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Bulletin of Chemical Reaction Engineering & Catalysis, 19 (4) 2024, 710-721

Research Article

Extracellular Lipase from *Pseudomonas aeruginosa* SB-37: Production by Solid State Fermentation, Immobilization, and Characterization

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Received: 23th October 2024; Revised: 18th December 2024; Accepted: 21th December 2024
Available online: 23th December 2024; Published regularly: December 2024

Abstract

Native lipase is still promising as an industrial biocatalyst. This study aimed to investigate the production of native local lipase using solid state fermentation (SSF) methods, immobilization the lipase by Ca-alginate entrapment, and characterization based on substrate preferences. To obtain high lipase production using SSF methods, we optimized the type of agro-wastes substrates, fermentation time, oil induction percentage and volume of preculture percentage. The optimal condition for lipase production via solid-state fermentation involved a 7% (v/v) preculture of *Pseudomonas aeruginosa* SB-37, utilizing palm kernel meal as the substrate, supplemented with 6% (v/w) oil induction (soybean oil:tween 80 = 75:25) at 50 °C for 24 h. This gave a lypolitic activity value of 2 U/gds (gram dry weight substrates). Since the protein profile of extracellular lipase has a few protein bands, we perform direct immobilization on crude protein supernatant. Immobilization by Ca-alginate entrapment results in loading capacity and recovery activity values of 86.84% and 148%, respectively. The immobilized lipase retained 92% activity until four batch repetition and keep 40% activity at tenth batch. The highest hydrolytic activity of immobilized lipase was 0.9 U/g gel on the pNP_8 substrates. While the highest transesterification activity was observed with acetonitrile solvent and substrates of pNP_8 and isopropanol with the activity value at 0.6 U/g gel. This present study emphasized the feasibility of producing lipase as a biocatalysts using economical agro-industrial wastes and efficient immobilization using entrapment method

Keywords: Native lipase; SSF; agro-wastes; palm kernel meal; alginate entrapment

How to Cite: Haryati, T., Haryono, N.Y., Nugraheni, D., Fatmawati, F., Dewi, A.K., Syach, M.Z.E. (2024). Extracellular Lipase from *Pseudomonas aeruginosa* SB-37: Production by Solid State Fermentation, Immobilization, and Characterization. *Bulletin of Chemical Reaction Engineering & Catalysis*, 19 (4), 710-721 (doi: 10.9767/bcrec.20234)

Permalink/DOI: https://doi.org/10.9767/bcrec.20234

1. Introduction

Lipase or triacylglycerol ester hydrolase (EC 3.1.1.3) are enzymes with versatile biocatalytic activity, such as hydrolisis, esterification, transesterification, aminolysis, and interesterification. Lipase's demand, particularly from microbial sources, is still growing and is being used in many biotechnological industries today [1]. Native lipase, usually an extracellular lipase with high activity is still promising to be used as a biocatalyst [2]. The disadvantage of using lipase enzyme as a biocatalyst is the expensive production costs. Therefore, the

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utilization of agro-wastes residues as a lipase production media can become an alternative.

Utilization of agro-wastes residues as raw materials in the synthesis of value added products in compliance with the 3R bio refinery concept (reduce, reuse, and recycle). The circular economy postulate on sustainable use of natural resources through agro-waste management is also in line with this [3]. Agro-wastes residues contain valuable nutrients and can produce numerous bio products and biologically active molecules through valorization techniques. An example of agro-industrial waste management through valorization technique i.e. produce 2-furoic acid from rice husk [4]; conversion of citrus wastes into lactic acid [5]; agro-industrial wastes as a support for enzymes immobilization [6]; and agro-industrial wastes as a substrate in solid-state fermentation (SSF) for enzyme production [7,8].

Previous study showed that SSF with different agro-wastes can produce many microbial lipases from yeast, fungi, and bacteria [9]. Various agro-wastes such as Babassu oil cake, Palm oil wastes, Olive mill wastes, Wheat bran, and residues from Macauba were used as a SSF substrates for lipase production [10–13]. Agro-wastes residues are generated from crops such as husks, straws, shells, peel, and pulp; from animals such as bone and fish scales; and from industrial wastes such as palm kernel meal and oil cake [14]. To enhance lipase production, several parameters including carbon and nitrogen sources, inducers, pH, temperature, and aeration have been optimized [15].

In the present study, we aim to optimize the fermentation conditions for extracellular lipase production from *Pseudomonas aeruginosa* SB-37 using solid-state fermentation methods. This extracellular lipase was culture collection of Research Center for Applied Microbiology, Research and Innovation Agency (BRIN), Bogor, Indonesia. The optimized fermentation conditions have been investigated using liquid broth media [16]. Since this lipase is thermotolerant and has acid tolerance, it is promising to be used as industrial biocatalysts. The reason for this is an ability of extremophile lipase to meet harsh reaction setting in industrial application [17].

In order to prepare economical lipase as a biocatalyst, we also immobilized this extracellular lipase. There are many choices for lipase immobilization methods. Enzyme entrapment by alginate has the advantage of being rapid, non-toxic, inexpensive, and protecting the enzyme from harsh environments. Substrates and products can flow through the matrices pore [18]. Interaction between lipase and matrices during this entrapment, categorized as physical immobilization, will not disrupt the three-dimensional structure of lipase [19].

This study encompasses the production of a sustainable biocatalyst using solid-state fermentation (SSF). Lipase production optimization through SSF, employed entrapment methods with Ca-alginate matrices for immobilization, and characterized the immobilized lipase according to its substrate preferences for hydrolytic and transesterification activities.

2. Materials and Methods

2.1 Materials

Agro-industrial wastes like peanut meal, palm kernel meal, bone meal, and fish meal were used as substrates for lipase production in solid state fermentation. Soybean oil from the local market in Bogor, Indonesia. All other chemicals are analytical grade from Sigma Aldrich (Sigma, Chemicals, USA), Himedia (India), and Biorad (USA).

2.2 Microorganism and Cultivation Condition

Culture stock collections of *Pseudomonas aeruginosa* SB-37 from the Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN) [16]. For isolate rejuvination, 25 uL glycerol stock was spread on the nutrient agar media in petridish at 37 °C for 16 h in the following medium: 0.2% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) NaCl, and 1.5% (w/v) agar. For preculture preparation, a single colony from petridish culture was pick up by using a toothpick then grow at 37 °C, 150 rpm for 16 h in Basal Salt Media (BSM) contained (w/v): 1.3% nutrient broth, 0.01% $MgSO_4.7H_2O$, 0,01% $CaCl_2$, 0.75% v/v soybean oil and 0.25% v/v tween 80.

2.3 Lipase Production through Solid State Fermentation

For substrate optimization, 5 g of various agro-industrial waste (see Materials section) were ground and sieved through a 200 mesh sieve, then transferred into a 250 mL screw-capped jar, and then added 10 mL of BSM. The medium was autoclaved at 121 °C for 15 min, and then inoculated with pre-culture in an aseptic condition. Production of lipase was assayed at regular intervals of 24 h for 4 days [20]. To check the effect of % oil induction, SSF media was added with various % oil (v/v) levels at 1 %, 3%, and 5%. To check the effect of volume of preculture on lipase production, SSF media was inoculated with different volumes of preculture: 2, 7, and 12% (v/v).

2.4. Crude Lipase Extraction

For lipase extraction in the SSF media, 30 mL of 50 mM sodium biphospate buffer pH 6 was added and incubated in the shaking incubator for 90 min at 150 rpm and a temperature of 30 °C. Crude lipase supernatant was then filtered using a 0.45 µM cellulose filter and kept at 4 °C for further use.

2.5 Lipase Immobilization by Alginate Entrapment Methods

The lipase was immobilized by entrapment in calcium alginate beads [21]. $CaCl_2$ was dissolved in 100 ml of deionized water at a concentration of 3% (w/v). Sodium alginate 1.5% (w/v) was dissolved in 10 mL of crude lipase supernatant and added dropwise into the $CaCl_2$ solution using a syringe with a 23G needle while stirring with a magnetic stirrer at a speed of 400 rpm. The calcium alginate beads were made to stand for an hour at room temperature and were hardened by re-suspending into a fresh 10 ml $CaCl_2$ solution for 5 h at 4°C. Finally, these beads were washed with 30 mL deionized water and dried at room temperature for 24 h for use as a catalyst for hydrolysis and transesterification reactions. The protein concentration of free and unbound lipases was analyzed using the Bradford method [22].

$$Loading\ Efficiency\ (\%) = \frac{Immobilized\ Lipase\ (mg)}{Total\ Lipase\ in\ crude\ enzymes\ supernatant\ (mg)} \times 100\% \quad (1)$$

$$Activity\ recovery\ (\%) = \frac{Total\ activity\ of\ immobilized\ lipase\ (U)}{Total\ activity\ of\ lipase\ before\ immobilization\ (U)} \times 100\% \quad (2)$$

2.6 Lipase Activity Assay

2.6.1 Hydrolysis activity

Lipase activity was determined using the visible ultraviolet spectrum at 405 nm and various pNP-ester with different carbon chain length including para nitro phenyl butyrate (C4), para nitro phenyl octanoate (C8), para nitro phenyl decanoate (C10), para nitro phenyl laurate (C12), para nitro phenyl myristate (C14), para nitro phenyl palmitate (C16), and para nitro phenyl stearate (C18) as a substrates. 855 µL of sodium biphosphate buffer (50 mM, pH 6.0), 36 µL of ethanol, and 9 uL (10 mM) para nitro phenyl ester in acetonitrile were mixed with 300 µL of purified enzyme supernatant. Then incubated at 50°C for 15 min. To stop the reaction, the incubation mixture was added with 250 µL of 0.1 M disodium carbonate. The amount of enzyme that releases 1 µmol of PNP per min is determined as a unit of lipase activity under the test conditions [23].

2.6.2 Transesterification activity

Transesterification assay followed Fu method with slight modification [24]. 0.5 g immobilized lipase was mixed with 10 mM para nitro phenyl octanoate (C8) and 1 M various acyl acceptor (ethanol, isopropanol, n-butanol, and ethyl acetate) in 1 mL of n-hexane. The assay took place in a 2 mL tube under constant shaking at 55 °C, 180 rpm, for 15 min with a total reaction volume of 1 mL. Furthermore, 200 µL of the clear reaction mixture was directly mixed with 1 mL of Tris-buffer (50 mM Tris/HCl pH 7.0, 0.1% Triton X-100) in a 2 mL sterile tube. The conversion of para-nitrophenol into para-nitrophenyl esters was monitored using absorbance at 400 nm. The amount of paranitrophenol released in µmol/min is used to define lipase unit activity.

2.7 Data Analysis

The assays were carried out in triplicate and the data was converted into a mean with deviation standard using Microsoft Excel. The difference of each stages analyzed by one way analysis of variance, followed by Tukey with level significance of 95%.

3. Results and Discussion

3.1 Lipase Production by Solid State Fermentation

In order to get the best conditions for lipase production using solid state fermentation methods, several parameters such as: SSF media, fermentation time, % oil induction, and volume of preculture have been optimized. This optimization for lipase production was observed through lipase activity. The lipase activity in various SSF media is shown in Figure 1. Lipase activity showed the optimum value at 0.615 U/gds when using palm kernel meal as substrate. The activity decreased 42% when fish meal was used as substrate, while the lowest activity was shown at peanut meal and bone meal as substrates, with activity values of 0.278 and 0.223 U/gds, respectively. The best lipase production was achieved with palm kernel meal as substrate at 0.615 U/gds. This result was in agreement with previous research that the best lipase *Pseudomonas aeruginosa* PseA activity at 0.625 U/g in solid-state fermentation using *Jatropha curcas* seed cake as substrate [25]. Palm kernel meal is a product that is derived from oil palm extraction. It contains protein, carbohydrates, crude fiber, and oil [26]. Previous studies also showed lipase production from yeasts using SSF methods with palm kernel oil as a substrate [27,28].

To get the best time of lipase production, we varied the fermentation time from 24 to 96 h (Figure 2). The highest lipase activity was 0.944 U/gds at 24 h fermentation time, then decreased by 1.2 and 1.56 fold at 48 and 72 h, respectively. The 96 h fermentation time saw a minimum activity of 0.42 U/gds. The fermentation time is an important factor for microbial growth in the fermentation medium to express extracellular lipase, which is related to nutrients availability and microbial metabolites [29]. The optimal fermentation time was varied from 24 to 96 hours depending on the microbial species and strain type. This results are in agreement with the production of lipase from *Chaetomium globosum* via solid-state fermentation, with the optimal fermentation time being 24 hours [30]. Meanwhile, lipase production from *Pseudomonas aeruginosa* JCM5962(T) and *Pseudomonas* sp. BUP6 has an optimum fermentation time of 48 and 96 hours respectively [20,31].

The synthesis of extracellular lipases is strongly induced by the presence of oil/triglycerides in the growth medium of the microorganism [32]. Triglycerides contained fatty acids that can induce lipase promoter gene for expressing extracellular lipase [33]. There are always optimum value for oil induction, if too low causes lower expression of lipase, but if too high might decrease oxygen transfer in the substrates medium and give negative effect towards lipase

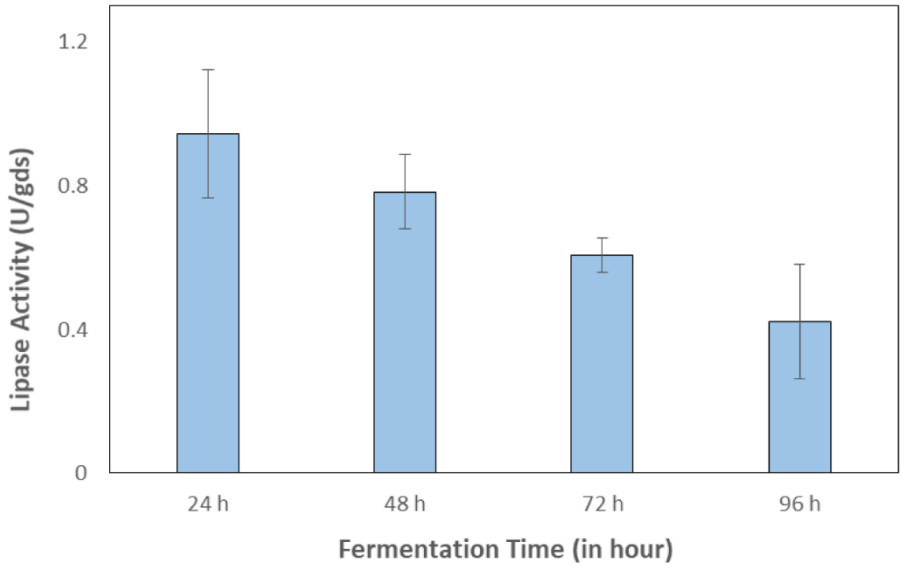

Figure 1. Effect of different agroindustrial wastes as SSF substrates media on lipase activity. 350 µL preculture of Pseudomonas aeruginosa SB-37 was inoculated onto different SSF substrates at 50 °C for 72 h. Lipase activity is represented as a unit per g of dry substrates.

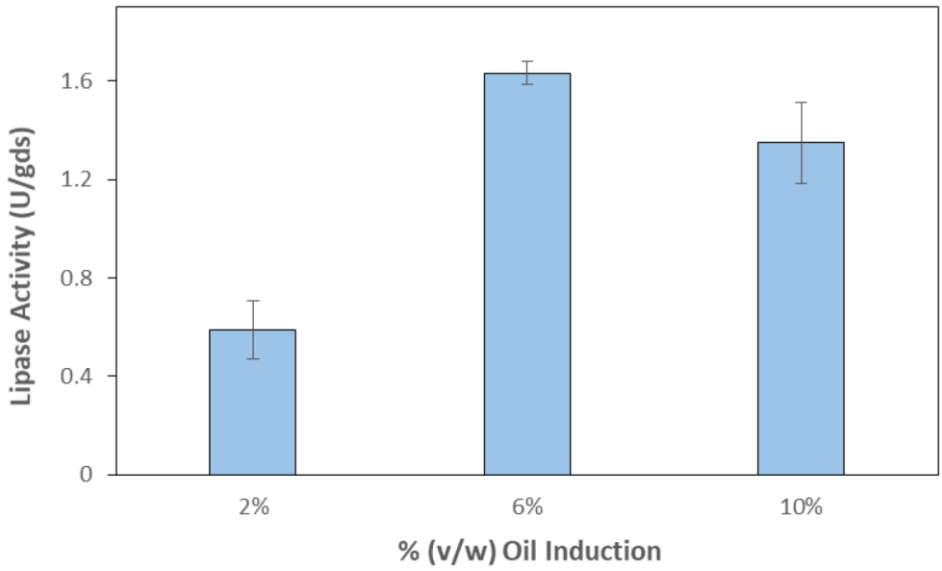

Figure 2. Lipase activity is influenced by the fermentation time. 350 µL preculture of Pseudomonas aeruginosa SB-37 was inoculated on palm kernel meal as a substrate at 50 °C for 24 to 96 h. Lipase activity is represented as a unit per g of dry substrates.

production [34,35]. Previous patents (Registered Number P00202315084) have optimized oil induction on the lipase production of *Pseudomonas aeruginosa* SB-37 based on liquid fermentation. The best condition was at 5% oil induction (soybean oil:tween 80 = 75:25) [16]. To understand the best oil induction on solid state fermentation, we varied the oil induction from 2 to 10% (v/w) (Figure 3). Lipase activity increased from 0.58 to 1.63 U/gds with increasing oil induction from 2 to 6% (v/w). However, it decreased to 1.34 U/gds at 10% oil induction. Oil addition to the fermentation medium can act as an induction agent for extracellular lipase and a carbon source for microbial growth. Various oils for lipase induction, such as: Canola oil, Palm oil, Castor oil, Rice bran oil, and Sunflower oil [36]. While surfactants like tween 80 can increase membrane permeability and facilitate enzyme export to the medium [37].

Extracellular lipase production also influenced by preculture/inoculum concentration, low inoculum concentration will secreting small number of lipase, while high inoculum concentration will compete for nutrients supply and give effect to lipase production [35]. To obtain the optimal volume of preculture for lipase production, we performed lipase production using SSF methods using various preculture volumes such as 2, 7, and 12% (v/v) (Figure 4). Lipase

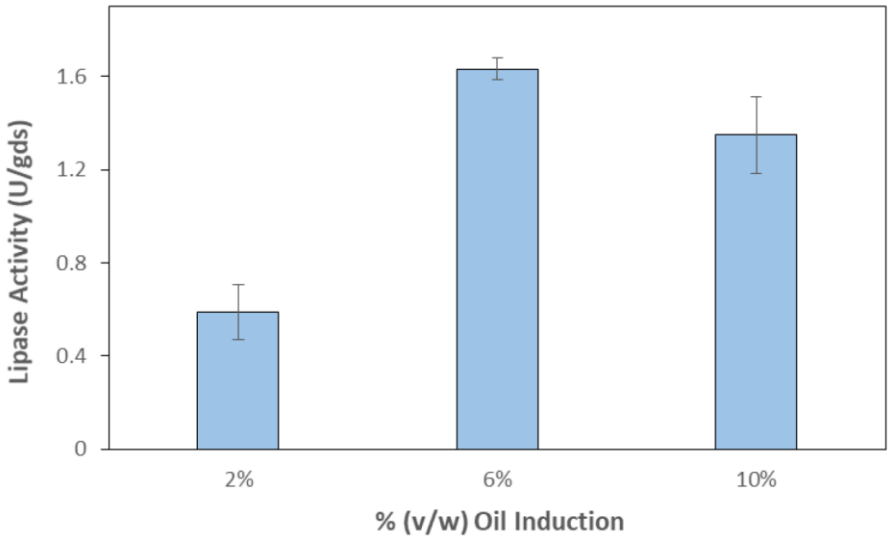

Figure 3. Effect of % oil induction (soybean oil:tween 80 = 75:25) on lipase activity. 350 µL preculture of Pseudomonas aeruginosa SB-37 was inoculated on palm kernel meal as a substrate with a varied % (v/w) oil induction from 2 to 10 % at 50 °C for 24 h. Lipase activity represented as Unit per g of dry substrates.

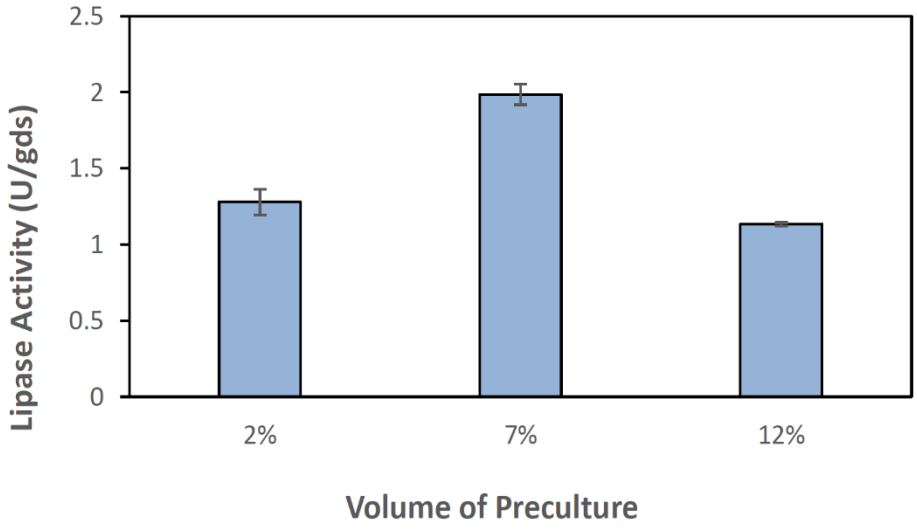

Figure 4. Effect of volume of preculture on lipase activity. Various volume preculture of Pseudomonas aeruginosa SB-37 at 2; 7; and 12% (v/v) was inoculated on palm kernel meal as a substrates at 6 % (v/w) oil induction (soybean oil:tween 80 = 75:25) at 50 °C for 24 h. Lipase activity represented as Unit per g of dry substrates.

activity increased from volume of preculture at 2 to 7% each, with 1.28 to 1.99 U/gds. The lowest lipase activity is at 1.13 U/gds with a volume of preculture of 12% (v/v). To achieve rapid and optimal lipase production, a sufficient volume of preculture is necessary. *Acinetobacter* sp. UBT1 and *Serratia marcescens* VITSD2 have optimal lipase production with preculture volume of each at 2 and 4% (v/v) respectively [38,39]. The optimized condition for lipase production using SSF methods was 7% (v/v) preculture of P aeruginosa SB-37 with palm kernel meal as a substrates, added with 6 % (v/w) oil induction (soybean oil:tween 80 = 75:25) at 50 °C for 24 hour.

3.2 Immobilization of Lipase Crude Supernatant by Encapsulation in Alginate

The crude lipase supernatant profile shows a dominant band around 25 to 35 kDa (Figure 5). This band is close to lipase A from the same species of *Pseudomonas aeruginosa* PAO1, categorized as a true lipase with a size of 29 kDa [40,41]. In order to make it a feasible biocatalyst for cost-effective reasons, we further immobilized lipase using alginate entrapment without purification.

Alginate is a polysaccharide derived from brown seaweed and bacteria, which are abundant as a natural resource in Indonesia [42]. Brown algae and bacteria generate it, and it is made up of α-l-guluronic acid (G) and ß-d-mannuronic acid (M) residues that are linked by 1,4-glycosidic bonds in a linear manner. These anionic polimers crosslink with Ca^{2+} to produce porous alginate beads [43].

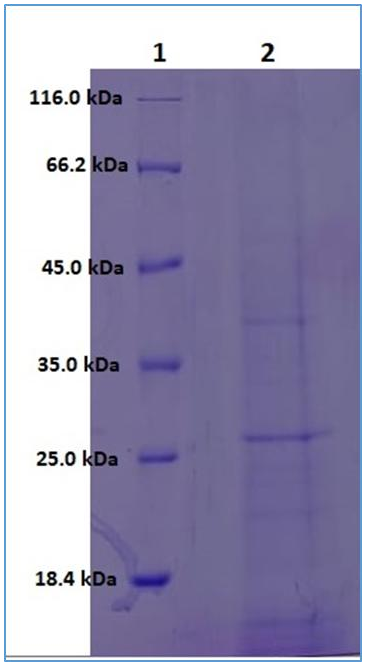

Figure 5. SDS-PAGE of the crude lipase supernatant extract obtained from Pseudomonas aeruginosa SB-37 under optimized solid state fermentation conditions. 1) Protein marker I Peqlab, 2) Crude lipase supernatant extract.

Since the protein profile of extracellular lipase has a few protein bands, we perform direct immobilization of crude protein supernatant. In this study, the alginate concentration was maintained at 1.5% w/v, resulting in a loading capacity of 86.84% and a recovery activity of 148% for immobilized lipase. Previous study showed that the loading capacity value of entrapment methods using alginate was 70% for extracellular lipase from *Pseudomonas* ADT3, 94.5% for *Mucor racemosus* lipase, and 100% for *Candida rugosa* lipase, respectively. While the recovery activity in this study was 148% higher than in the previous research, which was 94% [44–46]. High recovery activity in this study is thought to be caused by an appropriate alginate concentration. The decrease in alginate concentration can lead to an increase in beads' pores, which can enhance substrate and product diffusion [47,48]. This is in line with the previous study which indicated that the maximum activity of entrapped lipase was achieved at a concentration of 1.5% (w/v) alginate [49].

Immobilized lipase production stages are shown in Figure 6. Figure 6A shows the production of extracellular lipase using the SSF method with Palm kernel meal as a substrate. Then, extracellular lipase was extracted with 30 mM sodium biphospate buffer pH 6 to obtain crude lipase supernatant (Figure 6B). Finally, extracellular lipase was immobilized by entrapment methods on alginate beads (Figure 6C). This immobilized lipase was then used for the next experiment.

3.3 Characterization of Immobilized Lipase Based on Hydrolytic and Transesterification Activity

In order to better understand this immobilized lipase, we conducted both hydrolytic and transesterification assays. Hydrolytic activity

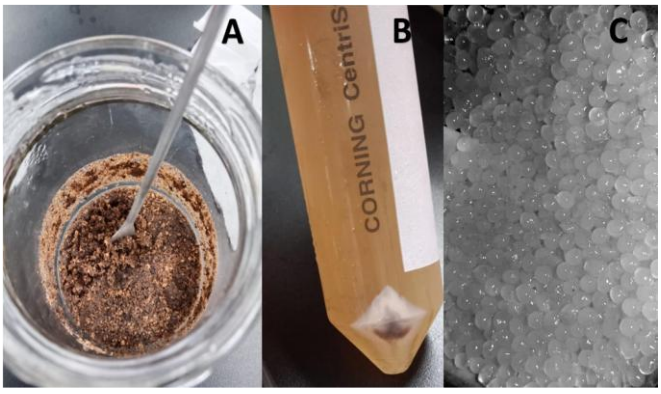

Figure 6. Stages in producing immobilized lipase, A) Palm kernel meal as SSF media, B) extracted lipase from SSF media, C) Immobilized lipase with entrapment on alginate beads.

assay was carried out on a several of para nitro phenyl ester substrates with varied carbon chain lengths from C4 to C18. The activities of immobilized extracellular lipase of *Pseudomonas aeruginosa* SB-37 are shown in Figure 7. Immobilized lipase units had an activity of 0.286, 0.245, and 0.299 U/g gel on pNP_4, pNP_8, and pNP_14, respectively. The highest hydrolytic activity was 0.933 U/g gel on the pNP_8 substrates. The second highest activity was 0.78 U/g gel on the pNP_10. The activity on the longer carbon chain was pNP_16 and pNP_18, each at 0.42 and 0.47 U/g gel, respectively. The previous study showed that the optimum hydrolytic activity of free extracellular lipase *Pseudomonas aeruginosa* SB-37 was on pNP_10 as substrate [16]. The optimal substrates of immobilized lipase were shifted towards the lowest carbon chain length because of steric hindrance in the immobilized lipase that affected substrate preferences [50].

To characterize the catalytic ability of immobilized lipase towards synthesis reactions, we performed a transesterification assay. The reaction was performed on the best substrate from previous hydrolytic activity, which is para nitro phenyl octanoate. Since short-chain alcohol, like methanol, has a negative effect on lipase activity [51,52], we perform optimization to get the best acyl acceptor. Transesterification activities of immobilized extracellular lipase of *Pseudomonas aeruginosa* SB-37 under various acyl acceptors are shown in Figure 8. The activity of immobilized lipase is 0.06 U/g gel when used ethanol as an acyl

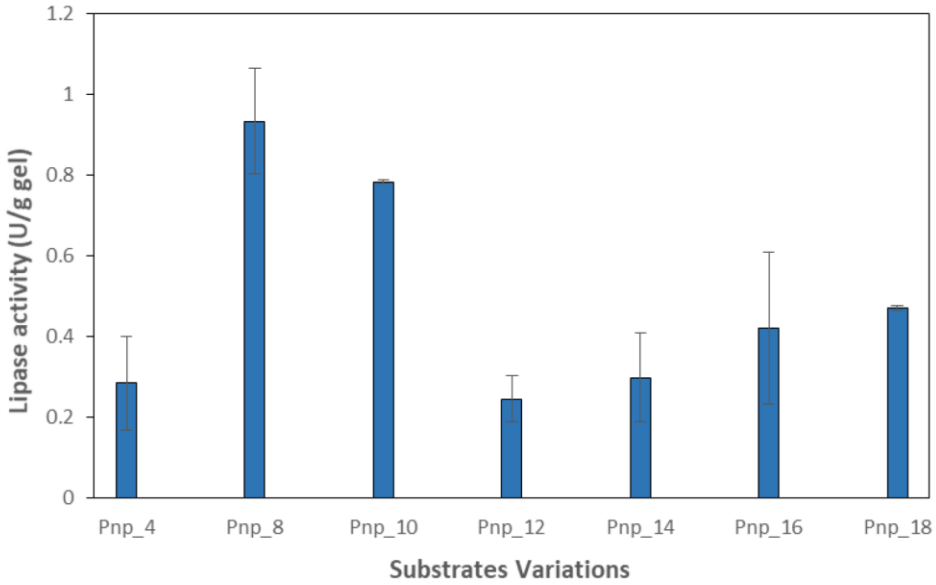

Figure 7. Hydrolytic activity assay presented as unit activity of immobilized lipase on substrates variation each represented paranitrophenyl butyrate, paranitrophenyl octanoate, paranitrophenyl decanoate, paranitrophenyl laurate, paranitrophenyl myristate, paranitrophenyl palmitate, and paranitrophenyl stearate). Unit activity define as µmoL/min/g supported alginate.

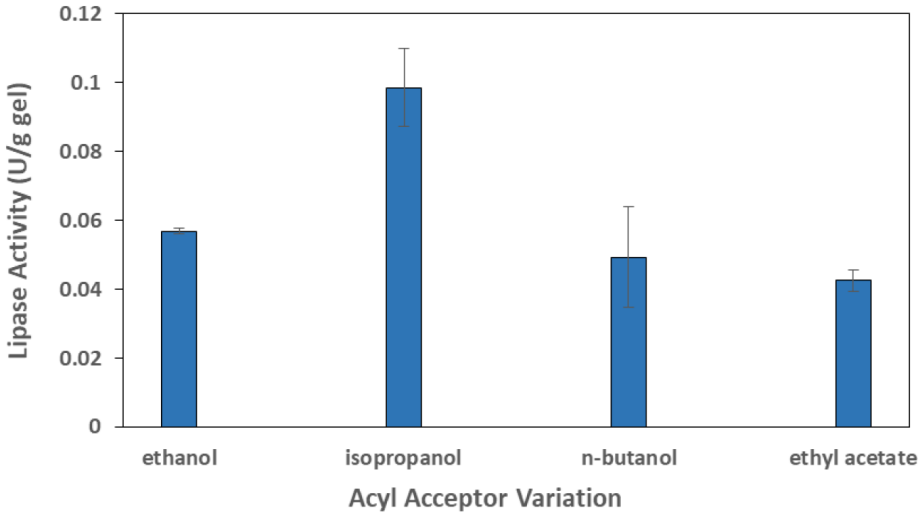

Figure 8. Transesterification activity assay presented as unit activity of immobilized lipase varies with acyl acceptor. Para nitro phenyl octanoate and various acyl acceptor in isooctane shaking at 150 rpm for 15 min at 50°C. Unit activity defined as µmoL pNP released /min/g supported alginate.

acceptor. Isopropanol was the best acyl acceptor with an activity value of 0.1 U/g gel. The activity values of n-butanol and ethyl acetate were 0.049 and 0.042 U/g gel, respectively. This result is in agreement with a previous study that the best acyl acceptor was isopropanol in the transesterification reaction with immobilized *Pseudomonas aeruginosa* [53].

To investigate the best organic solvents for transesterification activity, we perform an assay with para nitro phenyl octanoate as the substrate and isopropanol as the acyl acceptor in various organic solvents. The result displayed in Figure 9. Isooctane, which is a nonpolar organic solvent, provides the lowest activity at 0.05 U/g gel. The activity was higher for polar organic solvents at 0.58 and 0.44 U/g gel respectively, which were acetonitrile and acetone. The best activity was on a polar solvent acetonitrile. The previous study showed that the optimal activity of free extracellular lipase *Pseudomonas aeruginosa* SB-37 is in non-polar organic solvents such as n-heptane, n-hexane, and isooctane [16]. There is a change in the solvent preference for immobilized lipase due to entrapment on alginate beads. Lipase immobilized on alginate beads has a polar water environment. When a reaction occurs on an organic solvent, a polar solvent is easier to contact with that water environment to bring the substrate to the catalytic site. Unfortunately, non-polar solvents are difficult to contact with water environments, which have a 'like dissolve like' mechanism, making it difficult for the substrate to reach the catalytic site. This phenomenon has also been studied by Ng, C.H. and Yang, K.-L. [54], lipase in biphasic alginate beads contained hexadecane can be used as a biocatalyst for esterification in aqueous media.

3.4 Reusability of Immobilized Lipase

The reusability of the immobilized lipase was expressed as the percentage of residual transesterification activity (Figure 10). The immobilized lipase retained 92% activity at fourth batch, then declined until 40% activity at tenth batch, respectively. The loss of activity in immobilized enzyme may be due to the release of lipase during the reaction and washing. Another explanation is substrates and product accumulation on hydrogel beads that can hinder

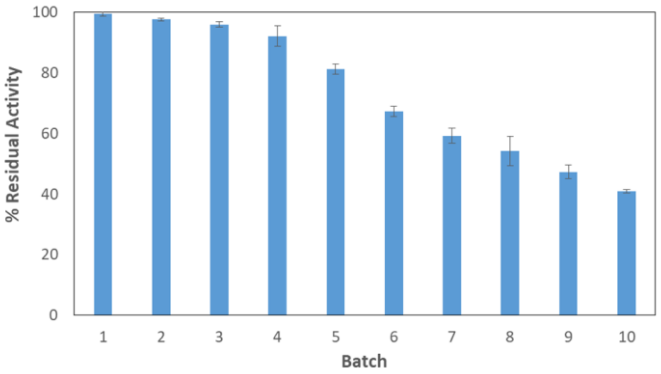

Figure 10. Reused of immobilized lipase. Specific activity calculated with para nitro phenyl octanoate and isopropanol as substrates in acetonitrile at 50 °C. Residual activity was measured by comparing the activity in a certain repetitions with the initial activity.

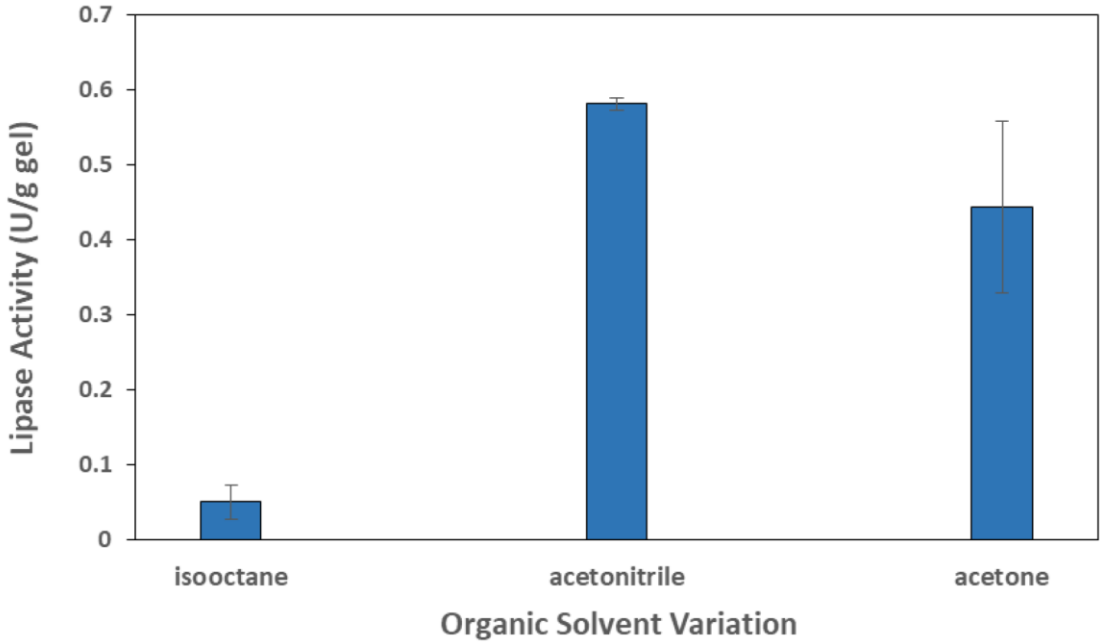

Figure 9. Transesterification activity assay presented as unit activity of immobilized lipase on variation in organic solvents. Para nitro phenyl octanoate and isopropanol as a acyl acceptor with various organic solvent shaking at 150 rpm for 15 min at 50°C. Unit activity define as µmoL pNP released /min/g supported alginate.

lipase catalysis [55]. This result in agreement with previous study of lipase entrapments using alginate-gelatin can retained activity of 40% at tenth cycle [56].

4. Conclusions

Extracellular local lipase from *Pseudomonas aeruginosa* SB-37 was successfully produced by SSF methods using palm kernel meal as substrate. The lipase supernatant was immobilized using entrapment methods with Ca-alginate beads. The immobilized lipase characterization proved that it is capable of catalyzing hydrolysis and transesterification reactions. This study briefly discusses the important value of converting agro-wastes residues into valuable lipase biocatalysts.

Acknowledgment

This work is supported by Joint Collaboration Research grants, Research Organization Life Science and Environment, National Research and Innovation Agency, SK Number: 1/III.5/HK/2024 and research project program from Research Organization Nanotechnology and Materials, National Research and Innovation Agency, SK Number: 20/III.10/HK/2024.

CRedit Author Statement

Author Contributions: T. Haryati: Conceptualization, Methodology, Resources, Data Curation, Writing, Review and Editing, Supervision, Project administration, Funding acquisition; N. Y. Haryono: Conceptualization, Methodology, Formal Analysis, Writing Draft Preparation, Supervision, Visualization; D. Nugraheny: Validation, Writing, Review and Editing, Data Curation; F. Fatmawati: Validation, Visualization, Data Curation; A.K. Dewi: Investigation, Writing Draft Preparation, Data Curation, Project administration; M.Z.E. Syach: Investigation, Writing Draft Preparation, Data Curation, Project administration. All authors have read and agreed to the published version of the manuscript.

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