P2.4	Yellow	Round	Smooth	Flat

3	P3 (Temperature 47°C, pH 8)	P3.1	Yellow ness	Round	Smooth	Flat
		P3.2	Crem	Round	Unorde red	Flat
		P3.3	Crem	Round	Smooth	Arise
		P3.4	Yellow ness	Round	Smooth	Arise

The results of amylase activity tests obtained 4 isolates of amylase-producing bacteria from 11 isolates **[Figure-1].**

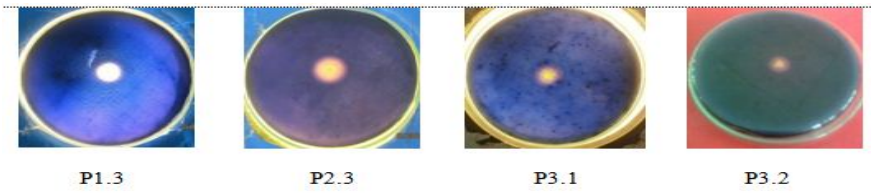

Fig: 1. Amylase producing bacteria

The amylolytic enzyme activity test was conducted qualitatively by considering the measurement of the amylolytic index (Table-2).

[TABLE-2]. Amylolytic index of bacterial isolates

No	Location	Isolates code	Clear zone diameter (mm)	Colony diameter (mm)	Amylolytic index (mm)
	P1 (Temperature 50°C, pH 8)	P1.1	-	-	-
		P1.2	1,61	0,42	3,83
		P1.4	-	-	-
	P2 (Temperature 48°C, pH 9)	P2.1	-	-	-
		P2.2	-	-	-
		P2.3	1,17	0,61	1,91
		P2.4	-	-	-
	P3 (Temperature 47°C, pH 8)	P3.1	1,59	0,47	3,38
		P3.2	1,02	0,42	2,42
		P3.3	-	-	-
		P3.4	-	-	-

3.2. Amylase activity test

Based on the research that has been done, obtained the results of different amylase activity of four different bacterial isolates. The highest amylase activity was obtained by P2.3 isolate that is 66.68 U/ml, followed by P1.3 isolate that is 64,54 U/ml, P3.2 isolate that is 43.76 U/ml, and P3.1 isolate that is 37.04 U/ml **[Figure-2].**

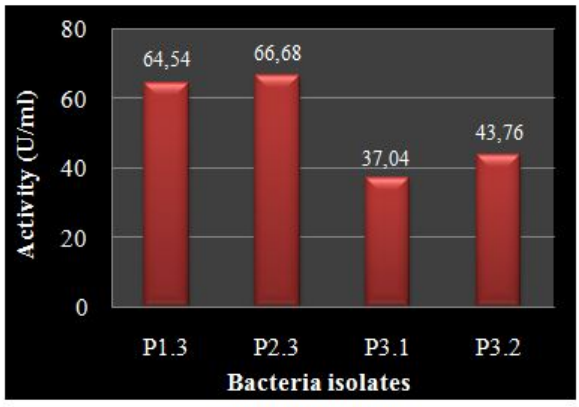

Fig: 2. Amylase activity test of four bacterial isolates

3.3. Characterization and biochemical properties of bacteria

The result of this study showed that the bacteria isolate P2.3 had white colonies, rounded shape, flat surface, and flat elevation. The result of Gram staining and biochemical properties showed that P2.3 isolate classified to Gram-positive and has spores **[Figure-3]**.

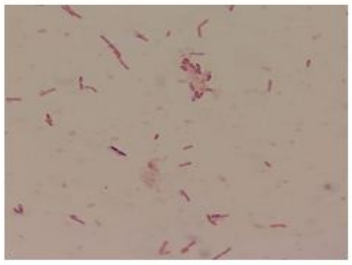

Fig: 3. The result of Gram staining P2.3 isolate

Based on the results of the test biochemical properties showed that isolate P2.3 belong to *Bacillus* sp.

[Table-3]. The result of a test of biochemical properties

No	Test of biochemical properties	Result
1	TSIA	Red/yellow
2	Catalase	+
3	Oxidase	-
4	Indole	-
5	Urea	+
6	Citrate	-
7	Lactose	-
8	Glucose	+
9	Sucrose	+
10	Mannitol	+
11	MR	+
12	VP	-
14	OF	-
15	Arabinose	-
16	Nitrate	+
17	Gelatine	+
18	Xylose	+

+ = Positive; - = Negative

3.4. Optimization medium of amylase production

An increase in inoculum size was found to improve the growth and production of α-amylase and reached a maximum of 10%. Increase in inoculum concentration above 10% was found to decline the amylase production **[Figure-4].**

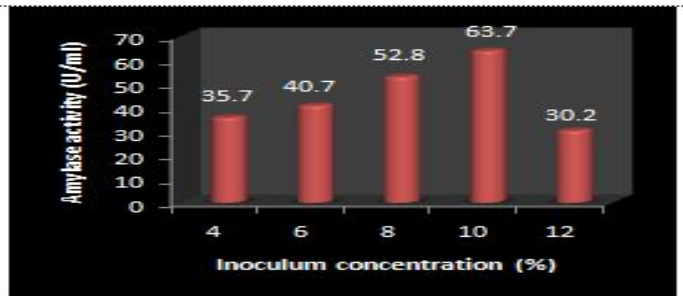

Fig:4. The effect of inoculum size on amylase production

All the nitrogen sources used (beef extract, peptone, yeast extract, urea, and tryptone) can increase amylase production, but the use of urea as a source of nitrogen can increase the production of amylase compared to other carbon sources **[Figure-5].**

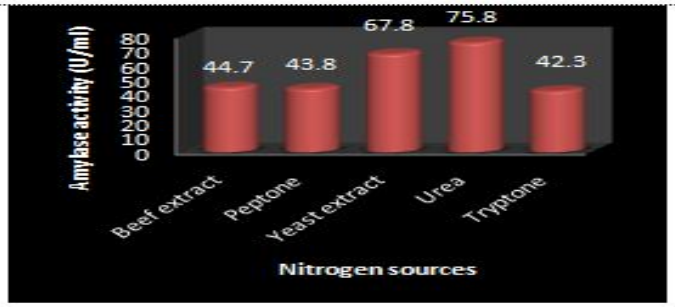

Fig:5. The effect of nitrogen source on amylase production

Four substrate types (rice, wheat, potatoes, and sago) are used for bacterial growth optimization and amylase production. The use of rice substrate in the production medium can increase the production of amylase compared to other substrates **[Figure-6].**

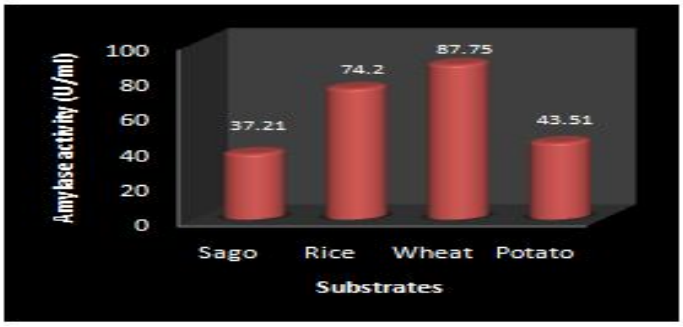

Fig:6. The effect of substrate on amylase production

[IV] DISCUSSION

The result of this study showed that the bacteria isolate P2.3 had white colonies, rounded shape, flat surface, and flat elevation **[Table-1].** Characterization of bacterial morphology derived from compost has also been done [11], the results obtained are CS3.1 bacterial isolates, irregular shapes, grooved edges, and elevated elevations. The results of characterization of bacterial isolates originating from hot springs in Jordan showed that isolates were grey, creamy, and white, opaque or translucent, rough or smooth, with regular or irregular edges. Colonies might appear finely wrinkled and adherent to the agar surface.

The amylolytic enzyme activity test was conducted qualitatively by considering the measurement of the amylolytic index. P1.2 isolate has amylolytic index is 3.83 mm, P2.3 isolate has amylolytic index is 1.91 mm. P3.1 isolate has amylolytic index is 3.38 mm, P3.2 isolate has amylolytic index is 2.42 mm **[Table-2].** The largest of the amylolytic index was 3.83 mm that produced of P1.2 isolate and the lowest activity was 1.91 mm that produced of P2.3 isolate.

11 isolates of thermophile bacteria originating from Pariangan hot spring 7 isolates did not produce amylase activity. Iodine solutions do not provide color with carbohydrate polymers of less than five monosaccharide groups, e.g glucose [12]. The media surrounding bacteria colonies that do not produce amylase will be blue when iodine solution drops. This shows that the starch in the medium is not degraded to simple sugars which means that bacteria do not produce

amylase. The small diameter of the clear zone formed from each isolate is different [13]. This is caused the ability to hydrolyze the starch of each isolate is different. The difference between clear zones is also due to differences in the amylolytic gene that each thermophile bacterium possesses. The magnitude of the resulting clear zone depends on the amount of glucose monomer produced from the process of starch hydrolysis [14]. The larger the number of glucose monomers produced the larger the clear zone formed around the colony.

The highest activity isolate was selected for further research that is P2.3 isolate **[Figure-2].** The presence of the enzyme activity of different values each isolate caused by enzymes produced each type of different microorganisms will produce different enzymes of the amount and sequence of amino acids that form the enzyme. The specific activity of different enzymes from *Bacillus* sp. probably due to the amount of enzyme and amino acid enzyme protein produced by each isolate of *Bacillus* sp. different from each other [15].

Based on Gram staining, the isolates were found mostly to be Gram-positive **[Figure-3].** Microscopic observation revealed spore-forming rod-shaped bacterium arranged in the chain [16].

The endospore produced by *Bacillus* aims protecting bacteria from a state that is not profitable such as drought, nutrient deficiency, freezing, as well as chemicals. The bacteria endospore types this is resistant to environmental changes, resistant to heat, and chemical disinfectants certain in a long [16]. If the environment is good, then endospores will experience sporogenesis and be forming a vegetative cell [17]. Based on the results of the test biochemical properties showed that isolate P2.3 belong to *Bacillus* sp.

The genus *Bacillus* was isolated from all explored sites, the presence of Bacillus in all sampled locations could be due to the ability of this genus to move at high rates and their resistance to harsh environmental conditions [18] in addition to its adaptation for hot surroundings [19]. *Bacillus* is known to produce a variety of extracellular enzymes and can be used in various industries [20]. The same study by **[21]** reported that *Bacillus* sp. was found in hot springs in Saudi Arabia that could produce amylases with high activity.

Optimization of various parameters and manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet industrial demands [22]. The fermentation profile of an organism is usually affected by the initial inoculum size. An increase in inoculum size was found to improve the growth and production of α-amylase and reached a maximum of 10%. Increase in inoculum concentration above 10% was found to decline the amylase production **[Figure-4].**

The same result is also obtained by **[23]**, 10% of inoculum concentration can increase amylase production. This might be due to a higher size of inoculums resulting in increased competition for carbon source and nutrients, which might lead to exhaustion of nutrient [24]. 1% of inoculum concentration may increase amylase production in *Proteus* sp [25]. Inoculum size at 2% might increase amylase activity *Brevibacillus borstelensis* R1 [26]. Inoculum concentration plays a role in increasing the growth and production of amylase. The nitrogen sources used (beef extract, peptone, yeast extract, urea, and tryptone) can increase amylase production, but the use of urea as a source of nitrogen can increase the production of amylase compared to other carbon sources **[Figure-5].** According to **[27]**, amylase production is increased by using soybean as a source of nitrogen. In contrast [28] to casein is the best source of nitrogen in the production of bacterial amylase.

Soya bean meal was found as the best nitrogen source for α-amylase production by *Bacllius* sp. I-3 [29]. Strains of *B. stearothermophilus* and *B.*

amylolyticus secreted maximum α-amylase in a medium supplemented with 1% peptone, 0.5% yeast extract and 0.5% maltose under vigorous shaking conditions [30].

Four substrate types (rice, wheat, potatoes, and sago) are used for bacterial growth optimization and amylase production. The use of rice substrate in the production medium can increase the production of amylase compared to other substrates **[Figure-6].** *Bacillus licheniformis* GHBB produces the maximum amylase by using wheat as a substrate [31]. Amylase from *Bacillus licheniformis* ATCC 9945a more quickly break down from cereal starch than tuber starch [32]. Natural substrates can be used by bacteria as a source of energy to produce amylases and can be obtained at lower prices [33]. The difference is thought to be influenced by the crystalline form of starch granules. Generally, tubers such as potatoes and cassava have a crystalline structure B while in cereals such as rice and corn have a crystalline structure A. Which crystalline B has higher water content and a relatively long chain of amylopectin which causes starch is more resistant to enzyme attacks [34].

[V] CONCLUSION

P2.3 bacterial isolates originating from Pariangan hot springs, West Sumatra, Indonesia belong to *Bacillus* sp. Amylase produced by P2.3 bacterial isolates increases with the concentration of inoculum 10%, urea as nitrogen sources, and wheat as a substrate. Amylase production can be improved by optimizing the growth medium in various conditions and genetic engineering techniques.

ACKNOWLEDGEMENT

Thank you to DRPM, Directorate General of Strengthening Research and Development. Ministry of Research, Technology and Higher Education. Contract number SP DIPA-042.06.1.401516/2018.

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