博 士 論 文
（Doctoral Thesis）

Basic and applied research on psychrophile-based simple biocatalysts for production of valuable chemicals

（低温菌シンプル酵素触媒を用いた有用化学品生産に関する基礎及び応用研究）

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2021 年 9 月
（September 2021）

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主論文

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Chapter 1 General introduction

Replacing conventional petroleum-based chemical production with more sustainable bio-based one has been an attractive challenge for effective use of natural resources in chemical industry. Bio-based chemical processes are usually more environmentally sustainable and effective than petrochemical processes since the catalysts used in bio-based chemical processes, enzymes, are capable of catalyzing reactions at moderate temperatures and produce few undesirable by-products. Thus, there is no need to deploy expensive, high pressure tolerant, corrosion resistant equipment and complex downstream processes. Conversions by purified enzymes and whole-cell biocatalyst are the two major procedures to apply enzymes in chemical processes. Advances in recombinant protein synthesis have dramatically reduced the cost of enzyme purification from cells. It was reported that recombinant enzymes take up over 50% of the enzyme market in 2011 (1).

Purified enzymes are applied to a wide range of industry including food (2, 3), textiles (4), detergent (5, 6), and pharmaceutical (7, 8) usually used directly or immobilized by other supportive materials to improve its cyclability and robustness. Since the cost of enzymes purification from cells accounts for a significant portion of overall production costs (9), and not all industries require high-grade purified enzymes, bio-conversion by recombinant enzymes without purification from their host cells appears to be a more economic approach.

Whole-cell biocatalyst, is a less expensive way to incorporate enzymes into chemical processes since the costs of enzymes purification from cells are avoided. It was

estimated that whole-cell biocatalyst costs approximately a tenth of the cost of purified enzyme (10). Whole-cell biocatalyst also provides a more protective reaction environment to the enzymes comparing with purified enzymes which are directly exposed to the reactor. It also allows multi-step reactions in one pot to produce complex products from simple substrate or regenerate some expensive cofactors such as $NAD(P)^+$ and NAD(P)H for saving the production cost.

Other applications of whole-cell biocatalyst include catalyzing the reactions at conditions with limited or even without any solvent to improve the final titer of the products by lyophilized whole cells (11, 12) and incorporating immobilized cells into bioreactors (13).

Whole-cell biocatalyst could be constructed based on all kinds of cells, traditionally, *Escherichia coli* (14-16) and *Saccharomyces cerevisiae* (17, 18) are typically used as the host for enzymes from prokaryotic and eukaryotic creatures respectively. Although whole-cell biocatalyst seems more promising for chemical industry than purified enzyme because it is less expensive and easy to be prepared on a large scale. There are two major challenges remained to be overcome. First, as compared to purified enzyme, whole-cell biocatalyst has advantages in the regeneration of expensive cofactors like $NAD(P)^+$ and NAD(P)H by multi-step reactions. Since these cofactors are involved in a variety of other biochemical reactions, suppressing or eliminating those undesirable side reactions becomes a major challenge to increase the yield of target products. There are mainly two approaches to overcome this obstacle: knocking out host enzymes which are competitive with heterologous enzymes, and over-expressing heterologous enzymes.

Tao et al. constructed a whole-cell biocatalyst based on *E. coli* co-expressing GlcNAc 2-epimerase and Neu5Ac aldolase to produce N-acetyl-D-neuraminic acid. The yield was 1.6-fold improved by knocking out *nagE* gene of the host cell to eliminate the side reaction (19). However, overexpressing heterologous proteins can sometimes be burdensome to host cells (20).

Psychrophiles are able to live and replicate in extreme cold, including temperatures below the freezing point of water (21). Some interesting characteristics of psychrophilic microorganisms have been confirmed as the host of whole-cell biocatalyst. Tajima et al. constructed a psychrophile-based simple biocatalyst (PSCat) to produce aspartic acid from fumaric acid with nearly 100% yield by eliminating the side reaction within the host cell through heat treatment at a moderate temperature (22). It was found that fumarase of the psychrophilic bacterium, *Shewanella livingstonensis* Ac10, was inactivated by heat treatment at 50 °C for 15 min. Therefore, total amount of substrate (fumaric acid in this case) was able to be consumed through desirable reaction by aspartate ammonia-lyase (aspartase) leading to a nearly 100% yield. Traditionally, this kind of side reactions are avoided by overexpressing heterologous enzymes, but as mentioned before, overexpression is not always available. The possibility of side reactions elimination easily provides us a new tool to construct whole-cell biocatalysts. The foundation of elimination of side reactions is that metabolic enzymes of psychrophile are assumed to be less thermostable than mesophilic enzymes and could be inactivated at temperatures which have less effects upon those mesophilic ones. However, basic knowledge of psychrophilic enzymes, especially their thermostabilities

is insufficient. Current research presented an investigation on the thermostability of a number of organic acid relevant enzymes in *S. livingstonensis* Ac10, most of them had a reasonable limited thermostability and were inactivated at 50 °C, but an NADP-dependent malic enzyme (SL-ME) was found with unexpected high thermostability which will be discussed in chapter 2.

Second, the cell membrane that acts as a cage to trap useful enzymes also can be a barrier for substrate to enter the cells. This causes significant issues, full capacity of the enzymes cannot be utilized because of inefficient substrate access, and certain conversion processes can be disabled due to the inability of substrate to obtain access to the enzymes in the cells. In order to solve the problem, surfactants and organic solvents are widely applied to destroy the part of membrane structure deliberately. P. Fontanille and C. Larroche tested several reagents including toluene, chloroform and Triton X-100 treatment to permeabilize *Pseudomonas rhodesiae* cells for production of isonovalal. It was found that Triton X-100 treatment showed the highest improvement on isonovalal production rate (23). Changing membrane structure through lipoprotein mutation to facilitate substrate uptake is also a feasible way (24). Besides, surface display of the enzymes is also proven an effective approach to enhance the accessibility between the enzyme and the substrate (25).

Psychrophile-based simple biocatalyst can also help to solve the permeability issue. As mentioned before that the cell membrane sometimes acted as a barrier for substrate uptake. In chapter 3, a novel method to permeabilize the cell membrane of psychrophilic bacterium through heat treatment was described. The heat-permeabilized

PSCat was able to convert citrate to itaconate at a faster rate than mesophilic one based on *E. coli*. Through current research, the author is aiming to not only explore basic enzymatic characteristics of psychrophilic enzymes but also construct a practical production process for valuable chemicals using psychrophilic cells as hosts of biocatalyst.

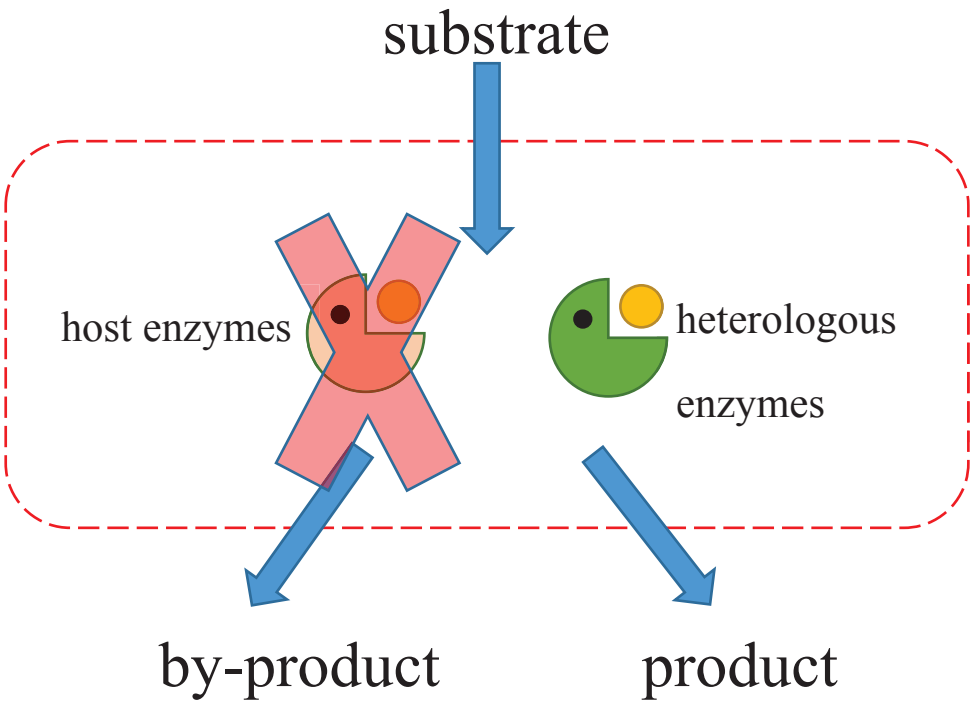

Fig. 1.1 Basic concept of psychrophile-based simple biocatalyst. In psychrophile, the host metabolic enzymes could be inactivated at temperatures which have less effects on mesophile-origined heterologous enzymes. In the meantime, the cell membrane of psychrophile is easy to be permeabilized through heat treatment, this will allow substrate to enter into the cells more efficiently thus accelerating the conversion rate.

Chapter 2 Effects of heat treatment on basic metabolic enzyme activities of *Shewanella livingstonensis* Ac10 and discovery of an NADP-dependent malic enzyme with unexpected thermostability

2.1 Introduction

Cold adaption enzymes in psychrophiles have attracted the interest of biochemists and bioengineers due to their unusually high specific activities at low temperatures and the mechanism that underpins them (26, 27). In general, cold adaptation enzymes, have more flexibility in their active sites to catalyze reactions at low temperatures while their thermostabilities have to be compromised to some degree (28).

Taking advantage of the low thermostability of psychrophilic enzymes, psychrophile-based simple biocatalysts has been developed. A psychrophilic bacterium, *Shewanella livingstonensis* Ac10 was selected as the host organism. Psychrophile-based simple biocatalyst relies on easy inactivation of psychrophilic host enzymes for the efficient conversion of valuable chemicals at high yields. Mesophilic or thermophilic enzymes were heterologously expressed in PSCats, in which psychrophilic enzymes, usually leading to undesired byproducts, were heat-inactivated through heat treatment at a moderate condition to efficiently obtain target products catalyzed by the exogenous enzymes. Thus, it is important to have a general concept about how thermostable the psychrophilic enzymes are and in what conditions can those homologous enzymes be inactivated without decreasing the yield of target products.

However, some unusual cases have been identified, such as thioredoxin in the psychrophilic bacterium *Pseudoalteromonas haloplanktis* (29) and isocitrate dehydrogenase in *Desulfotalea psychrophila* (30), both of which are stable at temperatures well above their optimum growth temperatures. Such irregular enzymes need to be identified and considered in advance before constructing a PSCat.

NADP-dependent malic enzyme (NADP-ME, EC: 1.1.1.40) is an NADP-dependent enzyme that catalyzes the reversible oxidative decarboxylation of L-malate to pyruvate in the meantime converting $NADP^+$ to NADPH. NADP-ME has been identified and separated from many creatures, including mammals, plants, and bacteria (31-33). It is important for organic acid metabolism and photosynthesis in plants (34). It also provides NADPH for long-chain fatty acid synthesis in mammals, fungus, and lipogenesis creatures like algae (35, 36).

NADP-ME in thermophiles such as *Sulfolobus solfataricus* and *Thermococcus kodakarensis* has also been found. *Sulfolobus solfataricus* NADP-ME catalyzes the reaction at extreme conditions and showed a less preference to the reaction direction. On the other hand, NADP-ME in mesophiles such as *Mortierella alpine*, an oleaginous fungus, has a reasonably limited thermostability and was completely inactivated above 45 °C (37).

In this chapter, a survey for thermostabilities of several metabolic enzymes in *Shewanella livingstonensis* Ac10 was performed. During the survey, a thermostable NADP-ME (SL-ME) was unexpectedly discovered and its unusual thermostability was investigated as well as its kinetic characteristics. Research on thermostability of enzymes in psychrophiles is limited. Exploring such irregular examples will increase our understanding of enzyme temperature adaptations and could lead to the development of more attractive enzymes in the future.

2.2 Experimental procedures

2.2.1 Medium and reagents

Lysogeny Broth (LB; Tryptone 10 g/L, NaCl 10 g/L, Yeast Extract 5 g/L) was used for culture of *E. coli* strains. Agar powder 20 g/L was added for solid medium. Tryptic Soy Broth (TSB; Difco, US) was used for culture of psychrophile. DNA polymerase KOD-Plus and DNA ligase Ligation High v2.0 were purchased from Toyobo Co. LTD (Osaka, Japan). Restriction endonucleases, FastDigest, were purchased from Thermo Fisher Scientific (Massachusetts, US). All other reagents are special grade chemicals if it is not mentioned.

2.2.2 Strains and culture conditions

Bacterial strains used in the current section are described in Table 2.1. Lysogeny Broth and Triptic Soy Broth (Difco Laboratories, Detroit, Michigan, US) were used to cultivate *E. coli* strains at 37 °C and *Shewanella livingstonensis* Ac10 at 18 °C with 50 mg/L of rifampicin, respectively. 50 mg/L kanamycin was added for recombinant *E. coli* strains.

2.2.3 Gene cloning and plasmid construction

E. coli DH5α and *S. livingstonensis* Ac10 genome DNA were used as template for gene amplification. The bacteria cultures were collected by the centrifugation at 20,400 × g for 2 min. Collected cells were resuspended in 540 μL GTE (glucose 50 mM, Tris 25 mM, EDTA 10 mM, pH = 8.0) containing 10 g/L lysozyme. After incubation at 37°C for 2 hours, 5 μL of 10

mg/mL Proteinase K and 60 μL of 10% w/v SDS were added to the mixture, and it was incubated at 50 °C for 1 hour. Cooling down suspension on ice was mixed with 600 μL phenol/chloroform solution with shaking for 10 min. After centrifugation at 20,400 ×g for 5 min, the supernatant was moved to new micro tube. Phenol/chloroform treatment was repeated to increase the purity of DNA. To precipitate the DNA, 40 μL of 3M CH_3COOK and 1 mL of 99.5% ethanol were added into 400 μL of supernatant and it was kept at -20 °C for 30 min. The precipitate obtained by the centrifugation at 20,400 ×g for 5 min was washed with 70% w/v ethanol and dissolved with 50 μL of TE (Tris-HCl 10 mM, EDTA 1 mM; pH = 8.0) containing 50 μg/mL RNase.

Since the genome sequence of *S. livingstonensis* Ac10 has not been released, primers for amplification of the malic enzyme gene in the Ac10 strain (SL-ME) were designed using the genome sequence of *Shewanella frigidimarina* NCIMB 400, a closely related species. Sfri_3757 is the name of a putative NADP-ME gene in *S. frigidimarina* NCIMB 400. Primers, plasmids, and strains used for cloning are described in Table 2.1. SL-ME gene was amplified by Toyobo KOD Plus polymerase following the manufacturer's instructions. The PCR product was then purified using FastGene PCR/Gel extraction kit (Nippon Genetics, Tokyo, Japan) and digested by EcoRI and XhoI FastDigest restriction enzymes (Thermo Fisher Scientific, Massachusetts, US). DNA fragments were separated by electrophoresis on a 1% w/v agarose gel and the gel bands containing the DNA fragments were cut out and extracted again. The PCR product was ligated with pET28b (+) fragments digested by the same restriction enzymes.

At last the ligation product was added into 100 μL *E. coli* competent cell suspension. The mixture was then kept on ice for 30 min. After that, the *E. coli* was heat-shocked at 42 °C for

30 seconds then cooled down on ice immediately. After 1 min, 1 mL of LB was added, then the mixture was incubated at 37 °C for 1 hour. After the incubation, the mixture was spread on a LB plate containing kanamycin to select *E. coli* harboring the plasmid. Twelve hours later, colonies on the plate were picked up and cultivated. The plasmid was extracted and confirmed by restriction enzyme digestion to confirm the insertion of the DNA fragments. NADP-ME from *E. coli* (MaeB) was cloned following the same procedure.

2.2.4 Cell extraction preparation and activity assays

S. livingstonensis Ac10 was first pre-cultivated in TSB with 100 mg/L ampicillin and 50 mg/L rifampicin for 48 hours. The cultures were then used to inoculate in 200 mL TSB in 500 mL Erlenmeyer flask (1%, v/v). The *S. livingstonensis* Ac10 cells were then collected by centrifugation at 4°C, 5,000 × g for 10 min and the cell extraction was prepared through sonication.

The cell extract was heat-treated at 50 or 60 °C before analyzing. Absorbances were monitored by a UV-Vis spectrometer (Thermo Fisher Scientific, Massachusetts, US) at 340 nm for analyzing of NADP-ME, lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH), aconitase, isocitrate dehydrogenase (IDH) and 2-oxoglutarate dehydrogenase (OGDH), 412 nm for citrate synthase, and 235 nm for SCS activities. All analyses were performed at 18 °C in 1 mL reaction mixture, enzyme activities of the cell extract without any heat treatment were regarded as 100%.

The reaction mixture of analyses was described as below:

For the NADP-ME, the reaction mixture contained 1 mM $NADP^+$, 10 mM L-malate, and 100 mM Tris-HCl (pH = 9.0). For the LDH, the reaction mixture contained 0.015 mM NADH, 10 mM pyruvate, 25 mM potassium phosphate buffer (pH = 7.0). For the PDH, the reaction mixture contained 0.2 mM TPP, 0.1 mM CoA, 1.0 mM $MgCl_2$, 0.3 mM DTT, 2.5 mM NAD^+, 100 μg BSA, 3.0 mM Lipoamide, 0.025 mM FAD^+, 5.0 mM pyruvate, and 50 mM phosphate buffer (pH = 8.0). For the aconitase, the reaction mixture contained 20 mM citrate, 1 mM $MnSO_4$, 100 mM Tris-HCl (pH = 9.0), 1.6 U IDH (Oriental Yeast, Tokyo, Japan), and 1 mM NAD^+.

For the IDH, the reaction mixture contained 1 mM NAD^+, 20 mM isocitrate, 50 mM $MgCl_2$ and 100 mM Tris-HCl (pH = 8.5). For the OGDH, the reaction mixture contained 0.5 mM NAD^+ , 2.5 mM 2-oxoglutarate, 0.25 mM $MgCl_2$, 0.04 mM CoA, 2.5 mM cysteine, and 75 mM potassium phosphate buffer (pH = 7.0). For the citrate synthase, the reaction mixture contained 0.5 mM oxaloacetic acid, 0.3 mM actyl-CoA, 0.1 mM 2-nitrobenzoic acid and 100 mM Tris-HCl (pH = 9.0). For the succinyl-CoA synthase, the reaction mixture contained 10 mM succinate, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM CoA, 50 mM KCl, 0.4 mM ATP, and 50 mM Tris-HCl (pH = 9.0).

2.2.5 Protein purification

Recombinant *E. coli* BL21 (DE3) cells were harvested by centrifugation at 4 °C, 5,000 × g for 5 min and stored at –80 °C. Cells were then resuspended in binding buffer (20 mM phosphate, 20 mM imidazole, 0.5 M NaCl, pH = 7.4) and lysed by French pressure cell press at 20000 psi

(American Instrument Co., Inc., MD, US). The cell extract was then loaded to a Ni column (GE Healthcare, Illinois, US), imidazole (200 mM for SL-ME, 400 mM for *E coli* NADP-ME, MaeB) was applied to strip the target protein which was further purified through size exclusion chromatography (SEC) with a AKTA explorer FPLC system equipped with a sephadex G-200 gel filtration column (GE Healthcare, Illinois, US). After that, the product was concentrated with ultra centrifugal filters (Merck, New Jersey, US) and stored in TMG buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 50% (v/v) glycerol (38).

2.2.6 Enzyme assay for NADP-MEs

NADP-ME activities were measured in 1 mL reaction mixture containing 50mM Tris-HCl, pH 7.5, 50 mM L-malate, 10 mM $MnCl_2$ and 0.2 mM $NADP^+$ at 30 °C. A UV-Vis spectrometer (Thermo Fisher Scientific, Massachusetts, US) was used to monitor Absorbance at 340 nm in real-time. Water bath (TAITEC, Koshigaya, Japan) were used for heat-treatment.

2.2.7 Stability measurement through circular dichroism

The thermostability of the *S. livingstonensis* Ac10 and *E. coli* NADP-ME was measured by monitoring circular dichroism (CD) spectra using a J-820 CD spectrometer (JASCO, Tokyo, Japan). 10 μM of protein solutions dialyzed against 20 mM sodium phosphate buffer (pH = 7.5) were analyzed. The CD spectra from 200 to 250 nm was scanned at 25 °C in a quartz cuvette of a path length of 1 mm. The CD ellipticity change at 222 nm was monitored from 30 to 90 °C

with the heating rate of 1.0 °C/min. The resulting raw data were subjected to nonlinear least-squares fitting, as described by Uchiyama et al. (39). The data points obtained were corrected for the slope of the base lines for the native and denatured forms, and were normalized to calculate the fraction of the denatured protein. The resulting fraction was plotted as a function of temperature.

Table 2.1 Plasmids and strains used in this research

Strains, plasmids and primers	**Genotype or phenotype**	**Resource**
Strains		
S. livingstonensis Ac10	Psychrophilic bacterium, Rif^r	(40)
E. coli DH5α	Cloning host *deo*R, *sup*E44, *hsd*R17(r_k^-, m_k^+), *pho*A, *rec*A1, *end*A1, *gyr*A96, *thi-1*, *rel*A1, D(*lac*ZYA-*arg*F)U169, f80d*lac*ZDM15, F^-, l^-	TOYOBO
E. coli BL21(DE3)	Suitable for high-level T7 expression of recombinant proteins. F– *omp*T *hsdSB* (rB–, mB–) *gal dcm* (DE3)	TAKARA
Plasmids		
pET28b (+)	Expression vector with T7 promoter, Kan^r,	MERCK

Table 2.1 (continued)

Primers		
sfri_3757_F	CATATGATGTCAGATATTCGTCA ACAAGCTCT	This study
sfri_3757_R	CTCGAGCACTTTGTATTCAGTC GGTA	This study
maeB_F	ATGCGAATTCATGGATGACCAG TTAAAACAAAGTG	This study
maeB_R	ATGCCTCGAGCAGCGGTTGGGT TTGCGCTTCTACC	This study

2.3 Results

2.3.1 Thermostability of *S. livingstonensis* Ac10 metabolic enzymes

Several TCA cycle relevant enzymes including lactate dehydrogenase (LDH), aconitase, pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), oxoglutarate dehydrogenase (OGDH), citrate synthase succinyl-CoA synthase (SCS) and SL-ME were tested for their thermostabilities at 50 and 60 °C. The result was showed in Table 2.2, most of them showed reasonable limited thermostabilities and lost most of their activities after the heat treatment except SL-ME. SL-ME kept largely active even after heat treatment at 60 °C.

Table 2.2: Relative activities of serval organic acid metabolic enzymes in *S. livingstonensis* Ac10 after heat treatment (HT)

Enzyme / HT (°C)	SL-ME	LDH	PDH	Aconitase	IDH	OGDH	Citrate synthase	SCS
50	91.0%	N.D.	0.2%	N.D.	0.02%	N.D.	2.8%	N.D.
60	72.6%	N.D.	N.D.	N.D.	N.D.	N.D.	2.4%	N.D.

2.3.2 Cloning, expression, and purification of *S. livingstonensis* Ac10 NADP-ME

Since SL-ME is clearly a unique enzyme in terms of thermostability, further investigation was performed. A plasmid for over-expressing recombinant SL-ME in *E. coli* was constructed based on pET-28b (+). NADP-ME gene of *S. livingstonensis* Ac10 was sequenced (DDBJ: LC627056). It has a high degree of similarity to *S. frigidinamrina* Sfri_3757, and the amino acid sequences of the proteins are identical. The deduced SL-ME amino acid sequence is highly similar to other NADP-MEs in *Shewanella* species such as *S. frigidimarina* (identical), which was same as the cases in cytochrome c5 and cytochrome c' from *S. livingstonensis* and *S. frigidimarina* (41, 42), *S. benthica* (NCBI: WP_005500301.1, 84% identity), *S. violacea* (NCBI: WP_013049565.1, 84% identity), *S. amazonensis* (NCBI: WP_011758568.1, 87% identity), and *S. oneidensis* (GenBank: PZP30003.1, 84% identity). *S. benthica* and *S. violacea* belong to Group I *Shewanella* species, which are usually psychrophilic and piezophilic. Later two belong to Group II *Shewanella* species are mesophilic, and not piezophilic (43). High sequence identity of SL-ME to the counterparts of other *Shewanella* species in both Group I and II indicates that NADH-MEs are widely conserved among the species. SL-ME also shares

a relatively high identities with a truncated MaeB (44) from N-terminal to A468 (52.8% identities). A completely same MaeB putative NADP-binding domain was confirmed (193-GAAGAAAIA-202) in SL-ME, but the residues in the vicinity of this domain are largely different (Fig. 2.1)

The purification of recombinant SL-ME began with a nickel column, followed by size exclusion chromatography. SL-ME is a 100 kD homodimer in solution, as determined by SDS–PAGE and native PAGE (Fig. 2.2). Surprisingly, this homodimer is very stable that the loading buffer containing SDS and 2-mercaptoethanol failed to fully denature it.

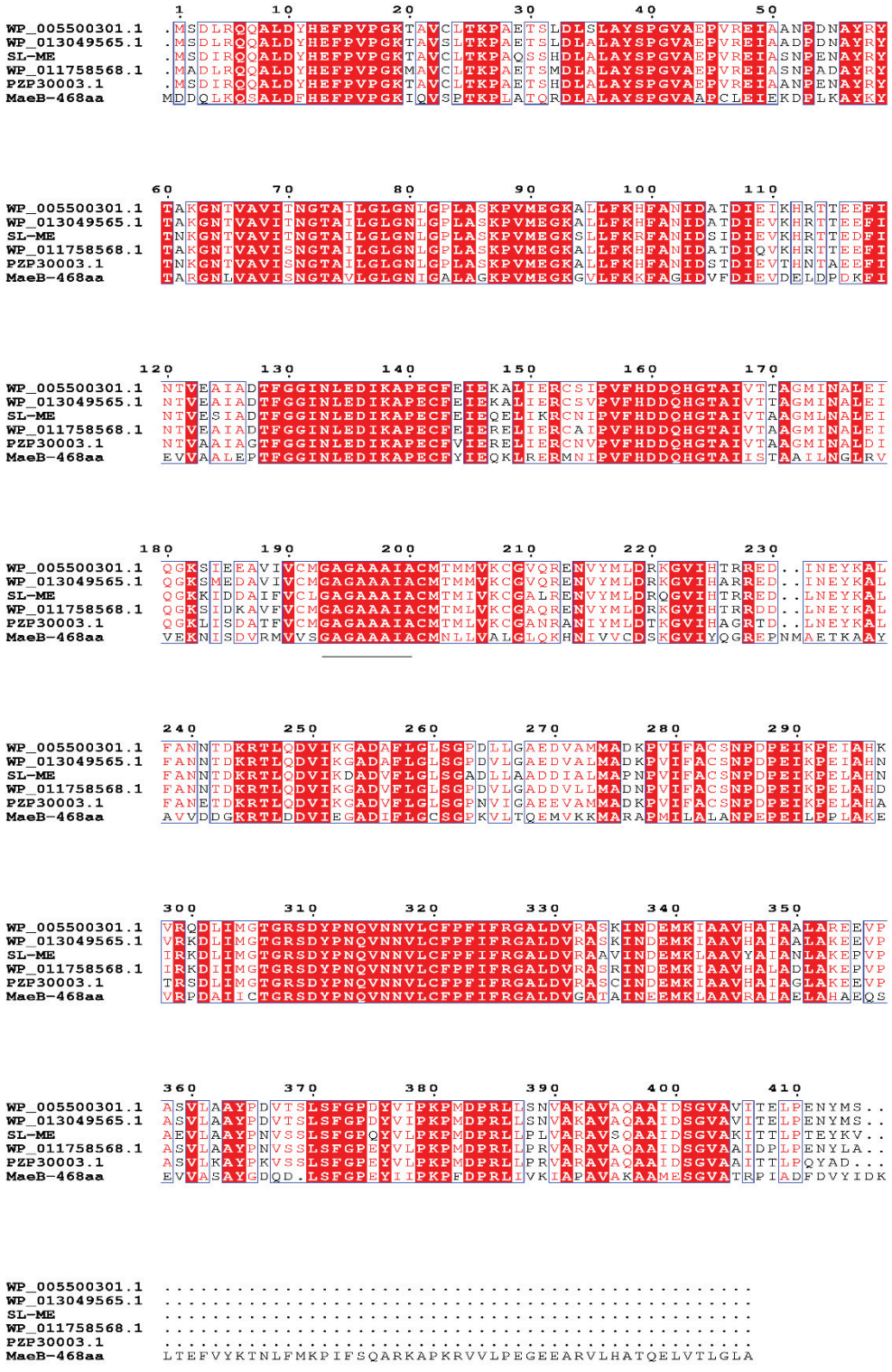

Fig. 2.1 Sequence alignment of SL-ME, NADP-MEs from *S. benthica* (WP_005500301.1), *S. violacea* (WP_013049565.1), *S. amazonensis* (WP_011758568.1), *S. oneidensis* (PZP30003.1) and MaeB (N-terminal to A468). Putative NADP-binding domain is marked by underline.

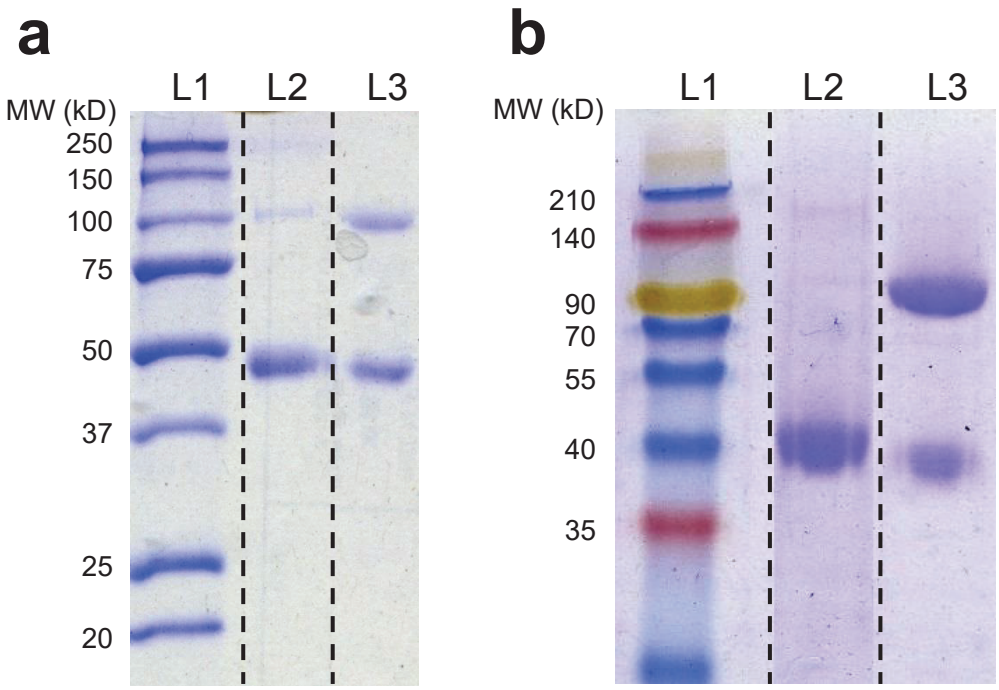

Fig. 2.2 SDS–PAGE result of purified recombinant SL-ME (a). L1, Molecular weight marker; L2, fractions of gel filtration, treated in 4 M urea at 100 °C for 5 min before running; L3, fractions gel filtration without any additional treatment. Native PAGE result of purified recombinant SL-ME (b). L1, Molecular weight marker; L2, fractions of gel filtration, treated by in 4 M urea at 100 °C for 5 min before running; L3, fractions gel filtration without any additional treatment. Relevant lanes have been rearranged in both panels for the clarity.

2.3.3 Kinetic characteristics of SL-ME

SL-ME kinetic parameters were measured. The reactions were carried out at 30 °C with various concentrations of L-malate and $NADP^+$. K_{cat} (S^{-1}) and K_m (mM) for L-malate were 11.8 and 7.3, respectively, while K_{cat} and K_m for $NADP^+$ were 10.7 and 0.2 (Fig. 2.3).

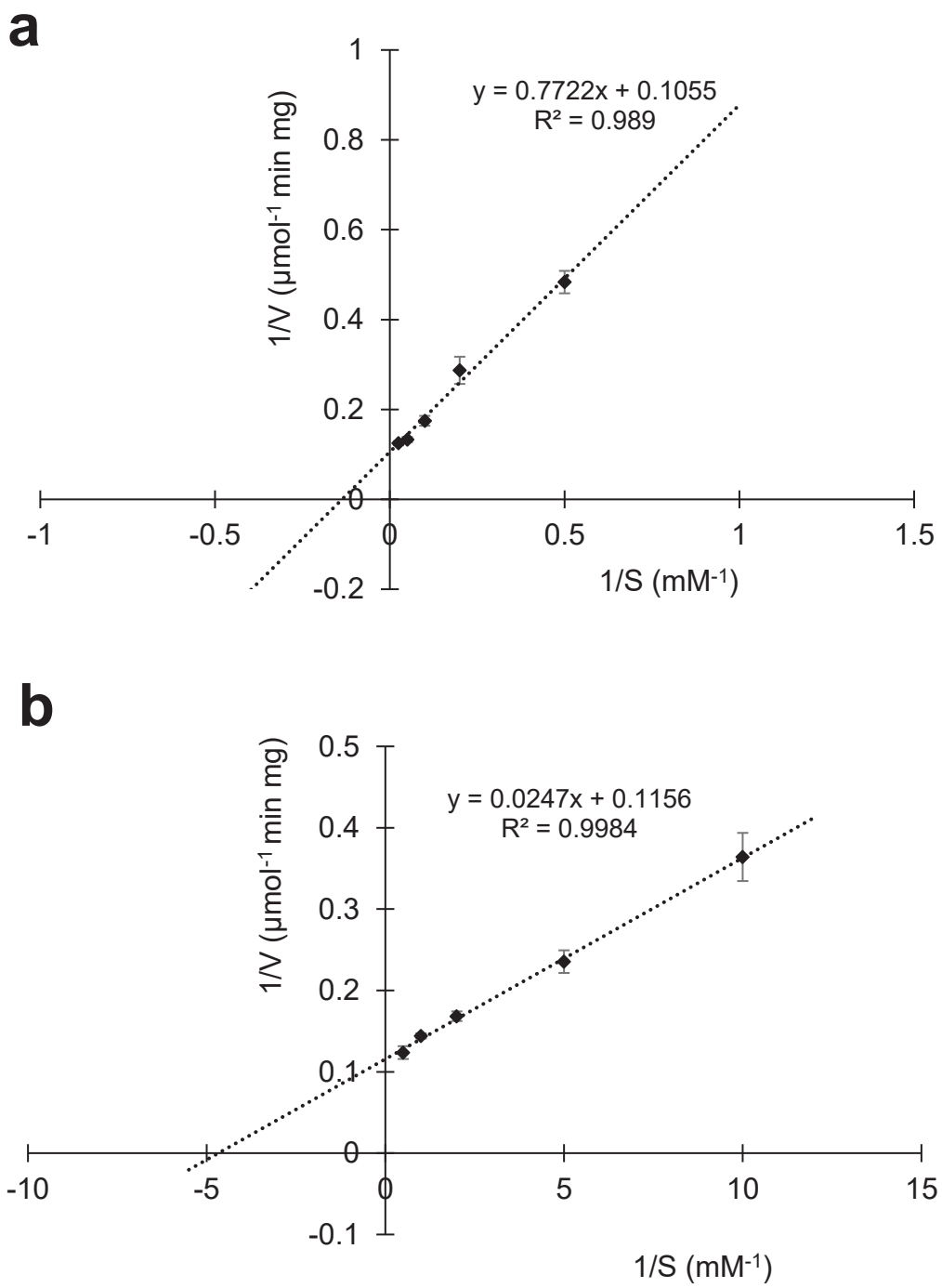

Fig. 2.3 Kinetic parameters of SL-ME, Lineweaver-Burk plot was used to determine the K_m and V_{max} of the enzyme. Error bars represent the standard deviation of the mean (n = 3).

2.3.4 Effects of temperatures on the activity of SL-ME

The activity of SL-ME was measured at temperatures ranging from 4 to 80 °C. As a result, though 70 °C is the ideal temperature for the reaction, the psychrophilic enzyme retains 8.9% and 91.9% of its original activity at 4 and 80 °C, respectively (Fig. 2.4a). For comparison, the effects of temperature on *E. coli* MaeB were investigated. This time, unlike the thermostability, the psychrophilic and mesophilic NADP-ME behaved differently. It is worth noting that although SL-ME has higher specific activities at temperatures above 70 °C, it is less active than MaeB at temperatures below 30 °C where *S. livingstonensis* Ac10 grows (Fig. 2.4b).

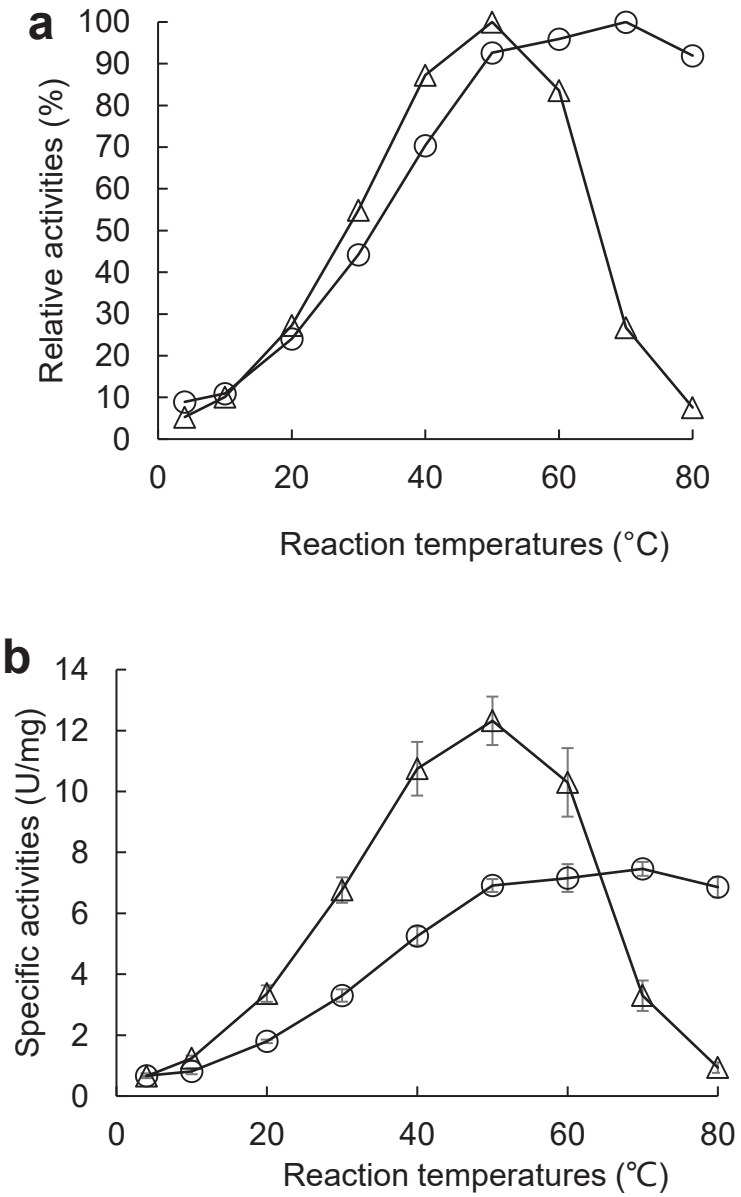

Fig. 2.4 Effects of temperature on the activity of SL-ME and MaeB. The reaction was performed at various temperatures ranging from 4 to 80 °C. Relative activities (a) and specific activities (b) were showed. Circle, *S. livingstonensis* Ac10 NADP-ME; triangle, *E. coli* NADP-ME. Error bars represent the standard deviation of the mean (n = 3).

2.3.5 Thermostability of SL-ME activity

Purified enzyme solution was incubated at different temperatures ranging from 20 to 80 °C to examine the thermostability of the recombinant SL-ME. The results indicated that the NADP-ME activity of *S. livingstonensis* Ac10 remained stable at temperatures up to 60 °C (Fig. 2.5a). The thermostability of NADP-ME from *E. coli* (MaeB) was also investigated as a contrast. Surprisingly, SL-ME had comparable thermostability to its mesophilic counterpart. The thermostability of SL-ME at 60 °C was explored further by incubating the protein at 60 °C for 0.5, 1, 2, and 3 hours, with no activity loss observed (Fig. 2.5b).

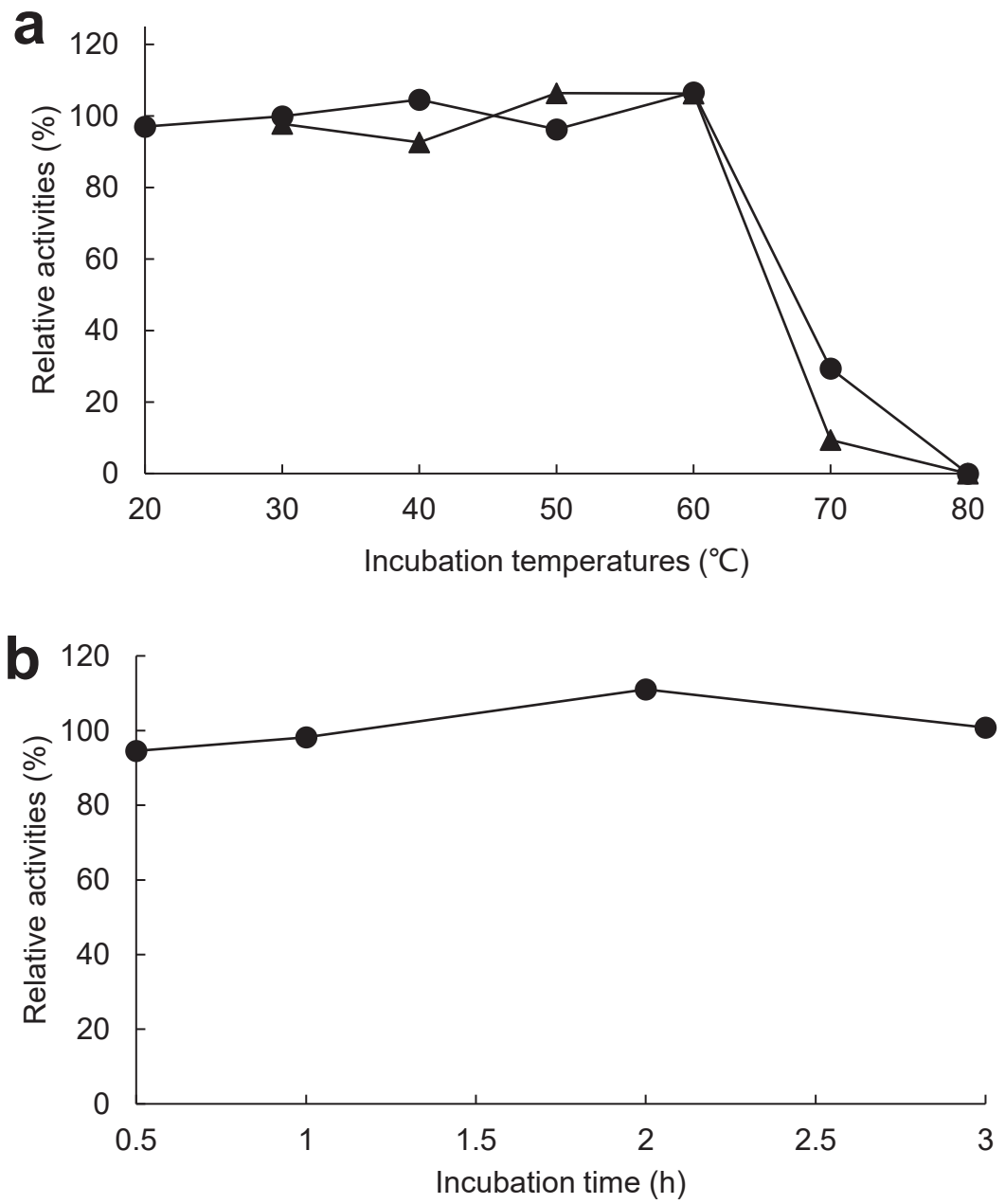

Fig. 2.5 Relative activities of SL-ME and MaeB incubated at various temperatures for 0.5 h (a), circle, SL-ME; triangle, MaeB. Thermostability of the SL-ME at 60 °C with long incubation time (b). Specific activity of enzymes kept on ice was defined as 100%.

2.3.6 Thermal denaturation of SL-ME

The effect of temperature on the structural stability of SL-ME and *E. coli* MaeB was verified by CD measurements. Both proteins showed negative peaks at 208 and 222 nm (Fig. 2.6a), indicating a predominantly α-helical structure. Denaturation curves for SL-ME and MaeB were obtained by detecting the CD ellipticity at 222 nm. The transition temperature mid-point during the thermal denaturation (Tm) of SL-ME was 71.9 ± 0.5 °C (from three independent experiments, ± standard deviation), which was significantly higher than that of *E. coli* MaeB (64.6 ± 0.4 °C). Thus, despite its origin, the psychrophilic NADP-ME has a more stable overall structure than its mesophilic counterpart (Fig. 2.6b). The density rate of SL-ME reached 0.14 at 69.5 °C, in contrast MaeB reached this level at 58.5 °C, which means SL-ME started to denature at a much higher temperature than its mesophilic counterpart. Both SL-ME and MaeB exhibited loss of reversibility at 70 °C (Fig. 2.6c). These results were consistent with the activity measurements for both enzymes, which showed only ~20% activity when exposed to 70 °C.

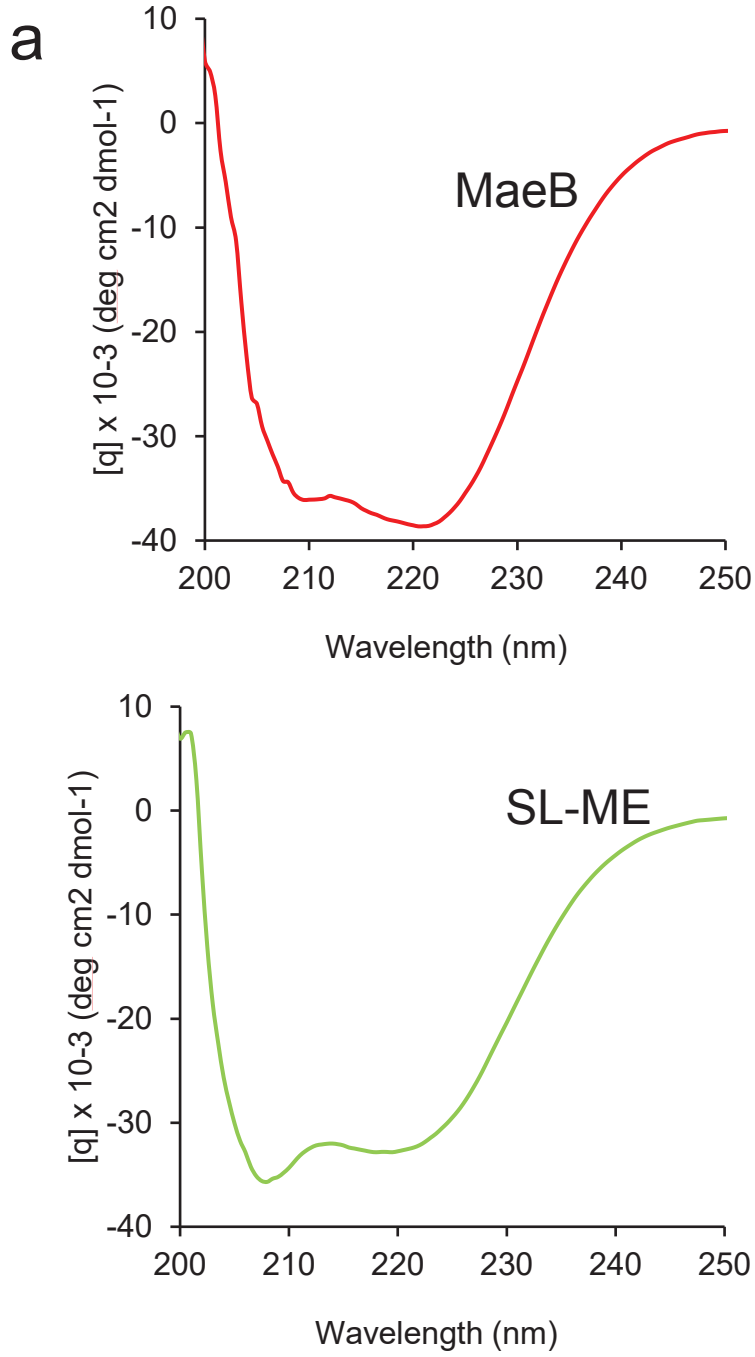

Fig. 2.6 Thermal denaturation analysis for SL-ME and MaeB by circular dichroism. (a) Circular dichroism scans at 25 °C.

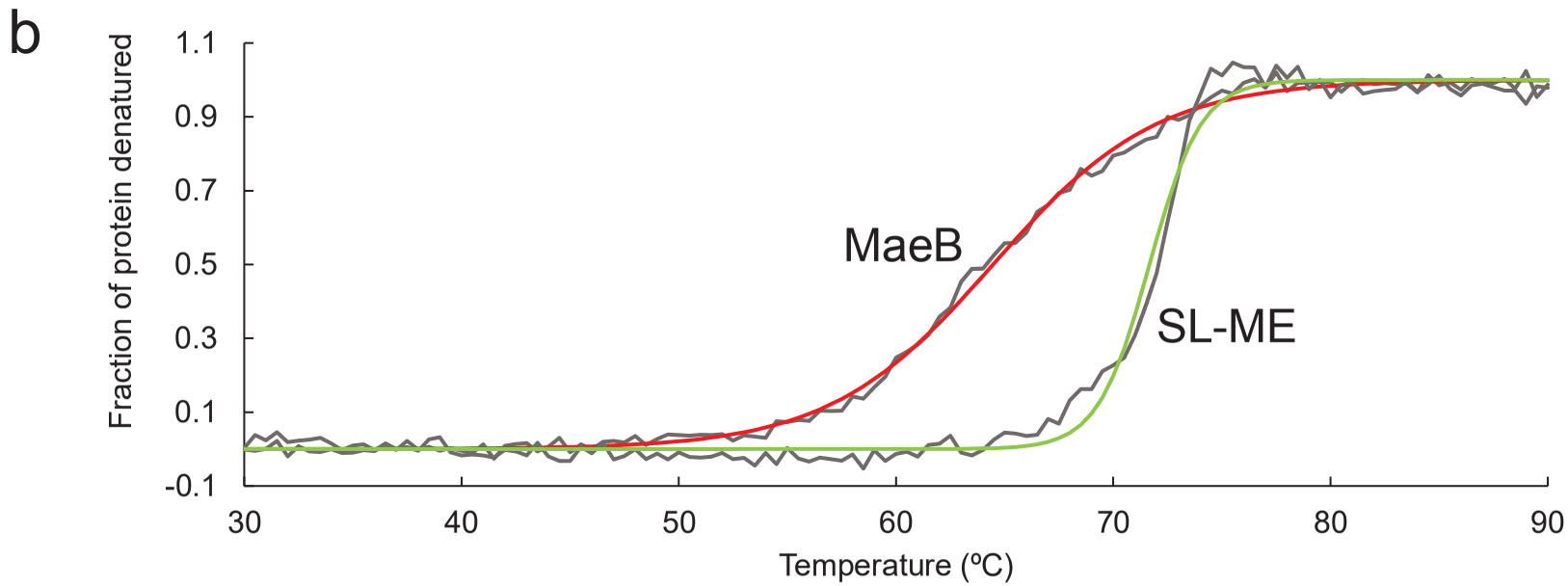

Fig. 2.6 Thermal denaturation analysis for SL-ME and MaeB by circular dichroism. (b) Representative normalized denaturation curves for SL-ME and MaeB. The raw data is shown by the gray lines.

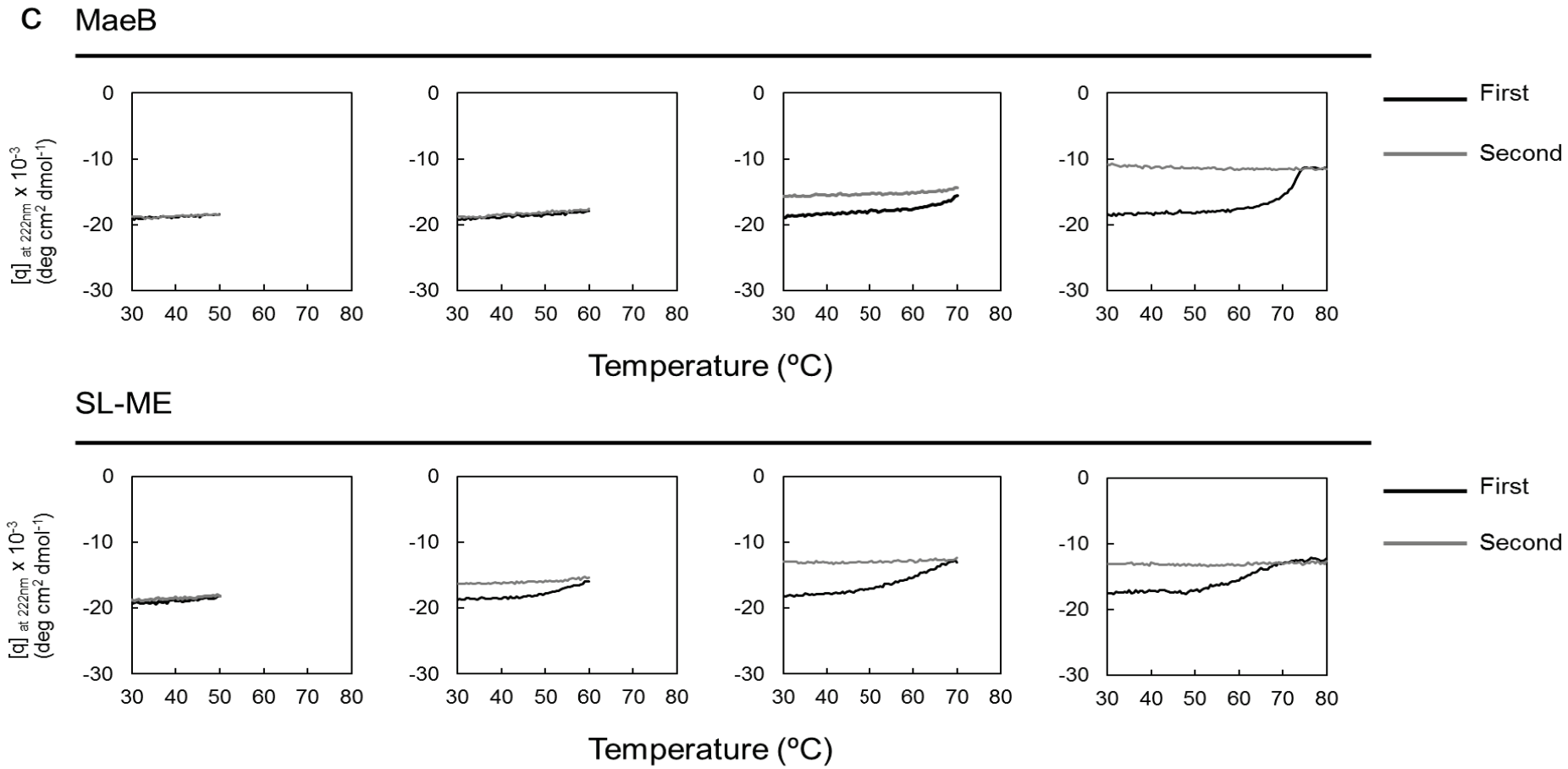

Fig. 2.6 Thermal denaturation analysis for SL-ME and MaeB by circular dichroism. (c) Reversibility checks of SL-ME and MaeB using circular dichroism (CD) after thermal denaturation. Samples were increased to 50, 60, 70, and 80 °C at a rate of 1.0 °C/min.

2.4 Discussion

Psychrophiles need cold adaptive enzymes to survive at low temperatures. Although psychrophilic enzymes are generally considered to have relatively low functional temperatures, investigations are still needed to prove this hypothesis. It is important for PSCat to inactivate homologous enzymes through heat treatment to achieve a high production yield. Thus, several organic acid metabolic enzymes were tested for their thermostabilities at 50 and 60 °C. Most of them are as heat-vulnerable as expected, except SL-ME. An NADP-ME from psychrophile (SL-ME) with abnormal thermostability was discovered in this study. The result strongly suggested that not all the enzymes in psychrophiles need to be cold-adapted. Although this does not change the foundation of PSCat, cautions still need to be made when introduce new pathways in *S. livingstonensis* Ac10 with reactions related to $NADP^+$ and NADPH regeneration. SL-ME shares high identities with other NADP-MEs in non-psychrophilic *Shewanella* species like *S. amazonensis* and *S. oneidensis*, it is possible that SL-ME is not engaged in cold adaptation in *S. livingstonensis* Ac10, but it is still important for *Shewanella* species to survive. A highly conservative NADP-binding domain is confirmed in SL-ME and this may explain the reason why SL-ME cannot use NAD^+ as a cofactor for its activity (data not shown) just like MaeB. While low degree of conservation around the NADP-binding domain may have an influence on their different temperature adaptations.

Leuenberger et al. had examined the thermostability of mesophile and thermophile on a proteomic scale (45). Thermophiles were discovered to achieve thermo adaptation by unequally improving protein thermostabilities. For psychrophiles, a similar mechanism may be

considered.

SL-ME is stable and functioning at a wide temperature range. Although it has a greater optimal operating temperature than its *E. coli* equivalent, this is unlikely to be physiologically significant because *S. livingstonensis* Ac10 cannot survive at such high temperatures.

SL-ME appears to be involved in *S. livingstonensis* Ac10's organic acid metabolism, as enzyme activity was observed in the cell extract. The role of SL-ME in *S. livingstonensis* Ac10 metabolism is waiting to be explored and mechanism behind its unusual thermostability is still unclear and needs to be explored in the future.

Chapter 3 Efficient itaconic acid production by a psychrophile-based simple biocatalyst

3.1 Introduction

Whole-cell biocatalysts have broad applications in the chemical and pharmaceutical industry. Compared to the purified enzymes, whole-cell biocatalysts for the production of biological material have been researched extensively in past years. Allowing multistep reactions is one of the advantages of whole-cell biocatalysts. Based on the results from chapter 2, an NADP-dependent malic enzyme remains active after heat treatment, which means any new reaction containing NADPH regeneration may be influenced by the SL-ME. Aiming to show the potential of PSCats, a bioconversion process without NADP(H) involvement was selected in this chapter as the target process of PSCat.

Itaconic acid is an unsaturated dicarboxylic acid with broad industrial applications. It is also a potential substitute for acrylic acid or methacrylic acid (46), a component for polymer production (47). Itaconic acid can be produced from glucose through fermentation by *Aspergillus*. *terreus* (48). Two enzymes are crucial for this process, aconitase and *cis*-aconitic acid decarboxylase (CAD). The fact that aconitase and CAD are present in different organelles (cytoplasm and mitochondria respectively) may limit the itaconic acid production by *A*. *terreus* (49). Using whole-cell biocatalyst to produce itaconic acid can be an alternative way to obtain a higher titer, purity, and productivity because all enzymes will be expressed in the cytoplasm and metabolic flux is not vital for itaconic acid biosynthesis. Kim et al. developed a whole-cell biocatalyst based on *E. coli* to produce itaconic acid (50). As mentioned in chapter 1, the cell

membrane of whole-cell biocatalyst often serves as a barrier to substrate influx. For example, *Pseudomonas pseudoalcaligenes* cells cannot convert maleate to D-malate because the cell membrane inhibits substrate uptake (51). Many methods have been developed to improve cell membrane permeability, such as solvent treatment, detergent treatment, and genetic engineering to change the constitution of cell membrane. In the research of Kim et al., detergent-permeabilized cells have achieved itaconic acid productivity of 2.19 g/L/h. However, additional chemical reagents may increase the complexity and the cost of downstream processing. Therefore, a simple method is required to improve the membrane permeability of whole-cell biocatalysts. The cell membrane of psychrophiles is very flexible due to the membrane lipid composition (52), allowing the passive transport of tiny hydrophilic molecules. Therefore, using psychrophile as the host for whole cell catalyst could be a potential solution for permeability issue.

In this chapter, a new approach to solve the permeability issue of whole-cell biocatalyst was described. A whole-cell biocatalyst based on a psychrophilic bacterium, *S. livingstonensis* Ac10, was constructed to produce itaconic acid from citric acid. Two enzymes vital for itaconic acid production from citric acid, aconitase B from *E. coli* and CAD from *A. terreus*, were expressed in *S. livingstonensis* Ac10 cells which were then heated at a moderate temperature to increase permeability. The optimum temperature for heat treatment and the performance of the biocatalyst was determined upon recycling it several times. An attempt to perform itaconic acid production at a high concentration of substrate was also made, aiming to obtain high titer of the product, but it was unsuccessful probably because of the product inhibition. After that the CAD was engineered through site-directed mutation to remove the product inhibition, no promising

mutants have been confirmed yet.

Mechanism behind the permeability improvement caused by heat treatment was also explored. A relative species of *S. livingstonensis*, *S. frigidimarina* was found to behave differently after heat treatments at the same conditions, thus it may provide some clues about the permeability improvement. Lipids composition was considered to be the key reason and both the membrane lipid composition of *S. livingstonensis* and *S. frigidimarina* was investigated. No difference in their lipid composition was confirmed and the possible mechanism of the permeability improvement remained unclear.

3.2 Experimental procedures

3.2.1 Medium and reagents

See chapter 2.

3.2.2 Gene cloning and plasmid construction

Procedures for molecular cloning is the same as described in chapter 2 if not mentioned.

Aconitase B gene (*acnB*, EG12316) from *E. coli* and *cis*-aconitic acid decarboxylase (*cadA*, ATEG_09971) gene from *A. terreus* were amplified through PCR using KOD plus Neo polymerase (TOYOBO, Osaka, Japan) following the procedures described in chapter 2, Templates of PCR amplification for *acnB* and *cadA* were genomic DNA of *E. coli* and a plasmid pUCIDT-AMP: 1_ATEG_09971 containing a codon-optimized chemical synthesis *cadA* gene of *A. terreus*, respectively. The PCR products of *acnB* and *cadA* were ligated into pHA12 at

restriction sites between EcoRI and KpnI and between SmaI and HindIII, respectively. Genes, primers and plasmids used in this research are listed in Table 3.1.

The plasmid containing *acnB* and *cadA* was first introduced into *E. coli* S17-1 following the method described in chapter 2 then into *S. livingstonensis* Ac10 by conjugation; *E. coli* S17-1 cells harboring the recombinant plasmid were cultured at 37 °C overnight, and *S. livingstonensis* Ac10 was cultured at 18 °C for 30 hours. They were then collected by centrifugation at 3,300 ×g for 3 min and washed twice with LB to remove antibiotics. After resuspending the *E. coli* and *S. livingstonensis* Ac10 cells with 250 μL LB respectively, the suspension was mixed together and spotted on a LB plate (without any antibiotics). The plate was then incubated at 18°C. After one day, 1 mL sterilized water was used to wash the plate, then the suspension was diluted 10, 100, and 1,000 times respectively. One hundred microliters of each group of diluted cell suspension was spread on plates containing ampicillin and rifampicin. After incubation at 18 °C for 5 days, the colonies on the plates were picked up, cultured and the plasmid was extracted to confirm the DNA fragments.

For expressing CAD in *E. coli* BL21 (DE3) for purifying, the gene was cloned following the same procedure described in chapter 2.

3.2.3 Strains and culture conditions

For recombinant *S. livingstonensis* Ac10 strains, 100 mg/L ampicillin was added in the medium. To overexpress *cadA* and *acnB*, *S. livingstonensis* Ac10 was transformed with plasmid pHA12-cadA-acnB, and their cloned genes were incorporated into plasmid pHA12 and expressed in *S.*

livingstonensis Ac10 upon induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). To generate cells overexpressing the target proteins, transformed Ac10 cells were cultured in 4 mL of TSB medium for 36 h and then inoculated into 200 mL of TSB culture medium (1% inoculum). The cultures were incubated in 500-mL Erlenmeyer flasks under constant agitation at 120 rpm on a rotary shaker. After 24 h of culturing, gene expression was induced by adding 100 μM IPTG.

3.2.4 Preparation of the cell suspension

Recombinant *S. livingstonensis* Ac10 cells were harvested by centrifugation at 5,000 × g for 10 min at 4 °C after culturing, washed twice, and resuspended in 50 mM phosphate buffer (PB, pH = 6.5). The dry weight of cells was measured via dissociation at 70 °C overnight.

3.2.5 Preparation of the cell extract

The cell suspension of recombinant *S. livingstonensis* Ac10 was disrupted via ultrasonication, with 1-s pulse/1-s intervals 600 times, using an ultrasonic disintegrator (Digital Sonifier 450; Branson, Connecticut, US). The supernatant was then harvested via centrifugation at 10,000 × g for 30 min at 4 °C.

3.2.6 Itaconic acid production and organic acid analysis

The cell suspension was heat-treated at various temperatures and transferred to 1 mL reaction mixture containing 200 mM PB (pH = 6.5) and 50 mM citric acid, final concentration of cells in the reaction mixture was approximately 35 mg/ml. The reaction was performed at various temperatures as described later. The reaction was terminated by heat treatment at 80 °C for 15

min or mixed with an equal volume of phenol-chloroform and vortexed for 5 minutes. A water bath (SM-05 N; Taitec, Saitama, Japan) was used for reactions and heat treatment. Citric acid, *cis*-aconitic acid, and itaconic acid were detected via high-performance liquid chromatography (HPLC; Jasco, Tokyo, Japan) system equipped with a UV detector at 210 nm and a column (Shodex RSpak KC-811; Showa Denko, Kanagawa, Japan) with a guard column (Shodex RSpak KC-G; Showa Denko). The products were flowed at 45 °C using 0.1% (v/v) phosphoric acid as a mobile phase at a flow rate of 0.7 mL/min. Citric acid was also measured by citric acid analysis kit (R-Biopharm AG, Germany) because some metabolites in the cells have close retention time to citric acid thus may disturb the quantification of it.

3.2.7 Protein purification

See chapter 2

Table 3.1 Strains and plasmids used in chapter 3

Strains, plasmids and primers	**Genotype or phenotype**	**Resource**
Strains		
S. firgidimarina DSM 12253	Host strain for whole-cell biocatalyst, Rif^r	Lab stock
S. livingstonensis Ac10	See chapter 2	See chapter 2
Plasmids		
pHA12	Expression vector, Amp^r	(53)

Table 3.1 (continued)

pUCIDT-AMP: 1_ATEG_09971	Contains codon-optimized CAD gene based on *A. terreus* CAD gene *cadA*	This study (chemical synthesis by I. D. T)
Primers		
AcnB_F	ATGCCCCGGGAGAGATGAACA[a] GTGCTAGAAGAATACCGTAA	This study
AcnB_R	ATGCAAGCTTAAACCGCAGTCT GGAAAATCA	This study
CAD_F	ATGCGAATTCGGAGAGATGAAC A ATGACTAAGCAATCAGCAGA	This study
CAD_R	ATGCGGTACCTTATACCAGCGG CGATTTTAC	This study

a: Sequences with underline refer to SD sequence.

3.2.8 Docking simulation

Docking simulation of the CAD and itaconic acid was performed on AutoDock (v1.5.6) – Vina (v1.1.2) using a putative 3D structure of *A. terreus* CAD generated by I-TASSER (54) based on mouse CAD (PDB: 6R6T). The result was visualized with PyMol (v1.7.5.0).

3.2.9 Library construction

All polar amino acid residues 6.5 Å from itaconic acid were considered as potential binding

sites. Besides, putative active site of mouse CAD were also selected. All 15 residues were subjected to site-specific saturation mutagenesis, generating all possible 285 mutants.

The mutations were prepared by amplifying full-length plasmid expressing CAD based on pET-28b(+) mentioned before, primers used for mutation generating are described in Appendix, procedures for molecular cloning were the same with those in formal chapters. All mutant genes were expressed by *E. coli* BL21 (DE3).

3.2.10 Library screening

A high throughput screening method based on Mizoroki-Heck reaction to detect itaconic acid in the reaction mixture was modified to screen variants (55). Briefly, A 96-well plate containing 200 μL LB with 50 mg/L kanamycin for each well was used to cultivate all variants at 37 °C for 2 ~ 3 hours, 200 rpm. IPTG was then added to a final concentration of 2 mM and the plate was transferred to 18 °C, cultivated overnight.

The culture was then transferred to a 96-well PCR plate and harvest by centrifugation at 2,500 × g for 5 min. Cells were washed once by 20 mM PIPES (pH = 6.5) and lysed by Bacterial Protein Extraction Reagent (Thermo Scientific, US) following the manufacturer procedure. After that, first round screening was performed by mixed cell extracts (with debris) to a reaction mixture containing 50 mM PIPES and 2 mM *cis*-aconitic acid. The plate was sealed and immersed in 37 °C water bath to get a more average heat transformation (56) for 10 min. The reaction was then stopped by adding 4 μL concentrated HCl and 10 μL supernatant was collected after centrifugation at 4,000 × g for 5 min. For itaconic acid detecting, 10 μL

supernatant 10 μL, 180 mM indobenzene in DMSO, 2 μL, 4.5 mM $Pd(OAc)_2$ was added to each well of a 96-well plate. The plate was then sealed and heated by water bath at 80 °C for 1 hour. After the reaction, 2 μL HCl was added to adjust pH before adding 50 μL, 5% soluble starch and 50 μL, 5% $NaNO_2$ to the reaction mixture. The plate was freezed at −20 °C to fix the color. Then the purple color can be measured at 595 nm by a Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific, US).

All mutants with comparable activities to the wild type were picked up for second round screening. In the second round screening, cell extract of each mutant was prepared as described at chapter 2. The cell extract was then mixed with reaction mixture containing 200 mM PIPES (pH = 6.5) and 500 mM *cis*-aconitic acid to produce itaconic acid, which was measured by HPLC after the reaction.

3.2.11 Extraction of total lipids and separation of phospholipids

Extraction of total lipids were performed based on methods described by Fang et al. (57). *S. livingstonensis* Ac10 and *S. firgidimarina* DSM 12253 were cultivated at 18 °C for 72 h, Cells were harvest through centrifugation at 5,000 × g for 10 min, washed twice by 20 mM Phosphate Buffer (pH = 6.5), freeze dried and stored at −80 °C.

For total lipids extraction, freeze dried cells were mixed with extraction solvent containing dichloromethane (DCM), methanol and 50 mM potassium phosphate buffer (pH = 7.4) at a ratio of 1:2:0.8. For 55mg of cells, 9.5 ml extraction liquid was added, shaken to mix and then stored at 4 °C in dark place to for overnight. After that, 2.5 mL DCM and 2.5 mL potassium phosphate buffer (pH = 7.4) were added, stored at 4 °C overnight.

The mixture was centrifugated at 5,000 × g for 10 min to separate layer, organic phase (lower layer) was collected and 0.5 g Na_2SO_4 was added to dry the liquid (total volume about 3 mL). A No.2 filter was used to filter the liquid. After that the total lipid was stored at −80 °C until further processing.

For phospholipid separation, total lipid sample was first dried by N_2 then dissolved again in 1 mL $CHCl_3$. Total lipid was separated into 3 classes by a silicic acid column (Wakogel, C-200; Wako, Osaka, Japan), neutral lipids, glycolipids and phospholipids. Since cell membrane is the research target, phospholipids were collected and applied to further experiments. The total lipid was first loaded on to the column, 8 mL solvent was then applied to elute different kind of lipids, $CHCl_3$ for neutral lipids, acetone for glycolipids and methanol for phospholipids. The phospholipid dissolved in methanol was dried by N_2 stream and stored at −80 °C.

3.2.12 Preparation of Fatty Acid Methyl Esters (FAME) and analysis of membrane phospholipids composition

Preparation of FAME were performed. Briefly, dried phospholipid was dissolved in 0.5 mL methanol and toluene mixture (1:1, v/v). 0.5 ml of 0.2 M KOH in methanol was added to the mixture. The mixture was then incubated at 37 °C for 15 min in water bath. After that, the reaction mixture was cooled down in room temperature and mixed with 2 mL $CHCl_3$, 2 mL H_2O and 0.5 mL of 0.2 M acetic acid, centrifuged for 5 min at 3,000 × g. Organic phase (lower phase) was collected, dried by N_2 stream and stored at −80 °C for further experiments.

3.2.13 Gas Chromatography / Mass Spectrometry (GC/MS) analysis

Dried FAME was dissolved in hexane and analyzed by a GC/MS system, Aglient Technologies 7890A GC system equipped with a DB-WAX column interreacted with 7000 GC/MS Triple Quad (Aglient Technologies, California, US). The oven temperature was programmed from 50 °C for 1 min, then to 200 °C at 25 °C/min, and to 230 °C at 3 °C/min, kept for 18 min. The detector temperature was set at 280 °C and the injection port temperature was 250 °C.

3.3 Results

In this chapter, a recombinant plasmid based on a broad range host, pHA12, was constructed and introduced into *S. livingstonensis* Ac10 to express CAD and AcnB (Fig. 3.1). Aconitase B converts citric acid to *cis*-aconitic acid, which is then converted to itaconic acid by CAD.

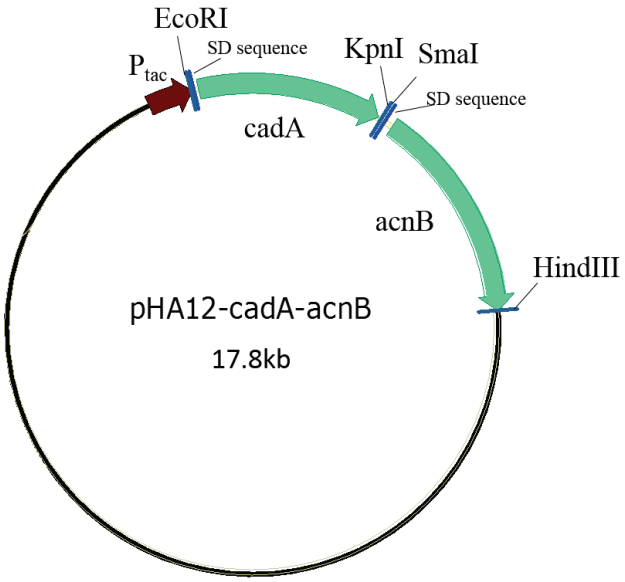

Fig. 3.1 Physical map of the expression vector, pHA12-cadA-acnB.

3.3.1 Cell membrane behaves as a barrier for substrate uptake

First, to clarify if itaconic production was really blocked by cell membrane barrier, an *E. coli* S17-1 strain harboring the recombinant plasmid mentioned above expressing AcnB and CAD was tested for itaconic acid production. The cell suspension was mixed with 50 mM citric acid to produce itaconic acid at 37 °C. The cells were also disrupted by sonication to remove the cell membrane completely for itaconic acid production. As the result showed in Fig. 3.2, the cell extract produced 37.6 mM itaconic acid in 3 hours while the intact cells only produced 4.3 mM, revealing a severe problem on substrate uptake. Because itaconic acid production of the cell

extract represents the production without any influence from cell membrane, the ration of intact cells productivity to the cell extract productivity is considered be used as an indicator for membrane permeability for subsequent experiments.

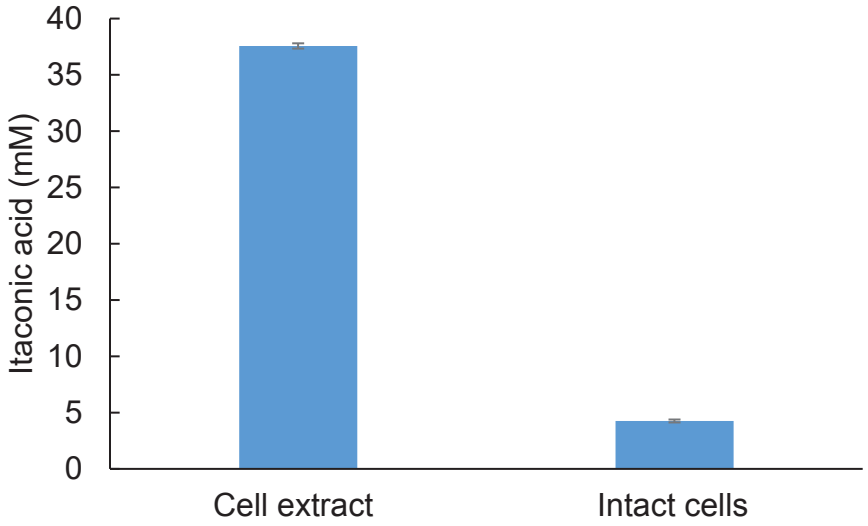

Fig. 3.2 Itaconic acid production by cell extract and intact cells of recombinant *E. coli*

Error bars represent the standard deviation of the mean (n = 3).

3.3.2 Effects of heat treatment on improvement of membrane permeability

Itaconic acid productivity of recombinant *S. livingstonensis* Ac10 cells was measured to evaluate membrane permeability. As a comparison, itaconic acid productivity of recombinant *E. coli* S17-1 constructed for conjugation was also measured at the same conditions. The percentage productivity of intact cells to that of their cell extracts was calculated (Fig. 3.3). When the conversion was performed at 28 °C for 1 hour without heat treatment, itaconic acid productivities were only 2.7% and 2.6% of their cell extracts *(E. coli* S17-1 vs *S. livingstonensis* Ac10 cells). It suggests that the cell membrane behaved as a powerful barrier for citric acid uptake. However, the productivity ratio of recombinant *S. livingstonensis* cells

after heat treatment at 40 °C for 15 min was improved to 14.2% while only a slight increase (up to 3.7%) was detected in recombinant *E. coli* cells treated at the same condition.

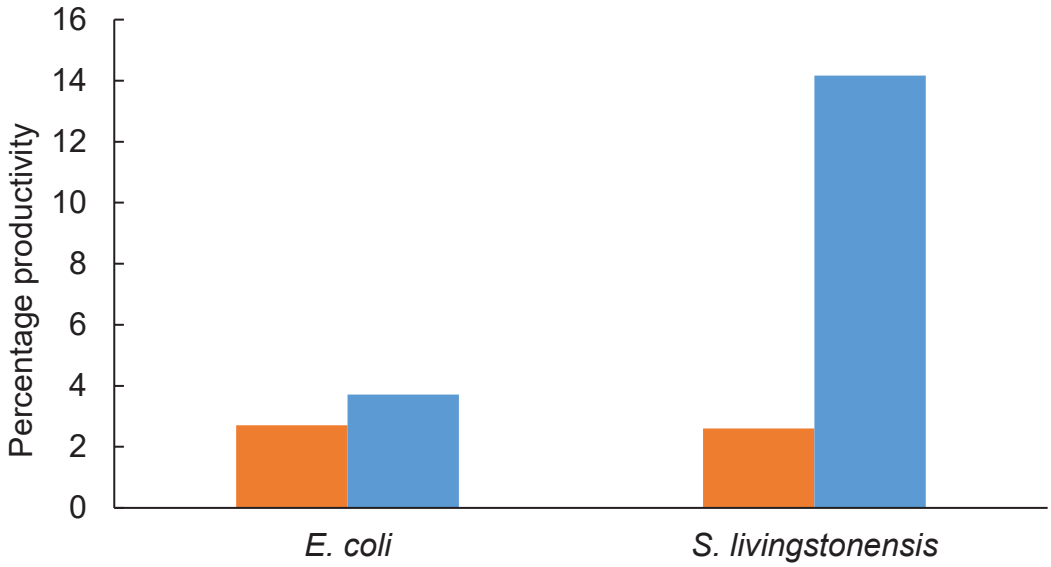

Fig. 3.3 Percentage productivity of recombinant *E. coli* and *S. livingstonensis* Ac10 cells to their cell extract respectively. Orange, cells without heat treatment. Blue, cells with heat treatment (40 °C, 15 min).

3.3.3 Optimal conditions for heat treatment

Optimal conditions for heat treatment were investigated to improve the permeability of the cell membrane and to achieve high itaconic acid productivity. *S. livingstonensis* cells expressing CAD and AcnB were collected and treated at various temperatures ranging from 35 °C to 50 °C for 15 minutes before they were mixed with citric acid to produce itaconic acid. The results showed that heat treatment at 45 °C significantly enhanced itaconic acid productivity. As shown in Fig. 3.4a, the productivity in the cells with heat treatment improved approximately 6-fold (0.22 to 1.41 g/L/h) compared to that in the cells without heat treatment.

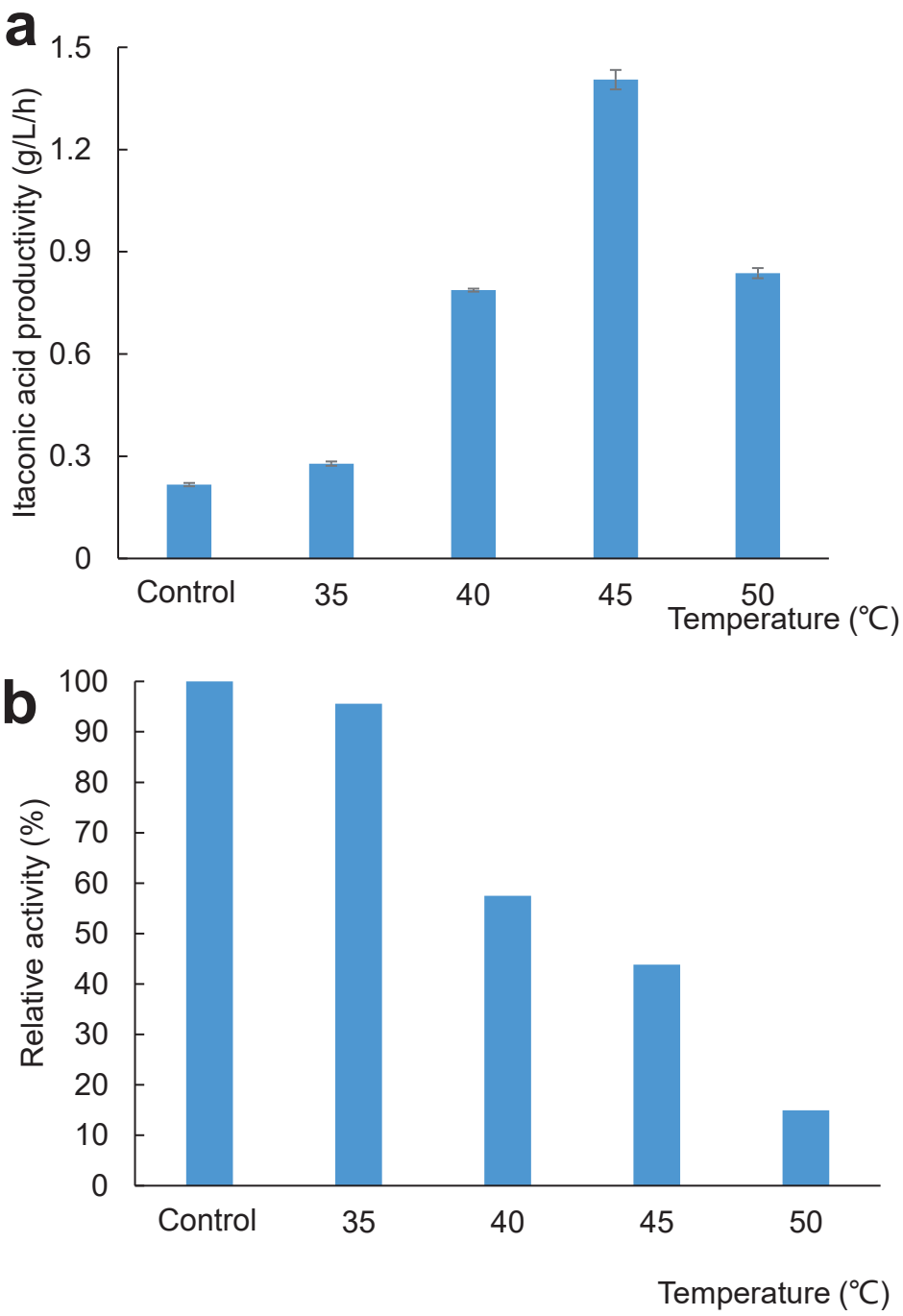

Fig. 3.4 Itaconic acid productivity of recombinant *S. livingstonensis* Ac10 cells which were treated at various temperatures. The control group comprises cell suspensions kept at 28 °C before the reaction (a). Thermostability of crude enzyme treated at various temperatures, the conditions for heat treatment and reaction are the same with the experiment described before (b).

Further increase of heat treatment temperature provides no help for improving itaconic acid productivity because CAD is possibly unstable at such high temperatures (58). To clarify the influence of heat treatment on the enzyme activity, cell extracts were also tested in the same condition. Surprisingly, only 43.85% of the enzyme activity was remained when the cell extracts were treated at 45°C for 15 min (Fig. 3.4b).

To detect whether any enzyme leakage caused by heat treatment, the cell suspension after heat treatment incubated at 28°C for 1 hour to simulate the process of the itaconic acid production. After incubation, the cell suspension was separated into supernatant and sediment (cells) by centrifugation (5,000 × g, 10 min, 4°C). Both productivities of the supernatant and the cells were measured by mixed with 50 mM citric acid and 200 mM PB (pH = 6.5) and incubated at 28°C for 1 hour. The results (Fig. 3.5) showed that the permeability of *S. livingstonensis* Ac10 cell membrane was indeed improved because itaconic acid productivity in the sediment of the cell suspension was significantly enhanced by heat treatment at 45 °C for 15 min compared with cells without heat treatment (0.21 to 0.80 g/L/h). Slight itaconic acid productivity (0.11 g/L/h at 45 °C) was detected in the supernatant, indicating that a limited amount of enzymes leaked out from the cells.

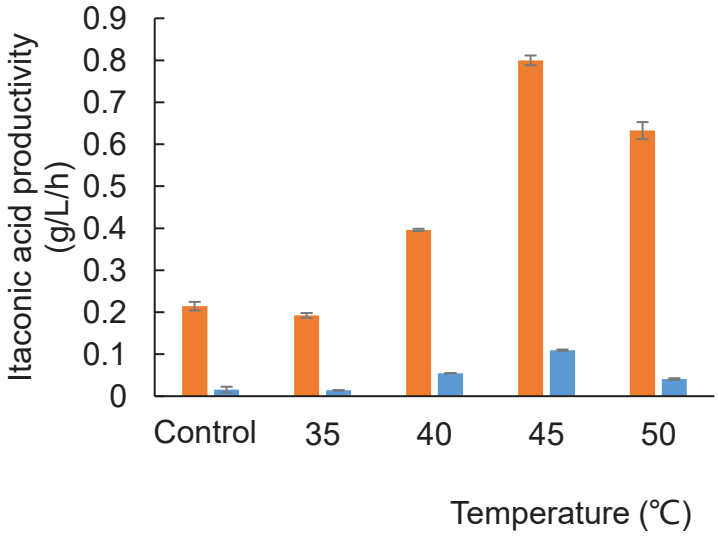

Fig. 3.5 Itaconic acid productivity of the supernatant and the sediment (cells). Orange bars, sediment. Blue bars, supernatant.

Optimal time for heat treatment was also investigated. Recombinant *S. livingstonensis* Ac10 cell suspension and cell extracts were treated at 45°C for 5 to 30 minutes. Itaconic acid production was then performed in conditions described above. The result indicated that long heat treatment time did improve the productivity, but the improvement was limited after 10 minutes (Fig. 3.6a). While cell extracts heat-treated for 10 and 15 minutes retained 87.0% of activities (Fig. 3.6b), and heat treatment longer than 15 minutes damaged the enzyme activity (75.9% for 20 minutes and 70.1% for 30 minutes). Heat treatment for 15 minutes was applied for all production experiments later considering facts that 15 minutes did help to increase the productivity compared with shorter time period and time period longer than 15 minutes influenced the enzyme activity but did not improve the overall productivity too much.

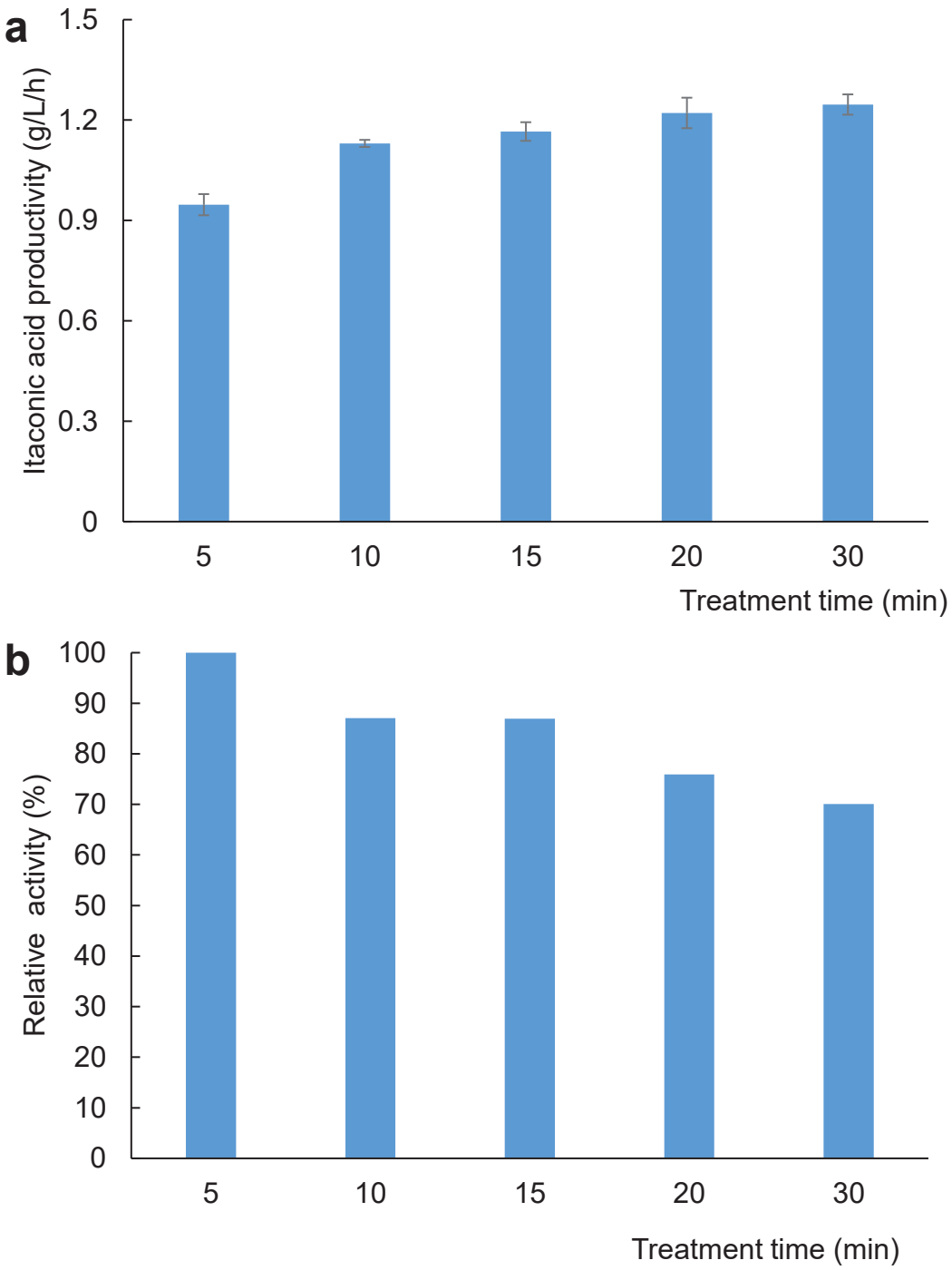

Fig. 3.6 Itaconic acid productivity of recombinant *S. livingstonensis* Ac10 cells treated at 45 °C for various time periods. Error bars represent the standard deviation of the mean (n = 3) (a). Relative activities of cell extracts, the conditions for heat treatment and reaction are the same with the experiment described before (b).

After exploring the optimal conditions for heat treatment, itaconic acid production was performed by this whole-cell biocatalyst heat-permeabilized at optimal conditions. Recombinant cells were heat-treated at 45°C for 15 min and then reacted with 50 mM citric acid as substrate at 28°C (Fig. 3.7). The yield (itaconic acid production / citric acid & *cis*-aconitic acid consumption) was 110.69%, probably because some substrates were absorbed by the cells thus could not be observed in the reaction mixture.

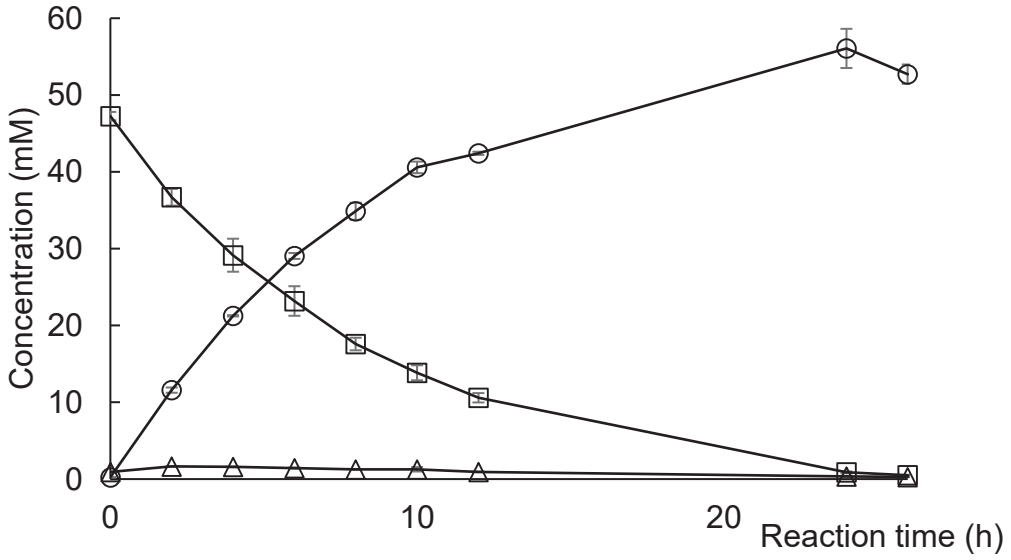

Fig. 3.7 Time-profile of itaconic acid production by heat-permeabilized *S. livingstonensis* Ac10 cells at 45°C for 15 min. Rectangle, citric acid; circle, itaconic acid; triangle, *cis*-aconitic acid.

3.3.4 Optimal reaction temperature for itaconic acid production

Next, Itaconic acid production was performed at higher temperatures ranging from 35 °C to 55 °C for 1 hour with 500 mM citric acid as substrate. Maximal itaconic acid productivity appeared at 50 °C (Fig. 3.8a). The productivity declined when the reaction temperature was over 50 °C and a middle product, *cis*-aconitic acid accumulated (Fig. 3.8b). This suggested that CAD might be inactivated. The cells were also tested for recycling after the reaction at 50 °C for 6 hours (Fig. 3.9a), only 0.16 g/L/h productivity was detected while 5.70 g/L/h for the first batch (Fig. 3.9b). Considering the poor recyclability of cells at 50 °C, itaconic acid production was still performed at 28 °C despite better reaction rate.

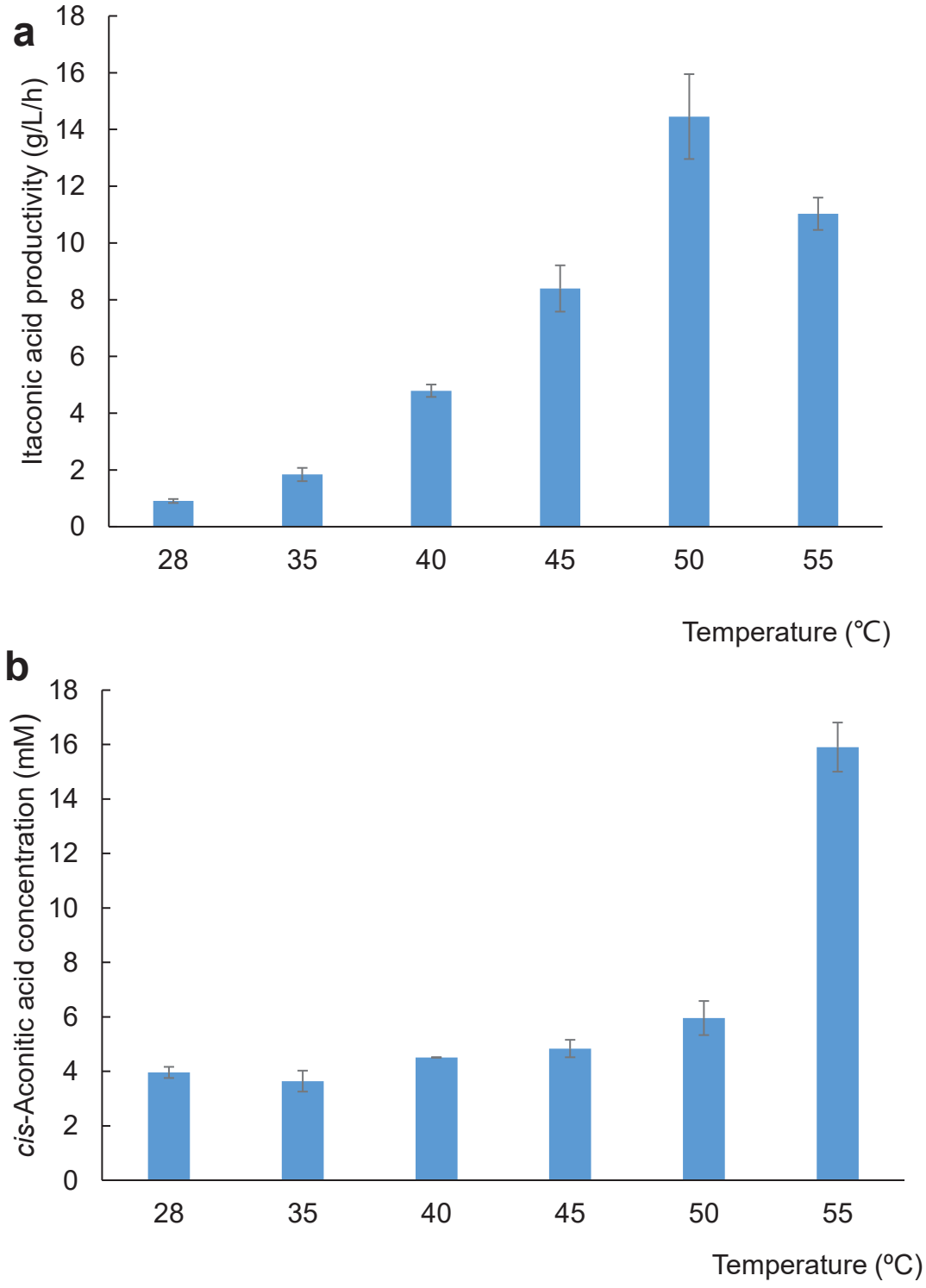

Fig. 3.8 Optimal reaction temperature for itaconic production by recombinant *S. livingstonensis* Ac10 cells after heat permeabilization (a). *cis*-Aconitic acid accumulation after production (b). Error bars represent the standard deviation of the mean (n = 3).

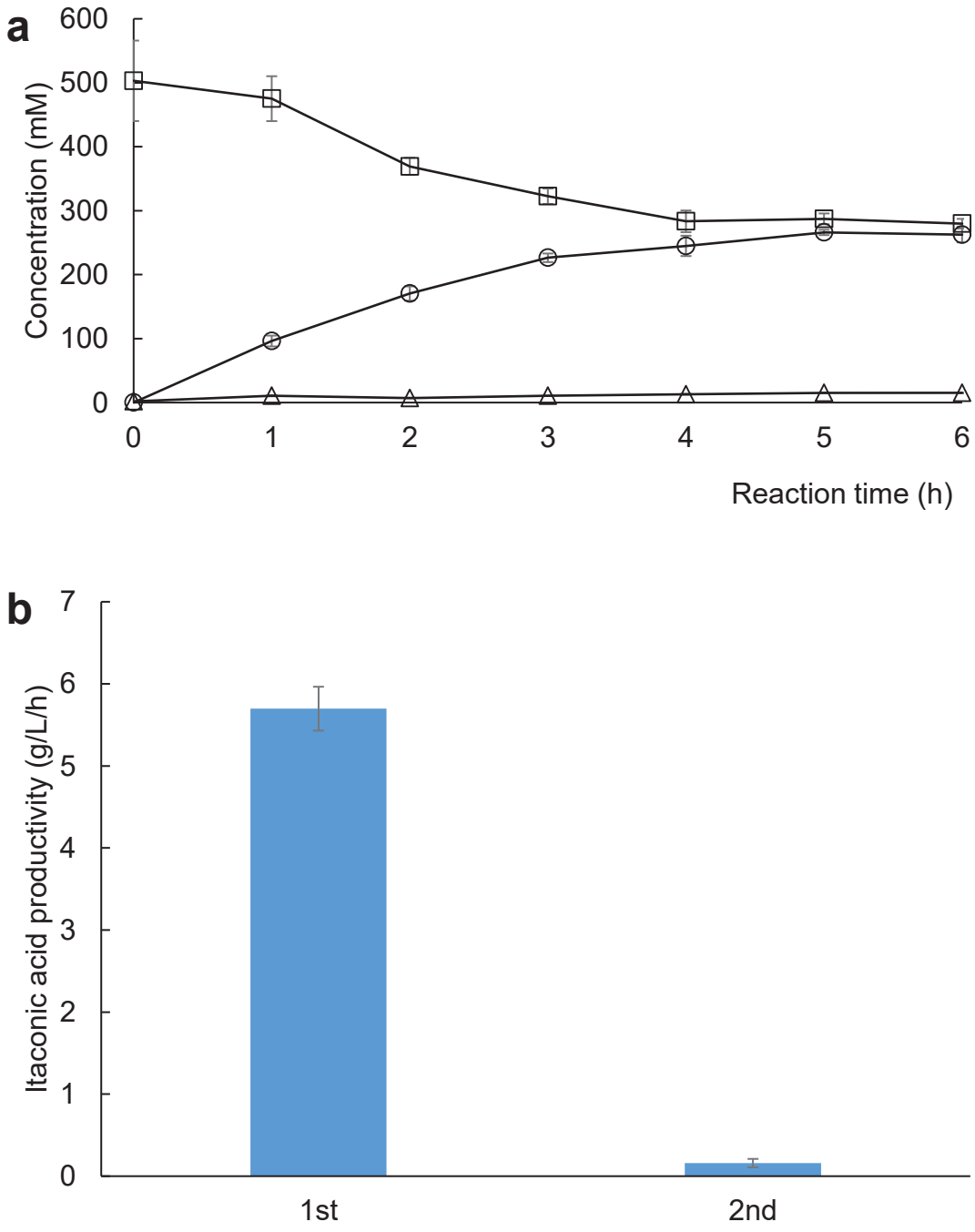

Fig. 3.9 Time-profile of itaconic production at 50 ºC by recombinant *S. livingstonensis* Ac10 cells after heat permeabilization (a). Rectangle, citric acid; circle, itaconic acid; triangle, *cis*-aconitic acid. Itaconic acid productivity of recycled cells (b). Error bars represent the standard deviation of the mean (n = 3).

3.3.5 Recycling of cells after heat treatment

After confirmation of no severe enzyme leakage after heat treatment, the sustainability of heat -treated *S. livingstonensis* Ac10 cells was investigated in terms of itaconic acid production. Recombinant cells were heated at 45 ºC for 15 min and applied for itaconic acid production at 28 °C in 15 mL tubes with 5 mL reaction mixture containing 50 mM citric acid. After a 10-h reaction, cells were separated via centrifugation and mixed with new reaction mixture. Concentration of itaconic acid was recorded at both start and end points of each batch so that itaconic productivity for each batch could be calculated. The result showed that after heat treatment, itaconic acid productivity of recombinant cells reached 0.47 g/L/h, and the productivity remained almost stable during the first four batches (Fig. 3.10). Notably, 67.3% of the productivity was observed in the fifth batch, revealing that the cell membrane integrity was still preserved to some extent after reactions. In contrast, when recombinant cells were washed by 50 mM PB buffer (pH = 6.5) once after each reaction, the productivity declined dramatically after the second batch suggesting that the cell membrane was fragile after heat treatment and may be disrupted by mechanical impact of pipetting.

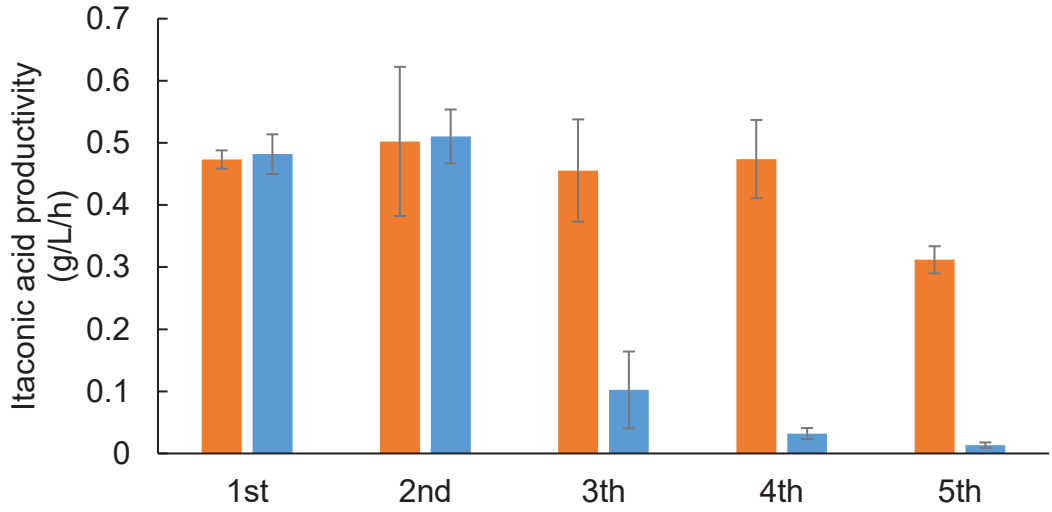

Fig. 3.10 Itaconic acid production by recycled recombinant *S. livingstonensis* Ac10 cells after heat permeabilization. Cells were separated from the reaction mixture every 10 hours via centrifugation and resuspended by the reaction mixture. Orange bars, cells without washing after each batch; Blue bars, cells washed with phosphate buffer (50 mM, pH = 6.5) after each batch. Error bars represent the standard deviation of the mean (n = 6).

3.3.6 Itaconic acid production was limited by product inhibition

It was estimated that costs of downstream process in itaconic acid production take up nearly half of the whole cost (59). Therefore, if high final concentration is achieved, it will significantly decrease the production cost. In 3.3.4, 500 mM citric acid was added to perform the itaconic acid production, but only approximately half of them were converted to itaconic acid, suggesting that the process may be inhibited by itaconic acid. To clarify if the reaction was inhibited by itaconic acid, cell suspension of PSCats was mixed with reaction mixture containing 500 mM citric acid and various concentrations of itaconic acid. As showed in Fig. 3.11, itaconic acid production decreased with more itaconic acid added in advance.

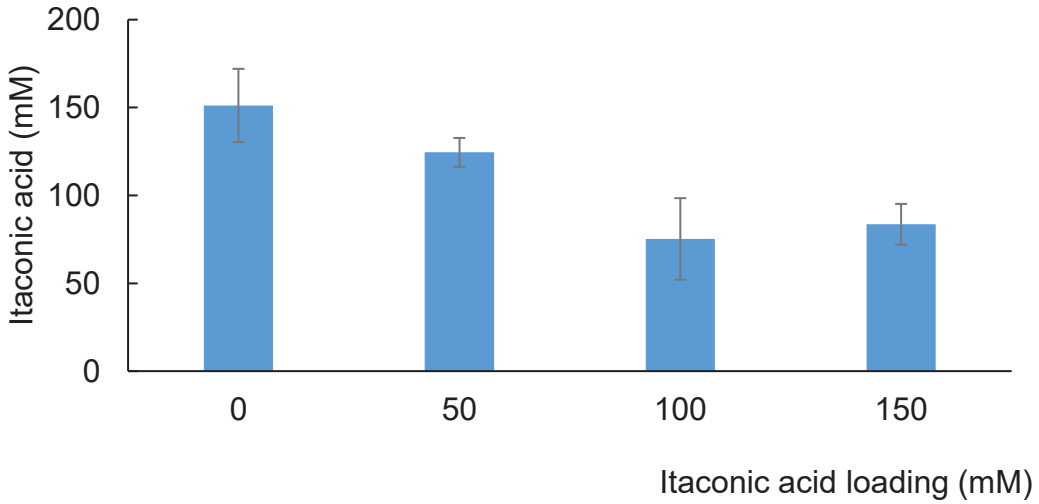

Fig. 3.11 Itaconic acid production by PSCat at 37 °C for 6 hours with various concentrations of itaconic acid added in advance. Error bars represent the standard deviation of the mean (n = 3).

One of the crucial enzymes involved in itaconic acid production, CAD was then cloned, expressed, and purified from recombinant *E. coli* to further confirm the inhibition. Product inhibition was also confirmed on purified CAD (Fig. 3.12).

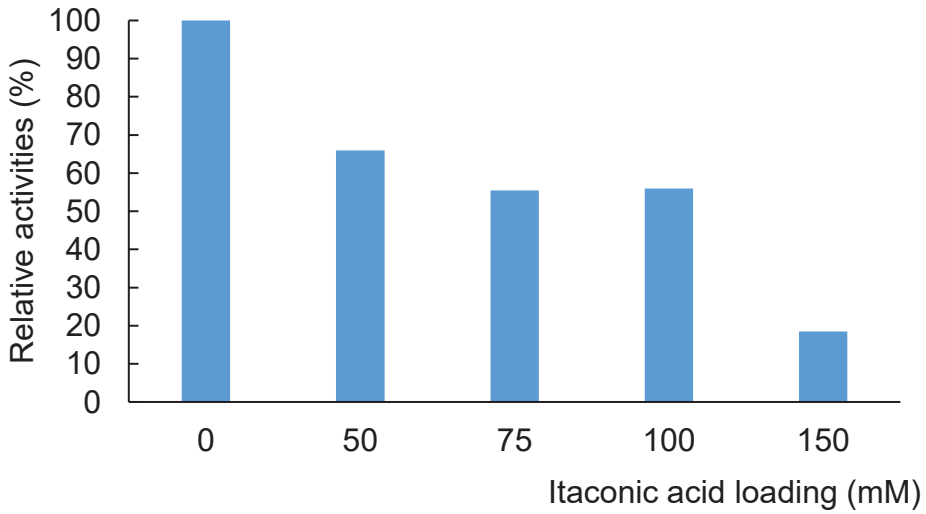

Fig. 3.12 CAD activities in existence of extra itaconic acid in the reaction mixture.

3.3.7 Identification of residues related to itaconic acid binding

Two pockets were found in the CAD (Fig. 3.13), pocket A is a putative active site, and pocket B is a putative itaconic acid binding site identified by docking simulation. There are 15 polar amino acid residues in these two pockets. To clarify if they are really associated with substrate or product binding, alanine substitutions were constructed and tested for enzyme activities. The result (Table 4.1) shows that most of the alanine mutants were completely inactivated and only S374A showed comparable activities, suggesting that these residues are indeed involved in substrate or product binding which means they are potential targets for protein engineering.

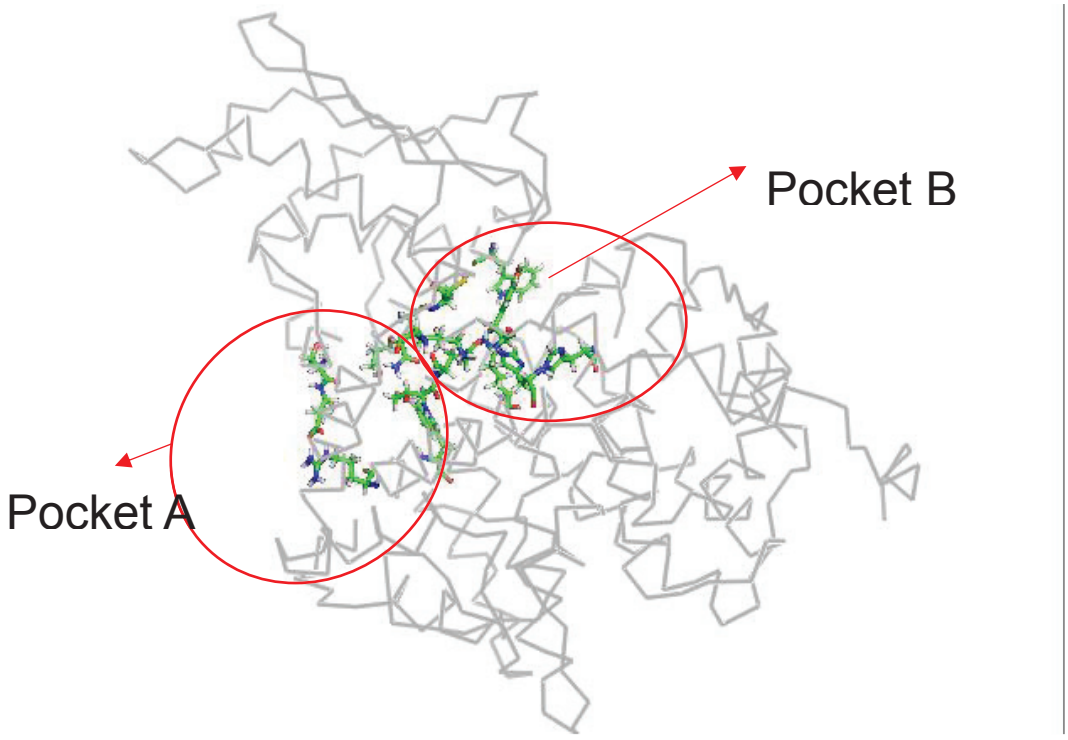

Fig. 3.13 Two putative pockets involved in substrate or product binding in *A. terreus* CAD, residues in pocket A: R54, W57, T99, S348, Q352, S374, E375; Residues in pocket B: E100, Y104, H111, H168, K217, K288, C293, W339.

Table 3.2 Activities of alanine substitutions of each residue in the pockets:

Pocket A:

	R54A	**W57A**	**T99A**	**S348A**	**Q352A**	**S374A**	**E375A**
Activities	ND	ND	ND	25.6%	ND	71.1%	ND

Pocket B:

	E00A	**Y104A**	**H111A**	**H168A**	**K217A**	**K288A**	**C293A**	**W339A**
Activities	ND	ND	ND	ND	ND	ND	ND	ND

3.3.8 Library screening

All possible variants of 15 residues were constructed, among those which are already tested, 11 of them (R54H, R54K, T99I, T99C, E100W, H168L, K217Q, W339N, S348V, S374N, E375P) showed comparable activities to the wild type but none of them can produce more itaconic acid from 500 mM *cis*-aconitic acid than the wild type.

3.3.9 *Shewanella frigidimarina* based whole-cell biocatalyst is not permeabilized by heat treatment

At first, a whole-cell biocatalyst based on a related species of *S. livingstonensis* was also constructed. Itaconic acid productivity of *S. frigidimarina* based whole-cell catalyst can also be improved by heat treatment. However, unlike *S. livingstonensis*, severe enzyme leakage was observed even without heat treatment (Fig. 3.14). This provides a clue for the mechanism of permeability change on *S. livingstonensis* because the two strains are assumed to be similar as described in chapter 2. By investigating the difference in membrane of two species, the mechanism of permeability improvement may be revealed.

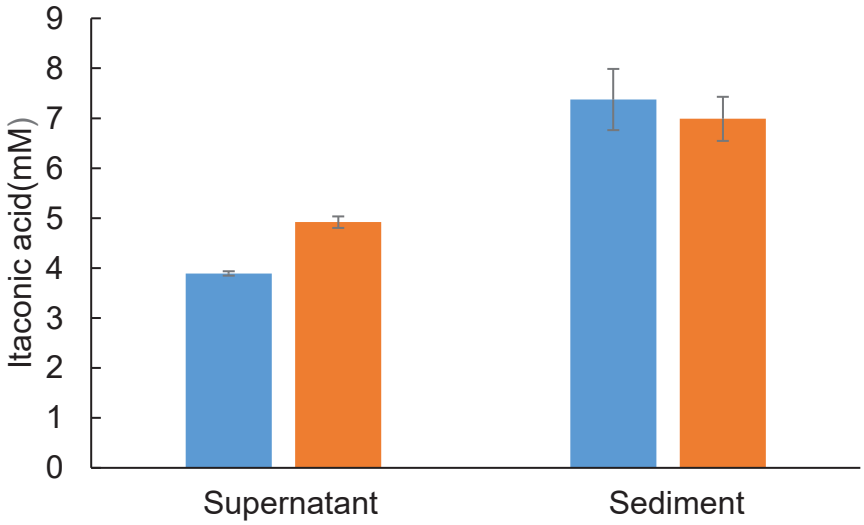

Fig. 3.14 Itaconic acid production by *S. frigidimarina* based whole-cell catalyst. Blue bar, without heat treatment, orange bar, with heat treatment at 40 °C for 5 min. Error bars represent the standard deviation of the mean (n = 3).

3.3.10 FAME composition of *S. frigidimarina* and *S. livingstonensis*

Phospholipids composition of *S. frigidimarina* and *S. livingstonensis* Ac10 was analyzed. The results are showed in Fig. 3. 15, of all five FAMEs detected by GC/MS, none of them showed a clear difference in amount between *S. frigidimarina* and *S. livingstonensis*, indicating that it is not the difference in phospholipids that causes the different behavior of these two strains after the heat treatment. The mechanism behind permeability improvement is therefore still unclear.

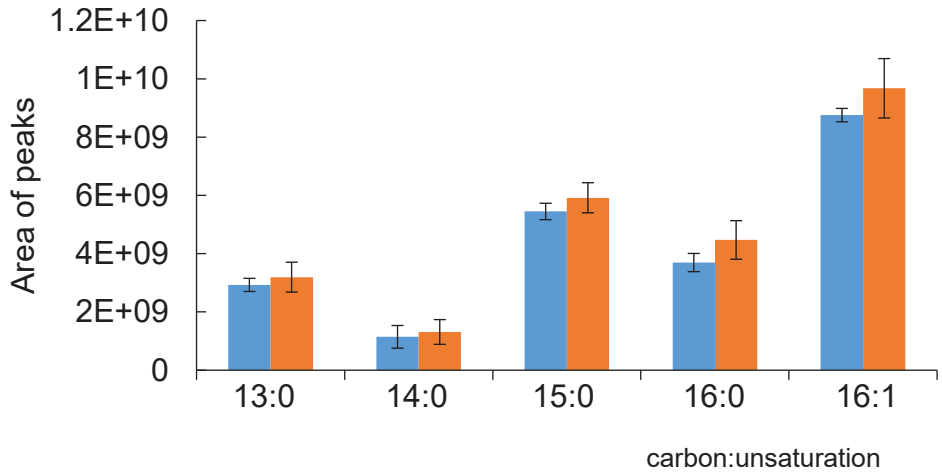

Fig. 3.15 FAME analysis result of *S. frigidimarina* and *S. livingstonensis*. Blue bar, *S. livingstonensis*, orange bar, *S. firigidimarina*.

3.4 Discussion

One of the major obstacles to the application of whole-cell biocatalyst is that cell membrane of the whole-cell biocatalyst host often serves as a barrier for substrate influx. Present chapter described a simple method to resolve the permeability issue by constructing whole-cell biocatalyst based on psychrophile. The results in this chapter indicate that the cell membrane of *S. livistonensis* Ac10 could be permeabilized at moderate conditions. The improvement on permeability was so effective that even the enzyme was partly inactivated, itaconic acid productivity of the whole-cell biocatalyst still increased significantly.

Generally speaking, high membrane fluidity is required for psychrophiles to grow and reproduce at a low temperature. However, it seems that it is not high membrane fluidity that facilitates citric acid influx because when recombinant *S. livingstonensis* Ac10 cells were intended for itaconic acid production without heat treatment, no significant difference in permeability was observed in comparison with *E. coli* cells. The reason underlying the improvement in membrane permeability in *S. livingstonensis* Ac10 after heat treatment remained unexplored. Some previous studies provide evidence to support this improvement in membrane permeability. The cell membrane of *E. coli* could be permeabilized at 55 °C due to membrane lipid loss, thereby increasing the porosity (60, 61); Similar phenomenon may occur at a lower heat treatment temperature for the psychrophilic cell membrane considering different lipid composition. Moreover, some membrane-bound molecules in psychrophiles may also be related to the membrane fluidity. For example, carotenoids are reportedly associated with regulating the membrane fluidity of some psychrophiles (62). A type of these pigment molecules in the cell membrane may dissociate from the cell membrane due to heat treatment,

thereby increasing the porosity of the cell membrane and resulting in more efficient membrane translocation of the substrate.

Based on current results, it is possible to recycle cells after heat treatment, but low reaction temperature and avoiding furious mechanical impacts are necessary because the cell membrane becomes vulnerable after heat treatment. The cell membrane integrity still remained, and a large part of itaconic acid productivity was preserved after the heat treatment when the cells were treated probably. Considering that it is difficult to recycle the cells permeabilized by surfactant because of cell lysis, psychrophile-based biocatalyst may be more suitable for industrial application to lower down the cost of culturing. Placing heat-treated in a more stable environment such as crosslinking the cells with the surface of specific materials (63) may help to improve the performance of them. The reaction temperature is another essential factor in influencing membrane integrity. 28 °C was chosen because *S. livingstonensis* Ac10 cannot grow at a temperature beyond 30 °C, the reaction temperature was slightly lower than it rather than the optimal temperature for itaconic acid production. The mechanism underlying the improvement in membrane permeability due to heat treatment remained unexplored, to intensify membrane tolerance to heat treatment and the reaction temperature, it is crucial to understand them in the future.

Product inhibition was confirmed both on the whole-cell biocatalyst and purified CAD, and it may be the reason for underachievement of high titer production. Considering the similarity of citric acid and itaconic acid on structure and chemical property, it may be difficult to separate them after the reaction. An attempt to engineer CAD to remove the product inhibition through directed-mutation was described.

Protein engineering is another powerful tool to eliminate or at least alleviate product inhibition (64). Traditionally, a random mutation library of the target protein was generated through Error-Prone PCR, the mutations were then tested in a high-throughput screen method to select desirable features. With the development of computer science and bioinformatics, it becomes possible to simulate interactions between enzymes and small molecules for example their substrates or inhibitors and delimit the scale of residue mutations. In this way, a library containing a small number of mutations could be constructed therefore reduce the labor for screening significantly (65).

Since crystal structure of mouse CAD has been identified recently (66), a docking simulation between CAD and its inhibitor, itaconic acid was performed, based on the docking result a library containing 285 mutants was constructed and some of them were tested for their itaconic acid tolerance. Unfortunately, no tested mutations showed better performance than the wild type.

Phospholipids composition of *S. livingstonensis* was analyzed and compared to a related species, *S. frigidimarina*, which cannot be permeabilized by heat treatment but lose some enzymes because the cell membrane is damaged. No difference in phospholipids composition was found between these two species and the mechanism of heat permeabilization remained unclear.

Chapter 4 General discussion and conclusion

Enzymes are essential components for biomass-based chemical industry, and whole-cell biocatalyst is one of the most effective tools human beings have to introduce enzymes into industry field. However, despite those great achievements which have been made, the fields for enzymatic reactions to step in and replace traditional processes are still limited. Pyschrophile-based simple biocatalyst is a new approach to solve some of these problems. The present research focuses on two limitations of whole-cell biocatalyst, side reactions caused by host homologous enzymes and cell membrane barrier which blocks the substrate influx in the whole-cell biocatalysts.

In chapter 2, an overall investigation of enzyme thermostability in *S livingstonensis* Ac10 was made. An NADP-dependent malic enzyme (SL-ME) with unexpected thermostability was found. It has operational temperatures even higher than its mesophilic counterpart, an NADP-dependent malic enzyme from *E. coli* (MaeB). SL-ME was cloned and expressed in *E. coli*, purified SL-ME was used to investigate its enzymatic characteristics including kinetic parameters, thermostability measured both by specific activities after heat treatment and by CD ellipticity at 222 nm, as well as its operational temperature range. The results clearly show that SL-ME does not share any similarity in terms of thermostability and functional temperature range with other homologous enzymes in *S. livingstonensis* Ac10. Based on sequence alignment with malic enzyme genes from other *Shewanella* species, malic enzyme genes are highly conserved among those species no matter in mesophilic or psychrophilic bacteria suggesting that malic enzyme may play a crucial role in *Shewanella* species.

Psychrophilic bacteria were assumed to be heat-vulnerable and that is the reason why they were chosen as the host for whole-cell biocatalysts. By all of 8 organic acid relevant enzymes investigated, only SL-ME is thermostable and the rest of them are all inactivated at moderate temperatures. Therefore, the concept that expressing mesophilic enzymes in psychrophile and then inactivate host homologous enzymes is proven to be realistic. Discovery of thermostable malic enzyme in psychrophilic bacterium suggests that there may be some reactions that could be distracted by homologous enzymes and knowledge on the host enzyme's thermostability will pave the way for us to design and introduce new pathways to the host cell.

In chapter 3, a new approach was developed to solve the permeability issue of whole cell biocatalyst. A whole-cell biocatalyst based on psychrophilic bacterium *S. livingstonensis* Ac10, was constructed. The cells express two crucial enzymes, aconitase B from *E. coli* and *cis*-aconitic acid decarboxylase from *A. terreus*, for itaconic acid production from citric acid. Cells were heat-treated before application for itaconic acid production.

Optimal conditions for heat treatment to improve the membrane permeability and preserve the enzymatic activity simultaneously. Optimal reaction temperature for itaconic acid production was also explored. The optimal reaction temperature was 50 °C but the cells lost their recyclability after reaction at such a high temperature.

Itaconic acid productivity was improved approximately 6 folds by heat treatment at 45 °C for 15 minutes. And the cells could be recycled up to 5 times after the reaction. According to Kim et al. (48), citric acid is difficult to be taken by *E. coli* based whole-cell biocatalyst. To solve this problem, they used detergent to lyse the cells while in the meantime the membrane integrity is lost. Considering the price of itaconic acid and its role as a basic building block for chemical

industry, low production is definitely desirable. Therefore, it is favorable to recycle cells after the production.

Compared to other methods to improve membrane permeability such as surfactant treatment or solvent treatment, heat treatment is more feasible for industrial application because it does not introduce any additional molecules in the reaction mixture thus simplifies downstream process for purifying the product. In this chapter, an attempt to produce a high titer of itaconic acid was made but failed, probably because of the product inhibition. Current chapter is aiming to remove the product inhibition through directed mutation of CAD.

Final titer of the product is also an important parameter which need to be considered when constructing a real-world process because a high titer will significantly decrease the downstream cost. Product inhibition has been the main obstacle for bioconversion (67). One of the most well-known product inhibition cases is Acetone Butanol Ethanol (ABE) production, in which the production of multiple solvents, especially butanol (68) is strictly limited. Taking the advantage of volatility of the solvent, a gas stripping approach has been developed to remove products from the reaction mixture simultaneously. The final titer has been increased significantly by gas stripping, final titer of ABE production by *Clostridium beijerinckii* BA101 can be increased from 27-29 g/L to an astonishingly 165g/L. Since citric acid and itaconic acid are not as different as glucose and organic solvent involved in ABE production, protein engineering was selected to remove or at least alleviate the product inhibition. Unfortunately, no promising mutants were found indicating the catalyzing efficiency of *A. terreus* CAD may already reach the optimal range. Other methods may need to be considered to improve the tier of itaconic acid such as In Situ Product Removal (ISPR) Strategies (69).

The present study demonstrates the potential of psychrophilic bacterium *S. livingstonensis* Ac10 as the host for whole-cell biocatalyst to produce itaconic acid from citric acid and the feasibility of increasing the permeability of the cell membrane by heat treatment at a moderate condition. Based on the present knowledge, no previous study has focused on the methods to increase cell membrane permeability of psychrophiles.

In summary, current thesis explored the potential of *S. livingstonensis* Ac10 as a host for whole-cell biocatalyst as well as develop a promising process for producing valuable chemicals. By investigating several key enzymes involved in organic acid metabolism, the fact that most of them could be heat inactivated has been confirmed. Which supports the very basic concept of psychrophile-based simple biocatalyst, e.g., introducing heterologous enzymes from mesophiles into psychrophiles, and then inactivate homologous enzymes in hosts to eliminate side reactions thus improve the yield of target products. There is also an unexpected discovery of a thermostable malic enzyme in *S. livingstonensis* Ac10. The enzyme was characterized but the mechanism laying behind its irregular thermostability along with its role in metabolism is still unclear. Itaconic acid was selected as the target product because its production is reportedly impeded by the cell membrane barrier and no ideal solutions have been found yet. To improve substrate permeability, cells were treated by heat and, under the optimal condition for heat treatment and enzyme activity, productivity of itaconic could be increased significantly and the cells could be reused after the production. A better understanding of the membrane structure and the mechanism to increase its permeability is necessary for further development of this approach and its industrial application.

Acknowledgements

This doctoral thesis is submitted to the Graduate School of Advanced Science of Matter, Hiroshima University. The research was conducted in the Laboratory of Biomolecular Technology, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, during the academic periods from 2018 to 2021.

I would like to express the most profound appreciation to my former advisor, Prof. Junichi Kato, and current advisor Prof . Takahisa Tajima, who have been supporting me before and throughout my Doctoral course and thesis. Their knowledge and patience have always been surprising and encouraging me. The guidance of their intelligentsia gave me the opportunity to pursue my research in my own way. Their wise support conveyed the spirit of adventure in regard to research and teaching me. Without their trust and persistent help, this project would not have been possible.

My sincere thanks also go to Prof. Yoshihiro Sambongi and Assistant Professor Sotaro Fujii in Graduate School of Integrated Sciences for Life, Hiroshima University for their valuable guidance, assistance, and technical skills. I will never forget his influential role and kindness in making this project a success.

I would like to thank all the staff of the Laboratory of Biomolecular Technology (Associate Professor Makoto Fujie, Assistant Professor Takeru Kawasaki, and Assistant Professor Akiko Hida) for their valuable discussion and encouragement.

I would also like to thank my fellow researchers, Mr. Sho Nakano, Ms. Miho Fujino and Mr. Takumi Koda), who helped me a lot in this project. I thank Professor Tatsuo Kurihara at Kyoto

University for providing *S. livingstonensis* Ac10.

I would also like to thank my dissertation committee members, Professor Yutaka Nakashimada and Professor Akio Kuroda, who offered insightful comments.

Special thanks to my friend Dr. Mohammad Mojarrad who has been working with me in the same place for 3 years. I'm lucky to have him suffering with me.

Finally, I want to thank my family for supporting and encouraging me to make this possible.

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Appendix

Primers used for mutation library construction

Primer name	Sequence (5’ - 3’)
Ala substitution	
R54A_F	ATGGGTTGGCGCTGCTGTG
R54A_R	GCGCAGGCAATACCGTCTAA
W57A_F	TGCCAGCTAGTGAGAAATATG
W57A_R	CACGAGCGCCAACCCATGC
T99A_F	AGCTGCTGAATTAGACGATTATC
T99A_R	TGAATGAATGCACTATTAG
E100A_F	AGCTACCGCTTTAGACGATTATC
E100A_R	TGAATGAATGCACTATTAGTC
Y104A_F	AGACGATGCTCATAGTGAAGCAC
Y104A_R	AATTCGGTAGCTTGAATGAATGC
H111A_F	AAGCACCTTTAGCTAGTGCTAGTAT
H111A_R	CACTATGATAATCGTCTAATTC
H168A_F	ACGGATGGGCTTGCGGAGCGGT
H168A_R	TATTAAGCAAGTCAGAGCCGT
K217A_F	GTATGGTTGCTCGCGTTCAGCA
K217A_R	CTCCATATTGGGCCGACATCA
K288A_F	ATCCGTATCGCTCTTTACGCATG
K288A_R	GGTAAAGGTATGCCAAAAGCT

Primers used for mutation library construction (continued)

C293A_F	ACGCATGTGCTGGTTTAGTCCATG
C293A_R	AAAGTTTGATACGGATGGTAAAG
W339A_F	ACATTGTGGTGCTATTCCTGAAGAGC
W339A_R	GAATTAGAGGCGGTTGATAAC
S348A_F	CGATTAGCGCTATTGCCGGTCAAAT
S348A_R	GGCGCTCTTCAGGAATCCA
Q352A_F	GGTGCTATGTCAGTGGCCTAT
Q352A_R	GGCAATGCTGCTAATCGGGCG
S374A_F	CAATTTGCTGAATTTGATGATAAT
S374A_R	GGCTAAAAGACATTGTTGGT
E375A_F	ATTTTCAGCTTTTGATGATAAT
R54	
R54F_F	GGGTTGGCGCTTTCGTGCCATGGAG
R54F_R	CTCCATGGCACGAAAGCGCCAACCC
R54L_F	GGGTTGGCGCTCTGGTGCCATGGAG
R54L_R	CTCCATGGCACCAGAGCGCCAACCC
R54I_F	GGGTTGGCGCTATTGTGCCATGGAG
R54I_R	CTCCATGGCACAATAGCGCCAACCC
R54M_F	ATGGGTTGGCGCTATGGTG
R54S_F	GGGTTGGCGCTAGCGTGCCATGGAG

Primers used for mutation library construction (continued)

R54S_R	CTCCATGGCACGCTAGCGCCAACCC
R54T_F	GGGTTGGCGCTACGGTGCCATGGAG
R54T_R	CTCCATGGCACCGTAGCGCCAACCC
R54K_F	ATGGGTTGGCGCTAAGGTG
R54Y_F	ATGGGTTGGCGCTTATGTG
R54H_F	GGGTTGGCGCTCATGTGCCATGGAG
R54H_R	CTCCATGGCACATGAGCGCCAACCC
R54Q_F	ATGGGTTGGCGCTCAGGTG
R54N_F	ATGGGTTGGCGCTAACGTG
R54D_F	GGGTTGGCGCTGACGTGCCATGGAG
R54D_R	CTCCATGGCACGTCAGCGCCAACCC
R54E_F	ATGGGTTGGCGCTGAGGTG
R54W_F	ATGGGTTGGCGCTTGGGTG
R54C_F	GGGTTGGCGCTTGCGTGCCATGGAG
R54C_R	CTCCATGGCACGCAAGCGCCAACCC
R54P_F	GGGTTGGCGCTCCGGTGCCATGGAG
R54P_R	CTCCATGGCACCGGAGCGCCAACCC
R54G_F	GGGTTGGCGCTGGCGTGCCATGGAG
R54G_R	CTCCATGGCACGCCAGCGCCAACCC
R54V_F	ATGGGTTGGCGCTGTGGTG

Primers used for mutation library construction (continued)

W57	
W57F_F	TGCCATTCAGTGAGAAATATG
W57L_F	TGCCACTGAGTGAGAAATATG
W57I_F	TGCCAATTAGTGAGAAATATG
W57M_F	TGCCAATGAGTGAGAAATATG
W57S_F	TGCCAAGCAGTGAGAAATATG
W57T_F	TCGTGTGCCAACGAGTGAGAAATAT
W57T_R	GCGCCAACCCATGCGCAGGCAAT
W57K_F	TGCCAAAGAGTGAGAAATATG
W57Y_F	TGCCATATAGTGAGAAATATG
W57H_F	TGCCACATAGTGAGAAATATG
W57Q_F	TGCCACAGAGTGAGAAATATG
W57N_F	TGCCAAACAGTGAGAAATATG
W57D_F	TGCCAGACAGTGAGAAATATG
W57E_F	TGCCAGAGAGTGAGAAATATG
W57C_F	TGCCATGCAGTGAGAAATATG
W57R_F	TGCCACGTAGTGAGAAATATG
W57P_F	TGCCACCGAGTGAGAAATATG
W57G_F	TGCCAGGCAGTGAGAAATATG
W57V_F	TGCCAGTGAGTGAGAAATATG
T99	

Primers used for mutation library construction (continued)

T99F_F	TCATTCAAGCTTTCGAATTAGACGA
T99F_R	TCGTCTAATTCGAAAGCTTGAATGA
T99L_F	AGCTCTGGAATTAGACGATTATC
T99I_F	TCATTCAAGCTATTGAATTAGACGA
T99I_R	TCGTCTAATTCAATAGCTTGAATGA
T99M_F	AGCTATGGAATTAGACGATTATC
T99S_F	AGCTAGCGAATTAGACGATTATC
T99K_F	AGCTAAGGAATTAGACGATTATC
T99Y_F	AGCTTATGAATTAGACGATTATC
T99H_F	AGCTCATGAATTAGACGATTATC
T99Q_F	AGCTCAGGAATTAGACGATTATC
T99N_F	AGCTAACGAATTAGACGATTATC
T99D_F	AGCTGACGAATTAGACGATTATC
T99E_F	AGCTGAGGAATTAGACGATTATC
T99W_F	AGCTTGGGAATTAGACGATTATC
T99C_F	AGCTTGCGAATTAGACGATTATC
T99R_F	AGCTCGTGAATTAGACGATTATC
T99P_F	AGCTCCGGAATTAGACGATTATC
T99G_F	AGCTGGCGAATTAGACGATTATC
T99V_F	TCAAGCTGTGGAATTAGACGAT
T99V_F	TGAATGAATGCACTATTAG

Primers used for mutation library construction (continued)

E100	
E100F_F	AGCTACCTTCTTAGACGATTATC
E100L_F	AGCTACCCTGTTAGACGATTATC
E100I_F	AGCTACCATTTTAGACGATTATC
E100M_F	AGCTACCATGTTAGACGATTATC
E100S_F	AGCTACCAGCTTAGACGATTATC
E100T_F	AGCTACCACGTTAGACGATTATC
E100K_F	AGCTACCAAGTTAGACGATTATC
E100Y_F	AGCTACCTATTTAGACGATTATC
E100H_F	AGCTACCCATTTAGACGATTATC
E100Q_F	AGCTACCCAGTTAGACGATTATC
E100N_F	AGCTACCAACTTAGACGATTATC
E100D_F	AGCTACCGACTTAGACGATTATC
E100W_F	AGCTACCTGGTTAGACGATTATC
E100C_F	AGCTACCTGCTTAGACGATTATC
E100R_F	AGCTACCCGTTTAGACGATTATC
E100P_F	AGCTACCCCGTTAGACGATTATC
E100G_F	AGCTACCGGCTTAGACGATTATC
E100V_F	AGCTACCGTGTTAGACGATTATC
Y104	
Y104F_F	AGACGATTTCCATAGTGAAGCAC

Primers used for mutation library construction (continued)

Y104L_F	AGACGATCTGCATAGTGAAGCAC
Y104I_F	AGACGATATTCATAGTGAAGCAC
Y104M_F	AGACGATATGCATAGTGAAGCAC
Y104S_F	AGACGATAGCCATAGTGAAGCAC
Y104T_F	AGACGATACGCATAGTGAAGCAC
Y104K_F	AGACGATAAGCATAGTGAAGCAC
Y104H_F	AGACGATCATCATAGTGAAGCAC
Y104Q_F	AGACGATCAGCATAGTGAAGCAC
Y104N_F	AGACGATAACCATAGTGAAGCAC
Y104D_F	AGACGATGACCATAGTGAAGCAC
Y104E_F	AGACGATGAGCATAGTGAAGCAC
Y104W_F	AGACGATTGGCATAGTGAAGCAC
Y104C_F	AGACGATTGCCATAGTGAAGCAC
Y104R_F	AGACGATCGTCATAGTGAAGCAC
Y104P_F	AGACGATCCGCATAGTGAAGCAC
Y104G_F	AGACGATGGCCATAGTGAAGCAC
Y104V_F	AGACGATGTGCATAGTGAAGCAC
H111	
H111F_F	AAGCACCTTTATTCAGTGCTAGTAT
H111F_R	ATACTAGCACTGAATAAAGGTGCTT

Primers used for mutation library construction (continued)

H111L_F	AAGCACCTTTACTGAGTGCTAGTAT
H111L_R	ATACTAGCACTCAGTAAAGGTGCTT
H111I_F	AAGCACCTTTAATTAGTGCTAGTAT
H111M_F	AAGCACCTTTAATGAGTGCTAGTAT
H111S_F	AAGCACCTTTAAGCAGTGCTAGTAT
H111S_R	ATACTAGCACTGCTTAAAGGTGCTT
H111T_F	AAGCACCTTTAACGAGTGCTAGTAT
H111T_R	ATACTAGCACTCGTTAAAGGTGCTT
H111K_F	AAGCACCTTTAAAGAGTGCTAGTAT
H111K_R	ATACTAGCACTCTTTAAAGGTGCTT
H111Y_F	AAGCACCTTTATATAGTGCTAGTAT
H111Q_F	AAGCACCTTTACAGAGTGCTAGTAT
H111Q_R	ATACTAGCACTCTGTAAAGGTGCTT
H111N_F	AAGCACCTTTAAACAGTGCTAGTAT
H111N_R	ATACTAGCACTGTTTAAAGGTGCTT
H111D_F	AAGCACCTTTAGACAGTGCTAGTAT
H111E_F	AAGCACCTTTAGAGAGTGCTAGTAT
H111W_F	AAGCACCTTTATGGAGTGCTAGTAT
H111W_R	ATACTAGCACTCCATAAAGGTGCTT
H111C_F	AAGCACCTTTATGCAGTGCTAGTAT
H111R_F	AAGCACCTTTACGTAGTGCTAGTAT

Primers used for mutation library construction (continued)

H111R_R	ATACTAGCACTACGTAAAGGTGCTT
H111P_F	AAGCACCTTTACCGAGTGCTAGTAT
H111P_R	ATACTAGCACTCGGTAAAGGTGCTT
H111G_F	AAGCACCTTTAGGCAGTGCTAGTAT
H111V_F	AAGCACCTTTAGTGAGTGCTAGTAT
H168	
H168F_F	ACGGATGGTTCTGCGGAGCGGT
H168L_F	ACGGATGGCTGTGCGGAGCGGT
H168I_F	ACGGATGGATTTGCGGAGCGGT
H168M_F	ACGGATGGATGTGCGGAGCGGT
H168S_F	ACGGATGGAGCTGCGGAGCGGT
H168T_F	ACGGATGGACGTGCGGAGCGGT
H168K_F	ACGGATGGAAGTGCGGAGCGGT
H168Y_F	ACGGATGGTATTGCGGAGCGGT
H168Q_F	ACGGATGGCAGTGCGGAGCGGT
H168N_F	ACGGATGGAACTGCGGAGCGGT
H168D_F	ACGGATGGGACTGCGGAGCGGT
H168E_F	ACGGATGGGAGTGCGGAGCGGT
H168W_F	ACGGATGGTGGTGCGGAGCGGT

Primers used for mutation library construction (continued)

H168C_F	ACGGATGGTGCTGCGGAGCGGT
H168R_F	ACGGATGGCGTTGCGGAGCGGT
H168P_F	ACGGATGGCCGTGCGGAGCGGT
H168G_F	ACGGATGGGGCTGCGGAGCGGT
H168V_F	ACGGATGGGTGTGCGGAGCGGT
K217	
K217F_F	GTATGGTTTTCCGCGTTCAGCA
K217L_F	GTATGGTTCTGCGCGTTCAGCA
K217I_F	GTATGGTTATTCGCGTTCAGCA
K217M_F	GTATGGTTATGCGCGTTCAGCA
K217S_F	GTATGGTTAGCCGCGTTCAGCA
K217T_F	GTATGGTTACGCGCGTTCAGCA
K217Y_F	GTATGGTTTATCGCGTTCAGCA
K217H_F	GTATGGTTCATCGCGTTCAGCA
K217Q_F	GTATGGTTCAGCGCGTTCAGCA
K217N_F	GTATGGTTAACCGCGTTCAGCA
K217D_F	GTATGGTTGACCGCGTTCAGCA
K217E_F	GTATGGTTGAGCGCGTTCAGCA
K217W_F	GTATGGTTTGGCGCGTTCAGCA
K217C_F	GTATGGTTTGCCGCGTTCAGCA

Primers used for mutation library construction (continued)

K217R_F	GTATGGTTCGTCGCGTTCAGCA
K217P_F	GTATGGTTCCGCGCGTTCAGCA
K217G_F	GTATGGTTGGCCGCGTTCAGCA
K217V_F	GTATGGTTGTGCGCGTTCAGCA
K288	
K288F_F	ATCCGTATCTTCCTTTACGCATG
K288L_F	ATCCGTATCCTGCTTTACGCATG
K288I_F	ATCCGTATCATTCTTTACGCATG
K288M_F	ATCCGTATCATGCTTTACGCATG
K288S_F	ATCCGTATCAGCCTTTACGCATG
K288T_F	ATCCGTATCACGCTTTACGCATG
K288Y_F	ATCCGTATCTATCTTTACGCATG
K288H_F	ATCCGTATCCATCTTTACGCATG
K288Q_F	ATCCGTATCCAGCTTTACGCATG
K288N_F	ATCCGTATCAACCTTTACGCATG
K288D_F	ATCCGTATCGACCTTTACGCATG
K288E_F	ATCCGTATCGAGCTTTACGCATG
K288W_F	ATCCGTATCTGGCTTTACGCATG
K288C_F	ATCCGTATCTGCCTTTACGCATG
K288R_F	ATCCGTATCCGTCTTTACGCATG

Primers used for mutation library construction (continued)

K288P_F	ATCCGTATCCCGCTTTACGCATG
K288G_F	ATCCGTATCGGCCTTTACGCATG
K288V_F	ATCCGTATCGTGCTTTACGCATG
C293	
C293F_F	TTTACGCATGTTTCGGTTTAGTCCA
C293F_R	TGGACTAAACCGAAACATGCGTAAA
C293L_F	ACGCATGTCTGGGTTTAGTCCATG
C293I_F	ACGCATGTATTGGTTTAGTCCATG
C293M_F	ACGCATGTATGGGTTTAGTCCATG
C293S_F	ACGCATGTAGCGGTTTAGTCCATG
C293T_F	ACGCATGTACGGGTTTAGTCCATG
C293K_F	ACGCATGTAAGGGTTTAGTCCATG
C293Y_F	ACGCATGTTATGGTTTAGTCCATG
C293H_F	ACGCATGTCATGGTTTAGTCCATG
C293Q_F	ACGCATGTCAGGGTTTAGTCCATG
C293N_F	ACGCATGTAACGGTTTAGTCCATG
C293D_F	ACGCATGTGACGGTTTAGTCCATG
C293E_F	ACGCATGTGAGGGTTTAGTCCATG
C293W_F	ACGCATGTTGGGGTTTAGTCCATG
C293R_F	ACGCATGTCGTGGTTTAGTCCATG

Primers used for mutation library construction (continued)

C293P_F	ACGCATGTCCGGGTTTAGTCCATG
C293G_F	ACGCATGTGGCGGTTTAGTCCATG
C293V_F	ACGCATGTGTGGGTTTAGTCCATG
W339	
W339F_F	ACATTGTGGTTTCATTCCTGAAGAGC
W339L_F	ACATTGTGGTCTGATTCCTGAAGAGC
W339I_F	ACATTGTGGTATTATTCCTGAAGAGC
W339M_F	ACATTGTGGTATGATTCCTGAAGAGC
W339S_F	ACATTGTGGTAGCATTCCTGAAGAGC
W339T_F	ACATTGTGGTACGATTCCTGAAGAGC
W339K_F	ACATTGTGGTAAGATTCCTGAAGAGC
W339Y_F	ACATTGTGGTTATATTCCTGAAGAGC
W339H_F	ACATTGTGGTCATATTCCTGAAGAGC
W339Q_F	ACATTGTGGTCAGATTCCTGAAGAGC
W339N_F	ACATTGTGGTAACATTCCTGAAGAGC
W339D_F	ACATTGTGGTGACATTCCTGAAGAGC
W339E_F	ACATTGTGGTGAGATTCCTGAAGAGC
W339C_F	ACATTGTGGTTGCATTCCTGAAGAGC
W339R_F	ACATTGTGGTCGTATTCCTGAAGAGC
W339P_F	ACATTGTGGTCCGATTCCTGAAGAGC

Primers used for mutation library construction (continued)

W339G_F	ACATTGTGGTGGCATTCCTGAAGAGC
W339V_F	ACATTGTGGTGTGATTCCTGAAGAGC
S348	
S348F_F	CGATTAGCTTCATTGCCGGTCAAAT
S348L_F	CGATTAGCCTGATTGCCGGTCAAAT
S348I_F	CGATTAGCATTATTGCCGGTCAAAT
S348M_F	CGATTAGCATGATTGCCGGTCAAAT
S348T_F	CGATTAGCACGATTGCCGGTCAAAT
S348K_F	CGATTAGCAAGATTGCCGGTCAAAT
S348Y_F	CGATTAGCTATATTGCCGGTCAAAT
S348H_F	CGATTAGCCATATTGCCGGTCAAAT
S348Q_F	CGATTAGCCAGATTGCCGGTCAAAT
S348N_F	CGATTAGCAACATTGCCGGTCAAAT
S348D_F	CGATTAGCGACATTGCCGGTCAAAT
S348E_F	CGATTAGCGAGATTGCCGGTCAAAT
S348W_F	CGATTAGCTGGATTGCCGGTCAAAT
S348C_F	CGATTAGCTGCATTGCCGGTCAAAT
S348R_F	CGATTAGCCGTATTGCCGGTCAAAT
S348P_F	CGATTAGCCCGATTGCCGGTCAAATGTCA
S348G_F	CGATTAGCGGCATTGCCGGTCAAAT

Primers used for mutation library construction (continued)

S348V_F	CGATTAGCGTGATTGCCGGTCAAAT
Q352	
Q352F_F	GGTTTCATGTCAGTGGCCTAT
Q352L_F	GGTCTGATGTCAGTGGCCTAT
Q352I_F	GGTATTATGTCAGTGGCCTAT
Q352M_F	GGTATGATGTCAGTGGCCTAT
Q352S_F	GGTAGCATGTCAGTGGCCTAT
Q352T_F	GGTACGATGTCAGTGGCCTAT
Q352K_F	GGTAAGATGTCAGTGGCCTAT
Q352Y_F	GGTTATATGTCAGTGGCCTAT
Q352H_F	GGTCATATGTCAGTGGCCTAT
Q352N_F	GGTAACATGTCAGTGGCCTAT
Q352D_F	GGTGACATGTCAGTGGCCTAT
Q352E_F	GGTGAGATGTCAGTGGCCTAT
Q352W_F	GGTTGGATGTCAGTGGCCTAT
Q352C_F	GGTTGCATGTCAGTGGCCTAT
Q352R_F	GGTCGTATGTCAGTGGCCTAT
Q352P_F	GGTCCGATGTCAGTGGCCTAT
Q352G_F	GGTGGCATGTCAGTGGCCTAT
Q352V_F	GGTGTGATGTCAGTGGCCTAT

Primers used for mutation library construction (continued)

S374	
S374F_F	CAATTTTTCGAATTTGATGATAAT
S374L_F	CAATTTCTGGAATTTGATGATAAT
S374I_F	CAATTTATTGAATTTGATGATAAT
S374M_F	CAATTTATGGAATTTGATGATAAT
S374T_F	CAATTTACGGAATTTGATGATAAT
S374K_F	CAATTTAAGGAATTTGATGATAAT
S374Y_F	CAATTTTATGAATTTGATGATAAT
S374H_F	CAATTTCATGAATTTGATGATAAT
S374Q_F	CAATTTCAGGAATTTGATGATAAT
S374N_F	CAATTTAACGAATTTGATGATAAT
S374D_F	CAATTTGACGAATTTGATGATAAT
S374E_F	CAATTTGAGGAATTTGATGATAAT
S374W_F	CAATTTTGGGAATTTGATGATAAT
S374C_F	CAATTTTGCGAATTTGATGATAAT
S374R_F	CAATTTCGTGAATTTGATGATAAT
S374P_F	CAATTTCCGGAATTTGATGATAAT
S374G_F	CAATTTGGCGAATTTGATGATAAT
S374V_F	CAATTTGTGGAATTTGATGATAAT
E375	

Primers used for mutation library construction (continued)

E375F_F	ATTTTCATTCTTTGATGATAAT
E375L_F	ATTTTCACTGTTTGATGATAAT
E375I_F	ATTTTCAATTTTTGATGATAAT
E375M_F	ATTTTCAATGTTTGATGATAAT
E375S_F	ATTTTCAAGCTTTGATGATAAT
E375T_F	ATTTTCAACGTTTGATGATAAT
E375K_F	ATTTTCAAAGTTTGATGATAAT
E375Y_F	ATTTTCATATTTTGATGATAAT
E375H_F	ATTTTCACATTTTGATGATAAT
E375Q_F	ATTTTCACAGTTTGATGATAAT
E375N_F	ATTTTCAAACTTTGATGATAAT
E375D_F	ATTTTCAGACTTTGATGATAAT
E375W_F	ATTTTCATGGTTTGATGATAAT
E375C_F	ATTTTCATGCTTTGATGATAAT
E375R_F	ATTTTCACGTTTTGATGATAAT
E375P_F	ATTTTCACCGTTTGATGATAAT
E375G_F	ATTTTCAGGCTTTGATGATAAT
E375V_F	ATTTTCAGTGTTTGATGATAAT

*Residues in red are substitutions.

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