Potential of waste activated sludge to accumulate polyhydroxyalkanoates and glycogen using industrial wastewater/liquid wastes as substrates

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ABSTRACT

Recovery of the organics in industrial wastewaters/liquid wastes as polyhydroxyalkanoate (PHA) and/or glycogen (GLG) in waste activated sludge is a useful strategy to not only improve the resource value of waste activated sludge but also reduce the energy and cost of waste disposal and wastewater treatment. This study aimed to evaluate the potential of activated sludge to accumulate PHA and GLG using complex substrates (actual and simulated industrial wastewaters/liquid wastes) in addition to various simple organic substrates (organic acids, saccharides, and glycerol). The 24 h PHA and GLG accumulation experiments resulted in the accumulation of up to 25.5%, 6.0% and 14.1% of polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and GLG, respectively, from simple substrates, and up to 9.8%, 0.1% and 14.6%, respectively, from complex substrates. The results indicated that activated sludge can accumulate PHA and GLG even from complex wastewater substrates, although the accumulated PHA and GLG levels were not sufficiently high. The results also indicated that the PHA and GLG accumulation abilities of activated sludge from complex substrates can be drastically enhanced by a short-term acclimation to the corresponding substrate. This study will present the practical implications for value-added resource production through the combined use of waste activated sludge and industrial wastewaters.

Key words | glycogen, industrial wastewater/liquid wastes, polyhydroxyalkanoates, waste activated sludge

INTRODUCTION

Wastewater treatment by employing activated sludge processes has significantly contributed to the improvement and maintenance of the quality of water environments and to the mitigation of environmental risks to humans and other wild animals. However, it inevitably consumes a large amount of energy and concurrently generates a large amount of waste sludge whose disposal requires large energy input. To realize a low-carbon/sustainable society, wastewater treatment must also become sustainable. Therefore, extensive efforts have been made especially regarding the enhancement of energy saving (e.g., improvement of instruments and operation) and of energy production (e.g., anaerobic digestion of waste sludge and through the cultivation of microalgae or aquatic plants). Furthermore, the production of value-added resources from wastewater substances via catalytic functions inherent to waste activated sludge is a possible strategy to realize sustainable wastewater treatment.

Polyhydroxyalkanoates (PHAs) and glycogen (GLG) are the main intracellular storage compounds of carbon and energy in diverse bacteria and can be regarded as value-added resources possibly produced by activated sludge. PHAs are the only bioplastics completely synthesized by bacteria and are completely biodegradable and biocompatible thermoplastic substances (Reddy et al. 2007). PHAs have been regarded as a promising alternative to petroleum-based plastics in various applications (Reddy et al. 2003). Furthermore, it has been reported that the accumulation of PHA in waste activated sludge can enhance the production of biogas (Wang et al. 2015; Soda et al. 2016) and short-chain fatty acids
The accumulation of PHA and GLG by activated sludge bacteria has been extensively studied, and their very efficient accumulation has been proven from not only simple substrates (organic acids, saccharides, and glycerol) but also complex substrates (Moralejo-Gárate et al. 2013, 2014; Kourmentza et al. 2017; Valentino et al. 2017). However, most of the previous studies applied the activated sludge-derived mixed microbial cultures enriched with efficient PHA and GLG accumulating bacteria mostly via the aerobic dynamic feeding method. By contrast, limited studies have investigated the PHA and GLG accumulation abilities of native activated sludge in municipal wastewater treatment plants (Takabatake et al. 2002; Mengmeng et al. 2009; Cavaillé et al. 2013; Sakai et al. 2015; Inoue et al. 2018, 2019). In particular, the abilities of non-enriched activated sludge to accumulate PHA and GLG from complex substrates, such as industrial wastewaters/liquid wastes, remain unclarified, although the use of such complex substrates is valuable for not only the improvement of the resource value of waste activated sludge but also the reduction of the energy and cost of the treatment of both wastewaters/liquid wastes and waste activated sludge.

Therefore, this study aimed to evaluate the abilities of activated sludge to accumulate PHA and GLG from industrial wastewater/liquid wastes as well as from simple substrates (organic acids, saccharides, and glycerol). Furthermore, short-term acclimation was attempted to enhance the PHA and GLG accumulation from complex substrates.

MATERIALS AND METHODS

Activated sludge sample

The activated sludge samples used in this study were collected from a municipal wastewater treatment plant in Osaka, Japan. The municipal wastewater treatment plant receives mainly domestic wastewater and a small amount of industrial discharge. It employs the conventional activated sludge process for secondary biological treatment with the planned maximum inflow volume of 82,400 m³/d. The collected samples were transported to the laboratory on ice and washed twice with nitrogen- and phosphorus-deficient basal salt medium (BSM) with pH 7.0 ± 0.3 (Inoue et al. 2018) prior to use in the experiments.

Substrates

Simple and complex substrates were used for the PHA and GLG accumulation experiments in this study. Acetate, propionate, butyrate, citrate, malate, pyruvate, lactate, glucose, fructose, sucrose, and glycerol were selected as the simple substrates. Actual industrial wastewaters or liquid wastes used as the complex substrates were molasses (sample I1), sweet-bean-making factory wastewater (sample I2), waste glycerol (sample I3), plum processing factory washing wastewater (sample I4), and plum processing factory seasoning wastewater (sample I5). Orange (sample I6) and apple juices (sample I7) were also used as simulated juice factory wastewaters.

PHA and GLG accumulation experiments

The ability of activated sludge to accumulate PHA and GLG from simple and complex substrates was evaluated using batch experiments. The sludge sample was inoculated at a mixed liquor suspended solids (MLSS) concentration of 1,000 mg/L into 500 mL Erlenmeyer flasks containing 200 mL of the aforementioned nitrogen- and phosphorus-deficient BSM supplemented with each of the 11 simple substrates or the seven complex substrates at an initial concentration of 1,000 mg-C/L. BSM was used even in the experiments with complex substrates to avoid underestimation of their potential as the organic substrate in PHA and GLG accumulation due to the lack of some trace elements. After the pH was adjusted to 7.0 ± 0.3, the cultures were aerobically incubated at 28 °C and 120 rpm for 24 h, and the contents of polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), and GLG in the sludge were determined periodically. All the experiments were conducted in duplicate.

To examine the possibility of improving the PHA and GLG accumulation ability from complex substrates, short-term acclimations were also performed prior to the aforementioned PHA and GLG accumulation experiments. Sample I6 (orange juice sample) was used as the representative complex substrate, and the following medium was used
for acclimation: sample I6, 324 mg-C/L, NH₄Cl 47.3 mg-N/L, KH₂PO₄ 38.6 mg-P/L, MgSO₄·7H₂O 68.4 mg/L, KCl 26.84 mg/L, allythiourea 5 mg/L, and trace element solution 1.5 mL/L (Inoue et al. 2019). Activated sludge was aerobically incubated at 28 °C and 120 rpm for 24 h; thereafter, the activated sludge was transferred to a fresh medium, and the same incubation was repeated up to a total of 14 batch cycles before use in the PHA and GLG accumulation experiments.

**Analytical methods**

The concentrations of MLSS, total and volatile solids (TS and VS, respectively), total nitrogen, and total phosphorus were measured according to standard methods (APHA 2012).

The concentrations of the simple substrates used and dissolved organic carbon (DOC) were measured to evaluate the consumption of the simple and complex substrates, respectively. Furthermore, the major organic constituents (organic acids and saccharides) in the complex substrates were determined. Prior to measurements, the samples were centrifuged (1,500 × g, 10 min, room temperature) and filtered through a 0.45 μm cellulose acetate filter (Advantec, Tokyo, Japan). The concentrations of the simple substrates were determined using high-performance liquid chromatography (HPLC) analysis with a Shimadzu LC-10Avp system (Shimadzu, Kyoto, Japan) equipped with an RID-10A refractive index detector (Shimadzu) and an Aminex HPX-87H or HPX-87P column (300 × 7.8 mm; Bio-Rad, Hercules, CA). The DOC concentrations were determined using a Shimadzu TOC-VCSH (Shimadzu).

The contents of PHB and PHV in the sludge samples were determined using a gas chromatograph GC2014 (Shimadzu) equipped with a flame ionization detector and an InertCap WAX-HT capillary column (20 m × 0.25 mm ID, 0.25 μm df; GL Sciences, Tokyo, Japan) after pretreatment via methanolysis decomposition as described previously (Michinaka et al. 2007; Sakai et al. 2015). The GLG contents were determined using the HPLC analysis for glucose as described above, after the pretreatment described in a previous study (Moralejo-Gárate et al. 2011) with the following minor modifications: the tube containing the sample after the addition of 0.6 N HCl was vigorously shaken prior to heating, and the sample after pretreatment was filtered through a 0.45 μm hydrophilic polytetrafluoroethylene membrane filter (Advantec) prior to HPLC analysis. Based on the analytical results, PHB, PHV and GLG contents (%) were calculated as the wt% of the weight of freeze-dried sludge that was subjected to PHA and GLG analyses.

**RESULTS AND DISCUSSION**

**PHA and GLG accumulation from simple substrates**

Before the experiments, the contents of PHB, PHV, and GLG in activated sludge were all less than 3%. After the PHA and GLG accumulation experiments for 24 h using each of the 11 simple substrates, the contents of PHB, PHV, and GLG increased by 2.0% to 25.5%, 0% to 6.0%, and 0.9% to 14.1%, respectively (Figure 1). Based on the substrate consumption and PHA and GLG accumulation during the experiments, the yields of PHB, PHV, and GLG from each of the substrates tested were calculated to be 0.03 to 0.35 C-mol 3-hydroxybutyrate/C-mol substrate, 0 to 0.09 C-mol 3-hydroxyvalerate/C-mol substrate, and 0.01 to 0.41 C-mol glucose/C-mol substrate, respectively.

Among the tested substrates, PHB was preferentially produced from acetate, butyrate, propionate, and lactate (Figure 1). In particular, over 20% of PHB was accumulated from butyrate and pyruvate within 24 h. The PHB accumulation from acetate for 24 h observed here was within the range obtained in previous studies using various activated sludge samples (Takabatake et al. 2002; Sakai et al. 2015). Noticeable PHV accumulation of 6.0% was observed only from propionate. This was corroborated by the findings in previous studies using enrichment cultures that reported a preferential induction of PHV synthesis when propionate was used as the substrate (Dionisi et al. 2004; Jiang et al. 2011). In contrast, 12.2% to 14.1% of GLG accumulation was observed from glucose, fructose, and sucrose, although the PHB accumulation from these substrates was 5.8% to 7.1%. When glycerol was used as the substrate, PHA and GLG were accumulated only marginally (5.1% in total). Low PHA accumulation from glycerol may be because limited bacteria are capable of utilizing glycerol for PHA production, as observed in our previous study (Inoue et al. 2016). These results indicated that PHB and GLG would be the main intracellular storage substances produced from organic acids and saccharides, respectively, by activated sludge in municipal wastewater treatment plants.

The differences in the preference and contents of PHA and GLG accumulated from 11 different substrates can be interpreted from PHA and GLG synthesis pathways (Supplementary Material Figure S1). For example, higher PHB accumulation from butyrate (25.5%) than from acetate.
(14.7%) can be elucidated by the difference in their utilization in the PHB synthesis pathway. According to the pathway, butyrate is transformed directly to PHB via acetocacetyl-CoA, whereas acetate is transformed to acetyl-CoA via β-oxidation. The produced acetyl-CoA is utilized both for PHB synthesis and in the tricarboxylic acid cycle. Moreover, a slightly higher PHB accumulation from pyruvate (22.1%) than from lactate (16.3%) would be because pyruvate is transformed directly to acetyl-CoA, but lactate is transformed to acetyl-CoA via pyruvate. By contrast, noticeable PHV accumulation only from propionate would be because propionyl-CoA, which is required for PHV synthesis, is produced directly from propionate but through multistep transformations from the other substrates tested here.

### PHA and GLG accumulation from industrial wastewater/waste liquids

The characteristics of the complex substrates (actual or simulated industrial wastewaters and liquid wastes) used in this study are summarized in Table 1. They contained carbon, nitrogen, and phosphorus with the C:N:P ratios of 100:0.2–2.5:0–3.0. Their compositions with abundant carbon and deficient nitrogen and phosphorus appeared suitable for the accumulation of PHA and GLG. In addition, their

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Wastewater/waste type</th>
<th>Collection date</th>
<th>pH</th>
<th>DOC (g-C/L)</th>
<th>C:N:P ratio</th>
<th>VS/TS ratio</th>
<th>Major organic constituents (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>Molasses</td>
<td>January 2016</td>
<td>5.1</td>
<td>972.6</td>
<td>100:2.5:0.4</td>
<td>0.89</td>
<td>Sucrose 44%, fructose 10%, glucose 7%</td>
</tr>
<tr>
<td>I2</td>
<td>Sweet-bean-making factory wastewater</td>
<td>April 2016</td>
<td>4.9</td>
<td>2.096</td>
<td>100:0.1:3.0</td>
<td>0.79</td>
<td>ND</td>
</tr>
<tr>
<td>I3</td>
<td>Waste glycerol</td>
<td>May 2016</td>
<td>10.0</td>
<td>9.360</td>
<td>ND</td>
<td>ND</td>
<td>Glycerol 35%</td>
</tr>
<tr>
<td>I4</td>
<td>Plum processing factory washing wastewater</td>
<td>July 2016</td>
<td>3.1</td>
<td>1.340</td>
<td>100:0.2:0.3</td>
<td>0.19</td>
<td>Citrate 42%, acetate 20%</td>
</tr>
<tr>
<td>I5</td>
<td>Plum processing factory seasoning wastewater</td>
<td>July 2016</td>
<td>2.5</td>
<td>79.72</td>
<td>100:0.5:0.1</td>
<td>0.74</td>
<td>Citrate 10%, acetate 3%</td>
</tr>
<tr>
<td>I6</td>
<td>Orange juice</td>
<td>May 2016</td>
<td>4.2</td>
<td>46.82</td>
<td>100:0.3:0.3</td>
<td>0.89</td>
<td>Glucose 24%, fructose 23%, sucrose 7%</td>
</tr>
<tr>
<td>I7</td>
<td>Apple juice</td>
<td>July 2016</td>
<td>4.0</td>
<td>45.16</td>
<td>100:0.4:0.1</td>
<td>0.96</td>
<td>Fructose 36%, glucose 22%, sucrose 7%</td>
</tr>
</tbody>
</table>

ND, not determined.

Figure 1 | PHA and GLG accumulation from the simple substrates in activated sludge after experiments for 24 h.
organic compositions were as follows. Sample I1 was rich in saccharides (i.e., glucose, fructose, and sucrose). Sample I2 was inferred to contain mainly saccharides and starch, although its components could not be identified in detail. In sample I3, glycerol was the main component. Samples I4 and I5 contained acetate and citrate with higher contents in sample I4. Samples I6 and I7 were both rich in saccharides including glucose, fructose, and sucrose.

Figure 2 shows the accumulation of PHA and GLG from seven complex substrates during experiments for 24 h. PHB, PHV, and GLG were accumulated at contents of 0.5% to 9.8%, 0% to 0.1%, and 1.1% to 14.6%, respectively, with total contents of 4.5% to 24.4%. PHV was hardly accumulated from any substrates. The highest content of PHA and GLG was obtained from sample I1 (PHB at 9.8% and GLG at 14.6%), followed by sample I6 (PHB at 9.7% and GLG at 11.3%). These results indicate that organics in various industrial wastewaters can be transformed to PHA and GLG by waste activated sludge, although the accumulated PHA and GLG contents are not sufficiently high.

When samples I1, I6, and I7 were used as the substrate, GLG was accumulated at higher content compared with PHB (Figure 2). Given the aforementioned evidence that GLG was accumulated preferably from saccharides (Figure 1) and that samples I1, I6, and I7 were rich in saccharides (Table 1), these results are reasonable. By contrast, PHB was produced preferably from samples I3 and I4 compared with GLG. The reason for preferable PHB production from sample I4 would be because the sample was rich in organic acids (Table 1), which are preferable substrates for the production of PHB, rather than GLG, whereas the reason for preferable PHB production from sample I3 is unknown because it was rich in glycerol (35%) and the other organic constituents were not clarified in this study. In contrast, very low PHB accumulation from sweet-bean-making factory wastewater (sample I2) was surprising because the sample was expected to be rich in saccharides and starch, which can be transformed to not only GLG but also PHB (Figure 1). This might be due to a high phosphorus concentration in the sample (C:N:P = 100:0.1:3.0) compared with those of the other samples, which would mitigate the nutrient-deficient stress, inhibiting the growth of microorganisms and consequently reducing PHB production.

**Possibility to enhance PHA and GLG accumulation ability via short-term acclimation**

The effect of short-term acclimation on the improvement of PHA and GLG accumulation ability from complex substrates was examined. Here, sample I6 was used as the representative complex substrate because it was considered a favorable substrate for PHA and GLG accumulation based on the second highest PHA and GLG contents in the above experiments. Figure 3 shows the results of PHB and GLG accumulation by activated sludge without acclimation and after one, two, and 14 cycles of acclimation for 24 h. During PHA and GLG accumulation experiments for 24 h, most of the organics (849–923 mg-C/L (81% to 93% of the initially supplemented organics)) were consumed when activated sludge was acclimated, although DOC consumption was only 530 mg-C/L (50% of the initially supplemented organics) without acclimation. In particular, substrate consumption rate during the initial 3 h was drastically increased after acclimation: 0.017 mg-C/mg-MLSS/h without acclimation, and 0.047, 0.046, and 0.10 mg-C/mg-MLSS/h with acclimation.
MLSS/h with acclimation for one, two, and 14 cycles, respectively. Regarding PHB and GLG accumulation, the maximum PHB and GLG contents in activated sludge without acclimation were 8.9% and 23.5% after 24 h, respectively. After acclimation, activated sludge accumulated 15.1% to 16.9% of PHB after 12 h, and the PHB accumulation ability of activated sludge was considerably improved even after one cycle of acclimation. On the other hand, activated sludge after 14 cycles of acclimation accumulated 48.7% of GLG after 3 h, while acclimation for one or two cycles did not improve the GLG accumulation ability of activated sludge. These results suggested that the abilities of activated sludge to accumulate both PHB and GLG can be considerably improved by acclimation. This study showed that activated sludge in municipal wastewater treatment plants has considerable potential to produce PHA and GLG, which are intracellular storage polymers.

In previous studies, enhanced PHA accumulation in activated sludge was achieved through the enrichment of PHA-accumulating microorganisms via the aerobic dynamic feeding method, even using complex substrates (Valentino et al. 2017). However, the need for a long period of enrichment (e.g., months) is a significant obstacle preventing the practical application of continuously generated waste activated sludge. By contrast, the results of this study indicated that acclimation to complex substrates even for a short term of several days can drastically enhance the PHA and GLG accumulation ability of activated sludge. Although further study is required to optimize the acclimation conditions, it would be a useful option for enhanced PHA and GLG production with the combined use of waste activated sludge and industrial wastewater/liquid wastes.

CONCLUSIONS

This study showed that activated sludge in municipal wastewater treatment plants has considerable potential to produce PHA and GLG, which are intracellular storage polymers.
compounds synthesized by diverse bacteria and can be applied as value-added resources in industries, from various industrial wastewater/liquid wastes as well as simple substrates. The results also indicated that short-term acclimation can be a key option to enhance the PHA and GLG accumulation abilities of activated sludge considerably. The findings of this study will be helpful for the realization of value-added resource production with the combined use of waste activated sludge and industrial wastewater/liquid wastes.

By contrast, further study with different types of activated sludge and industrial wastewater/liquid wastes should be conducted to generalize the usefulness of the proposed strategy and to determine its applicable range (types of activated sludge and industrial wastewater/liquid wastes). Through such studies, the relationship between microbial community composition and PHA and GLG accumulation potential of activated sludge should be also unveiled. Such knowledge would be useful for selecting activated sludge useful for PHA and GLG production and for judging the need of acclimation of the activated sludge prior to use for PHA and GLG production. The effects of cultivation conditions on PHA and GLG accumulation by activated sludge are also important issues to be addressed in order to optimize the PHA and GLG production from industrial wastewater/liquid wastes by activated sludge.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available at https://dx.doi.org/10.2166/wst.2020.059.

REFERENCES


