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Efficient aspartic acid production by a psychrophile-based simple biocatalyst

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Abstract We previously constructed a Psychrophile-based Simple bioCatalyst (PSCat) reaction system, in which psychrophilic metabolic enzymes are inactivated by heat treatment, and used it here to study the conversion of aspartic acid from fumaric acid mediated by the activity of aspartate ammonia-lyase (aspartase). In *Escherichia coli,* the biosynthesis of aspartic acid competes with that of L-malic acid produced from fumaric acid by fumarase. In this study, *E. coli* aspartase was expressed in psychrophilic *Shewanella livingstonensis* Ac10 heat treated at 50 °C for 15 min. The resultant PSCat could convert fumaric acid to aspartic acid without the formation of L-malic acid because of heat inactivation of psychrophilic fumarase activity. Furthermore, alginate-immobilized PSCat produced high yields of aspartic acid and could be re-used nine times. The results of our study suggest that PSCat can be applied in biotechnological production as a new approach to increase the yield of target compounds.

Keywords Alginate · Biomass · Aspartic acid · Psychrophilic bacteria · Immobilization

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Introduction

Whole cell biocatalysis is an attractive approach widely used in biotechnology for the production of various compounds because it enables multi-step reactions including the biosynthesis of stereospecific isomers at moderate temperatures and pressure in a one-pot process [13]. However, host metabolic enzymes occasionally interfere with the intended enzymatic biosynthesis such that the target molecules are further metabolized in side reactions, resulting in the decrease of the main product yield. Although metabolic pathways can be engineered by disrupting the expression of unnecessary metabolic enzymes, these approaches are often too complicated and time consuming for practical application. In our previous study, we constructed an innovative biocatalyst, Psychrophile-based Simple bioCatalyst (PSCat) [16]. The approach is based on the expression of mesophilic enzymes in psychrophilic bacteria, which are then heat treated at moderate temperatures; as a result, intrinsic bacterial enzymes are inactivated, while recombinant mesophilic enzymes retain their catalytic activity. Thus, PSCat is expected to efficiently convert substrates into target compounds without the interference of by-products synthesized by natural bacterial enzymes.

PSCat has been successfully applied to produce high yields of 3-hydroxypropionaldehyde from glycerol by heat treatment at 45 °C [16]. PSCat has significant advantages over conventional methods in that it is easy to prepare and implement since the method only requires heat treatment of bacterial cells. However, we did not directly evaluate by-products of bacterial metabolism because the psychrophilic bacteria used as a host cannot assimilate glycerol, and their respiratory activity was lost after the treatment at 45 °C for 15 min.

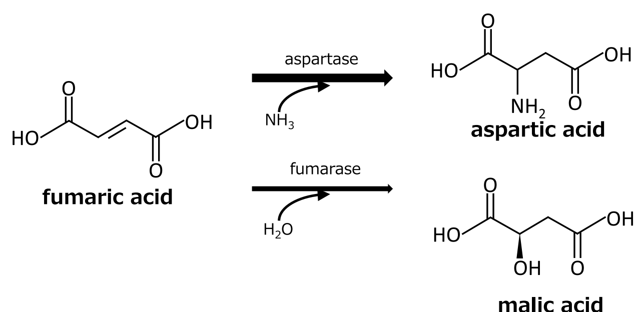

Fig. 1 Fumaric acid metabolism. Fumaric acid can be converted to aspartic acid (target product) by the activity of aspartase and to L-malic acid (by-product) by the activity of fumarase

Aspartic acid is an important four-carbon (C4) amino acid used for the production of a synthetic sweetener aspartame and amino analogs of C4 building-block chemicals such as 1,4-butanediol, tetrahydrofuran, and gamma-butyrolactone. Aspartic acid has been selected as a top value-added chemical from biomass by the US Department of Energy [19]. The biosynthetic production of aspartic acid involves the use of purified or bacterially overexpressed aspartase (L-aspartate ammonia-lyase: EC 4.3.1.1), which catalyzes the formation of aspartic acid from fumaric acid and ammonia (Fig. 1). Aspartic acid is produced by partially purified enzymes or whole bacteria including immobilized cells such as *E. coli* bound to polyurethane carriers. However, in intact cells, a fraction of the fumaric acid is lost because of the activity of constitutively expressed bacterial fumarase that converts fumaric acid to L-malate [3, 6]. To circumvent this problem, mutant bacterial strains optimized for efficient aspartic acid biosynthesis and low fermentation levels of by-products have been constructed [15]. Another approach is to use purified recombinant enzymes. Thus, free and immobilized thermostable aspartase of *Bacillus* sp. YM55-1 expressed in *E. coli* could yield over 430 mM aspartic acid after a 24-h fermentation [2]. However, enzyme purification involves steps such as mechanical cell disruption, removal of cell debris by prolonged centrifugation, and fractionation by ammonium sulfate, which are major bottlenecks for the industrialization of the process [14].

The aim of this study was to generate an efficient biocatalyst by a simple procedure. To achieve this, we constructed PSCat expressing *E. coli* aspartase in *Shewanella livingstonensis* Ac10 and analyzed the production of aspartic acid as well as the formation of by-products. Heat treatment conditions were optimized to inactivate native bacterial fumarase and prevent malate synthesis from fumaric acid. For sustainable use of the catalyst, PSCat was immobilized on alginate and tested in repeated cycles of aspartic acid production.

Materials and methods

Plasmid construction

Standard procedures were used for plasmid DNA amplification, restriction enzyme digestion, ligation, and transformation [11]. The *E. coli aspA* gene was amplified by PCR from genomic DNA of the *E. coli* DH5α strain using primers aspA_F (5′-GGAGAGATGAACAATGTCAAACAACATTCGTATCGAAG-3′) and aspA_R (5′-ATGCAAGCTTTTACTGTTCGCTTTCATCAGTATAGC-3′) and KOD plus Neo polymerase (TOYOBO, Ohtsu, Japan) according to the manufacturer's recommendations. PCR mixtures (50 µL) contained 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgSO_4$, 1 × KOD plus Neo PCR buffer (Takara, Otsu, Japan), 1 U KOD plus Neo (TOYOBO), and 110 ng *E. coli* DH5α genomic DNA. Thermocycling conditions were as follows: a denaturation step at 94 °C for 2 min, 30 cycles at 98 °C for 10 s, and a final step at 68 °C for 60 s. The PCR fragment was digested with *Sma*I and *Hind*III (TOYOBO) and inserted into the pHA12 plasmid to construct pHA12-aspA.

Bacterial strains and growth conditions

All *E. coli* strains were grown in Luria–Bertani (LB) broth or LB agar at 37 °C in the presence of 100 mg/L ampicillin when required for selection. *S. livingstonensis* Ac10-Rif^r was grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) with 50 mg/L rifampicin at 18 °C.

To overexpress *E. coli* aspartase in *S. livingstonensis* Ac10-Rif^r, the *E. coli aspA* gene was cloned into the pHA12 vector [1] and amplified in *E. coli* DH5*α* (TOYOBO). The plasmid was introduced into *S. livingstonensis* Ac10-Rif^r by transconjugation using *E. coli* S17-1 [7], and transformed *S. livingstonensis* cells (Ac10-Rif^r/pHA12-aspA) were selected using rifampicin (50 mg/L) and ampicillin (100 mg/L). The expression of aspartase was induced with 1 mM IPTG. To prepare Ac10/pHA12-aspA cells for a resting cell reaction, bacteria grown for 2 days in TSB were inoculated (1 %) into 100 mL of TSB in Erlenmeyer flasks and grown on a rotary shaker under constant agitation of 120 rpm. Cell growth was recorded by measuring culture optical density at 660 nm (OD_{660}), as well as cell dry weight.

Sonication

Bacteria were collected by centrifugation at 3220×*g* for 5 min at 4 °C and washed twice with ice-cold sonication buffer containing 100 mM Tris–HCl (pH 7.5), 20 mM KCl, 5 mM $MnSO_4$, 20 mM $MgCl_2$, 1 mM EDTA, and 2 mM

dithiothreitol. The pellets were suspended in 5 mL of the same buffer ($OD_{660} = 40$), and disrupted by 600 cycles of 1-s pulse/1-s intervals using an ultrasonic disintegrator (Digital Sonifier 450; Branson, Danbury, CT, USA).

Aspartic acid production

For whole cell-based production, recombinant *S. livingstonensis* was collected as described above and washed twice with 70 mM Tris–HCl buffer (pH 9). Cell suspensions or sonicated cells were heat treated at 45, 50, or 55 °C, and 5 mM $MgCl_2$ was added; the samples were then kept on ice until the reaction. The enzymatic reaction started by the addition of equal volume of 860 mM fumarate-NH_3 solution (pH 9) to cell suspension following incubation at 37 °C for 3 h. After enzymes were inactivated by heat treatment at 80 °C for 15 min, cell supernatant was obtained by centrifugation. Heat treatment and enzymatic reactions were performed in the water bath (SM-05 N, Taitec, Koshigaya, Japan).

Bacteria immobilization

Bacteria (0.56 g dry weight) were mixed with 50 mL of 20 g/L sodium alginate and added dropwise into 0.5 % $CaCl_2$ using a syringe with a 22-gauge needle to form a bead-type gel. The beads were stirred about 30 min and washed with 0.2 % $CaCl_2$. For aspartic acid production, 2 mL of 860 mM fumarate-NH_3 solution and 20 μL of 1 M $MgCl_2$ were added to 3 g of beads in 2 mL of 70 mM Tris–HCl buffer (pH 9). The beads with immobilized bacteria were kept in Tris–HCl buffer (pH 9) containing 0.2 % $CaCl_2$ at 4 °C until the next reaction.

HPLC analysis

Fumaric and malic acids were quantified using an RI detector and ion exclusion column (RSpak KC-811, 8.0 mm ID × 300 mm L; Shodex, Tokyo, Japan). The products were eluted at 60 °C using 0.1 % (v/v) phosphoric acid as a mobile phase at a flow rate of 0.7 mL/min. Aspartic acid was measured by the derivatization with 2,4-dinitrofluorobenzene (DNFB) as previously described with some modifications [2]. Briefly, 10 μL of 1 M $NaHCO_3$ was added to 25 μL of the sample and reacted with 40 μL of 37.6 mM DNFB in acetone at 40 °C for 90 min. After the reaction was stopped with 10 μL of 1 N HCl, the samples were diluted with 800 μL of 20 % acetonitrile, and the spectra were obtained at 360 nm using an HPLC system equipped with a UV detector and a reverse-phase column ODS-80TM (Tosoh, Tokyo, Japan). Aspartic acid was eluted at 40 °C using 0.095 % trifluoroacetic acid in milliQ water as mobile phase A and 80 % acetonitrile containing 0.1 % trifluoroacetic acid as mobile phase B at a flow rate of 1 mL/min; 31.3–48.8 % phase B gradient was applied. Aspartate at the concentration from 1.25 to 10 mM was used as a standard for quantitative analysis.

Results

Aspartic acid production by sonicated cell suspension and PSCat

To examine the effect of heat treatment at different temperatures on aspartic acid production by recombinant aspartase and the activity of native metabolic enzymes, *E. coli aspA* was expressed under the control of the *tac* promoter in psychrophilic *S. livingstonensis* Ac10- Rif^r (Ac10-Rif^r/pHA12-aspA). In our previous study, most of the respiratory activity in Ac10- Rif^r was lost by the treatment at 45 °C [16]; however, we could not obtain direct evidence of metabolic enzyme inactivation since Ac10- Rif^r cannot assimilate glycerol and did not generate any by-products via the activity of native enzymes. Therefore, we disrupted bacteria by sonication to determine the conditions of PSCat generation by evaluating the activity of recombinant aspartase and native metabolic enzymes after heat treatment at moderate temperatures. First, sonicated cells were either heated or not at 45, 50, and 55 °C for 15 min and reacted with 430 mM (50 g/L) fumarate-NH_3 as a substrate at 37 °C; then, aspartic, L-malic, and fumaric acids in the sample were measured by HPLC. L-malic acid was confirmed as the major by-product (Fig. 2). Heat untreated Ac10-Rifr

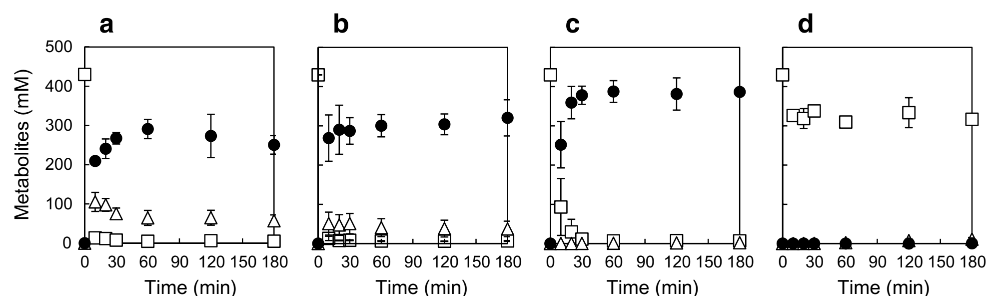

Fig. 2 Aspartic acid reactions using sonicated cell suspensions without heat treatment (**a**), and with heat treatment at 45 °C (**b**), 50 °C (**c**), and 55 °C (**d**). *Closed circles*, aspartic acid; *open triangles*, L-malic acid; *open squares*, fumaric acid. *Error bars* represent standard deviation of the mean ($n = 3$)

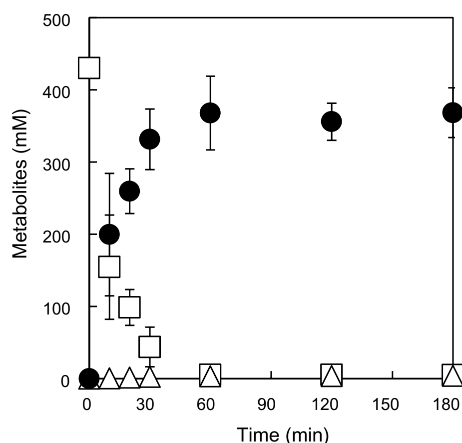

Fig. 3 Aspartic acid production by PSCat after heat treatment at 50 °C for 15 min. *Closed circles*, aspartic acid; *open triangles*, L-malic acid; *open squares*, fumaric acid. *Error bars* represent standard deviation of the mean ($n = 3$)

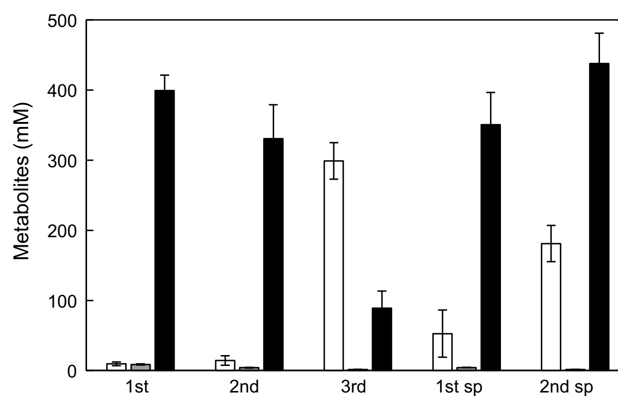

Fig. 4 Aspartic acid production by PSCat and bacterial supernatant in repeated reactions after 2 h. PSCat, three cycles (1st, 2nd, and 3rd); supernatant, two cycles (1st sp and 2nd sp). *Black*, aspartic acid; *gray*, L-malic acid; *white*, fumaric acid. *Error bars* represent standard deviation of the mean ($n = 3$)

bacteria produced only 200 mM aspartic acid and 100 mM L-malic acid from fumaric acid, indicating that 25 % of fumaric acid was converted to L-malic acid as a by-product. Heat treatment at 45 °C still resulted in the production of 50 mM L-malic acid together with more than 300 mM of aspartic acid, whereas the exposure to 50 °C almost eliminated the synthesis of L-malic acid and increased that of aspartic acid to 380 mM. Further temperature increase to 55 °C blocked the production of both aspartic and L-malic acids. These results indicate that fumarase was inactivated at 50 °C and aspartase at 55 °C. When the time of heat treatment was extended to 30 min, aspartic acid production was decreased to 250 mM because of partial aspartase inactivation (data not shown). Thus, the conditions to obtain PSCat by heat treatment for aspartic acid production were determined as 50 °C for 15 min.

Next, aspartic acid production was investigated in whole cell PSCat prepared by heating aspartase-expressing *S. livingstonensis* Ac10- Rif^r at 50 °C for 15 min (Fig. 3). PSCat could produce aspartic acid from fumaric acid in 60 min, although the rates of fumaric acid consumption and aspartic acid production were slightly slower than those in sonicated cells. In addition, similar to heat treated sonicated cells, PSCat did not synthesize L-malic acid.

Repeated use of PSCat for aspartic acid production

To evaluate the sustainability of PSCat application, recombinant bacteria treated at 50 °C were re-used for aspartic acid production. The results indicate that aspartic acid synthesis was slightly decreased in the second cycle and sharply dropped in the third cycle when fumaric acid was mostly detected in the reaction mixture (Fig. 4). When centrifuged cell lysate was re-used, the reaction mixture after the first and second cycle demonstrated the accumulation of more than 350 mM aspartic acid (Fig. 4). If initial concentrations of aspartic acid (193 and 249 mM for the first and second cycle, respectively) were subtracted from the final yield, the production amounted to 159 and 189 mM of aspartic acid, respectively. These results suggest that the recombinant intracellular aspartase in PSCat leaked to culture medium through the cell membrane damaged by heat treatment at 50 °C.

To prevent aspartase leakage from bacteria after heat treatment, heated cells were entrapped in sodium alginate as an immobilization carrier (cell density was adjusted to that in free cell reactions) and 2-h batch reactions were repeated 10 times (Fig.5). Batch reactions 1–2, 3–5, 6–9, and 10 were performed on separate days, and bead-immobilized cells were kept in Tris–HCl buffer with 0.2 % $CaCl_2$ at 4 °C until the next day. Aspartic acid was stoichiometrically produced from fumarate with high conversion yields (95.2–99.3 %) in batches 2–9, although aspartic acid production in batch 1 was slightly lower because of absorption by the beads. In batches 1–5, most aspartic acid was synthesized within 1 h; in subsequent batches, the aspartic acid production rate became slower (2 h) and the yield gradually decreased to 82.6 % in batch 10.

Discussion

In this study, we show that PSCat could convert fumaric acid to aspartic acid without by-product formation. To repress host basic metabolism, psychrophilic bacteria expressing a mesophilic enzyme (aspartase) were heat treated at 50 °C for 15 min, which effectively blocked the

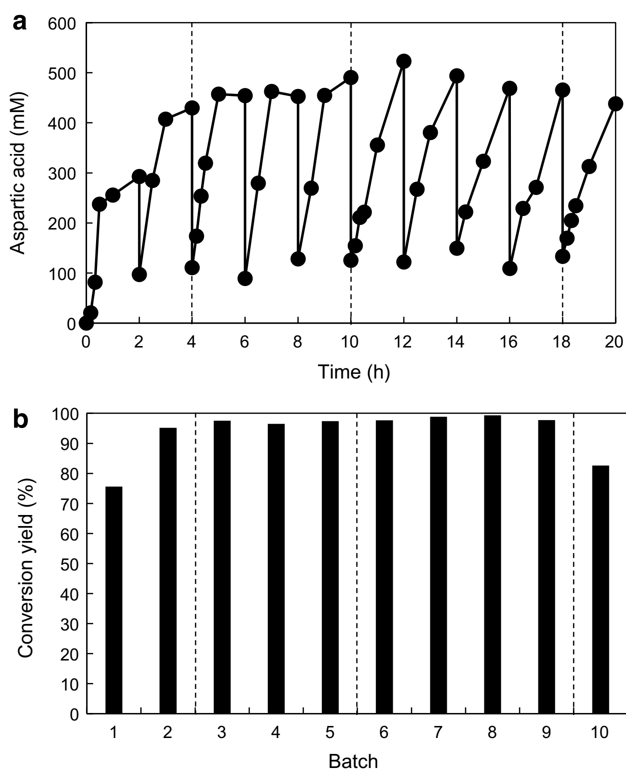

Fig. 5 Repeated cycles of aspartic acid production using PSCat immobilized on alginate. **a** Concentration of aspartic acid in the reaction mixture. **b** Conversion yield (%) of aspartic acid [aspartic acid production (mM)/fumaric acid consumption (mM) × 100]. The reactions performed on the same day are separated by *dashed lines*

activity of psychrophilic fumarase to produce L-malic acid, a major by-product of aspartate biosynthesis. In previous studies, bacterial strains have been applied for the industrial production of aspartic acid [2, 4, 12, 15, 17]; however, the preparation of biocatalysts for efficient bioconversion was laborious and time consuming because fermentation had to be regulated to suppress fumarase activity [15]. In addition, recombinant enzyme extraction and purification were required to eliminate the influence of metabolic enzymes [2, 4, 12, 17]. Compared with other methods to prevent by-product formation, heat treatment of recombinant bacteria is a simple and easy way to obtain an efficient biocatalyst, such as PSCat.

The PSCat-based method has significant advantages over other approaches in that it uses moderate temperatures to treat psychrophilic bacteria expressing mesophilic enzymes. Although the variety and number of mesophilic bacteria significantly exceed those of psychrophilic microorganisms [16], mesophiles cannot be used in heat inactivation-based systems because higher temperatures are required to repress their cell metabolism. Thus, Ueda et al. [18] have reported that 50 % of fumarase activity in *E. coli* still remains after treatment at 49 °C or 51 °C. In this study, we used *S. livingstonensis* Ac10- Rif^r isolated from Antarctic seas as a biocatalyst host, because its optimal growth temperature is 18 °C, while at 30 °C it does not grow [10]. Our results indicate that *S. livingstonensis* Ac10- Rif^r heated at 50 °C completely lost its fumarase activity. The activity of psychrophilic enzymes is decreased at temperatures over 40 °C as evidenced by stability curves indicating that the native state and conformational structure of natural metabolic enzymes in psychrophiles can be destroyed at moderate temperature regimes [5]. However, although psychrophilic enzymes are rarely stable at moderate temperatures, some activity may remain; for example, aspartase of *Cytophaga* sp. KUC-1 isolated from Antarctic seawater retained 20 % activity after incubation at 50 °C for 60 min [9]. Therefore, the activity of specific competing metabolic enzymes in psychrophilic host cells should be validated, as has been done in this study for fumarase. Fumarase was completely inactivated after 15 min at 50 °C, although most metabolic enzymes were considered to be inactivated after 10 min at 45 °C based on the loss of respiratory function [16].

Heat treatment conditions also depend on the thermostability of recombinant enzymes overexpressed in psychrophilic cells. The relative activity of native aspartase in *E. coli* was decreased to 40–47 % of that in control cells by heat treatment at 50 °C for 10 min [4, 8]. To prevent partial inactivation of aspartase at 50 °C, higher temperature-resistant enzymes such as *Bacillus* sp. YM55-1 [8] or *Cytophaga* sp. KUC-1 [9] aspartases could be used, although the activity of recombinant *E. coli* aspartase in this study was stable after heat treatment at 50 °C for 15 min and remained so in immobilized PSCat over 9 reuse cycles. However, aspartase activity in sonicated heat treated PSCat was 1.22 mmol aspartic acid/min/mg protein, which was lower than that in induced *E. coli* (117 mmol aspartic acid/min/mg protein) [15]. Low enzyme expression and/or activity in psychrophiles may limit the reaction rate when PSCat is applied in multi-step reactions catalyzed by several enzymes.

PSCat entrapped in the alginate carrier could be repeatedly used for the efficient production of aspartic acid. In biocatalysis, alginate is a popular carrier for immobilization of recombinant enzymes and cells. The production rate of aspartic acid in the first batch reaction by immobilized PSCat was 42.6 mmol/h/g dry weight, while that by free cell PSCat was 61.4 mmol/h/g dry weight. However, free cell-based PSCat could not efficiently produce aspartic acid in repeated reactions because of the leakage of recombinant enzyme through the membrane damaged by cell heat treatment at 50 °C. In immobilized PSCat, the leak of aspartase could be prevented, enabling repeated use of the catalyst (up to 9 cycles) for the efficient production of aspartic acid at high yield. Immobilized bacteria also have a significant

advantage over free cells in the separation of catalysts from the reaction mixture in repeated reactions. The conversion yield in this study (95.2–99.3 %) was higher than that demonstrated by *E. coli* aspartase immobilized on alginate (85 %) [3].

The PSCat reaction system has the potential to be used for efficient biocatalysis because it does not generate by-products synthesized by host metabolic enzymes and is easily prepared by heat treatment at moderate temperatures. Thus, PSCat can be successfully applied for efficient multi-step bioconversion process designed with thermostable mesophilic enzymes, which would retain catalytic activity after heat treatment at moderate temperatures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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