Journal of Bioscience and Bioengineering
VOL. 117 No. 3, 269–274, 2014

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

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Received 17 June 2013; accepted 27 August 2013
Available online 22 September 2013

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.

[**Key words:** *Sphingomonas* sp.; Alginate; Pyruvate; Macroalgae; Marine biomass]

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, 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 from glucose has primarily proceeded, 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.

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http://dx.doi.org/10.1016/j.jbiosc.2013.08.016

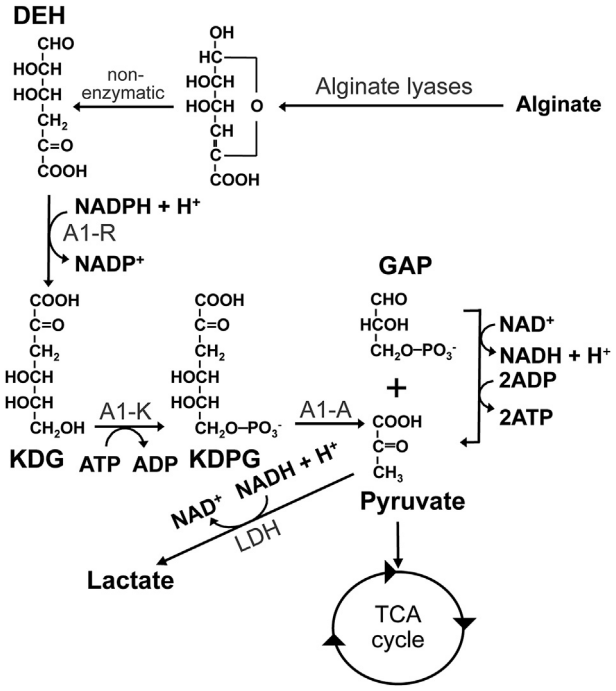

FIG. 1. Alginate metabolism in the *Sphingomonas* sp. A1 wild-type strain (16,17). Compounds are indicated in bold, and enzymes are in gray. Abbreviations: DEH, 4-deoxy-L-erythro-5-hexoseulose uronic acid; KDG, 2-keto-3-deoxy-D-gluconate; KDGP, 2-keto-3-deoxy-phosphogluconate; GAP, glyceraldehyde 3-phosphate;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

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 obtained 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