LIQUID BIPHASIC FLOTATION (LBF) SYSTEM: INTEGRATION OF FERMENTATION AND RECOVERY PROCESS OF LIPASE FROM BURKHOLDERIA CEPACIA

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Abstract: Conventional trend of biomolecules production involves the upstream fermentation process and the downstream comprising separation and purification processes. The extensive market growth of industrial biotechnology over the past decade has created the demand for the development of novel and innovative upstream and downstream techniques for proteins and enzymes. Integrating the upstream and downstream processes in a single system appears to have immense potential in enhancing the efficiency of many bioprocesses. Liquid Biphasic Flotation (LBF) is a new recovery method that has been successfully used as alternative for liquid-liquid extraction process of biomolecules. This method is based on the incorporation of the principles of Aqueous Two Phase System (ATPS) and Solvent sublation (SS). The aim of this research is to integrate the fermentation process of lipase from *Burkholderiacepacia* and subsequently extract lipase from the fermentation broth via LBF system. From the findings, it has been demonstrated fermentation using LBF system enables the bacteria to grow faster and produce higher yield of lipase compared to the conventional shaker method. Subsequent lipase separation from the fermentation broth was successfully extracted with efficiency reaching 82.67% and with lipase yield of 80.67%. The combination of upstream and downstream processes in a single system enables the acceleration of product formation, improve the product yield, and facilitate downstream processing. In addition, high separation efficiency, simple operation and low environmental impact can be attained from this single system unit. This integration LBF system is potentially attractive application for biomolecules fermentation and separation that possibly open new opportunities for industrial production.

Index Terms: Upstream; Downstream; Bioprocess;Liquid biphasic flotation (LBF); Lipase

I. INTRODUCTION

Lately, production of bio-based products has become an emerging field in the bioprocessing industry. Most of the industrial microbiological processes are carried out in the batch system. For example, the process of enzyme production has to undergo several steps of procedures from upstream to downstream. This may cause for low concentration of products obtained in this process. Continuous process benefits compared to batch process in terms of higher efficiencies, lower capital costs, minimal maintenance and operation requirements, and improved process control[1].

Liquid biphasic flotation (LBF) system or similarly knows as aqueous two-phase flotation (ATPF) is an emerging and effective purification technique that combines solvent sublation and aqueous two-phase extraction (ATPE) system to purify bioproducts from the fermentation broth is an alternative method replacing multiple steps of downstream process[2]. The key principle of LBF system is the adsorptions of surface-active compounds on the bubbles surfaces of an ascending gas stream. This bubbles then bubbles dissolved in an organic solvent phase placed on top of the aqueous solution[3]. Many applications of LBF have been reported to separate and purify products such as puerarin, baicalin, lincomycin and many more[4]. This method has advantages of easy separation, low quantity of organic solvent, high separation efficiency and simple operation.

In this study, we are proposing the idea to combine the upstream and downstream bioprocess as a continuous system. With the aim to enhance lipase production, higher productivity and lower operation requirements, a process of integration of fermentation and purification of lipase via LBF system is investigated. The objective of this work is to perform fermentation and subsequent lipase separation using in a single system. The initial study is to demonstrate that fermentation can be done in the flotation system and the next part, is to study the separation efficiency and lipase yield from the continuous process of the LBF system.

II. MATERIALS AND METHODS

Materials

Olive oil, ammonium sulphate[(NH4)2SO4], trisodium citrate [Na3C6H5O7], magnesium sulphate [MgSO4],di-potassium hydrogen phosphate (K2HPO4), monopotassium phosphate (KH2PO4), sodium carbonate (Na2CO3),Bradford reagent, glucose, ethanol, methanol, 1-propanol, 2-propanol, acetonitrile were purchased from R&M Chemicals. 4-nitrophenyl dodecanonate (p-NPL) was purchased from NacalaiTesque (Kyoto, Japan) whereas calcium chloride [CaCl2], gum arabic and nutrient broth were acquired from Sigma-Aldrich (St. Louis, USA). All the chemicals and solvents were of analytical grade consumption.

Apparatus

The LBF system consists of glass column of 7cm (i.d.) diameter, 15cm height and capacity of 500mL. The bottom of the column is equipped with a sintered disk of G4 porosity (size of pore approximately10µm) attached to a compressed air system that is used to source the bubbles to the column. The flowmeter used to measure the flowrate is a rotameter RMA-26-SSV with a range of 25-250 cc/min (Dweyer, USA) connected to the compressor by a regulator.

Methods

Microorganism and fermentation in shaker

B. cepacia strains ST8 was used in this research for producing lipase. For the inoculum, the bacteria were incubated in a nutrient broth 1 % (w/v) for 16 h. Fermentation was conducted in a 250 ml shake flask that consisted 150 ml of medium. Fermentation medium comprised of nutrient broth, 0.325 % (w/v); glucose, 1 % (w/v); CaCl2, 0.1 % (w/v); olive oil, 1 % (v/v); and gum arabic, 1 % (w/v). The pH of the medium was adjusted to 7.0 by the addition of NaOH solution. 5 % (v/v) of the pre-grown inoculum was added to the fermentation medium and the culture was subjected to a shaking water bath with an agitation speed of 200 rpm at room temperature.

Fermentation and separation using LBF system

B. cepacia from the similar inoculum that is prepared for the shaker was used. Fermentation was conducted in the LBF system that consisted 150 ml of medium. The culture was subjected to flotation with the flowrate 100 cc/min at room temperature for 24 hours. After 24h, salt and alcohol were added into the system for two-phase partitioning.

Optimization of fermentation using LBF system

This study was done in a series of batches. The comparison of fermentation in conventional shaker and LBF system was performed to compare the cell growth and lipase production. One variable at a time (OVAT) method was employed to explore the influences of the parameters on the bacteria growth and lipase production. Optimization of glucose concentration and flow rate of the flotation system were performed. The initial volume of fermentation medium was 150 mL, 1% of glucose concentration and the flow rate was set at100 cc/min. Compressed air was maintained at 0.5 bar and the air is filtered using sterile air filter with size 0.2 micron and was fit to the neck of the flotation tank.

Optimization of separation parameters using LBF system

Following fermentation using the same system, lipase was extracted from the broth using two-phase partitioning method by adding salt and alcohol. Salt is added to the flotation system and was mixed and subsequently alcohol was added to form two-phases. The total volume of phase separation process were 290mL, aqueous phase was 250mL, 40mL of alcohol phase, 50% (w/w) of 1-propanol, and 250 g/L ammonium sulphate [(NH4)2SO4] was used as the initial parameters. The initial concentration of crude lipase (CL) was 40% (w/w) and 50 cc/min of flowrate was used for the separation process. The experiment was carried out at room temperature and the time interval for lipase extraction using flotation was set at 15 minutes. The samples from top and bottom phases were collected at the end of the experiment. The recovered lipase will be at the top alcohol phase, the alcohol can be removed from the solution via rotary evaporation.

Analytical method

Lipase Assay

The activity of lipase was measured using a UV-vis spectrometer (model UV-1800, Shimadzu, Japan) at room temperature and the absorbance was taken at 405 nm after 30min of reaction time. The quantity of pNP emitted from the pNPL hydrolysis was calculated via the standard curve of pNP prepared previously. One unit (U) of lipase activity denotes to the volume of lipase needed for releasing 1 µmol of p-NP per minute. Cell concentration was quantified by measuring an absorbance at 600 nm. The results were expressed as a mean of triplicate readings.

Protein Assay

The protein concentration was measured using Bradford assay described previously [5] with bovine serum albumin (BSA) as standard.

Calculation of separation efficiency (E), purification factor (PF), selectivity (S), and lipase yield (Y)

The separation efficiency (E) which defines the concentration of lipase being recovered in alcohol phase was calculated using Eq (1):

$$E=[1- C_w/C_{wi}] \times 100\% \quad (1)$$

where C_w signify the concentration of lipase in the bottom phase at time t, while C_{wi} represents the initial concentration of lipase in aqueous phase.

Purification factor (PFT) was defined as the ratio of specific activity (SA) of lipase in the top alcohol phase to the lipase (SA) in the crude feedstock, as in Eq. (2):

$$P_{FT}= \text{(SA of top phase)/(SA crude feedstock)} \quad (2)$$

SA represents the ratio of enzyme activity of lipase to the protein concentration as shown in Eq. (3)

SA= (Enzyme activity (U))/(Protein (mg)) (3)

Selectivity was expressed by using the ratio of the enzyme partition coefficient (Ke) to the protein partition coefficient (Kp) as in the Eq. (4)

$$S = K_e/K_p = E_T/E_B \times P_B/P_T \quad (4)$$

P_T and P_B denote the protein concentrations (mg/mL) at the top and bottom phases, correspondingly whereas E_T and $E_B$signify the lipase concentrations (U) at the top and bottom phases individually.
The yield of lipase in the top organic phase after extraction was calculated using Eq. (5)

Y (%)= (Lipase activity in top phase)/(Lipase activity of crude feedstock)×100 (5)

III. RESULTS AND DISCUSSION

Comparing fermentation between aeration and shaker system

The synthesis and production of biological products such as enzymes actively involves in various bioprocesses. Microbial fermentation is a major process for the production of biological products especially in large scale. Oxygen transfer is an important factor in the fermentation process as air is vital for the microbial growth, which consequently affects the proteins and enzyme productions[6]. The traditional method of fermentation is usually done in a shake flask or a bioreactor whereby the transfer of oxygen is done by means of agitation.

The conventional fermentation technique has few limitations such as low product yield and product inhibition[7]. In order to overcome these limitations and to improve the efficiency of upstream and downstream processes we are proposing the idea to use the aeration via air bubbles flotation system for fermentation process. Flotation unit operation has wide unexplored potential in biotechnology. As the first step toward integration of the fermentation and subsequent extraction of lipase from the fermentation broth via LBF system, preliminary investigation of fermentation in the flotation system was assessed. This preliminary study is important to demonstrate that fermentation process can be performed using a flotation system. This study compares the cell growth of *B. cepacia* during fermentation by means of flask shaker and by flotation gas bubbles.

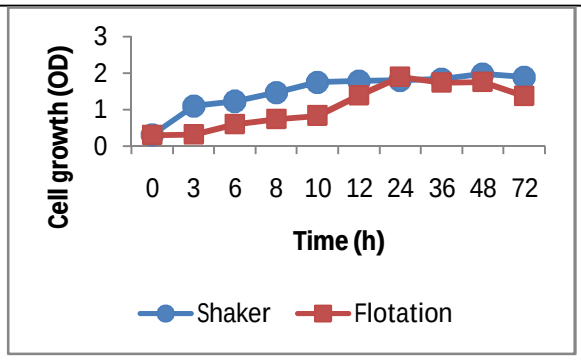


Figure 1: Comparison of cell growth during fermentation between shaker at 37 °C and flotation at room temperature.

Figure 1, shows the difference in the cell growth of B. *Cepacia* during fermentation via two different systems. For the fermentation via shaker, the temperature was control at 37 °C however, for the flotation system the temperature is set to room temperature, as the flotation system's design does not have temperature controlling facility.

From Figure 1, it is obvious that the bacterium is capable to grow in a flotation system. There is difference in the bacteria growth between shaker and flotation, as illustrate in the figure above the concentration of bacteria was high in the shaker for the first 12 h compared to flotation. At 24 h, the cell growth of bacteria in the flotation is higher compared to shaker. The bacteria continue to grow in the shaker and reach a maximum OD of 1.977 at 48 h and decreases at 72 h meanwhile, the bacteria in flotation system reaches its maximum OD growth of 1.897 at 24 h and decreases after that. The bacteria in flotation reached its maximum growth at 24 h while for the shaker it took up to 48 h and even though it decreases at 72 h the cell growth was considerably higher than flotation unit.

This preliminary study shows that despite the difference in temperature and mode of fermentation, the bacteria able to grow. The bacteria in the flotation system grows slower compared to the shaker and reached the maximum growth at 24 h, this may be due to the temperature difference. The optimum temperature for the bacteria growth is at 37°C [8]however, in the flotation unit the fermentation was done at room temperature, which influence the growth of bacteria. The bacteria growth in flotation took shorter period to reach its maximum growth due to efficient oxygen transfer in the medium. For respiration and the growth of bacteria in the fermentation medium, microorganism consumes oxygen, which is dissolved in the liquid media [6]. In most of the fermentation process, aeration and agitation is the utmost important parameters that are implemented. Fermentation in shake flask is based on agitation mode and the oxygen transfer from the medium is lower compared to the oxygen transfer based on aeration. With the aid of gas bubbles in the

flotation system, the oxygen passes across the gas liquid interface, then the bulk of liquid, and the end into the microbial cell[9].

Comparison of cell growth and lipase production

In this study, we are comparing the cell growth and lipase production from both fermentation system using the temperature as constant parameter. Fermentation was done for both flotation and shaker system at room temperature to compare the cell growth and lipase production.

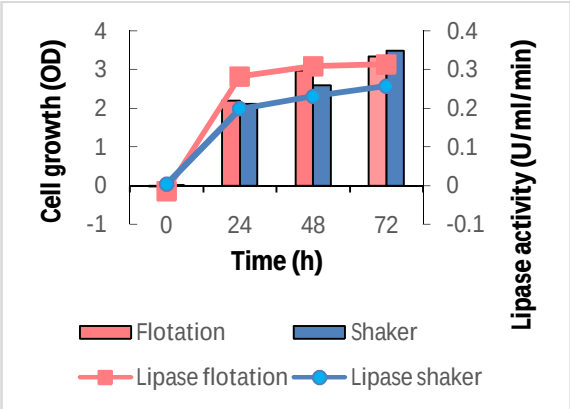


Figure 2: Comparison of cell growth and lipase production from fermentation of flotation and shaker at room temperature.

From Figure 2, it can be distinctly seen that at room temperature the cell growth of bacteria in flotation is better than shaker for the first 48 h and there is a slight decrement at 72 h. The decrement may due to the accumulation of cells in the flotation unit that might block the flowing of gas bubbles. This trend is absent in the shaker as the cells growth increases from 24 h to 72 h.

The lipase production was measured in terms of lipase activity, more lipase was produced from the flotation system compared to shaker. The lipase production in flotation system increases from 24 h 0.281 U/mL/min to 72 h which is the highest 0.313 U/mL/min. As for the lipase production from the shaker, similar increasing trend was noticed from 24 h 0.198 U/mL/min and at 72 h 0.257 U/mL/min. This result shows that with aeration system, cell growth and the lipase production was enhanced. This methodology of fermentation via aeration enables subsequent separation and purification of lipase via LBF. This aeration system helps the incorporation of upstream and downstream process in a single system.

Separation of lipase from fermentation broth via liquid biphasic flotation (LBF)

To improve the bioprocess of lipase production, integration of upstream and downstream is studied in this section. From the previous section, fermentation of B.cepacia via aeration system has demonstrated to produce high cell growth and lipase production. In order to reduce the number of process steps for downstream of lipase, subsequent lipase separation from the flotation system is investigated. Following fermentation process, lipase was separated from the broth using liquid biphasic flotation (LBF), which involves gas bubbles from the flotation system. The working principle of LBF involves the absorption of surface-active components in this case lipase on the surface of air bubbles of an ascending gas stream in aqueous solution or salt solution [10]. This air bubbles will then dissolved in the organic top phase. Once fermentation is completed, salts are added into the broth and subsequently, alcohol is poured into the flotation system that creates two-phases. The air bubbles from the flotation will aid the lipase separation from the salt-rich bottom phase to the alcohol top phase.

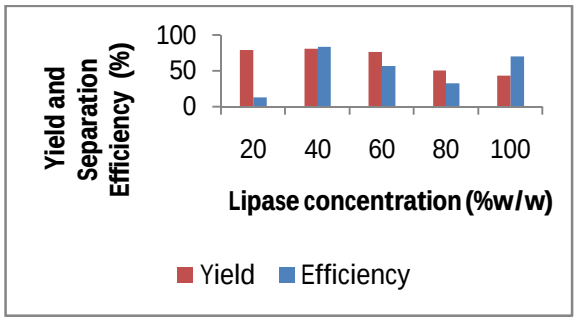


Figure 3: Effect of crude lipase concentration on the separation efficiency and lipase yield using liquid biphasic flotation (LBF).

The concentration of crude feedstock portrays an important role in LBF. Increasing crude load benefits the recovery processes via LBF technique. Few findings have indicated that the amount of crude feedstock could significantly influence the partitioning behaviour of protein [11].

In this study, we have studied lipase separation from 20-100 % w/w of crude feedstock. Initial fermentation is done via aeration using the LBF system and for the subsequent lipase separation, different concentration of salts is added to make the aqueous phase. From Figure 3, it can be seen that the yield and separation efficiency of lipase demonstrates the highest with 82.67 % and 80.67 % respectively at 40 % w/w of crude feedstock concentration.

Higher crude loading results in decrease of aqueous two-phase flotation (ATPF) performance due to the alteration in volume ratio and the partition behaviour of target protein [12]. In addition, increasing the concentration of crude feedstock will lead to increase in contaminants in the system and causes accumulation of precipitate at the interface that consequently reducing the performance of lipase separation [13]. This study has proven that both fermentation and subsequent separation of lipase can

be done via the similar flotation system and the optimal crude lipase concentration for the best performance is found to be at 40% w/w.

CONCLUSION

Lipase originated from B. cepacia was successfully fermented and separated via liquid biphasic sublationflotation (LBF). This study for the first time reports on the growth of bacteria and further purification process of lipase in a system that was aided by aeration. The optimized crude lipase concentration for the purification process was found to be at 40% w/w that achieved a separation efficiency reaching 82.67% and lipase yield of 80.67%. The integration of fermentation process into the sublation separation process can be a stepping-stone to ground-breaking innovations, researches and possible applications in the enzyme bioprocessing industry.

REFERENCES

[1] Gutiérrez LF, Sánchez ÓJ, Cardona CA. Analysis and design of extractive fermentation processes using a novel short-cut method. Ind Eng Chem Res

[2] Show PL, Tan CP, Anuar MS, Ariff A, Yusof YA, Chen SK, et al. Direct recovery of lipase derived from Burkholderia cepacia in recycling aqueous two-phase flotation. Sep Purif Technol 2011;80:577–84.

[3] Bi PY, Dong HR, Yuan YC. Application of aqueous two-phase flotation in the separation and concentration of puerarin from Puerariae extract. Sep Purif Technol 2010;75:402–6.

[4] Lee SY, Khoiroh I, Ling TC, Show PL. Aqueous Two-Phase Flotation for the Recovery of Biomolecules. Sep Purif Rev 2016;45:81–92.

[5] Show PL, Ooi CW, Anuar MS, Ariff A, Yusof YA, Chen SK, et al. Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt. Sep Purif Technol 2013;110:112–8.

[6] Najafpour GD. Gas and Liquid System (Aeration and Agitation)□□. Biochem. Eng. Biotechnol., Elsevier; 2015, p. 51–102.

[7] Sinha J, Dey PK, Panda T. Aqueous two-phase: the system of choice for extractive fermentation. Appl Microbiol Biotechnol 2000;54:476–86.

[8] Lau HL, Ariff a, Woo KK, Ling TC, Hii SL. Production and optimization of alkalostable lipase by alkalophilic Burkholderia cenocepacia ST8. African J Biotechnol 2011;10:7002–9.

[9] Bailey J, Bailey J, Ollis D. Biochemical Engineering Fundamentals. 2nd ed. McGraw-Hill Science/Engineering/Math; 1986.

[10] Mathiazakan P, Shing SY, Ying SS, Kek HK, Tang MSY, Show PL, et al. Pilot Scale Aqueous Two-Phase Floatation for Direct Recovery of Lipase Derived from Burkholderia cepacia strain ST8. Sep Purif Technol 2016;171:206–13.

[11] Selvakumar P, Ling TC, Walker S, Lyddiatt A. A practical implementation and exploitation of ATPS for intensive processing of biological feedstock: A novel approach for heavily biological feedstock loaded ATPS. Sep Purif Technol 2010;75:323–31.

[12] Ooi CW, Tey BT, Hii SL, Ariff A, Wu HS, Lan JCW, et al. Direct purification of Burkholderia Pseudomallei lipase from fermentation broth using aqueous two-phase systems. Biotechnol Bioprocess Eng 2009;14:811–8.

[13] Amid M, Shuhaimi M, Islam Sarker MZ, Abdul Manap MY. Purification of serine protease from mango (Mangifera Indica Cv. Chokanan) peel using an alcohol/salt aqueous two phase system. Food Chem 2012;132:1382–6.

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