Bacterial pyruvate production from alginate, a promising carbon source from marine brown macroalgae

Running title: Bacterial pyruvate production from alginate

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Abstract

Marine brown macroalgae is a promising source of material for biorefining, and alginate is one of the major components of brown algae. Despite the huge potential availability of alginate, no system has been reported for the production of valuable compounds other than ethanol from alginate, hindering its further utilization. Here we report that a bacterium, *Sphingomonas* sp. strain A1, produces pyruvate from alginate and secretes it into the medium. High aeration and deletion of the gene for D-lactate dehydrogenase are critical for the production of high concentrations of pyruvate. Pyruvate concentration and productivity were at their maxima (4.56 g/l and 95.0 mg/l/h, respectively) in the presence of 5% (w/v) initial alginate, whereas pyruvate produced per alginate consumed and % of theoretical yield (0.19 g/g and 18.6%, respectively) were at their maxima at 4% (w/v) initial alginate. Concentration of pyruvate decreased after it reached its maximum after cultivations for 2 or 3 days at 145 strokes per minute. Our study is the first report to demonstrate the production of other valuable compounds than ethanol from alginate, a promising marine macroalgae carbon source.

Introduction

Among red, green, and brown marine macroalgae, brown macroalgae are the largest and therefore the most desirable sources for biorefining. Macroalgae have several advantages as a crop: they are more productive than land crops; do not require arable land, irrigation water, or fertilizer; and they contain no lignin. (1-4). One of the major components in brown algae is alginate: the brown algae *Laminaria japonica* and genera *Sargassum* and *Turbinaria* contain up to 40% alginate by dry weight (5, 6). Alginate is a linear polysaccharide consisting of *β*-D-mannuronate (M) and its C5 epimer α-L-guluronate (G), arranged as polyM, polyG, and heteropolymeric random sequences (polyMG) (7).

Two systems for ethanol production from alginate have been established using bioengineered bacteria, including *Sphingomonas* sp. strain A1 (8) and *Escherichia coli* (9). The former system depends on the ethanologenic *Sphingomonas* sp. strain A1, which lacks the gene for D-lactate dehydrogenase (LDH) and also carries the genes for pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Zymomonas mobilis* on a broad-host-range plasmid pKS13 (10); this strain can produce up to 13 g/l ethanol from alginate (8). The latter system is dependent on an ethanologenic *E. coli* strain carrying genes for alginate utilization, PDC, ADH, and containing several deletions; this strain can produce 37 g/l ethanol from a mixture of mannitol and alginate derived from brown algae (kombu; *Saccharina japonica*) (9). Although these systems for ethanol production from alginate have been established (8, 9), no system has been reported for the production of valuable compounds other than ethanol from alginate.

Pyruvate is widely used as a starting material in the biosynthesis of pharmaceuticals (e.g., L-tryptophan, L-tyrosine, alanine, and L-DOPA) and is

employed for production of crop-protection agents, polymers, cosmetics, and food additives (11). Chemical production of pyruvate has been achieved by dehydration and decarboxylation of tartrate (12). However, this process is not cost-effective (11); hence, biotechnological pyruvate production has attracted attention. To date, successful biotechnological production of pyruvate has primarily proceeded from glucose, using bacteria such as *E. coli* and *Corynebacterium glutamicum* and yeasts such as *Saccharomyces cerevisiae* and *Torulopsis glabrata* (11, 13-15).

In *Sphingomonas* sp. strain A1, alginate is depolymerized by endo- and exo-alginate lyases into unsaturated uronic acid, which is non-enzymatically converted to 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) (Fig. 1). DEH is reduced to non-toxic 2-keto-3-deoxy-D-gluconate and metabolized to pyruvate, which could be further metabolized via the TCA cycle (16, 17) (Fig. 1). In ethanologenic *Sphingomonas* sp. strain A1, pyruvate is converted into ethanol by PDC and ADH (8). In this study, we found that *Sphingomonas* sp. strain A1 that lacks LDH gene secretes pyruvate into the medium, thus opening the door to marine biorefineries that could cost-effectively produce several valuable compounds from marine biomass.

MATERIALS AND METHODS

Strain and cultivation The *Sphingomonas* sp. A1 wild type (WT) strain is a Gram-negative bacterium that is able to assimilate alginate (16). The LDH gene of *Sphingomonas* sp. A1 WT strain was disrupted by inserting kanamycin-resistant cassette into LDH gene on the genome, resulting in the *Sphingomonas* sp. A1 *ldh* strain (MK2651) (8). Strain MK3567 is the *Sphingomonas* sp. A1 *ldh* strain carrying an empty broad host range plasmid, pKS13 (10).

Alginate medium contains sodium alginate (from brown algae; average MW, 300 kDa; ratio of M to G, 3:1; Nacalai Tesque, Japan), 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 $MgSO_4 \cdot 7H_2O$, 0.01% w/v yeast extract (pH 8.0) (8). For cultivation of MK3567, 20 mg/l tetracycline (Tet) and 25 mg/l kanamycin (Kan) were included in the media; for cultivation of MK2651, 25 mg/l Kan alone was included. For solid medium, alginate medium containing 0.5% w/v alginate was solidified with 1.5% w/v agar. For precultivation, fresh A1 bacteria grown on alginate solid medium were inoculated into liquid alginate medium containing 0.8% w/v alginate and precultured at 30°C for 24 h at 145 strokes per minute (spm) on a Personal Lt-10F shaking water bath (Taitec, Japan). Cells in the preculture were inoculated into 100 ml liquid alginate medium containing 5% w/v alginate (5% alginate medium) in a 300-ml Erlenmeyer flask (MK3567) or in 20 ml liquid alginate media containing 0.8, 2, 3, 4, 5, or 6% w/v alginate (0.8, 2, 3, 4, 5, or 6% alginate medium) in a 200-ml flask (WT and MK2651), to reach an OD_{600} of 0.1; bacteria were then cultivated further at 30°C at 50, 95, or 145 spm, unless otherwise specified. The supernatant of the culture was harvested after centrifugation of the culture at 20,000 × g, at 4°C for 5 min. Cell dry weight (CDW) of *Sphingomonas* sp. A1 was calculated from the OD_{600} using a ratio of 0.38 $g_{(CDW)}\ l^{-1}$ per OD_{600}. This ratio was obtained from 4 experiments in which MK2651 strain was cultivated in liquid 100 ml alginate medium containing 0.8% w/v alginate in a 300-ml flask at 30˚C and 145 spm for 1 day.

Metabolome analysis Metabolome analysis of the supernatant of the cultures described above was performed by Human Metabolome Technologies, Inc.,

using capillary-electrophoresis time-of-flight mass spectrometry (CE-TOFMS) in the anion and cation detection modes (8).

HPLC analysis For the analyses of pyruvate, 2-oxoglutarate, and 2-oxoisovalerate, HPLC analysis was conducted using an HPLC equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) and a RID-10A detector (Shimadzu, Japan). Other conditions were as follows: effluent, filtered and degassed 5 mM H_2SO_4; flow rate, 0.65 ml min^{-1}; column temperature, 65.5°C.

Concentration of oxygen in liquid medium Concentration of oxygen dissolved in liquid medium was measured using a Fibox 3 oxygen sensor (Presens, Regensburg, Germany) and an oxygen-sensor spot (Presens) attached to the bottom of a 300-ml Erlenmeyer flask. In this study, the saturated concentration of oxygen dissolved in liquid medium at 30°C was considered to be 7.5 mg/l (18).

TLC analysis Authentic compounds (5 µl, 2% w/v sucrose or glucose) and the cultures (5 µl) were spotted onto TLC glass plates with silica gel 60 F_{254} (Merck, USA), dried, developed with a solvent system consisting of 1-butanol, acetate, and water (3:2:2, v/v/v) and visualized by heating the TLC plate at 130°C for 5 min after spraying it with 10% (v/v) sulfuric acid in ethanol (17).

Other analytical methods The concentration of pyruvate in the 4-day supernatants of initial cultivation of MK3567 strain was determined with metabolome or HPLC analysis as above. In the other case, the concentration of pyruvate of the supernatant of the culture was determined using pyruvate assay kit (Roche

Diagnostics). A standard curve was prepared for each assay. Alginate concentrations in the cultures were determined by the carbazole sulfuric acid method, using sodium alginate as the standard (19).

RESULTS AND DISCUSSION

Production of pyruvate by *Sphingomonas* sp. strain A1 Metabolome analysis of the intracellular compounds of the *Sphingomonas* sp. A1 WT strain has been previously described (8). In the previous analysis, the WT strain was cultivated in liquid alginate medium containing 0.5% or 3% w/v alginate, and intracellular accumulation of lactate was observed (8).

In this study, we performed metabolome analysis of the supernatants of the *Sphingomonas* sp. A1 *ldh* strain carrying an empty plasmid pKS13 (10) (MK3567) cultivated in 100 ml liquid alginate medium containing 5% w/v alginate in 300-ml Erlenmeyer flasks for 1, 2, or 4 days at 95 spm (Table 1). The *Sphingomonas* sp. A1 *ldh* strain is the host for the ethanologenic *Sphingomonas* sp. A1 and lacks the LDH gene (8). The *ldh* strain still shows approximately 20% of LDH activity compared to WT strain (8). We initially conducted this analysis as a control for the analysis of the supernatants of ethanologenic *Sphingomonas* sp. A1 strain. The ethanologenic strain is *Sphingomonas* sp. A1 *ldh* strain carrying PDC genes and ADH gene on a broad host range plasmid pKS13 (8). This is the reason why we initially used the *ldh* strain carrying pKS13 (MK3567), not *ldh* strain without plasmid (MK2651).

Of the 61 metabolites that were quantitatively identified, only pyruvate and 2-oxoglutarate exceeded 10 mM; pyruvate concentrations were 1.2, 20.2, and 26.2 mM (0.11, 1.8, and 2.3 g/l, respectively), and 2-oxoglutarate concentrations were 0.09, 1.6, and 14.4 mM (0.01, 0.23, and 2.1 g/l, respectively), in supernatants of 1, 2, and 4-days cultures. 2-Oxoisovalerate was the compound that was produced in the third highest amounts: 0.018, 1.2, and 2.5 mM (0.00, 0.14, and 0.29 g/l, respectively) in 1, 2, and 4-day cultures.

Because pyruvate has been regarded as an intermediate in the alginate-metabolic pathway (16, 17) (Fig. 1), we did not expect to detect high concentrations of extracellular pyruvate. Due to high industrial demand for pyruvate (11) and the huge potential availability of alginate as source for biorefining, we chose to further characterize pyruvate production from alginate using *Sphingomonas* sp. strain A1.

Aeration is important for the production of pyruvate from alginate

Sphingomonas sp. A1 *ldh* strain carrying an empty plasmid pKS13 (MK3567) was cultivated under the same conditions used for metabolome analysis, but at 50, 95, or 145 spm for 4 days, during which we monitored the concentrations of oxygen, pyruvate, and alginate and the growth of this strain. The culture was aerated highly at 145 spm, moderately at 95 spm, and slightly at 50 spm.

Oxygen concentrations of the culture were kept at basal levels during cultivation at 50 and 95 spm, whereas the concentration increased to saturation at 6.0–7.5 mg/l at 145 spm (Fig. 2a). Cultivation at 145 spm also gave the maximum concentrations of pyruvate, whereas cultivation at 95 spm and 50 spm resulted in moderate and no pyruvate production, respectively. Growth was in accord with the pyruvate production, and consumption of alginate was lowest at 50 spm, but approximately equal at 95 and 145 spm. Thus, high aeration is important for pyruvate production. The 4-day supernatants were also analyzed by HPLC, and the maximum production of pyruvate at 145 spm was again confirmed (3.33, 0.49, and 0 g/l pyruvate at 145, 95, and 50 spm; data not shown). At 145, 95, and 50 spm, production of 2-oxoglutarate (0.87, 0.14, and 0 g/l) was lower than that of pyruvate, and no production of 2-oxoisovalerate was detected (data not shown).

Effect of initial alginate concentrations on production of pyruvate To determine the optimum initial concentration of alginate, *Sphingomonas* sp. A1 *ldh* strain (MK2651) that carries no plasmid was cultivated at 145 spm in 20 ml liquid alginate media containing 0.8, 2, 3, 4, 5, or 6% alginate in 200-ml Erlenmeyer flasks for 6 days, during which we measured the concentrations of pyruvate and alginate and the growth of this strain (Fig. 3). Productivity (mg/l/h), $Y_{P/S}$ (g/g) (an yield of pyruvate produced [ΔP; g/l] per alginate consumed [ΔS; g/l]), % of theoretical yield, CDW (ΔX; g/l), $Y_{X/S}$ (g/g) (an yield of CDW [ΔX; g/l] per alginate consumed [ΔS; g/l]), $Y_{P/X}$ (g/g) (an yield of pyruvate produced [ΔP; g/l] per CDW [ΔX; g/l]) were calculated based on the obtained data. Theoretical yield was taken to be 100% when 100 g pyruvate was produced from 100 g consumed alginate, because 2 mol of pyruvate (MW of 88) is theoretically produced from 1 mol of DEH (MW of 176) (Fig. 1).

As shown in Fig. 3 and Table 2, only a limited amount of pyruvate was produced in alginate medium containing 0.8% w/v alginate, whereas pyruvate concentration (ΔP; g/l) and productivity (mg/l/h) were at their maxima in the presence of 5% initial alginate. $Y_{P/S}$ (g/g) and % of theoretical yield were at their maxima at 4% initial alginate. CDW (ΔX; g/l) and $Y_{X/S}$ (g/g) were at their maxima at 3% initial alginate. $Y_{P/X}$ (g/g) was at its maximum at 6% initial alginate. Growth rates over 2 days were higher in 2 and 3% alginate media than other media (Fig. 3c). Consumption of alginate was also confirmed by TLC (Fig. 3d).

The maximum pyruvate concentration observed in this experiment (4.56 g/l; Fig. 3a, Table 2) was higher than those observed in Fig. 2b (2.80 g/l), although cells were cultivated in liquid 5% alginate medium at 145 spm at 30°C in both cases. We

attributed this to differences between the strains (MK3567 and MK2651 strains) and the scales of the cultures. In Fig. 2, *Sphingomonas* sp. A1 MK3567 strain (the *ldh* strain carrying an empty plasmid pKS13) was cultivated in 100 ml medium in 300-ml Erlenmeyer flasks, whereas *Sphingomonas* sp. A1 MK2651 strain (the *ldh* strain without plasmid) was cultivated in 20 ml medium in 200-ml flasks in Fig. 3.

Effect of LDH disruption on production of pyruvate This study was undertaken with the *Sphingomonas* sp. A1 *ldh* strains (MK3567 and MK2651 strains), which is host of the ethanologenic *Sphingomonas* sp. strain A1 (8). Since pyruvate is a substrate of LDH, disruption of LDH could enhance production of pyruvate. To confirm this, we compared production of pyruvate by the WT strain (Fig. 4, open symbols) at 145 spm in liquid alginate medium containing 5% w/v alginate to production by *Sphingomonas* sp. A1 *ldh* strain (MK2651) without plasmid (Fig. 4, closed symbols; Table 2). Although the consumption rates of alginate and growth of the *ldh* strain were approximately the same as those of the WT (Fig. 4, b and d), the maximum pyruvate concentration of WT strain was lower than that of the *ldh* strain (Fig. 4a). Moreover, concentration of pyruvate dropped rapidly in the WT strain (Fig. 4a). In accord with its lower production of pyruvate, the WT strain produced D-lactate, whereas the *ldh* strain produced only a limited amount of D-lactate (Fig. 4c). No production of L-lactate was observed in either the WT or *ldh* strains (Fig. 4c). These data demonstrate that disruption of the LDH gene clearly increased pyruvate production.

Assimilation of pyruvate by *Sphingomonas* sp. strain A1 We observed that the concentration of pyruvate decreased after reaching its maximum value,

whereas that of D-lactate did not decrease and remained saturated during cultivation (Fig. 2b, Fig. 3a, and Fig. 4ac), suggesting that *Sphingomonas* sp. strain A1 utilizes pyruvate, but not D-lactate, as a carbon source. Accordingly, we observed that the *Sphingomonas* sp. A1 WT strain utilized pyruvate as a carbon source, but not D-lactate or other organic acids (citrate, succinate, L-lactate, D-Lactate, fumarate, L-malate, DL-isocitrate, and acetate); this strain grew better in the presence of pyruvate than in the presence of glucose (Fig. 5a).

Moreover, we demonstrated that *Sphingomonas* sp. A1 *ldh* strain (MK2651) metabolized both alginate and pyruvate; both of which were utilized simultaneously (Fig. 5c). When alginate was present alone at a concentration of 0.8% (w/v), it was more rapidly consumed by the *ldh* strain than 0.8% (w/v) pyruvate alone or 0.8% (w/v) alginate and 0.8% (w/v) pyruvate (Fig. 5c), indicating that pyruvate partially suppresses the utilization of alginate. Growth of the *ldh* strain in the presence of both 0.8% (w/v) alginate and 0.8% (w/v) pyruvate was approximately the same as in the presence of 0.8% (w/v) alginate alone, but faster than in the presence of 0.8% (w/v) pyruvate alone (Fig. 5c). We also investigated whether the *ldh* strain would produce pyruvate in 5% (w/v) alginate medium initially containing 0.5% (w/v) [5.0 g/l] pyruvate, and compared the results with those obtained from the *ldh* strain cultivated in 5% (w/v) alginate medium with no initial pyruvate (Fig. 5d). We observed that the concentration of pyruvate dropped rapidly and never exceeded 5.0 g/l, and that alginate was utilized more moderately than in the absence of initial pyruvate (Fig. 5d). These observations demonstrate that pyruvate partially suppresses not only utilization of alginate as shown in Fig. 5c, but also production of pyruvate.

Collectively, we propose that when the *Sphingomonas* sp. A1 WT strain metabolizes alginate, it secretes pyruvate and reutilizes the secreted pyruvate, which

could partially suppress further utilization of alginate. This could at least partially explain why *Sphingomonas* sp. A1 *ldh* strain ceased to utilize alginate after 2–3 days during production of pyruvate from alginate (Fig. 2c, 3b).

Furthermore, we surmise that *Sphingomonas* sp. strain A1 produce the cellular structure molecules from pyruvate such as through gluconeogenesis and pentose-phosphate pathways. *Sphingomonas* sp. strain A1 contains all genes for gluconeogenesis and pentose-phosphate pathways on its genome (our unpublished data). However, the reason why other organic acids than pyruvate could not be the carbon source has remained to be elucidated. It should be noted that *Sphingomonas* sp. strain A1 carries the genes for TCA cycle and in particular the gene that shows 54% identity (e value of e-130) with dctA (*Escherichia coli* C4-dicarboxylic acid transporter) (20).

Toward production of higher amounts of pyruvate by *Sphingomonas* sp. strain A1 Several studies have reported the production of pyruvate from glucose by microorganisms, including the yeast *S. cerevisiae* IFO0538 (37 g/l pyruvate) (11), the yeast *T. glabrata* IFO0005 (68 g/l) (11), engineered *E. coli* (62 g/l) (14), and engineered *C. glutamicum* (44 g/l) (13); these systems produced more pyruvate than our system in this study produced from alginate. However, the literature regarding the production of pyruvate from carbon sources other than glucose is limited (11, 13, 14); no studies have reported production of pyruvate from alginate.

Growth of the *Sphingomonas* sp. A1 WT and *ldh* strains was not inhibited by extracellular pyruvate up to ~12 g/l (Fig. 5b). Thus, the *ldh* strain could produce at least ~12 g/l pyruvate as a result of several genetic modifications such as that relieves the partial suppression of alginate utilization by pyruvate, described above. In

addition, other genetic modifications could enhance tolerance of extracellular pyruvate.

As noted above, pyruvate is widely used in agriculture and industry (11); because pyruvate is an important intermediate in metabolic map, several valuable compounds could be produced from it. Furthermore, alginate is abundant and widely available. Therefore, the use of alginate to produce pyruvate could be of tremendous economic importance. Our study is the first report demonstrating the production of a valuable compound from alginate, opening the way to marine biorefineries that could cost-effectively produce several compounds from marine biomass, a promising carbon source.

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Figure legends

FIG. 1. Alginate metabolism in the *Sphingomonas* sp. A1 wild-type strain (16, 17). Compounds are indicated in bold, and enzymes are in gray. Abbreviations: DEH, 4-deoxy-L-erythro-5-hexoseulose uronic acid; KDG, 2-keto-3-deoxy-D-gluconate; KDGP, 2-keto-3-deoxy-phosphogluconate; GAP, glyceraldehyde 3-phosphate; A1-R, NADPH-dependent DEH reductase; A1-K, KDG kinase; A1-A, aldolase; LDH, D-lactate dehydrogenase.

FIG. 2. Aeration is important for the production of pyruvate from alginate. Concentrations of oxygen (a), pyruvate (b), alginate (c), and growth (d; OD_{600}) of the culture of the *Sphingomonas* sp. A1 *ldh* strain carrying pKS13 (MK3567). This strain was cultivated at 50 (circles), 95 (squares), and 145 (triangles) spm in 100 ml 5% liquid alginate medium [5% w/v alginate, 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 $MgSO_4 \cdot 7H_2O$, 0.01% w/v yeast extract (pH 8.0), 20 mg/l Tet, and 25 mg/l Kan] in 300-ml Erlenmeyer flasks at 30°C. b–d; Averages and standard deviations (SD) of three independent experiments are shown.

FIG. 3. Effect of initial concentration of alginate on production of pyruvate. The *Sphingomonas* sp. A1 *ldh* strain (MK2651) without plasmid was cultivated at 145 spm in 20 ml liquid alginate media [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 $MgSO_4 \cdot 7H_2O$, 0.01% w/v yeast extract (pH 8.0), and 25 mg/l Kan] containing 0.8% (open circles), 2% (open squares), 3% (closed diamonds), 4% (open diamonds), 5% (closed triangles), or 6% (closed circles) w/v alginate in 200-ml Erlenmeyer flasks at 30°C for 6 days during which concentrations of pyruvate (a) and alginate (b) and growth of the cultures (c) were measured. Averages and SD of

three independent experiments are shown (a–c). (d) Aliquots (5 μl) of cultures in the indicated media, cultivated for the indicated number of days, were also analyzed by TLC. Spots at the original positions represent alginate. The 0-day culture of 5% alginate medium was too viscous to spot.

FIG. 4. Comparison of the *Sphingomonas* sp. A1 WT and *ldh* strains. WT (open triangles) and *ldh* strains (closed triangles; MK2651 strain) were cultivated in 20 ml liquid 5% alginate media as in Fig. 3 for 6 days, during which concentrations of pyruvate (a), alginate (b), and D-lactate (c) and growth of the cultures (d) were measured. For the cultivation of WT strain, Kan was not included. Data of *ldh* strain were the same with those in Fig. 3. No production of L-lactate was detected. Means and SD of three independent cultivations are presented.

FIG. 5. Utilization of pyruvate (a) and tolerance to pyruvate (b) of the *Sphingomonas* sp. A1 WT strain, and simultaneous metabolism of alginate and pyruvate by the *Sphingomonas* sp. A1 *ldh* strain (c, d). (a) The WT strain was precultured as described in MATERIALS AND METHODS, collected, washed once with 2 mM sodium-potassium phosphate (pH 6.9), resuspended in the same buffer, and inoculated to reach an OD_{600} of 0.1 into 1.0 ml of liquid 0.8% alginate (Alg) medium, medium containing no alginate (None), or medium in which 0.8% alginate was replaced with 0.8% (w/v) of another carbon source: citrate (Cit), succinate (Suc), L-lactate (L-Lac), D-Lactate (D-Lac), fumarate (Fum), L-malate (Mal), DL-isocitrate (isoCit), acetate (Ace), glucose (Glc), or pyruvate (Pyr). The cells were cultivated for 24 h at 30°C, and OD_{600} was measured. (b) WT (open triangles) and *ldh* (closed triangles) strains were inoculated to reach OD_{600} of 0.1 into 1.0 ml of liquid 0.8% alginate medium as

above containing the indicated concentrations of pyruvate (final pH of the media; pH 8.0–8.2) as in Fig. 6a. The cells were cultivated for 24 h at 30°C, and OD_{600} was measured. (c) The *ldh* strain (MK2651) grown on alginate solid medium was suspended in 2 mM sodium-potassium phosphate and inoculated at an OD_{600} of 0.1 into 3.0 ml of liquid medium containing 0.8% (w/v) alginate (squares), 0.8% (w/v) pyruvate (circles), or both 0.8% (w/v) alginate and 0.8% (w/v) pyruvate (triangles). The cells were cultivated for 33 h at 30°C, during which the concentration of pyruvate (left; closed symbols), the concentration of alginate (left; open symbols), and OD_{600} (right) were measured. Means and maximum and minimum values of two independent cultivations are presented. (d) The *ldh* strain (MK2651) was cultivated as in Fig. 4 in 20 ml liquid 5% alginate medium without (closed triangles) or with (open triangles) an initial pyruvate concentration of 0.5% [5.0 g/l]. Concentration of pyruvate (left), concentration of alginate (middle), and OD_{600} (right) were measured. Data for the *ldh* strain cultivated without initial pyruvate were the same as those shown in Fig. 3 and 4. Means and SD of three independent cultivations are presented (a, b, d).

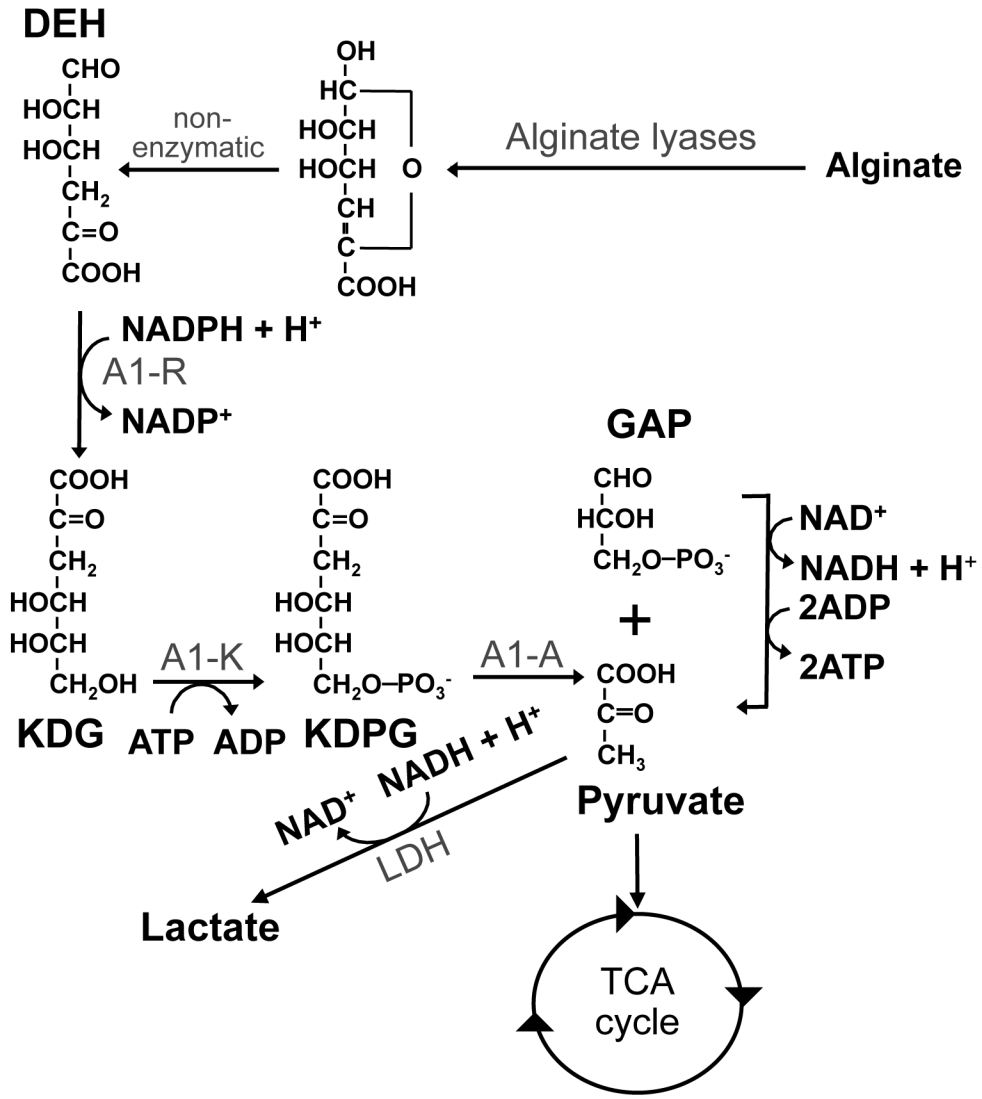
DEH
non-
enzymatic
Alginate lyases
Alginate
NADPH + H+
A1-R
NADP+
GAP
NAD+
NADH + H+
2ADP
2ATP
A1-K
A1-A
KDG
ATP
ADP
KDPG
NAD+
NADH + H+
LDH
Pyruvate
Lactate
TCA
cycle

FIG. 1

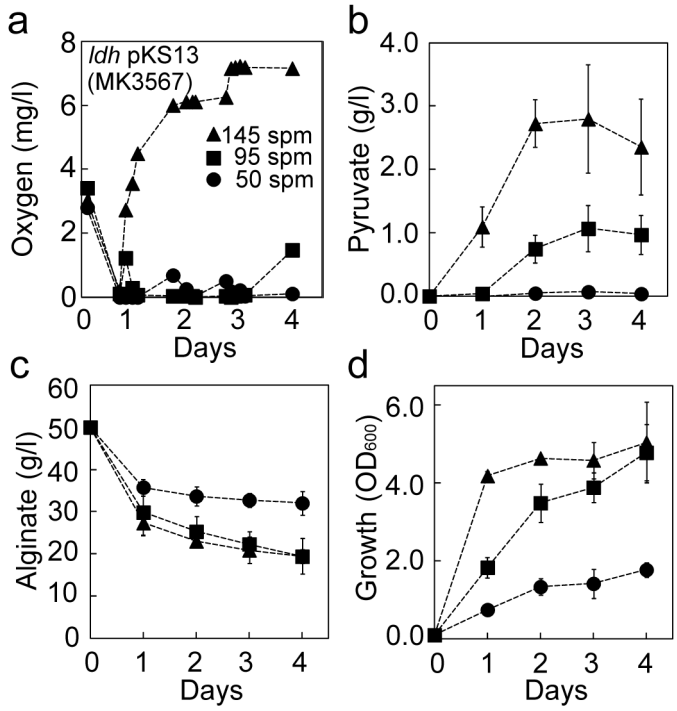
a
b
c
d
ldh pKS13
(MK3567)
145 spm
95 spm
50 spm
Oxygen (mg/l)
Pyruvate (g/l)
Alginate (g/l)
Growth (OD600)
Days
Days
Days
Days

FIG. 2

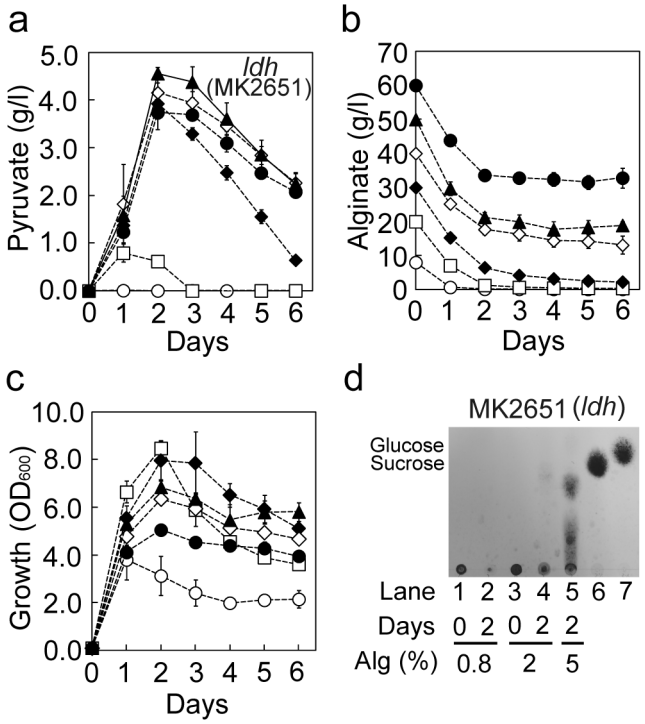
a
b
c
d
Pyruvate (g/l)
Alginate (g/l)
Growth (OD600)
Days
ldh
(MK2651)
MK2651 (ldh)
Glucose
Sucrose
Lane 1 2 3 4 5 6 7
Days 0 2 0 2 2
Alg (%) 0.8 2 5

FIG. 3

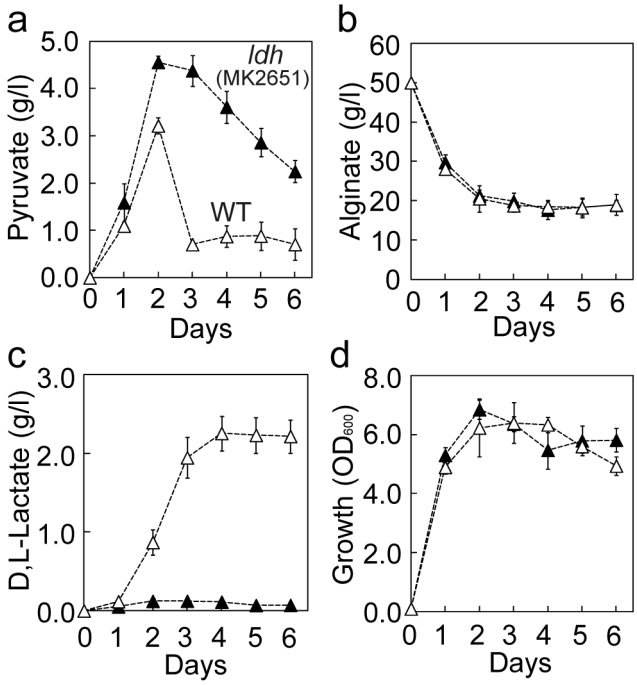
a
Pyruvate (g/l)
5.0
4.0
3.0
2.0
1.0
0.0
0 1 2 3 4 5 6
Days
ldh
(MK2651)
WT
b
Alginate (g/l)
60
50
40
30
20
10
0
0 1 2 3 4 5 6
Days
c
D,L-Lactate (g/l)
3.0
2.0
1.0
0.0
0 1 2 3 4 5 6
Days
d
Growth (OD600)
8.0
6.0
4.0
2.0
0.0
0 1 2 3 4 5 6
Days

FIG. 4

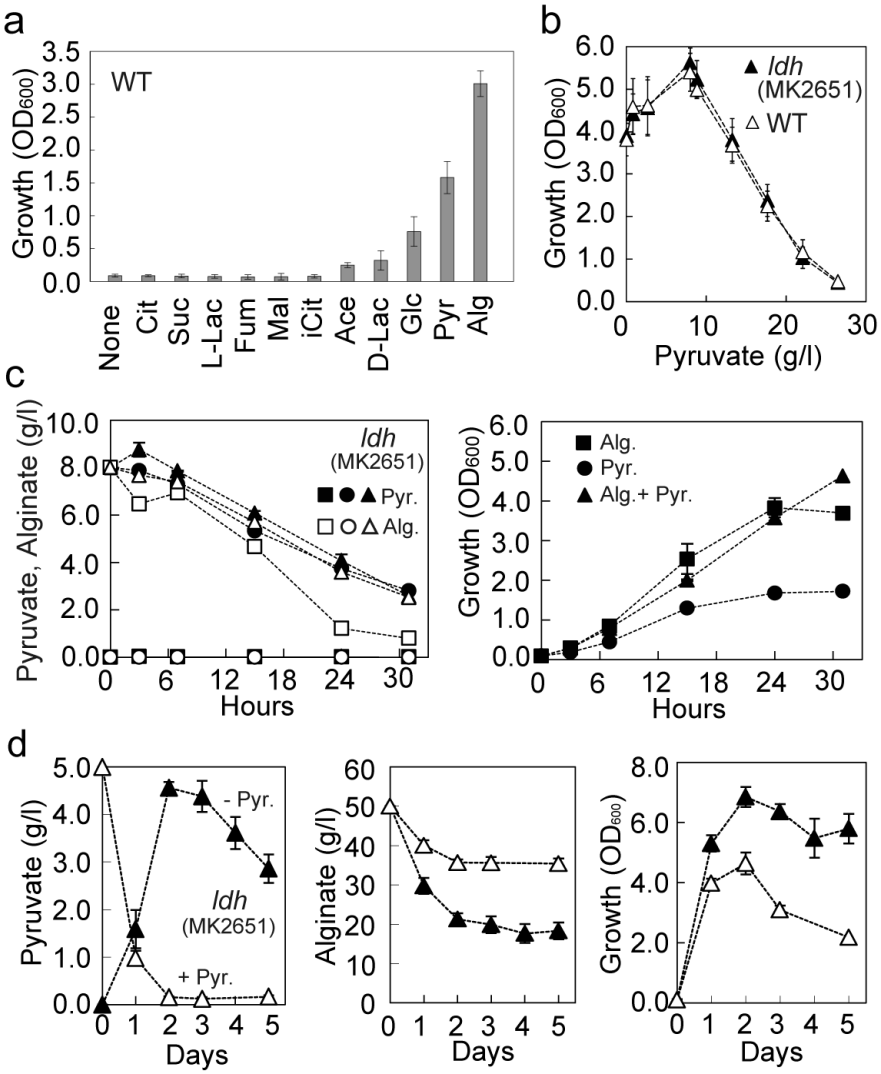
a
WT
Growth (OD600)
None
Cit
Suc
L-Lac
Fum
Mal
iCit
Ace
D-Lac
Glc
Pyr
Alg
b
ldh
(MK2651)
WT
Growth (OD600)
Pyruvate (g/l)
c
Pyruvate, Alginate (g/l)
ldh
(MK2651)
Pyr.
Alg.
Hours
Alg.
Pyr.
Alg.+ Pyr.
Growth (OD600)
Hours
d
Pyruvate (g/l)
- Pyr.
ldh
(MK2651)
+ Pyr.
Days
Alginate (g/l)
Days
Growth (OD600)
Days

FIG. 5

1

TABLE 1. Concentrations of metabolites in the supernatants of cultures [a]

	Concentrations (μM)		
Metabolites	1 day	2 days	4 days
Glyoxylic acid	122	107	39
Glycolic acid	106	64	72
Pyruvic acid	1,148	20,227	26,218
Lactic acid	43	33	53
3-Hydroxybutyric acid	24	100	645
Fumaric acid	83	472	1,268
2-Oxoisovaleric acid	18	1,217	2,505
Succinic acid	416	579	431
Malic acid	175	525	1,379
2-Oxoglutaric acid	88	1,602	14,418
Glycerol 3-phosphate	2.8	4.6	7.1
cis-Aconitic acid	5.5	21	119
3-Phosphoglyceric acid	6.3	19	35
Isocitric acid	4.7	10	66
Citric acid	49	94	536
6-Phosphogluconic acid	N.D.	N.D.	N.D.
Sedoheptulose 7-phosphate	N.D.	N.D.	N.D.
CMP	N.D.	N.D.	N.D.
UMP	N.D.	N.D.	N.D.
AMP	N.D.	N.D.	12
GMP	N.D.	N.D.	N.D.
CDP	N.D.	N.D.	N.D.
UDP	1.7	3.9	7.6
ADP	N.D.	N.D.	N.D.
GDP	N.D.	N.D.	N.D.
Gly	1.0	2.8	0.7
Putrescine	0.8	0.7	4.5
Ala	18	62	1.1
β-Ala	N.D.	1.6	N.D.
GABA	0.4	0.7	0.7
Choline	178	206	206
Ser	11	9.0	3.0
Cytosine	2.3	4.4	28
Creatinine	0.11	0.13	0.7
Pro	0.9	1.0	23
Val	68	25	2.5
Betaine	4.8	2.2	17
Homoserine	0.7	0.6	0.06
Thr	42	72	N.D.
Betaine aldehyde	0.8	0.8	0.6
Ile	1.9	0.2	0.12
Leu	8.7	2.1	0.5
Asp	0.6	0.3	0.8

TABLE 1. Continued.

Hypoxanthine	N.D.	N.D.	N.D.
Anthranilic acid	N.D.	N.D.	0.3
Ornithine	0.4	0.2	0.13
Adenine	N.D.	N.D.	N.D.
Gln	1.2	N.D.	N.D.
Lys	N.D.	N.D.	0.4
Glu	2.0	0.6	4.2
Met	N.D.	N.D.	N.D.
His	N.D.	N.D.	N.D.
Phe	N.D.	N.D.	N.D.
Arg	N.D.	N.D.	0.4
Tyr	N.D.	N.D.	N.D.
Thymidine	9.1	13	46
Cytidine	0.2	0.3	1.6
Adenosine	1.1	0.3	0.3
Inosine	1.2	1.3	3.6
Guanosine	0.3	0.6	2.5
S-Adenosylmethionine	0.3	0.5	0.3

[a] *Sphingomonas* sp. A1 *ldh* strain carrying pKS13 (MK3567) was cultivated in 100 ml liquid 5% alginate medium [5% w/v alginate, 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 $MgSO_4 \cdot 7H_2O$, 0.01% w/v yeast extract (pH 8.0), 20 mg/l Tet, and 25 mg/l Kan] in a 300-ml Erlenmeyer flask at 30°C and 95 spm for 1, 2, or 4 days.

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TABLE 2. Effect of initial alginate concentration on pyruvate production. [a]

Initial alginate (g/l)	ΔP (g/l) [b]	Productivity (mg/l/h) [b]	ΔS (g/l) [b]	$Y_{P/S}$ (g/g) [c]	Theoretical yield (%) [d]	ΔX (g/l) [b]	$Y_{X/S}$ (g/g) [e]	$Y_{P/X}$ (g/g) [f]
8	0.01	0.42	7.3	0.001	0.10	1.44	0.20	0.01
20	0.79	32.9	12.9	0.06	6.1	2.53	0.20	0.31
30	3.93	81.7	23.5	0.17	16.7	3.02	0.13	1.30
40	4.16	86.7	22.3	0.19	18.6	2.41	0.11	1.72
50	4.56	95.0	28.7	0.16	15.9	2.60	0.09	1.75
60	3.74	77.9	26.4	0.14	14.2	1.92	0.07	1.95
50 (WT) [g]	3.21	67.1	29.5	0.11	10.9	2.37	0.08	1.36

[a] Data were calculated based on data in Fig. 4. The *ldh* and WT strains were cultivated at 145 spm in 20 ml liquid 5% alginate media [5% w/v alginate, 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 $MgSO_4 \cdot 7H_2O$, 0.01% w/v yeast extract (pH 8.0)] in 200-ml Erlenmeyer flasks.

[b] Concentrations of produced pyruvate (ΔP), productivity (mg/l/h), consumed alginate (ΔS), or CDW (ΔX) in 1-day culture under initial alginate of 8 and 20 g/l, or those in 2-day culture under initial alginate of 30, 40, 50, and 60 g/l.

[c] Yield of pyruvate produced (ΔP) per alginate consumed (ΔS) (g/g).

[d] Theoretical yield of pyruvate produced (ΔP) per alginate consumed (ΔS) (%). Theoretical yield was taken to be 100% when 100 g pyruvate was produced from 100

g consumed alginate, because 2 mol of pyruvate (MW of 88) is theoretically produced from 1 mol of DEH (MW of 176) (Fig. 1).

[e] Yield of CDW (ΔX) per alginate consumed (ΔS) (g/g).

[f] Yield of pyruvate produced (ΔP) per CDW (ΔX) (g/g).

[g] *Sphingomonas* sp. A1 WT cells were cultivated in the presence of 50 g/l alginate.

1

2