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SHORT COMMUNICATION

Yeast Isolation for Bioethanol Production

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We have isolated 12 yeast isolates from five different rotten fruits by using a yeast glucose chloramphenicol agar (YGCA) medium supplemented with tetracycline. From pre-screening assay, four isolates exhibited higher substrate (glucose-xylose) consumption efficiency in the reaction tube fermentation compared to *Saccharomyces cerevisiae* dan *Saccharomyces ellipsoids* as the reference strains. Based on the fermentation process in gooseneck flasks, we observed that two isolates (K and SB) showed high fermentation efficiency both in sole glucose and mixed glucose-xylose substrate. Moreover, isolates K and SB produced relatively identical level of ethanol concentration compared to the reference strains. Isolates H and MP could only produce high levels of ethanol in glucose fermentation, while only half of that amount of ethanol was detected in glucose-xylose fermentation. Isolate K and SB were identified as *Pichia kudriavzeevii* (100%) based on large sub unit (LSU) ribosomal DNA D1/D2 region.

Key words: yeast, glucose-xylose substrate, ethanol

INTRODUCTION

The utilization of ethanol as an alternative fuel has escalated recently because of some conceivable reasons. Ethanol is a clean and renewable type of fuel which can be produced economically and environmentally friendly (Tian *et al.* 2009). Many agricultural by-products can be used as potential raw material for bioethanol production. The production of bioethanol from agricultural by-products is very prospective because the raw materials do not compete with other food-source materials which contain sugar and starch. Ethanol can be made from oil or biomass conversion by microbes through a fermentation process (Ohgren *et al.* 2006).

Saccharification of agricultural by-products can be done either through acid hydrolysis or enzymatic hydrolysis. Taherzadeh and Karimi (2007) reported that enzymatic hydrolysis is more beneficial than acid hydrolysis. This is due to the absence of sugar degradation into Hydroxy Methyl Furfuraldehyde (HMF) or furfural, milder reactions (low temperature, neutral pH), potential for high results in a reaction, and low maintenance expense (no corrosive instruments are used). Zhao *et al*. (2008) had also stated that enzymatic hydrolysis only uses low energy input, has low polution effects and no side products such as furfural or HMF are detected.

The application of yeast for ethanol conversion from starch-containing materials or other sugar sources, including cassava, sweet potato, sago palm, and unfermented palm juice, has been conducted previously. Yet, the application of lignocellulose materials, such as corn cob, for ethanol production should be carried out in detail because the hemicellulose and lignin content may influence the final products. The hydrolysis reaction of hemicellulose generates xylose (pentose sugar/C5) which can not be converted into ethanol by commercial yeast (*Saccharomyces cerevisiae).* Therefore, in this study we screen for yeast which will be able to convert glucose and xylose mixed-substrates.

MATERIALS AND METHODS

Yeast Isolation. Yeast isolates were recovered from five different rotten fruits, including (i) apple, (ii) watermelon, (iii) melon, (iv) papaya, and (v) pineapple. About 1 gram of each fruit was used as yeasts source and further serially diluted in reaction tube using NaCl 0.85% solution until 10^{-4} of dillution. About 100 µl of each of the last two serial dillutions was then spread on top of yeast malt extract agar (YMEA) with a composition consisting of 5 g/l malt extract agar and 23 g/l yeast extract agar.

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Then, the composition was further incubated for 48 hours at 30 °C. Expected yeast isolates were then purified and screened by using a selective medium.

Yeast Screening. Pre-screening of yeast isolates was conducted by using the selective yeast glucose chloramfenicol agar (YGCA with the following composition: glucose 20 g/l, yeast extract 5 g/l, chloramphenicol 0.1 g/l, agar 15 g/l) supplemented with tetracycline (0.05 g/l). Each isolate was simply streaked on the YGCA medium and subsequently incubated for 96 hours at 30 °C. All grown isolates were screened as yeast culture and further assayed for their capability in converting glucose and xylose mixed substrate (1:1) without aeration at 30 °C. For this assay, yeast isolates were previously prepared in a potato dextrose agar (PDA) medium and further subcultured in a potato dextrose broth (PDB) medium for 24 hours. About 10% (v/v) of the yeast culture was then inoculated in a reaction tube containing pure glucose-xylose (10%:10%) enriched with a solution of sodium phosphate potassium (0.04%) and ammonium sulphate (0.15%). The culture was fermented in a closed system, whereas the utilization of the substrate was periodically monitored during fermentation, by using the DNS method (Miller 1959). Selected isolates were chosen particularly based on substrate consumption compared to the reference yeast isolates (*S. ellipsoides* and *S. cerevisiae*) and assayed for bioethanol production afterwards.

Yeast Identification. Yeast isolates were partially identified by using large sub unit (LSU) ribosomal DNA D1/D2 region. DNA isolation was conducted by employing a DNA extraction kit of Nucleon PHYTOpure (Amersham Life Science). Primer NL1 (5'-CATATCAATAAGCGG AGGAAAG-3') and primer NL4 (5'-GTCCGTGTTTCAA GACGG-3') (O'Donnell 1993) were used for PCR amplification. PCR products were subsequently purified based on the polyethyleneglicol (PEG) precipitation method (Hiraishi *et al*. 1995) and followed with a sequencing process using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequences were further used for taxa identification using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and compared to the GenBank database.

Bioethanol Production. The production of bioethanol was carried out in a modified erlenmeyer flask (250 ml). The flask was connected with a water-containing measuring tube by a plastic hose and reversely submersed in a plastic tank containing water. Two different substrates were used for this fermentation assay: sole glucose (10%) and a mixture of glucose-xylose (10%:10%). The fermentation medium was also enriched with a solution of sodium phosphate potassium (0.04%) and ammonium sulphate (0.15%), as was also used in previous assays. About 10% (v/v) of the yeast culture in the PDB medium was transferred to the fermentation medium within the modified erlenmeyer flask and subsequently incubated in a waterbath shaker (120 rpm) at 30 °C. By using this modified flask, the CO_2 content yielded from the fermentation reaction would be released through the plastic hose which further lowers the water volume in the measuring tube. The reduction in the volume of water was proportional with the volume of CO_2 formed during fermentation and simply used to calculate the content of CO_2. Besides the content of CO_2, several different observations were also conducted during fermentation such as the substrate consumption [using the dinitrosalicilic acid (DNS) method] (Breuil & Saddler 1985), and the ethanol content (using gas chromatography).

Estimation of the Ethanol Content using Gas Chromatography (GC). Injection volume for the GC (Agilent Technologies 6890N) assay was done in a rate of 0.8 ml/min within the capiler column (HP-Innowax, length 60 m, diameter 0.25 mm and film thickness 0.25 µm) with helium (He) as the carrier gas. The GC system was attached with a flame ionization detector (FID, 250 °C), while the temperature of the injection port was controlled at 200 °C. The ethanol content was calculated by comparing the retention time of the sample to the ethanol standard.The standard curve was made by using pure ethanol with methanol as the solvent.

RESULTS

Yeast Isolation. By using the YMEA medium, 23 isolates were recovered. The presence of bacterial morphology was observed around the targeted yeast. Therefore, further screening assay using selective medium was necessary to obtain pure yeast colonies.

Yeast Screening. Further screening in the YGCA medium supplemented with tetracycline resulted in 12 isolates being able to show the yeast morphological character. Therefore, those 12 isolates were continuosly assayed for their capability to use the glucose-xylose substrates in simple fermentation within the reaction tube. After 72 hours of fermentation, the presence of yeast growth (indicated by the development of gas bubbles, the turbidity, and the aroma of yeast) was observed for all experiments. All isolates were capable of using the substrate with varying substrate consumption efficiency. However, only four isolates showed a higher level of substrate consumption compared to the reference strains (Table 1).

Bioethanol Production. Four selected isolates from previous assays were observed for their bioethanol production. The observation was done by quantifying the alteration of the CO_2 volume every 3 hours for the first 12 hours and every six hours for a further 48 hours (Table 2). The production of CO_2 differed among isolates.

Table 1. Yeast isolates with higher level of substrate consumption than reference strains during glucose:xylose (10%:10%) fermentation

Isolate	Origin	Morphological characters	$\Delta S/S_0$
Reference	*S. cerevisiae*	Yellowish, watery	0.34
Reference	*S. elipsoides*	Yellowish, watery	0.33
K	Apple	White, wide, without core	0.39
H	Papaya	White, small spot	0.40
MP	Melon	Yellowish-white, watery	0.33
SB	Watermelon	White, wide	0.64

Interestingly, all isolates produced their highest content of CO_2 at 18 hours of fermentation, and the number descended afterwards.

Estimation of Ethanol Content using Gas Chromatography (GC). The ethanol content during fermentation was different among isolates, yet the content of ethanol from glucose fermentations were higher than that of glucose-xylose fermentations, with the exception of isolate K (Table 3).

Based on Table 3, we noticed that isolate K and SB exhibited a relatively identical ethanol content from either glucose or glucose-xylose fermentations. Therefore, we assumed that those two isolates were capable in using both glucose or xylose, in particular, during ethanol fermentation. Moreover, the accumulation of CO_2 of those two isolates seemed to be equivalent with the production of ethanol (Figure 1). Interestingly, the volume of the augmented CO_2 from isolate SB using either glucose or the mixed substrate was found to be relatively similar, while isolate K showed a larger volume of CO_2 using the mixed substrate compared to the sole glucose substrate. In contrast, isolates H and MP yielded higher ethanol content using the sole glucose substrate compared to the glucose-xylose mixed substrate which only resulted in half of the yield. Therefore, we assumed that the two isolates could only convert glucose in mixed substrate fermentation.

Table 2. The content of CO_2 (ml) during fermentation using glucose and glucose-xylose (1:1) mixed substrate

Hour	CO_2 Volume (ml)							
	Isolate							
	K		SB		H		MP	
	G	GX	G	GX	G	GX	G	GX
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	5.0	26.3	7.5	12.5	3.5	12.5	6.0	16.3
6	9.0	45.0	15.0	55.0	8.0	17.5	19.0	18.0
9	15.0	48.5	37.5	25.5	27.3	22.5	40.0	34.5
12	18.5	18.5	32.5	30.8	32.8	52.5	37.5	43.8
18	47.5	92.5	106.0	109.8	106.3	102.5	133.8	95.0
24	35.0	52.5	50.0	21.5	53.8	63.8	62.5	61.3
30	35.0	35.0	34.0	20.0	33.8	53.8	46.3	42.5
36	38.0	30.0	33.0	17.5	23.8	32.5	35.0	30.0
42	26.0	12.5	17.0	10.0	13.8	15.0	16.3	17.5
48	16.0	10.0	9.5	10.0	26.3	17.5	23.3	15.0

G: Pure glucose, GX: glucose-xylose mixed substrate.

Table 3. The content of ethanol during glucose and glucose-xylose fermentation by selected yeast isolates using GC analysis

Isolate	$\Delta S/S_0$		Ethanol content (%)	
	Glucose	Glucose-xylose	Glucose	Glucose-xylose
K	0.52	0.50	1.09	1.32
H	0.58	0.53	3.13	1.51
SB	0.48	0.44	1.68	1.14
MP	0.45	0.50	2.49	1.58

Yeast Identification. Based on homology analysis, isolate K and SB both, similarly, possessed a high percentage of *Pichia kudriavzeevii* (100%).

DISCUSSION

Generally, yeast cells use monosaccharide for their growth, yet only a few of the monosaccharide compounds can be converted into ethanol. D-glucose is the best susbtrate for either the growth of yeast cells or fermentation for ethanol production. Mosier *et al.* (2005) and Hisamatsu *et al.* (2006) reported that hexose sugar, including glucose, galactose and mannose, can be fermented by many wild microorganisms, yet pentose sugar, such as xylose and arabinose, can only be fermented by a small number of wild microorganisms, frequently resulting in low ethanol yield. The yeast *Issatchenkia orientalis* MF 121 is one of the potential isolate for fermenting those types of substrates. This isolate is acid tolerant (pH 2) and halotolerant (5%), however MF 121 cannot use susbtrate D-xylose, D-galactose, or cellobiose for ethanol production. This research was conducted to explore the sources of yeast isolates and further determine their activity in assimilating glucose-xylose mixed susbtrates because the content of these two sugar compound are relatively high within agricultural by-products.

Fruits are food materials that contain a high level of glucose. It is suitable for yeast cells to grow on because yeast grows optimally in simple sugars, including glucose, and can even grow in complex sugars, such as sucrose (Chavan *et al.* 2009; Ocon *et al.* 2010). Yeast cells are both saprophyte and parasite. The damage to fruits due to

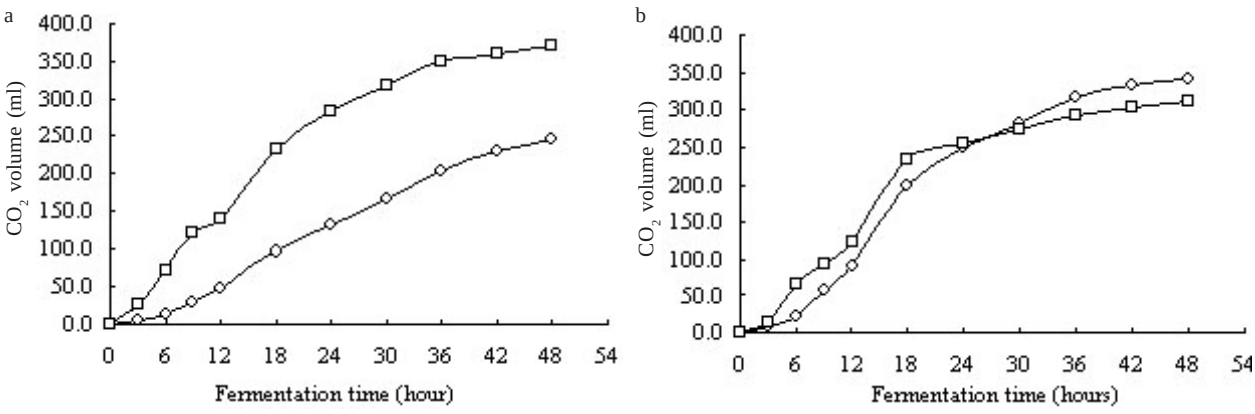

Figure 1. The accumulation of augmented CO_2 derived from 48 hours of glucose fermentation (–○–) and glucose-xylose fermentation (–□–) by isolate K (a) and isolate SB (b).

physicall collisions may cause structural defects in the fruit's tissue. This condition will lead to the colonization of yeast arround those defective areas. Yeast-causing damage is often indicated by the development of both an acid and alcohol aroma and the development of a unique layer in the surface area of the fruit, such as a defect in the fruit extract. The colonization of yeast will lead to the rotting of the fruit.

Stringini *et al*. (2008) had isolated yeast from both the fruits and leaves of papaya, cacao, banana, soil, sugar cane extract, and other sources from agricultural wastes. 151 isolates were isolated by using two isolation methods, YPD medium and enrichment mediums, one of which used chloramphenicol for inhibiting the bacterial growth.

In this study, for the pre-screening assay, YGCA medium added with tetracycline to was used to inhibit the growth of bacteria and molds. The growth of the yeast isolates varied with some isolates growing better than others. In fact, several isolates were unable to grow in the YGCA medium. We assumed those extinguish isolates were bacteria, since bacteria are commonly unable to grow in chloramphenicol and tetracycline-containing medium.

Three isolates from apple, papaya and melon exhibited relatively similar substrate consumption efficiency compared to the reference strains. In fact, one isolate from watermelon had two times the substrate consumption efficiency compared to the reference strains. Therefore, it was assumed that the capability of the isolate to convert susbtrate during the fermentation process was high because the level of substrate left was low. However, the high efficiency of substrate consumption may not be an indication of substrate convertion to bioethanol, since it could be a result of the substrate utilization for cell growth and the development of side products such as acids and flavour compounds.

Based on the results, isolates K and SB produced a relatively identical level of ethanol content compared to the reference strains. It was suggested that those two isolates could convert two type of substrate into ethanol, indicating their capability in utilizing xylose. Meanwhile, isolates H and MP could only produce high levels of ethanol in glucose fermentation, while only half of that amount was detected in glucose-xylose fermentation. Therefore, it was assumed that these two isolates could only used glucose for ethanol production.

The quantification of CO_2 content is pricipally based on indirect ethanol calculation as described in the chemical reaction of the fermentation as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (1)$$

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \quad (2)$$

From the reaction above, the ethanol content which are produced from either glucose (i) or xylose (ii) are equivalent with the yielded glucose. Bonciu *et al*. (2010) had also observed that the alteration of CO_2 volume, which were produced during fermentation, can be used to calculate the bioethanol content after fermentation. However, the alteration of CO_2 volume can not directly be used for determining bioethanol content from hydrolysate inuline. The bioethanol content is mainly determined by multiplying the volume of CO_2 with the coefficient 1.045 as in the Gay-Lussac equation.

The observation of the CO_2 volume was conducted for both pure glucose fermentation and glucose-xylose fermentation. These observations were mainly used as additional data for the two potential isolates. Based on the results, it was observed that these two isolates could produce CO_2 during fermentation. In addition, it was also suggested that the volume of CO_2 was equivalent with the ethanol content. As the duration of fermentation increased, the level of CO_2 also escalated, yet after a certain period of incubation the increasing level of CO_2 was not significant. It can be assumed that these two isolates were able to convert sugar into ethanol through enzymatic reactions. Moreover, the ethanol production was found todecrease as the level of substrate decreased.

At the begining of the fermentation step, yeast cells need oxygen for growth, yet after the accumulation of CO_2, the reaction turns anaerobic. During anaerobic growth, yeast cells metabolize glucose into ethanol mostly through Embden Meyerhoff Parnas. Each mol of glucose will generate two moles of ethanol, CO_2 and ATP. Therefore, theoritically, each gram of glucose yields 0.51 g of ethanol. In fact, the production of ethanol is less than 90-95% because most of the nutrition is used to synthesize biomass and maintain the reaction. Moreover, side reactions can also occur resulting in glycerol and succinate with 4-5% of substrate consumption. Ethanol can also inhibit the sustainability of the fermentation reaction as its level reaches 13-15%, yet it depends on temperature and type of yeast.

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