Molecular breeding and biochemical characterization of an oleaginous fungus *Mortierella alpina* for prostaglandin $F_{2\alpha}$ production

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CONTENTS

ABBREVIATIONS

ARA	arachidonic acid
ATMT	*Agrobacterium tumefaciens*-mediated transformation
CBXB	carboxin
COX	cyclooxygenase
DGLA	dihomo-γ-linoleic acid
DNA	deoxyribonucleic acid
GLA	γ-linoleic acid
GY	glucose and yeast extract
LA	linoleic acid
LB	Luria Bertani
LCFA	long-chain fatty acid
LCMS	liquid chromatography mass spectrophometry
MCFA	medium-chain fatty acid
OA	oleic acid
PA	palmitic acid
PCR	polymerase chain reaction
PGs	prostaglandins
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
$PGF_{3\alpha}$	prostaglandin $F_{3\alpha}$
PGG_2	prostaglandin G_2

PGH_2	prostaglandin H_2
PGHs	prostaglandin-endoperoxide G/H synthase
PGI_2	prostacyclin I_2
PUFA	polyunsaturated fatty acid
SA	stearic acid
UV	ultraviolet
VLCFA	very long-chain fatty acid

GENERAL INTRODUCTION

Prostaglandins are acidic lipids which can be enzymatically produced by most mammalian cell types in response to mechanical, chemical or immunological stimuli. Since their discovery, prostaglandins have drawn the attention of both scientists and medical practitioners. It was found that these substances are involved in a variety of clinically important processes, including inflammation, fever and thrombosis (1-2). Some of them also found applications as medicines. For example, PGE_2 and $PGF_{2\alpha}$ are used as aparturifacients in the field of obstetrics and gynecology. $PGF_{2\alpha}$ is also approved as a promotor of intestinal movement after abdominal operations. However, its full pharmaceutical potential has not yet been established. Because of the application of prostaglandins as medicines, industrial methods for their production have been investigated in various ways. One possible method is a biotechnological approach. Chemical synthesis is most often used for the industrial production of prostaglandins today. Different synthetic methods are used, however, all of them suffer from serious disadvantages: many steps are required for the synthetic process; the yield of the prostaglandin is very low, and the cost of the prostaglandins is therefore very high.

Chemically, the prostaglandins are 20-carbon unsaturated carboxylic acids with a cyclopentane ring. The main classes are subdivided according to the number of double bonds in the side- chains. The most prominent precursor of prostaglandins (and other eicosanoids) is arachidonic acid (20:4, ω-6). The level of free ARA in cells is very low, but it is stored in high concentrations in an esterified form in membrane phospholipids. The released arachidonate is first converted in two steps to PGH_2. This reaction is catalyzed by prostaglandin-endoperoxide synthase (also known as fatty acid cyclooxygenase). The cyclooxygenase activity of COXs oxygenates arachidonic acid to produce prostaglandin G_2, a cyclopentane hydroperoxy endoperoxide; the peroxidase activity of COXs then reduces this to prostaglandin H_2. Recently,

a cyclooxygenase gene (GvCOX) from a red alga, *Gracilaria vermiculophylla* was cloned and introduced into *E. coli* for the production of $PGF_{2\alpha}$ (3). The GvCOX gene has been shown to function in *E. coli* without protein modification, although the animal COXs have been reported to be modified post-translationally (4-5). Until now several red algae such as *G. vermiculophylla* have been reported to highly accumulate PGs and ARA (6-8). Meanwhile, the GvCOX gene has also been introduced into the liverwort, *Marchantia polymorpha* and the transgenic was able to produce the $PGF_{2\alpha}$, PGE_2 and PGD_2 (9). Since several *Mortierella* strains were reported to be potential producers of ARA in 1987 (10-11), they have been shown to be some of the promising single-cell oil (SCO) sources rich in various types of C20 PUFAs (12-13). Among them, *M. alpina* 1S-4 has the unique ability to synthesize a wide range of fatty acids, and has several advantages not only as an industrial strain but also as a model for lipogenesis studies.

Thus, the author focused on the establishment of fermentative $PGF_{2\alpha}$ production through breeding of *M. alpina,* which is an arachidonic acid producing microorganisms, with COX from *G. vermiculophylla*.

Chapter I describes the production of prostaglandin $F_{2\alpha}$ by molecular breeding of an oleaginous fungus *Mortierella alpina.* Chapter II describes the development of *Mortierella alpina* transformants with an algal cyclooxygenase gene for higher production of prostaglandin $F_{2\alpha}$. Chapter III describes the optimization of fermentation conditions for higher production of prostaglandin $PGF_{2\alpha}$ by *Mortierella alpina* transformants with an algal cyclooxygenase gene.

CHAPTER I

Production of prostaglandin $F_{2\alpha}$ by molecular breeding of an oleaginous fungus *Mortierella alpina*

Introduction

Prostaglandins (PGs) are a group of biologically active lipids derived from C20 polyunsaturated fatty acids, including arachidonic acid (ARA, 20:4ω-6), dihomo-γ-linoleic acid (DGLA, 20:3ω-6), and eicosapentaenoic acid (EPA, 20:5ω-3). They work as hormone-like chemical messengers in homeostatic functions and pathogenic reactions, including inflammatory responses (14). Broad attention has been paid to the PGs, including prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), as therapeutic agents. Commercially available PGs are mostly supplied by organic chemical synthesis (15-16). However, chemical synthesis requires numerous steps, which results in high PG costs.

$PGF_{2\alpha}$ is biosynthesized from ARA via the cyclooxygenase (COX) enzyme system in mammals (Figure 1-1). The key enzyme, COX (EC. 1. 14. 99. 1), catalyzes two steps of reactions in the pathway, in which ARA is first converted into prostaglandin G_2 (PGG_2) and next PGG_2 is reduced to form prostaglandin H_2 (PGH_2) (17). PGH_2 can be converted into $PGF_{2\alpha}$ by the enzyme PGF synthase as well as into various types of prostaglandins or prostacyclins by specific synthases, which is responsible for various biological activities in PGs.

A COX gene (GvCOX gene) with high activity was recently cloned from a red alga, *Gracilaria vermiculophylla* (3). The GvCOX, when expressed in *Escherichia coli*, functions without any post-translational modification (3; 18-19) and its potential as a catalyst for PG production has been evaluated in *in vitro* reactions. Following the introduction of the GvCOX gene, transgenic liverworts were generated, which not only catalyzed the production of $PGF_{2\alpha}$ from ARA *in vitro* reactions but also produced prostaglandins *de novo* (9). However, the *de*

novo productivity in the transgenic liverworts is not higher than that in red algae, which naturally produce PGs.

Mortierella alpina is an oleaginous fungus belonging to subphylum Mucoromycotina (20). The fungus produces lipids rich in triacylglycerols with dry weights that constitute up to 50% ARA, which is commercially used for the fermentative production of ARA (21-22). I developed a method for transforming the fungus using the gene transferring system of a plant-infecting bacterium, *Agrobacterium tumefaciens* (23).

In the present study, I introduced the GvCOX gene into *M. alpina* using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method for the fermentative production of PGs.

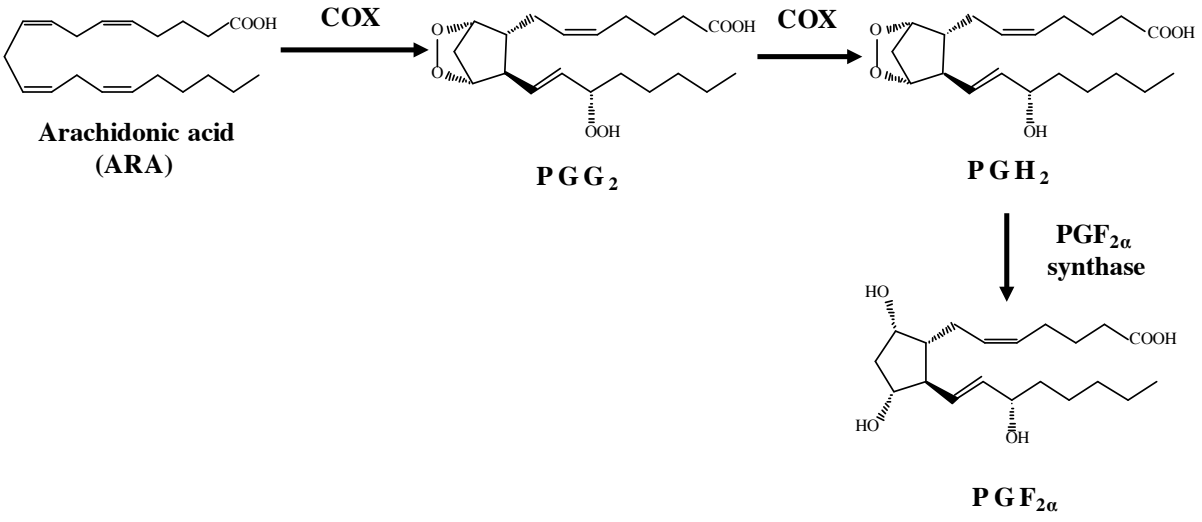

Figure 1-1. Schematic diagram of the reactions from ARA to $PGF_{2\alpha}$ in the COX enzyme system. PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; $PGF_{2\alpha}$ synthase, prostaglandin $F_{2\alpha}$ synthase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

MATERIALS AND METHODS

Strains and media

M. alpina 1S-4 *ura5*$^-$ strain has been previously described (24). The uracil auxotroph strain was used as the host strain for the transformation. Media used to culture the host and transformed strains in the present study were SC agar with and without uracil, Czapek–Dox agar supplemented with 0.05 mg/mL of uracil, and GY broth [2% (w/v) glucose and 1% (w/v) yeast extract], which have been previously described (23). *Escherichia coli* DH5α was used for DNA manipulation and grown on LB agar (25) containing 50 μg/mL kanamycin.

Plasmid construction

The cyclooxygenase gene of *Gracilaria vermiculophylla* (*GvCOX*) (3), a red alga, with optimized codon usage to reflect the codon bias in *M. alpina* obtained from the Kazusa database (http://www.kazusa.or.jp/codon/) was purchased from GENEART (Regensburg, Germany), with additional *Spe* I and *Bam*H I sites at the 5′ and 3′ flanking open reading frames, respectively. The gene (approx. 1.7 kb) was inserted downstream of *his550* promoter in plasmid pBIG35ZhGUSm (26) by *Spe* I and *BamH* I to give pBIG35ZhGvCOXm (Figure 1-2).

Transformation of *M. alpina* 1S-4 *ura5*$^-$ strain

M. alpina was transformed using the ATMT method as previously described (23). The colonies that grew on fresh uracil-free SC agar plates were inoculated onto GY broth and then evaluated as follows.

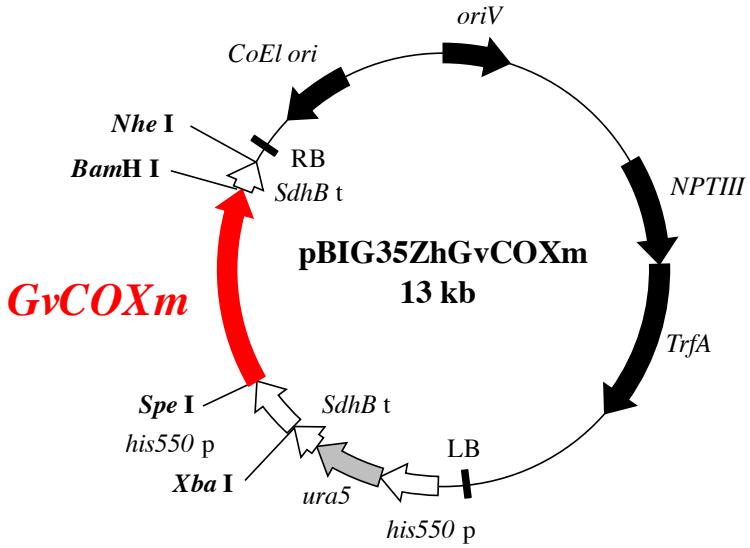

Figure 1-2. Schematic diagram of the plasmid pBIG35ZhGvCOXm with the GvCOX gene downstream of the histone promoter. *GvCOXm*, the codon-optimized GvCOX gene for *M. alpina*; *hispro550* p, *M. alpina* histone protein promoter; *SdhB* t, *M. alpina* SdhB gene transcription terminator; *ura5,* the orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *NPTIII,* neomysin phosphotransferase III gene; *TrfA,* TrfA locus, which produces two proteins that promote the replication of the plasmid; *ColE1 ori,* ColE1 origin of replication; *oriV,* pRK2 origin of replication; *RB* and *LB*, the right and left border, respectively, from Ti-plasmid of *A. tumefaciens*.

Evaluation of the transformants

The transformants were evaluated for their capacity to catalyze the reaction, in which ARA was converted to prostaglandins *in vitro* and those of fermentative prostaglandin production intra- and extracellularly.

For the ARA conversion *in vitro,* the individual transformant was cultivated in GY medium supplemented with 50 mg/L of uracil at 12 °C for 12 days. The cultivated cells were collected by filtration with No. 2 filter papers (Advantec, Toyo Roshi Co. Ltd., Tokyo, Japan) and mixed with 30.45 mg/L of ARA (Sigma, St. Louis, MI, USA) in a reaction buffer consisting

of 100 mM of Tris-HCl (pH7.5), 5 mM of EDTA, and 1 μM of Hematin. The reaction mixture was incubated at 23 °C for 1 hour. Lipids in the reaction mixture were extracted by mixing with ethyl acetate after the addition of 60 ng of $PGF_{2\alpha}$–d4 as an internal control. The organic phase obtained following centrifugation was transferred into a new tube, and the solvents were removed using a rotary evaporator with a diaphragm vacuum pump. The extracted lipids were resuspended in ethanol and stored at −80 °C until they were analyzed using liquid chromatography-mass spectrophotometry (LC-MS) as described later.

For the evaluation of fermentative production, the produced lipids were extracted from the cultured cells and media after cultivated in 15 mL GY medium supplemented with 50 mg/L of uracil at 12 ºC for 12 days. The cells and media were separated by filtration with No. 2 filter papers (Advantec, Toyo Roshi Co. Ltd.). The cultured cells were weighed and frozen at −20 °C. Chloroform and methanol volumes, double and equal to the flesh weight of the cells, respectively, were added and the solutions were mixed. After extraction at −20 °C for 16 hours, an equal volume of water was added, and the solutions were well mixed. The organic phase obtained by centrifugation was transferred into a new tube, and the solvents were removed using a rotary evaporator with a diaphragm vacuum pump. The extracted lipids were resuspended in ethanol and stored at −80 °C. The lipids in the filtrated media were extracted by adding twice the volume of the ethylacetate after acidification with hydrochloric acid. The solutions were then mixed well and centrifuged to separate the organic and water phases. The organic phase was evaporated as described above, and the extracted lipids were resuspended in ethanol and stored at −80 °C. The extracted lipids were analyzed using LC-MS (LC-MS 2020, Shimadzu, Kyoto, Japan) equipped with a 5C18-AR-II COSMOSIL packed column (4.6 mm i.d. x 150 mm, Nacalai Tesque, Kyoto, Japan). For the column using a 20%–100% acetonitrile gradient in 0.1% formic acid, in which the concentration of acetonitrile was increased to 20%–40% for 10 min, 40%–100% for 10 min, and maintained at 100% for 25 min

at a flow rate of 0.2 mL/min with the column temperature set at 40 °C. For the mass spectrometry conditions (negative ion mode), the electron spray ionization interface was performed at a heat block temperature of 200 °C and a desolvation-line temperature at 250 °C. The nebulizer gas flow rate was 1.5 L/min. Quantification was performed using standard calibration lines obtained from analyses of authentic standards. All PG standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

RESULTS AND DISCUSSIONS

The GvCOX gene was introduced into the *M. alpina* 1S-4 *ura5*$^-$ strain using the ATMT, in which a single copy of the gene would often be introduced into the genome. A total of 30 transformants grew on the SC without uracil plates. Initially, the COX activity in the transformants was tested *in vitro*. The COX activity was evaluated based on the amount of $PGF_{2\alpha}$ converted from ARA by the cells obtained from cultures in a similar volume (15 ml) of the medium. The transformants exhibited relatively high activity as shown in Figure 1-3. The transformants produced higher $PGF_{2\alpha}$ than the parental strain, which produces trace amounts of $PGF_{2\alpha}$ *de novo*, indicating that the introduced GvCOX gene conferred to *M. alpina* the ability to produce $PGF_{2\alpha}$. The highest concentration of $PGF_{2\alpha}$ was observed in GvMA#21 (approximately 800 μg/L). Notably, the transformants produced $PGF_{2\alpha}$ from ARA without the need of a reducing agent for the conversion of PGH_2 into $PGF_{2\alpha}$ following the *in vitro* reactions. Because several *Mortierella* species can produce PGs (27), the $PGF_{2\alpha}$-forming enzyme genes could also be present in *M. alpina*. Therefore, I evaluated the fermentative $PGF_{2\alpha}$ production by the transformants.

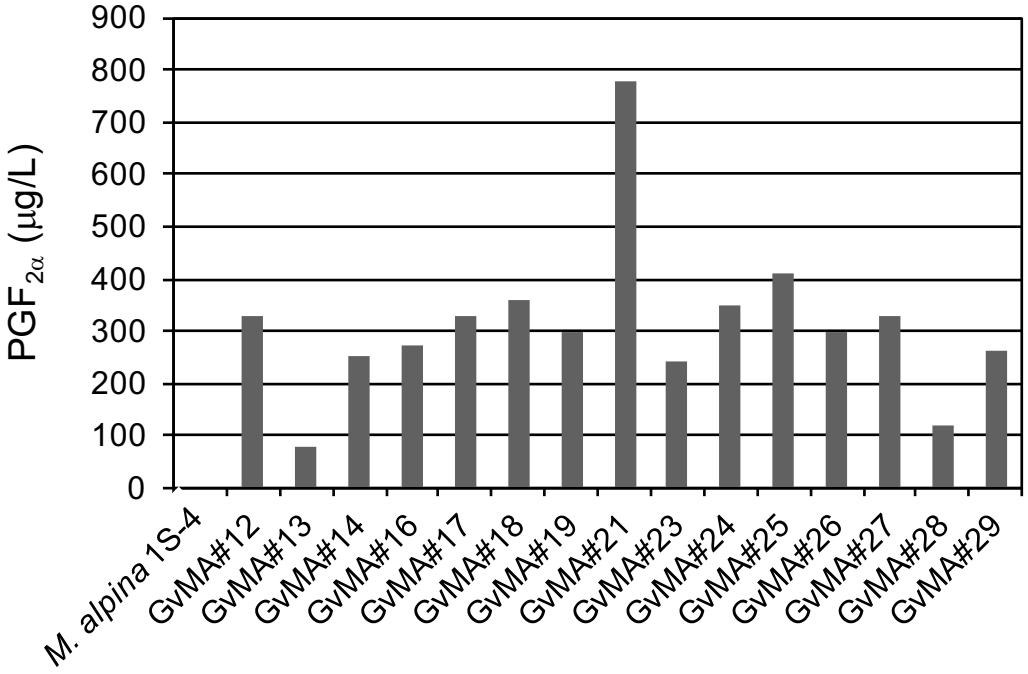

Figure 1-3. Evaluation of *in vitro* COX activity in the transformants. Resting cells were used to convert 30.45 mg/L of ARA to $PGF_{2\alpha}$

In a previous study, 90% of the total PGs produced *de novo* by several *Mortierella* species were found in the culture medium (27). Consequently, the transformants were initially tested to determine if they released $PGF_{2\alpha}$ into the media during cultivation. All transformants released much higher amounts of $PGF_{2\alpha}$ into the media than the host strain (Figure 1-4). Transformant *GvMA#21* produced the highest amounts of $PGF_{2\alpha}$, as predicted based on its *in vitro* activity. Another transformant, *GvMA#28*, produced $PGF_{2\alpha}$ at a comparable level to *GvMA#21*, although *GvMA#28* exhibited much lower activity in the *in vitro* conversion of ARA to $PGF_{2\alpha}$ (Figure 1-3). Culturing of *GvMA#21* and *GvMA#28* for 12 days resulted in a yield of 450 μg/L of fermentative $PGF_{2\alpha}$ production (Figure 1-4). The results demonstrated that ARA in the mycelia was converted into $PGF_{2\alpha}$ and released into the media.

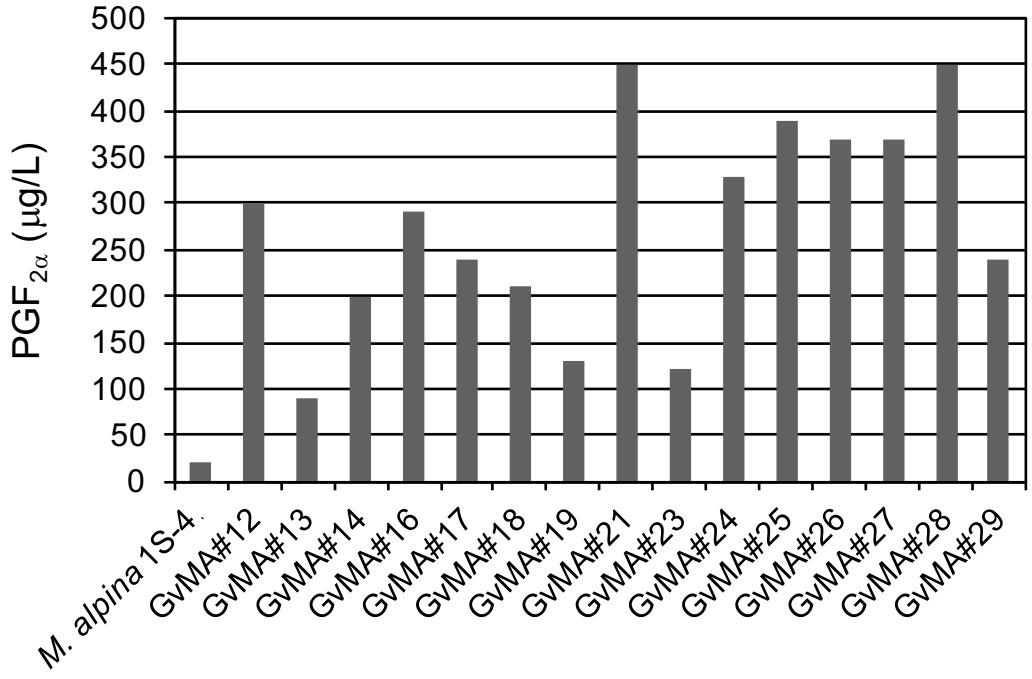

Figure 1-4. Evaluation of fermentative production of $PGF_{2\alpha}$ that was released into the media during the 12-day cultivation of the transformants.

The two transformants, *GvMA#21* and *GvMA#28*, were cultured afresh using similar methods and their fermentative production of PGs in mycelia (Figure 1-5) and in media (Figure 1-6) was investigated. A unit weight (1 g) of mycelia of the transformants *GvMA#21* and *GvMA#28* cultured for 12 days was estimated to contain 860 and 500 ng of $PGF_{2\alpha}$, respectively (Figure 1-5). The mycelia also contained $PGF_{1\alpha}$ and $PGF_{3\alpha}$ at levels of ~10% and ~5% of $PGF_{2\alpha}$, respectively (Figure 1-5). The two PGs were considered to be from DGLA and EPA stored in the mycelia (28), respectively. Additionally, $PGF_{2\alpha}$ was accumulated extracellularly in media with 609 and 6421 μg/L attributed to *GvMA#21* and *GvMA#28*, respectively (Figure 6). The media also contained $PGF_{1\alpha}$ at a level ~10% of $PGF_{2\alpha}$ but not $PGF_{3\alpha}$ (Figure 1-6). The values were calculated as the averages of six samples. To determine the reasons for the differences in $PGF_{2\alpha}$ accumulation in the results that are presented in Figures 4 and 6, and to

achieve more stable and higher production levels, I investigated the kinetics of $PGF_{2\alpha}$ accumulation in the media during the cultivation.

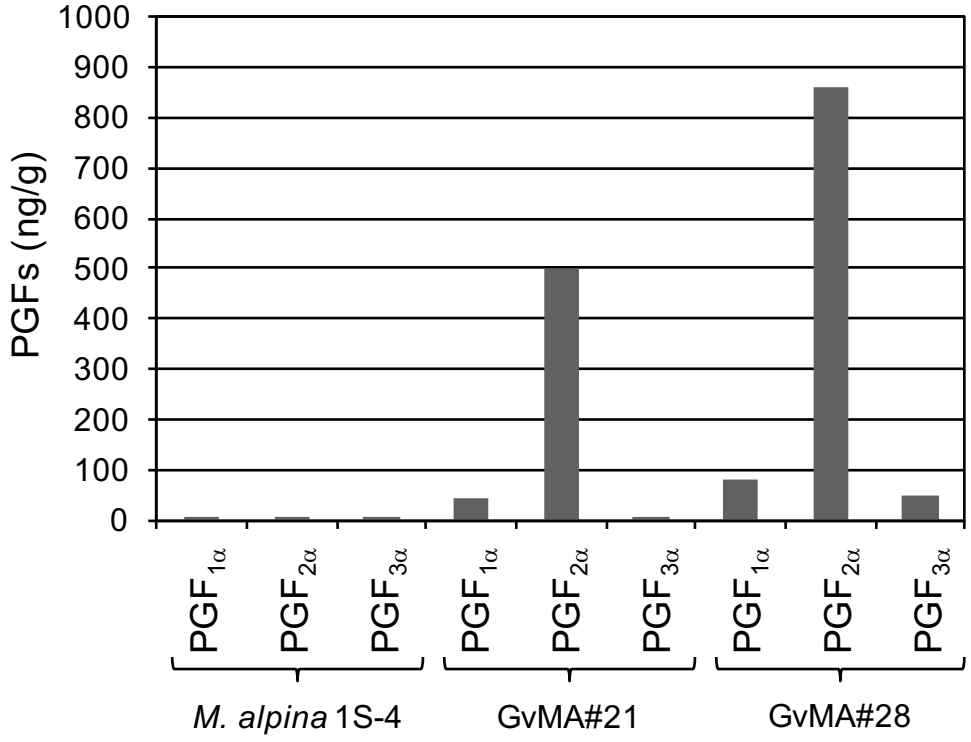

Figure 1-5. Fermentative intracellular production of prostaglandins $F_{1\alpha}$, $F_{2\alpha}$, and $F_{3\alpha}$ in the parental (*M. alpina* 1S-4) and transformant (*GvMA#21* and *GvMA#28*) strains.

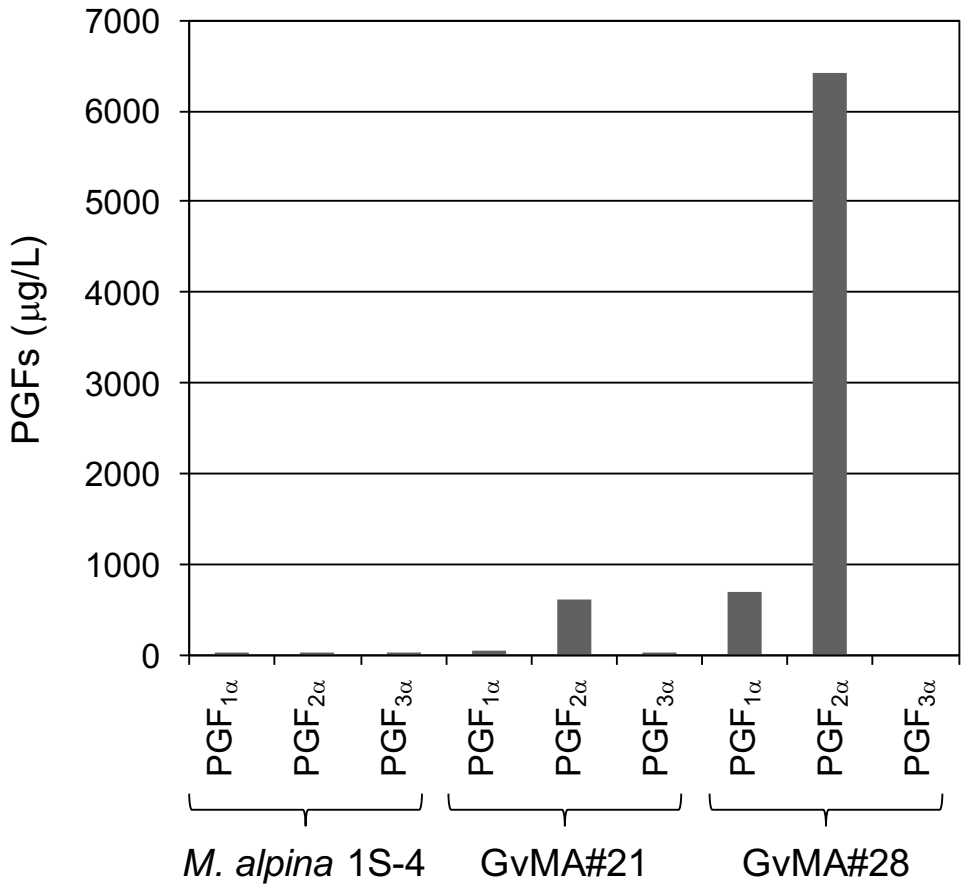

Figure 1-6. Fermentative extracellular production of prostaglandins $F_{1\alpha}$, $F_{2\alpha}$, and $F_{3\alpha}$ in the parental (*M. alpina* 1S-4) and transformant (*GvMA#21* and *GvMA#28*) strains.

Lipids in filtered media were extracted from the *GvMA#21* and *GvMA#28* cultures at 3, 5, 7, 9, and 12 days after inoculation, and the kinetics of $PGF_{2\alpha}$ accumulation were investigated (Figure 1-7). $PGF_{2\alpha}$ in the cultivation media of the transformants gradually increased from early cultivation days to day 7 and dramatically at day 9 until the end of cultivation, day 12. At day 12, the *GvMA#28* culture media contained $PGF_{2\alpha}$ at a 5-fold concentration than that in GvMA#21 (Figure 1-7). However, no substantial $PGF_{2\alpha}$ accumulation was observed in the media from the wildtype culture during the experimental period. The considerable change in the rate of $PGF_{2\alpha}$ accumulation might be caused by some changes that could have influenced

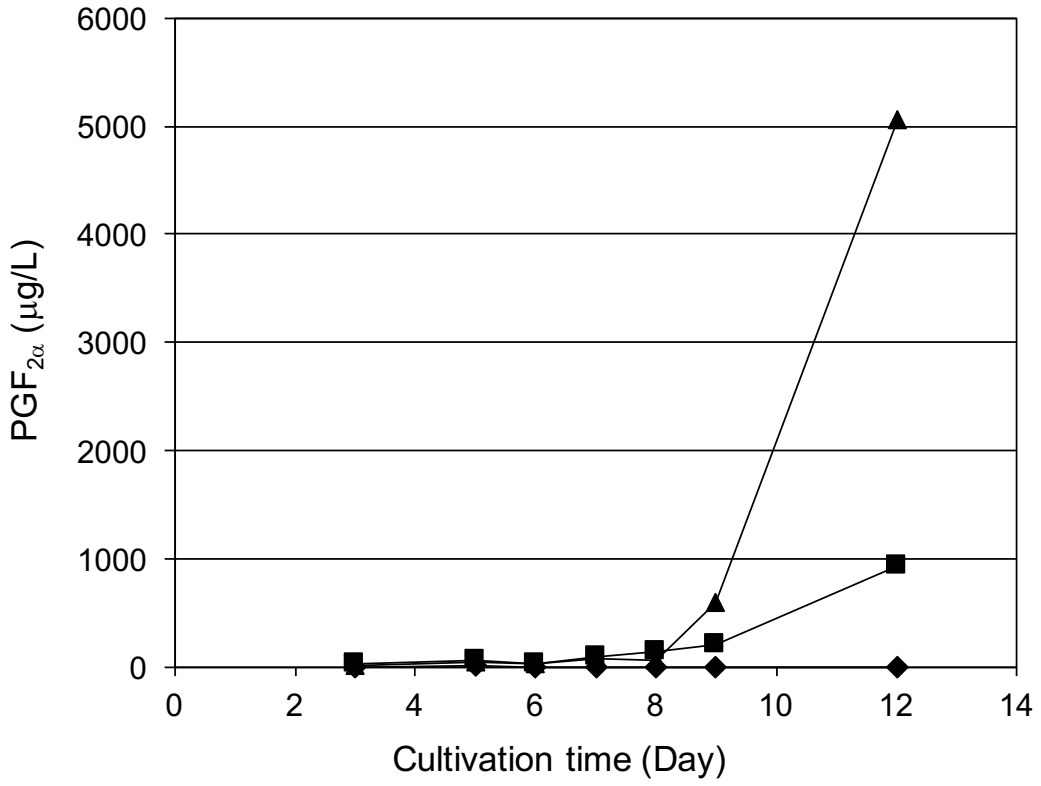

Figure 1-7. Time course analysis of extracellular $PGF_{2\alpha}$ production in 12 days of cultivation by the parental (◆, *M. alpina* 1S-4) and transformant (■, *GvMA#21* and ▲, *GvMA#28*) strains

metabolism rates during cultivation, e.g., depletion of nutrients. Similar to other COXs, GvCOX prefers free fatty acids as a substrate (18). However, free ARA is present in trace amounts in *M. alpina* whereas most ARA molecules are esterified in triacylglycerol. Therefore, it is probable that the GvCOX-mediated reactions would be under the influence of a lipid-controlling mechanism in *M. alpina*, including the release of free ARA. Activation of such mechanisms or conferring a mechanism for the release of free ARA would enhance $PGF_{2\alpha}$ production. In addition, the *de novo* accumulation of the $PGF_{2\alpha}$ in the culturing media as well as in the mycelia strongly suggests that $PGF_{2\alpha}$ could be produced from the COX product, PGG_2/PGH_2, by endogenous $PGF_{2\alpha}$-forming enzymes and exported to the media. Further studies will be required to elucidate such elements that influence $PGF_{2\alpha}$ production in *M. alpina*

transformants using the GvCOX gene. I demonstrated that the transformation of *M. alpina* by the GvCOX gene facilitated fermentative production of $PGF_{2\alpha}$. The fermentative system was considered as a high potential system that could be used to produce $PGF_{2\alpha}$, in which $PGF_{2\alpha}$ could be obtained from extracellular media.

It is likely that the amount of GvCOX influences transformant activity as demonstrated in the transformed liverworts (9). In the present study, GvCOX expression levels were not evaluated. Because stronger promoters have been found in *M. alpina*, e.g., *pp3* p or *pp6* p, (26), the effects of the substitution of the *his550* promoter with stronger promoters are currently under evaluation for the enhancement of $PGF_{2\alpha}$ production.

SUMMARY

Cyclooxygenase gene (*GvCOXm*) from the red alga, *Gracilaria vermiculophylla,* was introduced into *Mortierella alpina* 1S-4. A potent transformant, *GvMA#28,* in which the COX enzyme was driven by *his550* p, able to produce the $PGF_{2\alpha}$ extracellularly during cultivation.

CHAPTER II

Development of transgenic *Mortierella alpina* with an algal cyclooxygenase gene for higher production of prostaglandins $F_{2\alpha}$

Introduction

Prostaglandins (PGs) are acidic lipids which can be enzymatically produced by most mammalian cell types in response to mechanical, chemical or immunological stimuli. They are lipid autocoids derived from arachidonic acid in which stored and accumulated in an esterified form in membrane phospholipids. PGs are synthesized from arachidonate by the action of prostaglandins-endoperoxide G/H synthase (PGHs) (EC 1.14.99.1) or commonly known as cyclooxygenase (COX), generating intermediates such as PGG_2 and PGH_2, and eventually producing $PGF_{2\alpha}$ and several PGs by specific synthase. The enzymes are embedded in the luminal monolayer of the endoplasmic reticulum and inner membrane of the nuclear envelope (29-32). Generally, the COX-1 is constitutively expressed, whereas COX-2 expression is inducible (33-35). The cyclooxygenase reaction occurs in a hydrophobic channel in the core of the enzyme. In vitro, the peroxidase activity can operate independently of the cyclooxygenase (e.g. when the cyclooxygenase site is occupied by a nonsteroidal anti-inflammatory drug) (36) or during on-going cyclooxygenase catalysis (37). Due to the necessity of subcellular membrane organelles for post-translational glycosylation of animal PGHS, recombinant expression is limited to eukaryotic systems. Cloning and expression of a non-glycosylated form of PGHS of the red alga, *Gracilaria vermiculophylla* (gvPGHS), was published recently (3;9). These studies report the production of up to 3 mg/L $PGF_{2\alpha}$ in 7 h using recombinant *E. coli* and 22 mg $PGF_{2\alpha}$ per 500 g fresh weight liverworts, respectively.

The lipid production is not economically viable mainly due to the limitation in the productivity by the natural isolates. Various approaches need to be made to meet the

requirements including the genetic manipulation or engineering of selected isolates. Therefore, a continuous development of complementary methodologies is important for the genetic improvement. The microbial production of lipids containing polyunsaturated fatty acids (PUFAs) attracts many scholars in the past decade. Currently, several transformation methods are available for delivering the exogenous nucleic acid (DNA) to diverse fungi and yeast species (38) either by direct or indirect methods. The advancement of a transformation system for filamentous fungi is imperative for the cloning and mutagenesis of genes (39-40). A few standard procedures of random mutagenesis such as lithium acetate, $CaCl_2$-PEG, electroporation, microprojectile bombardment and *Agrobacterium*-mediated transformation have been applied. Over the past few years, the biolistic technology has been extensively utilized to introduce and express desirable traits in microbial, plants and animal cells (41-45). Thus, I successfully obtained a potent mutant which able to produce a high concentration of $PGF_{2\alpha}$ by the presence of the cyclooxygenase expression gene cassette driven by *pp3* promoter in the random genomic of *Mortierella alpina* 1S-4.

MATERIALS AND METHODS

Microorganisms and culture conditions

Two host strains, the uracil auxotroph strain, *Mortierella alpina* 1S-4 *ura5*$^-$ was previously described by (24). A *M. alpina* transformant harboring the GvCOX gene driven by *his550* promoter was described in (51). Both host strains were maintained on Czapek-Dox agar supplemented with or without 0.05 mg/mL of uracil. The competent cells, *E. coli* DH5α were grown on LB (Luria Bertani, 1951) containing 50 μg/mL of kanamycin and 50 μg/mL of carboxin. The fermentation medium, GY, was prepared as described by (23) and incubated at 28 ℃ with continuous shaking at 120 rpm.

Construction of plasmids

The *his550* promoter sequence in the plasmid pBIG35ZhGvCOXm (51) was replaced with that of the *SSA2* promoter with *Xba* I and *Spe* I recognition sites at the 5'- and 3'- ends of the promoter region, respectively, as described (26). The resulted plasmid, containing the GvCOX gene followed after the *SSA2* promoter, was named pBIG35SSA2pGvCOXm (Fig. 2-1).

The detail of plasmid construction was illustrated in the Figure 2-2. Three plasmids pBIG35ZhGUSm (26), pBIG35ZhGvCOXm (26) and pSDZNCBXB (28). All plasmids were amplified and transformed in the competent cell, *E. coli* DH5α. The plasmid pBIG35ZhGvCOXm contains the algal cyclooxygenase (GvCOXm) gene with *SSA2* promoter was digested with *Spe* I and *BamH* I restriction enzymes. The corresponding PCR fragment, GvCOXm gene was replaced with the GUSm gene, in the plasmid vector pBIG35ZhGUSm. The expression cassette (*pp3* p-*GvCOXm*-*Sdh*B t) was digested with *Xba* I and *Nhe* I, and ligated to plasmid pSDZNCBXB, which previously digested with *Xba* I restriction enzyme and treated with alkaline phosphatase (*E. coli BAP*75). The constructed plasmid,

pSDZNCBXBGvCOXm was amplified and transformed in competent cells, *E. coli* DH5α and grown on GY agar containing 60 μg/mL of carboxin and the expression cassette was confirmed by gel electrophoresis.

Transformation of *M. alpina* 1S-4 *ura5*$^-$ strain using the ATMT method

The spores of *M. alpina* 1S-4 strains were transformed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method as described by (26). The transformants were isolated from uracil-free SC agar plates with multiple subcultures.

Ultraviolet (UV) mutagenesis

The spore suspension of *M. alpina* was harvested with sterile Tween 80 and filtered through Miracloth (Calbiochem), and about 10^7 of spores were exposed under UV lamp from 0 to 10 seconds. The distance between the petri plate and the UV lamp was adjusted to 15 cm. 1 mL of the irradiated sample was transferred into sterilized tubes and diluted at appropriate rates. The diluted spore suspensions were spread on GY agar plates supplemented with Rose Bengal and incubated for four days at 28 ℃.

Transformation of UV-derived mutant by microprojectile bombardment

The spores of *M. alpina* strains were transformed by pSDZNCBXBGvCOXm using the particle gene bombardment system as described by (24) with some modifications. The GY with 2% (w/v) agar (GY agar), supplemented with 60 and 80 μg/mL of carboxin was used as a selective medium. Incubated at 28 ℃ for 5 days after been bombarded, the individual transformant was subcultured on GY agar without carboxin consecutively three times, in order to select a stable transformant. Mycelial from the third culture was finally inoculated onto GY agar containing the initial respective concentration of carboxin and incubated at 28 ℃ for 5

days.

Analysis of prostaglandins

The experiments were conducted in triplicates and in the same batch. The fermentative broth from each flask was filtered through No. 2 filter papers (Advantec) and diluted at appropriate factor (normally 10 times). As for the glucose feeding at 5th or 7th day, the sampling was done on the next day after glucose was fed in. While for the addition of fatty acids, the sampling was done on day-6, -8 and -10. The $PGF_{2\alpha}$ was analyzed by LC-MS as previously described by (51).

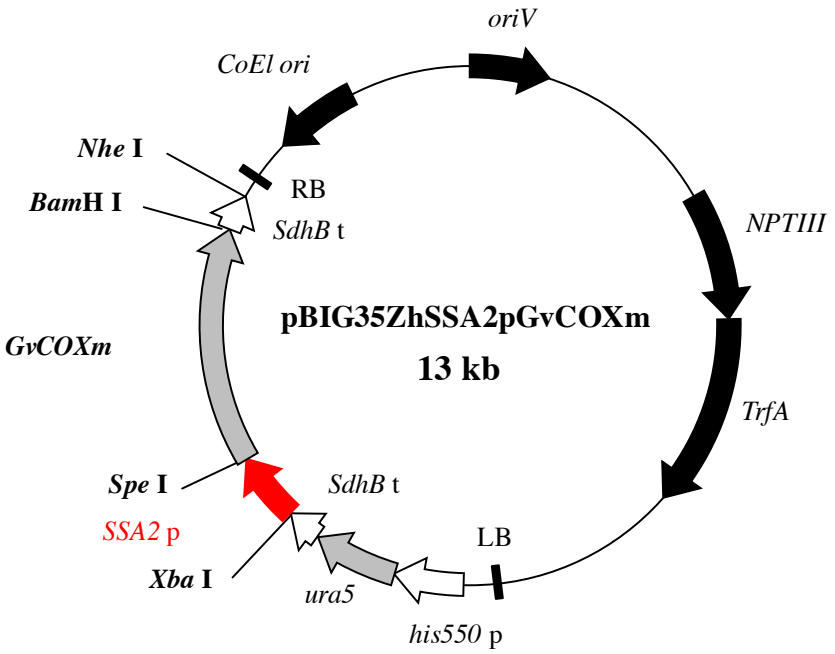

Fig. 2-1 Schematic diagram of the plasmid pBIG35SSA2pGvCOXm with the *SSA2* promoter on the upstream of GvCOX gene. *GvCOX*m, the codon-optimized GvCOX gene for *M. alpina*; *SSA2* p, *M. alpina* ATP binding protein promoter; *SdhB* t, *M. alpina SdhB* gene transcription terminator; *his550* p, *M. alpina* histone protein promoter; *ura5,* the orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *NPTIII,* neomysin phosphotransferase III gene; *TrfA,* TrfA locus, which produces two proteins that promote replication of the plasmid; *ColE1 ori,* ColE1 origin of replication; *oriV,* pRK2 origin of replication; *RB,* right border; *LB,* left border.

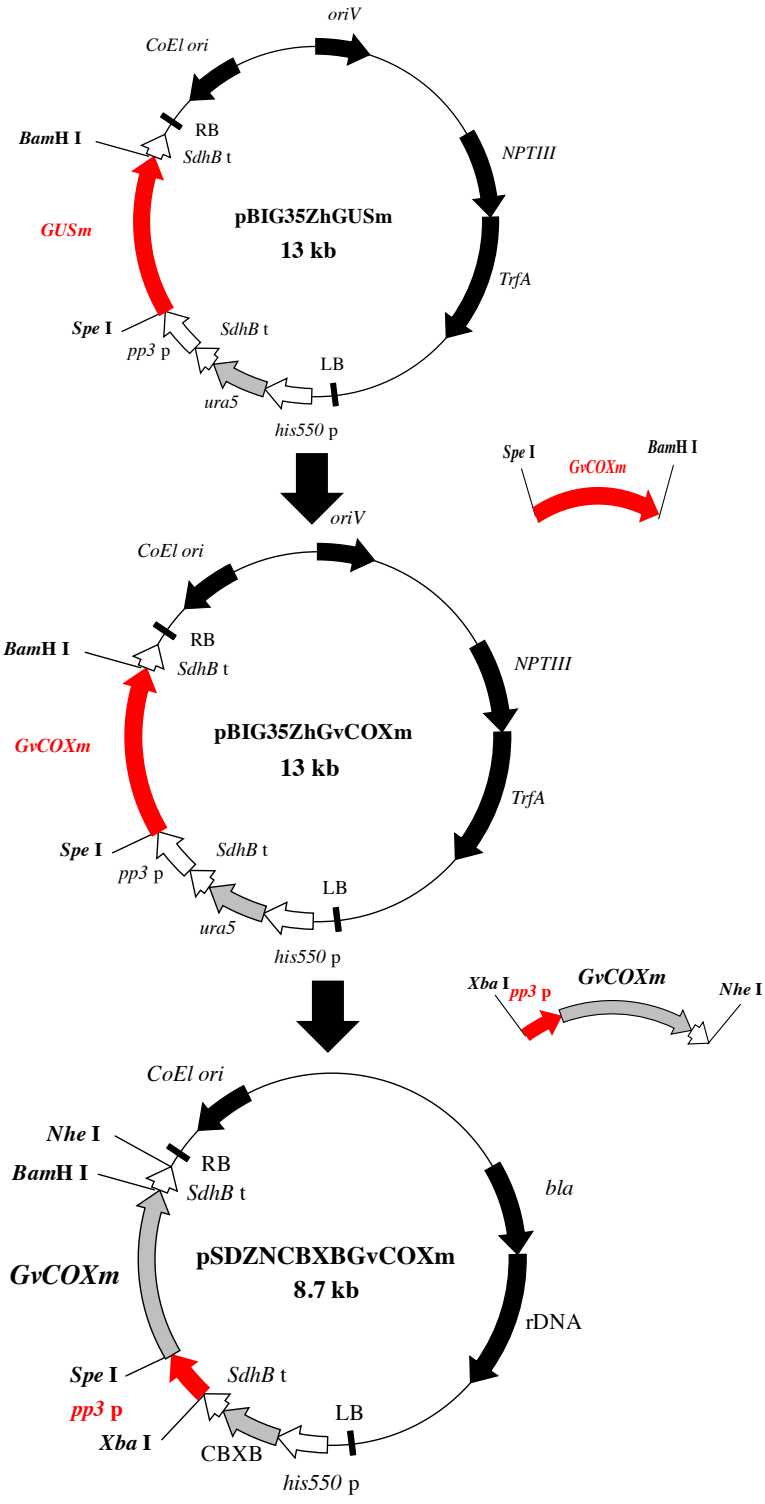

Fig. 2-2 Construction of plasmid pSDZNCBXBGvCOXm with the 1.7kb of *GvCOXm* gene from vector pBIG35ZhGUSm and pBIG35ZhGvCOXm for the transformation of *Mortierella alpina* 1S-4. *GvCOXm,* the codon-optimized *GvCOX* gene for *M. alpina*; *GUSm,* codon-optimized *β*–glucuronidase for *M. alpina*; *his550* p, *M. alpina* histone H4.1 promoter short fragment; *ura5*, orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *SdhB* t, *M. alpina SdhB* transcription terminator; *pp3* p, predicted protein 3 promoter; *NPTIII*, neomycin phosphotransferase III gene; *TrfA,* TrfA locus which produces two proteins that promote replication of the plasmid; *rDNA*, *M. alpina* rDNA gene fragment; *CBXB*, carboxin resistant gene; *LB*, left border; *RB*, right border.

Results and Discussions

Production of $PGF_{2\alpha}$ by transformants containing GvCOX gene driven by the *SSA2* promoter

Several promoters with different activities are available to introduce endogenous and foreign genes into *M. alpina* (26). Introduction of the GvCOX gene with a histone promoter *his550* into this fungus resulted in the fermentative production of $PGF_{2\alpha}$, in which $PGF_{2\alpha}$ accumulated for the most part in extracellular media in the culture. In order to increase the productivity, new transformants were created, in which the GvCOX gene expression was driven by the *SSA2* promoter from *M. alpina*. This *SSA2* promoter is a constitutive promoter with approximately 2.2-fold higher activity than the *his550* promoter when evaluated by the GUS activity in *M. alpina* transformants (26). Among 55 transformants obtained by the ATMT transformation method, a transformant *SSA2#23* produced the highest amount of $PGF_{2\alpha}$ (5603 μg/L) in the extracellular media for 12 days cultivation, while one of other transformants *SSA2#4* produced moderate amount of $PGF_{2\alpha}$ (3170 μg/L).

The kinetics of the $PGF_{2\alpha}$ production by these transformants was compared with that by the previous transformant *GvMA#28,* in which the GvCOX gene was driven by the *His* promoter (Fig. 2-3). The concentrations of $PGF_{2\alpha}$ in the extracellular media of *SSA2#4* and *SSA2#23* cultures gradually increased for 12 days while those of *GvMA#28* cultures were low for 9 days but increased rapidly at day 12 as described previously (51). In this experiment, the $PGF_{2\alpha}$ was accumulated in media with 5763±309.6 μg/L (n=3) attributed to *SSA2#23* at day 12, which was about twice as much as *GvMA#28*. Although this concentration was not higher than the previously described one by the *GvMA#28*, Fig.2-3 demonstrated that *SSA2* promoter had advantages over *his550* promoter to yield $PGF_{2\alpha}$ in *M. alpina* transformants under the similar condition. In transgenic liverworts, the accumulation level of PGs was correlated with the expression level of introduced GvCOX gene (9). Similarly, it is suggested that $PGF_{2\alpha}$ could

be produced more in *M. alpina* by intensifying the expression level of the GvCOX gene by using stronger promoter.

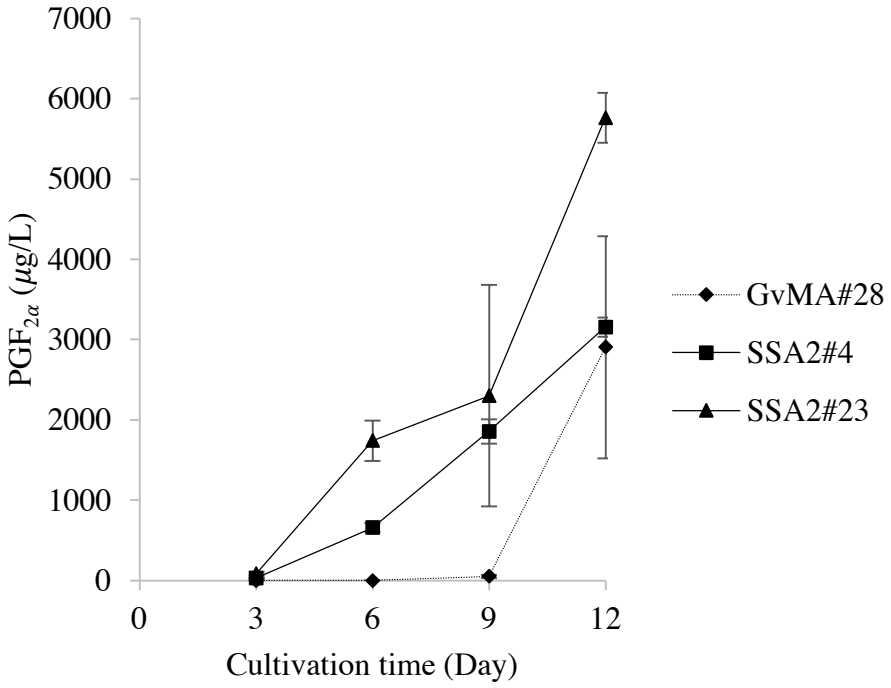

Fig. 2-3 Comparison of time-course analysis accumulation of $PGF_{2\alpha}$ in the extracellular media culturing transformants, *GvMA#28* with the *his550* promoter-driven GvCOX gene and *SSA2#4* and *SSA2#23* with the *SSA2* promoter-driven GvCOX gene. This experiment was done with 3 duplicates for each transformant. Standard error was indicated at individual points.

UV mutagenesis of transformant *SSA2#23*

Filamentous fungi have intrinsically versatile production abilities with regards to their durable and strong productivity as host microorganism, which tremendously contribute to the fermentation industry. UV mutagenesis and selection for oleaginous fungi resulted in varying potencies on diverse species and strains for improvement of lipid productivity. It also exhibits integrity stability as a host in numerous genetic manipulations and mutagenesis, notably to lipid-producing microorganisms. A prevalent oleaginous fungi species and strain could grow and produce lipids from a simple sugar such as glucose as a carbon source in the fermentation medium. The spores of transformant *SSA2#23* were irradiated by ultraviolet periodically for 10 seconds and, aseptically transferred and spread on the selective agar for the selection of growth of mutated spore. As a result, the spores of SSA2#23 were sensitive to the UV radiation where no survival of cells has been observed after long radiation time from 6 to 10 seconds. The fatality rate was increased with an increasing in exposure time. The survival rate of cells was decreased from 53 to 7% at 10^3 dilutions. The results indicated that the long-time UV radiation was the most lethal, suggesting that the severity of the DNA damage depends on the exposure time. Thirteen UV-derived mutants were isolated based on the physical morphological differences and subcultured onto potato dextrose agar (PDA) for further experiments.

As shown in Fig. 2-4, UV#7 showed the highest concentration of $PGF_{2\alpha}$ with 2.26 μg/mL. Whilst, the least $PGF_{2\alpha}$ was obtained by *UV#8* with only 0.37 μg/mL. The dry cell weight of *UV#7* showed the lowest amount with 0.0577g and the highest amount was achieved by *UV#10* with 0.0669 g. Interestingly, the deficient in dry cell weight of *UV#9* among other mutants has no significant effect on the production of $PGF_{2\alpha}$ (Fig. 2-5). The significant difference indicates that the increased UV exposure reduces the ability of mutant to produce the product. Akileswaran (46) reported that *Phanerochaete chrysosporium,* the most uracil auxotroph with UV radiation mutagenesis are the *ura5*-deficient strain. The *ura5* gene in *M.*

alpina 1S-4 may easily undergo mutation with any mutagen. Furthermore, the addition of Rose Bengal in the agar for mutant selection has benefited in eliminating all the actinomycetes and most bacteria, and reduced spreading of mold colonies (47). In *Lipomyces starkeyi and Rhodotula glutinis,* the lipid productivity has promisingly improved by UV irradiation random mutagenesis and selection (48-49) Therefore, random mutagenesis and selection could be a promising strategy for improving lipid production.

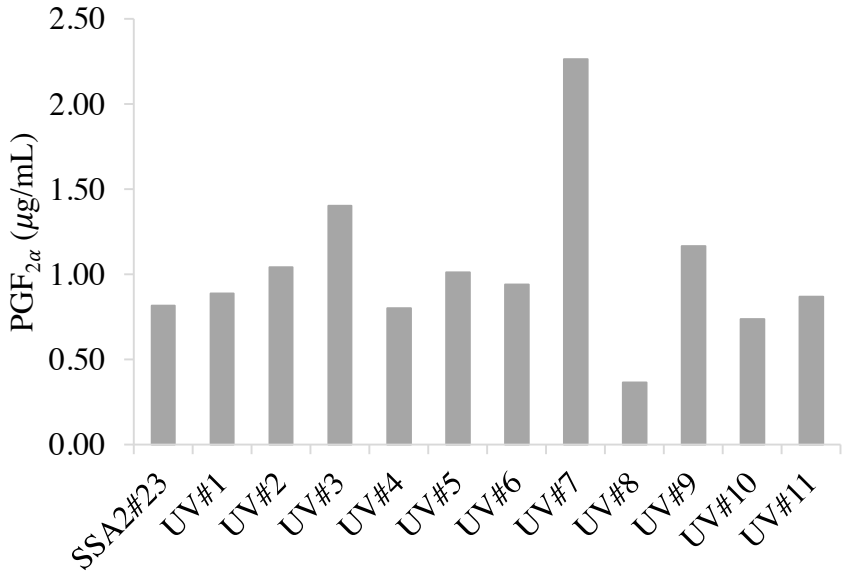

Fig. 2-4 Production of $PGF_{2\alpha}$ by UV-mutated strains at day 6th of cultivation

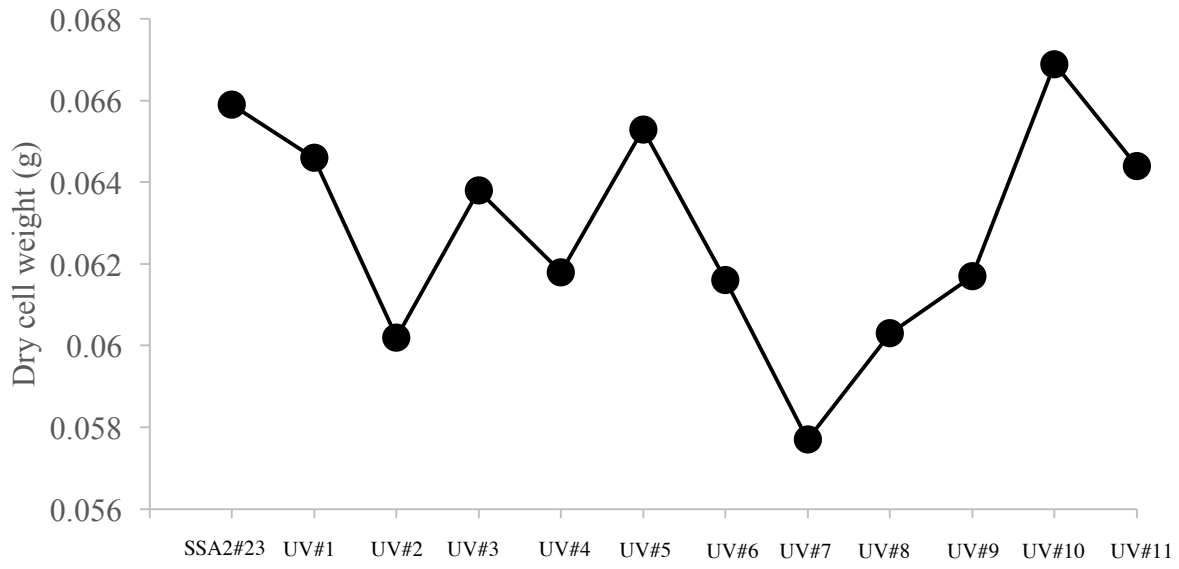

Fig. 2-5 Dry cell weight of the UV-derived mutants at day 6th of cultivation

Transformation of the UV-derived mutant (*UV#7)* with pSDZNCBXBGvCOXm by particle gene bombardment

Microprojectile bombardment is another method available for genetic transformation of *M. alpina*（24). This transformation method has the high probability of introducing multiple copies of genes, which can lead to various undesirable side effects such as gene silencing, low stability or altered genetic expression. In this study, tungsten particles coated with a plasmid pSDZNCBXBGvCOXm were bombarded into the dried spores of the host strain *UV#7* to obtain novel *M. alpina* transformants with very high productivity of $PGF_{2\alpha}$. The GvCOX gene expression was expected to be driven by the *pp3* promoter from hypothetical multiple insertion sites in addition to that driven by the *SSA2* promoter.

One mitotically stable transformant was obtained and labelled as *CB#1*. The transformant was evaluated on the production of $PGF_{2\alpha}$ at 2 days intervals. Based on the time

course analysis of the $PGF_{2\alpha}$, the highest concentration was attained at 30.07 μg/mL by *CB#1* at day-10 (Fig. 6). The results of *CB#1* was the leading breakthrough on the production of prostaglandin $F_{2\alpha}$ by oleaginous fungi, *M. alpina* strain.

Furthermore, two different concentrations (60 and 80 μg/mL) of carboxin were tested for the selection of the potential transformants. For the screening of antibiotics resistance, 60 μg/mL of carboxin was favorable for the transformant. (23) reported that the growth of zygomycetes, *M. alpina* 1S-4 was inhibited at 100 μg/mL of carboxin whilst basidiomycetes require lower concentration of carboxin. This highly effective fungicide has been used in a dominant drug-resistant transformation system for basidiomycetes. Thus, in this study, the carboxin (CBXB) gene was cloned into the constructed plasmid pSDZNCBXB and introduced to the host strains as a drug-resistant marker gene. Nonetheless, the transformation systems for *M. alpina* has been well developed in the past few years. Takeno (24) reported that the homologous *ura*5 gene has been transformed in *M. alpina* spores by microprojectile bombardment resulted in transformants with high accumulation of arachidonic acid than the wild strain. Moreover, the inclusion of fungal cyclooxygenase gene in the random genome of *M. alpina* has reinforced the capability of derived transformant to produce more products.

Recently, Jung-Ung An (50) reported that the biotransformation of bacterium *Myxococcus xanthus* produces hepoxilin, Trioxilin, and prostaglandins by lipoxygenase (LOX) and other enzymes in the cytochrome P450 using PUFAs as substrates. Growing interest in biotechnological research demands the development of novel strategies to manipulate and incorporate specific sequences into fungi to improve their characteristics in an easy, safe, reliable and reproducible form. Some techniques have been successfully established for a few types of fungi, but a great deal of research remains to be performed to effectively exploit these technologies in a wide variety of species and to increase the efficiency and reproducibility of genetic transformations.

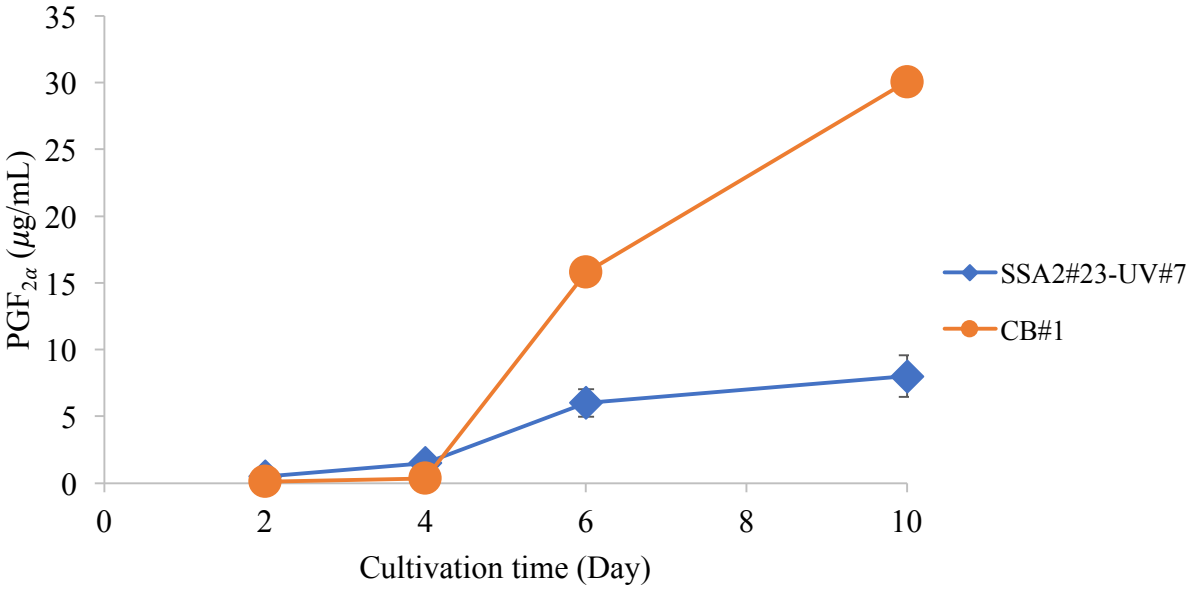

Fig. 2-6 Time-course production of prostaglandin $F_{2\alpha}$ by *SSA2#23* and *CB#1* during the fermentation period

SUMMARY

Transformant *SSA2#23* in which the *GvCOX* gene was driven by *SSA2* promoter, was obtained by the ATMT and able to produce 5.76 μg/mL of $PGF_{2\alpha}$. *SSA2#23-UV#7* obtained by UV-irradiation to *SSA2#23* exhibited 2.8-fold increased production of $PGF_{2\alpha}$ as compared with *SSA2#23*. A potent transformant, *CB#1* was obtained by particle gene bombardment transformation of *SSA2#23-UV#7* in which the *GvCOX* gene was driven by *pp3* promoter. With stronger promoter *pp3* and hypothetical multi copy numbers, transformant CB#1 showed the highest productivity of $PGF_{2\alpha}$ with 3.7-fold increase as compared with *SSA2#23-UV#7* (30.1 μg/mL of $PGF_{2\alpha}$).

CHAPTER III

Optimization of fermentation conditions for higher production of prostaglandin $F_{2\alpha}$ by *Mortierella alpina* transformants with an algal cyclooxygenase gene

Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a derivative of arachidonic acid (ARA) and has various biological activities. An enzyme, cyclooxygenase (COX) plays a crucial role in conversion from ARA to $PGF_{2\alpha}$. Recently, transformants from ARA-rich oleaginous fungus *Mortierella alpina* introduced a COX gene from a red alga *Gracilaria vermiculophylla* (GvCOX) were created and proved to produce $PGF_{2\alpha}$ fermentatively (51). (In Chapter II, this fermentative $PGF_{2\alpha}$ production system was improved by mutagenesis mediated by genetic manipulations and UV-irradiation, and a transformant *CB#1* was obtained as the best transformant). As the expected concentration of GvCOX in cells increased, more $PGF_{2\alpha}$ released into the media. However, the amount of $PGF_{2\alpha}$ was far less than ARA accumulated as a triglyceride form in *M. alpina* cells.

Biomass productivity and fatty acid accumulation of the microorganism can be increased by manipulating nutritional compositions in culturing media and culture conditions. This can be an advantage of fermentative production of $PGF_{2\alpha}$, as compared to the molecular breeding. Thus, the effect of several fermentation conditions such as different concentrations of glucose, fed-batch of 2% glucose, addition of palmitic and stearic acids in free, methyl and ethyl ester forms, and addition of other fatty acids with carbon chain length between 12 to 24 were investigated for the production of $PGF_{2\alpha}$

Materials and Methods

Microorganisms and culture conditions

Three different strains, *M. alpina* 1S-4, transformant *SSA2#23* and *CB#1* were used in this study. The latter two transformants have been created from uracil auxotroph of *M. alpina* 1S-4 (51; and Chapter II). These transformants can produce much more $PGF_{2\alpha}$ than the parental strain, while *CB#1* can produce more $PGF_{2\alpha}$ than *SSA2#23* (Chapter II). All strains were maintained on the potato dextrose agar (PDA) at 28 ℃. The GY medium consisted of 2% (w/v) of glucose and 1% (w/v) of yeast extract was used as a basic fermentation medium. The culture conditions were set up at 28 ℃ for the incubation temperature, continuous shaking at 120 rpm and cultivated in 20 mL Erlenmeyer flask for 10 days. The glucose concentration in the GY medium was changed to 1% or 3% (w/v) to study the effect on the production of $PGF_{2\alpha}$.

Additives to the medium

Fatty acids added to the medium were listed in Table 3-1. They were initially prepared as 1mg/mL of ethanol solutions and added individually into the medium to be 10 μg/mL at final concentration. Palmitic (free), oleic and linoleic acids were purchased from Sigma-Aldrich; palmitic (methyl and ester forms) were purchased from Wako Chemicals; and other fatty acids were purchased from Tokyo Chemical Industry Co. Ltd.

Analysis of the $PGF_{2\alpha}$ production released into the media

The experiments were conducted in triplicates and in the same batch. The fermentative broth from each flask was filtered through No. 2 filter papers (Advantec) and diluted at appropriate factor (normally 10 times). As for the glucose feeding at 5^{th} or 7^{th} day, the sampling was done on the next day after glucose was fed in. While for the addition of fatty acids, the

sampling was done on day-6, -8 and -10. The $PGF_{2\alpha}$ was analyzed by LC-MS as previously described by (51).

Table 3-1 Different types and forms of fatty acids used in this study

Common name	Number of carbon	Form of fatty acid		
Lauric acid	12	Free	-	-
Palmitic acid	16	Free	Methyl	Ethyl
Stearic acid	18	Free	Methyl	Ethyl
Oleic acid	18:1	Free	-	-
Linoleic acid	18:2	Free	-	-
Arachidic acid	20	Free	-	-
Tetracosanoic acid	24	Free	-	-

Results and Discussions

Optimization of initial glucose concentrations

Glucose concentrations play an important role both in the biomass and arachidonic acid production of *M. alpina* 1S-4 (10-11; 52). The effects of glucose concentration in the initial media on the fermentative production of $PGF_{2\alpha}$ for 10 days were studied. The *CB#1* strain was cultured in the media in which three concentrations, 1, 2, and 3% (w/v) of glucose was with 1% (w/v) yeast extract. Time course accumulation of $PGF_{2\alpha}$ in the culture medium of *CB#1*

was shown in Fig. 3-1. For 10 days culturing, *CB#1* produced the highest concentration of $PGF_{2\alpha}$, 30.07 μg/mL, when cultured in the medium contained 2% glucose, which was about 3 times higher than the concentration of $PGF_{2\alpha}$ produced in the 1 and 3% glucose-containing media. Meanwhile, the best glucose concentration for the $PGF_{2\alpha}$ production by *SSA2#23* differed from that by *CB#1* (Fig. 3-1 and 3-2). although the highest concentration of $PGF_{2\alpha}$ by *SSA2#23* for 10 days was attained by using the 3% glucose medium (9.45 μg/mL). This was not significantly different from the concentration of $PGF_{2\alpha}$ attained by the 1% glucose medium. Thus, the best glucose concentration should be determined depending on the transformant. Nonetheless, the medium consisted of 2% (w/v) glucose and 1% (w/v) yeast extract was used as a basic medium in the following studies.

Effect of the batch feeding of glucose on the $PGF_{2\alpha}$ production

Next, effects of the glucose added during the culture on the $PGF_{2\alpha}$ production was investigated. Glucose was added at day 5^{th} or 7^{th} to recover the glucose concentration up to ~ 2% (w/v). The batch feeding at either point of time resulted in dramatic increase of the $PGF_{2\alpha}$ production (Fig. 3-3 and 3-4). As for *CB#1*, the $PGF_{2\alpha}$ produced for 10 days was increased to 201.33 and 191.26 μg/mL by adding glucose on 5^{th} and 7^{th} day, respectively. While those in the basic medium without glucose addition was achieved at 30.07 μg/mL of $PGF_{2\alpha}$ production by *CB#1* (Fig. 3-3). The increase of the $PGF_{2\alpha}$ production had been started at the next day of the batch feeding. Similar results were obtained by using *SSA2#23* (Fig. 3-4). Thus, the increase of the $PGF_{2\alpha}$ production occurring by the batch feeding of glucose is considered to be a common phenomenon in the *M. alpina* transformants expressing the GvCOX gene. By adding of glucose at day 5^{th} and 7^{th} individually is theoretically expanding the exponential phase of the microorganisms and subsequently increasing the amount of $PGF_{2\alpha}$.

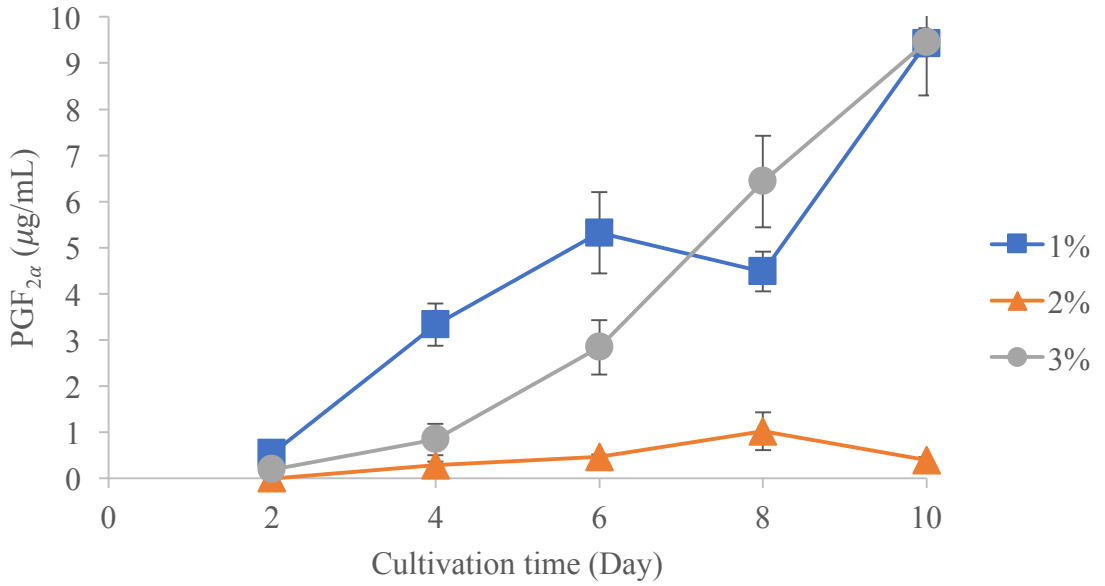

Fig. 3-1 Time-course of production of $PGF_{2\alpha}$ in 1, 2 and 3% of glucose by *SSA2#23*

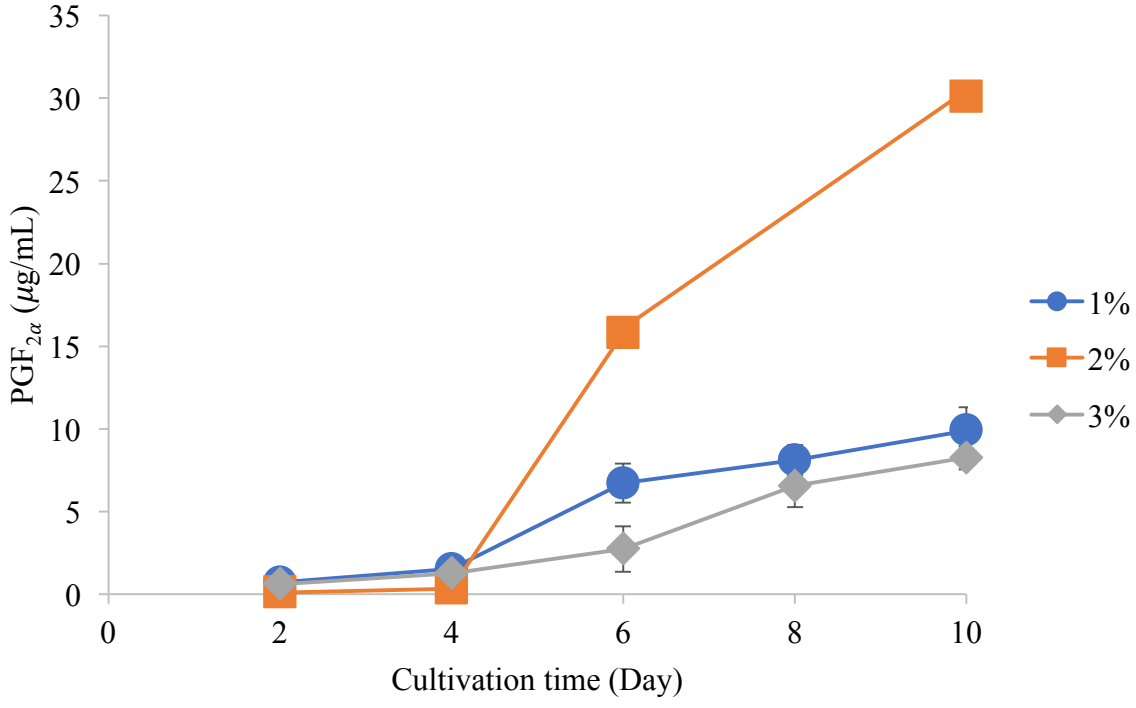

Fig. 3-2 Time-course of production of $PGF_{2\alpha}$ in 1, 2 and 3% of glucose by *CB#1*

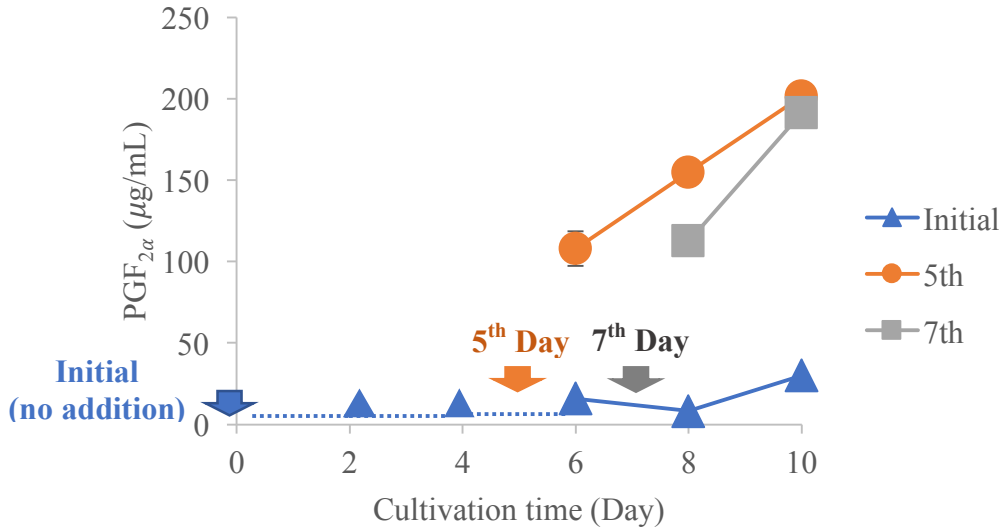

Fig. 3-3 Addition of 2% glucose at day 5th and 7th for the production of $PGF_{2\alpha}$ by *CB#1*

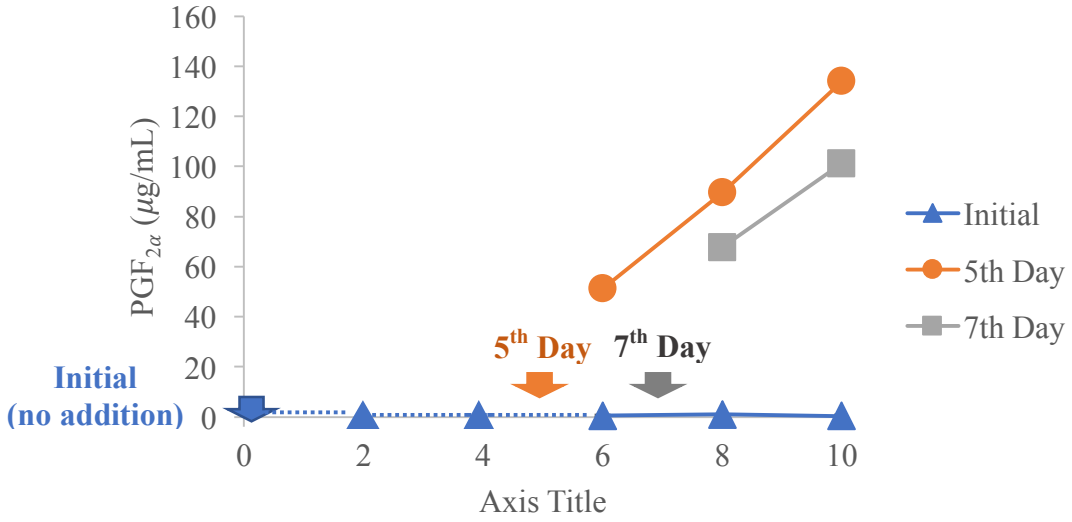

Fig. 3-4 Addition of 2% glucose at day 5th and 7th for the production of $PGF_{2\alpha}$ by *SSA2#23*

Effects of addition of fatty acids on the production of $PGF_{2\alpha}$

Several fatty acids, such as palmitic, stearic, oleic, and linoleic acids are intermediates in the ARA-synthesizing pathway in *M. alpina.* This pathway could be stimulated by increasing of those fatty acids as glucose that can be a starting material of ARA synthesis. Thus, it was also tested if the addition of those fatty acids to the basic medium would affect the extracellular production of $PGF_{2\alpha}$ by *CB#1*. Interestingly, however, human COX enzymes are allosterically regulated by binding of different free fatty acids. The COX-1 activity is inhibited by the most common fatty acids including palmitic and stearic acids, while that of COX-2 is stimulated relatively specifically by palmitic acid (53-54). GvCOX is considered as act as a tetramer (18) and there is no knowledge if this enzyme has allosteric functions, different from the human COX enzymes. Nevertheless, it was interesting to investigate the effects of free fatty acids on the fermentative $PGF_{2\alpha}$ production.

At first, palmitic and stearic acids in free, methyl and ethyl ester forms were individually added to the basic medium and tested the effects on the production of $PGF_{2\alpha}$ for 10 days (Fig. 3-5). The addition of these fatty acids resulted in enhanced production of $PGF_{2\alpha}$. As compared with the case without adding the fatty acids, far more $PGF_{2\alpha}$ was accumulated in the extracellular media as the culturing proceeded. Either fatty acids caused the best increase of $PGF_{2\alpha}$ with a free form rather than methyl- and ethyl ester forms (Table 3-1). Namely, *CB#1* produced $PGF_{2\alpha}$ for 10 days with the concentration of 253.04 μg/mL and 260.89 μg/mL in the medium initially added with palmitic and stearic acids, respectively. The enhancement of the $PGF_{2\alpha}$ production became obvious earlier with palmitic acid than with stearic acid (Fig. 3-5). Similar enhancement of the extracellular production of $PGF_{2\alpha}$ was observed by using SSA2#23 (Fig. 3-6 and Table 3-1). It was notable that this strain produced 240.07 μg/mL of $PGF_{2\alpha}$ for 10 days in the medium added with free stearic acid, corresponding to the level of $PGF_{2\alpha}$ produced by CB#1, although *SSA2#23* produced about one-sixth times as much $PGF_{2\alpha}$

produced by CB#1 in the basic medium. In addition, the parental strain, *M. alpina* 1S-4 produced negligible (but less than 10 μg/mL) amount of $PGF_{2\alpha}$ when cultured in the medium with fatty acids (Fig. 3-7 and Table 3-1). These results might suggest that the native $PGF_{2\alpha}$ synthesis in *M. alpina* was activated by the addition of fatty acids. Nevertheless, the increment of $PGF_{2\alpha}$ extracellularly accumulated in the culture of the GvCOX-expressing transformants was much more than the level expected from the amount of dosage which might be observed in the cultures of 1S-4. Thus, the fatty acids might enhance the expression and/or activity of GvCOX enzyme. Analyses of the mRNA/protein and the Cox activity will be necessary. Those results might be able to clarify the allosteric character of GvCOX with some other experimental data. Besides, the longevity and/or changes of the added fatty acids in the cultures should be studied.

Further, addition of several other fatty acids including lauric (C12), oleic (C18:1), linoleic (C18:2), arachidonic (C20), and tetracosanoic (C24) acids were individually studied in regard to the effect on the extracellular production of $PGF_{2\alpha}$ by culturing of CB#1 for 10 days. These fatty acids can divide into three different categories which are medium-chain fatty acids (MCFA) with 6 to 12 carbons, long-chain fatty acids (LCFA) with 13 to 21 carbons, and very long-chain fatty acids (VLCFA) with 22 or more carbons. Oleic and linoleic acids have unsaturated chain with one and two double bonds, respectively. As shown in Fig. 3-8, all fatty acids tested caused the increase of the extracellular production of $PGF_{2\alpha}$ by *CB#1*. Among them, when oleic acid was added, the highest production of $PGF_{2\alpha}$ was attained with the concentration 290.61 μg/mL. Similarly, these fatty acids caused the increase of the extracellular production of $PGF_{2\alpha}$ by *SSA2#23* (Fig. 3-9). However, as for this strain, the addition of lauric acid was most effective, which resulted in 164 μg/mL $PGF_{2\alpha}$ production at day 8^{th}. As for the *M. alpina* 1S-4, the production of $PGF_{2\alpha}$ was less affected by the addition of fatty acids. Only the addition of oleic acid in free form was observed to enhance the

production of $PGF_{2\alpha}$ in the culture medium of *M. alpina* 1S-4 (Fig. 3-10). The enhancement by tetracosanoic acids suggest that the ability of fatty acids to enhance the production is not related to the ARA-synthesis pathway. Those fatty acids can affect the growth of biomass, total amount of lipids, fatty acid composition, and GvCOX activity. Besides, they also can affect the membrane permeability and cause metabolic disorders.

This condition could be suggested that the microorganisms are capable to consume the fatty acids in the free form is higher than the one of the microorganisms capable to grow on TAGs or methyl-esters (55). Furthermore, an important point that should be considered in relation with the utilization of hydrophobic materials as microbial substrates (or co-substrates with other low-molecular weight hydrophilic compounds like sugars or glycerol) refers to the fact that the fatty compounds found in the medium in several cases may 'enhance' the accumulation of cellular lipids by the relevant micro-organisms employed, while growth on the hydrophilic material employed as the sole substrate under nitrogen-limited conditions, was not accompanied by noticeable single-cell oil (SCO) production (56-63).

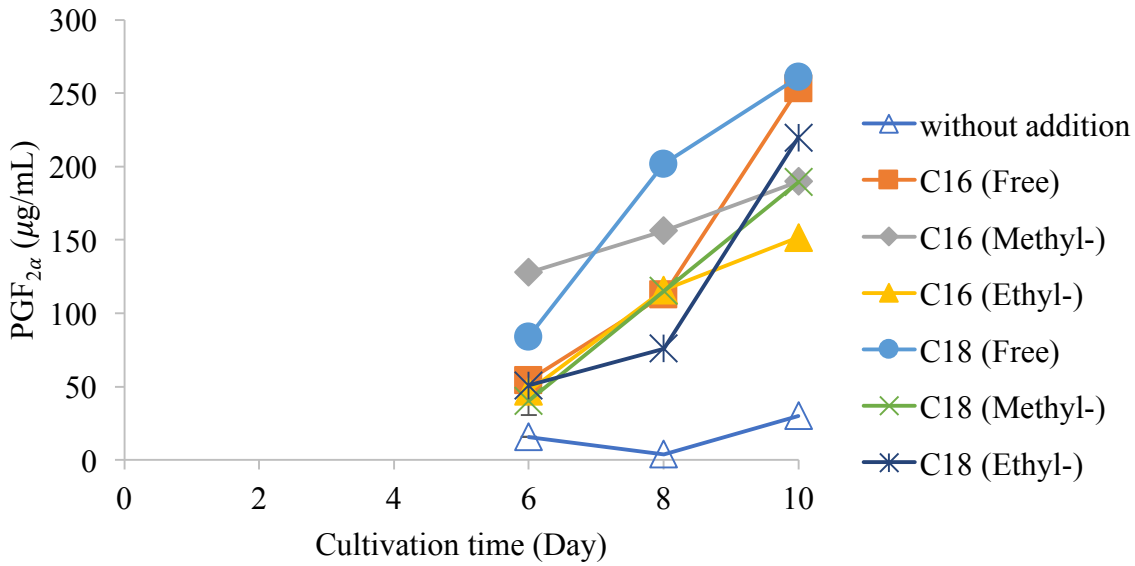

Fig. 3-5 Addition of fatty acids in free, methyl and ethyl forms on the production of $PGF_{2\alpha}$ by CB#1

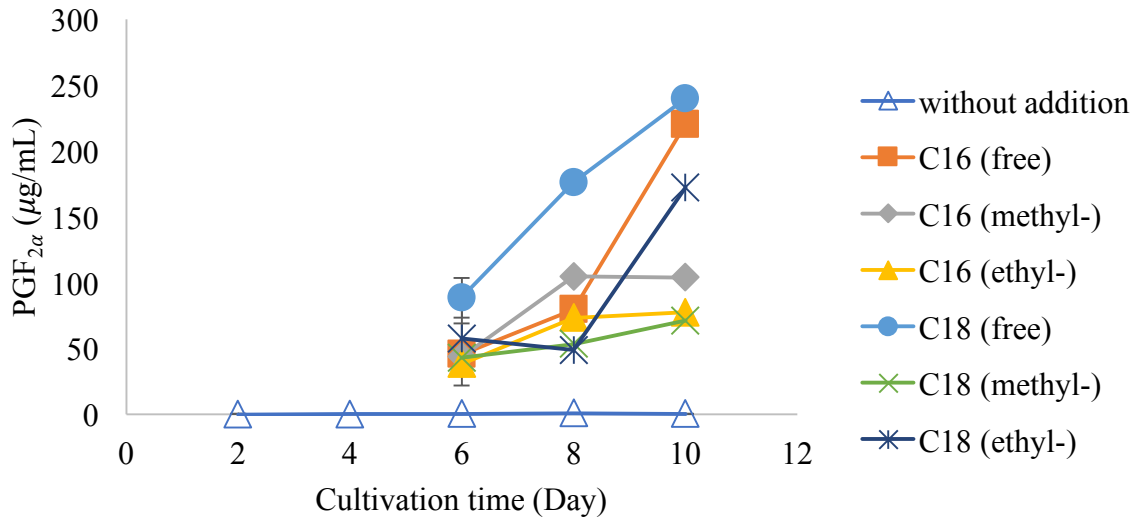

Fig. 3-6 Addition of fatty acids in free, methyl and ethyl forms on the production of $PGF_{2\alpha}$ by SSA2#23

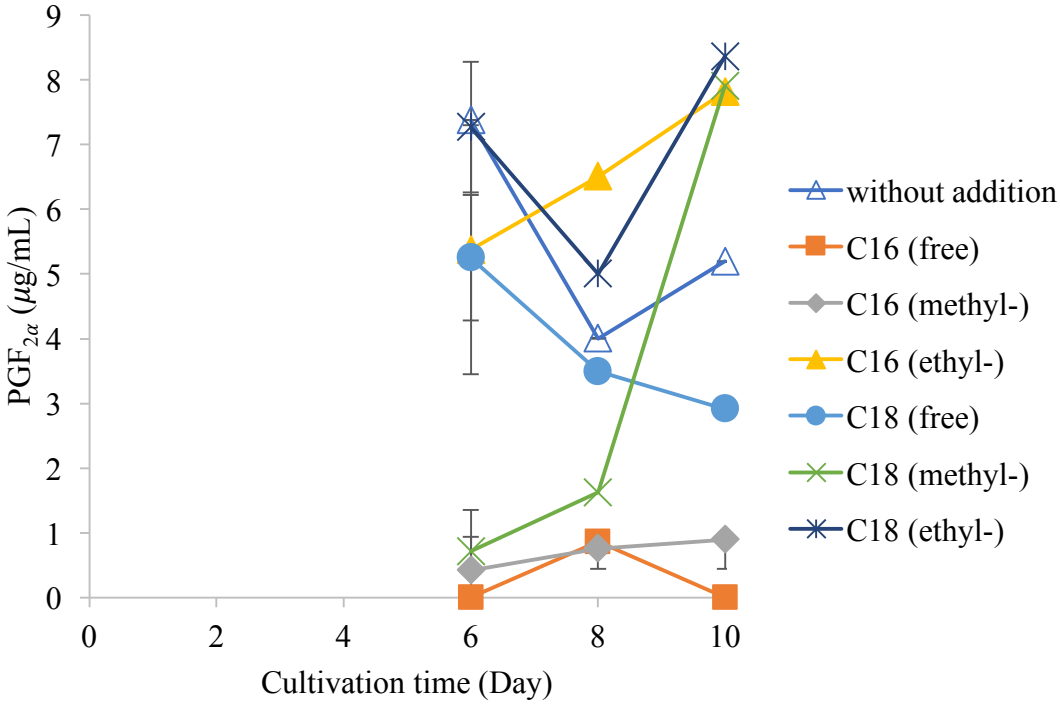

Fig. 3-7 Addition of fatty acids in free, methyl and ethyl forms on the production of $PGF_{2\alpha}$ by *M. alpina* 1S-4

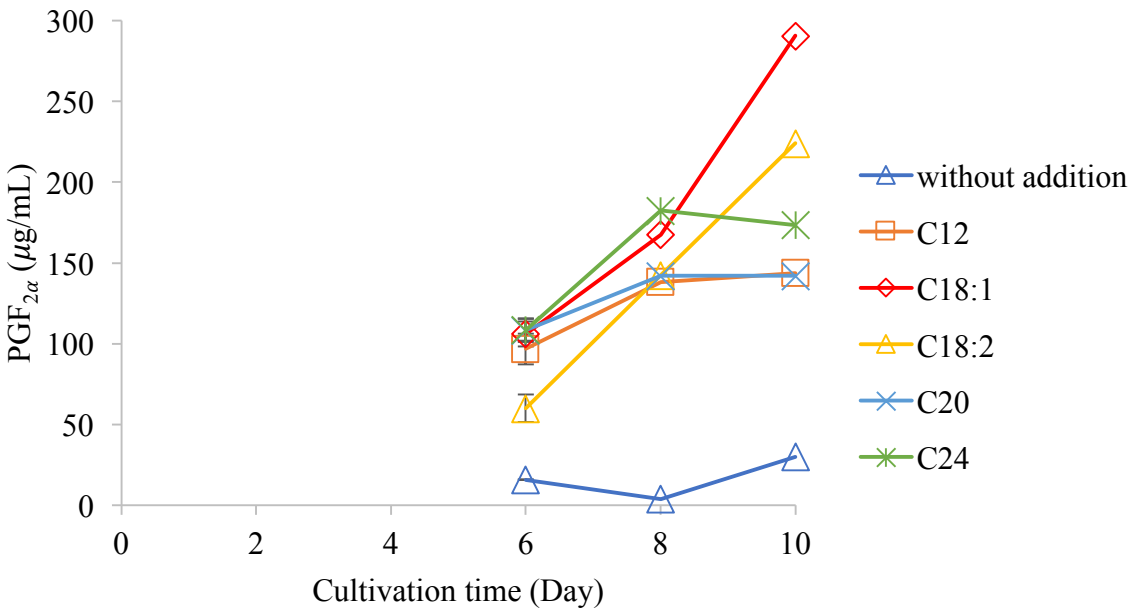

Fig. 3-8 Addition of MCFA, LCFA and VLCFA on the production of $PGF_{2\alpha}$ by CB#1

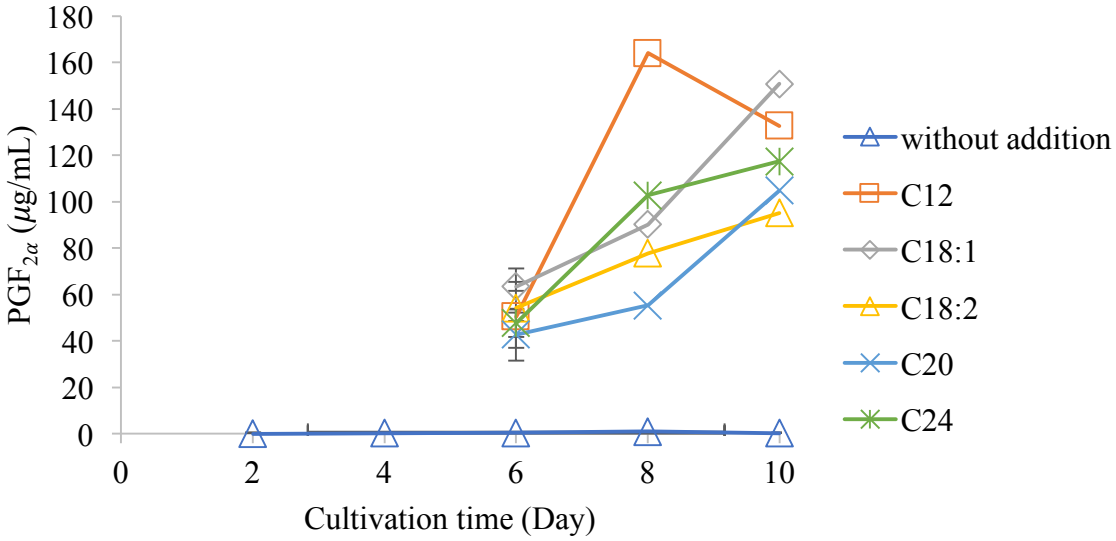

Fig. 3-9 Addition of MCFA, LCFA and VLCFA on the production of $PGF_{2\alpha}$ by SSA#23

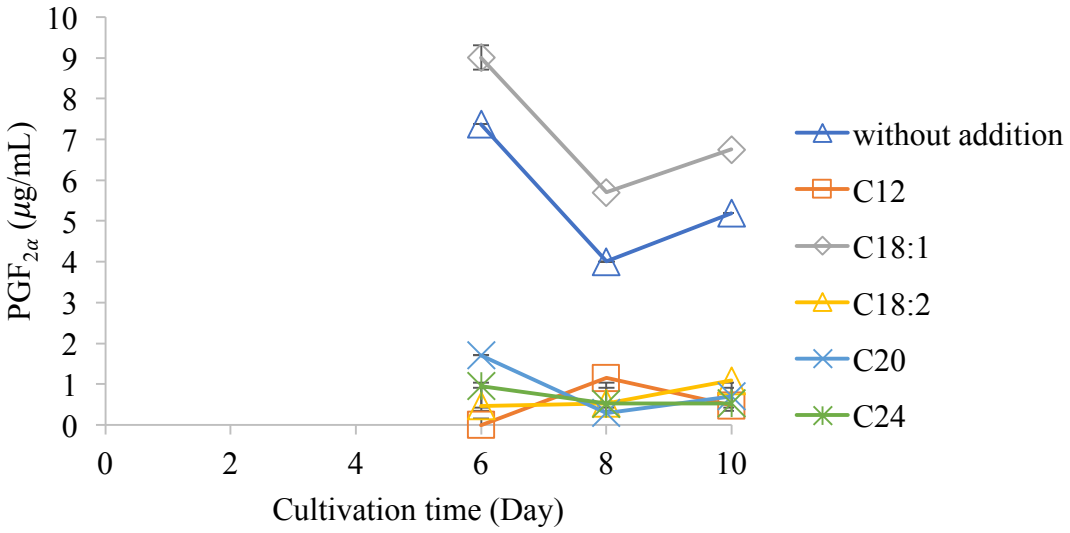

Fig. 3-10 Addition of MCFA, LCFA and VLCFA on the production of $PGF_{2\alpha}$ by *M. alpina* 1S-4

SUMMARY

Medium composition affects the production of $PGF_{2\alpha}$;

- Initial glucose concentration in the medium affected the production of $PGF_{2\alpha}$ by transformant *CB#1*.
- Batch feeding of glucose during the culture increased the production of $PGF_{2\alpha}$.
- Addition of a free fatty acid with 10 μg/mL in the free form significantly affected the production of $PGF_{2\alpha}$.

Transformant *CB#1* showed 291 μg/mL of $PGF_{2\alpha}$ production in the medium with an addition of oleic acid in 10 days of cultivation.

CONCLUSIONS

CHAPTER I

$PGF_{2\alpha}$ was produced by *M. alpina* transformed by the *GvCOX* gene. A potent transformant, *GvMA#28* was able to accumulate consistently the $PGF_{2\alpha}$ in the extracellular in which driven by *his550* promoter. These findings will provide a constructive information in improving the product yield of prostaglandins especially for $PGF_{2\alpha}$.

CHAPTER II

Two promising transformants were obtained using stronger promoter activity, *SSA2*, namely *SSA2#4* and *SSA2#23*. The production of $PGF_{2\alpha}$ was attained at 1.14-fold higher by *SSA2#23* than the *his550* promoter. Next, the spores of *SSA2#23* were irradiated with UV, resulted the breeding of *SSA2#23-UV#7*. This UV-mutated strain produced 1.3-fold higher of $PGF_{2\alpha}$ than the *SSA2#23* for 6 days. By changing stronger promoter, *pp3,* the $PGF_{2\alpha}$ was increased to 30.07 μg/mL, in which subsequently increasing the *GvCOX* activity by multi copies of the genes via particle gene bombardment.

CHAPTER III

Glucose concentration affected the extracellular production of $PGF_{2\alpha}$ by *M. alpina* transformants introduced the GvCOX gene. The best glucose concentration in the initial medium was 2% (w/v) for 1% (w/v) yeast extract, and batch feeding during the culture also caused the increase of $PGF_{2\alpha}$. on the other hand, the extracellular production of $PGF_{2\alpha}$ increased by adding several fatty acids to the initial medium. the addition of oleic acid resulted in 290 g/mL $PGF_{2\alpha}$ production by the most promising transformant *CB#1* for 10 days.

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