Cloning and expression of Baeyer-Villiger monooxygenase from *Microbacterium esteraromaticum* SBS1-7

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Abstract

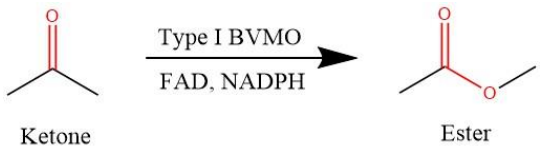
Type I Baeyer-Villiger monooxygenase (Type I BVMO) has been intensively studied due to its high potential for industrial applications as it can catalyze a Baeyer-Villiger oxidation (oxidation of ketone). Discovery of a BVMO with novel biocatalytic activities is, therefore, of importance for industrial applications. As enzymes from genus *Microbacterium* are rarely studied in detail, in this study, a Type I BVMO named as BVMO1MES (discovered in a genome of a unique BTEX- and naphthalene-degrading *Microbacterium esteraromaticum* SBS1-7) was cloned and subsequently expressed in *Escherichia coli*. In order to obtain the maximum BVMO1MES in soluble form, an expression was performed in various *E. coli* hosts including BL21 (DE3), SHuffle® and origami B. Moreover, various factors affecting an expression of a soluble target protein - including (1) an IPTG concentration (0.05, 0.1, 0.25 and 0.5 mM), (2) an induction start point (OD_{600} = 0.4, 0.8 and 1.2), (3) an induction temperature (16 and 20°C) and (4) an induction time (12, 16 and 24 h) - were optimized in detail. The highest amount of soluble BVMO1MES could be obtained from *E. coli* BL21 (DE3) clone when an induction was performed in a 50-mL culture at OD_{600} of 1.2 with 0.1 mM IPTG at 20°C for 12 h. Catalytic activity of a crude extract containing BVMO1MES was evaluated using a substrate benzaldehyde. Fragmentation pattern obtained from a gas chromatography–mass spectrometry (GC-MS/MS) confirmed a formation of an expected product (phenyl formate).

Keywords**:** *aldehyde oxidation, benzaldehyde, Type I BVMO*

1. Introduction

Type I Baeyer-Villiger monooxygenase (Type I BVMO) has been intensively studied for industrial applications since 1976 (Donoghue, Norris and Trudgill, 1976). The main activity of type I BVMO is Baeyer-Villiger oxidation (also known as an oxidation of ketone), a chemical reaction originally discovered in 1899 (Baeyer and Villiger, 1899), in which ketone is converted into ester (or lactone) as shown in Figure 1. An oxygen atom is inserted into a carbonyl group of ketones using FAD and NADPH as cofactor and coenzyme, respectively. This ability was employed to industrially produce valuable chemicals such as nylon-9 monomer (Milker et al., 2017) and β,δ-trimethyl-ε-caprolactones (TMCL), precursors for plastic and coating industries (Delgove et al., 2018). Discovery of a BVMO with novel biocatalytic activities (e.g., novel substrates, stability, etc.) is, therefore, of importance for industrial applications. *Microbacterium* is not a general host reported for BVMO, therefore, there is a high possibility to discover a novel BVMO in this bacterium.

Figure 1 A schematic diagram of an enzymatic Baeyer-Villiger oxidation



Microbacterium esteraromaticum SBS1-7 was recently reported for its ability to degrade BTEX and naphthalene (Wongbunmak et al., 2017). *In silico* analysis of the protein revealed that the BVMO found in a genome of *M. esteraromaticum* SBS1-7 (referred as BVMO1MES) belongs to a group of Type I BVMO (Figure 2A). Among known BVMOs, BVMO1MES exhibits the highest sequence similarity of only 46%

with an unusual Type I BVMO ethionamide monooxygenase (EtaA) from *Mycobacterium tuberculosis* H37Rv (Figure 2B), an enzyme capable of activating the prodrug ethionamide (Fraaije et al.,2004).

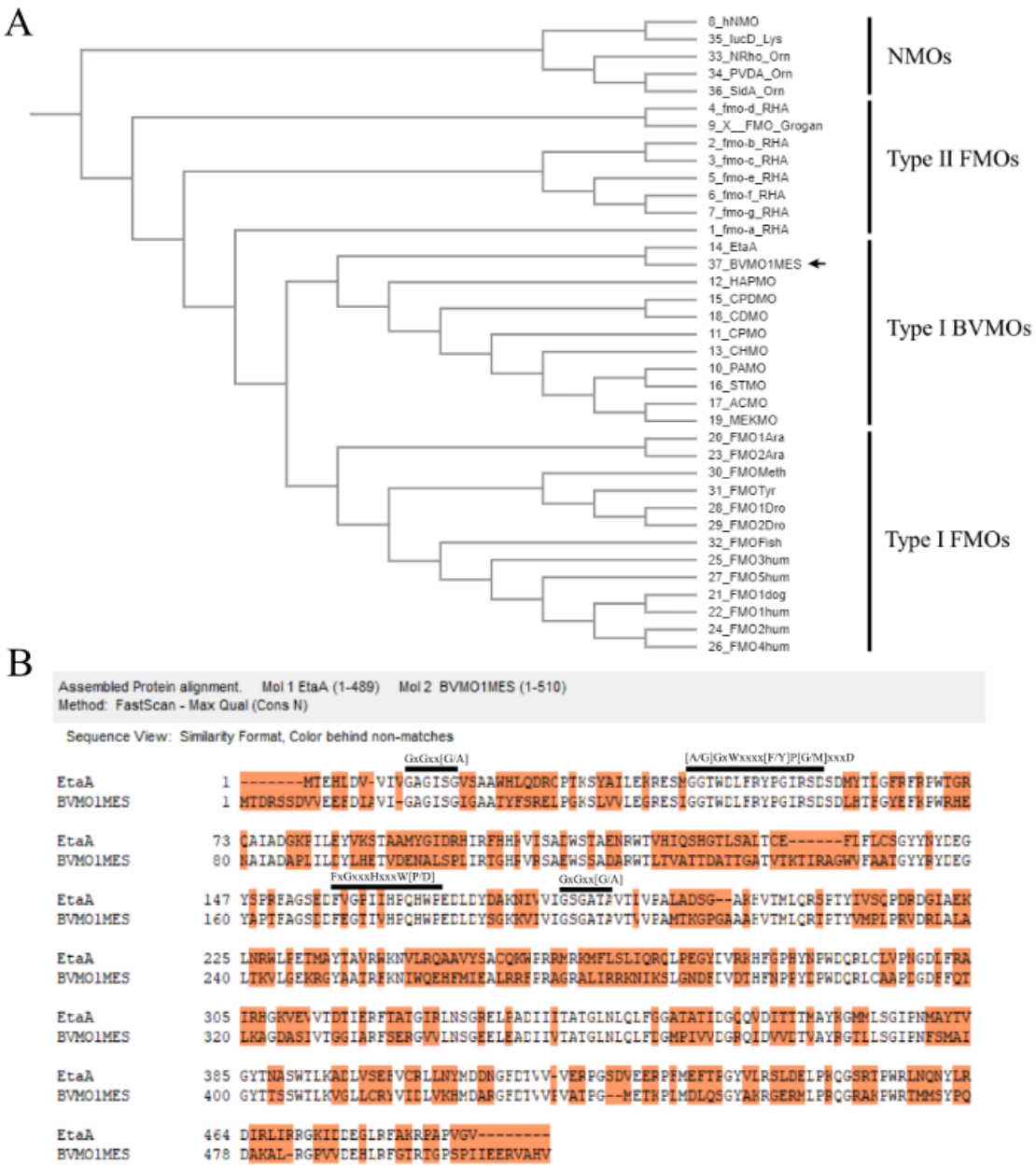


Figure 2 A) Phylogenetic tree of BVMO1MES and well-known class B flavin-dependent proteins. B) amino acid alignment between BVMO1MES and EtaA (NP_218371.1). Black arrow indicates the enzyme used in this study. BVMO fingerprint motifs are indicated above the amino acid sequence.

To confirm the substrate preference of this enzyme for a biocatalysis purpose, a recombinant BVMO1MES must be cloned and expressed in soluble form. In this study, *bvmo1mes* gene was expressed

using a pET system using isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer. To maximize an expression of BVMO1MES in a soluble form (functional protein with correct folding), an *E. coli* host as well as induction conditions (including an inducer concentration, an induction temperature, an induction start point and induction time) were systematically optimized. An enzyme activity assay was also performed to confirm the biocatalytic activity of the expressed BVMO1MES.

2. Objectives

This preliminary study aimed to establish a suitable induction condition for maximizing of a soluble BVMO1MES expression in a 50-mL culture as well as to confirm its biocatalytic activity.

3. Materials and Methods

3.1 Chemicals and media

All chemicals used in this study were of reagent-grade from known manufacturers including Sigma-Aldrich (USA), Merck (Germany), Asia Pacific Specialty Chemical (Australia), LOBA Chemical (India), and Univar (Australia). Bacterial cultivation medium components were purchased from LabM (UK). Lysogenic Broth (LB) medium, containing 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, was used for culture maintenance and cultivation. When necessary, 50 µg/mL ampicillin was supplemented for cultivation of *E. coli* DH5α, BL21 (DE3) and SHuffle®. In case of *E. coli* origami B, 30 µg/mL kanamycin was used instead. For solid medium preparation, 15 g/L of Bacto agar was added.

3.2 Cloning of a full-length *bvmo1mes*

Previously, a full-length sequence of *bvmo1mes* (GenBank Accession No. MZ097906) was retrieved from the genome of SBS1-7 by Wongbunmak (2020). For purification purpose in the future, 6xHis tag was integrated into the N-terminus of *bvmo1mes* gene. Forward primer (BVMO1-F-His-NdeI) was designed from position 1-19 of *bvmo1mes* gene whereas a reverse primer (BVMO1-R-BamHI) was designed from position 1513-1533 of the gene. The plasmid pET19b/BVMO1MES (Wongbunmak, 2020) was used as a template for amplification of a full-length *bvmo1mes* integrated with a 6x his-tag using Vent® polymerase (New England Biolabs, UK). The blunt-end PCR fragment (1,568 bp) was ligated with pJET1.2 (Thermo Scientific, US) and the resulting plasmid was referred as pJET1.2-*bvmo1mes*-his.

A BVMO1MES-expressing plasmid was constructed by introducing a *bvmo1mes*-containing fragment into the plasmid pET21a+ (Novagen, Germany). Briefly, the *bvmo1mes*-containing fragment (1.5 kb) was retrieved from a double digestion of pJET1.2-*bvmo1mes*-his using FastDigest NdeI and FastDigest BamHI (Thermo Scientific, US). The linearized pET21a+ and *bvmo1mes*-containing fragment were ligated. The resulting plasmid (pET21a+-*bvmo1mes*-his, Figure 3) was introduced into *E. coli* DH5α for propagation purpose. The plasmid extracted from the positive transformants, giving an expected PCR product (1.5 kb) from a colony PCR using primer BVMO1-F-His-NdeI and BVMO1-R-BamHI, was sent for DNA sequencing by Macrogen (using universal primer for T7 promoter and T7 terminator).

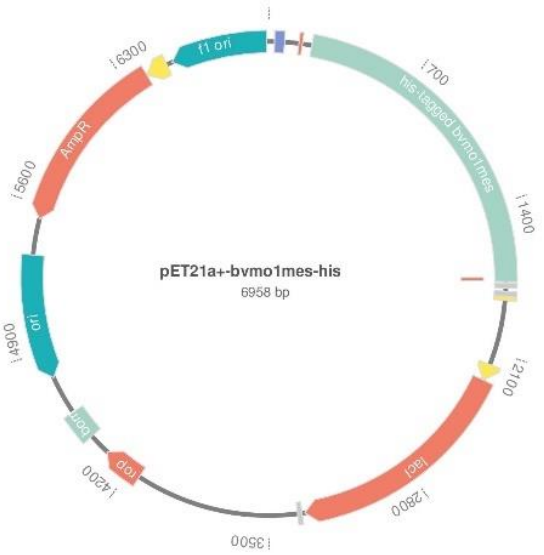


Figure 3 An expression plasmid used in this study

3.3 Overexpression of recombinant BVMO1MES

BVMO1MES-expressing plasmid was introduced into various expression hosts (*E. coli* BL21 (DE3), SHuffle® and Origami B). A flask-scale cultivation was conducted with 50 mL of LB medium supplemented with required antibiotic(s) in a 250 mL-round bottom flask using a shaking incubator (JSSI-100C, JSR, Korea) at 37°C, 200 rpm. In this study, a round-bottom flask was used instead of a baffled-flask (which promotes faster growth). As a consequence, a slightly extended cultivation time allowed us to harvest the cells at a specified OD_{600} more accurately. Soluble target proteins obtained from each host were compared to select the most appropriate host.

Protein expression was induced when a desired OD_{600} was reached by an addition of IPTG. In order to optimize the expression of recombinant BVMO1MES, several factors (i.e., IPTG concentration, state of the cells at an induction start point and induction temperature & time) were optimized one-by-one (Table 1).

Table 1 Optimization of induction conditions

No.	Factors	Levels
1	IPTG concentration)mM(	0.05, 0.1, 0.25 and 0.5
2	OD_{600} at an induction start point	0.4, 0.8 and 1.2
3	Induction temperature)°C(	16 and 20
4	Induction time) h(	12, 16 and 24 h

To visualize the protein, a mixture of cell-free supernatant and non-denaturing bromophenol blue dye (protein loading dye) was boiled (3-5 min) and loaded into NuPAGE™ 4-12% Bis-Tris Gel (Invitrogen, USA). Loading volume was adjusted to have either a fixed amount of total protein per well or a fixed volume of sample per well. SDS-PAGE was performed using 1xMES buffer at 125 V until the dye front reached the bottom of the gel (approximately 1.5 h). PageRuler™ Pre-stained Protein (Thermo Scientific, USA) was used as a protein marker according to the manufacturer's recommendation. In this study, a densitometric technique was employed for quantification of BVMO1MES in a cell-free extract. Briefly, the concentration of BVMO1MES (as µg-BVMO1MES per µL of the cell-free extract) was calculated from the densitometry data by using bovine serum albumin (BSA; Amresco, USA) as a standard. If necessary, the cell-free extract was concentrated further using a spin filter (Vivaspin, MWCO 10kDa; Sartorius, Germany) until the retentate contained 1 µM of BVMO1MES per µL.

3.4 Bioconversion using cell-free extract

Benzaldehyde was used to evaluate BVMO1MES activity. Bioconversion was performed in a 4-mL-glass vial with a reaction mixture (1 mL) containing Tris-HCl buffer (pH 8.0), 5 mM of benzaldehyde, 2 µM of enzyme, 2%(v/v) of DMSO (as a co-solvent), 10 µM FAD, 0.2 mM of NADH and 0.2 mM of NADPH. The reaction was performed at 30°C, 200 rpm for 3 h. The reaction mixture was extracted by using 300 µL of ethyl acetate for 5 min and the solvent phase (upper phase) was collected after a centrifugation at 11,000x*g* for 5 min. The solvent phase was analyzed using a GC/MS-MS (Agilent 5975C TAD Series GC/MSD, USA) equipped with DB-WAX column (30 m x 0.25 mm x 0.25 µm, Agilent, USA). The temperature profile was set as follow: held for 3 min at 50°C, increased to 80°C at a rate of 10°C·min^{-1}, increased to 230°C at a rate of 25°C·min^{-1}, increased to 250°C at a rate of 20°C·min^{-1} and then held for 5 min. As a control experiment, an abiotic control containing all components except a cell-free extract was performed in parallel.

4. Results and Discussion

4.1 Cloning of a full-length *bvmo1mes*

The modified *bvmo1mes* gene was successfully amplified from the plasmid pET19b/BVMO1MES. Purified fragment was integrated into a blunt-end cloning vector pJET1.2. Subsequently pJET1.2-*bvmo1mes*-his was introduced into a competent *E. coli* DH5α. *E. coli* DH5α clone no. 2 was confirmed for the presence of *bvmo1mes* fragment by colony PCR using pJET1.2-specific primers as well as a restriction analysis (NdeI/BamHI double digestion). Moreover, the gene sequence of clone no. 2 matched perfectly (100% similarity) with that of an original *bvmo1mes* sequence in the genome.

The full-length *bvmo1mes* fragment in the cloning vector pJET1.2 was retrieved and transferred into an expression vector pET21a(+). The plasmid pET21a(+)-*bvmo1mes*-his was introduced into a competent *E. coli* DH5α for propagation purpose. Restriction analysis (NdeI/BamHI double digestion) revealed that 6 of 18 clones (clone no. 1, 10, 11, 15, 17 and 18) gave the correct fragment size of 1553 bp. The expression vector pET21a(+)-*bvmo1mes*-his was extracted from clone no. 1 and subsequently introduced into the expression hosts of choice (*E. coli* BL21 (DE3), SHuffle® and Origami B). Colony PCR using *bvmo1mes*-specific primers indicated the presence of *bvmo1mes*-his fragment in all 14 transformants (Figure 4).

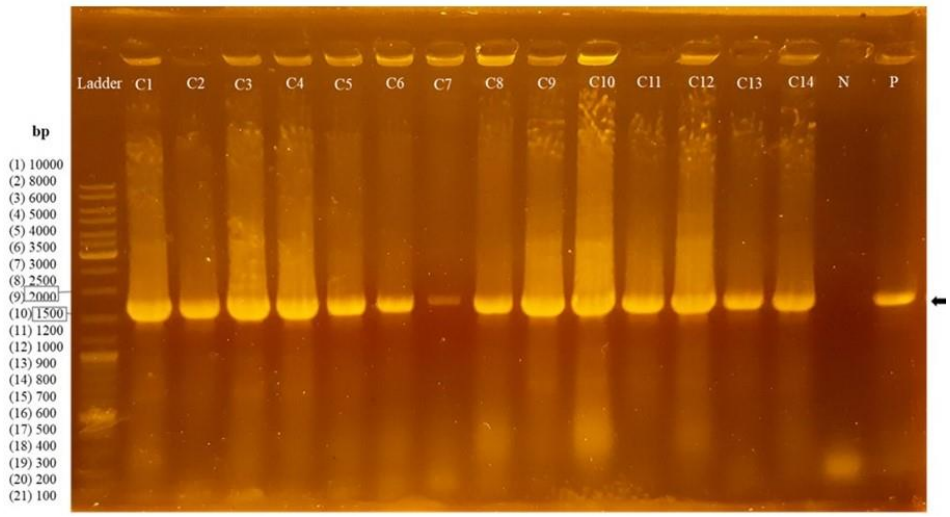


Figure 4 The colony PCR result of 14 *E. coli* BL21 (DE3) transformants after a transformation with an expression plasmid, pET21a(+)-*bvmo1mes*-his. Black arrow indicates the expected size of PCR product (1,533 bp).

4.2 Overexpression of BVMO1MES

All 14 clones of *E. coli* BL21 (DE3) harboring pET21a(+)-*bvmo1mes*-his were cultivated until OD_{600} reached a value of 0.8. They were induced by 0.1 mM IPTG at 20°C for 16 h. The expected protein (57.03 kDa) was observed in all tested transformants, but *E. coli* BL21 (DE3) clone no. 5, 6, 8 and 11 gave the most

abundant target protein (insoluble and soluble protein). Subsequently, these 4 clones were analyzed for their soluble and insoluble protein fraction separately. The most intense band of soluble BVMO1MES was observed in clone no. 11, therefore, *E. coli* BL21 (DE3) clone no. 11 was selected for further use.

As the amount of soluble target protein observed was lower than expected and might not sufficient for the bioconversion experiment, other *E. coli* expression hosts including origami B and SHuffle® were also evaluated. *E. coli* origami B and SHuffle® were genetically modified for improving soluble protein production, especially for disulfide bond-containing protein. Even though there was no disulfide bond in BVMO1MES, both *E. coli* hosts were investigated as they may have a positive effect on production of soluble BVMO1MES. It has been reported that the disulfide bond isomerase (DsbC) in *E. coli* SHuffle® not only enhanced disulfide bond formation, but also exhibited chaperone activity assisting the reactivation of the denatured D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme with no disulfide bond (Chen et al., 1999). Unfortunately, as shown in Figure 5, lower target protein was obtained comparing to that of *E. coli* BL21 (DE3). It is widely accepted that different expression hosts may exhibit different levels of plasmid stability and plasmid copy number. Plasmid stability is influenced by vector and host genotype (Rai and Podh, 2001), while the plasmid copy number is affected by the host's genetic background (Hughes and Welker, 1989). Therefore, different amount of recombinant protein expression can be expected from different hosts.

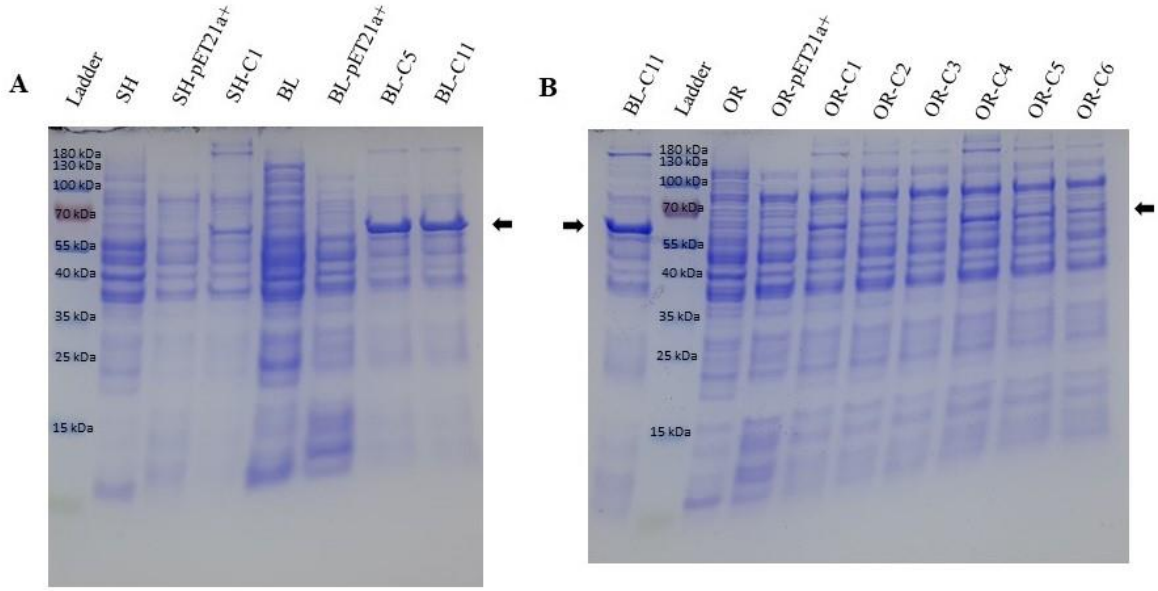


Figure 5 Comparison of a soluble his-tagged BVMO1MES. A) soluble protein expressed from *E. coli* SHuffle® (SH; clone no. 1) and *E. coli* BL21 (DE3) (BL; clone no. 5 and 11) B) soluble protein expressed from *E. coli* origami B (OR; clone no. 1 to 6) and *E. coli* BL21 (DE3) (BL; clone no. 11) (Black arrow indicates the expected band of a soluble his-tagged BVMO1MES, 57.03 kDa. The gel was loaded with 8 µg of total soluble protein per well.)

Several parameters including IPTG concentration, induction start point, induction temperature and induction time were optimized in order to maximize the soluble his-tagged BVMO1MES. IPTG is a very strong inducer that puts a high metabolic burden on the cells. High IPTG concentration was reported to induce massive insoluble protein production, resulting in a low soluble protein (Fruchtl, Sakon and Beitle, 2015). IPTG concentration normally used for an expression of BVMOs using a pET system was in a range of 0.1-1 mM with 0.1 mM as the most frequently used concentration (Kotani et al., 2006; Bisagni, Hatti-Kaul and Mamo, 2014; Bordewick et al., 2018). In this study, the result indicated that an IPTG concentration of 0.1 and 0.25 mM gave a comparable amount of soluble his-tagged BVMO1MES. Thus, an IPTG concentration of 0.1 mM was selected to minimize the cost and reduce the negative effect from IPTG.

In the literature, an induction for an expression of BVMOs usually starts when an OD_{600} was between 0.3 to 0.9, an early and mid-logarithmic growth stage. In this study, the result revealed no significant difference between different induction start points. Nonetheless, as the late-log stage gave the highest cell density (OD_{600}=1.2), an induction at a late-log phase was selected in order to maximize the amount of target protein obtained from each cultivation.

Lowering of an expression temperature has been expected to improve the solubility of recombinant protein due to the decelerated processes in cell including transcription, translation, cell division and aggregation of protein. This hypothesis has been proven in many studies including that of Vera and team in which an active protein expressed at 16°C was higher than those at 30 and 37°C (Vera et al., 2007). For an expression of soluble BVMOs using IPTG, a low induction temperature (15-25°C) was frequently used (Leipold, Wardenga and Bornscheuer, 2011; Bisagni et al., 2014; Bordewick et al., 2018). In this study, an induction at 20°C gave significantly higher amount of target soluble protein than that at 16°C. Therefore, an induction temperature of 20°C was employed for the rest of this study. It should be noted that, comparing to 16°C, an induction at 20°C would be less energy-intensive and more practical for a large-scale cultivation in a bioreactor.

An induction time frequently reported for an expression of BVMOs is in a range of 3 to 24 h depending on the induction temperature used. In this study, an induction time of 12 h consistently gave the highest amount of soluble target protein (Figure 6). When an induction was prolonged more than 12 h, the band intensity of the soluble BVMO1MES was significantly lower over time. This phenomenon was similar to the report by Woo and his team in which the obtained BVMO decreased during a prolonged induction, unlike the co-expressed alcohol dehydrogenase (ADH) that remained constant over time (Woo et al., 2018). Denaturation of the expressed protein was suspected as a cause of this phenomenon. In this study, an induction time of 12 h was selected and employed further.

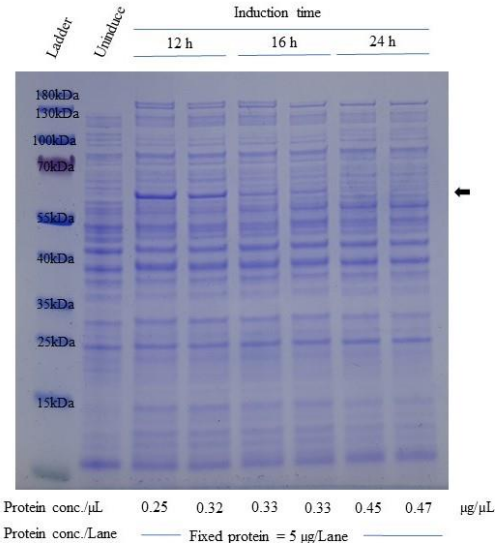


Figure 6 Effect of an induction time (12, 16 and 24 h) on an expression of soluble his-tagged BVMO1MES (Black arrow indicates the expected band of his-tagged BVMO1MES, 57.03 kDa. The gel was loaded with 5 µg of total soluble protein per well.)

4.3 Enzyme activity assay

A reaction was performed using 2 µL of cell-free extract per mL of reaction mixture (corresponding to 2 µM of BVMO1MES per mL of reaction mixture). A conversion of benzaldehyde (Figure 7) was evaluated in this study. Gas chromatography – mass spectrometer/mass spectrometer (GC-MS/MS) was used to identify the product generated from the reaction.

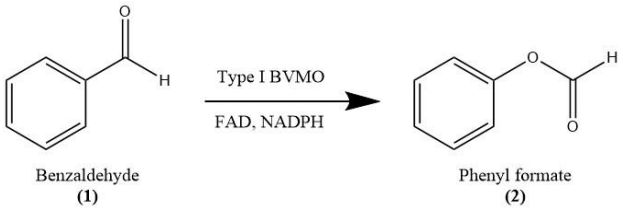


Figure 7 A conversion of benzaldehyde **(1)** into phenyl formate **(2)** by an enzymatic Baeyer-Villiger oxidation

The substrate (benzaldehyde) and a co-solvent used for dissolving the substrate (DMSO) came out at a retention time (RT) of 16.4 and 17.6 min, respectively. Comparing to a chromatogram of an abiotic control, a small putative product peak could be detected at an RT of 4.313 min (Figure 8A). A comparison of a fragmentation pattern (especially the first 3 highest ions) was performed to confirm the formation of product **(2)**. The fragmentation pattern of substrate **(1)** and product **(2)** are shown in Figure 8B and 8C, respectively (NIST Mass Spectrometry Data Center, 2014). The putative product (RT of 4.313 min) exhibited the product-specific ion (*m/z* = 94) as shown in Figure 8D. It should be noted that *m/z* = 94 is not present in the fragmentation pattern of substrate, DMSO, and ethyl acetate (used as an extracting solvent). In conclusion, the expected product was detected only from the reaction with BVMO1MES, confirming that the conversion was enzymatic. Although a reaction performed with a cell-free extract prepared from *E. coli* harboring an empty plasmid was not included in this study, *E. coli* has been a preferred host for recombinant BVMOs expression because it does not contain any native BVMO homologue, and therefore contribute no background enzymatic activity towards ketone substrates (Fürst et al., 2019).

Benzaldehyde-catalyzing ability may allow BVMO1MES to accept benzaldehyde derivatives, for example, diformylcalcium which can be converted into calcium formate (an animal supplement which is rising in demand). In the future, BVMO1MES will be evaluated with a wider range of substrates, including various benzaldehyde derivatives, to reveal its substrate preference and potential applications.

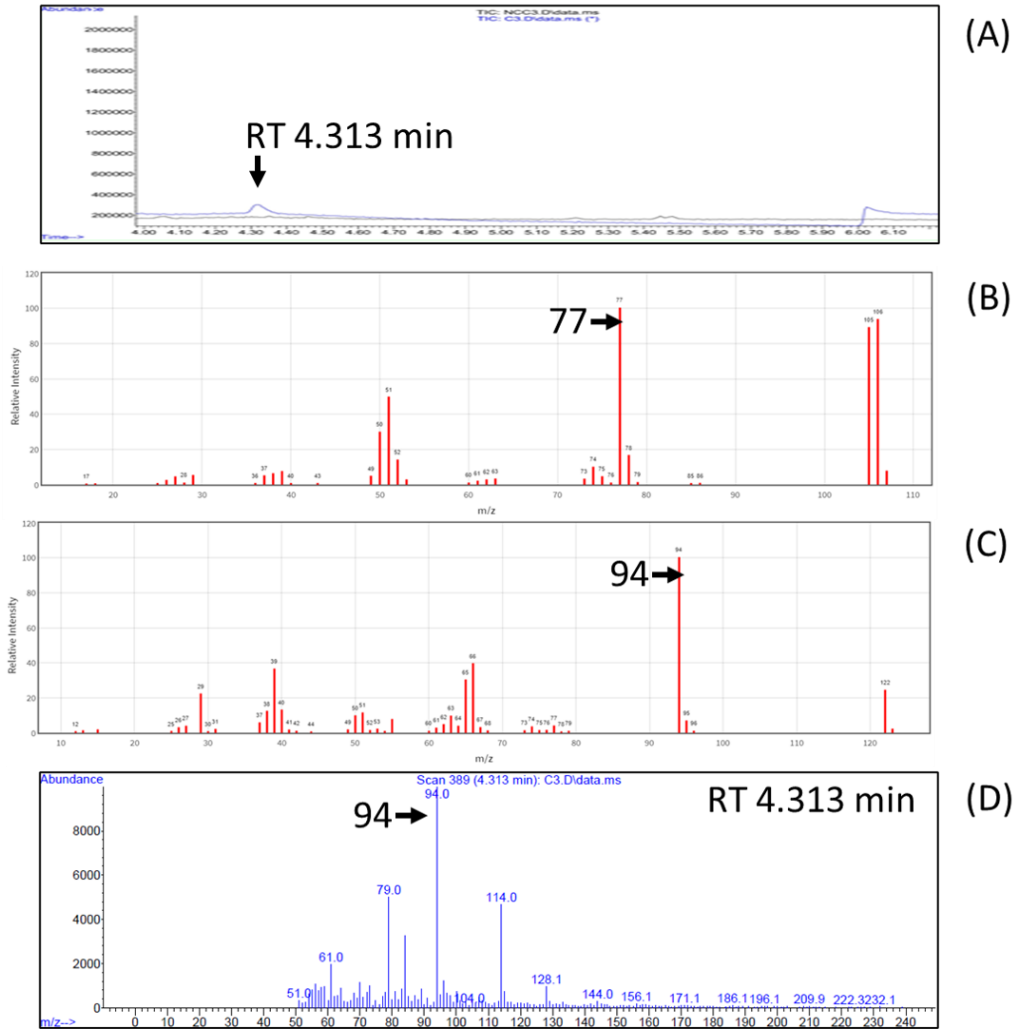


Figure 8 Overlaid chromatogram of an abiotic control (*Black solid line*) and a sample from a reaction using BVMO1MES (*Blue solid line*) (A). Expected product was detected only in the sample. Mass spectrum of benzaldehyde (B; substrate), phenyl formate (C; product), and a putative product obtained from the reaction using BVMO1MES (D; Retention time 4.313 min).

5. Conclusion

In this study, BVMO1MES was successfully cloned and expressed in *E. coli* BL21 (DE3). An induction was performed at a 50-mL scale in a shake-flask with an optimum induction condition as follow; 0.1 mM IPTG (OD_{600} of 1.2) at 20°C for 12 h. The cell-free extract containing the his-tagged BVMO1MES converted the substrate benzaldehyde into the expected product phenyl formate, indicating that the expressed BVMO1MES was fully functional.

6. Acknowledgements

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PhD) (Grant No. PHD/0086/2560). Academic scholarships have been granted to AW by the Faculty of Science, Mahidol University.

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