Asian Journal of Chemistry; Vol. 23, No. 4 (2011), 1841-1845

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Conversion of Starch into Ethanol by *Saccharomyces cerevisiae* Strain Transformed with α-Amylase Gene from *Rhizopus oryzae*

S. LI and Z.-X. WANG*

Research Center of Bioresource and Bioenergy, School of Biotechnology, Jiangnan University, Wuxi, P.R. China

*Corresponding author: Tel/Fax: +86 510 85918121, E-mail: zxwang@sytu.edu.cn

(*Received*: 21 September 2010; *Accepted*: 27 December 2010) AJC-9429

The direct production of ethanol from starch by a recombinant *Saccharomyces cerevisiae* strain, W303-1A, which expressed the *Rhizopus oryzae* α-amylase gene, was carried out by shake-flask fermentation. The effects of several factors, such as α-amylase activity, inoculum size, initial glucose supply and glucoamylase supplementation, on starch utilization and ethanol production have been investigated. The α-amylase produced by the recombinant strain was sufficient to obtain complete degradation of starch. However, the starch-degradation rate and ethanol-production efficiency were low. In the presence of exogenous glucoamylase in the starch-containing cultures, the ethanol-production levels of the recombinant strain were significantly improved and the ethanol yields increased up to 3.75 g/L, which was approximately three times higher than that observed for the native strain and the biomass obtained with the recombinant strain was two times that observed for the native strain.

Key Words: *Saccharomyces cerevisiae*, α-Amylase, Ethanol production.

INTRODUCTION

As an alternative fuel, bioethanol has great potential in petroleum-based transportation due to its renewable, clean and environmentally friendly properties[1,2]. Bioethanol produced from economically renewable biomass, such as sugar, starch, or lignocellulosic materials, appear to be efficient and sustainable[2]. Although lignocellulosic biomass has been shown to be one of the most promising sources of substrate for the fermentative production of ethanol, with some breakthroughs having been made in the recent past, sugar and starchy materials cannot be replaced in the near- or even the long term, due to economic and technical problems. Currently, the ethanol produced in the United States is mainly based on cornstarch; in China, there is an increasing interest in the production of ethanol from cassava starch.

Three steps are involved in the traditional industrial-scale ethanol production from starchy materials *i.e.*, liquefaction of starch with α-amylase, which hydrolyzes the α-1, 4 linkages in starch molecules at random; saccharification with glucoamylase, which hydrolyzes the α-1, 4 and α-1, 6 linkages in the liquefied starch; and fermentation of the resultant sugars to ethanol by the action of microorganisms[3,4]. Among the ethanol-producing microorganisms, *Saccharomyces cerevisiae* is most commonly used in the industrial production of ethanol from starchy materials because of its high fermentative capability and high ethanol yield[4,5]. However, the wild-type *S. cerevisiae* strains cannot directly utilize starchy materials because they lack the ability to produce starch-decomposing enzymes[3]. Thus, both α-amylase and glucoamylase are required for efficient hydrolysis of starch and production of ethanol in the industrial process that uses *S. cerevisiae* strains[5].

To simplify the fermentation process and reduce the costs caused by addition of enzymes, many researchers have resorted to imparting additional amylolytic activities to yeast strains by the heterologous expression of α-amylase and/or glucoamylase genes[3-5]. For instance, Knox *et al.*[6] evaluated the ethanol produced by three *S. cerevisiae* strains that were transformed with different combinations of foreign yeast-amylase genes and confirmed that the rate of starch hydrolysis was the rate-limiting step in the fermentation of starch. Shigechi *et al.*[5] reported that *S. cerevisiae* strains that coexpressed *Rhizopus oryzae* glucoamylase and *Bacillus stearothermophilus* α-amylase could produce ethanol directly by fermentation of corn starch cooked at 80 °C and the ethanol concentration thus obtained was almost equal to that produced from corn starch cooked at 120 °C. Yamada *et al.*[7] proposed a better energy-saving approach to produce ethanol directly from raw corn starch through fermentation with *S. cerevisiae* coexpressing *R. oryzae* glucoamylase and *Streptococcus bovis* α-amylase. In addition, direct production of ethanol from starch could also be carried out by coculturing *S. cerevisiae* with other amylolytic strains[4,8,9].

In this study, we propose a method for the direct production of ethanol from soluble starch by a genetically modified strain of *S. cerevisiae*, which was therefore able to produce the α-amylase of *R. oryzae*. The improved ethanol yields obtained by the action of the recombinant strain in cultures containing starch and exogenous glucoamylase was 3.75 g/L, which was very close to that obtained in cultures containing only glucose (4.2 g/L) and three times higher than that observed in native strain cultures.

EXPERIMENTAL

Strains, plasmids and culture medium: The strains were used, *Escherichia coli* JM109 [endA1, recA1, gyrA96, yhi1, hsdR17 (rk-, mk+), relA1, supE4, Δ(proAB, lac), F'(proAB, $lacI^q$,lacZΔM15), traD]; and *S. cerevisiae* W303-1A (MTAa leu2-3,112 ura3-1 trp1-92 his3-11, 15ade2-1 can1-100 GAL SUC mal). The recombinant plasmid pPIC9K-*RoAmy*, containing the *R. oryzae* α-amylase coding sequence, was constructed in our previous work. Plasmid pYX212 (URA3, triosephosphate isomerase (TPI) promoter) was used for the expression of α-amylase from *R. oryzae* (RoAmy). All the strains and plasmids used were stored and maintained by the Culture and Information Center of Industrial Microorganisms of China Universities (http://cicim-cu.jiangnan.edu.cn/).

E. coli was grown at 37 °C in Luria-Bertani medium containing 100 μg of ampicillin per milliliter. *S. cerevisiae* was grown at 30 °C in YPD medium containing 20 g peptone, 10 g yeast extract (Oxoid Ltd., London, England) and 20 g glucose per liter. *S. cerevisiae* transformants were selected on YDA plates, which contained 3.4 g yeast nitrogen base without amino acids, 10 g $(NH_4)_2SO_4$, 20 g glucose, 0.16 g leucine, 0.16 g tryptophan, 0.16 g histidine, 0.16 g adenine and 15 g agar per liter. For ethanol fermentation, YPD and YPS (20 g peptone, 10 g yeast extract and 20 g soluble starch per liter) media were used.

Construction of expression plasmid: A 2.0-kb fragment, containing the *S. cerevisiae* α-mating factor prepro sequence, the mature RoAmy coding sequence and the 3' alcohol oxidase-1 transcription termination fragment, was amplified from pPIC9K-*RoAmy* using the primers 5'-CTGAAGCTTATGA-GATTTCCTTCAAT-3' and 5'-GTAGTCGACGATAAGCT-TGCACAAACGAAC-3'. The amplification yielded a copy of the gene flanked by *Hin*dIII and *Sal*I sites provided by the two primers (underlined), respectively. The amplified gene fragment was gel-purified and digested with the two indicated enzymes and inserted at the corresponding site of the *S. cerevisiae* expression vector pYX212; this procedure yielded a recombinant plasmid, designated as pTaRA, in which the RoAmy was under the control of the TPI promoter, as shown in Fig. 1. The plasmid was propagated in *E. coli* and the inserted gene fragment was sequenced to confirm that no mutation had occurred.

Transformation of *S. cerevisiae* by electroporation: The *S. cerevisiae* W303-1A strain was inoculated into 10 mL of YPD medium in a baffled flask and grown in a shaking incubator at 30 °C overnight. An aliquot of the overnight culture (0.2 mL) was transferred to 100 mL of fresh medium in a 500 mL baffled flask and cultivated to an optical density (OD_{600}) = 1.0-1.5. The cells were harvested by centrifugation

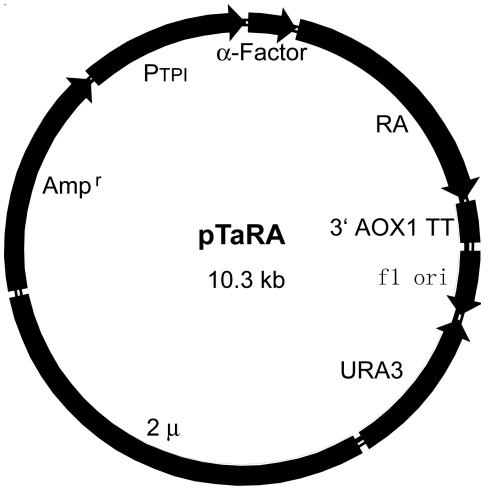

Fig. 1. Map of the recombinant expression plasmid pTaRA

at 1500× g for 5 min at 4 °C and washed thrice with ice-cold water and once with ice-cold 1 M sorbitol, followed by resuspension with an appropriate volume of ice-cold 1 M sorbitol. Eighty μL of the competent yeast cells thus obtained were mixed with 2 mg of recombinant plasmid (pTaRA) DNA. The mixture was transferred into an ice-cold 2 mm electroporation cuvette and incubated on ice for 5 min before pulsing the cells with field strength of 750 V/mm for 10 ms. The electroporated cells were suspended in 1 mL of 1 M sorbitol and spread on YDA plates. After incubating the plates at 30 °C for 3-4 days, the transformants were selected for further study.

Fermentation conditions: The recombinant strains were inoculated into 10 mL of YPD medium in a 250 mL baffled flask and grown in a shaking incubator at 200 rpm and 30 °C overnight. A 0.2 mL aliquot of the overnight culture was transferred to 50 mL of fresh medium in a 250 mL baffled flask and cultivated to an OD_{600} = 2.0-3.0 and the precultures were inoculated into 50 mL of YPD or YPS medium to obtain a final OD_{600} = 0.2. To investigate the effect of inoculum size on fermentation of starch, the initial ODs of the cultures were adjusted to values ranging from 0.1-0.4. For aerobic fermentation, the recombinant yeast was cultivated in a shaking incubator at 200 rpm at 30 °C; for anaerobic fermentation, the recombinant yeast was grown at 30 °C without shaking.

Analytical techniques: The growth of the recombinant yeast was measured in terms of the OD at 600 nm. The α-amylase activity was determined as follows: 1 mL of 1 % soluble starch was mixed with 0.25 mL of citric acid-Na_2HPO_4 buffer (0.2 M, pH 5.0) and incubated at 55 °C for 5 min, followed by the addition of 0.1 mL of the enzyme solution. It was further incubated for 5 min. The reducing sugar formed was determined according to a method described previously[10], using maltose as a standard. One unit of α-amylase activity was defined as the amount of enzyme that released 1 mg reducing sugar per minute under the above conditions. The starch concentration was quantified according to the method proposed by Xiao *et al.*[11]. The ethanol concentration was determined using high-performance liquid chromatography. A Shodex SUGAR column (SH1011, 8.0 × 300 mm, Thomson Instrument Co. and Shodex, Inc.) was used, with 5 mM H_2SO_4 as the mobile phase; the flow rate was 0.8 mL/min.

RESULTS AND DISCUSSION

Expression of α-amylase in *S. cerevisiae*: After selection and purification of transformants on YDA plates, 12 colonies were randomly replicated in YPDS plates (YPD medium, with an additional 5 g soluble starch and 15 g agar per liter) and grown at 30 °C for 3-4 days, before being stained with weak iodine solution (20 g KI and 2 g I_2 per liter). Clear zones were formed around all the selected strains, except for the host control, as shown in Fig. 2. Clones #8 and #11 showed larger clearance zones, whereas clones #1 and #3 showed smaller zones in the plate assay. However, this was not correlated with the corresponding amylase activities obtained during the culture under aerobic shake-flask fermentation mode at 200 rpm, as shown in Fig. 3. Clone #6 showed the highest α-amylase activity of 1330 units/L, whereas clone #4 yielded only 890 units/L.

Fig. 2. Test of the amylase activity of 12 selected clones of the pTaRA transformation into *S. cerevisiae* W303-1A. The colony labeled "C" of non-transformed control host W303-1A cells show no clearance zone indicating the absence of amylase activity

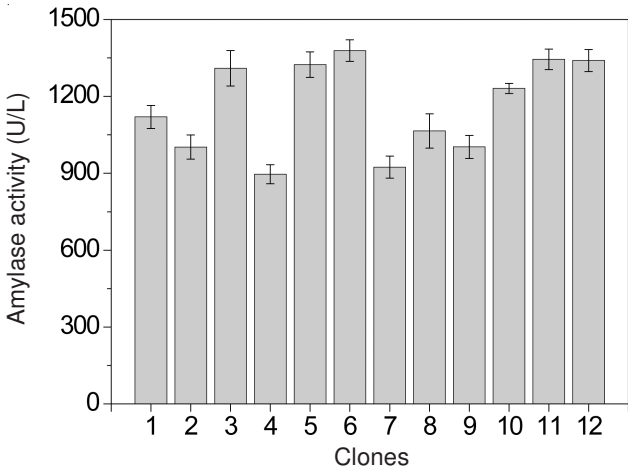

Fig. 3. Amylase activities by aerobic fermentation in YPD culture supernatants of recombinant W303-1A clones

Effect of the α-amylase activities produced in the cultures: To evaluate the effect of the α-amylase activities on starch fermentation, the host strain and two clones of the recombinant strain (#4 and #6), which displayed apparently different α-amylase expression levels, were used. The amount of α-amylase expressed by the two clones grown in YPS medium by anaerobic fermentation (Fig. 4) was relatively low compared to that obtained in the YPD medium under aerobic conditions (Fig. 3). The growth patterns of the host strain and the two clones grown in YPS medium are shown in Fig. 5. Both the clones gave an exponential growth phase in YPS medium, but the host strain appeared to have no apparent growth. The starch concentration did not decrease in the culture of the host yeast that lacked amylolytic activity and the culture of clone #6, which exhibited higher α-amylase activity, degraded starch more efficiently than clone #4. This proved that the starch-hydrolysis rate was highly correlated with the α-amylase activities of the clones.

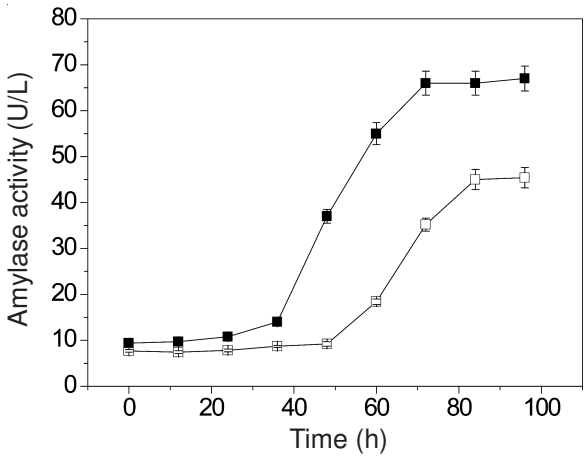

Fig. 4. Amylase activities produced by anaerobic fermentation in YPS culture supernatants. Open and solid symbols represent clone #4 and clone #6, respectively

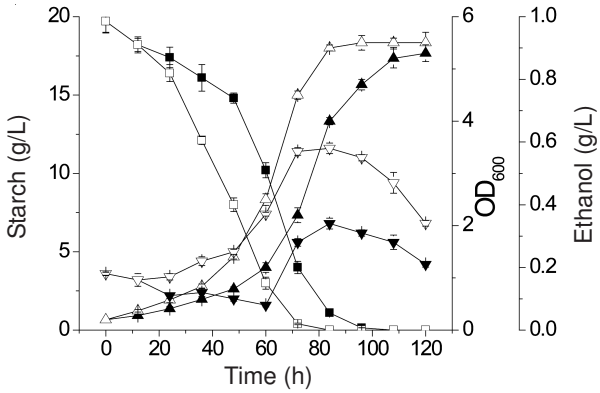

Fig. 5. Anaerobic fermentation of two clones with different amylase expression levels in YPS medium. Symbols represent starch concentrations (squares), cell densities (triangles) and ethanol productivities (inverted triangles) in the cultures of clone #4 (solid) and clone #6 (open), respectively

Although the two clones yielded a similar biomass finally, the growth curve of clone #6 had a shorter lag phase and reached the stationary phase *ca.* 24 h earlier than that of clone #4. This might have been caused by the greater glucose-release rate found in the culture of clone #6. Apart from the additional ethanol present in the cultures (about 0.2 g/L) introduced by inoculation, negligible amounts of ethanol were produced in the cultures, probably because the sugar-uptake

rate of the recombinant yeast exceeded the rate of sugar released from starch, thus leaving behind too little glucose to initiate the Crabtree effect.

Effect of initial inoculum size: Because previous reports have indicated that inoculum size may affect microbial growth[12] and that the starch fermentation rate could be improved by increasing the initial cell density[13], the effect of the initial inoculum size of recombinant yeast (clone #6) on starch fermentation was investigated in this study. As shown in Fig. 6, cultures with higher inoculum levels yielded higher starch-hydrolysis rates, higher yeast-growth rates and shorter lag times. Especially when the inoculum levels were increased to 0.4 OD_{600}, growth was initiated at a high rate and the stationary phase was reached *ca.* 20 and 60 h earlier than the growth observed with inoculum sizes of 0.2 and 0.1 OD_{600}, respectively. For all the three inoculum levels tested, a relatively low growth rate was observed between 12 and 48 h during the starch-fermentation processes, which turned into a rapid growth period between 48 and 80 h. This observation indicated that although most of the starch was degraded in the first 48 h, sufficient fermentable sugar was not formed to support yeast growth at a high rate. A possible explanation for the growth lag during starch decomposition might be the limitations on the formation of fermentable sugar by α-amylase because the initial products of starch degradation catalyzed by α-amylase were dextrins and oligosaccharides, in addition to small amounts of glucose. Furthermore, negligible amounts of ethanol were produced by all the three tested cultures (data not shown).

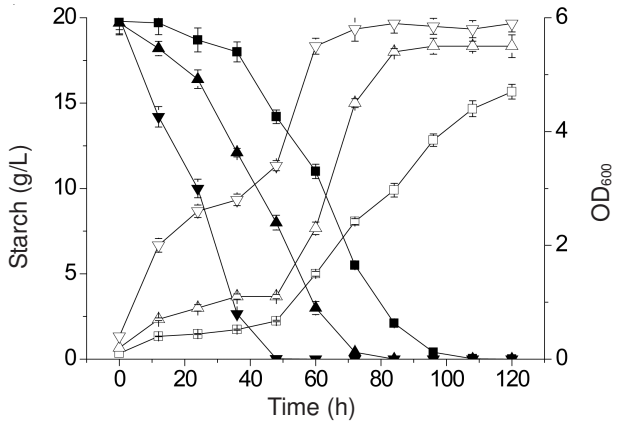

Fig. 6. Effect of initial cell density on starch degradation efficiency under anaerobic fermentation with clone #6 in YPS medium. Symbols represent initial cell density of 0.1 (squares), 0.2 (triangles) and 0.4 (inverted triangles). Solid and open symbols represent starch concentrations and cell densities, respectively

Effect of initial glucose supplementation in the medium: The growth of the recombinant strain in YPS medium has an apparently long lag phase and negligible ethanol was produced in the cultures (Figs. 5 and 6), which might be due to the low fermentable sugar available for initiating yeast growth and the poor glucose accumulation[14]. Thus, to shorten the lag phase and obtain good ethanol productivity, glucose was supplemented to a final concentration of 5 g/L, whereas the concentration of soluble starch was adjusted to 15 g/L to maintain a total content of carbon sources at 20 g/L (named as 15S-YPD medium). In another experiment, as a contrast, the starch was completely substituted by 20 g/L glucose (YPD medium). As shown in Fig. 7, the ethanol production paralleled the initial glucose concentration and was dramatically increased by the addition of glucose. For example, the ethanol production was 1.2 g/L in 15S-YPD and 3.4 g/L in YPD in the first 12 h, which was much higher than that obtained in YPS (Fig. 5). Although the lag time was shortened by the addition of 0.5 g/L glucose and the recombinant yeast showed a higher growth rate in the first 12 h, the yeast still entered a stage of relatively low growth rate between 12 and 48 h, which was observed in all YPS cultures. The starch-hydrolysis rate was slightly elevated in 15S-YPD compared to YPS with the same inoculum size (OD_{600} = 0.2; Fig. 6). However, the yeast-growth and the ethanol-production limitations caused by the low rate of fermentable-sugar release in 15S-YPD or YPS cultures were not eliminated by the addition of glucose.

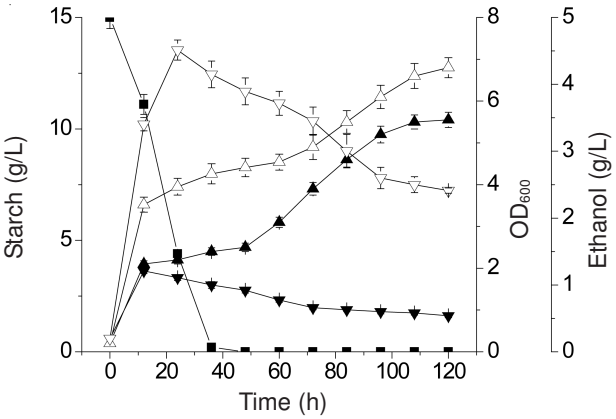

Fig. 7. Effect of initial glucose supplementation on anaerobic ethanol fermentation with clone #6. Symbols represent starch concentrations (squares), cell densities (triangles) and ethanol productivities (inverted triangles) in the cultures of 15S-YPD (solid) and YPD medium (open), respectively

Effect of glucoamylase supplementation on starch fermentation: Glucoamylase (EC3.2.1.3) is an exoglucosidase that catalyzes the release of glucose units from the non-reducing ends of maltodextrin chains[15]. As demonstrated by Birol *et al.*[16] and de Moraes *et al.*[17], the combined use of glucoamylase and α-amylase can accelerate the digestion of a starch chain or a large oligosaccharide to glucose. As shown in Fig. 8, the starch was decomposed at a higher rate by the addition of glucoamylase for both recombinant and host strains and the starch was hydrolyzed completely in 24 h. After the addition of glucoamylase, the ethanol production was increased to 3.75 g/L, which was much higher than that obtained in the absence of glucoamylase and close to that obtained in YPD culture (4.42 g/L, Fig. 7). Moreover, no apparent lag time existed between 12 and 48 h during the above starch-fermentation processes. However, the starch-hydrolysis rate of the host strain was relatively low in the presence of glucoamylase, with the highest ethanol production being only 1.4 g/L. This indicated that the addition of glucoamylase not only increased the starch-hydrolysis rate, but also enhanced glucose accumulation, which triggered the Crabtree effect and subsequently resulted in the production of higher levels of ethanol. In addition, compared to the recombinant yeast cultured in YPD or YPS medium

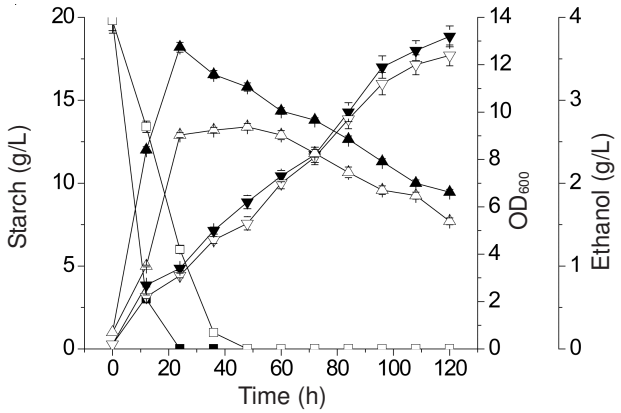

Fig. 8. Effect of exogenous glucoamylase supplementation on anaerobic ethanol fermentation with clone #6 in YPS medium. Symbols represent starch concentrations (squares), cell densities (triangles) and ethanol productivities (inverted triangles) in the cultures of recombinant yeast (solid) and host yeast (open), respectively

without glucoamylase, the biomass (OD_{600}) of the recombinant yeast grown in YPS supplemented with glucoamylase was almost double. A better growth was observed for the recombinant yeast in YPS with additional glucoamylase, which might have been caused by the improved starch-hydrolysis efficiency and/or the presence of some special sugars that might have been released from starch in the presence of the fungal α-amylase and glucoamylase.

Conclusion

In this study, the recombinant *S. cerevisiae* strain W303-1A, which exhibited *R. oryzae* α-amylase activity, was studied for application in the direct ethanol production from soluble starch. Although W303-1A is a laboratory strain and the ethanol-production ability is not as powerful as other industrial yeast strains, the positive influence of additional α-amylase activity on starch-fermentation and ethanol-production characteristics can be exploited for the direct production of ethanol from starchy materials on an industrial scale. Present results showed that the recombinant W303-1A strain could be cultured with soluble starch as the single carbon source and the α-amylase produced thereafter was sufficient to obtain the complete degradation of starch. However, the starch-hydrolysis rate was relatively low and negligible amounts of ethanol were produced. Although the starch-decomposing rate could be improved by increasing the inoculum size, ethanol-production levels were still low, because the glucose released by the action of the single α-amylase was not sufficient for the continuous production of both yeast and ethanol. This led us to try supplementation with glucoamylase in the culture medium, whereby both starch-hydrolysis efficiency and ethanol-production capacity of the recombinant yeast cultures were significantly increased, with levels much higher than those observed in the host strain cultures. In addition, the better growth rate of recombinant yeast in the starch-containing medium in the presence of *R. oryzae* α-amylase and glucoamylase might lead to the cultivation of yeast strains with high cell density.

ACKNOWLEDGEMENTS

This work was supported by Chinese Science and Technology Support Research for the 11th 5-year Plan Program (Grant 2006AA020204).

REFERENCES

1. O'.J. Sanchez and C.A. Cardona, *Bioresour. Technol.*, **99**, 5270 (2008).
2. K.A. Gray, L. Zhao and M. Emptage, *Curr. Opin. Chem. Biol.*, **10**, 141 (2006).
3. A. Kosugi, A. Kondo, M. Ueda, Y. Murata, P. Vaithanomsat, W. Thanapase, T. Arai, Y. Mori, *Renew. Energy*, **34**, 1354 (2009).
4. G. Verma, P. Nigam, D. Singh and K. Chaudhary, *Bioresour. Technol.*, **72**, 261 (2000).
5. H. Shigechi, K. Uyama, Y. Fujita, T. Matsumoto, M. Ueda, A. Tanaka, H. Fukuda and A. Kondo, *J. Mol. Catal. B-Enzym.*, **17**, 179 (2002).
6. A.M. Knox, J.C. du Preez and S.G. Kilian, *Enzyme Microb. Technol.*, **34**, 453 (2004).
7. R. Yamada, Y. Bito, T. Adachi, T. Tanaka, C. Ogino, H. Fukuda and A. Kondo, *Enzyme Microb. Tech.*, **44**, 344 (2009).
8. M.M. Abouzied and C.A. Reddy, *Appl. Environ. Microbiol.*, **52**, 1055 (1986).
9. B.Y. Jeon, S.J. Kim, D.H. Kim, B.K. Na, D.H. Park, H.T. Tran, R. Zhang and D.H. Ahn , *Biotechnol. Bioprocess. Eng.*, **12**, 566 (2007).
10. G.L. Miller, *Anal. Chem.*, **31**, 426 (1959).
11. Z. Xiao, R. Storms and A. Tsang, *Anal. Biochem.*, **351**, 146 (2006).
12. K.P. Koutsoumanis and J.N. Sofos, *Int. J. Food Microbiol.*, **104**, 83 (2005).
13. H. Shigechi, J. Koh, Y. Fujita, T. Matsumoto, Y. Bito, M. Ueda, E. Satoh, H. Fukuda and A. Kondo, *Appl. Environ. Microbiol.*, **70**, 5037 (2004).
14. K.Ö. Ülgen, B. Saygili, Z.Önsan and B. Kirdar, *Process Biochem.*, **37**, 1157 (2002).
15. P. Sutthirak, S. Dharmsthiti and S. Lertsiri, *Process Biochem.*, **40**, 2821 (2005).
16. G. Birol, Z.I. Onsan, B. Kirdar and S.G. Oliver, *Enzyme Microb. Tech.*, **22**, 672 (1998).
17. L.M.P. de Moraes, S. Astol-filho and S.G. Oliver, *Appl. Microbiol. Biotechnol.*, **43**, 1067 (1995).