

Bioethanol Production from Empty Fruit Bunch using Direct Fermentation by an Actinomycete *Streptosporangium roseum*

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Abstract. Study on the production of bioethanol using palm oil empty fruit bunch (EFB) has been performed using actinomycete *Streptosporangium roseum*. Positive result of bioethanol production was recorded using Iodoform test followed by confirmation with GC-FID using a polar capillary column (PEG-type, 10m x 0.53, with autosampler) and n-propanol as internal standard. The first and second round distillation has produced azeotrope (85-15% ethanol-water) and the third round has concentrated the ethanol to 96.1%. Therefore, the process was accomplished by using molecular sieves that selectively absorbed the final excess water. Direct fermentation using *Streptosporangium roseum* has shown to be a very potential way to catalyst for the synthesis of bioethanol from EFB.

1. Introduction

The mainstream of oleochemical industry has moved away from the used organic solvents and emulsifiers and being replaced by a diversity of direct reactions on mixed waste-substrates and biomasses with the help of range of microbes. The product varies into forms of biofuels and bioethanols which sometimes refer to as agro fuels; the liquid or gaseous fuels derived from catalysis reaction takes place in fermentation. In the second generation of biofuels, production of bioethanols derived mostly from excess of starch crops like sugarcane bagasse, corn and paddy husks. Feedstock for bioethanol production may as well comes from other non-derived major food sources for example grasses and trees. Potential of bioethanol as fuels for vehicles is not a dream since long ago.

It can be used in many kinds of vehicles in its pure form even though its critical used is as additive in gasoline [1] that helps to improve vehicle emissions and increase octane formation. Direct fermentation perhaps, is one way to run fermentation in lesser time cycles compare to enzymatic-non-cell related fermentations. This process will start with plant materials follow by conversion to ethanol which involves identification of starting plant material, isolation and development of bacterial and fungal strains, and design of appropriate protocols for efficient conversion of plant material to sugar monomers [2]. This paper will be discussing the production of a lignocellulosic bio-ethanol as an alternative energy production of biodiesel using Malaysian local isolate in a direct fermentation using empty fruit bunch

2. Material and Methods

2.1 Substrate preparation, culture maintenance and inoculum preparation

Empty fruit bunch (EFB) was collected from The Malaysian Palm Oil Board Malaysia (MPOB). The EFB was dried in an oven at 50°C for 48 hours to reduce moisture content



followed by grinding to 0.5 mm size, sieving and weighing. The EFB was sterilized at 121°C for 20 min [3]. *Streptosporangium roseum* is a collection from our local isolated species with high lipase and cellulose activity and growing at 65°C with pH 7.0. It was maintained at 4°C on slant agar. The inoculum was prepared in nutrient broth (NB) with cell concentration maintained at 5.0×10^5 cells/ml [4].

2.2 Solid state fermentation

The solid-state fermentation (SSF) procedure was adapted from a study conducted by Lim *et al.* [10]. The 5.0 g of grinded sterilized EFB was added with 0.075 g of yeast extract which amounted to 1.5% (w/w) and 0.05 g (1% w/w) of carboxymethylcellulose (CMC), followed by inoculation of 5.0×10^5 cells/ml of *Streptosporangium roseum* cell suspension. The amount of 8.0 ml of sterile distilled water was added to adjust the moisture concentration to 160% (v/w). Using a sterile spatula, the mixture was then mixed thoroughly and incubated at 60°C. Samples were prepared in triplicates and were withdrawn daily for 7 days. A small quantity of raw and fermented EFB was observed under the Scanning Electron Microscope (SEM). The samples was first mounted on aluminium stubs and placed on a sputter coater to coat the sample with a thin layer of gold before observing under the SEM [5].

2.3 Analysis

The sugar extraction was modified from Simple Contact Method [5]. The fermented EFB was added with 100.0 ml distilled water (dH₂O) and shake at 150 rpm (2 hours, 30°C) to allow sugar diffusion into the water. Then, the mixture content was filtered using Whatman No 1 filter paper (150 mm diameter) to separate the liquid from the solid followed by centrifuging at 5000 rpm, 4°C for 30 minutes using Sigma Refrigerated Benchtop. The supernatant from the centrifugation was analyzed for glucose content method using Dinitrosalicylic (DNS) acid adapted from Suresh [6]. The same procedure was carried out with stock sugar solution for Glucose Standard Curve.

2.4 Enzyme assay

Lipase activity was assayed by olive oil emulsion according to Nik Raikhan [7]. Substrate was prepared by homogenizing 30 mL of olive oil with 70 mL of emulsification reagent [8, 9]. One unit of lipase represented the release of 1µmol of fatty acid per min respectively under the above assay condition [8]. All assays are repeated thrice to gain the mean $\pm$ sd of three applications.

2.5 Bioethanol production, iodoform test and ethanol estimation

Method has been adapted and modified from Kuan *et al.* [10]. 5.0 g of fermented EFB and 1.0 ml of deionized water were mixed in a 25 mL Erlenmeyer flask, followed by the addition of n-hexane (25%, w/w of the fermented EFB) as a co-solvent. The reaction mixture was incubated in the temperature range of 66°C using a water bath with orbital shaking at 150 rpm for 48 hours. The n-hexane was removed using a rotary evaporator (Büchi KRvrTD 65/45, Flawil, Switzerland) with the bath temperature at 60°C. Then, 1.0 ml of distillate was added into 2.5 ml iodine along with 1.0 ml NaOH in a test tube. Positive result is recorded with cloudy formation and yellow precipitation and antiseptic smell after few minutes. For the ethanol estimation, standard curve of 1-10% ethanol in double distilled water was prepared with final volume of 25.0 ml followed by distillation. 10-15 ml of distillates was added into 25.0 ml of 3.4% chromic acid. The volume was added to 50.0 ml using double distilled water. The mixture was heated at 80°C for 15 minutes and absorbance was determined at 580 nm. Absorbance reading of samples was also determined at 580 nm and the standard curve was used to decide the ethanol percentage.

2.6 Gas chromatography (GC) and distillation

Method has been modified from Helena *et al.* [11]. The GC used was a HP 5890 Series II with FID. The analyses were performed using a polar capillary column (PEG-type, 10m x 0.53, with autosampler) and n-propanol as internal standard. The temperature of the FID was 260°C, and the injector temperature was 70°C. The oven temperature was set up to 35°C for 5 min, followed by an increase to 175°C at 8°C per minute. The carrier gas was helium with a flow of 5.0 mL/min. The injection of the sample was performed using a 10 µL Hamilton syringe with a removable needle (needle gauge 22S). The volume of injection was 1.0 µL, with a split ratio of 1:50. The distillation process is illustrated in Figure 5.

3. Results and Discussion

Figure 1 depicts the growth of *Streptosporangium roseum* on solid state fermentation and concentration of glucose extracted. The actinomeycete cells feed on the inducer; CMC as the carbon source, and releasing extracellular enzymes that has the ability to breakdown and digest the polysaccharides into monosaccharide, which in this case was used to convert the empty fruit bunch (EFB) into glucose. The cellulose activity was recorded as 25.8 U/ml, quite a high amount for SSF. Grinding the EFB was done to assist the break into smaller pieces of the EFB and to increase the surface area for microorganism to act upon. In the industrial approach, the size reduction of biomass is vital to provide a large surface area for enzyme to act on during enzymatic hydrolysis [12]. Smaller size of biomass increases the accessibility of cellulosic component to be degraded by *Streptosporangium roseum*. The production of reducing sugar was monitored every day for a week. Upon centrifugation during sugar extraction, the biomass was separated and the supernatant which contained the glucose was further tested using the Dinitrosalicylic (DNS) acid test. The dinitrosalicylic acid reagent assay utilizes the dinitrosalicylic acid salt, Rochelle salt, phenol, sodium bisulphite and sodium hydroxide. The sudden heat treatment will force the reaction to a halt, as enzyme loses its activity when exposed to extreme temperature and pH. The pH of the mixture has also changes due to the addition of DNS reagent into the mixture [13]. The reducing sugars contain free carbonyl group (C=O).

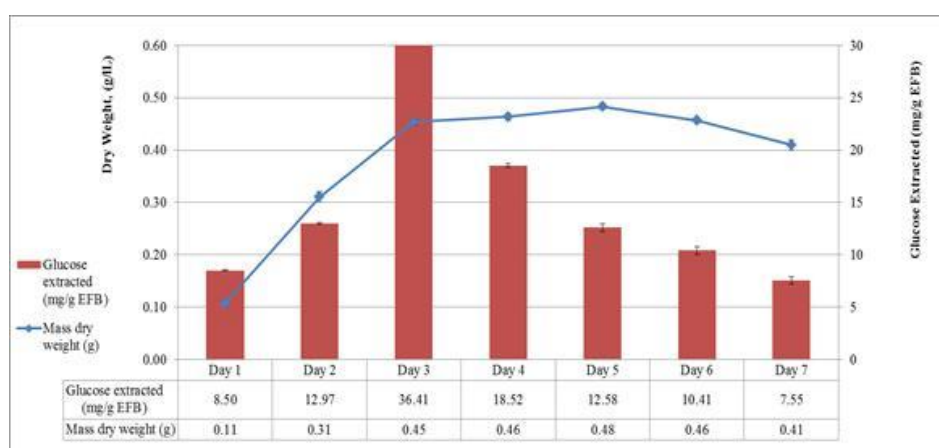


Figure 1: Growth profile of *Streptosporangium roseum* on EFB and concentration of glucose extracted for 7 days of cultivation. The solid substrate fermentation was carried out at room temperature, $60 \pm 2^\circ\text{C}$, 0.5 mm EFB, 160% (v/w) moisture content, 1.5% (w/w) of yeast extract, 1.0% (w/w) carboxymethyl cellulose, inoculum size of 2.0 ml of 1.0×10^9 spores/ml.

Figure 2(A) and 2(B) illustrates the surface area of EFB before and after fermentation, respectively. The sample was coated with metals to provide an electrically conductive layer, to restrain surface charges, and to minimize the damage caused by the radiation as well as increasing electron emission [14]. After 7 days of fermentation (Figure 2B) EFB was covered with *Streptosporangium roseum* spores with particles of produced glucose. During this stage, the *Streptosporangium roseum* has produced an enzyme that degrades the parenchyma cells that contains the cellulose and hemicellulose materials into fermentable sugars. These alterations were caused by the enzymatic hydrolysis process in which the enzyme was produced by the *Streptosporangium roseum* itself [5]. The crude lipase from *Streptosporangium roseum* has showed an excellent activity of 31.60 U/ml.

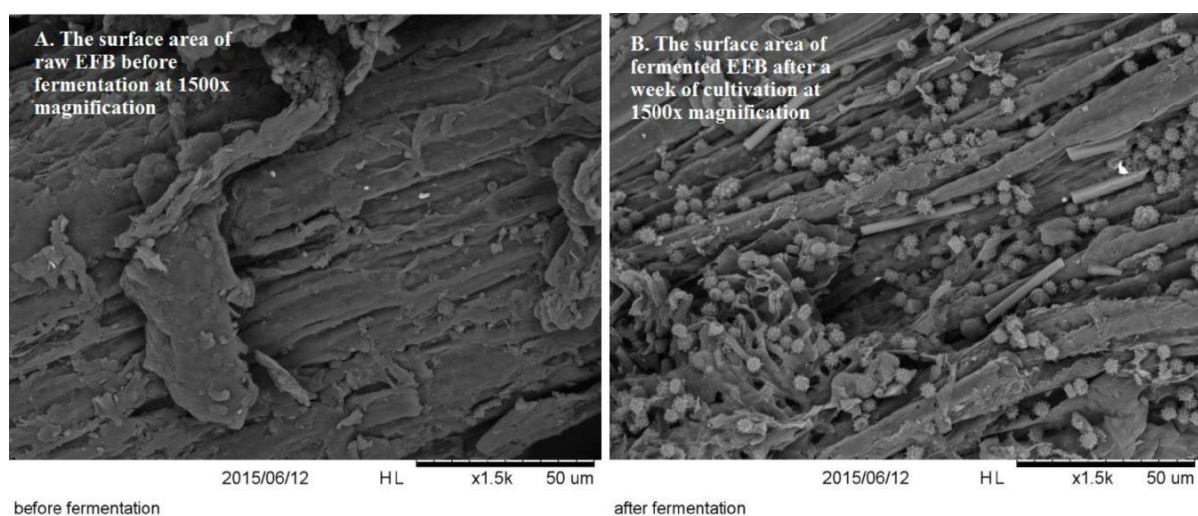
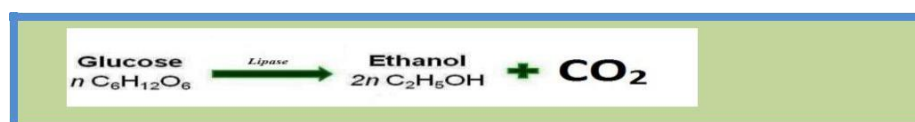


Figure 2: Scanning Electron Microscope (SEM) magnifications for (A) surface area of raw EFB before fermentation by *Streptosporangium roseum* at 1500x and (B) the surface area of the already fermented EFB with tiny particles of produced glucose or namely glucose extracted from empty fruit bunch.

3.1 Bioethanol production

Lipase from *Streptosporangium roseum* has been introduced to catalyst the journey of glucose-to-ethanol conversion through a specific fermentation with the below equation (Equation 1).



Ethanol (C₂H₅OH) produced from EFB by lipase *Streptosporangium roseum* has been detected positive with iodoform test (cloudy formation with yellow precipitation and antiseptic smell) and was confirmed by GC-FID. Chromatogram in Figure 3 shows the azeotrope of ethanol-water in the distillate sample with clear peaks. Details on the produced ethanol will be presented as quantitative data in other academic report and will not be discussed in detail here. C₂H₅OH was separated from the mash by distillation. It was a physical process where H₂O and components of C₂H₅OH were separated by differences in boiling point. The final collected ethanol in the ‘ethanol barrel’ was detected to be having a formation of an azeotrope, a mixture of two different liquids and in this case, was the mixture of the ethanol and water. The addition of deionized water into the reaction mixture was done

because lipase requires a certain amount of water to be active [15] and to catalyze the reaction. Since the mixture has a lower boiling point than either of the components, the water cannot be totally separated by a one time-run distillation process.

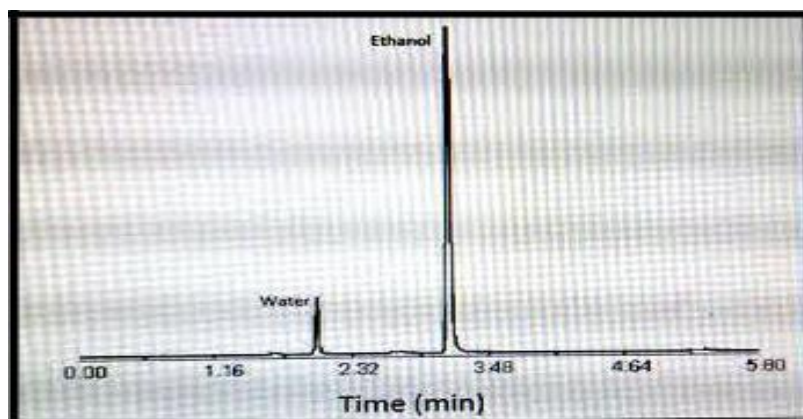


Figure 3: Representative chromatogram obtained from azeotrope of ethanol-water in the 'ethanol barrel' with peak identification and retention times of the quantified compounds.

Therefore, this extraction has been repeated 3 times with the collected azeotrope (85-15% ethanol-water). In the phenomenon of boiling, pure water boils and becomes condense as water vapor at 100°C. Since alcohol boils at 78°C, most of the vapor will be alcohol at this temperature because water does not boil until 100°C. This leaves less alcohol and more water in the azeotrope so the temperature continues to climb as the solution boils. Repeating this distillation has helped to overcome the issue. Figure 4 illustrates the process. The production of pure, water-free (anhydrous) ethanol requires a dehydration step following distillation.

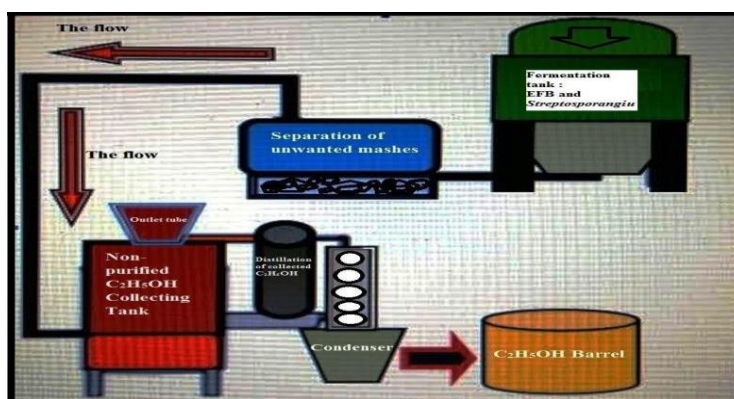


Figure 4: Distillation was used to separate a composite mixture into the base element of the produced ethanol. The process has involved the change of state from liquid to gas, and then subsequent condensation to return the now separated ethanol into liquid state again.

Dehydration, a relatively complex step in ethanol fuel production, is accomplished using molecular sieves that selectively absorb water on the basis of the difference in molecular size between water and ethanol to concentrate the ethanol from 96.1% to 100%. The percentage of ethanol and water products is subjected to minor changes depending on the lipase efficiency and then followed by the distillation process. Theoretically, the maximum conversion

efficiency of glucose to ethanol is 55-57 percent on a weight basis. However, some glucose is used by the lipase enzyme for the production of metabolic products other than ethanol. In practice, between 42 to 49 percent of glucose could be converted to ethanol. By taking a 45%-fermentation conductivity of lipase *Streptosporangium roseum* in catalytic efficiency, 1,000 kilograms of fermentable sugar (glucose extracted from EFB will be producing about 570 liters of pure ethanol. This means if we are aiming for 1,000 liters of ethanol, we will need to use about 1,800 kilograms of EFB. The whole mash or fermentation products typically will contain between 50 and 100 grams of ethanol per liter which is about 5.0 to 10% weight per volume assuming that fermentation is a 100% complete.

4. Conclusion

The present research shows that EFB has the potential to be developed as an economical alternative energy source in a glucose form using lipase *Streptosporangium roseum*. This lipase has shown to be a very potential enzyme as catalyst for the synthesis of bioethanol and has been proved to catalyze the system successfully. The team is now working with the properties of the synthesized biodiesel, and planning for entrapping the enzyme for reuse method.

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