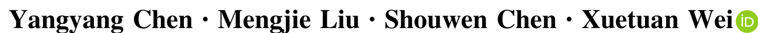
Biotechnol Lett (2017) 39:529–533
DOI 10.1007/s10529-016-2270-5



Decreased formation of branched-chain short fatty acids in *Bacillus amyloliquefaciens* by metabolic engineering

Yangyang Chen · Mengjie Liu · Shouwen Chen · Xuetuan Wei

Received: 1 November 2016 / Accepted: 8 December 2016 / Published online: 20 December 2016
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Abstract
Objectives To reduce the unpleasant odor during 1-deoxynojirimycin (DNJ) production, the genes of leucine dehydrogenase (*bcd*) and phosphate butyryltransferase (*ptb*) were deleted from *Bacillus amyloliquefaciens* HZ-12, and the concentrations of branched-chain short fatty acids (BCFAs) and DNJ were compared.
Results By knockout of the *ptb* gene, 1.01 g BCFAs kg^{-1} was produced from fermented soybean by HZ-12Δ*ptb*. This was a 56% decrease compared with that of HZ-12 (2.27 g BCFAs kg^{-1}). Moreover, no significant difference was found in the DNJ concentration (0.7 g kg^{-1}). After further deletion of the *bcd* gene from HZ-12Δ*ptb*, no BCFAs was detected in fermented soybeans with HZ-12Δ*ptb*Δ*bcd*, while the DNJ yield decreased by 26% compared with HZ-12.
Conclusions HZ-12Δ*ptb* had decreased BCFAs formation but also maintained the stable DNJ yield, which contributed to producing DNJ-rich products with decreased unpleasant smell.

Keywords *Bacillus amyloliquefaciens* · Branched-chain short fatty acids · Leucine dehydrogenase · Metabolic engineering · Phosphotransbutyrylase

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2270-5) contains supplementary material, which is available to authorized users.

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Introduction

1-Deoxynojirimycin (DNJ), a structural analogue of glucose, possesses α-glucosidase inhibitory activity, which creates its anti-diabetic effect. Development of DNJ-rich products show potential in the prevention and treatment of diabetes (Liu et al. 2016). DNJ can be synthesized by *Bacillus* spp. and *Streptomyces species* (Kang et al. 2011; Onose et al. 2013; Seo et al. 2013). A DNJ productive strain, *Bacillus amyloliquefaciens* HZ-12 has been used to prepare DNJ-rich product by solid fermentation of soybeans (Cai et al. 2016). However, a heavily unpleasant odor is formed during solid fermentation which has a serious influence on food sensory quality and environment. Thus the problem of unpleasant odor has to be solved.

Branched-chain short fatty acids (BCFAs), mainly including isobutyric acid and isovaleric acid, are the main odor ingredients in fermented soybeans (Takemura et al. 2000). Biochemically, BCFAs are synthesized by the branched-chain amino acid degradation

pathway (Fig. 1), mainly catalyzed by branched-chain amino acid aminotransferases, leucine dehydrogenase, branched-chain α-keto acid dehydrogenase complex, phosphotransbutyrylase, acyl kinase, branched-chain α-keto acid decarboxylase and aldehyde dehydrogenase (Sirobhushanam et al. 2016; Smit et al. 2009). In *Bacillus subtilis*, deletion of leucine dehydrogenase gene (*bcd*) could eliminate 99% of BCFAs concentration (Takemura et al. 2000). In *Clostridium acetobutylicum* and *Clostridium tyrobutyricum*, phosphate butryltransferase encoded by *ptb* gene was confirmed to be a rate-limiting enzyme, and knockout of *ptb* then inhibited butyric acid synthesis (Yu et al. 2011). Herein, the genes of *ptb* and *bcd* were deleted from *B. amyloliquefaciens* HZ-12 by a markerless knockout method, and the concentrations of BCFAs and DNJ were determined to evaluate the gene-deficient candidates comprehensively.

Materials and methods

Strains, plasmids, primers and growth media

Strains and plasmids involved in this study are listed in Supplementary Table 1. The primers were designed according to the *B. amyloliquefaciens* genome sequence (Supplementary Table 2). LB medium (10 g peptone l^{-1}, 5 g yeast extract l^{-1} and 10 g NaCl l^{-1}) was used for culture of *Escherichia coli* DH5α and *B. amyloliquefaciens* HZ-12 (CCTCC M2015234).

Chemicals

T4 DNA ligase, restriction enzymes and DNA marker were from Takara Bio (Dalian, China). TransStart Fast *Pfu* DNA polymerase was purchased from TransGen Biotech (Beijing, China). All other chemicals were supplied by Sinopharm Chemical Reagent (Shanghai, China).

Construction of knockout plasmids

Gene knockout vectors were constructed according to Qi et al. (2014) and Qiu et al. (2014). Firstly, two homologous arms (about 500 bp) around *ptb* gene were amplified from the total DNA of *B. amyloliquefaciens* HZ-12 with primers of *ptb*F1/*ptb*R1 and *ptb*F2/*ptb*R2. Secondly, two homologous arms were fused through the Splicing-with-Overlapping-Extension PCR method (SOE-PCR) using the primers of *ptb*F1 and *ptb*R2. The fused fragment finally was cloned into T2(2)-ori vector at the restriction sites of *Xba*I and *Bam*HI, further verified through gene sequencing performed by Beijing Genomics institution (China), and named as T2(2)-oriΔ*ptb*. The knockout plasmid of T2(2)-oriΔ*bcd* was obtained by the same method.

Construction of gene-deficient strains

Gene knockout was performed by homologous recombination (Qi et al. 2014). Taking *ptb* gene knockout as an example, *B. amyloliquefaciens* HZ-12 competent cells were electrotransformed with T2(2)-oriΔ*ptb*, then the transformants were picked by kanamycin-resistance screening (20 μg ml^{-1}), verified by PCR with primers of T_2F and T_2R. Positive transformants were transferred onto LB medium containing 20 μg kanamycin ml^{-1}, and incubated at 45 °C and 180 rpm for 8 h to obtain single-crossover strains with kanamycin resistance, further verified by PCR with Δ*ptb* single crossover primers of T_2F and ptbYR. Single-crossover colonies were inoculated into LB liquid medium, cultured at 37 °C for 8 h several times, then sprayed onto LB plates to screen the kanamycin-sensitive cells, which were verified by PCR with primers of *ptb*YF and *ptb*YR to obtain the *ptb* knockout strain of HZ-12Δ*ptb*. The double gene deficient strain of HZ-12Δ*ptb*Δ*bcd* was constructed by above method based on HZ-12Δ*ptb*.

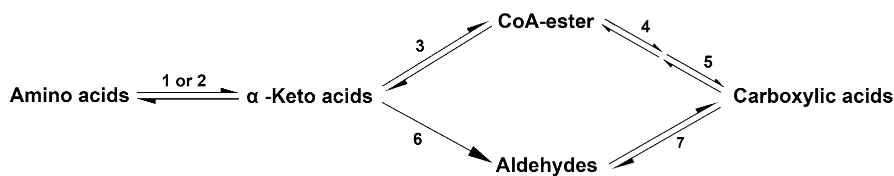

Fig. 1 Biosynthesis pathway of BCFAs in bacteria (Smit et al. 2009). Branched-chain amino acids aminotransferase (*1*), leucine dehydrogenase (*2*), branched-chain α-keto acid dehydrogenase complex (*3*), phosphotransbutyrylase (*4*), acyl kinase (*5*), branched-chain α-keto acid decarboxylase (*6*) and aldehyde dehydrogenase (*7*)

Table 1 BCFAs concentrations of different strains

Strain	Isobutyric acid (g kg^{-1})	Isovaleric acid (g kg^{-1})	Total BCFAs (g kg^{-1})
HZ-12	0.82 ± 0.06	1.45 ± 0.12	2.27 ± 0.17
HZ-12△*ptb*	ND	1.01 ± 0.07	1.01 ± 0.07
HZ-12△*ptb*△*bcd*	ND	ND	ND

Data are expressed as mean ± standard errors of three replicates; *ND* stands for "not detected"

Solid fermentation

Cells of HZ-12, HZ-12Δ*ptb*, and HZ-12Δ*ptb*Δ*bcd* were inoculated into 50 ml LB medium, cultured at 37 °C and 180 rpm until the OD_{600} of broth reached 4.2. Cells were transferred into 50 g sterile soybeans with 60% (v/w) water, cultured at 37 °C for 48 h. Samples were collected, added with distilled water, and rotated at 180 rpm to obtain the extracts, which were used to measure cell density, DNJ and BCFAs concentrations.

Analytical methods

Cells were counted by the plate colony-counting method. The DNJ concentration was determined by HPLC (Yatsunami et al. 2008). BCFAs were determined by GC (Zhou et al. 2016).

Results and discussion

Identification of HZ-12Δptb and HZ-12ΔptbΔbcd

Using the primers of *ptb*YF and *ptb*YR, the amplified DNA product from assumed *ptb*-deficient strain was about 1551 bp, while the DNA fragment amplified from HZ-12 was about 2304 bp, which harbored the *ptb* gene (Fig. 2a). Moreover, the DNA product amplified from *ptb* knockout strain was sequenced and analyzed and no other mutation occurred except the deleted *ptb* gene fragment (data not shown), confirming that the *ptb* gene was deleted, named as HZ-12Δ*ptb*. Deletion of leucine dehydrogenase gene from *B. subtilis* eliminated 99% of BCFAs concentration during natto fermentation (Takemura et al. 2000). Therefore, the leucine dehydrogenase gene (*bcd*) was further deleted from HZ-12Δ*ptb*. Similarly, the amplified DNA product (about 1730 bp) from assumed Δ*ptb*Δ*bcd* strain was shorter than that of Δ*ptb* strain (about 2569 bp), and the difference in DNA length was identical to that of *bcd* gene (Fig. 2b), indicating that the Δ*ptb*Δ*bcd* strain was constructed.

Determination of BCFAs concentrations

Table 1 shows the BCFAs concentrations produced from different strains. 0.82 g isobutyric acid kg^{-1} and 1.45 g isovaleric acid kg^{-1} were detected in fermented soybeans with strain HZ-12, which resulted in an unpleasant odor. Similar results have been reported for natto fermented by *B. subtilis* (Takemura et al. 2000). After knockout of *ptb*, the total content of BCFAs produced by HZ-12Δ*ptb* (1.01 g BCFAs kg^{-1}) was 56% lower than that of HZ-12 (2.27 g BCFAs kg^{-1}). Moreover, no isobutyric acid was detected and the unpleasant smell was decreased significantly. After

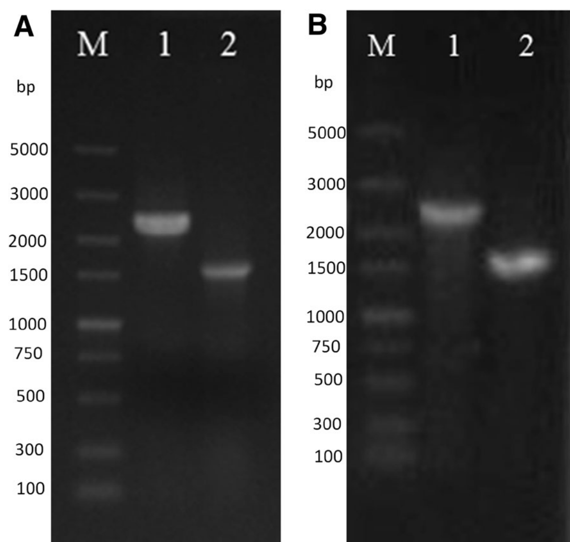

Fig. 2 Confirmation of defective mutants by PCR amplification. **a** DL5000 marker (M) and PCR products amplified from strain HZ-12 (Lane 1) and HZ-12△*ptb* (Lane 2) using primers of *ptb*YF and *ptb*YR; **b** DL5000 marker (M) and PCR products amplified from strain HZ-12△*ptb* (Lane 1) and HZ-12△*ptb*△*bcd* (Lane 2) using primers of *bcd*YF and *bcd*YR

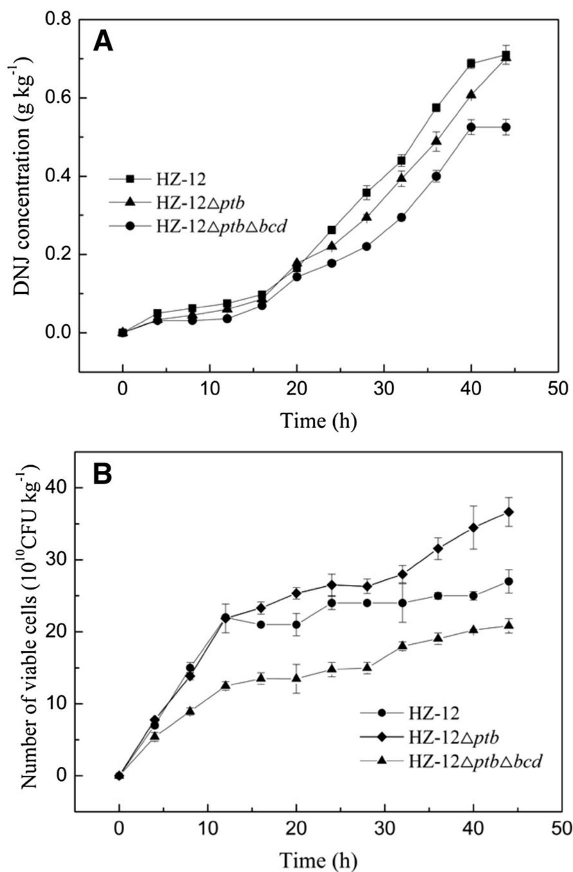

Fig. 3 Effects of gene deletion on DNJ production process. Time profiles of DNJ biosynthesis (**a**) and cell growth (**b**) from different strains during the fermentation process. Data were expressed as mean ± standard errors of three replicates

further deletion of *bcd*, no isobutyric acid or isovaleric acid was detected from HZ-12Δ*ptb*Δ*bcd* (Table 1), and similar results were reported in fermented soybeans with *B. subtilis* (Takemura et al. 2000). It is the first report that single knockout of *ptb* or double deletion of *ptb* and *bcd* can decrease BCFAs production using *B. amyloliquefaciens*. The results confirm that phosphotransbutyrylase and leucine dehydrogenase were key enzymes involved in BCFAs biosynthesis in *B. amyloliquefaciens*.

DNJ production and cell growth during solid fermentation

DNJ fermentation processes of HZ-12, HZ-12Δ*ptb*, and HZ-12Δ*ptb*Δ*bcd* were compared. Deletion of *ptb* gene showed no significant influence on DNJ yield (Fig. 3a), and the maximum DNJ yield of HZ-12Δ*ptb* reached 0.7 g kg^{-1}, which was as high as previous reports (Cho et al. 2008; Paek et al. 1997; Seo et al. 2013). On the other hand, knockout of *ptb* was beneficial to the cell growth compared with HZ-12 (Fig. 3b). However, double deletion of *ptb* and *bcd* significantly reduced the DNJ yield, and the maximum DNJ yield of HZ-12Δ*ptb*Δ*bcd* decreased by 26% compared with that of HZ-12, which might be due to the decreased viable cells (Fig. 3b). To balance the DNJ yield and BCFAs content, HZ-12Δ*ptb* is considered to be more suitable for preparation of DNJ-rich products with low odor.

Acknowledgments This work was funded by the National Natural Science Foundation of China (31501468) and the Fundamental Research Funds for the Central Universities (2662016PY121).

Supporting information Supplementary Table 1—Strains and plasmids used.
Supplementary Table 2—Primers used for PCR.

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