Original Article

Cascade bioethanol productions from glucose and mannitol in saccharified kombu Laminariaceae by anaerobic and aerobic fermentations with two kinds of yeast

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Abstract

In this paper, cascade bioethanol productions from glucose and mannitol in the saccharified kombu (Laminariaceae) by two kinds of yeast were examined respectively. Mannitol exists as monosaccharide and glucose is polymerized to polysaccharides–cellulose, hemicellulose, and laminaran in brown algae. There have been only a few reports about ethanol production from mannitol by yeasts. Therefore, screening of mannitol-fermenting yeasts was carried out from both coastal waters and culture collections of the research institute in Japan. All sixteen NBRC strains of *Saccharomyces paradoxus* were found to produce 22-35 g/L of bioethanol aerobically from 100 g/L of mannitol at 30 ℃ for 5-7 days. Thus, the cascade bioethanol production system was constructed from the saccharified solution of kombu with anaerobic fermentation with marine-derived *Saccharomyces cerevisiae* C-19 (Takagi et al., 2015; Obara et al., 2015; Takagi et al., 2012a) followed with aerobic fermentation with *S. paradoxus* NBRC10553. In this system, 1.1 g/L of bioethanol was produced with the strain C-19 in the first step and 7.9 g/L of bioethanol was produced with the strain NBRC10553 in the second step from the saccharified kombu.

Key words

Bioethanol, Kombu Laminariaceae, Mannitol, *Saccharomyces cerevisiae*, *Saccharomyces paradoxus*

1. Introduction

Since the beginning of the industrial revolution in the 18th century, global warning with an increase of CO_2 in the atmosphere has gradually occurred, and the reason is thought to be overuse of fossil fuels such as coal, petroleum, and natural gas. Furthermore, due to a tsunami caused by the Great East Japan Earthquake followed by a nuclear power plant accident, the operation of most power plants and development of new nuclear power generation has been suspended in Japan since 2011. Under the present circumstances, recyclable energies such as solar photovoltaics, wind power, and biofuels are thought to have a carbon neutral process without CO_2 increase in the atmosphere and are expected as near future technologies. Above all, in recent years, bioethanol is increasingly being supplied to the market of car fuel in the world. In 2012, each share of bioethanol around the world was 63 % in the U.S.A., 25 % in Brazil, and only 0.03 % in Japan, respectively. In both the U.S.A. and Brazil, most bioethanol has been produced from agricultural biomass such as corn, sugarcane, wheat, rye, and sugar beet (Quintero et al., 2008; Vidmantiene et al., 2006; Wang et al., 2002; Ogbonna et al., 2001). However, the use of these crops as raw materials for bioethanol production usually competes with the needs of those as foods for people and livestock. In addition, the constancy of the material supply in a year seems to be lacking because of using only parts of these agricultural products. Otherwise, cellulose biomass sources, such as trees, weeds, and waste woods, are being investigated as potential substrates for bioethanol production because they do not compete with a food supply need and because large quantities of this type of biomass are currently available in the world.

Cellulose biomass generally tend to be considered from only terrestrial origins, however, the biomass from aquatic origins are also worthy of consideration. Studies have been made on the production of bioethanol from various kinds of biomass such as seaweeds; wakame *Undaria pinnatifida* (Takagi et al., 2015; Obara et al., 2015; Takagi et al., 2012a), anaaosa *Ulva* sp. (Takagi et al., 2015; Takagi et al., 2012a), sizime *Costaria costata* (Takagi et al., 2015; Takagi et al., 2012a), an alien water plant; water hyacinth *Eichhornia crassipes* (Takagi et al., 2012b; Obara et al., 2014; Ogawa et al., 2008), and paper, or wood scrap (Obara et al., 2015; Obara et al., 2014; Obara et al., 2012) by fermentation with marine-derived yeasts. Among aquatic biomass, brown algae, one of the seaweeds, are abundant, and grow widely and rapidly in the shallow sea. They are harvested for food supply, however, much of their processing wastes are usually discarded. Thus, use of the wasted brown algae as biomass material seems to be economical and ecological. Studies have already been made on the efficient bioethanol production from glucose containing in the saccharified seaweeds or alien water plant by marine-derived yeasts (Takagi et al., 2015; Obara et al., 2015; Takagi et al., 2012a; Takagi et al., 2012; Obara et al., 2014; Ogawa et al., 2008). However, the brown algae such as the kombu Lami-

nariaceae, and the wakame *Undaria pinnafida* contain a high concentration of mannitol which cannot be fermented by even *S. cerevisiae* and most of yeasts with high fermentative activities of glucose. Therefore, isolation of novel mannitol-fermenting yeasts and construction of a bioethanol production system from mannitol in addition to glucose by those strains would be expected.

2. Materials and methods

2.1 Yeast culture

Firstly, three yeast strains, *Debaryomyces hansenii* 0794, *Pichia angophorae* 10016, and *Saccharomyces paradoxus* 0259 were purchased from the culture collections of NITE Biological Research Center (NBRC) because the strains were reported to have mannitol-assimilation/fermentation activities (Ota et al., 2013 and Ota et al., 2011). Sixteen strains of *S. paradoxus* 0263, 1804, 1805, 10552, 10553, 10554, 10555, 105556, 10609, 10695, 102001, 102002, 102003, 102004, 102005, and 102006 were also purchased from NBRC in order to examine mannitol- assimilation/fermentation activities.

Secondly, natural yeasts were isolated from the coasts of the Sanriku district in Miyagi prefecture, Miura peninsula in Kanagawa prefecture of Japan and mannitol-fermenting strains from the isolates were screened by a method as explained below. Coastal waters, muds, and seaweeds were collected in the fields and rapidly transferred to a laboratory in Tokyo University of Marine Science and Technology at cold temperature. The samples for cultivation from them were prepared as follows; the coastal waters were filtrated through membrane filter (pore size 0.2 μm), microorganisms were enriched by about 100 folds, and used as the samples for inoculation. Both muds and weeds were mixed with physiological saline (0.85 %w/v NaCl) at a ratio of about 1:1 and used as the samples. Portions of the samples were inoculated onto YPD solid medium (D-glucose, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L; chloramphenicol, 0.2 g/L; agar, 15 g/L) and incubated at 25 ℃ for several days. Microbial cells of grown colonies after isolation procedure were observed under a microscope, and yeasts or yeast-like cells morphologically were preserved at both 4 ℃ and −80 ℃.

2.2 Qualitative analyses about assimilation/fermentation of mannitol/glucose by the yeasts

To prepare testing yeasts, the cells from a fresh YPD solid medium were inoculated into each test tube containing 10 ml of the YPM_2 liquid medium (mannitol, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L) or the YPD_2 liquid medium (glucose, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L). In the fermentation test, each test tube containing a Durham pipe was anaerobically incubated at 25 ℃ for 2 weeks using AnaeroPack system (Mitsubishi Gas Chem. Co. Inc.), and gas produced with the yeasts into the pipe was observed with the naked eye. In the mannitol-assimilation test, the cells from a fresh YPD solid medium were inoculated into each test tube containing 10 ml of the YPM_2 liquid medium (mannitol, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L) and aerobically incubated at 25 ℃ in a water bath shaker at 120 rpm for 2 weeks. Mannitol-assimilating yeasts were screened by observing white flocculates of the cells in the upper layer of the YPM_2 medium using the modified method of Ota et al., 2013.

2.3 Quantitative analysis about fermentation of mannitol by the yeasts

The yeast isolates with mannitol-assimilation activities were tested for fermentation of mannitol. Their cells from a fresh YPD solid medium were inoculated into each test tube with L-form containing 10 ml of the YPM_2 liquid medium, and incubated at 30 ℃ in the water bath shaker at 120 rpm for 24 h. Each 10 ml of the yeast culture was transferred to the 100 ml of the YPM_2 liquid medium in 500 ml of each Erlenmeyer flask, and incubated at 30 ℃ in the water bath shaker at 120 rpm for 24 h. After the centrifugation at 3,000 rpm for 15 min, the collected cells were washed with saline, and centrifuged again. The washing procedure was repeated twice and the yeast cell pellet was obtained. A portion of the pellet was inoculated to 10 ml of the YPM_2 liquid medium (mannitol, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L) or the YPM_{10} liquid medium (mannitol, 100 g/L; peptone, 20 g/L; yeast extract, 10 g/L) at optical density 0.5 at 660 nm in the test tube with L-form, and incubated at 30 ℃ in a water bath shaker at 120 rpm.

Aerobic fermentation with the yeasts was carried out for 8 days and the amounts of mannitol, glucose, and ethanol was measured by a Shimadzu prominence HPLC system (Shimadzu Co.). The condition of the HPLC was as follows; Refractive Index Detector (RID-10A, Shimadzu Co.), column (Shim-pack SPR-Pb, particle: 8 μm, size: 250 × 7.8 mm, Shimadzu Co.), mobile phase: distilled water (Wako Pure Chem. Ind.), flow rate: 0.6 ml/min, column temp.: 80 ℃.

2.4 Saccharification of raw materials

Kombu and wakame are cultivated by coastal farms in the Tohoku district of Japan. Their root fractions are usually disposed of and the blades were also discarded in case of injuring their qualities and declining the commodity values. In this study, both the root fractions and the blades were collected, frozen, quickly transferred to the Tokyo University of Marine Science and Technology in Tokyo, maintained at -80 ℃ in the laboratory, and used as raw materials. In the case of saccharification, the frozen materials were broken into small pieces by a grinder, reduced to powder by an electric mortar, and dried at 70℃ overnight. 3 g of the dried powder were added to 100 ml of 3 % (v/v) sulfuric acid in a 200 ml flask and the mixture was autoclaved at 121 ℃ for 1 h. The acid hydrolysate was neutralized with NaOH up to pH 4.6 and 900 IU of

cellulase GC220 (Genencor International Inc., USA) was added to the supernatant. The suspension was maintained at 50°C and stirred at 300 rpm for 24 h. After saccharification of the materials, the suspension was centrifuged at 3,000 rpm for 10 min, the precipitate was removed, and the supernatant was prepared as the saccharified solution.

2.5 Cascade bioethanol productions from glucose and mannitol in the saccharified solution by the yeasts

The kombu saccharified solution was concentrated five times by an evaporator and the concentrate was used as the substrate for the bioethanol productions. In the case of the cascade process, *S. cerevisiae* C-19 was used for anaerobic fermentation and *S. paradoxus* NBRC0259 was used for aerobic fermentation. In the first step, strain C-19 was inoculated into the test tube containing 10 ml of the substrate, anaerobically fermented at 30 ℃ for 24 h, and the amounts of monosaccharides and ethanol were measured by HPLC. In the second step, the fermented suspension with the C-19 was centrifuged at 3,000 rpm for 15 min and the cells were removed. Strain NBRC0259 was inoculated into the test tube containing the supernatant, aerobically fermented at 30 ℃ for 7 days, and the amounts of the monosaccharides and ethanol were measured by HPLC.

3. Results

3.1 Isolation of natural yeasts and qualitative analyses about assimilation/fermentation of mannitol/glucose by the yeasts

Table 1 shows the number of yeast isolates from various coasts. 285 strains were obtained in the Sanriku distrct of Miyagi prefecture, 587 strains in Miura peninsula of Kanagawa prefecture, and 251 strains in Boso peninsula of Chiba prefecture. The total isolates from the coasts were 1,123 strains and they were preserved at −80 ℃ as the yeast library. The number of the yeasts fermenting glucose in the library was as follows; 13 strains from Sanriku, 77 strains from Miura, and 20 strains from Boso. The total glucose-fermenting yeasts were 110 strains. No yeasts fermenting mannitol were isolated in the library using CO_2 accumulation test in the Durham pipe. The mannitol-assimilating yeasts isolated by observing cell flocculation were as follows; 28 strains from Sanriku, 78 strains from Miura, and 9 strains from Boso. The total mannitol-assimilation yeasts were 115 strains. From 115 strains with mannitol-assimilation activities, the yeasts with mannitol-fermentation activities were screened (detailed data not shown) and the 4 strains were isolated as follows; *Debaryomyces hansenii* M11, M22, and M27 and *Candida sake* T13, which were identified by 26S rDNA D1/D2 domain sequence.

3.2 Quantitative analysis about fermentation of mannitol by the yeasts

Figure 1(a) and (b) shows the change of mannitol and ethanol concentration in the YPM_2 medium during aerobic fermentation with M11, M22, M27, and T13. The mannitol

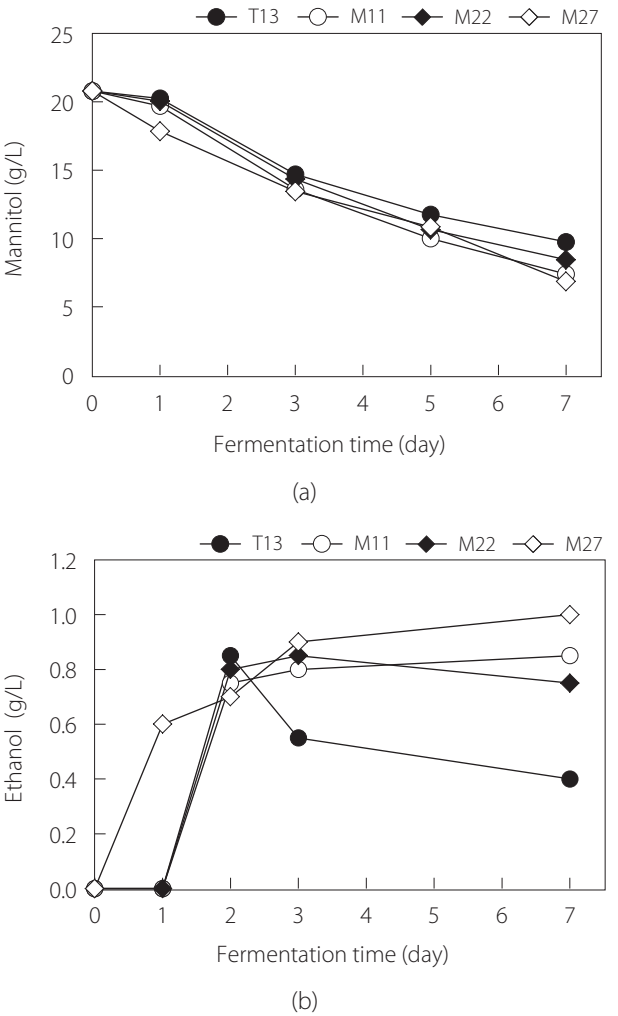

Figure 1: Ethanol fermentation from mannitol in the YPM_2 liquid medium with the selected yeasts by aerobic condition

Note: The amounts of (a) mannitol and (b) ethanol produced with the yeasts during 7 days.

Table 1: Number of yeast isolates from various coasts

Sampling site	No. of isolates	Gas-forming	Flocculation
Sanriku district in Miyagi	285	13	28
Miura peninsula in Kanagawa	587	77	78
Boso peninsula in Chiba	251	20	9
Total	1,123	110	115

concentration decreased from 20 to 9.8-6.1 g/L in 7 days with these strains. M27 had the highest activity for the mannitol-fermentation and produced 1.0 g/L of ethanol in 7 days. M11, M22, and T13 also produced 0.75-0.85 g/L of ethanol in 3-7 days. Otherwise, anaerobic fermentation in the YPM_2 medium with those strains were carried out and little change of mannitol and ethanol in the liquids was found (data not shown).

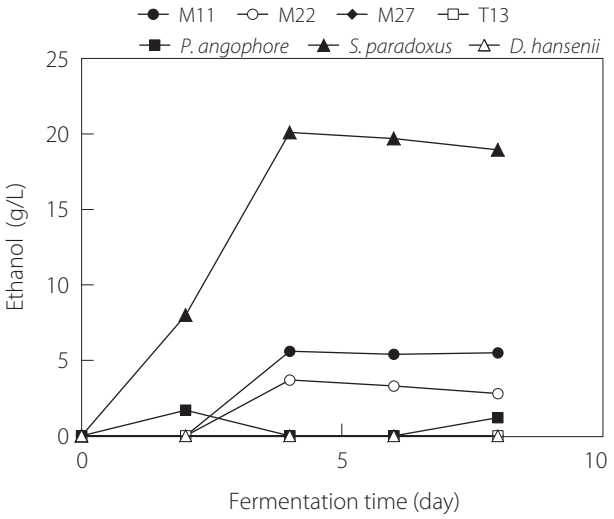

Figure 2: Comparison of ethanol productivities from mannitol in the YPM_{10} liquid medium with the selected yeasts and NBRC yeasts by aerobic condition

The phenomena seemed to be due to mannitol dehydrogenase catalyzing the reaction from mannitol to fructose only under aerobic condition (Ota et al., 2013).

Therefore, mannitol fermentation in the YPM_{10} medium under aerobic condition was carried out using the strains; *Debaryomyces hansenii* M11, M22, M27, and NBRC0794, *Candida sake* T13, *P. angophoae* NBRC10016, and *S. paradoxus* NBRC0259 in this study. *S. paradoxus* NBRC0259 produced 20.0 g/L of ethanol and it had higher fermentative activities than other strains as shown in Figure 2. Thus, the mannitol fermentation was conducted using other 16 strains of *S. paradoxus* preserved at NBRC in the next step.

Figure 3 shows mannitol fermentations by 16 strains of *S. paradoxus*. In 7 days, 100 g/L of mannitol decreased to 0-27 g/L and the ethanol concentration increased from 0 to 22-35 g/L. Therefore, the specie of *S. paradoxus* was found to have high mannitol-fermenting activity. Above all, NBRC0263, 10553, and 10554 strains produced about 35 g/L of ethanol in 5 days and they were selected for the candidates of cascade bioethanol productions.

3.3 Comparison of both glucose/mannitol amounts in the various saccharified materials

Table 2 shows a comparison of monosaccharides and salt amounts in the saccharified solutions of the seaweeds. The root fractions of the wakame and the kombu contained 0.06-

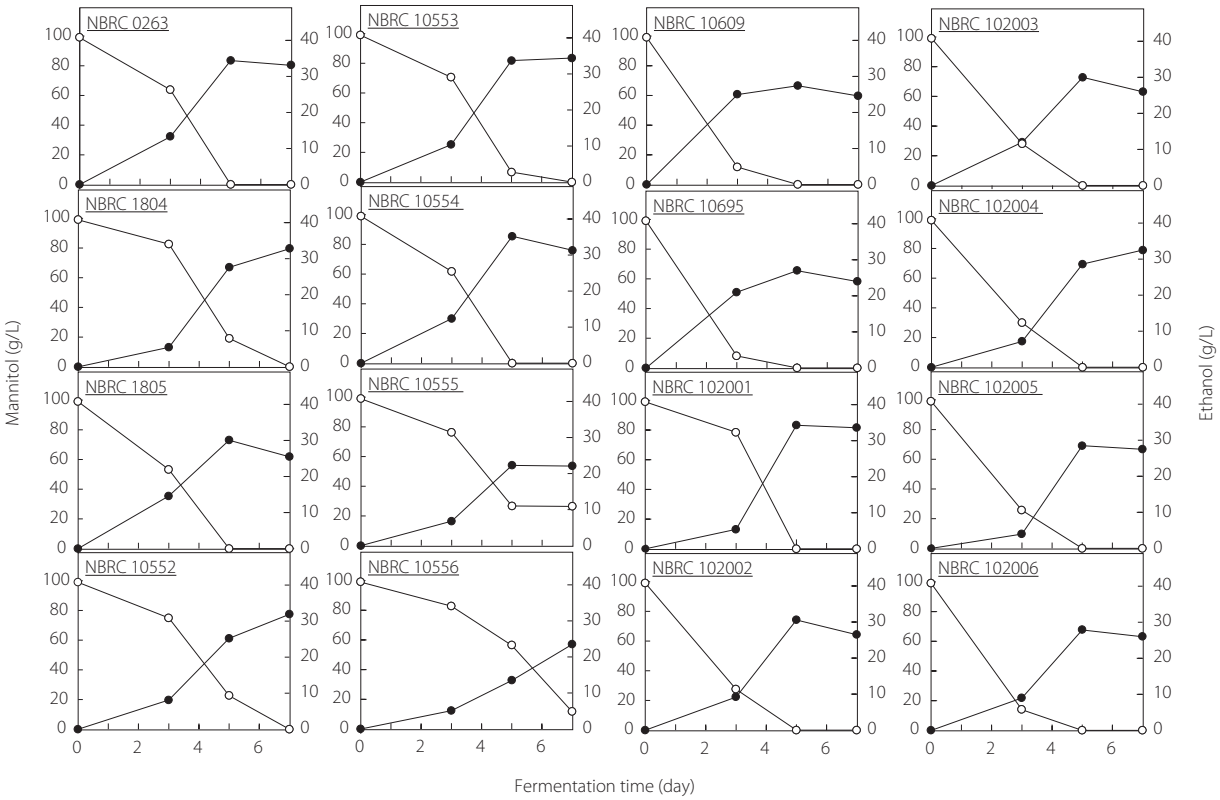

Figure 3: Ethanol fermentations from mannitol in the YPM_{10} liquid medium with sixteen *S. paradoxus* strains at NBRC
Note: Open circles; mannitol, closed circles; ethanol.

Table 2: Comparison of the monosaccharides and salt concentration in the saccharified solutions of the seaweeds

Seaweed	Part	Glucose (g/g dried seaweed)	Mannitol (g/g dried seaweed)	Salt (%)
Wakame	Root	0.061	0.032	1.32
Kombu	Blade	0.047	0.192	0.82
Kombu	Root	0.064	0.052	0.94

0.07 g glucose and 0.03-0.06 g mannitol/g weed. The blade of the kombu contained 0.047 g glucose/g weed and 0.192 g mannitol/g weed. The root fractions of the wakame and the kombu contained 0.94-1.32 g salt/g weed. The blade of the kombu contained 0.82 g salt/g weed. Thus, the blade of the kombu is thought to be suitable for bioethanol production.

3.4 Cascade bioethanol productions from glucose and mannitol in the saccarified solution by the yeasts

Figure 4(a), (b), and (c) show the aerobic fermentation of the saccharified solution of the kombu blade with *S. cerevisiae* C-19 or *S. paradoxus* NBRC0259. The glucose concentration decreased from 7 to 0 g/L by the C-19 or the NBRC0259 in 3 days as shown in Figure 4(a). The mannitol concentration decreased from 26 to 22 g/L with the C-19 or to 2 g/L with the NBRC0259 in 7 days as shown in Figure 4(b). No ethanol was produced with the C-19 and 1.6 g/L of ethanol was produced with the NBRC0259 in 7 days as shown in Figure 4(c).

Figure 5(a), (b), and (c) show anaerobic fermentation of the saccharified solution of the kombu blade with *S. cerevisiae* C-19 in the first step of cascade fermentation. The mannitol decreased a little, the glucose concentration decreased from 4.8 to 0 g/L, and ethanol produced 1.1 g/L with C-19 within 1 day.

Figure 5(d) shows aerobic fermentation of the first step products with *S. paradoxus* NBRC0259, 0263, 10553, or 10554 in the second step fermentation. The ethanol produced 6.0-7.9 g/L in 3 days with the four strains and NBRC10553 was found to have the highest ethanol productivity.

4. Discussion

Our studies have been made on the isolation of high glucose-fermenting yeasts from various hydrospheres and application them to bioethanol production (Obara et al., 2015a, Obara et al., 2015b, Obara et al., 2014a, Obara et al., 2014b, Obara et al., 2012, Ogawa et al., 2008, Takagi et al., 2015, Takagi et al., 2012a, Takagi et al., 2012b). However, there are only a few studies about mannitol-fermenting microorganisms. In the case of wild type bacteria, Wang et al. (2013) reported bioethanol production from mannitol by a newly isolated bacterium, *Enterobacter* sp. JMP3. In an anaerobic condition, it produced 1.15 mol ethanol/mol mannitol. The

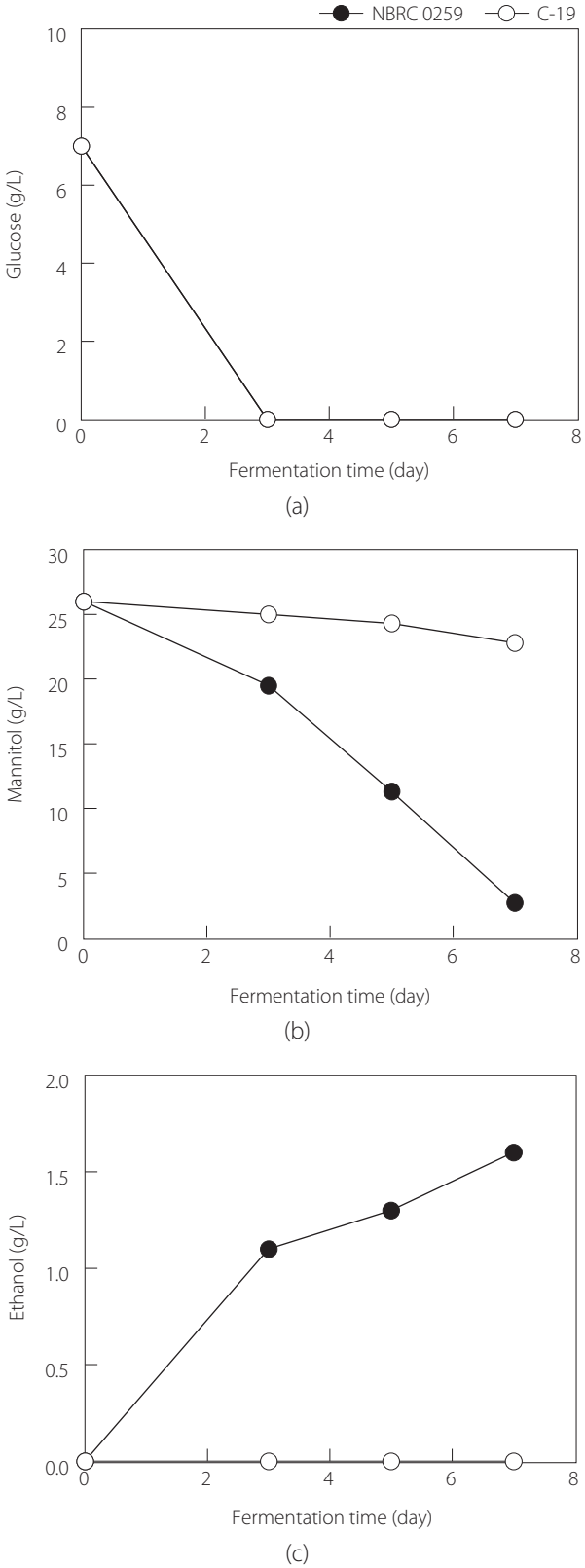

Figure 4: Comparison of ethanol fermentations from the saccharified solution of the kombu blade with *S. paradoxus* NBRC 0259 and *S. cerevisiae* C-19 by aerobic condition

Note: The measurements of the amounts of (a) glucose, (b) mannitol and (c) ethanol during 7 days.

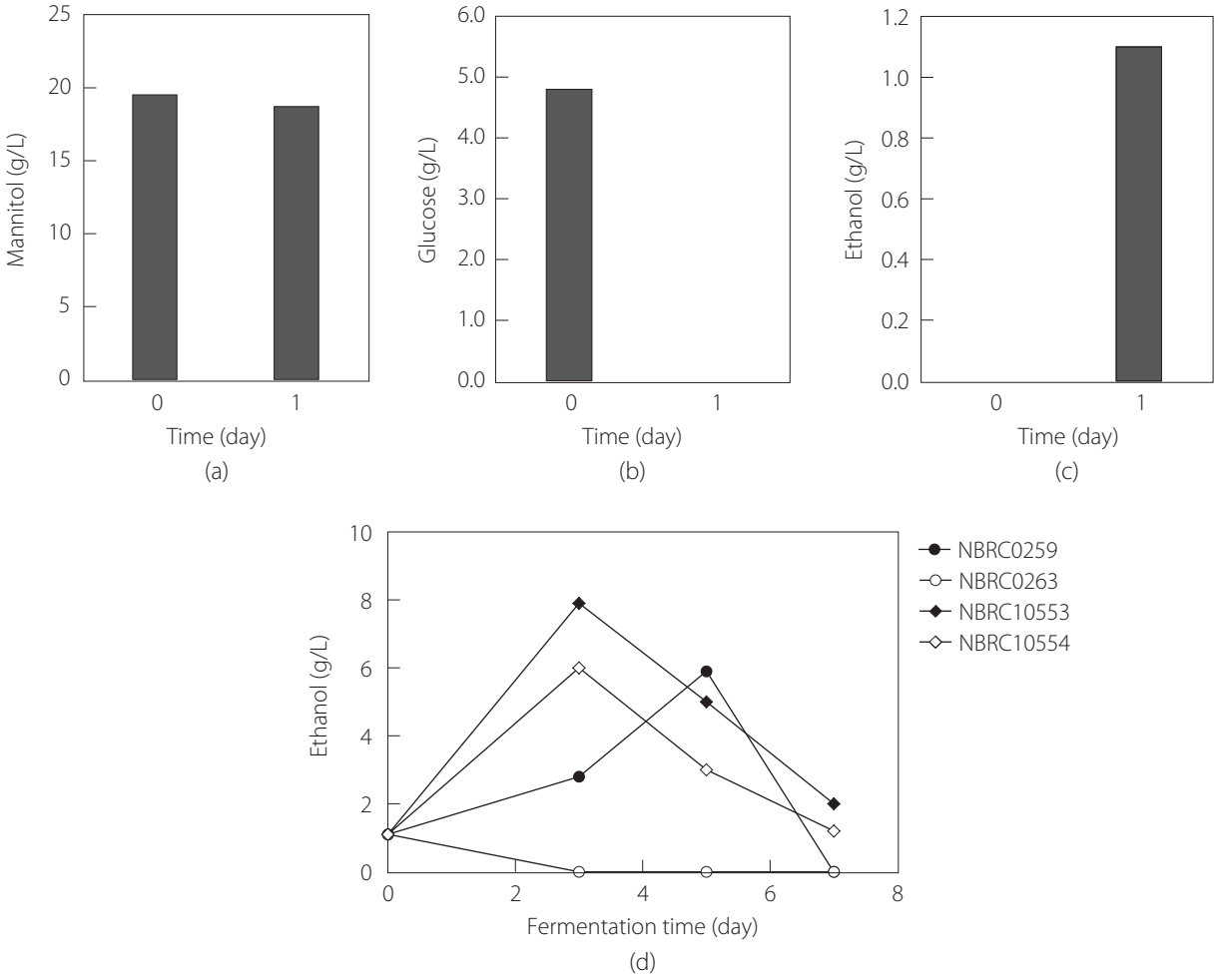

Figure 5: Cascade bioethanol productions using the saccharified solution of the kombu blade by a two-step fermentation system

Note: The measurements of the amounts of (a) mannitol, (b) glucose and (c) ethanol after the anaerobic fermentation using the saccharified kombu with *S. cerevisiae* C-19. (d) Comparison of ethanol fermentations from mannitol after aerobic fermentation with four strains of *S. paradoxus*.

mannitol was revealed to be more favorable than glucose for ethanol production. Horn et al. (2000a) also reported ethanol production from mannitol by *Zymobacter palmae* ATCC51623, whose strictly anaerobic fermentation was not observed in the synthetic mannitol media, but it produced 0.38 g ethanol/g mannitol under an O_2-limiting condition in the same medium. Using genetically modified bacterium, Kim et al. (2011) studied ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. The strain cloned the ethanol production genes from *Zymomonas mobilis*, utilized both glucose and mannitol, and produced 0.4 g ethanol/g carbohydrate from *Laminaria japonica*. However, high concentrated fermentation could not be attained using the bacteria because their phenotype were common in lower tolerance against osmotic pressure than that of yeasts. Otherwise, Horn et al. (2000b) reported *Pichia angophorae* CBS5320 was able to utilize laminaran and mannitol for ethanol production from seaweed extracts. Ota et al. (2013) also studied the production of ethanol from mannitol by *S. paradoxus* NBRC0259. However, it remains unknown whether its phenotype was specific to the strain or the specie.

In this study, all sixteen NBRC strains of *S. paradoxus* aerobically produced 22-35 g/L of the bioethanol from 100 g/L of the mannitol at 30 °C for 5-7 days and the yeasts' phenotype was found to be specific to the specie. Therefore, the cascade bioethanol production system was constructed from the saccharified kombu with anaerobic fermentation by marine-derived *Saccharomyces cerevisiae* C-19 followed with aerobic fermentation by *S. paradoxus* NBRC10553 and 7.9 g/L of bioethanol was produced in this system.

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