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CULTIVATION CONDITIONS FOR PROTEASE PRODUCTION BY A THERMO-HALOSTABLE BACTERIAL ISOLATE PLS A

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Abstract. Polyextremophiles have increasingly been utilised to produce thermostable enzymes with better stability in multiple extreme conditions. This study reports the screening results of four new bacterial isolates (PLS A, PLS 75, PLS 76 and PLS 80), isolated from an under water hot springs, in producing thermo-halostable protease enzyme. Optimum cultivation conditions for the protease production were also studied. Screening of protease-producing isolates was conducted using Thermus solid medium enriched with 3% skim milk and 0.5% casein. The growth of the isolates showing protease activity was monitored by measuring the cell dry weight and protease activity during 24 h cultivation period. The activity was also measured at various cultivation conditions, i.e. temperature, pH and salt concentrations. Amongst the four isolates, only PLS A showed the ability to produce protease. The optimum cultivation conditions for protease production were observed at 65°C, pH 7 for 18 h incubation. The activity increased with the addition of 1% NaCl concentration (0.085 Unit/mL). The ability of PLS A isolate to produce thermo-halostable protease was encouraging as they could potentially be used in industries requiring the enzyme with multiple extremes.

Keywords*:* Thermophilic, halophilic, protease, thermozyme

I. INTRODUCTION

Microorganisms are found in the entire biosphere, including environments with extreme temperature, pressure, salinity and pH. Microorganisms with the ability to adapt to extreme environments are called extremophiles, while those adaptive to more than one extreme are called poliextremophiles [1]. Extremophiles living in extreme temperatures are psychrophilic (0 - 20°C), thermophiles (45 - 80°C) and hyperthermophiles (above 80°C). Based on the environmental pH, extremophiles are grouped into acidophilic (pH < 5) and alkaliphiles (pH > 9) [2,4]. Those that are able to live in high salt content are called halophiles. Based on the response to the salt concentration, halophiles are divided into several categories, i.e. non-halophiles (< 0.2M), slight halophiles (0.2 - 0.5M), moderate halophiles (0.5 - 2.5M), borderline extreme halophiles (1.5 - 4.0M), extreme halophiles (2.5 - 5.2M) and non-halophilic halotolerant microorganisms that can tolerate the presence of salt [3]. Extremophiles can produce extracellular enzymes that are useful for research and industrial applications. Amongst polyextremophiles, thermophiles and halophiles are the most interesting groups. The former can produce thermostable hydrolytic enzymes, such as proteases, lipases, amylases, and cellulases, which are capable of working at high temperature. The high temperature is often preferred in industrial processes because it increases the solubility of the reactants, particularly polymer. Also, the risk of contamination from mesophilic microorganisms decreases [1,4]. Halophiles survive at high salt concentrations because of their ability to adjust the osmotic pressure of the cells to match the environment. In doing so, halophiles accumulate salts in the cells. Halophilic enzymes adapt to such situation by having many negatively charged amino acids (Asp and Glu) on their surface to avoid precipitation. This condition is useful because halophiles enzymes could be used in the non-water solvent reactions [5,6,7]. Proteases play significant role in polypeptides hydrolysis to produce oligopeptides and amino acids. The protease is the main enzyme in food, leather, detergents, meat tenderizers, protein hydrolysates and pharmaceutical industries, as well as in diagnostic, waste management and silver recovery [8]. As Indonesia is known as a maritime country, the exploration of marine microorganisms will give huge contributions for bio-based industrial development. We therefore isolated microorganisms from an under water hot

spring in Pria Laot Sabang Indonesia. The pool of the microorganisms is very likely polyextremophiles. This study screened four strains for protease-producing potential. The optimum cultivation conditions for the enzyme production were observed. The results may be used as the basis for further researches to produce proteases with better stability for industrial purposes.

II. METHODOLOGY

Sampling was performed on the seabed, approximately 15 m below the sea surface, in Pria Laot village, Sabang, Weh Island, Indonesia. The area temperature was high as it had fumaroles, releasing gas. The sand was collected using a 30 cm-PVC pipe with blinds on both ends, while the water was collected using sterile bottles with cap. The samples were collected from the area with the highest onsite temperature.

Analysis of water chemical parameters

The water sample was analysed to determine ions of calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}), lead (Pb^{2+}), copper (Cu^{2+}), chloride (Cl^-) and sulphate (SO_4^{2-}) contents. Volumetric method was used to analyse Ca^{2+} and Mg^{2+} while Fe^{3+}, Pb^{2+} and copper Cu^{2+} were analysed by Atomic Absorption Spectrophotometry. Argentometry and spectrophotometry techniques were used to analyse Cl^- and SO_4^{2-}, respectively.

Cultivation of bacterial strains

Sand and water sample were transferred into liquid media of Peptone Water (PW) to culture the microorganisms. PW was a minimum medium made by diluting 1% peptone and 0.05% sodium chloride with sterile seawater. The medium was enriched with 0.25% glucose. The pH adjusted to 7.2 ± 0.2 at 25°C. Separately, 25 g of sand and 25 mL of water sample were added to 250 mL sterile seawater. After thorough shaking, 5 mL of each sample was transferred into flasks containing 25 mL sterile PW medium. The culture was incubated at 70°C and 150 rpm for 7 d. About 0.5 mL of the broth from the flasks showing growth were then transferred to solid PW medium and further incubated at 70°C. If the solid medium showed growth of more than one colony, then every single colony was transferred to a new solid PW medium. All procedures were performed aseptically. Each single colony was separately regenerated in modified Thermus solid media (0.2% yeast extract, 0.8% peptone, 0.2% NaCl and 3% agar), with the addition of 0.1% glucose dissolved in sterile seawater. Each colony was marked alphabetically or using Arabic numeral.

Screening of protease-producing isolates

Four isolates showing good growth were screened for protease-producing capability on a modified solid Thermus medium, with the addition of 0.1% glucose, 3% skim milk and 0.5% casein dissolved in sterile seawater. The medium was incubated at 70°C for 24 h.

Microbial growth study

Based on the screening results, the most potential strain was incubated in Thermus media (without agar), with the addition of 0.1% glucose and 2% casein in sterile seawater. The medium was then incubated at 70°C, pH 7 for 48 h to study the optimum time for *protease* production. The cell dry weight and protease activity were monitored in time intervals during the incubation period.

The effect of temperature, pH and salt addition on protease production

The optimum temperature for protease production was determined by inoculating the most potential isolate in a Thermus medium prepared as described above, at various temperature (60, 65, 70°C). The pH was adjusted to 7 using phosphate buffer (disodium hydrogen phosphate or sodium dihydrogen phosphate) and incubated at 150 rpm for optimum incubation time based on the data of growth curve. The protease activity was then analysed. The optimum pH for protease production was determined similarly, using various pH (5, 7 and 9). The pH media were adjusted by adding corresponding phosphate buffers. It was incubated at optimum incubation time and temperature. The protease activity was then analysed. To study the effect of salt concentrations on protease production, the media were made as previously described. Sodium chloride of various concentrations (1, 2, 3, 4%) was added into the media to reach final concentrations of 4.5% (0.79M), 5.5% (0.96M), 6.5% (1.14M), and 7.5% (1.32M). The molarity calculation was based on the assumption that the seawater was singly sodium chloride (density 1.025 g mL^{-1}) of 35 g NaCl per kg solution. They were then incubated at optimum incubation time, temperature and pH. The protease activity was then determined.

Protease assay

The protease activity was assayed by Anson Method [9], using a tyrosine standard curve (20, 30, 40, 50, 60, 70 μg/mL). One unit of protease activity was defined as the amount of the enzyme required to produce 1 μmol of

tyrosine per minute under the assay conditions.

III. RESULTS AND DISCUSSION

Water analysis

Analysis of the chemical components in the water sample was to indicate the chemical environment of the sampling location (Fig. 1). This could be used to improve the culture medium to promote better growth.

Figure 1. Sampling location on the undersea fumaroles in Pria Laot Sabang

As expected, the water sample was rich in chloride, which were 21-fold higher than in typical seawater. Surprisingly the concentration of iron, magnesium, chloride and sulphate were only 35, 0.34, 20 and 9.4%, respectively, of those in typical seawater [10], despite the water sample produced a strong sulphur scent. Lead and copper were also detected in the sample (Table 1). Salinity of seawater is assumed generally uniform and the compositions vary only slightly across the globe [11]. It seems there is huge differences in several inorganic matters in the seawater from the sampling location. This suggests that the water might provide unique physiological conditions for the isolates. However, PW and Thermus media used in this study were suitable to culture the microorganisms. Therefore, there was no additional growth element added to the medium, except glucose in a very small amount (0.1 - 0.25%) to accelerate biomass production.

Table 1. Chemical parameter of the water sample from sampling location

Parameter	Average value* (mg/L)
Calcium (Ca^{2+})	8666 ± 3031
Magnesium (Mg^{2+})	4284 ± 783
Iron (Fe^{3+})	0.199 ± 0.046
Lead (Pb^{2+})	0.0255 ± 0.0046
Copper (Cu^{2+})	0.0058 ± 0.0030
Chloride (Cl^{-})	3885 ± 200
Sulphate (SO_4^{2-})	252 ± 27

*Values are average from 2 repeats ± SD

Isolation and regeneration of microorganisms

There was no observed microbial growth on the medium inoculated with the culture from the sand sample. The growth was only shown by microorganism in the water sample. The use of PW medium was to select robust microorganisms that can grow on a very minimal medium that provides only the exact essential nutrients needed for microorganisms to grow [12]. As PW only contained 1% peptone as the carbon and nitrogen sources, it was expected to only suit very adaptable strains. After regeneration on PW medium, only four isolates exhibited good growth (namely PLS A, PLS 75, PLS 76 and PLS 80), which were further regenerated in modified Thermus media. All isolates showed yellowish white round colonies (Fig. 2).

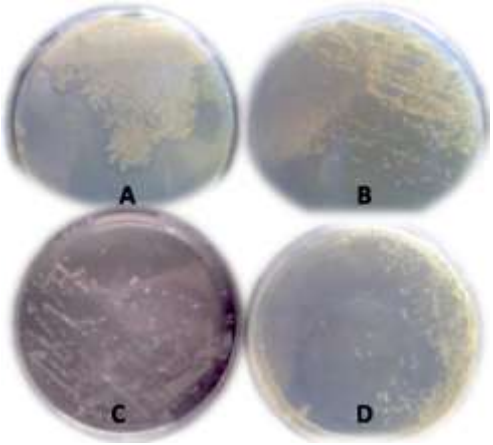

Figure 2. Colonies of four PLS isolate on Thermus solid media at 70 °C for 24 hours (a) PLS 76; (b) PLS 75; (c) PLS A; (d) PLS 80.

Screening of protease

Proteolytic activity of the four isolates was determined by growing them on a solid medium containing casein. A clear zone around the colony was an indicator of casein hydrolysis by the enzyme. Only PLS A showed a positive result. (Fig. 3). It was therefore used for microbial growth study.

PLS A growth study

As incubation time, inoculum size, pH and temperature affect metabolites synthesis, the growth curve of PLS A was studied to define the best fermentation time for protease production. The protease activity and the biomass (as cell dry weight) were determined. Fig. 4 shows that PLS A experienced a very short lag phase. The culture was already adaptable to the medium

because the initial culture was propagated in similar medium and conditions. The growth curve also showed a very short stationary phase between 20 – 22 h. The biomass concentration declined significantly afterward.

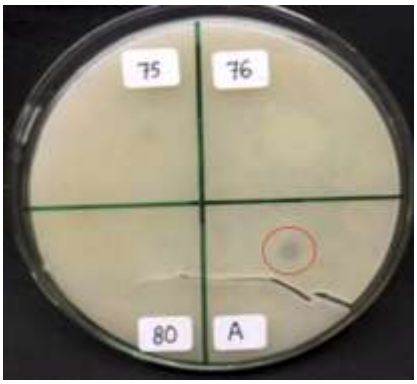

Figure 3. Proteolytic activity of PLS 75, 76, 80 and A Isolate in a Thermus medium, enriched with casein and skim milk after incubation at 70°C for 24 h.

The protease, as produced by the presence of casein in the medium, showed an activity trend similar to the biomass production with an early-onset however. The optimum protease activity was observed at 18 h (0.016 U/mL). The protease activity decreased subsequently. A comparable result is reported for *Bacillus thermoglucosidicus AF-01* showing optimum biomass production at 24 h but optimum proteases activity at 18 h [13]. Optimum cultivation time for enzyme production varies, depending on type of microorganism and culture conditions such as inoculum size, pH and temperature [14]. Highest enzyme production is normally achieved at stationary phase. Optimum protease production is reported as early as 9 h [15] or as late as 96 h [16].

The effect of temperature on protease production

The effect of cultivation temperature (60, 65 and 70°C) on protease production was investigated during 14 - 22 h fermentation. The results showed that the optimum cultivation time for protease production was 18 h at all temperature. The highest protease activity was produced at 65°C (0.035 U/mL). The activity was decreased slightly when at 60°C (0.029 units/mL). A slight increase of the temperature caused a significant different in the activity as the activity at 70°C was only half of that at 65°C (Fig. 5A). The optimum temperature for protease production by thermophiles may vary depending on the microorganisms and not necessarily correspond to the environmental conditions from which the microorganisms are isolated. In this study, the temperature at the sampling location was approximately 90°C, while the optimum temperature for protease production was 65°C. A *Bacillus* sp. isolated from soil sample around a detergent industry produces optimum protease in a media containing rice bran at a temperature of 55°C [17]. Similarly, a *Bacillus* sp. isolated from soil and waste in the area of milk processing industry and slaughter houses produces an optimum protease at 50°C [18]. For both instances, the production temperature is higher than that of the sampling locations.

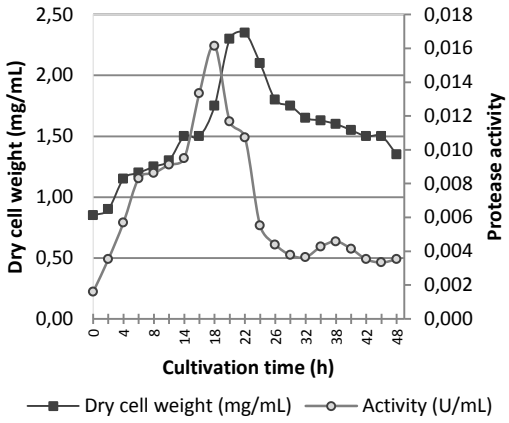

Figure 4. The growth-curve of PLS A isolate. The cultivation was conducted in Thermus medium at 70°C, pH 7, 150 rpm for 48 h. Values are average from 2 repeats.

The effect of pH on protease production

PLS A was able to produce optimum protease at 18 h cultivation time in all pH values. However it was better being produced in neutral to alkaline media. The pH 7.0 medium produced protease with high activity as early as 14 h (0.033 U/mL), while the optimum was observed at 18 h (0.035 U/mL). At pH 9.0 and 18 h, the optimum activity (0.031) U/mL differed only about 10% than that at pH 7.0. However, the activities before and after the optimum time at this pH were significantly lower. The protease produced at pH 5.0 showed significantly reduced activities at all cultivation time compared to the other two conditions (Fig. 5B). Protease could be produced at acidic condition, mostly from non-microbial origin [19]. However, bacterial proteases are typically produced at neutral [20] or alkaline pH [17]. Contrary to the temperature condition, the optimum pH for protease production is closely related to the enzyme optimum activity. Alkaline proteases are the most common type of protease used in industrial processes. It is used in various industries including detergent, textile, leather, food, pharmaceuticals and waste industries [8].

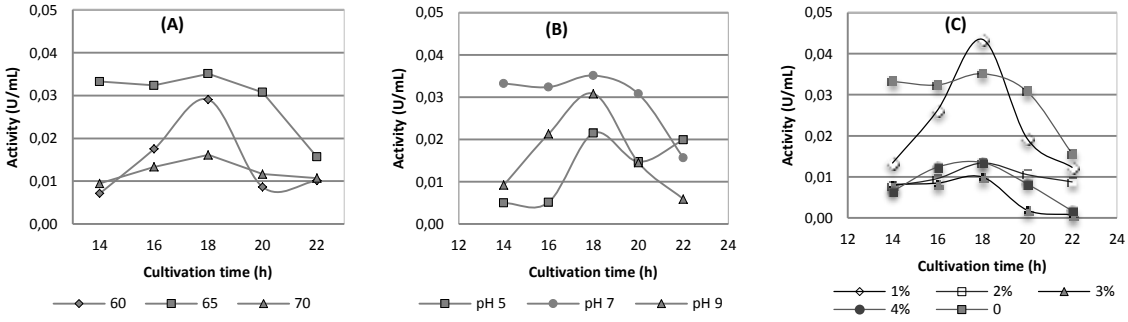

Figure 5. Optimum cultivation conditions for protease production. (A) Variation of temperature at pH 7.0 (B) Variation of pH at 65°C, (C) Addition of NaCl at 65°C and pH 7.0. Values are average from 2 repeats.

The effect of NaCl addition on protease production

The effect of salt addition was determined by adding various concentrations of sodium chloride (1, 2, 3 and 4%). As the typical seawater concentration is approximately 3.5% (in the form of NaCl), the final salt concentrations of the medium were 4.5, 5.5, 6.5 and 7.5%, respectively. PLS A showed the highest protease activity at 1% salt addition (0.043 U/mL), corresponding to 0.79 M of the total salt. The value was significantly higher than in the other media. The addition of more than 1% salt significantly reduced the activity (Fig. 5C). Similar result has been reported for protease from *Bacillus* strain NPST-AK that shows optimum growth and enzyme production in media containing 0 – 5% of NaCl. The strain is however still able to grow over broad range of NaCl concentrations (0 – 20%) [21]. The activity of the protease produced by halophilic microorganisms is stable in organic solvents [22]. The enzyme can therefore be used to catalyse reactions in non-aqueous solutions. Thus, it has a great application potential in industry.

CONCLUSION

PLS A was able to produce extracellular thermo-halostable protease. The optimum cultivation conditions in Thermus medium was at 65°C and pH 7 for 18h, indicating that PLA protease was a neutral thermostable enzyme. As it showed the highest protease activity at the addition of 1M NaCl, PLS A was a moderately halophilic microorganism.

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