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Title

Production of ethanol from mannitol by the yeast strain *Saccharomyces paradoxus* NBRC 0259

Running title

PRODUCTION OF ETHANOL FROM MANNITOL

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Key words.

macroalgae; yeast; mannitol; ethanol; flocculation; *Saccharomyces paradoxus*

ABSTRACT

Mannitol is a promising marine macroalgal carbon source. However, organisms that produce ethanol from mannitol are limited; to date, only the yeast *Pichia angophorae* and the bacterium *Escherichia coli* KO11 have been reported to possess this capacity. In this study, we searched a yeast strain with a high capacity to produce ethanol from mannitol and selected *Saccharomyces paradoxus* NBRC 0259 for its ability to produce ethanol from mannitol. This ability was enhanced after a 3-day cultivation of this strain in medium containing mannitol; the enhanced strain was renamed *S. paradoxus* NBRC 0259-3. We compared the ability of strain NBRC 0259-3 to produce ethanol from mannitol and glucose, under several conditions, with those of *P. angophorae* and *E. coli* KO11. As a result, we concluded that *S. paradoxus* NBRC 0259-3 strain is the most suitable yeast strain for the production of ethanol from mannitol.

Marine biomass, including macroalgae, is a promising source of biofuels (1, 2). The major advantages of macroalgae with respect to biofuels production are (i) the greater productivity of macroalgae over land crops; (ii) the lack of a requirement of arable land for algal cultivation, avoiding irrigation of water, use of fertilizer, etc.; and (iii) the absence of lignin in macroalgae (2-4). Macroalgae comprise green, red, and brown algae (4); high contents of carbohydrates have been reported in red and brown algae. It is very important to develop a method for producing biofuels from the carbohydrates in these algae.

The major carbohydrate constituents of brown algae are mannitol, the sugar alcohol corresponding to mannose (5), and alginate, a linear polysaccharide consisting of two monosaccharides, β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G), in which the two monosaccharides are arranged in three different configurations: polyM, polyG, and heteropolymeric random sequences (polyMG) (6). The brown alga *Laminaria japonica* contains up to 30% mannitol and 25% alginate; these and subsequent figures represent percentage of dry weight (7). Zubia *et al*. reviewed the contents of mannitol (up to 33%) and alginate (up to 40%) in several brown algae of genera *Sargassum* and *Turbinaria* (8). Horn *et al*. also reported that the brown alga *Laminaria hyperborea* contains 25% mannitol (9).

In order to utilize brown algae as a source for bioethanol, it will be necessary to develop systems for production of ethanol from both mannitol and alginate. Two such systems have been established (10). The first of these utilizes the ethanologenic bioengineered *Sphingomonas* sp. strain A1 (ethanologenic strain A1) that can produce 13 g/L ethanol from alginate (10). Unfortunately, *Sphingomonas* sp. strain A1 is not able to assimilate mannitol (11). The other established system utilizes a bioengineered ethanologenic *Escherichia coli* strain that is able to produce 37 g/L ethanol from brown algae (kombu; *Saccharina japonica*) containing a mixture of mannitol and alginate (12).

Despite the higher yield in the bacterial system, bacteria are generally sensitive to ethanol (9, 13); therefore, yeast is considered to have several advantages over ethanologenic bacteria, including high tolerance to ethanol and other inhibitory compounds (14). Two bacterial strains, *Zymobacter palmae* and *E. coli* KO11, can produce ~13 and 26 g/L ethanol from mannitol, respectively; however, both bacteria are sensitive to 50 g/L ethanol (9, 13). Therefore, it would be ideal to establish a system for ethanol production from alginate and mannitol that utilizes a bioengineered yeast, e.g. *Saccharomyces cerevisiae*.

As an initial step, we envisioned a two-step fermentation in which alginate is first converted to ethanol by the ethanologenic strain A1, and the remaining mannitol is then converted to ethanol by yeast. Ethanol has been produced from mannitol by some yeast strains, e.g., *S. cerevisiae* polyploid strain BB1 (5 g/L) and *Pichia angophorae* (14.4 g/L) (9, 15, 16). By contrast, however, other *S. cerevisiae* strains, e.g., polyploid BB2, haploid S288C, and haploid *Sc41 YJO*, are unable to assimilate mannitol for growth (15, 17). Therefore, we decided to search for yeast suitable for production of ethanol from mannitol.

MATERIALS AND METHODS

Microorganisms

The yeast strains used in this study are listed in Table 1. Ethanologenic *Sphingomonas* sp. strain A1 (EPv104), carrying eight copies of the *Zymomonas mobilis* pyruvate carboxylase gene and one copy of the *Z. mobilis* alcohol dehydrogenase gene on plasmid pKS13, was described previously (10). *E. coli* strain KO11 (ATCC 55124) was purchased from the American Type Culture Collection. *P. angophorae* (CBS5830) (9) was purchased from CBS-KNAW Fungal Biodiversity Centre. These strains were stocked at -80˚C in the presence of 17% (v/v) glycerol.

Media

Complete synthetic medium without carbon source (SC-C) (pH 5.6) consisted of the following (in g/L): yeast nitrogen base w/o amino acid (Becton, Dickinson and Company, Sparks, MD), 6.7; -Leu Do supplement (Clontech, Palo Alto, CA), 0.69; and L-Leucine (Nacalai Tesque, Kyoto, Japan), 0.1. SC-C medium was supplemented with 20 g/L glucose (for SC medium), 20 g/L mannitol (for SM medium) or 3% (v/v) glycerol (for SG medium). Yeast extract/peptone medium (YP) (pH 5.6) consisted of the following (in g/L): yeast extract, 10; tryptone, 20. The YP medium was supplemented with 20 g/L glucose (for YPD medium), 20 g/L mannitol (for YPM medium), or 3% (v/v) glycerol (for YPG medium), until otherwise stated. LB medium (pH 7.2) consisted of the following (in g/L): yeast extract, 5; tryptone, 10; NaCl, 10. LB medium was supplemented with 20 g/L mannitol (for LBM medium) and 20 g/L glucose (for LBD medium). Solid media were generated by addition of 20 g/L agar (Nacalai Tesque) to the appropriate liquid media. YP and LB media were sterilized by autoclaving the base media separately from the carbon sources. Stocks of 10-fold concentrated YP and 10-fold concentrated LB were sterilized by filtration with 0.2-μm pore size. When necessary, cells were grown under anaerobic conditions using the AnaeroPack Anaero (Mitsubishi Gas Chemical, Tokyo, Japan).

ρ^0 yeast strains were produced by treating yeast with 25 μg/ml ethidium bromide (18). In *S. cerevisiae*, strains completely lacking mitochondrial genomes are denoted ρ^0, whereas strains harboring intact mitochondrial genomes are ρ^+ (18). ρ^0 strains fail to grow on YPG or YPM medium, which contain only nonfermentable carbon sources, due to their inability to perform respiration.

To prepare an A1 supernatant, ethanologenic *Sphingomonas* sp. strain A1 (EPv104) was cultured for 3 days at 30°C on a shaker (Personal Lt-10F, Taitec, Tokyo, Japan) at 95 strokes per minute (spm) in a liquid medium containing 50 g/L alginate as described (10). After 3 days of cultivation, the culture was centrifuged at 20,000×*g* for 10 min. The pH of the resulting supernatant was adjusted to pH 5.8 using HCl, yielding the "A1 supernatant". YP-A1 medium consisted of 22.5 ml A1 supernatant and 2.5 mL 10-fold concentrated YP (pH 5.8). YP2M-A1 and YP5M-A1 media were 25 ml YP-A1 media containing 0.5 g (20 g/L) and 1.25 g (50 g/L) mannitol, respectively. LB5M-A1 medium contained 22.5 ml A1 supernatant, 2.5 ml 10-fold concentrated LB, and 1.25 g (50 g/L) mannitol.

Cultivation

For cultivation of ethanologenic yeasts in liquid medium, fresh cells grown on solid YPM media were suspended in sterilized water (SDW) and added to 50 ml liquid YPM medium to give an OD_{600} of 0.1. Cultivation was conducted at 30°C in a 100 ml Erlenmeyer flask on a shaker (Personal Lt-10F) at 95 spm, unless otherwise stated. After 1 day of cultivation, cells were collected, washed once with SDW, suspended in SDW, and added to fresh 50 ml YPM or YPD medium to give an OD_{600} of 0.1; cultivation was continued at 30°C and 95 spm. For cultivation of *E. coli* KO11, LBM and LBD were used instead of YPM and YPD. *E. coli* cells were washed and suspended in SDW containing 10 g/L NaCl, and were cultured at 30°C and 95 spm. When YP2M-A1, YP5M-A1, and LB5M-A1 media were used, cultivation was conducted in 25 ml liquid medium in a 50 ml Erlenmeyer flask on a shaker (Personal Lt-10F) at 95 spm and 30°C.

Analytical methods

Ethanol concentrations in culture supernatants obtained by centrifugation (5 min, 20,000×*g*, 4°C) were determined using the ethanol assay F-kit (Roche Diagnostics, Basel, Switzerland). Concentrations of glucose and mannitol were determined using an HPLC equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad) and a RID-10A detector (Shimadzu, Kyoto, Japan). Other conditions were as follows: effluent, filtered and degassed 5 mM H_2SO_4; flow rate, 0.65 ml min^{-1}; and column temperature, 65.5°C. Detection limits for glucose and mannitol were 0.2 g/L.

RESULTS AND DISCUSSION

Identification of yeast strains producing ethanol from mannitol

SC and SM media are synthetic media containing each of 20 g/L glucose and 20 g/L mannitol as a carbon source, respectively; SC-C is a synthetic medium containing no carbon source. To identify yeast strains capable of producing ethanol from mannitol, we first searched for yeast strains that could utilize mannitol for growth. Of the 45 strains tested, 15 grew better on SM solid media than on SC-C solid or liquid media (Table 1). Among these 15 strains, six (*Saccharomyces paradoxus* NBRC 0259, *Kuraishia capsulata* NBRC 0721, *Kuraishia capsulata* NBRC 0974, *Ogataea glucozyma* NBRC 1472, *Ogataea minuta* NBRC 1473, and *Debaryomyces hansenii* NBRC 0794) produced at least 26 mg/L ethanol in SM liquid medium and at least 1.0 g/L ethanol from SC liquid medium without shaking (i.e., at 0 spm) (Fig. 1A). Of these six strains, *S. paradoxus* strain NBRC 0259 produced the highest amount of ethanol from mannitol in this condition (Fig. 1A) and in YPM liquid medium at 95 spm (Fig. 1B, Table 2) (19). Moreover, *S. paradoxus* strain NBRC 0259 consumed the highest amount of mannitol, exhibited the highest ethanol productivity and yield among these six strains in YPM liquid medium at 95 spm (Table 2), and also exhibited the highest tolerance to 50 g/L ethanol (Fig. 1C) (19).

Ethanologenic strain A1 can produce ethanol from alginate, but not from mannitol (10). An ideal yeast strain would be capable of producing ethanol from mannitol after the ethanologenic strain A1 had finished producing ethanol from alginate. To identify such a strain, we cultivated the ethanologenic strain A1 in a liquid medium containing 50 g/L alginate for 3 days and centrifuged to obtain the supernatant (A1 supernatant) which contained ~10.0 g/L ethanol and other unknown metabolic compounds. We then investigated whether the six yeast strains could produce ethanol from mannitol in YP2M-A1 (A1 supernatant plus YP and 20g/L mannitol) medium as described in MATERIALS AND METHODS. As shown in Fig. 1D, only *S. paradoxus* NBRC 0259 produced ethanol under this severe condition, suggesting that this strain is highly tolerant to the toxic compounds generated by ethanologenic strain A1 (Fig. 1D). Therefore, we selected *S. paradoxus* NBRC 0259 for further study.

Ethanol production from mannitol by *S. paradoxus* NBRC 0259

S. paradoxus NBRC 0259 exhibited Ca^{2+}-dependent flocculation, especially in the presence of glucose (Fig. 2A), whereas the other five strains did not. Yeast flocculation is a reversible, non-sexual cell aggregation in which cells adhere to each other in a Ca^{2+}-dependent manner to form flocs; it has been used in the brewing industry as a simple and cost-effective way to separate yeast cells from fermentation products (20). *S. paradoxus* strains have been isolated from natural and fermentative habitats (e.g., tree bark, oak tree bark, pulque fermentation, and wine fermentation) and are tolerant to ethanol (21, 22). *S. paradoxus* has also been regarded as an attractive model for population-genetic and genomic studies (23).

The polyploid *S. cerevisiae* strain BB1 needs oxygen to utilize mannitol, and exhibits high respiratory activity when growing in SM medium (15). Oxidation of mannitol to fructose by mannitol dehydrogenase is predicted to produce excess NADH (Fig. 2B); hence, it has been proposed that yeasts require respiration in order to assimilate mannitol (15). As shown in Fig. 2C, *S. paradoxus* NBRC0259 did not grow on SM medium under anaerobic conditions irrespective of the presence or absence of intact mitochondria, whereas a ρ^0 strain that lacks the mitochondrial genome failed to grow on SM medium even under aerobic conditions, demonstrating that *S. paradoxus* NBRC0259 requires oxygen and respiration to assimilate mannitol (19).

S. paradoxus NBRC 0259 maintained on YPD solid medium tended to lose the capacity to grow on YPM or YPG solid medium (five of six single colonies tested). Therefore, *S. paradoxus* NBRC 0259 ρ^+ strain was streaked from glycerol stock on YPM solid medium and grown on this medium, rather than YPD solid medium, in order to avoid losing its capacity to grow on YPM or YPG medium, i.e., to avoid becoming ρ^0. To monitor the effects of recent handling, *S. paradoxus* NBRC 0259 grown on YPM solid medium was pre-cultured in either YPM or YPD liquid medium, and then further cultured in YPM liquid medium. The ethanol productivity of cells pre-cultured in YPM liquid medium was significantly higher than that of cells pre-cultured in YPD liquid medium (Fig. 2D; closed and open circles). Thus, we chose YPM liquid medium as the medium for pre-culture.

Because *S. paradoxus* NBRC 0259 requires oxygen to assimilate mannitol, we examined the effects of aeration conditions on ethanol production. *S. paradoxus* NBRC 0259 pre-cultured in YPM liquid medium was cultured in YPM or YPD liquid medium at various shaking speeds (0, 95, and 145 spm) (Fig. 2E). At 145 spm in YPM medium, the strain exhibited the best growth, but no ethanol production. At 0 spm in YPM medium, the strain hardly grew and produced low ethanol concentration. At 95 spm in YPM medium, the strain displayed moderate growth and the highest ethanol production. Thus, moderate aeration by shaking at 95 spm was chosen as the aeration condition for production of ethanol from mannitol (19). This strain produced higher amounts of ethanol from glucose than those from mannitol at 0 and 95 spm, although it flocculated in YPD medium (Fig. 2A, E).

S. paradoxus NBRC 0259 produced ethanol less efficiently from mannitol than from glucose; ethanol production began after 2 or 3 days of cultivation (Fig. 2D, E). The original *S. paradoxus* NBRC 0259 cells that had been grown for 3 days in YPM liquid medium were frozen in the presence of 17% (v/w) glycerol and maintained at −80°C; this isolate was named *S. paradoxus* NBRC 0259-3. NBRC 0259-3 strain was streaked from glycerol stock on YPM solid medium, grown on this medium, pre-cultured in YPM liquid medium, and cultivated in YPM liquid medium at 30˚C and 95 spm to monitor the ethanol production. We observed that this strain started to produce ethanol more quickly than the original NBRC 0259 isolate (Fig. 2D; closed triangles); it also flocculated in the presence of glucose (data not shown). Hence, we selected NBRC 0259-3 strain for further study. We speculate that some epigenetic events, making yeasts ready to assimilate mannitol, possibly occurs during initial cultivation of original NBRC 0259 strain in YPM liquid medium.

Comparisons of the capacity to produce ethanol from mannitol

We compared the ethanol tolerance and ethanol productivity of *S. paradoxus* NBRC 0259-3 with those of two other microbes previously reported to produce ethanol from mannitol, *P. angophorae* (9) and *E. coli* KO11 (13). In contrast to the case of *S. paradoxus* NBRC 0259, these microbes' abilities to produce ethanol from mannitol were not enhanced after 3 days of cultivation in YPM or LBM (data not shown).

Among the three organisms, *S. paradoxus* NBRC 0259-3 exhibited maximum tolerance to 50 g/L ethanol (Fig. 3A). The three strains produced approximately the same amounts of ethanol from mannitol and glucose and also exhibited approximately the same productivity and yield (Fig. 3B, Table 2). However, in the presence of the A1 supernatant, in which ethanologenic strain A1 had produced approximately 10 g/L ethanol from a liquid medium containing 50 g/L alginate, KO11 did not produce ethanol from mannitol, whereas both yeasts did (Fig. 3C), indicating that *E. coli* KO11 is sensitive to the metabolites produced from alginate by ethanologenic strain A1, while yeasts are tolerant. In the presence of a high concentration of glucose or mannitol (100 g/L), *S. paradoxus* NBRC 0259-3 produced higher amount of ethanol from mannitol than *P. angophorae* did and higher amount of ethanol from glucose than *E. coli* KO11 did (Fig. 3D). In the presence of both 20 g/L glucose and 20 g/L mannitol (total sugars, 40 g/L), all three organisms utilized mannitol to produce ethanol, although glucose was consumed faster than mannitol (Fig. 3E).

Thus, *S. paradoxus* NBRC 0259-3 exhibited the highest tolerance to ethanol; high production of ethanol from mannitol in the presence of A1 supernatant; and high production of ethanol from high concentrations of glucose and mannitol (Fig. 3, Table 2). Based on these observations, we concluded that *S. paradoxus* NBRC 0259-3 is the most suitable yeast strain for the production of ethanol from mannitol, a promising marine macroalgal carbon source (19).

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paradoxus, Genetics, **166**, 43-52 (2004).

FIGURE LEGEND

FIG. 1. Properties of ethanologenic yeasts. (A) Ethanol production by ethanologenic yeasts cultured in 1.0 ml SM (gray bar) and SC (closed bar) liquid media without shaking. Scales on left and right sides indicate concentrations of ethanol from mannitol and glucose, respectively. (B) Ethanol production by ethanologenic yeasts cultured in 50 ml YPM liquid medium at 95 spm. (C) Ethanol tolerance of ethanologenic yeasts. Yeasts were inoculated into 1.0 ml YPM liquid medium with or without 50 g/L ethanol to give OD_{600} of 0.1 and grown for 1 day at 95 spm. OD_{600} of the culture with ethanol is shown as relative growth, taking that of the culture without ethanol as 1.0. (D) Ethanol production of ethanologenic yeasts cultured in 25 ml YP2M-A1 liquid medium (containing the A1 supernatant) at 95 spm. (A-D) Means and maximum and minimum values of two independent experiments are shown. Strains (A–D): 1, diamond, *Saccharomyces paradoxus* NBRC 0259; 2, triangle, *Kuraishia capsulata* NBRC 0721; 3, X, *Kuraishia capsulata* NBRC 0974; 4, circle, *Ogataea glucozyma* NBRC 1472; 5, +, *Ogataea minuta* NBRC 1473; 6, square, *Debaryomyces hansenii* NBRC 0794.

FIG. 2. Properties of *S. paradoxus* NBRC 0259. (A) Ca^{2+}-dependent flocculation. *S. paradoxus* NBRC 0259 strain was cultured for 1 day in 5 ml YPD or YPM liquid media, transferred to test tubes, and held for 10 min (left). To the culture, 500 mM EDTA was added to reach a final concentration of 50 mM, and then the culture was vortexed and held for 10 min (center). Cells were collected, washed once with SDW, resuspended in 10 mM $CaCl_2$, and held for 10 min (right). Flocculated NBRC 0259 cells were dispersed in 50 mM EDTA and flocculated again in 10 mM $CaCl_2$, demonstrating

Ca^{2+}-dependent flocculation. (B) Reaction catalyzed by mannitol dehydrogenase (19). (C) Growth of ρ^0 and ρ^+ strains of *S. cerevisiae* BY4742 and *S. paradoxus* NBRC 0259 on SM and SC solid media in the presence (+) or absence (−) of oxygen (O_2) after 4 days. (D) *S. paradoxus* NBRC 0259 cells pre-cultured for 1 day in YPM (closed circle) or YPD liquid medium (open circle), and *S. paradoxus* NBRC 0259-3 cells pre-cultured for 1 day in YPM liquid medium (closed triangle), were inoculated to YPM liquid medium and cultivated at 95 spm for the indicated periods. (E) Effect of shaking speed on ethanol production of *S. paradoxus* NBRC 0259 strain in YPM (closed symbols) or YPD (open symbols) liquid medium. This strain was pre-cultured in YPM liquid medium for 1 day and further cultured in YPM liquid medium with the indicated shaking speed (triangle, 145 spm; circles, 95 spm; squares, 0 spm). This strain produced no ethanol in YPM medium at either 0 or 145 spm. (D, E) Means and maximum and minimum values of two independent experiments are shown.

FIG. 3. Comparison of the ability to produce ethanol from mannitol. (A) Ethanol tolerance of the three organisms. Microbes were inoculated to 1.0 ml YPM (1, NBRC 0259-3; 2, *P. angophorae*) or LBM (3, *E. coli* KO11) liquid medium containing 0 and 50 g/L ethanol to give an OD_{600} of 0.1 and were grown for 1 day at 95 spm. OD_{600} of the culture containing ethanol is shown as relative growth, taking that of the culture without ethanol as 1.0. (B) Ethanol production of NBRC 0259-3 (triangles), *P. angophorae* (squares), and *E. coli* KO11 (diamonds) in YPM (closed symbols) or YPD (open symbols) liquid media. (C) Ethanol production by the three ethanologenic organisms as in (B) in YP5M-A1 or LB5M-A1 consisting of A1 supernatant, 50 g/L mannitol, and YP or LB. (D) Ethanol production of NBRC 0259-3 (left, NBRC), *P.*

angophorae (center, Pan), and *E. coli* KO11 (right, KO11) in YP plus 100 g/L glucose (open circles) or 100 g/L mannitol (closed circles). In the case of *E. coli* KO11, LB was used instead of YP. (E) Sugars (glucose, open circles; mannitol, closed circles) and ethanol (closed squares) in cultures of the three organisms, as in (D). The organisms were grown in YP (NBRC 0259-3 and *P. angophorae*) or LB (*E. coli* KO11) liquid medium containing both 20 g/L glucose and 20 g/L mannitol (total sugars, 40 g/L). (A-E) Means and maximum and minimum values of two independent experiments are shown.

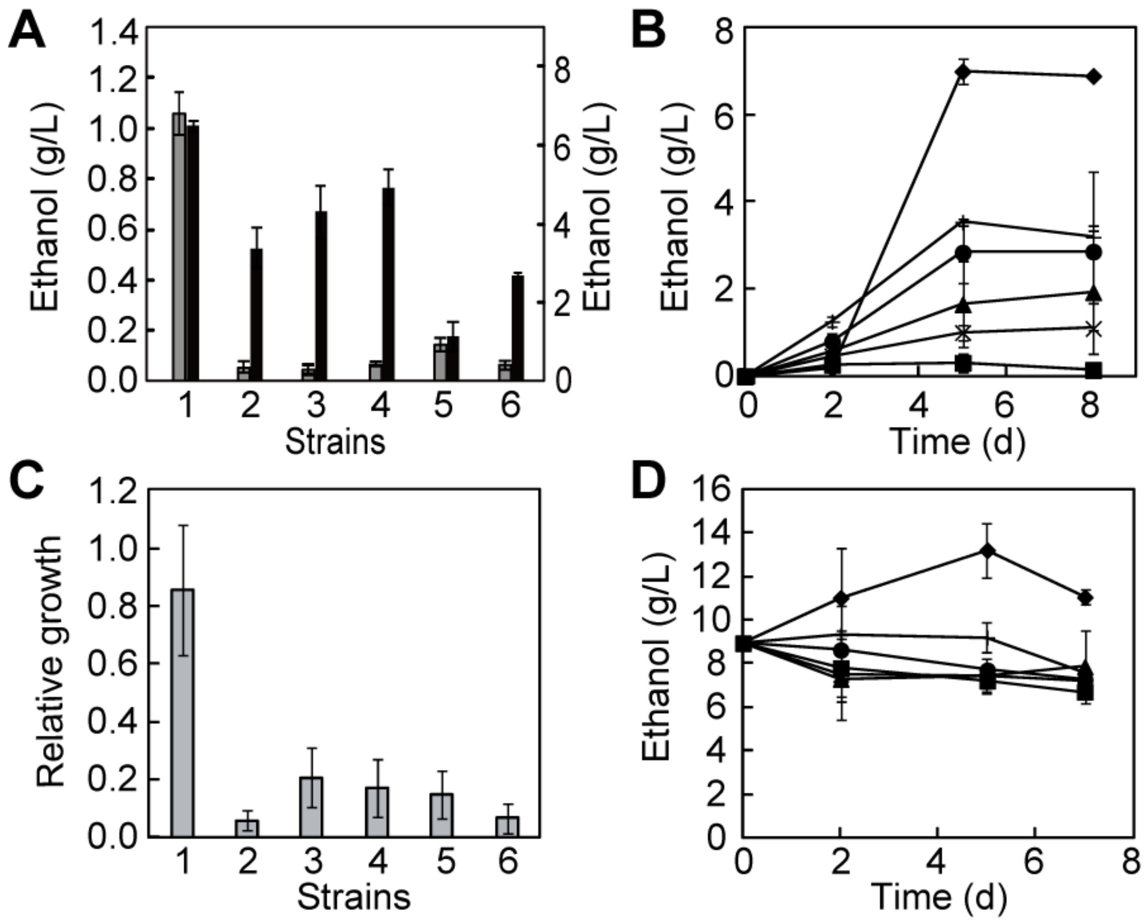
A
Ethanol (g/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
8
6
4
2
0
1 2 3 4 5 6
Strains
B
Ethanol (g/L)
8
6
4
2
0
0 2 4 6 8
Time (d)
C
Relative growth
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6
Strains
D
Ethanol (g/L)
16
14
12
10
8
6
4
2
0
0 2 4 6 8
Time (d)

FIG. 1.

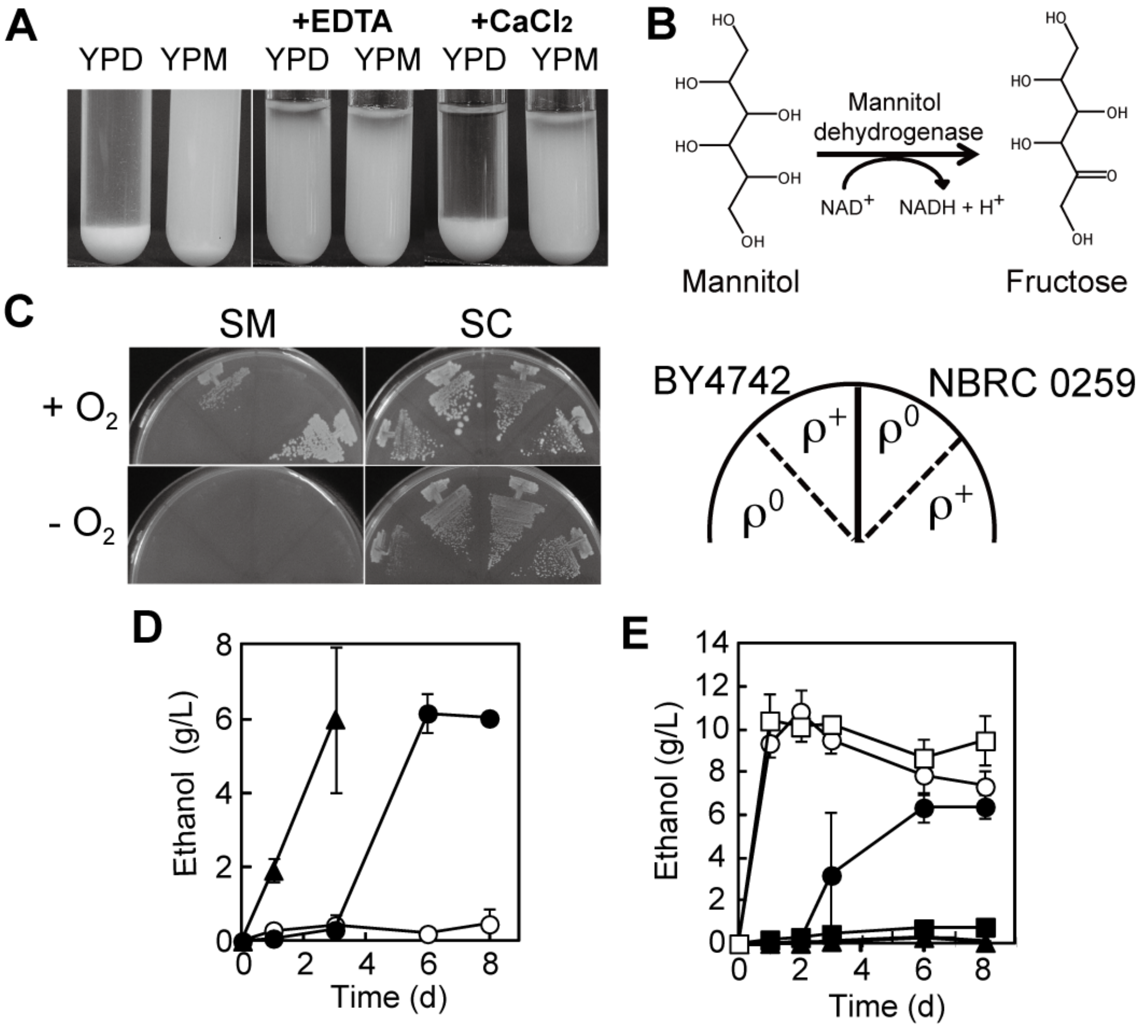
A
YPD YPM
+EDTA
YPD YPM
+CaCl2
YPD YPM
B
Mannitol dehydrogenase
NAD+
NADH + H+
Mannitol
Fructose
C
SM
SC
+ O2
- O2
BY4742
NBRC 0259
ρ+
ρ0
ρ0
ρ+
D
Ethanol (g/L)
Time (d)
E
Ethanol (g/L)
Time (d)

FIG. 2.

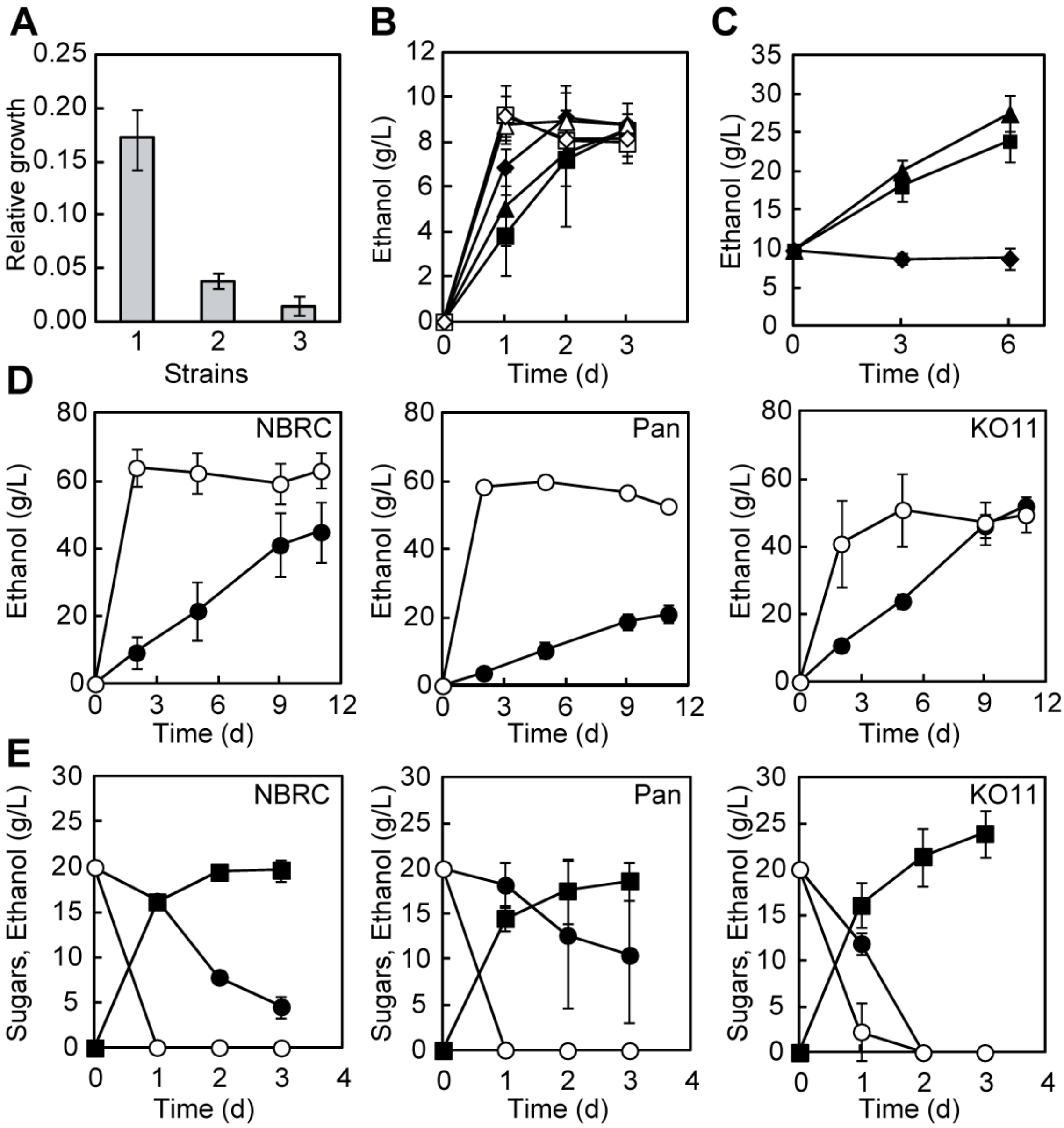
A
B
C
D
E
Relative growth
Strains
Ethanol (g/L)
Time (d)
NBRC
Pan
KO11
Sugars, Ethanol (g/L)

FIG. 3.

TABLE 1. Yeast strains used in this study

Yeasts [a]	AKU No.	Other No.	Growth [b]	Ethanol [c]
Saccharomyces cerevisiae BY4742		ATCC 201389	-	-
American yeast (Fleischmann baker's yeast)	4001		-	-
American yeast (American whiskey yeast)	4004		-	-
Saccharomyces sake Chuyu	4011		-	-
Saccharomyces sake Hozan	4013		-	-
Saccharomyces sake Ozeki	4014		-	-
Saccharomyces sake Sakaizumi	4016		-	-
Saccharomyces sake Fukumusume	4017		-	-
Saccharomyces sake Unryu	4019		-	-
Saccharomyces sake Sawanotsuru	4022		-	-
Wine yeast	4036		-	-
Beer yeast (Kirin)	4037		-	-
Baker's yeast (Oriental)	4039		-	-
München beer yeast	4042		-	-
Saccharomyces carlsbergensis	4044		-	-
Saccharomyces cerevisiae	4100		-	-
Saccharomyces logos	4101		-	-
Kazachstania unispora	4106	NBRC 0215	-	-
Saccharomyces fragilis	4108	IFO 0228	-	-
Saccharomyces sp.	4110		-	-
Naumovozyma castellii	4111		-	-
Saccharomyces cerevisiae	4136	NBRC 1346	-	-
Saccharomyces cerevisiae	4150	IAM 4512	-	-
Schizosaccharomyces pombe	4220	NBRC 0346	-	-
Saccharomyces paradoxus	4135	NBRC 0259	+	+
Zygosaccharomyces japonicus	4242	IFO 0595	+	-
Pichia polymorpha	4250	IFO 0195	+	-
Pichia farinosa	4262	NBRC 0193	+	-
Pichia haplophila	4263	NBRC 0947	+	-
Pichia saitoi	4266	IAM 4945	+	-
Hansenula saturnus	4301	IFO 0177	+	-
Kuraishia capsulata	4305	NBRC 0721	+	+

Wickerhamomyces silvicola	4313	NBRC 0807	+	-
Kuraishia capsulata	4326	NBRC 0974	+	+
Ogataea glucozyma	4330	NBRC 1472	+	+
Ogataea minuta	4332	NBRC 1473	+	+
Debaryomyces hansenii	4357	IFO 0023	+	-
Debaryomyces hansenii	4359	NBRC 0794	+	+
Naumovia castellii	4127	NBRC 0285	-	-
Hanseniaspora valbyensis	4405	NBRC 0115	-	-
Sporidiobolus salmonicolor	4440	NBRC 1035	-	-
Yarrowia lipolytica	4598	NBRC 0746	+	-
Yarrowia lipolytica	4599	NBRC 1195	-	-
Candida solani	4612	NBRC 0762	-	-
Candida albicans	4633	NBRC 1269	+	-

[a] These strains were maintained on YPD solid medium, suspended in SDW, added to 1.0 ml liquid medium in a test tube to an OD_{600} of 0.1, and cultivated at 30°C without shaking for 3 days.

[b] Strains that exhibited better growth on SM solid and liquid media than on SC-C media are shown by plus (+); strains that did not show better growth on SM media than SC-C media are by minus (-).

[c] Ethanologenic yeasts that produced ethanol after 3 days of cultivation in SM liquid medium are indicated by plus (+); yeast that produced no ethanol in this condition are by minus (-).

TABLE 2. Comparison of ethanol production

Strains	Substrate [a]	Cultivation time (h) [b]	Substrate consumption (g/L) [c]	Ethanol production (g/L) [d]	Productivity (g/L/h) (= d/b)	Yield (g ethanol /g substrate) (= d/c)
S. paradoxus NBRC 0259	Mannitol	120	20.0	7.3	0.06	0.36
K. capslata NBRC0721	Mannitol	120	14.0	2.6	0.02	0.19
K. capslata NBRC0974	Mannitol	120	7.6	1.2	0.01	0.15
O. glucozyma NBRC1472	Mannitol	120	10.2	2.1	0.02	0.21
O. minuta NBRC1473	Mannitol	120	6.7	3.5	0.03	0.52
D. hansenii NBRC 0794	Mannitol	120	4.6	0.1	0.00	0.02
S. paradoxus NBRC 0259-3	Mannitol	72	20.0	8.9	0.12	0.44
P. angophorae	Mannitol	72	20.0	9.3	0.13	0.46
E. coli KO11	Mannitol	72	20.0	9.2	0.13	0.46
S. paradoxus NBRC 0259-3	Glucose	24	20.0	10.2	0.43	0.51
P. angophorae	Glucose	24	20.0	11.5	0.48	0.57
E. coli KO11	Glucose	24	20.0	11.0	0.46	0.55

[a] Initial concentration of substrate is 20 g/L.

[b] The cultivation time at which the highest concentration of ethanol was produced.

[c] Substrate consumed during the cultivation time (b).

[d] Ethanol produced during the cultivation time (b).