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To cite this article: M Suci et al 2018 IOP Conf. Ser.: Earth Environ. Sci. 105 012126

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# Lipase production from Bacillus subtilis with submerged fermentation using waste cooking oil

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Abstract. Bacterial lipase has been developed lately because of its advantage to produce with large scale. Culture of Bacillus subtilis was grown to produce lipase in Waste Cooking Oil (WCO) using submerged fermentation (SmF) method. The enzyme activity of the culture was improved by using different concentration of inoculum, substrate, nitrogen source, inducer, and  $Ca^{2+}$  ion at 30°C for 84h fermentation. Lipolytic activity of crude lipase was determined using titrimetric method with hydrolysis reaction. Maximum activity of lipase (4.96 U/mL) was found at 5% (v/v) inoculum, 4% (v/v) WCO, 0.5% (w/v) yeast extract, 0.25% (v/v) olive oil, and 10 mM Ca<sup>2+</sup> that present in medium culture. Later, the crude lipase has been dried with spray dryer and resulting 17.33 gr of dry lipase powder per 500 mL crude lipase.

#### **1. Introduction**

Lipases (E.C. 3.1.1.3) have been known as one of enzyme which find usage in a wide array of industrial applications, such as detergent, food technology, textile, pharmacy, cosmetic, biodiesel, and agrochemical industrial [1]. Lipases are ubiquitous hydrolytic enzymes that catalyze the breakdown of fats and oils into free fatty acids, monoacylglycerols, diacylglycerols, and glycerol [2]. Among bacterial lipases, those from Bacillus shows interesting properties that make them potential for biotechnological applications. As cells have adapted to survive in such extreme climatic conditions, their enzymes and lipases in particular have evolved and exhibit exceptional properties [3]. Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus, and *Bacillus alcalophilus* are the most common bacterial lipases that being used [4]. Bacterial lipases are mostly extracellular and are produced by submerged fermentation (SmF) [5].

In the present paper, we describe the effect of inoculum concentration and nutritional factors for optimum production of Bacillus subtilis lipase. Nutritional factors were carried out by using different concentrations of carbon source, nitrogen source, inducer, and Ca<sup>2+</sup> metal ion.

#### 2. Materials and Methods

#### 2.1. Chemicals

The chemicals were of reagent grade and purchased from commercial suppliers (Merck, Sigma-Aldrich). Waste cooking oil was collected from household waste with maximum usage is twice. The olive oil for enzyme assays and skim milk were supplied from the local market.

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## 2.2. Microorganism

A lipase producing bacterial culture was purchased from InaCC - LIPI Cibinong. The culture was periodically sub-culture in Nutrient Agar (NA) containing agar 15 g/L, meat extract 1 g/L, peptone 5 g/L, sodium chloride 5 g/L, yeast extract 2 g/L with final pH 7.1 $\pm$ 0.2 (25°C). The stock culture was stored at -4°C.

#### 2.3. Culture media and conditions

Pre-culture was maintained on Luria Bertani broth medium. The stock culture was inoculated into 50 mL pre-culture medium and incubated at  $30^{\circ}$ C for 24 h. The cells were inoculated into 50 mL production medium where the concentration of inoculum, substrate, nitrogen source, inducer, and Ca<sup>2+</sup> metal ion were used to find out the highest lipolytic activity. The standard operating conditions and the composition of production medium were referred to the previous study [6]: peptone, yeast extract, substrate in phosphate buffer (0.1 M pH 7.5) with temperature 30°C and stirring rate 150 rpm. The culture was grown in 100 mL Erlenmeyer flasks on shaker water bath for 84 h fermentation. The optimum condition of variation mentioned previously, will be the basic to scale up the lipase production.

### 2.4. Enzyme assays

The culture broth was centrifuged at 4000 rpm for 45 minutes. The supernatant was filtered and used as the crude enzyme for the estimation of lipase activity. Lipase activity was assayed by alkali titration using olive oil as substrate [7]. Olive oil (10% m/v) was emulsified with polyvinyl acetate (5% m/v) in 0.05 M phosphate buffer pH 7.5. A 2 mL sample of crude enzyme was added to 18 mL of this emulsion. After incubation in a shaker for 15 minutes at 37 °C and 150 rpm, the reaction was interrupted by the addition of 20 mL of ethanol. The solution was titrated with 0.05 M NaOH in the presence of phenolphthalein as indicator. Reaction blanks were run in the same way, but the sample was adding after the addition of the ethanol solution. The lipase activities were performed in duplicate. A unit lipase activity (U) was defined as the amount of enzyme that catalyzes the release of fatty acid per min under the conditions mentioned above.

#### 2.5. Protein analysis

Protein concentration was measured spectrophotometrically at 750 nm by the Lowry method [8] using bovine serum albumin as protein standard.

#### 2.6. Dry lipase extract

Skim milk (12% m/v) was added into 500 mL crude enzyme and stirred until homogenous. The mixture was dried using spray dryer ( $T_{in}=80^{\circ}C$ ,  $T_{out}=130^{\circ}C$ ) to obtain dry lipase powder from *B*. *subtilis* [9].

#### 3. Results and discussion

#### 3.1. Growth and lipase production profile

*B. subtilis* were grown at medium, which has been specified to find out the optimum incubation period. Maximum lipase activity of 4.72 U/mL was observed on 84 hours of production with the average optical density 2.452 at 600 nm. On the third day, decline in growth of *B. subtilis* and lipase activity was observed (figure 1). The decrease of *B. subtilis* growth may be due to over populated culture and fixed amount of nutrient with which the organism starts liberating proteolytic enzyme, enhancing self-consumption [10]. On the other hand, the decrease of lipase production at the later stage could be possibly due to pH inactivation, proteolysis, or both [11]. Maximum lipase activity on the 80 hours of fermentation also has been reported from *Bacillus sp.* [12]. Therefore, these conditions were employed for cultures used in future experimentation.

IOP Conf. Series: Earth and Environmental Science 105 (2018) 012126 doi:10.1088/1755-1315/105/1/012126



Figure 1. Lipase production and growth profile of Bacillus subtilis. The production was carried out at 30°C under shaking condition (150 rpm) in presence of 1% waste cooking oil, 0.3% yeast extract, 0.5% peptone, 0.25% olive oil, and 10 mM CaCl<sub>2</sub>.2H<sub>2</sub>O in phosphate buffer 0.1 M pH 7.5. •, cell growth;  $\blacktriangle$ , lipase activity.

#### 3.2. Selection of inoculum concentration

The inoculum was added to the production medium in varying concentrations (1, 3, 5, 7 and 9%). The results showed that the optimum lipase production was observed in case of 5% inoculum with lipase activity about 1.83 U/mL (figure 2). Above 5% concentration, decline in lipase activity was observed. Beside due to over populated culture, this might be due to limited nutrient and dissolved oxygen to support better bacterial growth and lipase production [13]. Similar results were reported [14] that lipase production by *Bacillus sp.* FH5 was at optimum condition when concentration of inoculum (5%) was used.

5



4 (Jul) 3 2 1 1 1 2 3 4 5 Substrate Concentration (%)

**Figure 2.** Effect of inoculum concentration on lipase production

**Figure 3.** Effect of substrate concentration on lipase production

#### 3.3. Selection of substrate concentration

The waste cooking oil (WCO) as the carbon source was added to the production medium in varying concentrations (1, 2, 3, 4 and 5%). The results showed that the optimum lipase production was observed in case of 4% substrate with lipase activity about 4.7 U/mL (figure 3). The increasing concentration of WCO increased the lipase activity, but it has also been observed that after 4% concentration, the lipase activity has decreasing. This may be attributed to the substrate inhibition (Feedback inhibition) [10]. Waste oil was theoretically beneficial for lipase yield [15]. However, results showed that a high concentration of oily substrate is unfavorable for lipase yield and its activity. This might be due to other carbon source or inducer present. On the previous study [16] found

that the high concentration of oil usually decreased the transfer rate of oxygen to the fermentation medium, which reduces the specific growth rate and further reduces the yield of the products.

#### 3.4. Selection of nitrogen source concentration

Yeast extract as the nitrogen source was added to the production medium in varying concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%). The results showed that the optimum lipase production was observed in case of 0.5% (w/v) yeast extract with lipase activity about 4.75 U/mL (figure 4). The increasing concentration of yeast extract increased the lipase activity. Yeast extract, apart from acting as a nitrogen source also supplies vitamins and trace metals, thereby affecting the growth of the organism and thus increasing lipase production [17]. Yeast extract can act alone or in combination with other nitrogen sources. In the present study, yeast extract was added with 0.5% (w/v) peptone as organic nitrogen source.





source concentration on lipase production



#### 3.5. Selection of inducer concentration

Olive oil as the inducer was added to the production medium in varying concentrations (0.1, 0.15, 0.2, 0.25, and 0.3%). The results showed that the optimum lipase production was observed in case of 0.25% (v/v) olive oil with lipase activity about 4.92 U/mL (figure 5). The increasing of olive oil above 0.25% obtained the decline of lipase activity. Previous study [18] found that inducer from olive oil more effective to induce production of lipase. This might be due to lipid content in olive oil, tributyrin, which is structurally easily digested by microorganisms. However, the increasing of inducer was not proportional to the number of enzyme produced. This might be due to the ratio of carbon and nitrogen in the medium. Generally, the activity of intra and extracellular lipases increases with increasing lipid concentrations, although excessive levels in the growth medium may be cytotoxic [19].

# 3.6. Selection of $Ca^{2+}$ metal ion concentration

 $CaCl_2.2H_2O$  as the  $Ca^{2+}$  metal ion was added to the production medium in varying concentrations (10, 15, 20, 25, and 30 mM). The results showed that the optimum lipase production was observed in case of 10 mM CaCl<sub>2</sub>.2H<sub>2</sub>O with lipase activity about 4.96 U/mL (figure 6). The increasing of metal ion  $Ca^{2+}$  obtained the decline of lipase activity. Several enzymes need metal ion to increase their activity [20]. However, those metal ions can act as an activator or inhibitor in a certain condition. On the previous study [21] found that the enzyme production was reasonably improved in the presence of  $Ca^{2+}$  which is significant in maintaining cell wall rigidity, stabilizing oligometric proteins and covalently bounding protein peptidoglycan complexes in the outer membrane.

IOP Conf. Series: Earth and Environmental Science **105** (2018) 012126 doi:10.1088/1755-1315/105/1/012126



Figure 6. Effect of Ca<sup>2+</sup> metal ion concentration on lipase production

# 3.7. Scale up production of enzyme

The production of lipase was scaled up from a flask (100 mL) to fermenter (20 L). The fermenter design is based on previous study. Pre-culture was grown in 1 L LB Broth medium and inoculated to 20 L production medium. The optimum condition from previous study was used with following composition: 5% (v/v) inoculum, 4% (v/v) WCO, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.25% (v/v) olive oil, 10 mM CaCl<sub>2</sub>.2H<sub>2</sub>O in phosphate buffer (0.1 M pH 7.5). The operating conditions are temperature 30°C and agitation rate 150 rpm during 84-hour fermentation. The culture broth was centrifuged at 4000 rpm for 45 minutes to obtain the supernatant. Furthermore, the supernatant was dried using spray dryer to get the dry lipase powder. Lipase activity was carried out with titrimetric and Lowry method through dry lipase enzyme and commercial enzyme (*Candida rugosa* lipase). The activity was 28 U/g solid and 694 U/g solid. While the specific activity was 1.44 U/mg protein and 12.5 U/mg protein respectively.



**Figure 7.** Comparison of lipase activity (U/g solid)

**Figure 8.** Comparison of lipase activity (U/mg protein)

# 4. Conclusion

The use of submerged fermentation for lipase production using waste cooking oil as substrate presented satisfactory results. A lipase *B. subtilis* was growth dependent as revealed by nutritional studies. The growth and lipase production are shown to be promoted by 5% (v/v) inoculum, 4% (v/v)

WCO, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.25% (v/v) olive oil, 10 mM CaCl<sub>2</sub>.2H<sub>2</sub>O in phosphate buffer (0.1 M pH 7.5) at 30°C under shaking condition (150 rpm). The maximum lipolytic activity was 4.96 U/mL in 84-hour fermentation. The activity in dry form decrease due to several factors but increase in specific activity. It is important to figure out the best purification method and immobilization to increase yield and activity of lipase.

# 5. Acknowledgement

The authors gratefully acknowledge KEMENRISTEK DIKTI, Universitas Indonesia, and USAID SHERA for supporting this research.

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