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Optimisation of fermentation conditions for bioethanol production from oil palm trunk sap by *Saccharomyces cerevisiae*

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ABSTRACT

Aims: Oil palm trunk (OPT) can be a potential biomass from replanting activities for biomass-to-liquid (BTL) particularly in bioethanol production. The OPT contains higher carbohydrates compared to other oil palm biomass, thus has better advantages as feedstock for biofuel. To realise this, the feasibility of using oil palm trunk (OPT) sap as a substrate for bioethanol fermentation was explored via optimising the various culture conditions (pH, temperature, inoculum size, nitrogen source, dilution effect and growth medium) using *Saccharomyces cerevisiae*.
Methodology and results: A total of six parameters were tested for optimising bioethanol production i.e. pH, temperature, inoculum size, nitrogen source, dilution effect and types of medium. Results showed that the optimum conditions for OPT sap in bioethanol production were at pH 4.0, temperature of 30 °C, inoculum size of 10 % (v/v), without requirement of nitrogen supplementation and substrate dilution. A fermentation period of 24 h was best for bioethanol production and resulted in bioethanol production, formation rate and yield of 47.5 g/L, 1.98 g/h and 0.50 g/g, respectively.
Conclusion, significance and impact study: The study has clearly demonstrated that high efficient bioethanol production from OPT sap is possible but it is susceptible to various fermentation influencing parameters. This study could establish an effective and sustainable utilisation of waste OPT especially its sap as a lignocellulosic biomass supplement from the oil palm industry for second generation biofuel production.

Keywords: Oil palm trunk sap, fermentation, *Saccharomyces cerevisiae*, bioethanol

INTRODUCTION

Oil palm (*Elaeis guineensis*), a perennial crop, is only felled every 25 years of its life cycle for replanting. Besides producing oil, the palm also produces other forms of biomass in abundance. During replanting, an estimated 209200 ha of oil palm trees were felled from 5.23 million ha of oil palm planted area in 2013 (MPOB, 2013). Currently, some of the felled oil palm trunks (OPT) are transported to the intended destination e.g. plywood factory, where the trunks are processed to remove the bark and sap so that the biomass produced can be used to manufacture furniture. Typically, the sap of OPT will not be utilised and will be discarded as waste. According to Kosugi and Mori (2007), freshly felled OPT may contain up to 70-85% of sap based on the weight of the whole trunk. The amount of fermentable sugars was found nearly 10% from the sap of the inner trunk. The squeezed sap contained an abundance of fermentable sugars and could be directly converted into bioethanol by microbial fermentation. Ethanol production via fermentation of sugars may provide an economically competitive source of energy by substituting gasoline.

Ethanol fermentation is a complex biochemical process with yeast or bacteria utilising fermentable sugar as substrate for their growth and converting them into ethanol, carbon dioxide and other metabolic products (Asyraf *et al.*, 2011). Among the important factors affecting ethanol fermentation, culture conditions play a significant role in microbial growth as well as ethanol production. During ethanol fermentation, most of the yeast cells suffer from various stresses, including sugar concentration, nutrient deficiency, temperature and pH (Yah *et al.*, 2010). Thus, this study was carried out to explore the potential of OPT sap for bioethanol production aiming at optimising the fermentation conditions i.e. pH, temperature, inoculum size, nitrogen source, dilution effect and growth medium utilising the sap of OPT in order to obtain high bioethanol yield.

MATERIALS AND METHODS

Materials

The sap of OPT used in this study was supplied by Epaga Venture and was sampled at Segamat Johor in Malaysia.

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The sap was extracted by mechanical pressing from different parts of OPT (i.e. inner, outer) as depicted in Figure 1. Samples were sterilized at 110 °C for 15 min and kept in a -4 °C freezer prior to fermentation to avoid microbial contamination.

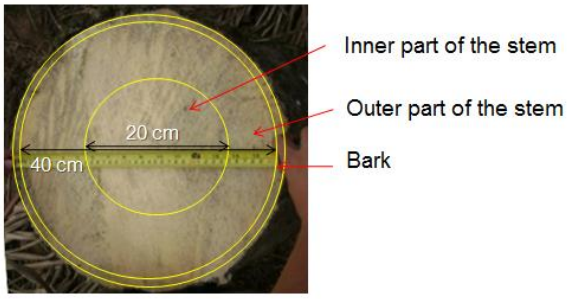

Figure1: Sample of sap of oil palm trunk.

Optimisation of fermentation of OPT sap

Fermentation of OPT sap was carried out using *Saccharomyces cerevisea* ATCC 24860 in shake flask. A single colony of freshly prepared *S. cerevisiae* was grown overnight in 10 mL yeast-peptone-glucose (YPD) [consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] broth. This culture was used to inoculate 100 mL OPT sap in a 250-mL Erlenmeyer flask, agitated at 150 rpm for 96 h. A total of six parameters were evaluated to optimise bioethanol production i.e. pH, temperature, inoculum size, nitrogen source, substrate concentration and type of medium. In each of the experiment conducted, samples were harvested at predetermined time intervals, filtered and analysed for ethanol and sugar contents.

Effect of initial pH

The effect of pH on fermentation of OPT sap was carried out by varying the pH from 3.5 to 6.0 at 0.5 interval. The OPT sap was adjusted to the desired pH using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) prior to inoculating with standardized *S. cerevisiae.* It was incubated at 30 °C with an agitation rate of 150 rpm for 96 h.

Effect of temperature

The effect of temperature on fermentation of OPT sap was carried out by varying the temperature at 30 °C, 35 °C and 40 °C. The sap sample was incubated at the optimum pH obtained from the pH optimization study.

Effect of inoculum size

The effect of inoculum size on fermentation of OPT sap was carried out by varying the inoculum concentration from 5% to 20% (v/v). The sap sample was incubated at the optimum pH and temperature obtained from above.

Effect of nitrogen sources

The effect of nitrogen source on fermentation of OPT sap was studied in five different nitrogen sources i.e. yeast extract, meat extract, peptone, urea and ammonium chloride (NH_4Cl) at 1% (w/v). The sap sample was incubated at the above optimum fermentation conditions.

Effect of substrate concentration

The substrate concentration was adjusted by diluting OPT sap with sterilized distilled water to the desired medium concentration ranging from 25% to 90% (v/v). The undiluted sample was 100% (v/v). The sap sample was incubated at the above optimum fermentation conditions.

Effect of type of medium

Three types of medium were selected to compare their performance on fermentation of OPT sap i.e. defined medium (0.5% w/v KH_2PO_4, 0.2% w/v $(NH_4)_2SO_4$, 0.04% w/v $MgSO_4 \cdot 7H_2O$, 0.15% w/v yeast extract, 5% w/v glucose); rich medium (YPD; 1% w/v yeast extract, 2% w/v peptone, 5% w/v glucose), minimal medium (6% w/v glucose, 1% w/v fructose). The sap sample was incubated in the respective medium at the above optimum fermentation conditions.

Product analysis

The moisture content of OPT sample was determined by drying in an oven at 105 °C for 24 h. The fermentable sugar content of OPT sap and the bioethanol concentration were determined using high performance liquid chromatography (HPLC) (Waters 2707); Sugar Pack™ column: 6.5 x 300 mm, detector temperature: 35 °C, column temperature: 75 °C, flow rate: 0.5 mL/min and injector volume of 1 µL. The ethanol yield ($Y_{p/s}$) was calculated based on experimental ethanol produced and expressed as g ethanol per total g of sugar utilised (g/g) (Eq. 1) and the ethanol formation rate was calculated based on ethanol yield obtained against maximum production time, Δt (Eq. 2). The fermentation efficiency was calculated as ethanol produced against theoretical maximum ethanol yield from sugar (i.e. 0.51 g ethanol per g sugar) (Eq. 3).

$$\text{Ethanol yield } (Y_{p/s}) = \frac{\text{(Ethanol, g/L)}}{\text{(Glucose, g/L)}} \quad \text{(Eq. 1)}$$

$$\text{Ethanol formation rate} = \frac{\text{(Ethanol, g/L)}}{\Delta t} \quad \text{(Eq. 2)}$$

$$\text{Efficiency (\%)} = \frac{\text{(Ethanol, g/L)}}{\text{(Glucose, g/L)} \times 0.51} \times 100 \quad \text{(Eq. 3)}$$

Cell concentration in fermentation broth was determined by spectrophotometer (Genesys 20, Thermo Scientific, USA) at 600 nm absorbance. The medium was diluted with distilled water 1:1 accordingly.

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Statistical analysis

All the experiments related to fermentation parameters were carried out in triplicate and the data was analysed using Minitab®16 by performing an analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Level of statistical significance was set at 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Sugar compositions

The composition of fermentable sugars in the sap from inner and outer parts of OPT are shown in Table 1. The sap extracted from the inner part of OPT showed the highest concentration of fermentable sugar containing mainly glucose, i.e. 89.3 g/L. The higher sugar content in the inner part may be due to the presence of greater amount of soft parenchyma tissue compared to the outer part of OPT.

Table 1: Compositions of fermentable sugars in the sap from different parts of oil palm trunk.

Part of OPT	Moisture content (%)	Glucose (g/L)	Fructose (g/L)	Total fermentable sugars (g/L)
Inner	67.10	89.30	4.61	93.91
Outer	66.58	16.06	0.91	16.97

Bioethanol production from OPT sap

Effect of initial pH

The effect of different pH on bioethanol production is shown in Figure 2. The highest bioethanol yield was obtained at pH 3.5 with a maximum ethanol concentration of 46.47 ± 1.62 g/L followed closely by pH 4.0 with 46.30 ± 2.79 at 24 h of fermentation. However, the effect between these pH values was not statistically significant (Table 2). Fermentation at pH 4.0 performed better in longer fermentation period compared to other pH values tested. Therefore, this pH was used in all the following experiments for optimisation. Since the pH of OPT sap was well within the range, it was used without requiring further pH modification.

It has been reported that pH could influence significantly on fermentation, mainly on yeast growth, fermentation rate and by-product formation (Sheela *et al.*, 2008). During yeast growth, an acidic intracellular pH must be preserved and maintained for optimal microbial performance, due to the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cells need to invest more energy to either pump in or pump out H^+ in order to maintain the optimal intracellular pH, failing which the yeast may not function normally. In this case, the yeast cell will not be able to grow and produce ethanol efficiently (Narendranath *et al.*, 2001).

The pH affects the functioning of microbial cells enzyme and the transport of nutrients into the cell (Chooklin *et al.*, 2011). The results obtained from this study showed that the most suitable pH for bioethanol production from OPT sap was at pH 3.5-4.0. Increasing pH value could reduce bioethanol production as well as glucose consumption rate. Higher pH increases the permeability of the cell membrane resulted in the reduction of sugar conversion rate and ethanol yield. Besides, formation of undesired product such as glycerol and organic acid in less acidic pH may also take place during the fermentation process (Pramanik, 2003). The results obtained was in agreement with Manikandan *et al*., (2008) who also reported that yeast growth and fermentation process performed the best in slightly acidic environment.

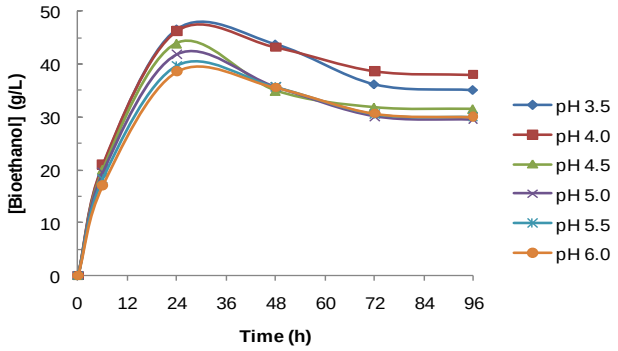

Figure 2: Bioethanol production from oil palm trunk sap at different pH.

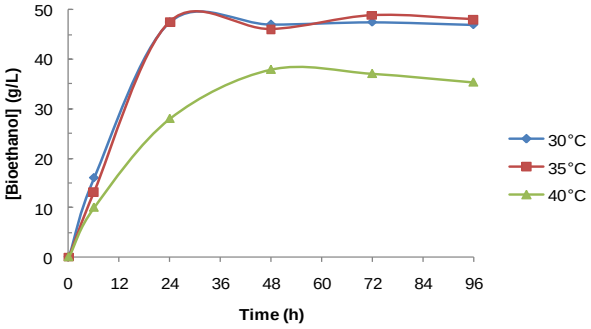

Figure 3: Bioethanol production from oil palm trunk sap at different reaction temperatures.

Effect of temperature

The effect of different temperature on bioethanol production is shown in Figure 3. The highest yield of bioethanol was obtained at 30-35 °C after 24 h of fermentation with 47.53 ± 0.19 and 47.54 ± 0.09 g/L, respectively. However, the effect between these temperatures was not statistically significant (Table 2). Fermentation at 40 °C, on the other hand, showed a significant fall in ethanol yield and productivity ($p < 0.05$). Clearly, higher temperature was found unsuitable and less

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efficient than lower temperature for bioethanol production by *S. cerevisiea*, as was indicated after 24 h of fermentation at 40 °C producing only 27.93 ± 0.52 g/L ethanol corresponded to 58% fermentation efficiency. This finding is in agreement with that studied by Yah and co-workers (2010), showing similar trend in bioethanol production at temperature >40 °C using different feedstock such as sugarcane molasses, baggases, corncorb and sweet sorghum hydrolysate by *S. cerevisiea*. A decline in bioethanol yield at ≥40 °C might be due to the inactivation of enzyme in ethanol production pathways, leading to loss of enzyme activity and lipid metabolisms, disruption of enzyme, alteration of membrane structure and decreased functionality, hence resulted in low bioethanol production (Pramanik, 2003; Sharma *et al.*, 2007; Sener *et al.*, 2007; Beltran *et al.*, 2008). In this study, an incubation temperature of 30 °C was chosen to optimise bioethanol production from OPT sap.

Table 2: Effect of different fermentation parameters (i.e. pH, temperature, inoculum size and nitrogen source on bioethanol production from oil palm trunk (OPT) sap at 24 h of fermentation.

Fermentation parameters		Ethanol concentration (g/L)	Productivity (g/L/h)	Ethanol yield, $Y_{p/s}$ (g/g)	Fermentation efficiency (%)
pH	3.5	46.47 ± 1.62 ^{a}	1.936	0.495	96.8
	4.0	46.30 ± 2.79 ^{a}	1.929	0.493	96.5
	4.5	43.91 ± 3.48 ^{ab}	1.830	0.468	91.5
	5.0	41.80 ± 3.56 ^{b}	1.742	0.445	87.1
	5.5	39.71 ± 2.48 ^{b}	1.655	0.423	82.8
	6.0	38.59 ± 2.00 ^{b}	1.608	0.411	80.4
Temperature (°C)	30	47.53 ± 0.19 ^{a}	1.980	0.50	99.0
	35	47.54 ± 0.09 ^{a}	1.981	0.50	99.0
	40	27.93 ± 0.52 ^{b}	1.164	0.30	58.1
Inoculum size (%,v/v)	5	37.52 ± 4.36 ^{a}	1.563	0.40	78.2
	10	45.84 ± 1.11 ^{a}	1.910	0.49	95.5
	15	41.06 ± 2.28 ^{a}	1.711	0.44	85.6
	20	42.90 ± 1.07 ^{a}	1.788	0.46	89.4
Nitrogen source	YE	45.12 ± 0.36 ^{a}	1.880	0.50	97.0
	ME	43.16 ± 1.07 ^{a}	1.798	0.47	92.8
	Peptone	42.90 ± 0.96 ^{a}	1.788	0.47	92.2
	Urea	38.08 ± 1.64 ^{b}	1.587	0.42	81.9
	NH_4Cl	42.38 ± 0.75 ^{a}	1.766	0.47	91.1
	Control	43.60 ± 0.78 ^{a}	1.817	0.48	93.8

Values are mean concentration ± standard deviation of triplicate determination. Means bearing different letter in a column are significantly different ($p < 0.05$).

Effect of inoculums size

As excessive inoculum in the medium compromises fermentation rate, ethanol production and recovery (Jones *et al.*, 2007), a suitable and optimal inoculums size is critical to achieve more efficient bioethanol production from OPT sap. Figure 4 shows bioethanol production profile from OPT sap at different initial inoculum size. The highest bioethanol concentration of 45.84 ± 1.11 g/L was obtained from OPT sap inoculated with 10% (v/v) *S. cerevisiae* for 24 h. Increasing the inoculum size beyond 10% (v/v) resulted in a declined bioethanol yield. However, the effect of different inoculum size on bioethanol production in this study was statistically insignificant ($p > 0.05$) as shown in Table 2. From this finding, the inoculum size of 10% (v/v) that showed the highest bioethanol formation rate of 1.91 g/L/h and 96% fermentation efficiency was used to optimise bioethanol production from OPT sap.

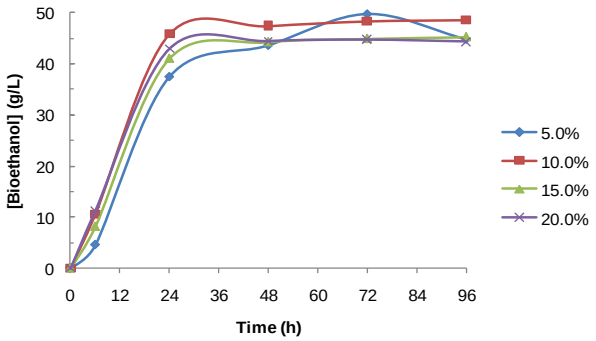

Figure 4: Bioethanol production from oil palm trunk sap at different inoculum sizes.

Effect of nitrogen source

An investigation on fermentation of OPT sap with various nitrogen supplements and non-supplemented control

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revealed that nitrogen sources i.e. yeast extract (YE), meat extract (ME), peptone and NH_4Cl did not significantly influence bioethanol production (Figure 5). However, YE could provide a better positive attribute for the process than the control and other supplementation as it has tendency to shorten the fermentation period due to a higher productivity i.e. 1.88 g/L·h (Table 2). On the other hand, addition of urea had significantly reduced bioethanol production ($p < 0.05$) as was evident by a lower growth curve, productivity and fermentation efficiency (Figure 4, Table 2). Conclusively, urea is not a suitable additive for bioethanol production from OPT sap.

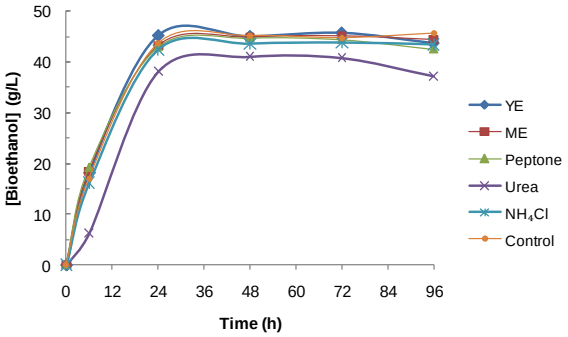

Figure 5: Bioethanol production from oil palm trunk sap at different nitrogen sources.

An additional nitrogen source is necessary to accelerate the growth and multiplication of yeast, and it influences the ethanol tolerance of yeast and the ultimate ethanol productivity (Bafrncova *et al.*, 1999). However, in this study, the addition of various nitrogen sources to OPT sap did not show any significant influence on bioethanol production. Bafrncova and co-workers (1999) also demonstrated that an additional nitrogen source was not able to increase bioethanol productivity, although it increased glucose consumption rate by up to three times than that of the control. Since the added YE showed negligible increase in bioethanol production from OPT sap, its addition was not necessary.

Effect of substrate concentration

The ability of *S. cerevisiae* to utilise OPT sap as the sole carbon source was studied by using different concentrations of OPT sap during fermentation (Figure 6). The undiluted raw sap gave the highest yield of bioethanol since the initial sugars concentration was the highest among the others. This showed that *S. cerevisiae* was able to utilise 100% (v/v) OPT sap without much problem encountered in the reaction concerning product inhibitory and or insufficient dispersion of substrate in too concentrated medium (Lu *et al.*, 2008; Hodge *et al.*, 2008). The effect of different substrate concentration in fermentation efficiency of OPT sap was found insignificant ($p > 0.05$) as high ethanol yield could be achieved in all substrate concentrations (Table 3).

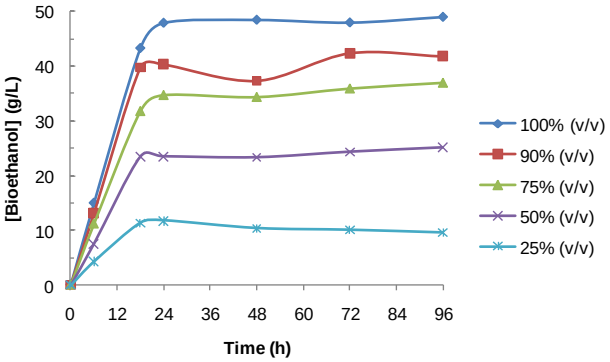

Figure 6: Bioethanol production from oil palm trunk sap at different substrate concentration.

Table 3: Effect of dilution and fermentation medium on bioethanol production from oil palm trunk (OPT) sap at 24 h of fermentation.

	Initial sugar concentration(g/L)	Ethanol concentration(g/L)	Productivity (g/L/h)	Ethanol yield, $Y_{p/s}$ (g/g)	Fermentation efficiency (%)
Dilution (%, v/v)					
100 (undiluted)	93.91	47.55 ± 0.48	1.981	0.50	99.1 [a]
90	86.82	39.99 ± 2.53	1.667	0.46	90.1 [a]
75	74.05	34.93 ± 0.81	1.455	0.47	92.3 [a]
50	49.64	23.70 ± 0.54	0.975	0.48	93.4 [a]
25	22.56	10.72 ± 0.91	0.447	0.48	93.0 [a]
Type of medium					
Defined (basal salts)	60.0	29.41 ± 0.94	1.225	0.49	96.1 [a]
Rich (YPD)	60.0	28.97 ± 0.49	1.207	0.48	94.7 [a]
Minimal (only C-source)	70.0	27.65 ± 2.54	1.152	0.40	77.5 [b]
OPT sap (control)	93.9	47.54 ± 0.27	1.981	0.50	99.3 [a]

Values are fermentation efficiency of triplicate determination. Means bearing different letter in a column are significantly different ($p < 0.05$).

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Distillation of fermentation broth to obtain purer ethanol is energy demanding especially in the case if the broth has lower ethanol concentration (Olsson and Hahn-Hagerdal, 1996). It is thus preferable to work on substrate having high concentrations, i.e. high fermentable sugars concentrations and its resulting high ethanol concentrations in industrial processes. In this study, it was evident that a sugar concentration up to 94 g/L did not give any negative effect to the yeast cell. Thus, the undiluted OPT sap can be directly used in ethanol fermentation as *S. cerevisiae* has high ethanol tolerance up to 10 % (v/v).

Type of medium

The performance of *S. cerevisiae* in reference media i.e. rich medium (YPD), defined medium (basal salts) and minimal medium (only carbon sources) *vs.* OPT sap for bioethanol productivity were shown in Figure 7. It demonstrated that OPT sap had sufficient nutrients to support fermentation by *S. cerevisiae*, and may not contain inhibiting substances. *S. cerevisiae* performed the best in OPT sap although the efficiency (99 %) was not significantly different as compared to those in the reference media i.e. YPD and defined medium. However, minimal medium showed the worst with only 78 % fermentation efficiency ($p < 0.05$) (Table 3). This was attributed in part to an absence of essential nutrients and nitrogen source for yeast growth thus lowering the yield of bioethanol. OPT sap, on the other hand, contains lots of amino acids (serine, alanine, glutamic acid and aspartic acid), organic acids (citric, malic and maleic acids), vitamins (vitamin B and C) and minerals (calcium, magnesium and chloride) (Kosugi *et al.,* 2010), hence it is a good medium for yeast growth in fermentation.

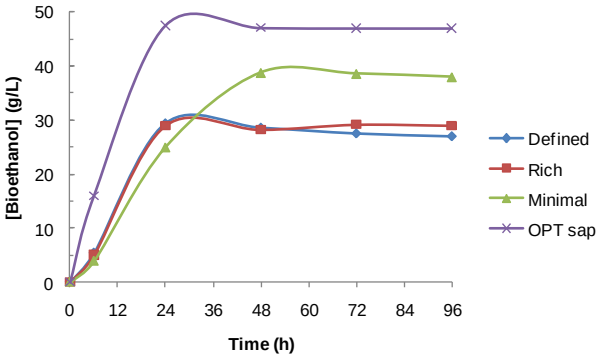

Figure 7: Bioethanol production from oil palm trunk sap and other reference media – rich, defined and minimal.

Table 4: Comparison of bioethanol production from various renewable substrates.

Strain	Substrate	Sugar conc. (g/L)	Ethanol yield, $Y_{p/s}$ (g/g)	Reference
S. cerevisiae (strain DTN)	Sugar beet thick juice	100	0.43	Razmovski and Vucurovic, 2012
S. cerevisiae (drying baking yeast)	Sweet sorghum stalk juice	110	0.39	Mairan *et al.*, 2011
S. cerevisiae (commercial Bakers yeast, *Mauripan)*	OPF juice	53	0.49	Zahari *et al.*, 2013
S. cerevisiae Kyokai no. 7	OPT sap	55	0.48	Kosugi *et al.*, 2010
S. cerevisiae ATCC 24860	OPT sap	94	0.50	This study

The OPT sap yielded 0.50 g ethanol/g sugars employing the optimised fermentation conditions. This value is slightly higher compared to that of ethanol produced from OPT sap in another study (Kosugi *et al.*, 2010) and other renewable liquid resources as reported previously using various different strains of *S. cerevisiae* (Table 4). The result suggests that OPT sap has a great potential as fermentation substrate for ethanol production. The procedure is rather straightforward; which does not require any additional nitrogen and nutrient supplementation, pH adjustment and can be operated at ambient temperature.

CONCLUSION

The study has clearly demonstrated that high efficient bioethanol production from OPT sap is possible but it is influenced by various fermentation parameters. A total of 63 g/kg of fermentable sugars from inner part of OPT can be converted into 40 L/tonne bioethanol employing the optimised fermentation conditions described in this study. This process has potential to be scaled up to either pilot or commercial production, making the process more economical. This study has also established that OPT sap from the plywood industries which has not been exploited commercially for any industrial application and is poorly disposed of could be utilised effectively for biofuel production.

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