

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

Running title: Bacterial pyruvate production from alginate

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1   **Abstract**

2           Marine brown macroalgae is a promising source of material for biorefining,  
3   and alginate is one of the major components of brown algae. Despite the huge  
4   potential availability of alginate, no system has been reported for the production of  
5   valuable compounds other than ethanol from alginate, hindering its further utilization.  
6   Here we report that a bacterium, *Sphingomonas* sp. strain A1, produces pyruvate from  
7   alginate and secretes it into the medium. High aeration and deletion of the gene for  
8   D-lactate dehydrogenase are critical for the production of high concentrations of  
9   pyruvate. Pyruvate concentration and productivity were at their maxima (4.56 g/l and  
10   95.0 mg/l/h, respectively) in the presence of 5% (w/v) initial alginate, whereas  
11   pyruvate produced per alginate consumed and % of theoretical yield (0.19 g/g and  
12   18.6%, respectively) were at their maxima at 4% (w/v) initial alginate. Concentration  
13   of pyruvate decreased after it reached its maximum after cultivations for 2 or 3 days at  
14   145 strokes per minute. Our study is the first report to demonstrate the production of  
15   other valuable compounds than ethanol from alginate, a promising marine macroalgae  
16   carbon source.

17

18

## 1    **Introduction**

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

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

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

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

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

17

## 18 MATERIALS AND METHODS

19

20         **Strain and cultivation** The *Sphingomonas* sp. A1 wild type (WT) strain  
21 is a Gram-negative bacterium that is able to assimilate alginate (16). The LDH gene  
22 of *Sphingomonas* sp. A1 WT strain was disrupted by inserting kanamycin-resistant  
23 cassette into LDH gene on the genome, resulting in the *Sphingomonas* sp. A1 *ldh*  
24 strain (MK2651) (8). Strain MK3567 is the *Sphingomonas* sp. A1 *ldh* strain carrying  
25 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% w/v KH<sub>2</sub>PO<sub>4</sub>, 0.1% w/v Na<sub>2</sub>HPO<sub>4</sub>, 0.01% w/v MgSO<sub>4</sub>·7H<sub>2</sub>O, 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<sub>600</sub> 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<sub>600</sub> using a ratio of 0.38 g (CDW) l<sup>-1</sup> per OD<sub>600</sub>. 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.,

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

3  
4 **HPLC analysis** For the analyses of pyruvate, 2-oxoglutarate, and  
5 2-oxoisovalerate, HPLC analysis was conducted using an HPLC equipped with an  
6 Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) and a RID-10A detector  
7 (Shimadzu, Japan). Other conditions were as follows: effluent, filtered and degassed 5  
8 mM H<sub>2</sub>SO<sub>4</sub>; flow rate, 0.65 ml min<sup>-1</sup>; column temperature, 65.5°C.

9  
10 **Concentration of oxygen in liquid medium** Concentration of oxygen  
11 dissolved in liquid medium was measured using a Fibox 3 oxygen sensor (Presens,  
12 Regensburg, Germany) and an oxygen-sensor spot (Presens) attached to the bottom of  
13 a 300-ml Erlenmeyer flask. In this study, the saturated concentration of oxygen  
14 dissolved in liquid medium at 30°C was considered to be 7.5 mg/l (18).

15  
16 **TLC analysis** Authentic compounds (5 µl, 2% w/v sucrose or glucose)  
17 and the cultures (5 µl) were spotted onto TLC glass plates with silica gel 60 F<sub>254</sub>  
18 (Merck, USA), dried, developed with a solvent system consisting of 1-butanol, acetate,  
19 and water (3:2:2, v/v/v) and visualized by heating the TLC plate at 130°C for 5 min  
20 after spraying it with 10% (v/v) sulfuric acid in ethanol (17).

21  
22 **Other analytical methods** The concentration of pyruvate in the 4-day  
23 supernatants of initial cultivation of MK3567 strain was determined with metabolome  
24 or HPLC analysis as above. In the other case, the concentration of pyruvate of the  
25 supernatant of the culture was determined using pyruvate assay kit (Roche

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

4

## 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 rpm (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.

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

7

8           **Aeration is important for the production of pyruvate from alginate**  
9 *Sphingomonas* sp. A1 *ldh* strain carrying an empty plasmid pKS13 (MK3567) was  
10 cultivated under the same conditions used for metabolome analysis, but at 50, 95, or  
11 145 spm for 4 days, during which we monitored the concentrations of oxygen,  
12 pyruvate, and alginate and the growth of this strain. The culture was aerated highly at  
13 145 spm, moderately at 95 spm, and slightly at 50 spm.

14           Oxygen concentrations of the culture were kept at basal levels during  
15 cultivation at 50 and 95 spm, whereas the concentration increased to saturation at  
16 6.0–7.5 mg/l at 145 spm (Fig. 2a). Cultivation at 145 spm also gave the maximum  
17 concentrations of pyruvate, whereas cultivation at 95 spm and 50 spm resulted in  
18 moderate and no pyruvate production, respectively. Growth was in accord with the  
19 pyruvate production, and consumption of alginate was lowest at 50 spm, but  
20 approximately equal at 95 and 145 spm. Thus, high aeration is important for pyruvate  
21 production. The 4-day supernatants were also analyzed by HPLC, and the maximum  
22 production of pyruvate at 145 spm was again confirmed (3.33, 0.49, and 0 g/l  
23 pyruvate at 145, 95, and 50 spm; data not shown). At 145, 95, and 50 spm, production  
24 of 2-oxoglutarate (0.87, 0.14, and 0 g/l) was lower than that of pyruvate, and no  
25 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 rpm 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 [ $\Delta P$ ; g/l] per alginate consumed [ $\Delta S$ ; g/l]), % of theoretical yield, CDW ( $\Delta X$ ; g/l),  $Y_{X/S}$  (g/g) (an yield of CDW [ $\Delta X$ ; g/l] per alginate consumed [ $\Delta S$ ; g/l]),  $Y_{P/X}$  (g/g) (an yield of pyruvate produced [ $\Delta P$ ; g/l] per CDW [ $\Delta X$ ; 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 ( $\Delta P$ ; 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 ( $\Delta X$ ; 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 rpm at 30°C in both cases. We

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

6

7       **Effect of LDH disruption on production of pyruvate** This study was  
8 undertaken with the *Sphingomonas* sp. A1 *ldh* strains (MK3567 and MK2651 strains),  
9 which is host of the ethanologenic *Sphingomonas* sp. strain A1 (8). Since pyruvate is  
10 a substrate of LDH, disruption of LDH could enhance production of pyruvate. To  
11 confirm this, we compared production of pyruvate by the WT strain (Fig. 4, open  
12 symbols) at 145 spm in liquid alginate medium containing 5% w/v alginate to  
13 production by *Sphingomonas* sp. A1 *ldh* strain (MK2651) without plasmid (Fig. 4,  
14 closed symbols; Table 2). Although the consumption rates of alginate and growth of  
15 the *ldh* strain were approximately the same as those of the WT (Fig. 4, b and d), the  
16 maximum pyruvate concentration of WT strain was lower than that of the *ldh* strain  
17 (Fig. 4a). Moreover, concentration of pyruvate dropped rapidly in the WT strain (Fig.  
18 4a). In accord with its lower production of pyruvate, the WT strain produced D-lactate,  
19 whereas the *ldh* strain produced only a limited amount of D-lactate (Fig. 4c). No  
20 production of L-lactate was observed in either the WT or *ldh* strains (Fig. 4c). These  
21 data demonstrate that disruption of the LDH gene clearly increased pyruvate  
22 production.

23

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

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

8           Moreover, we demonstrated that *Sphingomonas* sp. A1 *ldh* strain (MK2651)  
9 metabolized both alginate and pyruvate; both of which were utilized simultaneously  
10 (Fig. 5c). When alginate was present alone at a concentration of 0.8% (w/v), it was  
11 more rapidly consumed by the *ldh* strain than 0.8% (w/v) pyruvate alone or 0.8%  
12 (w/v) alginate and 0.8% (w/v) pyruvate (Fig. 5c), indicating that pyruvate partially  
13 suppresses the utilization of alginate. Growth of the *ldh* strain in the presence of both  
14 0.8% (w/v) alginate and 0.8% (w/v) pyruvate was approximately the same as in the  
15 presence of 0.8% (w/v) alginate alone, but faster than in the presence of 0.8% (w/v)  
16 pyruvate alone (Fig. 5c). We also investigated whether the *ldh* strain would produce  
17 pyruvate in 5% (w/v) alginate medium initially containing 0.5% (w/v) [5.0 g/l]  
18 pyruvate, and compared the results with those obtained from the *ldh* strain cultivated  
19 in 5% (w/v) alginate medium with no initial pyruvate (Fig. 5d). We observed that the  
20 concentration of pyruvate dropped rapidly and never exceeded 5.0 g/l, and that  
21 alginate was utilized more moderately than in the absence of initial pyruvate (Fig. 5d).  
22 These observations demonstrate that pyruvate partially suppresses not only utilization  
23 of alginate as shown in Fig. 5c, but also production of pyruvate.

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

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

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

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

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

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

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

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10

1 **Figure legends**

2 FIG. 1. Alginate metabolism in the *Sphingomonas* sp. A1 wild-type strain (16, 17).

3 Compounds are indicated in bold, and enzymes are in gray. Abbreviations: DEH,  
4 4-deoxy-L-erythro-5-hexoseulose uronic acid; KDG, 2-keto-3-deoxy-D-gluconate;  
5 KDGP, 2-keto-3-deoxy-phosphogluconate; GAP, glyceraldehyde 3-phosphate; A1-R,  
6 NADPH-dependent DEH reductase; A1-K, KDG kinase; A1-A, aldolase; LDH,  
7 D-lactate dehydrogenase.

8

9 FIG. 2. Aeration is important for the production of pyruvate from alginate.

10 Concentrations of oxygen (a), pyruvate (b), alginate (c), and growth (d; OD<sub>600</sub>) of the  
11 culture of the *Sphingomonas* sp. A1 *ldh* strain carrying pKS13 (MK3567). This strain  
12 was cultivated at 50 (circles), 95 (squares), and 145 (triangles) spm in 100 ml 5%  
13 liquid alginate medium [5% w/v alginate, 0.1% w/v (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% w/v KH<sub>2</sub>PO<sub>4</sub>,  
14 0.1% w/v Na<sub>2</sub>HPO<sub>4</sub>, 0.01% w/v MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01% w/v yeast extract (pH 8.0), 20  
15 mg/l Tet, and 25 mg/l Kan] in 300-ml Erlenmeyer flasks at 30°C. b–d; Averages and  
16 standard deviations (SD) of three independent experiments are shown.

17

18 FIG. 3. Effect of initial concentration of alginate on production of pyruvate. The

19 *Sphingomonas* sp. A1 *ldh* strain (MK2651) without plasmid was cultivated at 145  
20 spm in 20 ml liquid alginate media [0.1% w/v (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% w/v KH<sub>2</sub>PO<sub>4</sub>, 0.1%  
21 w/v Na<sub>2</sub>HPO<sub>4</sub>, 0.01% w/v MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01% w/v yeast extract (pH 8.0), and 25  
22 mg/l Kan] containing 0.8% (open circles), 2% (open squares), 3% (closed diamonds),  
23 4% (open diamonds), 5% (closed triangles), or 6% (closed circles) w/v alginate in  
24 200-ml Erlenmeyer flasks at 30°C for 6 days during which concentrations of pyruvate  
25 (a) and alginate (b) and growth of the cultures (c) were measured. Averages and SD of

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

5

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

13

14 FIG. 5. Utilization of pyruvate (a) and tolerance to pyruvate (b) of the *Sphingomonas*  
15 sp. A1 WT strain, and simultaneous metabolism of alginate and pyruvate by the  
16 *Sphingomonas* sp. A1 *ldh* strain (c, d). (a) The WT strain was precultured as described  
17 in MATERIALS AND METHODS, collected, washed once with 2 mM  
18 sodium-potassium phosphate (pH 6.9), resuspended in the same buffer, and inoculated  
19 to reach an OD<sub>600</sub> of 0.1 into 1.0 ml of liquid 0.8% alginate (Alg) medium, medium  
20 containing no alginate (None), or medium in which 0.8% alginate was replaced with  
21 0.8% (w/v) of another carbon source: citrate (Cit), succinate (Suc), L-lactate (L-Lac),  
22 D-Lactate (D-Lac), fumarate (Fum), L-malate (Mal), DL-isocitrate (isoCit), acetate  
23 (Ace), glucose (Glc), or pyruvate (Pyr). The cells were cultivated for 24 h at 30°C,  
24 and OD<sub>600</sub> was measured. (b) WT (open triangles) and *ldh* (closed triangles) strains  
25 were inoculated to reach OD<sub>600</sub> of 0.1 into 1.0 ml of liquid 0.8% alginate medium as

1 above containing the indicated concentrations of pyruvate (final pH of the media; pH  
2 8.0–8.2) as in Fig. 6a. The cells were cultivated for 24 h at 30°C, and OD<sub>600</sub> was  
3 measured. (c) The *ldh* strain (MK2651) grown on alginate solid medium was  
4 suspended in 2 mM sodium-potassium phosphate and inoculated at an OD<sub>600</sub> of 0.1  
5 into 3.0 ml of liquid medium containing 0.8% (w/v) alginate (squares), 0.8% (w/v)  
6 pyruvate (circles), or both 0.8% (w/v) alginate and 0.8% (w/v) pyruvate (triangles).  
7 The cells were cultivated for 33 h at 30°C, during which the concentration of pyruvate  
8 (left; closed symbols), the concentration of alginate (left; open symbols), and OD<sub>600</sub>  
9 (right) were measured. Means and maximum and minimum values of two independent  
10 cultivations are presented. (d) The *ldh* strain (MK2651) was cultivated as in Fig. 4 in  
11 20 ml liquid 5% alginate medium without (closed triangles) or with (open triangles)  
12 an initial pyruvate concentration of 0.5% [5.0 g/l]. Concentration of pyruvate (left),  
13 concentration of alginate (middle), and OD<sub>600</sub> (right) were measured. Data for the *ldh*  
14 strain cultivated without initial pyruvate were the same as those shown in Fig. 3 and 4.  
15 Means and SD of three independent cultivations are presented (a, b, d).

16

17

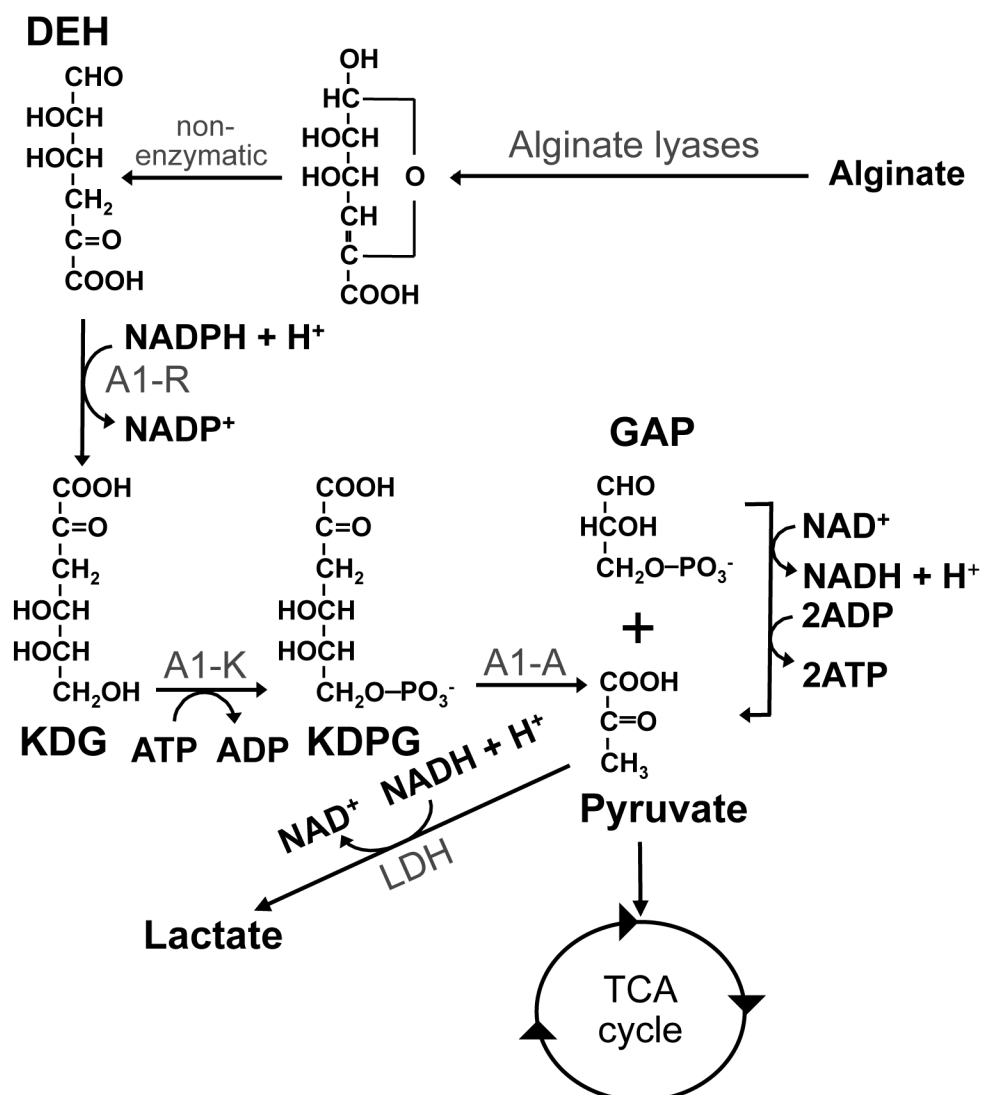
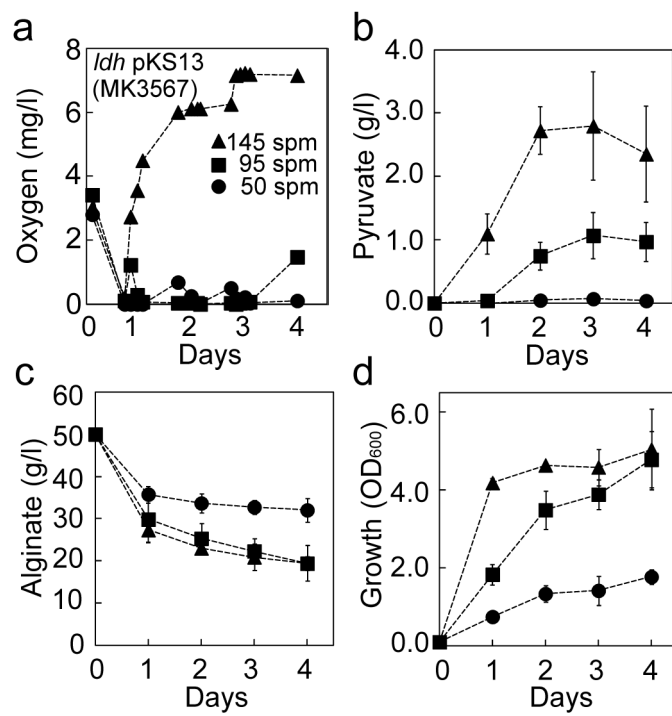
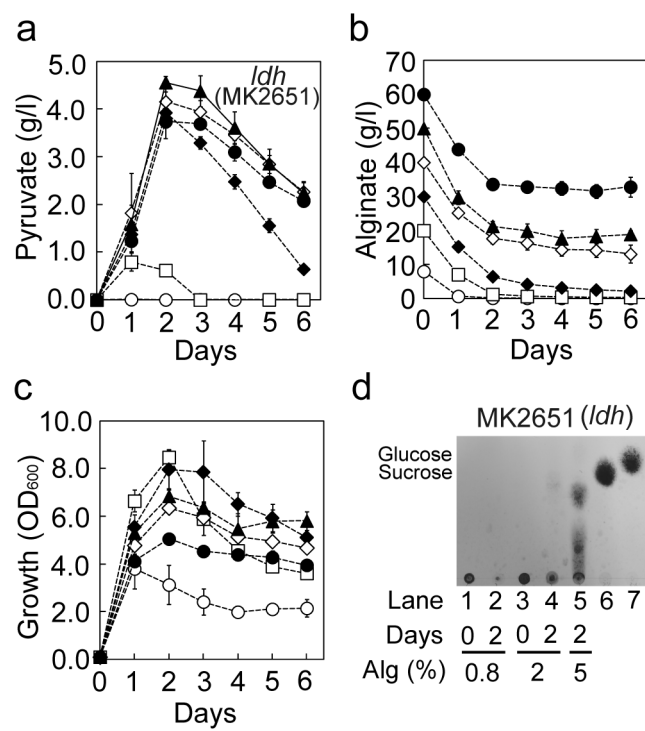


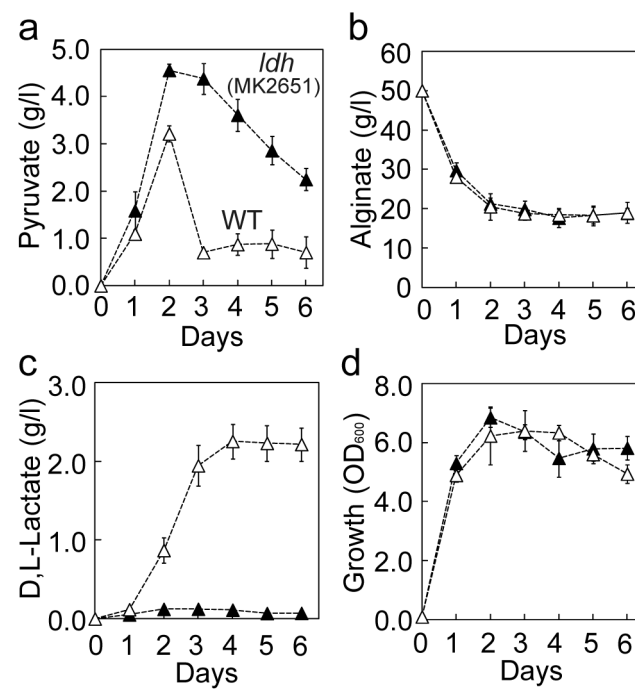
FIG. 1



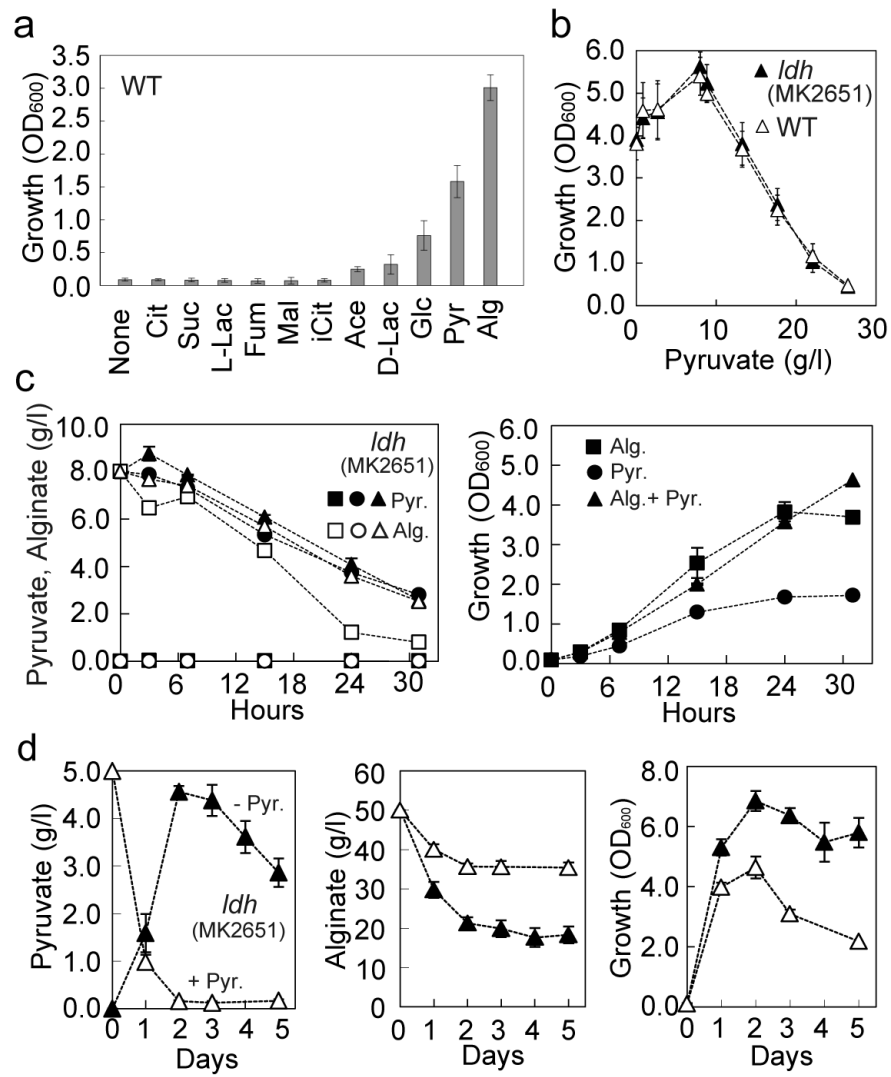
**FIG. 2**



**FIG. 3**



**FIG. 4**



**FIG. 5**

**TABLE 1.** Concentrations of metabolites in the supernatants of cultures <sup>a</sup>

| Metabolites               | Concentrations (μM) |        |        |
|---------------------------|---------------------|--------|--------|
|                           | 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    |
| <i>cis</i> -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  |

<sup>a</sup> *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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% w/v KH<sub>2</sub>PO<sub>4</sub>, 0.1% w/v Na<sub>2</sub>HPO<sub>4</sub>, 0.01% w/v MgSO<sub>4</sub>·7H<sub>2</sub>O, 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. <sup>a</sup>

| Initial<br>alginate<br>(g/l) | $\Delta P$<br>(g/l) <sup>b</sup> | Produ<br>ctivity<br>(mg/l/<br>h) <sup>b</sup> | $\Delta S$<br>(g/l) <sup>b</sup> | $Y_{P/S}$<br>(g/g) <sup>c</sup> | Theoretical<br>yield (%) <sup>d</sup> | $\Delta X$<br>(g/l) <sup>b</sup> | $Y_{X/S}$<br>(g/g) <sup>e</sup> | $Y_{P/X}$<br>(g/g) <sup>f</sup> |
|------------------------------|----------------------------------|-----------------------------------------------|----------------------------------|---------------------------------|---------------------------------------|----------------------------------|---------------------------------|---------------------------------|
| 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<br>(WT) <sup>g</sup>      | 3.21                             | 67.1                                          | 29.5                             | 0.11                            | 10.9                                  | 2.37                             | 0.08                            | 1.36                            |

<sup>a</sup> Data were calculated based on data in Fig. 4. The *ldh* and WT strains were cultivated at 145 rpm in 20 ml liquid 5% alginate media [5% w/v alginate, 0.1% w/v (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% w/v KH<sub>2</sub>PO<sub>4</sub>, 0.1% w/v Na<sub>2</sub>HPO<sub>4</sub>, 0.01% w/v MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01% w/v yeast extract (pH 8.0)] in 200-ml Erlenmeyer flasks.

<sup>b</sup> Concentrations of produced pyruvate ( $\Delta P$ ), productivity (mg/l/h), consumed alginate ( $\Delta S$ ), or CDW ( $\Delta X$ ) 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.

<sup>c</sup> Yield of pyruvate produced ( $\Delta P$ ) per alginate consumed ( $\Delta S$ ) (g/g).

<sup>d</sup> Theoretical yield of pyruvate produced ( $\Delta P$ ) per alginate consumed ( $\Delta S$ ) (%). Theoretical yield was taken to be 100% when 100 g pyruvate was produced from 100

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

<sup>e</sup> Yield of CDW ( $\Delta X$ ) per alginate consumed ( $\Delta S$ ) (g/g).

<sup>f</sup> Yield of pyruvate produced ( $\Delta P$ ) per CDW ( $\Delta X$ ) (g/g).

<sup>g</sup> *Sphingomonas* sp. A1 WT cells were cultivated in the presence of 50 g/l alginate.

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