

Potential of *Candida glabrata* from ragi as a bioethanol producer using selected carbohydrate substrates

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Abstract. Vincent M, Johnny Q, Adeni DSA, Suhaili N. 2021. Potential of *Candida glabrata* from ragi as a bioethanol producer using selected carbohydrate substrates. *Nusantara Bioscience* 13: 1-10. The flexibility and efficiency of fermenting microorganisms to convert substrates to ethanol are important factors in achieving high bioethanol yields during ethanolic fermentation. In this study, *Candida glabrata*, a common yeast found in fermented food, was evaluated in terms of its capability to produce ethanol using different types of carbohydrates, which included simple saccharides (glucose, maltose, sucrose), polysaccharides (starch and cellulose) and complex carbohydrates (total sago effluent, TSE). Our results indicated that *C. glabrata* was able to efficiently produce ethanol from glucose at 79.84% TEY (Theoretical Ethanol Yield). The ethanol production from sucrose was low, which was only 6.44% TEY, while no ethanol was produced from maltose. Meanwhile, for complex carbohydrate substrates such as starch and cellulose, ethanol was produced only when supplementary enzymes were introduced. Simultaneous Saccharification and Fermentation (SSF) of starch dosed with amylases resulted in an ethanol yield of 55.08% TEY, whilst SSF of cellulose dosed with cellulases yielded a TEY of 31.41%. When SSF was performed on TSE dosed with amylases and cellulases, the highest ethanol production was recorded within 24 h, with a yield of 23.36% TEY. Lactic acid and acetic acid were found to be at minimal levels throughout the fermentation period, indicating an efficient ethanol conversion. A notable increase in *C. glabrata* biomass was observed in cultures fed with glucose, starch (with supplementary amylases), and TSE (with supplementary amylases and cellulases). The current study indicates that *C. glabrata* can be used for bioethanol production from glucose, polysaccharides, and complex starchy lignocellulosic substrates such as TSE via SSF.

Keywords: Bioethanol, *Candida glabrata*, *Metroxylon sagu*, simultaneous saccharification and fermentation, total sago effluent

INTRODUCTION

The interest in producing renewable fuels has increased tremendously over recent years due to the instabilities of fossil fuel supplies and increasing global demands (Wei et al. 2015; Wong and Vincent 2019; Mohammad et al. 2020). Alternative liquid biofuel, such as bioethanol, is seen as the current choice of such renewable fuel to supplement and substitute petroleum-based fuel, due to its sustainability and carbon dioxide neutrality (Vincent et al. 2015; Hung et al. 2018). Compared to conventional gasoline, bioethanol is highly attractive as it offers cleaner combustion that is friendlier towards the environment. Economically, the bioethanol production and supply chain are also desirable as it creates many jobs and financial opportunities for both urban and rural areas (Ștefănescu-Mihăilă 2016).

In mass bioethanol production, substrate selection plays a major role. It is one of the main cost factors for the ethanol industry (Vincent et al. 2015). There are currently many feedstock sources that are used as substrates for bioethanol production (Techaparin et al. 2017; Ahorsu et al. 2018; Mohammad et al. 2020). When substrates such as lignocellulosic biomass are used to produce bioethanol, this type of bioethanol is termed as second-generation bioethanol (Zhang et al. 2016). It is projected that in the future, second-generation bioethanol will replace first-generation bioethanol, which is mostly produced from food-based materials, because of its low cost and feedstock

abundance (Vincent et al. 2015; Ștefănescu-Mihăilă 2016). In bioethanol production, the typical bioprocess engaged is Simultaneous Saccharification and Fermentation (SSF) as this procedure offers higher reaction rates, higher yields, and greater ethanol concentrations compared to its closest counterparts such as Separate Hydrolysis and Fermentation.

The efficiency of ethanol production is also influenced by the species of microorganisms used. The desired microorganism should be robust and capable of converting substrates to ethanol effectively. The most common examples of ethanol producers are *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Fusarium oxysporum* (Ali et al. 2016; Vincent et al. 2018; Mohammad et al. 2020). Among these, *S. cerevisiae* has been mostly used in alcoholic fermentation due to its ethanol productivity. However, this particular species has several limitations. For example, poor stress tolerance and incapability to ferment xylose and arabinose, the main sugars released from hemicelluloses. Therefore, several genetic engineering studies to improve sugar utilization, ethanol production, and other applications have been explored (Carrasco et al. 2013; Pagliardini et al. 2013; Wong and Vincent 2019). Another approach is to search for new fermenting yeasts that may perform better or are more flexible than *S. cerevisiae* (Vincent et al. 2018).

Another yeast species that has similar characteristics to *S. cerevisiae* is *Candida glabrata*, which can be commonly

found in fermented foods (Tsuyoshi et al. 2005) and starters, such as *ragi*, a traditional fermented food starter (Kofli and Davaon 2010; Hajar et al. 2012; Vincent et al. 2018). In this study, *C. glabrata* was isolated from the samples of *ragi* in Sarawak and was identified by using PCR and commercial identification kit. According to Kwon-Chung and Bennett (1992), *C. glabrata* was firstly classified under the genus *Torulopsis* due to its lack of pseudohypha production but in 1978, it was confirmed that the ability to produce pseudohyphae was not a reliable distinguishing factor for members of the genus *Candida*; and thus it was classified as a member of *Candida* genus, along with over 200 species. The *C. glabrata* cell size was reported to be small compared to other *Candida* species and is in the range of 1 to 4 μm (Fidel et al. 1999). In terms of ethanol production, *C. glabrata* is reported to be suitable for bioethanol fermentation as it possesses higher stress tolerance to acid and high temperatures in addition to impressive ethanol conversion capabilities (Watanabe et al. 2010). According to Merico et al. (2007), *C. glabrata* was reported to yield high ethanol percentages when grown under anaerobic conditions. Furthermore, *C. glabrata* is suitable for SSF since the optimum temperature for amyolytic and cellulolytic activities is above 40 °C.

Presently, limited information is available in the systematic studies of the ethanolic fermentation involving *C. glabrata*, especially when biomass is used as substrates. Biomass such as total sago effluent (TSE) can be found abundantly in Sarawak, Malaysia. TSE is a waste or residue from sago (*Metroxylon sagu*) industries that are usually disposed of into nearby rivers. According to Vincent et al. (2020), about 7 tons of TSE is discharged

into the rivers per day from typical sago starch factories. This waste needs to be handled properly to avoid water pollution (Mohammad et al. 2020). TSE consists of sago hydrolysate, which is the liquid part while the solid component is called sago hampas. It is reported to contain high amount of lignocellulosic materials, which can be found in the solid sago hampas, making it suitable to be used as a substrate for bioethanol production (Hung et al. 2018). The hydrolysate can also contribute to bioethanol production as it contains starch that can be converted into fermentable sugars (Vincent et al. 2015). Therefore, the main objective of this study is to investigate the abilities of *C. glabrata* in producing ethanol from a variety of carbohydrates, ranging from common simple carbohydrates (glucose – monosaccharide, sucrose, and maltose – disaccharides) to polymeric carbohydrates (starch and cellulose) and a complex carbohydrate mixture (total sago effluent).

MATERIALS AND METHODS

Experimental setup

Figure 1 shows an overview of the experimental procedures in this study.

Collection of total sago effluent

TSE was collected from Herdsen Sago Mill Sdn Bhd in Pusa, Sarawak (Figure 2). The TSE samples were stored at -20 °C prior to its usage.

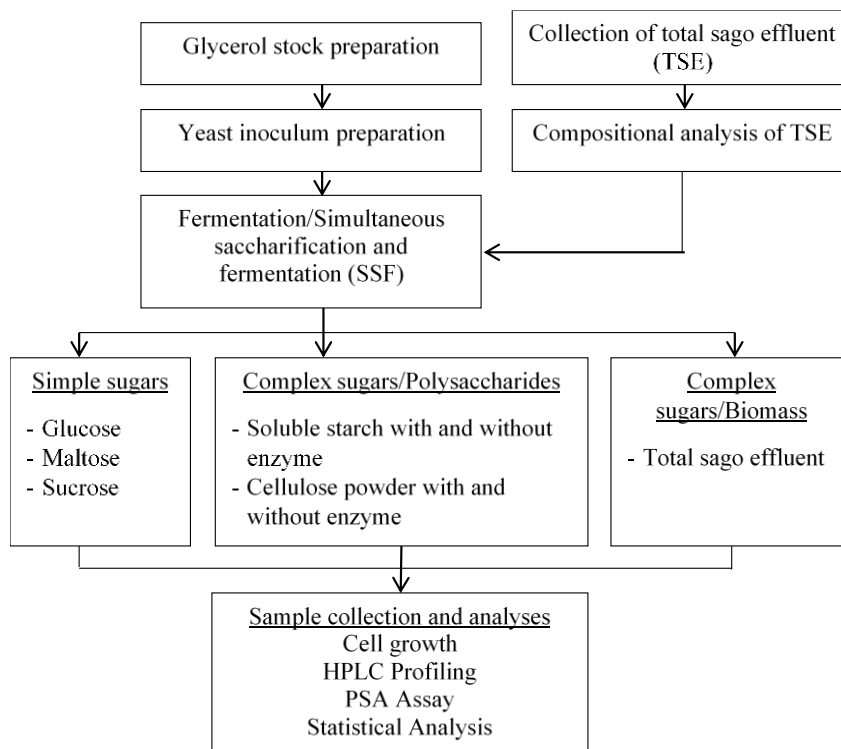


Figure 1. Overall experimental setup



Figure 2. Total sago effluent (TSE) released from the sago mill in Pusa, Sarawak, Malaysia

Characterization of sago hampas

The moisture content of TSE sample was determined by measuring the weight loss from the initial weight after oven-drying (Shel Lab, USA) at 105°C for 3 days. Prior to the characterization process, the TSE was oven-dried before it was ground to fine powder. The powdered samples were subjected to starch and fiber analysis by using the Phenol-Sulphuric Acid assay (PSA), Acid detergent Fiber (ADF), Neutral Detergent Fiber (NDF), and Klason Lignin Determination (Goering and van Soest 1970). The ash content was determined using furnace incineration, where the sample was subjected to dry ashing at 550°C for 6 hours in an ashing muffle furnace (KC, 40/13. ThermConcept, Germany).

Glycerol stock preparation

The *C. glabrata* strain used in this study was isolated from samples of ragi, collected from Kuching, Sarawak (Malaysia) (Vincent et al. 2018). Isolation of yeast single colonies was carried out on Rose Bengal Chloramphenicol Agar (RBDC) (Hi Media, India) and the single colonies obtained were grown in YM broth (Sigma, USA) by shaking at 150 rpm (NB-101MT Multi Shaker, N-Biotek, Korea) at ambient temperature. For long-term storage, the stock culture was prepared by mixing culture medium with 20% (v/v) glycerol (R&M Marketing, UK) and were stored at -20°C for preservation.

Candida glabrata inoculum preparation

For *C. glabrata* inoculum preparation, the cultures were taken from the glycerol stocks. The cultures were grown overnight in YM broth at ambient temperature with constant shaking at 150 rpm. The cultures were then centrifuged (BK-1032J Low-Speed Centrifuge, Biobase, China) for 6 min at 4500 rpm. The resulting *C. glabrata* cell pellets were used as inocula for fermentations in this work.

Fermentation –using glucose, maltose and sucrose

Fermentation medium was prepared in 500 mL Schott bottles containing 150 mL of mixed solution of 1.5 g/L of

yeast extract (Bacto, USA), 3.0 g/L of bacteriological peptone (Bendosen Laboratory Chemicals, Malaysia) and 5% (w/v) of either glucose (R&M Chemical, United Kingdom), maltose (R&M Chemical, United Kingdom) or sucrose (Bendosen Laboratory Chemicals, Malaysia) in 0.05 M citrate buffer, pH 5.0. The pH of the media was adjusted to the optimum pH level for fermentation at 4.8, before it was autoclaved (Model No. 25X Electric Pressure Steam Sterilizer, All American, United State) for 15 min at 121°C. Prior to the fermentation, the harvested cells, which were in the range of 1.29×10^8 cells/mL to 2.86×10^8 cells/mL were added aseptically into the sterilized fermentation media. Fermentations were carried out, anaerobically for 5 days, at ambient temperature with a constant shaking of 150 rpm. For each type of sugar, the fermentations were done in triplicates (n=3).

Fermentation –using starch and cellulose

The two sets of media consisting of polysaccharides namely 5% (w/v) starch (Bendosen Laboratory Chemicals, Malaysia) and 5% (w/v) cellulose (Nacalai Tesque, Japan) were prepared separately. After the pH adjustment to 4.8, the media were autoclaved in order to sterilize and also to gelatinize the starch. Next, the harvested *C. glabrata* cells and 75 µL of each amylase (Sunson, China), α -amylase (EC.3.2.1.1) and glucoamylase (EC.3.2.1.3) were added into the sterile starch solution, while 2.0 mL of cellulase (50 FPU) (Sunson, China) was added into the sterile cellulose solution. Cellulase enzymes used consisted of acid cellulase, beta-glucosidase, and xylanase. Another set of sterile starch and cellulose solution was prepared but no enzyme was added to those media. The fermentations were conducted under the same conditions of the fermentation with simple sugars.

Fermentation – using total sago effluent (TSE)

TSE fermentation medium was prepared by mixing 1.5 g/L yeast extract, 3.0 g/L peptone, and 0.05 M citrate buffer, with pH of 5, in a 500 mL Schott bottle and the solution was topped up to 150 mL with TSE. Following the pH adjustment

to 4.8 and sterilization, about 75 μL of each amylase (α -amylase and glucoamylase) and 2.0 mL of cellulase were added in order to start the SSF of TSE. SSF was done in triplicates ($n=3$) according to the same conditions as the fermentation described earlier.

Sample collection and *Candida glabrata* cell count

About 1.5 mL of culture broth was pipetted out at 0, 6, 12, 24, 36, 48, 72, 96 and 120 h during the fermentation. Prior to HPLC analyses, cell counting was performed using hemocytometer (Optik Labor, United Kingdom) and the viable cells were observed under a light microscope after methylene blue staining. The remaining supernatant was centrifuged (WiseSpin CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 13,500 rpm for 3 min and was filtered through a 0.45 μm nylon membrane filter (Whatman, NJ, USA) to eliminate any solid residues. The filtrate was used for phenol-sulfuric acid assay (PSA) and High-Performance Liquid Chromatography (HPLC) analyses.

Phenol-sulfuric acid assay (PSA)

Total carbohydrate content of the samples was determined based on PSA assay. The assay was performed by measuring the optical density of the samples at 490 nm using a spectrophotometer (SP-880 Metertech, Taiwan). The amount of total carbohydrate present in the samples was determined based on a standard curve, which was plotted prior to the assay.

Analytical analysis

Quantification of ethanol, sugars, lactic acid and acetic acid was performed using High-Performance Liquid Chromatography (HPLC) (Waters 2695 Separations Module, Alliance HPLC System, United States) The HPLC system was equipped with a column heater, refractive index detector (2414 RI Detector) and computer controller. The protocols for separation and analyses of ethanol and other fermentation constituents were according to Vincent et al. (2015). Theoretical Ethanol Yield (TEY) of each substrate was determined based on ethanol produced and was calculated as follows:

$$\text{TEY} = (\text{Ethanol produced (g/L)} / \text{conversion factor} \times \text{initial concentration of sugar}) \times 100$$

Where,

Conversion factor for sugar to ethanol = 0.511

Conversion factor for biomass to sugar = 1.1

Statistical analysis

Error bars were determined based on the standard deviation from the mean value of triplicate experiments. The data was statistically analyzed using One-way Analysis of Variance (ANOVA) and Tukey's *Post Hoc* test (SPSS Statistics Software version 22), and the differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Candida glabrata cell growth using different carbohydrate feedstock

In the current study, the ability of *C. glabrata*, isolated from local wine starter (ragi), in utilizing and fermenting different carbohydrates (mono-, di-, polysaccharides and complex mixture), represented by glucose, sucrose, maltose, starch, cellulose, and total sago effluent (TSE), to bioethanol and other byproducts were examined. Similar to other common yeasts, *C. glabrata* utilizes these different classes of carbohydrates differently, either for primary cell growth or for conversion to other secondary metabolites, alcohols, or organic acids.

A rapid and good specific growth rate is crucial for efficient fermentation and high yield of ethanol (Chang et al. 2018). For this reason, the cell population growth of *C. glabrata* in all substrates was monitored in order to gauge its growth profile. Based on the results from Figure 3, *C. glabrata* cells were propagating rapidly in the first 12 h in all fermentations. However, a notable increase in the first 6 h was only observed in fermentations employing glucose, starch with enzyme and TSE while the growth in other fermentations was relatively slower. After 48 h, a gradual decline in cell concentration was observed in all fermentations, due to substrate limitation and/or ethanol toxicity. The same observations were reported in studies elsewhere (Jönsson et al. 2013). Throughout the fermentation period, the trend of change of sugar uptake and alcohol production profiles was found to coincide with the logarithmic phase of the growth profile.

Ethanol production from glucose, maltose and sucrose

Figure 4 shows the time course of ethanol production during the fermentation by *C. glabrata* using different carbon sources. The ethanol production in fermentations using glucose increased sharply from 0 to 48 h, with the maximum production of 20.40 g/L (79.84% TEY) achieved at 72 h. After 72 h, the production of ethanol decreased slowly over time. At the later stage of fermentation, the ethanol concentration decreased in most of the fermentation because the yeast required energy to maintain growth. According to Li et al. (2017), the yeast consumed some ethanol to obtain energy due to the small amount of residual sugars in the media. From Figure 4, low ethanol yield was observed in the fermentation using sucrose, with the peak production of only 1.64 g/L at 6 h. On the other hand, no ethanol was detected in fermentations using maltose throughout the 5 days of incubation. This showed that *C. glabrata* does not produce particular enzyme to break down maltose. In the meantime, only low ethanol produced in fermentations using sucrose, which was probably caused by certain conditions such as concentration of starting materials, pH, or temperature during the fermentation.

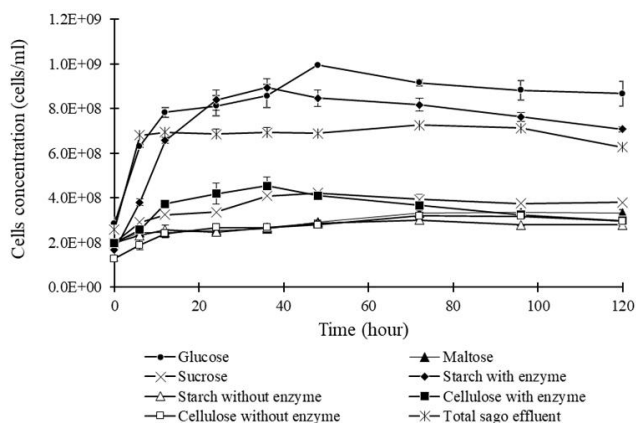


Figure 3. Time course of cell concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

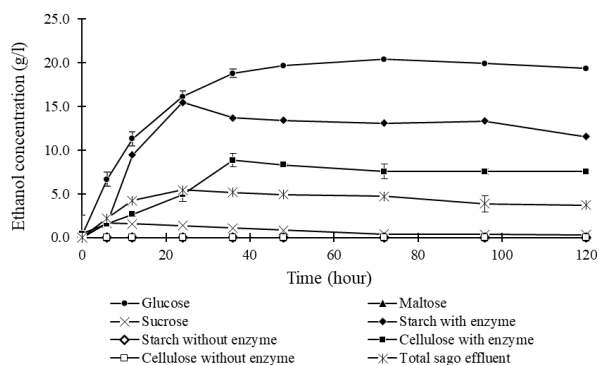


Figure 4. Time course of ethanol concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

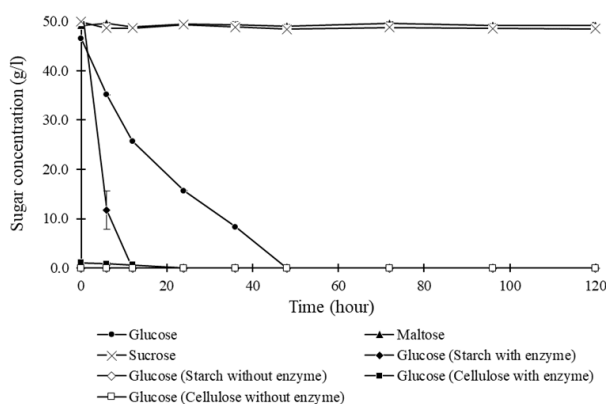


Figure 5. Time course of sugar concentration during bioethanol fermentation by *C. glabrata* using different carbon sources (simple sugars and complex sugars). The data points represent the average of three independent experiments (n=3)

The high efficiency of conversion of glucose to ethanol by *C. glabrata* was also documented previously by Barnett et al. (2000). Our study has shown that *C. glabrata* was capable of fermenting glucose efficiently. According to Mosier (2005), hexoses such as glucose can be fermented by most microorganisms species, especially yeasts. Glucose is also considered the best substrate for growing yeast cells and also ethanol fermentation (Ruriani et al. 2012). Conversion of glucose to ethanol at 79.84% of the theoretical value obtained in this work is considered high yield. The maximum yield is usually lower than 90% because it is usually limited by nutrients or substrates used to synthesize biomass and maintain the reactions. Another similar study conducted by Techaparin et al. (2017) on ethanol production from glucose by *C. glabrata* reported only a 42% conversion. By comparison, the *C. glabrata* strain used in this work showed higher efficiency of ethanol production, which renders its practicality for industrial applications.

Figure 5 shows the profile of sugar consumption during fermentation by *C. glabrata* using different substrates. The profile depicts a steady decrease of glucose concentration during the 48-hour incubation. However, the concentration of maltose and sucrose remained unchanged at approximately 48.0 g/L throughout the fermentation period. This observation is in agreement with the reports on the extremely narrow carbon utilization range of *C. glabrata* when compared to other yeasts (Turner and Butler 2014).

Simultaneous saccharification and fermentation (SSF) of starch, cellulose, and total sago effluent (TSE)

The ability of *C. glabrata* to saccharify starch, cellulose, and TSE for ethanol production was also evaluated in this work. Both starch and cellulose are classified as glucose-based polymers, with the latter more abundant in nature. However, these two carbohydrates differ in the orientation of the linkages between the glucose subunits (Jacques et al. 2003). As shown in Figure 5, the initial glucose concentration in fermentation medium containing starch was completely depleted after 12 h of fermentation, which was in line with the increase of ethanol (15.23 g/L) was achieved at 24 h. On the other hand, no glucose was detected in the fermentation medium containing starch when the supplementary enzymes were not added.

The results indicated that supplementary amylases are important in the alcoholic fermentation of *C. glabrata* as no ethanol was produced in the absence of the enzymes. These results can also be correlated with the sugar consumption profile (Figure 5) where glucose was only detected in media that was dosed with amylases. The conversion of starch to glucose occurred most efficiently on the first day of SSF as the maximum production of ethanol was recorded at 24 h, along with the depletion of glucose in the fermentation media. Our findings suggest that *C. glabrata* does not produce amylolytic enzymes to breakdown starch in the fermentation broth.

When cellulose powder was used as a carbon source, ethanol was only detected in the medium containing

supplementary cellulases. The ethanol concentration increased gradually over the 36 h of fermentation, peaking at about 8.83 g/L, as shown in Figure 4. After that, the ethanol yield started to remain constant until the end of the SSF period. On the contrary, in the absence of supplementary enzymes, no ethanol yield was recorded throughout the SSF period, as shown in Figure 4. This outcome indicates that cellulases are crucial for the conversion of cellulose to ethanol, as supported by the findings by Fox et al. (2012) where the cellulase action on the insoluble cellulosic substrates is modeled as a heterogenous biocatalytic reaction. The reaction involves endo- and exocellulases that either interact directly with soluble cello-oligosaccharides or form complexes with insoluble cellulose chains. Cello-oligosaccharides are saccharides that consist of 2 to 6 glucose by β -1-4-linkages, containing mainly cellobiose (Zhao et al. 2009). This study further confirms the inability of *C. glabrata* to breakdown cellulose and requires extracellular cellulases to convert cellulose into fermentable sugar.

SSF was also performed on TSE. The compositional analyses of TSE prior to SSF are presented in Table 1 and Table 2. The TSE used in the current study consisted of sago effluent hydrolysate (water component) and sago hampas (solid component), with 97.14% was the water component as shown in Table 1. Approximately 2.23% of the hydrolysate was starch, which can be converted to fermentable sugar through enzymatic hydrolysis. Further analysis of sago hampas was carried out to determine the exact amount of lignocellulosic contents. As outlined in Table 2, dried sago hampas used in this study contained 52.03% starch, 27.82% cellulose, 5.32% hemicellulose and 3% lignin. The results obtained in this work are comparable to a previous report by Hung et al. (2018), where sago hampas were reported to contain 55.4% starch, 23.6% cellulose, 9.1% hemicellulose, and 4.0% lignin. Furthermore, the starch content recorded in this study, which was in the range of 30% - 50% was comparable with that reported by Awg-Adeni et al. (2010). The variation of lignocellulosic composition can be associated with the difference in the sago species, growth conditions and maturity (Kim et al. 2010; Tye et al. 2011). The amount of cellulose and hemicellulose in biomass is the key in biofuel production, as they are convertible into bioethanol (Malherbe and Cloete 2003).

Figure 6 shows the profile of total soluble carbohydrates and sugars released during SSF of TSE. The initial total carbohydrate concentration was recorded at 20.57 g/L. At 6 h, the total carbohydrate content declined steeply to 4.98 g/L, giving a total reduction of approximately 75%. At 12 h, the concentration decreased further to 4.02 g/L and remained relatively constant until the end of the SSF period. The initial glucose concentration was 16.47 g/L at 0 h, before it decreased to 0.56 g/L at 6 h and became completely depleted after 12 h. For cellobiose, the initial concentration was 4.32 g/L and it decreased to 1.62 g/L and became plateau until the end of the incubation. Another wood sugar, arabinose was also detected in the TSE samples with the concentrations fluctuated slightly between 0.8 and 0.9 g/L, before peaking

at 1.10 g/L at 120 h. There was no xylose detected at the initial stage of SSF until at 12 h when a concentration of 0.19 g/L was detected. The xylose concentration remained between 0.25 and 0.27 g/L throughout the remaining SSF period. A trace amount of arabinose with a concentration of 0.40 g/L was detected and the amount was found to remain constant throughout the incubation. Xylose and arabinose are the most abundant pentose sugars in hemicellulose (Bettiga et al. 2009). These sugars are released from xyloglucan, xylan, arabinan and arabinogalactan (substructures of pectin) from polysaccharides in plant cell (Battaglia et al. 2011).

Table 1. Compositional analysis of total sago effluent TSE comprising of sago hydrolysate and dried sago hampas.

Compositions	Amount (%)	
Sago hydrolysate (water component)	Water	94.91 \pm 0.09
	Free Starch	2.23 \pm 0.86
	Starch	1.49 \pm 1.11
Dried sago hampas (solid component)	Cellulose	0.79 \pm 1.23
	Hemicellulose	0.15 \pm 2.00
	Lignin	0.09 \pm 0.09
	Ash	0.06 \pm 0.06
	Others	0.28 \pm 0.00

Table 2. Compositional analysis of dried sago hampas The percentages was based on dry weight, n=3.

Compositions	% w/w
Starch	52.03 \pm 1.11
Cellulose	27.82 \pm 0.62
Hemicellulose	5.32 \pm 0.96
Lignin	3.00 \pm 0.05
Ash	1.95 \pm 0.03
Others	9.88 \pm 0.07

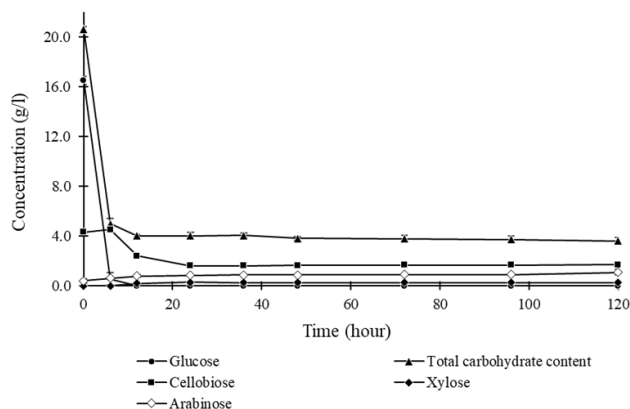


Figure 6. Time course of total carbohydrate content and sugar concentration (glucose, cellobiose, xylose, arabinose) during SSF of TSE. The data points represent the average of three independent experiments (n=3).

As shown in Figure 6, xylose was only detected at 12 h and the peak was found at 24 h. This showed that the breakdown of hemicellulose only started to occur at 12 h and onwards. In contrast, a trace amount of arabinose was detected from the beginning until the end of SSF. The degradation of pectin during the autoclaving process of media preparation may have resulted in the accumulation of arabinose at this stage. The concentration of arabinose was found to increase during the first 12 h, indicating the active breakdown of hemicellulose by xylanase into xylose and arabinose. After 24 h, the concentration of xylose and arabinose remained constant until the end of SSF implying the inability of *C. glabrata* to utilize both sugars. This is in parallel with a study by Barnett et al. (2000) and Ruriani et al. (2012) that reported the selective fermentation of *C. glabrata*. The same studies further reported that yeast cells basically use monosaccharides for their growth, but only a few of the monosaccharide compounds such as glucose, galactose and mannose can be converted into ethanol. Pentose sugars (five-carbon sugars) such as xylose and arabinose, can only be fermented by a small number of wild microorganisms, often with a low ethanol yield (Mosier 2005).

A complete breakdown of cellulose into glucose involves extracellular cellulases. Cellobiose, a disaccharide, is usually the intermediate product. Based on our results, the highest concentration of cellobiose was found in the beginning of SSF indicating the enzymatic hydrolysis of cellulose into cellobiose. Subsequently, β -glucosidases, a part of cellulase enzymes will cleave cellobiose into 2 glucose units (Lynd et al. 2002). The depletion of cellobiose denotes the effective enzymatic activity in the fermentation broth. However, cellobiose was only detected in low concentrations, and this in turn resulted in a low yield of glucose.

Based on Figure 6, a steep decrease of total sugar content during the first 6 h indicated that the substrates present in the fermentation broth were rapidly fermented into ethanol by *C. glabrata*. The same observations were also reported by Hung et al. (2018) who stated that not all sugars can be converted to ethanol. In addition, the results obtained in this study showed the presence of other unknown hexose sugars in the fermentation broth besides glucose.

Ethanol was produced at the early stage of SSF, with a concentration of 2.22 g/L recorded at 6 h, as shown in Figure 4. The increase in ethanol yield is highly correlated with the steep decrease in glucose concentration. After the depletion of glucose at 6 h, the ethanol concentration continued to increase until its maximum production at 24 h. The highest ethanol concentration was 5.44 g/L. This corresponds to an estimated 21.25% TEY, which is significantly lower than that reported by Hung et al. (2018) (79.65% TEY). The author reported a higher TEY because they used only sago hampas as substrate, while in this study, the mixture of sago effluent and sago hampas was used. Compared to sago hampas, TSE contains less lignocellulosic materials and thus produced less ethanol.

Analyses of fermentation by-products – Lactic acid and acetic acid

Figure 7 depicts the profile of lactic acid during fermentation by *C. glabrata* using different carbon sources. Lactic acid commonly comes from lactic acid bacteria (LAB). A sharp increase of lactic acid was observed in fermentations using glucose as a substrate with a peak of 3.71 g/L at 24 h, which was caused by the presence of LAB in the media throughout the fermentation. On the other hand, the lactic acid concentration was relatively low in fermentations that use other carbon sources.

Figure 8 illustrates the concentration of acetic acid during the fermentation by *C. glabrata* using different carbon sources. Overall, the level of acetic acid in all fermentations was recorded to be less than 0.5 g/L. Similar to lactic acid, acetic acid also comes from bacteria, acetic acid bacteria (AAB). A previous study stated that acetic acid also can be found in hydrolysates and it basically comes from acetyl side-chains in hemicellulose. According to Vincent et al. (2015), acetic acid is an important indicator of the efficiency of biomass hydrolysis where an increase in acetic acid concentration in the fermentation broth denotes a continuous degradation of the lignocellulosic components of TSE throughout the SSF period.

Although the presence of organic acids is common in typical fermentation systems, the concentrations of lactic acid and acetic acid must, however, be closely monitored to avoid a reduction in the ethanol production due to the changes in the pH of the fermentation media (Lin et al. 2012). According to Narendranath (2003), the presence of these acids in high concentrations can also be indicator of contamination, which could be caused by Lactobacilli. In this study, the overall concentrations of both lactic acid and acetic acid in the cultures were relatively low (<0.5 g/L). This indicated that the fermentations were free from contaminations caused by bacteria, except for the media containing glucose.

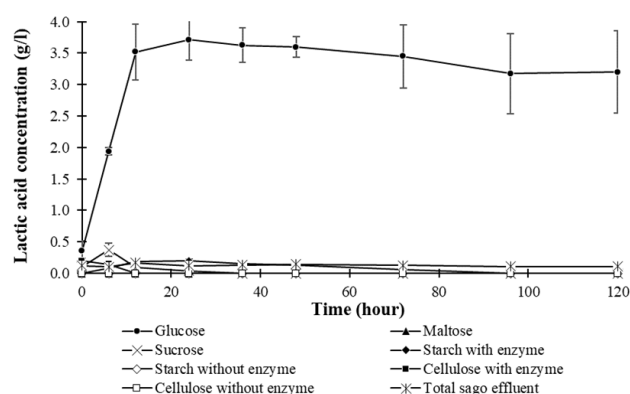


Figure 7. Time course of lactic acid concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average values of three independent experiments (n=3).

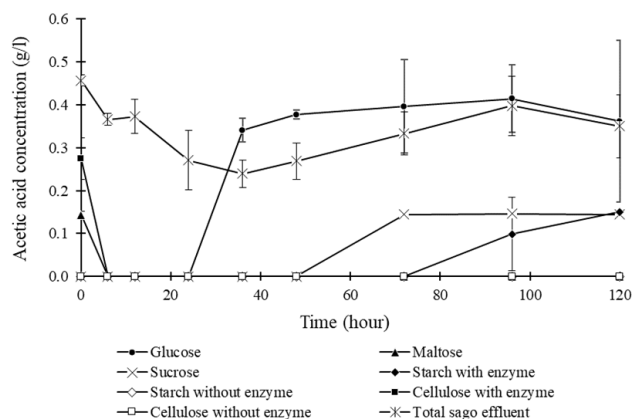


Figure 8. Time course of acetic acid concentration during bioethanol fermentation by *C. glabrata* using different carbon sources. The data points represent the average of three independent experiments (n=3)

Table 3. Maximum ethanol production and yield achieved in fermentation by *C. glabrata* using different substrates.

Substrate	Maximum production of ethanol (g/L)	Experimental theoretical yield (%)
Glucose	20.40 ± 0.30	79.84 ± 1.18a
Maltose	0.00 ± 0.00	0.00 ± 0.00b
Sucrose	1.64 ± 0.08	6.44 ± 0.30b
Starch with enzyme	15.23 ± 2.11	55.08 ± 7.50d
Starch without enzyme	0.00 ± 0.00	0.00 ± 0.00c
Cellulose with enzyme	8.83 ± 0.76	31.44 ± 3.31c
Cellulose without enzyme	0.00 ± 0.09	0.00 ± 0.00b
Total sago effluent	5.54 ± 0.09	21.25 ± 0.89e

Note: *Data are mean of triplicates ± S.D (Different superscript letters in the same column show significant differences, *p*-value < 0.05)

Table 3 outlines the overall maximum ethanol production and yield achieved in fermentation by *C. glabrata* using different substrates. The highest ethanol production was recorded in fermentations using glucose as a substrate. A maximum ethanol concentration of 20.4 g/L was produced, which corresponded to a theoretical yield of 79.84%. This clearly showed that in comparison to other carbon sources examined in this study, glucose is the best substrate for ethanol production by *C. glabrata*. The second best substrate was found to be starch when it was supplemented with enzymes. The maximum ethanol concentration achieved was 15.23 g/L and with a theoretical yield of 55.08%. In contrast, there was no ethanol yielded in fermentations using maltose, starch, and cellulose. Among all substrates tested in this work, only glucose and sucrose were consumed by *C. glabrata* to produce ethanol. Meanwhile, it is observed that soluble starch and cellulose were consumed only with the aids of the supplementary enzymes, with the former gave a higher yield. As for the SSF of TSE, the maximum ethanol production was 5.44 g/L (21.25% TEY). Although the ethanol concentration was relatively low compared to that

achieved using other carbon sources, the results do reflect the ability of *C. glabrata* to grow on TSE. It is expected that higher yield of ethanol can be achieved upon further optimization of the SSF process.

In conclusion, the results from this study indicated that *C. glabrata* was capable to ferment glucose efficiently (79.84% of theoretical yield), making it as compared to other common fermenting yeasts such as *S. cerevisiae*, which was reported to yield TEY of 40 to 49% when fermenting 4 to 5% (w/v) glucose (Govindaswamy and Sane 2010; Kumar et al. 2011). Although *C. glabrata* is incapable of fermenting other sugars such as xylose and arabinose as well as polysaccharides like starch and cellulose without the aid of supplementary amylases and cellulases, the strain does show a promising potential to be used for fermenting starchy industrial waste such as TSE.

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