

Development of a simple isolation method for yeast *Saccharomyces cerevisiae* with high fermentative activities from coastal waters

Nobuo Obara (Graduate School of Marine Science and Technology, Tokyo University of Marine Science Technology, nobara0@kaiyodai.ac.jp)

Nozomi Oki (Graduate School of Marine Science and Technology, Tokyo University of Marine Science Technology)

Masahiko Okai (Graduate School of Marine Science and Technology, Tokyo University of Marine Science Technology, mokai01@kaiyodai.ac.jp)

Masami Ishida (Graduate School of Marine Science and Technology, Tokyo University of Marine Science Technology, ishida@kaiyodai.ac.jp)

Naoto Urano (Graduate School of Marine Science and Technology, Tokyo University of Marine Science Technology, urano@kaiyodai.ac.jp)

Abstract

In this study, the development of a simple isolation method for obtaining yeast *Saccharomyces cerevisiae* with high fermentative activities from coastal waters was examined. 824 isolates were obtained on YPD₂ (glucose 2 %) solid medium from coastal waters of both Tokyo Bay and Sagami Bay in Japan. In the first screening from the isolates, 221 of the 824 yeasts had fermentative activities in YPD₂ liquid medium by a Durham pipe test. In the second one, 55 of the 221 yeasts had fermentative activities in YPD₃₀ (glucose 30 %) liquid medium by the same test. In the third one, exact amounts of gas produced by the 55 strains were rapidly measured by a syringe test and the gas productivities of the 29 strains were higher than that of a marine-derived *Saccharomyces cerevisiae* C19 which was isolated and used for bioethanol production in previous studies (Takagi et al., 2015; Obara et al., 2015; Obara et al., 2012). Above all, the best 4 strains with the highest gas producing activities among the 29 ones were also identified as *Saccharomyces cerevisiae*. Thus, the yeast species *S. cerevisiae* was found to be simply isolated from coastal waters by the method as above. In addition, ethanol productivities of 10 strains randomly selected from the 29 ones were measured. There was an almost linear relationship between ethanol and gas productivities in the 10 strains. Therefore, bioethanol productivities of the yeast isolates from coastal waters could be speculated by measuring amounts of gas produced by the syringe test.

Key words

yeast, *Saccharomyces cerevisiae*, fermentation, bioethanol, coastal water

1. Introduction

Today, there are about 500 species of yeasts in 60 genera and about 1,000 species of yeast-like organisms in the world. Although the fungi are multicellular, growing as filaments called hyphae, the yeasts or the yeast-like cells have morphological terms that refer to one-celled fungi. The yeasts have already been used for various industrial purposes and basic studies on molecular biology, genetics in addition to traditional baking and alcoholic fermentations. Among the yeast species, *Saccharomyces cerevisiae* has been thought to be one of the most important micro-organisms for humans because most of the yeasts applying to various fermentation technologies were identified as the *S. cerevisiae* which had been secured as safe for foods by experience for a long period. The *S. cerevisiae* strains have the highest fermentative activities among the yeast species and they are found in various natural environments such as flowers, trees, animals, soils, hydrosphere and artificial environments such as foods, drinks (Barnet et al., 2000). Since the beginning of the 21st century, various studies on marine-derived yeasts except for *S. cerevisiae* have been reported (Sreedevi et al., 2008; Chen et al., 2009; Mastuda et al., 2008; Zhang et al., 2010). Otherwise, only a few

studies have been reported about isolation and application of marine-derived *S. cerevisiae* as a main target (Saravanakumar et al., 2013). In our previous studies, a marine-derived *S. cerevisiae* C19 with the highest fermentative activity among many yeast isolates was isolated and applied to the ethanol production from various biomass (Takagi et al., 2015; Obara et al., 2015; Obara et al., 2012). Although several hundreds of yeast strains were isolated from the coastal waters of Tokyo Bay in Japan, only one strain of *S. cerevisiae* could be obtained (Obara et al., 2012). Therefore, it is expected that a more rapid and simple isolation method of *S. cerevisiae* from coastal waters would be developed.

2. Materials and methods

2.1 Yeast isolation

Using sterilized plastic bottles, 2l bottles of sea water were collected from Station 1: the coast under the Rainbow Bridge in Tokyo Bay on June 18 in 2013, from St. 2: the Hakkeijima coast of Yokohama city in Tokyo Bay on June 24 in 2013, and from St. 3: the Hayama Port of Miura Peninsula in Sagami Bay on September 3 in 2013 as shown in Figure 1.

The coastal water was passed through a membrane filter (pore size; 0.2 μm) and micro-organisms in the water was concentrated at about 100 folds. A portion of the concentrated substance was spread on YPD₂ solid medium (D-glucose, 20 g/l; peptone, 20 g/l; yeast extract, 10 g/l; chloramphenicol,

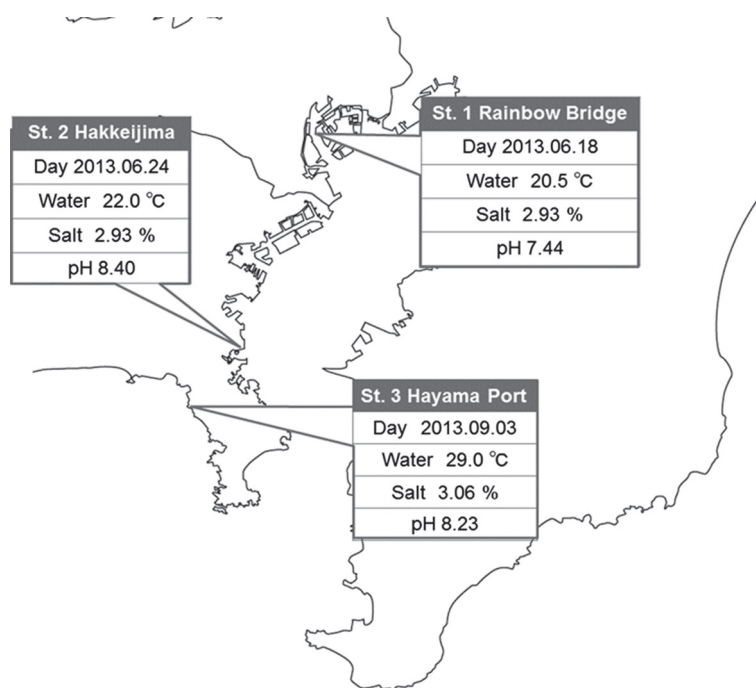


Figure 1: Stations collecting coastal waters

0.2 g/l; agar, 15 g/l) and incubated at 25 °C for 2 weeks. Each growth colony of the micro-organism was picked up and its cells were observed under a microscope. In the first screening, the yeast or yeast-like cells from morphology were sorted from them and maintained at 4 °C.

2.2 Assay of fermentative activity by the yeast

In the second screening, each isolate was inoculated into each test tubes containing 10 ml of the YPD₂ liquid medium (D-glucose, 20 g/l; peptone, 20 g/l; yeast extract, 10 g/l; chloramphenicol, 0.2 g/l) with a Durham pipe and incubated statically at 25 °C for 2 weeks. The yeast fermentation in the culture was examined by the naked eye based on the storage of gas from the cells in the Durham pipe. In the third screening, each isolate from the second one was inoculated into test tubes containing 10 ml of the YPD₃₀ liquid medium (D-glucose, 300 g/l; peptone, 20 g/l; yeast extract, 10 g/l; chloramphenicol, 0.2 g/l) with the Durham pipe and incubated statically at 25 °C for 2 weeks. The yeast fermentation in the culture was also examined by the same test as in the second one.

2.3 Assay of gas productivity by the yeast

Amounts of the gas produced by the yeasts from the third screening were measured by a syringe test as shown in Figure 2. The procedure was as follows: (1) Each yeast with fermentative activity in the third screening was inoculated to 10 ml of YPD₂ liquid medium whose optical density at 660 nm became 0.1 by suspending the cells. (2) The suspension was transferred to 50 ml of the plastic syringe. (3) The air in the

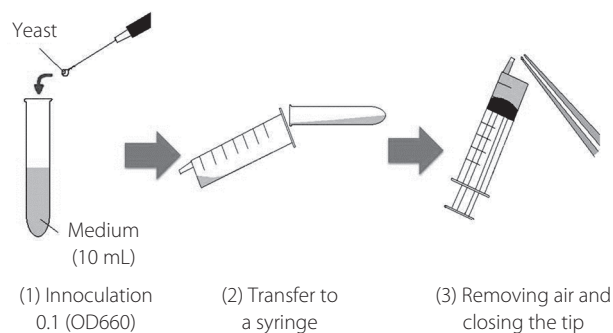


Figure 2: Assay system for gas production by the yeast

syringe was removed and its tip was closed by flash heating. The processed syringe containing the suspension of the yeast-YPD₂ medium was incubated statically at 25 °C and the amount of gas produced by the cells was measured by its storage in the syringe every 3 h for 24 h.

2.4 Yeast identification

The standard *S. cerevisiae* used in this study was the strain C19, which was isolated from Tokyo Bay and identified by 28S rDNA D₁/D₂ domain sequence analysis (DNA Data Bank of Japan: accession no. AB67255) (Obara et al., 2012). The new yeast isolates from coastal waters were also identified by 28S rDNA D₁/D₂ domain sequence analysis and BLASTIN program survey (Altshul et al., 1997).

2.5 Carbon dependence of yeast fermentation

Fermentation of various saccharides (arabinose, fructose, galactose, glucose, maltose, mannose, raffinose, sucrose, and

xylose) by the yeasts isolated in this study and C19 were tested, following the procedure described in our previous study (Ueno et al., 2002). Ten ml of a minimum culture for the yeasts containing 20 g/l of each saccharide (Wako Pure Chem. Ind. Ltd., Japan) and 6.7 g/l of yeast nitrogen base (YNB) without amino acids (Sigma-Aldrich, USA) was prepared in the test tube with the Durham pipe. Each yeast strain was inoculated into the medium and the mixture was incubated statically at 25 °C for 2 weeks. The carbon dependence of yeast fermentation in the culture was also examined by the storage of gas in the Durham tube.

2.6 Assay of the ethanol production by the yeasts

The yeast pellets (0.1 g) were inoculated into 10 ml of YPD₂ in the test tube. Fermentation by the yeast was carried out at 25 °C for 24 h under anaerobic condition using the Anaero Pack System (Mitsubishi Gas Chem. Co. Inc., Japan). The amounts of ethanol in the fermentative suspension were measured using an enzyme assay F-kit (Roche, Basel, Switzerland). All procedure in this study is summarized in Figure 3.

3. Results

3.1 Yeast isolation

Figure 4 shows the number of the yeasts isolated from the Stations 1, 2, and 3. In the first screening, 487 isolates were obtained from St. 1, 281 from St. 2, and 56 from St.3. A total of 824 strains were obtained from the three stations. In the second screening, 87 isolates were obtained from St. 1, 121 ones were from St. 2, and 13 ones were from St. 3. The proportion of the yeast with fermentative activities to the yeasts isolated on the YPD₂ solid medium was 17.9 % at St.1, 43.1 % at St. 2, and 23.2 % at St. 3. Many yeasts with fermentative activities were found in St. 2 but the reason remains unknown. However, there are many sea weeds-Anaosa- in the Hakkeijima coast of Yokohama city in Tokyo Bay and many fermentative

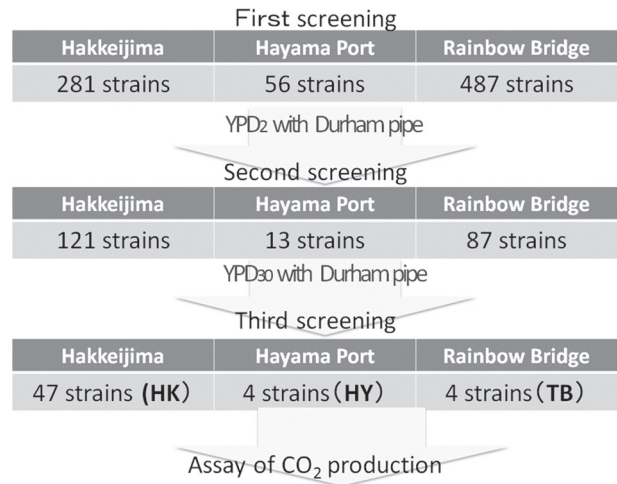


Figure 4: Number of the yeasts isolated from 1st to 3rd screening

yeasts also seemed to be living in this ecosystem.

In the third screening, 4 isolates were obtained from St.1, 47 ones from St. 2, 4 ones from St. 3. The proportion of fermentative yeasts in YPD₃₀ to that in YPD₂ was 4.6 % from St. 1, 38.8 % from St. 2, and 30.8 % from St. 3. The number and proportion of fermentative yeasts in YPD₃₀ was also high in St. 2.

3.2 Assay of gas productivity by the yeast

The amount of gas produced by the yeast was measured by its storage in the syringe. Figure 5 shows the gas productions of the yeasts: the 55 isolates and the strain C19. The 29 strains from the 55 ones had higher gas productivities than the strain C19 in YPD₂ at 25 °C. Above all, the best 4 strains with the highest gas productivities were found to be the strains: HK6, HK9, HK21, and HK27 isolated from St. 2.

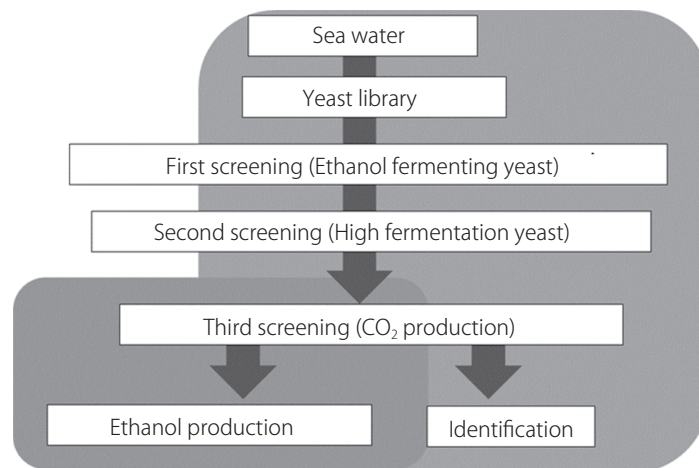


Figure 3: Flow chart for isolation and characterization of the yeasts

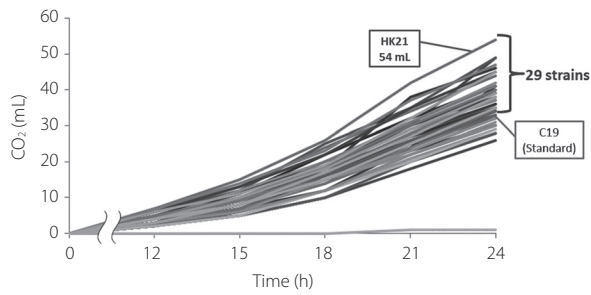


Figure 5: CO₂ production by a syringe test

3.3 Carbon dependence of fermentation and identification of the yeast

Table 1 shows the carbon dependence of yeast fermentation in the selected strains HK6, HK9, HK21, and HK27 and the strain C19. All the strains had fermentative activities of fructose, glucose, maltose, mannose, raffinose, and sucrose and no activities of arabinose, galactose, and xylose. The strains HK6, HK9, HK21, and HK27 were found to have the same carbon dependence of fermentation as *S. cerevisiae* C19.

Table 2 shows the results of the identification about the selected strains HK6, HK9, HK21, and HK27. All the 4 strains were found to be *S. cerevisiae* and a simple method for isolation of *S. cerevisiae* from coastal waters could be developed in this study.

Table 1: Carbon dependency for yeast fermentation

	HK6	HK9	HK21	HK27	C-19
Glucose	++	++	++	++	++
Arabinose	-	-	-	-	-
Maltose	++	++	++	++	++
Raffinose	+	+	+	+	+
Galactose	-	-	-	-	-
Sucrose	++	++	++	++	++
Fructose	++	++	++	++	++
Mannose	++	++	++	++	++
Xylose	-	-	-	-	-

Notes: CO₂ production at 0-7 days: ++, at 7 days -: +, No CO₂ production: -

Table 2: Identification of yeast with the highest gas producing activities

Strain No.	Yeast species
HK21	<i>Saccharomyces cerevisiae</i>
HK6	<i>Saccharomyces cerevisiae</i>
HK9	<i>Saccharomyces cerevisiae</i>
HK27	<i>Saccharomyces cerevisiae</i>

3.4 Relationship between ethanol productivity and gas productivity of the yeast

Figure 6 shows ethanol productivities of the selected strains HK10, HK17, HK18, HK20, HK21, HK26, HK41, HK42, and HK43, and the strain C19. The ethanol produced by the

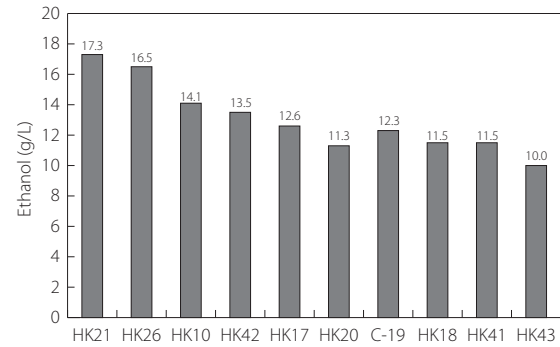


Figure 6: Ethanol production by the selected yeasts in the syringe for 24 h

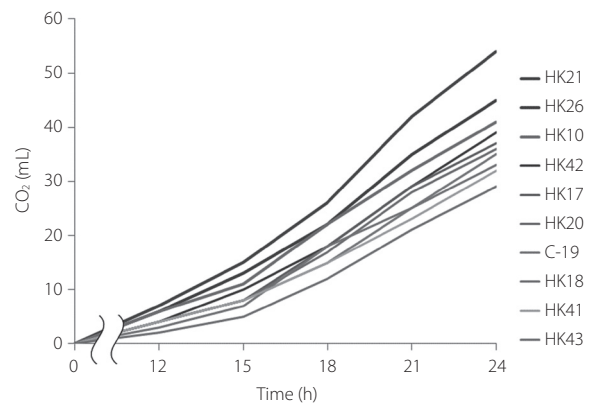


Figure 7: Gas production by the selected yeasts in the syringe for 24 h

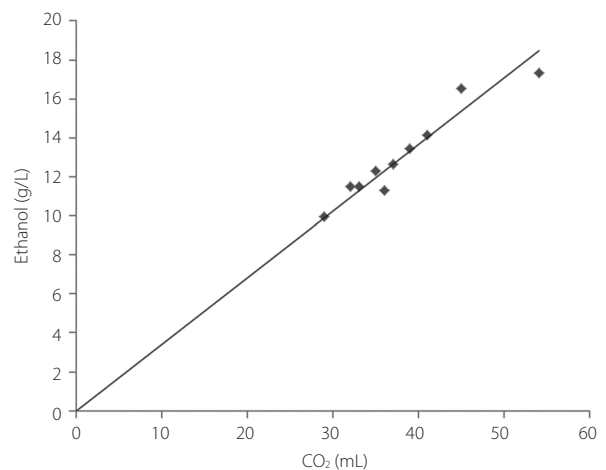


Figure 8: Relationship between ethanol production and gas production by the selected yeasts

yeasts were 10.0 – 17.3 g/l for 24 h and 5 out of the 9 strains had higher ethanol productivities than the strain C19. Figure 7 shows the gas productivities of the selected strains HK10, HK17, HK18, HK20, HK21, HK26, HK41, HK42, and HK43, and the strain C19. Six strains had higher gas productivities than the strain C19. Figure 8 shows the relation between ethanol and gas productivities of the selected 9 strains and C19. An almost linear relationship between ethanol and gas productivities in the 10 strains was found. The ethanol productivities by the yeasts could be speculated from the gas productivities by themselves.

4. Discussion

Bread was made with baker's yeast in about BC2,000 and beer was made by brewer's yeast in about BC1,500 in Mesopotamia. Sake was also made by sake yeast from ancient times in Japan. The suitable yeasts for various industries have been isolated and bred according to the kinds of fermentation for a long time. All industrial yeasts have the same characters of high fermentative and alcohol-tolerant activities. After the 18th century, identification methods for yeasts were developed and most of the yeasts used in the fermentation industries were found to be the species of *Saccharomyces cerevisiae*. From ancient to modern times, *S. cerevisiae* has been the most important yeast species in the history of humans. Although the *S. cerevisiae* was generally isolated from terrestrial origins, it can also be found from marine origins and applied to various purposes (Takagi et al., 2015; Obara et al., 2015; Obara et al., 2012; Saravanakumar et al., 2013; Ogawa et al., 2008a; Ogawa et al., 2008b; Takagi et al., 2012; Obara et al., 2014). However, it has been very difficult to isolate the *S. cerevisiae* from marine origins because their population density is very low in the sea. In this study, three step screenings of high fermentative yeasts were carried out and it could be successful in producing a concentration of the *S. cerevisiae* cells from all yeasts from coastal waters. The *S. cerevisiae* from coastal waters has salt/osmotic tolerance and could be applied to the high concentration of bioethanol production (Takagi et al., 2015; Obara et al., 2015; Obara et al., 2012). Further studies are directed towards the application of the marine-derived *S. cerevisiae* to various fermentation industries.

References

- Altshul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, Vol. 25, No.17, 3389-3402.
- Barnet, J. A., Payne, R. W. and Yarrow, D. (2000). *Yeast: Characteristics and identification*, 3rd Ed.. Cambridge University Press.
- Chen, Y. S., Yanagida, F. and Chen, L. Y. (2009). Isolation of marine yeasts from coastal waters of northeastern Taiwan. *Aquatic Biology*, Vol. 8, 55-60.
- Mastuda, K., Guo, X. F., Uryu, N., Hagiwara, T. and Watabe, S. (2008). Isolation of marine yeasts collected from the Pacific Ocean showing a high production of γ -aminobutyric acid. *Bioscience, Biotechnology, and Biochemistry*, Vol. 72, No.12, 3265-3272.
- Obara, N., Okai, M., Ishida, M. and Urano, N. (2015). Bioethanol production from mixed biomass (waste of wakame processing and paper shredding) by fermentation with marine-derived *Saccharomyces cerevisiae*. *Fisheries Science*, in press.
- Obara, N., Okai, M., Furukawa, A., Ishida, M. and Urano, N. (2014). Effect of various parameters (sugar/salt conc. and yeast sp.) on ethanol production from aquatic biomass with yeast. *Studies in Science and Technology*, Vol. 3, No. 2, 145-150.
- Obara, N., Ishida, M., Hamada-Sato, N. and Urano, N. (2012). Efficient bioethanol production from paper shredder scrap by a marine derived *Saccharomyces cerevisiae* C-19. *Studies in Science and Technology*, Vol. 1, No. 2, 49-54.
- Ogawa, G., Ishida, M., Usui, Y. and Urano, N. (2008a). Ethanol production from the water hyacinth *Eichhornia crassipes* by yeast isolated from various hydrospheres. *African Journal of Microbiology Research*, Vol. 2, 110-113.
- Ogawa, G., Ishida, M., Shimotori, K. and Urano, N. (2008b). Isolation and characterization of *Saccharomyces cerevisiae* from hydrospheres. *Annals of Microbiology*, Vol. 58, No. 2, 262-264.
- Saravanakumar, K., Senthilraja, P. and Kathiresan, K. (2013). Bioethanol production by mangrove-derived marine yeast, *Saccharomyces cerevisiae*. *Journal of King Saud University - Science*, Vol. 25, 121-127.
- Sreedevi, N. K. and Rosamma, P. (2008). Marine yeasts – a review. *Yeast*, Vol. 25, No. 7, 465-483.
- Takagi, T., Uchida, M., Matsushima, R., Kodama, H., Takeda, T., Ishida, I. and Urano, N. (2015). Comparison of ethanol productivity among yeast strains using three different seaweeds. *Fisheries Science*, in press.
- Takagi, T., Uchida, M., Matsushima, R., Ishida, M. and Urano, N. (2012). Efficient bioethanol production from water hyacinth *Eichhornia crassipes* by both preparation of the saccharified solution and selection of the fermenting yeasts. *Fisheries Science*, Vol. 78, Issue 4, 905-910.
- Ueno, R., Urano, N. and Kimura, S. (2002). Effect of temperature and cell density on the ethanol fermentation by a thermotolerant, aquatic yeast strain isolated from hot spring environment. *Fisheries Science*, Issue 3, Vol. 68, 571-578.
- Zhang, T., Chi, Z., Chi, Z., Parrou, J. L. and Gong, F. (2010). Expression of the inulinase gene from the marine-derived *Pichia guilliermondii* in *Saccharomyces cerevisiae* sp. WO and

ethanol production from inulin. *Microbial Biotechnology*,
Vol. 3, No. 5, 576-582.

(Received: March 10, 2015; Accepted: April 30, 2015)