Chiang Mai J. Sci. 2019; 46(3) : 461-468
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Simultaneous Saccharification and Fermentation for Ethanol Production from Rice Straw by *Candida shehatae* and *Saccharomyces cerevisiae*

Marika Ngamsirisomsakul [a,b], Prawphan Yuvadetkun [a,d], Alissara Reungsang [a,c] and Mallika Boonmee Kongkeitkajorn* [a]

[a] Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand.

[b] Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand.

[c] Research Group for Development of Microbial Hydrogen Production Process, Khon Kaen University, Khon Kaen 40002, Thailand.

[d] Cellulosic Biomass Technology Co., Ltd., Kumphawapi, Udonthani 41110, Thailand.

*Author for correspondence; e-mail: mallikab@kku.ac.th

Received: 26 July 2018
Revised: 2 October 2018
Accepted: 4 October 2018

ABSTRACT

Ethanol production from rice straw using simultaneous saccharification and fermentation (SSF) was presented in comparison with its variation, semi-SSF (sSSF) in order to explore any potential improvement in ethanol production and conversion of cellulosic sugars. Neither significant differences in term of ethanol concentration nor improvement in overall productivity when using sSSF and SSF were observed in this study. Similar ethanol concentration of approximately 12 g/L was obtained in all cases using *Candida shehatae* ATCC 22984, *Saccharomyces cerevisiae* TISTR 5339 and co-cultivation of both strains, although lower productivity was evident when using *C. shehatae*. Furthermore, sequential fermentation of *C. shehatae* followed by *S. cerevisiae* with added cellulase had demonstrated its potential application in fermentation that fully converted xylose and glucose into ethanol.

Keywords: ethanol, lignocellulosic ethanol, rice straw, SSF, sequential fermentation

1. INTRODUCTION

Ethanol is an important chemical derived from yeast or bacterial fermentation. It is now widely used as fuel or as a fuel additive to petrol. Ethanol could be blended with petrol in various fractions; from 10% for general use and up to 85% for use in flexible-fuel vehicles. Sugar- and starch-based raw materials are common in ethanol production. However, as there are focuses on second-generation biofuels, ethanol from lignocellulosic biomass has also been in attention.

Main sugars obtained from hydrolysis of the biomass are xylose from hemicellulosic fraction, and glucose from cellulosic fraction. Utilization of the main sugars from hydrolysis has been in interests of many research groups. Approaches included the use of

both wild type and genetically engineered microorganisms [1-5]. A number of wild type yeasts have been reported for their abilities to ferment both glucose and xylose into ethanol. These yeasts include *Scheffersomyces stipitis*, *Candida shehatae*, *Candida tropicalis* and *Pachysolen tannophilus*.

Fermentation strategies usually employed in ethanol production have been separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). SSF has an advantage of combining saccharification and fermentation step, hence total process time was reduced. Recently, a variation of SSF has been introduced. The strategy involves enzymatic hydrolysis step in the fermentation vessel prior to inoculation. This additional step helps to overcome the SSF drawback that hydrolysis temperature is higher than fermentation temperature. It was also designed to help reducing the broth viscosity in SSF with high substrate loading which caused mixing difficulty [6]. This fermentation strategy has been referred to in various names including semi-simultaneous saccharification and fermentation (sSSF or SSSF), prehydrolysis and simultaneous saccharication and fermentation (PSSF) and same vessel saccharication and fermentation (SVSF) [7-9].

This research aimed to assess and compare ethanol production from rice straw by using 2 types of yeasts; a common yeast (*S. cerevisiae*) and a xylose-fermenting yeast (*C. shehatae*). SSF strategy was employed with a variation of semi-simultaneous saccharification and fermentation (sSSF). Comparisons between SSF and sSSF strategies were made to demonstrate if the prehydrolysis step would help improving the ethanol production by improving the mixing, hence better mass transfer, at a specified solid loading in this study. The results from this study would provide an assessment of using rice straw as an alternative lignocellulosic raw material in ethanol production. Furthermore, characteristics of the fermentation would also provide valuable information for further developments of lignocellulosic ethanol production

2. MATERIALS AND METHODS

2.1 Preparation of Rice Straw and Fermentation Medium

Rice straw was collected from Demonstration Farm of Faculty of Agriculture, Khon Kaen University, Thailand. It was cut and sieved through 10-mesh screen before being dried at 60 °C. Prior to fermentation, 20 g of dried rice straw was pretreated with 200 mL of 2% sulfuric acid at 10% substrate loading. They were heated at 121 °C for 10 min using an autoclave. After cooling, the slurry had its pH adjusted to pH 5.0 with sodium hydroxide pellets. After sterilising at 121 °C for another 15 min, autolyzed yeast powder (FM801, Angel Yeast, China) was added to the whole slurry at 5 g/L dosage, with or after enzyme addition. This mixture was used as the medium (designated as whole slurry medium) in fermentation step.

2.2 Culture Inoculum Preparation

Candida shehatae ATCC 22984 and *Saccharomyces cerevisiae* TISTR 5339 were used for ethanol production. Inocula of both yeasts were prepared in YMG medium which consisted of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose. Seed cultures were incubated at 30 °C with 200 rpm shaking for 24 h. Ten percent of the first seed was transferred to a fresh medium with 20 g/L glucose and incubated at the same condition for another

24 h. The cells would be used as an inoculum.

2.3 Semi-simultaneous Saccharification and Fermentation (sSSF)

Both yeasts were either cultivated separately or co-cultivated. Prior to inoculation, 10 FPU/g (rice straw) of Cellic® CTec2 (Novozyme, Denmark) enzyme was added into 200 mL of the whole slurry medium (section 2.1). The mixture was incubated at 50 °C in water bath for 24 h. The pre-hydrolyzed medium was inoculated with 10 % v/v of *S. cerevisiae*, *C. shehatae* or 5% v/v of double-concentrated inocula of both yeasts. All cultures were maintained at 30 °C in an incubator, shaking at 100 rpm.

2.4 Simultaneous Saccharification and Fermentation (SSF)

In SSF, the enzyme was added together with an inoculum at the beginning of fermentation. Inoculations in single strain cultivations and co-cultivation of both yeasts were the same as that carried out in sSSF. A sequential cultivation of 2 yeasts was also performed in SSF where 5% v/v of double-concentrated *C. shehatae* was firstly added into whole slurry medium without enzyme addition. After 48 h, another 5% v/v of double-concentrated *S. cerevisiae* was added with the same dosage of enzymes as in other cultivations. All cultures were also maintained at 30 °C, 100 rpm.

2.5 Analytical Methods

Number of cells was determined by cell count and the value reported as CFU/mL. High performance liquid chromatography (Shimadzu, Japan) was used for analysis of glucose, xylose and ethanol. HPLC was equipped with Aminex HPX-87H column (Bio-Rad, USA) and Refractive Index detector (Shimadzu, RID-6A, Japan). Five millimolars sulfuric acid was used as the mobile phase at a flow rate of 0.75 mL/min.

Fermentations were carried out in duplicates. Results on ethanol concentration were presented as mean values and standard deviations. Mean comparisons were carried out using 2-tail Student's t-test at 95% confidence level.

3. RESULTS AND DISCUSSIONS

3.1 Single Strain Fermentations

In cultivation of *C. shehatae*, similar patterns of sugars usage and ethanol production were observed for both sSSF and SSF (Figure 1A). During the first 24 h (the prehydrolysis period), sSSF showed higher glucose accumulation due to the action of added cellulase without sugars being used to form ethanol since the inoculum had not been added. Lower glucose accumulation was evident in SSF as *C. shehatae* used the sugar to produce ethanol from the beginning of the fermentation. Maximum ethanol concentration was resulted after 72 h of the operation, with 12.2 ± 0.5 g/L ethanol from sSSF and 11.6 ± 0.5 g/L ethanol from SSF. Although xylose continued to drop from this point but there was no further ethanol formation and no xylitol was detected. Results from cell count had indicated that xylose was used for cell growth after ethanol reached its maximum as the cell count of *C. shehatae* continuously increased after 72 h with decreasing xylose concentration (Figure 2).

Ethanol productions in sSSF and SSF by *S. cerevisiae* were not significantly different to those produced by *C. shehatae*. Ethanol of 12.0 ± 0.6 g/L by sSSF and 11.8 ± 0.2 g/L by SSF were obtained but with much faster fermentation time. Maximum ethanol was reached after 48 h of operation in both cases with most of the ethanol (~95% of total ethanol produced) being produced during the first 24 h of fermentation in

SSF (Figure 1B). Although maximum ethanol in sSSF was obtained after 48 h, the time taken for maximum production was 24 h after inoculation. Therefore, there was no obvious advantage in using sSSF over SSF in this study as the overall operation time was the same. In SSF by *S. cerevisiae*, glucose accumulation was very low throughout fermentation as compared with that of *C. shehatae*, which indicated a more efficient utilization of glucose by *S. cerevisiae*.

During fermentations by *S. cerevisiae*, decrease in xylose was observed and xylitol was detected with approximately 6 g/L xylitol at the end of fermentation. Although *S. cerevisiae* does not utilize xylose, its hexose-uptake system (including uptake of glucose) could provide uptake of xylose [10] resulting in decreasing trend in xylose during fermentation. *S. cerevisiae* was also reported to have the genetic prerequisites for xylose metabolism but it is repressed by glucose [11].

Although there was insignificant difference in ethanol concentration when producing using sSSF and SSF in this study, a significant change in physical appearance of the whole slurry medium was evident. When pre-hydrolyzed the medium with enzyme, the medium became less viscous and the content was easier to mix by magnetic stirrer without any improvement in ethanol production [9, 12-13]. The results implied that sSSF could be beneficial for the fermentation with very high solid loading because reduced viscosity would allow for more addition of biomass, hence substrate for the fermentation. They also suggested that the solid loading of 10% used in this study was considerably too low to display any advantages of the pre-hydrolysis step.

3.2 Co-cultivation of *S. cerevisiae* and *C. shehatae*

Co-cultivation of *S. cerevisiae* and *C. shehatae* was carried out on a basis that *S. cerevisiae* is a superior strain in fermenting glucose into ethanol and *C. shehatae* has an ability to ferment xylose to ethanol. The fermentation results in Figure 1C showed profiles that almost resembled the *S. cerevisiae* fermentations with rapid glucose use within 24 h of inoculations in both sSSF and SSF. Similar ethanol concentrations were also produced, which were 12.0 ± 0.4 g/L by sSSF and 11.9 ± 0.3 g/L by SSF.

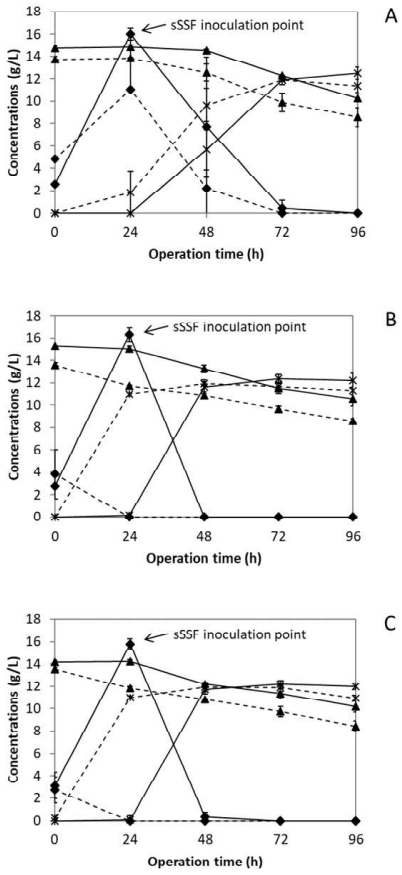

Figure 1. Ethanol and sugars profiles during sSSF (line) and SSF (broken line) of (A) *C. shehatae*, (B) *S. cerevisiae* and (C) co-cultivation of both strains in whole slurry of 10% rice straw; ◆ = glucose, ▲ = xylose and * = ethanol.

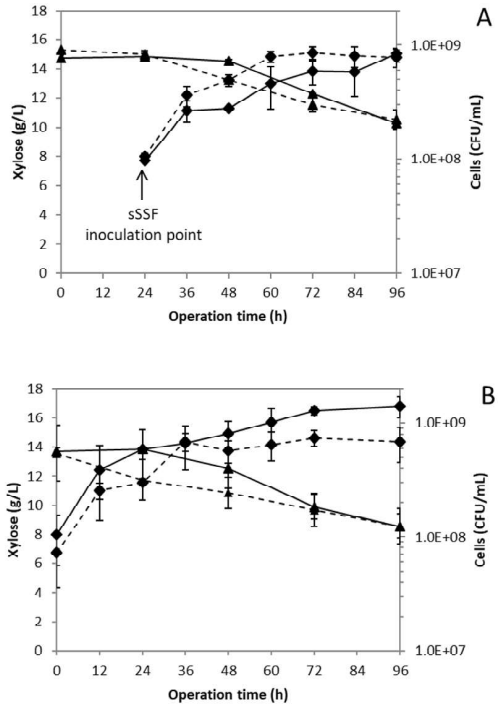

Figure 2. Growth of *C. shehatae* (line) and *S. cerevisiae* (broken line) in relation to xylose consumption during (A) sSSF and (B) SSF of the yeasts in whole slurry of 10% rice straw; ◆ = cell number and ▲ = xylose.

Co-cultivation of *S. cerevisiae* and *C. shehatae* did not provide an expected result that higher ethanol would be produced, as both xylose and glucose would be utilized. In co-cultivation, glucose was quickly taken up by *S. cerevisiae* as soon as it was released by the cellulase. *C. shehatae* was expected to utilize xylose when glucose concentration in the medium was at a low level as evident in previous study where xylose was reported as not being utilized until glucose concentration in the medium became lower than 5 g/L [14]. No further use of xylose as occurred in this study could be caused by the presence of sulphate ions from the acid used in pretreatment step. Presence of ions could increase osmotic stress on the cells and inhibit xylose consumption especially when xylose is a sole carbon source [1]. In a culture using mixed glucose and xylose by genetically modified *S. cerevisiae*, specific xylose consumption was reduced by approximately 75% with the presence of 0.5 M sodium sulphate [1]. In contrast, xylose consumption rate by *S. stipitis* was not different with or without addition of sodium sulphate when xylose was a sole carbon source [15]. However, sodium sulphate in that particular study was only 0.025 M. Higher sulphate supplementation (3%) was demonstrated to reduce xylose utilization rate [16]. Although there was no direct explanation on xylose utilization of xylose-fermenting yeasts in mixed sugars environment with the presence of sulphate, effect of ethanol concentration on xylose utilization ability of xylose-fermenting yeast could be greater with the presence of sulphate in the fermentation medium.

3.3 Fermentation with Sequential Inoculation of *C. shehatae* Followed by *S. cerevisiae*

As both single strains and co-cultivation of the yeasts did not yield the expected results that both glucose and xylose were utilized, attempts on using sequential inoculation technique were made. Firstly, *C. shehatae* was inoculated into whole slurry medium that contained mainly xylose to allow for its ability to convert xylose into ethanol. After 24 h, *S. cerevisiae* was inoculated together with cellulase addition. As the enzyme released glucose, it was immediately taken up by *S. cerevisiae* and converted into ethanol. This method employed advantages of *C. shehatae* as being a xylose-fermenting yeast and also of *S. cerevisiae* with its superior performances in both sSSF and SSF as shown in results of previous sections. A preliminary test on fermentation of dilute acid hydrolysate from 10% rice straw

by *C. shehatae* had shown that approximately 6.5 g/L ethanol could be produced from that fraction, which contained approximately 15 g/L xylose and 3 g/L glucose.

The profiles in Figure 3 demonstrated that when using 10% rice straw to prepare whole slurry medium, xylose was not utilized as expected. This was mainly due to a small liquid fraction presented when *C. shehatae* was inoculated. This appearance was different to SSF of single cultures and co-cultures where the mixture gradually appeared more fluid during the first 24 h. At 10% rice straw, ethanol started to produce after 24 h when enzyme was added with *S. cerevisiae*. Maximum ethanol was reached after 72 h and the concentration was 10.5 ± 0.3 g/L which was significantly lower than previous fermentations with single strains and co-cultivation (Table 1). The lower ethanol concentration could be the result of inefficient sugar usage during the first 24 h of fermentation. As the profiles in Figure 3 suggested, glucose in the medium was slightly utilized by *C. shehatae* but no ethanol was being produced. This reduction in glucose without ethanol formation could contribute to the small but significantly difference in final ethanol concentration.

In order to prove the concept of sequential fermentation, rice straw loading was decreased to 5%. By using lesser rice straw, the fermentation mixture had more fluidity. Xylose was utilized and ethanol was produced from the beginning of the fermentation as more fluid was present and facilitated the fermentation process (Figure 3). Xylose was fully utilized in 48 h and maximum ethanol of 8.1 ± 0.1 g/L was obtained after 72 h or 48 h after *S. cerevisiae* inoculation.

Summary of all fermentation results in Table 1 showed that there was no significant difference in ethanol produced when using SSF and sSSF, with an average ethanol produced of 12 g/L. Slightly lower ethanol concentration was observed in sequential fermentation using the same amount of substrate due to the reason of insufficient liquid fraction to facilitate the fermentation as explained earlier. Significant increase in ethanol yield ($g/g_{rice\ straw}$) was observed when decreasing the substrate loading by half in sequential fermentation. This increasing yield could be due to ethanol that was produced from xylose by *C. shehatae* in the first 24 h of fermentation.

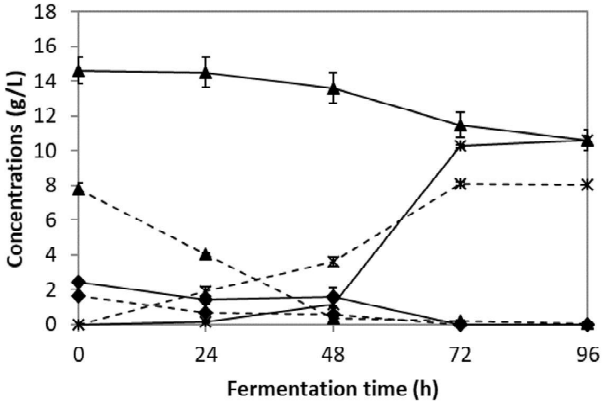

Figure 3. Ethanol and sugars profiles during sequential SSF of *C. shehatae* and *S. cerevisiae* (inoculated 24 h following *C. shehatae* inoculation) using whole slurries of 10% (line) and 5% (broken line) rice straw as substrates; ◆ = glucose, ▲ = xylose and * = ethanol.

Table 1. Ethanol produced from rice straw by SSF of *C. shehatae* ATCC 22984 and *S. cerevisiae* TISTR 5339 using various inoculation techniques. All experiments used 10% rice straw as substrate unless otherwise stated.

Inoculation techniques	Ethanol from SSF			Ethanol from sSSF		
	(g/L)	($g/g_{rice\ straw}$)	(g/L.h)	(g/L)	($g/g_{rice\ straw}$)	(g/L.h)
C. shehatae ATCC 22984	11.6 ± 0.5^{aA}	0.094	0.156	12.2 ± 0.5^{aA}	0.098	0.168
S. cerevisiae TISTR 5339	11.8 ± 0.2^{aA}	0.095	0.242	12.0 ± 0.6^{aA}	0.097	0.166
Co-cultivation	$11.9 \pm 0.3^{aA.}$	0.096	0.242	$12.0 \pm 0.4^{aA.}$	0.096	0.165
Sequential fermentation	10.5 ± 0.5^{B}	0.084	0.143	-	-	-
Sequential fermentation (5%)	8.1 ± 0.1	0.143	0.137	-	-	-

Note: 1) Reported values were average values of 2 replicates.

2) Capital letters compared concentration data in the same column. Small letters compared concentration data in the same row.

4. CONCLUSIONS

Simultaneous saccharification and fermentation (SSF) had been successfully applied in fermentation using the whole broth prepared from dilute acid hydrolysis of rice straw. Prehydrolysis of rice straw in sSSF did not provide any obvious advantages over SSF in this study. In order to achieve fermentation with total sugar utilization, sequential fermentation of *C. shehatae* and *S. cerevisiae* using the whole broth (slurry) showed a good potential for the purpose. Suitable percent loading of substrate that provides sufficient fluidity of the slurry was a key success factor in this case.

ACKNOWLEDGEMENTS

This research work was 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 NRU543030). Additional supports from TRF Senior Research Scholar (Grant No. RTA590008) was also appreciated.

REFERENCES

[1] Casey E., Mosier N.S., Adamec J., Stockdale Z., Ho N. and Sedlak M., *Biotechnol. Biofuels*, 2013; **6**: 83. DOI 10.1186/1754-6834-6-83.

[2] Farias D., de Andrade R.R. and Maugeri-Filho F., *Appl. Biochem. Biotechnol.*, 2014; **172**: 361-379. DOI 10.1007/s12010-013-0546-y.

[3] Oberoi H.S., Vadlani P.V., Brijwani K., Bhargav V.K. and Patil R.T., *Process Biochem.*, 2010; **45**: 1299-1306. DOI 10.1016/j.procbio.2010.04.017.

[4] Sun W.L. and Tao W.Y., *Afr. J. Microbiol. Res.*, 2010; **4**: 1105-1109.

[5] Zhao L., Zhang X. and Tan T., *Biomass Bioenerg.*, 2008; **32**: 1156-1161. DOI 10.1016/j.biombioe.2008.02.011.

[6] Ohgren K., Vehmaanpera J., Siika-Aho M., Galbe M., Viikari L. and Zacchi G., *Enz. Microb. Technol.*, 2007; **40**: 607-13. DOI 10.1016/j.enzmictec.2006.05.014.

[7] Karagoz P. and Ozkan M., *Bioresour. Technol.*, 2014; **158**: 286-293. DOI 10.1016/j.biortech.2014.02.022.

[8] Shen J. and Agblevor F.A., *Bioproc. Biosyst. Eng.*, 2011; **34**: 33-43. DOI 10.1007/s00449-010-0444-4.

[9] Lopez-Linares J.C., Romero I., Cara C., Ruiz E., Moya M. and Castro E., *Fuel*, 2014; **122**: 112-118. DOI 10.1016/j.fuel.2014.01.024.

[10] Hamacher T., Becker J., Gardonyi M., Hahn-Hagerdal B. and Boles E., *Microbiology*, 2002; **148**: 2783-2788. DOI 10.1099/00221287-148-9-2783.

[11] Richard P., Toivari M.H. and Penttila M., *FEBS Lett.*, 1999; **457**: 135-138. DOI 10.1016/S0014-5793(99)01016-9.

[12] Goncalves F.A., Ruiz H.A., Nogueira C.D.C., dos Santos E.S., Teixeira J.A. and de Macedo G.R., *Fuel*, 2014; **131**: 66-76. DOI 10.1016/j.fuel.2014.04.021.

[13] Larsen J., Peterson M.O., Thirup L., Li H.W. and Iversen F.K., *Chem. Eng. Technol.*, 2008; **31**: 765-772. DOI 10.1002/ceat.200800048.

[14] Yuvadetkun P. and Boonmee M., *Sains Malays.*, 2016; **45**: 581-587.

[15] Agbogbo F.K. and Wenger K.S., *Biotechnol. Lett.*, 2006; **28**: 2065-2069. DOI 10.1007/s10529-006-9192-6.

[16] Huang C.F., Lin T.H., Guo G.L. and Hwang W.S., *Bioresour. Technol.*, 2009; **100**: 3914-3920. DOI 10.1016/j.biortech.2009.02.064.