Optimisation of Bioethanol Yield from Oil Palm Trunk Sap

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Abstract*—This paper presents the effect of nutrients addition and fermentation microorganism on bioethanol yield from oil palm trunk sap. Six microorganisms namely, *Saccharomyces cerevisiae* ATCC 9763, *Saccharomyces cerevisiae* ATCC 26602, baker's yeast, *Kluyveromyces marxianus* ATCC 46537, *Zymomonas mobilis* ATCC 29501 and *Escherichia coli* ATCC 10536 were screened for ethanol production at fixed temperature, pH, agitation and inoculum size. The sugar and ethanol content were determined using a high performance liquid chromatography (HPLC). The results showed that *K. marxianus* produced the highest ethanol yield (60.9%) at a shorter fermentation time (16h) compared to the other strains. Six nutrients, namely, ammonium sulphate, di-ammonium hydrogen phosphate, magnesium sulphate, β-alanine, calcium chloride and potassium dihydrogen phosphate were screened using this strain and the highest ethanol yield (98.62%) was achieved in fermentation supplemented by magnesium sulphate and β-alanine. Subsequently, the optimisation study using a reseponse surfae methodology found the optimum value of magnesium sulphate was 7.93 g/L and 0.90 g/L for β-alanine. Under the optimum conditions, the predicted ethanol concentration was 34.58 g/L while the experimental value (35.50 g/L) was in agreement with the predicted value with 2.66% error.

Keywords— Bioethanol; Oil Palm Trunk Sap; Nutrient Supplementation; Response Surface Methodology

I. INTRODUCTION

Malaysia has an abundant amount of oil palm biomass arising from replanting activities involving old oil palm tree aged above 20 years-old. According to Malaysian Palm Oil Board, about 10% of the total 5.23 million hectares of oil palm (*Elaeis guineensis*) plantation in Malaysia must be replanted yearly due to decrease in oil productivity of old trees besides the difficulty in harvesting their fruit [1,2]. This means about 70 million old palm trees will be felled annually in Malaysia, which generates over 15 million tons of oil palm trunks [3]. In most cases the felled trees are left to rot on the plantation grounds, although some oil palm trunks were used for plywood manufacturing. The plywood manufacturing only utilises the header portion (the outer layer) of the felled oil palm trunk, whilst the soft inner portion is normally discarded. This soft inner portion contains a huge amount of sugar-rich sap that can be fermented into bioethanol [4].

The most common fermenting microorganism used for bioethanol production is yeast, particularly *S. cerevisiae*, which is also known as baker's yeast [5]. *S. cerevisiae* is often chosen for ethanol production due to its excellent fermenting capacity, the capacity to grow rapidly under anaerobic conditions and high tolerance to ethanol [6]. The potential of oil palm trunk saps as feedstock for bioethanol production has been examined by Kosugi et al. [4] and Yamada et al. [7]. Limited study on oil palm trunk sap (OPTS) fermentation using various ethanol-producing strains in exception of those by Norhazimah and Faizal [8], who studied the effect of single and co-culture strain on ethanol production from OPTS. They studied several strains of yeast and bacteria, namely, *S. cerevisiae*, *S. cerevisiae Kyokai no. 7* ATCC 26622, *S. cerevisiae* JCM 2220 ATCC 9804, *Zymomonas mobilis* JCM 10190 ATCC 29191, *Zymobacter palmae* JCM 21091 ATCC 51623, and *Pichia stipitis* JCM10742 ATCC 58376. They reported high ethanol yield (over 0.4 g ethanol/g sugar) was obtained from fermentation using any of the yeast studied. Meanwhile, all the bacteria strains tested produced low ethanol yield below 0.24 g ethanol/g sugar. No study concerning OPTS fermentation to bioethanol using *K. marxianus* ATCC 46537, *Z. mobilis* ATCC 29501 and *E. coli* ATCC 10536 is available in the literature, and hence screening their suitability is one of the objectives of this work. Three yeast species, namely, *S. cerevisiae* ATCC 9763, *S. cerevisiae* ATCC 26602 and baker's yeast are also tested for comparison purpose. Moreover, limited study on the influence of nutrient addition to the bioethanol yield from oil palm trunk sap is available in the literature, in exception of the one by Nor Syahirah et al. [9]. However, the recent work by Nor Syahirah et al. [9] is limited to *Saccharomyces cerevisiae*, whereas this work includes the effect of microorganism types. The OPTS has a sufficient amount of micronutrients (i.e. Mo, Na, Ca, Zn and vitamins) for the growth of yeast [4], however, the content of macronutrients existed naturally in the OPTS is too low to effectively enhance bioethanol production [9]. Addition of

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nutrient such as magnesium sulfate (MgSO4), ammonium sulfate ($(NH_4)_2SO_4$), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), β-alanine ($C_3H_7NO_2$), calcium chloride ($CaCl_2$) or potassium dihydrogen phosphate (KH_2PO_4) are known to increase yield of bioethanol from sugar fermentation [10-14]. Thus, the primary objective of the current work is to study the effects of multiple nutrient additions to the bioethanol yield. Subsequently, optimization of the amount of nutrient addition was studied using response surface methodology.

II. Materials and Methods

A. Preparation of Oil Palm Trunk Sap

The OPTS in this work was obtained from a 30 year-old oil palm trunk from the Federal Land Development Authority (FELDA) Jengka 14, Pahang. The outer harder portion of the trunk was peeled to obtain the softer sap-rich core. The trunk core was then chopped into smaller pieces of about 20 × 20 × 1 cm. Thereafter, the OPT core was squeezed using a heavy-duty sugar cane juice machine to obtain the sap. The sap was centrifuged at 4629 ×g for 15 min at 4 °C (Eppendorf 5810R, Germany), filtered and stored in the freezer at -20 °C before used for fermentation.

B. Chemicals and Fermentation Strain

The sulphuric acid was obtained from Merck (Darmstadt, Germany). Yeast extracts, peptone, ammonium sulfate, diammonium hydrogen phosphate, calcium chloride, potassium dihydrogen phosphate, magnesium sulphate and standard sugars for HPLC analysis, such as glucose, sucrose and fructose were obtained from Fisher Scientific (Leicestershire, UK). In addition, the galactose standard sugar and β-alanine were obtained from Acros (New Jersey, USA) while the Baker's yeast in powder form was obtained from AB MAURI (Balakong, Malaysia). The remaining strains of *Saccharomyces cerevisiae* ATCC 9763, *Escherichia coli* ATCC 10536, *Saccharomyces cerevisiae* ATCC 26602, *Kluyveromyces marxianus* ATCC 46537, *Zymomonas mobilis* ATCC was obtained from the American Type Culture Collection, USA (www.atcc.org).

C. Pure Culture and Inoculum Preparation

Six strains of microorganisms were used in this work which is *Saccharomyces cerevisiae* ATCC 9763, *Escherichia coli* ATCC 10536, *Saccharomyces cerevisiae* ATCC 26602, *Kluyveromyces marxianus* ATCC 46537, *Zymomonas mobilis* ATCC and baker's yeast. All strains were prepared by streaking on new agar plates and incubated (Infors-HT, Switzerland) at 30 °C up to 3 days. The strains were kept at 4 °C and sub-cultured every month on new nutrient agar to maintain the continuity of pure culture supply.

Inoculums were prepared by transferring one loop of pure culture into 100 ml of sterile nutrient broth. All strains were incubated for 12-18h at 30 °C and 150 RPM until a standard initial concentration (optical density of 1.5-1.7 at 600 nm) was reached. The standard optical density of 1.5-1.7 corresponds to a stationary phase in the microbial growth deduced by studying the microbial growth curve. Cells were harvested via centrifugation in 50 ml conical centrifuge tubes for 10 minutes at 6300 ×g.

D. Microorganism Screening Fermentation

All fermentations were performed using the 250 ml Erlenmeyer flask with batch cultures of 100 ml working volume. The feedstock for fermentation, oil palm sap (± 90 g/L) was added into fermentation flasks. After sterilization by autoclave, the flask was aseptically inoculated with 10% (v/v) of microorganism suspensions. Subsequently, the fermentations were incubated at 30 °C, agitated at 150 rpm and subjected to fermentation up to 72 hours. All experiments were performed in duplicate. Sample aliquots of 4 ml were taken aseptically at 0h, 12h, 24h, 36h, 48h, 60h and finally 72h. The sample was centrifuged for 10 min at 10621 ×g and filtered through a 0.2 µm nylon syringe filter. The samples were stored at -20 °C freezer until further analysis.

E. Experimental Design and Statistical Analysis

Experimental design and statistical analysis were performed using the design-expert version 8.0.6 software (Stat-Ease Inc, Minneapolis, USA) with risk factor (α) values of 0.05 (95% level of confidence). The adjusted coefficient of regression (R^2_{adj}) value above 0.75 is considered acceptable. Variables with P-value lower than 0.05 were considered to have a significant effect on the response. The physical parameters were fixed at pH 5.5, agitation of 150 RPM, temperature of 30 °C and fermentation time of 16h. All experiments were carried out in duplicates and the results are reported in terms of mean values ± standard deviation.

A two-level factorial experiment matrix was set to identify the important factors and to estimate their significance in ethanol production. The model predicts a linear relation of $Response = a + biXi$ where only main effects are taken into consideration. The response represents a dependent variable, i.e. ethanol production (g/l), a is the model interception, X_i represents different levels of independent variables and b_i is a coefficient predicted by the equation. In this work, six independent variables were selected which are the amount of ammonium sulphate (A), diammonium hydrogen phosphate (B), magnesium sulphate (C), β-alanine (D), calcium chloride (E) and potassium dihydrogen phosphate (F). Nutrient screening for ethanol production were performed in an Erlenmeyer flask added with different nutrients according to the experimental design as shown in Table 1.

TABLE I. Two-level Factorial Design Experiments to Investigate The Effects Of Six Nutrients (A, B, C, D, E and F) on Ethanol Production

Factor	Name	Units	Low (-1)	Centre point (0)	High (1)
A	$(NH_4)_2SO_4$	g/L	0	2.5	5
B	$(NH_4)_2HPO_4$	g/L	0	2.5	5
C	$MgSO_4$	g/L	0	2.5	5
D	$C_3H_7NO_2$	g/L	0	0.25	0.5
E	$CaCl_2$	g/L	0	0.4	0.8
F	KH_2PO_4	g/L	0	1	2

F. Cell Dry Weight Measurement

The initial centrifuge tubes were marked, dried to constant weight and their weight were recorded. During fermentation, the cells were harvested at interval times by centrifugation for 10 minutes at 10621 ×g and washed three times with distilled water and re-centrifuged. The cell pellet was dried at 60 °C to constant weight and the weight of each tube was recorded.

G. Determination of Sugar Content

The sugar components (sucrose, glucose and fructose) were analysed using HPLC Agilent Technology 1200 Series with Zorbax Carbohydrate Analysis Column, 4.6 mm ID × 150 mm (5 µm). The mobile phase used was 75:25 of acetonitrile:water with a flow rate of 1.4 ml/min at 30 °C detected using a refractive index detector (RID). The volume injected was 10 µl and the run time was 10 minutes for each sample.

H. Determination of Ethanol Content

Ethanol analyses were performed using HPLC Agilent Technology 1200 Series equipped with autosampler, degasser and a RID-10A refractive index detector. A Rezex 8 µm ROA-Organic Acid H^+ (8%) LC Column 150 × 7.8 mm (Phenomenex, Torrance, CA, USA) was used with SecurityGuard™ cartridges (Phenomenex, Torrance, CA, USA) as a guard column. The temperature was heated up to 65 °C, and 0.005 N H_2SO_4 was used as eluent at a flow rate of 0.6 ml/min. The volume injected was 20 µl. Run time was 15 minutes and 50% methanol was used in the autosampler needle washing solution to avoid bacterial contamination.

III. Results and Discussion

A. Microorganisms Growth Curve

The preliminary experiment was conducted prior to microorganisms screening to check the growth of each microorganism. The six strains were *Kluyveromyces marxianus* ATCC 46537, *Saccharomyces cerevisiae* ATCC 9763, *Saccharomyces cerevisiae* ATCC 26602, *Escherichia coli* ATCC 10536, *Zymomonas mobilis* ATCC 29501 and baker's yeast. The experiments were carried out at an inoculum size of 10 % (v/v), pH of 5.5, agitation speed of 150 RPM, incubation temperature of 30 ºC and fermentation time of 24 h. All strains were screened in nutrient agar medium except for *Escherichia coli* ATCC 10536 which was carried out in nutrient broth. The growth curve for each strain was plotted by measuring their cell dry weight against time as shown in Fig. 1.

Fig. 1 shows that all strains develop well in their standard medium. The kinetic parameters of each strain were calculated and presented in Table 2 to compare the growth rate between each strain. The X_{max} is the maximum cell dry weight and the value of specific growth rate (µ) was calculated from the plot of ln (X) versus time. The specific rate of substrate utilization (q_s) and cell doubling time (t_d) were determined by using the following equation:

$$q_s = \mu X_{max} / Y_{x/s} \quad (1)$$

$$t_d = \ln 2 / \mu \quad (2)$$

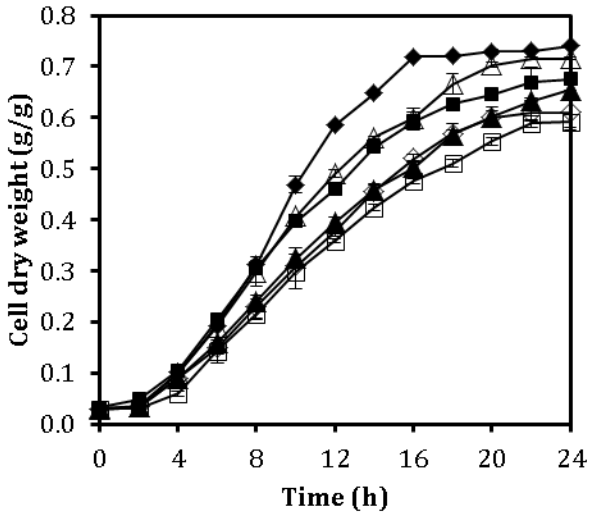

Fig. 1. The growth curve by each strain. Symbols represent: (□) *Z. mobilis* ATCC 29501, (◊) *E. coli* ATCC 10536, (▲) Baker's yeast, (Δ) *S. cerevisiae* ATCC 9763, (■) *S. cerevisiae* ATCC 26602 and (♦) *K. marxianus* ATCC 46537.

TABLE II. Kinetic Parameters of Each Strain in Their Standard Fermentation Media

Strains	X_{max}	µ	q_s	t_d
S. cerevisiae ATCC 26602	6.75	0.19	9.8	3.72
Baker's yeast	6.54	0.19	9.95	3.65
E. coli ATCC 10536	6.10	0.19	9.94	3.67
K. marxianus ATCC 46537	7.40	0.22	11.23	3.22
S. cerevisiae ATCC 9763	7.15	0.20	10.49	3.46
Z. mobilis ATCC 29501	5.91	0.19	10.19	3.58

B. Microorganisms Screening

Fig. 2 shows a representative fermentation profile of OPTS using *K. marxianus* ATCC 46537 without nutrient addition. The total sugar concentration decreased quickly in the first 36 hours where all sugars were totally consumed by *K. marxianus* ATCC 46537. A rapid production of ethanol was observed within 12h and slightly decreased after 24h. The highest ethanol concentration was obtained at 24h which was 28.09 ± 1.29 g/l. The maximum cell dry weight was achieved at 24h with 7.18 ± 0.32 g/g and retained until the end of the fermentation period.

Similar trends can be observed for all four strains in ethanol production where there were rapid productions of ethanol in the first 24h and maintained constant until 36 except for strain *K. marxianus* ATCC 46537 which decreased at 36h. Then, a slight decrease of ethanol yield can be observed after 48h. Walker [15] described the reduction of ethanol occurred due to glucose derepression. Glucose repression in yeasts describes a long-term regulatory adaptation to degrade glucose exclusively to ethanol and carbon dioxide. Therefore, when the yeast grows on high concentrations of glucose, fermentation accounts for the majority of glucose consumption. In batch culture, however,

when the levels of glucose consumption drop, cells will progressively become derepressed, resulting in induction of respiratory enzyme synthesis. This, consecutively, results in oxidative consumption of accumulated ethanol causing depletion of ethanol yield if further fermentation continues. The *K. marxianus* ATCC 46537 was chosen for further optimization because it can achieve a maximum ethanol concentration in shorter fermentation time beside capable of producing high ethanol yields.

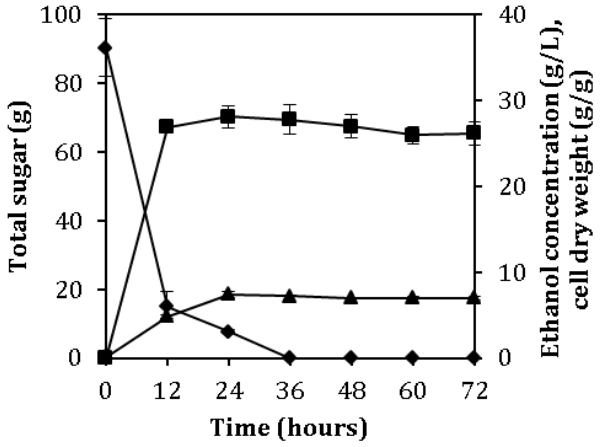

Fig. 2. Fermentation profile of un-supplemented OPTS by *K. marxianus* ATCC 46537. Symbols represent: (♦) total sugar, (■) ethanol concentration and (▲) cell dry weight.

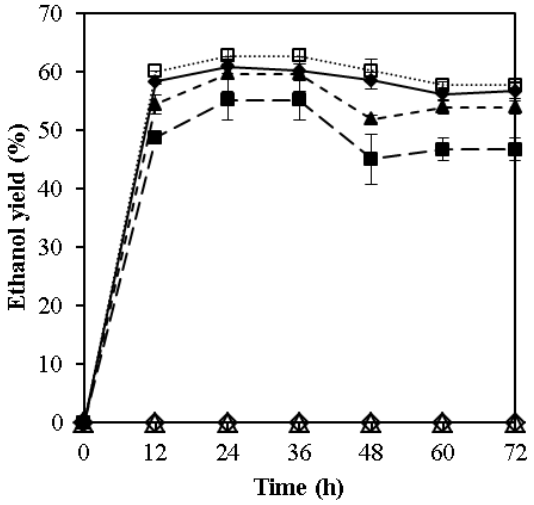

Fig. 3. Time course of ethanol yield by each strain for non-supplemented OPTS fermentation. Symbols represent: (□) *S. cerevisiae* ATCC 26602, (◊) *Z. mobilis* ATCC 29501, (▲) *S. cerevisiae* ATCC 9763, (Δ) *E. coli* ATCC 10536, (■) Baker's yeast and (♦) *K. marxianus* ATCC 46537.

Fig. 3 shows the theoretical yield of ethanol produced by each strain throughout the experiment. There are four strains that showed positive results, namely *K. marxianus* ATCC 46537, baker's yeast, *S. cerevisiae* ATCC 9763 and *S. cerevisiae* ATCC 26602. The other two strains, *Z. mobilis* ATCC 29501 and *E. coli* ATCC 10536, did not produce ethanol. The maximum ethanol yield was obtained within 24h of a fermentation period for all the four strains that showed positive results. The highest ethanol yield was produced by strain *S. cerevisiae* ATCC 26602 (62.6%), followed by *K. marxianus* ATCC 46537 (60.9%). Meanwhile, *S. cerevisiae* ATCC 9763 produced 59.6% of ethanol yield and baker's yeast produced 55.2% of ethanol yield.

TABLE III. ETHANOL PRODUCTION (G/L), PRODUCTIVITY (G/L/H), THEORETICAL YIELD (%) AND CELL DRY WEIGHT (G/G) BY FOUR ETHANOL PRODUCING STRAINS AT 24H

Microorganisms	Ethanol Concentration (g/l)	Productivity (g/l/h)	Ethanol yield (%)	Cell dry weight (g/g)
K. marxianus ATCC 46537	28.09 ± 1.29	1.17	60.9	7.18 ± 0.32
Baker's yeast	25.45 ± 3.50	1.06	55.2	6.50 ± 0.00
S. cerevisiae ATCC 9763	27.51 ± 2.95	1.15	59.6	8.95 ± 0.14
S. cerevisiae ATCC 26602	28.90 ± 1.41	1.20	62.6	6.75 ± 0.35

Table 3 shows a comparison of ethanol production, productivity, theoretical yield and cell dry weight of all ethanol producing strains tested after 24h. The highest ethanol concentration, 28.90 g/l, equivalent to a productivity of 1.20 g/l/h and 62.6% of theoretical yield, was achieved by *S. cerevisiae* ATCC 26602, followed by *K. marxianus* ATCC 46537 that produced 28.09 g/l ethanol, productivity of 1.17 g/l/h and 60.9 % of the theoretical yield. The baker's yeast has the lowest ethanol concentration, which was 25.45 g/l, or a productivity of 1.06 g/l/h and a yield 55.2 % of the theoretical yield. The highest cell dry weight, 8.95 g/g was produced by *S. cerevisiae* ATCC 9763 whereas the lowest was 6.50 g/g produced by baker's yeast. Meanwhile, *S. cerevisiae* ATCC 26602 and *K. marxianus* ATCC 46537 produced cell dry weight of 6.75 g/g and 7.18 g/g, respectively.

C. Screening of Nutrients using Two-Level Factorial Design

The development of a fermentation medium based on industrial substrates to enhance ethanol yield from OPTS fermentation is economically desirable. The nutrient screening experiments were carried out at an inoculum size of 10 % (v/v), pH of 5.5, agitation speed of 150 RPM, incubation temperature of 30 ºC and fermentation time of 16h. Table 4 shows the experimental data as well as the values predicted by the models constructed using one response variable which is the final ethanol concentration. The quantity of ethanol produced by using *K. marxianus* ATCC 46537 varied from 22.95 to 37.71 g/l. The Run 6 and Run 3 showed the minimum and maximum ethanol production, respectively. The mean of the ethanol produced by *K. marxianus* ATCC 46537 using selected supplements with selected concentration was 30.56 g/l.

Regression analysis showed that the models for final ethanol concentration is adequate with $R^2 = 0.91$ and adjusted $R^2 = 0.84$. The significance of each coefficient was determined by student's t-test. The p-value was used as an indicator of the statistical significance of the test. The result of the model using the final ethanol concentration as the response is presented in Table 5. Significant linear terms were *B*, *C* and *D*

while *A*, *E* and *F* are non-significant terms. The interaction coefficient of *AB*, *AC* and *ABD* presented were significant too. Other interaction coefficient of *AD*, *AE*, *AF*, *BD*, *BF* and *ABF* exhibited non-significant terms. Taking accounts the coefficient estimate, only *C* and *D*, whose probability values are below 0.05 showed a positive effect on ethanol production in oil palm sap fermentation.

TABLE IV. THE EXPERIMENTAL DATA OF FINAL ETHANOL CONCENTRATION AND VALUES PREDICTED BY THE MODELS

Run	A	B	C	D	E	F	Ethanol yield (%)	Ethanol concentration (g/l)	
								Experiment	Predicted
1	5	0	5	0.5	0	0	89.51	34.31	34.3
2	5	5	5	0	0.8	0	75.40	29.09	28.97
3	5	5	5	0.5	0.8	2	98.62	37.71	35.87
4	5	0	5	0	0	2	84.15	31.55	33.52
5	0	5	5	0.5	0	2	74.80	28.31	28.99
6	5	5	0	0	0	2	59.86	22.95	23.07
7	0	5	0	0	0.8	2	69.75	26.68	27.17
8	0	0	0	0.5	0	2	96.94	36.84	36.63
9	0	0	0	0	0	0	76.78	28.93	29.34
10	2.5	2.5	2.5	0.25	0.4	1	86.83	32.54	31.14
11	0	5	0	0.5	0.8	0	77.85	29.34	28.65
12	2.5	2.5	2.5	0.25	0.4	1	80.26	29.85	31.14
13	5	0	0	0	0.8	0	79.81	29.59	27.62
14	5	0	0	0.5	0.8	2	74.03	28.4	28.4
15	0	5	5	0	0	0	72.04	28	27.51
16	0	0	5	0	0.8	2	79.23	30.08	29.68
17	5	5	0	0.5	0	0	72.67	28.13	29.97
18	2.5	2.5	2.5	0.25	0.4	1	81.94	31.11	31.14
19	0	0	5	0.5	0.8	0	96.45	36.75	36.97
20	2.5	2.5	2.5	0.25	0.4	1	79.64	31.05	31.14

TABLE V. THE COEFFICIENT ESTIMATE AND P-VALUE OF THE MODEL

Factor	Coefficient estimate	p-value
Intercept	30.56	
A-Ammonium sulphate	-0.2	0.5229
B-Ammonium phosphate	-1.64	0.0047*
C-Magnesium sulphate	1.56	0.0056*
D-Alanine	2.06	0.0020*
E-Calcium chloride	0.54	0.1341
F-Potassium phosphate	-0.1	0.7445
AB	0.9	0.0357*
AC	1.39	0.0084*
AD	-0.14	0.661
AE	0.44	0.1987
AF	0.04	0.9025
BD	0.04	0.9015
BF	0.24	0.454
ABD	1.49	0.0066*
ABF	0.69	0.0755

* Significant ($p < 0.05$)

AB, *AC* and *ABD* also showed significant positive effects on ethanol production. Nonetheless, *A* does not have a significant effect on the ethanol yield, but *B* certainly negatively affecting the ethanol production. Overall, the results of the screening experiment show that addition of magnesium sulphate and alanine significantly improves the ethanol production. Potassium dihydrogen phosphate, calcium chloride, ammonium sulphate and diammonium hydrogen phosphate did not significantly increase ethanol yield. According to Birch and Walker [12], magnesium ion has an important role to protect yeast cells from ethanol and temperature-induced stress by preventing the increase in cell membrane permeability. The addition of inorganic salts such as Mg^{2+}, which also act as an activator of some enzymes in yeast cells will promote the ethanol yield [10, 13]. Thus, supplementation of fermentation media with magnesium has been shown to increase fermentation rate and ethanol productivity [10, 11, 14].

Meanwhile, there has been limited study on β-alanine addition as a supplement to ethanol fermentation yield. Williams *et al*. [16] has discovered that addition of β-alanine into the fermentation medium enhance the growth of yeast greatly. β-alanine can act as a growth promoting factor in yeast as the yeast likely utilize β-alanine by converting it into pantothenic acid [17]. Pantothenic acid is a vitamin required by yeast for the synthesis of coenzyme A which is necessary for formation of nitrogen-containing precursors, *O*-acetylserine and *O*-acetylhomoserine. Taherzadeh *et al*. [18] observed that addition of pantothenic acid enhanced the biomass yield of yeast.

D. Optimisation of Nutrients Addition

Central composite design (CCD) is the most common experimental design used in response surface methodology (RSM). The F-test analysis of variance (ANOVA) was used to check the statistical significance of a model equation. The averages of the duplicate measurements of the ethanol concentration and its predicted value are shown in Table 6. The polynomial model for ethanol yield was regressed by considering only the significant terms. The expanded equation is shown as follows:

$$Y = 34.54 + 0.22A - 0.35B + 0.22AB - 0.75A^2 - 0.80B^2 \quad (3)$$

Based on the experimental responses obtained in Table 6, the quantity of ethanol produced by *K. marxianus* ATCC 46537 ranged from 32.40 to 34.93 g/l. Run 3 and Run 7 had the minimum and maximum ethanol production, respectively. The ANOVA result of quadratic regression model for ethanol yield is described in Table 7. ANOVA of the regression model for ethanol yield verified that the model was significance with the F-value of 8.51 and a very low probability value (< 0.05). The calculated F-value is greater than the tabulated F-value showed that the model predicted the experimental results well and the estimated factors are valid. ANOVA (F-test) for the model explained the response of the dependent variable *Y*. The high F-value of the model (8.51) and non-significant lack of fit indicates that the model fits the experimental data well. The model p-value of 0.0069 indicates the model is valid. All linear terms (*A* and *B*) were not significant within 95% confidence level. The interaction coefficient of *AB* is not significant, however the quadratic terms of A^2 and B^2 were significant within 95% confidence level.

The isoresponse contour was constructed as a function of two factors at a time, holding all other factors at fixed levels, to determine the optimal level of each variable for maximum ethanol production. This surface plot is helpful to study both the main and the interaction effects of these two factors. The response values for the variables can be predicted from these plots. Fig. 4 shows the contour plot for the ethanol concentration for OPTS fermentation at varying nutrient

concentration. The ethanol concentration increased as the magnesium sulphate and β-alanine concentration increases until the optimum yield reached (magnesium sulphate at 7.63 g/l and β-alanine at 0.90 g/l), but the ethanol concentration decreased thereafter.

The required criteria were selected with maximum ethanol concentration as the target in the validation report. The choice of the solutions was automatically retrieved by the model. The magnesium sulphate concentration of 7.93 g/l and β-alanine of 0.90 g/l was selected based on desirability value of 0.86. The ethanol target is above 34.58 g/l with a lower limit of 32.40 g/l and upper limit of 34.93 g/l. The predicted value given by the model was 34.58 g/l and the actual value obtained from the average of five replicates experiment was 35.50 ± 0.40 g/l, corresponding to an acceptable error of 2.66%.

Costly organic additive such as yeast extract and peptone was proven to increase ethanol yield compared to "without additives" during the OPTS fermentation process [4]. The nutrients (inorganic salts supplement) used in this study are a lot cheaper compared to organic nutrients such as yeast extract and peptone. The supplement studied in this work may enhance ethanol production from the OPTS without significantly increasing the operating cost.

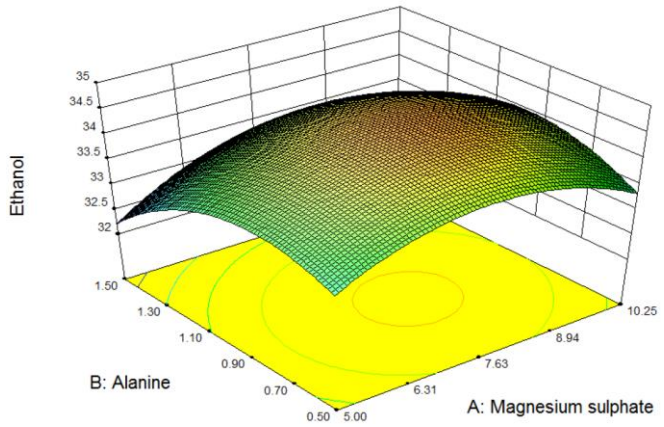

Fig. 4. Effect of magnesium sulphate and β-alanine concentration on ethanol production

IV. CONCLUSIONS

The results showed that *K. marxianus* produced the highest ethanol yield (60.9%) at a shorter fermentation time (16h) compared to the other strains. Six nutrients, namely, ammonium sulphate, diammonium hydrogen phosphate, magnesium sulphate, β-alanine, calcium chloride and potassium dihydrogen phosphate were screened using this strain and the highest ethanol yield (98.62%) was achieved in fermentation supplemented by magnesium sulphate and β-alanine. Subsequently, the optimisation study using a reseponse surfae methodology found the optimum value of magnesium sulphate was 7.93 g/L and 0.90 g/L for β-alanine. Under the optimum conditions, the predicted ethanol concentration was 34.58 g/L while the experimental value (35.50 g/L) was in good agreement with the predicted value with 2.66% error.

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