Sains Malaysiana 45(4)(2016): 581–587

# Ethanol Production Capability of *Candida shehatae* in Mixed Sugars and Rice Straw Hydrolysate

(Keupayaan Pengeluaran Etanol *Candida shehatae* dalam Campuran Gula dan Hidrolisat Jerami Padi)

PRAWPHAN YUVADETKUN & MALLIKA BOONMEE*

ABSTRACT

*Ethanol fermentations by* Candida shehatae *TISTR 5843 at low (20 g/L) and high (80 g/L) sugar concentrations with various glucose to xylose ratios were investigated. Glucose was a preferred substrate as it was consumed first at a faster consumption rate. The type of sugar and ratio between glucose and xylose did not have an effect on ethanol produced. The average ethanol concentrations were 7.99 g/L when using 20 g/L sugar and 27.82 g/L when using 80 g/L sugar. Small amounts of xylitol and glycerol as by-products were presented when using 20 g/L sugar. Xylitol appeared to be the main by-product at high xylose concentration with elevated concentrations as xylose is increased. When using rice straw hydrolysate containing 34.75 g/L glucose and 21.29 g/L xylose, 19.37 g/L ethanol was produced with the ethanol yield and ethanol productivity at 0.49 g/g and 0.20 g/L.h, respectively. However, xylose was not completely consumed after fermentation was complete.*

*Keywords:* Candida shehatae*; ethanol; fermentation; lignocellulose; xylose*

ABSTRAK

*Penapaian etanol oleh* Candida shehatae *TISTR 5843 pada kepekatan gula rendah (20 g/L) dan tinggi (80 g/L) dengan pelbagai glukosa untuk nisbah xilosa dikaji. Glukosa adalah substrat pilihan kerana ia telah digunakan pertama pada kadar penggunaan yang lebih cepat. Jenis gula dan nisbah antara glukosa dan xilosa tidak mempunyai kesan ke atas etanol yang dihasilkan. Purata kepekatan etanol adalah 7.99 g/L apabila menggunakan 20 g/L gula dan 27.82 g/L apabila menggunakan 80 g/L gula. Sedikit xylitol dan gliserol hadir sebagai produk sampingan apabila menggunakan 20 g/L gula. Xylitol hadir menjadi produk sampingan utama pada kepekatan xilosa tinggi dengan kepekatan tinggi sebagai xilosa ditambah. Apabila menggunakan jerami padi hidrolisat mengandungi 34.75 g/L glukosa dan 21.29 g/L xilosa, 19.37 g/L etanol telah dihasilkan dengan hasil dan produktiviti etanol masing-masing pada 0.49 g/g dan 0.20 g/L.h. Walau bagaimanapun, xilosa tidak digunakan sepenuhnya selepas penapaian lengkap.*

*Kata kunci:* Candida shehatae*; etanol; lignoselulosa; penapaian; xilosa*

## INTRODUCTION

Ethanol is an important alternative and renewable fuel which may be produced via fermentation by microorganisms. Currently, the ethanol industry relies on sugary and starchy raw materials for its production. As the world demand for ethanol has increased by 400% in the past 7 years, from 2 mtoe (million ton of oil equivalent) in 2008 to 8 mtoe in 2014 (James et al. 2010), there is now real concerns about shortage of raw materials for ethanol production. Research on raw materials used in ethanol production has been carried out with emphasis on alternative sugar plants, wastes and unused fractions from agricultural products.

Lignocellulosic material is an alternative raw material for ethanol production. Upon hydrolysis, xylose and glucose are the main sugars released from its hemicellulose and cellulose fractions. Despite the success of GM yeast and bacteria in producing ethanol from xylose, interests in using non-modified microorganisms are still evident. Xylose could be transported into yeast cells and converted to xylitol by xylose reductase and then to xylulose by xylitol dehydrogenase. Xylulose kinase then converts xylulose to xylulose 5-phosphate through the pentose phosphate and Embden-Meyerhof-Parnas pathways to produce pyruvate and then ethanol. This pathway will yield 5 g of ethanol from 3 g of xylose (McMillan 2013). *Pichia stipitis* (now *Scheffersomyces stipitis*), *Candida shehatae* (now *Scheffersomyces shehatae*) and *Pachysolen tannophilus* are the wild type strains that can utilize both glucose and xylose as substrates for ethanol fermentation. Reports on ethanol produced from xylose by these yeasts ranged from 20 to 57 g/L with ethanol yield between 0.2 and 0.46 g/g depending on the strain (Abbi et al. 1996; Farias et al. 2014).

The present study employed *C. shehatae* TISTR 5843, a xylose-fermenting yeast, for ethanol production. Two concentrations of sugars, 20 and 80 g/L (nominal values) at various glucose-to-xylose ratios were used. Rice straw hydrolysate containing mixed sugars was also used as a substrate in ethanol production. The results were compared with the fermentation using glucose and xylose.

## Materials and Methods

### Rice straw hydrolysate preparation

Rice straw hydrolysate was prepared by pre-treating 15% dry rice straw in 2% (v/v) sulfuric acid at 121ºC for 10 min. The pH of acid-treated slurry was adjusted to pH5.0 by NaOH pellets and further hydrolyzed by the enzyme Cellic®CTec2 (Novozyme, Denmark) at the rate of 10 FPU/g (rice straw). Enzyme hydrolysis was carried out at 50ºC for 24 h. Liquid fraction was then separated by filtration with filter paper and stored at 4ºC before use.

### Microorganism, inoculum preparation and fermentation

*Candida shehatae* TISTR 5843 obtained from Thailand Institute of Scientific and Technological Research was maintained at -20ºC in YM medium containing 20% (v/v) glycerol. YM medium consisting of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and xylose or glucose was used in all fermentations.

Single colonies on YM agar were used to prepare inoculums. Inoculum was propagated twice in 10 g/L followed by 20 g/L xylose at 30ºC, 200 rpm for 24 h. Fermentation was started by inoculating 10% inoculum into a 250-mL Erlenmeyer flask with 150 mL working volume. Initial sugar concentrations of 20 and 80 g/L (nominal values) with glucose to xylose ratios of 4:0, 3:1, 1:1, 1:3 and 0:4 were used in this study.

When using rice straw hydrolysate as substrate, the medium was prepared by mixing the hydrolysate with 10% of concentrated YM-medium without sugars such that the final concentrations of the medium compositions were the same as mentioned above. All fermentations were carried out at 30ºC with shaking at 100 rpm.

### Analytical methods

Biomass concentration was determined from optical density at 600 nm ($OD_{600}$) by spectrophotometer and converted to biomass concentration by calculation using dry cell weight (DCW) at the end of fermentation. Reducing sugar in rice straw hydrolysate was analysed using dinitrosalicylic colorimetric assay. Ethanol, sugars (glucose or xylose) and by-products (glycerol and xylitol) were analysed using high performance liquid chromatography (HPLC) (Shimadzu, Japan). The analysis employed Aminex HPX-87H column (Bio-Rad, USA) and Refractive Index detector (Shimadzu, RID-6A, Japan). Sample injection volume was 20 µL. The column temperature was maintained at 40°C. Five millimolar sulfuric acid was used as the mobile phase at a flow rate of 0.75 mL/min.

### Statistical analysis

Each set of experiment was carried out in three replicates and data presented as an average value with standard deviation. T-test was used in comparisons at 95% confidence level.

## Results

### The fermentation at low sugar concentration of 20 g/L

The results of ethanol fermentation when using 20 g/L total sugar concentration with different glucose to xylose ratios are summarized in Table 1. Ethanol was produced at similar concentrations and yields regardless of the glucose to xylose ratios. The average ethanol concentration and yield were 7.99 g/L and 0.42 g/g (glucose + xylose utilized).

TABLE 1. Summary of fermentation results when using 20 g/L and 80 g/L sugar concentrations as substrate

| | Sugar concentration (g/L) | Glucose : xylose ratio 4:0 | 3:1 | 1:1 | 1:3 | 0:4 |
|---|---|---|---|---|---|---|
| Ethanol | 20 | 8.31±0.54$^{a}$ | 8.32±0.83$^{a}$ | 7.47±0.68$^{a}$ | 7.42±0.81$^{a}$ | 8.95±0.59$^{a}$ |
| (g/L) | 80 | 27.50±1.42$^{a}$ | 26.89±4.24$^{a}$ | 28.96±1.26$^{a}$ | 28.38±0.64$^{a}$ | 27.39±1.56$^{a}$ |
| Xylitol | 20 | - | 0.41±0.04$^{a}$ | 0.54±0.12$^{a}$ | 0.54±0.10$^{a}$ | 0.48±0.10$^{a}$ |
| (g/L) | 80 | - | 2.65±0.41$^{d}$ | 5.23±0.80$^{c}$ | 7.17±0.56$^{b}$ | 12.18±0.58$^{a}$ |
| Glycerol | 20 | 0.74±0.04$^{a}$ | 0.67±0.05$^{ab}$ | 0.58±0.10$^{b}$ | 0.48±0.02$^{bc}$ | 0.39±0.03$^{d}$ |
| (g/L) | 80 | 3.38±0.08$^{a}$ | 2.91±0.33$^{b}$ | 2.66±0.31$^{b}$ | 2.69±0.16$^{b}$ | 2.64±0.14$^{b}$ |
| $Y_{ethanol/sugar}$ | 20 | 0.43±0.06$^{a}$ | 0.43±0.05$^{a}$ | 0.39±0.05$^{a}$ | 0.40±0.07$^{a}$ | 0.44±0.04$^{a}$ |
| (g/g) | 80 | 0.38±0.03$^{a}$ | 0.39±0.08$^{a}$ | 0.39±0.05$^{a}$ | 0.37±0.00$^{a}$ | 0.37±0.03$^{a}$ |
| $Y_{xylitol/xylose}$ | 20 | - | 0.10±0.01$^{a}$ | 0.06±0.01$^{a}$ | 0.04±0.01$^{b}$ | 0.03±0.00$^{b}$ |
| (g/g xylose) | 80 | - | 0.18±0.01$^{a}$ | 0.14±0.01$^{ab}$ | 0.12±0.02$^{b}$ | 0.16±0.01$^{a}$ |
| $Q_{ethanol}$ | 20 | 0.52±0.03$^{a}$ | 0.26±0.03$^{b}$ | 0.23±0.02$^{b}$ | 0.23±0.03$^{b}$ | 0.28±0.01$^{b}$ |
| (g/L.h) | 80 | 0.86±0.04$^{a}$ | 0.37±0.06$^{b}$ | 0.40±0.02$^{b}$ | 0.39±0.01$^{b}$ | 0.38±0.02$^{b}$ |
| $Q_{glucose}$ | 20 | 1.19±0.05$^{a}$ | 0.88±0.01$^{b}$ | 0.78±0.02$^{c}$ | 0.39±0.02$^{d}$ | - |
| (g/L.h) | 80 | 1.79±0.04$^{a}$ | 1.66±0.08$^{ab}$ | 1.56±0.13$^{b}$ | 0.78±0.02$^{c}$ | - |
| $Q_{xylose}$ | 20 | - | 0.18±0.01$^{d}$ | 0.34±0.02$^{c}$ | 0.50±0.03$^{b}$ | 0.68±0.01$^{a}$ |
| (g/L.h) | 80 | - | 0.22±0.02$^{d}$ | 0.51±0.05$^{c}$ | 0.88±0.02$^{b}$ | 1.15±0.04$^{a}$ |

1. Results were average values from three replicates. 2. Superscripted alphabets compared the data in the same row. The same alphabet indicates that the values were not significantly different at 95% confidence levels

Ethanol productivity was highest when the medium contained solely glucose. The productivities decreased by approximately 50% when introducing xylose into the medium. Glycerol was a by-product in all fermentations whereas xylitol was presented only when xylose was present. However, both by-products were produced in a small amount when compared with ethanol.

The profiles of fermentations at 20 g/L sugars are presented in Figure 1. Ethanol and by-products were produced in parallel to growth of yeast and utilization of sugars. When using glucose as a sole sugar (Figure 1(a)), glucose was completely consumed after 16 h of fermentation. Using only xylose in the medium resulted in longer fermentation time (Figure 1(e)). Xylose was completely utilized after 28 h which was 1.8 times slower than using glucose. Introducing xylose into glucose medium, even in a small amount at the glucose to xylose ratio of 3:1 (Figure 1(b)), affected the overall sugar utilization rate as xylose also took 28 h to be utilized. Increasing xylose fractions in the sugar mixture resulted in similar fermentation time (Figure 1(c)-1(d)). When both glucose and xylose were presented, glucose was a preferred sugar completely consumed after 12-15 h of fermentation. Figure 1(b) and 1(c) shows that xylose was not consumed when there was glucose presented in the medium. In Figure 1(d) where initial glucose was low (~5 g/L), xylose consumption was observed while glucose was presented but at low concentration.

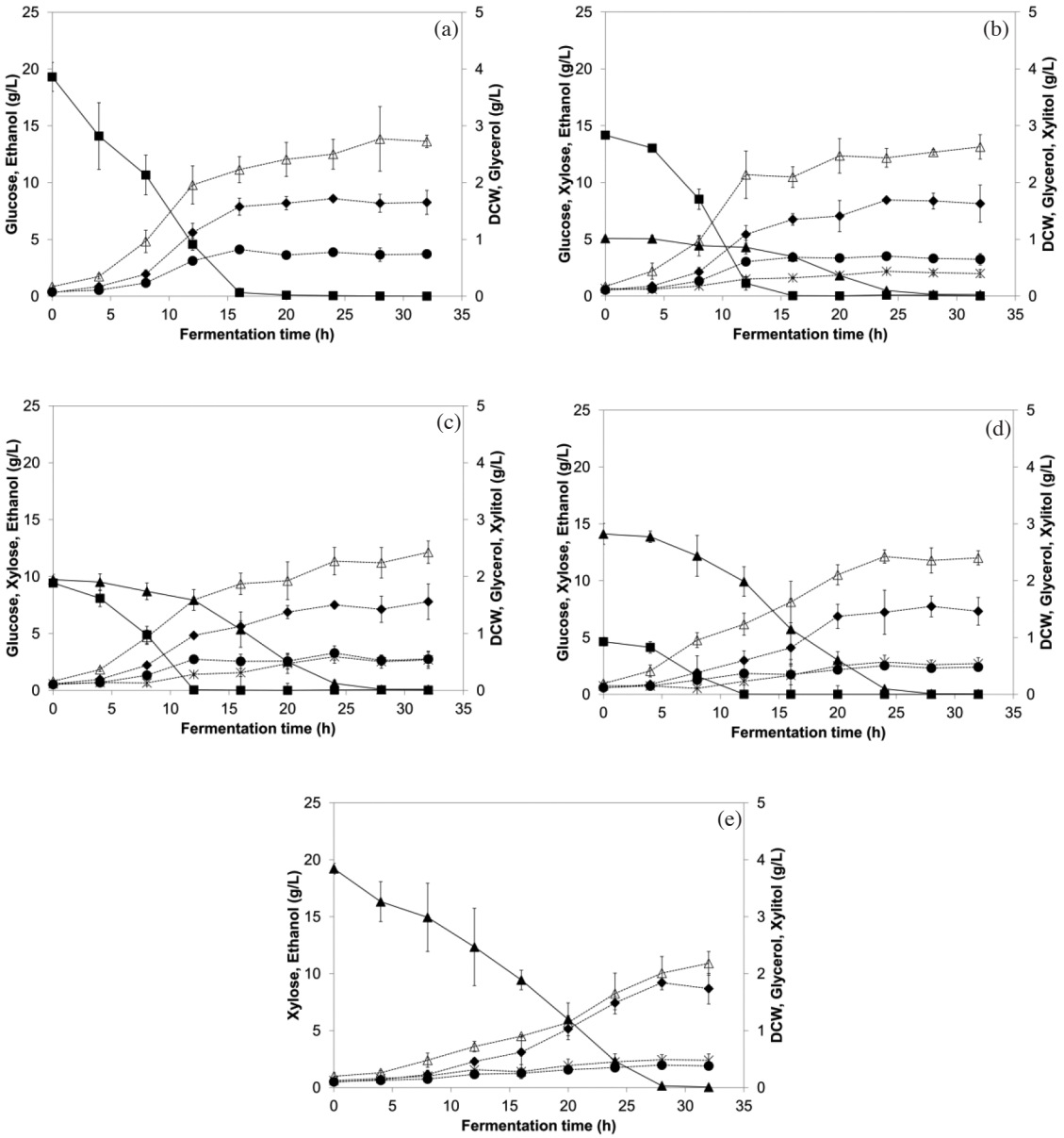


FIGURE 1. Fermentation profiles in medium with 20 g/L sugars with glucose:xylose ratios at 4:0 (a), 3:1 (b), 1:1 (c), 1:3 (d) and 0:4 (e); ▲ xylose, ■ glucose, ♦ ethanol, △ DCW, ● glycerol, ∗ xylitol

ETHANOL FERMENTATION AT HIGH SUGAR CONCENTRATION OF 80 g/L

The results of ethanol fermentation when using 80 g/L total sugar concentration at different glucose to xylose ratios are also summarized in Table 1. Higher ethanol concentrations were obtained with slight decrease in ethanol yields when compared with the fermentations at 20 g/L sugars. Insignificant differences in ethanol concentrations and yields were observed for different ratios of glucose to xylose. The average ethanol concentration and yield were 27.82 g/L and 0.38 g/g (glucose + xylose utilized). The ethanol productivities were higher than those at 20 g/L although a similar trend was observed. The presence of xylose reduced ethanol productivity by approximately 55%. As higher ethanol concentrations were obtained due to higher sugar concentration, by-products (glycerol and xylitol) were also increased. However, different by-products pattern was observed in this condition when compared with at 20 g/L sugar. At 80 g/L sugars, xylitol increased with higher xylose fraction in the medium, while glycerol was produced in a similar amount regardless of glucose to xylose ratios. On average, glycerol produced was about 10% of the ethanol present. On the other hand, xylitol produced varied from none when only glucose was in the medium to 44% of ethanol produced when xylose was the sole sugar.

The fermentation profiles when using only glucose or xylose are shown in Figure 2(a) and 2(e). Similar to fermentation at 20 g/L sugar, glucose was consumed faster than xylose. Complete consumption of glucose occurred after 32 h of fermentation, while xylose took longer at after 56 h. In the medium with mixed sugars, glucose was also

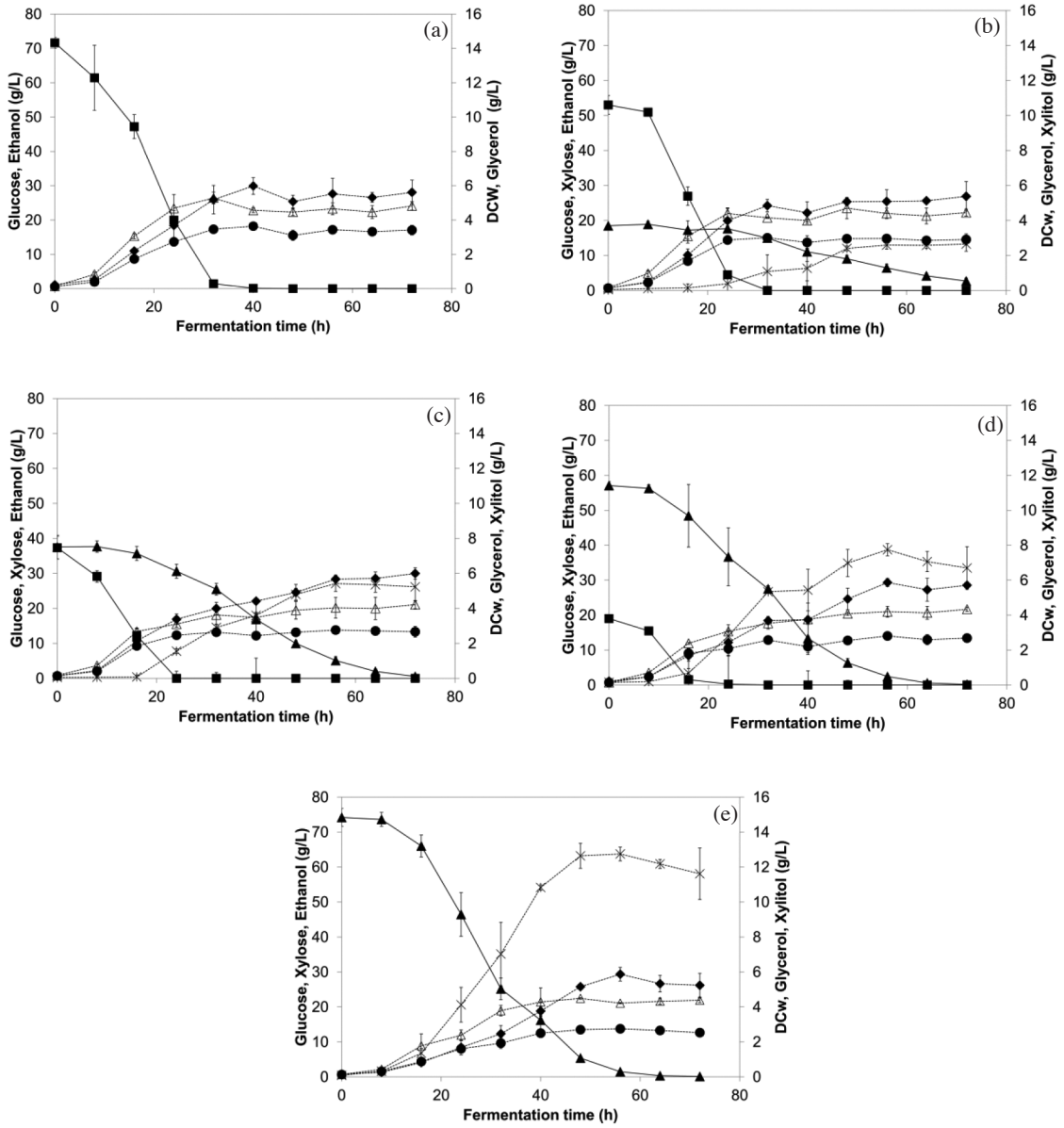


FIGURE 2. Fermentation profiles in medium with 80 g/L sugars with glucose: xylose ratios at 4:0 (a), 3:1 (b), 1:1 (c), 1:3 (d) and 0:4 (e); ▲ xylose, ■ glucose, ♦ ethanol, △ DCW, ● glycerol, ∗ xylitol

a preferred sugar for the yeast. Xylose was consumed only when glucose concentration was low, which could clearly be seen in Figure 2(b)-2(d). Unlike in the fermentations with 20 g/L sugars where xylose was completely consumed after 28 h regardless of its initial concentrations in the medium, it took longer for xylose to be utilized (64-70 h) when presented together with glucose (Figure 2(b)-2(d).

### ETHANOL FERMENTATION USING RICE STRAW HYDROLYSATE

The rice straw hydrolysate contained 83.5 g/L reducing sugars with 34.75 g/L glucose and 21.29 g/L xylose (approximate glucose to xylose ratio of 1.6:1). The fermentation profiles (Figure 3) showed incomplete utilization of reducing sugars. Glucose was used before xylose as in fermentations with mixed sugars presented earlier. However, the complete consumption occurred much later at after 72 h of fermentation or about 3 times longer than when using mixed sugars. Xylose was used after glucose was approximately 10 g/L. Its utilization rate was much slower than that in the mixed sugars experiments. Xylose was not completely consumed after fermentation was ended at 240 h. The concentration also levelled off at the end of fermentation. Maximum ethanol produced was 19.37 g/L with ethanol yield and ethanol productivity of 0.49 g/g and 0.20 g/L.h at 96 h. After 120 h, ethanol showed slightly decreasing trend while xylose was still being utilized. For by-products formation, glycerol was produced in a small amount but no xylitol was detected.

## DISCUSSION

In single sugar fermentation, *C. shehatae* TISTR 5843 had 43 and 36% faster fermentation rate in glucose than those in xylose at 20 and 80 g/L sugars. The yeast also preferred glucose over xylose in the mixed sugar fermentation. These preferences in glucose utilization are common in other pentose-fermenting yeasts. *P. stipitis* and *Pa. tannophilus* also showed faster fermentation rate when using glucose than that when using xylose (Agbogbo et al. 2006; Zhao et al. 2008). In this study, the critical level of glucose prior to xylose utilization was approximately 5 g/L or 0.5% w/v. While this critical concentration was not evident in engineered yeast strains for ethanol production, it is common for other wild strains of xylose-fermenting yeasts (Agbogbo et al. 2006; Panchal et al. 1988).

Accumulation of xylitol was significantly observed when high concentration of xylose was present in the fermentation (only at 80 g/L initial sugars). Higher xylose was reported to induce xylose reductase and xylitol dehydrogenase activities (Winkelhausen & Kuzmanova 1998). Moreover, xylitol accumulation could result from lower dissolved oxygen due to high media concentrations. With such conditions, NADH accumulates and inhibits xylitol dehydrogenase, the enzyme that converts xylitol to D-xylulose, resulting in accumulation and secretion of xylitol (Winkelhausen & Kuzamanova 1998). More concentrated media was also reported to have higher xylitol accumulation (Kastner et al. 1999a, 1999b).

Ethanol production using rice straw hydrolysate was similar or superior to that in other studies using similar substrates and native xylose-fermenting yeasts (Table 2). However, incomplete consumption of sugars was evident. The unused portion of sugars was xylose and some other types of reducing sugars (Figure 3). Glucose and xylose utilization was inconsistent with the results from fermentation using pure sugars where both sugars in hydrolysate should be fully utilized. Combined effect of accumulated ethanol and inhibitory compounds in the medium could have resulted in the incomplete consumption of xylose in hydrolysate observed in this study. Inhibitory compounds such as sulphate, furfural and acetic acid have shown to reduce sugar utilization and hence ethanol productivities (Cho et al. 2010; Huang et al. 2009). Although critical concentrations have not been reported for *C. shehatae*, 65% decrease in ethanol productivities of *P. stipitis* was evident when increasing sulphate concentration from 1.5% to 3% (Huang et al. 2009). Low concentrations

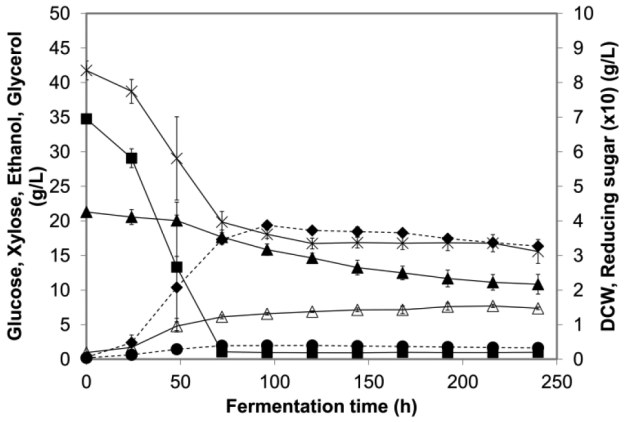


FIGURE 3. Fermentation profiles in medium with rice straw hydrolysate; ▲ xylose, ■ glucose, ♦ ethanol, △ DCW, ● glycerol, * reducing sugar

TABLE 2. The ethanol production from lignocellulosic materials by several microorganisms

| Microorganisms | Raw materials | Sugars (g/L) | | | Ethanol | | | References |
|---|---|---|---|---|---|---|---|---|
| | | Reducing | Glucose | Xylose | (g/L) | $Y_{p/s}$ (g/g) | $Q_p$ g/(Lh) | |
| *P. stipitis* BCRC 21777 | Rice straw | n.r. | 41.2 | 21.4 | 26.8 | 0.47 | 0.50 | Chen et al. 2012 |
| *P. stipitis* | Rice straw | n.r. | 5# | 18# | 11# | 0.44# | 0.23# | Lin et al. 2012 |
| *P. stipitis* KCCM12009 | Yellow Poplar | n.r. | 59.5 | 29.7 | 28.7 | 0.48 | 0.40 | Cho et al. 2010 |
| *P. stipitis* DSM 3651 | Wheat straw | n.r. | 31.82 | 13.75 | 17.37 | 0.41 | 0.10 | Toquero & Bolado 2014 |
| *C. shehatae HM 52.2* | Rice hull | n.r. | 34.1 | 12.7 | 15.77 | 0.40 | 0.16 | Hickert et al. 2013 |
| *C. shehatae* TISTR 5843 | Rice straw | 83.5 | 34.75 | 21.29 | 19.37 | 0.49 | 0.20 | This study |

Note: * genetically engineered strain; # obtained from the graph or calculation; n.r = no data reported; $Y_{p/s}$ = ethanol yield based on sugar utilized

of furfural (0.5 g/L) and acetic acid (0.7 g/L) were also reported to reduce the rate of ethanol production in *P. stipitis* (Cho et al. 2010; Huang et al. 2009).

## CONCLUSION

The ethanol fermentation on mixed sugars of glucose and xylose at various ratios at low and high sugar concentrations by *C. shehatae* TISTR 5843 were investigated. The presence of xylose in glucose medium did not affect levels of ethanol produced but lowered ethanol productivities. At high sugar concentrations, accumulation of xylitol was more pronounced than that in lower sugar concentrations with increasing xylitol at higher xylose fraction. Rice straw hydrolysate can thus be used as substrate for ethanol production by *C. shehatae* TISTR 5843. The slow fermentation rate and incomplete xylose utilization may be caused by combined effect of ethanol and inhibitors presented in the hydrolysate. As the hydrolysate contained unused sugar fraction, more studies on various approaches to reduce inhibition factors in pre-treatment steps to improve sugar utilization would be pursued. Improvement in hydrolysis of rice straw to obtain higher titre of sugar will also increase ethanol produced.

## ACKNOWLEDGEMENTS

This research works were financially supported by the National Research Council of Thailand (NRCT), the Higher Education Research Promotion and National Research University Project of Thailand through Biofuels Research Cluster of Khon Kaen University (Project Number NRU543051). Ms. Prawphan receives a financial support of her Ph.D. from Khon Kaen University (Grant Number BiF-2553-Ph.d-02).

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Prawphan Yuvadetkun
Graduate School
Khon Kaen University
Khon Kaen 40002
Thailand

Prawphan Yuvadetkun & Mallika Boonmee*
Department of Biotechnology
Faculty of Technology
Khon Kaen University
KhonKaen 40002
Thailand

Mallika Boonmee*
Fermentation Research Center for Value Added Agricultural Products
Khon Kaen University
Khon Kaen 40002
Thailand

* Corresponding author; email: mallikab@kku.ac.th

Received: 12 May 2015
Accepted : 5 November 2015