Production of pyruvate from mannitol by mannitol-assimilating pyruvate decarboxylase-negative *Saccharomyces cerevisiae*

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Abstract

Mannitol is contained in brown macroalgae up to 33% (w/w, dry weight), and thus is a promising carbon source for white biotechnology. However, *Saccharomyces cerevisiae*, a key cell factory, is generally regarded to be unable to assimilate mannitol for growth. We have recently succeeded in producing *S. cerevisiae* that can assimilate mannitol through spontaneous mutations of Tup1-Cyc8, each of which constitutes a general corepressor complex. In this study, we demonstrate production of pyruvate from mannitol using this mannitol-assimilating *S. cerevisiae* through deletions of all three pyruvate decarboxylase genes. The resultant mannitol-assimilating pyruvate decarboxylase-negative strain produced 0.86 g/L pyruvate without use of acetate after cultivation for 4 days, with an overall yield of 0.77 g of pyruvate per g of mannitol (the theoretical yield was 79%). Although acetate was not needed for growth of this strain in mannitol-containing medium, addition of acetate had a significant beneficial effect on production of pyruvate. This is the first report of production of a valuable compound (other than ethanol) from mannitol using *S. cerevisiae*, and is an initial platform from which the productivity of pyruvate from mannitol can be improved.

Key words: pyruvate, mannitol, brown macroalgae, pyruvate decarboxylase, *Saccharomyces cerevisiae*

Introduction

Mannitol is a sugar alcohol derivative of mannose and a promising carbon source for white biotechnology, since brown macroalgae contains mannitol at up to 33% (w/w, dry weight) [1-3]. The budding yeast *Saccharomyces cerevisiae* is a key cell factory that is used for production of a wide range of industrial products [4]. However, *S. cerevisiae* including the S288C reference strain is generally thought to be unable to assimilate mannitol for growth [5]. However, we [6] and Enquist-Newman *et al.* [7] have recently succeeded in producing *S. cerevisiae* that can utilize mannitol, thus opening a new way to produce valuable compounds from mannitol.

Enquist-Newman *et al.* overexpressed genes for mannitol dehydrogenase and mannitol transporter and produced a *S. cerevisiae* strain that assimilates mannitol [7]. We also produced a *S. cerevisiae* strain that assimilates mannitol using spontaneous mutations of Tup1-Cyc8, each of which constitute a general corepressor complex that regulates many genes [6]. We demonstrated production of ethanol from mannitol using this *S. cerevisiae* strain [6]. These mannitol-assimilating *S. cerevisiae* strains may also have potential for production of other valuable compounds from mannitol.

Pyruvate is widely used for production of crop-protection agents, polymers, cosmetics, and food additives, and as a starting material in the biosynthesis of pharmaceuticals (e.g., L-DOPA, alanine, L-tryptophan, and L-tyrosine) [8, 9]. Pyruvate production from glucose has been achieved using a pyruvate decarboxylase (Pdc)-negative *S. cerevisiae* TAM strain [10]. The goal of this study was to produce pyruvate from mannitol using our mannitol-assimilating *S. cerevisiae* strain [6].

Materials and Methods

Strains and Media

The *S. cerevisiae* strains used in the study are listed in Table 1. *ADE2* of MK4416 was removed and *TRP1* was replaced with *trp1Δ63* using plasmid YRp14-*trp1Δ63* (ATCC 77148) [11], resulting in the MK5316 strain. *PDC1* and *PDC6* of MK5316 were eliminated [11] and *PDC5* was deleted [12], resulting in the MK5376 strain. Detailed information is described in Supplementary Methods. Standard yeast media were used [13], including SG, SM, SGE (ethanol 0.15%), and SGE (ethanol 0.3%) media consisting of 0.67% w/v yeast nitrogen base w/o amino acids (BD), complete amino acids/nucleosides (Clontech), and carbon sources: 2% v/v glycerol (SG), 2% w/v mannitol (SM), 2% v/v glycerol plus 0.15% v/v ethanol [SGE (ethanol 0.15%)], and 2% v/v glycerol plus 0.3% v/v ethanol [SGE (ethanol 0.3%)]. Glycerol (30% v/v) and sodium acetate (27.1% w/v, pH 9.1; equal to 20% w/v acetate) were each autoclaved separately from the other components and ethanol was also separately sterilized by filtration (0.20-μm pore size). Liquid medium was solidified at 2% w/v agar. Amino acids/nucleosides are removed from the medium when necessary. 5-Fluoroorotic Acid (FOA) for the counter-selection of yeast was added to the solid medium at 1 mg/ml (26). The MK5316 and MK5376 strains were maintained on SGE (0.3% ethanol) solid medium at room temperature and stored in the presence of 17% v/v glycerol at -80°C. Cultivation was conducted with a Personal Lt-10F (Taitec, Tokyo, Japan).

Pyruvate Production from Mannitol

Pyruvate production was conducted as follows, unless otherwise stated. The

MK5376 strain was precultured on SM solid medium, transferred to SM liquid medium (5 mL in a 100-mL Erlenmeyer flask), and cultured at 30°C at 145 strokes per min (spm) for approximately 24 h. Cells precultured in each medium were transferred to SM liquid medium (10 mL in a 100-mL Erlenmeyer flask) and further cultured at 0, 95, or 145 spm at 30°C.

Analytical Methods

Cultures were centrifuged at 20,000 g for 5 min at 4°C and each component in the supernatant was analyzed. Ethanol was assayed using an Ethanol Assay F-kit (Roche). The concentration of mannitol was determined using an HPLC equipped with an Aminex HPX-87H (300 × 7.8 mm) (Bio-Rad) column (65°C, elution with 5 mM H_2SO_4 at 0.65 ml/min) and a RID-10A detector (Shimadzu) [6]. Pdc activity was assayed as described elsewhere [14]. The protein concentration was determined using a Bradford reagent assay (Sigma) [15] with bovine serum albumin as a standard.

Results and Discussion

Growth Phenotype of Mannitol-assimilating Pdc-negative *S. cerevisiae*

We previously found that *S. cerevisiae* BY4742 cells capable of assimilating mannitol arise spontaneously from wild-type BY4742 cells during prolonged culture in mannitol-containing medium due to spontaneous mutations in genes encoding Tup1 or Cyc8, which constitute a general corepressor complex [6]. Of the strains that acquired the ability to assimilate mannitol, the MK4416 strain had a spontaneous partial deletion in *CYC8* (Table 1) and showed salt tolerance, as well as high ethanol productivity [6], and was chosen as the mannitol-assimilating strain in

this study. Auxotrophy for Ade and Trp was introduced into the MK4416 strain to give the MK5316 strain, which was used as the parental mannitol-assimilating strain. The three genes for Pdc in *S. cerevisiae* (*PDC1*, *PDC5*, and *PDC6*) [16] were deleted in the MK5316 strain to give the Pdc-negative and mannitol-assimilating MK5376 strain (Table 1), which was confirmed to have no Pdc activity (Supplementary Results).

The *S. cerevisiae* Pdc-negative strain in a T2-3D or CEN.PK 113-7D background cannot grow in the presence of glucose in a defined liquid or solid medium, but can grow in a glucose-limited chemostat culture in the presence of C2 compounds (ethanol or acetate) [10, 14]. This requirement for C2 compounds was attributed to a deficiency of this Pdc-negative strain to synthesize cytosolic acetyl-CoA [17, 18]. On the defined medium, the Pdc-negative MK5376 strain exhibited no growth in the presence of glucose as reported [10, 14], but showed growth in the presence of glycerol, glycerol plus ethanol, pyruvate, and, in particular, with mannitol alone (Supplementary Fig. S1). Cytosolic acetyl-CoA would be supplied from functional mitochondria when the MK5376 strain assimilates mannitol of which assimilation requires functional mitochondrial respiration [6]. The Pdc-negative MK5376 strain in the BY4742 background showed poorer growth in a complex medium than in a defined medium (Fig. S1), in contrast to previous findings for the Pdc-negative strain in a T2-3D background [17].

Pyruvate Production from Mannitol

In a previous attempt to produce pyruvate from glucose using *S. cerevisiae*, a Pdc-negative strain in a CEN.PK113-7D background was evolved to a TAM strain

[16]. The TAM strain was independent of C2 compounds and tolerant to glucose, and produced 135 g/L pyruvate with an overall yield of 0.54 g of pyruvate per g of glucose [10]. To produce pyruvate from mannitol using our MK5376 strain, the conditions for preculture of MK5376 strain were first examined. SG and SM media gave better pyruvate production than SGE (0.15 or 0.3% ethanol) media, although growth was not affected by these four media (Fig. 1A). Thus, we chose SM for preculture. Among the tested shaking speeds of 0, 95, and 145 spm, a speed of 0 spm (i.e., a static batch culture) resulted in the best production of pyruvate from mannitol (Fig. 1B). Using these conditions (preculture in SM medium and pyruvate production using a static batch culture), the pyruvate and ethanol productivity of the mannitol-assimilating Pdc-negative MK5376 strain was compared with that of the parental Pdc-positive MK5316 strain (Fig. 2). The parental strain produced no pyruvate, but the Pdc-negative strain produced 0.86 g/L pyruvate through consumption of 1.12 g/L mannitol after cultivation for 4 days, with an overall yield of 0.77 g of pyruvate per g of mannitol (the theoretical yield was 79%). This was a higher yield, but lower productivity, compared to the TAM strain (yield of 0.54 g of pyruvate per g of glucose, 135 g/L pyruvate production, consumption of 250 g/L glucose for 4 days) [10]. The difference in pyruvate productivity between the Pdc-negative strain MK5376 and the TAM strain could be attributed to fact that MK5376 metabolized mannitol less efficiently than TAM metabolized glucose, as indicated shown by the amount of sugar consumption (1.12 g/L mannitol vs 250 g/L glucose) and biomass formation (A_{600} of 1.7 [MK5376] vs. A_{600} of 50 [TAM strain]) after 4 days of cultivation (Fig. 2) [4]. The Pdc-negative strain MK5316 must acquire the enhanced ability to metabolize mannitol, e.g., through adaptive

159 evolution, as in the case of TAM.

160

161 Effect of Acetate on Pyruvate Production

162 The mannitol-assimilating Pdc-negative MK5376 strain showed no requirement
163 for C2 compounds, but there is a possibility that C2 compounds helped with supply
164 of cytosolic acetyl-CoA and enhanced pyruvate production. As expected, addition
165 of acetate up to 0.3% w/v had a significant effect on pyruvate production. First, the
166 strain produced pyruvate at shaking speeds of 95 and 145 spm (Fig. 1C), whereas
167 no pyruvate was produced at 95 and 145 spm in the absence of C2 compounds (Fig.
168 1B). Second, the strain produced a larger amount of pyruvate in the presence of
169 acetate compared to that in the absence of acetate (Fig. 1BC). After long-term
170 cultivation (20 days) at 0 spm, 2.71 g/L pyruvate was produced through
171 consumption of 2.2 g/L mannitol and 0.88 g/L acetate (Fig. S2). The mechanism
172 underlying this beneficial effect of acetate is unclear and further challenges are
173 needed to improve productivity of pyruvate. However, this is the first
174 demonstration of production of a valuable compound, other than ethanol, from
175 mannitol using *S. cerevisiae*.

176

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239

240 **Table 1.** *S. cerevisiae* strains used in this study

Strains	Descriptions	Sources
S. cerevisiae		
BY4742	MATα *his3Δ1 his3Δ1leuΔ0 lys2Δ0 ura3Δ0*	Euroscarf
MK4416 [a]	BY4742 *cyc8* (Δ1139-1164/ Q380ASCKTGRKX)	(14)
MK5286	MK4416 *ade2Δ0*	This study
MK5316	MK4416 *ade2Δ0 trp1Δ63*	This study
MK5327	MK4416 *ade2Δ0 trp1Δ63 pdc6Δ0*	This study
MK5336	MK4416 *ade2Δ0 trp1Δ63 pdc6Δ0 pdc1Δ0*	This study
MK5376	MK4416 *ade2Δ0 trp1Δ63 pdc6Δ0 pdc1Δ0 pdc5Δ::URA3*	This study

241 [a] MK4416 strain spontaneously lacks the central small region (1139-1164 nt) of
242 *CYC8* (total, 2,901 nt) resulting in a nonsense mutation in which a stop codon was
243 created after a short new peptide (^{380}ASCKTGRK^{387}) [6]. MK4416 strain acquired
244 the ability to assimilate mannitol.

245

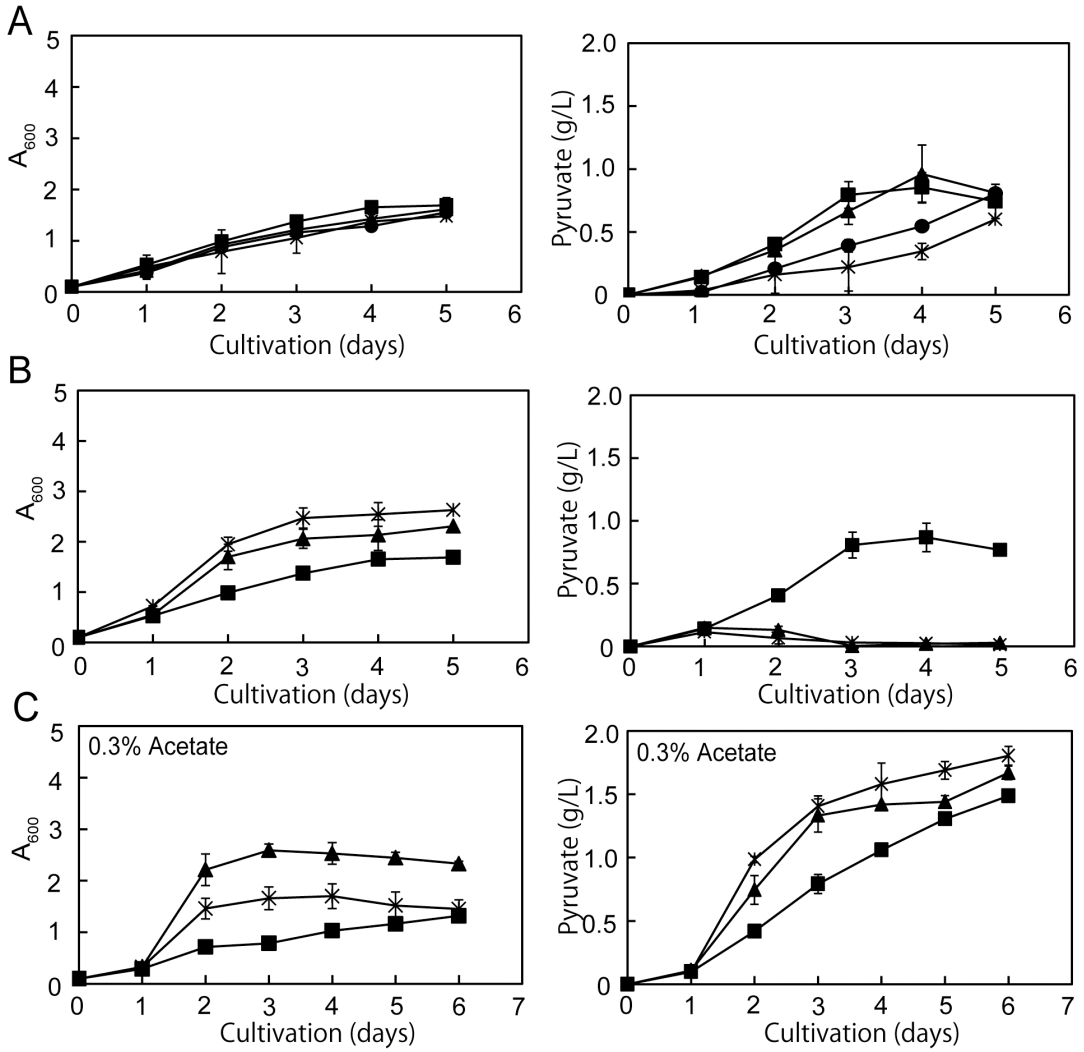

Fig. 1. Production of pyruvate from mannitol by the mannitol-assimilating Pdc-negative MK5376 strain. Growth (left) and pyruvate concentration (right) for the MK5376 strain (A) precultured in several media, (B) cultured at several shaking speeds in SM medium, and (C) cultured in SM medium plus 0.3% w/v acetate. Pyruvate production was conducted as described in the Materials and Methods, except that (A) the MK5376 strain was precultured on SGE (0.3% ethanol) (closed circles), SGE (0.15%EtOH) (asterisks), SG (closed triangles), and SM (closed squares) solid media, further precultured in the same liquid media, and statically cultured at 0 spm at 30°C; (B) cultivation was conducted at 0 (closed squares), 95

255 (asterisks), and 145 (closed triangles) spm at 30°C; and (C) cultivation was
256 conducted as in (B), but in the presence of 0.3% w/v acetate. (A-C) Averages and
257 maximum and minimum values are presented (n=2). (A) For precultivation in SM
258 medium, averages ± standard deviations (SD) are presented (n=6).
259
260

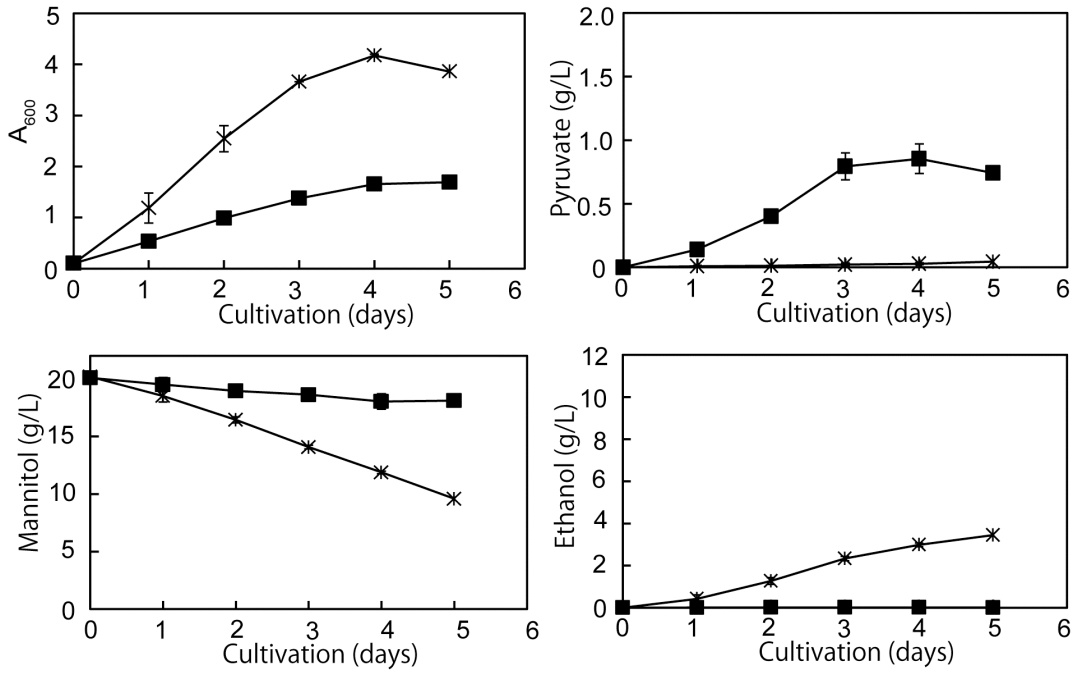

Fig. 2. Comparison of growth profiles between the Pdc-negative MK5376 strain and the Pdc-positive parental MK5316 strain. Pyruvate production was conducted as described in the Materials and Methods. Averages and maximum and minimum values (n=2) are presented, except that the growth and pyruvate concentration for the MK5376 strain are averages ± SD (n=6), and concentrations of ethanol and mannitol for the MK5376 strain are averages ± SD (n=3).

Supplementary information for

Production of pyruvate from mannitol by mannitol-assimilating pyruvate decarboxylase-negative *Saccharomyces cerevisiae*

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Supplementary Methods

Strains

Detailed information for plasmids and primers used in this study is provided in Tables S1 and S2, respectively. Disruption of *ADE2*, *TRP1*, *PDC6*, and *PDC1* in strain MK4416 was performed as previously described [1]. Briefly, the upstream region (5'-1,993 bp to 5'-33 bp; ~2 kbp) and the downstream region (3'-69 bp to 3'-1,959 bp; ~2 kbp) of *ADE2* were PCR amplified from genomic DNA of BY4742 using primers 1 and 4, for the upstream region and primers 2 and 3 for the downstream region. Using the amplified fragments as templates, a single DNA fragment (4 kbp, the upstream region followed by the downstream region) was PCR amplified using primers 1 and 2 and inserted into the EcoRI site of YIplac211 using the In-fusion kit (Clontech), yielding pMK5265. After linearization of pMK5265 by EcoRI digestion, strain MK4416 was transformed with linearized pMK5265. Transformants were selected on SC-U solid medium, purified again on the same medium, and the Ade$^-$ derivative was selected on SC+FOA solid medium, yielding strain MK5286 (MK4416 *ade2Δ0*). Deletion of *ADE2* was confirmed by genomic PCR using primers 1 and 2.

TRP1, *PDC6*, and *PDC1* were deleted using the same method. *TRP1* of MK5286 was deleted using pMK5300, and the Trp$^-$ derivative was named MK5316 (MK5826 *trp1Δ*63). pMK5308 and pMK5329 were constructed as described above for pMK5265 and linearized with EcoRI. Strain MK5316 was transformed with linearized pMK5308, and transformants were selected on SC-U solid medium and then MK5327 strain (MK5316 *pdc6Δ0*) was selected. Deletion of *PDC6* was confirmed with genomic PCR using primers 6 and 7 and by sequencing of the

resultant PCR products using primers 10 and 11. Similarly, strain MK5336 (MK5327 *pdc1Δ0*) was obtained after transformation of strain MK5327 with linearized pMK5329. Deletion of *PDC1* was confirmed by genomic PCR using primers 12 and 13, and also by sequencing the resultant PCR products using primer 16.

Deletion of *PDC5* was conducted as described [2]. Briefly, the upstream region of *PDC5* (5'-999 bp to 5'-4 bp; 1 kbp) and the *URA3* locus (1,037 bp) were PCR amplified from genomic DNA of BY4742 and pRS416, respectively, using primers 24 and 25 for the upstream region and primers 26 and 27, for *URA3*. Using the resultant DNA fragments as templates, a DNA fragment (*PDC5::URA3*; 2,110 bp) was amplified using primers 24 and 27 and introduced into strain MK5336. Transformants were selected on SGE-U（0.15% EtOH) solid medium, yielding Pdc-negative strain MK5376 (MK5336 *pdc5Δ0*). Deletion of *PDC5* was confirmed by genomic PCR using primers 24 and 27, and also by sequencing the resultant PCR products using primers 5 and 28.

Supplementary Results

Pdc activity of cell extracts of parental MK5316 and Pdc-negative MK5376

Pdc activity of cell extracts of parental MK5316 was linear with respect to the amount of protein in the reaction mixture (5.8–23.3 μg; Table S3). Based on these data, specific activity of Pdc of MK5316 was calculated as 2.04 U/mg, slightly lower than the reported activity (3.1 U/mg) in *S. cerevisiae* strain T2-3D [3]. In contrast to MK5316, cell extracts of Pdc-negative MK5376 exhibited lower activity and no linearity. Even when 115.2 μg protein was present in the reaction mixture,

the specific activity was calculated as 0.01 U/mg. Thus, we concluded that the specific activity of MK5376 was undetectable.

Table S1. Plasmids used in this study

Plasmid	Descriptions	Sources
YIplac211	Amp^r, *URA3*	Gietz *et al.* [5]
pRS416	Amp^r, *URA3*, *CEN*	Christianson *et al.* [6]
pMK5265	The DNA fragment PCR-amplified using primers 1, 2, 3, and 4 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 556 bp downstream of *ADE2*.	This study
pMK5300	Purchased from ATCC as ATCC77148 (YRp14/*trp1Δ63*).	ATCC
pMK5308	The DNA fragment PCR-amplified using primers 6, 7, 8, and 9 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 1,132 bp upstream of *PDC6*.	This study
pMK5329	The DNA fragment PCR-amplified using primers 12, 13, 14, and 15 was inserted into the EcoRI site of YIplac211 using the In-fusion kit. Only one EcoRI site is present 406 bp downstream of *PDC1*.	This study

Table S2. Primers used in this study

Nos	Primers	Sequences	Descriptions
1	YIpE15bF_ ADE2-1992 F	taccgagctcgaattTACG ATGTGGATGAGGG AG	For amplification of the upstream region of *ADE2* (5'-1,993 bp to 5'-33 bp) [a].
2	YIpE15bR_ ADE2+1959 R	gacggccagtgaattGTG ACATCTAGACGCT CACAAG	For amplification of the downstream region of *ADE2* (3'-69 bp to 3'-1,959 bp) [a].
3	ADE2-33F_ +69F	GTACATCCTACTA TAACAATCAAGGT TATGATTACATCA AATGTG	For amplification of the downstream region of *ADE2,* defined above. Sequence complementary to primer 4 is underlined.
4	ADE2-33R	CTTGATTGTTATA GTAGGATGTAC	For amplification of the upstream region of *ADE2,* defined above.
5	URA3_3_25 R	GTCGAAAGCTACA TATAAGGAAC	For sequencing.
6	YIpE15bF_ PDC6-1996F	taccgagctcgaattGCAG TGTCTGGTGTACC AC	For amplification of the upstream region of *PDC6* (5'-1,996 b to 5'-86 b) [a].
7	YIpE15bR_ PDC6+1970 R	gacggccagtgaattACTT GAATTGTTCCTTC AC	For amplification of the downstream region of *PDC6* (3'-112 b to 3'-1,970 b) [a].

Table S2. *Continued.*

8	PDC6-86F_+112F	GGCTGTTTGAAGCCATTCTATCCTAAATACTACGTTATCGCCG	For amplification of the downstream region of *PDC6,* defined above. Sequence complementary to primer 9 is underlined.
9	PDC6-86R	GATAGAATGGCTTCAAACAGCC	For amplification of the upstream region of *PDC6,* defined above.
10	PDC6-308F	GCCCACAACTTATCAAGTG	For sequencing.
11	PDC6+427R	GCCAAAGAGATGAGCCAAAG	For sequencing.
12	YIpE15bF_PDC1-1932F	taccgagctcgaattGTATTGCAAGTGGTAGTAC	For amplification of the upstream region of *PDC1* (5'-1,932 bp to 5'-27 bp) [a].
13	YIpE15bR_PDC1+1998R	gacggccagtgaattGAACAGTTGTAGTAGCAC	For amplification of the downstream region of *PDC1* (3'-79 bp to 3'-1,998 bp) [a].
14	PDC1-27F_+79F	CTACTCATAACCTCACGCCTGCACTGTCACTTACCATG	For amplification of the downstream region of *PDC1* (3'-79 bp to 3'-1,998 bp). Sequence complementary to primer 15 is underlined.
15	PDC1-27R	GCGTGAGGTTAT	For amplification of the upstream

		GAGTAG	region of *PDC1* (5'-1,932 bp to 5'-27 bp).

Table S2. *Continued.*

16	PDC1-209 F	CAGCTTATGGTG ATGGCACA	For sequencing.
24	PDC5-999	GCCACGCTGATA GATATCCCG	For amplification of the upstream region of *PDC5* (5'-999 bp to 5'-4 bp).
25	PDC5-4c	GTTCTTCTTGTTA TTGTATTGTG	For amplification of the upstream region of *PDC5* above
26	PDC5URA 3-2	CACAATACAATA ACAAGAAGAACtt tttacttttgaaggttatagat gtttaggtaaataattGAT TCGGTAATCTCC GA	For amplification of the *URA3* locus (1,037 bp) from pRS416. Sequence complementary to primer 25 is underlined. The short downstream sequence of *PDC5* (3'-41 b to 3'-80 b) is in lower case.
27	PDC5-40U c	aatacacaaacgttgaatcat gagttttatgttaattagcGG GTAATAACTGAT ATAATT	For amplification of the *URA3* locus from pRS416, defined above.
28	PDC5_+36 R	CACAAACGTTGA ATCATGAG	For sequencing.

[a] Sequence around *Eco*RI of YIplac211 is in lowercase.

Table S3. Pdc activities of strains MK5316 and MK5376

Strains	Amounts of proteins in the reaction mixture (μg)	Activity (ΔmA_{340}/min)
MK5316	5.8	178.4
	11.6	271.9
	23.3	503.0
MK5376	11.6	5.0
	115.2	11.9

Activity of MK5316 was assayed within 3 min, whereas activity of MK5376 was assayed after a 10 min reaction.

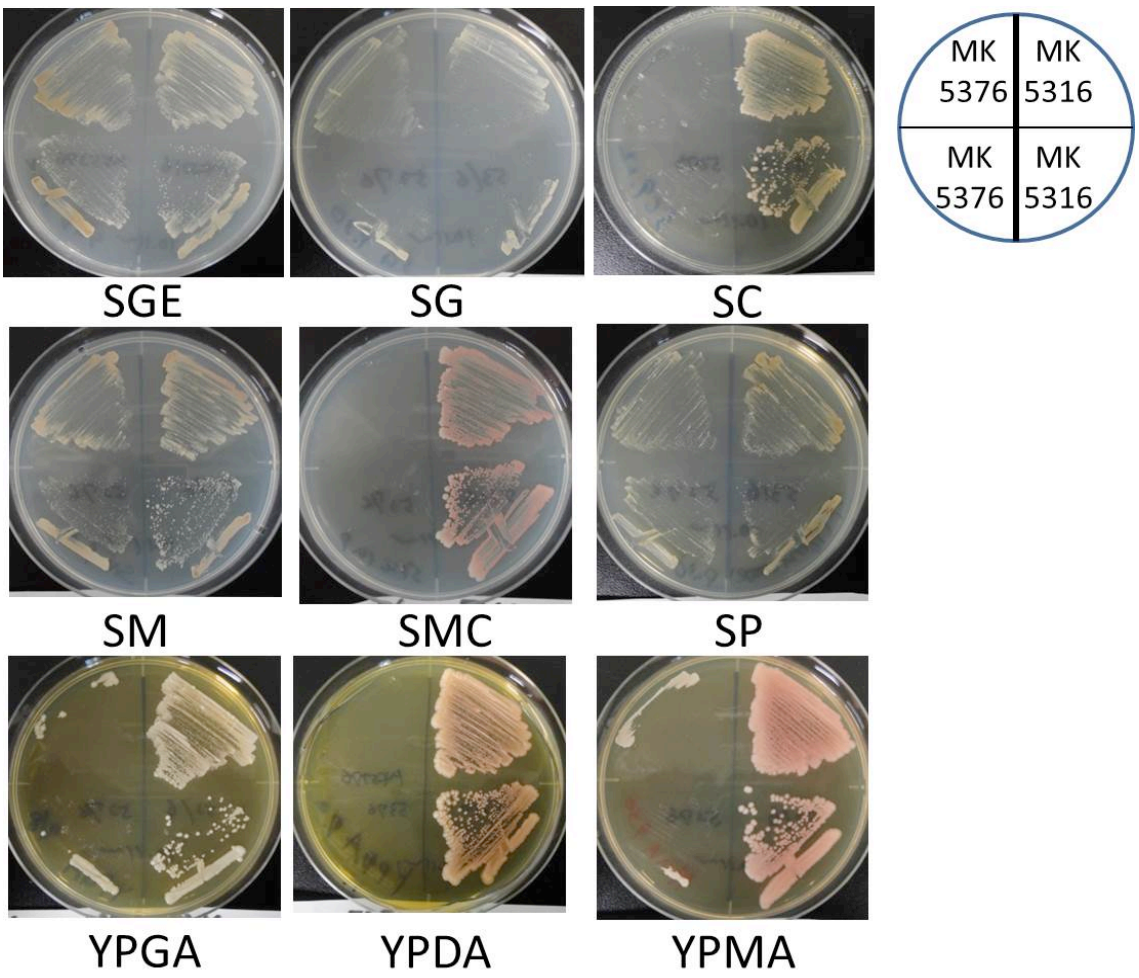

Fig. S1. Growth phenotypes of the parental MK5316 strain and Pdc-negative MK5376 strain. MK5316 and MK5376 strains pre-grown on SGE solid medium were streaked with the indicated solid media and incubated at 30°C for 5 days. SGE, SG, SC, SM, SMC, and SP consisted of 0.67% w/v yeast nitrogen base w/o amino acids (BD), complete amino acids/nucleosides (Clontech), 2% w/v agar, and carbon sources: 2% v/v glycerol plus 0.15% v/v ethanol (SGE), 2% v/v glycerol (SG), 2% w/v glucose (SC), 2% w/v mannitol (SM), 2% w/v mannitol plus 0.3% w/v glucose (SMC), and 2% w/v sodium pyruvate. YPGA, YPDA, and YPMA solid media contained 2% w/v yeast extract, 2% w/v tryptone, 147 mg/L adenine, 2% w/v agar (pH 5.6), and carbon sources: 3% v/v glycerol (YPGA), 2% w/v glucose (YPDA), and 2% w/v mannitol (YPMA). Representative results are shown.

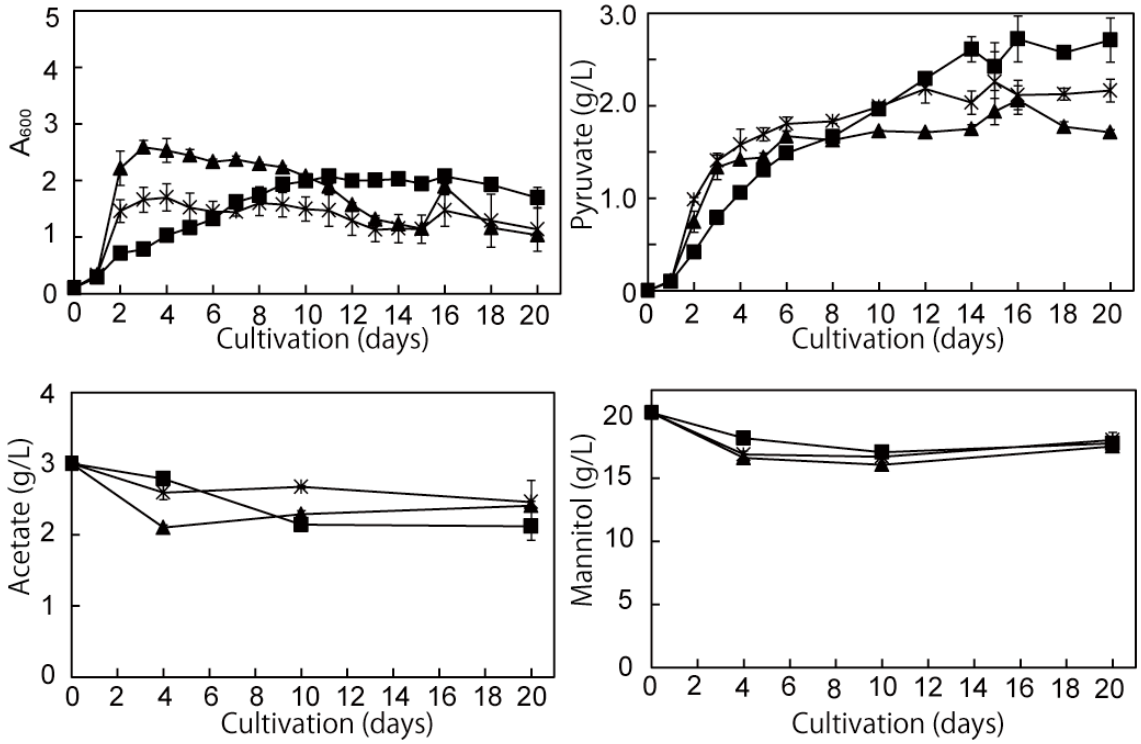

Fig. S2. Production of pyruvate from 2% w/v mannitol plus 0.3% w/v acetate. Production was conducted as in Fig. 1C and continued for longer periods, as indicated. Production of ethanol was not detected.

Supplementary References

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3. Flikweert MT, VanderZanden L, Janssen WMTM, Steensma HY, VanDijken JP, Pronk JT. Pyruvate decarboxylase: An indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast 1996; 12:247-57.