Development of a hybrid bio-conversion system consisting of *in vivo* fermentation and *in vitro* enzymatic conversion

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Research objective

The biocatalytic manufacturing of chemical compounds can be divided into two groups. The first is fermentation, in which the metabolic activity of living microorganisms is used, and the second is enzymatic conversion, which employs only specific enzyme(s) isolated from living cells as catalytic elements. Fermentation is preferred for the conversion of cheap agricultural materials (e.g., starch and glucose) into primary metabolites, such as alcohols, organic acids, and amino acids, and is frequently implemented on a large scale. However, a certain fraction of the substrate is consumed for cell proliferation and maintenance; therefore, the product yield is not always high. Meanwhile, enzymatic conversion is applicable even to chemically synthesized substrates, and is also able to achieve a high product yield owing to the absence of unwanted side reactions. However, it often requires costly and time-consuming procedures for the isolation and purification of enzyme(s) from living cells.

In the present study, we aimed to develop a new biocatalytic chemical manufacturing system that has the advantages of both fermentation and enzymatic conversion. In the new system, we can switch the process from the fermentation step (at 30–40°C) to the enzymatic conversion step (at 50–70°C) by simply shifting the reaction temperature. Enzymes involved in the later step are obtained from thermophilic microorganisms, and their genes are expressed in mesophilic microorganisms, such as *Escherichia coli* and *Corynebacterium glutamicum*, which are capable of fermentative production of a primary metabolite. By shifting the reaction temperature, host-derived mesophilic enzymes are thermally inactivated, and only recombinantly-produced thermophilic enzymes remain in active forms. Consequently, the fermentatively produced metabolite can be converted into a target product at a high yield (Figure 1). In the present study, we demonstrated the one-pot production of L-pipecolic acid (L-PA) via fermentative production of L-lysine from glucose to prove the concept of this hybrid system (Figure 2).

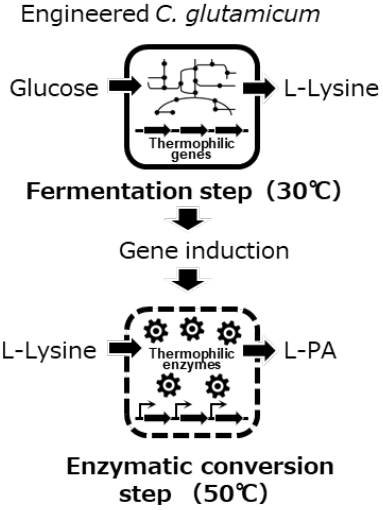

Figure 1 Outline of the hybrid bio-conversion system developed in this study.

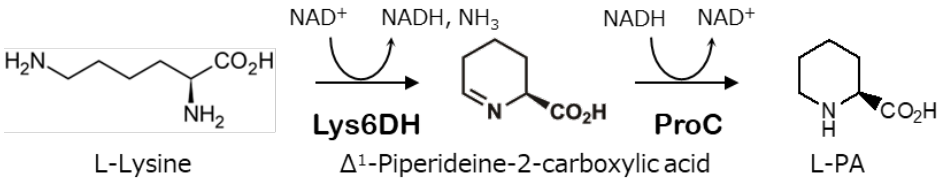

Figure 2 Enzymatic conversion of L-lysine to L-PA

Methods

Homology searches were performed against the genome sequences of thermophiles stored in our laboratory to explore thermophilic genes encoding enzymes involved in the conversion of L-lysine to L-PA. Genes encoding lysine 6-dehydrogenase (Lys6DH) of *Geobacillus stearothermophilus* (1) and pyrroline-5-carboxylate reductase (ProC) of *Sulfolobus solfataricus* (2) were used as query sequences. Selected genes were expressed in *E. coli*, and the resulting recombinant proteins were semi-purified by heat treatment of the cell extract. The heat-purified enzymes were assayed, and the one that exhibited the highest specific activities was used for further study. Genes encoding these enzymes were codon-optimized for expression in *C. glutamicum* and integrated into a gene expression plasmid vector, pECXT99A. The vector was then introduced into a metabolically engineered L-lysine-producing strain, *C. glutamicum* GRLys1 (3). The resulting transformant was aerobically cultivated at 30℃ in 50 mL of CGXII medium supplemented with 4% glucose. After confirmation of glucose depletion in the culture medium, the cultivation temperature was increased to 50℃ and NADH was added to the medium at a final concentration of 1 mM. L-Lysine and L-PA in the culture medium were determined by HPLC after derivatization with phenyl isothiocyanate.

Results

A homology search revealed that numerous thermophiles possess genes encoding putative Lys6DH and ProC in their genomes. Among them, five genes were selected for Lys6DH and ProC, and recombinant enzymes were prepared and assayed. Consequently, Lys6DH derived from *Geobacillus* sp. 30 and ProC from *Desulfurobacterium thermolithotrophum* were selected owing to their high specific activities among the tested enzymes. Genes encoding these enzymes were tandemly aligned, introduced into an expression vector, and then expressed in *C. glutamicum* GRLys1. When the resulting transformant was cultivated at 30℃ in the CGXII medium, 39 mM L-lysine was accumulated in the medium after cultivation for 48 h. Meanwhile, no detectable amount of L-PA was found in the medium. A fraction of the cells was harvested from the culture medium and subjected to enzyme assay at 50℃. The assay showed that both Lys6DH and ProC activities were detectable in the crude extract of *C. glutamicum* GRLys1. This indicates that genes encoding thermophilic Lys6DH and ProC were

functionally expressed in *C. glutamicum* but could not exert their catalytic activity at a reaction temperature of 30℃. The cultivation temperature was then increased to 50℃, and NADH was added to the mixture at a final concentration of 1 mM. The mixture was further incubated for 48 h, resulting in the accumulation of 13 mM L-PA.

Conclusion

In the present study, fermentative production of L-lysine by metabolically engineered *C. glutamicum* followed by the enzymatic conversion of the resulting L-lysine to L-PA was achieved in a one-pot reaction. However, the conversion yield of L-PA from L-lysine in the enzymatic conversion step remained moderate (33 mol%). Further studies will be performed to address this issue, for example, by enhancing thermophilic enzyme gene expression levels using engineered promoters and ribosome binding sites, and by increasing the cell concentration (and thereby enzyme concentration) by employing a fed-batch operation in the fermentation step.

References

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