Growth Conditions for Alpha-amylase-producing Bacterium PLS 75 Strain Isolated from a High Temperature and Saline Area

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Abstract: Extreme habitat has been explored to find microorganisms that are capable of producing industrial enzymes with better activity and stability. Four thermo-halophilic bacteria strains isolated from undersea fumaroles were screened for their ability to produce extracellular hydrolytic enzymes. The most potent α-amylase producer was PLS 75 strain. The strain was Gram-negative with uniform basil-chained shaped. Homology analysis of the 16S rRNA gene fragments shows that the strain was closely related to an uncultured bacterium isolate, supporting the fact that the strain was difficult to culture. Fermentation study shows that PLS 75 produced the highest biomass (5.48 mg/ml) and α-amylase activity (4.43 U/ml) when incubated at 60 °C, pH 7 for 31 h. The results provided information on PLS 75 culture conditions that may be used to produce the enzyme for the study of its attributes and catalytic activity.

1 INTRODUCTION

Microbiological research has shifted more attention to extremophilic microorganisms. Their ability to grow well in extreme conditions makes them unique. Not only as of the source of distinct metabolites, but they also a subject of adaption mechanism studies (Elleuche et al., 2015). Extremophiles are microorganisms inhabiting and living in extreme conditions, such as high temperature (thermophile and hyperthermophiles), high acidity (acidophiles), high alkalinity (alkaliphiles), high pressure (piezophiles), high radiation (radiophiles) and high metal content (metalophiles). Habitats of extremophiles widely diverse, from natural to artificial environments. The former includes hydrothermal and geothermal area, acid soil, soda lake, high salt lake, deep ocean, while the latter includes nuclear reactor and toxic chemical waste (Dalmaso et al., 2015).

Enzymes produced by extremophiles are in-demand biocatalysts due to their high activity and stability in the harsh industrial process conditions. Most enzymes are hampered due to high temperature, pH and salinity requirement in the process. Therefore, extremozymes are preferable for their resistance to high temperature and chemical denaturation (Souza et al., 2012).

Among the enzymes, thermostable hydrolases are the most used enzymes in industries requiring elevated temperature (Elleuche et al., 2014; Dalmaso et al., 2015). Hydrolytic enzymes (carbohydrase, protease, lipase) are accounted for about 80% of the total enzyme market in the US, with a sales value approaching USD 3 billion in 2019 (Grand View Research, 2019).

α-Amylase (1,4-D-Glucan glucanohydrolase, EC 3.2.1.1) is a carbohydrase enzyme that catalyses the hydrolysis of α-1,4-glycosidic bonds in starch to produce simpler sugar molecules, such as dextrin, maltose, and glucose (Reddy et al., 2003). The enzyme has been widely used in food, textiles, paper, detergents, bioethanol production and pharmaceuticals industries (Souza and Magalhaes, 2010). α-amylases in starch industries are required to remain active at high temperature, particularly those involved in gelatinisation (100-110 °C) and liquefaction (80-90 °C).

Our group has previously isolated some microbial strains from shallow sea fumaroles (Iqbalsyah et al., 2018; Iqbalsyah et al., 2019a). The area provides extreme environments of high temperature and salinity, which is an ideal habitat for the extremophiles to produce enzymes with

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unique features. Therefore the objective of this study was to screen the microbial strains, which were able to produce hydrolytic enzymes, particularly α-amylase. The potential strain was phenotypically and genotypically identified. As it is essential to imitate the conditions of the native sampling area for further experiments in the laboratory, the optimum fermentation time, temperature and pH for the production of α-amylase by the strain was studied. The results could be used to produce the enzyme for the study of its attributes and catalytic activity.

2 MATERIALS AND METHOD

2.1 Microorganism

This study used four microbial strains, namely PLS 75, PLS 76, PLS 80 and PLS A, that were the stock culture of the Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences Syiah Kuala University. The strains were previously isolated from the sea bead of fumaroles in Pria Laot and initially cultivated from the sand sample on Thermus medium.

2.2 Screening of Hydrolytic Enzymes

PLS 76, PLS 75, PLS 80 and PLS A was grown on on separate agar plates to evaluate their ability to produce protease, lipase, amylase and cellulase. Media of ½ Thermus solid, containing 0.4% peptone, 0.2% yeast extract, 1% NaCl, 0.25% glucose, 3% bacto agar, was used. The medium was added with 3% skim milk and 0.5% casein for protease screening; 1% olive oil, 0.5% tween 80 and 200 µl of 0.001% of Rhodamine B for lipase screening; 1% soluble starch for α-amylase screening and 1% carboxymethylcellulose (CMC) for cellulase screening. All media were incubated for 24 h at 70 °C.

Protease activity was indicated by a clear zone around the colonies. Lipase activity was identified by orange fluorescent around the colonies under UV light. Strains with amylase activity showed by a clear zone after staining with potassium iodium iodate. Strains with cellulase activity were identified by a clear zone formed after being washed by 1 M sodium chloride.

2.3 Identification of Microorganism

The cells of the most potent strain in producing α-amylase were centrifuged at 5000 ×*g* for 10 minutes. The pellet was used for morphology identification by Gram-staining and Scanning Electron Microscope (SEM).

The strain genotype was analysed from the 16S rRNA gene sequence. Genomic DNA was isolated from the pellet by using a DNA isolation kit (Genetika Science). It was then used as a template to amplify the 16S rRNA gene fragments using a pair of primers, i.e. Com 1F 5'-CAGCAGCCGCGGTAATAC-3' and Com-1R (5'-CCGTCAATTCCTTTGAGTTT-3') (Schwieger & Tebbe, 1998). The amplification consisted of 30 cycles of denaturation at 95 °C for 5 minutes and annealing at 55 °C for 1 minute, as well as an extension at 72 °C for 2 minutes. The amplicons were subjected to agarose electrophoresis. The 16S rRNA gene was sequenced by direct sequencing method and compared with GenBank entries by BLAST search (www.ncbi.nml.nih.gov). Alignment and phylogenetic analysis were done using ClustalW and Mega 6 software (Tamura et al., 2013), respectively.

2.4 Growth Curve Study

The most potent α-amylase-producing strain was incubated in ½ T medium pH 7 (using 0.1M phosphate buffer) enriched with 0.25% glucose and 1% soluble starch for 48 h at 70 °C and 150 rpm. The media used in the experiments were diluted in sterile seawater to maintain the native salt conditions. The medium was inoculated with the strain to a level of 1 x 10^6 cells/ml. The growth was measured by cell dry weight.

The α-amylase activity was assayed by measuring the amount of reducing sugar produced from the starch hydrolysis by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), using a standard curve of glucose (1–10 µg/mL) measured at 540 nm. One enzyme unit (U) is defined as the amount of enzyme required to produce 1 µmol of reducing glucose per minute under the assay conditions. All measurements were done in triplicates.

2.5 Effect of Temperature, pH and Salt on Biomass and α-amylase Production

The optimum temperature for growth and α-amylase production was determined by inoculating the strain in ½ T medium in 0.1 M buffer phosphate pH 7, enriched with 0.25% glucose and 1% soluble starch. The incubation was conducted at 60, 65 and 70 °C and 150 rpm until optimum incubation time.

The optimum pH for α-amylase production was determined by varying the pH of the media. The pH was adjusted using 0.1M sodium acetate (pH 5), 0.1M dipotassium hydrogen phosphate (pH 7) and 0.1M glycine-NaOH (pH 9.0). The culture was incubated at optimum incubation time and temperature.

The effect of salt addition on α-amylase production was determined by adjusting the ½ T media to contain 1%, 2%, 3%, 4% sodium chloride. The media was already dissolved in seawater, so the final salt concentrations of the media were higher. The culture was incubated at the optimum incubation time, temperature and pH.

All experiments of temperature, pH and salt variation were done in triplicates. Cell dry weight and α-amylase activity were determined as described in Section 2.4.

3 RESULTS AND DISCUSSION

3.1 Enzyme Assay

All strains produced α-amylase, with PLS 75 showed the most profound activity. Only PLS A was able to produce protease. PLS 80 was the only strain that showed a lipase activity. Meanwhile, PLS 75 exhibited better cellulase activity than PLS 80 (Table 1). PLS 75 was therefore selected for further α-amylase production experiment.

Table 1: Screening results of the isolated strains for hydrolytic enzymes activity.

Strain	Enzyme			
	Amylase	Protease	Lipase	Cellulase
PLS A	+	++	-	-
PLS 75	+++	-	-	++
PLS 76	+	-	-	-
PLS 80	++	-	+	+

3.2 Identification of PLS 75

Gram-staining result shows that PLS 75 was a Gram-negative bacterium. The SEM image supports the information that PLS has a uniform long rod-chained shaped, with roughly 5-10 mm in length (Figure 1).

The size of PLS 75 strain chromosomal DNA was well over 10 kbp. The primers used in the amplification of the 16S rRNA gene fragments (Com 1F and Com 2R) were able to produce gene fragments of nearly 500 bp (Figure 2).

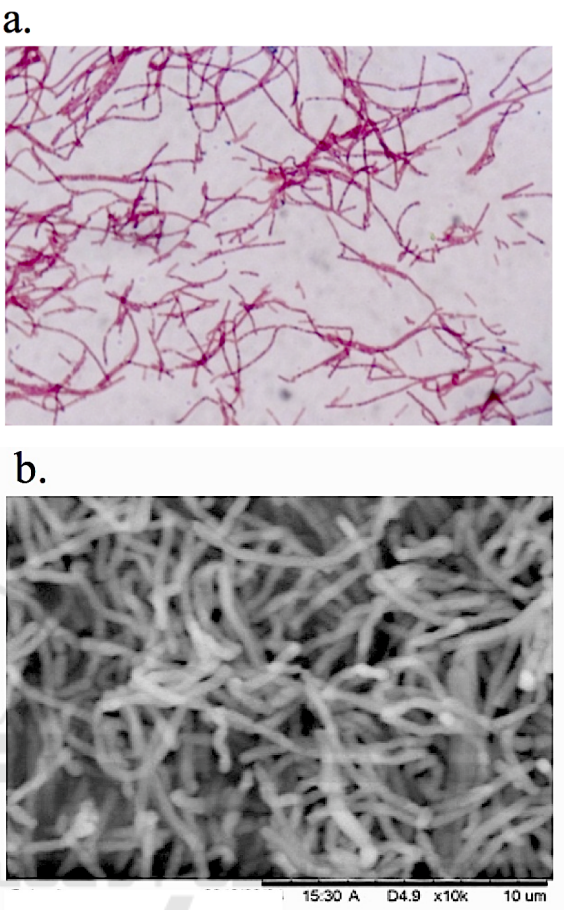

Figure 1: a. Gram Staining result; b. SEM image of PLS 75 with 10000× magnifications.

The phylogenetic tree was constructed from some homologous genes in the GenBank (Figure 3). PLS 75 has the closest homology with Uncultured bacterium clone YE-DC-B41 with sequence similarity of 98%. Until the intact 16s rRNA gene (1500 bp) is amplified and sequenced, the PLS 75 species is still indefinable.

Growing PLS 75 was indeed difficult. The ½ Thermus (½ T) medium needed enrichment with a small amount of glucose to promote biomass production. Thermus medium is designed for culture Thermus genera. The use of ½ T medium in this study was to isolate more robust microorganisms that can survive in minimum nutrition supply. The use of enriched media (for example LB or NB) was avoided, as they would host common microorganisms.

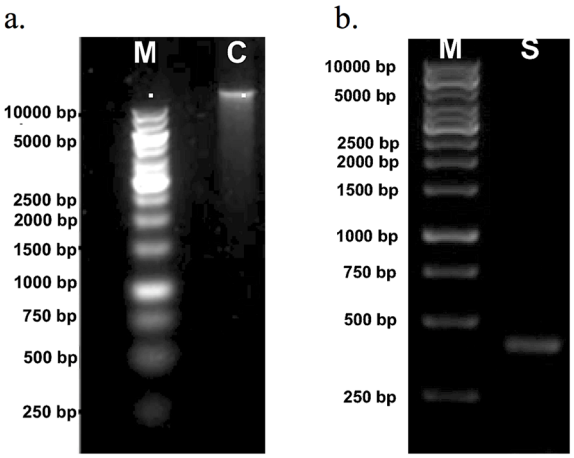

Figure 2: a. Electrophoresis results of chromosomal DNA of PLS 75; b. Electrophoresis results of 16S rRNA gene fragments of PLS 75. M = DNA ladder, C = Chromosomal DNA, S = 16S rDNA fragments.

3.3 Growth Curve of PLS 75

The growth curve study was conducted to determine the optimum fermentation time for PLS 75 to produce α-amylase. Fermentation was done at 70 °C on ½ T medium containing starch. The starch induced the production of the enzyme. Excreted α-amylase then catalysed the starch digestion. The products were further used for biomass and product formation.

Figure 4 shows that the lag phase occurred up to 8 h, indicated by the relatively stagnant amount of biomass. PLS 75 was in the exponential phase between 8 to 30 h, marked by an increase in the biomass. The highest amount of biomass produced at 30 h (3.66 mg/ml). PLS 75 then entered the stationary phase up to 42 h. The death phase occurred afterwards.

Similar to biomass, the highest α-amylase activity was also detected at the end of the exponential phase (30 h). The activity then dropped significantly. α-amylase production did not follow the trend of biomass. There were fluctuations before 24 h of fermentation. Nevertheless, the enzyme was still produced to utilise the starch in the medium. The enzyme production declined when the biomass decreased, implying the nutrition already exhausted.

The behaviour of microorganisms during metabolites production depends on the strain, the availability of the nutrients and the growth conditions. Generally, α-amylase is optimally produced at stationary phase (Malhotra et al., 2000; Liu et al., 2008; Annamalai et al., 2011). However, some are produced optimally in the exponential phase (Moshfegh et al., 2013; Berekaa et al., 2007). It has been suggested that the addition of glucose as the carbon source in the media may lead to catabolite repression that suppresses α-amylase production (Berekaa et al., 2007). The same suppression might also occur in this study, as a small amount of glucose was added to the fermentation medium.

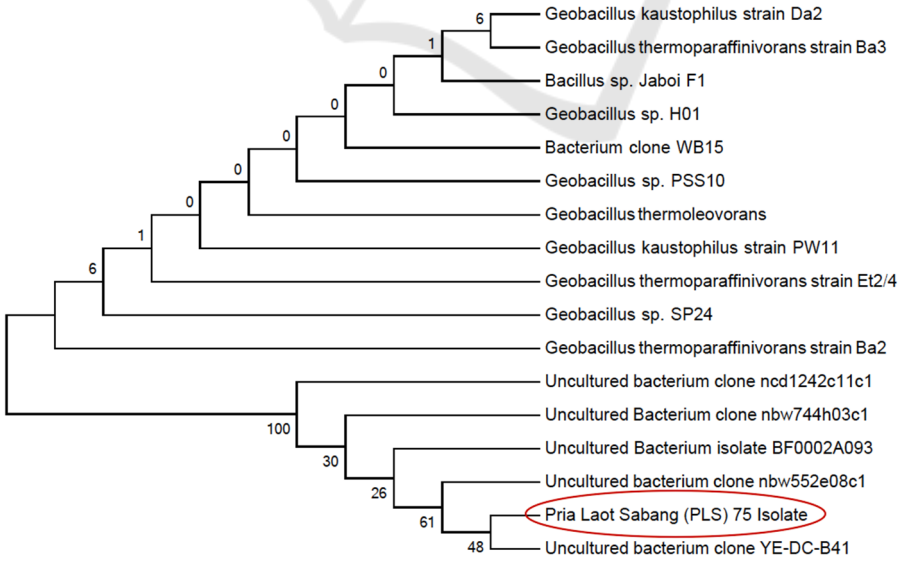

Figure 3: Phylogenetic relationship of the 16S rRNA gene (480 bp) from PLS 75 with 17 most related gene sequence. The phylogenetic tree was constructed using the neighbour-joining method of the Mega 6 software with 1000 bootstrap replicates.

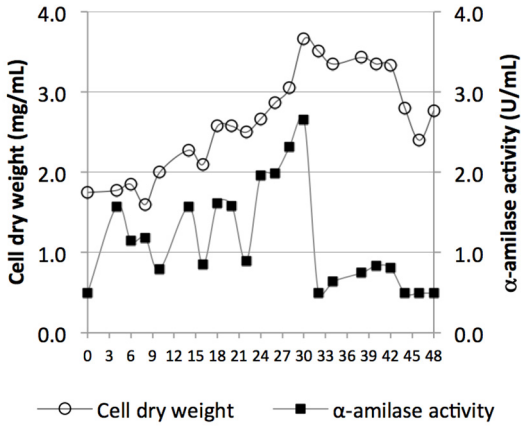

Figure 4: Growth curve of PLS 75 in ½ T medium enriched with 0.25% glucose. The incubation was conducted at temperature 70 °C, pH 7 and 150 rpm. The data was an average of triplicates ± SD.

3.4 Effect of Incubation Temperature on Biomass and α-amylase Production

The effect of temperature on biomass and α-amylase production was studied at 60 °C, 65 °C, and 70 °C in a narrow time window (27 – 32 h). Figure 5a shows that the biomass, measured as cells dry weight, was best produced at 60 °C. The highest biomass concentration (5.5 mg/ml) was observed at 60 °C and 28 h. The biomass at 70 °C was roughly half of that at 60 °C. Optimum α-amylase activities of all temperature were observed at a slightly delayed time. The highest activity (4.43 U/ml) was achieved at 60 °C and 31 h. The optimum time was also applicable to all other temperature (Figure 5b).

The number of cells and enzyme activity is generally interrelated. On the one hand, the increase in cells mass will produce more enzymes. On the other hand, biomass formation is catalysed by enzymes. Enzyme production increases with increasing temperature until a certain level. A temperature above the optimum causes a decrease in enzyme activity. It gives adverse impacts on metabolic activity, which reduce growth and product formation (Sundarram and Murthy, 2014). High temperature affects enzyme activity in two ways, i.e. changing the reaction rate constant and causing thermal denaturation of the enzyme (Demirkan et al., 2017). However, cells formation and α-amylase production are controlled by different regulatory mechanisms (Baysal et al., 2003).

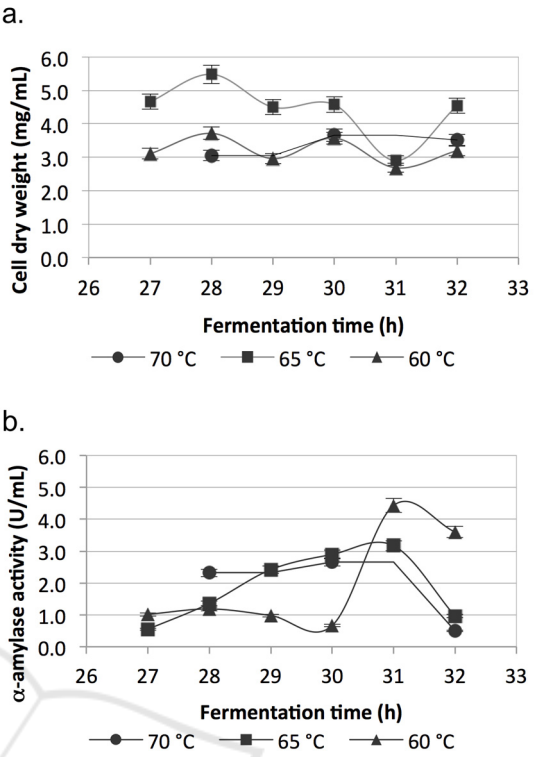

Figure 5: Effect of fermentation temperature on; a. Biomass production; b. α-amylase activity. The incubation was conducted at pH 7 and 150 rpm. The data was an average of triplicates ± SD.

The results are in agreement with several studies employing thermophilic bacteria. Devi et al. (2010) reported that the optimum incubation temperature for two of *Bacillus* spp. strains to acquire high α-amylase activity was in the range 60-80 °C. The enzyme is already produced in the exponential phase and reaches a maximum at the stationary phase. Meanwhile, a thermo-halophilic *Bacillus sp.* NRC22017 produces optimum α-amylase at 45 °C. A 10 °C increase in the temperature reduces the enzyme activity by two-third (Elmansy et al., 2018).

3.5 Effect of pH of Medium on Biomass and α-amylase Production

The effect of pH on biomass and α-amylase production was studied at pH 5, 7 and 9. The biomass at pH 5 and 7 was about twice as much as that at pH 9 (Figure 6a). The highest biomass (3.57 mg/ml) was observed at pH 7 and 30 h. The highest α-amylase activity (4.4 U/ml) was also produced at pH 7 and 31 h. The enzyme activity at acidic and alkaline pH was low (Figure 6b).

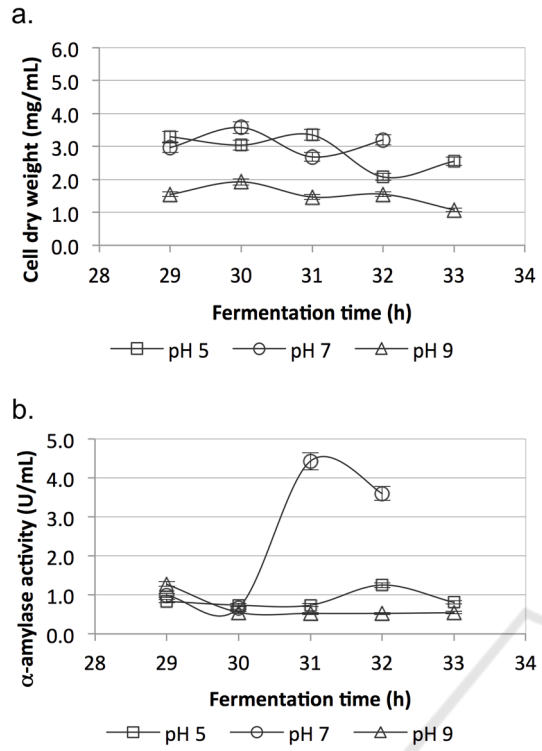

Figure 6: Effect of pH of the medium on; a. Biomass production; b. α-amylase activity. The incubation was conducted at temperature 60 °C and 150 rpm. The data was an average of triplicates ± SD.

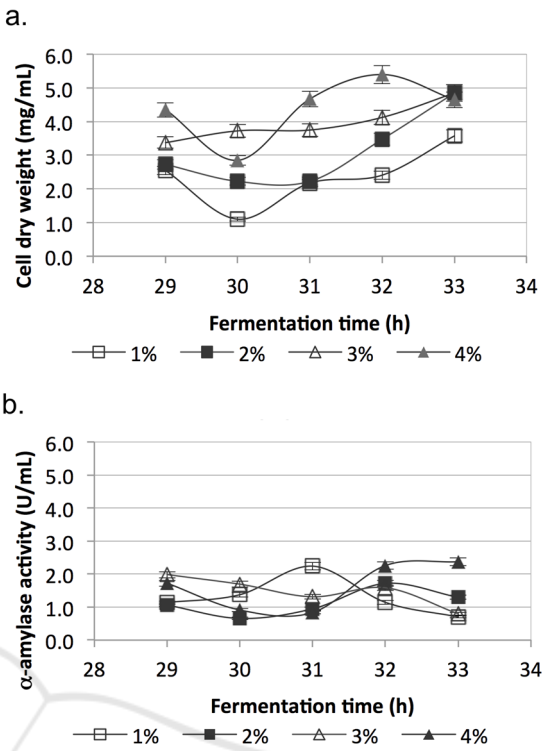

Figure 7: Effect of NaCl addition on; a. Biomass production; b. α-amylase activity. The salt addition was made to a final concentration of the media. The incubation was conducted at temperature 60 °C, pH 7 and 150 rpm. The data was an average of triplicates ± SD.

We have previously reported that the highest activity of the purified amylase from PLS 75 strain was at pH 5 (Iqbalsyah et al., 2018). Other molecules might have affected the activity as the enzyme was still in the crude form. Most microbial α-amylases perform the best catalytic activity at low pH (Zhang et al., 2017). However, some may work across a wide range of pH (Ghorbel *et al.*, 2009).

3.6 Effect of Salt Addition on Biomass and α-amylase Production

On the top of the available salts in the medium, as it was dissolved in seawater, various concentrations of sodium chloride were added to the media to contain an additional 1%, 2%, 3% and 4% to study the halophilic character of PLS 75 strain. The highest biomass concentration (5.40 mg/mL) was observed with the addition of 4% salt (Figure 7a). The highest α-amylase activity (2.38 U/mL) was also observed at the same salt concentration, although the difference was inconsequential (Figure 7b).

The results suggest that the α-amylase from PLS 75 strain was an extreme halophilic enzyme as it was still active in the presence of 2.5–5.2M NaCl. This result is in agreement with our previous report of PLS A strain isolated from the same area in producing protease (Iqbalsyah *et al.*, 2019a). This result suggests that the enzyme may be able to escape denaturation in high salt concentrations. It can thus be useful to catalyse reactions in non-aqueous solution (Moreno et al., 2013).

The fumaroles from which the PLS 75 strain was isolated had an onset temperature around 80-100 °C, a neutral pH and a salt concentration of around 3.5 M. Magnesium, chloride, iron, lead and copper ions are much higher than those in typical seawater (Iqbalsyah *et al.*, 2019b). The different chemical composition than the typical seawater implies unique physiology of PLS 75 strain.

4 CONCLUSION

Four poly-extremophilic strains isolated from undersea fumaroles in Pria Laot Sabang were able to secrete extracellular hydrolytic enzymes. PLS 75 was the most potent strain to produce thermostable α-amylase and cellulase. The strain had a close relationship with uncultured microorganism after 16s rRNA gene sequence analysis. The results of growth condition experiments show that PLS 75 optimally produced thermostable α-amylase at 60°C and pH 5 for 30 h incubation. It resisted high salt concentration. This study provided preliminary information on PLS 75 cultivation conditions. may The results may be used for scaling-up of the enzyme production for various uses.

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REFERENCES

Annamalai, N., Thavasi, R. and Vijayalakshmi, S. and Balasubramanian, T. 2011, 'Extraction, purification and characterisation of thermostable, alkaline tolerant α-amylase from *Bacillus cereus*', *Indian Journal of Microbiology* 51 (4), 424–429.

Baysal, Z., Uyar, F. and Aytekin, C. 2003, 'Production of α-amylase by thermotolerant *Bacillus subtilis* in the presence of some carbon, nitrogen-containing compounds and surfactants', *Annals of Microbiology* 53 (3), 323–328.

Berekaa, M. M., Soliman, N. A. and Abdel-Fattah, Y. R. 2007, 'Partial characterisation and cloning of thermostable α-amylase of a thermophile *Geobacillus thermoleovorans* YN', *Biotechnology* 6, 175–183.

Dalmaso, G. Z. L., Ferreira, D. and Vermelho, A. B. 2015, 'Marine extremophiles a source of hydrolases for biotechnological applications', *Marine Drugs* 13 (4), 1925–1965.

Demirkan, E., Sevgi, T. and Başkurt, M. 2017, 'Optimisation of physical factors affecting the production of the α-amylase from a newly isolated *Bacillus* sp. M10 strain', Karaelmas Fen ve Mühendislik Dergisi 7 (1), 23–30.

Devi, L. S., Khaund, P. and Joshi, S. R. 2010, Thermostable α -amylase from natural variants of *Bacillus* sp. prevalent in Eastern Himalayan range. *African Journal of Microbiology Research* 4 (23), 2534-2542.

Elleuche, S., Schafers, C., Blank, S., Schroder, C. and Antranikian, G. 2015, 'Exploration of extremophiles for high-temperature biotechnological processes', *Current Opinion in Microbiology* 25, 113–119.

Elmansy, E. A., Asker, M. S., El-Kady, E. M., Hassanein, S. M and El-Beih F. M. 2018, 'Production and optimisation of α-amylase from thermo-halophilic bacteria isolated from different local marine environments', *Bulletin of the National Research Center* 42: 31.

Ghorbel, R. E. Maktouf, S., Massoud, E. B., Bejar, S., and Chaabouni, S. E. 2009, 'Thermostable amylase from *Bacillus cohnii* US147 with broad pH applicability', *Applied Biochemistry and Biotechnology* 157, 50–60.

Grand View Research 2019, Enzymes market size, share and trends analysis report by product (carboydrases, proteases, lipases), by application (industrial, specialty), by end use, by region, and segment forecasts, 2019 - 2025. [online] Available at: https://www.grandviewresearch.com/industry-analysis/enzymes-industry [Accessed 26 August 2019].

Iqbalsyah, T. M., Fajarna, F. and Febriani 2018, 'Purification and partial characterisation of α-amylase produced by a thermo-halophilic bacterium isolate PLS 75', *Biosaintifika* 10 (3), 574–580.

Iqbalsyah, T. M., Malahayati, M. Atikah, A. and Febriani, F. 2019a, 'Cultivation conditions for protease production by a thermo-halostable bacterial isolate PLS A', *Jurnal Natural* 19 (1), 18–23.

Iqbalsyah, T. M., Malahayati, Atikah and Febriani 2019b, 'Purification and partial characterisation of a thermo-halostable protease produced by *Geobacillus* sp. strain PLS A isolated from undersea fumaroles', *Journal of Taibah University for Science* 13 (1), 850–857.

Liu, X.D. and Xu, Y. 2008, 'A novel raw starch digesting α-amylase from a newly isolated *Bacillus* sp. YX-1: Purification and Characterisation', Bioresources Technology 99, 4315–4320.

Malhotra, R., Noorwez, S. and Satyanarayana, T. 2000, 'Production and partial characterisation of thermostable and calcium-independent α-amylase of an extreme thermophile *Bacillus thermooleovorans* NP54', *Letters in Applied Microbiology* 31, 378–384.

Miller, G. L. 1959, 'Use of Dinitrosalicylic Acid reagent for determination of reducing sugar', *Analytical Chemistry* 31 (3), 426–428.

Moreno, M. de L., Perez, D. and Garcia, M. T. Mellado, E. 2013, 'Halophilic bacteria as a source of novel hydrolytic enzymes', *Life* 3, 38–51.

Moshfegh, M., Shahverdi, A. R., Zarrinni, G. and Faramarzi, M. A. 2013, 'Biochemical characterisation of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xingjiangense'*, *Extremophiles* 17, 677–687.

Reddy, N. S., Nimmagadda, A. and Rao, K.R.S.S. 2003, 'An overview of the microbial α-amylase family', *African Journal of Biotechnol*ogy 2 (12), 645–648.

Schwieger, F. and Tebbe, C.C. 1998, 'A New Approach to Utilise PCR-Single Stranded Conformation

Polymorphism for 16S rRNA Gene-Based Microbial Community Analysis, *Applied Environmental Microbiology* 64, 4870-4876.

Souza, P. M. de and Magalhaes, P. de O. 2010, Application of microbial α-amylase in industry - A review', *Brazilian Journal of Microbiol*ogy 41, 850–861.

Sundarram, A. and Murthy, T. P. K. 2014, 'α-Amylase production and application: A review', *Journal of Applied and Environmental Microbiology* 2 (4), 166–175.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar S. 2013, 'MEGA6: Molecular evolutionary genetics analysis version 6.0', *Molecular Biology and Evolution* 30, 2725–2729.

Zhang, Q., Han, Y. and Xiao, H. 2017, 'Microbial α-amylase: A biomolecular overview', *Process Biochemistry* 53, 88–101.