



## Biosynthesis of lipase by *Burkholderia cenocepacia* ST8 using waste cooking oil as feedstock

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### ABSTRACT

**Aims:** Every year, an estimated 25 million tons of waste oil are produced worldwide, and the generation of waste oil is one of the biggest global environmental problems. The incorporation of oil as a substrate for lipase production has been studied and shown to have a positive impact on its production. *Burkholderia* sp. is one of the major lipase-producing bacteria with their ability in bioremediation of oil-contaminated soil. This study aims to compare the production of lipase by *Burkholderia cenocepacia* ST8 using waste cooking oil and unused cooking oil as feedstock.

**Methodology and results:** The effect of different types of waste cooking oil (sunflower oil and palm oil) and concentration (1-3%) of waste cooking oil, agitation speed (100-400 rpm) and initial dissolved oxygen concentration (10-50%) on lipase production by *B. cenocepacia* ST8 under batch fermentation mode were investigated. The major fatty acids of which had been consumed were determined using gas chromatography. Results showed that 2% (v/v) of single used sunflower cooking oil produced the highest lipase activity of 138.86 U/mL with a productivity of 2.10 U/mL/h; agitation speed of 300 rpm produced the highest lipase activity of 183.56 U/mL with a productivity of 3.06 U/mL/h while 30% initial concentration of dissolved oxygen produced a lipase activity of 176.45 U/mL with a productivity of 2.94 U/mL/h. Oleic acid and linoleic acid were found to be the most consumed by *B. cenocepacia* ST8 among other fatty acids.

**Conclusion, significance and impact of study:** This study shows that 2% (v/v) single used sunflower cooking oil was the better type and optimum concentration of carbon source for the production of lipase by the fermentation of *B. cenocepacia* under 300 rpm and 30% initial concentration dissolved oxygen. The incorporation of 2% (v/v) single used sunflower cooking oil may be a great alternative to reduce the cost for the production of lipase as well as reducing the amount of waste oil generation.

**Keywords:** Sunflower oil, palm oil, agitation speed, dissolved oxygen, kinetic study

### INTRODUCTION

For over three centuries, triglyceride hydrolysing enzymes have been extensively investigated. Lipases (triacylglycerol acylhydrolase, EC. 3.1.1.3) in the recent years particularly have become one of the increasingly important enzymes in the field of organic synthesis due to their distinct characteristic of acting at the interface between an aqueous and a non-aqueous phase (Amin and Bhatti, 2014). Long-chain triacylglycerols are the common substrates of lipases which have generally low solubility in water; and the reaction is catalyzed at the lipid-water interface. Lipases have shown to possess the exceptional capability of performing the reverse reaction which further leads to esterification, interesterification,

alcoholysis and acidolysis (Rajendran *et al.*, 2009; Sharma and Kanwar, 2014).

There are many applications of lipases such as fat and oil processing, food processing, biodiesel production, detergent formulation, organic synthesis, textile production and pharmaceutical production. In the manufacturing of food, the liberation of fatty acids into food products have incorporated the use of lipases for the hydrolysis processes. The fatty acid obtained contributes to the flavors, fragrance and colors of the food product. As to what degree these aspects are seen with the food product rely upon the length of the carbon chain, the location of the fatty acid and the level of unsaturation (Barros *et al.*, 2010). The ripening of cheese such as Parmesan as well as the lipolysis of butter and cream

have shown to be accelerated with the use of lipases produced from *Lactobacillus* (Sharma and Kanwar, 2014).

Lipases are ubiquitous enzymes and may be produced by a variety of different animals, plants as well as microorganisms. Even so, lipases produced by microorganisms are still preferred due to them having less complicated extraction procedures compared to those extracted from animals and plants. They also have enzymatic properties which are diverse as to also being substrate specific (Ramakrishnan *et al.*, 2013). *Burkholderia cenocepacia* is one of the major lipase-producing bacteria and have been broadly studied for over two decades particularly for its applications in different industries. The unique properties of having enzyme activity at wide range of pH, thermal stability and high enantioselectivity allows it to be a more favorable lipase-producing bacterium compared to other microorganisms (Wang *et al.*, 2009; Sánchez *et al.*, 2017).

The importance of lipases has steadily been growing in the recent years as industrial catalysts. Its outstanding characteristics have increased the market value in the biotechnology industry. The participation of lipases in the worldwide enzyme industry market has grown significantly and it is believed that they will acquire importance comparable to that of the proteases, which currently represents 25% to 40% of industrial enzyme sale (Hasan *et al.*, 2006). There have been reports on the demand of enzymes to undergo an increase of 4.6%, reaching US \$7.2 billion in 2020. The main regions which will bring about this increase are the developing countries such as those in Asia/Pacific and South America regions (Li *et al.*, 2012).

To upkeep the constant increase of demand of lipase, it is vital to optimize the growth condition to maximize its yield. One of the essential nutrients for fermentation processes is carbon source as it provides the source of energy for cell growth and product formation. Past research has incorporated the use of replacement substrates for carbon source such as palm oil, coconut oil, olive oil and sunflower oil, rice bran oil, soybean oil, corn oil and engine oil. Sunflower oil as the substrate resulted in the highest lipase activity by *B. cenocepacia* in shake flask culture at 179 U/mL while palm oil as substrate produced a lipase activity of 86.75 U/mL. Glucose and fructose were some of the simple sugars supplied but did not stimulate the production of lipase (Lau *et al.*, 2011).

As *B. cenocepacia* is an aerobic bacterium, the agitation speed and dissolved oxygen levels affects the production of lipase. Aerobic bacteria require a well oxygenated environment for them to grow well. Past research showed that higher agitation speed causes higher dissolved oxygen levels which promotes lipase production (Sathish Kumar *et al.*, 2013). An increase in agitation speed showed that the nutrient dispersion throughout the medium is improved and the availability of nutrients increases thus promoting cell growth (Rathi *et al.*, 2002; Potumarthi *et al.*, 2008). Research has also shown that fatty acids play an important role in obtaining

microbial lipases. Csutak *et al.* (2018) reported that oleic acid and their esters resulted in higher lipolytic activity compared to palmitic acid.

It was reported that 378 million litres of waste cooking oil may be generated per day in the USA. In the European countries, approximately 700,000-1,000,000 tonnes per year is produced while in Asian countries, an estimated 40,000 tonnes of waste cooking are produced per year (Chhetri *et al.*, 2008; Ganesh *et al.*, 2012). The large amount of waste cooking oil produced creates an issue for its collection, treatment and disposal. Past research has shown that waste cooking oil produced positive results for lipase production by *Bacillus subtilis* and *Yarrowia lipolytica* W29. This bioprocess contributes to a circular economy where a low-cost substrate is used, lipase is produced and less wastes are generated (Lopes *et al.*, 2018).

There have been studies on the incorporation of different oils for lipase production but not many on the use of waste oil. As the generation of waste oil poses a great threat to the environment, the use of waste oil may help to reduce pollution and save the environment. The usage of waste oil is also believed to have a positive effect on the production of lipase. Based on previous literature, the impacts of agitation speed and dissolved oxygen concentration on the production of lipase by *B. cenocepacia* ST8 has not been extensively studied. Hence, the objective of the study was to investigate the effect of different types (sunflower oil and palm oil) and concentration (1-3%) of waste cooking oil, agitation speed (100-400 rpm) and initial dissolved oxygen concentration (10-50%) on lipase production by *B. cenocepacia* ST8 under batch fermentation mode. Poly-nitrophenyl-laurate spectrophotometric assay was used to analyze the lipolytic activity. Gas chromatography analysis was used to determine the fatty acid which has been consumed by *B. cenocepacia* ST8 in lipase production.

## MATERIALS AND METHODS

### Microorganism

*Burkholderia cenocepacia* ST8, which is isolated from forest soil was obtained from School of Industrial Technology, Universiti Sains Malaysia and used throughout the experiment. The strain was kept at a temperature of  $-80\text{ }^{\circ}\text{C}$  in 25% (v/v) glycerol stock solution. *Burkholderia cenocepacia* ST8 was sub-cultured by inoculating one loop onto nutrient agar and incubated in a shaking incubator at  $37\text{ }^{\circ}\text{C}$  for 24 h. Inoculum was prepared by inoculating a single colony of *B. cenocepacia* ST8 into 100 mL of nutrient broth in 250 mL Erlenmeyer flask, and incubated in the shaking incubator at  $37\text{ }^{\circ}\text{C}$ , 200 rpm for 12 h.

### Medium formulation

*Burkholderia cenocepacia* ST8 was cultured in the fermentation medium with its main components composing of carbon source and nitrogen source. The

basic medium formulation was adapted from Liew *et al.* (2015) which comprised of 1% (v/v) of Tween 80, 0.325% (w/v) of nutrient broth, 1% (w/v) gum arabic and 0.1% (w/v) calcium chloride (CaCl<sub>2</sub>). Sunflower oil and palm oil were purchased from local grocery shop and the waste cooking oil (single and multiple used of sunflower oil and palm oil) were collected from kitchen waste. In this study, 1-3% (v/v) of single and multiple used sunflower oil or 1-3% (v/v) of single and multiple used palm oil were used as the carbon source. Multiple used oil is equal to 10 times used oil for deep fry. Pretreatment of waste cooking oil was performed to filter out the solid impurities by filtration with filter paper of grade 6 S/N using vacuum pump. Throughout the fermentation process, Tween 80, nitrogen source, gum arabic and calcium chloride were controlled at a fixed concentration. The pH level of the fermentation medium was adjusted to 9.0 using 3M sodium hydroxide (NaOH) and 3 M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>).

### Fermentation in shake flask and stirred tank bioreactor

Ten percent (v/v) of the inoculum of *B. cenocepacia* ST8 was transferred into the fermentation medium with 200 mL working volume in shake flask and 1 L working volume in stirred tank bioreactor. The fermentation process was conducted for 192 h in shake flask and 72 h in stirred tank bioreactor at 37 °C. Different concentration of used palm oil, used sunflower oil (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) and 1% unused cooking oil as control were used in shake flask fermentation. For bioreactor fermentation, different agitation speed (100-400 rpm) and dissolved oxygen concentration (10-50%) were tested. The bioreactor (Bioflo 115; Eppendorf Malaysia) was agitated by two six bladed Rushton turbine (impeller diameter = 0.052 m) and the temperature, pH and dissolved oxygen tension (DOT) were monitored by a control module system. Liew *et al.* (2015) had previously studied agitation speed up to 300 rpm and reported that higher agitation may result in higher lipase activity. The control was supplied with 2% (v/v) of unused sunflower oil under an agitation speed of 200 rpm and initial dissolved oxygen at 30% air saturation. In this study, the agitation speed was varied from 100 to 400 rpm (impeller tip speed =  $\pi ND$ , ranging from 0.27 to 1.09 m/s, respectively) according to the requirement of the experiment with a fixed dissolved oxygen concentration of 30%. For the study of DOT, the DOT level was varied at the range from 10% to 50% with a fixed agitation speed of 200 rpm. DOT level was measured by a polarographic DO electrode (Mittler Toledo, Switzerland) and recorded throughout the fermentation. Sampling was done at every 6 h interval using a sterile syringe throughout the 72 h fermentation.

### Analytical methods cell concentration

During fermentation, samples were withdrawn at various time intervals for analysis. Cell growth was observed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. The correlation between

dry cell weight (DCW) and OD was estimated from several batch experiments using the Equation 1:

$$\text{Dry cell weight } \left(\frac{g}{L}\right) = OD \text{ at } 600 \text{ nm} \times 4.8855$$

(Equation 1)

### Lipase activity

The supernatants were used for lipase activity determination. After the removal of the cell pellets for cell concentration determination, 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) is defined as the amount of enzyme releasing 1  $\mu$ mol p-nitrophenol (pNP) per minute. The enzyme assay includes sample solution 0.1% (v/v), 0.01 M pNPL (4-Nitrophenyl dodecanoate) (dissolved in absolute ethanol) 0.1% (v/v) and 0.05 M phosphate buffer 0.8% (v/v). The mixture was incubated at 37 °C for 30 min then the reaction was stopped by adding 0.25 mL of 0.1 M of Na<sub>2</sub>CO<sub>3</sub>. The assay mixture was centrifuged at 6000 rpm for 15 mins and the absorbance was measured at 410 nm. Blank reading was obtained by replacing sample solution with medium broth. The correlation between lipase activity and OD was calculated using pNP as standard curve with Equation 2:

$$\text{Lipase activity } \left(\frac{U}{mL}\right) = \frac{OD \text{ at } 410 \text{ nm}}{0.0002} \times \text{dilution factor}$$

(Equation 2)

### Gas chromatography

Fatty acid methyl esters (FAME) were prepared according to O'Fallon *et al.* (2007) method. A total amount of 20 mL oil sample was added in a 50 mL centrifuge tube. Then, 0.7 mL 10 N KOH in water and 5.3 mL methanol were added. The tube was incubated in a 55 °C water bath for 90 min with vigorous shaking every 20 min to properly permeate, dissolve and hydrolyze the sample. The tube was cooled to room temperature under running tap water and 0.58 mL of 24 N H<sub>2</sub>SO<sub>4</sub> was added. The tube was mixed by inversion until precipitated K<sub>2</sub>SO<sub>4</sub> was formed. The tube was incubated again in a 55 °C water bath for 90 min with 5 sec of hand shaking every 20 min. The tube was cooled under running tap water to room temperature. After that, 3 mL of hexane was added and vortexed for 5 min. The hexane layer containing FAME was placed in chromatography vial to be analysed in gas chromatography. The samples were analysed with gas chromatograph equipped with a capillary fused column 30 m x 0.25 mm i.d. column coated with a 0.25  $\mu$ m film of HP-5 and helium as carrier gas. The injector and detector temperature were set at 250 °C and 280 °C, respectively and split ratio of 15:1 was used. Oven temperature was programmed as follows: holding at 150 °C for 0.5 min, 150 °C to 180 °C at rate of 10 °C/min, 180 to 220 °C at rate of 1.5 °C/min, 220 °C to 260 °C at rate of 30 °C/min and hold at 260 °C for 5 min.

**Table 1:** Kinetic parameter values of lipase production using single and multiple used sunflower oil.

Kinetic parameters	Single used sunflower oil concentration (%)						Multiple used sunflower oil concentration (%)					
	Control	1.0	1.5	2.0	2.5	3.0	1.0	1.5	2.0	2.5	3.0	
Maximum cell concentration (g/L)	4.22 ± 0.26	3.89 ± 0.24	4.49 ± 0.23	6.26 ± 0.19	4.17 ± 0.22	3.03 ± 0.25	3.69 ± 0.33	4.17 ± 0.27	3.71 ± 0.34	3.11 ± 0.27	2.20 ± 0.28	
Maximum lipase activity (U/mL)	96.25 ± 4.23	81.73 ± 5.34	107.28 ± 5.68	138.86 ± 3.32	88.80 ± 3.11	61.64 ± 5.42	74.48 ± 5.36	102.63 ± 4.31	79.32 ± 5.47	67.69 ± 6.39	51.72 ± 6.33	
Lipase productivity (U/mL/h)	1.46	1.35	1.63	2.10	1.35	0.93	1.03	1.56	1.01	0.81	0.62	

**Table 2:** Kinetic parameter values of lipase production using single and multiple used palm oil.

Kinetic parameters	Single used palm oil concentration (%)						Multiple used palm oil concentration (%)					
	Control	1.0	1.5	2.0	2.5	3.0	1.0	1.5	2.0	2.5	3.0	
Maximum cell concentration (g/L)	3.08 ± 0.21	3.48 ± 0.22	4.69 ± 0.24	3.23 ± 0.21	2.47 ± 0.17	2.11 ± 0.25	3.25 ± 0.28	4.22 ± 0.20	3.18 ± 0.13	2.85 ± 0.19	2.37 ± 0.25	
Maximum lipase activity (U/mL)	82.87 ± 3.33	87.88 ± 5.09	94.35 ± 4.72	70.56 ± 5.87	56.41 ± 5.63	42.32 ± 4.28	89.53 ± 5.28	101.32 ± 4.21	84.47 ± 5.79	72.61 ± 6.42	61.24 ± 5.26	
Lipase productivity (U/mL/h)	1.26	1.33	1.43	0.98	0.78	0.59	1.24	1.54	1.17	1.10	0.73	

### Statistical analysis

Data obtained were statistically using SPSS software, IBM SPSS Statistics version 25 (IBM, New York). One-way analysis of variance (ANOVA) was used to compare the means among test samples at 95% confidence level ( $\alpha = 0.95$ ).

## RESULTS AND DISCUSSION

### Effect of waste sunflower oil on lipase production

The effect of single and multiple used (Table 1) sunflower oil concentrations at 1.0%, 1.5%, 2.0%, 2.5% and 3.0% were studied. In this study, the increase in sunflower oil concentration especially beyond 2% led to a decrease in cell concentration as well as lipase activity. This was observed from both single used and multiple used sunflower oil. It has been previously revealed that high levels of substrate concentration have detrimental effects on the production of lipase. This could be due to the inhibitory action caused by the increase of fatty acids concentration which was released during the hydrolysis of triglycerides. Thus, an optimum concentration needs to be supplied to be able to obtain maximum lipase production (Kalyani and Saraswathy, 2014).

In this study, the highest lipase activity was 138.86 U/mL and maximum lipase productivity was 2.10 U/mL/h when 2% (v/v) single used sunflower oil was used as carbon source. From Lau *et al.* (2011), the incorporation of 1.0% sunflower oil as the carbon source was able to produce a lipase activity of 179 U/mL by the fermentation of *B. cenocepacia* in shake flask culture. However, the lipase productivity obtained was 1.72 U/mL/h which is lower than the results in this study. This shows that the

maximum lipase activity was produced at a shorter amount of time.

Maximum lipase activity and lipase productivity obtained from the single used sunflower oil were higher than that of multiple used sunflower oil. This may be due to the change of the amount of the fatty acid components present in the oil after being used at a high temperature. Sunflower oil consists of high levels of unsaturated fatty acids thus it is easier to be broken down and become more readily available to uptake as they are thermodynamically less stable than saturated fatty acids (Rustan and Drevon, 2005). The multiple used sunflower oil would have a lower amount of fatty acids as most would have been broken down after being used at a high temperature for multiple times.

### Effect of waste palm oil on lipase production

The effect of single used and multiple used (Table 2) palm oil concentrations of 1.0 %, 1.5 %, 2.0 %, 2.5% and 3.0 % were studied. The results obtained from palm oil were very much alike to that of sunflower oil where an increase in palm oil concentration, especially beyond 1.5 % led to a decrease in cell concentration as well as lipase activity for both single used and multiple used oil. In this study, the repression of lipases synthesis in the single used and multiple used palm oil (Table 2) might be due to fatty acids level in palm oil. Palm oil consists of saturated fatty acids, monounsaturated fatty acids as well as polyunsaturated fatty acids. The major fatty acids in palm oil are oleic acid, linoleic acid, palmitic acid, stearic acid and myristic acid. The amount of saturated and unsaturated fatty acids in palm oil are almost equal (Koushki *et al.*, 2015). Palm oil has higher levels of saturated fatty acids compared to sunflower oil. This

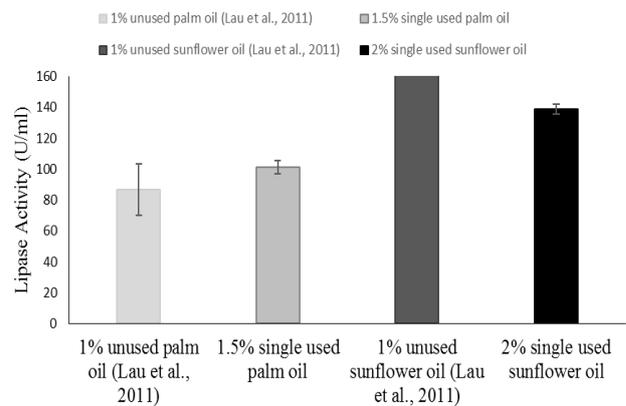
means that palm oil has a greater oxidative stability hence they are not so easily broken down at high temperatures (Matthäus, 2007).

The maximum lipase activity obtained from this study was higher than the results observed in previous studies. Rathi *et al.* (2002) reported a maximum lipase activity of 60 U/mL by the fermentation of *B. cepacia* using palm oil. Another study incorporated 1.0% palm oil as the carbon source and was able to produce a lipase activity of 86.75 U/mL by the fermentation of *B. cenocepacia* in shake flask culture (Lau *et al.*, 2011). The lipase productivity in this study was also higher than that of past reports. Lau *et al.* (2011) reported that the lipase productivity was 0.58 U/mL/h when medium was incorporated with palm oil. However, this study showed a three-fold increase in lipase productivity of 1.54 U/mL/h with the incorporation of 1.5% multiple used palm oil. This shows that this concentration was the optimum concentration to produce the maximum lipase activity in less amount of time. A similar pattern was previously reported whereby the effect of punnakka oil concentrations of 1.0% to 5.0% on lipase production by *Staphylococcus aureus* was investigated. The study resulted with an increase in lipase activity from 1.0% to 2.0% with maximum lipase activity achieved when 2.0% of punnakka oil was supplied in the medium. Punnakka oil concentrations of 3.0% to 5.0% showed adverse effects on the lipase activity (Kalyani and Saraswathy, 2014).

#### Lipase production between waste sunflower oil and waste palm oil

From the kinetic parameter values obtained, single used sunflower oil was able to produce a higher lipase activity of  $138.86 \pm 3.32$  U/mL compared to single used palm oil which produced a maximum lipase activity of  $101.32 \pm 4.21$  U/mL. These results are similar with previous studies done by Lau *et al.* (2011). The study incorporated the use of replacement substrates for carbon source such as palm oil, coconut oil, olive oil and sunflower oil, rice bran oil, soybean oil, corn oil and used engine oil. Carbon source is one of the most important nutrients for fermentation processes as it provides the main source of energy for the growth of the cell and product formation. Figure 1 showed the comparison of lipase activity among Lau *et al.* (2011) using unused palm oil and sunflower oil with single used waste sunflower oil and waste palm oil from this study. The maximum lipase activity by *B. cenocepacia* was obtained with sunflower oil (1.0%, v/v) and tween 80 (1.0%, v/v) as substrate in a shake flask culture at 179 U/mL while a maximum lipase activity of 86.75 U/mL was achieved with palm oil (1.0%, v/v) and tween 80 (1.0% v/v) as substrate (Lau *et al.*, 2011).

Fairly positive results of lipase production were also achieved by different bacterial species; for example, *Aspergillus niger* cultivated using sunflower oil as the carbon source which resulted with a lipase activity of



**Figure 1:** Comparison of maximum lipase activity by *B. cenocepacia* between 1% unused palm oil and sunflower oil (Lau *et al.*, 2011) with 1.5% of single used palm oil and 2.0% of single used sunflower oil were incorporated as carbon source.

61.30 U/mL (Gerber *et al.*, 2013) and *B. cepacia* cultivated with palm oil resulting with lipase activity of 60 U/mL (Rathi *et al.*, 2002). Previous research stated that the fatty acids components of sunflower oil such as linoleic, linolenic and oleic acids were able to increase the productivity of lipase by *Pseudomonas mephitica* (Ghosh *et al.*, 1996).

#### Effect of aerobic conditions on lipase production in stirred tank bioreactor

After fermentation in shake flask culture, a concentration of 2% (v/v) single used sunflower oil was found to produce the highest cell concentration, lipase activity and lipase productivity. Thus, it was incorporated into the fermentation media as the main carbon source in the 3-L stirred tank bioreactor for further optimization of aerobic conditions.

Based on Table 3, the highest lipase activity of  $183.56 \pm 4.33$  U/mL and maximum productivity of 3.06 U/mL/h were obtained when an agitation speed of 300 rpm was used. On the other hand, 30% dissolved oxygen concentration resulted in a lipase activity of  $176.45 \pm 5.33$  U/mL and maximum productivity of 2.94 U/mL/h (Table 4). Both parameters produced a higher lipase activity and a higher productivity than the control. These values were obtained after 60 h of fermentation. There was a 61.67% and 55.41% increase in lipase activity as well as a 77.91% and 70.93% increase in maximum productivity, respectively, as compared to the control.

Low agitation speed of 100 rpm resulted in the lowest cell concentration and lipase production. This could be due to oxygen transfer limitation, hindering culture medium homogenization and reducing lipid availability or uptake by the cells.

On the other hand, high agitation speed of 400 rpm also produced low lipase activity and cell concentration.

**Table 3:** Kinetic parameter values of lipase production by *B. cenocepacia* ST8 using different agitation speeds with 2% (v/v) single used sunflower oil in 3 L stirred tank bioreactor.

Kinetic parameters	Agitation speed (rpm)				
	Control	100	200	300	400
Maximum cell concentration (g/L)	3.73 <sup>a</sup> ± 0.27	3.64 <sup>a</sup> ± 0.21	5.28 <sup>b</sup> ± 0.19	6.77 <sup>c</sup> ± 0.23	6.02 <sup>d</sup> ± 0.11
Maximum lipase activity (U/mL)	113.54 <sup>a</sup> ± 4.25	77.43 <sup>b</sup> ± 5.04	141.72 <sup>c</sup> ± 5.33	183.56 <sup>d</sup> ± 4.33	140.94 <sup>c</sup> ± 4.69
Maximum productivity (U/mL/h)	1.72	1.61	2.36	3.06	2.61

Both cell concentration and lipase activity were determined from three separate fermentations and reported as mean ± standard deviation. The mean values in the same row followed by a different letter are significantly different ( $p < 0.05$ ).

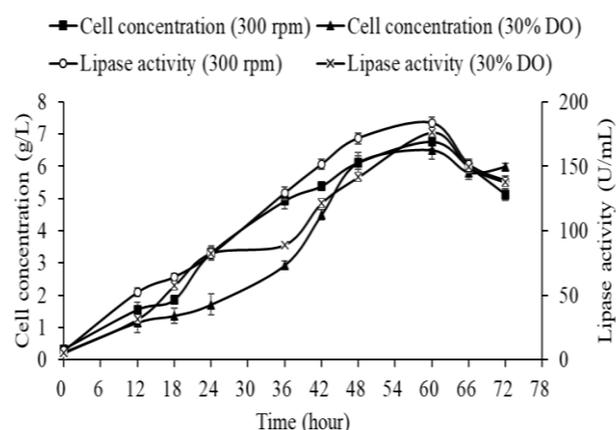
**Table 4:** Kinetic parameter values of lipase production by *B. cenocepacia* ST8 using different initial dissolved oxygen concentrations with 2% (v/v) single used sunflower oil in 3 L stirred tank bioreactor.

Kinetic parameters	Initial dissolved oxygen concentration (%)			
	Control	10	30	50
Maximum cell concentration (g/L)	3.73 <sup>a</sup> ± 0.27	3.93 <sup>a</sup> ± 0.27	5.28 <sup>b</sup> ± 0.19	4.76 <sup>c</sup> ± 0.29
Maximum lipase activity (U/mL)	113.54 <sup>a</sup> ± 4.25	108.26 <sup>b</sup> ± 3.21	176.45 <sup>c</sup> ± 5.33	101.34 <sup>b</sup> ± 2.87
Maximum productivity (U/mL/h)	1.72	2.00	2.94	1.88

Both cell concentration and lipase activity were determined from three separate fermentations and reported as mean ± standard deviation. The mean values in the same row followed by a different letter are significantly different ( $p < 0.05$ ).

This could probably be caused by the mechanical or oxidative stress due to the influence of shear stress by blade tips of the impeller and decrease in external pH (Alonso *et al.*, 2005). This result agrees to the report by Liew *et al.* (2015) where the highest lipase activity (11.18 U/mL) was produced at agitation speed of 300 rpm. However, their study did not further investigate on higher agitation speed. Agitation is a crucial parameter in aerobic fermentation as higher agitation increases the dissolved oxygen concentration as well as the nutrient dispersion in the culture.

The trend of lipase activity (Figure 2) was similar in stirred tank bioreactor when 2% of single used waste sunflower cooking oil was incorporated under an agitation speed of 300 rpm and initial dissolved oxygen concentration of 30%, respectively. The highest cell concentration and lipase activity was produced after 60 h of fermentation. It can be observed that the production of lipase began to increase rapidly from the early exponential growth phase which was after the 12<sup>th</sup> h. Both cell concentration and lipase activity began to decrease after 60 h. Based on Figure 2, the effect of agitation speed on cell growth and lipase activity is more significant compared to aeration rate. This is due to agitation plays an important mixing and shearing role in fermentation processes. It not only improves mass and oxygen transfer between the different phases, but also maintains homogeneous chemical and physical conditions in the medium by continuous mixing. On the other hand, aeration determines the oxygenation of the fermentation process, and contributes to mixing of the fermentation broth, especially where mechanical agitation speeds are low. Besides this, it is obvious from the given lipase activity, carbohydrate utilization, and cell growth data that mixing was crucial for better oxygen and nutrient transfer rate during entire period of operation.



**Figure 2:** Time profile of lipase production by *B. cenocepacia* ST8 in stirred tank bioreactor when 2% of single used sunflower cooking oil was incorporated under an agitation speed of 300 rpm and initial dissolved oxygen concentration of 30%, respectively.

Mixing is important for maximal production of microbial lipase by optimizing the mixing rates in batch fermentation and can be imparted by means of both aeration and agitation for better oxygen mass transfer rate and for better product formation. Past research has shown that the role of oxygen is important for the metabolism of lipid and cell growth. In addition to that, the production of lipase by various microorganisms depends on the oxygen

availability (Alonso *et al.*, 2005). An increase in agitation speed and dissolved oxygen concentration led to a decrease in cell concentration as well as lipase activity. Shear stress causes cell disruption, especially at higher agitation speed (beyond 300 rpm) resulting in the release of other enzymes, including esterases and proteases (Alonso *et al.*, 2005). This statement is supported by Freire *et al.* (1997), an increase in protease release into the culture medium was observed for *Penicillium restrictum* when stirring speed was raised from 50 to 300 rpm. This is similar to previous studies using different microorganisms. *Penicillium chrysogenum* is cultured with olive oil as its main carbon source which obtained a maximum lipase activity of 205 U/mL at 400 rpm after 72 h of fermentation (Shafei *et al.*, 2011). This was higher than the lipase activity attained at 200 rpm which was 160 U/mL after 96 h of fermentation. When the agitation speed was increased to 600 rpm, a decrease in lipase activity was observed. Another study compared the effects of different agitation speeds on lipase production by *Y. lipolytica*. It was reported that the maximum lipase activity of 5300 U/L was obtained with an agitation speed of 200 rpm after 240 h of fermentation which was higher than the lipase activity obtained at 300 rpm after 120 h of fermentation with a value of 1500 U/L (Alonso *et al.*, 2005).

In a separate report, fermentation using *Candida cylindracea* NRRL Y-17506 under an agitation speed of 200 rpm and 100% dissolved oxygen concentration showed an enhanced production of lipase (Krastanov *et*

*al.*, 2008). Another study compared different agitation speeds of 150-300 rpm for lipase production by *Rhizopus oligosporus*. It was found that 250 rpm produced the highest lipase activity after 24 h of fermentation with the media being supplied with olive oil and glucose as carbon sources (Iftikhar *et al.*, 2010). The agitation and aeration rates not only affect the productivity of the microbial process but also affect the overall energy requirement of the production process.

#### Fatty acid analysis by gas chromatography

Sunflower oil consists of saturated fatty acids, monounsaturated fatty acids as well as polyunsaturated fatty acids. The major fatty acids found are linoleic acid, oleic acid, palmitic acid and stearic acid. Another study which investigated the lipase production by *Y. lipolytica* NCIM 3589 showed that the presence of sunflower oil had a positive effect on the production of lipase. It was explained that the high level of lipase production may be supported by the polyunsaturated fatty acids component found in sunflower oil (Babu and Rao, 2007). Based on the results obtained from the gas chromatography, linoleic acid and oleic acid were found to be the fatty acids most taken up by *B. cenocepacia* ST8 for lipase production. In Table 5, the concentration of oleic acid and linoleic acid were decreased with the increase of agitation speed in stirred tank bioreactor. This indicated that increased agitation speed enhanced the nutrient dispersion, resulted in the availability of oleic acid and linoleic acid to the cells.

**Table 5:** Fatty acid composition before and after 72 h of fermentation under different agitation speeds with 2% single used sunflower oil and 30% initial dissolved oxygen concentration.

Agitation speed (rpm)	Fatty acid composition (mol c/g)			
	Before fermentation		After fermentation	
	Linoleic acid	Oleic acid	Linoleic acid	Oleic acid
Control	5.04 <sup>a</sup> ± 3.13 × 10 <sup>-6</sup>	1.02 <sup>a</sup> ± 2.43 × 10 <sup>-5</sup>	4.57 <sup>a</sup> ± 5.03 × 10 <sup>-6</sup>	7.63 <sup>a</sup> ± 3.46 × 10 <sup>-6</sup>
100	4.96 <sup>a</sup> ± 3.22 × 10 <sup>-6</sup>	1.00 <sup>a</sup> ± 1.86 × 10 <sup>-5</sup>	4.56 <sup>a</sup> ± 3.66 × 10 <sup>-6</sup>	9.47 <sup>b</sup> ± 5.72 × 10 <sup>-6</sup>
200	7.59 <sup>b</sup> ± 2.87 × 10 <sup>-6</sup>	2.08 <sup>b</sup> ± 2.94 × 10 <sup>-5</sup>	5.94 <sup>b</sup> ± 4.19 × 10 <sup>-6</sup>	1.47 <sup>c</sup> ± 2.13 × 10 <sup>-5</sup>
300	6.28 <sup>c</sup> ± 2.42 × 10 <sup>-6</sup>	1.88 <sup>b</sup> ± 2.07 × 10 <sup>-5</sup>	6.12 <sup>c</sup> ± 4.78 × 10 <sup>-6</sup>	7.22 <sup>d</sup> ± 5.54 × 10 <sup>-6</sup>
400	6.36 <sup>c</sup> ± 3.51 × 10 <sup>-6</sup>	1.56 <sup>a</sup> ± 2.18 × 10 <sup>-5</sup>	2.84 <sup>d</sup> ± 3.23 × 10 <sup>-6</sup>	4.78 <sup>e</sup> ± 4.17 × 10 <sup>-6</sup>

Fatty acid composition was determined by gas chromatography. The values represent mean ± standard deviation. The mean values in the same row followed by a different letter are significantly different ( $p < 0.05$ ).

**Table 6:** Fatty acid composition before and after 72 h of fermentation under different initial dissolved oxygen concentrations with 2% single used sunflower oil and 300 rpm agitation speed.

Initial dissolved oxygen concentration (%)	Fatty acid composition (mol c/g)			
	Before fermentation		After fermentation	
	Linoleic acid	Oleic acid	Linoleic acid	Oleic acid
Control	5.04 <sup>a</sup> ± 3.13 × 10 <sup>-6</sup>	1.02 <sup>a</sup> ± 2.43 × 10 <sup>-5</sup>	4.57 <sup>a</sup> ± 5.03 × 10 <sup>-6</sup>	7.63 <sup>a</sup> ± 3.46 × 10 <sup>-6</sup>
10	7.22 <sup>b</sup> ± 8.67 × 10 <sup>-6</sup>	1.88 <sup>a</sup> ± 3.23 × 10 <sup>-5</sup>	6.12 <sup>b</sup> ± 5.34 × 10 <sup>-6</sup>	6.28 <sup>b</sup> ± 7.23 × 10 <sup>-6</sup>
30	6.22 <sup>c</sup> ± 5.86 × 10 <sup>-6</sup>	1.54 <sup>a</sup> ± 3.10 × 10 <sup>-5</sup>	1.11 <sup>c</sup> ± 3.89 × 10 <sup>-6</sup>	4.81 <sup>c</sup> ± 5.13 × 10 <sup>-6</sup>
50	4.23 <sup>d</sup> ± 4.43 × 10 <sup>-6</sup>	2.65 <sup>b</sup> ± 4.18 × 10 <sup>-5</sup>	3.84 <sup>d</sup> ± 4.31 × 10 <sup>-6</sup>	5.81 <sup>c</sup> ± 4.55 × 10 <sup>-6</sup>

Fatty acid composition was determined by gas chromatography. The values represent mean ± standard deviation. The mean values in the same row followed by a different letter are significantly different ( $p < 0.05$ ).

Under an agitation speed of 300 rpm resulted in a  $7.22 \times 10^{-6}$  mol c/g concentration of oleic acid after 72 h of fermentation which is a 61.59% decrease. The concentration of linoleic acid after 72 h of fermentation was  $6.12 \times 10^{-6}$  mol c/g which was a slight decrease of 2.55%. In Table 6, the culture under 30% initial dissolved oxygen concentration resulted in a  $4.81 \times 10^{-6}$  mol c/g concentration of oleic acid after 72 h of fermentation which is a 66.77% decrease. The concentration of linoleic acid after 72 h of fermentation was  $1.11 \times 10^{-6}$  mol c/g which was a significant decrease of 82.15%. The fatty acid uptake correlates to the kinetic parameter values of the culture under an agitation speed of 300 rpm and 30% dissolved oxygen concentration as these two produced the highest cell concentration and lipase activity in bioreactor.

It was reported that the low levels of lipase activity could be due to the low content of unsaturated fatty acid esters. As the total amount of C18:n fatty acids increased, the production of lipase also increased (Lakshmi *et al.*, 1999). There have been previous reports on the optimization for the production of lipase by *Burkholderia* sp. using olive oil as the primary carbon source (Liu *et al.*, 2006; Lo *et al.*, 2012). Olive oil, similar to sunflower oil, contains a substantial amount of oleic and linoleic acid.

In another study, waste cooking oil (vegetable oil) with high levels of oleic acid and linoleic acid showed positive results in lipase activity obtained by cultivating *Y. lipolytica* W29 (Lopes *et al.*, 2018). Thus, C18:n fatty acids such as linoleic acid and oleic acid could act as important substrates for production of lipase. Sustainability assessment tools such as life cycle assessment (LCA) may be conducted to further investigate the sustainability aspects of this process which utilizes waste cooking oil feedstock (Rosen, 2018). The application of LCA helps to promote the sustainable design and redesign of products and processes, leading to reduced overall environmental impacts and the reduced use and release of nonrenewable or toxic materials (Brusseau *et al.*, 2019).

## CONCLUSIONS

In this study, results showed that highest lipase activity was obtained with 2% of single used sunflower cooking oil which was  $138.86 \pm 3.32$  U/mL at 66 h of fermentation with a productivity of 2.10 U/mL/h while 1.5% of single used palm cooking oil produced a maximum lipase activity of  $101.32 \pm 4.21$  U/mL with a productivity of 1.53 U/mL/h at 66 h of fermentation. With 2% (v/v) single used sunflower oil, the highest lipase activity of 183.56 U/mL with a productivity of 3.06 U/mL/h was obtained after 60 h of fermentation by *B. cenocepacia* ST8 under batch fermentation when an agitation speed of 300 rpm was used. Meanwhile, an initial dissolved oxygen concentration of 30% was able to produce the highest lipase activity of 176.45 U/mL with a productivity of 2.94 U/mL/h after 60 h of fermentation. It was observed through the gas chromatography results that oleic acid and linoleic acid were the fatty acids which were taken up during

fermentation process for the production of lipase by *B. cenocepacia* ST8.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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