博士論文

Development of psychrophile-based simple biocatalysts for simultaneous biosynthesis of 1,3-propanediol and 3-hydroxypropionic acid

（低温菌シンプル酵素触媒による 1,3-プロパンジオールと 3-ヒドロキシプロピオン酸の共生産）

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Chapter I

General Introduction

3-Hydroxypropionic acid (3-HP) is a three-carbon organic molecule and one of the attractive building-block chemicals. This chemical could be converted into various valuable compounds such as acrylic acid, methyl acrylate, and acrylamide due to two functional groups (a carboxyl group and a β-hydroxyl group). The most chief derivative from 3-HP is 1,3-propanediol (1,3-PDO) (Matsakas *et al*., 2018).

1,3-PDO is a C3 dihydroxy compound. It is mainly used as a monomer for producing plastic (poly-tri-methyl-terephthalate (PTT)) with superior properties in biodegradability and biocompatibility. Furthermore, this chemical has a vast range of applications in the cosmetics, food, pharmaceutical, and textile industries (Ko *et al*., 2017; Vivek *et al*., 2016).

The market potentials of 3-HP (10 billion USD) and 1,3-PDO (600 million USD) make them a treasured target for industrial production (Ko *et al*., 2017). A variety of chemical methods have been developed for synthesizing 3-HP and 1,3-PDO. However, the environmental constraints and high cost led these methods to be unsuitable for industrial production (Seok *et al*., 2018; Rodriguez *et al*., 2016). Therefore, the biological synthesis of 3-HP had been scrutinized for an alternative solution for industrial production.

A considerable number of efforts have been carried out to synthesize 3-HP and 1,3-PDO via biological methods, individually (Yun *et al*., 2018; Li *et al*., 2016; Moscoviz *et al*., 2016, Zhao *et al*., 2015; Huang *et al*., 2013). Owing to the yield of production of them from glycerol, the limited regeneration and cellular availability of the cofactors (NAD^+ and

NADH), and the requirement of the exogenous supply of high-cost coenzyme B_{12}, the bacterial production of these compounds remains challenging (Ko *et al*., 2017; Ashok *et al*. 2011). More importantly, excessive accumulation of undesirable by-products such as lactate, ethanol, and acetate caused titer coproduction of these compounds was much lower than the individual production (Ko *et al*., 2017).

Some researches have suggested the simultaneous production of 3-HP and 1,3-PDO in a single cell to solve the challenges mentioned above (Ashok *et al*., 2011; Huang *et al*., 2012). The coproduction of 3-HP and 1,3-PDO makes the balance between NADH and NAD^+. To put it another way, NADH generated from 3-HP production can be used to produce 1,3-PDO (Kumar *et al*., 2012). Moreover, the accumulation of 3-HPA has a toxic effect on cellular physiology (Park *et al*., 2017), and this solution can be used for reducing the concentration of 3-HPA owing to two enzymes (*ald* and *dha*) consuming the toxic intermediate simultaneously (Kumar *et al*., 2013). Ashok *et al*. (2011) could produce 16.0 g 3-HP and 16.8 g 1,3-PDO per liter with a cumulative yield of 51% on glycerol carbon in 24 h under micro-aerobic conditions at pH 7.0 in a 5-l bioreactor (the recombinant *Klebsiella pneumoniae* Δ*dhaT* (*puuC*) strain). Moreover, Huang *et al*. (2013) reported that the recombinant strain *K. pneumoniae*/pUC18*kan-aldHEC* could produce 48.9 g/L 3-HP and 25.3 g/L 1,3-PDO with a coproduction yield of 0.66 mol/mol in 28 h under micro-aerobic conditions. Although these experiments have been significant achievements, we still need more creative methods to produce these compounds.

Psychrophile-based Simple bioCatalyst (PSCat) is an innovative approach that consists of selecting suitable pathways for production of

valuable compound from mesophile or thermophile bacteria, and introduction and expressions of these pathways in psychrophile host bacteria. After that, intrinsic host bacterial metabolic pathways are inactivated by treating cells at high temperature (40-50°C), while recombinant mesophilic enzymes retain their catalytic activity (Figure 1.1) (Luo *et al*., 2020; Tajima *et al*., 2013, 2015, 2018). This method can be solution to the primary challenges mentioned.

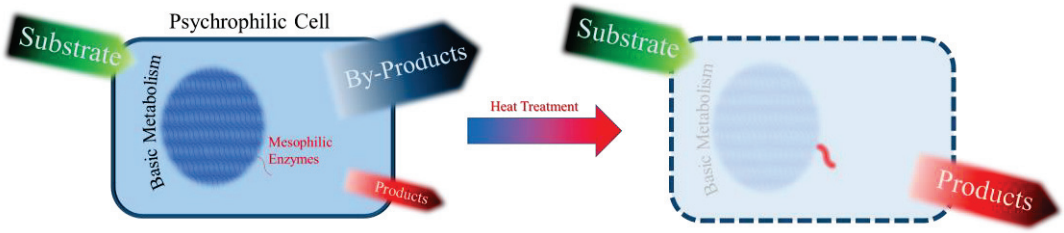

Figure 1.1. Schematic concept of PSCat. PSCat consists of expressions of suitable mesophilic or thermophile enzymes in psychrophile host bacteria. After that, intrinsic host bacterial metabolic pathways are inactivated by heat treatment (40-50°C), while recombinant mesophilic enzymes retain their catalytic activity. After heat treatment, the mesophilic or thermophile enzymes could convert all substrate to target products.

This method has been developed in the past few years. Specifically, it has been used to examine different bacterial species as cell factories to achieve more efficient performance to produce valuable compounds. For example, Luo *et al*. (2020) produced itaconic acid with this method and productivity was 1.41 g $L^{-1}h^{-1}$ in this experiment. Tajima *et al*. (2013) could produce 3-hydroxypropionaldehyde with a high production rate of 8.85 mmol 3-HPA/g dry cell/h by PSCat. Therefore, this method could be considered for the production of both compounds.

First, finding a suitable metabolic pathway from a mesophilic or thermophilic microorganism is needed to achieve that goal. *Klebsiella*

pneumoniae, due to having the reductive pathway to produce both compounds, is a promising choice (Matsakas *et al*., 2018). The conversion of glycerol to 3HP and 1,3-PDO using the reductive pathway in this bacterium requires fewer intermediates reaction steps and enzymes than the oxidative pathway (Matsakas *et al*., 2018). The reductive pathway chiefly consists of two routes. A dehydration reaction is catalyzed by the glycerol dehydratase (DhaB) enzyme, converting glycerol to 3-hydroxypropionaldehyde (3-HPA). Then, the 3-HPA intermediate is oxidized to 3-HP by the action of aldehyde dehydrogenase (PuuC) enzyme (Figure 1.1). Coenzyme B_{12} is required for the active enzyme of glycerol dehydratase, and oxygen can inactivate this enzyme. More importantly, for efficient oxidation, the aldehyde dehydrogenase reduces NAD^+ to NADH from 3-HPA to 3-HP (Matsakas *et al*., 2018; Park *et al*., 2017; Ma *et al*., 2010). 3-HPA intermediate can also be used as a substrate to produce 1,3-PDO. The 3-HPA is reduced and converted to the 1,3-PDO by catalysis of NADH-dependent 1,3-propanediol oxidoreductase (DhaT) enzyme (Figure 1.2).

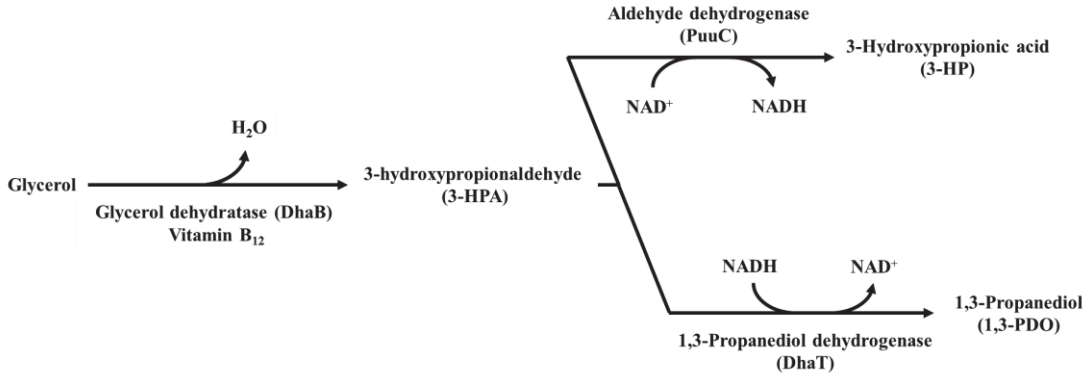

Figure 1.2. Schematic diagram of glycerol metabolism in *Klebsiella pneumonia*.

This study will examine the potential of the PSCat approach in the

coproduction of 3-HP and 1,3-PDO. For the PSCat approach all psychrophile host enzymes are inactivated by heat treatment, while recombinant mesophilic enzymes are active after heat treatment and can synthesize target products. Therefore, undesirable by-products cannot be produced in this method. Moreover, expression of each enzymes in different psychophysics host bacteria was considered as a solution for the development of PSCat. This new method could eliminate the insufficient activity of glycerol dehydrogenase, aldehyde dehydrogenase, and 1,3-propanediol oxidoreductase problem. Because the new approach give us more control to all part of reaction and based on enzyme activity we can add more enzyme to the reaction.

More importantly, the competition for cofactors and coenzymes between intrinsic enzymes and recombinant enzyme can be entirely eliminates and it enables efficient production of the valuable compounds. In this study, genes encoding glycerol dehydratase (DhaB: conversion of glycerol to 3-HPA), 1,3-propanediol oxidoreductase (DhaT: conversion of 3-HPA to 1,3-PDO) and aldehyde dehydrogenase (PuuC: conversion of 3-HPA to 3-HP) derived from *K. pneumoniae* were introduced into *Shewanella* sp. to examine the PSCat method for simultaneous production of 3-HP and 1,3-PDO.

Chapter II

Development a new method of Psychrophile-based Simple bioCatalyst

2.1. Summary

1,3-Propanediol (1,3-PDO) is a valuable compound with a substantial potential market in many industries. This study evaluates the abilities of the Psychrophile-based Simple bioCatalyst (PSCat) approach to biosynthesize 1,3-PDO. This whole-cell biocatalyst has a potential platform that replaces the chemical-based production counterparts. The two genes (*dhaB* and *dhaT*) involved in the metabolic pathway were expressed together and individually in the psychrophilic host bacterium. The intracellular psychrophilic enzymes and metabolic flux were deactivated using heat treatment at 45°C for 15 min. After individual gene expression, 1,3-PDO production reached 25.0 mM. When genes were expressed together, this compound's yield decreased by approximately 2.5 times (reached 10.2 mM). When the cofactor regeneration system was included in the biocatalyst, the productivity was boosted (31.1 mM). Hence, individual expression of genes and the cofactor regeneration system was verified in the PSCat approach. Nonetheless, further researches are essential to develop and optimize for industrial production.

2.2. Introduction

1,3-Propanediol (1,3-PDO) is a C3 dihydroxy compound often used as a monomer for producing poly-tri-methyl-terephthalate (PTT) with superior properties such as biodegradability and biocompatibility. Furthermore, this chemical has a vast range of applications in various industries, such as food and pharmaceutical industries (600 million USD

market potential) (Ko *et al*., 2017; Vivek *et al*., 2016). The U.S. Department of Energy has identified it as a priority production candidate (Werpy *et al*., 2004).

Though various methods by using chemical process have been developed for 1,3-PDO synthesis, their environmental constraints and high cost render them unsuitable for industrial production. (Seok *et al*., 2018). Therefore, synthesizing 1,3-PDO from glycerol by using microorganisms has become a prospective solution.

Klebsiella pneumoniae is an acclaimed bacterium that can convert glycerol to 1,3-PDO. The production pathway in this bacterial species consists of two reductive steps (Matsakas *et al*., 2018): First, glycerol dehydratase catalyzes a dehydration reaction upon activation with vitamin B12 and convert glycerol to 3-hydroxypropionaldehyde (3-HPA). Then 3-HPA is reduced to 1,3-PDO by 1,3-PDO dehydrogenase. For efficient reduction, 1,3-PDO dehydrogenase must oxidize NADH to NAD^+ (Figure 2.1).

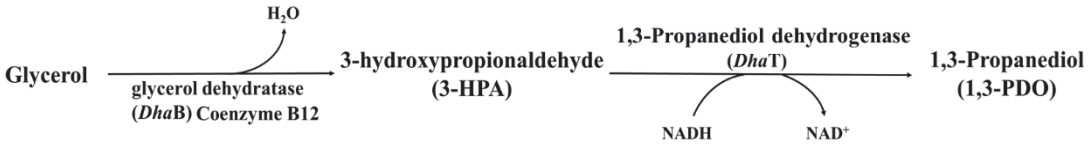

Figure 2.1. Schematic diagram of 1,3-PDO production from glycerol.

Despite considerable efforts to use biological methods for synthesizing 1,3-PDO (Yun *et al*., 2018; Li *et al*., 2016; Moscoviz *et al*., 2016), this compound's production remains challenging. Some of the challenges are the requirement of exogenous, high-cost vitamin B_{12}, the limited regeneration and cellular availability of the cofactor (NADH), and the low production yield of 1,3-PDO from glycerol (Ko *et al*., 2017; Ashok *et al*., 2011). More importantly, the industrial application of these strains

potentially gives rise to public health concerns; due to the pathogenicity of most strains in the genus *Klebsiella* (Matsakas *et al*., 2018; Vivek *et al*., 2016). Therefore, the development of innovative approaches for the production of 1,3-PDO should be considered.

The Psychrophile-based Simple bioCatalyst (PSCat) reaction system consists of selecting proper pathways producing treasure compounds from thermophilic or mesophilic bacteria. First, the selected mesophilic pathway is expressed in psychrophile host bacteria. Next, intrinsic bacterial metabolism is inactivated via the high-temperature treatment of the cell suspension. The recombinant target mesophilic enzymes are able to retain their catalytic activity after high-temperature treatment. This innovative method has been developed to examine different psychrophile species of bacteria as prospective cell factories, thus achieving greater efficiency in producing valuable compounds (Luo *et al*., 2020; Tajima *et al*., 2013, 2015, 2018).

In the conventional PSCat method, all genes involved in chemical production are expressed under single promoter's control. This method has some benefits, such as no production of undesirable by-products and high yield. However, limitations, such as the number of expressed genes in the psychrophilic host bacteria, led us to develop a new approach to this system.

In the new approach of PSCat, each gene involved in chemical production is expressed separately in the psychrophilic host bacteria. For the desired compound production, the recombinant bacteria were heated to inactivate the psychrophile host enzymes. Finally, PSCat cells are mixed at the right proportion, and the substrate is added to start the production reaction.

This study will examine the potential of the conventional and new PSCat approach in the production of 1,3-PDO. All psychrophilic host enzymes are inactive during the PSCat approach, and only recombinant mesophilic enzymes can synthesize target products. Therefore, undesirable by-products are not produced via this method. In this study, genes encoding glycerol dehydratase (DhaB: for the conversion of glycerol to 3-HPA) and 1,3-PDO dehydrogenase (DhaT: for the conversion of 3-HPA to 1,3-PDO) derived from *K. pneumoniae* were introduced in *Shewanella livingstonensis* Ac10 and *Shewanella frigidimarina* DSM 12253 to examine the new PSCat method for the production of 1,3-PDO.

2.3. Materials and methods

2.3.1. Bacterial strains, plasmids, and culture conditions

S. livingstonensis-Rif^r and *S. frigidimarina*-Rif^r were used as hosts for the construction of recombinant strains. Luria–Bertani medium (LB medium), containing yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L, was used as the culture medium for *Escherichia coli*. Tryptic soy broth medium culture (TSB; Difco Laboratories, Detroit, MI, USA) was used to culture *S. livingstonensis*-Rif^r and *S. frigidimarina*-Rif^r with 50 mg/L rifampicin at 18°C. Ampicillin (100 mg/L) was added to the culture media in order to maintain the presence of plasmids in transformed bacteria.

For production tests and enzyme activity, recombinant host bacteria were inoculated into 100 mL of TSB media (1%) in 250 mL Erlenmeyer flasks. Three days was considered for the duration of culture and growth of recombinant bacteria. The flasks were incubated in a rotary shaking incubator at 18°C at 120 rpm for one day. IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to culture media at 1 mM to

induce the expression of all recombinant enzymes after one day. All Erlenmeyer flasks were kept for a further two days.

2.3.2. Plasmid construction

A GeneAtlas G thermal cycler (ASTEC CO., Ltd., Fukuoka, Japan) was used to amplify the *dhaB* and *dhaT* genes from *K. pneumoniae* T7 and *K. pneumoniae* NBRC14940, respectively. Besides, the formate dehydrogenase (FDH) gene from *Candida boidinii* was amplified with this thermal cycler. The KOD -Plus- Neo polymerase (TOYOBO, Osaka, Japan) was used according to the manufacturer's recommendations to amplify these genes. All PCR-amplified fragments were inserted into the pHA12 plasmid to construct pHA12-*dhaB-dhaT* (pBT), pHA12-*dhaB-dhaT-fdh* (pBTf), pHA12-*dhaB* (pB), pHA12-*dhaT* (pT), and pHA12-*fdh* (pf). The genes, primers, and plasmids used in this study are listed in Table 1 and Figure 2.2.

The *E. coli* DH5α (TOYOBO) was used to amplify all constructed plasmid vectors. The constructed vectors were extracted from *E. coli* DH5α and used to transform *E. coli* S17-1 (Simon *et al*., 1983). Plasmids were introduced into *S. livingstonensis*-Rif^r and *S. frigidimarina*-Rif^r via trans-conjugation using recombinant *E. coli* S17-1. All transformed *Shewanella* spp. cells were selected by using rifampicin (50 mg/L) and ampicillin (100 mg/L). The broad-host-range expression vector pHA12 (Aria et al., 1991) was used to overexpress exogenous genes in *Shewanella* spp.

Table 2.1. Bacterial strains, plasmids, and primers used in this study.

Strains, plasmids and primers	Relevant characteristics	Source or reference
E. coli strains		
DH5α	Cloning host *supE* 44 Δ*lac* U169 (*Φ* 80 *lac*Z ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A 96 *thi*-1 *relA* 1	TOYOBO
S17-1	*rec* A *pro hsdR* RP4-2-Tc::Mu-Km::Tn7	(Simon *et al*., 1983)
S. livingstonensis strains		
Ac10-Rif^r	Parent strain, the rifampin-resistant mutant of Ac10	(Kawamoto *et al*., 2007)
Ac10-B	Ac10-Rif^r harboring pHA12-*dhaB*	This study
Ac10-T	Ac10-Rif^r harboring pHA12-*dhaT*	This study
Ac10-f	Ac10-Rif^r harboring pHA12-*fdh*	This study
Ac10-BT	Ac10-Rif^r harboring pHA12-*dhaB*-*dhaT*	This study
Ac10-BTf	Ac10-Rif^r harboring pHA12-*dhaB*-*dhaT*-*fdh*	This study
S. frigidimarina-strains		
DSM 12253	Type strain	DSMZ
Sf-Rif^r	Parent strain, the rifampin-resistant mutant of DSM 12253	This study
Sf-B	Sf-Rif^r harboring pHA12-*dhaB*	This study
Sf-T	Sf-Rif^r harboring pHA12-*dhaT*	This study
Sf-f	Sf-Rif^r harboring pHA12-*fdh*	This study
Plasmids		
pHA12	Amp^r, expression vector	(Arai *et al*., 1991)
pHA12-*dhaB*	Amp^r, pHA12 containing *K. pneumoniae dhaB*	(Tajima *et al*., 2013)
pHA12-*dhaT*	Amp^r, pHA12 containing *K. pneumoniae dhaT*	This study
pHA12-*fdh*	Amp^r, pHA12 containing *C. boidinii fdh*	This study
pHA12-*dha*B-*dhaT*	Amp^r, pHA12 containing *K. pneumoniae dhaB* and *dhaT*	This study
pHA12-*dha*B-*dhaT*-*fdh*	Amp^r, pHA12 containing *K. pneumoniae dhaB* and *dhaT*, *C. boidinii fdh*	This study

Continuation of Table 2.1.

Primers		
Forward - *dhaB*	5′-ATGGGAATTCGGAGAGATGAACAATGAAAAGATCAAAACGATTTGCAG-3′*	(Tajima *et al*., 2013)
Reverse - *dhaB*	5′-ATGCTCTAGATTAGCTTCCTTTACGCAGCT-3′	(Tajima *et al*., 2013)
Forward - *dhaT*	5′-GAGGGAATTCGGAGAGATGAACAATGAGCTATCGTATGTTTGATTATC-3′*	This study
Reverse - *dhaT*	5′-AGACTCTAGATCAGAATGCCTGGCGGAAAA-3′	This study
Forward - *fdh*	5′-GCAGGCATGCAAGCTTGGAGATAGATAATGAAGATTGTCTTAGTTCTTTATGAT-3′	This study
Reverse - *fdh*	5′-GGCCAGTGCCAAGCTCTATTTCTTATCGTGTTTACCGTAA-3′	This study

* Sequences with underline refer to SD sequence.

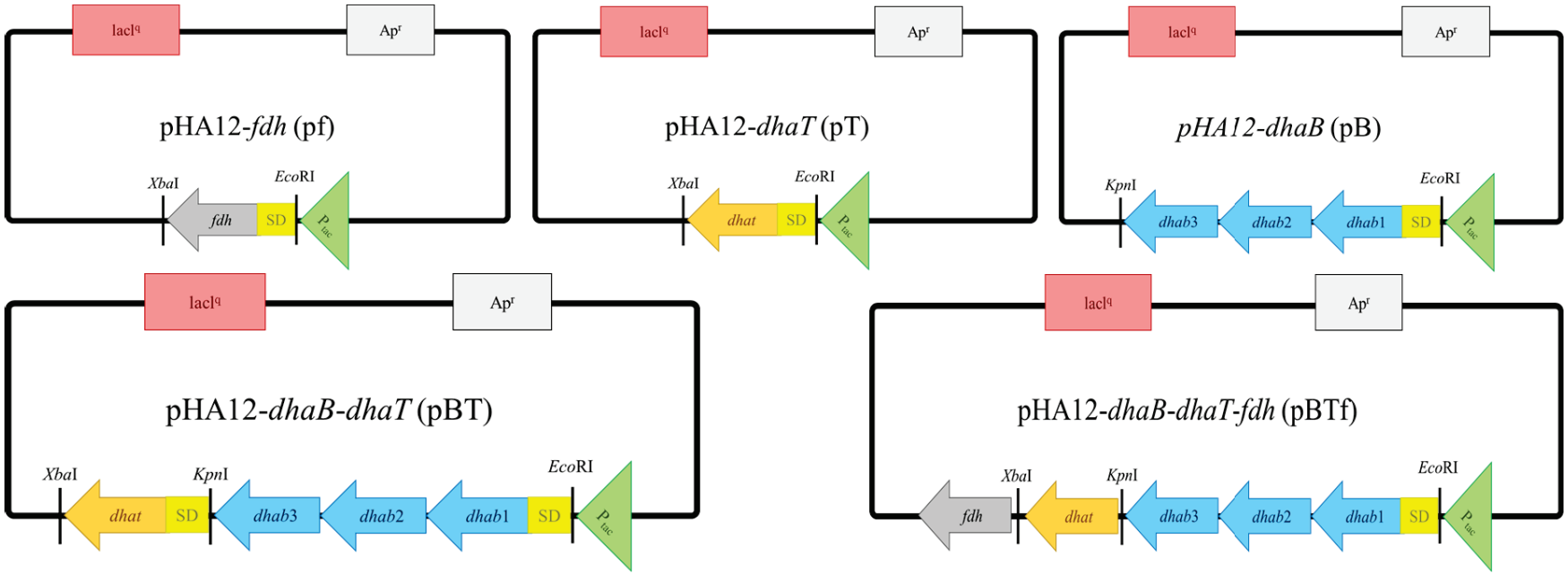

Figure 2.2. The schematic picture of the plasmids were used in this study. The transcription of cloned genes cloned gene in this expression vector was induced by IPTG addition (Arai *et al*., 1991).

2.3.3. Enzymatic activity

The enzymatic activity of all recombinant enzymes was measured. Transformed bacteria were prepared according to the previously described method. Cells in three-day bacteria culture were collected by using centrifugation at 3,300×*g* for 5 min, at 4°C. The pellets of bacteria were washed twice with 70 mM sodium phosphate buffer (pH=8).

The pellets were suspended in 5 mL of 50 mM sodium phosphate buffer containing 1 mM Dithiothreitol (DTT). The cell suspension was sonicated for 600 cycles of 1-s pulse/1-s intervals, using an ultrasonic disintegrator (Digital Sonifier 450; Branson, Danbury, CT, USA). The cell debris was removed by using centrifugation at 20,400×*g* for 30 min, at 4°C. The supernatant, which contains enzymes, was appropriately diluted with 50 mM sodium phosphate buffer. Cofactors and substrates for DhaT was added to start reaction. DhaT activity was measured according to the previously published report by González-Pajuelo *et al*. (Gonz´alez-Pajuelo *et al*., 2006). An Evolution 260 Bio UV–Vis spectrophotometer (Thermo Fisher Scientific Inc., Rockford, IL, USA) was set at 340 nm and used to measure NADH's decrease. Enzymatic activity was calculated using the following formula: Specific activity of enzyme (μmol/min/mg protein) = $\Delta Absorbance_{340} \times Vt \times 60 \times 1000 / (\Delta t \times 6.3 \times 10^3 \times d \times P \times Vs)$, where Vt = Total reaction volume (mL), Δt = time (Second), d = Optical path length (cm), P = Protein concentration (mg/ml) and Vs = volume of culture used in the assay (mL).

The total amount of protein was assessed, and the crude enzyme solution was diluted at an appropriate magnification to obtain data. Ten microliters of each solution were added to 990 μL of Bio-Rad protein assay solution (BIO-RAD, Hercules, CA, USA) diluted five-fold. The mixture

was incubated at 20°C for 5 min, and then absorbance was measured at 595 nm. The amount of protein in the crude enzyme solution was calculated from the prepared protein calibration curve. Serial dilution of bovine serum albumins solution (BSA; Katayama Chemical Industry, Osaka, Japan) (0.1~0.5 mg/mL) was used to plot out the protein calibration curve.

2.3.4. Production of target compounds

In general, for whole cell-based biosynthesis of 3-HPA and 1,3-PDO, the recombinants *S. livingstonensis* and *S. frigidimarina* were cultured according to the previously mentioned method herein. After 72 h, the grown recombinant bacteria were harvested and washed twice with sodium phosphate buffer (70 mM, pH=8).

Cell suspensions were heat-treated at 45°C for 15 min. The enzymatic reaction was started via the addition of 20 mM glycerol, 15 mM vitamin $B_{12,}$ 50 mM KCl (only for the production of 3-HPA), and 0, 5, 10, and 15 mM NADH (only for the production of 1,3-PDO) to the cell suspension and subsequently the reaction mixtures were incubated at an optimal temperature (37°C) for 1 h. Next, the reactions were terminated via heat treatment at 80°C for 15 min, and supernatant was obtained by centrifugation (20,400×*g* for 5 min at 4°C). A water bath (SM-05 N, Taitec, Koshigaya, Japan) was used for heat treatment and enzymatic reactions.

The modified colorimetric method described by Circle *et al*. (1945) was used to evaluate the quantification of 3-HPA. Briefly, 200 µL of the sample was added to 150 µL of 0.05 N HCl solution containing 10 mM tryptophan dissolved. Next, 600 µL of 37% HCl was mixed and the mixed solution was incubated at 37°C for 20 min. The absorbance was then measured at 560 nm using an Ultrospec 2100 UV-Visible Spectrophotometer (Biochrom, Massachusetts, United States). Acrolein

(0–1 mM) was used to create the standard curve.

A high-performance liquid chromatography system (HPLC; Jasco, Tokyo, Japan) with a RI detector was used to analyze the amount of 1,3-PDO and residual glycerol. A column (Shodex RSpak KC-811; Showa Denko, Kanagawa, Japan) and a guard column (Shodex RSpak KC-G; Showa Denko) was considered for these measurements. The target compounds were flowed at 60°C, using 0.1 % (v/v) phosphoric acid as a mobile phase at a 0.7 mL/min flow rate.

2.4. Results

Tajima *et al*. (2013) previously studied the potential of PSCats method to produce 3-HPA via using the psychrophile *S. livingstonensis* Ac10. 3-HPA is a toxic intermediate compound in the production of the 1,3-PDO pathway in *K. pneumoniae*. I attempted to survey the efficient conversion of glycerol to 1,3-PDO by both the PSCats method.

2.4.1. Enzymatic activities

To acquire the optimal condition of the PSCat process, I measured the activity of enzymes in both host bacterial cells. The *dhaB* gene cluster (*dhaB*1, *dhaB*2, and *dhaB*3) from *K. pneumoniae* T7 and the *dhaT* from *K. pneumoniae* NBRC 14940 were cloned into broad-host-range plasmid pHA12. The DhaB amino acid sequences had 100% identity to the corresponding proteins in *K. pneumoniae* MGH 78578. Four different nucleotide sequences were found for the *dhaT* gene in NCBI database. However, all of them express DhaT with the same amino acid sequence. The translated amino acid sequences of cloned *dhaT* shared complete identity with the other amino acid sequences in NCBI databases (Table 2.2).

Table 2.2. The nucleotide alignment results of the *dhaT* gene from *K. pneumoniae* NBRC14940 (LC550353) with other sequences of this gene

Accession number	CP050843.1	CP034321.1	CP000647.1	CP025630.1	CP025092.1	CP020061.1
Identities	1164/1164	1164/1164	1161/1164	1161/1164	1161/1164	1160/1164
Percentage of Identities	100.00%	100.00%	99.74%	99.74%	99.74%	99.66%
Disputes	-	-	258, 750, 1044	258, 543, 879	258, 543, 879	258, 543, 879, 1152

The translated amino acid sequences of amplified *fdh* gene shared complete identity with the translated amino acid sequences of AJ245934.1. The nucleotide sequences of *dhaT* and *fdh* gene that were determined in this study were submitted to the DDBJ database under the accession numbers: LC550353 (*dhaT*), LC549526 (*fdh*).

The specific temperatures and durations of treatment were investigated to establish the optimal condition for heat treatment. The whole crude enzyme solution from all recombinant strains was prepared. The 100 times dilution of crude enzyme solution was applied to the test. The enzyme mixture was heated at different temperatures (40, 45, 50, and 55°C) for 10 and 15 min to inactivate the psychrophilic host enzymes. Heating the crude enzyme solution at 45°C for 15 minutes caused all psychrophilic host enzymes were inactivated in this project. Therefore, no activity was measured in the absence of substrate, while activity was observed following adding the substrate. This condition was used as the pre-heat treatment in this part of the study (Figure 2.3).

Recombinant Ac10-BT, Ac10-T, and Sf-T cells were prepared for the measuring of 1,3-PDO dehydrogenase activity. The final concentration of NAD^+ was 0.5 and 1 mM for testing the activity of DhaT in all

recombinant cells. The recombinant Ac10-BT expressed both DhaB and DhaT under the control of the *tac* promoter. The Ac10-BT strain showed enzyme activity 29.8 ± 3.1 and 28.0 ± 2.7 μmol/min/mg protein, when 0.5 and 1 mM NAD+, respectively, was added to the reaction (Figure 2.3). The wild type Ac10 and DSM 12253 strains showed no activity for 1,3-PDO dehydrogenase at 37 °C (Figure 2.3).

The activity of DhaT was assessed in the recombinant Ac10-T and Sf-T cells, which only expressed DhaT. The recombinant Ac10-T strain showed the DhaT activity (172.7±35.2 μmol/min/mg protein) when the concentration of NAD^+ was 0.5 mM. The enzyme activity was 121.6±13.8 μmol/min/mg protein in recombinant Sf-T strain (Figure 2.3). The activity of DhaT rose in both Ac10-T and Sf-T strain when the amount of cofactor (1 mM) added to the reaction was increased (Figure 2.3).

Cofactor regeneration is one of the effective strategies to reduce the cost of chemical productions. To implement the cofactor regeneration, FDH was expressed in both psychrophile host bacteria. The test conditions for the preparation of whole crude enzyme solution and heat treatment were the same as measuring DhaT activity. Two millimolar of NAD^+ was added to the reaction as the cofactor's final concentration in this step. The activity of FDH was 103.5 and 109.9 μmol/min/mg protein in the FDH single expression recombinant Ac10-f and Sf-f strain, respectively (Figure 2.4).

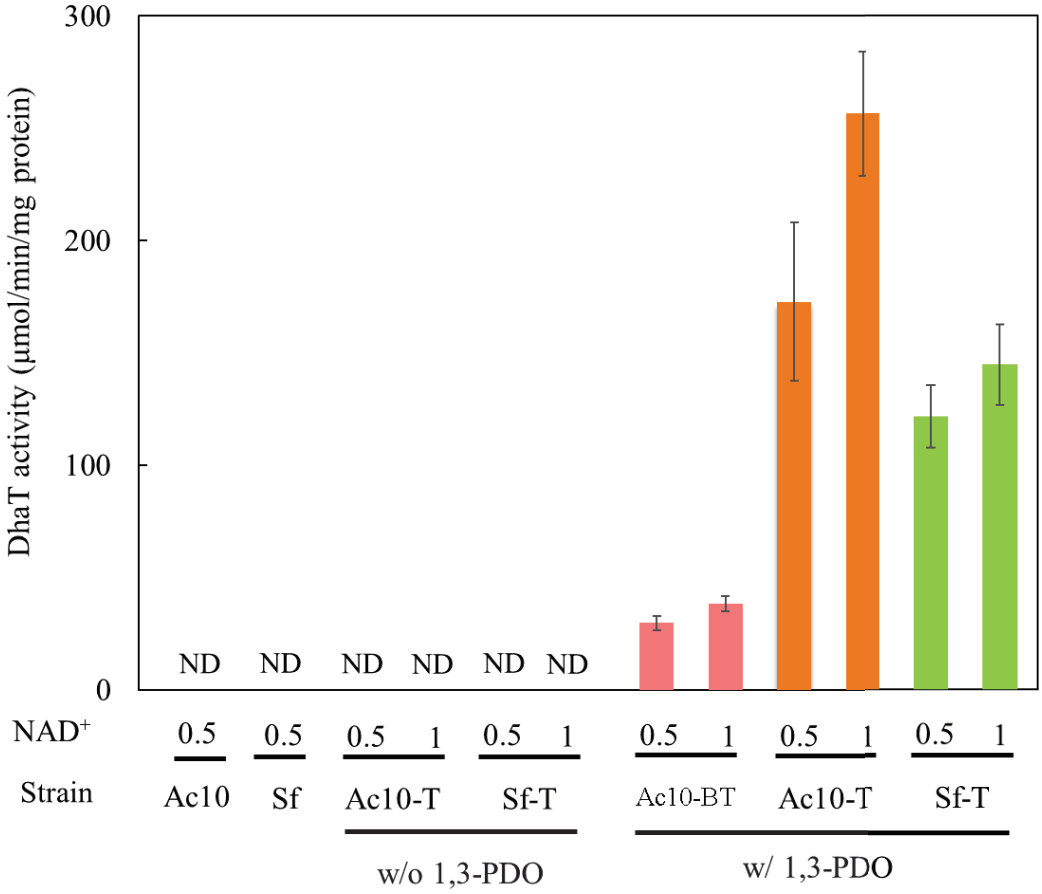

Figure 2.3. The activity of DhaT at 37°C. The reaction catalyzed by the DhaT was propane-1,3-diol + NAD^+ = 3-hydroxypropanal + NADH + H^+. The crude enzyme solution was heated at 45°C for 15 min, and the substrate and cofactor were added as per the reaction. w/o, without substrate; w/ with the substrate. Error bars represent the standard deviation of the mean (n = 3).

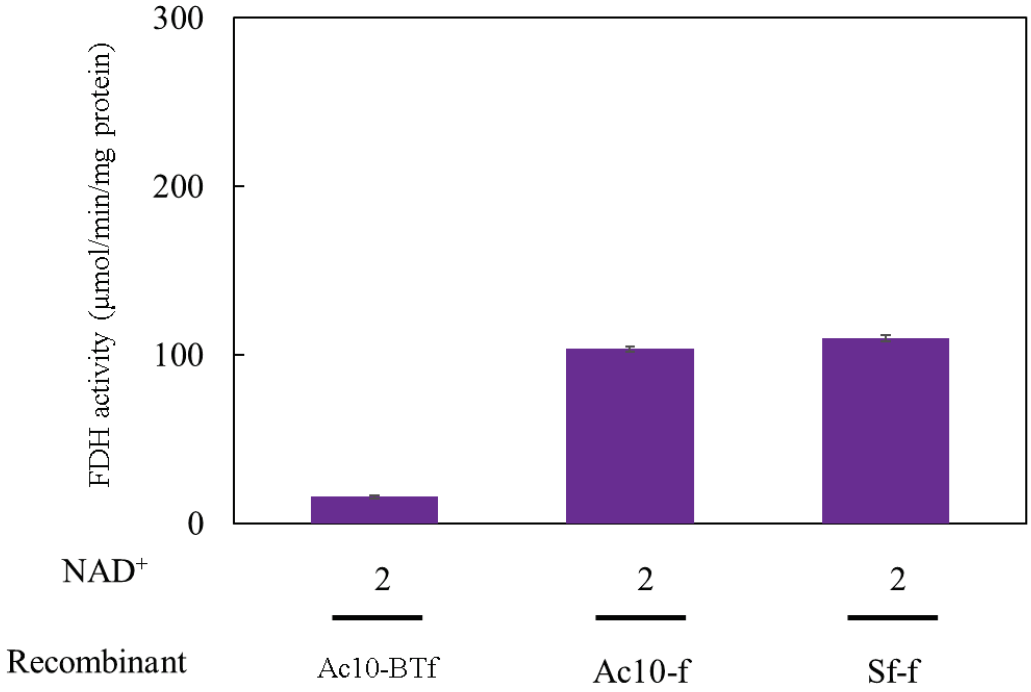

Figure 2.4. The activity of FDH at 37°C. Error bars represent the standard deviation of the mean (n = 3).

2.4.2. Production of 3-HPA

To check the potential of PSCat for 3-HPA production, the recombinant Ac10-B and Sf-B were used. The reaction mixture contained 22.3 g dry cell/L, 20 mM glycerol, and 15 μM vitamin B_{12}. The production of 3-HPA in the Ac10-B strain was 19.5±0.4 mM after 30 minutes, and 19.9±1.2 mM in the Sf-B strain after only 15 minutes (Figure 2.5A and 2.5B). The activity of DhaB was measured at both recombinant host cells based on the production of 3-HPA. The DhaB activity at the recombinant Ac10-B was not significantly higher than the recombinant Sf-B. Therefore, I considered the activity of this enzyme in both recombinant host cells in the same range.

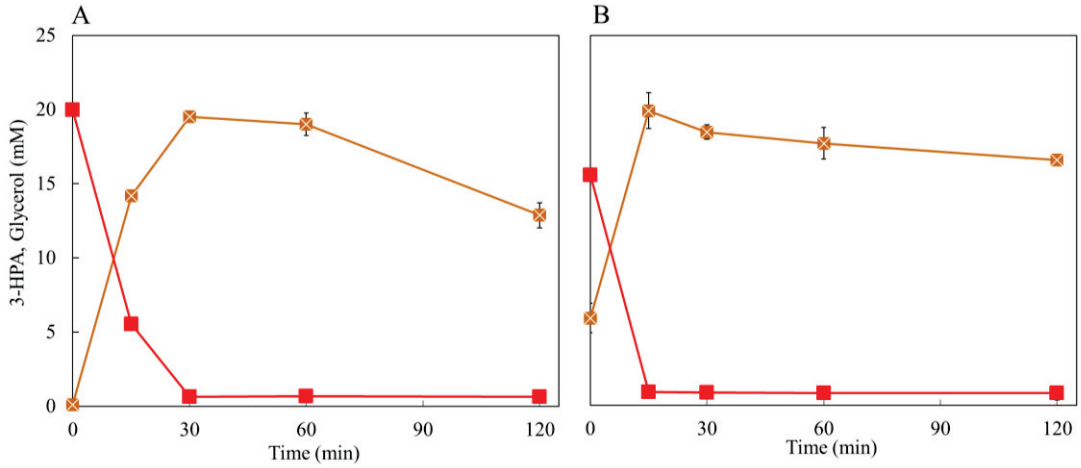

Figure 2.5. 3-HPA prodcution by recombinant Ac10-B cells (A) and recombinant Sf-B cells (B) at 37°C. The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B_{12} (μM) was 20, 22.3, and 15, respectively. Square with a cross inside (Brown), 3-HPA; Squares (Red), glycerol. Error bars in all figures represent the standard deviation from the mean (n = 3).

2.4.3. Production of 1,3-PDO

1,3-PDO productivity was examined using both PSCat methods. In all production tests, the prepared cell suspension was heated at 45°C for 15 min and then added glycerol as substrate, NADH as the cofactor, and vitamin B_{12}. The final concentration of vitamin B_{12} was formulated to 15 μM for all tests.

2.4.3.1. The conventional PSCat method

For the initial examination, the wild type Ac10, Ac10 with pHA12 (Ac10-pHA12), and the recombinant Ac10-BT strains were used. The *dhaB* and *dhaT* genes in the recombinant Ac10-BT were simultaneously expressed at the same time under the control of the *tac* promoter. Cultivated cell concentration was set at 5.57 g dry cell/L and 20 mM glycerol and 5 mM NADH were added for this assay. Although the wild type Ac10 and the recombinant strain with Ac10-pHA12 produced no 1,3-PDO at 37 °C, the Ac10-BT strain produced 4.6 mM in 60 min reaction.

Observation of 1,3-PDO production confirmed the suitability of the conventional PSCat method for application to production of 1,3-PDO. To increase the amount of 1,3-PDO production, some possible strategies, such as increasing the number of bacterial cells or increasing the cofactor concentration, were examined.

I considered increasing the amount of the Ac10-BT cell as a first strategy to improve 1,3-PDO production. Increasing the number of recombinant cells means an increase in the amount of 1,3-PDO production enzyme present in the reaction. The concentration of substrate and cofactor was 20 mM and 5 mM, respectively. The results demonstrated that the production was increased until the cell concentration reached 22.3 g dry cell/L. At this concentration of Ac10-BT, the production of 1,3-PDO reached 12.7 mM (Table 2.3).

Table 2.3. Production of 1,3-PDO in different Ac10-BT cell concentration at 37°C. Glycerol = 20 mM, NADH = 5 mM.

Cell (g dry cell /L)	1,3-PDO (mM)
2.8	2.0
5.6	4.7
11.2	8.1
22.3	12.7
44.6	13.0

To improve 1,3-PDO production, I considered increasing the NADH cofactor concentration as the next strategy. The cultivated cells concentration was set to 5.57 g dry cell/L, and glycerol concentration was 20 mM. Every 15 min, 5 mM of NADH was added to the reaction mixture, and the final cofactor concentration reached 20 mM after 60 min. The amount of 1,3-PDO production and the amount of residual glycerol were

measured over time (Table 2.4). Through the sequential adding of NADH, the amount of 1,3-PDO production increased by 5.5 mM and reached 10.2 mM.

Table 2.4. Production of 1,3-PDO in different concentration of NADH at 37°C. Glycerol = 20 mM, Ac10-BT cell = 5.6 g dry cell /L.

Reaction time (min)	Glycerol (mM)	1,3-PDO (mM)
0	20.0	0.0
15	13.0	4.2
30	9.3	8.0
45	7.8	10.2
60	6.8	10.2

2.4.3.2. The new PSCat method

The higher expression of genes involved in chemical production is, the higher yield of chemical product is. Therefore, to achieve the higher expression, the *dhaB* gene cluster and *dhaT* were cloned into the pHA12 vector individually. Both psychrophile host bacteria (*S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253) were transformed with the constructed vectors. Then, recombinant Ac10-B, Ac10-T, Sf-B, and Sf-T strains (Table 2.1) were examined for enzymatic activity.

I expected that the mesophilic enzyme expression levels would increase in recombinant cells because of each gene's expression under the control of a *tac* promoter. Moreover, I optimized 1,3-PDO production by adjusting mixing ratio of these four recombinant strain cells.

Cell density of each cell suspension was adjusted to approximately 22.3 g dry cell/L. Different volumes and combinations of each recombinant cell were added to the reaction mixture to find the production's best combination. In the first test, I added the same amount of each recombinant

cell to the reaction mixture (DhaB:DhaT = 1:1). In the next experiment, I added one of the bacteria at a level two times higher than the other (DhaB:DhaT = 2:1 and 1:2). The different cofactor concentrations were tested (0, 5, 10, and 15 mM), and the concentration of glycerol was set at 20 mM.

The results indicated that increasing the cofactor concentration increased the volume of production in all combinations and ratios (Figure 2.6A, 2.6B, and 2.6C). The increasing rate in 1,3-PDO production varied according to the combination and ratio of bacteria. I assumed the potential cofactor reservoir to be approximately 8 mM in host psychrophilic bacterial cells (Figure 2.6A, 2.6B, and 2.6C). This is due to approximately 8 mM of 1,3-PDO was produced when no cofactors were added to the reaction. Furthermore, adding a greater volume of the DhaB-containing recombinant bacteria had a positive effect on 1,3-PDO production (Figure 2.6A, 2.6B, and 2.6C). These results indicate that the ratio of DhaB:DhaT (2:1) had greater potential to produce 1,3-PDO. More importantly, I investigated the highest achievable volume of production when Ac10-B and Sf-T were added to the reaction. The production of 1,3-PDO when the final concentration of NADH was 15 mM, and two volumes of Ac10-B and one volume of Sf-T were added to the reaction, reached 19.7 mM (Figure 2.6C). Therefore, the combination of Ac10-B and Sf-T, with 2:1 ratio of DhaB:DhaT, was selected for further experiments in this study.

The production of 1,3-PDO and glycerol residues were examined over time. The 2:1 ratio of DhaB:DhaT was mixed by using recombinant Ac10-B and Sf-T and heated at 45°C for 15 min. I added 20 mM glycerol, 15 mM NADH, and 15 μM vitamin B_{12}. After 30 min, almost all substrates were converted to 1,3-PDO, and the amount of production reached to 19.44

mM (Figure 2.7). The yield (1,3-PDO production/glycerol consumption) was around 97%. Next, I increased the concentration of glycerol and assessed its effect on production. When the final concentration of glycerol in the reaction reached 30 mM, 1,3-PDO production reached 25.02 mM, after 60 min (Figure 2.8), the productivity of this compound was 1.90 g $L^{-1}h^{-1}$.

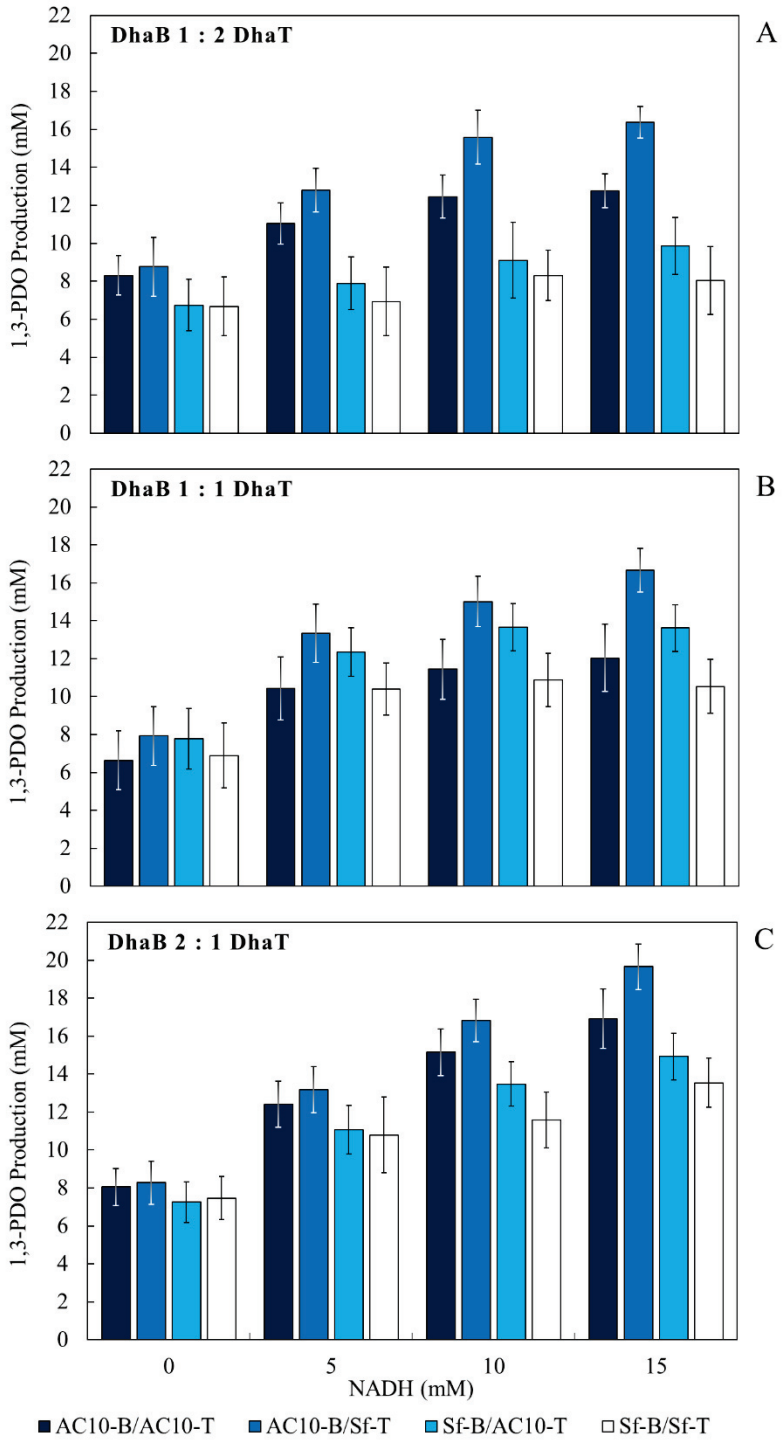

Figure 2.6. 1,3-PDO productivity of recombinant cells at various ratios, combinations, and NADH concentrations at 37°C. The volume ratio of the recombinant cell suspension of DhaB to DhaT was 1:2 (A), 1:1 (B), and 2:1 (C). The initial concentration of glycerol and cells was 20 mM and 22.3 g dry cell/L, respectively. Error bars represent standard deviation from the mean (n = 3).

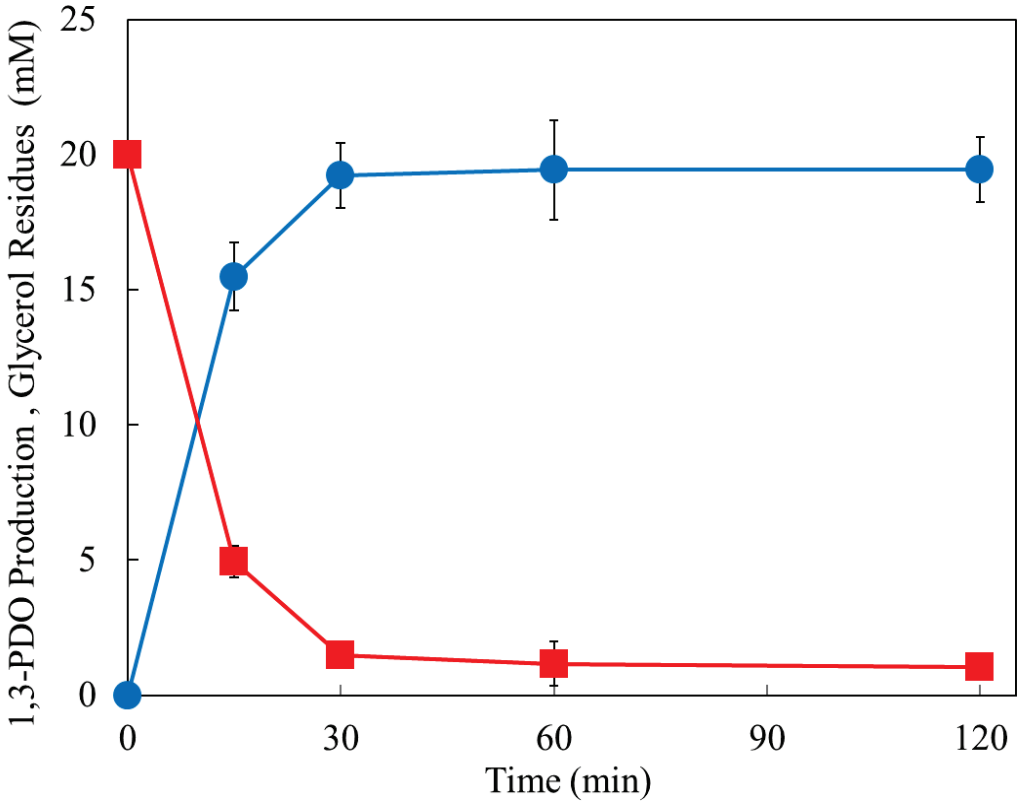

Figure 2.7. 1,3-PDO productivity of recombinant Ac10-B/Sf-T at 37°C. The initial concentration of glycerol (mM), cell (g dry cell/L), and NADH (mM) are 20, 22.3, and 15. Circles (Blue), 1,3-PDO; squares (Red), glycerol. Error bars represent the standard deviation of the mean (n = 3).

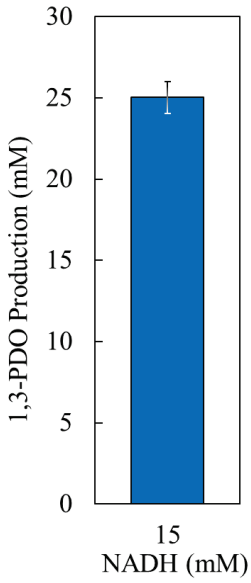

Figure 2.8. 1,3-PDO productivity of recombinant Ac10-B/Sf-T at 37°C. The initial concentration of glycerol (mM), cell (g dry cell/L), and NADH (mM) are 30, 22.3, and 15 (B), respectively. Error bars represent the standard deviation of the mean (n = 3).

2.4.4. Production of 1,3-PDO with NADH regeneration

The cellular cofactor (NADH) availability is one limitation for efficient 1,3-PDO production. As such, a cofactor regeneration system may reduce production costs. Here, I coupled FDH with DhaT to create a cofactor regeneration system (Figure 2.9).

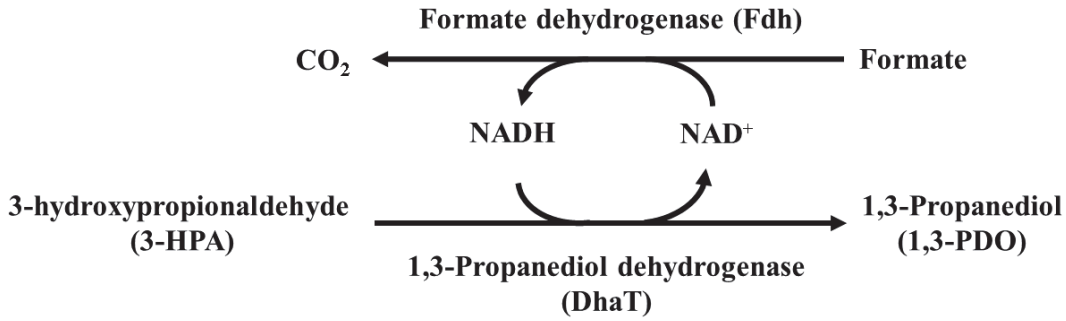

Figure 2.9. Schematic diagram of the cofactor regeneration system between FDH and DhaT.

2.4.4.1. The conventional PSCat method

The reaction of 3-HPA reduction to 1,3-PDO with NADH regeneration was designed. The Ac10-BTf was cultured and prepared in this step of the project. In the recombinant Ac10-BTf, the DhaB, DhaT, and FDH were co-expressed under the control of the *tac* promoter in *S. livingstonensis* Ac10-Rif^r. Sodium formate (20 mM) was used as a substrate for FDH. The concentration of glycerol and NADH was 20 mM and 5 mM, respectively. As a result, 18.7 mM was produced at a cell concentration of 33.5 g dry cell/L (Table 2.5).

Table 2.5. Production of 1,3-PDO in different Ac10-BTf cell concentration at 37°C. Glycerol = 20 mM, sodium formate = 20 mM, NADH = 5 mM.

Cell (g dry cell /L)	1,3-PDO (mM)
22.3	11.8
33.5	18.7

2.4.4.2. The new PSCat method

To make the cofactor regeneration system in the new PSCat method, the *fdh* gene was cloned and expressed in Ac10-f under the *tac* promoter's control. Some preliminary tests were designed and done to find the best condition. The preliminary test results indicated that the volume of recombinant bacteria with FDH should be lower than the volume of SF-T. Therefore, the ratio of 2:1:0.1 was chosen for Ac10-B:Sf-T:Ac10-f, respectively. Same as the other part of the project, the recombinant bacteria were set 22.3 g dry cell/L. As substrates, I considered 40 mM glycerol and 30 mM sodium formate. I added glycerol, 15 mM NADH, and vitamin B_{12} after heat-treatment at 45 ◦C for 15 min to achieve the highest production level. The reaction tube was kept at 37°C for 60 min, and after that, sodium

formate and heated recombinant Ac10-f were added. The control group comprised of cell suspensions, cofactor, coenzyme, and only glycerol. 1,3-PDO production reached around 26 mM in both control and reaction tubes after 60 min. I added both substrates after 120 min, and the production increased and reached 31.09 mM in the reaction tube. At the same time, no increase was observed in the control group. Following this, the production level stabilized (Figure 2.10).

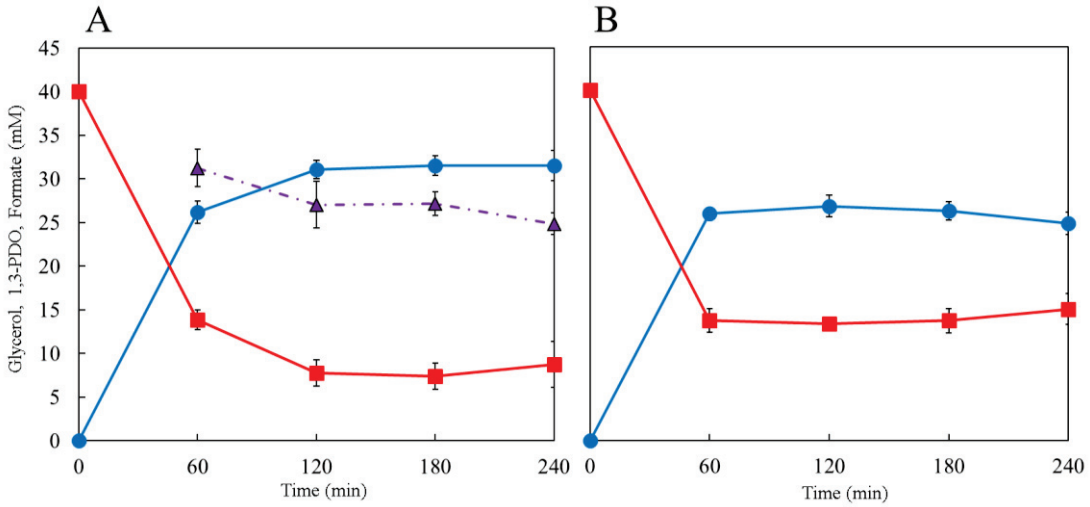

Figure 2.10. 1,3-PDO production of recombinant Ac10-B/Sf-T/Ac10-f with (A) or without (B) formate at 37°C. The initial concentration of glycerol, cell, and NADH is 40 mM, 22.3 g dry cell/L and 15 mM, respectively. After 60 min reaction, 30 mM sodium formate was added. Circles (Blue), 1,3-PDO; squares (Red), glycerol; triangles (Purple), formate. Error bars represent the standard deviation of the mean (n = 3).

2.5. Discussion

In this study, I expressed enzymes involved in producing 1,3-PDO in the psychrophilic bacterial cells and used psychrophile-based simple biocatalyst (PSCats) to produce valuable chemicals. Although several compounds were efficiently biosynthesized with *S. livingstonensis* Ac10

via this method (Luo *et al.*, 2020; Tajima *et al.*, 2018; Tajima *et al.*, 2015). Other species of *Shewanella* had not been used and may also have the potential to be utilized in the PSCat system. Moreover, the production of 1,3-PDO had not been examined with this method. Therefore, I examined the production of 1,3-PDO in *S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253 via this method. The *S. frigidimarina* DSM 12253 was utilized as a psychrophile host for the first time in this study. Finding its optimal production conditions was essential to start the production reaction.

The 45°C for 15 min was adequate to inactivate the psychrophilic host enzymes and metabolic flux in both S. *livingstonensis* Ac10 and *S. frigidimarina* DSM 12253. All metabolites and psychrophilic host enzymes were present in the mixture because of utilizing whole-cell suspension for enzymatic activity. Therefore, if the host enzyme remained active in the control tube after heating, any cofactor concentration changes would be recorded using the spectrophotometer. I could not detect any tube activity without the substrate required for the mesophilic enzyme at 45°C. Hence, this condition was chosen as a pre-heat treatment for this study. Previous researches have indicated that the same temperature may be suitable for the inactivation of psychrophilic enzymes (Luo *et al*., 2020; Tajima *et al*., 2013). Most mesophilic and all thermophilic enzymes can maintain the structures at the 45°C. Thus, the PSCat can be considered for use with most of the valuable compounds. The optimum temperature for DhaB (EC 4.2.1.30) (Wang *et al*., 2007), DhaT (EC 1.1.1.202; Lama *et al*., 2015), and FDH (EC 1.17.1.9; Netto *et al*., 2017) is 37°C. Therefore, the reaction condition was examined at this temperature in this study.

Altering the enzyme concentration affects enzyme activity (Curtis *et al*., 2002). If I add more enzyme to an in vitro system such as PSCat

method, it can increase the activity of the enzyme. In the recombinant Ac10-BT and Ac10-BTf, the *tac* promoter controlled the expression of two and three genes, respectively. The first gene (*dhaB*) in the gene cluster showed the highest expression, and the last gene showed the lowest expression. The enzyme concentration of DhaT in the cell suspensions of recombinant Ac10-T and Sf-T was higher than the recombinant Ac10-BT and Ac10-BTf. Therefore, DhaT showed a higher level of enzyme activity in the cell suspensions of recombinant Ac10-T and Sf-T. FDH also experiences the same situation. The *fdh* was the third gene in the gene cluster of pHA12-*dhaB*-*dhaT*-*fdh*, so its expression was lower than those in the Ac10-f and Sf-f.

I induced the *tac* promoter with IPTG in all recombinant bacteria and expected to find gene overexpression in this test. However, the activity of DhaT in recombinant Ac10-T was higher than recombinant Sf-T. I believe this difference is due to the growth potential of recombinant bacteria. Owing to the better growth of the recombinant Ac10-T, the concentrations of total protein in the recombinant Ac10-T was higher than recombinant Sf-T (Table 2.6). Therefore, as the enzyme concentration in the recombinant Sf-T was lower than in recombinant Ac10-T, and I expected less enzymatic activity.

Table 2.6. Optical density and concentration of total Protein for recombinant bacteria

	Optical density (660 nm)			
	Ac10-B	Sf-B	Ac10-T	Sf-T
Average	2.01±0.07	1.81±0.10	2.05±0.08	1.81±0.12

Continuation of Table 2.6.

	Total Protein	
	Ac10-T	Sf-T
Average	2.95±0.39	2.49±0.25

The use of PSCat provides several benefits such as catalytic efficiency and low impact on the environment. Researchers previously reported these benefits in the field of whole-cell biocatalysts (Anteneh and Franco; 2019). Besides, the PSCats method can overcome the challenge of the cell membrane's resistance to the substrate influx in the whole-cell biocatalysts (Luo *et al*., 2020). Luo *et al*. (2020) showed that the cell membrane of *S. livingstonensis* Ac10 could be permeabilized with moderate heat treatment. More importantly, the psychrophilic hosts' basic metabolism could be inactivated because of heat treatment (Table 2.7). Therefore, only recombinant mesophilic enzymes could access all molecules in the cells, such as cofactors and coenzymes. This feature, along with the absence of non-target compounds production, helps to improve the production yield.

Table 2.7. NADH consumption in the wild type strains with or without heat treatment.

	With heat treatment		Without heat treatment	
		Activity (µmol/min/mg protein)		Activity (µmol/min/mg protein)
S. livingstonensis Ac10 wild type	No activity observed	0	Activity observed	435.9±27.1
S. frigidimarina DSM 12253 wild type	No activity observed	0	Activity observed	135.0±12.5

I examined both a novel psychrophilic host and a strategy for the further development of the PSCat method. Previous researchers utilized the psychrophile *S. livingstonensis* Ac10 as a host (Kawamoto *et al*., 2007) to produce 3-HPA (Tajima *et al*., 2013), aspartic acid (Tajima *et al*., 2015), fructose for fermentation (Tajima *et al*., 2018) and itaconic acid (Luo *et al*., 2020) with conventional PSCats method. In all these studies, researchers made one gene cassette containing one or more genes and expressed them under *tac* promoters' control. This approach's limitations are the simultaneous expression of all genes and their limits on the number of genes.

In this study, I expressed each gene individually in two psychrophilic host bacteria. This new strategy allowed me to control all parts of the project. For example, I could control the desired volume and perfect time at which to add each enzyme to the reaction. More importantly, I had no limit to the number of genes I was able to express in the reaction. The results of this study showed that the individual expression of genes improved the production of 1,3-PDO on a laboratory scale (from 10.2 mM to 19.4 mM). Thus, PSCat with the individual expression of genes can be considered for an efficient multi-step bioconversion process.

The new strategy of PSCat demonstrated that a 2:1 ratio of DhaB:DhaT, added to recombinant Ac10-B and Sf-T, had the highest efficiency. Previous research indicated that glycerol dehydratase activity is the limiting factor for the production of 1,3-PDO (Daniel *et al*., 1998; Tong *et al*., 1991). Two arguments may explain this limitation: the role of vitamin B12 in the catalytic cycle and glycerol's inhibitory activity as a substrate for DhaB. Therefore, the overexpression of *dhaB* could potentially solve the challenge of the production of the target compound.

Zheng *et al*. (2006) reported that the accumulation of toxic 3-HPA could lead to a serious impairment of *K*. *pneumoniae* growth and the instability of expression plasmid following overexpression of glycerol dehydratase. Many studies have shown that 3-HPA, as an intermediate metabolite, has a lethal effect on living cells (Sankaranarayanan *et al*., 2017; Zaushitsyna *et al*., 2017). Consequently, the NADH-linked 1,3-PDO dehydrogenase quickly reduced 3-HPA to 1,3-PDO in most 1,3-PDO native producing bacteria (Chatzifragkou *et al*., 2011).

The new strategy has easilly resolved the challenge presented by the contradictory relationship of overexpression of *dhaB* and 3-HPA toxicity. One of the advantages of this approach is that all psychrophile host cells are dead after heat treatment. Therefore, there are no inhibitory effects from the toxicity of 3-HPA on cell growth and cell viability. My results demonstrated that increasing the volume of DhaB had a central role in raising the production of 1,3-PDO. Adding a greater volume of the heated recombinant bacteria with *dhaB* means an increase in the volume of DhaB enzyme and simulation of the reaction's over-expression state.

I checked the production rate of 3-HPA and the results showed that when the concentration of recombinant cells had set at the 22.3 g dry cell/L, the production reaches around 20 mM. Tajima *et al*. (2013) showed that the production rate of 3-HPA was 7.3 and 8.0 mmol 3-HPA/g dry cell/h, when using 19.5 and 28.0 g dry cell/L, respectively. I calculated the enzyme activity of DhaB based on our results and data from Tajima *et al*. (2013; Data not shown). Both calculations were revealed that the activity of DhaB was not significantly higher than the activity of DhaT. Therefore, the DhaB and DhaT activities are considered in the same range. I believed that because of the limitation of DhaB, which I have mentioned previously in

this paper, the 1:1 ratio (DhaB:DhaT) could not produce 1,3-PDO actively. The highest achievable volume of production in this ratio was around 17 mM. Therefore, I examined the ratio 2:1 (DhaB:DhaT), and the results of this ratio demonstrated the highest production in this study.

Furthermore, due to the slower growth of the recombinant Sf-B, I expected a lower 3-HPA production rate in this strain. My findings showed that regardless of the ratio and type of recombinant bacteria with *dhaT*, the presence of recombinant Ac10-B always increased the yield of productivity. The highest production (25.0 mM after 60 min) was accomplished when the recombinant Ac10-B and Sf-T were mixed at the ratio of 2:1 DhaB:DhaT.

Furthermore, the production rate of 1,3-PDO in the combination of recombinant Ac10-B and Sf-T was always higher than in other combinations. I concluded the less growth of recombinant *S. frigidimarina* (Table 1), and consequently, the less enzymatic activity of DhaT, caused the right balance between 3-HPA production and consumption rate. Hence, the inherent production potential of 1,3-PDO increased with this new strategy of PSCat. The target compound's production was 10.2 mM when expressed all genes in the one gene cluster in *S. livingstonensis* Ac10. However, the production of 1,3-PDO reached 25.0 mM after 60 min when using different host bacteria to express each gene individually.

Due to high number of applications of 1,3-PDO in the new polymer industry, cosmetics, personal care and cleaning products, it is considered a specialty platform chemical (Rodriguez *et al*., 2016). The genera of *Klebsiella*, *Clostridium*, *Lactobacillus*, and *Citrobacter* biologically have the ability to convert glycerol to 1,3-PDO (Ju *et al*., 2020; Yun *et al*., 2018; Celinska *et al*., 2015). However, some limitations, such as the production

of undesirable by-products, and the accumulation of the toxic intermediate 3-HPA through fermentation (Yun *et al*., 2018).

In this chapter, I have already described 3-HPA toxicity. Because of the deactivation of metabolic flux in the psychrophile host cells after heat treatment, I could not find any by-products in this project. More importantly, the yield of 1,3-PDO production, using the PSCat approach was around 97 % in only 60 minutes when I added 20 mM glycerol to the reaction. The yield of production in this short time is higher than many previous reports (Zhou *et al*., 2019; Yun *et al*., 2018).

DhaT is an NADH-dependent enzyme (Zhao *et al*., 2009). Based on the present results, suppling the NADH indicated a positive impact on the enzyme activity and the production of 1,3-PDO. However, the cost of a complementary coenzyme is one of the limiting points in the production of 1.3-PDO (Yun *et al*., 2018). I considered checking the regeneration of cofactors system to achieve the highest production amount while decreasing the cost.

The regenerated system was examined in relation to 1,3-PDO production in recombinant Ac10-BTf. However, the activity of FDH was low in the Ac10-BTf; the cofactor regeneration system activated and increased the production of 1,3-PDO. The individual expression of FDH in psychrophilic host cells causes a rise in the amount of enzyme. Therefore, the enzymatic activity of FDH increases significantly.

One of the central advantages of the novel strategy of PSCat is the addition of the desired amount of enzyme to the reaction. I expected to increase my target metabolite production by adding a higher volume of FDH compared to other enzymes in the reaction. Nevertheless, the NADH regenerate system did not make active when I added 2 and 1 volume of

heated Ac10-*fdh* in two separate experiments. Hence, based on the result of the Ac10-BTf test, I considered added lower the volume of FDH in the reaction to decrease the activity of FDH. The 2:1:0.1 ratio of DhaB:DhaT:FDH was chosen to assess the regeneration system. The system could increase the production of 1,3-PDO from approximately 25 mM to 31.1 mM only after 120 min. However, the yield of 1,3-PDO production with the regeneration system was 77.7% in 120 min. Previous studies showed that lower activity of FDH, compared to production enzymes increases the production of the target compound (Wang *et al*., 2013; Kaup *et al*., 2003).

The disadvantages of FDH, which mention in some studies, are its relatively low specific activity (Van der Donk and Zhao., 2003). Serval efforts have been implemented to improve the kinetic properties of FDH (Tishkov and Popov., 2006). Because of the ability to add the desired amount of enzyme, the new strategy of PSCat rectifies these challenges. For further advance, optimization of the regeneration system is necessary for the cofactor. Moreover, the coenzyme system should be developed for vitamin B_{12}.

Chapter III

Effective co-production of 3-hydroxypropionic acid and 1,3-propanediol

3.1. Summary

3-Hydroxypropionic acid (3-HP) and 1,3-propanediol (1,3-PDO) have a tremendous potential market in many industries. This study evaluates the simultaneous bioproduction of 3-HP and 1,3-PDO, using the new Psychrophile-based Simple bioCatalyst (PSCat) reaction system. The new method of PSCat is based on the expression of each gene in the psychrophilic host bacteria, individually. Heat treatment at 45°C for 15 min deactivated the intracellular metabolic flux. The production process was started after adding adequate amounts of a substrate, cofactor, and coenzyme. In the solo production process, the production of 3-HP reached 62.0 mM per hour, and productivity was 5.40 g $L^{-1}h^{-1}$. The solo production of 1,3-PDO was 25.0 mM per hour, and productivity was 1.94 g $L^{-1}h^{-1}$. In the simultaneous production process, productivity was boosted, and the production of 3-HP increased by 13.5 mM and 1,3-PDO by around 5 mM, respectively. Hence, the feasibility of the simultaneous biosynthesis system for these compounds was verified in the PSCat approach. Furthermore, this simultaneous biosynthesis system has increased the efficiency of the process.

3.2. Introduction

3-Hydroxypropionic acid (3-HP) and 1,3-propanediol (1,3-PDO) are a three-carbon organic molecule. They are chiefly consumed as a monomer in the production of poly-tri-methyl-terephthalate (PTT) plastic.

The PTT has superior properties, such as biodegradability and biocompatibility (Matsakas *et al*., 2018; Ko *et al*., 2017; Vivek *et al*., 2016). The market potentials of 3-HP (10 billion USD) and 1,3-PDO (600 million USD) make them a treasured target for industrial production (Ko *et al*., 2017). Both compounds were in the list of high-value bio-based chemicals or materials released by the U.S. Department of Energy (Werpy *et al*., 2004).

Using microorganisms in the synthesis of both compounds is an economical and eco-friendly solution for industrial production. Several reports have shown that *K. pneumoniae* could convert glycerol to 3-HP and 1,3-PDO (Vidra and Németh, 2018; Rodriguez *et al*., 2016). In *K. pneumoniae*, the production pathway of 3-HP and 1,3-PDO mainly consists of two routes. The dehydration reaction by glycerol dehydratase enzyme catalyzes 3-hydroxypropionaldehyde (3-HPA) from glycerol. Glycerol dehydratase requires vitamin B_{12} for it activity. In the first route, aldehyde dehydrogenase (PuuC) enzyme oxidizes 3-HPA to 3-HP (Figure 3.1). For efficient oxidation, NAD^+ is reduced to NADH by aldehyde dehydrogenase along with oxidation from 3-HPA to 3-HP. The second route involves the production of 1,3-PDO through reduction of 3-HPA. The 3-HPA is reduced by NADH-dependent 1,3-propanediol dehydrogenase enzyme (Figure 3.1) (Matsakas *et al*., 2018; Vidra and Németh, 2018; Park *et al*., 2017; Ma *et al*., 2010).

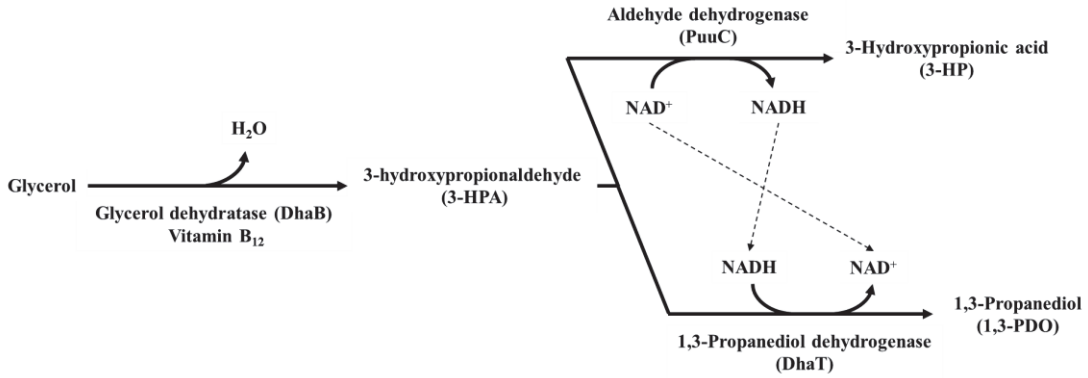

Figure 3.1. Schematic diagram of 3-HP and 1,3-PDO production from glycerol.

Individual production of 3-HP and 1,3-PDO by using microorganisms that possess the intrinsic reductive pathway or are genetically manipulated are conventional approaches (Yun *et al*., 2018; Li *et al*., 2016; Moscoviz *et al*., 2016; Zhao *et al*., 2016; Huang *et al*., 2013). Due to the limitation of the cofactors' cellular availability, the low yield of production, and the requirement of the exogenous supply of high-cost vitamin B_{12}, the bacterial synthesis of these compounds remains challenging (Ko *et al*., 2017; Ashok *et al*., 2011). The coproduction of 3-HP and 1,3-PDO or development of innovative approaches for producing each target compound will be the solutions to overcome these challenges.

Simultaneous production of 3-HP and 1,3-PDO in a single cell could be a solution to the challenges mentioned earlier (Huang *et al*., 2012; Ashok *et al*., 2011). The simultaneous production of both compounds balances NADH and NAD^+ and makes the cofactor regeneration system (Kumar *et al*., 2012). The titer of coproduction of these compounds was much lower than in their separate production, because of excessive accumulation of undesirable by-products such as lactate, ethanol, and acetate (Ko *et al*., 2017). Therefore, considering a new technique could assist in solving the remaining challenges.

Psychrophile-based Simple bioCatalyst (PSCat) is an innovative whole cell-based production method that could conquer many of the challenges mentioned. This technique can be the solution for the accumulation of undesirable by-products, low yield, and mesophilic bacteria's possible pathogenicity. Chapter II describes the development of the new approach to PSCat, and I showed that this method could produce 1,3-PDO with 97% yield.

This study examined the potential of the new PSCat approach for the production of 3-HP and coproduction of 3-HP and 1,3-PDO. In this study, *Shewanella livingstonensis* and *Shewanella frigidimarina* were transformed via genes encoding glycerol dehydratase (*dhaB*; conversion of glycerol to 3-HPA) 1,3-propanediol dehydrogenase (*dhaT*; conversion of 3-HPA to 1,3-PDO) and aldehyde dehydrogenase (*puuC*; conversion of 3-HPA to 3-HP) derived from *K. pneumoniae*. Then, the best condition of PSCat was examined for producing 3-HP alone and along with 1,3-PDO.

3.3. Materials and methods

3.3.1. Bacterial strains, plasmids, and culture conditions

Previous in chapter II, I describe the bacterial strains, plasmids, and culture conditions. However, to review again were chosen *S. livingstonensis* Rif^r and *S. frigidimarina* Rif^r as psychrophilic hosts to construct recombinant strains. The *E. coli* was transformed with all genes individually, and all recombinant *E. coli* strains were cultured in Luria–Bertani medium (LB) medium (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L) at 37°C. The psychrophilic bacteria were cultured in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) with 50 mg/L rifampicin at 18°C. I added 100 mg/L ampicillin to the culture media

to maintain the plasmids' presence in the transformed bacteria. For enzyme activity and production tests, 100 mL of TSB medium was prepared in 250 mL Erlenmeyer flasks, and 1% of the recombinant psychrophilic host bacteria were inoculated in it. The flasks were incubated in a rotary shaker incubator at 18°C and 120 rpm for 1 day. Isopropyl β-D-1-thiogalactopyranoside (IPTG), at a final concentration of 1 mM, was added to the cultured bacteria to express all recombinant enzymes. All Erlenmeyer flasks were kept for further 2 days at the incubator shaker.

3.3.2. Plasmid construction

As mentioned in the previous chapter, the *S. livingstonensis* Rif^r and *S. frigidimarina* Rif^r was transformed by the genes involved in the 1,3-PDO production (*dhaB*, and *dhaT*). Herein, I amplified the *puuC* from *K. pneumoniae* using the thermal cycler Gene Atlas G (ASTEC Co., Ltd., Fukuoka, Japan). The KOD plus Neo polymerase (TOYOBO, Osaka, Japan) was applied according to the manufacturer's recommendations to amplify the *puuC* gene. The pHA12 vector, as a broad host range expression vector was considered to overexpress this gene in both psychrophilic host bacteria (Arai *et al*., 1991). The pHA12-*puuC* (pU) was constructed by inserting the PCR fragments into the pHA12 vector. The constructed vector was amplified in *E. coli* DH5α (TOYOBO). The extracted vector from *E. coli* DH5α was used and transformed *E. coli* S17-1. Trans-conjugation was used to introduce the plasmid from *E. coli* S17-1 into *S. livingstonensis* Rif^r and *S. frigidimarina* Rif^r. All transformed cells were selected using rifampicin (50 mg/L) and ampicillin (100 mg/L). Gene, primer, and plasmid used in this study are listed in Table 2.1, 3.1, and Figure 2.2, 3.2.

Table 3.1. Bacterial strains, plasmids, and primers used in this study

Strains, plasmids, and primers	Relevant characteristics	Source or reference
S. livingstonensis strains		
Ac10-U	Ac10-Rif^r harboring pHA12-*puu*C	This study
S. frigidimarina strains		
Sf-U	Sf-Rif^r harboring pHA12-*puu*C	This study
Plasmids		
pHA12	Amp^r, expression vector	(Arai *et al*., 1991)
pHA12-*puuC*	Amp^r, pHA12 containing *K. pneumoniae puu*C	This study
Primers		
Forward - *puuC*	5′-AATTGAATTCGGAGAGATGAACAATGATGAATTTTCAGCACCTGGC -3′	This study
Reverse - *puuC*	5′-CGGTTCTAGATCAAGACTCCAGGGCAATCC-3′	This study

* Sequences with underline refer to SD sequence.

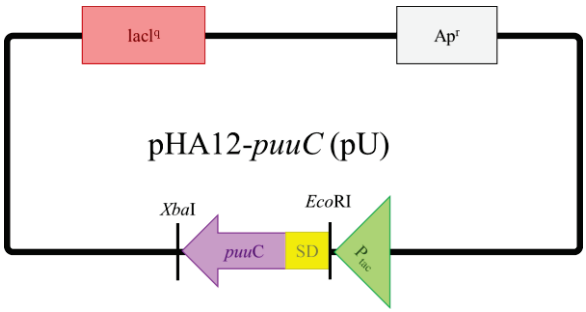

Figure 3.2. The schematic picture of pU plasmid was used in this study.

3.3.3. Enzymatic activity

A temperature of 40°C was considered for the simultaneous production of 1,3-PDO and 3-HP. Therefore, the enzymatic activity was measured for all recombinant enzymes at 40°C. Three-day culture of transformed bacteria was prepared according to the previous described

method. Well-grown transformed bacteria were collected by using centrifugation at 3,300 ×*g* for 5 min at 4°C, and washed twice with 70 mM sodium phosphate buffer (pH 8).

To suspend the pellets, five milliliters of 50 mM sodium phosphate buffer containing 1 mM DTT was used. An ultrasonic disintegrator (Digital Sonifier 450; Branson, Danbury, CT, USA) was set at 600 cycles of 1-s pulse/1-s intervals to sonicate the cell suspension. Cell debris was removed by centrifugation at 20,400 ×*g* for 30 min at 4°C.

DhaT activity was measured according to a published paper by González-Pajuelo *et al*. (2006) (González-Pajuelo *et al*., 2006). However, the temperature is set at 40°C to measure enzymatic activity. The supernatant containing proteins was diluted with 50 mM sodium phosphoric acid into the cuvette. NAD^+ and substrates for the DhaT enzyme were added to the reaction after heat treatment. For measuring the activity of PuuC, I used the protocol followed by Raj *et al*. (2010) (Raj *et al*., 2010). Herein, similar to DhaT, the activity of PuuC was measured at 40°C. The substrate and NADH were added to the reaction after heat treatment.

The activity was determined by measuring the oxidation of NADH to NAD^+ at 340 nm. Thermo Evolution 260 Bio (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to measure NADH's increase. The enzyme activity was calculated using the following formula: Activity of enzyme (μmol/min/mg protein) = (ΔAbsorbance340 × Vt × 60 × 1000) / (Δt × 6.3 × 103 × d × P × Vs), where Vt = total reaction volume (mL), Δt = time (second), d = optical path length (cm), P = protein concentration (mg/mL), and Vs = volume of culture used in the assay (mL).

To assess the data for the total amount of protein, the crude enzyme

solution was diluted. Ten microliters of diluted solution were added to 990 µL of Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) diluted five-fold. The mixture was incubated at 20°C for 5 min, and then absorbance was measured at 595 nm. The bovine serum albumins (BSA; Katayama Chemical Industry, Osaka, Japan) were used to prepare serial dilution (0.1~0.5 mg/ml). The process of protein assay was done. The obtained data were used to plot out the protein calibration curve. The protein amount was calculated from the prepared protein calibration curve.

3.3.4. Production of target compounds

For the production of 3-HP, 1,3-PDO, and 3-HPA, the recombinants *S. livingstonensis* and *S. frigidimarina* were cultured as mentioned earlier. After 72 h, the well-grown recombinant bacteria were harvested and washed twice with sodium phosphate buffer (70 mM, pH 8).

The enzymatic reaction was started after cell suspensions were heat-treated at 45°C for 15 min. Adding glycerol (20 mM for the production of 1,3-PDO and 3-HPA, and 80 mM for the production of 3-HP), 15 µM vitamin B_{12}, 50 mM KCl (only for the production of 3-HPA), and 0, 5, 10, and 15 mM of the appropriate cofactor (for the production of 3-HP and 1,3-PDO) to the cell suspension was followed after heat treatment. The reaction tube was incubated at 40°C for 1 h. The production process was stopped after 1 h by heat treatment of the tube at 80°C for 15 min. The cell supernatant was obtained by centrifugation (20,400 ×*g* for 5 min at 4°C). Heat treatment and enzymatic reactions were carried out in a water bath (SM-05 N, Taitec, Koshigaya, Japan).

3.3.5. Metabolites analysis

The quantification of 3-HPA was described in the previous chapter. Briefly, 150 µL of a solution containing 10 mM tryptophan dissolved in

0.05 N HCl was added to 200 μL of the sample. Subsequently, 600 μL of 37% HCl was mixed with other parts and incubated at 37°C for 20 min. The Ultrospec 2100 UV-Visible Spectrophotometer (Biochrom, MA, USA) was set at 560 nm. Acrolein (0–1 mM) was used to obtain the standard curve.

A high-performance liquid chromatography system (HPLC; Jasco, Tokyo, Japan) with a guard column (Shodex RSpak KC-G; Showa Denko, Kanagawa, Japan) was used to analyze the metabolites. The amount of 3-HP was measured by the UV-2070 Plus detector. The RI-1531 detector detected 1,3-PDO and residual glycerol. The target compounds were flowed at 30°C using 0.1 % (v/v) phosphoric acid as the mobile phase at a 0.7 mL/min flow rate.

3.4. Results

The new PSCat approach's potential was examined to produce 1,3-PDO using the psychrophile *S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253 at 37°C (Chapter II). Two important features of the new PSCat strategy are control of all parts of the production process and no limitation on the expression of the number of genes. Therefore, I endeavored to examine glycerol's efficient conversion to 1,3-PDO and 3-HP simultaneously with this new strategy.

3.4.1. Enzymatic activities

The activity of all production enzymes was measured in both psychrophilic host cells at 40°C. The *dhaT* and *puuC* genes from *K. pneumoniae* NBRC 14940 and the *dhaB* gene cluster (*dhaB*1, *dhaB*2, and *dhaB*3) from *K. pneumoniae* T7 were cloned into pHA12. The nucleotide sequence of cloned *puuC* was entirely identical to that of the *K.*

pneumoniae FDAARGOS_775 gene. The *puuC* nucleotide sequence was submitted to the DDBJ database under the accession number LC551792.

The crude enzyme solution for all recombinant strains was prepared to obtain optimal conditions for heat treatment. Enzyme solution was diluted 5, 10, and 100 times and heated at 40, 45, 50, 55, and 60°C for 10 and 15 min to inactivate the psychrophilic host enzymes. One hundred times dilution of crude enzyme solution showed the best results (data not shown). The activities of PuuC and DhaT were expedited at 40°C.

3.4.1.1. PuuC activity

Aldehyde dehydrogenase (PuuC) activity was measured in recombinant Ac10-U and Sf-U cells. To inactivate all psychrophilic host enzymes, the prepared recombinant cell suspensions were heated at 45°C for 15 min. However, this heat treatment was not enough, and some activity was still observed (Figure 3.3). The rise in temperature was not practical to inactivate all psychrophilic enzymes because of the decrease in the activity of PuuC. Therefore, 45°C for 15 min was preferred as a pre-heating treatment to keep the highest activity of the PuuC enzyme for the rest of the investigation. Two different final concentrations of NADH (0.5 and 1 mM) were examined in this step. At first, when the concentration of NADH was set at 0.5 mM, the recombinant Ac10-U strain showed 46.1±8.5 μmol/min/mg protein activity for PuuC. The activity of this enzyme was 39.4±6.2 μmol/min/mg protein when the recombinant Sf-U strain was added to the reaction (Figure 3.3). In both the Ac10-U and Sf-U strains, the activity of PuuC increased with an increase in the amount of NADH (1 mM) added to the reaction (Figure 3.3).

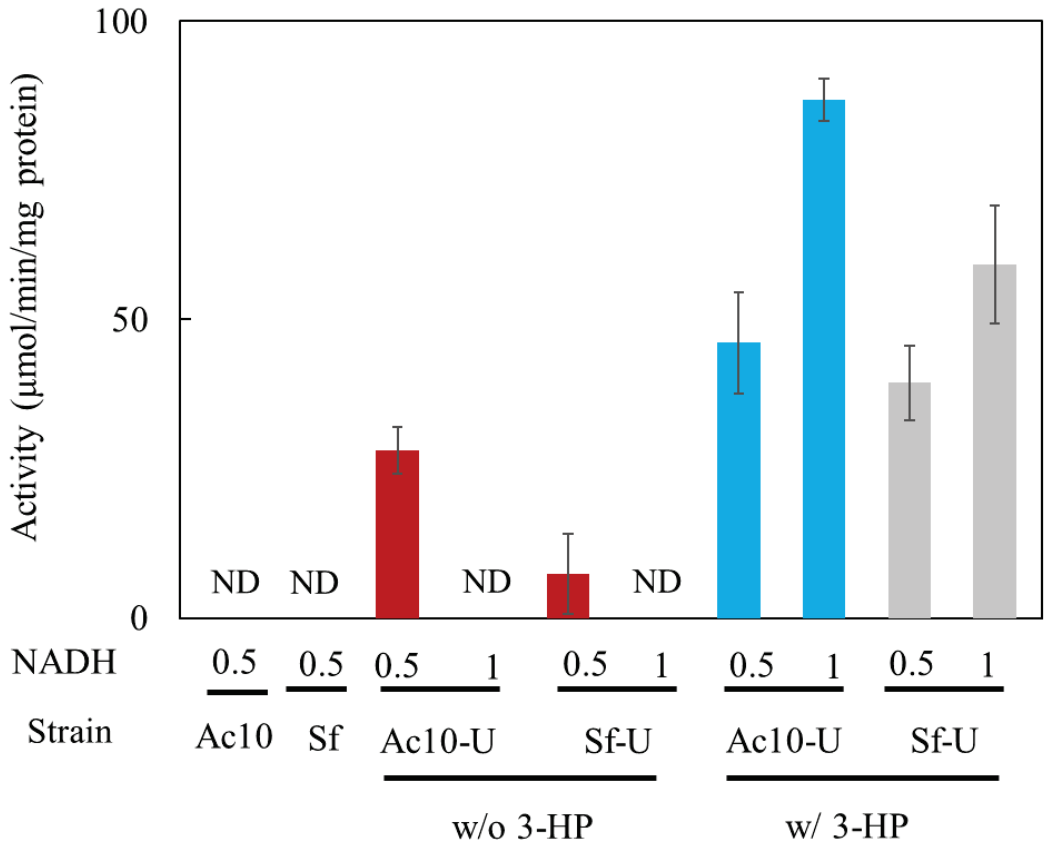

Figure 3.3. The activity of PuuC at 40°C. The reaction catalyzed by the DhaT was 3-Hydroxypropionic acid + NADH + H^+ = 3-hydroxypropanal + NAD^+. The crude enzyme solution was heated at 45°C for 15 min, and the substrate and cofactor were added as per the reaction. w/o, without substrate; w/ with the substrate. Error bars represent the standard deviation of the mean (n = 3).

3.4.1.2. DhaT activity

In chapter II, I reported the activity of the DhaT enzyme at 37°C. Due to the changing of the temperature for both simultaneous production of 3-HP and 1,3-PDO, I have to assess the activity of this enzyme at 40°C. To measure 1,3-PDO dehydrogenase activity at 40°C, recombinant Ac10-T and Sf-T cells, which only expressed DhaT under the control of the *tac* promoter, were cultured and crude enzyme solution were prepared. All

psychrophilic host enzymes were inactivated by heating the cell suspension at 45°C for 15 min. The final concentrations for NAD^+ were set at 0.5 and 1 mM in this step. I observed the activity of DhaT in recombinant Ac10-T was higher than that of recombinant Sf-T at both concentrations of NAD^+ (Figure 3.4). The activity of this enzyme in the recombinant Sf-T strain was 143.2± 19.8 and 104.5±14.3 µmol/min/mg protein when 1 and 0.5 mM cofactor was added to the reaction, respectively (Figure 3.4).

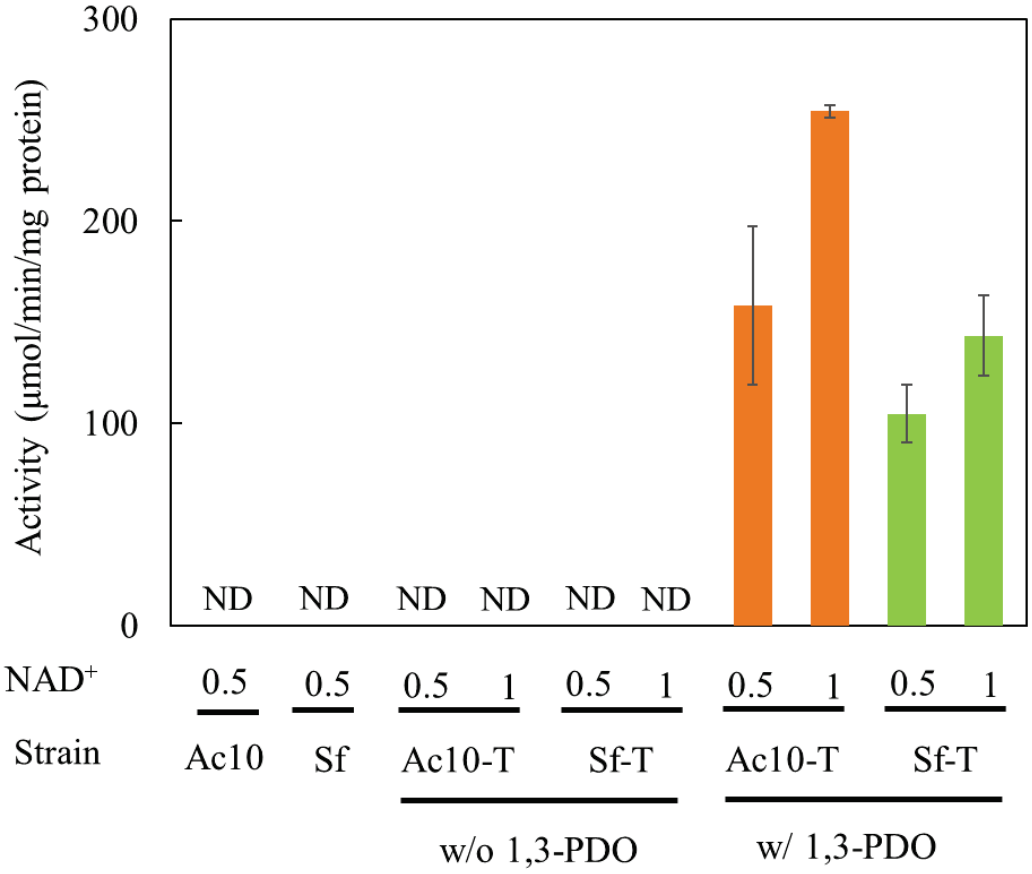

Figure 3.4. The activity of DhaT at 40°C. The reaction catalyzed by the DhaT was propane-1,3-diol + NAD^+ = 3-hydroxypropanal + NADH + H^+. The crude enzyme solution was heated at 45°C for 15 min and then added substrate and cofactor tailored to the reaction. w/o, without substrate; w/ with the substrate. Error bars represent the standard deviation of the mean (n = 3).

3.4.2. Production of 3-HP and 1,3-PDO individually

At first, the individual production of 3-HP and 1,3-PDO was examined at 40°C. The genes involved in the production process were cloned into the pHA12 vector and expressed them individually in two psychrophilic host bacteria. Due to one gene's expression under the control of the *tac* promoter, I expected the production enzyme expression levels to rise in both recombinant cells. More importantly, I could adjust the enzymatic activities by the blend ratio of cultivated cells. This feature is a result of the expression of each gene individually. Therefore, I expected to find both compounds' maximum production when optimizing the volume of cultivated cells added to the reaction.

Cell density was adjusted to around 22.3 g dry cell/L, before the production process with recombinant host cells. The prepared cell suspension was heated at 45°C for 15 min. Subsequently, glycerol as substrate, NAD^+ or NADH as the cofactor, and vitamin B_{12} were added. The final concentration of vitamin B_{12} was 15 μM for all tests. Reaction mixture were incubated at 40°C. I considered the parent *S. livingstonensis* Ac10 Rif^r, *S. frigidimarina* DSM 12253 Rif^r, and the recombinant Ac10-pHA12 strains as the negative control. No production of 3-HP or 1,3-PDO was observed in the parent strains and the recombinant Ac10-pHA12 strains.

3.4.2.1. Production of 3-HP

The *dhaB* gene cluster and *puuC* were individually cloned into pHA12. *S. livingstonensis* and *S. frigidimarina* were transformed with the pHA12 recombinant plasmid. The recombinant Ac10-B, Ac10-U, Sf-B, and Sf-U (Table 2.1 and 3.1) was used to produce 3-HP.

To determine the optimum combination for production, different

volumes and combinations of each transformed cell were examined. At the beginning of the experiment, I mixed the same amount of each transformed bacterium to the reaction (DhaB:PuuC = 1:1). In the subsequent tests, one of the bacteria's content was kept two or three times higher than the other one to examine the different ratios of transformed bacteria in the production of the 3-HP (DhaB:PuuC = 3:1, 2:1, 1:2, and 1:3). The substrate concentration was 80 mM, and different concentrations (0, 5, and 10 mM) of cofactor were tested (Figure 3.5).

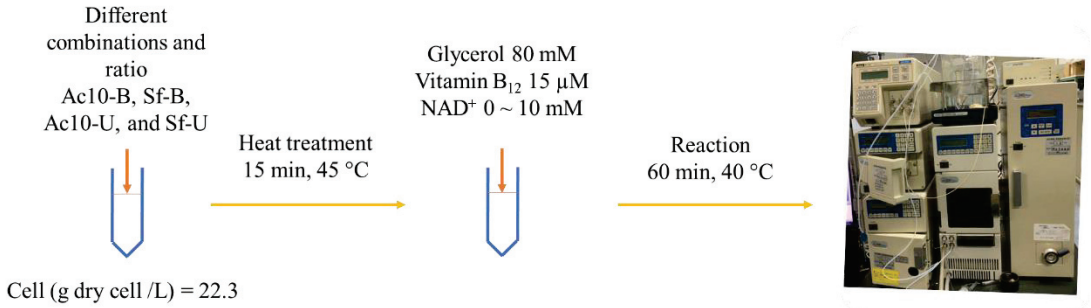

Figure 3.5. The schematic picture of the 3-HP individual production experiment.

The results indicated that in all combinations and ratios, raising the concentration of NAD^+ had a positive effect on the production of 3-HP (Figure 3.6 A-E). Based on the combination and ratio of bacteria, the increase in production was inconsistent. In all combinations and ratios, when no cofactors were added to the reaction, the production of 3-HP was approximately 20 mM (Figure 3.6 A-E). Furthermore, increasing the volume of the recombinant bacteria with DhaB resulted in an increase in production volume. I observed that the highest volume of production was achieved by adding 10 mM cofactor and DhaB:PuuC at a ratio of 2:1 to the reaction (57.2±7.8 mM; Figure 3.6 D). The ratio of DhaB:PuuC (2:1) was chosen and examined the production of 3-HP when the final concentration

of NAD^+ was 15 mM. The latest test results indicated that the production of 3-HP reached 62.0±4.0 mM (Figure 3.7). Therefore, the combination of Ac10-B and Sf-U with the ratio of DhaB:PuuC (2:1) was selected for further experiments in this study.

I examined the production of 3-HP and glycerol residues over time. The recombinant Ac10-B and Sf-U with the ratio DhaB:PuuC (2:1) was used, and after heating at 45°C for 15 min, 15 mM NAD^+, 15 μM vitamin B12, and 80 mM glycerol were added to the mixture. The amount of 3-HP produced was measured over time. After 60 min of reaction, this compound's productivity was 5.40 g L^{-1} h^{-1}, and 61.5±4.2 mM was generated (Figure 3.8).

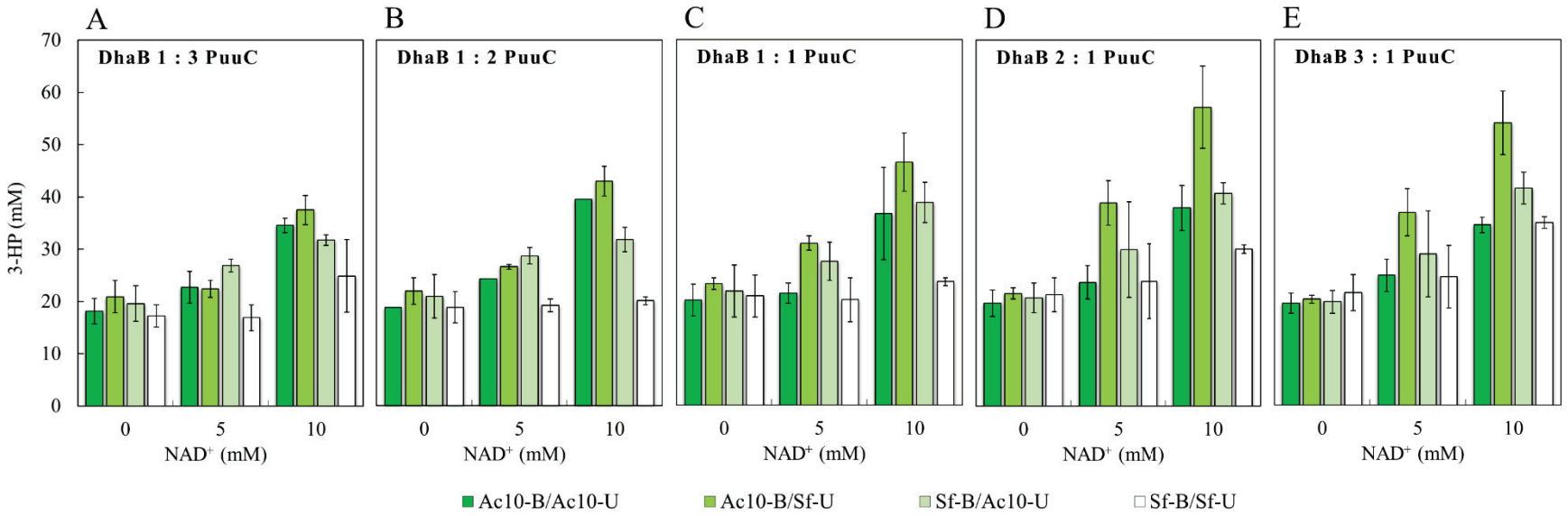

Figure 3.6. 3-HP productivity of recombinant cells at various ratios, combinations, and concentrations of NAD^+ at 40°C. The volume ratio of the recombinant cell suspension of DhaB to PuuC was 1:3 (a), 1:2 (b), 1:1 (c), 2:1 (d), and 3:1 (e). The initial concentration of glycerol and cells was 80 mM and 22.3 g dry cell/L, respectively. Error bars in all figures represent the standard deviation from the mean (n = 3).

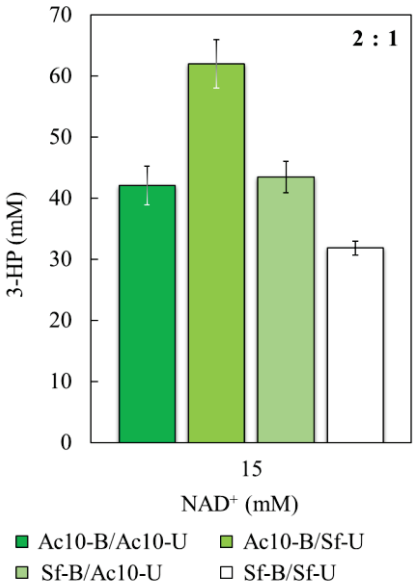

Figure 3.7. 3-HP production of recombinant cells at ratio DhaB:PuuC (2:1), 15 mM concentrations of NAD^+, and various combinations at 40°C. The initial concentration of glycerol (mM), and cells (g dry cell/L), was 80, 22.3, and 15, respectively. Error bars in all figures represent the standard deviation from the mean (n = 3).

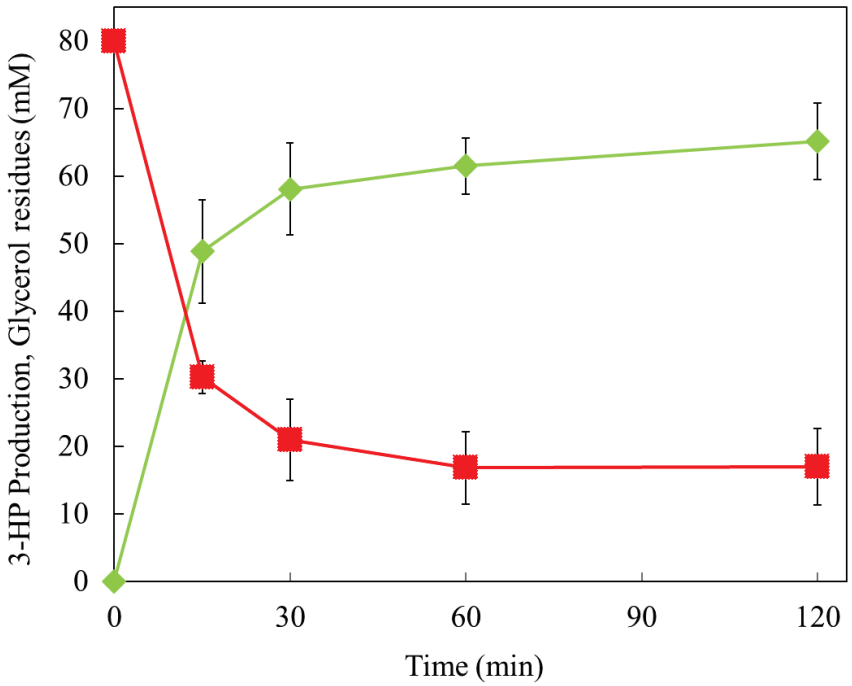

Figure 3.8. 3-HP productivity of recombinant Ac10-B/Sf-U at 40°C. Diamond (Green), 3-HP; squares (Red), glycerol. Error bars in all figures represent the standard deviation from the mean (n = 3).

3.4.2.2. Production of 1,3-PDO

The genes (*dhaB* gene cluster and *dhaT*) which involved in the 1,3-PDO production pathway were individually cloned into the pHA12 plasmids. Both psychrophilic host bacteria were transformed with the constructed vector (Mojarrd *et al*., 2020). Ac10-B, Ac10-T, Sf-B, and Sf-T (Table 2.1) recombinant strains, were used to synthesize 1,3-PDO.

As I mentioned in chapter II for the 1,3-PDO production experiment at 37°C, different volumes and combinations of each transformed bacteria were added to the reaction (DhaB:DhaT = 2:1, 1:1, and 1:2). The different concentrations of the cofactor were tested (0, 5, 10, and 15 mM) and the concentration of glycerol as the substrate was 20 mM (Figure 3.9).

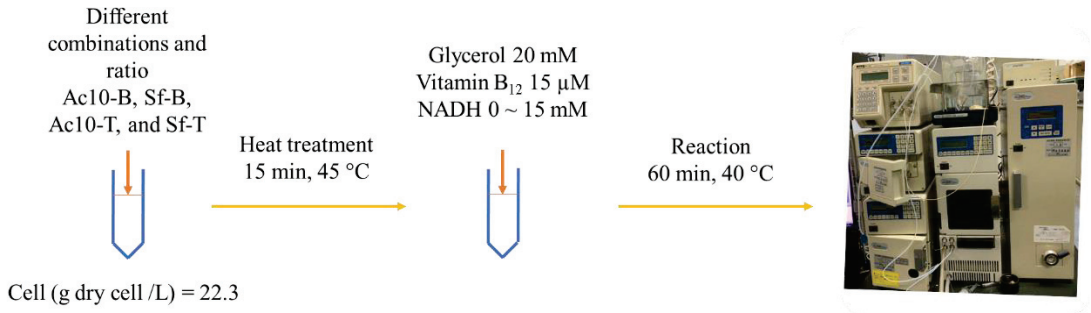

Figure 3.9. The schematic picture of 1,3-PDO individual production experiment.

The results demonstrated that increasing the concentration in all combinations and ratios contributed to an increase in 1,3-PDO production (Figure 3.10 A-C). The best concentration of cofactor for production in all combinations and ratios was 15 mM. Furthermore, increasing the volume of the recombinant bacteria with DhaB had a positive impact on the production of the target compound (Figure 3.10 A-C). The results showed that the combination of Ac10-B:Sf-T with ratio 2:1 had a better potential to

produce 1,3-PDO. This combination with ratio 2:1 demonstrated 19.7±0.8 mM production when the final concentration of NADH was 15 mM. Therefore, the combination Ac10-B:Sf-T (2:1) was selected for further experiments in this study.

The ratio DhaB:DhaT (2:1) when using the combination Ac10-B:Sf-T was considered to examine 1,3-PDO production and glycerol residues over time. Both Recombinant bacteria were mixed and heated at 45°C for 15 min. Subsequently, 15 mM NADH and 15 μM vitamin B_{12}, and 20 mM glycerol were added to the reaction. After 60 min of reaction, all substrates were consumed and 20.0±0.6 mM of 1,3-PDO was generated (Figure 3.11). This compound's productivity was 1.94 g L^{-1} h^{-1}, and the yield (1,3-PDO production/glycerol consumption) was 100%. For the next test, the final concentration of glycerol in the reaction was reached 30 mM. The production of 1,3-PDO was reached 25.02 mM, after 60 min (Figure 3.12).

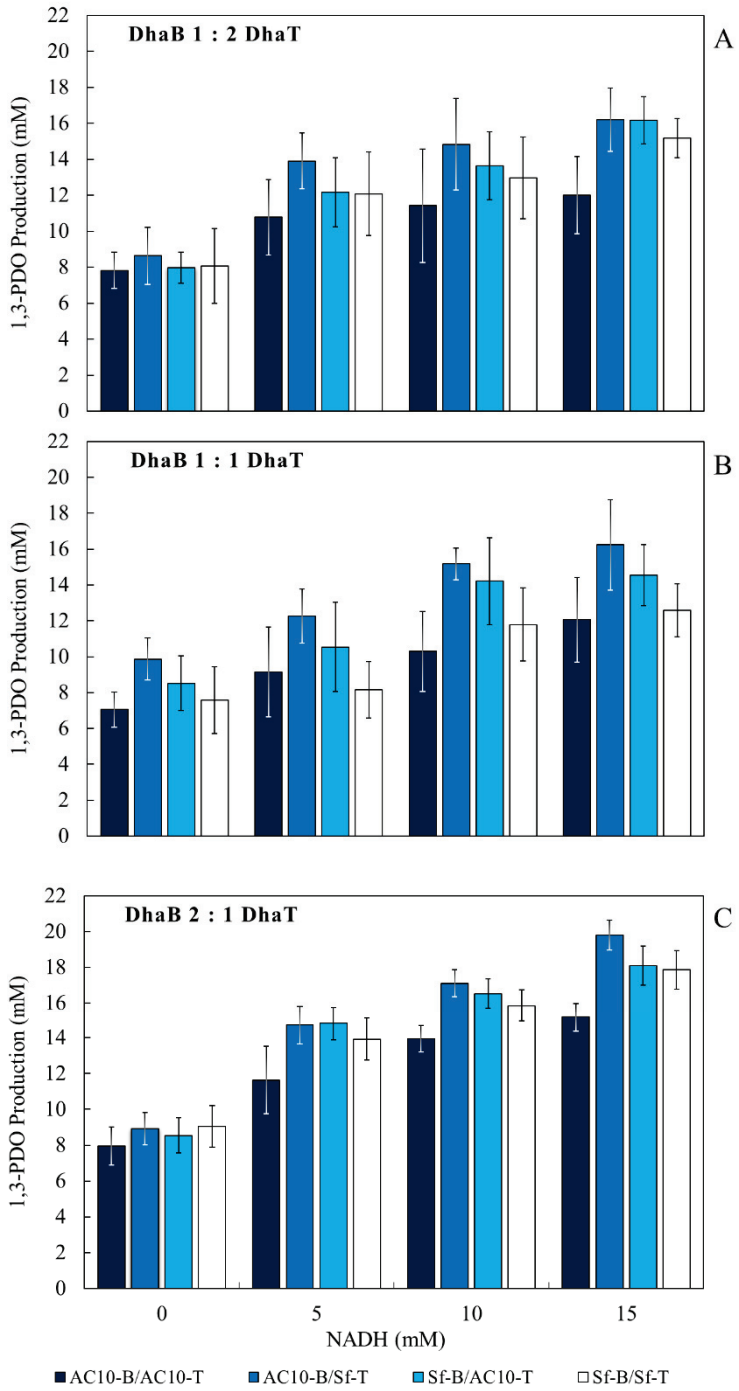

Figure 3.10. 1,3-PDO productivity of recombinant cells at various ratios, combinations, and NADH concentrations at 40°C. The volume ratio of the recombinant cell suspension of DhaB to DhaT was 1:2 (A), 1:1 (B), and 2:1 (C). The initial concentration of glycerol and cells was 20 mM and 22.3 g dry cell/L, respectively. Error bars in all figures represent the standard deviation from the mean (n = 3).

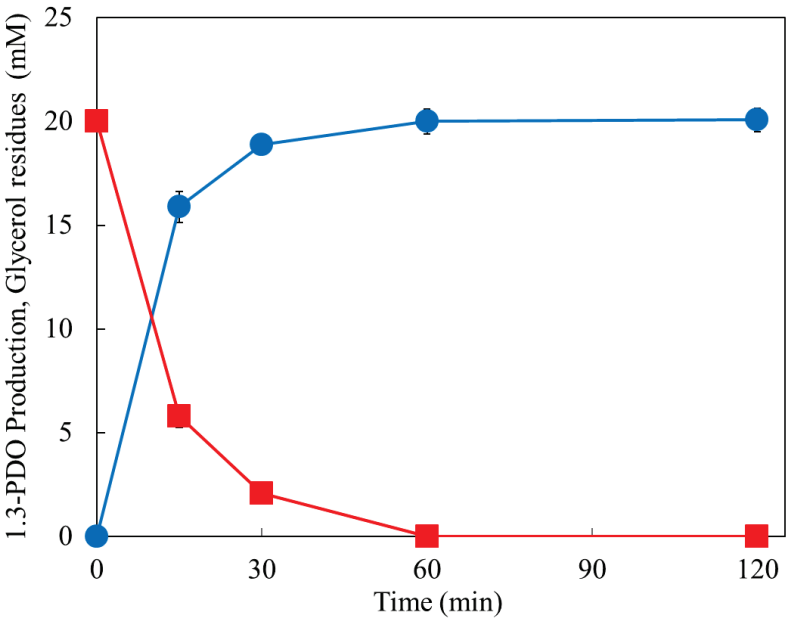

Figure 3.11. 1,3-PDO productivity of recombinant Ac10-B/Sf-T overtime at 40°C. The initial concentration of glycerol (mM), cell (g dry cell/L), and NADH (mM) are 20, 22.3, and 15. Circles (Blue), 1,3-PDO; squares (Red), glycerol. Error bars represent the standard deviation of the mean (n = 3). Error bars represent the standard deviation of the mean (n = 3).

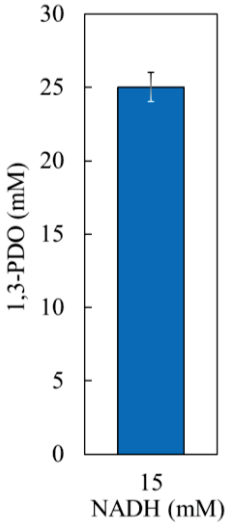

Figure 3.12. 1,3-PDO productivity of recombinant Ac10-B/Sf-T at 40°C. The initial concentration of glycerol (mM), cell (g dry cell/L), and NADH (mM) are 30, 22.3, and 15. Error bars represent the standard deviation of the mean (n = 3).

3.4.2.3. Production of 3-HPA

For 3-HPA production, the recombinant Ac10-B and Sf-B were considered to check the potential of PSCat. The concentration of well-growth recombinant cell was set to 22.3 g dry cell/L, and enhanced with 20 mM glycerol and 15 µM vitamin B_{12}. The production of 3-HPA in the Sf-B strain was 19.5±0.3 mM after 30 minutes, and 16.7±0.4 mM in the Ac10-B strain after only 15 minutes (Figure 3.13). The activity of DhaB was measured for both recombinant host cells based on the production of 3-HPA. The activity of this enzyme for the recombinant Ac10-B was not significantly higher than that of recombinant Sf-B. Therefore, I considered this enzyme activity in both recombinant host cells to be in the same range.

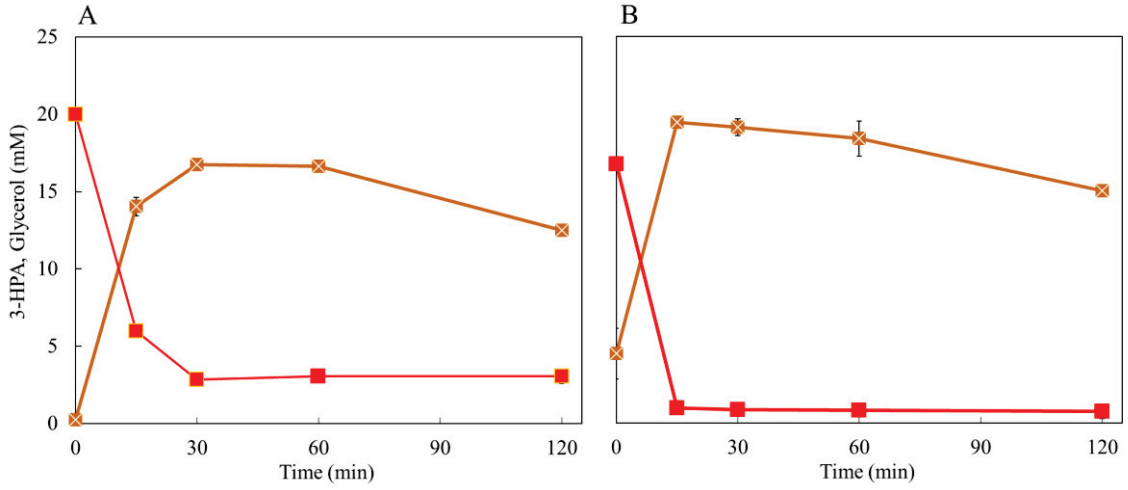

Figure 3.13. 3-HPA productivity of recombinant Ac10-B cells (A) and recombinant Sf-B cells (B) at 40°C. The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B12 (µM) was 20, 22.3, and 15, respectively. Square with a cross inside (Brown), 3-HPA; Squares (Red), glycerol. Error bars in all figures represent the standard deviation from the mean (n = 3).

3.4.3. Simultaneous production of 3-HP and 1,3-PDO

One limitation of the efficient production of 3-HP and 1,3-PDO is limited cellular cofactor (NADH and NAD^+) availability. PuuC and DhaT enzymes need oxidized and reduced forms of the cofactor. Therefore, these enzymes could create a cofactor regeneration system (Figure 3.1). To achieve that goal, the DhaB, PuuC, and DhaT were expressed individually under the tac promoter's control in both psychrophilic host bacteria. The preliminary test results indicated that adding all recombinant cells to the reaction at the beginning of the production process leads to reduced 3-HP production and did not increase 1,3-PDO production. Therefore, I produced both compounds based on individual production (ratio 2:1) for 60 min, separately. Then, the contents of both production microtubes were mixed together. After mixing, 30 mM glycerol was added to the production microtubes, and the concentration of the coenzyme was adjusted (Figure 3.14). The results of this test demonstrated that the mixing process triggered the inactivation of DhaB (Figure 3.15 A~B).

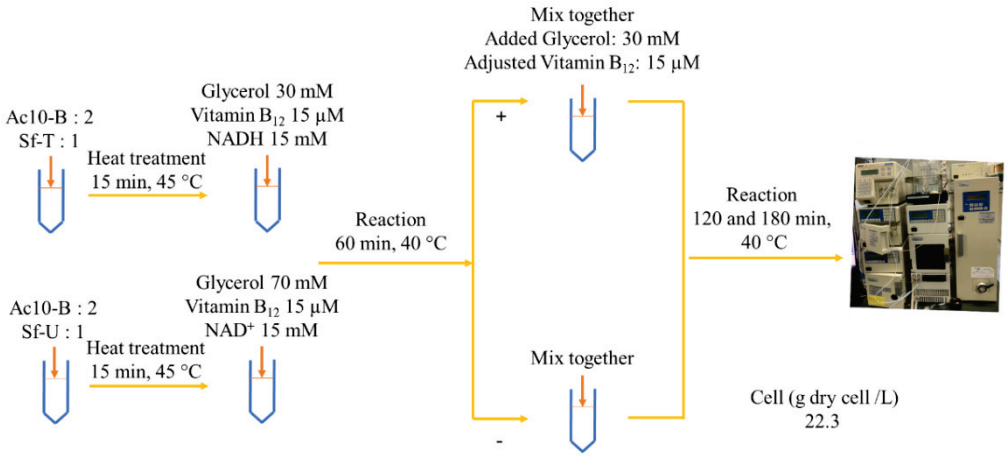

Figure 3.14. The schematic picture of the simultaneous production of 3-HP and 1,3-PDO experiment. The ratio was 4:1:1(DhaB:PuuC:DhaT).

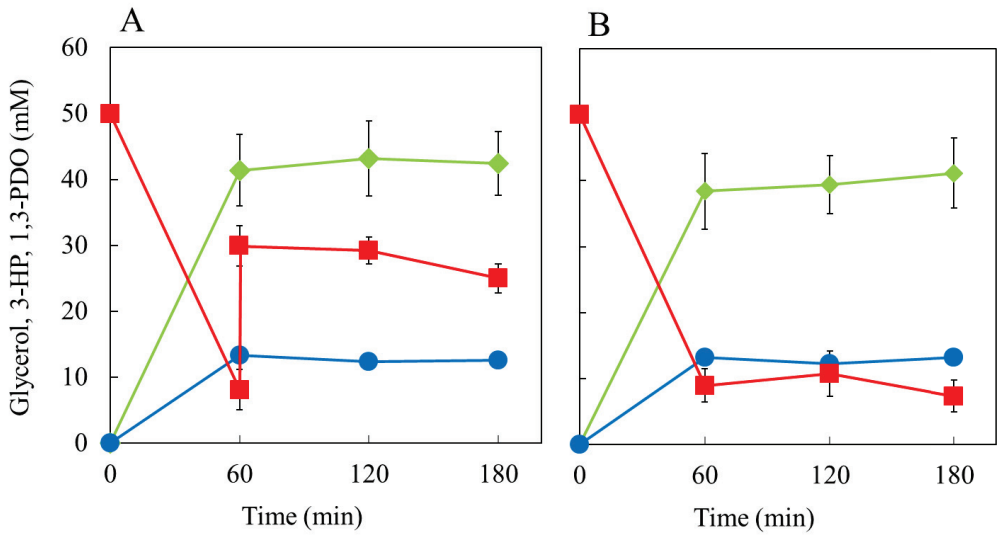

Figure 3.15. Coproduction of 3-HP and 1,3-PDO. The ratio 4:1:1(DhaB:PuuC:DhaT) (A) the production tube (B) the control tube (w/o addition of glycerol and vitamin B_{12} after 1 hour). The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B_{12} (µM) was 70, 22.3, and 15, respectively, for 3-HP production for the first hour of production. The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B_{12} (µM) was 30, 22.3, and 15, respectively for 1,3-PDO production for the first hour of production. The contents of both production tubes were mixed and 30 mM glycerol was added after 1 hour. Diamond (Green), 3-HP; Circles (Blue), 1,3-PDO; Squares (Red), glycerol. Error bars in all figures represent the standard deviation from the mean (n = 3).

In the last phase of this experiment, to create stable production conditions, a 4:2:1 ratio was chosen for Ac10-B:Sf-U:Sf-T. In this part, after mixing the contents of both production microtubes, I added two volumes of pre-heated recombinant Ac10-B and one volume of Sf-U to the reaction. I also adjusted the concentration of vitamin B_{12} and added 30 mM glycerol as a substrate base to the new volume in the production tube. For the control group, after an hour, I added only more cell suspensions (Figure 3.16). After an hour of mixing, 3-HP and 1,3-PDO production reached approximately 30 mM and 16 mM in the control and reaction tubes, respectively. After 120 min, the results showed that the production increased for both compounds. In the reaction tube, the production reaching 48.3 mM for 3-HP and 24.3 mM for 1,3-PDO. As a reminder, I added more substrates and adjusted vitamin B_{12} concentration in the reaction tube. More importantly, no increase was observed in the control group. Following this, the rate of production stabilized (Figure 3.17 A, B).

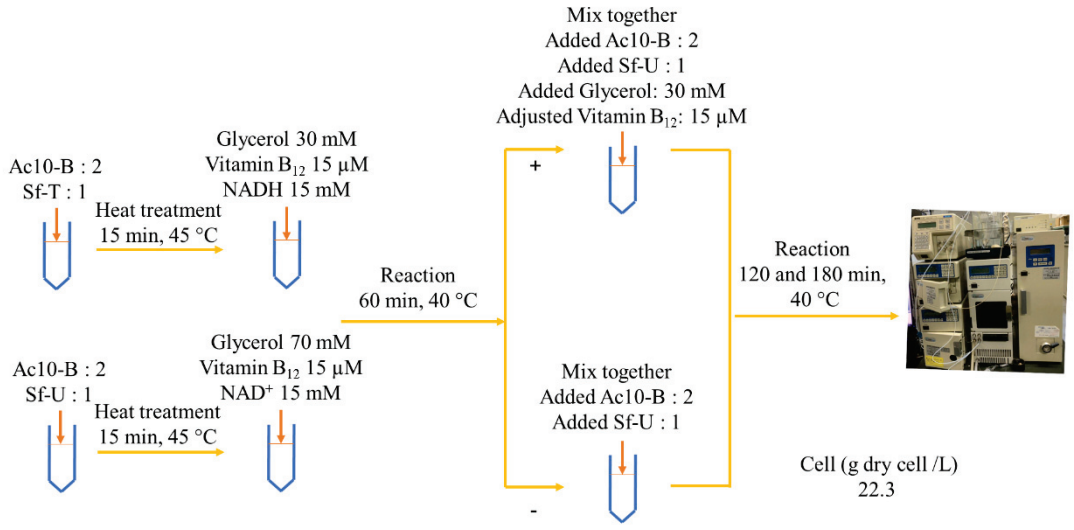

Figure 3.16. The schematic picture of the simultaneous production of 3-HP and 1,3-PDO experiment. The ratio was 4:2:1(DhaB:PuuC:DhaT).

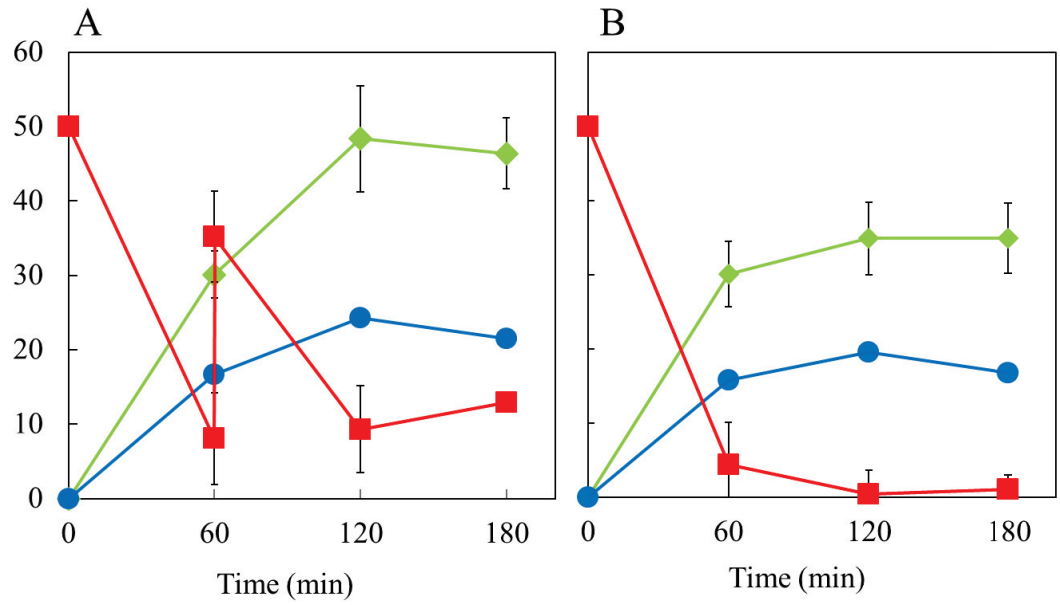

Figure 3.17. Coproduction of 3-HP and 1,3-PDO. The ratio 4:2:1(DhaB:Puu:DhaT) (A) the production tube (B) the control tube (w/o addition of glycerol and vitamin B12 after 1 hour). The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B12 (μM) was 70, 22.3, and 15, respectively, for 3-HP production for the first hour of production. The initial concentration of glycerol (mM), cells (g dry cell/L), and vitamin B12 (μM) was 30, 22.3, and 15, respectively, for 1,3-PDO production for the first hour of production. Both production tubes were mixed, and two volumes Ac10-B, one volume Sf-PuuC, and 30 mM glycerol were added after 1 hour. Diamond (Green), 3-HP; Circles (Blue), 1,3-PDO; Squares (Red), glycerol. Error bars in all figures represent the standard deviation from the mean (n = 3).

3.5. Discussion

In this study, two valuable chemicals were simultaneously biosynthesized with a new approach of the PSCat system. Several experiments showed that some compounds had been efficiently biosynthesized using *S. livingstonensis* Ac10 via the conventional PSCat method (Luo *et al*., 2020; Tajima *et al*., 2013, 2015, 2018). The potential of the new approach of the PSCat system was demonstrated for the production of 1,3-PDO at 37°C in the previous chapter (Mojarrd *et al*., 2020). In this

new approach, each gene involved in the metabolic pathway is expressed separately in the psychrophilic host bacteria. The psychrophilic host enzymes were inactivated by heat treatment of the recombinant bacteria. Finally, the heated bacteria's right proportions were mixed, and the substrate was added to start the production reaction.

No production of undesirable by-products and high yield are the benefits of both new and conventional PSCat method. More importantly, the new method does not limit the number of genes and the simultaneous expression of all genes in the psychrophilic host bacteria. Furthermore, because of the necessity of reduced and oxidized forms of the cofactor by DhaT and PuuC, respectively, a co-production of 3-HP and 1,3-PDO could initiate a cofactor regeneration system. Therefore, I examined the production of 3-HP and 1,3-PDO in *S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253 using the new PSCat method. For starting the production reaction, finding optimal production conditions was essential.

The optimal temperature for the enzymatic activity of PuuC (EC 1.2.1.3) is 45°C (Raj *et al*., 2010) whereas DhaB (EC 4.2.1.30; Wang *et al*., 2007) and DhaT (EC 1.1.1.202; Lama *et al*., 2015) is 37°C. The range of temperature which the two producing enzymes (DhaT and PuuC) can tolerate was between 30 and 55°C (Raj *et al*., 2010; Lama *et al*., 2015). Therefore, I considered the 40°C for both enzymes. This temperature was close to the optimum temperature for their activity. In this study, the temperature required to inactivate host enzymes was also investigated.

The metabolic flux and psychrophilic host enzymes in both recombinant bacteria cell with *dhaT* could be inactivated at 45°C for 15 min. Nevertheless, the psychrophilic host enzymes in the recombinant host cells with *puuC* was not inactivated at 45°C. At this temperature, I detected

little activity in the control tube (the tube deficient of the substrate required for mesophilic enzymes) (Figure 3.3). Previous researches in PSCat had indicated that different temperatures might be used for the inactivation of psychrophilic enzymes (Tajima *et al*., 2015, 2018). In those researches, different genes were expressed in psychrophilic cells. For example, the host psychrophilic enzymes in the transformed *S. livingstonensis* Ac10 with the *aspA* gene were deactivated at 50°C for 15 min (Tajima *et al*., 2015). The temperature required to inactivate psychrophilic enzymes was affected by the enzyme expressed in the host cell.

In this study, due to the overexpression of PuuC in the recombinant psychrophilic host bacteria, they faced the growth limitation, especially for recombinant Sf-U. However, the total protein concentration in both recombinant Ac10-U and Sf-U was the same (Table 3.2 and Table 3.3). Based on these results, the overexpression of PuuC may cause some changes in the amount and type of proteins present in the cell. Because of the overexpression of *puuC*, the falling growth in *K. pneumoniae* was reported (Ashok *et al*., 2011). They presumed that this situation occurred due to the inhibition of protein overexpression and an imbalance in the metabolic flux ratio between reductive and oxidative pathways. Moreover, many researches had shown that efforts to produce large quantities of a single protein ended in boosted quantities of heat-shock proteins in bacterial cells (Kurland and Dong, 1996). These pieces of evidence could explain why the heat treatment at 45°C for 15 min was insufficient to deactivate all host enzymes in Ac10u and Sf-U.

Table 3.2. Optical density (660 nm) for recombinant bacteria

	Ac10-T	Sf-T	Ac10-U	Sf-U
Average	2.05±0.08	1.81±0.12	1.93±0.07	1.53±0.11

Table 3.3. The concentration of total Protein for recombinant bacteria (mg/ml)

	Ac10-T	Sf-T	Ac10-U	Sf-U
Average	2.95±0.39	2.49±0.25	2.88±0.26	2.81±0.25

Altering concentration of enzyme affects enzymatic activity. PSCat is an in vitro system, and in this system, enzyme activity can be controlled by adding more or less enzyme to the reaction mixture (Curtis *et al*., 2002). I used the *tac* promoter to express target genes, and because of that, I expected to achieve gene overexpression in this study. After analyzing the results, I found that the activity of DhaT in recombinant Ac10-T was higher than that in recombinant Sf-T. The results indicated that the recombinant Ac10-T generally grows more efficiently compared with the recombinant Sf-T. Further, the total protein concentration in the recombinant Ac10-T was higher than that in recombinant Sf-T (Table 3.2 and Table 3.3). This could explain the higher activity of DhaT in recombinant Ac10-T. In chapter II the same result was reported for the activity of DhaT at 37°C. A comparison of the enzymatic activity results in this chapter with the previous chapter showed that the activity of this enzyme at 40°C was slightly lower than that at 37°C when the 0.5 mM cofactor was added to the reaction.

Besides, the activity of the PuuC in recombinant Ac10-U was higher than that in recombinant Sf-U. I believe that the ability of the recombinant Ac10-U to grow faster led to increased activity of this enzyme. The intrinsic difference between *S. frigidimarina* and *S. livingstonensis*

may have caused the Ac10 strain to tolerate the effects of PuuC overexpression.

The new strategy of PSCat is to express each gene individually in the psychrophilic host bacteria. This new method permits me to control timing and volume of enzyme addition to the reaction mixtures. Besides, I had no limitation to the number of genes that I was able to express in the reaction. Therefore, I could examine the different combinations and ratios of bacteria to assess the best conditions for both chemicals' production. The results of individual production showed that addition of recombinant Ac10-B/Sf-T and Ac10-B/Sf-U with the 2:1 ratio of DhaB:DhaT and DhaB:PuuC had the highest level of production.

Adding a greater volume of PSCat cells to the reaction mixture could increase enzyme concentration in the new approaches of PSCat. Previous research has indicated that glycerol dehydratase is a key and rate-limiting enzyme in the 3-HP and 1,3-PDO pathways (Jiang *et al*., 2015; Daniel *et al*., 1998; Tong *et al*., 1991). The main reasons for this limitation are inhibitory effect of glycerol as a substrate on DhaB and the requiremnet of vitamin B_{12} in the catalytic cycle. Therefore, overexpression of *dhaB* is one of the solutions to overcome this challenge. However, the toxicity of 3-HPA, which is the product of this enzymatic reaction, could severely affect the growth and survival of bacteria (Sankaranarayanan *et al*., 2017; Zaushitsyna *et al*., 2017). In the new approaches of PSCat, I can easily add more DhaB enzyme to the reaction mixture. After heat treatment, both psychrophilic host cells were dead. Because of that, the lethal impact of 3-HPA on living cells had no inhibitory effect on the PSCat process.

The production of 3-HP and 1,3-PDO production are affected by the volume of DhaB enzyme. This study showed that rising the volumes of

DhaB had a positive impact on both chemicals' production amount. However, the balance between 3-HPA consumption by PuuC and DhaT and its production by DhaB is also essential (Chatzifragkou *et al*., 2011). Hence, adding three volumes of the heated *dhaB*-containing recombinant bacteria to the reaction for the production of 1,3-PDO and 3-HP upset this balance.

The combination of Ac10-B/Sf-U and Ac10-B/Sf-T was always higher than that in other combinations that I examined. The activity of both production enzymes (PuuC and DhaT) in the recombinant *S. frigidimarina* DSM 12253 was lower than these enzymes' activity in the *S. livingstonensis* Ac10. I believe that these fewer activities caused the right balance between 3-HPA consumption and production rate. These conditions lead to reaching the production of 3-HP to 61.5 mM and 1,3-PDO to 25.0 mM, after 60 min.

The production of 1,3-PDO with 1:1 ratio of DhaB:DhaT was not effective in this test. The activity of DhaB and DhaT was at the same range in this project. Because of the mentioned limitation of DhaB, a higher activity of DhaB was needed to produce 1,3-PDO efficiently. Therefore, I considered the 2:1 ratio of DhaB:DhaT. Adding Ac10-B, Sf-T with this ratio, and 30 mM glycerol to the reaction, the product reached 25.0 mM after 60 min. In chapter II, the same amount of production for 1,3-PDO was reported in the new PSCat method at 37°C. The yield of production in this short duration (only 60 min) is higher than in many previous reports (Zhou *et al*., 2019; Yun *et al*., 2018).

The new PSCat method showed significant potential for the biosynthesis of 3-HP. A high product concentration (above 100 g L^{-1}), productivity (over 2 g L^{-1} h^{-1}), and substrate-to-product yield (above 50%)

are required for bio-based production of 3-HP to be economically feasible (Vidra and Németh, 2018). The results showed that the yield and productivity of 3-HP were 75% and 5.4 g L^{-1} h^{-1} in this experiment, respectively. I believe that such a high output was possible because of the cofactor regeneration system between the PuuC enzyme and psychrophilic host enzymes. My results demonstrated that some of the host enzymes remained active after heat treatment. These psychrophilic enzymes could regenerate the cofactors and increase the availability of NAD^+ for the production of 3-HP. The activity of the psychrophilic host enzyme was lower than that of the PuuC enzyme. Some previous studies have reported that the activity of production enzymes is higher in cofactor regeneration systems compared to other enzymes in the cofactor regeneration system (Wang *et al*., 2013; Kaup *et al*., 2003).

Accumulation of 3-HP in the reaction causes the reduced acidity of the reaction medium. This is one limitation in the production of 3-HP (Vidra and Németh, 2018). This low acidity causes the enzyme's activity in an in vitro system, such as a PSCat and the pH-based growth inhibition for living producer bacteria. The activity of PuuC was utterly abolished at pH 6.0 (Raj *et al*., 2010). Therefore, I believe that because of the decrease in the PuuC enzyme activity, the yield of production could not reach 100%, and some glycerol remained unused in the reaction.

The K_M and K_{cat}/K_M for PuuC enzyme are lower than the DhaT enzyme when 3-HPA is used as a substrate (Raj *et al*., 2010; Lama *et al*., 2015). In this study, I found that the DhaT enzyme activity was higher than that of the PuuC enzyme in the recombinant *S. frigidimarina* with *dhaT* and *puuC*. Therefore, in co-production of both chemicals, when all recombinant cells were added to the reaction at the beginning of the production process,

all 3-HPA was consumed by DhaT and reached the maximum production potential, but 3-HP production was reduced.

To develope the co-production of both compounds, I added two volumes of the recombinant Sf-U and only one volume of Sf-T. Under these conditions, the lack of enzymatic activity was compensated because of the amount of PuuC was higher than DhaT in the reaction. This is the central advantage of the novel PSCat strategy. In this strategy, I can control enzymes' activity based on the desired amount of enzyme, which I added to the reaction.

The amounts of 3-HP and 1,3-PDO produced were much higher when I produced them individually after 1 hour. Because of mixing the microtube content, all compound concentrations were constant while the reaction volume increased at the moment of mixing. Therefore, the HPLC results showed that concentrations of all compounds and residual substrates decreased after mixing. I checked concentrations of the produced compounds before mixing, and I could not find any significant difference between them and individual production results.

The co-production results of 3-HP and 1,3-PDO with ratio 4:2:1 (DhaB:Puu:DhaT) showed an increase for both compounds. I believe that this increase might have two reasons. First, during the individual production of both target compounds, most of the cofactor is converted to other forms and accumulates in the production microtube. The mixing of the microtubes' contents caused an increase in the concentration of suitable cofactor for each enzyme in the reaction tube. This condition is similar to the addition of more cofactors to the reaction. In this project, I proved that adding more cofactors to the reaction positively affects production. The second reason is that the cofactor regeneration system might occur between

DhaT and PuuC. Further, I know that some psychrophilic host enzymes are still active and could increase the rate of oxidation of NADH to NAD^+. Therefore, I believe that NADH's concentration was lower than that of NAD^+, which might explain why the production of 3-HP increased more than the production of 1,3-PDO. Further studies are needed to optimize the cofactor regeneration system and to develop a DhaB regeneration system.

Chapter IV

General discussion and conclusion

Psychrophile-based Simple bioCatalyst (PSCat) is an innovative approach to produce valuable chemical with high yield. In addition to catalytic efficiency, this method has other benefits, such as no production of undesirable by-products and low impact on the environment. In previous reports the psychrophile *S. livingstonensis* Ac10 (Kawamoto *et al*., 2007) was used as a host to produce 3-hydroxypropionaldehyde (Tajima *et al*., 2013), aspartic acid (Tajima *et al*., 2015), itaconic acid (Luo *et al*., 2020), and to convert mannitol to fructose efficiently (Tajima *et al*., 2018). Moreover, the PSCats method can overcome the challenge of the cell membrane's resistance to the substrate influx in the whole-cell biocatalysts. Luo *et al*. (2020) showed that the cell membrane of *S. livingstonensis* Ac10 could be permeabilized with moderate heat treatment. However, the PSCat has some limitations which they need to improve.

All genes involved in the desirable metabolic pathway are expressed under single promoter's control in the “conventional PSCat” method. Under this condition, the number of genes expressed in the psychrophile host bacteria is limited. When a gene cluster is expressed using a single promoter, there is a tendency that expression of the first gene in the cluster is highest while that of the last gene is lowest. Curtis *et al*. (2002) reported that altering enzyme concentration affects enzyme activity. Adding more enzyme to an in vitro system such as the PSCat method can increase the enzyme activity. Therefore, finding the solution to increase expression of all genes in the cluster is a priority for developing the better PSCat system.

To achieve the highest amount of enzyme in the psychrophilic host

cells, I developed the “new approach for PSCat”. The main idea is based on the expression of each enzyme involved in the production of desirable chemicals, individually. The expression of each gene under the control of one promoter makes the highest amount of each enzyme in the host cell. To synthesize the target compound, the necessary step is only heating the recombinant psychrophilic bacteria to inactive intrinsic host enzymes. Then, mix the recombinant bacteria and add the substrate to produce target compounds. Furthermore, individual expression of genes enables users to more efficiently control over the production process.

In this study, I endeavor to examine the new PSCat method's efficiency compared to the conventional PSCat method. I considered producing 1,3-PDO in *S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253 via this method. The production of 1,3-PDO had not been examined with this new method. Although several compounds were efficiently biosynthesized with *S. livingstonensis* Ac10 via this method (Lue *et al*., 2020; Tajima *et al*., 2018; Tajima *et al*., 2015). Some other species of *Shewanella* had not been used, but they might also have the potential to be utilized in the PSCat system. Therefore, The *S. frigidimarina* DSM 12253 was examined as a psychrophile host for the first time in this study.

One of the most important features of PSCat is deactivation of psychrophilic host metabolic flux and enzymes. This is simply deactivation done by heat treatment. After heat treatment, only recombinant mesophilic enzymes could access all molecules in the cells, such as cofactors and coenzymes. Therefore, undesirable by-products cannot be produced in this method. The previous research in PSCat has indicated that different temperatures may be used to inactive psychrophilic enzymes (luo *et al*., 2020; Tajima *et al*., 2013, 2015, 2018). In those researches, different

temperatures such as 45°C for 15 min or 50°C for 15 min were examined to achieve inactivation of psychrophilic enzymes. The same bacterial strain (*S. livingstonensis* Ac10) had been used to produce different compounds on the PSCat projects and the different genes ware expressed in that bacterium. I believe that temperature to inactive intrinsic enzymes should be determined based on exogenous enzymes involved in production of chemicals. The important part is that most mesophilic and all thermophilic enzymes can maintain their activity at temperature range (40 - 55°C). Thus, most of the valuable compounds can be produced by using the new or conventual PSCat method.

Production of 1,3-PDO was examined with the new and conventional PSCat methods. Before starting the production test, I identified optimal production conditions. The psychrophilic host enzymes in both recombinant bacteria cells with *dhaT* were inactivated at 45°C for 15 min. I considered this condition as heat treatment for the production of 1,3-PDO. However, in the recombinant psychrophilic host cells with *puuC*, the intrinsic metabolic flux was not inactivated at 45°C. At this temperature, I detected a little activity in the tube deficient of the substrate required for mesophilic enzymes (the control tube) (Figure 3.3). The overexpression of PuuC in the recombinant psychrophilic host bacteria had an inhibitory effect on the recombinant cell growth, especially for recombinant Sf-U. Despite this slow growth, the total protein concentrations in both recombinant Ac10-U and Sf-U were same (Table 3.2 and Table 3.3). Ashok *et al*. (2011) reported that the overexpression of *puuC* resulted in decreased growth in *K. pneumoniae*. They presumed that this situation occurred due to an imbalance in the metabolic flux ratio between reductive and oxidative pathways. Moreover, many researches have shown

heat-shock proteins in bacterial cells could be boosted because of the production of large quantities of a single protein (Kurland and Dong, 1996). Based on these results, the overexpression of PuuC may cause some changes in the amount and type of proteins present in the cell. I believe the fluctuations in the amount and type of proteins could explain why the heat treatment at 45°C for 15 min was insufficient to deactivate all host enzymes in Ac10u and Sf-U.

The conventional PSCats method was used to biosynthesize 1,3-PDO. For this purpose, the gene cluster of *dhaB* and *dhaT* was made. Expression of both genes was under the control of one *tac* promoter. This approach's limitations are the simultaneous expression of all genes and limited number of genes. The results indicated that the production of 1,3-PDO was not efficient. The production of 1,3-PDO with the conventional PSCats method was only 12.7 mM. The expression of each gene individually in two psychrophile host strains improved the production of 1,3-PDO at 37°C (19.4 Mm). Thus, the new PSCat method (the individual expression of genes in multiple psychrophile host strains) can be considered for an efficient multi-step bioconversion process. More importantly, in this new strategy, the desired volume of the PSCat cells and perfect timing to add each enzyme to the reaction could be controlled by the user.

The limiting factor for the production of 1,3-PDO is the dehydratase activity of glycerol dehydratase (Daniel *et al*., 1998; Tong *et al*., 1991). This limitation may be explained by two arguments: the requirement of vitamin B12 in the catalytic cycle, and the inhibitory activity of glycerol as a substrate for DhaB. Many researchers overexpressed of *dhaB*, to solve the challenge of the production of

1,3-PDO. However, 3-HPA, which is a reaction product by DhaB, has a lethal effect on living cells (Sankaranarayanan *et al*., 2017; Zaushitsyna *et al*., 2017; Zheng *et al*. 2006). The new strategy has effortlessly resolved the challenge caused the contradictory relationship of overexpression of *dhaB* and 3-HPA toxicity. First, after heat treatment, recombinant psychrophile host cells are dead. Therefore, cell growth and viability are not impacted by the toxicity of 3-HPA. Second, in the new strategy of PSCat, the volume of enzyme added to the reaction, is entirely under the user's control. Adding a greater volume of the heated recombinant bacteria with *dhaB* causes increased volume of DhaB enzyme, simulating the over-expression state.

The DhaT (1,3-PDO producing enzyme) is an NADH-dependent enzyme (Zhao *et al*., 2009). In this project, I proved that supplying NADH had a positive impact on the enzyme activity and the production of 1,3-PDO. However, the cost of coenzymes is one of the limiting points in the production of 1.3-PDO (Yun *et al*., 2018). Therefore, I considered checking coupling with the regeneration system using FDH. The cofactor regeneration system increased the production of 1,3-PDO in the conventional PSCat (11.8 mM). Although the production was increased with the regenerate cofactor system, the efficiency was not high. I used the new strategy of PSCat and added the different volumes of recombinant bacteria with *dhaB*, *dhaT,* and *fdh* to the reaction. The 2:1:0.1 ratio of DhaB:DhaT:FDH was chosen to assess the regeneration system. The system could increase the production of 1,3-PDO from 25 mM to 31.1 mM only after 120 min. Previous studies showed that lower activity of FDH, compared to production enzymes, increases the target compound (Wang *et al*., 2013; Kaup *et al*., 2003).

The new PSCat approach's potential was proved, and 1,3-PDO was

produced by using the psychrophile *S. livingstonensis* Ac10 and *S. frigidimarina* DSM 12253 at 37°C (Chapter II). For the next step, I endeavored to examine efficient simultaneous conversion of glycerol to 1,3-PDO and 3-HP with this new strategy. For starting the simultaneous production reaction, I considered 40°C for reactions. The optimal temperature of PuuC (EC 1.2.1.3) is 45°C (Raj *et al*., 2010), whereas those of DhaB (EC 4.2.1.30; Wang *et al*., 2007) and DhaT (EC 1.1.1.202; Lama *et al*., 2015) are 37°C. The range of temperature which the DhaT and PuuC enzymes can tolerate was between 30 and 55°C (Raj *et al*., 2010; Lama *et al*., 2015). Therefore, 40°C is close to the optimum temperature for all enzymes.

Vidra and Németh (2018) reported that production of more than 100 g L^{-1}, over 2 g L^{-1} h^{-1} or above 50% yield in the synthesis of 3-HP could be considered as economic production. The new PSCat method showed significant potential for the biosynthesis of 3-HP. The results showed that the yield and productivity of 3-HP were 75% and 5.4 g L^{-1} h^{-1} in this experiment, respectively. This high output was possible because of the cofactor regeneration system between the production enzyme (PuuC) and psychrophilic host enzymes. I have already discussed about some of the host enzymes remained active after heat treatment. These psychrophilic enzymes could regenerate the NAD^+ for the production of 3-HP. Less activity of these psychrophilic enzymes compared to PuuC was helped to make the regeneration system. Some previous studies have reported that the activity of production enzymes is higher in cofactor regeneration systems compared to other enzymes in the cofactor regeneration system (Mojarrd *et al*., 2020; Wang *et al*., 2013; Kaup *et al*., 2003).

After achieving success in production of 1,3-PDO with the cofactor

regeneration system, I used the new strategy of PSCat for co-production of 1,3-PDO and 3-HP. The DhaT and PuuC enzymes need two forms of cofactors. Therefore, adding proper volume of each enzyme could valance the cofactor cycling between these two enzymes. I examined the different volumes of recombinant bacteria with *dhaB*, *dhaT,* and *PuuC* for simultaneous production of 1,3-PDO and 3-HP. The co-production of 3-HP and 1,3-PDO with ratio 4:2:1 (DhaB:Puu:DhaT) resulted in the best production for both compounds. As I have already described in chapter III, I individually produced each chemical in the first hour. Then the contents were mixed, and extra fresh enzyme and substrates were added to the reaction mixtures. I believe that there might be two reasons for the increase in both chemical production with ratio 4:2:1. First, during the individual production of both target compounds, most of the cofactor is converted to other forms and accumulates in the production microtube. The mixing of the microtubes' contents caused an increase in the concentration of suitable cofactor for each enzyme in the reaction tube. This condition is similar to the addition of more cofactors to the reaction. In this project, I demonstrated that adding more cofactors to the reaction has a positive effect on production. The second reason is that the cofactor regeneration system might occur between DhaT and PuuC. Further, I know that some psychrophilic host enzymes are still active and could increase the rate of oxidation of NADH to NAD^+. Therefore, I believe that the concentration of NADH was lower than that of NAD^+, which might explain why the production of 3-HP increased more than the production of 1,3-PDO. Further studies are needed to optimize the cofactor regeneration system, and to develop a DhaB regeneration system.

In conclusion, I believe my newly developed method of PSCat will

help to increase the yield of chemical production. This new method has high adaptability. The amount and timing of the addition of enzymes involved in the reaction pathway are entirely up to the users. Moreover, the possibility of producing compounds that have a complex metabolic pathway can be tested with a new PSCat strategy. However, more researches are needed to be done on the cofactor and coenzyme regeneration system to make the new approach of PSCat usable for industrial-scale chemical production.

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公表論文
（Articles）

(1) Efficient production of 1,3-propanediol by psychrophile-based simple biocatalysts in *Shewanella livingstonensis* Ac10 and *Shewanella frigidimarina* DSM 12253.
Mohammad Mojarrad, Keisuke Hirai, Koji Fuki, Takahisa Tajima, Akiko Hida, Junichi Kato
Journal of Biotechnology, **323** (1), 293-301 (2020).

(2) Psychrophile-based simple biocatalysts for effective coproduction of 3-hydroxypropionic acid and 1,3-propanediol.
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