Pilot-Scale Production of Lipase Using Palm Oil Mill Effluent as a Basal Medium and Its Immobilization by Selected Materials
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Abstract: A pilot-scale production of lipase using palm oil mill effluent (POME) as a fermentation basal medium was carried out, and parameters for immobilization of the produced lipase were optimized. Lipase production in a 300-L bioreactor was performed using two proposed strategies, constant power per volume (P/V) and constant tip speed. Moreover, lipase immobilization on different materials was also investigated. Lipase production was performed using liquid-state bioconversion of POME as the medium and Candida cylindraceae as the inoculum. The fermentation medium was composed of 1% total suspended solids (TSS) of POME, 0.5% (w/v) peptone, 0.7% (v/v) Tween-80, and 2.2% inoculum. The medium composition was decided on the basis of the medium optimization results of a previous study. The fermentation was carried out for 48 h at 30°C and pH 6. The maximum lipase production was 5.72U/mL and 21.34 U/mL, obtained from the scale-up strategies of constant tip speed and P/V, respectively. Four accessible support materials were screened for their potential use in immobilization. The most suitable support material was found to be activated carbon, with a maximum immobilization of 94%.

Key words: lipase, Candida cylindraceae, stirred tank bioreactor (300L), scale up strategy, immobilization

1 INTRODUCTION

Malaysia accounts for 57.7% of the world’s total production of palm oil, as reported by the Malaysian Palm Oil Board (MPOB) [1]. As the largest supplier, annual oil production figures were estimated to be 8.3 and 16.3 million tons in 2000 and 2008, respectively [2]. The number of oil mills has increased tremendously over the years, with a total of 423 mills producing approximately 89 million tons of fresh fruit bunches per year [3]. The main by-product of crude palm oil production is a liquid sludge waste, known as palm oil mill effluent (POME). More than 2.5 tons of POME is generated during the production of 1 ton of crude palm oil [4]. POME is a viscous brown liquid, predominantly organic and non-toxic with a highly unpleasant odor, and contains fine suspended solids in the pH range between 4 and 5 [5]. The effluent discharges from the palm oil industry have generated pollution problems, like oxygen depletion and other related effects on watercourses, due to high content of total solids, suspended organic solids, and dissolved organic matter, among others. POME consists of high concentrations of carbohydrates, proteins, nitrogenous compounds, lipids, and minerals [6, 7]. This composition makes POME a suitable substrate for microbial fermentation processes that produce different bioproducts. Hence, POME was used as the basal medium for a pilot-scale lipase production in this study.

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that breakdown fats and oils with a subsequent release of free fatty acids, diacylglycerols, monoglycerols, and glycerol. Currently, lipases are also extensively studied for their potential industrial applications. They can be obtained from animals, plants, and many naturally occurring or genetically engineered microorganisms, both in endogenous and exogenous forms. Apart from lipases derived from plant and animal sources, these enzymes are extensively produced by microbial fermentation for different applications. This is because microbes can be easily cultivated, and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions [8-10]. The enzyme also contributes to the multibillion-dollar lipid technology bioindustry. Lipase has been used in in situ lipid metabolism and ex situ multifaceted industrial appli-
Candida cylindracea is a well-known industrial lipase-producing yeast. Lipase produced by C. cylindracea has been one of the most widely used enzymes in research owing to its high activity in hydrolytic reactions and its use in synthetic chemistry. Salihu et al. found that C. cylindracea lipase, produced using POME as a basal medium, had an activity of 20.3 U/mL under optimized conditions. To our knowledge, research on pilot-scale lipase productions using POME as a basal medium is yet to be conducted. However, POME has been used successfully as a fermentation medium for the production of high-activity lipase at laboratory levels. Prior to implementing a large-scale POME-based lipase production, it is important to develop pilot-scale strategies to overcome the constraints of process optimization. Among the different scale-up strategies available, constant tip speed and constant power per volume (P/V) were used to evaluate the effects of agitation and aeration rates on lipase production.

The produced lipase was then immobilized with different support materials in order to improve its suitability for industrial applications. Enzyme immobilization is an economical method to reuse enzymes. Immobilized enzymes with their catalytic activities retained, are confined or localized physically in a defined space and can be used repeatedly. Various types of support materials have been used in immobilization studies. Parameter selection of the best support material will influence immobilization performance. Support material to be used as an immobilization of enzyme should has the following properties: permeability; insolubility; large surface area; high rigidity; suitable shape and particle size; resistance to microbial attachment; and chemical, mechanical, and thermal stability.

Recent studies on immobilization have shown that cellulose, activated carbon, coconut fiber, and rice husk were successfully utilized as support materials. Several support materials (cellulose, activated carbon, and empty fruit bunch [EFB]) were selected as support materials for immobilization in this study on the basis of the results obtained in previous studies. Mesoporous carbon materials are very suitable for use in large molecule adsorption, including battery capacitors, catalyst supports, biomedical engineering, and adsorbents for bulky pollutants.

### 2 MATERIALS AND METHODS

#### 2.1 Material collection, preparation, and fermentation

POME, used as the substrate in this study, was collected from West Oil Mill of Sime Darby Sdn. Bhd. in Carey Island, Malaysia, in clean containers, immediately brought to the laboratory, and stored at 4°C. The detailed composition of POME is shown in Table 1. Candida cylindracea ATCC 14830 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). A 4-d-old potato dextrose agar plate culture of C. cylindracea was suspended in 10 mL of sterile distilled water, and this suspension was used as the inoculum for pre-cultures. Incubations were carried out at 28°C for 48 h at 150 rpm in 100 mL of seed culture medium. The seed culture was then transferred into a 300-L stirred tank fermenter (Biosys, Fermetec Resources, Kuala Lumpur, Malaysia) with 150L of POME-based media containing 0.45% (w/v) peptone and 0.65% (v/v) Tween-80 for a 48-h fermentation period. Apart from the agitation and aeration rates (to be optimized in this study), all other process parameters such as inoculum size, temperature, and pH were kept constant at 2.2% (v/v), 30°C and 6, respectively. The reactor was equipped with a Rushton impeller with a 6-flat-blade design. The fermentation parameters for temperature, aeration, agitation, pH, foam control, and pressure control were monitored using a digital measurement and control system.

| Table 1 Approximate compositions of palm oil mill effluent (POME). |
|-----------------|-----------------|-----------------|
| *Materials       | Sivapalan and Ripin (8) | Rashid (9)     |
| pH              | 4.89            | 4.5             |
| N               | 47              | 194             |
| P               | –               | 223             |
| K               | 3,365           | 4157            |
| Ca              | 71              | 446             |
| Cu              | –               | 1.65            |
| Mg              | 442             | 946             |
| Hg              | –               | <0.001          |
| Mn              | –               | 5.41            |
| Zn              | –               | 2.83            |
| Fe              | –               | 105             |
| B               | –               | –               |
| NO₃             | 92              | –               |
| SO₄             | 322             | –               |
| Oil             | 10,500          | 8,632           |
| SS              | 20,650          | –               |
| TS              | 41,200          | –               |
| TVS             | –               | –               |
| BOD             | 35,100          | –               |
| COD             | 61,000          | 98,000          |
| DO              | –               | 0.05            |

*unit in ppm except for pH
10^7 spores/mL to a maximum cell count of 4.15 × 10^7 spores/mL.

2.2 Total suspended solids (TSS) measurement in POME-based medium

The standard method recommended by the American Public Health Association (APHA, 1989) was used to measure the concentration of TSS. Filter papers were dried at 105°C and weighed. Fifty milliliters of POME was then filtered using the pre-weighed filter papers. After filtration, the filter papers were oven-dried for 16 h at 105°C. The difference in the weight of the dried filter papers before and after filtering the POME sample was used as a measure of the TSS concentration.

2.3 Colorimetric assay for lipase activity

Lipase activity was assessed according to the method previously described by Gopinath et al. The cell-free extract from the fermentation broth was assayed quantitatively using p-nitrophenyl palmitate (pNPP). The substrate solution was prepared by mixing 10 mL of isopropanol and 30 mg of pNPP with 90 mL of 0.05 M phosphate buffer (pH 8.0) that contained 207 mg of sodium deoxycholate and 100 mg of gum arabic. A total of 2.4 mL of freshly prepared substrate solution was dispensed into each test tube. Thereafter, 0.02 mL of enzyme solution, using an appropriate enzyme dilution, was added to initiate hydrolysis. After 15 min of incubation at 37°C, the optical density at 410 nm was measured and normalized to an enzyme-free control. One lipase unit (U) was defined as the amount of enzyme that liberated 1 μmol p-nitrophenol per milliliter per minute under the standard assay conditions. All the enzyme assays were performed in triplicate, and the average values were calculated.

2.4 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) estimation was performed with a range of COD digestion reagents (0–1500 mg/L) by using a digester and a bench top spectrophotometer (Hach Company, USA). Two milliliters of the sample was added into each digestion vial and capped tightly, and the contents were heated at 150°C for 2 h, during which the vials were inverted intermittently at least 2–3 times to mix the contents. A blank was prepared by subjecting 2 mL of distilled water to the same treatments. After the 2-h incubation, the vial cooled to room temperature before analysis.

2.5 Lipase immobilization

Filtration processes were performed to produce partially purified lipase. The filtration separated lipase from the microbial cells and other debris. The cell-free filtrate was obtained after centrifuging the fermentation broth at 5000 × g for 10 min. The filtrate was then subjected to microfiltration by using a hollow fiber membrane cartridge in the QuickStand bench top system. A 0.45-μm membrane with an effective surface area of 0.011 m² was used for microfiltration. All experiments were performed at a room temperature (28°C ± 2°C). Next, the partially purified lipase was immobilized using physical adsorption techniques. The process was performed by adding 0.2 g of each support material to 25 mL of partially purified lipase (22 U/mL) at room temperature (28°C ± 2°C) with an agitation rate of 300 rpm and a contact time of 2 h. The immobilized lipase was then centrifuged at 10,000 rpm for 10 min, and the supernatant was separated. The concentration of adsorbed lipase was determined by measuring the lipase activity of the supernatant.

2.6 Effect of time on lipase immobilization

In order to effectively facilitate immobilization, it was important to determine the optimum immobilization time. The optimum immobilization time was determined by incubating lipase with the support material over different time intervals until the lipase immobilization reached its equilibrium, beyond which further incubation yielded no increase in residual lipase activity. The maximum immobilization occurred in 2 h. Therefore, for further experiments, the optimum time for lipase immobilization was fixed at 2 h.

3 RESULTS AND DISCUSSION

3.1 Selection of scale-up strategy

The first scale-up strategy, in which the tip speed was kept constant as an agitation-based parameter, relating to shear rate produced by impellers with respect to the fermentation media, was considered. The following equation presents the scale-up strategy when tip-speed is constant:

\[ N_2D_2 = N_1D_1 \]  

where \( N_2 \) and \( N_1 \) represent the agitation speeds in 300-L and 30-L bioreactors, respectively; \( D_2 \) and \( D_1 \) are diameters of the 300-L and 30-L bioreactors, respectively.

Using the above equation, the optimum agitation rate used was 98 and 225 rpm in the 300-L and 30-L bioreactors, respectively. The aeration during the fermentation process was maintained by varying superficial gas velocity (\( V_s \)). On the basis of the lab-scale data and constant \( V_s \), the parameter was calculated using the following equation:

\[ V_s = Q_{gas}/A_c \]  

where \( V_s \) is superficial gas velocity, \( Q_{gas} \) represents gas volumetric flow rate, and \( A_c \) is the cross-sectional area of the vessel. For the \( V_s \) to remain constant, 0.3vvm aeration was used in the 300-L bioreactor. Considering the relationships in equations 1 and 2, the starting operating conditions for the first scale up strategy used in the 300-L bioreactor...
were 30°C, 98 rpm, and 0.3vvm for temperature, agitation, and aeration, respectively. The lab-scale study found that higher agitation (>300 rpm in a 2-L bioreactor) yielded higher lipase production within a shorter fermentation period (36 h). Therefore, another approach using a constant P/V strategy, where the agitation rate is increased in order to escalate the lipase production, was also considered. The formula describing the constant P/V strategy is as follows:

\[ P/V = \frac{D^3}{N^3} \]  

Using the above formula, the agitation rate in the 300-L bioreactor was ultimately increased to 130 rpm.

The lipase activity was determined every 6 h during fermentation (Fig. 1) for the constant P/V strategy only. The results showed that lipase production slowly increased, reached its peak (21.34 U/mL) at 36 h of fermentation, and decreased thereafter, which was similar to the results obtained when lipase was produced in the 2-L bioreactor. For the constant tip speed strategy, lipase production was found to be 5.72 U/mL within 36 h of fermentation. No lipase activity was observed with other periods of fermentation (36-48 h) and not shown in Fig. 1. Therefore, most parameters were determined for the constant P/V strategy only.

The dissolved oxygen (DO) value was also measured at various time points over a 48-h period for data comparison with that of other researchers. Longet et al. observed that when the DO was kept above 20% saturation, by intermittent adjustment of the agitation speed (≥400 rpm) and raising the aeration rate from 0.5 to 1.0 vvm, there was an enhancement in the production of an extracellular lipase from Serratia marcescens ECU1010 in a 5-L fermenter\(^22\). Both agitation and aeration were found to influence dissolved oxygen (DO) concentration in the culture medium. Tan et al.\(^23\) showed that a high agitation speed (400 rpm) increased the DO in the medium and enhanced the lipase production of the mutant strain Candida sp. 99-125; the maximum activity of the produced lipase was higher than that seen with a low agitation speed of 200 rpm. With regard to aeration conditions, lipase produced using an aeration rate of 0.5 vvm had an activity higher than that of lipase produced using an aeration rate of 1 vvm. These results suggest that the DO saturation level is important since excess air did not stimulate lipase production\(^23\).

The results in Fig. 2 show that the constant P/V strategy yielded more a stable DO value than the constant tip speed strategy. DO values were maintained above 60% when using constant P/V strategy. These observations are similar to those of a previous study conducted by Takac et al., in which high-speed agitation maintained the DO level above 20% and enhanced lipase yield\(^22\). Their results showed that considerable lipase activity (21.34 U/mL) was obtained using the constant P/V strategy. The initial pH of raw POME obtained from the sampling area was 4.75 and was adjusted to 6.0 before initiating the fermentation process. The pH was maintained at 6.0 while using the constant tip speed scale-up strategy. A low lipase activity was observed at this pH; therefore, the pH was not controlled while using the constant P/V strategy. The fermentation medium pH in the 300-L stirred tank bioreactor was recorded every 6 h for 2 d. The results showed that the pH fluctuated during fermentation owing to microbial utilization of the medium components. There was a slight decrease in the pH of the medium after 12 h, and it increased to a pH of 8.26 after 24 h (Fig. 3). A similar pattern was observed by Rajendran et al. when Rhizopus arrhizus (MTCC 2233) was used as the inoculum\(^25\). In their study, the decrease in pH was attributed to organic acid production during enzyme production, while the increase in pH was attributed to the presence of free amino acids in the medium. Salihu et al. also reported that the lipase produced using a POME-based medium could be characterized as alkaline\(^26\). The pH increase observed in the bioreactor might have been caused by favorable conditions for alkaline lipase production by Candida cylindracea ATCC 14830.

The COD data generated using operational parameters (agitation and aeration) in the constant P/V strategy show

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**Fig. 1** Production of lipase with varying fermentation time at constant P/V strategy.

**Fig. 2** Dissolve oxygen pattern during fermentation at various fermentation time.
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an environment adequate for the utilization the organic substances in POME media and the production of lipase. During lipase production, COD values were determined, and reduction in these values was measured at regular intervals over a 48-h fermentation period (Fig. 4). The results show the continuous utilization of organic matter in POME during 48 h of fermentation.

3.2 Immobilization of lipase with different supporting materials

The support material was selected on the basis of a literature review and the local availability of the materials. Low-cost support materials are preferred for industrial applications; they can be organic (chitin, chitosan) or inorganic (CaCO₃) [26]. The organic support materials could come from lignocellulosic agro-industrial wastes, such as sawdust, wood chips/shavings, straw, spent grains [27], rice straw [28, 29], cellulignin [30], rice husk [30], or coconut fiber [31]. Another abundant raw material is oil palm empty fruit bunch (EFB). As several cellulosic materials have already been considered as immobilization support materials, EFB, a similar material, was considered as a good alternative support material. The other support materials evaluated were rice husk, commercial cellulose, and activated carbon.

The ability of the support materials to aid in the immobilization process was investigated.

The result shows that activated carbon had an immobilization activity of 94% (Fig. 5). Hence, activated carbon was selected as the support material for the entire immobilization process in this study. The optimum immobilization time was determined by incubating 2% (w/v) activated carbon with 25 mL of partially purified lipase over different time intervals until the immobilization reached equilibrium (Fig. 6). Lipase immobilization was examined every 10 min for 1 h, and then every 1 h for up to 24 h. The immobilization process reached its equilibrium after 2 h. The highest carbon adsorption of 1956.7 U/g, which was up to 94% immobilization, was observed after 2 h (Fig. 6). This high immobilization percentage could be related to the pore structure of activated carbon. Mitchel found that the enzyme immobilization on inorganic matrices retains the stability of enzymes under extreme environmental conditions [32]. Among the carrier matrices studied, porous inorganic materials with high specific surface areas and large porous volumes were considered for enzyme immobilization [33, 34].

Fig. 3 pH value at various fermentation times using constant P/V strategy.

Fig. 4 Reduction (%) and content of COD (g/L) at varying fermentation period.

Fig. 5 Selection of different support materials at various times for 24 hours of immobilization.

Fig. 6 Equilibrium time of lipase immobilization on activated carbon.
4 CONCLUSIONS

This study explores the strategies to be implemented in a pilot-scale plant production of lipase. The findings indicate that pilot-scale lipase production is possible using a POME-based medium in a 300-L bioreactor, by implementing the two proposed strategies. A considerable amount of enzyme was recovered using the constant P/V strategy. Lipase activities measured after 48-h fermentation periods were 5.72 U/mL and 21.94 U/mL for constant tip speed and P/V strategies, respectively. Furthermore, the immobilization time was found to be 2 h when activated carbon was used as the support material. The overall results of this study provide new information regarding the process scale-up strategy that can be implemented for industrial applications. It also provides a good platform for pilot-scale lipase production. Efforts need to be made to develop cost-effective processes that increase lipase recovery. The study findings reflect the bright prospects of bioconverting POME into a valuable product (lipase) at the industrial level.

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