Korean Society for Biotechnology and Bioengineering Journal 30(3): 125-131 (2015)
http://dx.doi.org/10.7841/ksbbj.2015.30.3.125 ISSN 1225-7117 / eISSN 2288-8268

연구논문

The Application of Thermotolerant Yeast *Kluyveromyces marxianus* as a Potential Industrial Workhorse for Biofuel Production

Jae-Bum Park[1], Jin-Seong Kim[1], Seung-Won Jang[1], Eunsoo Hong[2], and Suk-Jin Ha[1]*

Received: June 2015 / Accepted: 23 June 2015

Abstract: *Kluyveromyces marxianus* is a well-known thermotolerant yeast. Although *Saccharomyces cerevisiae* is the most commonly used yeast species for ethanol production, the thermotolerant *K. marxianus* is more suitable for simultaneous saccharification and fermentation (SSF) processes. This is because enzymatic saccharification usually requires a higher temperature than that needed for the optimum growth of *S. cerevisiae*. In this study, we compared the fermentation patterns of *S. cerevisiae* and *K. marxianus* under various temperatures of fermentation. The results show that at a fermentation temperature of 45°C, *K. marxianus* exhibited more than two fold higher growth rate and ethanol production rate in comparison to *S. cerevisiae*. For SSF using starch or corn stover as the sole carbon source by *K. marxianus*, the high temperature (45°C) fermentations showed higher enzymatic activities and ethanol production compared to SSF at 30°C. These results demonstrate the potential of the thermotolerant yeast *K. marxianus* for SSF in the industrial production of biofuels.

Keywords: *Kluyveromyces marxianus*, Thermotolerant, Simultaneous saccharification and fermentation, Biofuel

1. INTRODUCTION

Due to increasing environmental concerns and need for energy security, biofuel production has become an attractive alternative to the finite fossil fuels [1-3]. The production of biofuels from non-edible cellulosic biomass is a cost effective way to compete with conventional gasoline production, which has been optimized for over a century [4]. In contrast to starch hydrolyzed into only hexose sugars, cellulosic biomass consists of hexose and pentose, which indicates that both sugars need to be efficiently utilized for the commercial production of cellulosic biofuels. *Saccharomyces cerevisiae* has been widely used in the production of cellulosic biofuels due to an efficient glucose fermentation capability. Moreover, *S. cerevisiae* has been engineered for the efficient utilization of xylose, which is the most abundant pentose sugar from cellulosic biomass [5-7]. The utilization of hexose and pentose sugars has been developed through several well-defined strategies, including cell surface display and cellobiose/xylose co-fermentation [8, 9]. In addition to laboratorial *S. cerevisiae* strains, an industrial *S. cerevisiae* strain has been engineered for cellulosic biofuel production [10-13].

Cellulosic biomass needs to be hydrolyzed into monomeric sugars before ethanol fermentation. The hydrolysis of cellulosic biomass requires pretreatment with either an acidic or alkali catalyst, followed by enzymatic hydrolysis usually with cellulases and cellobiases [14, 15]. One of the major problems to arise during separate hydrolysis and fermentation (SHF), is feedback inhibition from the production of monomeric sugars. For commercial production of cellulosic biofuels, simultaneous saccharification and fermentation (SSF) are required, as well as the efficient utilization of both hexose and pentose sugars [16]. The overall rate limiting step for SSF is usually the enzymatic hydrolysis step, which generally have high optimum temperatures [17]. Therefore, SSF is carried out under low temperatures by genetically engineered *S. cerevisiae* because the high optimal fermentation temperatures over 35°C are unsuitable for the growth of the non-thermotolerant *S. cerevisiae*.

However, the recent use of the thermotolerant yeast, *Kluy-*

[1]Department of Bioengineering and Technology, Kangwon National University, Chuncheon 200-701, Korea
Tel: +82-33-250-6277, Fax: +82-33-243-6350
e-mail: sjha@kangwon.ac.kr

[2]Department of Molecular Science and Technology, Graduate School of Interdisciplinary Program, Ajou University, Suwon 443-749, Korea

veromyces marxianus, for the production of biofuels and commercially valuable chemicals from renewable resources provides an alternative to *S. cerevisiae* [18-20]. The thermotolerant *K. marxianus* has several advantages over the mesophilic yeast *S. cerevisiae* in a number of practical aspects, for example; reduced cooling costs, improved enzymatic hydrolysis for SSF, a high growth rate, the ability to utilize various substrates and low contamination levels [21, 22]. The genome of *K. marxianus* KCTC17555 has been completely sequenced, allowing for the development of several genetic engineering tools, which have greatly facilitated the metabolic engineering of *K. marxianus* [23-25].

In this study, we have verified the thermotolerance of *K. marxianus* strains, using spotting growth assays and flask fermentation. Due to the advantages of thermotolerant growth, *K. marxianus* strain exhibited higher fermentation rates in SSF when compared to *S. cerevisiae* grown at high fermentation temperatures. High temperature fermentation is a significant benefit of the *K. marxianus* strains, which can be exploited for the production of biofuels and commercially valuable chemicals from cellulosic biomass.

2. MATERIALS AND METHODS

2.1. Strains and culture conditions

The strains used in this study included three *K. marxianus* strains (CBS1555, CBS712, and ATCC36907), four *S. cerevisiae* laboratory strains (CEN.PK 2-1D, L2612, BY4742, and D452-2) and one *S. cerevisiae* industrial strain (JAY270). To prepare inoculums for fermentation, yeast cultures were routinely grown in YP medium (10 g/L yeast extract, 20 g/L Bacto peptone), containing 20 g/L of glucose. Cultures were shaken at 200 rpm and incubated at 30°C. Cells were then harvested at mid-exponential phase, washed twice with sterilized water and used for inoculations.

2.2. Fermentation experiments

Fermentation experiments were performed using 50 mL of YP medium, containing 80 g/L of glucose in 250 mL, with an initial optical density at 600 nm (OD_{600}) of 1 under oxygen-limited conditions. For SSF with starch and glucoamylase, 80 g/L of starch was added as a substrate and 1200 U of amyloglucosidase was added. For fermentation with corn stover hydrolyzate, the enzyme treated corn stover hydrolyzate was used as the sole medium. For SSF with pretreated corn stover, 310 U of cellulase, and 70 U of cellobiase were added to pretreated corn stover. All of the flask fermentation experiments were repeated three times independently.

2.3. Spotting growth assay

Yeast cells were grown to saturation (~ 2×10^8 cells/ml) at 30°C with shaking at 200 rpm. Ten-fold serial dilutions were prepared for each strain, ranging from 10^7 cells/mL to 10^3 cells/mL. A 5 μL volume of each dilution was spotted onto YPD agar plates, containing 20 g/L of glucose. The plates were then incubated at either 30°C or 45°C for 2 days.

2.4. Preparation of corn stover hydrolyzate

The corn stover hydrolyzate was prepared by the following procedure;: 1) The corn stover was washed with tap water, chopped (20-30 cm strips), air-dried and milled to particle size smaller than 1 mm. 2) Corn stover particles were then immersed in 1% NaOH (w/v), at a solid-liquid ratio of 1:10 and at 50°C for 48 h. The corn stover particles were then washed with water and adjusted to pH 5.0 using 50 mM citrate buffer (pH 4.8). 3) The pretreated corn stover particles were dried in an oven at 60°C until they reached constant weight. 4) The pretreated corn stover particles were then soaked in 50 mM citrate buffer (pH 4.8), with a solid content of 10% (w/v). The diluted cellulase (Celluclast Conc BG., Daejung, Seoul, Korea), and cellobiase (Novozyme 188, Sigma) enzymes were added to a final concentration of 310 endo-glucanase unit (EGU)/g-dry substrate and 70 cellobiase units (CBU)/ g-dry substrate, respectively. Enzyme hydrolysis was performed at 50°C and 180 rpm for 48 h.

2.5. Enzymatic assay of amyloglucosidase

The enzymatic activity of amyloglucosidase from *Aspergillus niger* (Sigma), was assayed using a reaction mixture containing 0.5 mL of 5% soluble starch solution prepared in sodium acetate buffer (pH 4.0) with 0.5 mL of appropriately diluted amyloglucosidase. The enzyme substrate mixture was incubated at various temperatures, from 30°C to 85°C for 5 min. The reducing sugars liberated were determined using HPLC analysis. All experiments were carried out in triplicate and the mean data are presented.

2.6. Analytical methods

Cell growth was monitored by optical density (OD) at 600 nm using a UV-visible spectrophotometer (Biomate 5, Thermo, Rochester, NY). Glucose, xylose, xylitol, glycerol, acetate and ethanol concentrations were determined by high performance liquid chromatography (HPLC 1200 Series, Agilent Technologies, Santa Clara, CA), equipped with a refractive index detector, using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50°C.

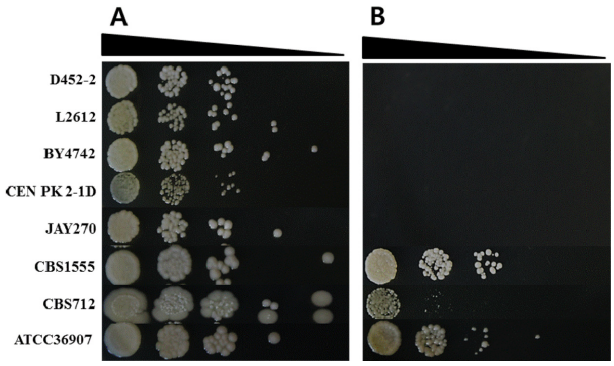

Fig. 1. Spotting growth assays of the four *S. cerevisiae* laboratory strains (CEN-PK 2-1D, L2612, BY4742, and D452-2), the *S. cerevisiae* industrial strain (JAY270), and three *K. marxianus* (CBS1555, CBS712, and ATCC36907) strains on YPD agar plate containing 20 g/L of glucose at 30°C(A) or 45°C (B). Yeast cells were five-fold serially diluted (from left to right) and spotted onto plates.

3. RESULTS AND DISCUSSION

3.1. Comparison of *K. marxianus* and *S. cerevisiae* growth rates at 30°C and 45°C

In comparison to *S. cerevisiae*, *K. marxianus* is a thermotolerant yeast. To verify the thermotolerant growth characteristics of *K. marxianus* strains, spotting growth assays were performed at 30°C or 45°C with the four *S. cerevisiae* laboratory strains (CEN.PK 2-1D, L2612, BY4742, and D452-2), the *S. cerevisiae* industrial strain (JAY270), and the three *K. marxianus* strains (CBS1555, CBS712, and ATCC36907). As expected, there were no significant differences in the growth rates when grown at 30°C (Fig. 1A). However, at 45°C the growth of the two *K. marxianus* strains (CBS1555 and ATCC36907) were comparable to their growth at 30°C, with the exception of the *K. marxianus* strain CBS712, which grew poorly at 45°C. The five *S. cerevisiae* strains did not grow at 45°C (Fig. 1B).

For flask fermentations carried out in YPD80 medium (YPD medium supplemented with 80 g/L of glucose) at 30°C, the three *K. marxianus* strains consumed all the glucose within 12 h, producing around ~32-33 g/L of ethanol, which corresponded to ~0.40-0.41 g/g ethanol yield (Fig. 2A). In the case of the five *S. cerevisiae* strains, only the industrial *S. cerevisiae* JAY270 consumed all glucose within 12 h, producing 34 g/L of ethanol. The four *S. cerevisiae* laboratory strains did not consume all the glucose within the 12 h period, producing slightly less ethanol (~22-30 g/L) compared to the industrial and thermotolerant yeast strains. The *S. cerevisiae* CEN.PK 2-1D showed the lowest ethanol production (22 g/L) together with the lowest glucose consumption rate. Interestingly, the ethanol yields for the five *S. cerevisiae* strains (0.43~0.45 g/g) were higher than those from three *K. marxianus* strains (0.41~0.43 g/g) at 30ºC. However, at the fermentation temperature of 45°C (Fig. 2B), the five *S. cerevisiae* strains demonstrated a much reduced rate of glucose consumption and ethanol production. Only the *S. cerevisiae* L2612 and JAY270 showed relatively high production of ethanol among the five *S. cerevisiae* strains at 45ºC. However, the three *K. marxianus* strains showed almost comparable ethanol production at 45°C when compared to ethanol production at 30°C (Fig. 2). The spotting growth assays and high temperature flask fermentation results demonstrate that that the three *K. marxianus* strains maintain fermentation capabilities up to 45°C in contrast to the *S. cerevisiae* strains. When the fermentation temperature was increased to 50°C, fermentation rates of the three *K. marxianus* strains were significantly reduced (data not shown). The thermotolerant advantage of *K. marxianus* strains at 45°C can potentially be applied to SSF, as hydrolytic enzymatic activities (amylases

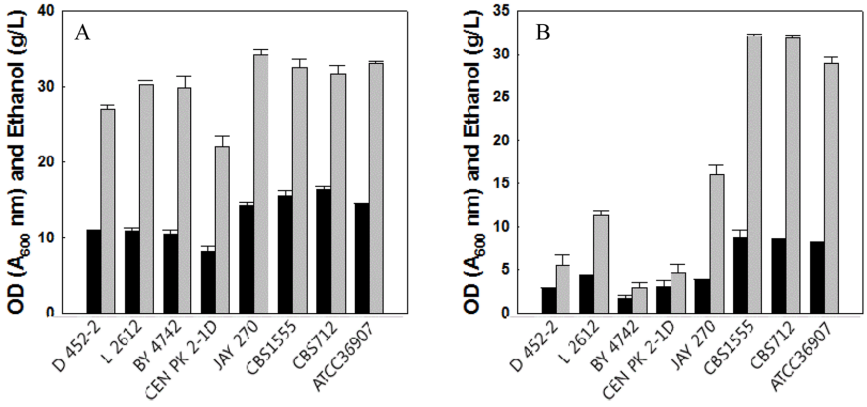

Fig. 2. Fermentation results of the four *S. cerevisiae* laboratory strains (CEN-PK 2-1D, L2612, BY4742, and D452-2), the *S. cerevisiae* industrial strain (JAY270), and the three *K. marxianus* strains (CBS1555, CBS712, and ATCC36907) on YPD medium containing 80 g/L of glucose at 30°C (A), or 45°C (B) for 12 h. Symbols: OD (black bar) and ethanol (gray bar).

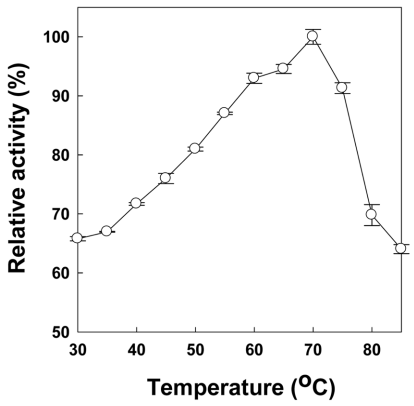

Fig. 3. Relative enzymatic activities of the amyloglucosidase from *Aspergillus niger* at various reaction temperatures between 30°C and 90°C.

or cellulases) are enhanced at higher temperatures.

3.2. Improvement of the *K. marxianus* SSF fermentation rate with high temperature

Simple SSFs were prepared using starch as a substrate and amyloglucosidase from *Aspergillus niger* as the hydrolysis enzyme. The *K. marxianus* strain CBS1555 and the two *S. cerevisiae* strains (L2612 and JAY270) were chosen for comparisons as these strains exhibited the highest fermentation rates at 45°C (Fig. 2B). Before performing the simple SSF, the enzymatic activity of amyloglucosidase at various reaction temperatures was established (Fig. 3). As the reaction temperature increased from 30°C to 70°C, amyloglucosidase activity increased, however, the amyloglucosidase activity was significantly inhibited at temperature above 70°C. A comparison of amyloglucosidase activity at 30°C and 45°C, showed that activities were increased from 65% to 76%. These results suggested that the fermentation rate of SSF at 45°C might be improved by at least 17% compared to fermentation arte carried out at 30°C.

When SSFs using starch and amyloglucosidase were performed at 30°C with *K. marxianus* CBS1555 and the *S. cerevisiae* strains L2612 and JAY270 (Fig. 4), all the strains showed similar starch degrading rates as the same amount of amyloglucosidase was added. However, *S. cerevisiae* L2612 had a reduction in the rate of glucose consumption and ethanol production (Fig 2B). At 45°C, the starch degrading rates were enhanced compared those from 30°C, due to an increase in enzymatic activity of amyloglucosidase. As expected, *S. cerevisiae* L2612 was not able to consume glucose with 68 g/L of glucose remaining after 12 h because *S. cerevisiae* L2612 is very sensitive to high fermentation temperature. In the case of *S. cerevisiae* JAY270, 20 g/L of glucose remained after 8 h, with no

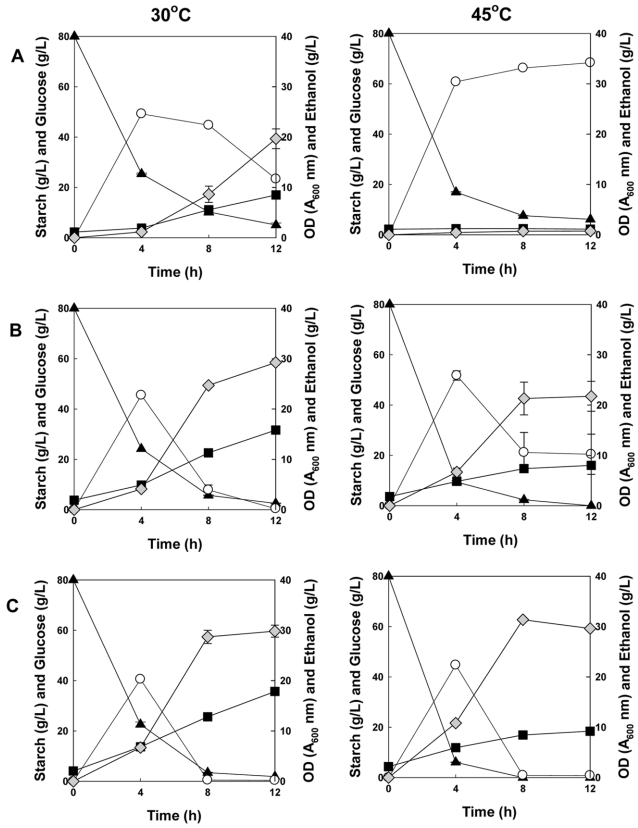

Fig. 4. SSF fermentation results with YPS medium containing 80 g/L of starch as the sole carbon source and enzymatic hydrolysis with amyloglucosidase. (A) *S. cerevisiae* L2612, (B) *S. cerevisiae* JAY270, (C) and *K. marxianus* CBS1555 at 30°C or 45°C for 12 h. Symbols: OD (square), glucose (circle), and ethanol (diamond).

more glucose consumption a further 4 h. This indicated that that glucose consumption and ethanol production had been inhibited, due to the high fermentation temperature of 45°C. *K. marxianus* CBS1555 consumed all the glucose and produced 31 g/L of ethanol within 8 h. At 45°C, the ethanol concentration (31 g/L) and productivity (3.92 g/L·h) of *K. marxianus* CBS1555 were higher than those from 30°C (30 g/L and 2.48 g/L·h, respectively), due to lower cell growth and higher amyloglucosidase activity at 45°C.

3.3. SSF at 45°C results in efficient ethanol production from pretreated corn stover

Usually cellulosic biomass hydrolysates possess several fermentation inhibitors such as furfural or 5-hydroxymethylfurfural (HMF). Because of these inhibitors, the fermentation rates were significantly reduced when cellulosic biomass hydrolysates were used as a carbon source. In order to compare the resistance to fermentation inhibitors, fermentation experiments were performed using corn stover hydrolyzate as a sole med-

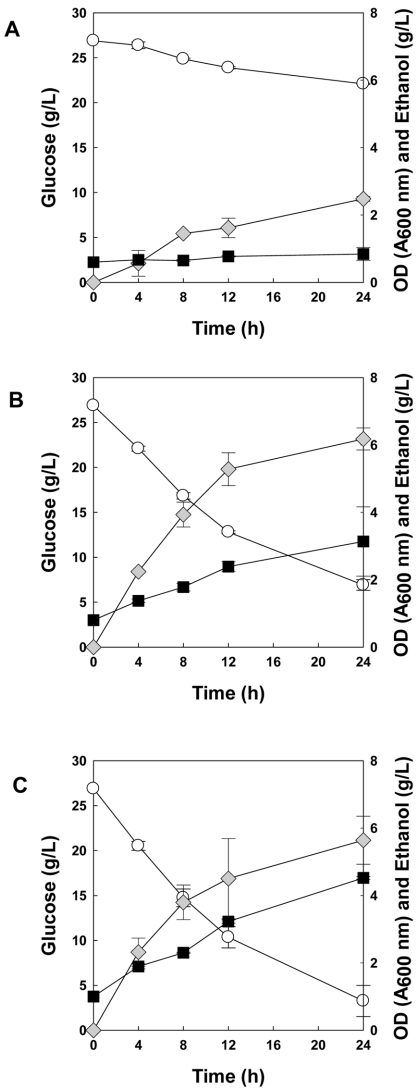

Fig. 5. Fermentation results with corn stover hydrolysates as the sole medium. (A) *S. cerevisiae* L2612, (B) *S. cerevisiae* JAY270, (C) *K. marxianus* CBS1555 at 30°C. Symbols: OD (square), glucose (circle), and ethanol (diamond).

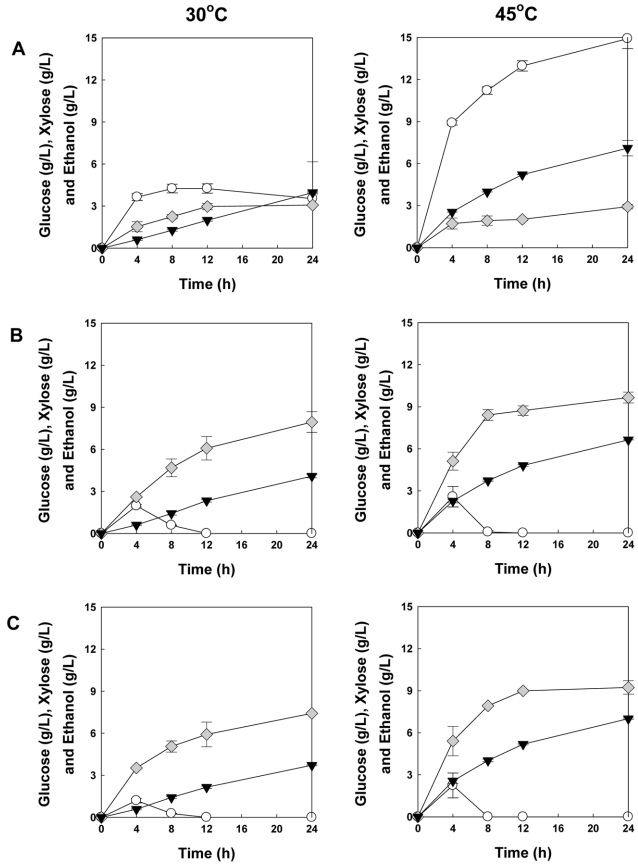

Fig. 6. SSF fermentation results with corn stover as the sole medium and with hydrolysis using an enzyme mixture (cellulase and cellobiose). (A) *S. cerevisiae* L2612, (B) *S. cerevisiae* JAY270, (C) and *K. marxianus* CBS1555 at 30°C or 45°C. Symbols: glucose (circle), xylose (reverse triangle), and ethanol (diamond).

ium at 30°C. The results show that *S. cerevisiae* L2612 showed a reduction in the rate of glucose consumption and ethanol production. Approximately 5 g/L of glucose was consumed and 2.5 g/L of ethanol produced at 24 h by *S. cerevisiae* L2612 (Fig. 5A). These results indicate that *S. cerevisiae* L2612 is very sensitive to inhibition from corn stover hydrolyzate. *S. cerevisiae* JAY270 consumed 20 g/L of glucose and produced 6.2 g/L of ethanol at 24 h. This corresponded to a 0.31 g/g yield (Fig. 5B). The *K. marxianus* CBS1555 strain consumed slightly more glucose (23 g/L) but produced less ethanol (5.6 g/L) at 24 h in comparison to *S. cerevisiae* JAY270 (Fig. 5C). As shown in the YPD80 fermentation results (Fig. 2), the *K. marxianus* CBS1555 exhibited lower ethanol yields than *S. cerevisiae* JAY270, as the *K. marxianus* CBS1555 cell growth was higher than *S. cerevisiae* JAY270 (Fig. 1). These results suggest that the *K. marxianus* CBS1555 and the *S. cerevisiae* JAY270 have equivalent resistance to fermentation inhibitors found in corn stover hydrolysate.

SSF fermentation using corn stover as the sole medium with hydrolysis performed by an enzyme mixture of cellulase and cellobiose was carried out by *K. marxianus* CBS1555 and *S. cerevisiae* L2612 and JAY270 at 30°C or 45°C (Fig. 6). The *S. cerevisiae* L2612 was not able to efficiently consume glucose or produce ethanol, regardless of fermentation temperature. This is because the *S. cerevisiae* L2612 is not resistant to fermentation inhibitors found in corn stover hydrolyzate, as shown in Fig. 5. However, an increase in glucose accumulation was shown at 45°C in comparison to 30°C, due to higher hydrolysis activities of the cellulase and cellobiose at 45°C (Fig. 6A). In addition, faster xylose accumulations were shown at 45°C compared to xylose accumulation at 30°C, as *S. cere-*

visiae L2612 is not able to consume xylose. In the case of *S. cerevisiae* JAY270 and *K. marxianus* CBS1555, a significant increase in ethanol production were seen at 45°C, in comparison to ethanol production at 30°C. This indicates that high ethanol production could be achieved at high fermentation temperatures. The *K. marxianus* CBS1555 and the *S. cerevisiae* JAY270 showed similar ethanol productivity at 30°C and 45°C. This is because *K. marxianus* CBS1555 and *S. cerevisiae* JAY 270 both have resistance to fermentation inhibitors found in corn stover hydrolyzate (Fig. 5). As the fermentations were finished quickly due to low glucose concentrations in SSF using corn stover and hydrolysis enzyme mixture, the thermotolerant advantage of *K. marxianus* CBS1555 was not shown.

4. CONCLUSION

Thermotolerant yeast are suited for SSF type process, as optimum temperatures for enzymatic saccharification are higher than the temperature required for the growth of *S. cerevisiae*. Results from the spotting growth assay and glucose fermentation, demonstrated that *K. marxianus* exhibited higher rates of growth and ethanol production at 45°C, in comparison to *S. cerevisiae*. For SSF using starch or corn stover, fermentations at 45°C showed higher enzymatic activities and ethanol production, compared to fermentations performed at 30°C. The thermotolerant *K. marxianus* offers a potential advantage for the industrial production of biofuels using SSF.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2014R1A1 A4A01004700). This study was supported by 2014 Research Grant from Kangwon National University (No. C1010952-01-01).

REFERENCES

1. Huang, G., F. Chen, D. Wei, X. Zhang, and G. Chen (2010) Biodiesel production by microalgal biotechnology. *Appl. Energ*. 87: 38-46.
2. Najafi, G., B. Ghobadian, T. Tavakoli, and T. Yusaf (2009) Potential of bioethanol production from agricultural wastes in Iran. *Renew. Sust. Energ. Rev.* 13: 1418-1427.
3. Swana, J., Y. Yang, M. Behnam, and R. Thompson (2011) An analysis of net energy production and feedstock availability for biobutanol and bioethanol. *Bioresour. Technol*. 102: 2112-2117.
4. Farrell, A. E., R. J. Plevin, B. T. Turner, A. D. Jones, M. O'Hare, and D. M. Kammen (2006) Ethanol can contribute to energy and environmental goals. *Science*. 311: 506-508.
5. Ho, N. W. Y., Z. Chen, and A. P. Brainard (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl. Environ. Microbiol.* 64: 1852-1859.
6. Jin, Y. S., H. Ni, J. M. Laplaza, and T. W. Jeffries (2003) Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. *Appl. Environ. Microbiol.* 69: 495-503.
7. Toivari, M. H., A. Aristidou, L. Ruohonen, and M. Penttila (2001) Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: Importance of xylulokinase (*XKS1*) and oxygen availability. *Metab. Eng.* 3: 236-249.
8. Ha, S. J., J. M. Galazka, S. R. Kim, J. H. Choi, X. Yang, J. H. Seo, N. L. Glass, J. H. Cate, and Y. S. Jin (2011) Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc Natl Acad Sci U S A.* 108: 504-509.
9. Nakamura, N., R. Yamada, S. Katahira, T. Tanaka, H. Fukuda, and A. Kondo (2008) Effective xylose/cellobiose co-fermentation and ethanol production by xylose-assimilating *S. cerevisiae* via expression of beta-glucosidase on its cell surface. *Enzyme Microb. Technol.* 43: 233-236.
10. Aeling, K., K. Salmon, J. Laplaza, L. Li, J. Headman, A. Hutagalung, and S. Picataggio (2012) Co-fermentation of xylose and cellobiose by an engineered *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* 39: 1597-1604.
11. Ha, S. J., S. R. Kim, H. Kim, J. Du, J. H. D. Cate, and Y. S. Jin (2013) Continuous co-fermentation of cellobiose and xylose by engineered *Saccharomyces cerevisiae*. *Bioresour. Technol*. 149: 525-531.
12. Matsushika, A., H. Inoue, K. Murakami, O. Takimura, and S. Sawayama (2009) Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. *Bioresour. Technol*. 100: 2392-2398.
13. Sedlak, M. and N. Ho (2004) Production of ethanol from cellulosic biomass hydrolysates using genetically engineered *Saccharomyces*; yeast capable of cofermenting glucose and xylose. *Appl. Biochem. Biotechnol.* 114: 403-416.
14. Davison, B., J. Lee, M. Finkelstein, J. McMillan, D. Schell, J. Farmer, and M. Newman (2003) Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. Humana Press
15. Kumar, R., and C. E. Wyman (2009) Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies. *Biotechnol. Bioeng.* 102: 457-467.
16. Ghosh, P., N. B. Pamment, and W. R. B. Martin (1982) Simultaneous saccharification and fermentation of cellulose: effect of beta-d-glucosidase activity and ethanol inhibition of cellulases. *Enzyme Microb. Technol.* 4: 425-430.
17. Wyman, C. E., S. R. Decker, M. E. Himmel, J. W. Brady, C. E. Skopec, and L. Viikari (2005) Hydrolysis of cellulose and hemicellulose. Polysaccharides: Structural Diversity and Functional Versatility (second ed.). 995-1033.

18. Cheon, Y., J.-S. Kim, J.-B. Park, P. Heo, J. H. Lim, G. Y. Jung, J.-H. Seo, J. H. Park, H. M. Koo, K. M. Cho, J.-B. Park, S.-J. Ha, and D.-H. Kweon (2014) A biosynthetic pathway for hexanoic acid production in Kluyveromyces marxianus. *J. Biotechnol.* 182??83: 30-36.
19. Lark, N., Y. Xia, C.-G. Qin, C. S. Gong, and G. T. Tsao (1997) Production of ethanol from recycled paper sludge using cellulase and yeast, *Kluveromyces marxianus*. *Biomass Bioenergy.* 12: 135-143.
20. Loser, C., T. Urit, A. Stukert, and T. Bley (2013) Formation of ethyl acetate from whey by *Kluyveromyces marxianus* on a pilot scale. *J. Biotechnol.* 163: 17-23.
21. Fonseca, G., E. Heinzle, C. Wittmann, and A. Gombert (2008) The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl. Microbiol. Biotechnol.* 79: 339-354.
22. Limtong, S., C. Sringiew, and W. Yongmanitchai (2007) Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour. Technol*. 98: 3367-3374.
23. Heo, P., T.-J. Yang, S.-C. Chung, Y. Cheon, J.-S. Kim, J.-B. Park, H. M. Koo, K. M. Cho, J.-H. Seo, J. C. Park, and D.-H. Kweon (2013) Simultaneous integration of multiple genes into the *Kluyveromyces marxianus* chromosome. *J. Biotechnol.* 167: 323-325.
24. Jeong, H., D.-H. Lee, S. H. Kim, H.-J. Kim, K. Lee, J. Y. Song, B. K. Kim, B. H. Sung, J. C. Park, J. H. Sohn, H. M. Koo, and J. F. Kim (2012) Genome Sequence of the Thermotolerant Yeast *Kluyveromyces marxianus* var. *marxianus* KCTC 17555. *Eukaryotic Cell.* 11: 1584-1585.
25. Pecota, D. C., V. Rajgarhia, and N. A. Da Silva (2007) Sequential gene integration for the engineering of *Kluyveromyces marxianus*. *J. Biotechnol.* 127: 408-416.