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Title: Regulation of pH attenuates toxicity of a byproduct produced by an ethanologenic strain of *Sphingomonas* sp. A1 during ethanol fermentation from alginate

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ABSTRACT

Marine macroalgae is a promising carbon source that contains alginate and mannitol as major carbohydrates. A bioengineered ethanologenic strain of the bacterium *Sphingomonas* sp. A1 can produce ethanol from alginate, but not mannitol, whereas the yeast *Saccharomyces paradoxus* NBRC 0259-3 can produce ethanol from mannitol, but not alginate. Thus, one practical approach for converting both alginate and mannitol into ethanol would involve two-step fermentation, in which the ethanologenic bacterium initially converts alginate into ethanol, and then the yeast produces ethanol from mannitol. In this study, we found that during fermentation from alginate, the ethanologenic bacterium lost viability and secreted toxic byproducts into the medium. These toxic byproducts inhibited bacterial growth and killed bacterial cells, and also inhibited growth of *S. paradoxus* NBRC 0259-3. We discovered that adjusting the pH of the culture supernatant or the culture medium containing the toxic byproducts to 6.0 attenuated the toxicity toward both bacteria and yeast, and also extended the period of viability of the bacterium. Although continuous adjustment of pH to 6.0 failed to improve the ethanol productivity of this ethanologenic bacterium, this pH adjustment worked very well in the two-step fermentation due to the attenuation of toxicity toward *S. paradoxus* NBRC 0259-3. These findings provide information critical for establishment of a practical system for ethanol production from brown macroalgae.

INTRODUCTION

Macroalgae has several advantages as a promising source of biofuels: it is more productive than land crops; its cultivation requires no arable land, irrigation water, or fertilizer; and it contains no lignin.[1-5] As a group, the macroalgae include the red, green, and brown macroalgae. One of the major carbohydrates in brown algae is alginate, a linear polysaccharide consisting of β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G), in which the two monosaccharides are arranged as polyM, polyG, and heteropolymeric random sequences (polyMG).[6] The brown algae *Laminaria japonica* and genera *Sargassum* and *Turbinaria* contain up to 40% w/v alginate.[7, 8] The other major carbohydrate in brown algae is mannitol, a sugar alcohol corresponding to mannose.[9] *L. japonica* contains up to 30% w/v mannitol.[8] In a review, Zubia *et al*. reported mannitol content up to 33% w/v in several brown algae of genera *Sargassum* and *Turbinaria*,[7] and Horn *et al*. reported that the brown alga *Laminaria hyperborea* contains 25% w/v mannitol.[10]

Two systems for producing ethanol from alginate have been established using bacteria.[11, 12] One system utilizes a bioengineered ethanologenic strain of *Sphingomonas* sp. A1 (ethanologenic strain MK3353), which carries genes for pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Zymomonas mobilis* and has acquired the ability to produce ethanol from alginate[11]; however, *Sphingomonas* sp. A1 is unable to assimilate mannitol.[13] The other system is a bioengineered ethanologenic *Escherichia coli* strain that carries genes for alginate utilization; this strain can produce ethanol from a mixture of mannitol and alginate derived from brown algae (kombu; *Saccharina japonica*).[12] In addition, we have recently established a system for ethanol production from mannitol that utilizes the yeast *Saccharomyces paradoxus* NBRC 0259-3, which

unlike *Saccharomyces cerevisiae* can naturally assimilate mannitol.[14] Thus, one practical approach for converting both alginate and mannitol into ethanol would involve two-step fermentation, in which the ethanologenic strain MK3353 initially converts alginate into ethanol, and then the yeast produces ethanol from mannitol.

In order to establish a practical system for production of ethanol from alginate and mannitol, it is necessary to achieve greater understanding of ethanol production by the ethanologenic strain MK3353. In this study, we found that this bacterium secretes toxic byproducts that inhibit the growth of both itself and *S. paradoxus* NBRC 0259-3, and kill the bacterium itself, during ethanol fermentation from alginate. We also discovered that adjusting the media pH to 6.0 attenuates this toxicity and enhances the two-step fermentation process.

RESULTS

Process of ethanol production from alginate. In order to understand the process of ethanol production from alginate, the ethanologenic strain MK3353 (*Sphingomonas* sp. A1 lacking in lactate dehydrogenase [LDH] gene and carrying genes for PDC and ADH in the broad–host range plasmid pKS13)[11] and the control strain MK3567 (*Sphingomonas* sp. A1 lacking in LDH gene and carrying pKS13 alone) were cultivated in liquid alginate medium containing 5% w/v alginate at 30°C and 95 strokes per minutes (spm). Growth (A_{600} of the culture), viability (cfu), ethanol concentration in the supernatant (hereafter, we refer to supernatant from a culture of *Sphingomonas* sp. A1 as "A1-supernatant"), and alginate concentration in the culture were determined (Fig. 1).

The ethanologenic strain MK3353 started to produce a large quantity of ethanol after 1 day of cultivation, as reported,[11] but the control strain MK3567 not. Ethanol

concentration reached a maximum after 3 days of cultivation, and did not increase thereafter (Fig. 1B). Accordingly, a larger amount of alginate was consumed by the ethanologenic strain MK3353 than the control strain MK3567, indicating that introduction of the genes encoding PDC and ADH improved alginate metabolism. The ethanologenic strain MK3353 consumed almost all alginate after 3 days of cultivation (Fig. 1C). The pH of the culture of the ethanologenic strain MK3353 became slightly alkaline (from pH 8.0 at day 0 to pH 8.7 at day 3 and pH 9.0 at day 4), whereas that of the control MK3567 strain was not (from pH 8.0 at day 0 to pH 6.3 at day 1 and pH 7.2 at day 4) (data not shown). Notably, the ethanologenic strain MK3353 started to lose viability after 2 days of cultivation, and completely lost viability at day 5. We attributed the loss of ethanol-production capacity to this loss of cell viability. No remarkable morphological change was observed by transmission electron microscopy (TEM) or scanning electron microscopy (SEM) analyses of the ethanologenic strain MK3353 or the control strain MK3567 after 1, 2, or 3 days of cultivation (Supplementary Fig. S1).

Toxic byproducts produced by the ethanologenic strain MK3353. The data described above suggested that the loss of ethanol-production ability and the decrease in viability were caused by complete consumption of alginate. However, this possibility was ruled out by the observation that addition of alginate (1 g/day) each day after 2 days of cultivation had no effect on ethanol concentration or viability of the ethanologenic strain MK3353 (data not shown).

Another possibility is that ethanol itself caused the loss of cell viability. However, when the ethanologenic strain MK3353 was cultivated in liquid alginate medium containing 5% w/v alginate and an initial concentration of 1.0% w/v (10 g/l) ethanol,

there was no loss of viability, and ethanol was still produced from alginate, although the added ethanol did delay cell growth (Supplementary Fig. S2). Thus, the reduction in viability could not be attributed to the ethanol produced by the ethanologenic strain MK3353.

We then hypothesized that some toxic byproduct other than ethanol might be produced by the ethanologenic strain MK3353, but not the control strain MK3567. To test this idea, we examined the growth of the ethanologenic strain MK3353 in liquid alginate media containing 0.4% w/v alginate and 0, 10, 25, or 50% v/v of A1-supernatant from 1-, 2-, 3-, or 4-day culture of the ethanologenic strain MK3353 or the control strain MK3567 (Fig. 2A). Growth inhibition was observed in the presence of A1-supernatants from 2-, 3-, and 4-day cultures of the ethanologenic strain MK3353, but not A1-supernatant from 1-day culture of the ethanologenic strain MK3353 strain or A1-supernatants from any cultures of the control strain MK3567. Moreover, the inhibitory effect was dependent on the concentration of A1-supernatant. Collectively, these data indicate that toxic byproducts were produced specifically by the ethanologenic strain MK3353 (i.e., in a manner dependent on the presence of the genes encoding PDC and ADH), and that the concentration of these byproducts increased over the cultivation period.

A1-supernatant from 4-day culture of the ethanologenic strain MK3353 killed the bacterial cells, whereas A1-supernatant from 4-day culture of the control strain MK3567 did not (Fig. 2B). Inhibition of the growth of *S. paradoxus* NBRC 0259-3 was also observed in the presence of A1-supernatant from 3-day culture of the ethanologenic strain MK3353, but not in the presence of A1-supernatant from 3-day culture of the control strain MK3567 (Fig. 2C); however, A1-supernatant of 3-day culture of the

ethanologenic strain MK3353 did not kill *S. paradoxus* NBRC 0259-3 cells (Fig. 2D).

Attenuation of the toxic effects of byproducts secreted by the ethanologenic strain MK3353. We examined the effect of pH on the toxic effects of A1-supernatant from cultures of the ethanologenic strain MK3353. The pH of A1-supernatant from 3-day culture was approximately 8.7. We adjusted the pH to 4.0, 5.0, 6.0, 7.0, and 8.0; all samples were adjusted to the same final volume by addition of sterilized water, as necessary, to rule out effects of dilution. As shown in Fig. 3A, growth inhibition of the ethanologenic strain MK3353 was not observed in the presence of A1-supernatant at pH of 5.0 or 6.0. Moreover, the killing effect of A1-supernatant at pH 6.0 was much weaker than that of A1-supernatant at pH 5.0 (Fig. 3B). Growth inhibition of *S. paradoxus* NBRC 0259-3 was also not detected in the presence of A1-supernatants at pH of 6.0 (or at 4.0 and 5.0) (Fig. 3C). These data indicate that adjustment of pH to 6.0 attenuated the toxicity of the byproducts in A1-supernatants.

Ethanol production by the ethanologenic strain MK3353 with continuous adjustment of pH to 6.0. The data described above led us to predict that continuous adjustment of culture pH to 6.0 would improve ethanol productivity from alginate. To test this prediction, we performed ethanol fermentation in liquid alginate medium containing 5% w/v alginate (25 ml) using the ethanologenic strain MK3353. The pH of the culture was adjusted to pH 4.0, 5.0, 6.0, 7.0, or 8.0 every day (Fig. 4A). As expected, adjustment of pH to 6.0 improved cell viability (Fig. 4B); however, ethanol production was not improved by this adjustment (Fig. 4C), indicating that the cells were viable for a longer period at pH 6.0, but still lost the ability to produce ethanol from alginate.

161 Addition of alginate (0.625 g or 1.25 g) after 3 days of cultivation had no effect on
162 ethanol concentration or cell viability of the ethanologenic strain MK3353 (data not
163 shown). Therefore, we speculate that although the toxic effects of byproducts in the
164 culture were attenuated by pH adjustment, these byproducts could still inhibit the
165 reactions involved in production of ethanol from alginate.
166
167 **Effects of pH adjustment on two-step fermentation.** Finally, we examined the effect
168 of adjusting pH to 6.0 on the two-step fermentation that we demonstrated previously.[14]
169 In our earlier study, we adjusted the pH of A1-supernatant of 3-day culture to 5.8, the
170 same as that of the yeast extract/peptone (YP) that was added to A1-supernatant to
171 support the growth of yeast.[14] In this study, we adjusted the pH of A1-supernatant of
172 3-day culture to 6.0, based on the results described above, and prepared YPM-A1
173 medium (final pH 6.1) by mixing 22.5 ml of A1-supernatant adjusted to pH 6.0, 2.5 ml
174 of 10-fold concentrated YP (pH 5.6 in this study), and 0.5 g mannitol. We also prepared
175 YPM-A1 medium (final pH 7.3) by mixing 22.5 ml of A1-supernatant without pH
176 adjustment (pH 8.7), 2.5 ml of 10-fold concentrated YP (pH 5.6), and 0.5 g mannitol.
177 The final mannitol concentrations in both media were 2% w/v. As controls, we also
178 prepared YPM (pH 6.1) and YPM (pH 7.3).
179 We cultivated *S. paradoxus* NBRC 0259-3 in these four media and monitored
180 ethanol production from mannitol (Fig. 5). As expected, *S. paradoxus* NBRC 0259-3
181 grew and produced ethanol in YPM-A1 medium (pH 6.1), but not at all in YPM-A1
182 medium (pH 7.3) (Fig. 5). Because *S. paradoxus* NBRC 0259-3 grew similarly in YPM
183 (pH 6.1) and YPM (pH 7.3), and produced only slightly less ethanol in YPM (pH 7.3)
184 than in YPM (pH 6.1) (Fig. 5), we concluded that the severe difference in growth and

ethanol production between YPM-A1 medium (pH 6.1) and YPM-A1 medium (pH 7.3) was not due to the pH difference alone, but was rather largely due to the attenuation of toxicity resulting from the pH adjustment. These observations demonstrate that adjustment of pH to 6.0 is also very effective in the two-step fermentation to produce ethanol from alginate and mannitol.

DISCUSSION

Due to the huge potential availability of marine macroalgae, and hence of alginate and mannitol, a system for production of ethanol from these carbohydrates would be of great value. Despite the importance of this goal, however, only two systems for production of ethanol from alginate have been established to date: one using engineered *Sphingomonas* sp. A1, and another using engineered *E. coli*.[11, 12]

The ethanologenic strain MK3353, a bioengineered strain of *Sphingomonas* sp. A1, carries genes for PDC and ADH from *Z. mobilis*.[11] In this strain, the PDC and ADH genes are controlled by a potent intrinsic promoter identified using DNA microarrays. In addition, the gene for LDH was deleted from the genome, because when this gene is present, lactate accumulates as the main byproduct of ethanol production.[11] Consequently, the ethanologenic strain MK3353 has acquired the capacity to produce as much as 13 g/L ethanol from 60 g/L alginate after a 72 h-fermentation.[11] However, as noted above, this bacterium is unable to assimilate mannitol.[13] To construct the engineered *E. coli* strain BAL1611, (i) an alginate-lyase secretion system, (ii) the genes for alginate degradation, transport, and metabolism, and (iii) the genes for PDC and ADH from *Z. mobilis* were introduced into an *E. coli* strain in which several genes (*pflB-focA*, *frdABCD*, and *ldhA*) had been deleted from the genome. Due to the intrinsic

209 ability of *E. coli* to assimilate glucose and mannitol, the resulting engineered strain is
210 able to produce 35–41 g/L ethanol after 150 h-fermentation from extracts (total mass,
211 140 g) of *S. japonica* containing various carbohydrates such as alginate, mannitol, and
212 glucose.[12]

213 Several bacterial species and strains are capable of producing ethanol from
214 mannitol: the bacteria *Zymobacter palmae* (13 g/L ethanol from 38 g/L mannitol after
215 70 h-fermentation), *E. coli* KO11 (25.8 g/L ethanol from 90 g/L mannitol after 120
216 h-fermentation), and the aforementioned *E. coli* strain (BAL1611).[9, 12, 15] Ethanol has
217 been produced from mannitol by some yeast strains, e.g., the *S. cerevisiae* polyploid
218 strain BB1 (5 g/L ethanol from 50 g/L mannitol after 60 h-fermentation) and *Pichia*
219 *angophorae* (10 g/L ethanol from 38 g/L mannitol after 75 h-fermentation).[10, 16] By
220 contrast, however, other *S. cerevisiae* strains, e.g., polyploid BB2, haploid S288C, and
221 haploid *Sc41 YJO*, are unable to assimilate mannitol for growth.[16, 17] Recently, we
222 demonstrated that the *S. paradoxus* strain NBRC 0259-3 is more suitable for the
223 production of ethanol from mannitol than *P. angophorae* and *E. coli* KO11.[14] *S.*
224 *paradoxus* strain NBRC 0259-3 was derived from the original NBRC 0259 strain by
225 cultivation for 3 days in medium containing mannitol, resulting in acquisition of higher
226 capacity to produce ethanol from alginate: *S. paradoxus* NBRC 0259-3 strain produced
227 40 g/L ethanol from 100 g/L mannitol after an 11-day fermentation.[14] We also
228 succeeded in a two-step fermentation in which the ethanologenic strain MK3353
229 initially converts alginate into ethanol, and then the yeast *S. paradoxus* NBRC 0259-3
230 produces ethanol from mannitol. In that system, we adjusted the pH of A1-supernatant
231 of 3-day culture to 5.8, the same as that of the YP added to A1-supernatant to support
232 growth of the yeast.[14]

In order to establish a practical system for production of ethanol from alginate as well as mannitol, it is necessary to achieve greater understanding of ethanol production by these systems. In this study, we found that during fermentation of the ethanologenic *Sphingomonas* sp. A1 strain MK3353 from alginate, the culture became slightly alkaline, and the bacterium secreted toxic byproducts that inhibited the growth of both itself and *S. paradoxus* NBRC 0259-3, and also killed the bacterial cells. However, we discovered that this toxicity could be attenuated by adjusting the pH of toxic culture supernatant or culture medium to 6.0. This worked very well in the two-step fermentation, due to the reduction in toxicity toward *S. paradoxus* NBRC 0259-3. To our knowledge, this is the first report of the formation of toxic byproducts during ethanol fermentation from alginate.

In ethanol production from lignocellulosic biomass, inhibitory compounds including aldehyde inhibitors, ketone inhibitors, organic acid inhibitors, and phenol-based inhibitors are generated during the thermo-chemical pre-treatment of the biomass.[18] Several detoxification methods have been described, including physical treatments (evaporation and use of membranes), physicochemical treatments (ion exchange resins, neutralization, overliming, use of activated charcoal, and extraction with organic solvents), and biological treatments (use of enzymes and microorganisms).[19] In our ethanol fermentation from alginate, we used commercially supplied sodium alginate, and therefore did not need to pretreat brown macroalgal biomass. Because generation of toxic compounds was dependent on the presence of the PDC and ADH genes, it is possible that the toxic compounds were derived from the metabolic conversion of alginate to ethanol (Fig. 1).

Although the toxic compounds derived from ethanol fermentation from alginate

remain to be identified, our findings provide a rationale for the success of our previous two-step fermentation method, in which we adjusted the pH of A1-supernatant of 3-day culture to 5.8, the same as that of YP.[14] Furthermore, because yeasts generally prefer acidic conditions (as shown in Fig. 5), and acidic conditions generally prevent bacterial contamination, attenuation of the toxicity of A1-supernanant by adjustment of the pH to 6.0 should be beneficial in the context of practical two-step fermentation. Therefore, such an approach should aid in the establishment of a practical system for ethanol production from brown macroalgae, a promising carbon source for bioethanol.

MATERIALS AND METHODS

Strains and cultivation. The ethanologenic *Sphingomonas* sp. A1 strain MK3353, that was previously called EPv104, lacks the LDH gene and carries the *Z. mobilis* genes for PDC and ADH on a broad–host range plasmid, pKS13.[11] The control *Sphingomonas* sp. A1 strain, MK3567, is also lacks in LDH gene, but carries pKS13 alone (i.e., without the *Z. mobilis* genes). *Sphingomonas* sp. A1 was transformed by triparental mating with *E. coli* DH5α carrying pRK2013 as a helper.[20] *S. paradoxus* NBRC 0259-3 is a derivative of the original *S. paradoxus* strain NBRC 0259, which can naturally assimilate mannitol.[14] *S. paradoxus* NBRC 0259-3 has a higher capacity than the parental strain for production of ethanol from mannitol.[14]

Alginate medium consists of sodium alginate from brown algae [average molecular weight (MW), 300 kDa; ratio of mannuronate to guluronate, 3:1; Nacalai Tesque], 0.1% w/v $(NH_4)_2SO_4$, 0.1% w/v KH_2PO_4, 0.1% w/v Na_2HPO_4, 0.01% w/v $MgSO_4 \cdot 7H_2O$, and 0.01% w/v yeast extract (pH 8.0).[11] Antibiotics [20 mg/l tetracycline (Tet) and 25 mg/l kanamycin (Kan)] were included in media for cultivation of the ethanologenic strain

MK3353 and the control strain MK3567. For solid medium, 1.5% w/v agar and 0.5% w/v alginate were included. For liquid media, 0.4, 0.8, or 5% w/v alginate was included. For ethanol production, fresh cells of the ethanologenic strain MK3353 grown on alginate solid medium were inoculated into liquid medium containing 0.8% w/v alginate and precultured at 30°C for 24 h at 145 spm. Cells in the preculture were inoculated into liquid alginate medium containing 5% w/v alginate (100 and 25 ml media in a 300- and 100-ml Erlenmeyer flasks, respectively), at an initial A_{600} of 0.1, and subsequently cultivated at 30°C and 95 spm.[11] After 3 days of cultivation, the culture was centrifuged at 20,000×*g* for 10 min, and the resultant supernatant was referred to as "A1-supernatant". When necessary, the pH of A1-supernatant was adjusted.

YP medium consisted of 1% w/v yeast extract and 2% w/v tryptone (pH 5.6). For YPM medium, YP medium was supplemented with 2% w/v mannitol. For solid medium, 2% w/v agar was included. YP medium was sterilized by autoclaving prior to addition of carbon sources. Ten-fold concentrated YP (pH 5.6) was sterilized by passage through a filter with 0.2-μm pores. YPM-A1 medium consisted of 22.5 ml of A1-supernatant (pH-adjusted if necessary), 2.5 ml 10-fold concentrated YP (pH 5.6), and 0.5 g mannitol. For ethanol production, *S. paradoxus* NBRC 0259-3 was grown as reported previously.[14] Yeast supernatant was obtained by centrifugation of the culture at 20,000×*g* at 4°C for 5 min.

Analytical methods. Concentration of ethanol in A1-supernatant or yeast supernatant was determined using assay kits (Roche) according to the manufacturer's instructions. A standard curve was prepared for each assay using an ethanol standard solution. Alginate concentration in the culture was determined by the carbazole–sulfuric acid

305 method, using sodium alginate as a standard.[21] To determine colony-forming units (cfu),
306 cultures were diluted in 1× P solution (1.1 mM KH_2PO_4 plus 1.1 mM Na_2HPO_4), and
307 streaked on solid alginate medium containing 20 mg/l Tet and 25 mg/l Kan.
308
309 **TEM.** The culture was mixed with an equal volume of 100 mM sodium and potassium
310 phosphate, pH 7.4 (PB) plus 4% paraformaldehyde and 4% glutaraldehyde, and then
311 incubated at 4°C for 1 h. After centrifugation at 2,000×*g* for 2.5 min, the collected cells
312 were again suspended in PB plus 2% glutaraldehyde and fixed overnight. The cells were
313 rinsed three times with PB, followed by post-fixation with 2% osmium tetroxide in PB.
314 The fixed cells were dehydrated with ethanol, infiltrated with propylene oxide, placed
315 into a 7:3 mixture of propylene oxide and Quetol-812 (Nisshin EM, Tokyo, Japan), and
316 incubated overnight with the lid open to volatilize propylene oxide. The cells were then
317 transferred to 100% resin and polymerized at 60°C for 48 h. Ultra-thin sections
318 (approximately 70 nm thick) were cut with a diamond knife using an Ultracut UCT
319 ultramicrotome (Leica). Sections were placed on copper grids and stained with 2%
320 uranyl acetate, followed by lead staining (Sigma). The sections were examined using a
321 JEM-1200EX microscope (JEOL, Tokyo, Japan) at 80 kV.
322
323 **SEM.** Cells were fixed and dehydrated as for TEM. The dehydrated cells were
324 substituted with tert-butyl alcohol and vacuum-dried using a DAP-6D dry vacuum
325 pump (Ulvac Kiko) with slow decompression. After drying, the samples were coated
326 with a thin layer (30 nm) of osmium using an NL-OPC80NS plasma coater (Nippon
327 Laser & Electronics Laboratory). The samples were observed using a JSM-6340F
328 scanning electron microscope (JEOL) at an electron voltage of 10.0 kV.

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400 **FIGURE LEGENDS**

401 **Figure. 1.** Process of ethanol production from alginate. The ethanologenic strain
402 MK3353 (closed symbol) and the control strain MK3567 (open symbol) were
403 precultured, inoculated, and cultivated as described in MATERIALS AND METHODS
404 in liquid alginate medium (100 ml) containing 5% w/v alginate at 30°C and 95 spm.
405 Growth (A_{600} of the culture) (A), ethanol concentration in A1-supernatant (B), alginate
406 concentration in the culture (C), and number of viable cells per 10 µl culture (cfu) (D)
407 were determined. Means and standard deviation (SD) of three independent experiments
408 are shown.

409

410 **Figure. 2.** Growth-inhibitory and killing effects of A1-supernatants. (A)
411 Growth-inhibitory effects of A1-supernatants on the ethanologenic strain MK3353.
412 MK3353 was precultured as described in MATERIALS AND METHODS; inoculated
413 into liquid alginate medium (1.0 ml) containing 0.4% w/v alginate plus 0, 10, 25, or
414 50% v/v of A1-supernatant from 1- (black bar), 2- (hatched bar), 3- (gray bar), or 4-day
415 (white bar) culture of MK3353; grown for 24 h at 145 spm and 30°C; and then the A_{600}
416 was measured. (B) Killing effects of A1-supernatant on MK3353. MK3353 was
417 cultured as in (A), except that the bacteria were grown for 8 h in media containing 0%
418 (open symbol) or 50% (closed symbol) v/v of A1-supernatant from 4-day culture of
419 MK3353. During cultivation, viability was determined as described in MATERIALS
420 AND METHODS. (C) Growth-inhibitory effect of A1-supernatant on *S. paradoxus*
421 NBRC 0259-3. Fresh *S. paradoxus* NBRC 0259-3 cells on YPM solid medium were
422 suspended in sterilized water (SDW) and inoculated into YPM (1.0 ml) containing 0, 10,
423 25, 50, or 90% v/v of A1-supernatant from 3-day culture of MK3353 or MK3567. The

culture was grown for 24 h at 145 spm and 30°C, and then A_{600} was measured. (D) Effect of A1-supernatant on viability of *S. paradoxus* NBRC 0259-3. *S. paradoxus* NBRC 0259-3 was cultured as in (C), except that *S. paradoxus* NBRC 0259-3 was grown for 8 h in the media containing 0% (open symbol) or 50% (closed symbol) v/v of A1-supernatant from 3-day culture of MK3353. During cultivation, viability was determined as described in MATERIALS AND METHODS. (A–D) Means and SD of three independent experiments are shown.

Figure 3. pH adjustment attenuates the growth-inhibitory and killing effects of A1-supernatant. (A) Attenuation of growth-inhibitory effect of A1-supernatant on the ethanologenic strain MK3353. MK3353 was cultured as described for Fig. 2A, but in liquid alginate medium containing 0.4% w/v alginate plus 0% (cont.) or 50% v/v of A1-supernatant from 4-day culture of MK3353, with pH adjusted with HCl as indicated, and then A_{600} was measured. (B) Attenuation of the killing effect of A1-supernatant on MK3353. MK3353 was cultured as described in (A); but with pH adjusted to 5.0 or 6.0 with HCl, or not adjusted (pH 8.7), in 25 ml liquid medium at 95 spm and 30°C. After 24 h of cultivation, viability was determined. (C) Attenuation of growth-inhibitory effect of A1-supernatant on *S. paradoxus* NBRC 0259-3. Fresh *S. paradoxus* NBRC 0259-3 cells on YPM solid medium were cultured as in Fig. 2C; but in YPM (1.0 ml) containing 0% (cont.) or 50% v/v of A1-supernatant from 3-day culture of MK3353, with pH adjusted with HCl as indicated, and then A_{600} was measured. (A–C) Means and SD of three independent experiments are shown.

Figure 4. Effect of continuous pH adjustment on ethanol production from alginate. (A)

pH profile of the culture. The ethanologenic strain MK3353 was grown in liquid alginate medium (25 ml) containing 5% w/v alginate at 95 spm and 30°C, as described in MATERIALS AND METHODS. The pH of the culture was adjusted with HCl or NaOH as indicated (open triangle, without pH adjustment; open square, pH 4.0; open diamond, pH 5.0; closed triangle, pH 6.0; closed square, pH 7.0; closed diamond, pH 8.0) every 24 h. (B) Viability of the cells in the cultures. (C) Ethanol concentrations in the cultures. (A–C) Means and SD of three independent experiments are shown.

Figure 5. Effect of pH adjustment on the two-step fermentation. *S. paradoxus* NBRC 0259-3 maintained on solid YPM medium was precultured in 50 ml YPM liquid medium at 30°C in a 100-ml Erlenmeyer flask on a shaker (Personal Lt-10F) at 95 spm.[14] After 1 day of cultivation, cells were collected, washed once with SDW, suspended in SDW, and added to 25 ml YPM (open triangles, pH 7.3; or open squares, pH 6.1) or 25 ml YPM-A1 medium (closed triangles, pH 7.3; or closed squares, pH 6.1) in a 50-ml Erlenmeyer flask to an initial A_{600} of 0.1; cultivation was continued at 30°C and 95 spm. YPM-A1 medium consists of 2.5 ml 10-fold concentrated YP, 0.5 g mannitol, and 22.5 ml of A1-supernatant from 3-day culture of the ethanologenic strain MK3353 without pH adjustment (resulting in a final pH of 7.3; closed triangles) or with adjustment to pH 6.0 (resulting in a final pH of 6.1; closed squares). A_{600} indicating cell growth (A) and ethanol concentration (B) were measured. (A, B) Means and maximum and minimum values of two independent experiments are shown.

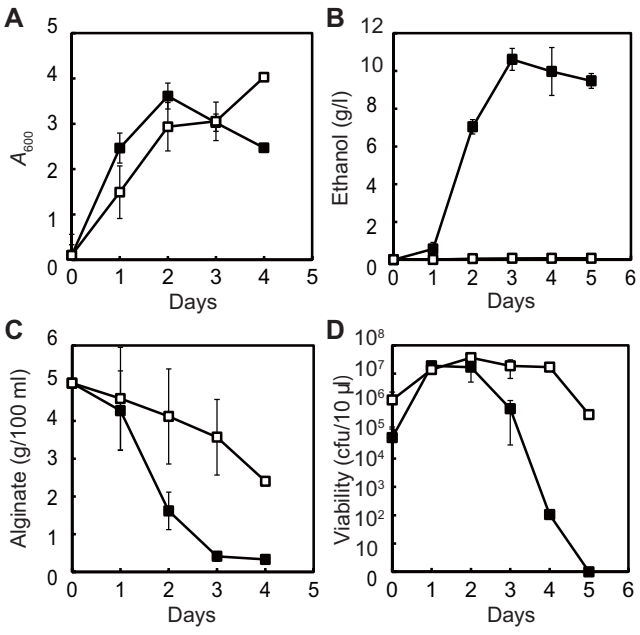

A
A_{600}
Days
B
Ethanol (g/l)
Days
C
Alginate (g/100 ml)
Days
D
Viability (cfu/10 µl)
Days

Fig. 1

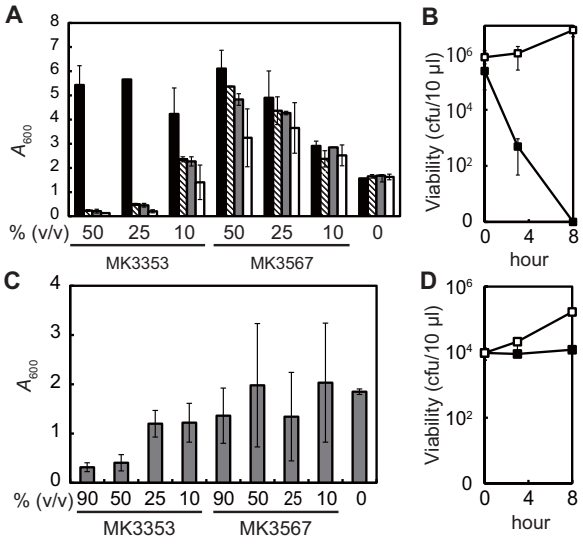

A
B
C
D
A_{600}
Viability (cfu/10 µl)
% (v/v)
50 25 10 50 25 10 0
MK3353
MK3567
90 50 25 10 90 50 25 10 0
hour

Fig. 2

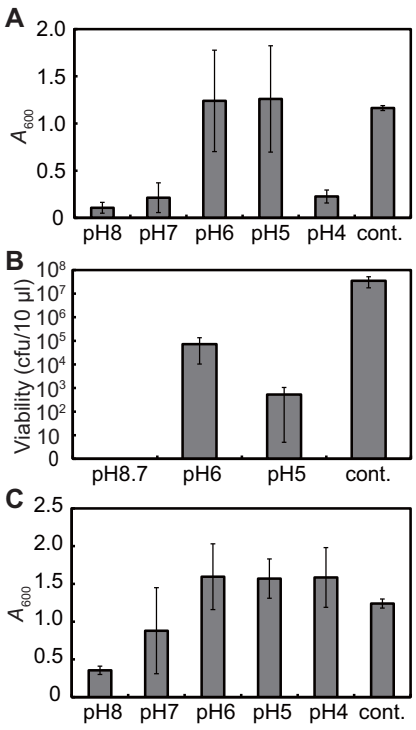

A
2.0
1.5
1.0
0.5
0
A_{600}
pH8
pH7
pH6
pH5
pH4
cont.
B
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
Viability (cfu/10 µl)
pH8.7
pH6
pH5
cont.
C
2.5
2.0
1.5
1.0
0.5
0
A_{600}
pH8
pH7
pH6
pH5
pH4
cont.

Fig. 3

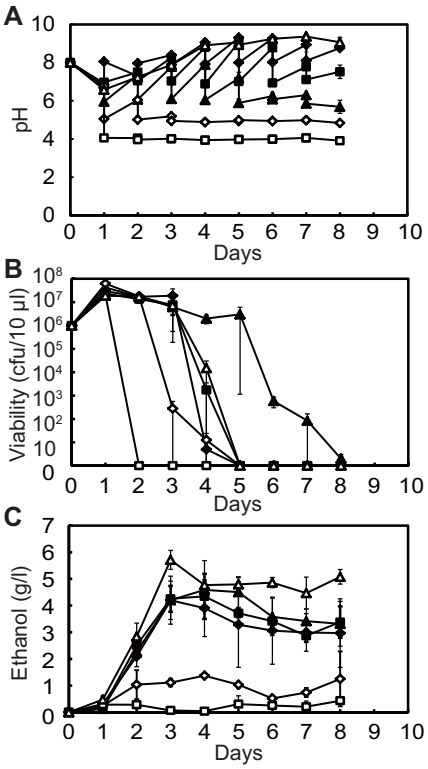


Fig. 4

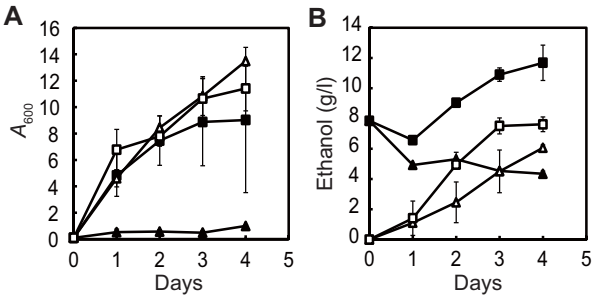
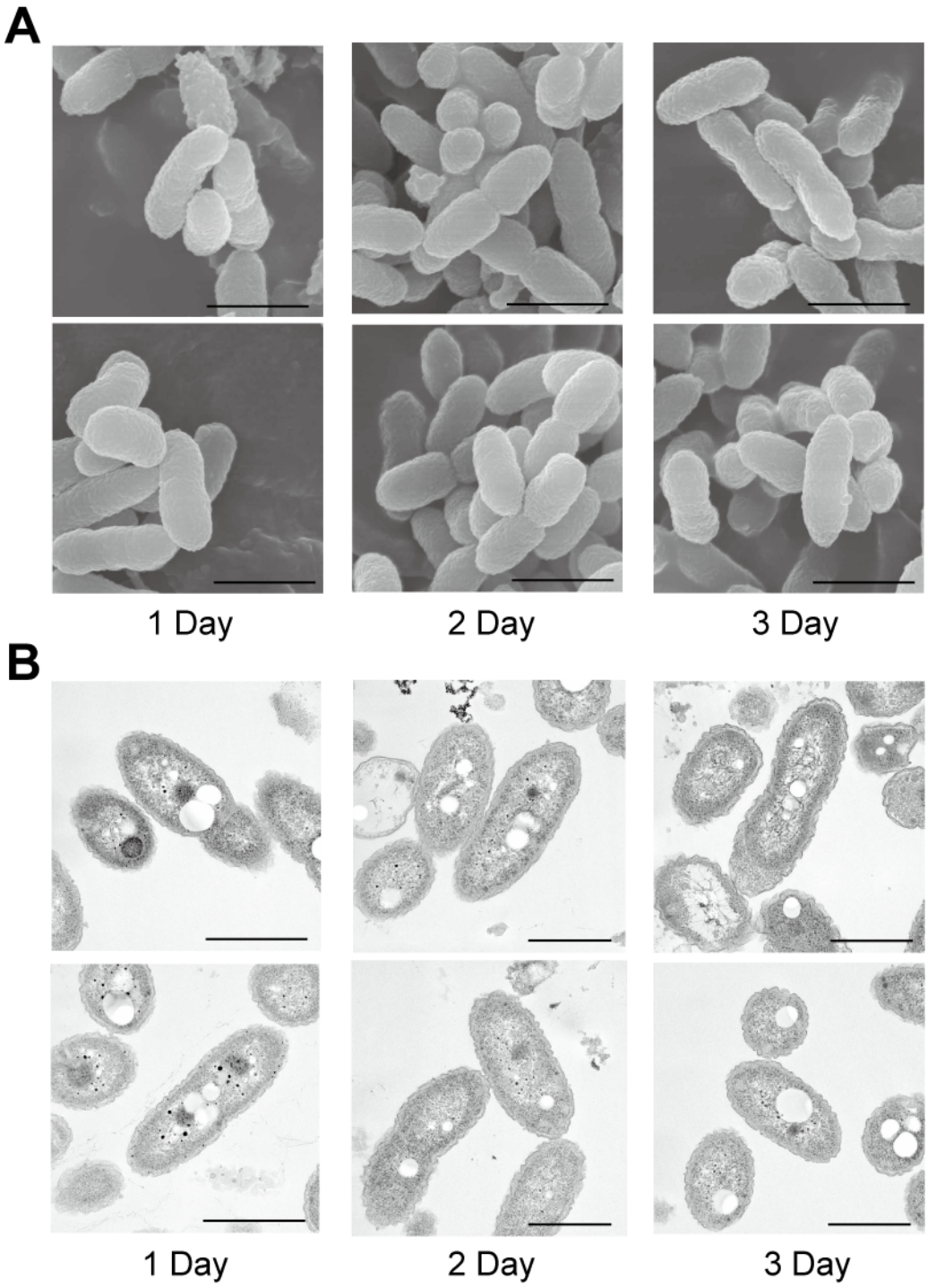
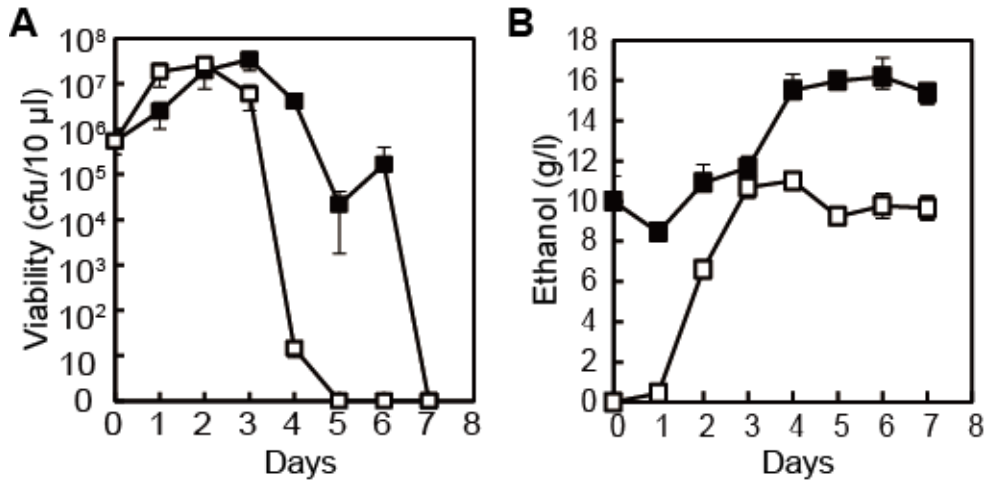

A
16
14
12
10
8
6
4
2
0
A_{600}
0 1 2 3 4 5
Days
B
14
12
10
8
6
4
2
0
Ethanol (g/l)
0 1 2 3 4 5
Days

Fig. 5



Supplementary Fig. S1. SEM (A) and TEM (B) observations of cells of the ethanologenic strain MK3353 (upper panels) and the control strain MK3567 (lower panels). Cells were precultured, inoculated, and cultivated as described in MATERIALS AND METHODS in liquid alginate medium (100 ml) containing 5% w/v alginate at 30°C and 95 spm for the indicated periods. SEM and TEM observations were conducted

as described in MATERIALS AND METHODS. Scale bars: 1 μm for SEM, 0.5 μm for TEM.



Supplementary Fig. S2. Initial presence of ethanol causes no reduction in cell viability of ethanologenic strain MK3353. The ethanologenic strain MK3353 was cultivated as in Fig. S1 in the presence of an initial concentration of 0% w/v (open symbols) or 1.0% w/v [10 g/l] (closed symbols) ethanol. Number of viable cells per 10 µl culture (viability; cfu) (A) and ethanol concentration in A1-supernatant (B) were determined. Means and standard deviation (SD) of three independent experiments are shown.