Dual production of poly(3-hydroxybutyrate) and glutamate using variable biotin concentrations in *Corynebacterium glutamicum*

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Abstract

We previously synthesized poly(3-hydroxybutyrate) [P(3HB)] in recombinant *Corynebacterium glutamicum*, a prominent producer of amino acids. In this study, a two-step cultivation was established for the dual production of glutamate and P(3HB) due to the differences in the optimal concentration of biotin. Glutamate was extracellularly produced first under the biotin-limited condition of 0.3 μg/L. Production was then shifted to P(3HB) by addition of biotin to a total concentration of 9 μg/L. The final products obtained were 18 g/L glutamate and 36 wt% of P(3HB).

Poly(3-hydroxybutyrate) [P(3HB)], a biodegradable and biocompatible plastic, has received considerable research attention (1). P(3HB) and its derivatives can be used for medical applications and as food containers (2, 3). Most bacteria production studies have been carried out using gram-negative bacteria (4). However, the P(3HB) accumulated by gram-negative bacteria is not suitable for medical and food container applications, owing to possible endotoxin contamination. There have been a few attempts to remove endotoxins during the preparation of polyhydroxyalkanoate (PHA), but traces amount of endotoxin always remained in the material (5, 6). We developed an endotoxin-free P(3HB) production system using a gram-positive bacterium, *Corynebacterium glutamicum* (7).

C. glutamicum has been extensively employed for the industrial production of glutamate used in food, feed and pharmaceutical products for several decades (8-12). We attempted to produce intracellular P(3HB) along with extracellular glutamate. Dual production of P(3HB) with other value-added materials is thought to address the problem of high production cost which is the main obstacle for P(3HB) commercialization. When P(3HB) is produced with a valuable second material in the same batch of cells, cost-efficiency will increase. For example, Hori et al. demonstrated simultaneous production of polyhydroxyalkanoates and rhamnolipids, which is a type of

biosurfactant. (13)

C. glutamicum is a biotin auxtrophic bacterium in which glutamate production is induced under biotin-limited conditions. However, in this study, P(3HB) production was found to be induced by the presence of biotin. In order to establish an effective dual production system of P(3HB) and glutamate in *C. glutamicum*, biotin was manipulated as the key component to switch production from glutamate to P(3HB). This switching behavior is in response to the metabolic linkage between the glutamate and our proposed P(3HB) synthetic pathway. This two-step cultivation would enable both substances to be produced at an optimized concentration of biotin.

pPS-*CAB* harboring a promoter of *cspB* and *phaCAB* operon derived from *Ralstonia eutropha* was constructed as described previously (7) for the P(3HB) biosynthesis. pPS-*CAB* was digested with *Bst*EII and *Bam*HI, blunted with T4 DNA polymerase and then a 6.2-kb fragment was self-ligated to construct pPS, which harbors a promoter of *cspB* only as the control vector.

C. glutamicum ATCC13869 was used as a host for P(3HB) production. This strain was transformed with the plasmid vectors by electroporation as described previously (14). Two medias were used; a nutrient-rich medium (CM2G) (15) for pre-culture, and a minimal medium (MMTG) (15) for the main culture of *C.*

glutamicum to produce P(3HB) or/and glutamate. Kanamycin (50 μg/ml) was added to the seed-culture when needed.

The concentration of glutamate produced by recombinant *C. glutamicum* was determined using Yamasa L-Glutamic acid Assay Kit (Yamasa, Japan) according to the protocol recommended by the manufacturer. The P(3HB) content of the transformants was quantified by high-performance liquid chromatography (HPLC), as described previously (16).

Glutamate production in *C. glutamicum* is known to be modulated by biotin, a vitamin essential for cell growth (17). The specific activity of 2-oxoglutarate dehydrogenase complex (ODHC) decreased under the biotin-limited condition which eventually leads to extracellular overproduction of glutamate (18). Furthermore, a decrease in the cellular concentration of the Acetyl-CoA carboxylase (AccBC) subunit DtsR1 caused by biotin limitation was reported to reduce ODHC activity, which leads to glutamate overproduction (19). Therefore, biotin is able to switch the carbon flux of acetyl-CoA towards glutamate biosynthesis or fatty acid biosynthesis (Fig. 1). Concentration of biotin could affect the P(3HB) production as well, because acetyl-CoA is also a starting substance required for P(3HB) biosynthesis. Thus, we investigated the effects of biotin concentration on P(3HB) and glutamate production in the recombinant

strains.

Figure 2A shows glutamate and P(3HB) productions by wild-type and recombinant strains of *C. glutamicum* grown under various biotin concentrations, respectively. The production of glutamate by the wild-type strain was induced under biotin-limited conditions. Glutamate production reached 23 g/L when 0.3 μg/L of biotin was added. However, glutamate production decreased drastically with increasing concentrations of biotin, and was mostly suppressed when more than 9 μg/L of biotin was added. In contrast, P(3HB) production by the recombinant strain occurred for 3 μg/L or higher biotin concentration. The recombinant strain reached maximum P(3HB) production (23 wt%) when 9 μg/L of biotin was added and remained constant under an excess amount of biotin (450 μg/L). This result indicates that *C. glutamicum* produced glutamate under the biotin-limited conditions and accumulated P(3HB) under the biotin-supplied conditions. Because the optimal concentration of biotin for glutamate and P(3HB) productions is largely different, these substances are not produced simultaneously per se; however, a two-step production should be rather favorable to produce glutamate and P(3HB). Thus, we attempted to establish a two-step production system, in which glutamate is produced in the first stage of fermentation and biotin is added to produce P(3HB) in the second stage.

In order to establish the dual production system, we investigated the effect of introducing P(3HB) biosynthetic genes on glutamate production. Table 1 shows that the levels of glutamate produced by the recombinant strains of *C. glutamicum* harboring the *phaABC* genes were equal to that of the control vector strain, indicating that glutamate production is not negatively affected by introducing P(3HB) biosynthetic genes. Therefore, we concluded that 0.3 μg/L of biotin is optimal for glutamate production in the first stage of fermentation, while 9 μg/L of biotin would be the most efficient for inducing intracellular accumulation of P(3HB) in the second stage of fermentation.

Furthermore, the time-course of glutamate production was monitored to determine the optimal timing to switch into the P(3HB) production phase. Glutamate production reached its maximum at 72 hours and remained constant after that (data not shown). Therefore, we chose 72 hours as the time to switch the fermentation to P(3HB) production.

Considering these factors, we created a two-step production system of glutamate and P(3HB) as a strategy to optimize the production levels of both substances. As shown in Fig. 2B, glutamate production was induced by biotin limitation (0.3 μg/L) at the early stage. When the glutamate production reaches its peak after 72 hours of culture, 9 μg/L biotin and 60 g/L glucose were added to induce intracellular

accumulation of P(3HB). Slight decrease was observed in glutamate production from 72 to 96 hours, but remained constant throughout the rest of the fermentation. The extracellularly produced glutamate might have been consumed during cell growth triggered by the addition of biotin. Using biotin as the metabolic flux switch, we obtained 18 g/L of glutamate and 36 wt% of P(3HB) as the final products. The role of biotin as a switch of the carbon flux towards P(3HB) biosynthesis is clearly shown by the drastic increase in P(3HB) production after biotin addition.

To date, changes in the metabolic flux between glutamate and P(3HB) biosynthetic pathways have not been discussed in terms of biotin concentration. In this study, we investigated the production of P(3HB) with or without glutamate production by manipulating the biotin concentration as a switch between glutamate and P(3HB) production. It should be noted that P(3HB) accumulated in the dual production system (36 wt%, Fig. 2B) increased 1.5-fold compared to that of the single production system (23 wt%, Fig. 2A). Even though glucose was added twice during the fermentation, it is unlikely the reason for higher P(3HB) content is glucose, because nearly all glucose was consumed during the glutamate production phase (less than 1 g/L at 72 h, data not shown). Therefore, these results suggest that glutamate excreted into the medium improved P(3HB) production in the second stage. On the other hand, Liu et al

performed simultaneous production of P(3HB) and glutamate in *C. glutamicum* and reported that P(3HB) accumulation increased glutamate production (20). However, in our system, glutamate and P(3HB) were separately produced by changing the biotin concentration, and therefore the positive effect of glutamate on P(3HB) production was clearly demonstrated.

We propose a prototype dual production process for glutamate and P(3HB) as illustrated in Fig. 2C. This system consists of two-step fermentation based on regulation of the biotin concentration, which is a key component that controls the production of both compounds in *C. glutamicum*.

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Table 1 Glutamate production by *C. glutamicum* harboring pPS vector and pPS-CAB operon in different biotin concentrations

Biotin	Glutamate (g/L)[a]	
(μg/L)	pPS	pPS-*CAB*
0.3	20.1 (±2.3)	17.4 (±2.5)
3	1.7 (±0.5)	1.8 (±1.0)
30	ND[b]	ND[b]

[a]The data was presented based on three independent experiment. [b]ND: Not detected.

Figure captions

Fig. 1 Metabolic linkage between glutamate synthetic pathway and P(3HB) synthetic pathway in *C. glutatamicum*. The letters in boxes indicate the enzymes or complex relevant to these two synthetic pathway.

AccBC: Acetyl-CoA carboxylase; ODHC: 2-oxoglutarate dehydrogenase complex; PhaA: β-ketothiolase; PhaB: NADPH-dependent acetoacetyl-CoA reductase; PhaC:

PHA synthase.

Fig. 2 Glutamate and P(3HB) production in *C. glutamicum* with different concentration of biotin. Black bars and white bars indicate glutamate concentration and P(3HB) content, respectively. Cells were grown in MMTG medium at 30°C for 72 h. The data was presented based on three independent experiments.

Fig. 3 Dual production of glutamate and P(3HB) by *C. glutamicum* harboring pPS-CAB. Recombinant *C. glutamicum* was cultivated in MMTG medium with 0.3 μg/L of biotin for initial 72 h and then 9 μg/L of biotin and 60 g/L of glucose were added to the culture medium. Cells were grown at 30°C. Open circles, open squares and open triangles indicate glutamate concentration, P(3HB) content and dry cell weight, respectively. The data was presented based on three independent experiments.

Fig. 4 The scheme of two-step production system for glutamate and P(3HB) established in *C. glutamicum* by regulating the concentration of biotin. First *C. glutamicum* harboring pPS-CAB was cultivated in MMTG medium with 0.3 μg/L of biotin to extracellularly produce glutamate. Then, 9 μg/L of biotin and 60 g/L of glucose were added to the culture medium to induce the P(3HB) accumulation for 72 h .

Fig. 1

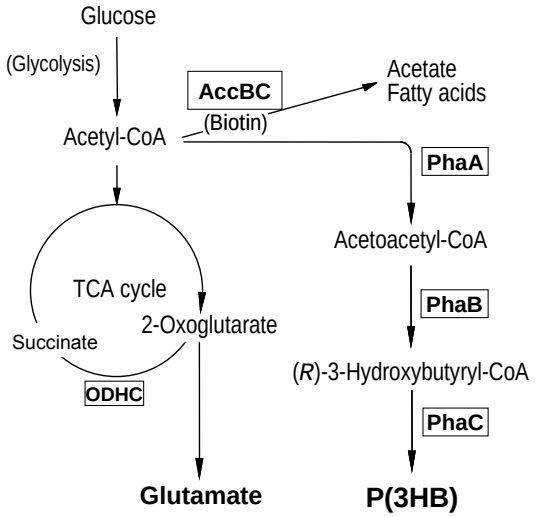

Glucose
(Glycolysis)
AccBC
(Biotin)
Acetate
Fatty acids
Acetyl-CoA
PhaA
Acetoacetyl-CoA
TCA cycle
PhaB
2-Oxoglutarate
Succinate
(R)-3-Hydroxybutyryl-CoA
ODHC
PhaC
Glutamate
P(3HB)

Fig. 2

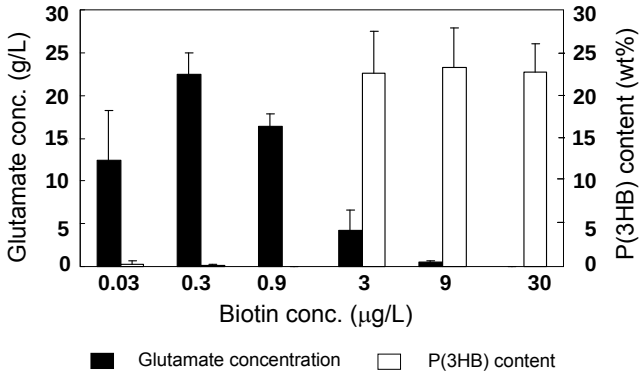

Glutamate conc. (g/L)
P(3HB) content (wt%)
0
5
10
15
20
25
30
0.03
0.3
0.9
3
9
30
Biotin conc. (μg/L)
Glutamate concentration
P(3HB) content

Fig. 3

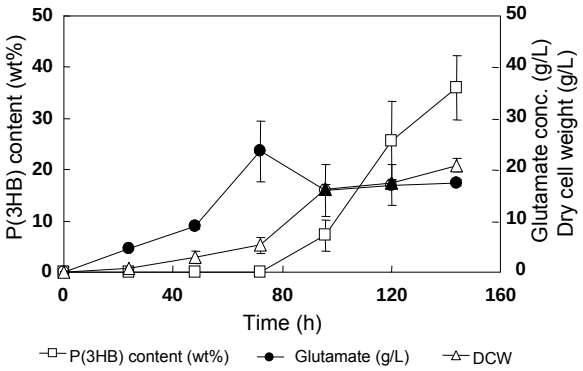
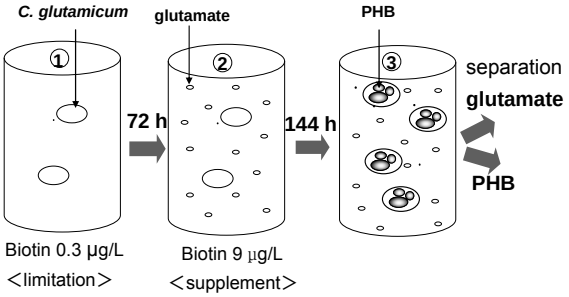

P(3HB) content (wt%)
Glutamate conc. (g/L)
Dry cell weight (g/L)
Time (h)
0
10
20
30
40
50
0
40
80
120
160
P(3HB) content (wt%)
Glutamate (g/L)
DCW

Fig. 4


C. glutamicum
glutamate
PHB
①
②
③
72 h
144 h
separation
glutamate
PHB
Biotin 0.3 μg/L
＜limitation＞
Biotin 9 μg/L
＜supplement＞