Conversion of Waste Cooking Oil to Glycerol by Halal Microbial Lipase

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Abstract. Environmental concern over discharge of waste cooking oil (WCO) has been on the rise. This is particularly alarming since the chain of fast food restaurants in Malaysia is thriving and therefore escalate the usage of cooking oil. Therefore, there is a challenge to manage the abundance of WCO generated by this industry. Interestingly, WCO presents as economical and readily available substrate for the conversion to biodiesel and surplus of crude glycerol which has numerous applications particularly in the food industry to manufacture artificial sweetener. However, concern arises among the Muslim populations on the source of enzyme lipase which is applied for the conversion of WCO to glycerol since the commercially available lipase for this purpose often originates from porcine. Therefore, the present study embarks on the concept of sustainability by converting waste cooking oil by halal microbial lipase to glycerol. Lipase from *Rhodococcus* sp. strain NAM81 demonstrated high affinity towards the substrate ($K_m = 1.9349 \% (v/v)$) and accelerated the rate of olive oil conversion ($V_{max} = 0.602 \text{mU/mg/hour}$). The findings of WCO conversion by lipase was comparable to positive control using chemical oxidation indicating the applicability of the enzyme in industry. Therefore, production of high titre of rhodococci lipase will be attempted for future study.

1. Introduction
Malaysia has established a robust position for various food cuisines, especially variable deep-fried dishes such as the popular fried bananas, French fries and fried chickens. In fact, a study conducted by Chee et al. [1] found that almost 18 participants out of 35 respondents in the survey consumed fried food three to five times per week indicating the popularity of fried cuisines. Hence, large amount of cooking oil is required in the food preparation, which simultaneously yields a similar portion of utilized cooking oil which can then be recycled. Malaysia has been listed as one of the countries producing large quantities of WCO as reported by Sanjid et al. [2] yielding close to 1 million tonnes per year. Large market in Malaysia for used cooking oil is because the price of cooking oil here is subsidised by the government making it as the cheaper starting material for the production of biodiesel [3]. According to the Star News (July, 2017), this waste cooking oil (WCO) is being exported to the European country 500 to 3,000 tonnes each time for the conversion WCO to biodiesel. Interestingly,
besides biodiesel, generation of glycerol is also encountered during the conversion of WCO which can
occur through the activity of an enzyme known as lipase [4].
Lipase is among the 15 different enzymes and their mixtures extracted from animal sources according
to European Union (EU) enzyme database [5]. This enzyme which can otherwise alternatively
produced through fermentation is widely used in the food industry [5]. The utilization of porcine
pancreatic lipase in biotransformation reactions has received widespread attention due to many reasons
namely economical in comparison to other commercially available microbial and animal lipases,
readily and sufficiently obtainable, possesses resistance to high thermal exposure and the ability to
retain activity in dry condition [6]. However, lipases from porcine are deemed non-halal since pork or
porcine derivatives are considered as haram sources [7]. In addition, lipases extracted from animal
origins have presence of bitter tasting amino acids, the presence of residual animal hormones or
viruses and the undesirable effects in the processing of vegetarian diets [8]. Therefore, halal lipases
from microorganisms serve important roles in industrial processes such as yoghurt and cheese
fermentation and this case in converting WCO to glycerol. Microbial lipase are the most persuasive
sources due to their diverse enzymatic properties, substrate specificity, chemoselectivity,
regioselectivity, stereoselectivity, ease of culture handling, availability and ease of mass production
[9].
Despite the great potential of microbial lipases, their use in industry is sometimes restricted as a result
of inconsistent yield of the enzyme when the extraction is attempted repeatedly and the occurrence of
low titre of the lipase [10]. Many recent studies have been concentrated on the use of bacterial strains
namely Acinetobacter, Aeribacillus, Aneurinbacillus, Bacillus, Burkholderia, Chromobacterium,
Colwellia, Desulfotalea, Enterobacter, Enterococcus and Geobacillus [11]. However, the needs to
screen for more lipase-producing bacterial strain is of great need to increase the potential of the
enzyme for actual application in the industry. Rhodococcus emerges as one the bacterial strains
exhibiting huge potential for the production of lipase due to its robustness and wide catabolic
expression and the presence of hydrophobic feature in this genus could greatly assist the conversion of
WCO [12]. Therefore, the present study attempted the use of locally isolated Rhodococcus sp. strain
NAM81 to produce halal lipase for the conversion of WCO to biodiesel and glycerol. Surplus
formation of crude glycerol from biodiesel production via the activity of halal lipase can be used for
subsequent application in the food industry. Purified glycerol is a high-value and commercial chemical
with thousands of uses, providing great opportunities for new applications such as in the
manufacturing of artificial sweetener. The kinetic of lipase activity and the evaluation of glycerol
produced by Rhodococcus sp. strain NAM81 were carried out in the present study.

2. Materials and methods

2.1 Chemicals
Chemicals used in this study were purchased from Sigma (USA), Systerm (Malaysia), Fisher
Scientific (Singapore) or Merck (Germany).

2.2 Bacteria and seed culture preparation
Rhodococcus sp. strain NAM81 was obtained from Culture Collection Unit of Institute of Bio-IT,
Selangor and were grown on nutrient agar (20 g/L) plates and incubated at 30 ℃. The seed culture was
prepared by inoculating a loopful of the bacteria from nutrient agar into 100 mL of nutrient broth (8
g/L) and incubated overnight in an incubator shaker (Jeio Tech SI-600R, Korea) at 30 ℃ and160 rpm.

2.3 Lipase production medium
An amount of 100 mL of minimal salt medium (MSM) was used as the production medium with
composition as described by Jayesree Nagarajan et al. [4] which was inoculated with 12 % of pre-
prepared inoculum (optical density in the range of 0.6 to 0.8 which denotes the exponential phase of
the bacterial growth) and incubated overnight at 30 ℃ and160 rpm in an incubator shaker.
2.4 Enzyme extraction
Intracellular enzyme crude was extracted by first collecting the cells by centrifugation at 4400 rpm for 30 minutes (Eppendorf 5702R, South Asia). Upon spinning, the supernatant was discarded and the cell pellets were washed with 0.05 M potassium phosphate buffer (pH 7) twice and resuspended in 2 mL of buffer. The cells were incubated with 3 mL of 0.1 % Triton X-100 for 30 minutes at room temperature after a brief vortex to soften and solubilize the aggregated cells, but only when different inducers were used. The cells were then subjected to cryogenic grinding. The samples were frozen in liquid nitrogen for 40 seconds before grinding. The cells were then transferred into a pre-cooled mortar placed in an ice box and grinded for 10 minutes continuously. The lysed samples were then centrifuged at 4400 rpm for 20 minutes. The resulting supernatant was used as intracellular crude and subjected to Bradford assay and lipase activity analysis.

2.5 Spectrophotometry for protein content and lipase activity analysis
Total protein content of the crude was measured by mixing 50 μL of the crude with 1.5 mL of Bradford’s reagent. The absorbance of the mixture was measured at 595 nm (Bio-mate 3, Thermo Scientific, USA) with 1.5 mL Bradford reagent mixed with 50 μL distilled water as blank. The resulting OD was compared with standard curve prepared for Bradford’s assay for bovine serum albumin (BSA) with concentration range of 0.125-1.5 mg/mL to determine the total protein. Lipase activity of crude was analyzed with fresh reaction substrate prepared by mixing 1:1 ratio of 0.1M Tris-HCl buffer and 420 μM p-nitrophenyl substrate. To this mixture, 200 μL of crude was added and incubated at room temperature for 10 minutes after a brief vortex to initiate reaction. After 10 minutes, the absorbance of the mixture was taken at 410 nm and the resulting optical density was used to calculate the released p-nitrophenol based on a standard curve prepared for p-nitrophenol with concentration ranging from 0.005-0.05 μmol/mL. One unit (U) of enzyme activity represents amount of lipase releasing 1 μmol p-nitrophenol per minute. Lipase specific activity is also calculated with reference to total protein content.

2.6 Kinetic study
The kinetic parameters were determined by monitoring the rate of substrate conversion by lipase extracted from Rhodococcus sp. strain NAM81 and immobilized in calcium alginate. Crude lipase was mixed with 2 % (w/v) sodium alginate in 1:1 ratio. The resulting mixture was extruded in 0.2 M CaCl2 solution overnight and subsequently washed in 0.9 % (w/v) sodium chloride before use. Crude lipase amounting to 1.025 mU/mL was used for immobilization and approximately 30 beads were used for the olive oil conversion. Kinetics parameters, namely K_m and V_max for lipase were calculated using non-linear regression fitting of the Lineweaver-Burk transformation of the Michaelis-Menten equation using Prism 5 with at least eight initial substrate concentrations.

2.7 Conversion of WCO using halal lipase from Rhodococcus sp. strain NAM81
WCO was obtained from a restaurant situated near the university and filtered to remove solid food particles before being subjected to conversion. The extracted lipase was then immobilized in calcium alginate as mentioned above using approximately 100 beads.

2.8 Statistical analysis
All experiments were conducted in triplicate to ensure the authenticity of the data in terms of error bars using Excel application. The standard errors were almost negligible and therefore were not included in the graphs. Kinetic data was analysed by employing GraphPad software version 5.02.
3. Results and discussion

3.1 Kinetic study

The conversion of substrate by lipase is primarily an intracellular enzymatic process and thus further investigations have been attempted in the present study to establish the kinetics of the biotransformation of olive oil as substrate by lipase produced by *Rhodococcus* sp. strain NAM81. Figure 1 illustrates the data of the rate of conversion of olive oil based on the substrate concentration. Experimental protocols with different initial concentrations of olive oil ranging from 0.2 % (v/v) to 1.5 % (v/v) were carried out in order to determine the maximum rate of biotransformation of olive oil and the maximum concentration tolerance of the immobilized lipase of *Rhodococcus* sp. strain NAM81 to oil olive in an agitated culture at 160 rpm.

![Figure 1. Kinetics of olive oil biotransformation by immobilized cells of *Rhodococcus* sp. strain NAM81 in calcium alginate using Michaelis-Menten kinetic model.](image)

Michaelis-Menten (Figure 1) and Lineweaver-Burk (Figure 2) graphs were plotted for the purpose of establishing the kinetic parameters for the biotransformation of olive oil. From the experimental data, the maximum specific rate of biotransformation ($V_{\text{max}}$) and the value of apparent Michaelis-Menten constant ($K_m$) were estimated at 0.602 mU/mg/hour and 1.9349 % (v/v) for immobilized lipase of *Rhodococcus* sp. strain NAM81 in calcium alginate, respectively. It was quite clear that the value of $V_{\text{max}}$ was relatively high when the technology of immobilization was adopted. This finding indicated that the catalytic conversion of olive oil improved significantly upon immobilization as greater $V_{\text{max}}$ value specifies higher rate of olive oil conversion [13].

A low $K_m$ signifies a strong binding as the $K_m$ is a measure of the enzyme substrate complex. The application of calcium alginate as the entrapment matrix might act a barrier for immediate conversion of olive oil leading to lower $K_m$ value compared to that of free cells [14] which in turned favoured for higher affinity to the substrate. Based on the collision theory equations, the apparent value of $K_m$ can be lowered when the size of the carrier is reduced leading to increased rate of formation of the products [15]. Figure 1 also revealed that the immobilized lipase of *Rhodococcus* sp. strain NAM81 in calcium alginate could tolerate high substrate concentration whereby substrate inhibition effect occurred only at olive oil concentrations above 1 % (v/v).
3.2 Conversion of WCO by immobilized lipase of Rhodococcus sp. strain NAM81 in calcium alginate

WCO obtained from restaurant nearby the university was filtered to remove solid food particles before being subjected to conversion by immobilized lipase of Rhodococcus sp. strain NAM81 in calcium alginate. Figure 3 shows the positive indication of the formation of crude glycerol as designated by the presence of dark brown solution. A positive control using chemical catalyst was performed and the results were comparable to the use of immobilized lipase of Rhodococcus sp. strain NAM81 suggesting the halal catalyst has a huge potential to be used industrially for the conversion of WCO to valuable products. These findings simultaneously provided solutions for waste management and promoted the use of green catalyst for industrial application.

**Figure 3.** Glycerol formation catalysed by rhodococci lipase.
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