

Lipase *Pseudomonas aeruginosa* NR.22 (Ps.NR.22) in Ethanol Production by Using Agricultural Waste

Aziyatulshafiqa Ali^{*1}, Nik Raikhan Nik Him²

Faculty of Chemical Engineering,

Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor.

MALAYSIA

Abstract—Ethanol is known as the most commonly used in biofuel, chemical solvents and preservatives. The demand of ethanol is rising due to the need for replacing fossil fuels in transportation sector. In this study, pineapple waste is used as an agricultural waste with *Pseudomonas aeruginosa* NR.22 as an active microorganism to produce ethanol. Unit operation that used in the study is Gas Chromatography (GC) and Fourier Transform Infrared Spectroscopy (FTIR). The objectives of the study are to produce ethanol from agricultural waste by lipase *Pseudomonas aeruginosa* NR.22 and to detect the concentration of ethanol. The study is conducted based on several method which is pretreatment of biomass, enzymatic hydrolysis, fermentation and product recovery. The whole fermentation process was carried out for 72 hours at 37°C with initial pH of 6.8. Initial pH of the culture broth indicates as one of the most important environmental parameters that will affect production of lipase and bacterial growth. Initially, 5% (v/v) of 24 hours *Pseudomonas aeruginosa* NR.22 inoculum containing 5×10^6 cell/mL were used in this study. The value of lipase activity was 22.5 ± 0.02 U/mL with the oil removal recorded to be 58.2% with bacterial biomass of 5.95 ± 0.001 g/mL. From the result, it was found that the *Pseudomonas aeruginosa* NR.22 is able to utilize pineapple peels to produce ethanol without the need of supplying other nutrient. Ethanol yield at 72 hours fermentation reached 20.06 g/L with 31% purity. The untreated and treated substrates were analyzed by FTIR to check the modification of the structure appeared after this pretreatment which guides to efficient saccharification. The FTIR spectroscopic analysis of pineapple peels which classified under three conditions: untreated, treated and after saccharification of samples.

Keywords— Pineapple peel; lipase; *Pseudomonas aeruginosa* NR.22; pretreatment; ethanol production;

Thus, renewable sources might be an alternative way to minimize the need of fossil fuels. E10 (gasoline blended with 10% (v/v) ethanol) is primarily used in United States in the motor gasoline production. Almost all of which is now blended with 10% (v/v) ethanol in the recent years. However, higher ethanol blends such as flex fuel (E85) and gasoline blended with 15% (v/v) ethanol (E15) is limited.

Therefore, production of bioethanol from lignocellulosic biomass, especially from agricultural waste is preferred to resolve the food versus fuel issues in the developing countries [3]. According to Food and Agriculture Organization (FAO) statistics, during the year 2013-2014, pineapple production for Malaysia was estimated to be form from 244 353 tonnes to 335 725 tonnes per year. Pineapple peel waste has been used in this study as an agricultural waste. Utilization of pineapple peels, rich in holocellulose content (60-85%) which is an attempt towards valorisation of the waste for bioethanol production. Since Malaysia known as one of the major pineapple producers in the world, their residual and disposal have become an environmental interest. This is because there is little or no productive use of these residue thus causing environmental problems such as pollution groundwater and spread of disease [1].

There are various methods involves to produce ethanol from agricultural waste which is pretreatment of biomass, enzymatic hydrolysis, fermentation and product recovery. The pretreatment processes involved in the study are biological and non-biological technique. Non-biological pretreatment techniques of the biomass consist of physical (milling and grinding, microwave), physiochemical (steam explosion) and chemical (acid hydrolysis) where the techniques do not involve any microbial treatments whereas biological pretreatment are carried by microorganisms which is bacterial. There are a many kind of microorganisms that can be used to produce ethanol such as *Neurospora* sp. (Irfan, et al. [4]), *Bacillus subtilis* (Eman [5]), *A. ellipticus* (Ingale, et al. [6]), *Candida shehatae*, *Saccharomyces cerevisiae* (Prema, et al. [7]) and *Enterobacter aerogenes* (Choonut, et al. [8]). However, active microorganism used in the study is *Pseudomonas aeruginosa* NR.22 in the production of ethanol using agricultural waste. Thus, the main objective of the study is to produce ethanol from agricultural waste by lipase *Pseudomonas aeruginosa* NR.22.

Gas Chromatography (GC) application has been used in the study in order to analyze the amount of ethanol being produced, the amount of fermentable sugars in the fermentation broth and also the concentration of unwanted by-product produced during the fermentation process. Furthermore, Fourier Transform Infrared (FTIR) analysis of the substrates is used in order to indicate fundamental result in the modification of the structure appeared after pretreatment which advance to efficient saccharification.

1. Introduction

Ethanol is known as the most commonly used in biofuel, chemical solvents and preservatives. Ethanol becomes important due to climate changes and global warming issue that rising throughout years [1]. Nowadays, the declining fossil fuels, escalating population, rising pollution and global warming due to energy crisis has triggered the society to comprehend the need for renewable sources. The limitation of reserved fossil fuel will affected the import of transport fuel to the world [2].

2. Materials and Methodology

Reagent, chemicals and pH

All reagents and chemical used were from various sources and technical grade as listed. Nutrient broth (Merck), olive oil (pure, Naturel), polyvinyl alcohol ($[-CH_2CHOH-]_n$, Aldrich), *Pseudomonas* agar (Microbiology), ethanol (CH_3CH_2OH analytical grade, R&M), acetone (CH_3COCH_3 analytical grade, R&M), sodium hydroxide (NaOH, R&M), sodium phosphate dibasic (S3264, $\geq 98.5\%$ Na_2HPO_4 , Sigma), sodium phosphate monobasic (S0751, $\geq 99.0\%$ NaH_2PO_4 , Sigma-Aldrich), hydrochloric acid (HCl, 37%, Bendosen) and phenolphthalein 1% alcoholic (R&M).

Pretreated of substrates

Pineapple (*Ananas comosus*) were procured from the local market (Pasar Tani Stadium Shah Alam). The pineapple peels were washed by using pure water to make sure there is no impurities left that will affect the result of the experiment. Then, the peels were chopped and sun-dried before drying in the oven at temperature $105^\circ C$ for 1 hour in order to make sure there is no moisture content that may affect the efficiency of the sample during experiment. The samples were then grind and sieved in order to obtain powder form. The pineapple peel powders in about 40.05g were mixed with 50mL of 10% (w/v) HCl solution. The samples were then filtrated and the residues were heated in the oven for 15 minutes at $105^\circ C$. This method was adapted and modified using method by Eman [5].

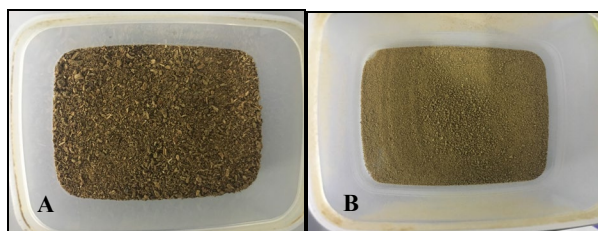


Fig. 1. A. Before pretreatment of pineapple peel powder; B. After pretreatment of pineapple powder

Microorganism and inoculum preparation

Pseudomonas aeruginosa NR.22 (Ps.NR.22) was obtained from the Biotechnology Laboratory, Chemical Engineering Universiti Teknologi Mara Shah Alam. The stock cultures were grown on nutrient agar at pH 6.8. Inoculated slopes were incubated at $35^\circ C$ for 24 hours and then stored at $4^\circ C$ in sealed universals. They were periodically sub-cultured by transfer onto fresh agar medium. The nutrient broth was sterilized for 15 minutes and then cooled down. Three loops of the microorganism culture of *Pseudomonas aeruginosa* NR.22 was added into 250mL Erlenmeyer flask containing 100mL nutrient broth. The mixture was then kept in the incubator shaker at $35^\circ C$ for 24 hours. The amount of 5% (v/v) containing 5.0×10^6 cells/mL was used later for fermentation process.

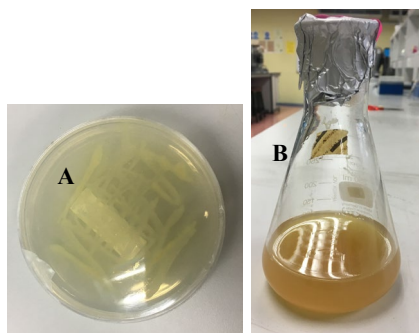


Fig. 2. A. After 24 hours incubation of *Pseudomonas aeruginosa* NR.22 agar; B. 24 hours *Pseudomonas aeruginosa* NR.22 inoculum

Lipase assays.

Lipase assay method was measured using polyvinyl alcohol (PVA) and olive oil emulsion as substrate as describe by Ota and Yamada [9] with some modification. One lipase unit was characterized as the amount of the enzyme that released 1 μ mol fatty acid per minute under standard assay conditions.

Fermentation and centrifugation process

The fermentation procedure was carried out by adding 5% (v/v) containing 5.0×10^6 cells/mL of 24 hours inoculum and 5.0g of pineapple peels powder sample into 250mL Erlenmeyer flask containing 100mL nutrient broth. The batch fermentation was carried out for 72 hours at $37^\circ C$ under continuous stirring at 150rpm using incubator shaker in order to allow it ferment completely. The mixture was then centrifuged at 13 000rpm for 10 minutes in order to get the supernatant. The sample is drawn periodically from the fermented flask in order to determine pH value, bacterial growth and ethanol productivity.

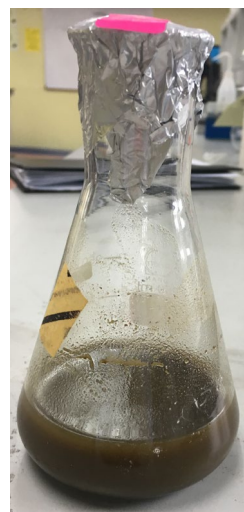


Fig. 3. After 72 hours fermentation period

Bacterial growth and pH value

The optical density (OD) of bacterial broth was estimated periodically by using spectrophotometer at 560nm. The pH of the supernatant was measured by using pH meter.

Fourier Transform Infrared Spectroscopy of substrates

Fourier Transform Infrared Spectroscopy (FTIR) measurement was used to check the alteration of chemical in treated and untreated samples in the modification of the structure appeared after pretreatment which direct to efficient saccharification. The spectrum was recorder with 32 scans in the frequency range of $4000-515cm^{-1}$ with a resolution of $4cm^{-1}$ using method by Irfan, et al. [4] with some modification.

Estimation of ethanol

Modification of method by Eman [5] has been used for GC. The ethanol content was measured by using gas chromatography (HP 5890) equipped with flame ionization detector ND Hydrodex- β -PM 25m $\times$ 0.25mm column, 0.25 μ m film with permethylated-cyclodextrin; Macherey & Nagel. The temperature of the injector was set at $150^\circ C$ whereas detector temperature was set at $200^\circ C$. The temperature of oven was initially maintained at $40^\circ C$ for 1 minute and then increased to $130^\circ C$ at a gradient of $20^\circ C$ per minute. Carrier gas used was helium with ethanol as the internal standard.

3. Result and discussion

In this study, substrates like pineapple peels were used for

ethanol production by *Pseudomonas aeruginosa* NR.22 in 250mL Erlenmeyer flask at 37°C for three days of fermentation period. The first step for the production of ethanol is pretreatment of substrate which reduce cellulose crystallinity and improves the efficiency of the recovery and purification of the products. Physical and chemical pretreatment are the significant types of pretreatment techniques employed. Pretreatment of lignocellulosic biomass are necessary in order to break down lignin thus increase the accessibility of enzymes and microbes to carbohydrates. Firstly, pineapple peels were subjected to physical pretreatment by reduction of size and forming into powder and the chemical pretreatment by acid hydrolysis are then applied by treated with 10% (w/v) HCl to remove the lignocellulosic component in order to expose the simple sugar which help the utilization of enzyme followed by heating in the microwave oven for 15 minutes at 105°C.

Bacterial growth and pH value after 72 hours fermentation was recorded as shown in Figure 4. From the bacterial growth values, the bacteria reached a maximum growth within 48 hours with the value of 1.647. However, the inoculum pH decreased during 72 hours fermentation from an initial pH 6.8 at 0 hour decrease to pH 4.44 which indicating essential production of acid. Initial pH of the culture broth indicates as one of the most important environmental parameters that will affect production of lipase and bacterial growth. The pH value of the culture

decreased provide a close indicator for the completion of fermentation and appears to correspond with maximum ethanol production. In addition, carbon is known as the primary nutrient for bacteria.

Lipase catalyze the hydrolysis of tri-acyl glycerol to glycerol and free fatty acids. Initial pH of the culture broth indicates as one of the most important environmental parameters that will affect production of lipase and bacterial growth. The optimal pH of lipase often to be in range of 7 to 9. Pineapple peels and olive oil were used as the substrate and inducer for lipase production. Initially, 5% (v/v) of 24 hours *Pseudomonas aeruginosa* NR.22 inoculum containing 5×10^6 cell/mL were used in this study. In this study, there is no additional carbon source is used to support the growth of bacterial and production of lipase. The result shows the lipase activity and percentage of oil removal with biomass of 5.95 ± 0.001 g/L at 72 hours fermentation of 5% (v/v) *Pseudomonas aeruginosa* NR.22 cells. The value of lipase activity was 22.5 ± 0.02 U/mL with the oil removal recorded to be 58.2%. It shown that the level of enzyme production corresponds to the degree of oil removal thus means isolated bacteria is capable in removing oil.

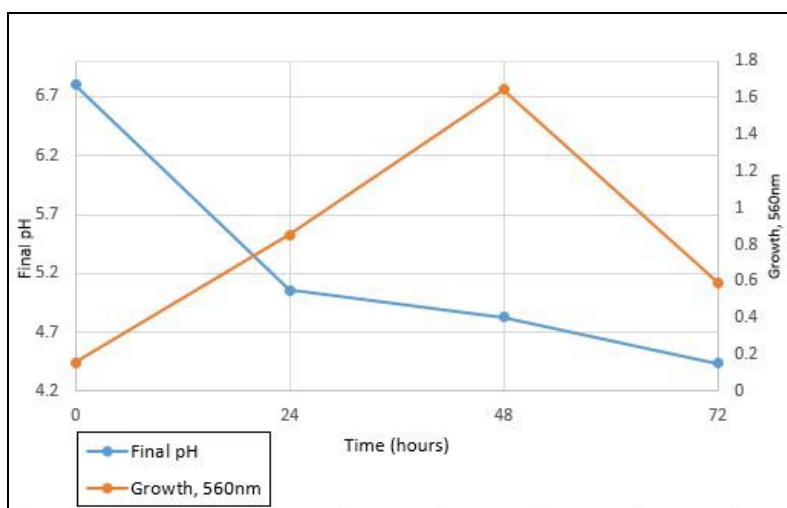


Fig. 4. *Pseudomonas aeruginosa* NR.22 growth and pH value within 72 hours fermentation

Besides, the untreated and untreated substrates were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) to check the modification of the structure appeared after this pretreatment which guides to efficient saccharification. FTIR also known as an efficacy technique for the surface and interface characterization of lignocellulosic with other substances. The FTIR spectroscopic analysis of pineapple peels which classified under three conditions: untreated, treated and after saccharification of samples.

Figure 5 shows the spectrum of untreated fibers having a characteristic absorption peak at 1023.67cm^{-1} and 1623.38cm^{-1} corresponds to C-O and C=C stretching frequencies. These peaks were not as sharp as shown in treated pineapple peels which revealing the dislocation of cellulose, hemicellulose and lignin components. However, the prominent peaks at 673.19cm^{-1} represents C=C bending frequency in hemicellulose which is due to glucosidic linkage between the sugar unit in hemicellulose and cellulose while peak in range of 3285.03cm^{-1} represents the stretching vibration of O-H in lignin.

Next, Figure 6 illustrated the FTIR spectroscopic analysis of pretreated pineapple peels. The dominant peak at 1031.66cm^{-1} corresponds to C-O stretching in lignin, cellulose and hemicellulose. Small peak at 1237.06cm^{-1} communicate to aromatic skeletal vibrations and ring breathing in the C-O

stretching in lignin. The absorption at 1622.10cm^{-1} represent C=C stretching in cellulose, hemicellulose and lignin whereas the band at 2920.82cm^{-1} in treated pineapple peels arises from C-H stretching in lignin. The strong band at 3331.22cm^{-1} is depicting the stretching of hydroxyl group (OH) in treated pineapple peels.

After pretreatment, the substrate was saccharified by *Pseudomonas aeruginosa* NR.22 for 72 hours at 37°C. Figure 7 indicated that the absorption at 3304.23cm^{-1} correspond to the stretching vibration of O-H in lignin whereas small peak at 2344.63cm^{-1} was assigned to O=C=O stretching. It shows that there is presence of glucose in the saccharified pineapple peels. The absorbance at 1991.12cm^{-1} and 1639.70cm^{-1} are depicting C=C asymmetric stretching and C=C symmetry reduce intensity, respectively. These bands were expended so much in pretreated pineapple peels which reveals the dislocation of hemicellulose and lignin components.

In Figure 5 and 6, it clearly seen that there is no organic compound found at the wavelength $2800\text{--}1800\text{cm}^{-1}$ but showing spectrum of O=C=O and C=C after 72 hours fermentation as seen in Figure 7. However, Figure 5 and 6 showing sharp spectrum of C-O content related to the presence of glucose at wavelength $1200\text{--}800\text{cm}^{-1}$ but it broke down completely after 72 hours fermentation as seen in Figure 7.



Fig. 5. FTIR spectra of untreated pineapple peels

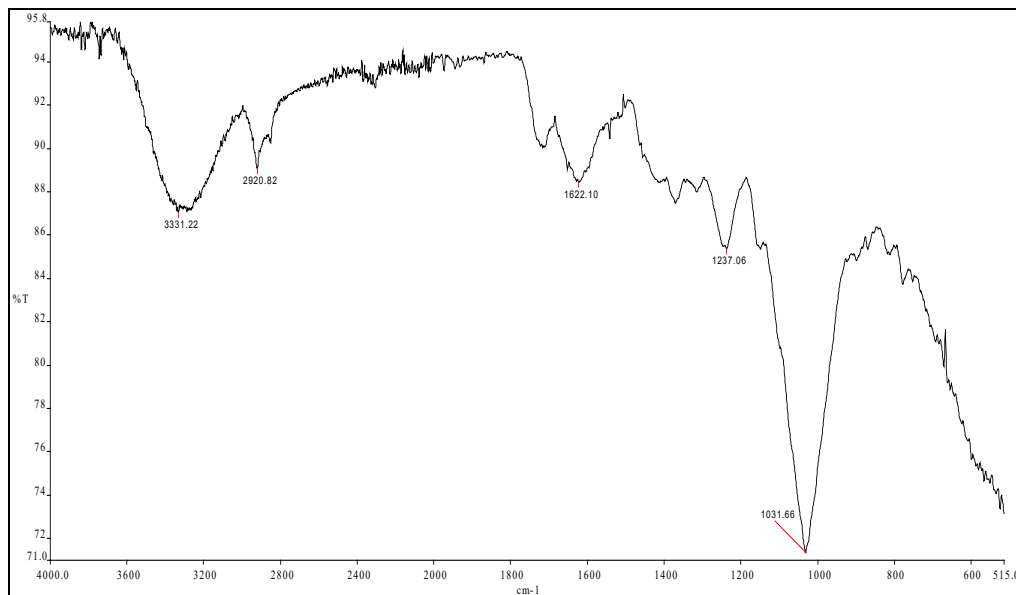


Fig. 6. FTIR spectra of treated pineapple peels

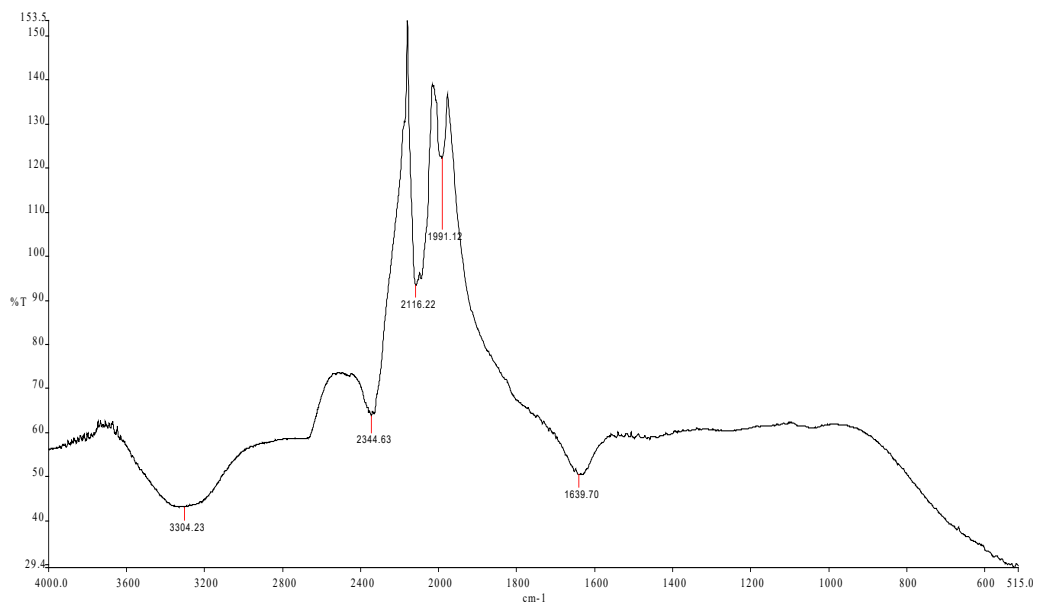


Fig. 7. FTIR spectra of saccharified pineapple peels

According to the estimation of ethanol by gas chromatography (GC), figure 8 indicated the production of ethanol from pineapple peels by *Pseudomonas aeruginosa* NR.22 in submerged 72 hours fermentation at 37°C. From the result, it was found that the *Pseudomonas aeruginosa* NR.22 is able to utilize pineapple peels to produce ethanol without the

need of supplying other nutrient. The result shown that ethanol compound is detected at 3.2342 minutes retention time. Ethanol yield at 72 hours fermentation reached 20.06g/L with 31% purity.

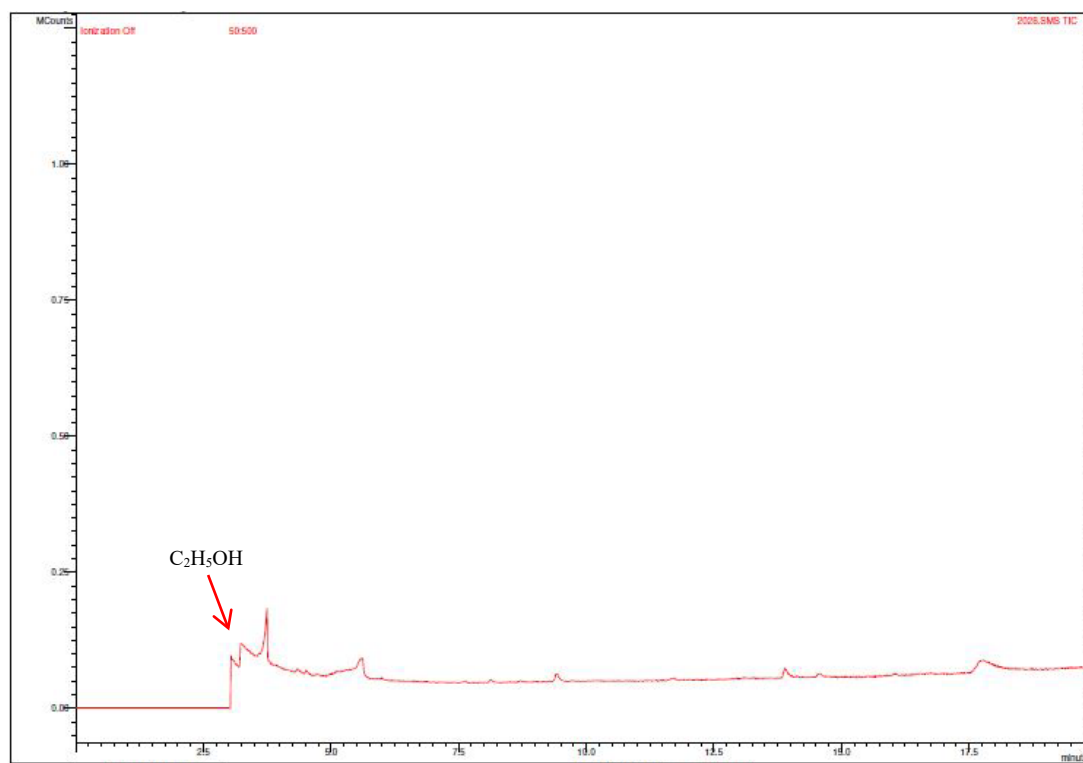


Fig. 8. Gas Chromatography analysis on 72 hours fermented samples

4. Conclusion

In conclusion, the potential of pineapple peels as raw material for ethanol production was studied. Moreover, *Pseudomonas aeruginosa* NR.22 has ability to utilize pineapple peels to produce ethanol without the need of supplying other nutrient and yet has proven to be the best lipase producer. The selection of operating conditions of ethanol production such as pH, bacteria and substrate play an important role in obtaining ethanol yield and purity. Initial pH of the culture broth indicates as one of the most important environmental parameters that will affect production of lipase and bacterial growth.

5. Acknowledgement

I would like to express my greatest appreciation and gratitude to my supervisor Dr. Nik Raikhan Nik Him for the unlimited advice, guidance and motivation. Also, my special thanks to Faculty of Chemical Engineering, Universiti Teknologi MARA (UiTM) for the study to take place.

6. References

- [1] A. B. Guerrero, I. Ballesteros, and M. Ballesteros, "The potential of agricultural banana waste for bioethanol production," *Fuel*, vol. 213, pp. 176-185, 2/1/ 2018.
- [2] N. Sarkar, S. K. Ghosh, S. Bannerjee, and K. Aikat, "Bioethanol production from agricultural wastes: An overview," *Renewable Energy*, vol. 37, no. 1, pp. 19-27, 2012/01/01/ 2012.
- [3] A. D. Chintagunta, S. Ray, and R. Banerjee, "An integrated

- bioprocess for bioethanol and biomanure production from pineapple leaf waste," *Journal of Cleaner Production*, vol. 165, no. Supplement C, pp. 1508-1516, 2017/11/01/ 2017.
- [4] M. Irfan, M. Nadeem, and Q. Syed, "Ethanol production from agricultural wastes using *Saccharomyces cerevisiae*," *Brazilian Journal of Microbiology*, vol. 45, no. 2, pp. 457-465, Sep 2014.
- [5] Z. G. Eman, "Bioconversion of orange peels for ethanol production using *Bacillus subtilis* and *Pseudomonas aeruginosa*," *African Journal of Microbiology Research*, vol. 7, no. 14, pp. 1266-1277, 2013.
- [6] S. Ingale, S. J. Joshi, and A. Gupte, "Production of bioethanol using agricultural waste: Banana pseudo stem," *Brazilian Journal of Microbiology*, vol. 45, no. 3, pp. 885-892, 10/09, 11/11/received, 03/14/accepted 2014.
- [7] D. Prema, M. L. Prabha, and G. Gnanavel, "Production of Biofuel using Waste Papers from *Pseudomonas aeruginosa*," *International Journal of ChemTech Research*, vol. 8, no. 4, pp. 1803-1809, 2015.
- [8] A. Choonut, M. Saejong, and K. Sangkharak, "The Production of Ethanol and Hydrogen from Pineapple Peel by *Saccharomyces Cerevisiae* and *Enterobacter Aerogenes*," *Energy Procedia*, vol. 52, no. Supplement C, pp. 242-249, 2014/01/01/ 2014.
- [9] Y. Ota and K. Yamada, "Lipase from *Candida parapolityca*," *Agricultural and Biological Chemistry*, vol. 30, no. 4, pp. 351-358, 1966/04/01 1966.