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Optimization of Alkaline Lipase Production from *Burkholderia cepacia* through Submerged Fermentation

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The hydrolysis of long chain triglycerides can be catalysed by a class of enzymes known as lipases. Currently, microbial lipases are receiving much attention because of their actual and potential application in industry. In this paper, the production of lipase from *Burkholderia cepacia* through submerged fermentation (SmF) has been optimized. Various parameters which include pH (4.4 – 9.0), agitation speed (100 – 300 rpm), incubation time (12 – 72 h) and mode of autoclave (mix or separate) were optimized. It was observed that the fermentation medium should first be mixed before autoclaved instead of mixing the fermentation medium after autoclave as the former has higher lipase production. The optimum operating condition for the fermentation to obtain maximum production of lipase (11.18 U/mL) was at pH 9.0, 300 rpm and incubation time of 72 h.

1. Introduction

The hydrolysis of long chain triglycerides can be catalyzed by a class of enzymes known as lipases. Currently, lipases are receiving much attention because of their actual and potential application in a wide variety of industries such as detergent, oleochemical, organic synthesis, dairy, fat and oil modification, pharmaceutical (Kamini et al., 2000). Lipases can be produced from various sources such as plants, microorganisms and animals (Chen et al., 1998). However, microbial lipase are commonly adapted as the lipase produced are extracellular, more stable (Ellaiah et al., 2004) and are able to catalyse a wide variety of reactions in both aqueous and non-aqueous phase (Saxena et al., 2003). The genera *Pseudomonas* and *Burkholderia* are often chosen to produce lipase amongst the various bacterial lipases being exploited due to the unique properties of high enantioselectivity and enzyme activity at a wide range of pH and temperature (Gupta et al., 2007). In order to meet the increasing commercial demand on lipase, it is important to optimize the growth condition for maximum production yield. Bacterial lipases are produced mainly by submerged culture (Sharma et al., 2001) due to the fact that cultural variables can be easily kept under control (Li and Zong, 2010). A wide variety of culture conditions that stimulate or suppress the production of bacterial lipases are described in the literature. However, there is no general procedure to improve the lipase production. Generally, bacterial lipases are greatly influenced by nutritional factors such as nitrogen and carbon sources, presence of lipids, and inorganic salts (Selva Mohan et al., 2012). However, physio-chemical factors such as temperature, pH, agitation and dissolved oxygen concentration also play an important role on affecting the production yield (Gupta et al., 2004). Hence in this paper, the production of lipase from *Burkholderia cepacia* (*B. cepacia*) through submerged fermentation (SmF) has been optimized. Various parameters which include pH (4.4 – 9.0), agitation speed (100 – 300 rpm), incubation time (12 – 72 h) and mode of autoclave (mix or separate) were optimized. To analyse the significance of the results obtained using analysis of variance (ANOVA), general factorial methodology was designed.

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2. Materials and Methods

2.1 Materials

Calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), Gum Arabic, Tween 80, Agar-agar powder, dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), ethanol (C_2H_6O) and ammonia (NH_3) solution were purchased from R&M Chemicals (Essex, UK). While nutrient broth and p-nitrophenyl laurate (pNPL) were purchased from Difco (Becton, Dickinson and Company, USA) and Sigma–Aldrich Co. (St. Louis, MO, USA) respectively. All of the chemicals and solvents were of analytical grade.

2.2 Media and Culture Condition

B. cepacia ST8 was used in this research for producing lipase (E.C. 3.1.1.3). The cultivation method used in Ooi et al. (2011) and on the direct recovery of lipases Show et al. (2011) has been adapted in this research. The bacteria were first incubated in a nutrient broth 1 % (w/v) for 16 h to develop inoculum. Fermentation was then conducted in a 250 mL Erlenmeyer flask. The basic medium for fermentation comprises of nutrient broth 0.325 % (w/v), $CaCl_2$ 0.1% (w/v), tween 80 1 % (v/v) and gum arabic 1 % (w/v). The pH of the medium was adjusted to 9.0 by the addition of NH_3 solution. 5 % (v/v) of the pre-grown inoculum was added to fermentation media. The culture was then maintained under agitation speed of 250 rpm at 37 °C in an incubator shaker for 72 h.

2.3 Lipase Activity Assay

The lipase activity assay was performed by using the spectrophotometric method by adapting method described by Ooi et al. (2011). One unit of lipase activity (U) was defined as the amount of enzyme releasing 1 µmol p-nitrophenol (pNP) per min.

The enzyme assay includes sample solution 0.1 % (v/v), 0.01 M pNPL (dissolved in C_2H_6O) 0.1 % (v/v) and 0.05 M phosphate buffer (pH 6.5) 0.8 % (v/v).The mixture was incubated at 37 °C for 30 min then the reaction was stopped by adding 0.025 mL of Na_2CO_3 (0.1 M). The assay mixture was then centrifuged 15 min at 6,000 rpm and the absorbance was taken at 410 nm. The amount of pNP released from the hydrolysis of pNPL was measured and calculated using the standard curve of pNP prepared earlier. The results of the enzyme assays were expressed as a mean of duplicate readings.

2.4 Process optimization

Various parameters were studied in order to obtain the best condition that produces maximum lipase from *B. cepacia*.

1. Effect of mode of autoclave (mixed or separate) :
   Two different sets procedure was used to prepare the fermentation media. The first set (**Mixed**) was prepared by mixing all the required nutrients in a conical flask followed by autoclave for 15 min at 121 °C. Next, 5 % (v/v) of the pre-grown inoculum was added to fermentation media. The second set (**Separate**) was prepared by autoclaving the nutrients required for fermentation individually. The autoclaved nutrients were then mixed into a conical flask before the addition of pre-grown inoculum.
2. Effect of agitation speed :
   In order to study the effect of agitation speed on the lipase production, the following parameters were kept constant; mode of autoclave (mixed), pH (6.0), temperature (37 °C), and incubation time (72 h). Three fermentation medium was cultured at 100, 200 and 300 rpm respectively.
3. Effect of pH :
   The pH of fermentation media was adjusted using NH_3 solution (0.87 M) and hydrochloric acid (HCl) (6 N). Four different pH were chosen in this study. Since the original pH of the fermentation medium was 6.0, an acidic condition (pH 4.47) and another two alkaline condition (pH 9.0 & pH 10.0) were prepared.
4. Effect of incubation time :
   The effect of incubation time on the lipase production was conducted simultaneously with the effect of pH. Samples were withdrawn from all four different pH at an interval of 24 h for a total duration of 96 h.

Data generated from each of the variables were analysed using the statistical software package Design-Expert® (version 6.0, Stat-Ease, Inc., Minneapolis, USA) which employed the use of analysis of variance (ANOVA). Significant differences between mean values were determined from the ANOVA analysis at a significance level of p-value < 0.05.

3. Results and discussion

3.1 Effect of mode of autoclave

Various physio-chemical factors such as temperature, pH and agitation have been studied in previous literature. However, no study has been done on the general procedure of culture media preparation. Therefore in this study, the effects of mixing the basic medium before and after autoclave on the lipase production of *B. cepacia* were studied. The results obtained were summarized in Table 1. The results obtained show that mixing the basic medium before autoclave has shown better results (133 % improvement) as compared to the separate autoclave. This can be attributed to the possibility of better homogeneity of the pre-mixed media while autoclaving. The ANOVA analysis shows that the effect of mode of autoclave on enzyme production is significant (p-value = 0.0264).

Table 1: Effect of mode of autoclave on lipase production at constant temperature (37 °C), incubation time (24 h) and pH of fermentation medium (pH 6.0)

Mode of Autoclave	Lipase Production (U/mL)
Mixed	0.07 ± 0.01
Separate	0.03 ± 0.004

3.2 Effect of agitation speed

Figure 1 shows the results obtained for the effect of agitation on lipase production. An enhancement in lipase production has been observed with the increment in agitation. A similar trend has been observed for *Burkholderia sp* cultivation in both shake flask (Rathi et al., 2002) and 14 L bioreactor (Gupta et al., 2007). Agitation is a crucial parameter in enhancing the production as higher agitation increases the dissolved oxygen concentration (Sathish Kumar et al., 2013). Besides, agitation improves the nutrient dispersion throughout the whole medium and hence enhances the nutrient availability for faster cell growth and therefore improves the lipase production (Potumarthi et al., 2008). In this study, 300 rpm produced most lipase (11.18 U/mL). This result was expected since *B. cepacia* is an aerobic bacteria therefore it will grow rapidly under high dissolved oxygen condition (Rathi et al., 2002). The impact of agitation on enzyme production was also analysed using ANOVA. At 5 % significance level, agitation was observed to be a significant factor with a p-value of 0.0001. However higher agitation speed will be investigated in future work.

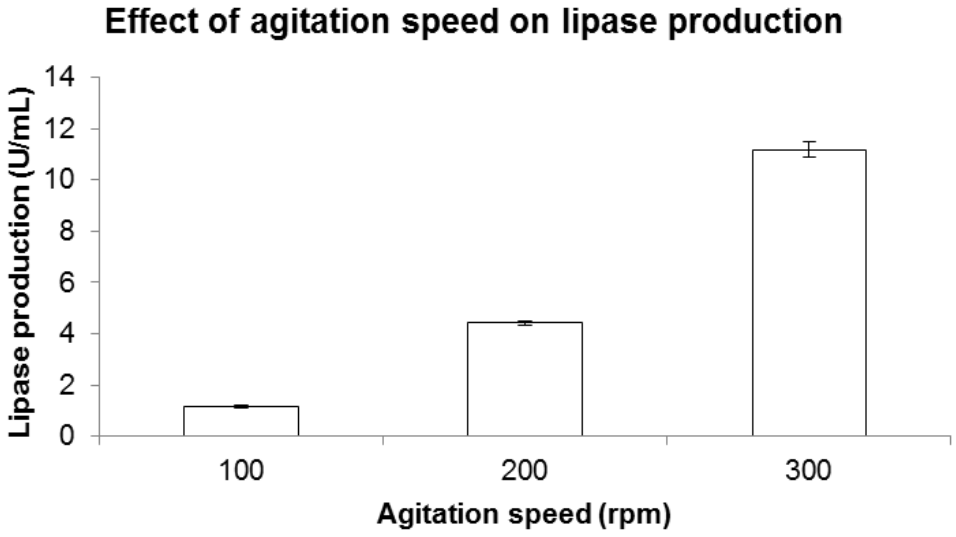

Figure 1: Effect of agitation on lipase production at constant temperature (37 °C), incubation time (72 h) and pH of fermentation medium (pH 6.0)

3.3 Effect of incubation time

Incubation time is the most important parameters that would significantly affect the overall production performance. An increase in production with time was observed until it reaches an optimum incubation time of 72 h (Figure 2). Beyond this time, no significant increment in enzyme production was observed. This may be attributed to the nutritional limitations or accumulation of fatty acids (Rathi et al., 2001). Oleic acid (fatty acid resulted from the hydrolysis of Tween 80) was shown to be an inhibitor to lipase production (Liu et al., 2011). Accumulation of oleic acid may lead to saponification (Chen et al., 1998) and hence inhibit the growth of lipase. The results obtained in this study were similar to those obtained by Liu et al. (2006) where 67 h was the optimum incubation time for *Burkholderia* sp. C20. The optimum incubation time obtained in this study was slightly longer than those obtained by Liu et al. (2006) as the culture condition in this study was performed at 37 °C which was lower than the reported optimum temperature of 55 °C.

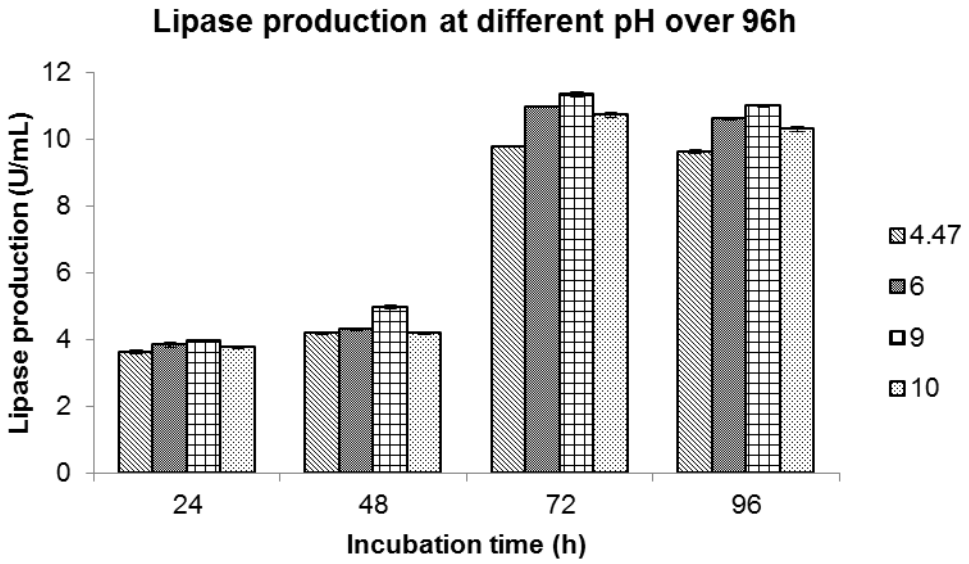

Figure 2: Effect of pH and incubation time on lipase production at constant temperature (37 °C) and agitation (300 rpm)

3.4 Effect of pH

The initial pH of the culture medium is of pH 6.0. In order to study the effect of initial pH on the production yield, the initial pH of the three other mediums were adjusted to 4.47 using hydrochloric acid, pH 9.0 and 10.0 using NH_3 solution respectively. All four medium were incubated for 96 h and samples were withdrawn in every 24 h interval. pH plays an important role in determining the category of an microorganisms. pH will affect the growth and metabolic activity of a microorganism (Amin and Bhatti, 2014). Therefore, the enzyme production and its stability can be affected by initial pH of the fermentation medium (Kumar et al., 2011). Each microorganism has a unique optimum pH to grow and act well in. In this study, the best lipase production (11.35 U/mL) was obtained when the initial pH of the medium was adjusted to pH 9.0. Acidic pH in the medium decreases the production as shown in Figure 2. This indicates that alkaline condition is more favourable for the growth of *B. cepacia*. The result obtained was consistent to those obtained by Liu et al. (2006) and Sharma et al. (2009) pH 9.0, where maximum lipase activity from *Burkholderia* sp. C20 and *Arthrobacter* sp. BGCC# 490 were observed at pH 9.0. Generally, majority bacterial lipases can be produced at an optimum neural (Gupta et al., 2004) or alkaline pH (Mladenoska and Dimitrovski, 2013).

The significance difference between each pH on the enzyme production was further studied through the Student's t-test as shown in Table 2. The data at 72 h was used in this analysis since it produced the highest yield of enzyme activity. It was observed that all of the pH shows significant difference as the Prob > |t| value obtained for each combination was less than 0.05.

Table 2: Student's t-test on effect of pH on enzyme production

Treatment	Mean difference	DF	Standard error	T for H_0 Coeff = 0	Prob > |t|*
4.47 vs 6	-1.2	1	0.084	-14.31	0.0001
4.47 vs 9	-1.56	1	0.084	-18.61	< 0.0001
4.47 vs 10	-0.95	1	0.084	-11.33	0.0003
6 vs 9	-0.36	1	0.084	-4.29	0.0127
6 vs 10	0.25	1	0.084	2.98	0.0406
9 vs 10	0.61	1	0.084	7.28	0.0019

*Values of "Prob > |t|" less than 0.05 indicate the difference in the two treatment means is significant; Values of "Prob > |t|" more than 0.05 indicate the difference in the two treatment means is insignificant

3.5 Optimization of operating conditions for submerged fermentation of *Burkholderia cepacia*

In summary, mixing the basic medium before autoclave and higher agitation speed (> 300 rpm) would facilitate in the enzyme production. Table 3 shows the ANOVA for the linear model on the effect of pH and incubation time on enzyme production.

Eq(1) shows the expression of the model for the predicted response Y where Y is the enzyme activity, A_1 ,A_2, A_3, A_4, B_1, B_2, B_3, and B_4 are the coded variables for pH 4.47, 6, 9, 10, incubation time 24 h, 48 h, 72 h, and 96 h respectively.

$$Y = 7.33 - 0.52A_1 + 0.10A_2 + 0.49A_3 - 3.52B_1 - 2.92B_2 + 3.38B_3 + 0.35A_1B_1 - 0.068A_2B_1 - 0.32A_3B_1 + 0.29A_1B_2 - 0.21A_2B_2 + 0.065A_3B_2 - 0.4A_1B_3 + 0.17A_2B_3 + 0.14A_3B_3 \quad (1)$$

It was observed that 99.97 % of the variations could be explained by the fitted model from the value of determination coefficient (R^2) of 0.9997. The R^2_{adj} value obtained was 0.9995 which implied that only 0.05 % of the total variations were not explained by the model. The statistical model was considered to be good since the R^2_{adj} value was close to R^2 value. The experimental values in this study were reliable as the C.V. (coefficient of variation) value was relatively low (1.03). The corresponding variables would be more significant if the F-value is much greater and the p-value is much lesser. The model terms will be considered as significant when the p-value is less than 0.05. Hence, the model in this study was significant as the p-value (< 0.0001) was less than 0.05 and the F-value (3972.10) was relatively high.

The optimum conditions for maximum enzyme production were then predicted to be at pH 9 and incubation time of 72 h. Under this condition, the predicted enzyme activity was 11.34 U/mL. Table 3 shows the predicted value and the actual value obtained for the selected optimum operating conditions. At the actual operating conditions, 11.18 U/mL of lipase has been obtained which was close to the predicted value.

Table 3: Selected optimum conditions and the predicted and actual experimental value of response at the optimum conditions

	pH	Incubation time (h)	Enzyme activity (U/mL)
Optimum conditions (predicted)	9	72	11.34
Actual conditions	9	72	11.18

4. Conclusions

Extracellular lipase from *B. cepacia* through submerged fermentation has been produced in present work. Various physio-chemical parameters affecting the lipase production were investigated and the best operating conditions to produce lipase from *B. cepacia* has been obtained in this study. It was observed that *B. cepacia* favors alkaline condition (pH 9.0). Furthermore, the culture medium required for the growth of *B. cepacia* was found to be best when autoclaved after mixing. Higher agitation improves the lipase production and in this study agitation at 300 rpm obtained highest lipase production. The optimum incubation time was obtained to be 72 h. At the optimum operating condition, 11.18 U/mL of lipase was produced in this study. In future studies, the effect of higher agitation speed (> 300 rpm) and temperature on the enzyme production will be investigated.

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