

# Partial Purification of Bacterial Lipase to Be Further Used In Biofuel Production as a Renewable Energy Source

Zeynab Amini<sup>1\*</sup>, Hoora Mazaheri<sup>1</sup>, and Ong Hwai Chyuan<sup>2</sup>

<sup>1</sup>Department of Biotechnology, Faculty of Bioscience and Medical Engineering, University of Technology Malaysia, Skudai, Malaysia, Email: [azeynab2@live.utm.my](mailto:azeynab2@live.utm.my), [mhoora2@live.utm.my](mailto:mhoora2@live.utm.my)

<sup>2</sup>Department of Mechanical Engineering, University of Malaya, 50603 Kuala Lumpur, Malaysia, Email: [onghc@um.edu.my](mailto:onghc@um.edu.my)

**Abstract:** *There is growing worldwide concern for the protection of environment as well as the conservation of non-renewable natural sources, mainly petrol and fossil fuel, eventually, the potential of developing alternative energy sources in order to replace with non-renewable natural sources has received large attraction in recent years. Enzymes as a wide group of macromolecules with extensive applications in industry are found in living organisms such as plants, animals, microbes, fungi and algae/microalgae. In this study lipase, used widely in the production of biofuel as a renewable energy, was studied and partially purified from a Bacillus sp. through ammonium sulphate precipitation, anion exchange chromatography and SDS-PAGE analysis.*

**Keywords:** *biofuel, biodiesel, Bacillus lipase, ammonium sulphate precipitation, anion exchange chromatography, SDS-PAGE analysis*

## 1. Introduction

Biodiesel is an alternative, environment friendly diesel fuel produced from a lipid feedstock and an alcohol in the presence of a catalyst. Biodiesel which is fatty acid methyl ester (FAME) principally contains triglycerides (TGs) and free fatty acids (FFAs). The main reaction in the production of biodiesel is transesterification. In this reaction oil or fat react with monohydric alcohol in the presence of a catalyst such as acid, base or enzyme [1]. Biodiesel contains several benefits such as being renewable, biodegradable and nontoxic which makes it more nature friendly. Although currently, biodiesel is mainly produced by chemical reactions because of their reaction time and high yields, there are some drawbacks to chemical production of biodiesel such as glycerol recovery, removing of salt residues and high energy cost. To overcome these problems use of the lipase as a catalyst in the transesterification reaction process has recently had great attraction for researchers [2].

Microbial enzymes are attractive to be used in industrial processes because of variety of catalytic activities, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media [3]. Enzymes are extensively used in industry as biocatalysts; they are proved to be environmental and economic sustainable [4]. Lipase forming a major group of biocatalysts profoundly used in biotechnological processes, because of the far ranging of substrates, was recognized as a valuable biocatalyst in food, pharmaceutical, detergent and finechemical. Lipase is a desired enzyme to be studied in biofuel because of its biotechnological applications [3]. Lipase can be used in transesterification or alcoholysis reaction for producing biodiesel as a biocatalyst because of its properties such as being efficient, highly selective, involving less energy consumption (reactions can be carried out in mild conditions), and producing less side products or waste which makes it more sustainable and environment friendly [5]. Although enzymes used in biodiesel production are widely available at the market, the cost of

enzymes is a problem to this approach, hence finding alternatives to produce lipase in bacteria in large scale as the catalyst to be further used in transesterification reaction to produce biodiesel was the main aim of this study.

Enzymes need to be stabilized in order to improve the stability and therefore the economic viability of the process, especially for their industrial uses. Feedstock used in biofuel production is estimated to make 70-88% of the total price of biodiesel, so it is important to use raw materials with appropriate physicochemical characteristics and large availability [6]. Biodiesel has several advantages over petroleum as listed below which makes it a sustainable alternative over petroleum fuel [7]:

- a. biodegradable and a renewable resource which reduces greenhouse gas emission
- b. Reduces the dependency on petroleum crude oil
- c. Lowers combustion emission profile
- d. Improves rural economy
- e. Proper performance of the engine and preventing engine modification
- f. Low toxicity.

The main aim of this study was to shed the light on potentials of application of lipases in production of biodiesel.

## 2. Methodology

Methodology included the steps below which are explained in the following.

1. Enzyme production
2. Crude enzyme preparation
3. Anion exchange chromatography
4. Protein concentrating

### 2.1. Enzyme Production

A *Bacillus sp.* was grown on nutrient agar media for 24 h in incubator, the temperature was adjusted to 37 °C. For the seed culture, one colony per plate was inoculated into 5 ml of nutrient broth and incubated at 37 °C in a shaking incubator for 20 h; the pH was adjusted to 7. 1 ml of the seed culture broth was transferred to 1 liter of fresh nutrient broth in a shaking incubator and incubated for 20 h at 37 °C.

### 2.2. Crude Enzyme Preparation

The culture nutrient broth was centrifuged at 4 °C for 30 minutes to remove insoluble materials. The pellet was removed and the supernatant was stored at 4 °C.

### 2.3. Anion Exchange Chromatography

The concentrated dialyzed sample was loaded in on a DEAE-Sepharose Fast Flow column in AKTA-prime system for ion exchange chromatography. The unbound proteins that were positively charged as well as the target protein were eluted away when the matrix was washed with 20 mM of Tris-HCl buffer, pH 8.4. The bound proteins which were negatively charged were then eluted by 0.2 M NaCl in 20 mM Tris-HCl buffer, pH 8.0. Fractions of 2 ml were collected at 4 °C at flow rate of 1 ml/min in order to gain the best separation of protein molecule over the DEAE matrix gel.

### 2.4. Protein concentrating

Total protein concentration for sample was measured by BCA (bicinchoninic acid) assay using BSA (Bovine Serum Albumin) as a standard. Spectrophotometer was used for reading the absorbance of samples at 590 nm wavelength in order to find the concentration of proteins in the samples.

### 2.5. SDS-PAGE

Two gels were used for this analysis. 12% w/v separating gel and 5% w/v stacking gel. Separating gel consisted of 4 ml of solution A (29.2 g acrylamide, 0.8 bis-acrylamide and 70 ml dH<sub>2</sub>O) , 2.5 ml of solution B (75 ml 2 M Tris-HCL pH 8.8, 4 ml 10% SDS and 21 ml dH<sub>2</sub>O), 3.5 ml of distilled water, 50 µL of 10% w/v ammonium persulphate and 10 µL of TEMED. Water was introduced to the top of the separating gel to prevent

bubbles formation and make the top of the gel plane. After separating gel was polymerized, sandwich was poured to remove the water. 5% w/v of stacking gel consisted of 0.67 ml of solution A, 1 ml of solution C (50 ml 1 M Tris-HCL pH 6.8 and 4ml 10% SDS, 46 ml dH<sub>2</sub>O), 2.3 ml of distilled water, 50 µL of 10% w/v ammonium persulphate and 5 µL of TEMED was introduced to the sandwich and comb was inserted. Stacking gel was allowed to polymerize for 30 minutes. Loading samples consisted of 30 µL of sample solution and 7.5 µL of sample buffer. Protein marker and the sample were gently loaded onto the wells and the system was run at 150 V for 80 min. Gel was finally dyed by coomassie blue.

### 3. Results and Discussion

#### 3.1. Purification of lipase Enzyme

All purification steps were carried out at 4 °C. 1 L of overnight culture was used for the ammonium sulphate precipitation. Cells were separated from the culture broth by centrifugation and then solid ammonium sulphate was added to make 20%, 40%, 60%, 80% and 90% saturation. Centrifugation steps were done for 30 minutes at 8000 g. The precipitate gained from each centrifugation round were collected and re-dissolved in 20 mM Tris-HCl buffer, pH 7.8. The protein solution was dialyzed against the same buffer and then applied to DEAE-Sepharose fast flow column in AKTA Prime system. The system was equilibrated with 20 mM Tris-HCl buffer, pH 7.8. The flow rate was adjusted to 1 ml and it was washed at a rate of 40 ml/h with 0.1 M of NaCl in the same buffer. Active fractions were pooled and concentrated by a 10 K ultra-filtration from MWCO. It was finally freeze-dried for the further procedure. Two peaks were observed in the chromatogram but in view of the fact that the concentrations of fractions were low, fractions 35 - 44 (F1) and 45 - 53 (F2) were pooled together respectively.

#### 3.2. Protein concentrating using BCA method

In biochemical investigations and protein purification determination of protein content is an essential step. Total protein concentration for the sample was measured by BCA (Bicinchoninic Acid) assay using BSA (Bovine Serum Albumin) as a standard. The principle of BCA assay is similar to the Lowry method since both depend on the formation of a Cu<sup>+</sup> protein complex under alkaline conditions, followed by reduction of the Cu to Cu<sup>+1</sup> [8]. The amount of reduction is proportional to the protein present. Cysteine, tryptophan, tyrosine and the peptide bond can reduce Cu to Cu<sup>+1+1</sup>. BCA makes a blue complex with Cu<sup>+2</sup> in alkaline solutions. BCA assay facilitates a basis to monitor the reduction of alkaline Cu by proteins and thus determines the protein concentration in a biochemical sample [9]. The fraction corresponded to pick 1 and pick 2 were pooled and named as fraction 1 and fraction 2 and their protein concentration was measured and the highest content fractions (fraction 1 and fraction 2) contained 1.15 and 0.79 mg/ml.

#### 3.3. Protein Molecular Weight Determination

Basically, the *Bacillus* lipases of gram-positive bacteria were classified into two subfamilies including lipases that have low molecular mass (19-20 kDa) found in *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* and lipases in the second subfamily(molecular mass around 43 kDa) including *Bacillus therothophilus* and *Bacillus stearothermophilus* [10]. In this study two bands which had the molecular weight about 43 kDa and 19kDa were observed in the SDS-PAGE gel which probably correspond to lipase. However further assays for the certain identification of enzyme such as mass-spectrometry is recommended to be carried out.

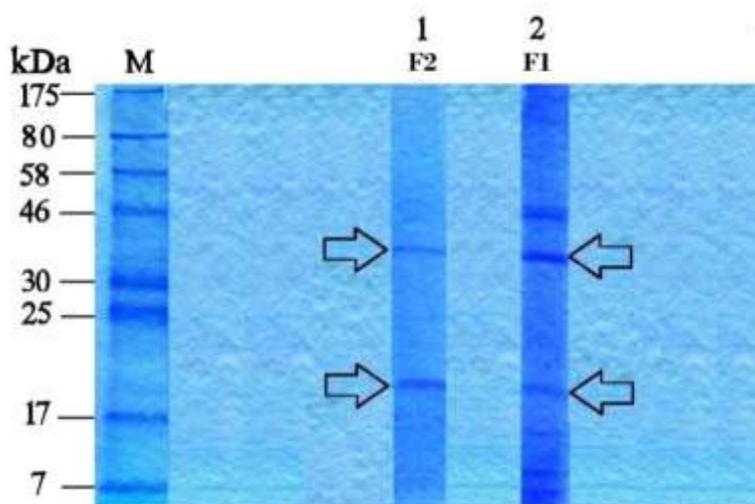


Fig. 1: lane 1: marker, lane 2: fraction 2, lane 3: fraction 1

#### 4. Conclusion

Based on the feasibility of production and purification of enzymes from bacteria, these microorganisms can be considered as great alternatives for the biofuel production. The enzymes found in bacteria with lipolytic properties (lipases) are suggested to be identified and studied for their lipolytic activities to be further used in the generation of biofuel. However, molecular methods for boosting the lipase production such as genetic engineering are highly recommended.

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