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Instructions for use

Single-step production of polyhydroxybutyrate from starch by using

α-amylase cell-surface displaying system of *Corynebacterium glutamicum*

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Abstract

Direct polyhydroxybutyrate (PHB) production from starch was for the first time achieved using engineered *Corynebacterium glutamicum* expressing PHB biosynthetic genes and displaying α-amylase on its cell surface. The engineered strain accumulated 6.4 wt% PHB from starch which was higher than that obtained from glucose (4.9 wt%).

Polyhydroxyalkanoates (PHA)s are biobased polymers synthesized by bacteria as intracellular carbon and energy storage materials which were first discovered by Lemoigne (1). A typical bacterial PHA is polyhydroxybutyrate (PHB) which has received much attention as a biodegradable polymer and can be produced from renewable and abundantly available biomass resources. For the past two decades, people began their interest in using engineered *Escherichia coli* platform to produce PHA with diverse structures by polymerizing different monomers into the copolymers, such as 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) etc. However, *E. coli* is a Gram-negative bacterium which is known to produce potentially harmful substances,

(*i.e.*, endotoxin and lipopolysaccharide) (2). In considering practical applications of polymers, especially for food grade and biomedical demands, the use of endotoxin-free Gram-positive bacteria is preferable. *Corynebacterium glutamicum* is an aerobic, Gram-positive, non-sporulating, bacterium with GRAS status that has been extensively employed for the industrial production of several food-grade amino acids, feed and pharmaceutical products for several decades (3). We previously reported production of PHB (4), poly(3HB-*co*-3HV) (5) and poly(lactate-*co*-3HB) (6) in engineered *C. glutamicum* harboring PHA biosynthetic genes, indicating the capability of polyester synthesis of this organism.

However, economic considerations of production costs suggest that the carbon source, mainly glucose used for polymer production has a high impact on the production cost of PHA, affecting their commercialization potential. There has been a considerable interest in using inexpensive substrates as an alternative to glucose in PHA production. One such substrate is starch which is a renewable carbon source available in large quantities. Nevertheless, prior to fermentation, starch need to be hydrolyzed into glucose by a two-step process, liquefaction and saccharification which need physicochemical and enzymatic treatment (7). For example, Chen et al. treated starch by an enzymatic reactive extrusion process for polymer production (8); hydrolyzed starch was used as

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substrate for PHB production in *Halomonas boliviensis* (9); similarly, hydrolyzed corn starch was also utilized for PHB synthesis using recombinant *E. coli* (10). The treatment before these fermentation makes using starch less economically viable. One strategy to decrease the costs of using starch for biopolymer production should be the direct utilization of starch instead of using enzyme-treated starch.

To meet this goal, the cell-surface display technology is a powerful platform, which allows peptides and/or proteins fusing with the anchoring motifs to be localized on the external surface of the cell membrane. The cell-surface display technology was established in yeast (11,12) and also applied to prokaryotes (13). The display of glycosidases enables microbes to hydrolyze and simultaneously utilize polysaccharides, such as starch, as a carbon source. Additionally, the displayed enzymes were reported to maintain the activity during long time cultivation (14) that could be an advantage over the enzyme-secreting system. Therefore in this study, we attempt to produce PHB from starch using an engineered *C. glutamicum* strain HPA displaying α-amylase from *Streptococcus bovis* strain 148 on the cell surface, which was demonstrated to produce L-lysine from starch (15). The gene encoding the fusion of α-amylase and anchor protein was integrated into the chromosomal DNA of *C. glutamicum*, which allows us to maintain the α-amylase activity without the addition of antibiotics. This is the first

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report for the direct production of PHB from starch.

To determine the applicability of starch for PHB synthesis, *C. glutamicum* HPA, in which the α-amylase gene is integrated into its genome (15), harboring plasmid pPSC$_{Re}$AB contain PHB biosynthetic genes (*phaCAB*) from *Ralstonia eutropha* (4) was cultivated on CM2G medium (16) at 30°C for 24h for preculture. The cells were collected by centrifugation then transferred into fresh MMTG medium (16) supplemented with 6 % starch or glucose as a carbon source. The soluble starch solution was autoclaved separately and combined with MMTG medium. 25 μg/mL kanamycin, 0.45 mg/L biotin to suppress the glutamate production (17) and 0.5 g/L L-homoserine, were added when necessary. Subsequently, the cells were cultivated at 30°C for 72 h for PHB production. The *C. glutamicum* ATCC13032, which is the parent strain of HPA, harboring the same plasmid was prepared as a control.

To confirm the functional expression of α-amylase during PHB production, samples were taken at intervals and the α-amylase activity was analyzed by using the α-amylase measurement kit (Kikkoman, Tokyo, Japan) as per the manufacturer's instructions. Fig. 1 shows that the engineered HPA strain exhibited a significant α-amylase activity (1.2−1.6 U/mL), whereas no activity was detected in ATCC13032 cells. The displayed α-amylase mostly retained its activity during the cultivation.

Next, the consumption of starch during PHB production by *C. glutamicum* HPA was analyzed. The analysis was based on a colorimetric method using the phenol−sulphuric acid reaction (18) to give the amount of total sugars corresponding to starch and starch hydrolysis products in the medium. The HPA cells dually expressing α-amylase and PhaCAB consumed starch at a comparable rate to that of glucose (Fig. 2), suggesting that the utilization of starch should be efficient to prompt PHB production. Some starch remained unutilized at the end of the culture possibly due to the inability of the α-amylase to hydrolyze the α-1,6-glycosidic bonds in starch (15).

The *C. glutamicum* HPA cells were lyophilized after cultivation and subjected to PHB analysis as described previously (19). In brief, the intracellular accumulated PHB is directly converted into crotonic acid by the treatment with concentrated sulfuric acid and quantified using HPLC. As shown in Table 1, the cells grown on starch accumulated higher PHB (6.4 wt%) than those grown on glucose (4.9 wt%). This indicated that the fermentable sugars resulting from starch hydrolysis by α-amylase induced PHB production. The productivity of PHB from starch (0.39 g/L) was slightly higher than that from glucose (0.35 g/L). In terms of stoichiometrical comparison, the carbon molarity of starch is 11% higher than the same weight of glucose. However, the conversion yield from starch to PHB was higher than that from glucose considering 14

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g/L starch remained unutilized (Fig. 2). In contrast, the dry cell weight obtained by using glucose (7.1 g/L) was higher compared to that using starch (6.1 g/L). This suggested that glucose was consumed for growth rather than for PHB production, presumably because the utilization of starch was slower than that of glucose (Fig. 2). The previous research has also indicated such benefit of using starch over glucose in *C. glutamicum* for L-lysine production (15). Therefore, the delayed consumption of starch could be beneficial for high-yield PHB production compared to glucose in *C. glutamicum* α-amylase cell-surface displaying system. The trace amount of PHB in the wild-type strain was due to a background peak in HPLC analysis. The wild-type strain was thought to not produce PHB, since *C. glutamicum* possesses no PHA synthase gene in its genome.

In conclusion, we succeeded in the production of PHB from soluble starch using the α-amylase cell-surface displaying *C. glutamicum.* Additionally, the use of starch can give a higher polymer yield compared with the utilization of glucose. Therefore, this system may be useful for the reduction of the cost of PHB production in a biorefinery for potential industrial scale applications, and could be applicable to various copolymer productions, such as lactate-based polyesters (20).

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Figure legends

FIG. 1. α-Amylase activity in the cultures of wild-type *C. glutamicum* (*squares*) and HPA strain harboring pPS$C_{Re}AB$ (*circles*). The activity includes supernatant and surface displayed α-amylase. Data are average ± standard deviation of three independent trials.

FIG. 2. Total sugar consumption of starch (*open circles*) and glucose (*diamonds*) by *C. glutamicu*m HPA harboring pPS$C_{Re}AB$. Data are average ± standard deviation of three independent trials.
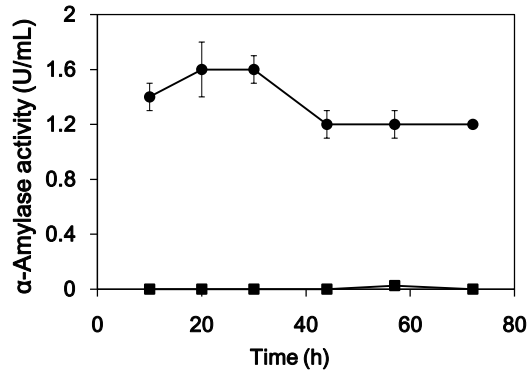
Fig. 1
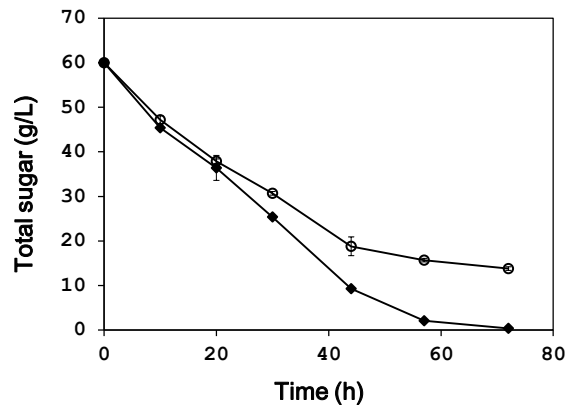
Fig. 2

TABLE 1. PHB production in *C. glutamicum* HPA grown on starch and glucose. [a]

Carbon source	Plasmids	CDW[b] (g/L)	PHB Content (wt%)	Polymer yield (g/L)
Glucose	pPS	7.4±0.4	0.4±0.1	0.03
Glucose	pPS$C_{Re}AB$	7.1±1.4	4.9±2.4	0.35
Starch	pPS$C_{Re}AB$	6.1±0.5	6.4±1.1	0.39

[a] Cells were grown on MMTG medium at 30°C for 72 h.

[b] CDW, cell dry weight. The polymer yield is CDW multiplied by PHB content.