

Optimization of Pre-Treated Oil Palm Frond Producing Bioethanol via Fermentation with *Escherichia Coli* KO11

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Abstract — Bioethanol as a fossil fuel substitute has drawn much attention as an alternative transportation fuel. Thus, the need for an alternative fuel from renewable resources contributes the sustainability that possibly to replace our dependence on fossil fuel. In this study, in order to investigate the high possibility of yield efficiency of bioethanol production will be mark on pre-treatment parameters. Ionic liquid (IL) pre-treatment has shown great potential as a novel pre-treatment technology with high sugar yields. It is an economical bioprocess to produce the fermentable sugars at laboratory scales using Oil Palm Frond (OPF) as substrate in Simultaneous Saccharification and Fermentation (SSF). The tolerance of *Escherichia coli* KO11 will be analyzed in SSF through oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER). The oil palm frond (OPF) pre-treat has been carried out using 1-ethyl-3-methylimidazolium chloride [EMIM][Ac] with different concentration content. The analysis of using from intermediate to low concentration of ionic liquid in the high production of bioethanol and the gas chromatography – flame ionization detector for bioethanol composition will be investigate.

Keywords—Ionic Liquid, Pre-Treatment, Simultaneous Saccharification and Fermentation, XRD, FTIR, RSM, GC-FID.

I. INTRODUCTION

Regarding to world issues on depleting fossil fuel, we are ought to face an arduous demands to find the alternatives way to cover up the existing petroleum sources that soon will be spend. A lot of efforts are in action to reduce these impact of global concerns, bioethanol is one of the production from the renewable resources that has created significant interest replacing the fossil fuel (Deenanath, 2013). Production of bioethanol has been continually increase since the last few years and it is a shocking moment when it has reached around 88.7 billion liters in 2011.

Bioethanol is mainly produced by the sugar fermentation process, even though it also can be manufacture by chemical process of reacting ethylene with steam. The main source of sugar required to produce ethanol comes from energy crops and other generation source. These fuel crops are normally grown specifically for energy use and include maize, corn and willow, wheat crops, sawdust, waste straw, reed canary grass, myscanthus, oil palm frond, oil palm trunk and sorghum plants. Bioethanol produces only carbon dioxide and water as the waster products on burning, and the carbon dioxide is released during the fermentation

process and the combustion as well equals to the amount removed from the atmosphere while the crop is growing.

The fermentation process from lignocellulosic materials that ideally converts cellulose into glucose monomers by microorganisms. The native cellulose is very protected by lignin matrix due to the strong existing connections (D'Almeida, 1988). The arrangement of these components inside the biomass makes it more complex structure than others (Alvira P, 2010). In order to improve the bioethanol production especially the yield, some trend has been to introduce a pre-treatment in the process that facilitate the hydrolysis mechanism and achieve a high rates of saccharification. The saccharification is typically accomplished by undergoes enzymatic hydrolysis process that has a high selectivity of enzymatic catalysis which gives a high sugar yields.

A pre-treatment process is a process that required to separate the lignin and hemicellulose contents from the cellulose nor dissolution where it is reduce the cellulose crystallinity and increase the porosity of the structure, hence improving the cellulose hydrolysis (Kuo, 2009). However, many pre-treatment method require harsh conditions and give rise to by-products, which decrease the sugar yields and inhibit the enzymatic hydrolysis and microbial biocatalysts. In recent times, ionic liquid has been discovered and widely used to dissolving cellulose structure. Moreover, it also offers several advantages over the conventional acid pre-treatment method (Nur Aainaa Syahirah Ramlia, 2014)

In this research theory, there are sort of several aspects that need to be attained and investigate the study of yield effectiveness of bioethanol production. The analysis of the biomass pre-treatment process by using bio-ionic liquid reactor where the main show is the presence of ionic liquid in pre-treatment process is been analyzed. Theoretically, the previous study investigate the uses of ionic liquid as they give a high efficiency of bioethanol production as the raw material been disrupt into main component efficiently and undergoes the fermentation process very well. The fermentation involved is the simultaneous saccharification and fermentation. The specific strain of *Escherichia coli* KO11 which is a recombinant bacteria being studied to investigate their effectiveness for utilizing the glucose in fermentation broth and bioethanol production. The high tolerance of the microorganism used is investigated as the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) being analyze. The fermentation parameters (OUR and CER) relatively monitored via BlueSens monitoring device.

II. PROBLEM STATEMENT

A. Demands of Bioethanol Production

Transportation energy is one of the most important aspects of a country's security and economic growth. As such, it presents a great opportunity to sustainable development in its future. The rising fossil fuel prices associated with growing demand for energy and environment are the main aspects that drives the high interest of renewable energy sources especially in biofuel.

Biofuel or also known as bioethanol which includes solid biomass, liquid fuels and various biogas is one of the most rapidly growing renewable energy technologies in recently. In Malaysia, the manufacturing bioethanol from agricultural and forestry waste is one of the ways Malaysia is hoping to reduce its share of carbon dioxide emissions (Choong, 2012). CO₂ is produced in the combustion of the fuel where it creates an obvious advantage over fossil fuels which only can emit CO₂ as well as other poisonous emissions. Blending bioethanol with gasoline also help to reduce greenhouse gases (GHG) emissions by oxygenate the fuel mixture so it burns more completely. Bioethanol can serve a wide range of uses in the pharmaceuticals, cosmetics, beverages and medical sectors as well as for industrial uses. The market potential for bioethanol is therefore not just limits to energy production or transport fuel but has another potential to supply the existing chemicals industry (H'ng, 2013).

B. Proposal of Ionic Liquid as Pre-Treatment

Ionic liquid has attracted high interest for the last decades with a diversified range of applications. The ionic liquid-based pre-treatments are significantly use for better yield fermentation that role as to disrupt the lignin complex within biomass or dissolution and generate a product that is readily to converted into fermentable sugars that suitable for the production of advanced biofuels. The findings provided of ionic liquids as tools to perform biomass pre-treatment and the advantageous use of their specific properties over the conventional pre-treatment processes (Andre M da Costa Lopes, 2013). Herein, the analysis of pre-treatment efficiency by using ionic liquid at different concentrations are investigate.

Theoretically, many researchers analyze the use of ionic liquid of pre-treatment at 70% and above concentration. It is a difficult to find the literature of below 70% of ionic liquid in pre-treatment being use. This is because ionic liquid relatively incurred a high cost due to their efficiency to produce high yield of bioethanol production. According to researcher studies, ionic liquid with cation especially EMIM cation has better relation with high flexibility and low in viscosity compared to BMIM cation. This is because the EMIM cation contributes high potential in dissolution of cellulose rather than BMIM cation. EMIM cation consists of chloride and acetate ion. In this case, acetate ion is more preferable to be used in industries compared to chloride due to chloride's properties that need high maintenance and other solving problem to overcome the issue. Hence, EMIM acetate or [EMIM][Ac] is favorable to be used in the pre-treatment process.

C. Tolerance of *Escherichia coli* KO11

Over decades, *Escherichia coli* has been studied regarding the multitude of factors that determine its physiology and the different phenotypes it can adopt. Moreover, it is not surprising that *Escherichia coli* has been widely used by applied microbiologists to try to steer the metabolism of this organism toward the biotechnological production. *Escherichia coli* have been reported as the highest contribution of yielding through the fermentation. Nevertheless, the yields and titers from the microbial fermentation

is usually held back by the accumulation of toxic end-product ethanol. As such, it is important to have the high tolerance of ethanol-tolerant microbes for large-scale bioethanol production.

III. METHODOLOGY

A. Oil Palm Frond (OPF) as Raw Material

All the oil palm biomass have a high content of cellulose, hemicellulose and lignin as well. These oil palm biomass is classified to 50% cellulose, 25% hemicellulose and 25% lignin contents respectively. Through researcher studies, the chemical composition between empty fruit bunch (EFB) and oil palm frond (OPF) are quite the same but the difference are only in their cellulose content. It assures that oil palm frond was the most popular among other oil palm biomasses due to their small percentage of undesired component composition such as lignin and ash. Hence, the high percentage of cellulose and hemicellulose are in benefit. This is because the cellulose will undergoes enzyme hydrolysis process where contributes to high yield of many production.

B. Ionic Liquid as Pre-Treatment

[EMIM][Ac] is used for pre-treatment of the untreated OPF. Before starting the pre-treatment process, the untreated OPF is analyze first with XRD and FTIR. The result of untreated OPF will compare with treated OPF.

Pre-treatment at different temperature of 90°C, 110°C and 130 °C for 3 hours			
Ionic Liquid Concentration	20%	40%	60%
Analysis Sampling (XRD and FTIR)	After biomass is treated, then every sample will be analyze with XRD and FTIR		

Table 1: Pre-Treatment at Different Parameter

C. Preparation of Agar and Broth Culture

The preparation of Luria Bertani medium involve two way of preparing which are agar and broth. The Luria Bertani agar is in solid form whereas Luria Bertani broth is in liquid form. These Luria Bertanis medium act as preparation for *Escherichia coli* KO11 (ATTC55124) to growth and make some new colony. In Luria Bertani agar, there are two concentration of chloramphenicol need to be prepare which are 40 mg/L and 600 mg/L. For Luria Bertani agar with 40 mg/L, 20g of Luria Bertani agar is mixed with 400 mL of distilled water and at the side, prepare 10g of glucose mixed with 100 mL of distilled water.

These separated preparation are then autoclaved for 3 hours at 121°C. After autoclave for 3 hours, the prepared agar and glucose are mixed up, then cooled for 2-3 minutes. After cooled down, 0.5 mL of chloramphenicol is added by using syringe. Previous time, the chloramphenicol that need to add in the mixture of agar and glucose is been added with 10 mL of 70% ethanol, to ensure the resistance of microorganism. The combined agar, glucose and chloramphenicol is then poured into petri dish. Then sealed and stored in freeze refrigerator at 4°C.

For Luria Bertani with 600 mg/L steps preparation also almost the same with 40 mg/L agar. The only difference between 40 mg/L and 600 mg/L is in 40 mg/L, the contents of chloramphenicol mixture is 0.4g of chloramphenicol with 10 mL of 70% ethanol whereas in 600 mg/L, the chloramphenicol mixture is 6g of chloramphenicol with 10 mL of 70% ethanol. In Luria Bertani broth, the preparation of the broth are prepare 12.5g of Luria

Bertani agar with 400 mL of distilled water and 10g of glucose with 100 mL of distilled water. Then these two separated preparation are autoclaved for 3 hours at 121°C. After 3 hours of autoclave, combine the agar and glucose, then cooled for 2 -3 minutes. Then after being cooled, placed them in incubator shaker at 37°C.

Agar		Time
1.	Prepare 40 mg/L and 600 mg/L respectively	-
2.	40 mg/L is streak with <i>Escherichia coli</i> KO11 (ATTC55124)	After streaked wait for 12 hours
3.	600 mg/L is streak with new grown colony <i>Escherichia coli</i> of 40mg/L that has been streaked previous 12 hours ago	After streaked wait for 12 hours
4.	The new colony in 600 mg/L will inoculate into broth medium	After inoculated, wait for 4 hours to get absorbance reading and then place them in shake flask

Table 2: Preparation of Agar Medium

D. Online Monitoring

A modern gas analysis has been introduced to the industry that allows for controlling the fermentation process in real-time and enables to get a greater insight the performance of your microbial cultures. The analyzer for oxygen and carbon dioxide has been designed for monitoring the cell growth processes in biotechnology. The shake flask experiment one of the development from BlueSsens that has been carried out for pre-culture conditions optimization. The software can visualize the result and calculate the main parameters that compromise with oxygen uptake rate (OUR) and carbon dioxide emission rate (CER) on both flask fermenters as well. It also has a better look in visualization of the process performance, increase of reliability and repeatability, better system optimization without limitation of the source and has a good prediction on scale up range.

E. Standard Curve of *Escherichia coli* KO11

In order to make a standard growth curve, 10% of 50 mL working volume with Luria Bertani broth which is mixed with glucose as a food source is being prepared and the growth curve of *Escherichia coli* is monitored by online monitoring software for the next 24 hours. By performing the standard curve of *Escherichia coli*, it is considered as the most important process before starting the fermentation process. This is because the standard curve of *Escherichia coli* will show the conventional bacteria growth curve and will be compare with the next experimental fermentation. Moreover, by performing the process it is easy to know as the fermentation is processing correctly just by looking at the fermentation reading whether by online monitoring software or absorbance reading from spectrometer as it is really grow at the right path. Hence, the standard curve of *Escherichia coli* need to perform correctly in order to eliminate the error reading for the next performing fermentation.

F. Washing Method

The treated OPF samples with all concentration will undergoes four times wash by centrifugation. The four times mixing of treated OPF with distilled water is centrifuge intervally. This to ensure there is no ionic liquid content in the treated OPF. After centrifugation, the treated OPF will be analyze by XRD.

G. X-Ray Diffractometer

XRD technique is applied before and after the pre-treatment of OPF. This technique is employed to identify the crystalline structure of the material and can provide an information on unit cell dimension. To start the XRD technique, firstly placed the sample on the glass plate and flatten the surface. It is to ensure for x-ray beam is easy to inject the surface. Then, the glass plate is placed inside the XRD equipment and set the condition in Rigaku software via computer connected. The voltage is 40 kV at current of 26 mA. The angle is $2\theta = 5^\circ - 35^\circ$ and 2.5 minutes per 2θ intervals. In order to calculate the crystallinity index, the peak height can be calculated by below equation.

$$CrI_{2\theta} = \frac{I_{2\theta} - I_{am}}{I_{2\theta}} \times 100\%$$

Where; $I_{2\theta}$ is highest peak and I_{am} is lowest peak.

H. Response Surface Methodology

In order to design the advanced set of experiment, the chosen method used is Box-Behnken. This is because the designs are only requiring fewer parameters compared to central composite design. The box-behnken design works in rotatable which contains the region of poor prediction quality like the CCL. Those hidden region may be used when there is occurrence of combined extreme factors. Hence, this design promotes the prevention of potential loss of the data in any cases. The design can be easier to make by using RSM software or known as Statistica where the box-behnken design can be implemented. The sample data is calculated by equation below as the equation will give the graph with curve line. If the crystallinity index number is not be divided, the graph will give the flat graph or with no curvature.

$$\frac{1}{CrI_{XRD}}$$

I. Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy or best known as FTIR is one of the most important techniques used to identify the polymeric and others material. It is based on the interaction between the material and electromagnetic radian of wavelengths in the infrared region (13300 – 20-1). The particle size of the sample should be less than 2 μm . For sample preparation, the standard method to prepare the sample is need to mix with KBr but for treated OPF, the sample is no need to mix with KBr. The sample is placed onto the glass plate and flatten the surface. Then, the sample is squeezed to form the transparent disc so that it can be measured directly.

J. Optimum Condition of Pre-Treatment Parameter

The treated OPF that already processed by washing method, all samples will be taken to the XRD analysis in order to get the crystallinity index number by XRD data. The crystallinity index data by all samples will be key in into software of response surface method (RSM) or known as Statistica to get the optimum condition for pre-treatment process. The pre-treatment, washing method process, XRD and FTIR analysis are then repeated again with the new optimum condition. The treated OPF of optimum condition will be used for the next process which is the fermentation.

K. Simultaneous Saccharification and Fermentation

In simultaneous saccharification and fermentation process or known as SSF, 500 mL of Erlenmeyer flask BlueSens are carried out with the mixing of several main items. 1 g of pellets of treated OPF is used for the fermentation process. The 50 mL working volume of Luria Bertani broth that has prepared beforehand is placed inside the shake flask. The subsequence of cryogenic culture of *Escherichia coli* KO11 (ATTC55124) that already inoculated inside the broth is prepare to undergoes the enzyme hydrolysis when the cellulase enzyme is been added.

These three mixture of 1g dried pellet treated OPF, cellulase enzyme and inoculated Luria Bertani broth are mixed for saccharification and fermentation process. At the first stage, the saccharification process is take place where the enzymatic hydrolysis is occurred between the treated OPF, inoculated broth and cellulase enzyme at 38°C for 12 hours. After 12 hours, the fermentation process is then carried out. The mixed broth is frequency shake at 250 rpm for 24 hours. As the fermentation start to begins, the BlueSens sensors are detecting the performance of oxygen and carbon dioxide in the shake flask through FermV software.

L. Gas Chromatography-FID

Gas Chromatography-Flame Ionization Detector or known as GC-FID is an analytical technique to analyze the volatile organic compounds or semi-volatile compounds aiding with hydrogen at 35 psi or air flame where sample is passed to oxidize organic molecules. Normally, instead of hydrogen, the helium gas at 80 psi and air at 60 psi also employed but it depends on the industry and laboratory needs. For GC-FID procedure, the sample which in aqueous form is inserted inside the small bottle and placed them in the provided rack at the upper body of GC-FID. The condition is set up through the Chrome-Card for Trace-Focus GC Data System software. The chosen methods and sequences inside the software have been selected. Then, shut the file and turned on the standby button. After the result came out, the data is collected and burned into disk.

IV. RESULTS AND DISCUSSION

A. Effectiveness of using Ionic Liquid as Pre-Treatment

1) Response Surface Methodology

At an early pre-treatment process, the oil palm fronds were investigated by different parameters of ionic liquid concentration, temperature and weight percentage. These parameters are the main factor design variables that can be implement through response surface methodology software or called as Statistica. In this software, the box behnken design has been chosen to design the set of experiment. The factor variables involved are ionic liquid concentration that compute between 20% of concentration until 60%, different temperature parameter ranging at minimum of 90°C and maximum at 130°C and oil palm frond weight percentage from 5 wt% to 15wt%. The calculated critical value at the optimum condition for oil palm frond parameter is falls at the 46.87994% of ionic liquid concentration, 11.57248 of oil palm frond weight percentage and temperature at 93.688°C. To standardize the critical value, the optimum condition is stated as table below. These values are being used to repeat the pre-treatment process and all analysis before undergoes the fermentation process.

Ionic Liquid Concentration, %	Temperature, °C	OPF, wt%
46	94	12

Table 3: Optimum Condition

After the critical values have been calculated, the graph of polynomial relationship is plotted. The polynomial relationship is referring to the distribution surface of factor variables given can be optimize and reach the optimum condition. As the distribution shows the surface with no curvature, it means the factor variables are difficult to be optimize whereas if the distribution surface is at hill with curvature the factor variables are said to be in line of optimum condition and can be improve until reaching the best critical values. The plotted graph below shows the distribution polynomial give a curvature distribution as the factor variables are success to be optimized. In order to create the graph of desirability distribution surface, the method used is spline fit.

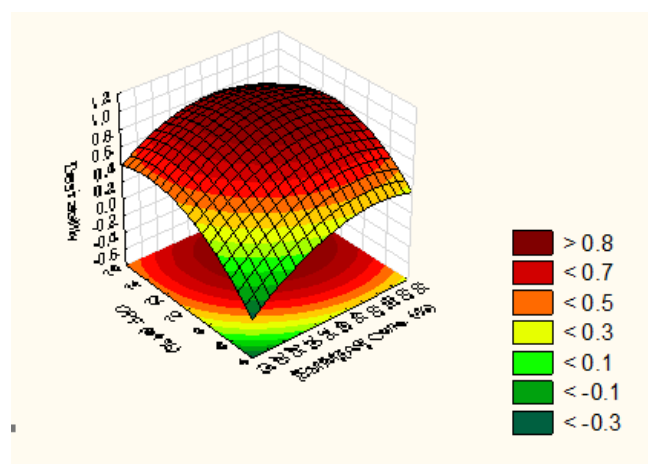


Figure 1: Desirability Surface of OPF factor variables

2) X-Ray Diffraction

After the XRD process end, the computer connected give a graph with several peak that indicates the crystallinity of the component inside the material. The voltage used is 40 kV at the current of 26 mA. The angle is $2\theta = 5^\circ - 35^\circ$ and 2.5 minutes per 2θ intervals

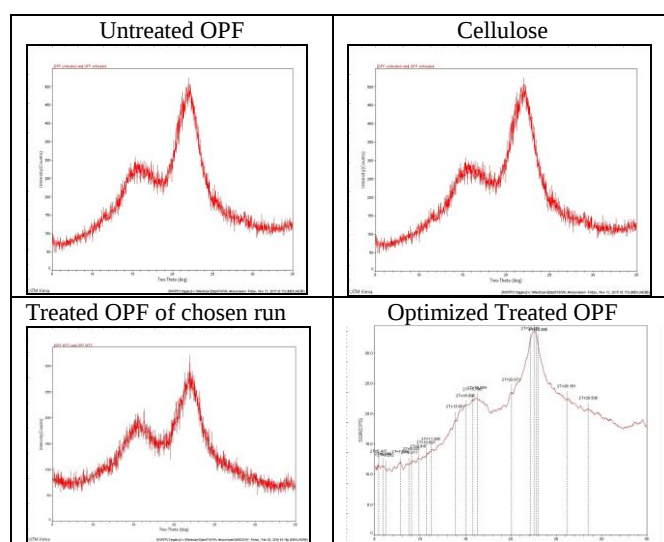


Figure 2: The Graph of Crystallinity Index Intensity

Each graph of crystallinity index number has been calculated. The calculated value is showing the crystallinity of the sample itself to be at their loose or still in crystal structure. For untreated OPF and cellulose, the percentage of crystallinity index number are assume to be high as the samples are not being treated with any pre-treatment to dissolubilize their structure. The treated OPF at chosen run of 20% ionic liquid concentration, 12wt% OPF and at temperature of 130°C.

Sample	CrI %
Untreated OPF	91.8%
Cellulose	95.65%
Treated OPF at 20 IL %, OPF 10 wt%, 130°C	82.5%
Treated OPF at Optimum Condition	84.76%

Table 4: Percentage of Crystallinity Index Number

3) Fourier Transform Infrared Spectroscopy

Regarding to the pre-treatment effectiveness, the dissolution of cellulose content can be measure by the FTIR analysis as to quantify the percentage of cellulose and their bonds stretch involved. These chemical functional groups can be observed by indirect indication of the carbonoxygen-hydrogen bonds of oil palm frond composition. Throughout the pre-treatment progression, the increment of cellulose content should be observed by the successful dissolution and the wavenumber shown where the wavenumbers involved indicates the possible functional groups present.

For treated oil palm frond at optimum condition, the FTIR analysis is focusing by broad band of from 620 to 3400 cm^{-1} . For the first spectra region, the wavenumber observed at 3336.73 cm^{-1} where the wavenumber range shows the O-H bonds in the cellulose content. For second region, the wavenumber is 1566.54 cm^{-1} where it gives indication of C=C stretching of the aromatic ring in lignin composition. In the third region, the observed wavenumber is in the range of 1401.74 cm^{-1} which the possible functional involved is C-H stretching of aromatic ring in cellulose composition. For the fourth region, the wavenumber indicates the C-H₂ wagging in the cellulose which lies in the 1335.87 cm^{-1} . The fifth region is implies the lignin composition where the behavior as same as the second region. The sixth and seventh region which are 1166.57 cm^{-1} and 1033.35 cm^{-1} respectively indicates the C-O-C asymmetric stretching in cellulose.

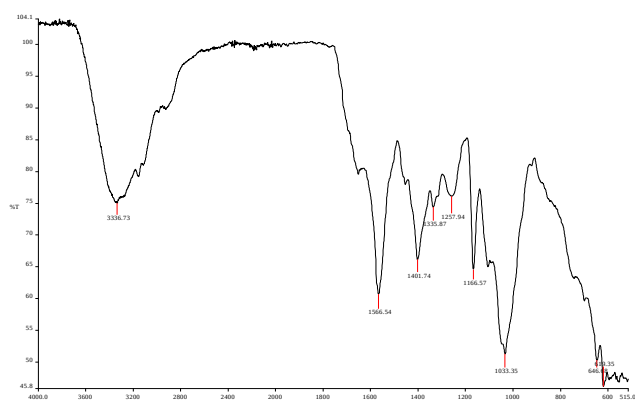


Figure 3: OPF at Optimum Condition

As to compare with untreated oil palm frond, the FTIR analysis observed the peak region of the broad band wavenumber is slightly different with the optimum condition but still almost at the same possible functional groups. The comparison spectra region can be seen in the figure 4. The region involved in the untreated OPF compromises the O-H stretching in the cellulose, C=C in lignin,

C=C stretching of the aromatic ring in the lignin and C-O-C asymmetric stretching in the cellulose.

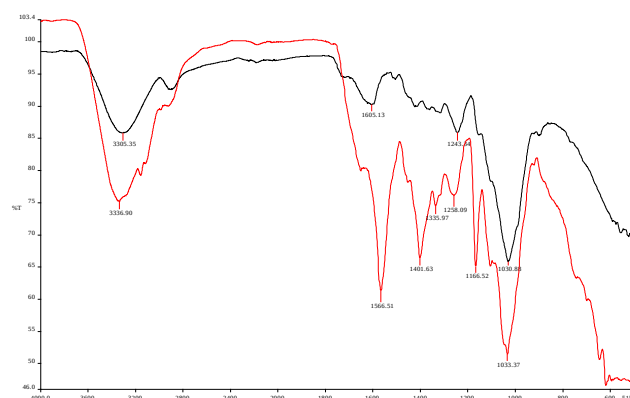


Figure 4: Untreated OPF and OPF at Optimum Condition

B. Standard Growth Curve of *Escherichia coli* KO11

In order to perform the standard growth curve of *Escherichia coli* KO11, the 500mL shake flask is used with the 10% of working volume of *Escherichia coli* KO11 that been inoculated by the best 3 colonies full loop streak from the agar plate. The duration of fermentation process is 24 hours in incubator shaker equipped with BlueSens online monitoring at the temperature of 37°C. The figure below represent the standard curve of *Escherichia coli* KO11 within 24 hours fermentation period. The plotted graph is based on the absorbance reading by spectrometer with the wavelength of 600nm with time.

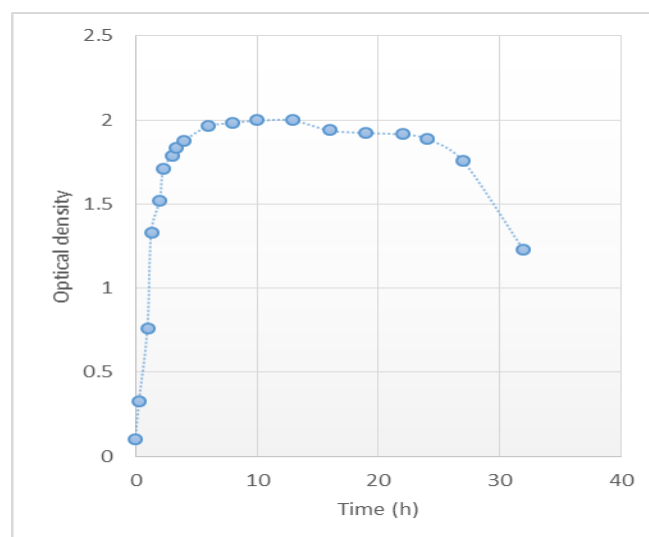


Figure 5: Standard Growth Curve of *Escherichia coli*

The graph indicates the bacteria is passing through each phase from undergone lag phase until death phase. The first hour shows that the bacteria is undergo the short lag phase and rapidly growth after 1 hour. The bacteria begins to grow steadily until the first 3 hour and its reach their maximum exponential or in order word is at their most active activity. Within the growing exponential phase, the bacteria is keep feeding on the oxygen and the glucose provided as nutrient source. The present of growing exponential phase indicate the success of cultivation of bacteria as the bacteria is managed to survive and adapt to the new environment. After 4 hours passes, the bacteria growth is said to at it maximum state where the bacteria can work their best for any conversion. At this state, the fermentation process shall be continue with the aid of active bacteria. It easy for the fermentation process to convert the sugar to the production of bioethanol.

C. Simultaneous Saccharification and Fermentation

1) Simultaneous Saccharification and Fermentation of Cellulose as Food Source

By performing the fermentation with the aid of cellulose as the direct raw material, the conversion towards bioethanol also can be used to be the standard parameter along with the graph of standard growth curve of *Escherichia Coli* KO11. The graph online monitoring shows the relation between oxygen uptake concentration in blue line by the bacteria in order to convert the raw material into product and the carbon dioxide releasing in red line. As the graph plotted automatically by BlueSens sensor shows the oxygen uptake is consumed vigorously by the bacteria at the exponential phase. This is because the raw material itself is the main food source of bacteria and the substrate concentration always at the maximum concentration.

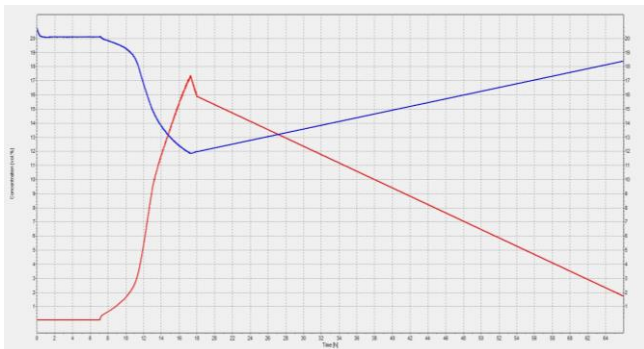


Figure 6: Online Monitoring Fermentation with Cellulose of Oxygen Uptake Concentration and Carbon Dioxide Releasing Concentration

The time length for bacteria to convert the substrate into end product is in the short time. This shows in the graph the lag phase only requiring at least 6 to 7 hour. The exponential phase starting at early time and incline rapidly towards the high peak of active phase at 17 hour. As to compare with the standard curve *Escherichia Coli* KO11, the growth curve at exponential phase is taking only 4 hours but in this fermentation it takes up to 10 hours before entering the stationary phase and death phase. Hence, the conversion of bioethanol is easily to be convert at the short time and producing high yield.

2) Simultaneous Saccharification and Fermentation of Treated OPF

The oil palm frond is a lignocellulosic biomass that commonly known has the high composition in cellulose which can be used to convert into bioethanol efficiently compared to other lignocellulosic biomass. The treated oil palm frond by ionic liquid pretreatment enhance the dissolution of cellulose present in the oil palm frond composition. However, the concentration of cellulose present in the lignocellulosic biomass could be in small percentage and the other composition such as lignin and hemicellulose can interrupt the conversion of the glucose from cellulose enzymatic hydrolysis into desired end product. As the fermentation start after enzyme hydrolysis and added together with bacteria at active phase has plotted the graph automatically by BlueSens sensor. The graph shows the oxygen uptake is consumed steadily by the bacteria at the exponential phase. However, when it comes to duration of fermentation process, it shows that the time length for bacteria to convert the substrate into end product is taking a bit longer compared to in standard cellulose fermentation. This shows in the graph as the lag phase requiring up to 14 hour. The exponential phase start slowly and steadily increase towards the high peak of active phase at 28 hour. However, as to compare the growth rate within the fermentation and the standard curve *Escherichia Coli* KO11, the fermentation process takes up to 14 hours which is extra

4 hours compared to cellulose fermentation before entering the stationary phase and death phase. Hence, the conversion of bioethanol is still producing at high yield but lack in duration conversion from the substrate into desired product due to inhibitor by product and adaptation towards harsh environment condition.

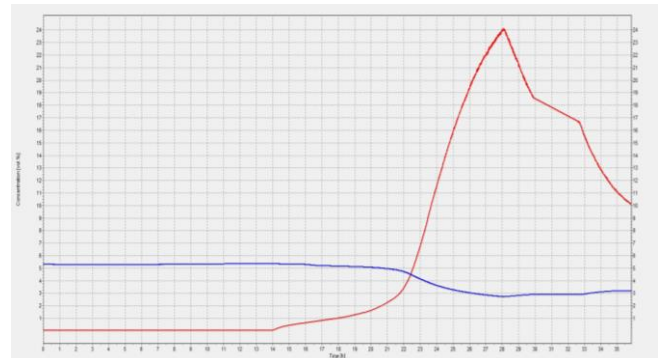


Figure 7: Online Monitoring Fermentation with OPF at Optimum Condition of Oxygen Uptake Concentration and Carbon Dioxide Releasing Concentration

3) The Comparison of OUR and CER between Cellulose as Food Source and Optimized Treated OPF

Based on the OUR and CER plotted on both cellulose and treated oil palm frond fermentation, the graph shows the oxygen uptake and carbon dioxide releasing for bacteria growth's benefits. The significant of sudden increment of OUR shows the bacteria has greatly consumed the oxygen for their growth and promotes to their exponential phase. For CER, the sudden decline may due to the accumulation of carbon dioxide and resulting from utilizing oxygen and glucose hydrolysis. The blank before lining of OUR and CER is happened due to the values were too small where the BlueSens system is not able to detect and read the data. In cellulose fermentation, the OUR at exponential phase start at 0.01 mol/Lh whereas the treated OPF start at 0.003 mol/Lh. At the end of exponential phase which is also at their active stage, the OUR for cellulose fermentation is 0.07 mol/Lh whereas the treated OPF at 0.022 mol/Lh. For CER, the increment rate within cellulose fermentation start at 0.007 mol/Lh and their highest accumulation of carbon dioxide at 0.12 mol/Lh. In treated OPF fermentation, the accumulation of carbon dioxide start at 0.0035 mol/Lh whereas the highest accumulation observed in the graph is 0.18 mol/Lh.

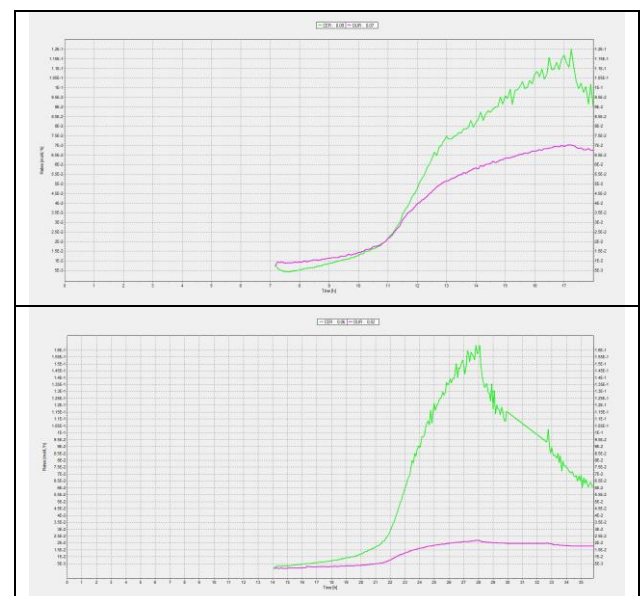


Figure 8: OUR and CER between Cellulose (1) and Optimized OPF Condition (2)

4) Respiratory Quotient (RQ) Comparison between Cellulose as Food Source and Optimized Treated OPF

The respiratory quotient (RQ) is the measurement of the ratio volume of carbon dioxide produced by the bacteria to the volume of oxygen consumed. This quotient is useful as the volume of carbon dioxide and oxygen produced depends on which fuel source is being metabolized. By measuring RQ is convenient as a way of to gain information about the source of energy for bacteria being used. Within cellulose fermentation, the highest RQ value is 2.5 at 17.2 hour whereas in treated optimum condition OPF the highest RQ value is 7.5 at 28 hour. At these hours of highest RQ value, the graph shows that the recombinant culture cell generating carbon dioxide at highest and consumption of oxygen is low.

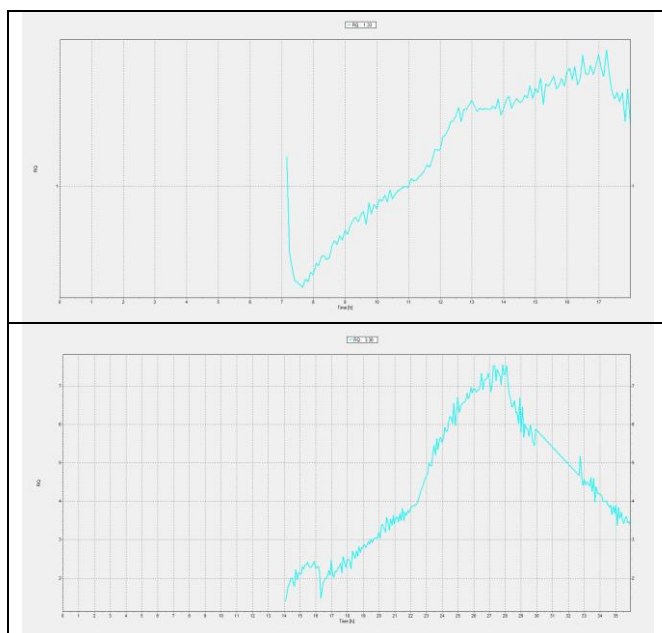


Figure 9: Respiratory Quotient (RQ) between Cellulose (1) and Optimized OPF Condition (2)

5) Gas Chromatography – Flame Ionization Detector

The GC-FID is one of the analysis that can offers a wide range of detection between 1 to 30% v/v. This analysis also has proven being one of the most suitable for fermentation process especially the bioethanol product. The analysis of bioethanol is conducted by using Trace GC ThermoFinnigan equipped with fused silica capillary column (Carbowax 20m). The GC is also equipped with an internal air compressor and hydrogen generator. Addition to that the nitrogen gas used as carrier gas. For sample introduction into GC analyzer, there are three ways to place the sample into the inlet. Within the analysis period, the sample is syringe up into microcapillary and directly syringe in the GC inlet right after push the prep run button. When push in the sample into the GC inlet need to be in fast manner so that the pressure inside the GC will not push out back the sample. For GC sample, there are 3 samples that being analyzed which are ethanol (70%), bioethanol (Oil Palm Frond) and bioethanol (Cellulose). The ethanol (70%) and bioethanol (Cellulose) will act as the standard and benchmark for the research study bioethanol from oil palm frond.

a) GC Analysis on Ethanol (70%) and Bioethanol (Cellulose)

According to the result obtained, both ethanol (70%) and bioethanol (Cellulose) happened at 6 minute of retention time. This prove that bioethanol by using cellulose has the exact composition with pure ethanol. The retention time of bioethanol is 6.0633 minute where the peak start at 6.0583 minute and end at 6.0850 minute. The peak height of ethanol is 8416 mVolt. For bioethanol (Cellulose), the retention time is 6.1483 minute. The peak start happened at 6.0950 minute and end at 6.5917 minute. The peak height of bioethanol (Cellulose) is 6060 mVolt. These minutes and peaks height will be put on as the bioethanol (Oil Palm Frond) benchmark.

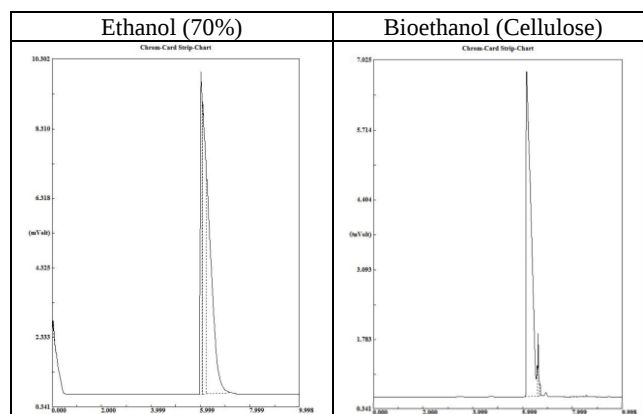


Figure 10: GC Analysis on Ethanol (70%) and Bioethanol (Cellulose)

b) GC Analysis on Bioethanol (Oil Palm Frond)

By achieving the main objective of the research scope is dependent on the composition similarity with the standard ethanol and bioethanol (Cellulose) composition. As the result obtained for bioethanol by oil palm frond, it shows that the peak also happened at minute of 6. This prove that the bioethanol (Oil Palm Frond) has nearly the same with the standard composition. The retention time is 6.2967 minute where the peak started at 6.1883 minute and end at 6.4783 minute. The peak height is 433 mVolt. Although the time starting a bit slightly difference by 0.13 minute, the peak of bioethanol (Oil Palm Frond) has proven that the composition of the biofuel and alcohol has the similarity.

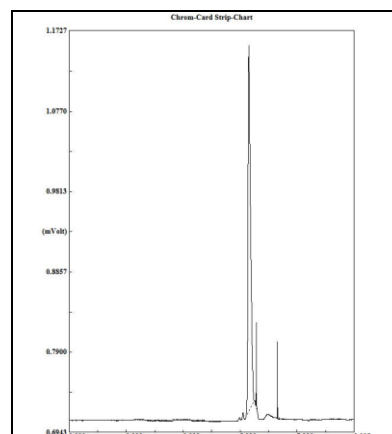


Figure 11: GC Analysis on Bioethanol (Oil Palm Frond)

V. CONCLUSION

The enhancement of pre-treatment process in order to get the efficient dissolution of cellulose is achieved as the ionic liquid used which is also a green solvent has successfully dissoluted the cellulose composition and has made easier for enzymatic hydrolysis process. With the aid of effective pre-treatment, the high cellulose concentration obtained from lignocellulosic biomass can be accomplished. The analysis used by XRD and RSM has contributed to give the optimum condition for pre-treatment process as to get the effective condition for fermentation process later on. In real fermentation of treated OPF at optimum condition has shown the conversion substrates from enzymatic hydrolysis into desired product of bioethanol also successfully achieved. In order to get yield of bioethanol and has the same composition with the standard ethanol, the analysis from GC-FID has been implemented. From the analysis, the bioethanol composition present within the sample from fermentation which referring to standard ethanol and bioethanol (Cellulose) are also achieved. As to get better yielding in bioethanol production, the type of pre-treatment with optimum condition must be analyzed and the selection of raw material that have high content of cellulose composition can contribute to high yield of bioethanol production.

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