STRATEGIES FOR ENHANCEMENT OF ISOBUTANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE* WILD TYPE

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ABSTRACT

Isobutanol is one of the effective biofuels that can be produced from renewable biomass through microbial fermentation. This alcohol is a better alternative compared to bioethanol in order to replace gasoline as transportation fuel. Yeast naturally produces isobutanol as an end product of valine catabolism via the Ehrlich pathway. However, production of isobutanol using yeast as production host produces low alcohol titers. Therefore, this study was conducted to increase the isobutanol production using several strategies on selected yeast based on the preliminary studies. Yeasts including *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799, *Pichia pastoris* KM71H, *Pichia pastoris* GS115 and *Pichia pastoris* X33 were investigated in respect of production yield and alcohol tolerance. The result of preliminary study showed that *Saccharomyces cerevisiae* produced isobutanol titers continuously during 96 hours of fermentation and has high alcohol resistance compared to other strains thus it was selected as the best producer. In order to improve the isobutanol production, optimization of medium compositions and operating conditions for fermentation was carried out using central composite design. Medium components affecting the isobutanol production were screened using fractional factorial design and the optimized medium compositions obtained are glucose of 140 g/l, yeast extract of 8 g/l and peptone of 8 g/l with the yield of isobutanol of 172 mg/l. The fermentation was further optimized in order to evaluate the effects of operating conditions. The optimum isobutanol of 200 mg/l was obtained with temperature of 28 °C, pH of 7, agitation of 179 rpm and inoculum size of 10%. Amino acids, keto acid and vitamins were added into the fermentation medium in order to enhance the alcohol production yield as isobutanol is the by-product of amino acid catabolism. Vitamins were required in the first step of alcoholic fermentation, thus by adding these materials into the fermentation medium, the alcohol yield could be increased. Valine, leucine and 2-ketoisovalerate with concentration of 0.05 to 1.5 g/l were added into the fermentation medium and the concentration of 1.5 g/l contributes a significant effect towards isobutanol production. The highest isobutanol concentration (856 mg/l) was obtained with the addition of 1.5 g/l 2-ketoisovalerate. Further investigation on the interaction effect of amino and keto acids was carried out and the highest production of isobutanol was 1058 mg/l which is 10-fold higher compared to the control, obtained with supplementation of valine and 2-ketoisovalerate combination. An amount of 0.05 to 0.5 g/l of biotin, niacin, thiamine pyrophosphate and para-aminobenzoic acid (PABA) was supplemented into the fermentation medium. Isobutanol concentration decreased with the addition of these vitamins individually. Supplementation of combination of vitamins mixture showed that the combination of niacin-PABA and biotin-niacin-PABA contributed to highest isobutanol production of 402 mg/l. Supplementation of amino acids and vitamins simultaneously into the medium broth resulted in the highest isobutanol production yield of 1027 mg/l with the addition of valine-2-KIV-niacin-PABA. The kinetics on microbial growth and product formation were studied using Monod and Leudeking-Piret equations. The values of μ_{max} and k_s obtained were 0.75 h^{-1} and 58 g/L, respectively. The isobutanol yield ($Y_{P/S}$), biomass yield ($Y_{X/S}$) and the correlation between isobutanol production and yeast growth ($Y_{P/X}$) were found to be 5.19 mg/g, 0.1 g/g and 50.0 mg/g, respectively. Throughout the study, it can be concluded that *Saccharomyces cerevisiae* is the best isobutanol production host and the alcohol titers can be increased via optimization and supplementation of amino acids and vitamins. With the improvement of technologies nowadays, the isobutanol production is expected to be increased in the future, thus encouraging the usage of this fuel in the transportation industry worldwide.

ABSTRAK

Isobutanol adalah salah satu bahan api bio yang boleh diperbaharui yang efektif dan boleh dihasilkan daripada biojisim melalui penapaian mikrob. Alkohol ini merupakan alternatif yang lebih baik berbanding bioetanol untuk menggantikan petrol sebagai bahan api pengangkutan. Yis secara semulajadinya menghasilkan isobutanol sebagai produk akhir daripada proses katabolisma valin melalui laluan Ehrlich. Walau bagaimanapun, pengeluaran isobutanol menggunakan yis sebagai hos pengeluaran hanya menghasilkan titisan alkohol yang sedikit. Oleh itu, kajian ini dijalankan untuk meningkatkan pengeluaran isobutanol dengan menggunakan beberapa strategi terhadap yis yang terpilih berdasarkan kajian permulaan. Beberapa jenis yis termasuk *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799, *Pichia pastoris* KM71H, *Pichia pastoris* GS115 dan *Pichia pastoris* X33 telah dikaji terhadap hasil pengeluaran isobutanol dan toleransi terhadap alkohol. Hasil kajian awal menunjukkan bahawa *Saccharomyces cerevisiae* menghasilkan titisan isobutanol berterusan sepanjang 96 jam proses penapaian dan ia mempunyai ketahanan alkohol yang lebih tinggi berbanding dengan spesis yang lain dan ini menjadikannya sebagai pengeluar terbaik. Untuk meningkatkan pengeluaran alkohol, pengoptimuman komposisi media dan keadaan operasi penapaian dilakukan menggunakan reka bentuk komposit berpusat. Komposisi media yang mempengaruhi pengeluaran isobutanol disaring menggunakan reka bentuk pecahan faktorial dan komposisi medium yang optimum diperoleh adalah 140 g/l glukosa, 8 g/l ekstrak yis dan 8 g/l pepton dengan hasil isobutanol adalah 172 mg/l. Penapaian ini dioptimumkan lagi untuk menilai kesan keadaan operasi, isobutanol optimum sebanyak 200 mg/l diperoleh dengan suhu pada 28 °C, pH 7, agitasi pada 179 rpm dan saiz inokulum pada 10%. Asid amino, asid keto dan vitamin telah ditambah ke dalam medium penapaian untuk meningkatkan hasil pengeluaran alkohol kerana isobutanol merupakan produk sampingan yang dihasilkan daripada katabolisma asid amino. Beberapa vitamin diperlukan dalam langkah pertama penapaian alkohol, oleh itu dengan penambahan bahan ini ke dalam media penapaian, hasil alkohol dapat ditingkatkan. Valine, leucine dan 2-ketoisovalerat dengan kepekatan 0.05 hingga 1.5 g/l telah ditambah ke dalam media penapaian dan kepekatan 1.5 g/l telah menyumbang kepada kesan yang signifikan ke arah pengeluaran isobutanol. Kadar kepekatan isobutanol tertinggi (856 mg/l) diperoleh dengan penambahan 1.5 g/l 2-ketoisovalerat. Kajian lanjut mengenai kesan interaksi asid amino dan asid keto telah dilakukan dan pengeluaran isobutanol tertinggi adalah sebanyak 1058 mg/l iaitu 10 kali ganda lebih tinggi berbanding dengan kawalan, diperoleh dengan kombinasi kombinasi valine dan 2-ketoisovalerat. Sejumlah 0.05 hingga 0.5 g/ l biotin, niacin, tiamin pirofosfat dan asid para-aminobenzoik (PABA) telah ditambah ke dalam media penapaian. Kepekatan isobutanol menurun dengan penambahan vitamin ini secara individu. Penambahan gabungan campuran vitamin menunjukkan bahawa kombinasi niacin-PABA dan biotin-niacin-PABA menghasilkan pengeluaran isobutanol tertinggi sebanyak 402 mg/l. Penambahan asid amino dan vitamin secara serentak ke dalam media penapaian menghasilkan pengeluaran isobutanol tertinggi sebanyak 1027 mg/l dengan penambahan valine-2-KIV-niacin-PABA. Kinetik pertumbuhan mikrob dan pembentukan produk dikaji menggunakan persamaan Monod dan Leudeking-Piret. Nilai μ_{max} dan K_s yang diperoleh masing-masing adalah 0.75 h^{-1} dan 58 g/L. Hasil isobutanol ($Y_{P/S}$), hasil biomassa ($Y_{X/S}$) dan korelasi antara pengeluaran isobutanol dan pertumbuhan yis ($Y_{P/X}$) masing-masing adalah 5.19 mg/g, 0.1 g/g dan 50.0 mg/g. Sepanjang kajian, dapat disimpulkan bahawa *Saccharomyces cerevisiae* adalah pengeluar isobutanol yang terbaik dan titisan alkohol dapat ditingkatkan melalui proses pengoptimuman dan penambahan asid amino dan vitamin. Dengan peningkatan teknologi pada masa kini, pengeluaran isobutanol dijangka meningkat pada masa akan datang sekaligus menggalakkan penggunaan bahan api ini dalam industri pengangkutan di seluruh dunia.

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LIST OF ABBREVIATIONS

ABE	-	Acetone-Butanol-Ethanol
ADH	-	Alcohol Dehydrogenase
ANOVA	-	Analysis of Variance
BAT	-	Branched-Chain Amino Acids Transferase
BIO	-	Biotin
CCD	-	Central Composite Design
CH_4	-	Methane
CO_2	-	Carbon Dioxide
DNS	-	Dinitrosalicyclic Acid
$FeSO_4.7H_2O$	-	Iron Sulphate Heptahydrate
ILV2	-	Acetolactate Synthase
ILV3	-	Dihydroxyacid Dehydratase
ILV5	-	Acetohydroxyacid Reductoisomerase
KDC	-	α -Ketoacid Decarboxylase
KH_2PO_4	-	Potassium Phosphate
LEU	-	Leucine
MPC	-	Mitochondrial Pyruvate Carrier
$MgSO_4.7H_2O$	-	Magnesium Sulphate Heptahydrate
N_2O	-	Nitrous Oxide
$(NH4)_2SO_4$	-	Ammonium Sulphate
NIA	-	Niacin
RSM	-	Response Surface Methodology
2-KIV	-	2-Ketoisovalerate
3-MB	-	3-Methyl-1-Butanol
PABA	-	Para-Aminobenzoic Acid
THI	-	Thiamine Pyrophosphate
VAL	-	Valine

LIST OF SYMBOLS

^{o}C	-	Degree Celsius
%	-	Percentage
>	-	More Than
<	-	Less Than
g/l	-	Gram per litre
mg/l	-	Milligram per litre
ml	-	Millilitre
v/v	-	Volume per volume
w/v	-	Weight per volume
rpm	-	Rotation per minute

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Environmental concerns such as depletion of fossil fuel reserves, escalating global energy demand and rising crude oil price have attracted various attention which leads to the rising interest in the production of liquid biofuel. The negative environmental impact encourages global movements towards reducing the use of fossil resources due to it being one of the major contributors in greenhouse gases through the production of methane (CH_4) and nitrous oxide (N_2O) which are dangerous. Besides, fossil fuel also raises the atmospheric concentration of harmful carbon dioxide (CO_2). Fossil fuels are produced from non-renewable sources which lead to the exhausted supply of fossil fuel. In this fuel dependent era, the demand for petroleum-based fuel is increasing due to the continuous rise of energy demand in transport and industry sectors consequently increased the crude oil price thus directly affecting the global economic development and growth.

Biofuel is an alternative fuel predominantly produced by renewable feedstock through biological process (Toogood and Scrutton, 2018). Production of biofuel from renewable sources is considered as the most sustainable alternative to fossil fuels as it provides positive impact towards the environment (Mandade *et al.*, 2016) and the economy. Biofuel can be found in the form of liquid, gasses and solid such as bioalcohol (Demirbas, 2008; Hacisaligoglu, 2009), biodiesel, Fischer-Tropsch diesel, methane and hydrogen. Biofuel has been widely used as transportation fuel due to its advantages and the current established biofuels are biodiesel and bioethanol. However, the new alternative which is isobutanol has been discovered to possess more benefits compared to the current ones.

Alcohol has a great potential in replacing or substituting gasoline as the transportation fuels. At the moment, the most establish vehicle fuel is ethanol due to its various advantages. However, butanol receives great attention as it possesses more benefits than ethanol. Compared to ethanol that contains 2 carbon atoms (C_2H_5OH), butanol is consists of 4 carbon atoms with the chemical formula of C_4H_9OH. Butanol can be differentiated into four isomers which are 1-butanol, 2-butanol, isobutanol and tert-butanol (Liu *et al.*, 2016). Among the isomers, 1-butanol and isobutanol are known to be the most suitable candidate as transportation fuel (Jin *et al.*, 2011). *Clostridium acetobutylicum* is the oldest natural producer of 1-butanol through the acetone-butanol-ethanol (ABE) fermentation while yeast has the ability to produce isobutanol naturally based on the Ehrlich pathway.

Currently, production of isobutanol from biomass as transportation fuel attracts public's attention worldwide (Ezeji *et al.,* 2014). This branched chain higher alcohol has a range of physical properties that is more suitable to be used as gasoline substitute than bioethanol. Isobutanol is proved to be a better candidate in replacing gasoline as vehicle fuel due to its high energy content (110000 BTU per gallon) compared to bioethanol (84000 BTU per gallon) and is similar to gasoline (115000 BTU per gallon) (Ha *et al.*, 2010). Besides, isobutanol possess several characteristics such as higher boiling point, low miscibility in water, lower vapor pressure, higher blending ability with gasoline as well as reducing the need to modify the current combustion engine (Li *et al.*, 2017; Wechgama *et al.*, 2017).

Clostridia species is the main producer in Acetone-Butanol-Ethanol (ABE) fermentation. *Clostridium acetobutylicum* is the oldest and the most established host for butanol production (Lee *et al.*, 2008). However, this bacteria is known to be unable to produce isobutanol titers naturally through fermentation. In addition to *Clostridia* species, *Escherichia coli* is also not a native producer of isobutanol. *Escherichia coli* is genetically tractable, well characterized and a fast growth microorganism but this organism is not a native producer of neither butanol nor isobutanol thus the expression of butanol synthesis genes are needed. As both of these microorganisms are not the native producer of isobutanol, metabolic and genetic engineering have been performed on them so that isobutanol is able to be produced (Atsumi *et al.*, 2008; Atsumi and

Liao, 2008; Lee *et al.*, 2009; Yazdani and Gonzalez, 2008). According to Chen *et al.* (2011) the use of host that does not produces butanol is quite complicated as extensive work for optimal expression is required. *Clostridia* species is known to be strictly anaerobic and has complex physiology which is difficult for genetic manipulation. *Clostridia* is intolerant to butanol concentration of above 2 %; which might affect the alcohol production titers. On the other hand, *Escherichia coli* possesses facultative anaerobic and its genetics are better studied with well-developed genetic modification tools. However, both *Clostridia* species and *Escherichia coli* produces only a small amount of isobutanol. Therefore, there is a need to identify other native hosts in isobutanol production.

Yeast has the ability to produce small amount of isobutanol naturally during fermentation process. According to Chen *et al.*, 2011, the heterologous pathway can be avoided using the natural production host in alcohol fermentation. Expression of heterologous pathway is a complicated process that relates to genetic and metabolic engineering. Baker's yeast, *Saccharomyces cerevisiae* is one of the most promising host in the production of biofuel (Ida *et al.,* 2015). Yeast exhibits numerous advantageous that make it appropriate to be used as fermentation host. Yeast is well known to have high tolerance with alcohols. For instance, *Saccharomyces cerevisiae* has the ability to grow in butanol concentration that is higher than 20g/L (Knoshaug and Zhang, 2009). Besides, yeast has high robustness making it able to resist harsh conditions during fermentation and is also tolerant to low pH resulting in low risk for contamination (Kondo *et al.* 2013). In addition, yeast possesses facultative characteristics thus the complex facilities for the fermentation is not required.

Several strategies have been conducted by the researchers to improve isobutanol production yield in fermentation. Most alternatives to enhance isobutanol titers involve modification of microbial genetic such as overexpression of related genes, re-localization of the pathway in the same compartment and deleting the genes that inhibited the product formation. The overexpression of genes including *ILV2*, *ILV5*, *ILV3* (Kondo *et al.* 2012), *BAT2* (Lee *et al.* 2012), *KDCs* and *ADHs* in valine biosynthetic pathway has been conducted to increase the isobutanol levels (Chen *et al.* 2011). The re-localization of valine biosynthesis in cytoplasm or the overexpression

of *KDCs* and *ADH*s in the mitochondria enables the improvement of isobutanol yield (Brat *et al.* 2012; Lee *et al.* 2012; Avalos *et al.* 2013). In addition, deletion of *BAT1* in *Saccharomyces cerevisiae* CEN.PK2-1C results in the increase of isobutanol titers by 14.2 folds. On the other hand, as the *BAT2* is deleted the isobutanol yield remains approximately similar to the wild type (Hammer and Avalos, 2017). Optimization is another strategy utilized in obtaining high alcohol yield during fermentation. Al-Shorgani *et al.* (2016) shows that the optimization of glucose concentration, butyric acid addition and C/N ratio affect the final butanol yield by *Clostridium acetobutylicum* YM1. The optimum medium compositions result in 12.16 g/l butanol titers, 31.3 % higher than the un-optimized medium.

Wild type yeast has a potential for improvement of the isobutanol production yield with the study on its metabolomics. In this study, a research on enhancement of isobutanol production by wild type yeast is proposed using strategies such as optimization of medium compositions and operating conditions as well as the supplementation of amino acids and vitamins into the fermentation medium.

1.2 Problem Statement

Yeast produces isobutanol as a by-product of Ehrlich pathway in fermentation with carbon source. However, the quantity of isobutanol yield produced is relatively in small quantities leading to various alternatives taken by researchers in an effort to increase the yield of isobutanol. It is expected for isobutanol titers by wild type yeast to be increased through fermentation's medium compositions and operating conditions manipulations. Optimization is a process of finding the best conditions to be applied in order to obtain the optimum response. The usage of the best conditions leads to the improvement of the response; the optimization strategy is contemplated to be the best method in enhancing the isobutanol production yield. Besides, the Ehrlich pathway of yeast shows amino and keto acids directly involved with the production of alcohol while vitamins are related to the microbial growth, thus isobutanol production is expected to increase with the application of these chemicals.

Several strategies could be conducted to increase isobutanol production yield by wild type yeast. Preliminary study of isobutanol production and alcohol tolerance by several types of yeast is conducted prior to identify the most suitable producer. Optimization of medium compositions and fermentation operating conditions is important to improve the product yield. The medium plays an important role in product formation during the fermentation process. Carbon source is the crucial nutrients for the living cells as it acts as the fundamental building block that serves as an energy source. Carbohydrate is the most common carbon source that has been used in microbial fermentation processes (Stanbury *et al.*, 1984). In addition, nitrogen source is also important as an energy source in certain microorganisms (Kampen, 2014). In addition to medium compositions, fermentation operating conditions also affect the incubation process. According to Togarepi *et al.* (2012), temperature directly affects the production rate of enzyme; temperature increment increases the rate of enzyme reactions up to the optimal temperature before being denatured. It is also known that in alcohol production, temperature strongly influences the biochemical reaction yeast and its metabolic pathways (Fleet and Heard, 1993). Moreover, another factor that affects the microbial growth and alcoholic fermentation is pH value. Regulating protein function and transporting nutrients into cells are few examples of the pH functions in the microbes (Togarepi *et al.*, 2012). Agitation is important in substrate mixing, mass transfer and heat transfer of the fermentation. This factor affects the cell growth, the morphology and the production of the metabolites.

Isobutanol production can also be improved by supplementation of amino acids, keto acids and vitamins into fermentation medium broth. The Ehrlich pathway of yeast presents the production of isobutanol through degradation of amino and keto acids. The research by Atsumi *et al.* (2008), shows that the addition of 2-ketoisovalerate increased the isobutanol production by *Saccharomyces cerevisiae*. Besides, vitamins relate directly to the microbial metabolism. Addition of biotin shifted the aerobic metabolism of yeast towards fermentation through ethanol titers increment and biomass concentration decrement (Parrondo *et al.*, 2009). Consequently, by having efficient strategies that could improve the yield, this matter could be achieved in parallel.

1.3 Objective of Study

The main objective of this research is to enhance the isobutanol production in wild type yeast through optimization and biological pathway strategies.

The specific objectives are:

i. To determine the most suitable yeast for isobutanol production in term of production yield and alcohol tolerance.

ii. To optimize the fermentation's medium compositions and operating conditions for optimum isobutanol production.

iii. To improve the isobutanol production yield by supplementation of amino acids, keto acid and vitamins.

iv. To evaluate the kinetic coefficients of isobutanol production from yeast in the optimum fermentation conditions.

1.4 Scopes of Study

In order to achieve the specific objectives of this study, several scopes and limitations are outlined:

i. The yeasts involved in the preliminary study are *Saccharomyces cerevisiae* (baker's yeast), *Kluyveromyces lactis* GG799 and *Pichia pastoris* (KM71H, GS115 and X33). Alcohols such as isobutanol, 3-methyl-1-butanol and butanol ranged between 0.5 to 2.5 % (v/v) were studied for alcohol tolerance.

ii. The medium compositions affecting isobutanol production were screened using the fractional factorial design and further optimized using Central

Composite Design (CCD). The medium involved in the screening process are glucose (20 – 100 g/l), peptone (1 – 5 g/l), yeast extract (1 – 5 g/l), $(NH4)_2SO_4$ (1-5 g/l), KH_2PO_4 (1 – 5 g/l), $MgSO_4.7H_2O$ (0.5 – 2.5 g/l) and $FeSO_4.7H_2O$ (0.01 – 0.05 g/l) while the medium for optimization process are glucose (80 – 140 g/l), peptone (4 – 8 g/l), yeast extract (4 – 8 g/l). The parameters for optimization of fermentation's operating conditions are temperature (28 – 40 ^oC), pH (4 – 7), agitation (50 – 200 rpm) and inoculum size (3 – 10 % v/v) using the Central Composite Design (CCD).

iii. Investigation of chemicals for isobutanol production enhancement on selected yeast. The chemicals involved include valine, leucine, 2-ketoisovalerate, biotin, thiamine phyrophosphate, niacin and 2- para amino benzoic acid with the range of 0.5 to 1.5 g/l amino acids and 0.01 to 0.5 g/l vitamins.

iv. The coefficients for microbial growth and product formation, Y, k_d, μ_{max} and k_s are determined using the Monod's and the Ledeuking – Piret's equations.

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APPENDIX A

SOLUTION PREPARATION

A1. Preparation of DNS Reagent

600ml of distilled water is being heated on hot plate at 100 ^{o}C with full speed stirring. 10 g of dinitrosalicyclic acid (DNS) was added into the hot water and allowed to dissolve. After that, an amount of 16 g of sodium hydroxide (NaOH) was gradually added and also allowed to dissolve. Then, 300 g of Rochelle salt (sodium potassium tartrate) was slowly added and stirred for about 20-30 minutes and warm at temperature 45 ^{0}C. The mixture was cooled down and dilute to 1000 ml before stored in Schott bottle.

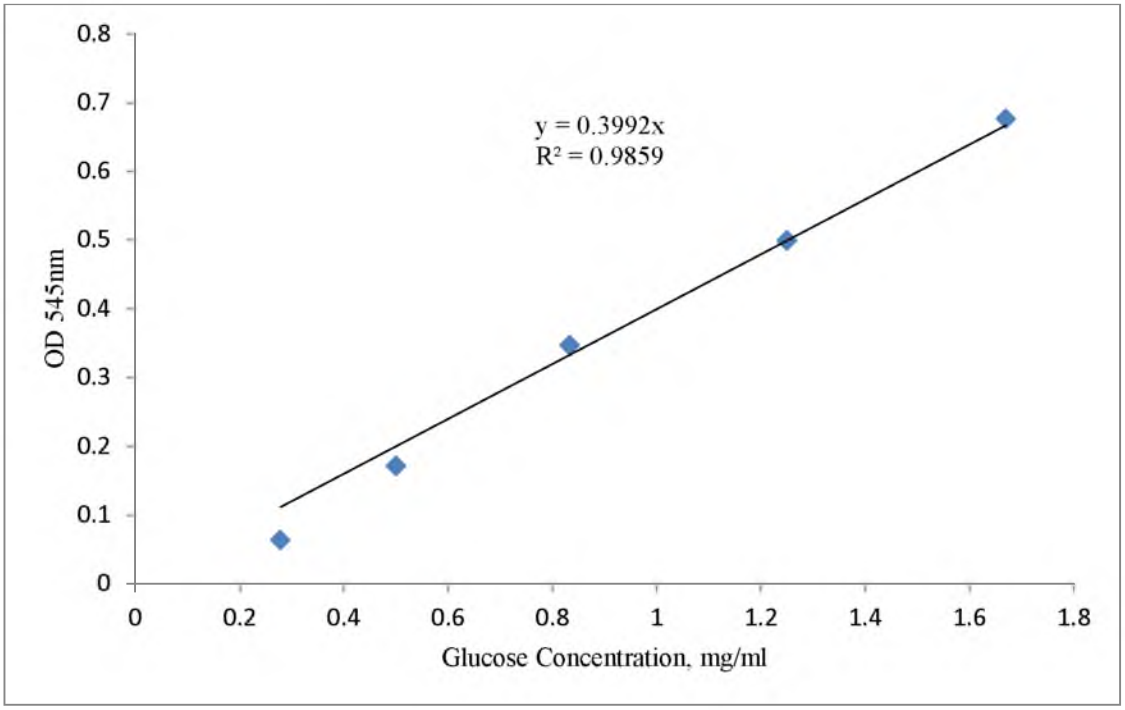
A2. Preparation of Sodium Citrate Buffer Solution

210 g of citric acid monohydrate was added into 750 mL of distilled water. Slowly, NaOH was added around 50 to 60 g until the pH reach 4.3. The solution was diluted to 1000 ml until reach pH 4.5 by adding NaOH. The prepared 1 Molar citrate buffer was diluted to 0.05 M concentration with pH adjusted to pH 4.8 and stored for further use.

APPENDIX B

STANDARD CURVE

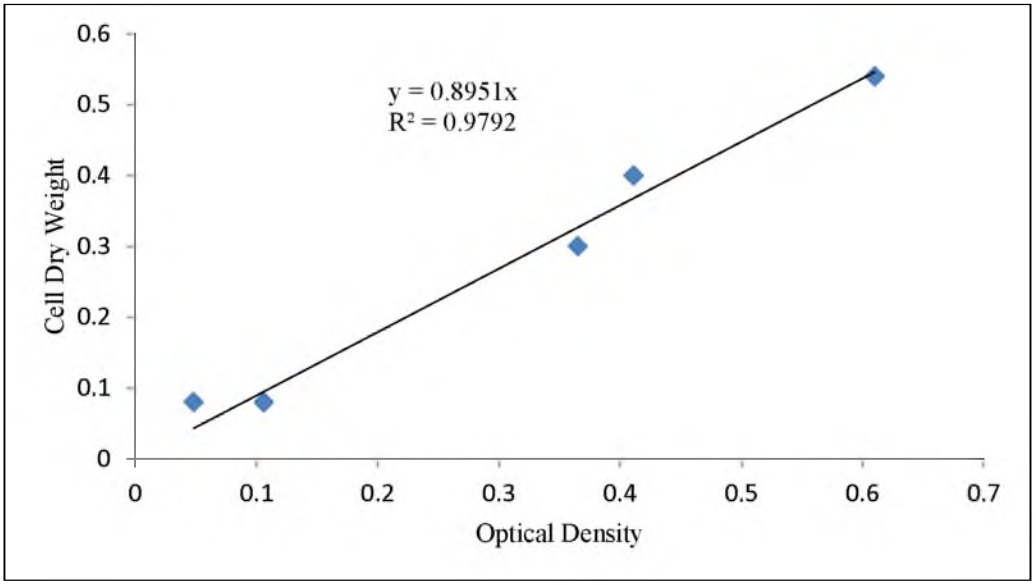
B1. Glucose Standard Curve



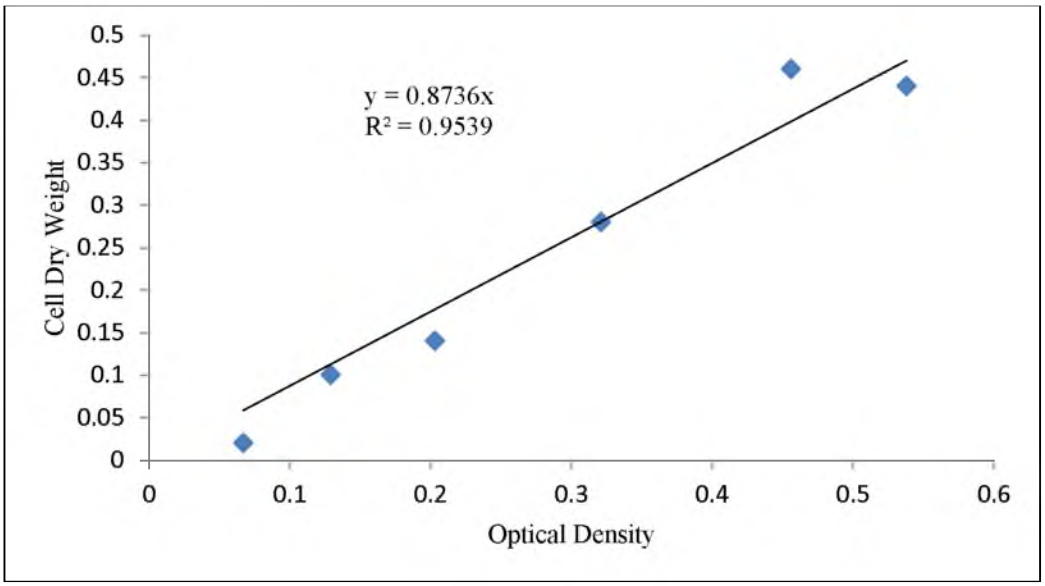
APPENDIX C

BIOMASS CURVE

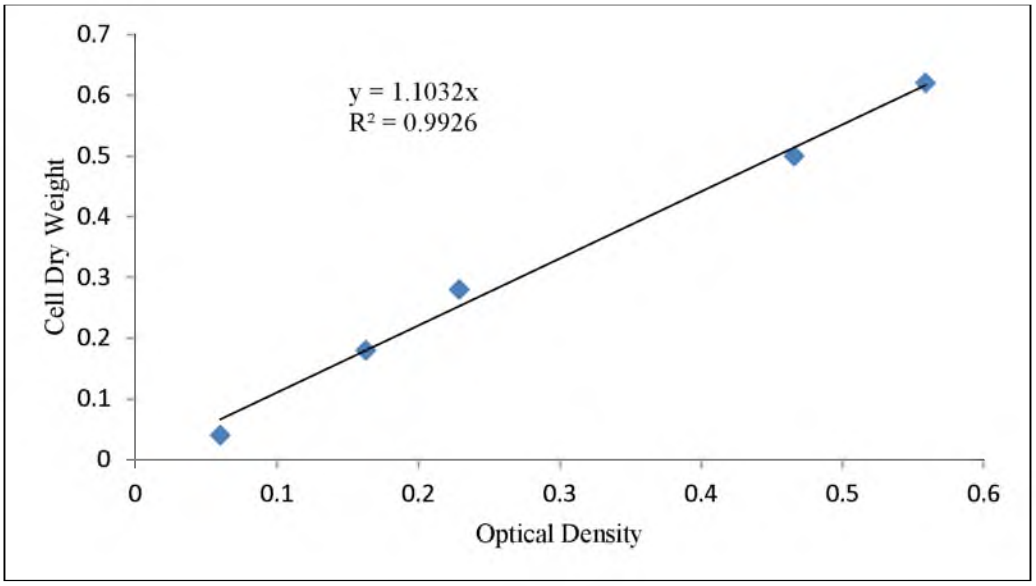
C1. *S. Cerevisiae*



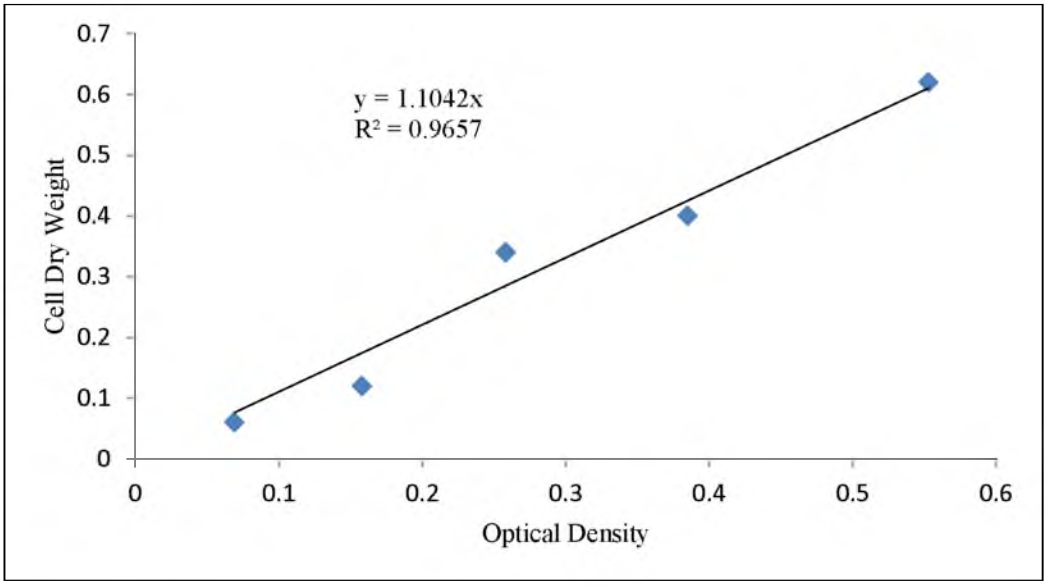
C2. *K. Lactis* GG799



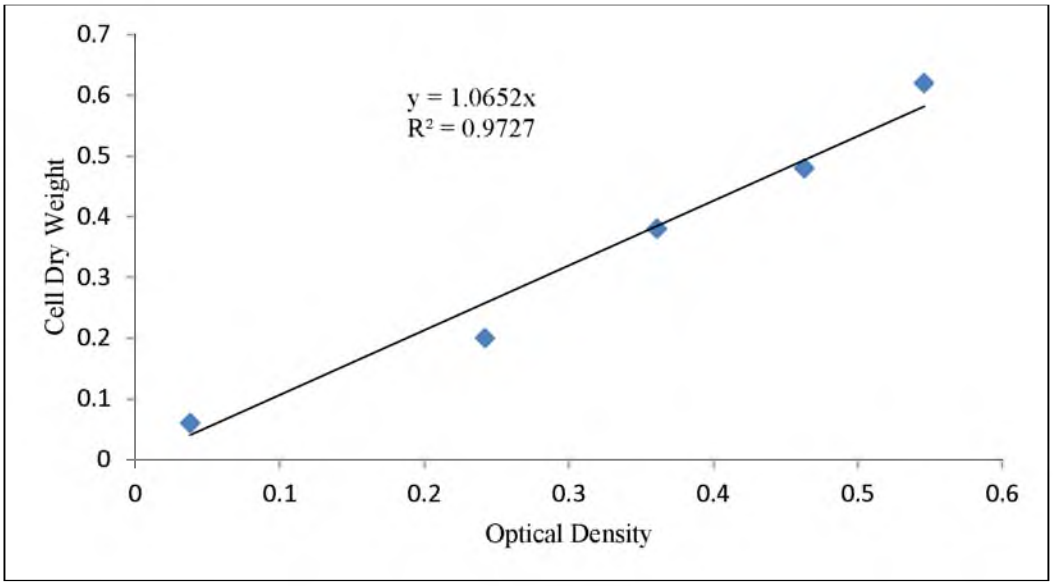
C3. *P. Pastoris* KM71H

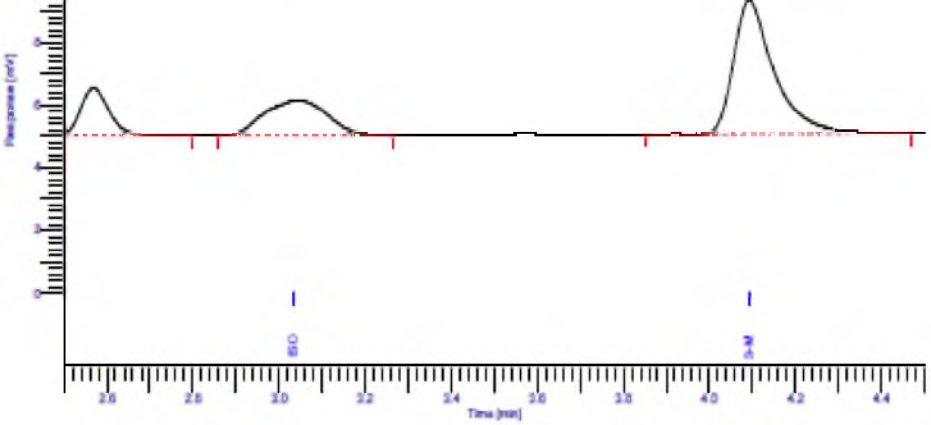


C4. *P. Pastoris* GS515



C5. *P. Pastoris* X33





ALCOHOL ANALYSIS REPORT

Peak #	Component Name	Time [min]	Area [µV*sec]	Height [µV]	Raw Amount ppm	Adjusted Amount ppm
1		2.570	7303.76	1537.36	0.0073	0.0073
2	isobutanol	3.038	11852.27	1154.09	92.4310	92.4310
3	3-methyl-1-butanol	4.099	28594.71	4896.47	233.2137	233.2137
			47750.75	6968.46	325.6520	325.6520

APPENDIX D

GC CHROMATOGRAM

D1. *S. cerevisiae*

Software Version	: 6.3.2.0646	Data	: 23/5/2015 12:07:41 PM
Operator	: UTM-GC	Sample Name	:
Sample Number	: 005	Study	: azah
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: Clarus 580	Channel	: A
Instrument Serial #	: None	A/D mV Range	: 1000
Delay Time	: 0.00 min	End Time	: 10.00 min
Sampling Rate	: 12.5000 pts/s		
Sample Volume	: 1.000000 ul		
Sample Amount	: 1.0000	Area Reject	: 0.000000
Data Acquisition Time	: 23/5/2015 10:25:19 AM	Dilution Factor	: 1.00
		Cycle	: 1

Raw Data File : C:\GC\Data\Azah\Screening Microorganism\21 May 2015\s.cerevisiae 48hrs 3.raw
Inst Method : C:\GC\Method\db-wax azah15_10 minutes from C:\GC\Data\Azah\Screening Microorganism\21 May 2015\s.cerevisiae 48hrs 3.raw
Proc Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
Calib Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
Report Format File: C:\GC\Method\Alcohol Analysis Report (300315).rpt
Sequence File : C:\GC\Sequence\db-wax azah15_10minutes.seq

D2. *K. lactis* GG799

Software Version	: 6.3.2.0646	Date	: 29/5/2015 12:19:02 PM
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Sample Number	: 008	Study	: azah
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: Clarus 580	Channel	: A
Instrument Serial #	: None	A/D mV Range	: 1000
Delay Time	: 0.00 min	End Time	: 10.00 min
Sampling Rate	: 12.5000 pts/s		
Sample Volume	: 1.000000 ul		
Sample Amount	: 1.0000	Area Reject	: 0.000000
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		Cycle	: 1

Raw Data File : C:\GC\Data\Azah\Screening Microorganism\21 May 2015\k.lactis GG799 48hrs 2.raw
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Calib Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
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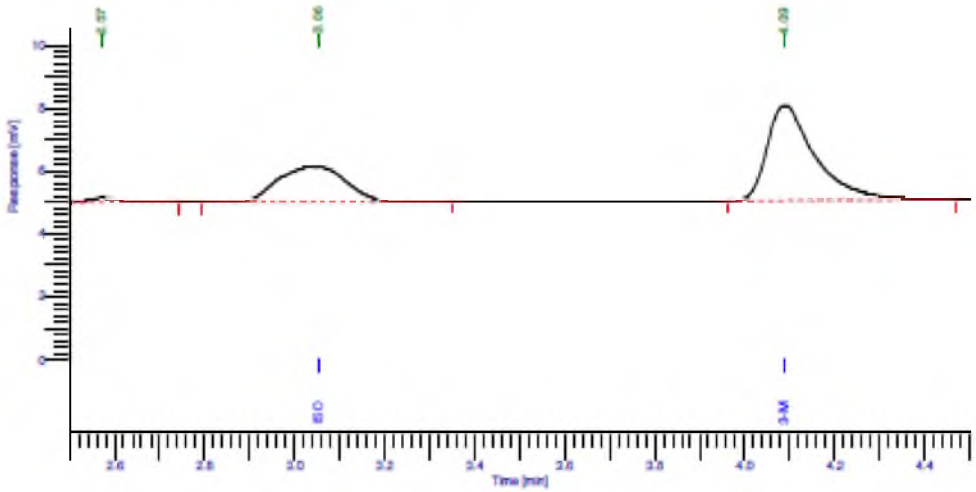


ALCOHOL ANALYSIS REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Raw Amount ppm	Adjusted Amount ppm
1		2.571	735.84	148.37	0.0007	0.0007
2	Isobutanol	3.056	11916.78	1136.48	92.9341	92.9341
3	3-methyl-1-butanol	4.089	22663.88	3028.05	184.8428	184.8428
			35316.50	4312.90	277.7776	277.7776

Missing Component Report

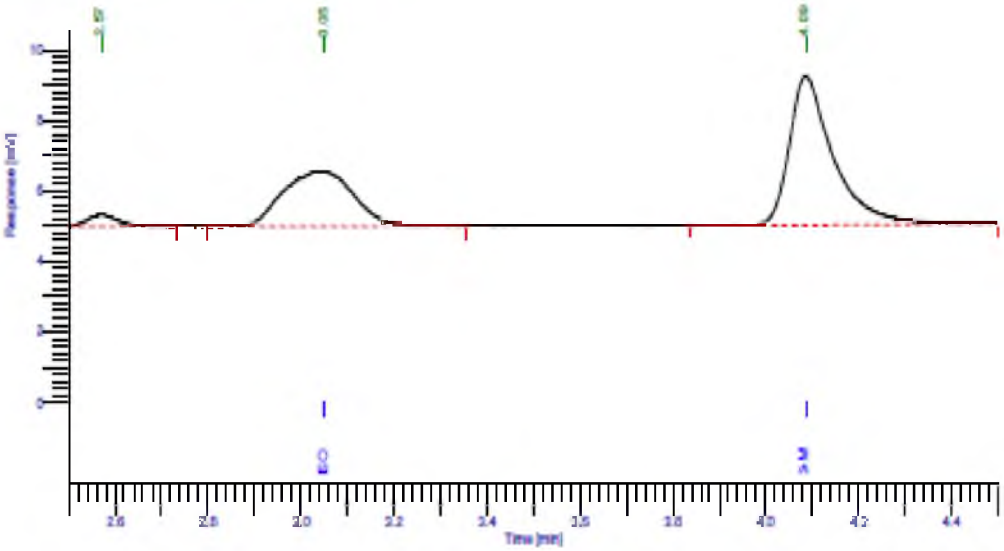
Component	Expected Retention (Calibration File)

All components were found

D3. *P. pastoris* KM71H

Software Version	: 6.3.2.0646	Date	: 29/5/2015 10:23:21 PM
Operator	: UTM-GC	Sample Name	:
Sample Number	: 012	Study	: azah
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: Clarus 580	Channel	: A
Instrument Serial #	: None	A/D mV Range	: 1000
Delay Time	: 0.00 min	End Time	: 10.00 min
Sampling Rate	: 12.5000 pts/s		
Sample Volume	: 1.000000 ul		
Sample Amount	: 1.0000	Area Reject	: 0.000000
Data Acquisition Time	: 29/5/2015 12:09:10 PM	Dilution Factor	: 1.00
		Cycle	: 1

Raw Data File : C:\GC\Data\Azah\Screening Microorganism\21 May 2015\p.pastoris KM71H 48hrs 9.raw
Inst Method : C:\GC\Method\db-wax azah15_10 minutes from C:\GC\Data\Azah\Screening Microorganism\21 May 2015\p.pastoris KM71H 48hrs 9.raw
Proc Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
Calib Method : C:\GC\Method\db-wax azah16 standard 30315.mth from
Report Format File: C:\GC\Method\Alcohol Analysis Report (30315).rpt
Sequence File : C:\GC\Sequence\db-wax azah15_10minutes.sq



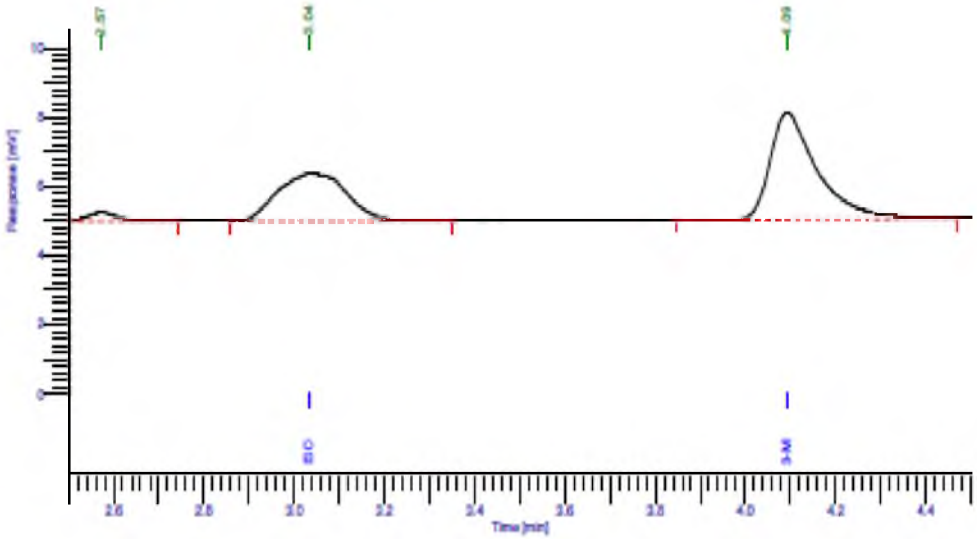
ALCOHOL ANALYSIS REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Raw Amount ppm	Adjusted Amount ppm
1		2.570	1523.57	325.29	0.0015	0.0015
2	isobutanol	3.050	16379.43	1545.46	127.7286	127.7286
3	3-methyl-1-butanol	4.066	27773.77	4225.33	226.5182	226.5182
			45681.77	6096.72	354.2484	354.2484

D4. *P. pastoris* GS515

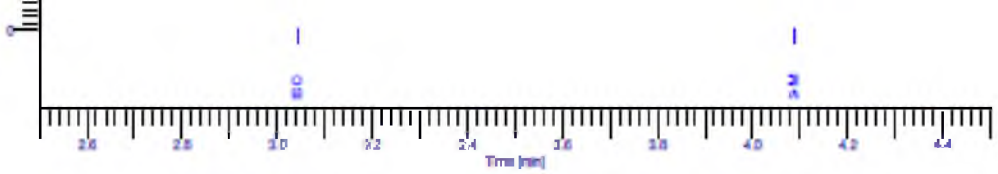
Software Version	: 6.3.2.0846	Date	: 23/5/2015 10:25:50 PM
Operator	: UTM-GC	Sample Name	:
Sample Number	: 013	Study	: azan
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: Clarus 580	Channel	: A
Instrument Serial #	: None	A/D mV Range	: 1000
Delay Time	: 0.00 min	End Time	: 10.00 min
Sampling Rate	: 12.5000 pts/s		
Sample Volume	: 1.000000 ul		
Sample Amount	: 1.0000	Area Reject	: 0.000000
Data Acquisition Time	: 23/5/2015 12:19:22 PM	Dilution Factor	: 1.00
		Cycle	: 1

Raw Data File : C:\GC\Data\Azan\Screening Microorganism\21 May 2015\p.pastoris GS115 48hrs 1.raw
Inst Method : C:\GC\Method\db-wax azan15_10 minutes from C:\GC\Data\Azan\Screening Microorganism\21 May 2015\p.pastoris GS115 48hrs 1.raw
Proc Method : C:\GC\Method\db-wax azan15 standard 230315.mth from
Calib Method : C:\GC\Method\db-wax azan15 standard 230315.mth from
Report Format File: C:\GC\Method\Alcohol Analysis Report (230315).rpt
Sequence File : C:\GC\Sequence\db-wax azan15_10minutes.seq



ALCOHOL ANALYSIS REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Raw Amount ppm	Adjusted Amount ppm
1		2.579	1190.59	254.41	0.0012	0.0012
2	isobutanol	3.036	14701.11	1398.03	114.6479	114.6479
3	3-methyl-1-butanol	4.094	22593.45	3076.03	184.2684	184.2684
			38484.95	4724.34	298.9175	298.9175



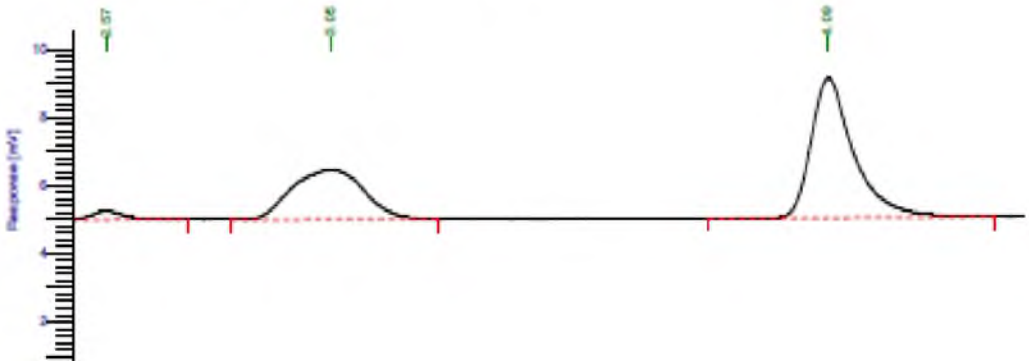
ALCOHOL ANALYSIS REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Raw Amount ppm	Adjusted Amount ppm
1		2.570	1192.79	258.91	0.3012	0.3012
2	isobutanol	3.04E	15460.43	1465.33	120.5695	120.5695
3	3-methyl-1-butanol	4.330	25796.37	4083.56	209.9015	209.9015
			42389.59	5807.37	330.4723	330.4723

D5. *P. pastoris* X33

Software Version	: 6.3.2.0646	Data	: 23/5/2015 10:29:16 PM
Operator	: UTM-GC	Sample Name	:
Sample Number	: 018	Study	: azah
AutoSampler	: NONE	Rack/Vial	: 0/0
Instrument Name	: Clarus 580	Channel	: A
Instrument Serial #	: None	A/D mV Range	: 1000
Delay Time	: 0.00 min	End Time	: 10.00 min
Sampling Rate	: 12.5000 pts/s		
Sample Volume	: 1.000000 ul		
Sample Amount	: 1.0000	Area Reject	: 0.000000
Data Acquisition Time	: 23/5/2015 1:41:06 PM	Dilution Factor	: 1.00
		Cycle	: 1

Raw Data File : C:\GC\Data\Azah\Screening Microorganism\21 May 2015\p.pastoris X33 48hrs 3.raw
Inst Method : C:\GC\Method\db-wax azah15_10 minutes from C:\GC\Data\Azah\Screening Microorganism\21 May 2015\p.pastoris X33 48hrs 3.raw
Proc Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
Calib Method : C:\GC\Method\db-wax azah15 standard 30315.mth from
Report Format File: C:\GC\Method\Alcohol Analysis Report (300315).rpt
Sequence File : C:\GC\Sequence\db-wax azah15_10minutes.seq



APPENDIX E

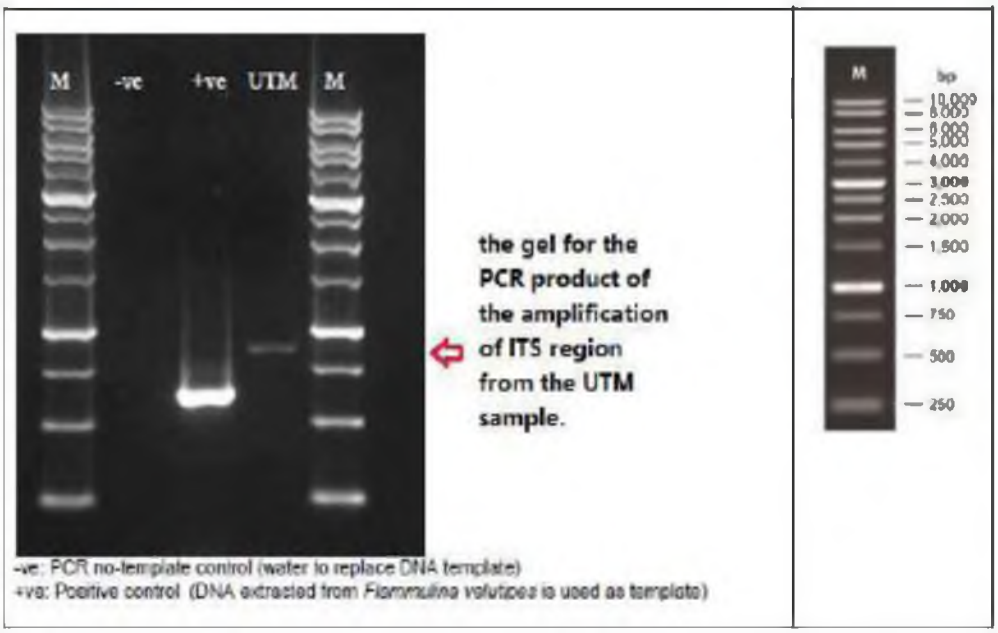
SPECIES BARCORDING REPORT

SAMPLE INFORMATION

Sample Name	:	Fungus_UTM
PCR Product	:	Internal transcribed spacer (ITS), ~ 700 bp

RESULTS

1. Gel Photo



The PCR product was cloned to the pJET1.2 vector. Then the plasmid was extracted and sequence by using our universal primer. After determine the sequencing result, we have help to trim off the vector sequence and determine the sequence of the insert (the sequence of the ITS region for sample UTM) as in the report. Then we performed BLAST for the insert sequence and find the top 10 results which have the highest Hits in the NCBI database. This result show the possibility of your sample is a "*Saccharomyces cerevisiae*".

APPENDIX F

LIST OF PUBLICATIONS

Indexed Journal

1. **Ramli, N. A**., Rahman, R. A., & Illias, R. M. (2017). Microbial growth kinetics in isobutanol production by *Saccharomyces cerevisiae*. *Chemical Engineering Transactions*, *56*, 793-798. DOI: 10.3303/CET1756133 (Index by Scopus)

2. **Ramli, N. A**., Rahman, R. A., Ngadi, N., & Samah, R. A. (2017). Optimisation of fermentation conditions for isobutanol production by *Saccharomyces cerevisiae* using response surface methodology. *Chemical Engineering Transactions*, *56*, 301-306. DOI: 10.3303/CET1756051 (Index by Scopus)

Non-Indexed Journal

1. **Ramli, N. A**., Rahman, R. A., & Illias, R. M. (2019). Enhancement of isobutanol and 3-methyl-1-butanol production yields in *Saccharomyces Cerevisiae* without genetic modification. *Journal of Energy and Safety Technology*, 1(2), 7 – 13.

2. **Ramli, N. A**., & Rahman, R. A. (2015). Isobutanol production and alcohol tolerance by yeast wild strain. *Advanced Materials Research*, 1113, 334-339.

3. **Ramli, N. A**., Rahman, R. A., & Ngadi, N. (2014). Production of 3-methyl-1-butanol by yeast wild strain. *International Journal of Biological, Veterinary, Agricultural and Food Engineering*, 8 (4), 400-403.

4. Pagarra, H., Rahman, R. A., Illias, R. M., & **Ramli, N. A**. (2014). Optimization of pectin extraction from *Nephrolepis biserrata* leaves using response surface methodology. *Applied Mechanics and Materials*, 625, 920-923.

Book Chapter

1. **Ramli, N. A**., Rahman, R. A., Ngadi, N., & Illias, R. M. Biobutanol: An Attractive Microbial Fuel. In: Rahman, R. A., & Ngadi, N. ed. *Biomass into Fuel and Chemical Derivatives. Malaysia*. UTM Press. 1-20; 2016.