Bacterial community structure and predicted alginate metabolic pathway in an alginate-degrading bacterial consortium

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Abstract

Methane fermentation is one of the effective approaches for utilization of brown algae; however, this process is limited by the microbial capability to degrade alginate, a main polysaccharide found in these algae. Despite its potential, little is known about anaerobic microbial degradation of alginate. Here we constructed a bacterial consortium able to anaerobically degrade alginate. Taxonomic classification of 16S rRNA gene, based on high-throughput sequencing data, revealed that this consortium included two dominant strains, designated HUA-1 and HUA-2; these strains were related to Clostridiaceae bacterium SK082 (99%) and Dysgonomonas capnocytophagoides (95%), respectively. Alginate lyase activity and metagenomic analyses, based on high-throughput sequencing data, revealed that this bacterial consortium possessed putative genes related to a predicted alginate metabolic pathway. However, HUA-1 and 2 did not grow on agar medium with alginate by using roll-tube method, suggesting the existence of bacterial interactions like symbiosis for anaerobic alginate degradation.
Land biomass including both agricultural crops and lignocellulosic components has been identified as a renewable energy resource, although several highly energetic crops, such as sweet potato or corn, are in direct competition with food production. Other plant materials, such as straw or remains from forest clearing tend to be rich in lignin, and are consequently less efficient as energy sources because of the high costs associated with converting this biomass into fermentative sugars (1, 2, 3). On the other hand, abundant elements of marine ecosystems, such as seaweed, are hardly utilized as a food resource and possess low or no lignin content, making them a suitable alternative to land biomass for energy generation (2, 4, 5, 6). Therefore, seaweed has been receiving increasing attention as third-generation biomass (7). Among marine seaweed biomass, brown algae have been highlighted as the most promising feedstock as a renewable resource (8, 9, 10).

Alginate is a linear polysaccharide composed by two uronic acids, β-D-mannuronate (M) and α-L-guluronate (G) covalently (1–4)-linked in different sequences (11, 12). Alginate is a major component of brown algae and represents at up to 40% of the organism’s dry weight, depending on the species (4, 13). As a consequence, comprehensive understanding of alginate metabolism is essential to ensure efficient biorefinery procedures to exploit this group as an energy source; however, several microorganisms are known to metabolize alginate (13, 14, 15), through an alginate degradation pathway mediated by alginate lyase. This enzyme catalyzes the depolymerization of endo-type alginate into oligomers via an endolytic β-elimination reaction (10, 11). The resultant oligomers are further degraded into unsaturated monomers through the action of an exolytic enzyme known as exotype.
alginate lyase (10, 11). These monomers are eventually metabolized to pyruvate and
glyceraldehydes-3-phosphate, which are key intermediates of the glycolytic pathway
(10, 11). On the other hand, to date, research on the anaerobic degradation of alginate by
bacterial communities has been limited, despite its potential importance in fermentative
production of valuable chemical compounds, such as short-chain fatty acids (16, 17),
and biofuels such as methane (18, 19). Methane production by anaerobic digestion of
macroalgae is a promising algal bioenergy option (18); it is known that the rate of
degradation (hydrolysis and acidogenesis) of polysaccharides is one of the rate-limiting
factors (20). In the case of brown algae, it was reported that the methane fermentation
rate is sometimes restricted by the degradation capacity of alginate (21). However little
is known regarding the mechanisms of alginate degradation by bacterial consortia.
Indeed, Seon et al. (17) reported one microbial community structure degrading alginate
to volatile fatty acids in an anaerobic condition. However, the information regarding the
pathway of anaerobic alginate degradation by microbial consortium has not been
clarified.

In this study, we established a bacterial consortium able to anaerobically degrade
alginate. This bacterial consortium has now been cultured for >5 years while
maintaining its alginate degradation ability. Here we describe not only the community
structure of this bacterial consortium but also its predicted alginate metabolic pathway
by metagenome analysis.
MATERIALS AND METHODS

Growth media and culture conditions Since its establishment, the consortium have been cultured at 30°C under anoxic conditions in a basal medium (pH = 6.8) containing 5.5 g of KH$_2$PO$_4$, 7.0 g of K$_2$HPO$_4$, 1.0 g of (NH$_4$)$_2$SO$_4$, 0.12 g of Na$_2$MoO$_4$·2H$_2$O, 0.039 g of Fe(NH$_4$)$_2$SO$_4$·6H$_2$O, 0.029 g of Co(NO$_3$)$_2$·6H$_2$O, 0.021 g of CaCl$_2$·2H$_2$O, 0.25 g of MgSO$_4$·7H$_2$O, 0.1 g of yeast extract, 0.1 g of tryptone, 10 ml of trace elements, 10 ml of Wolfe’s vitamin solution (22), and 1.0 mg of resazurin per liter of deionized water. Anoxic conditions were generated by using titanium (III) citrate solution as reducing agent. The basal medium was supplemented with 5.0 g/l of alginate or fructose as the sole carbon source. *Escherichia coli* HST08 (Takara Bio Inc., Shiga, Japan) was used for plasmid construction and 16S rRNA gene clone analysis. *E. coli* strains were cultured at 37°C in LB medium supplemented with appropriate antibiotics.

Enrichment of bacterial consortium Sand sample was collected from the beach on the gulf of Hiroshima, Japan. Approximately 1 g wet weight of sand sample was inoculated into 10 ml of basal medium and vigorously shaken. The suspensions (1 ml) were cultured for 48 h in 9.0 ml basal medium supplemented with 5.0 g/l alginate at 30°C. The sample where alginate consumption could be confirmed was further subcultured in basal medium with alginate.

Quantification of culture products Organic substances in culture medium were quantified by high-performance liquid chromatography (LC-2000 Plus HPLC; Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031 Plus; Jasco), Shodex
RSpak KC-811 column (Showa Denko, Kanagawa, Japan), and a guard column (Shodex RSpak KC-G; Showa Denko) at 60 °C. Ultrapure water containing 0.1% (v/v) phosphoric acid was used as the mobile phase at a flow rate of 0.7 mL/min. Crotonate was used as an internal standard.

Quantification of alginate We measured alginate concentration using a modified carbazole sulfuric acid method (23) and sodium alginate as standard. For this procedure, we added 0.06 ml of 4 M sulfuric acid solution to 0.5 ml of culture suspension and adjusted to pH 7.5 using KOH. We subsequently added 0.06 ml of 1 M borate buffer (adjusted to pH 8.4 using KOH) and mixed it vigorously. The solution was kept in boiling water for 6.5 min after adding 3 ml of H$_2$SO$_4$ and cooled on ice for 2 min. Furthermore, after adding 0.125 ml of 0.2% carbazole solution and vigorously mixing the solution; it was boiled for 10 min before measuring absorbance at 525 nm using a spectrophotometer.

Metagenome and 16S rRNA gene analyses based on high-throughput sequencing data Whole cells were harvested from the bacterial consortium when OD$_{660}$ reached 0.4–0.5. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany). A paired-end library of the whole genome was generated for metagenome sequencing by Hokkaido system science Co. Ltd. (Sapporo, Japan) using Illumina HiSeq 2000. High-throughput sequencing of 16S rRNA gene and analysis was performed using Roche GS FLX+, Hokkaido system science, in which the 16S rRNA gene fragments were amplified using PCR performed in KOD FX Neo (Toyobo, Tokyo,
Japan) with the tagged primer set Bact27f (5′-AGAGTTTGATCCTGGCTCAG) and Bact519r (5′-GWATTACCGCGGCKGCTG).

16S rRNA gene cloning analysis Genomic DNA from the bacterial consortium was extracted using NucleoSpin Tissue. 16S rRNA gene fragments were amplified using PCR performed in a KOD FX Neo with the primer set Bact27f and Bact1492r (5′-TACGGYTACCTTGTTACGACTT). The PCR fragments obtained were subsequently ligated into SmaI-digested pUC19, and the products were used to transform E. coli HST08. The 16S rRNA gene-harboring plasmids were extracted from E. coli transformants and sequenced by Eurofins Genomics (Tokyo, Japan). Nucleotide sequence similarities were determined using a BLAST search on DDBJ (http://www.ddbj.nig.ac.jp/) and Genetyx software (Genetyx, Tokyo, Japan).

PCR–RFLP (restriction fragment length polymorphism) and T(terminal)-RFLP 16S rRNA gene fragments from the bacterial consortium or the cloned plasmids was amplified by PCR performed in KOD FX Neo with the primer set Bact27f and Bact1492r and purified using MagExtractor-PCR&Gel Clean up (Toyobo). For T-RFLP, we used dye-labeled Bact27f primers. For PCR-RFLP, purified 16S rRNA gene fragments were digested with MboI and separated by electrophoreses in 2% agarose gel. For T-RFLP, 16S rRNA gene fragments were digested with HhaI and analyzed in a CEQ-2000 DNA sequencer (Beckman Coulter, Inc., Brea, CA).

Alginate lyase assays The bacterial consortium was cultured in basal medium supplemented with alginate. Cells were harvested by centrifugation (20,400 ×g for 10
min at 4°C) at OD600 = 0.3–0.5. Harvested cells were washed and suspended in 50 mM phosphate buffer (pH 6.8) and subsequently sonicated using Branson Digital sonifier (Branson Ultrasonics, Corp., Danbury, CT) with 2.0 s ‘On’ and 2.0 s ‘Off’ pulses (total 10 min) at 20% power amplitude. Cell-free extracts were obtained by centrifuging the sonicated cells (20,400 × g for 30 min at 4°C). The protein concentrations of the cell-free extracts were determined using the Bradford method. Alginate lyase activity was measured following the method reported by Park et al. (2012) with slight modifications. In brief, the cell-free extracts were added to a reaction solution containing 0.1% alginate and 50 mM phosphate buffer (pH 6.8). Enzyme activity was monitored by measuring the increase in A235 at 30°C. One unit was defined as the amount of enzyme required to achieve an increase in A235 of 0.1 per minute.

RESULTS AND DISCUSSION

Enrichment culture of bacterial consortium  Bacterial consortium that could anaerobically degrade alginate was obtained from sand from the beach on Hiroshima Gulf, Hiroshima, Japan. Subculture of the bacterial consortium was repeated using the basal medium supplemented with alginate as the sole carbon source. Although this bacterial consortium has been subcultured for >5 years, the level of alginate consumption activity remained stable (data not shown).

Figure 1 shows a typical time course of the batch culture of the bacterial consortium grown on alginate at 30°C. Alginate concentration decreased concomitantly with growth (OD600). When alginate was supplied as a the sole carbon source, the bacterial consortium consumed 4.82 ± 0.13 g/l of alginate and mainly produced acetate.
(23.5 ± 0.18 mM) after culturing for 5 days. In addition, small amounts of formate (1.89 ± 0.11 mM), propionate (1.54 ± 0.08 mM), ethanol (0.65 ± 0.09 mM), and CO2 (14.0 ± 1.09 mM) were produced.

**Taxonomic classification of 16S rRNA gene based on high-throughput sequencing data** To reveal the community structure of the bacterial consortium, we sequenced 16S rRNA gene by high-throughput sequencing techniques using Roche GS FLX+ to obtain a total of 12,690 reads with an average length of 462 base. All reads of 250 base or longer were used as input reads. Homology search of input reads was performed using BLAST against the 16S rRNA gene database from DDBJ. Taxonomy ID and names for genus, family and class were obtained, respectively. A total of 11,024 reads were used for community analyses.

Table 1 shows the results of the taxonomic classification of the bacterial consortium using 16S rRNA gene. These results revealed that the bacterial consortium was constituted by at least three genera of bacteria, *Clostridium*, *Dysgonomonas*, and *Citrobacter*. In particular, the BLAST search identified that the most abundant strains, representing 39.9% and 44.0% of the total bacteria in the consortium, were related to *Clostridiaceae* bacterium SK082 and *D. capnocytophagoides*, respectively. *Clostridiaceae* bacterium SK082 was identified as belonging to an unclassified *Clostridiaceae* species. The anaerobic microorganism AN-C16-KBRB isolated from bovine rumen was also closely related to *Clostridiaceae* bacterium SK082, with a 16S rRNA gene sequence similarity of 98% (24). AN-C16-KBRB can degrade cellulose, which possesses a chemical structure similar to alginate in addition to presenting bifunctional endo-/exo-type cellulase CelEdx16 (25, 26). For these reasons, *Clostridium*
is thought to play an important role in the degradation of polymers such as alginate. *D. capnocytophagoides* has been classified as a group DF-3 organism (dysgonic fermenter 3) by the Centers for Disease Control and Prevention (CDC) (27). The biochemical profile of *D. capnocytophagoides* appears to be similar to that of *Capnocytophaga*; in addition, *Dysgonomonas* spp. form a genetic cluster together with *Bacteroides forsythus* and *Bacteroides distasonis*, based on 16S rRNA gene sequences (28, 29). However, alginate metabolism by *Dysgonomonas* has not been reported.

16S rRNA gene cloning analysis and community dynamics of the bacterial consortium  As mentioned above, taxonomic classification based on high-throughput sequencing data reveals that the consortium is mainly composed by two dominant strains related to *Clostridiaceae* bacterium SK082 and *D. capnocytophagoides*. However, in both cases, fragment length was not long enough to allow for the identification of the specific strain. To this end, we conducted cloning and detailed sequence analysis of the 16S rRNA gene. 16S rRNA gene fragments derived from the bacterial consortium were ligated into *Sma*I digested pUC19 and cloned. The clones obtained were classified using PCR–RFLP analysis (Fig. 2A). Three taxonomic units were obtained based on the RFLP patterns, designated HUA-1, HUA-2, and HUA-3. Based on the level of sequence similarity for 16S rRNA gene sequence (1400 bp), HUA-1 (accession no. LC020431), HUA-2 (LC020432) and HUA-3 (LC020433) showed to be related to *Clostridiaceae* bacterium SK082 (99%, AB298754), *D. capnocytophagoides* (95%, AB548674), and *Citrobacter freundii* (98%, AB680314), respectively. Therefore, we obtained the 16S rRNA gene sequence for the two dominant strains (HUA-1 and HUA-2) and one minor strain (HUA-3) shown in Table 1.
We subsequently analyzed the band patterns derived from bacterial consortium obtained following PCR-RFLP of 16S rRNA gene (Figure 2B). The intensity of the band corresponding with HUA-3 was considerably lower than those of HUA-1 and HUA-2.

To elucidate the dynamics of the bacterial consortium during alginate degradation, we used T-RFLP as shown in Figure 3. In an initial cultivation phase (0 - 0.5 days), the abundances of HUA-1 were higher than that of HUA-2; however, after 1 day of cultivation, HUA-1 and HUA-2 abundances stabilized at similar levels. On the other hand, the abundance ratio of HUA-3 remained below 1% during cultivation. These results suggested that both HUA-1 and HUA-2 play important pivotal roles in the degradation of alginate.

To analyze HUA-1 and HUA-2 strains in more detail, we attempted to isolate these strains using the roll tube method (30) with agar medium containing alginate, which is similar to the medium used to subculture the bacterial consortium except the presence of agar. A 16S rRNA gene analysis, following isolation, revealed that all the small colonies formed belong to the HUA-3 strain. Although all the isolated strains identified as HUA-3 were cultured in a liquid medium containing alginate, none of them were confirmed to consume alginate (data not shown). The culture medium also contained yeast extract, which could have served HUA-3 as carbon source instead of alginate. Despite HUA-1 and HUA-2 were dominant strains in this consortium and its abundance reached 83.9 % in total as shown Table 1, these strains could not be isolated with agar medium containing alginate as a sole carbon source. Indeed, the growth of anaerobic alginate-degrading microorganisms on the agar medium may not be straightforward. However, the bacterial community structure and its alginate degradation ability have
now been maintained for more than 5 years. Hence, these results suggest that alginate metabolism in this consortium could have arisen as a consequence of anaerobic bacterial interactions like symbiosis, although the type of symbiosis such as cooperation, commensalism, or other interactions (31) is unknown.

**Predicted alginate metabolic pathway in the bacterial consortium** The possibility of a symbiotic metabolism of alginate in the bacterial consortium also gives rise to the possibility of a unique alginate metabolic pathway. The first step of alginate metabolism in bacteria is usually the breakdown of alginate into monomers through the action of alginate lyase (11, 32). To confirm the presence of alginate lyase in the consortium, we assessed alginate lyase activity. The level of enzymatic activity detected in cell-free extracts was 2.3 ± 0.3 U/mg protein. We did not detect alginate lyase activity in the supernatant derived from the culture medium. These results indicated the presence of an alginate degradation pathway mediated by alginate lyase in the bacterial consortium. To investigate whether the bacterial consortium possesses all of genes known to be involved in alginate metabolism, we performed a metagenome analysis of the bacterial consortium by high-throughput sequencing techniques using Illumina HiSeq 2000.

A total of 32,37,966 reads were obtained by high-throughput sequencing. As the first step of assembly, read data was assembled using program velet. Then, Meta Velet which is extension assemble program of velet was used for meta genome assembly. We used the bacterial Genome Annotation Pipeline (MiGAP) to annotate assembly data, and the annotated gene sequences were translated into amino acid sequences using
Genetyx software. A blastp search on DDBJ was subsequently conducted using the translated amino acid sequences.

Figure 4 shows a previously reported metabolic pathway to convert alginate into acetate in bacteria (10, 11). Usually, alginate is break down into monomers through the action of alginate lyases. Alginate monomers are then spontaneously converted into 4-deoxy-L-erythro-hexoseulose uronic acid (DEH), which is subsequently converted into 2-keto-3-deoxy-D-gluconic acid (KDG) and 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) by KDG reductase and KDG kinase, respectively. Furthermore, KDPG is degraded into Glyceraldehyde-3-phosphate (G3P) and pyruvate by KDPG aldolase. G3P is converted into pyruvate through the glycolytic pathway mediated by G3P dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate kinase. Acetate is eventually produced from pyruvate through acetyl-CoA and acetyl phosphate through the reactions mediated by phosphotransacetylase and acetate kinase.

The acetate production from G3P or pyruvate is typical metabolism in bacteria. Hence degradation of alginate to G3P and pyruvate by alginate lyase, KDG reductase, KDG kinase, and KDPG aldolase are more important steps in alginate degradation.

Metagenomic analysis revealed that the bacterial consortium possessed 10 putative alginate lyase genes (see Table S1), 9 putative KDG reductase genes (see Table S2), 6 putative KDG kinase genes (see Table S3) and 6 of putative KDPG aldolase genes (see Table S4). Putative alginate lyase, KDG kinase, and KDPG aldolase genes were also annotated using MiGAP. Both alginate lyase and heparinase belong to polysaccharide lyase families (33). The amino acid sequence of alginate lyase is known to be highly similar to that of heparinase II/III family proteins; in particular, exo-type alginate lyases possess a domain in the C-terminal region found in heparinase II/III family (34). For
this reason, putative heparinase II/III are also included as alginate lyase candidates in Supplemental Table 1.

Putative KDG reductase genes could not be annotated by MiGAP; therefore, we performed a local blast X using Genetyx software for known KDG reductase gene (A1-R) derived *Sphingomonas* sp. A1 (35). This search resulted in 9 putative genes. The amino acid sequence of the putative KDG reductase showed 28%–32% identity with the A1-R amino acid sequence. An additional BLAST search on the DDBJ server showed a high level of identity between the putative KDG reductase and known short-chain dehydrogenase/reductase (SDR) superfamily proteins. In fact, the A1-R gene belongs to the SDR superfamily of proteins. Figure 5 shows a multiple sequence alignment of A1-R and putative KDG reductases. Putative KDG reductases possess highly conserved sequence motifs characteristic of the SDR superfamily of proteins (35), such as a glycine-rich pattern in the cofactor binding Rossmann fold region (Thr-Gly-X-X-Gly-X-Gly) indicated in the figure by black arrows and a catalytic triad (Thy, Lys, and Ser) indicated by white arrows. Hence putative 9 proteins were considered to be KDG reductase. The enzymes involved in conversion from pyruvate to acetate were annotated by MiGAP and shown in Table 2.

In relation to the alginate to pyruvate pathway, the genes associating with *Clostridium* were the most abundant ones, as shown supplemental tables. Total 14 genes associated with *Clostridium* were confirmed. Among them, these are 6 putative alginate lyase genes (see Table S1), 4 putative KDG reductase genes (see Table S2), 3 putative KDG kinase genes (see Table S3) and 1 of putative KDPG aldolase genes (see Table S4). From the taxonomic classification of 16S rRNA gene based on high-throughput sequencing data, *Clostridium* was the dominant genus in the consortium. Since
abundance of HUA-1 reaches 94.5% in the strains classified as *Clostridium*, HUA-1 may possess some of these genes involved in alginate metabolism. Genes related to *Citrobacter*, comprising a series of minor strains in the consortium, were not found upstream of the alginate to pyruvate pathway; however, the downstream pathway contains several genes (KDG kinase and DKPG aldolase) belonging to *Citrobacter*. Thus, our results suggested that *Citrobacter* may be involved in the downstream section of the alginate degradation pathway. On the other hand, only one gene associated with *Dysgonomonas* was confirmed, despite a *Dysgonomonas* was dominant in the consortium. It has not been reported whether *Dysgonomonas* can degrade alginate. Therefore, contribution to alginate degradation of *Dysgonomonas* is unknown.

To the best of our knowledge, the pathway of anaerobic alginate degradation by microbial consortium has not been previously reported. In this study, metagenome analysis revealed that the bacterial consortium studied here possessed all candidate genes involved in the predicted metabolic pathway of the alginate to acetate. These novel findings aid the further analysis of anaerobic alginate degradation by bacterial consortia. However, anaerobic degradation mechanisms of alginate by bacterial consortia are not still understood. Therefore, further efforts are required to isolate and characterize the key bacteria from this consortium.

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Figure legends

FIG. 1. Bacterial growth and alginate consumption in cultures of the bacterial consortium. Each experiment was performed in triplicate. Values represent the mean ± standard deviation for each point. Basal medium supplemented with 5.0 g/l of alginate as the sole carbon source and 50 mM of phosphate buffer (pH 6.8) was used as the growth medium. Time course of growth (circle) and alginate consumption (triangle).

FIG. 2. (A) 16S rRNA gene analysis of the clones by PCR–RFLP and (B) PCR–RFLP analysis of the bacterial consortium.  (A) Lane 1: DNA size marker; Lane 2: PCR–RFLP HUA-3 band; Lane 3, 5, 6, 8, 9, and 10: HUA-1 bands; Lane 4 and 7: HUA-2 bands.  (B) Lane 1: DNA size marker; Lane 2: PCR–RFLP bands for the bacterial consortium; Lane 3: HUA-1 bands; Lane 4: HUA-2 bands; Lane 5: HUA-3 bands

FIG. 3. Community structure of the bacterial consortium revealed by T-RFLP. Black and white bars represent the abundance of HUA-1 (black bar) and HUA-2 (white bar), respectively, in the bacterial consortium.

FIG. 5. Multiple sequence alignment of A1-R and candidate KDG reductase genes present in the bacterial consortium. Numbers 2–1 to 2–9 coincide with the numbers in Table 3. Solid arrow shows the motif of the cofactor binding Rossmann fold region (Thr-Gly-X-X-Gly-X-Gly) from the SDR super family of proteins. Open arrows indicate the catalytic triad (Thy, Lys, and Ser).
Highlights

• Alginate-degrading bacterial consortium was obtained from sand on the beach.

• This consortium was mainly composed of two dominant genera.

• This consortium possessed all the candidate genes involved in the alginate metabolic pathway.
Table 1. Taxonomic classification of the species in the consortium, based on 16S rRNA gene sequence

<table>
<thead>
<tr>
<th>Related genera</th>
<th>Related strains</th>
<th>Designation</th>
<th>Similarity (%)</th>
<th>Accession no.</th>
<th>Abundance (%)</th>
<th>Total abundance (%)</th>
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<td><strong>Dysgonomonas</strong></td>
<td><em>D. capnocytophagoides</em></td>
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Table 2. Candidate genes encoding enzymes involved in acetate production from glyceraldehyde-3-phosphate.

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<th>Enzymes</th>
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<td>Phosphoglycerate mutase</td>
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<td>Pyruvate kinase</td>
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<td>Pyruvate dehydrogenase</td>
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<td>Phosphotransacetylase</td>
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<tr>
<td>Acetate kinase</td>
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</table>
Cell growth (OD$_{660}$)

Culture time (h)

Figure 1, Kita et al.
Fig. 2, Kita et al.
Fig. 3, Kita et al.
Fig. 4, Kita et al.