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BIOETHANOL FERMENTATION BY *KLUYVEROMYCES MARXIANUS* CONSIDERING THE EFFECT OF GLUCOSE IN OIL PALM FROND (OPF) JUICE CONCENTRATES

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Abstract

Bioethanol fermentation by *Kluyveromyces marxianus* in OPF juice considering the effect of glucose was studied. Experiments were performed in shake flask culture of batch mode. The OPF juice was concentrated using rotary evaporator to get desired concentration of glucose ranged 20-50 g/L, prior to fermentation. The highest bioethanol yield (0.513 g/g) was achieved at initial glucose concentration 40 g/L when the yeast was grown at 40°C, initial media pH 6, agitation speed 150 rpm for 60 h. Thus, showing that oil palm frond juice can be used as a chemical feedstock for bioethanol production.

Keywords: Oil palm frond juice, Fermentation, Bioethanol, *Kluyveromyces marxianus.*

1. Introduction

Fast growing of human population and economic development has increased global demand on petroleum-derived fuels consumption. Rapid emerging in industrialization and transportation sectors has led to energy crisis as well as affected the stability of ecosystems and climates changed [1, 2]. An excessive dependent on fossil fuel and global warming has geared research on an alternative biofuels [3, 4].

Bioethanol also known as grain alcohol is an alternative liquid biofuel which can be produced from various types of biomass feedstock [5-7], as well as process technologies [8, 9]. Biomass has recently drawn much attention as the most attractive renewable resource for bioethanol production in Malaysia, since it produced huge amount of wastes every year. As the world largest oil palm producer, Malaysia generated about 83 million tons (wet weight) of OPF

annually [10], of which most is regarded as wastes. Currently, the disposal of these solid-agro wastes is by direct decaying on plantation area or by burning on site; hence, this practice would initiate environmental problems [11].

As reported by a few researchers, bioethanol production from OPF is receiving much interest as the materials are renewable, cheap and abundantly found in nature [11, 12]. However, the problems in removing lignin from lignocellulosic material such as in OPF have impeded the potential of commercialization [13]. Besides, the cost of sugar recovery from the lignocellulose is remarkably high [14]. Therefore, readily available sugar from the OPF juice is urgently needed. Zahari et al. [15] stated that OPF juice consisted of higher concentration of fermentable sugar, almost 70% of the total free sugar which can be used directly for fermentation without modification. Glucose was found to be dominant sugar component in OPF juice, followed by sucrose and fructose. Moreover, the OPF juice is enriched with natural nutrients such an amino acids and higher percentage of carbon [15]. Razmovski and Vucurovic [16] reported that minerals, salts and macronutrient as well as micronutrient have a stimulatory and protective effect either on cell growth in fermentation or viability of producing microorganisms, in which can stimulate the bioethanol production rate and contributes to an efficient fermentation process.

Kluyveromyces marxianus is a facultative type of yeast which can assimilated glucose as a carbon source to produce bioethanol in either aerobic or anaerobic fermentation [17]. The OPF juice has a great potential to be used as a substrate for bioethanol production since the carbon source can be utilized directly without any pretreatment. Thus reducing the operating and chemicals cost for treating the OPF, accordingly. Herein, the aim of this study is to investigate the feasibility of OPF juice concentrate as a substrate for bioethanol production by *Kluyveromyces marxianus* in shake flask culture. Effect of different glucose concentration on the yeast growth and bioethanol yield will also be looked at.

2. Material and Methods

2.1. Raw materials

Fresh OPF were collected from a palm oil plantation belongs to Felda Trolak Selatan Berhad, Malaysia. The leaflets were removed from petioles then shredded into small chips and being compressed in compressing mills to obtain its juice. The small particulates in juice were removed by centrifugation and the supernatant was stored at -20°C until needed

2.2. Vacuum evaporation procedures

In order to get higher sugar concentration, raw OPF juice was concentrated using a rotary vacuum evaporator (Heidolph LABOROTA 4011- Digital Rotary Evaporator, Germany) at 80°C, and pressure 0.6 bars for two hours. The glucose concentration was analysed until a final concentration of 15°Brix was achieved.

2.3. Fermentation

2.3.1. Inoculum preparation

The *Kluveromyces marxianus* ATCC36907 inoculum was prepared by growing it on solid yeast mold agar (YMA) plates. The plates were incubated at 40°C for 48 h.

A loopful of yeast colonies was transferred to a 50 mL Erlenmeyer flask containing 10 mL of yeast mold (YM) medium and grown at 40°C in static condition for 24 h. This culture was then used to inoculate the production flask culture.

2.3.2. Fermentation process

The fermentation was performed in 200 ml Erlenmeyer flask containing 100 ml sterilized OPF juice concentrate. The inoculum (10% (v/v)) was inoculated into the concentrates and incubated at 150 rpm, 40°C for 60 hours. Sample was withdrawn every 6 hr.

2.4. Analytical methods

2.4.1. Bioethanol concentration and yield determination

Bioethanol concentration was determined using a gas chromatography system equipped with flame ionization detector (GC-FID) (Hewlette, 5890 Series II, Hewlett Packard, Alto, CA) and a column of 2 m length and 0.2 cm internal diameter (80/120 mesh Carbopack B-DA / 4% Carbowax 20 M, Supelco, USA). The oven was programmed to increase from 100°C (in 2 min) to 175°C at 10°C min^{-1}. The injector and detector temperature was set at 225°C. For the analysis, 2 µl of liquid sample was injected into GC-FID system. All analysis was done in triplicates. A calibration curve was made using dilutions of a standard solution of ethanol. Bioethanol yield attained from the experiments were calculated using Eq. (1):

$$Y_{P/S} = \frac{[Bioethanol]_{\max}}{0.511 \times [Glu\cos e]_{ini}} \quad (1)$$

where $Y_{P/S}$ is the bioethanol yield (g g^{-1}), $[Bioethanol]_{max}$ is the maximum bioethanol concentration obtained at the end of the fermentation period (g L^{-1}), $[Glucose]_{ini}$ is the concentration of glucose at t = 0 of the fermentation (g L^{-1}), 0.511 is the theoretical conversion factor from glucose to bioethanol based on stoichiometric biochemistry of yeast, P is a product and S is a substrate.

2.4.2. Glucose determination

The glucose content in OPF juice was analysed using an Agilent series 1200 infinity high performance liquid chromatography (HPLC) system equipped with a 385-ELSD (evaporative light scattering detector), and operated at 80°C. The column used was Hi-Plex Ca (300 mm x 7.7 mm) and the mobile phase was de-ionized water with flow rate at 0.6 ml min-1. 20 µL of sample was injected into the sampling port. All samples and standards were filtered using 0.45 µm syringe filter (Nylon membrane, Whatman) prior to analysis. All analysis was done in triplicates. The glucose concentrations were identified by comparing the retention time of sample peaks with those of glucose standards. The standard calibration curve was obtained using analytical grade of glucose (anhydrous).

2.4.3. Biomass determination

Biomass (g/L) was expressed as cell dry weight calibrated by optical density at 600 nm using a spectrophotometer (Agilent Technologies, Carry 60 UV-Vis

USA). The biomass obtained was determined by measuring the cell dry weight. 1 mL of sample was centrifuged at 4400 rpm, 4°C for 15 min in pre-weighted Eppendorf tubes. The samples were washed with distilled water and dried in an oven at 110°C for 48 h until constant weight were reached.

2.5. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) from duplicates samples. The results were statistically tested by t-test using statistical package in Microsoft Excel. Statistical significance was assumed at $p < 0.05$.

3. Results and Discussion

3.1. Proximate analysis of OPF juice concentrates

The proximate composition of OPF juice concentrates used in this study is given in Table 1. Based on HPLC analysis, glucose was the dominant sugar component at 55±0.22 g/L, accounting 72% of total fermentable sugar in OPF juice concentrates. In addition, sucrose was also found to be the second largest sugar component in OPF juice accounting 15.01±0.02 g/L. This is in agreement with the research of Zahari et al. [15] who reported that glucose was major sugar component in OPF juice at 53.95±2.86 g/L.

Table 1. Proximate analysis of OPF juice concentrates.

Component	OPF juice concentrates
pH	4 - 5
Glucose (g/L)	55 ± 0.22
Sucrose (g/L)	10.10±0.02
Fructose (g/L)	11.15±0.37
Nitrogen, N (%)	0.29±0.01
Carbon, C (%)	33.80±0.15
Hydrogen, H (%)	1.42±0.02
Sulfur, S (%)	0.35±0.01
Protein (%)	2.20±0.21
Total amino acid (µg/g)	168.20±1.90

*data is the mean±SD of duplicate samples (n = 2)

OPF juice obtained from Malaysia plantation area is heterogeneous in properties and varied in characteristics from one plantation to another. This could probably due to the plantation pruning practice, the quality of the fresh frond, the analytical technique used, the quality of juice storing condition and the methods of juice squeezing process [18].

3.2. Effect of initial glucose concentration in OPF juice concentrate

During microbial fermentation, the carbon source not only acts as a major constituent for building of cellular material, but it is also used for polysaccharides synthesis and as energy source [19-21]. In order to study the feasibility of OPF juice concentrate as a carbon source for bioethanol production by *Kluyveromyces marxianus*, different glucose concentration in the juice (20, 30, 40 and 50 g/L) were used.

Figure 1 shows the profiles of bioethanol production at different initial glucose concentration of OPF juice concentrates. It is found that bioethanol production for all glucose concentration increased exponentially up to 30 h. The highest bioethanol production was 20.5±0.035 g/L ($p > 0.05$) at 40 g/l of glucose presence in the juice. According to Siqueira et al. [22], an optimal concentration of reducing sugars used in fermentation process is capable to increase the production of bioethanol, efficiently.

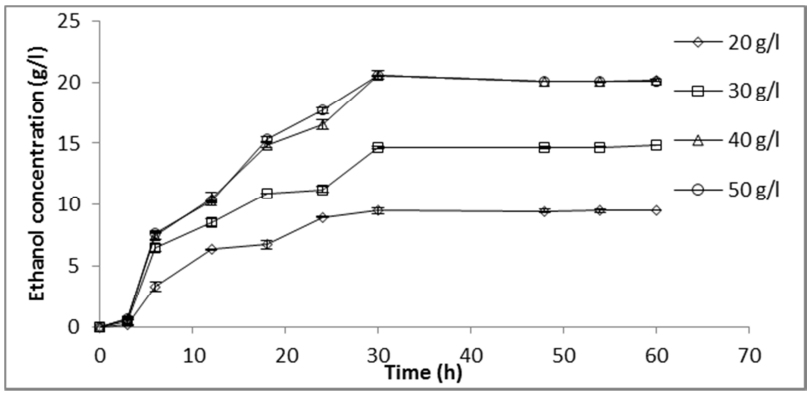

Fig.1. Effect of Initial Glucose Concentration on Bioethanol Production by *Kluyveromyces marxianus* using OPF Juice as a Substrate.

The growth profiles of the tested yeast are described in Fig. 2. At an early period of fermentation, a lag phase of 0 to 3 h was observed. According to Doran [23], during this period the maintenance energy was used by any organisms for cell adaptation in the fermentation medium, which caused the cell to grow slowly. After 10 h of the fermentation period, the yeast growth began to increase gradually. During this stage, an exponential phase was observed, in which the yeast cells replicated actively until late 30 h. With further fermentation time up to 60 h, the yeast growth remained constant.

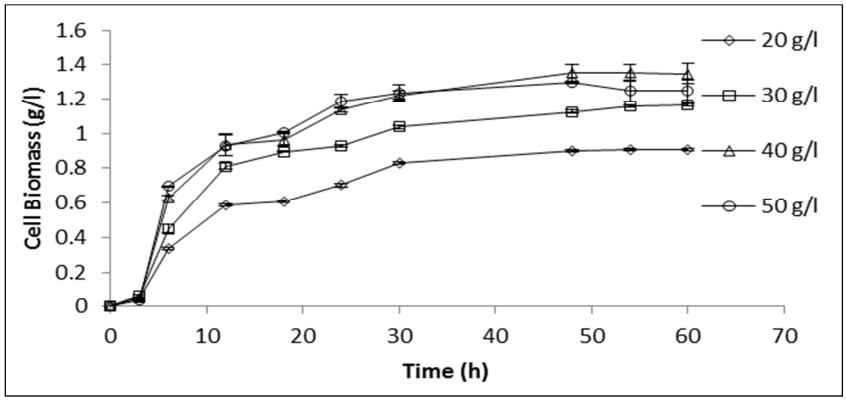

Fig. 2. Effect of Initial Glucose Concentration on Biomass by *Kluyveromyces marxianus* using OPF Juice as a Substrate.

Figure 3 illustrates the consumption of reducing sugar at various initial glucose concentrations of the juice. Glucose consumption was shown up to 50 h. The results also showed that with increased of glucose concentration; growth and bioethanol production increased, and then remained constant. This could be due to the fact that *Kluyveromyces marxianus* cells were metabolically inactive; however it still consumed glucose for maintenance and ATP formation. According to Doran [23], maintenance functions include cell motility, turnover of cellular components and adjustment of membrane potential and internal pH. Maintenance activities were carried out by living cells even in the absence of growth and product formation.

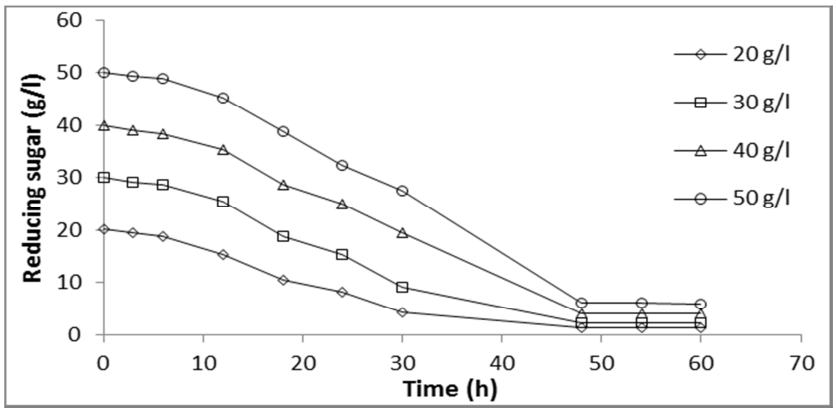

Fig. 3. Effect of Initial Glucose Concentration on Reducing Sugar Consumption by Kluyveromyces marxianus using OPF Juice as a Substrate.

Few researchers have reported that the maximum theoretical yield of bioethanol on a mass basic of glucose metabolism is 0.51 [22, 24]. As can be seen in Table 2, the maximum bioethanol yield (0.513 g/g) and biomass (1.35 g/L) were detected at initial glucose concentration in OPF juice concentrates at 40 g/L. Hence, indicated that the result obtained in the present lied within the theoretical range.

Table 2. Effect of initial glucose concentration in OPF juice concentrates on growth and bioethanol production.

Glucose concentration in OPF juice concentrates (g/l)	Maximum biomass, X_m (g/L)	Maximum bioethanol production, P_m (g/L)	Maximum bioethanol yield, $Y_{p/s}$ (g/g)
20	0.909	9.969	0.473
30	1.166	14.670	0.494
40	1.350	20.502	0.513
50	1.246	20.094	0.416

4. Conclusion

This study demonstrated that OPF juice concentrates, particularly from inner part of the fronds is a potential feedstock for bioethanol production by *Kluyveromyces marxianus*. The maximum biomass and bioethanol yield were attained at 1.35 g/L and 0.513 g/g for OPF juice concentrates with glucose concentration 40 g/L,

respectively. This showed that with million tonnes of OPF wastes generated in oil palm plantation, can be converted into second generation bioethanol as an alternative energy source.

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