Original paper

Optimization of a Molasses Based Fermentation Medium for Lipases from *Burkholderia* sp. Bps1 Based on Response Surface Methodology

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This study aimed to acquire a low-cost medium to produce high-yields of lipases from *Burkholderia* sp. Bps1 by using a statistic method so as to optimize the compositions of medium in the following two steps: 1) a single factor experiment (SFE) was conducted to screen the most significant factors affecting lipase activity to determine the optimal carbon source (molasses), nitrogen source (peptone) and inducer (palm oil); 2) a central composite design (CCD) and response surface methodology (RSM) were used to further optimize the previously mentioned factors to acquire an optimal combination consisting of 9.7 g/L molasses, 13.7 g/L peptone and 0.60 % (v/v) palm oil. The optimized lipases activity reached 153.537 U/mL which was 30 times greater than the activity of the primary enzyme. This research used a statistical method to improve the production of lipases from submerged fermentation and formed a theoretical foundation for the industrial production of lipases using low cost raw materials.

Keywords: lipase production, *Burkholderia* sp, optimization, central composite design, cane molasses

Introduction

Triacylglycerol lipase (EC 3. 1. 1. 3) is a special esterase which can hydrolyze triglyceride into fatty acids, diglyceride, monoglyceride and glycerin (Houde *et al.*, 2004). It has been widely applied in the fields of medicine, food, chemistry, cosmetics and textiles and other related industries because of its catalytic activities involving hydrolyzing triglyceride, esterification, trans-esterification and enantio separation (Hasan *et al.*, 2013; Houde *et al.*, 2004; Ribeiro *et al.*, 2011).

Lipase is a common enzyme that is present in different animals, plants, and microbes (Salihu *et al.*, 2011). Lipases from microorganisms as a type of non-aqueous biocatalyst have been the subject of much research interest (Hasan *et al.*, 2006; Kantak *et al.*, 2011; Shu *et al.*, 2007). Lipases from different microbes such as strains of *Pseudomonas* (PSL) and *Burkholderia* (BL) have beneficial properties including high thermo-stability, tolerance to organic solvents and strict

enantio-selectivity, which have led to broad ranging applications across the several fields including organic synthesis (Gupta *et al.*, 2007), detergent manufacturing, non-aqueous catalytics, preparation of biodiesels, enantio-separation and enzyme production (Borgström and Brockman, 1984; Wang *et al.*, 2008).

Lipases for industrial applications have been mainly obtained from *Pseudomonas* by the Amano Corporation in Japan, and from *Burkholderia* sp. by the companies in USA (Snellman *et al.*, 2002). However, limited industrial production results in expensive products of lipases. To cope with such a challenge, there is a pressing need for the mass production of lipases by developing low-cost raw materials and compositions (Salihu *et al.*, 2011).

In recent years, lipase research has mainly focused on molecular cloning, enzyme analysis, organic-phase enzymatic esterification, stereo-selectivity and relevant enantio-separation.

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However fermentation techniques for lipase production have not been extensively investigated.

In recent years, many lipases from the *Burkholderia* genus have been found, which are essential in acquiring high yields from diverse strains using varying fermentation techniques. In 1988, Lonon *et al.* (Lonon *et al.*, 1988) separated lipases from ten types of *Burkholderia* genera. The molecular mass of the purified lipases was greater, 215 kDa and optimum growth conditions involved pH 9.0 and a Tween 40 substrate.

Generally, the most favorable temperature for the growth of *Burkholderia* sp. ranges between 30–35 °C. However, Rath *et al.* (Rath *et al.*, 2002; Rath *et al.*, 2001) reported the most favorable growth temperature ranged between 45–50 °C for *B. cepacia* RGP210 when using RSM to optimize the compositions of lipases culture medium. Moreover, the group reported other factors which significantly affect lipase activities including induction time, inoculation amount and concentration of carbon sources. They also found that reducing the supplement of carbon sources could decrease fermentation time and that castor oil was an optimal inducer of *B. cepacia*. Furthermore, when the concentrations of Ca^{2+} and Mg^{2+} were 0.4 mmol/L and 0.6 mmol/L respectively, lipase yield reached its maximum. The optimal conditions for producing lipases were defined as fermentation for 15 hours in a 14 L fermentor to reach lipase activities of 160 U/mg.

Chien-Hung Liu *et al.* (Liu *et al.*, 2012) found that lipases from *Burkholderia* sp. exhibited good tolerance to temperature and pH values and the molecular mass obtained by zymogram analysis was 23 kDa. Yun Li *et al.* (Yin *et al.*, 2007) also used RSM to optimize fermentation conditions for the production of lipases from *B. cepacia* to increase the yield of lipases. The results indicated that olive oil, hydrolyzed soybean powder and initial pH are the main factors influencing the lipase yield. Additionally, Wang *et al.* (Wang *et al.*, 2008) utilized an optimized fermentation process of *B. cepacia* lipases using RSM to successively realize fermentation for the production of lipases using a 10 L fermentor. They obtained significant factors influencing urea concentration, inoculation amount and initial pH. Fernandes *et al.* (Fernandes *et al.*, 2007) reported that the activity of lipases produced by solid state fermentation for 72 hours by using corn bran as the culture medium and 5 % (v/m) corn oil as inducers could reach 108 U/g. In addition to lipases from *B. cepacia*, lipases from other types of *Burkholderia* genera were also detected.

In this study, the *Burkholderia gladioli* Bps1 from rotten onions was screened to further optimize the fermentation process of lipases. The optimal conditions for lipase production from *Burkholderia gladioli* Bps1 were obtained as pH 9.0 and 50 °C. The obtained lipases exhibit good tolerance to pH, temperature and short-chain alcohols, and therefore have significant potential in industrial applications.

Molasses is a byproduct of sugar production with 45–50 % sugar content and can be used as an economical carbon source

during microbial fermentation (Najafpour and Shan, 2003). Owing to the high organic matter content, molasses has attracted much attention and as a renewable carbon resource, it can be used for fermentation in the production of lipases.

The purpose of this study is to determine a base culture medium using low cost raw materials and compositions for the fermentation production of lipases. SFE was conducted to screen optimal carbon sources, nitrogen sources and inducers. Afterwards, RSM was used to obtain the optimal compositions of the culture medium to maximize production of lipases from the low-cost molasses based medium. This study is the first report on the use of molasses as a fermentation medium for *Burkholderia gladioli* in lipase production.

Material and Methods

Microorganisms, culture medium and conditions The strains of Bsp1 used in this experiment were screened from rotten onion, which were identified as the *Burkholderia* genus based on morphology, physiology and biochemistry (16SrDNA sequence of *Burkholderia gladioli* Bps1 GenBank database under Accession No. MF618254.1). The compositions of the original culture medium comprised 1 % (w/v) glucose, 0.5 % (v/v) olive oil, 1 % (w/v) peptone, 0.2 % (w/v) K_2HPO_4 and 0.05 % (w/v) MgSO_4 . 50 mL of medium was added into a conical flask with a volume of 250 mL and strain seed liquid at an inoculum concentration of 1 % (v/v) was introduced into the fermentation medium and cultured for 3 days at 30 °C at a rotational speed of 200 r/min in a shaking table test (Liu and Zhang, 2011).

Determination of lipase activity The activity of lipases was determined using *p*-nitrophenyl palmitate (pNPP) as a substrate based on the methods proposed by Pencreac'h and Baratti (Gupta *et al.*, 2002; Pencreac'h and Baratti, 1997; Singh *et al.*, 2006). The reaction system is described as follows: the isopropanol solution with 45 µL of 16.5 mM pNPP was mixed with 405 µL solution containing 50 mM Tris-HCl buffer consisting of 2 % Triton X-100 and 0.1 % arabic gum. After pre-heating at 50 °C for 5 minutes, the diluted liquid enzyme sample (the supernatant of the fermentation medium) was reacted for 10 minutes and a spectrophotometer method was used to measure the content of the released pNPP at a wavelength of 405 nm. The lipase activity unit U is defined as the amount of lipase consumed by the decomposition of pNPP for the production of 1 µmol of nitrophenol per minute. All experiments were performed in triplicate.

Optimized compositions of the experimental medium using SFE SFE was used to screen the compositions involving carbon sources, nitrogen sources and inducers of the experimental medium. Amongst these parameters, the most favorable compositions of producing high yields of lipase were determined using the fermentation technique. Carbon and nitrogen sources and inducers were used to replace corresponding compositions in the medium whilst other compositions were constant, aiming to find the optimal composition of medium. Standard deviation

Table 1. Variables and their levels for response surface methodology.

Variables	Symbol	Coded levels				
		-2	-1	0	1	2
Molasses (g/L)	A	2.0	6.0	10.0	14.0	18.0
Peptone (g/L)	B	3.0	9.0	15.0	21.0	27.0
Palm oil (%v/v)	C	0.1	0.3	0.5	0.7	0.9

was obtained based on the mean values of three parallel tests.

Central composite design According to the results obtained by SFE, molasses, peptone and palm oil were chosen as components of the culture medium. Central composite design (CCD) was then employed to determine the optimal concentrations of these components and analyze the primary effects and interactions between factors on lipase yield. In the experiment, the design with three-factors and five-levels ($-\alpha$, -1 , 0 , $+1$, and $+\alpha$), and 20 experimental sites is shown in Table 1. Eight factorials, six axial points and six central points are summarized in Table 2. Design Expert (8.0.6.0, Stat Ease Inc, Minneapolis, USA) was used to design the quadratic model and the analysis of multinomial coefficients. The quadratic linear regression equation is expressed as (Vijayaraghavan *et al.* 2014):

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{j=1}^3 \beta_{ij} X_{ij} \quad \dots \text{Eq. 1}$$

Where, Y is predicative response, x_i, x_{ij} denotes input variable influencing response Y , β_0 is offset term, β_{ii} is i th linear coefficient, β_{ii} is i th quadratic coefficient, and β_{ij} represents ij th interaction coefficient.

Statistical analysis was conducted on the correlation coefficient R , multiple correlation coefficient R^2 (the fitting degree of the quadratic model), Fischer's F and its probability p (F) (Batra *et al.*, 2014). The response surface curves were drawn based on the Quadratic model.

Results

The optimized compositions of the experimental medium based on SFE Selection of carbon sources Figure 1 illustrates the effects of different carbon sources on the activity of lipase from *Burkholderia* sp. Bps1. As shown in the figure, when molasses was used as a carbon source, the lipase activity reached its maximum, followed by malt extracts and sucrose (Fig.1 (a)). Paterson *et al.* (Paterson *et al.*, 2000) reported that as molasses contains rich sugar content, nitrogen sources and multiple trace elements, it can promote the growth of microbes. As seen from the Fig.1 (b), when the content of molasses in the medium was 5.0 g/L, the activity of lipases increased to the peak value. Furthermore, molasses is cheap and more widely applicable to mass production of lipase and could therefore be used as an optimal carbon source.

Selection of nitrogen source The effect of nitrogen sources (both organic and inorganic nitrogen sources) on the

Fig. 1. The effects of different carbon sources and the concentration on lipase activity of *Burkholderia* sp. Bps1.

Fig1a The effects of different carbon sources (10 g/L Glucose, Molasses, Sucrose, Malt extract, Maltose, Dextrin, Lactose, Soluble starch) on lipase activity of *Burkholderia* sp. Bps1.

Fig1b The effects of molasses and sucrose concentration on lipase activity of *Burkholderia* sp. Bps1 (■ = molasses, ● = sucrose).

activity of lipase from *Burkholderia* sp. Bps1 was investigated in this study. Given the molasses contained only 1 % nitrogen source, it was necessary to supplement this with nitrogen to increase lipase activities further. As shown in the Fig. 2 (a), when peptone was used as a nitrogen source, the lipase activity reached its maximum, followed by that achieved when using yeast extract and corn syrup powder. Fig. 2 (b) shows that the addition of 15 g/L peptone significantly increased lipase activity. This is because peptone is rich in protein, amino acids, vitamins and multiple unknown compositions which is highly conducive to the growth of microbial cells and lipase production (Tanyol *et al.*, 2014).

Fig. 2. The effects of different nitrogen sources and their concentrations on lipase activity of *Burkholderia* sp. Bps1.

(a) The effects of different nitrogen (10 g/L peptone, yeast extract, bean powder, corn syrup powder, NaNO₃, Urea, (NH₄)₂SO₄ sources on the lipase activity of *Burkholderia* sp. Bps1 when molasses was used as the carbon source at a concentration 10 g/L.

(b) The effects of different concentrations of nitrogen source on the lipase activity of *Burkholderia* sp. Bps1.

Selection of lipase inducers Lipases produced from microbes are mostly inducible enzymes (Sharma *et al.*, 2001). Oil was used as a necessary inducer for the production of lipases in our experiments. As shown in Fig.3 (a), oil as a compound carbon source and inducer has significant effect on the promotion of the lipase activity from *Burkholderia* sp. Bps1. Palm oil was shown to significantly enhance the microbial growth and lipase activity. As the concentration of palm oil is 0.5 % (v/v), lipases activity reaches 145.8 U/mL, as shown in Fig.3 (b). Moreover, as palm oil is inedible and inexpensive, it is likely to decrease production costs and is therefore chosen as the inducer for the fermentation medium.

Optimized compositions of the experimental medium based on RSM CCD was used to determine the optimal concentrations for different compositions in the medium for the lipase production from *Burkholderia* sp. Bps1. The experiment

Fig. 3. The effects of different inducers and their concentrations on lipase activity of *Burkholderia*.sp Bps1.

Fig.3a The effects of different inducers (0.5 %, v/v, including castor, colza, , soy bean, rice bran, olive, corn and palm oils) on lipase activity of *Burkholderia* sp. Bps1, when the carbon and nitrogen sources were molasses and peptone respectively, at concentrations of 10 g/L and 15 g/L.

Fig.3b The effects of inducers at different concentrations on the lipase activity of *Burkholderia*.sp Bps1.

was designed for 20 times, with variable range shown in Table 1. The experimental design and the change in lipases activity (50.45—147.96 U/mL) are displayed in Table 2.

The multiple correlation coefficient R^2 is 0.9525, indicating there is 95.25 % correlation existing between the quadratic linear regression equation and the response values. The results show that the second-order equation fits well with the CCD experimental results. Meanwhile, a low variation coefficient (C.V. = 6.03 %) further satisfactorily validates the accuracy and reliability of the model.

The experimental data were used in multiple regression analysis of variance. The experimental data in the CCD fit closely with the quadratic linear regression equation model. The fitted response relationships between the lipase activity (Y) and three variables based on the regression equations are expressed as Eq. 2:

Table 2. CCD matrix for the experimental design and predicted results for lipase production.

Standard order	FACTORS (%)			Experimental results (U/ml)	Predicted results (U/ml)
	Molasses (g/L)	Peptone (g/L)	Palm oil (%v/v)		
1	6.0	9.0	0.3	128.20	115.8423
2	14.0	9.0	0.3	90.297	87.30909
3	6.0	21.0	0.3	100.13	102.2303
4	14.0	21.0	0.3	100.06	100.333
5	6.0	9.0	0.7	142.58	138.8695
6	14.0	9.0	0.7	137.37	131.8425
7	6.0	21.0	0.7	118.44	117.9978
8	14.0	21.0	0.7	128.68	137.6067
9	2.0	15.0	0.5	118.34	123.8302
10	18.0	15.0	0.5	116.97	114.9059
11	10.0	3.0	0.5	108.38	118.9561
12	10.0	27.0	0.5	118.25	111.1084
13	10.0	15.0	0.1	50.45	55.22251
14	10.0	15.0	0.9	116.86	115.5233
15	10.0	15.0	0.5	148.31	146.2423
16	10.0	15.0	0.5	144.27	146.2423
17	10.0	15.0	0.5	143.16	146.2423
18	10.0	15.0	0.5	142.89	146.2423
19	10.0	15.0	0.5	147.96	146.2423
20	10.0	15.0	0.5	147.43	146.2423

Table 3. ANOVA data of the CCD.

Source	Sum of Squares	DF	Mean Square	F Value	p-value	Prob > F
Model	10947.52	9	1216.391	22.29647	< 0.0001	Significant
A-Molasses	79.64383	1	79.64383	1.459873	0.2547	
B-Peptone	61.58728	1	61.58728	1.128896	0.3130	
C-Palm oil	3636.19	1	3636.19	66.65144	< 0.0001	Significant
AB	354.7343	1	354.7343	6.502287	0.0289	Significant
AC	231.2587	1	231.2587	4.238976	0.0665	
BC	26.35173	1	26.35173	0.483028	0.5029	
A ²	1134.924	1	1134.924	20.80318	0.0010	Significant
B ²	1530.676	1	1530.676	28.05732	0.0003	Significant
C ²	5822.27	1	5822.27	106.7223	< 0.0001	Significant
Residual	545.5531	10	54.55531			
Lack of Fit	514.2612	5	102.8522	16.43435	0.0040	Significant
Pure Error	31.29185	5	6.25837			
Cor Total	11493.07	19				

$R^2 = 0.9525$, C.V. % = 6.03, Adeq Precision = 17.427

$$Y = 146.2423 - 2.2311 \times A - 1.9619 \times B + 15.0752 \times C + 6.659 \times A \times B + 5.3766 \times A \times C - 1.8149 \times B \times C - 6.7186 \times A^2 - 7.8025 \times B^2 - 15.2173 \times C^2 \quad \text{.....Eq. 2}$$

Where, the lipase activity Y changes with the variation of variables including molasses (A), peptone (B) and palm oil (C).

The ANOVA of the second order RSM model is presented in Table 3. The p value of the overall regression equation is smaller than 0.001, suggesting the model is very effective and

exhibits favorable fitting performance. The model was therefore employed to predict optimal compositions in the medium for the fermentation production of lipases. Furthermore, palm oil exerts the most significant effect on lipase activity ($p < 0.0001$), which is largely greater than the effects observed for molasses and peptone. Additionally, there is also a significant interaction between molasses and peptone,

Fig. 4. The effects of molasses, peptone and palm oil on lipase production by *Burkholderia* sp. Bps1 based on 3D response surface curves. (a) shows molasses and peptone at a fixed level of palm oil. (b) illustrates molasses and palm oil at a fixed level of peptone. (c) shows peptone and palm oil at a fixed level of molasses.

with a statistical significance of $p = 0.0289$, indicating the lipase activity was mainly determined by the concentration of palm oil, which is consistent with the SFE results (Himabindu *et al.*, 2006).

The signal-to-noise ratio of the model is 17.427, confirming the preferable reliability of the experimental results. The F value is 22.30, “ $Prob > F$ ” is smaller than 0.05, showing that the model is statistically significant. C , AB , A^2 , B^2 and C^2 are shown to have significant influence on the response values (Batra *et al.*, 2014).

RSM Plots Fig. 4 illustrates a three-dimensional response surface graph based on the quadratic linear regression equation for the interactions among factors including molasses, peptone and palm oil. The figure mainly demonstrates the effect of the interaction between two factors on the lipase activity when one of the factors is unchanged.

As shown in Fig. 4 (a), the contour is elliptical indicating there is significant interaction between the molasses and peptone as the lipase activity is enhanced with increasing concentrations of molasses and peptone. The lipase activity is highest when the concentrations of molasses and peptone increase to 10.0 g/L and 15.0 g/L. However, as the concentrations of molasses and peptone continue to increase, lipase activity is reduced.

The response surface graphs shown in Fig. 4 (b) illustrate the effect of the interaction between molasses and palm oil on lipase activity at a constant peptone concentration. When 10.0 g/L molasses and 0.6 % (v/v) palm oil were used, the lipase activity reaches its maximum value (Wang *et al.*, 2011). According to the diagonal direction of the elliptical contour, interaction between the two variables is not statistically significant, suggesting the effect of molasses concentration on lipase activity is dependent on the level of palm oil. The interaction between peptone and palm oil shown in Fig. 4 (c) agrees with that shown in Fig. 4 (b). As can be seen from the figure, lipase activity constantly increases as the concentration of palm oil increases from 5.0 to 6.0 g/L. However, when the concentration continues to increase, lipase activities are reduced. These data show the significant influence of the concentration of palm oil on lipase activity with a great interaction between the concentration of molasses and peptone which are dependent on palm oil.

To further verify the accuracy and reliability of the second-order equation, the theoretically optimum conditions obtained from the optimization results were verified. The optimum fermentation medium compositions calculated by RSM include: 9.7 g/L molasses, 13.7 g/L peptone, and 0.60 % (v/v) palm oil, and as estimated by the model, the derived lipase yield is 150.267 U/mL. The experiments with the above compositions consisting of medium were conducted in triplicate. After fermentation for 72 hours, the average value of three tests for the production of lipase using the fermentation technique was calculated to be 153.537 U/mL, which is in agreement with predicated value 150.267 U/mL. The results

reveal a smaller error of the theoretically predicted value compared with actual theoretical value, validating the performance of the model in simulating and predicating lipase activity.

Discussion

The best carbon sources, nitrogen sources, and inducers were found in this research. Carbon sources, such as molasses or sucrose, could significantly enhance lipase activity. As the main ingredient in molasses, sucrose is the key carbon source with regard to the lipase activity of Bps1. It has been reported (Gupta *et al.*, 2014) that sucrose and maltose are better than glucose as the carbon sources for three different kinds of fungus fermentation to produce lipase, including *Aspergillus ochraceous*, *Aspergillus fumigatus*, and *Penicillium purpurogenum*. It has also been found (Park *et al.*, 2013) that fructose exhibits higher rates and greater amounts of lipase production and cell growth than glucose on lipase production by *P. lyferdii* NRRL Y-7723. In comparison with sucrose, molasses includes a variety of oligosaccharides, nutrients and a small amount of nitrogen source material. The other components of molasses and sucrose in synergistic action are more conducive to improving the activity of lipase which attributed to the characteristics of the microorganisms. However, there have been no specific reports on the effects of carbon sources on the production of lipases to date (Park *et al.*, 2013).

After the response surface methodology (RSM) design of experiments, our results show that those inducers play the most important roles in the lipase production from Bps1. The Bps1 has almost no lipase activity when the medium contains no inducers. This is consistent with a previous report, but the activity of lipase is higher than that of other vegetable oils, which is different from the findings of previous reports. Most of the key factors affecting lipase production are related to olive oil, for example, other workers (Coradi *et al.*, 2013) used a mixture of castor oil cake and sugarcane bagasse supplemented with 1 % (v/w) olive oil. They eventually obtained the best activity of 1.4 U/mL among the cultures in SSF of *Trichoderma harzianum*; the study (Lo *et al.*, 2012) showed that olive oil, tryptone, and Tween-80 exert significant influences on lipase production by *Burkholderia* sp, which yield a maximum activity of 122.3 U/mL, probably because palm oil contains a much greater saturated fatty acid content than other vegetable oils (Yanlan, 2005). In our study, the probable reason for the higher lipase activity of Bps1 with palm oil may be that the saturated fatty acid composition of oil could be an important factor affecting the production of lipase. The reason can be further analyzed with respect to the study of the activity of lipase substrates.

Conclusions

On the basis of SFE, RSM was used to determine the optimum compositions of lipase medium from submerged

fermentation of *Burkholderia* sp.Bps1. Experimental results showed that palm oil is the primary factor influencing the production of lipases from *Burkholderia* sp. Bps1, followed by the interaction between concentrations of peptone and molasses. By conducting optimization using the statistic method, the results suggested that compared with initial culture medium, the production of lipases from submerged fermentation of *Burkholderia* sp.Bps1 reaches 153.537 U/mL on average, which is 30 times higher than the activity (5.04 U/mL) of the primary enzyme in the initial medium. The results reveal that RSM could greatly promote the optimization of the compositions of the medium for the production of lipase from submerged fermentation of *Burkholderia* sp.Bps1. The optimized compositions include 9.7 g/L molasses, 13.7 g/L peptone and 0.60 % (v/v) palm oil. The method used in this study for the production of lipase using agricultural wastes is capable of reducing environmental pollution by significantly decreasing the discharge of wastes.

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