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Batch Fermentation of Bioethanol from the Residues of *Elaeis guineensis*: Optimization and Kinetics Evaluation

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Abstract: Bioethanol was produced by *Saccharomyces cerevisiae* using oil palm trunk (OPT) sap supplemented with palm oil mill effluent (POME) in a shake flask culture. Four variables namely OPT sap to POME ratio, inoculum size, pH and incubation time were chosen to investigate their effects on production of bioethanol using One -Factor -at -a -Time (OFAT) method. The optimum process conditions were found to be at OPT sap to POME ratio of 30:20 and inoculum size of 4% v/v. The fermentation process exhibited better result in slightly basic media. The bioethanol concentration at the optimum condition was 12.52 g/l which equal to bioethanol yield of 44% g/g after four days of fermentation. It was found that the cell growth followed a sigmoidal trend. Logistic model, Luedeking-Piret model and Luedeking-Piret-like model were chosen and validated to describe the behavior of dry weight of the biomass, bioethanol production and glucose consumption, respectively. All selected models fitted well to experimental data, proven by high R^2 values (>0.9) and low RMSE values.

Keyword: Oil palm trunk sap · Palm oil mill effluent · Bioethanol fermentation · One-Factor-at-a-Time (OFAT) method · Logistic model · Luedeking-Piret model

INTRODUCTION

The worldwide production of palm oil (*Elaeis guineensis*) is rapidly increasing since past decades. In production of palm oil, palm oil mill effluent (POME) is generated in the mill at final stage of the process. For each tonne of crude palm oil (CPO) produced, an average of 0.9 - 1.5 m^3 POME is generated [1]. POME is a colloidal suspension with high biological oxygen demand (BOD), as well as chemical oxygen demand (COD). Currently, ponding system is the most common method used for the treatment of POME. However, this system gave negative effect on environment due to high green house gas emission [2]; thus, leads to secondary pollution. On the other hand, the economic life of oil palm is only about 25 years and be replanted after then [3]. During replanting, a large quantity of felled trunks is generated. Most of Oil Palm Trunk (OPT) were left abandoned or used as mulch[4]. However, there was an interesting discovery on OPT sap where it actually contained relatively high sugar content [5]. Since high glucose level is detected in OPT, it has a great potential to be used as a feedstock for the production of bioethanol.

Bioethanol is the main and most efficient biofuel; it is considered as raw material with wide range of applications. The great advantage of bioethanol is that can be produced from various feedstock including corn, sugar beet, sugarcane, red seaweed part, etc[6]. In environmental point of view, bioethanol may contribute to decrease air pollution and reduce carbon dioxide accumulation[7]. To date, *Saccharomyces cerevisiae* yeast is still regarded as the most potential microorganism for bioethanol fermentation due its robustness [8].

Kinetics models are very important in design and control of the microbial processes [9]. In this study, three

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unstructured models were selected and validated to describe the behavior of bioethanol production from OPT sap. However, OPT sap contained high sugar content and might cause high osmotic stress on yeast cells. Therefore, OPT sap was diluted with POME to the desired glucose concentration. In the cultured media, POME served as a source of supplementary nutrients. Based on the One-Factor-at-a-Time (OFAT) method, the effects of different culture conditions on production of bioethanol were investigated.

MATERIALS AND METHODS

Chemicals: Sodium hydrochloride, sulphuric acid, acetonitrile, phenol, sodium sulfite, glucose, ethanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Dinitrosalicylic acid was purchased from Sigma-Aldrich. All the chemicals were of analytical grade.

Preparation of Oil Palm Trunk Sap and Palm Oil Mill Effluent: OPTs were obtained from Felda Trolak, Perak, Malaysia. The trunks were squeezed at Forest Research Institute of Malaysia, Kepong, Kuala Lumpur using a hydraulic squeezer to obtain OPT sap; while, POME was collected from Serting Hilir Palm Oil Mill, Negeri Sembilan. Due to fast degradation, OPT sap and POME were stored in a freezer at -20°C. Prior use OPT sap and POME were defrosted to room temperature when needed.

Inoculum Preparation: Yeast (*S. cerevisiae*) used was an ordinary baking yeast (Mauri-pan Instant Yeast, AbMauri) purchased at a local sundry stall. Inoculum was prepared by mixing 10 g of yeast with 100 ml of sterile deionised water. The mixture was then vortexed for 30 s so that the mixture was well mixed and the yeast cells were evenly distributed.

Bioethanol Fermentation: Bioethanol fermentations were run in 250 ml cotton plugged shake flasks. The substrate (100 ml) was prepared with OPT sap and POME in a ratio of 50:0, 40:10, 30:20, 20:30, 10:40 and 0:50. Inoculum size (0, 1, 2, 3, 4, 5 and 6% w/v), pH (4.2, 5.0, 6.0, 7.0 and 8.0) and incubation time (1, 2, 3, 4, 5, 6 and 7 days) were also chosen as the process variables. The pH of the mixture was adjusted by adding 2 M sodium hydroxide and 0.5 M sulphuric acid solutions by use of a glass-electrode pH meter (Mettler Toledo Delta 320). Samples were harvested and analysed for biomass, glucose and bioethanol concentration.

Analytical Method: Total nitrogen was measured by the use of an elemental analyzer. The COD level was measured according to Eaton *et al*. [10]. Dry weight of the samples or biomass was analysed by drying in an oven at 110 °C until a constant weight was achieved.

DNS analysis was carried out to determine sugar concentration using method described by Das *et al*. [11] with slight modifications. A 3 ml DNS reagent solution (Dinitrosalicylic acid: 10 g, Phenol: 2 g, Sodium Sulfite: 0.5 g, Sodium hydroxide: 10 g, deionised water: 1 liter) was added to 3 ml of sample in a capped test tube. The sample was then heated to 90°C for 15 minutes to develop a red-brown color. A 1 ml of 40% w/v potassium sodium tartrate solution was then added to the sample to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was measured with a spectrophotometer (Human Corporation X-Ma 1200V) at wavelength 575 nm.

Bioethanol was measured by High Performance Liquid Chromatography (HPLC) (Perkin Elmer Series 200) equipped with autosampler and programmable multi-wavelength detector set at 210 nm. A 10 ml of sample was injected into a column (Kromasil 100Å - NH_2 column) with 80% acetonitrile and 20% deionised water as the mobile phase at a flow rate of 0.6 ml/min at 22°C.

Statistical Analysis: The yield of bioethanol was calculated and expressed as percentage by Equation (1).

$$Yield\ of\ ethanol\ (\%g/g) = \frac{bio\ ethanol\ (g/l)}{initial\ glucose\ (g/l)} 100\% \quad (1)$$

Selected Model and Model Parameters Estimation: Kinetics of the yeast growth was validated by the logistic equation which was associated with substrate independent condition. Luedeking-Piret equation was chosen for the kinetic of product formation, while a Luedeking-Piret-like equation was selected to model the glucose consumption of the culture. Derivations and profound discussions on these equations had been done by Elibol and Mavituna [9]. Experimental results were analyzed using the linearized equations of the selected kinetic models as summarized in Table 1.

Table 1: Selected kinetic models and their respective linear forms

Cell growth	Logistic Model	$\frac{dX}{dt} = \mu_m\left(1 - \frac{X}{X_m}\right)X$	(2)
	Integration from t=0, X=X_o	$X = \left(\frac{X_o e^{\mu_m t}}{1 - (X_o/X_m)(1 - e^{\mu_m t})}\right)$	(3)
	Linear form	$ln\left(\frac{X}{X_m - X}\right) = \mu_m t - ln\left(\frac{X_m}{X_o} - 1\right)$	(4)
Bioethanol production	Luedeking-Piret Model	$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$	(5)
	Linear form	$[P - \beta B(t)] = \alpha A(t)$	(6)
	in which,	$\beta = \frac{(dP/dt)_{stationary}}{X_m}$	(7)
		$A(t) = X_o\left(\frac{e^{\mu_m t}}{(1 - (X_o/X_m)(1 - e^{\mu_m t}))} - 1\right)$	(8)
		$B(t) = \frac{X_m}{\mu_m} ln\left(1 - \left(\frac{X_o}{X_m}\right)(1 - e^{\mu_m t})\right)$	(9)
Glucose consumption	Luedeking-Piret-like Model	$-\frac{dS}{dt} = \gamma\frac{dX}{dt} + \delta X$	(10)
	Linear form	$S_o - S - \delta D(t) = \gamma C(t)$	(11)
	in which,	$\delta = \frac{-(dS/dt)_{stationary}}{X_m}$	(12)
		$C(t) = X_o\left(\frac{e^{\mu_m t}}{(1 - (X_o/X_m)(1 - e^{\mu_m t}))} - 1\right)$	(13)
		$D(t) = \frac{X_m}{\mu_m} ln\left(1 - \left(\frac{X_o}{X_m}\right)(1 - e^{\mu_m t})\right)$	(14)

Model Fitting and Validation: Polymath 5.1 (CACHE Corpn. USA) was used for data analyzing. Statistical coefficient and statistical error parameters were determined using a non-linear regression (Levenberg-Marquardt method).

RESULTS AND DISCUSSION

Characteristics of Oil Palm Trunk Sap and Palm Oil Mill Effluent: The characteristics of OPT sap and POME are summarized in Table 2. OPT sap contained higher glucose content (49.12 g/l) as compared to POME (3.04 g/l). Both of them are acidic condition. POME contained low total soluble solid content. The total nitrogen contained in POME was higher as compared to OPT sap. Compared to the literature, glucose concentration in our OPT sap was slightly lower but the total nitrogen value in POME was in the range that was previously reported [12-15]. This could be due to different types of oil palm tree species, palm oil mill process, analytical techniques used and the method of OPT sap squeezing process.

Effect of Oil Palm Trunk Sap to Palm Oil Mill Effluent Ratio: Table 3 shows the effect of OPT sap to POME ratio on production of bioethanol by *S. cerevisiae*. The highest bioethanol yield was found to be 3.08% when the OPT to POME ratio was 30:20. An increase in volume of OPT sap in the substrate increased the concentration of glucose and subsequently improved the conversion of glucose to bioethanol. However, the conversion of glucose to bioethanol started to decline beyond the initial glucose concentration of 27.94 g/l. This could be due to osmotic stress and consequently caused the yeast to loss its viability and thus reduced the rate of fermentation [16].

In order to maintain the cell growth and survive in the adverse and fluctuating conditions, the yeast cells were equipped with certain mechanisms to identify the changes and build up appropriate responses accordingly [17]. The ability of the cells to survive in higher glucose concentration depends on its osmoregulation system where the cellular water content and tugor pressure were regulated [18].

Inoculum Size: Microorganisms perform at their highest efficiencies if the right proportion of the substrate were provided. Table 4 presents the effect of inoculum size on conversion of glucose to bioethanol. A linear relationship was observed between the inoculum size with the yield of bioethanol up to the size of 4.0% (v/v). Beyond this level, the yield of bioethanol produced started to decrease.

Table 2:The characteristics of OPT sap and POME

	OPT Sap			POME		
	Present study	Lokesh *et al*. [12]	Yamada *et al*. [13]	Present study	Wong *et al*. [14]	Ahmad *et al*. [15]
pH	4.2	acidic	-	4.1	4.15 - 4.45	4.7
Concentration of glucose (g/l)	49.12 ± 0.04	55.4 ± 1.53	93.0 - 111.8	3.04 ± 0.03	-	-
COD (mg/l)	15000 ± 500	-	-	48600 ± 500	45500 - 65000	50000
Total soluble solid (°Brix)	5.10 ± 0.05	-	-	4.00 ± 0.05	-	-
Total solid (mg/l)	35163± 610	-	30000	37173 ± 573	33790 - 37230	40500
Total nitrogen, TN (mg/l)	648	-	-	718	500 - 800	750

Values are means ± standard deviations (n=3)

Table 3: Effect of OPT sap to POME ratio

	Glucose concentration				
OPT sap :POME ratio	Initial (g/l)	Final (g/l)	Percentage of consumption (%)	Bioethanol production (g/l)	Percentage of bioethanol yield (%)
50:00	44.72 ± 0.04	42.70 ± 0.14	4.51 ± 0.31	1.00 ± 0.07	2.24 ± 0.16
40:10	36.32 ± 0.03	34.18 ± 0.10	5.91 ± 0.28	1.06 ± 0.05	2.92 ± 0.13
30:20	27.94 ± 0.03	26.22 ± 0.07	6.16 ± 0.25	0.86 ± 0.03	3.08 ± 0.12
20:30	19.54 ± 0.03	18.56 ± 0.06	5.01 ± 0.31	0.48 ± 0.03	2.46 ± 0.15
10:40	11.16 ± 0.04	10.70 ± 0.03	4.17 ± 0.27	0.22 ± 0.01	1.97 ± 0.13
00:50	2.76 ± 0.03	2.66 ± 0.01	3.71 ± 0.36	0.04 ± 0.05	1.44 ± 0.18

Values are means ± standard deviations (n=3)

Table 4: Effect of inoculum size

	Glucose concentration				
Inoculum Size (%v/v)	Initial (g/l)	Final (g/l)	Percentage of consumption (%)	Bioethanol production (g/l)	Percentage of bioethanol yield (%)
0.0	30.72 ± 0.03	30.71 ± 0.01	0.03 ± 0.02	0.00	0.00
1.0	30.12 ± 0.02	29.40 ± 0.15	2.40 ± 0.49	0.36 ± 0.07	1.20 ± 0.23
2.0	29.54 ± 0.01	28.32 ± 0.13	4.17 ± 0.44	0.60 ± 0.06	2.03 ± 0.20
3.0	28.98 ± 0.01	27.36 ± 0.10	5.64 ± 0.35	0.80 ± 0.05	2.76 ± 0.17
4.0	28.46 ± 0.03	26.70 ± 0.13	6.18 ± 0.46	0.88 ± 0.06	3.09 ± 0.21
5.0	27.94 ± 0.01	26.22 ± 0.12	6.16 ± 0.43	0.86 ± 0.06	3.08 ± 0.21
6.0	27.44 ± 0.03	25.76 ± 0.11	6.12 ± 0.40	0.84 ± 0.05	3.06 ± 0.18

Values are means ± standard deviations (n=3)

Inoculum size is a vital parameter that imposed significant influenced on fermentation process. Prolonged lag phase was observed in the fermentation process with a smal volume of inoculum [19] and may lead to stuck fermentation process [20]. High level of inoculum size could accelerate the fermentation process and resulted in high bioethanol yield and production of glycerol [19, 20]. Therefore, fermentation with high inoculum size will enhance the production of bioethanol with suitable fermentation time [19]. In present study, the most optimum inoculum size was found to be at 4.0 % (v/v), where the increment of inoculum size after this point no longer resulted in positive impact on production of bioethanol. This happened possibly due to stress related to high cell density. However, it is still remained unclear in what type of stress is encountered by the yeast when it was exposed to high cell density [21]. The most possible factor is depletion of essential nutrient in the culture media.

Effect of pH: Table 5 shows the effect of pH on conversion of glucose to bioethanol. The production of bioethanol improved with an increase in pH values. From the results, the most optimum pH for fermentation process in this study was 8.0. Any increase in pH value more than that resulted in negative impact on fermentation productivity.

pH exhibited a great impact on the activities of microorganism. It was crucial to carry out the fermentation process at optimum pH condition so as to obtain the highest yield. The intracellular pH of *S. cerevisiae* is

Table 5:Effect of initial pH

Initial pH (g/l)	Glucose concentration				
	Initial (g/l)	Final (g/l)	Percentage of consumption (%)	Bioethanol production (g/l)	Percentage of bioethanol yield (%)
4.2	28.46 ± 0.02	26.72 ± 0.13	6.09 ± 0.46	0.86 ± 0.07	3.02 ± 0.23
5.0	28.46 ± 0.02	26.72 ± 0.14	6.09 ± 0.49	0.88 ± 0.07	3.09 ± 0.25
6.0	28.46 ± 0.02	26.62 ± 0.14	6.44 ± 0.49	0.90 ± 0.05	3.16 ± 0.22
7.0	28.46 ± 0.02	26.64 ± 0.15	6.35 ± 0.52	0.90 ± 0.06	3.16 ± 0.22
8.0	28.46 ± 0.02	26.24 ± 0.15	7.78 ± 0.52	1.10 ± 0.06	3.87 ± 0.25
9.0	28.46 ± 0.02	26.26 ± 0.13	7.69 ± 0.46	1.08 ± 0.05	3.80 ± 0.23

Values are means ± standard deviations (n=3)

Table 6:Parameter values used

Parameter	Estimated from	Estimated Value	Simulated Value
S_o	Experimental data	28.46	31.82
μ_m	Slope of Equation (4)	0.7572	0.2302
X_o	Intercept of Equation (4)	43.68	41.66
X_m	Experimental data	45.03	46.21
α	Intercept of Equation (6)	1.6880	2.7808
β	Equation (7)	0.0087	0.0170
γ	Intercept of Equation (11)	7.2619	9.4479
δ	Equation (12)	0.0124	-0.0008

Table 7: Statistical error parameters

Parameter	Cell Growth	Product Formation	Glucose Consumption
R^2	0.904	0.905	0.918
RMSE	0.135	0.617	1.159

almost neutral. Intracellular diffusion and dissociation of weak acids through the plasma membrane are possible. In order to maintain the intracellular pH at neutral, cells utilized their metabolic energy to pump out the surplus H^+ ions. For that reason, the fermentation performance would be affected by the concentration of undissociated forms of weak acids [21]. In present study, higher amount of bioethanol was produced at the initial pH value of 8.0 compared to that of pH 7.0. This was due to decrease in pH value as the fermentation process was progressing as bioethanol is acidic. However, if a pH controlled system was applied, by maintaining the pH value at 7.0, it was possible to obtain better result than the pH at 8.0. From this study, it could be concluded that the bioethanol fermentation should be conducted with the medium of slightly basic if there was no pH control.

Incubation Time: The effect of incubation time on the bioethanol production is shown in Figure 1. A positive correlation was found between the incubation time and bioethanol produced as well as dry weight of the biomass. Cell growth followed the classical sigmoidal growth trend. The concentration of bioethanol and biomass exponentially increased with the extension of time, while a declining trend was observed in glucose concentration

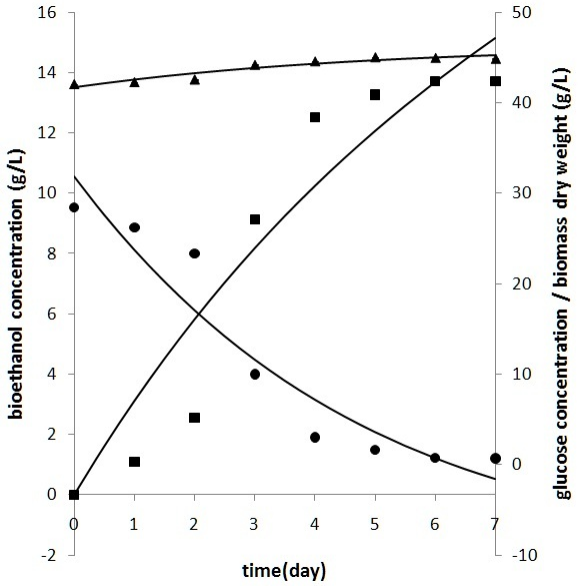

Fig. 1: Comparison of simulated value and experimental value (● Glucose concentration; □Bioethanol concentration; Δ Biomass dry weight) (dots: experimental values; line: simulated values)

particularly within the range of 0-4 days. Though, improvement in the yield was observed when the incubation time was longer than 4 days, the differences were not significant. The bioethanol yield at 4 days was 12.52 g/l which equal to 0.44 g of bioethanol produced per g glucose supplied.

Logistic model was chosen to describe the trend of cell growth, Luedeking-Piret model was selected to describe the bioethanol production; while the kinetic of glucose consumption was modeled using Luedeking-Piret-like model as suggested by Elibol and Mavituna [9]. Table 6 shows the estimated and simulated values of variables involved in these models. While, the statistical analyses of all models are presented in Table 7.

The results showed that all selected models were adequate in describing the experimental data, as evidenced by high R^2 values which were more than 0.9 for

all cases. Besides, low RMSE values were also obtained for all models. This indicated that the experimental and simulated values were in agreement and close to each other.

CONCLUSIONS

Four variables were chosen to investigate their effects on production of bioethanol by *S. cerevisiae*. The yield of bioethanol increased with an increase in OPT sap to POME volume up to the ratio of 30:20 and thereafter decreased. Increase in inoculum size during the fermentation process could also lead to enhancement of bioethanol yield. Nevertheless, beyond the inoculum size of 4% v/v, no significance improvement in yield was observed. Also, it was found that bioethanol fermentation resulted in high yield under slightly basic conditions. The production of bioethanol increased with an extension of incubation time, especially in the first 4 days. The bioethanol yield at optimum condition was 12.52 g/l; which was equal to bioethanol yield of 0.44 g/g. It is often very difficult to obtain a complete understanding of what is actually going on in a particular fermentation system. The presented models in this present study provided a good description on variations of biomass, bioethanol and glucose concentrations in a batch fermentation process.

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Nomenclature: glucose concentration (g 1^{-1})

t = Time (day)
X = Cell concentration (g dry weight $(1)^{-1}$)
X_o = The initial viable inoculum size (g dry weight $(1)^{-1}$)
X_m = The maximum attainable biomass concentration (g dry weight $(1)^{-1}$)

Greek Letters:

α = Growth-associated product formation coefficient (g g^{-1}).
β = Non-growth-associated product formation coefficient (g g^{-1} day^{-1})
γ, δ = Parameters in Luedeking-Piret-like equation for glucose consumption (g S (g cells)$^{-1}$, g S (g cells)$^{-1}$ day^{-1}, respectively)
μ = Specific growth rate (day^{-1})
μ_m = Maximum specific growth rate (day^{-1})

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چکیده

بیو اتانول توسط مخمر ساکارومیسس سرویسیه از شیره روغن کنده نخل (OPT)، به همراه پساب کارخانه روغن نخل (POME) در فلاسک شیکر دار تولید شد. چهار متغیر نسبت شیره OPT به POME، میزان تلقیح، pH و زمان انکوباسیون انتخاب شدند تا اثرات آنها در تولید بیو اتانول با استفاده از روش یک فاکتور در یک زمان (OFAT) مورد بررسی قرار گیرد. شرایط بهینه فرآیند برای نسبت شیره OPT به POME برابر ۳۰:۲۰ و میزان تلقیح ۴٪ حجمی تعیین شد. فرآیند تخمیر در محیط کشت اصلی نتیجه بهتری به نمایش گذاشته است. غلظت بیو اتانول در شرایط بهینه ۱۲.۵۲ g/l و بازده ۴۴٪ گرم/گرم بود که پس از چهار روز تخمیر بدست آمد. همچنین مشخص گردید که منحنی رشد سلول‌ها از یک روند S شکل پیروی می‌کند. برای توضیح رفتار وزن خشک سلولی، تولید بیو اتانول و مصرف گلوکز، مدل لوجستیک، مدل لودکینگ-پیرت و مدل شبه لودکینگ-پیرت انتخاب و مورد استفاده قرار گرفت. همه مدل‌های انتخاب شده به خوبی داده‌های تجربی را برازش نموده است ک ($R^2 > 0.9$) و مقادیر کم RMSE این ادعا را اثبات می‌کند.