Scalable Bio-Production of High Value Products in Bacteria

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Utah State University

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SCALABLE BIO-PRODUCTION OF HIGH VALUE PRODUCTS IN BACTERIA

by

Yukie Kawasaki

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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UTAH STATE UNIVERSITY
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2015
ABSTRACT

Scalable Bio-production of High Value Products in Bacteria

by

Yukie Kawasaki, Doctor of Philosophy

Utah State University, 2015

Major Professor: Dr. Jon Y. Takemoto
Department: Biology

Biliverdin IXα is a green bile pigment produced by enzymatic cleavage of a tetrapyrrole ring of heme by heme oxygenase. While biliverdin IXα is emerging as an effective cytoprotectant, the conventional method for producing biliverdin IXα by chemical conversion of animal bile is not suitable for large scale production. A novel scalable production method was pursued via bacterial fermentation. Recombinant *Escherichia coli* strains were obtained by sequence optimization and plasmid transformation of a cyanobacterial heme oxygenase gene. Further strain development was done by plasmid overexpression of a native *E. coli* flavodoxin gene as a possible electron donor for heterogeneous heme oxygenase. The resulting strains were grown in a fed-batch culture system optimized for biliverdin IXα production.

Syringomycin E is a lipodepsinonapeptide produced by certain strains of *Pseudomonas syringae* pv. *syringae* by nonribosomal peptide synthesis. Though syringomycin E had been considered a phytotoxin in the past, recent research results indicate that syringomycin E is a natural fungicide that is not toxic to animals and plants.
Syringomycin E is a potential fungicide especially for use in the organic agriculture sector. New strains of *P. syringae* pv. *syringae* were isolated through ultraviolet mutagenesis and screenings for enhanced capability to produce syringomycin E especially under agitated conditions. Fermentative production was conducted in a newly formulated medium and the product was purified through a large scale chromatography system using organic-compatible solvents. Purified syringomycin E was tested on cucumber seeds to examine its antifungal activity against a soil-borne pathogen *Pythium ultimum*. Syringomycin E was able to inhibit *Pythium* infection and protected seeds and seedlings without developing disease symptoms.

This dissertation research showed scalable production of two natural products, biliverdin IXα and syringomycin E in bacterial platforms. Strain development by gene recombination and mutation was done to obtain bacterial strains capable of overproducing desired metabolites. The resulting strains were grown in fermenters to maximize the yields under agitated conditions. Monitoring growth parameters and medium modifications were critical to achieve large scale production.
Scalable Bio-production of High Value Products in Bacteria

Yukie Kawasaki

Natural products are structurally and chemically diverse bioactive compounds produced by living organisms. The complex structures of natural products are often difficult to duplicate synthetically and thus industrial production of natural products most often depends on use of the host organisms. In this dissertation research, scalable production of natural products was pursued in bacterial platforms.

Biliverdin IXα is a natural anti-inflammatory compound which is emerging as a potent cytoprotectant in the medical field. Syringomycin E is a natural fungicide expected to be an effective agrofungicide in organic farming. Both biliverdin IXα and syringomycin E have limited supplies in the market. Genetic modification and mutation were performed to obtain bacterial strains capable of overproducing biliverdin IXα or syringomycin E. The resulting strains were grown in fermenters under optimized growth conditions for large scale production.

This research was conducted in the Department of Biology and Synthetic Bioproduct Institute at Utah State University. Novel production methods of two different natural products, biliverdin IXα and syringomycin E, were developed using bacteria as manufacturing platforms.
ACKNOWLEDGMENTS

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Jon Y. Takemoto for his guidance and support of my dissertation study and life in Logan. He has been a great mentor in many ways and helped me to grow as a scientist and also as a person. I deeply appreciate everything I learned from him.

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Yukie Kawasaki
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CPR</td>
<td>NADPH-cytochrome P450 reductase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>Fld</td>
<td>flavodoxin</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FNR</td>
<td>ferredoxin-NADP(^+) reductase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HO</td>
<td>heme oxygenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IMM</td>
<td>improved minimal medium</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-(\beta)-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISM</td>
<td>improved SRE medium</td>
</tr>
<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption/ionization time of flight</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitriloacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>potato dextrose broth</td>
</tr>
<tr>
<td>psi</td>
<td>pound-force per square inch</td>
</tr>
<tr>
<td>RL</td>
<td>rhamnolipid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>slpm</td>
<td>standard liter per minute</td>
</tr>
<tr>
<td>SRE</td>
<td>syringomycin E</td>
</tr>
<tr>
<td>SRM</td>
<td>syringomycin minimal medium</td>
</tr>
<tr>
<td>SYRA</td>
<td>1:3 mixture of SRE and rhamnolipid</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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CHAPTER 1
INTRODUCTION

Background

Natural products are compounds or substances derived from living organisms (47). Antibiotics, surfactants, hormones, enzymes, proteins, toxins and other functional molecules are synthesized to promote the survival of the producing organisms. Historically, natural products had been the major source of drugs on the market (24, 48, 63, 91). Though the use of synthetic chemicals has recently emerged, more than half of all commercial drugs are still natural products or compounds based on natural products (14, 34, 79, 124). Because of the complex structures of natural products (e.g., multiple chiral centers and labile connectivity), it is often difficult to establish economical chemical synthesis methods for their large scale production. Industrial production of natural products most often depends on use of the original host organisms (132).

To supply industrial quantities, fermentation methods have been developed for production of natural products (4, 29, 92, 96). The original definition of “fermentation” is “anaerobic energy-yielding metabolism of carbon sources in which organic molecules are involved as the final electron acceptors”. Another definition for this term is “any process in which organisms are grown, especially on a large scale, even if the final electron acceptor is not organic molecules and the growth is not carried out under anaerobic conditions” (86). The latter definition is commonly used in industrial
microbiology where large scale production of various useful natural products is pursued using yeasts and bacteria as biocatalysts.

In nature, microbes usually produce natural products in small amounts, just enough to satisfy their needs. For large scale microbial production of natural products, improvement of the producer strains, so-called strain development, is required to enhance desired metabolisms (1, 28, 90). Gene recombination is one approach to strain development which combines pre-existing genetic traits into new combinations (50, 100). Recent advances in recombinant DNA technologies allow isolation, cloning, synthesis, and insertion of target genes in innate hosts or in surrogate organisms for gene recombination. Mutation is another approach which involves mutagenesis (e.g., chemical and radiation mutation) and selection of strains for desired metabolic activities (99). This is a classic technique and efficient approach for strain development in a short period of time.

As the results of strain development by gene recombination and mutation, microbial strains can be obtained for overproduction of natural products. Next, a scale-up process of the production by the resulting strains from laboratory scale to industrial scale is important (103). While screening of microbial strains generally performed in shake flasks or tubes, industrial production of natural products are usually conducted in large capacity fermenters. Oxygen transfer rates, agitation efficiencies, and hydraulic pressures are highly affected by the size of culture broth (33, 103). Temperature, pH, dissolved oxygen level, agitation, aeration, air flow, and nutrient concentrations need to be monitored to have repeatable high-titer production in fermenters.
In this research, attempts were made to enhance large scale production of two natural products, biliverdin IXα and syringomycin E (SRE), by strain development and fermentative growth of the resulting strains.

Biliverdin IXα

**Heme degradation by heme oxygenase**

Heme proteins are metalloproteins containing heme, iron protoporphyrin IX, as the prosthetic group. Electron transfer (41, 42), gas transfer (6, 18, 58), substrate oxidation (88, 109), storage and transportation of metal irons (3, 20), ligand sensing (71, 123), and other important physiological tasks are conducted by heme proteins under aerobic conditions. Despite their critical roles, senescent heme proteins release pro-oxidant heme which mediates formation of oxygen free radicals and lipid peroxidation (16). In animals, pathological states such as sickled disease, ischemia reperfusions, and malaria induce severe hemolysis and myolysis result in high levels of free heme leading to organ, tissue, and cellular injuries (57, 68).

Heme oxygenase (HO, EC; 1.14.99.3) is primarily responsible for detoxifying cytotoxic free heme. There are two HO isozymes, stress-inducible HO1 concentrated in tissues of spleen and liver, and constitutive HO2 concentrated in neural tissues (30, 31, 128). HO cleaves the tetrapyrrole ring of heme at the α-methene bridge and forms equimolar amounts of biliverdin IXα, Fe^{2+}, and carbon monoxide (Fig. 1-1) (62, 68, 75, 131). Biliverdin IXα is a green bile pigment and subsequently converted into bilirubin IXα, a yellow pigment known to cause hyperbilirubinemia, by biliverdin reductase (EC:
1.3.1.24) of phagocytic cells (7, 94, 98). Bilirubin IXα is highly hydrophobic and binds to albumin for circulation and transportation to the liver, where bilirubin IXα-albumin bond is detached and then bilirubin IXα-glucuronic acid complex is formed for excretion from the body in bile and feces (37, 69). While biliverdin IXα is the final product of heme degradation in lower vertebrates, the conversion of biliverdin IXα into bilirubin IXα is an immediate reaction and biliverdin IXα is hardly detected in mammals.

On the other hand, HO1 has a critical role in the synthesis of light harvesting and light sensing apparatus in cyanobacteria (8, 23), some algae (9, 21), and higher plants (26, 107) since the product biliverdin IXα is the common precursor of bilichromes. In cyanobacteria and red algae, biliverdin IXα is converted into phycobilins by bilin reductases such as PcyA for light harvesting (8, 22). In higher plants, phytochromobilins, chromophores of phytochromes which function in light signaling, are synthesized from biliverdin IXα (119). Though many cyanobacteria possess the biliverdin reductase gene, bilirubin IXα hardly detected in these phototrophic organisms. Biliverdin reductase is considered to have a regulatory role in phycobiliprotein biosynthesis (55, 102).

**Therapeutic effects of biliverdin IXα**

Biliverdin reductase generates 250-300 mg bilirubin IXα per day in normal human adults (11). Bilirubin IXα is highly hydrophobic and >99% of bilirubin IXα is bound to albumin to become soluble in plasma, while unbound bilirubin IXα disrupts cell or mitochondrial membranes to cause cell lysis or mitochondrial dysfunctions (15, 59). Albumin-bound bilirubin IXα is reactive with peroxyl radicals and oxidized back to biliverdin IXα which can be re-reduced to bilirubin IXα by biliverdin reductase on the
external plasma membranes of macrophages and other cells (87). The biliverdin IXα/bilirubin IXα redox cycle is an important antioxidative mechanism which can protect cells from 10,000-fold excess of peroxyl radicals (7).

While biliverdin reductase is an important amplifier in the biliverdin IXα/bilirubin IXα redox cycle, biliverdin IXα-activated biliverdin reductase also undergoes phosphorylation on its cytoplasmic tail. The serine/threonine/tyrosine kinase activity initiates signaling cascades leading to anti-inflammatory and anti-apoptotic responses, including secretion of anti-inflammatory cytokines such as interferon-10 (70, 127, 129). Administration of biliverdin IXα, less toxic but more hydrophilic form than bilirubin IXα, is reported to be effective against ischemia and reperfusion injuries (5, 35, 85), diabetes (54, 117), lung injuries (66, 101), human immunodeficiency virus (84), and hepatitis (134). Biliverdin IXα is emerging as a potent cytoprotectant in the medical field.

Production of biliverdin IXα

Though there are raising interests in biliverdin IXα as a therapeutic reagent, scalable production methods of biliverdin IXα had not been well established. Recent commercially available BV is predominantly prepared by chemical conversion of bilirubin IXα extracted from animal bile (89). Bilirubin IXα is dehydrogenated with FeCl₃, benzoquinone or H₂O₂ under acidic conditions (81), or 2,3-dichloro-5,6-dicyanobenzoquinone in dimethyl sulfoxide (80). These chemical reactions usually generate trace amounts of non-bioactive biliverdin IIIα and XIIIα isomers, dimers, and other contaminants. Purification of biliverdin IXα isomer from the product mixture is difficult and gives low yields (49, 80–82).
In genetic engineering, *Escherichia coli* has been employed as the host organism for foreign gene transformation because of its favorable traits, quick growth, well studied metabolisms, and easier genetic manipulations (116). Although certain pathogenic strains of *E. coli* express HO to acquire iron from infected animal hosts, biliverdin IXα production in wild-type strains has not been reported (114, 115). There are some reports of heterogeneous HO1 expression in *E. coli* while the yields were not specified or low. Ishikawa *et al.* first performed the expression of rat HO1 in *E. coli* (56). This animal enzyme has a 23-amino acid non-polar tail at its C-terminus to bind to cellular membranes and for exchange of electrons with membrane bound NADPH-cytochrome P450 reductase (CPR) (23). Later, Wilks *et al.* reported that soluble rat HO1 truncated at its C-terminus is also catalytically active in *E. coli* (130). The activity of HO1 is strongly dependent on the presence of electron donors (62). Since CPR does not exist in *E. coli*, there must be substitute redox partners for heterogeneous rat HO1. The *Synechocystis* sp. PCC6803 HO1 has been also successfully expressed in *E. coli* (23). Unlike animal HO1, bacterial HO1 is not membrane anchored and does not accept electrons well from CPR (32). Cyanobacterial HO1 was considered as ferredoxin (Fd)/flavodoxin (Fld)-dependent but the specific Fd or Fld that supports the enzymatic activity of HO1 is not yet known (7).

The first scalable production of biliverdin IXα was reported by Chen *et al.* (19). Strain development of *E. coli* strain BL21(DE3) was done by gene recombination. A sequence encoding the *ho1* gene from *Synechocystis* sp. PCC6803 was modified for optimum expression in *E. coli* and transformed to obtain a recombinant *E. coli* strain.
BL21(mHO1). The resulting strain was grown in fermenters with optimization of growth parameters, such as carbon sources, resulting in biliverdin IXα yield up to an average of 23.5 mg·L⁻¹ culture broth.

Effects of different carbon sources on *E. coli* biliverdin IXα production

Carbohydrates are the most common energy sources of bacteria. Different carbohydrates affect different levels of glycolytic intermediates and thus different signal transduction processes (12, 74, 112). For example, glucose-lactose diauxie is a well-known catabolic repression associated with the *lac* operon in *E. coli* (73). Concentrations of glucose and lactose or synthetic analogs of lactose control the expression of genes. Inducible gene expression systems such as the *lac* operon are commonly used in recombinant DNA technologies and many expression vectors were constructed with them (110).

*E. coli* can utilize various carbohydrates, whereas glucose is the most easily accessible compound and often used in industrial fermentation of *E. coli* (40). However, expressions of heterogeneous proteins are often influenced by the glucose concentration of the medium broth. Medium optimization of carbon sources is necessary to maximize the production of target proteins or metabolites. Glucose, sucrose, lactose, mannitol, sorbitol and other sugars can be tested individually or in combinations. In the study of Chen *et al.* (19), recombinant *E. coli* strains BL21(HO1) and BL(mHO1) were obtained using a *lac* operon vector system. Optimization of carbon source was performed as a part of this dissertation study to enhance biliverdin IXα production.
**Redox partner of enzymatic activity of heme oxygenase**

The enzymatic activity of HO1 is strongly dependent on electrons derived from NADPH. In mammals, electron transfer from NADPH to HO1 is mediated by CPR which consists of four structural domains, the flavin mononucleotide (FMN)-binding domain, the connecting domain, the flavin adenine dinucleotide (FAD)-binding domain, and the NADPH-binding domain, connected in sequence from the N- to C- temini (126). The FMN-binding domain is similar to the structure of Fld, whereas the FAD-binding domain and NADPH-binding domain are similar to these of ferredoxin-NADP⁺ reductase (FNR). Electrons are transported from NADPH to CPR (first to the FAD prosthetic group and then to the FMN prosthetic group) and from CPR to HO1 for heme degradation (5, 7).

On the other hand, cyanobacterial HO1 uses Fd as the electron donor (23, 113). Cyanobacterial HO1 does not have an anchor tail on its C-terminus while amino acid residues around the heme binding site are highly conserved (e.g., 57% similarities between human HO1 and *Synechocystis* PCC 6803 HO1). Though catalytic activity of cyanobacterial HO1 was reported with CPR as the electron donor, its narrower heme-/electron donor-binding site prefers plant-type 2Fe-2S Fd (~10 kDa) rather than CPR (~77 kDa) (113). The electron flow is expected to be from NADPH to FNR (FAD prosthetic group), from FNR to Fd, and from Fd to HO1 (23, 67).

Replacement of Fd with Fld is reported under environmental stress (13, 39, 72). Fld is a small soluble flavoprotein (14-23 kDa) which has a non-covalently bound FMN molecule as a prosthetic group instead of an iron-sulfur cluster. Fld is isofunctional to Fd.
and able to mediate NADP⁺ reduction via FNR (72). In general, Fd is used as a major electron carrier while Fld is induced under adverse conditions. For instance, cyanobacteria express Fld under iron-deficient conditions to replace Fd (13, 39). Few organisms like *E. coli* and *Helicobacter pylori* are known to require Fld as a specific electron carrier for their survival (38, 51, 95). In recombinant *E. coli* strain BL21(mHO1) (19), the redox partner of heterogeneous mHO1 is unknown. However, it is predicted that the electron is provided from NADPH to FNR, from FNR to Fd/Fld, and from Fd/Fld to mHO1.

Syringomycin E

**Disease controls in organic agriculture**

The Federal Organic Foods Production Act of 1990 was a milestone for organic farming in the United States. This act established standards for producing and marketing organically produced commodities. In 2014, the sales of organic qualified products reached US $39.1 billion (122). The organic farming industry is a fast-growing sector of the US agriculture with rising concerns on environmental protection and food safety (93). However, with such an accelerated growth, disease control is emerging as a huge problem. Applications of synthetic chemicals are not allowed in organic farming whereas adaption of chemical compounds greatly contributes to the success of conventional agriculture. Demands for organic-compatible pesticides consisting of natural products, naturally occurring microbes, and mined minerals (121) are increasing.
Fungi and fungal-like organisms are the major cause of plant diseases (64). They produce thick-walled spores resistant to desiccation and other extreme environmental conditions. Spores can survive for many years and it is difficult to remove them from soil. Soil-borne pathogens such as *Fusarium*, *Rhizoctonia*, *Pythium*, and *Phytophthora* are known to cause damping-off diseases that can affect various vegetable seeds and seedlings (65). The outbreak of damping-off disease results in significant loss of yield and quality of produces. Seed treatment is a common approach to prevent damping-off disease and most commercially available seeds are treated with synthetic fungicides (17, 53, 60, 77, 97, 106, 120, 125). However, exclusion of synthetic chemicals limits the choice of disease control in organic farming. Most organic seeds in the market remain untreated. Though physical treatments (e.g., temperature and radiation treatments) are available, they possibly cause seed damages (43). There is a demand for organic-compatible fungicides for seed treatments.

**Syringomycin E as a secondary metabolite natural fungicide**

Besides ribosomal peptides, microorganisms synthesize small peptides by using multifunctional enzymes of 100–1700 kDa, the so-called nonribosomal peptide synthetases which utilize the multiple-carrier thiotemplate mechanism (32, 78, 105). Nonribosomal peptides have structural diversities, like branched/cyclic parts and non-proteinogenic amino acids, and they thus have diverse bioactivities. Certain strains of *Pseudomonas syringae* pv. *syringae* produces syringomycin E (SRE) (Fig. 1-2) via nonribosomal peptide synthesis (108). SRE consists of a hydrophobic 2-
hydroxydodecanoate tail and a hydrophilic nine-amino acid peptide ring (Fig. 1-2). The positive charge of the peptide ring gives SRE a high affinity for binding to the negatively charged sphingolipids on fungal plasma membrane (45, 111). The amphiphilic structure of SRE inserts into the target membrane to form voltage-gated ion channels slightly selective for anions (2, 52, 76). The resultant ionic imbalance caused by ion fluxes is lethal to fungi. Antifungal activity of SRE has been reported against phytopathogenic fungi and fungal-like organisms such as Fusarium, Rhizoctonia, and Phythium species (10, 27, 61). As a secondary metabolite natural product with a potent antifungal activity, SRE is a potential fungicide for disease control applications in organic farming (118).

Production of syringomycin E

For applications in agriculture, a scalable production method will be required to supply a large quantity of SRE. It is difficult to meet this goal with the current still batch culture preparation method, incubation of P. syringae pv. syringae B301D in potato dextrose broth supplemented with 0.4% of casamino acids at 28 °C for 10 days without shaking (44, 133). Though genetic modification is a common approach to obtain enhanced producer strains, genetically modified organisms are not allowed in organic food production (25, 46). Also, the SRE gene cluster of P. syringae pv. syringae encompass as approximately 55 kb (104) and handlings of large gene clusters usually entail difficulties (36). In this study, strain development of P. syringae pv. syringae was performed via ultraviolet (UV) radiation mutagenesis regarding the USDA organic standards (83). Strains were selected for their enhanced capability to produce SRE, especially in agitated conditions. Then optimization of fermentation parameters was
conducted to obtain organic-compatible SRE to be tested as a seed protectant fungicide against *Pythium* infection.

**Goals of This Study**

Many microorganisms are capable to produce bioactive natural products. Though these compounds are highly valuable to us, the amounts of product in wild-type strains are usually limited. The main goal of this dissertation research was to perform scalable production of high-value natural products, biliverdin IXα and SRE. Biliverdin IXα is an emerging cytoprotectant in human therapeutics and SRE is a possible fungicide in organic agriculture. Though they have potent bioactivities, large scale production protocols of biliverdin IXα and SRE have not well established. As is common in recent industrial microbiology, scalable production of biliverdin IXα and SRE was pursued by microbial strain development and fermentation optimization for the resulting strains. Bacterial strains capable of overproducing biliverdin IXα and SRE were obtained by gene recombination and mutagenesis, respectively. For quick production of biliverdin IXα with easier gene manipulations, modified cyanobacterial HO1 was plasmid transformed into *E. coli* (Chapter 2). To improve enzymatic activity of the heterogeneous gene, possible electron donors were overexpressed in the transformed cells (Chapter 3). For production of SRE as an organic-compatible agrofungicide, enhanced SRE producer strains of *P. syringae* pv. *syringae* were obtained by UV mutagenesis (Chapter 4). Productions of biliverdin IXα and SRE with the resulting strains were conducted in fermenters with optimization of growth parameters such as carbon sources.
References


FIG. 1-1. Heme degradation by HO and BV reductase. The three reactions shown in the box are conducted by HO while the conversion of biliverdin IXα into bilirubin IXα is conducted by biliverdin reductase.
FIG. 1-2. Structure of SRE. The structure consists of a hydrophobic 3-hydroxydodecanoic acid and hydrophilic nine amino acid peptide ring.
CHAPTER 2

*SCALABLE PRODUCTION OF BILIVERDIN IXα BY \textit{ESCHERICHIA COLI}*

Abstract

**Background.** Biliverdin IXα is produced when heme undergoes reductive ring cleavage at the α-methene bridge catalyzed by heme oxygenase. It is subsequently reduced by biliverdin reductase to bilirubin IXα which is a potent endogenous antioxidant. Biliverdin IXα, through interaction with biliverdin reductase, also initiates signaling pathways leading to anti-inflammatory responses and suppression of cellular pro-inflammatory events. The use of biliverdin IXα as a cytoprotective therapeutic has been suggested, but its clinical development and use is currently limited by insufficient quantity, uncertain purity, and derivation from mammalian materials. To address these limitations, methods to produce, recover and purify biliverdin IXα from bacterial cultures of \textit{Escherichia coli} were investigated and developed.

**Results.** Recombinant \textit{E. coli} strains BL21(HO1) and BL21(mHO1) expressing cyanobacterial heme oxygenase gene ho1 and a sequence modified version (mho1) optimized for \textit{E. coli} expression, respectively, were constructed and shown to produce biliverdin IXα in batch and fed-batch bioreactor cultures. Strain BL21(mHO1) produced roughly twice the amount of biliverdin IXα than did strain BL21(HO1). Lactose either alone or in combination with glycerol supported consistent biliverdin IXα production by

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strain BL21(mHO1) (up to an average of 23.5 mg·L⁻¹ culture) in fed-batch mode and production by strain BL21(HO1) in batch-mode was scalable to 100 L bioreactor culture volumes. Synthesis of the modified hol gene protein product was determined, and identity of the enzyme reaction product as biliverdin IXα was confirmed by spectroscopic and chromatographic analyses and its ability to serve as a substrate for human biliverdin reductase A.

**Conclusions.** Methods for the scalable production, recovery, and purification of biliverdin IXα by *E. coli* were developed based on expression of a cyanobacterial hol gene. The purity of the produced biliverdin IXα and its ability to serve as substrate for human biliverdin reductase A suggest its potential as a clinically useful therapeutic.

**Introduction**

Biliverdin is a linear tetapyrrole produced by ring cleavage of heme catalyzed by the enzyme heme oxygenase (HO) (E.C.C.1.14.99.3) [1]. In animals, heme cleavage by HO occurs selectively at the α-methene bridge to generate the most physiologically relevant biliverdin IXα isomer. Hence, the term “biliverdin” typically refers specifically to biliverdin IXα, and this usage is applied throughout in this paper. Biliverdin is best known as a degradative intermediate associated with erythrocyte and hemoglobin turnover. It is subsequently reduced via NADPH biliverdin reductase (E.C.C. 1.3.1.24) to bilirubin IXα that in turn is consecutively bound to serum albumin and glucuronic acid for excretion in bile. The overall process serves to eliminate heme, which is toxic when accumulated.
Bilirubin IXα is also known to associate with cell membranes where it quenches the propagation of reactive oxygen species (ROS) [2, 3] conferring protection to membrane lipids and proteins against oxidative damage. Thus, an additional function of biliverdin is to serve as the immediate source of bilirubin IXα that in turn acts as a cytoprotective antioxidant. It is not clear if biliverdin is oxidatively regenerated after bilirubin IXα reacts with ROS [4–7]. Though bilirubin IXα is an effective ROS quencher, biliverdin administered at tissue injury/inflammatory sites appears as effective a cytoprotectant as bilirubin IXα [8–13]. Biliverdin’s effectiveness has been attributed to its hydrophilicity and efficient conversion to bilirubin IXα [1]. In addition, biliverdin interaction with biliverdin reductase signals the downstream production of anti-inflammatory cytokine interferon-10 [14] and the nitrosylation-dependent inhibition of pro-inflammatory TLR4 expression [15]. Thus, biliverdin, acting together with biliverdin reductase, is increasingly recognized as a potential anti-inflammatory therapeutic agent [3, 16–18]. Examples of its cytoprotective effects in animal models include those for ischemia/reperfusion following liver [19] and small bowel [10] transplants, vascular injury [20], endotoxic shock [21], vascular intimal hyperplasia [9], and nephropathy [8]. In addition, biliverdin has been reported to inhibit *in vitro* replication of hepatitis C [22] and other viruses [23, 24] and to reverse parameters of type 2 diabetes in mice [25]. The growing list of potential clinical applications for biliverdin suggests a future need for high-quality preparations in ample quantity.

Biliverdin is also produced by microbes and plants [26–30]. In cyanobacteria, red algae, and plants, it serves primarily (and perhaps solely) as precursor to photosensitive
linear tetrapyrroles such as phycocyanobilin and phycoerythrobilin [31]. These in turn serve as chromophores for cyanobacterial and red algal light-harvesting phycobiliprotein complexes and the light-sensing receptor phytochrome [27, 32]. In these organisms, biliverdin IXα is the predominant isomer produced via HO enzymes with sequence homologies to mammalian HO1 [28, 33, 34].

To meet the projected pharmaceutical demand for biliverdin, high yield and low cost methods that provide the IXα isomer in high purity and preferably from non-mammalian sources are needed. Currently, commercial biliverdin is predominantly derived by chemical oxidation of bilirubin [35]. The source bilirubin (that occurs in conjugated form) is extracted from mammalian bile under acidic conditions that generate isomers (e.g. IIIα and XIIIα isoforms) and consequently lead to biliverdin preparations of unsuitable purity (e.g. as low as 38% biliverdin IXα [36]). Reported non-mammalian synthesis of biliverdin include *Escherichia coli* cultures expressing HO1 from rat [37, 38] and cyanobacteria [39] and yeast cultures supplemented with hemoglobin [40]. In these reports, the amounts of biliverdin produced are not documented or appear low. Biliverdin extracted from salmon bile is reported [41], but the potential for scalable production is not discussed.

Here, we report the use of *E. coli* to synthesize biliverdin and describe procedures for the scalable production of the IXα isomer. This was achieved by sequence optimization of the cyanobacterial *hol* gene for enhanced expression in *E. coli* and development of growth culture parameters that promote biliverdin production.
Materials and Methods

*E. coli* strains and vectors

One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies, Carlsbad, CA, USA) was used to construct the recombinant plasmids. BL21 Star™ (DE3) Chemically Competent *E. coli* (Life Technologies, Carlsbad, CA) was used for transformation and protein expression. Expression vector constructions were done with pET101/D-TOPO® (Life Technologies, Carlsbad, CA) and pJexpress 401 (DNA2.0, Menlo Park, CA).

Construction of expression vectors

**pET101-HO.** The heme oxygenase gene (*hol*) of *Synechocystis* PCC6803 was amplified by PCR of Biobrick gene part BBaI15008 (Registry of Standard Parts, The BioBricks Foundation, http://biobricks.org/ website) using forward primer 5’-CACC ATGAGTGTCAACTTAGCTTC-3’ and reverse primer 5’-CTAGCCTTCGGAGGTA GGCGA-3’ and cloned into pET101/D-TOPO® to generate plasmid vector pET101-HO1 (Figure 2-1.A) with expression under T7 lac promoter control according to instructions provided by Life Technologies (Carlsbad, CA) (TOPO® Cloning Reaction Method). The *hol* gene sequence was verified by DNA sequencing. The vector pET101-HO1 was transformed into BL21 Star™ (DE3) Chemically Competent *E. coli* to give *E. coli* strain BL21(HO1).

**pJexpress401-mHO1.** The *hol* gene sequence was codon optimized for expression in *E. coli* using DNA2.0 Algorithms (DNA2.0, Inc., Menlo Park, CA) (Figure
The coding sequence for hexahistidine was incorporated at the 5’ end to provide a 6× His tag at the N-terminus of the synthesized protein. The E. coli codon optimized gene (mho1) was synthesized and inserted into plasmid vector pJexpress401 by DNA2.0 Inc. (Menlo Park, CA). The resulting vector, pJexpress401-mHO1 (Figure 2-1.B), was transformed into BL21 Star™ (DE3) Chemically Competent E. coli to give E. coli strain BL21(mHO1).

**Testing carbon sources for biliverdin production**

Several carbon sources at different concentrations and in combination were examined for capabilities to support growth and biliverdin synthesis by E. coli strains BL21(HO1) and BL21(mHO1). Cultures were grown in 125 mL capacity Erlenmeyer flasks on a New Brunswick G76 rotary incubator shaker (30°C, 200 rpm) in 50 mL Luria-Bertani (LB) medium [42] with various single carbon sources that included sucrose (1% wt·vol⁻¹), mannitol (0.1, 1, 2, 5, 10 and 20% wt·vol⁻¹), sorbitol (1, 5, 10 and 20% wt·vol⁻¹), lactose (1, 2.5, 5 and 10% wt·vol⁻¹), succinate (2% wt·vol⁻¹), malate (2%) or combinations of carbon sources that included mannitol (1% wt·vol⁻¹) + glucose (1% wt·vol⁻¹), sucrose (1% wt·vol⁻¹) + glucose (1% wt·vol⁻¹), mannitol (1% wt·vol⁻¹) + sorbitol (2.5% wt·vol⁻¹), or mannitol (5% wt·vol⁻¹) + sorbitol (5% wt·vol⁻¹). Ampicillin or kanamycin (100 μg·mL⁻¹) was used for selection, and isopropyl-β-thiogalactopyranoside (IPTG) (0.5 mM) was added (at cell density with absorbance (1 cm) (A₆₀₀) of ~0.5) as inducer except when lactose was the carbon source. Growth was monitored at A₆₀₀ and the culture color was recorded when stationary phase growth was achieved (24 to 48 h). Biliverdin levels were estimated by absorbance spectroscopy.
using a mM extinction coefficient of 25 at 650 nm (1 cm light path length) using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

**Biliverdin production using bioreactor batch cultures**

For bioreactor inocula, *E. coli* strains BL21(HO1) and BL21(mHO1) were grown in 50 mL of LB medium plus 100 μg·mL⁻¹ ampicillin or kanamycin, respectively, in 250 mL capacity Erlenmeyer flasks with rotary shaking (225 rpm) at 37°C to an A₆₀₀ of 2 to 6 with LB medium as a blank control. Inoculum cultures (80 mL) were added to 2 L of modified New Brunswick Scientific (NBS) medium [43] with 2% (wt·vol⁻¹) lactose in place of glucose or modified ZY medium [44] composed of per L: 2 g lactose, 2.2 g glucose, 16 g glycerol, 15 g N-Z-Amine™ A or 10 g Hy Express System II (Sheffield™ Bio-Science, Norwich, NY), 10 g yeast extract (FisherScientific, Waltham, MA), 1 mL 2M MgSO₄, 50 mL of 20× NP solution (66 g (NH₄)₂SO₄, 136 g KH₂PO₄, 142 g Na₂HPO₄ in 1 L twice-distilled H₂O), and 1 mL 1000× trace elements solution (50 mL of 1% HCl, 0.675 g FeCl₃, 0.15 g CaCl₂, 0.1 g MnCl₂, 0.015 g ZnSO₄, 0.023 g CoCl₂, 0.015 g CuCl₂, 0.023 g NiCl₂, 0.025 g Na₂MoO₄, 0.007 g H₃BO₃ in distilled water to a final volume of 250 mL). Separately, 20× NP solution and 2 M MgSO₄ were autoclaved and 1000× trace elements solution was filter-sterilized, and the solutions were added to complete the preparation of modified ZY growth medium. Batch culture growth was conducted in a New Brunswick Scientific BioFlo® 310 Controller bioreactor using BioCommand software with a 5 L capacity vessel (New Brunswick Scientific Co., Endfield, CT). A
dissolved O$_2$ level of 40% was cascade controlled and monitored by gassing with O$_2$ (0-50%) and air (0.75-4 slpm). No antifoam was used. For fed-batch experiments, a 200 mL solution of 10% (wt·vol$^{-1}$) glycerol, 2% (wt·vol$^{-1}$) lactose, and with or without 5% (wt·vol$^{-1}$) peptone was continuously fed (8 mL·h$^{-1}$·L$^{-1}$) during exponential growth beginning 4 h after inoculation. Cell culture absorbance ($A_{600}$) was approximately 10 at 4 h and 29 at 11 h after culture inoculation. Growth was terminated approximately 25 h after inoculation. Green material (containing biliverdin) accumulated in foam above the culture liquid surface and on the inner surfaces of the bioreactor vessel and in the foam over-flow material that was siphoned into a flask outside the vessel. The pigmented material was collected using methanol or distilled water as necessary and the pH of the final suspension was lowered to 4.3 or 4.5, respectively, to promote biliverdin precipitation. The recovered material was centrifuged at 7477×g for 6 min, and the sedimtented blue-green material was suspended in methanol. Non-sedimenting biliverdin in aqueous fractions was recovered by readjusting the pH to 4.3 followed by re- centrifugation and suspension of the green pellet in methanol. The pooled methanolic solutions were placed on a rotating shaker (225 rpm) at room temperature for 15 min. The solution was centrifuged at 4500×g for 4 min to remove particles from solution. Fresh methanol was added to the pellet, and the extraction repeated. The extraction is further repeated with distilled methanol until the $A_{650}$ of a 1:10 dilution of the supernatant fluid is less than 0.5. The amounts of biliverdin recovered were quantitated by HPLC with comparisons to known amounts of authentic biliverdin IX$\alpha$ (Frontier Scientific, Inc., Logan, UT).
Larger (100 L) batch cultures of *E. coli* strain BL21(HO1) were grown at 37°C with NBS medium containing 2% (wt·vol⁻¹) lactose in a B. Braun UE-100D bioreactor (B. Braun Melsungen AG, Germany). Fed-batch mode was not used. *E. coli* strain BL21(HO1) inoculum cultures (4 L) were grown overnight at 37°C in LB medium in BioFlo® 310 bioreactors. Inoculum cultures (4 L) were added to 100 L growth medium and growth was terminated 24 h following inoculation. Biliverdin was collected, extracted and purified as described above for the 2 L bioreactor batch cultures.

**HO identification and activity**

**HO cell extraction.** Aliquots (48 to 400 mL) of bioreactor batch culture of *E. coli* strain BL21(mHO1) were collected at 2, 5, 10, 15, and 2 h after inoculation, centrifuged (4500×g, 5 min), and the supernatant liquid discarded. The sedimented cell pellets were stored at −20°C. The cells were extracted, and proteins were recovered from Ni-NTA columns using the QIAexpress® Ni-NTA fast Start Kit (QIAGEN, Valencia, CA) according to procedures described in the kit manual.

**SDS-PAGE.** Twenty μL of each protein solution from Ni-NTA column purification were added to 20 μL of SDS-PAGE sample buffer (Bio-Rad, Hercules, CA), heated for 10 min with boiling water, and centrifuged briefly. Supernatant liquid aliquots (30 μL) were loaded into wells of Bio-Rad Criterion Precast Gels and electrophoresed in a Bio-Rad Criterion precast Gel System. The gel was stained using Bio-safe™ Coomassie G-250 (Bio-Rad, Hercules, CA). Precision Plus Protein Prestained Standards (Bio-Rad Laboratories, Hercules, CA) was used for estimation of protein molecular size.
Identification. Ni-NTA column purified protein samples were reduced and alkylated with iodoacetamide. The resulting peptides were concentrated on a ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument (performed by Alphalyse, Inc., Palo Alto, CA). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination. MALDI TOF/TOF analyses were performed on 8 separate peptides for partial peptide sequencing. The MS and MS/MS spectra were combined and analyzed using Mascot software and NCBI protein databases.

HO activity. Harvested *E. coli* strain BL21(mHO1) and BL21 Star™ (DE3) cells were washed and suspended in assay buffer (50 mM Tris-HCl, pH 7.7, 10% wt·vol⁻¹ glycerol) and 1 mM EDTA, and disrupted three times using a French press cell operated at 18,000 psi. The lysate was centrifuged at 15,000×g, and the supernatant fraction was used for HO activity assays similar to published procedures [28, 45]. The enzyme reaction mixture (500 μL) contained assay buffer, 40 μM methemalbumin, 2.5 mM Tiron, 20 μg·mL⁻¹ ferredoxin, 0.02 units of ferredoxin reductase (Sigma-Aldrich, St. Louis, MO), and cell lysate (0.128 mg protein). The reaction was initiated with the addition of 0.2 mg of NADPH and the mixture was incubated at 37°C for 20 min in the dark. The mixture was then extracted and esterified [46] and biliverdin dimethyl ester was quantitated by HPLC using a Beckman C18 Ultrasphere column (4.6 mm×15 cm), elution with methanol, and absorbance measurement at 380 nm.
Biliverdin purification

Ammonium acetate (0.1 M, 1.5 L) was mixed with biliverdin in buffered methanol (60% 0.1 M ammonium acetate/40% methanol, vol·vol⁻¹, 1 L) and the mixture was loaded onto a glass column (4.0 mm×300 mm) packed with C18 silica beads (125Å pore, 55-105 μM diameter, Waters, Manchester, UK). The column was preconditioned by sequential elution with 200 mL of methanol and 200 mL of buffered methanol. After loading the sample, the column was washed with 100 mL buffered methanol solution. Biliverdin was eluted with 30% 0.1 M ammonium acetate/70% MeOH (vol·vol⁻¹) solution and collected as material in a green band. To 25 mL of eluted biliverdin material was gradually added 400 mL of 1 mM HCl with stirring. The solution was kept at −20°C for 1 h and then centrifuged for 15 min at 11325×g at 4°C. The supernatant fluid was removed, the biliverdin pellet was washed and suspended in 20 mL H₂O in a 50-mL capacity plastic centrifuge tube and centrifuged for 15 min at 4500×g at 4°C. The supernatant fluid was discarded, the biliverdin pellet was frozen at −80°C and then freeze-dried using a FreeZone Plus Freeze Dry System (Labconco, Kansas City, MO).

Biliverdin characterization

Absorbance spectra. Absorbance spectra (300 and 800 nm) were obtained using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

HPLC analysis. Biliverdin samples (20 μL were subjected to HPLC using a 4.6 mm×75 mm Symmetry® C18 column (Waters Co., Milford, MA) and a gradient of solvent A: 99.9% H₂O, 0.1% trifluoroacetic acid and solvent B: 99.9% methanol and
0.1% trifluoroacetic acid. The elution gradient program was: 100% solvent A, 1 min; 0-60% solvent B, 1 min; 60-100% solvent B, 8 min; 0-100% solvent A, 1 min; 100% solvent A, 4 min with a flow rate of 1 mL·min\(^{-1}\) using a Waters Alliance HPLC (Waters Co., Milford, MA).

**Proton NMR analysis.** NMR data was collected on a JEOL Eclipse 400MHz NMR (JEOL, Peabody, MA). Biliverdin samples were dissolved in DMSO-d\(_6\) (Cambridge Isotope Labs, Andover, MA).

**LC-MS analysis.** Biliverdin samples were analyzed on a NanoACQUITY UPLC (Waters Co., Milford, MA) and a Q-Tof Primer tandem mass spectrometer (Waters Co., Milford, MA). Samples (3 μL) were introduced into a Symmetry® C18 trapping column (180 μm×20 mm) with NanoACQUITY Sample Manager (Waters Co., Milford, MA) washed with 99% solvent A and 1% solvent B for 3 min at 15 μL·min\(^{-1}\). Solvent A was 99.9% H\(_2\)O, 0.1% formic acid and solvent B was 99.9% acetonitrile and 0.1% formic acid. Chemicals were eluted from the trapping column over a BEH300 C4 column with a 40 min gradient (1-4% solvent B, 0.1 min; 4-98% solvent B, 19.9 min; 98-85% solvent B, 2 min; 85% solvent B, and 10 min, 85-1% solvent B, 8 min) at a flow rate of 0.8 μL·min\(^{-1}\). MS scan time was 1.0 sec.

**NADPH biliverdin reductase activity**

The enzymatic conversion of biliverdin to bilirubin was measured using the Biliverdin Reductase Assay Kit (Sigma-Aldrich, St. Louis, MO). One mg of biliverdin produced by strain *E. coli* strain BL21(mHO1) was dissolved in 2 mL methanol, and 0.2 mL was mixed with 1 mL of the kit assay buffer. The kit-supplied recombinant human
biliverdin reductase A enzyme was suspended in 800 μL water, and 160 μL of the enzyme suspension was added to 480 μL of assay buffer. Biliverdin-containing kit assay buffer (50 μL), biliverdin reductase solution (200 μL), and NADPH solution (0.24 mg·mL⁻¹ NADPH in assay buffer, 750 μL) were combined and the absorbance spectrum between 300-800 nm was measured at 0, 15, 30, 45, 60, 90, 145, 240 and 360 min using a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

Results and Discussion

Effect of carbon source on biliverdin production

Several potential carbon sources, alone and in combination, were examined for their abilities to support biliverdin production by E. coli strains BL21(HO1) and BL21(mHO1) growing in LB medium (Table 2-1). Lactose at 2 and 2.5% (wt·vol⁻¹) alone or in combination with D-glucose yielded green cultures containing 2 to 4 mg·L⁻¹ biliverdin without IPTG addition. E. coli strain BL21(HO1) cultures grown with D-mannitol (alone or in combination with glucose or sorbitol) also yielded green cultures whereas other carbon compounds and combinations (Table 2-1) yielded brown or yellow-green and pale green cultures containing <0.2 and <2 mg·L⁻¹ biliverdin, respectively. Similarly, E. coli strain BL21(mHO1) produced enhanced amounts of biliverdin with lactose alone or in combination with D-glucose. These results show that certain carbohydrates and particularly lactose (2 to 2.5% wt·vol⁻¹), alone or in combination with D-glucose, and D-mannitol (2 to 5% wt·vol⁻¹) support higher levels of biliverdin.
production by *E. coli* strains BL21(HO1) and BL21(mHO1) growing in LB medium as compared to other carbon sources. With lactose, the addition of IPTG was not required for enhanced biliverdin production offering a practical and economic advantage for large-scale, commercial production of biliverdin.

**Bioreactor batch culture production of biliverdin**

Based on observations that lactose enhanced biliverdin production, modified ZY medium containing 2% wt·vol⁻¹ lactose was used to grow *E. coli* strains BL21(HO1) and BL21(mHO1) in 2 L volumes in a New Brunswick Scientific BioFlo® 310 Controller bioreactor. Consistent biliverdin production was achieved with 40% dissolved O₂, agitation between 280 and 500 rpm, and continuous feeding of lactose (2% wt·vol⁻¹) and glycerol (10% wt·vol⁻¹) in fed-batch mode initiated 4 h after culture inoculation (exponential growth phase). Biliverdin was visible as and collected in green material that accumulated in the foam above the culture liquid surface (Figure 2-3). Biliverdin was identified and quantitated by HPLC analyses of the collected material. *E. coli* strains BL21(HO1) and *E. coli* BL21(mHO1) produced between 2.5 and 5 mg (n=3, average 3.3 mg) and between 5.3 to 7.5 mg (n=9, average 6.4 mg) of biliverdin, respectively, per L of culture. Therefore, *E. coli* strain BL21(mHO1) produced nearly twice the amount of biliverdin than *E.coli* strain BL21(HO1) in the bioreactor cultures growing in modified ZY medium in fed-batch mode with lactose and glycerol. In contrast, the two strains produced approximately the same amounts of biliverdin when grown in LB medium with lactose (2.5% wt·vol⁻¹) in small shaker flasks (Table 2-1). When peptone was included in the fed-batch medium (together with lactose and glycerol), *E. coli* strain BL21(mHO1)
bioreactor cultures produced between 18.4 to 25.3 mg·L⁻¹ (n=11, average of 23.8 mg·L⁻¹) of biliverdin. *E. coli* strain BL21(HO1) produced between 3 and 3.9 mg·L⁻¹ (n=9, average of 3.3 mg·L⁻¹) in modified NBS medium in batch mode.

Biliverdin production was also achieved in 100 L bioreactor (Braun UE-100D) batch mode cultures of *E. coli* strain BL21(HO1) grown in NBS medium containing 2% (wt·vol⁻¹) lactose. Biliverdin yields ranging between 200 to 311 mg (n=5, average 212) were achieved. This rate of production was similar to that achieved by *E. coli* strain BL21(HO1) in the 2 L bioreactor (BioFlo® 310) batch mode cultures (average rate: 3.3 mg·L⁻¹) indicating that biliverdin production by *E. coli* strain BL21(HO1) is scalable to larger volumes and quantities.

**HO expression and activity**

When grown in 2 L bioreactor cultures, *E. coli* strain BL21(mHO1) cells contained Ni-NTA recoverable proteins with molecular size ~29 kDa and detectable initially between 2 to 5 h after inoculation and then until growth was terminated (25 h) (Figure 2-4). The proteins were equivalent in size to ho1 of *Synechocystis* PCC6803 with a 6× His tag (i.e. 28.7 kDa) and the gel excised protein showed sequence similarity to the cyanobacterial ho1 (31% sequence coverage, Mascot protein score =146). Extracts of *E. coli* strain BL21(mHO1) cells harvested at 25 h of bioreactor growth had HO activities of 80 pmol·hr⁻¹·mg protein⁻¹ whereas extracts from *E. coli* strain BL21 Star™ (DE3) cells showed no or barely detectable activities (<5 pmol·hr⁻¹·mg protein⁻¹). These results confirmed that *E. coli* strain BL21(mHO1) synthesized an HO enzyme when grown
under conditions that allowed accumulation of green pigment determined to be biliverdin (see below).

**Identification of biliverdin IXα**

The identity of the biliverdin extracted from *E. coli* strain BL21(mHO1) cultures as biliverdin IXα was indicated by comparisons to authentic biliverdin IXα using absorbance spectroscopy, HPLC, proton NMR spectroscopy (Figure 2-5) and mass spectroscopy (mass 582.2). The degree of purity was >98% based on HPLC profiles (biliverdin IXα retention time of 6.6 min, Figure 2-5.B).

**E. coli produced biliverdin as substrate for biliverdin reductase A**

Purified biliverdin produced by *E. coli* BL21(mHO1) was reduced to bilirubin IXα by recombinant human biliverdin reductase A and NADPH (Figure 2-6). Since human biliverdin reductase A specifically uses biliverdin IXα as substrate [1, 35] this result confirms the identity of the *E. coli* BL21(mHO1) produced biliverdin as the IXα isomer. It also suggests that the produced biliverdin has therapeutic potential because of its substrate interaction with the human enzyme and its facile conversion to bilirubin IXα.

**Conclusions**

Methods for the scalable production of biliverdin by *E. coli* cultures were developed. Production is enhanced with the use of an altered version of a cyanobacterial *hol1* gene that is sequence-optimized for *E. coli* expression. The produced biliverdin is
solely the physiologically relevant IXα isomer and is easily obtained at a high degree of purity (>98%). Its purity and ability to serve as substrate for human NADPH biliverdin reductase A suggest its potential as a clinically useful therapeutic for inflammatory diseases and conditions. When commercially produced for therapeutic applications, the biliverdin IXα preparations will undoubtedly require screening for and elimination of endotoxin contaminants that are a consequence and limitation of industrial scale production by E. coli cultures.

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Table 2-1  Biliverdin production by *E. coli* strain BL21(HO1) and BL21(mHO1) with growth in LB medium supplemented with various sources

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Carbon Source</th>
<th>Conc. %</th>
<th>IPTG(^a)</th>
<th>Pigment(^b)</th>
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</thead>
<tbody>
<tr>
<td>BL21(HO1)</td>
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\(^a\)Isopropyl-β-thiogalactoside (IPTG), 0.5 mM added (+), or not added (−).

\(^b\) Biliverdin concentrations were 2 to 4 mg·L\(^{-1}\) (green), <2 to >0.2 mg·L\(^{-1}\) (pale green) and <0.2 mg·L\(^{-1}\) (yellow green and brown).
Figure 2-1  Gene maps of expression vectors pET101 HO1 (A) and pJexpress401-mHO1 (B). *ho1* gene expression in (A) is controlled by T7 *lac* promoter which consists of a strong bacteriophage T7 promoter and a downstream 25 bp *lac* operator in pET101. For mho1 expression (B), an “IP-free” T5 promoter sequence was used with the lac operator placed downstream of the T5 promoter in the vector pJexpress 401.
Figure 2-2  Gene sequence of ho1 of cyanobacterium *Synechocystis* PCC6803 (red), *E. coli* expression optimized *mho1* gene sequence (blue) and the corresponding *ho1* protein sequence (black).
Figure 2-3 Biliverdin production from *E. coli* strain BL21(mHO1) growing in modified ZY medium in a New Brunswick BioFlo® 310 bioreactor. Biliverdin is subsequently extracted from the green-colored material that accumulates above the culture surface.
Figure 2-4 SDS-PAGE of eluted solutions from Ni-NTA columns of cell extracts derived from bioreactor cultures of *E. coli* strain BL21(mHO1) harvested at various times during growth on ZY medium. Gel lanes are: mixture of protein molecular size standards (a), and cell extracts from cultures harvested at 2 h (b), 5 h (c), 10 h (d), 15 h (e) and 25 h (f) after culture inoculation. Expression of MW ~29 kDa *ho1* is evident (right arrow) and not visible when derived from cells grown without lactose or with glucose (+IPTG) (not shown). Gel positions of 37 kDa and 25 kDa protein markers are indicated by arrows (left side).
Figure 2-5  Spectral and chromatographic analyses of biliverdin produced by bioreactor cultures of *E. coli* BL21(mHO1).  (A) Absorbance spectra of biliverdin produced by *E. coli* BL21(mHO1) (red) and commercial biliverdin IXα derived from an animal source (blue).  (B) HPLC chromatograms of biliverdin produced by *E. coli* BL21(mHO1) (top) and commercial biliverdin IXα derived from an animal source (bottom).  (C) One dimensional proton NMR (400 Mhz) spectrum of *E. coli* BL21(mHO1)-produced biliverdin in DMSO-d6. 1H NMR (400 MHz, DMSO) signal assignments are: 12.32 (s; 2H); 12.1 (s; 1H); 12.01 (s; 1H); 10.78 (s; 1H); 10.67 (s; 1H); 7.58 (s; 1H); 6.91 (t, J=15.6 Hz; 1H); 6.69 (t, J=15.2 Hz; 1H); 6.32 (d, J=12.2 Hz; 3H); 5.82 (d, J=10.8 Hz; 1H); 5.56 (d, J=11.2 Hz; 1H); 5.78 (s; 1H); 3.05 (m; 4H); 3.43 (m; 4H); 2.18 (s; 3H); 2.27 (s; 3H); 2.15 (s; 3H); 2.01 (s; 3H) and similar to those reported for biliverdin IXα derived from animal sources [36].
Figure 2-6  Absorbance spectra at various times during bilirubin formation from *E. coli* BL21(mHO1) produced biliverdin catalyzed by human recombinant biliverdin reductase. NADPH-dependent reduction was monitored spectrophotometrically for 6 h. The arrows indicate the direction of absorbance changes over time during the reaction.
CHAPTER 3
ENHANCED BILVERDIN IXα PRODUCTION BY RECOMBINANT
ESCHERICHIA COLI WITH OVEREXPRESSION OF ELECTRON
DONORS FOR TRANSFORMED HEME OXYGENASE

Introduction

Heme oxygenase (HO, EC:1.14.99.3) is an oxygen-dependent enzyme which cleaves the tetrapyrole ring of heme at the α-methene bridge and forms equimolar amounts of linear tetrapyrole biliverdin IXα, Fe²⁺, and carbon monoxide. There are two HO isozymes, stress-inducible HO1 concentrated in tissues (e.g., spleen and liver) and constitutive HO2 concentrated in neural tissues (10, 12, 63). Though HO is widely distributed in animals, insects, higher plants, cyanobacteria, algae, fungi, and some pathogenic bacteria, the product biliverdin IXα is found as a final product only in lower vertebrates. In cyanobacteria, biliverdin IXα is produced as a substrate in bilin chromophore synthesis for light-harvesting (3, 8). In mammals, HO1 is important in degradation of pro-oxidant heme from a series of hemeproteins, including hemoglobin from aged red blood cells (42, 57). Hemoglobin heme is mainly degraded by HO1, and the product biliverdin IXα is subsequently converted into bilirubin IXα by biliverdin reductase (EC: 1.3.1.24) of phagocytic cells in the spleen and liver (41). Bilirubin IXα is highly hydrophobic and binds to albumin for circulation and transportation to the liver, where the bilirubin IXα-albumin bond is broken and a glucuronic acid conjugate of biliverdin IXα is formed for excretion from the body in bile and feces (15, 28). Though
bilirubin IXα was once considered as a toxic waste product (18, 19, 45, 48), its property as a potent anti-oxidant at lower concentrations has been recognized to be of physiological importance (2, 31, 39, 49, 56, 58, 62). The bilirubin IXα-albumin complex can be oxidized by peroxyl radicals, which cause lipid peroxidation, to a biliverdin IXα-albumin complex in plasma and the extravascular space (33, 47, 49). One mol of bilirubin IXα-albumin can scavenge two moles of peroxyl radicals. Cell protection from 10,000-fold excess of peroxyl radicals has been reported with the biliverdin reductase amplified biliverdin IXα/bilirubin IXα redox cycle in vitro (2). Also, biliverdin reductase mediates serine/threonine/tyrosine kinase activity when it is bound with biliverdin IXα, and then initiates cell signaling pathways leading to anti-inflammatory responses and suppression of cellular pro-inflammatory events (29, 61). Administration of biliverdin IXα, a less toxic but more hydrophilic form than bilirubin IXα, has been reported to be effective against ischemia and reperfusion injuries (1, 14, 38), diabetes (22, 55), lung injuries (26, 44), HIV (37), and hepatitis (65).

The enzymatic activity of HO1 is the rate-limiting step in the degradation of senescent hemeproteins into biliverdin IXα which consumes seven electrons and strongly depends on the presence of electron donors (25, 30, 64). Both mammalian and cyanobacterial HO1 use NADPH as the electron source, though they have different mediator molecules for electron transportation. Mammalian HO1 has a hydrophobic tail on its C-terminus and is anchored to the microsomal membranes (46, 52). This localization allows electron transfer through NADPH-cytochrome P450 reductase (CPR). CPR consists of four structural domains, the flavin mononucleotide (FMN)-binding
domain, the connecting domain, the flavin adenine dinucleotide (FAD)-binding domain, and the NADPH-binding domain, connected in sequence from the N- to C- termini (60). The FMN-binding domain is similar to the structure of Fld, whereas the FAD-binding domain and NADPH-binding domain are similar to those of ferredoxin-NADP⁺ reductase (FNR). Electrons from NADPH flow first to the FAD prosthetic group of CPR and then to FMN prosthetic group of CPR and finally to HO1 for heme degradation (5, 7). A similar directional electron flow is also observed in cyanobacteria. Cyanobacterial HO1 does not have an anchor tail on its C-terminus while amino acid residues around the heme binding site are highly conserved (e.g., 57% similarities between human HO1 and Synechocystis PCC 6803 HO1) (51). Though catalytic activity of cyanobacterial HO1 was reported with CPR as the electron donor, its narrower heme-/electron donor-binding site prefers plant-type 2Fe-2S ferredoxin (Fd, ~10 kDa) rather than CPR (~77 kDa) (51). In cyanobacteria, electrons flow from NADPH to FNR which contains FAD as the prosthetic group, FNR to Fd, and then Fd to HO1 (9, 27).

Flavodoxin (Fld) is a small soluble flavoprotein (14-23 kDa) which has a non-covalently bound FMN molecule as a prosthetic group instead of an iron-sulfur cluster (13). It is isofunctional to ferredoxin and able to mediate NADP⁺ reduction via FNR (32). While Fd is distributed throughout all kingdoms from prokaryotes to animals, Fld is only found in prokaryotes and some eukaryotic algae (66). In general, Fd is used as a major electron carrier while Fld is induced under adverse conditions. For instance, cyanobacteria produce Fld under iron-deficient conditions to replace Fd in spite of their different structures, sizes, and redox cofactors (5, 17). Few organisms like Escherichia
coli and *Helicobacter pylori* are known to require Fld as a specific electron carrier for their survival (16, 21, 40).

In our previous study, cyanobacterial HO1 was expressed in *E. coli* BL21(DE3) and the new *E. coli* construct was grown in fermenters for scalable production of biliverdin IXα (6). Although some pathogenic strains of *E. coli* (e.g., O157:H7) have HO for iron acquisition, biliverdin IXα production in wild-type strains has not been reported (53, 54). Lack of native redox partners for the heterologous HO1 may limit biliverdin IXα production in the current recombinant of *E. coli* strain. The aim of this study was to obtain a strain with improved biliverdin IXα production by overexpression of the proteins, Fld or FNR.

Materials and Methods

**E. coli strain**

*E. coli* BL21(mHO1-GFP) was used as the host for overexpression of electron donors for enzymatic activity of transformed HO1 (6). Briefly, the *ho1* gene from *Synechocystis* sp. PCC6803 was sequence-optimized for expression (*mho1*) in *E. coli* and incorporated with green fluorescent protein at the 5’-terminus. The *mho1*-gfp gene was synthesized and inserted into plasmid vector pJexpress401 (DNA2.0 Inc., Menlo Park, CA). The resulting vector pJexpress401-*mho1*-gfp was transformed into One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (Life Technologies, Carlsbad, CA) to give *E. coli* strain BL21(mHO1). The resulting strain was grown in LB amended with
100 µg·mL"^{-1} kanamycin as the selectable marker. Calcium competent *E. coli* BL21(mHO1-GFP) was prepared and stored at −70°C (43).

**Plasmids for overexpressions of electron donors**

The plasmid pET11a-*fpr* (23, 24) (Fig. 3-1. A), pET11 vector with the *fpr* gene encoding FNR from *E. coli* strain JM109, was obtained as a gift from Dr. M. Waterman (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN) and the plasmid pDH122M (4) (Fig. 3-1. B), pTRC99A vector with the *fldA* gene encoding Fld, was kindly provided by Dr. M. Koutmos (Uniformed Service University of the Health Science, Bethesda, MD). Both plasmids have ampicillin as the selectable marker. Strain BL21(mHO1-GFP+FNR) and BL21(mHO1-GFP+Fld) were obtained by transformations of pET101-*fpr* and pDH122M into chemically competent cells of *E. coli* BL21(mHO1-GFP), respectively.

**Biliverdin IXα production in shake flasks**

For production of biliverdin IXα in baffled flasks with shaking, *E. coli* strain BL21(mHO1-GFP), BL21(mHO1-GFP+FNR), and BL21(mHO1-GFP+Fld) inocula were grown in test tubes containing 5 mL of LB broth amended with 100 µg·mL"^{-1} of kanamycin or kanamycin and ampicillin with rotary shaking (200 rpm) at 30°C overnight. Inoculum cultures were added into 100 mL New Brunswick Scientific (NBS) medium (59) supplemented with 2% lactose and 100 µg·mL"^{-1} corresponding selectable markers (kanamycin or kanamycin and ampicillin) in 250 mL capacity baffled flasks for biliverdin IXα production. Flasks were incubated at 37°C with rotary shaking (200 rpm) for 2 days.
until the green product appeared as rings above cultures on the inner wall of flasks.
Absorbance at 600 nm was measured at the end of growth. The production in flasks was
repeated three times for each strain.

**Biliverdin IXα production in a fed-batch fermenter system**

Fermentative production of biliverdin IXα was performed with strain
BL21(mHO1-GFP) and BL(mHO1-GFP+Fld) to show their scalability in fermenters.
Inocula were prepared in 250 mL capacity baffled flasks containing 50 mL of Yeast-Hy
ES II medium, 5 g·L⁻¹ Hy-Yeast® 444 (Sigma-Aldrich, St. Louis, MO) and 15 g·L⁻¹ Hy-
Express™ System II (Sheffield™ Bio-Science, Norwich, NY) mixed with 16 g·L⁻¹
glycerol, 25 g·L⁻¹ glucose, 1.5 mL·L⁻¹ trace element solution I (0.25 g CuSO₄·5H₂O, 2.4
g MnSO₄·H₂O, 0.30 g Na₂MoO₄·2H₂O, 2.5 g Ni(NO₃)₂, 1.5 g ZnSO₄, and 0.3 mL 6 N
H₂SO₄ in distilled water to make a final volume of 100 mL), 1.5 mL·L⁻¹ trace element
solution II (0.5 g NaCl, 0.475 g FeCl₃·6H₂O, 0.075 g CoCl₂·6H₂O, 0.05 g H₃BO₃, 0.29 g
CaCl₂·2H₂O, 0.3 mL 6 N H₂SO₄ in distilled water to make a final volume of 100 mL) 50
mL·L⁻¹ 20× NP stock solution (66 g (NH₄)₂SO₄, 136 g KH₂PO₄ and 142 g Na₂HPO₄ in
distilled water to make a final volume of 1 L), 0.5 g·mL⁻¹ MgSO₄, 0.4 mg·L⁻¹ thiamine,
and 100 µg·mL⁻¹ of kanamycin or kanamycin and ampicillin after autoclaving. The
inoculum cultures were grown at 30°C with rotary shaking (200 rpm) for 12 h. Biliverdin
IXα production was conducted in a New Brunswick BioFlo® 310 Benchtop Fermentor &
Bioreactor System (New Brunswick Scientific Co., Endfield, CT) with BioCommand®
Software (Eppendorf, Inc., Hauppauge, NY) and a 5-L capacity vessel filled with 3.5 L
modified ZY medium (6, 50), 2 g·L⁻¹ lactose, 2.2 g·L⁻¹ glucose, 16 g·L⁻¹ glycerol, 10 g·L⁻¹ Hy-Express™ System II, 5 g·L⁻¹ Hy-Yeast® 444, and 2 mM MgSO₄ mixed with 50 mL 20× NP stock solution and 1 mL·L⁻¹ of trace element solution III (0.2% HCl, 0.27 g FeCl₃, 0.08 g CaCl₂·2H₂O, 0.063 g MnCl₂·4H₂O, 0.011 g ZnSO₄·7H₂O, 0.01 g NiCl₂, 0.012 g Na₂MoO₄·2H₂O, and 0.003 g H₃BO₃ in 100 mL distilled water) after autoclaving. A dissolved oxygen level was maintained at 40.0% by program-controlled agitation (300-600 rpm) and aeration (1.0-3.0 slpm, 0-100% O₂ in air), and temperature was kept at 37°C. Feeding of 100 g·L⁻¹ glycerol, 50 g·L⁻¹ Hy-Express™ System II, and 20 g·L⁻¹ lactose was initiated 4 h after inoculation at 25 mL·h⁻¹. Green pigment accumulated in the foam above the bioreactor culture was collected by siphoning. The fermentation run was repeated three times for each strain.

**HPLC quantification of biliverdin IXα**

Product biliverdin IXα in growth medium was extracted with 0.1 M ammonium acetate in 20% methanol. After removal of cell debris by centrifugation at 10,000×g for 10 min, 1 mL of the supernatant was loaded on a 1mL capacity C18 Resprep® SPE Cartridge (Restek, Bellefonte, PA) pre-equilibrated by sequential elution with 1 mL methanol and 1 mL 0.1 M ammonium acetate. The cartridge was washed with 1 mL 0.1 M ammonium acetate, and biliverdin IXα was eluted with 1mL 30% 0.1 M ammonium acetate/70% methanol (vol·vol⁻¹) solution. The prepared sample was loaded to a Beckman System Gold RP-HPLC instrument (Beckman Coulter, Inc., Brea, CA) on a 150×4.6 mm Beckman Ultrasphere C18 column (Beckman Coulter, Inc., Brea, CA). The elution gradient of solvent A: 0.1% (v·v⁻¹) TFA in distilled water and solvent B: 0.1%
(ν·ν⁻¹) TFA in methanol was 0% solvent B, 1 min; 0-60% solvent B, 1 min; 60-100% solvent B, 8 min; 100% solvent B, 3 min with a flow rate of 1.0 mL·min⁻¹. The elution was monitored at 385 nm and fractions were collected separately every 1 min. The biliverdin IXα peak was identified and quantified based on comparisons with known amounts of commercial biliverdin IXα (Frontier Scientific, Inc., Logan, UT).

Protein extraction

Cultures of E. coli strain BL21(mHO1-GFP) and BL21(mHO1-GFP+Fld) were grown in 250 mL capacity baffled flasks containing 50 mL of NBS medium supplemented with 2% lactose and 100 µg·mL⁻¹ kanamycin or kanamycin and ampicillin. After 24 h incubation with shaking at 30°C, cells were harvested by centrifugation at 4,000×g for 20 min. Soluble proteins were extracted by acetone-precipitation and buffer-solubilization (11). Briefly, cells collected from 20 mL of 24 h old culture were sonicated in 4 mL homogenization buffer (50 mM Tris-acetate pH 8.0, 10 µM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM β-mercaptoethanol) on ice for 30 s. Homogenized samples were combined with 36 mL acetone and agitated for 1 h at 0°C. Insoluble materials in 90% (ν·ν⁻¹) acetone were collected by centrifugation at 15,000×g for 15 min and dried under a stream of air. Proteins were re-suspended in 2 mL sample buffer (50 mM Tris-acetate pH 8.0, 10 µM phenylmethylsulfonyl fluoride, and 1 mM EDTA) and agitated at 200 rpm and 0°C for 24 h. Then cell debris was removed by centrifugation at 10,000×g and 4°C for 20 min. The concentrations of crude soluble proteins were estimated by measurements of absorbance at 280 nm (A₂₈₀).
**SDS-PAGE**

Soluble protein extracts of *E. coli* strain BL21(mHO1-GFP) and BL21(mHO1-GFP+Fld) were subjected to SDS-PAGE (15% acrylamide gel). Wells were loaded with 2.5 or 12.5 µL of soluble protein extracts mixed with 2.5 or 12.5 µL 2× SDS-PAGE loading buffer and 3 µL BLUEstain™ Protein ladder (Gold Biotechnology, Inc., St. Louis, Mo). The gel was electrophoresed with Tris-glycine buffer using an ISCO Model 470 Power Supply (ISCO Inc., Lincoln, NE).

**Results**

**Biliverdin IXα production in shake flasks**

Biliverdin IXα product accumulated on the inner walls of baffled flasks was harvested with methanol at 48 h. Based on HPLC quantification of the methanol wash, the yields were 2.00±0.03 mg·L⁻¹, 1.20±0.15 mg·L⁻¹, 2.45±0.13 mg·L⁻¹ by strains BL21(mHO1-GFP), BL21(mHO1-GFP+FNR), and BL21(mHO1-GFP+Fld), respectively (Table 3-1. A). The strain BL21(mHO1-GFP+FNR) had 40.1±7.6% lower yield while strain BL21(mHO1-GFP+Fld) had 17.8±5.5% higher yield comparing to strain BL21(mHO1-GFP).

**Biliverdin IXα production in a fed-batch fermenter system**

Production of biliverdin IXα by *E. coli* strain BL21(mHO1-GFP) and BL21(mHO1-GFP+Fld) was conducted in a BioFlo® 310 Benchtop Fermentor & Bioreactor System. Both cultures of both strains had a common pattern of pH changes
during growth (Fig. 3-3). When *E. coli* was grown without feeding, the pH changed in a similar way at 0-7.5 h but kept decreasing after 7.5 h (data not shown). Feeding was started once the pH started to re-increase at 4 h, and initiation of green pigment production was observed at 7.5 h when the pH hit the bottom value of ~5.2. At 20 h, the estimated biliverdin IXα yields based on HPLC were 9.09±2.49 mg·L⁻¹, and 13.17±1.71 mg·L⁻¹ by BL21(mHO1-GFP) and BL21(mHO1-GFP+Fld), respectively (Table 3-1. A).

**Protein extraction and SDS-PAGE**

Soluble proteins were extracted from 24 h old cultures by acetone-precipitation and solubilized in buffer. The concentrations were estimated as 401.3±25.2 µg·mL⁻¹ for strain BL21(mHO1-GFP) and 419.3±7.0 µg·mL⁻¹ for strain BL21(mHO1-GFP+Fld) based on measurements of A₂₈₀ (n=3). Separation of soluble proteins was performed by SDS-PAGE in Tris-glycine buffer. The extract of strain BL21(mHO1-GFP+Fld) had a visible band at 20 kDa, while the extract of strain BL21(mHO1-GFP) had a diffused tail of 21 kDa band at this size (Fig. 3-2). The protein extract from *E. coli* strain BL21(mHO1-GFP+Fld) had overexpression of a ~20 kDa protein which is predicted as Fld (36).

**Discussion**

Plasmids pET11a-fpr and pDH122M were transformed into *E. coli* BL21(mHO1-GFP) for the purpose of providing potential electron donors, FNR and Fld, for enzymatic activity of heterogeneous mHO1. In baffled flasks, a 17.8±5.5% higher biliverdin IXα yield was achieved with overexpression of Fld with pDH122M while overexpression of
FNR with pET11a-fpr lowered biliverdin IXα yield by 40.1±7.6%. Also, the cell density measurements ($A_{600}$) of *E. coli* strain BL21(mHO1-GFP+Fld) was 13.0±5.9% higher than the measurements of strain BL21(mHO1-GFP) (Table 3-1. B). The biliverdin IXα production by BL21(mHO1-GFP+Fld) was scalable in a New Brunswick BioFlo® 310 Benchtop Fermentor & Bioreactor System. The fed-batch fermentation of strain BL21(mHO1-GFP+Fld) achieved 40.8±9.1% higher yield of biliverdin IXα and 26.1±2.0% higher $A_{600}$ comparing to strain BL21(mHO1-GFP).

The *fldA* gene belongs to the *soxRS* (superoxide response) oxidative stress regulon of *E. coli* and lack of the *fldA* gene can be lethal under both aerobic and anaerobic conditions (16). Overexpression of cyanobacterial Fld in *E. coli* was reported to confer resistances against oxidative stress inducers such as paraquat and atrazine (7). In this study, *E. coli* was grown to achieve high cell densities, and higher $A_{600}$ was achieved in the culture of *E. coli* strain BL21(mHO1-GFP+Fld) than the culture of strain BL21(mHO1-GFP). Though the aim to overexpress *fldA* was to increase electron transfer to mHO1, Fld possibly functioned as a cell protectant under highly stressed condition or by preventing protein denaturation of mHO1. Further study will be required to clarify the role of Fld flavoprotein in biliverdin IXα production.

The new *E. coli* construct described in this work revealed a 17-40% increase in biliverdin IXα production with overexpression of the *fldA* gene that encodes Fld. The biliverdin IXα production was shown to be scalable in a fermenter. Current commercially available biliverdin IXα is prepared by chemical oxidation of bilirubin IXα extracted from animal bile (34), a process that generates significant levels of
contaminants like biliverdin IIIα and XIIIα isomers (35). This chemical conversion requires further chromatographic procedures to obtain the biologically active biliverdin IXα isomer in pure form (20). On the other hand, enzymatic cleavage of heme by HO1 is a site-specific reaction and transformed \( E. coli \) solely produces the IXα isomer as reported in our previous study (6). Fermentative production of biliverdin IXα accumulates the product in the foam above the culture and greatly facilitates its purification. Biliverdin IXα biosynthesis by \( E. coli \) offers the potential for scalable production and high degrees of purity of this compound for medical applications.

References


TABLE 3-1. Biliverdin IXα yields and final $A_{600}$ in baffled flasks and fermenters

A. Biliverdin IXα yields estimated by HPLC quantifications

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<td>1.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BL21(mHO1-GFP+Fld)</td>
<td>2.56</td>
<td>2.60</td>
<td>2.20</td>
<td>12.62</td>
<td>13.71</td>
<td>18.20</td>
</tr>
</tbody>
</table>

B. Cell density measurements as $A_{600}$

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th>final $A_{600}$</th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>flask (48 h)</td>
<td>1st</td>
<td>2nd</td>
<td>3rd</td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>BL21(mHO1-GFP)</td>
<td>4.56</td>
<td>5.28</td>
<td>4.70</td>
<td>45.4</td>
<td>47.1</td>
<td>46.9</td>
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<tr>
<td>BL21(mHO1-GFP+FNR)</td>
<td>4.48</td>
<td>4.88</td>
<td>5.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BL21(mHO1-GFP+Fld)</td>
<td>5.68</td>
<td>5.54</td>
<td>5.15</td>
<td>56.1</td>
<td>58.7</td>
<td>61.0</td>
</tr>
</tbody>
</table>
FIG. 3-1. Gene maps of expression vector pET11a-fpr (A) and pDH122M (B)
FIG. 3-2. SDS-PAGE of soluble protein extracts. Gel lanes are protein standards (A), *E. coli* BL21(mHO1-GFP) extract (B=2.5 μL and C=12.5 μL), and BL21(mHO1-GFP+Fld) extract (D=2.5 μL and E=12.5 μL). The white arrows indicate ~21 kDa band and the black arrow indicates ~20 kDa band. The ~20 kDa band was observed with BL21(mHO1-GFP+Fld) extract.
FIG. 3-3. Growth curves ($A_{600}$) and pH changes in a bioreactor. The $A_{600}$ and pH measurements are shown as solid and dotted lines, respectively. A is from the growth of *E. coli* strain BL21(mHO1-GFP) (3rd experiment) and B is from the growth of strain BL21(mHO1-GFP+Fld) (3rd experiment).
CHAPTER 4

*PRODUCTION AND APPLICATION OF SYRINGOMYCIN E AS AN ORGANIC FUNGICIDE SEED PROTECTANT AGAINST PYTHIUM DAMPING-OFF

Abstract

Syringomycin E (SRE) is a cyclic lipodepsinonapeptide with potent antifungal activity produced by certain strains of *Pseudomonas syringae* pv. *syringae*. In this study, its potential as an organic-compatible agrofungicide and seed treatment against the soil-borne pathogen *Pythium ultimum* var. *ultimum* was examined. A variant of *P. syringae* pv. *syringae* strain B301D with enhanced SRE-producing capabilities was isolated and grown in a bioreactor with SRE yields averaging 50 mg·L⁻¹ in 40 h. SRE was extracted and purified through a large-scale chromatography system using organic-compatible processes and reagents. The minimum concentrations of the purified product required to inhibit 50 and 90% of *P. ultimum* oospore germination were determined as 31.3 and 250 µg·mL⁻¹, respectively. Drench treatment of cucumber seeds in *P. ultimum* infested potting medium (500 oospores·g⁻¹) with 50 µg·mL⁻¹ SRE resulted 90.2±4.5% sprouting while control water drench had 65.7±4.6% sprouting. Seed coating with 0.03% (w·w⁻¹) SRE allowed 65.7±4.6% seedlings to emerge on naturally ingested soil while 100.0±0.0% of non-coated seeds were depleted by *Pythium* infection. Organic-compatible SRE has potential as a novel organic fungicide seed protectant.

* Yukie Kawasaki, Claudia Nischwitz, Michelle Grilley, Justin Jones, Jason Brown, and Jon Y. Takemoto
Introduction

Strains of *Pseudomonas syringae* pv. *syringae* are either epiphytes or opportunistic pathogens on healthy or diseased plants. They produce two classes of non-ribosomal cyclic lipodepsipeptides commonly known as syringomycins and syringopeptins (Hutchison and Gross 1997; Little, Bostock, and Kirkpatrick 1998). Syringomycins are relatively small molecules (MW 1100-1300 Da) with potent antifungal activities, while syringopeptins are larger (MW 2200-2500 Da) and have both antimicrobial and phytotoxic activities (Fukuchi et al. 1992; Grgurina et al. 2005; Iacobellis et al. 1992; Raaijmakers et al. 2006; Takemoto 1992). Syringomycin E (SRE) is a predominant syringomycin analog and consists of a 3-hydroxydodecanoic acid and a nine amino acid peptide ring (Segre et al. 1989). This amphipathic structure interacts with the plasma membranes of fungi and forms lipidic ion channels (Feigin et al. 1996; Hama et al. 2000; Kaulin et al. 1998, 2005; Malev et al. 2002, 2008). SRE channels are voltage-dependent and slightly selective for anions. The resultant fluxes of H\(^+\), K\(^+\), and Ca\(^{2+}\) ions result in ionic imbalances that are lethal to the target cells (Hutchison et al. 1995; Schagina et al. 2003; Takemoto et al. 1989). Antifungal activities of SRE have been reported against many fungal species, including soil-borne pathogens such as *Pythium, Fusarium,* and *Rhizoctonia* (De Lucca et al. 1999).

Soil-borne fungal and oomycete phytopathogens are major causes of crop loss in the USA and are difficult to control (Bonanomi et al. 2007). *P. ultimum* is a widespread oomycete which mainly survives as oospores in temperate regions (Hendrix and Campbell 1973). Its oospores are resistant to desiccation and other extreme
environmental conditions, and they survive in soil for several years in the absence of suitable hosts (Martin and Loper 1999). The germinated oospores quickly grow and colonize host plants by producing hyphae. Oospore germination triggers damping-off disease of sprouting seeds (Al-Sheikh and Abdelzaher 2010; Hancock 1981). Natural plant resistance against damping-off disease is weak, and as a consequence *P. ultimum* infection has been a common problem in many fields and greenhouses (Benhamou and Bélanger 1998; Georgakopoulos et al. 2002). Soil drench and seed treatment with chemical fungicides are common approaches to prevent infections by *Pythium* and other soil-borne pathogens (Koch 1999; Taylor and Harman 1990). Phenylamides (e.g., metalaxyl, mfenoxan) and captan have been used to control *Pythium* infections (Casas et al. 1990; Hwang et al. 2001; Raaijmakers et al. 2009). However, for organic farming in the USA, these conventional synthetic fungicides are not allowed and losses of crops due to oomycetes and fungi are serious problems for this agricultural sector.

As a secondary metabolite natural product SRE is a potential natural fungicide for disease control applications (Takemoto et al. 2010). Its inhibitory activities against oosporic phytopathogens such as *Pythium* sp. make it particularly attractive to explore as a seed protectant against diseases caused by these pathogens. However, current SRE production and purification methods are not suitable for large scale production and organic farming applications. In the past, *P. syringae* pv. *syringae* B301D was principally used for the production of SRE in potato dextrose broth (PDB) medium (Gross and DeVay 1977a; Zhang and Takemoto 1987), syringomycin minimal (SRM) broth (Gross 1985; Mo and Gross 1991) or improved minimal medium (IMM) broth
(Surico et al. 1989), and provided SRE levels in milligram quantities after several days of growth, typically without agitation. The extraction and purification methods typically involved reagents (e.g. butanol, acetone, and methanol) that are not allowed by the USDA for products used for organic applications (USDA 2015). The goals of this study were to: 1) produce scalable amounts of SRE by methods that allow qualification for organic use certification (e.g. USDA), and 2) determine the capabilities of the produced SRE to serve as an effective seed protectant agrofungicide against *Pythium* infections.

**Materials and Methods**

**Isolation of enhanced SRE-producing strain *P. syringae pv. syringae* strain G10**

Logarithmically growing cultures of *P. syringae pv. syringae* strain B301D were spread onto potato dextrose agar plates at a concentration of $5 \times 10^3$ cells per plate. Plates were inverted without lids onto an ultraviolet light source (Transilluminator, UVP, Upland, CA), irradiated at 302 nm for 10 s, and then placed in the dark at 28°C for 2 days. Surviving colonies (survival rate was approximately 10%) were inoculated in 0.1 mL PDB supplemented with 0.4% (w·v$^{-1}$) casamino acids (PDB$\_CA$) in 96-well microtiter plates, 28°C. After growth, 0.05 mL of each culture was removed, extracted with an equal volume acetone acidified with 0.4% (v·v$^{-1}$) HCl, dried, and then suspended in 0.05 mL sterile water. Extracts were tested for antifungal activity by placing 0.02 mL of the suspended material onto a lawn of *Rhodotorula pilimanae* (Zhang and Takemoto 1987). The isolates with extracts showing the largest zones of growth inhibition were subjected to a second round of UV mutagenesis and selection, yielding *P. syringae pv. syringae*.
strain 2B6. Strain 2B6 underwent a third round of mutagenesis, as above, except ISM medium (see below) was used in place of PDB<sub>CA</sub> in the 96 well microtiter plate. Isolates showing greatest antifungal activity at this stage were subcultured by the addition of 0.02 mL of culture to 3 mL ISM in a test tube. Antifungal activity was tested again after 48 h incubation at 28°C on a rotary shaker and SRE levels quantified by HPLC. The strain which produced the greatest amount of SRE under these conditions was named <i>P. syringae</i> pv. <i>syringae</i> strain G10.

**Medium modification**

Modifications of growth medium, SRM (1% glucose, 0.4% histidine, 0.8 mM MgSO₄, 1 mM FeCl₃, and 0.8 mM potassium phosphate, pH7) (Gross 1985; Mo and Gross 1991) were performed to improve SRE production by <i>P. syringae</i> pv. <i>syringae</i> strains. The antifungal activities of <i>P. syringae</i> pv. <i>syringae</i> strain 2B6 culture extracts (estimated by visual inspection of <i>R. pilimanae</i> growth inhibition zones on potato dextrose agar) were examined as a function of different carbon compounds substituted for 1% (w·v⁻¹) glucose in SRM. Tested were glycerol, ribose, galactose, mannitol, lactic acid, acetate, or succinate, each at 1% (w·v⁻¹), glucose plus mannitol, each at 1 or 2% (w·v⁻¹), glucose, 1% (w·v⁻¹), plus fructose, 0.1% (w·v⁻¹), and arbutin, 100 µM, and glucose, 1% (w·v⁻¹), plus fructose, 0.1% (w·v⁻¹), and salicylate, 100 µM. The inclusion of 1% (w·v⁻¹) glucose plus 1% (w·v⁻¹) mannitol gave the highest antifungal activity, and this mannitol-supplemented SRM was named “improved SRE medium” or “ISM.” SRE production by <i>P. syringae</i> pv. <i>syringae</i> strain G10 was examined with growth in SRM, ISM, SRM plus 100 µM arbutin and 0.1% (w·v⁻¹) fructose, ISM plus 100 µM arbutin and
0.1% (w·v⁻¹) fructose, and SRM plus 100 µM salicylate and 0.1% (w·v⁻¹) fructose in agitated flask cultures.

**Scalable SRE production**

*P. syringae pv. syringae* strain G10 was initially grown in 500 mL of ISM with rotary shaking (200 rpm) at 28°C for 15 h. The 500 mL culture was used as inoculum for a 10 L ISM culture in a 10 L-working volume Winpact Bioreactor and Fermenter System (Major Science, Saratoga, CA) with conditions maintained at pH 6.7, 28°C, 2.5 slpm aeration, and 350 rpm stirring. Consumption of carbohydrates during growth was measured on a Shimadzu LC-10 HPLC system (Shimadzu, Columbia, MD) equipped with 802 BP-100 H⁺ column (300×7.8 mm, Benson Polymeric Inc., Sparks, NV) and a low temperature evaporative scattering detector ELSD-LTII (Shimadzu, Columbia, MD), using deionized water at a flow rate of 0.4 mL·min⁻¹ as mobile phase (Zhao et al. 2013). At 40 h after inoculating the bioreactor, SRE was only detected (by HPLC) in the foam that accumulated above the culture medium and not in the culture broth. SRE was collected by siphoning the foam material.

**Organic-compatible SRE purification**

Formic acid and isopropanol, both on the National List of Allowed and Prohibited Substances (USDA 2015), were selected as solvents for large-scale preparative purification of SRE. Formic acid was added to the collected foam at a concentration of 0.1% (v·v⁻¹), and the solution was centrifuged at 4000×g for 60 min to remove cell debris. The supernatant was adjusted to pH 3.5 and subjected to an ÄKTA avant 150
Liquid Chromatography System (GE Healthcare Life Sciences, Piscataway, NJ) equipped with a 6.4×100 mm Resource™ RPC C18 column (GE Healthcare Life Sciences, Piscataway, NJ). The gradient elution of SRE was performed using solvent A: 0.1% (v·v⁻¹) formic acid in water and solvent B: 0.1% (v·v⁻¹) formic acid in isopropanol at a flow rate of 2.2 mL·min⁻¹ with the following program: 0-20% solvent B, 3 min; 20-45% solvent B, 42 min. The elution was monitored at 240 nm and fractions were collected separately every 1 min. HPLC fractions eluting with 29-30% solvent B were determined to be SRE based on co-elution with a SRE standard. Purified SRE was lyophilized and stored at −20°C. A sample of the purified material was sent to the Proteomics and Metabolomics Facility, Colorado State University (Fort Collins, CO) for MALDI-TOF analysis.

**SRE quantification**

Cultures or SRE preparations were extracted in 50% (v·v⁻¹) acetone in water acidified with 0.4% (v·v⁻¹) HCl. The extract was diluted with water to make a final concentration of 25% (v·v⁻¹) acidified acetone in water and loaded on a C18 Resprep® SPE cartridge (Restek. Co., Bellefonte, PA). The cartridge was washed with 25% (v·v⁻¹) acidified acetone in water, and SRE was eluted with 100% acidified acetone. The eluent was dried under a stream of air and suspended in 60% (v·v⁻¹) acidified acetone in water for HPLC analysis. The prepared sample was loaded on a 100×4.60 mm GEMINI-NX C18 column (Phenomenex Inc., Torrance, CA). The elution gradient of solvent A: 0.1% (v·v⁻¹) trifluoroacetic acid (TFA) in water and solvent B: 0.1% (v·v⁻¹) TFA in isopropanol was 30% solvent B, 2 min; 30-80% solvent B, 15 min; 80-100% solvent B, 1 min; 100%
solvent B, 1 min with a flow rate of 0.5 mL·min⁻¹ on a Beckman System Gold RP-HPLC instrument (Beckman Coulter, Inc., Brea, CA). Absorbance was monitored at 240 nm. The amount of SRE was quantified by comparisons to a standard curve constructed with known amounts of SRE produced by the conventional method (Bidwai and Takemoto 1987; Zhang and Takemoto 1987).

**Activity test of SRE against *P. ultimum* oospores**

*P. ultimum* was grown in 12 mL of V8-cholesterol broth (Ayers and Lumsden 1975) in sterile petri dishes incubated in the dark at room temperature without shaking for 3 days. Mycelial mats which formed in the petri dishes were rinsed two times with sterile mineral solution (0.9 mM Ca(NO₃)₂, 0.1 mM MgSO₄, 0.7 mM KH₂PO₄, and 0.1 mM FeCl₃) and then incubated in sterile petri dishes filled with 12 mL sterile distilled water in the dark at room temperature for 7 days. Oospores formed in mycelial mats were harvested using a Dounce glass homogenizer followed by filtration through glass fibers to remove mycelial fragments. The oospore concentration was estimated with a hemocytometer. Oospores at a concentration of 10⁵ oospores·mL⁻¹ were incubated in PDB containing 0, 3.9, 15.6, 31.3, 62.5, 125, and 250 µg·mL⁻¹ of SRE or 0/0, 3.9/11.7, 15.6/46.8, 31.3/93.9, 62.5/187.5, 125/375, and 250/750 µg·mL⁻¹ of SYRA (SRE/RL) in a 96-well plate. Rhamnolipid (RL, Zonix™, Jeneil Biosurfactant Co., Saukville, WI), a commercial organic-compatible fungicide, was also tested at concentrations of 0, 11.7, 46.8, 93.9, 187.5, 375, and 750 µg·mL⁻¹. Oospore germination was observed with light microscopy after 4 h incubation at 24°C.
Soil drench application of SRE on cucumber seeds

The effect of SRE as an agrofungicide was tested on organic cucumber seeds (Snow Seed Organic, Salinas, CA) and against *P. ultimum* infection. Cucumber seeds were planted in 24-well plates with Sunshine Mix 4 potting medium (Sun Gro Horticulture Inc., Bellevue, WA) artificially infested with 0 or 500 *P. ultimum* oospores·g\(^{-1}\) (Cummings, Miles, and du Toit 2009). The potting medium was drenched with 4 mL·g\(^{-1}\) of sterile distilled water, 50 µg·mL\(^{-1}\) SRE, or 500 ppm RL. Each treatment was composed of 24 planted seeds. Germination and growth of cucumber seedlings were observed with incubation at 25°C with 12 h light and 12 h dark for 6 days in a growth chamber (Percival Scientific Inc., Perry, IA). The averages of germination rates and standard errors were calculated based on three repeats of experiments.

Coating application of SRE on cucumber seeds

Non-treated organic cucumber seeds (Snowseed Co., Salinas, CA) were dipped into 0.3 mg·mL\(^{-1}\) SRE in 5% heat-gelatinized starch and dried at 4°C. Eight of each non-coated and SRE-coated seeds were planted on 1.5% water agar to test effects of SRE on seeds. Then eight seeds of non-coated and SRE-coated cucumber seeds were placed in petri dishes filled with soil naturally infested by *P. ultimum* collected at the Utah State University Research Farm (Kaysville, UT). Seeds were incubated at 25°C in the dark for 6 days and the emerging seedlings were counted. The experiment was repeated three times and germination rates and standard errors were calculated. To confirm the cause of disease in naturally infected soil, non-germinated cucumber seeds were plated on 1.5%
water agar after surface sterilization by soaking in 20% bleach for 30 s and rinsing with sterile water. The growth on water agar was observed under microscope. The occurrence of *Pythium* was verified by PCR using the following methods: DNA was extracted from the recovered *Pythium* mycelial mats using the FastDNA Spin kit (MP Biomedical, Santa Ana, CA, USA) and following the manufacturer’s protocol. The universal fungal primers for the internal transcribed spacer (ITS) region, ITS 4 and ITS 5 (White et al. 1990), were used for PCR. PCR reactions consisted of 5 µL 10× PCR buffer, 5 µL Q solution, 1 µL dNTPs (Qiagen Inc., Valencia, CA, USA), 1 µL primer ITS 4 (100 pmol), 1 µL primer ITS 5 (100 pmol), 2 µL DNA and 0.25 µL Taq DNA polymerase. Nuclease free water was added to obtain a 50 µL final volume. The reaction was denatured at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a final extension for 10 min at 72°C. The PCR products were visualized in a 1% agarose gel stained with ethidium bromide. The resulting bands were cut out and the DNA extracted using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) following the manufacturer’s protocol, and sequenced at the University of Arizona Genetics Core facility (Tucson, AZ). The sequences were compared to other fungal sequences in the NCBI GenBank® database using the BLAST search function for species identification.

**Phytotoxicity test of SRE on carrot, hot pepper, lettuce, and tomato seeds**

Non-treated organic seeds of carrot, hot pepper, and lettuce (all from Burpee Garden Products Co., Warminster, PA, USA), and tomato (Territorial Seed Co., Cottage Grove, OR, USA) were coated with SRE in heat-gelatinized starch as described above.
Eight of each non-coated and SRE-coated seeds (carrot, hot pepper, lettuce, and tomato) were planted on 1.5% water agar to test phytotoxicity of SRE on different vegetable seeds.

Results

**Novel SRE-producing *P. syringae pv. syringae* strains and growth medium development**

*P. syringae pv. syringae* strain 2B6 cultures had the highest antifungal activities when grown in modified SRM containing 1% (w·v⁻¹) glucose and 1% (w·v⁻¹) mannitol (ISM) compared with activities observed with substitution of other carbon sources and compounds for glucose (Table 4-1). With ISM, *P. syringae pv. syringae* strain G10 (derived from *P. syringae pv. syringae* strain 2B6) produced 53 mg·L⁻¹ SRE, which is >35% higher than strain B301D (37-39 mg·L⁻¹) produced in agitated flask cultures (Table 4-2). The level of SRE production under these conditions was not improved with the addition of 100 μM arbutin plus 0.1% (w·v⁻¹) fructose to the growth medium, a combination previously shown to stimulate SRE production by strain B301D in SRM (Mo and Gross 1991).

**Scalable SRE production**

In an agitated and aerated 10 L capacity WinPact bioreactor and with ISM as growth medium, *P. syringae pv. syringae* strain G10 grew and produced foam on the culture surface. At the end of the bioreactor culture period at 40 h, 5 g·L⁻¹ of glucose remained, while the mannitol concentration remained steady between 9 and 10 g·L⁻¹.
throughout the culture period (Fig. 4-1). The bioreactor culture began to foam on the liquid surface between 15-30 h coincident with initial detection of SRE in the culture broth (at 0.036-0.06 mg·L⁻¹ SRE). At 40 h, SRE product was detected in the foam above the culture broth but not in the culture broth. The foam containing accumulated SRE was collected by siphoning. The SRE yield averaged 50 mg·L⁻¹ of culture.

SRE purification

The collected foam material (in liquid form) was adjusted to pH 3.5 with formic acid. It was then subjected to one-step C18 reverse phase chromatography. Approximately 75% of the SRE activity applied to the column was recovered in material eluting as a single chromatographic peak (Fig. 4-2). MALDI-TOF analysis of the sample showed a molar mass of 1225 Da (Fig. 4-3), the expected value for SRE (Monti et al. 2001).

Activity of organic-compatible SRE against *P. ultimum* oospores

*P. ultimum* oospores were exposed to different concentrations of SRE, SYRA and RL, and germination rates were determined (Fig. 4-4). SRE prevented 50 and 90% of the oospores at 31.3 and 250 µg·mL⁻¹ SRE, respectively. On the other hand, there were no significant differences between germination rates of non-treated and SYRA-treated oospores. RL-treated oospores also exhibited no inhibition of germination, though RL treatment did result in an increase in the appearance of tangled and branched germ tubes (Fig. 4-4).
Soil drench application of SRE on cucumber seeds

The germination of cucumber seed was counted after 6 days from soil drench treatment with water, SRE, and RL treatments. With water soil drench treatment, the germination rates of cucumber seeds were 82.0±5.2% (mean±standard error) and 65.7±4.6% in non-infested and infested potting medium, respectively. Drench treatment of SRE had a 93.3±2.3% germination rate in non-infested medium and a 90.2±4.5% germination rate in infested medium. There were no significant disease symptoms compared to the control water-treatment in non-infested potting medium. Pre-emergence Pythium damping-off disease incidence was prevented by soil drench treatment with 50 µg·mL⁻¹ SRE. The treatment with 500 µg·mL⁻¹ RL resulted in germination rates of 44.7±2.3% with oospores and 79.0±4.0% without oospores. RL did not inhibit P. ultimum oospore germination or mycelial infection on cucumber seedlings.

Coating application of SRE on cucumber seeds

SRE was coated on cucumber seeds at a level of 0.03% (w·w⁻¹). There was no significant difference between germination rates of non-coated (95.8±4.2%) and SRE-coated (95.8±4.2%) cucumber seeds. SRE-coated cucumber seeds sprouted at the rate of 76.1±11.5% on naturally infested soil, while non-coated seeds had a germination rate of 0.0±0.0%. Coenocytic hyphae were grown from surface sterilized non-germinated cucumber seeds on water agar plates after 24 h incubation at room temperature. Gene sequence analysis (18S rRNA) identified these isolates as P. ultimum var. ultimum (Fig. 4-5). SRE decreased Pythium damping-off onsets of cucumber seeds.
Phytotoxicity test of SRE on carrot, hot pepper, lettuce, and tomato seeds

The amount of SRE applied on carrot, hot pepper, lettuce, and tomato seeds were estimated as 0.12%, 0.09%, 0.07%, and 0.28% (w·w⁻¹), respectively. There was no significant difference between germination rates of non-coated seeds and SRE-coated seeds on water agar (Table 4-3), thus 0.03% to 0.28% (w·w⁻¹) SRE was not phytotoxic to seeds.

Discussion

In this work, scalable methods for SRE production and purification were designed to meet USDA organic standards (USDA 2015). The combination of using a revised growth medium (ISM), novel production method, and cultivation of *P. syringae* pv. *syringae* strain G10 in a bioreactor grown with aeration and agitation resulted in a greater yield of SRE (50-53 mg·L⁻¹) than previously reported (~15 mg·L⁻¹) (Gross 1985; Gross and DeVay 1977a; Mo and Gross 1991; Surico, Lavermicocca, and Iacobellis 1989; Zhang and Takemoto 1987). The inclusion of mannitol in the medium was key to increased yields of SRE in agitated cultures (Table 4-1), though it was not consumed during the growth of *P. syringae* (Fig. 4-2). Mannitol has been shown to alter the physiology of some bacteria. As an example, mannitol increases the antibiotic sensitivity of persisters in *P. aeruginosa* biofilms due to a combination of metabolic and osmotic changes (Barraud et al. 2013). Further research is necessary to discern the role of mannitol in the increased production of SRE by *P. syringae* pv. *syringae*. 
The aerated bioreactor culture used in this study permitted the partitioning and recovery of the SRE produced in foam that accumulated at and above the surface of the culture. This eliminated the need to separate and extract SRE away from cells and cellular debris which greatly facilitated its purification. A previously used and common SRE purification method required several days and involved culture extraction with solvents followed by Amberlite XAD-2 chromatography and HPLC using a C18 silica reverse phase column (Bidwai et al. 1987). In contrast, the currently described method involves extraction of the foam material and single step chromatographic purification through an ÄKTA avant 150 Chromatography System yielding purified SRE within a few hours. Also, the new purification process employs isopropanol and formic acid instead of acetone, methanol, and TFA (Bidwai et al. 1987). Isopropanol and formic acid are listed as “allowed” on the National List of Allowed and Prohibited Substances (USDA 2015) that will contribute to its consideration as an organic-compatible fungicide. Scalable production of an organic-compatible SRE product could help to meet the growing demand for effective fungicides in organic farming.

The product SRE inhibited germination of oospores, the major primary infection source in epidemics of *Pythium* damping-off disease. Most fungicides available in the market such as RL have little or no effect against *Pythium* oospores (Stasz and Martin 1988), and this lack of inhibitory effect was confirmed in the current study with *Pythium* oospores (Fig. 4-4). In contrast, SRE’s observed inhibitory activity against *Pythium* oospores offers the possibility for the control of damping-off disease. Soil drench and seed treatment with organic-compatible SRE inhibited *P. ultimum* infection during early
development of cucumber seedlings. In contrast, SYRA, which is a mixture of SRE and RLs, did not inhibit oospore germination. Although RLs are reported to enhance the antifungal activity of SRE against several fungal pathogens of grape (Takemoto et al. 2010), it reduced SRE’s inhibitory activity against *P. ultimum* oospores (Fig. 4-4). Thick-walled oospores are usually insensitive to fungicides (Stasz and Martin 1988). Zoosporic stages are susceptible to inhibition by RLs (Kim, Lee, and Hwang 2000; Maier and Soberón-Chávez 2000), but *P. ultimum* var. *ultimum* rarely produces sporangia and zoospores (Levesque et al. 2010).

On water agar no significant difference was observed between the germination rates of non-coated and SRE-coated seeds, and SRE did not stunt cucumber seedlings. These findings support accumulating evidence that SRE and similar cyclic lipononapeptides are not phytotoxic (Bull et al. 1997, 1998; Mendes et al. 2011; Pauwelyn et al. 2013; Williamson et al. 2008) despite their historic portrayal as phytotoxins (Bidwai and Takemoto 1987; Gross and DeVay 1977b; Iacobellis et al. 1992; Kauss et al. 1991). The syringomycins were originally described as antibiotic compounds against fungi and yeasts (Sinden et al. 1971). Structurally related cyclic depsipeptide compounds, principally syringopeptins, are biosynthetically produced simultaneously with the syringomycins by *P. syringae* pv. *syringae* (Ballio et al. 1991, 1994). The latter are more antibacterial and phytotoxic and less antifungal than the syringomycins (Bensaci et al. 2011). With impure cell extracts, the phytoxic properties of syringopeptins may be erroneously attributed to syringomycins. The relative amounts of
the syringomycins and syringopeptins likely influence the complex balance between the pathogenic and saprophytic life styles of *P. syringae pv. syringae*.

Antifungal but non-phytotoxic property of SRE predict that it is a candidate fungicide for use in organic farming. SRE is water soluble and physically stable up to 121°C, with UV light exposure, and at pHs below 8. Its shelf life is sufficient and comparable to most commercial fungicides. Importantly, SRE is not toxic to animals at concentrations at which it is fungicidal (Sorensen et al. 1998; Fiore et al. 2008) and despite being hemolytic (Sorensen, Kim, and Takemoto 1996). Formulated non-pathogenic SRE-producing strains of *P. syringae pv. syringae* (ESC10 and ESC11) are approved by the US Environmental Protection Agency for use as post harvest biological decay control agents (Bull, Stack, and Smilanick 1997; Bull et al. 1998).

References


Table 4-1  Influence of carbon compounds substituted for 1% glucose in SRM growth medium on antifungal activity of SRE-producing *P. syringae* pv. *syringae* strain 2B6.

<table>
<thead>
<tr>
<th>Glucose substitutes (1%, w·v⁻¹)</th>
<th>Antifungal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>++</td>
</tr>
<tr>
<td>glycerol</td>
<td>+</td>
</tr>
<tr>
<td>ribose</td>
<td>-</td>
</tr>
<tr>
<td>galactose</td>
<td>+</td>
</tr>
<tr>
<td>mannitol</td>
<td>+</td>
</tr>
<tr>
<td>lactic acid</td>
<td>-</td>
</tr>
<tr>
<td>acetate</td>
<td>-</td>
</tr>
<tr>
<td>succinate</td>
<td>-</td>
</tr>
<tr>
<td>glucose plus mannitol</td>
<td>+++</td>
</tr>
<tr>
<td>2× glucose plus 2× mannitol</td>
<td>++</td>
</tr>
<tr>
<td>glucose plus 100 µM arbutin and 0.1 % (w·v⁻¹) fructose</td>
<td>++</td>
</tr>
<tr>
<td>glucose plus100 µM salicylate and 0.1 % (w·v⁻¹) fructose</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 4-2  Influence of growth medium composition on SRE production by *P. syringae* pv. *syringae* strain G10 in agitated flask cultures

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>SRE yield (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRM</td>
<td>14</td>
</tr>
<tr>
<td>ISM</td>
<td>53</td>
</tr>
<tr>
<td>SRM plus 100 µM arbutin and 0.1% (w·v⁻¹) fructose</td>
<td>28</td>
</tr>
<tr>
<td>ISM plus 100 µM arbutin and 0.1% (w·v⁻¹) fructose</td>
<td>51</td>
</tr>
<tr>
<td>SRM plus 100 µM salicylate and 0.1% (w·v⁻¹) fructose</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 4-3  Germination rates of non-coated and SRE-coated seeds on water agar and naturally infested soil

<table>
<thead>
<tr>
<th></th>
<th>Germination rates (%)</th>
<th>non-treated</th>
<th>SRE-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>water agar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carrot</td>
<td>79.2±8.3</td>
<td>87.5±7.2</td>
<td></td>
</tr>
<tr>
<td>hot pepper</td>
<td>87.5±0.0</td>
<td>87.5±7.2</td>
<td></td>
</tr>
<tr>
<td>lettuce</td>
<td>95.8±4.2</td>
<td>100.0±0.0</td>
<td></td>
</tr>
<tr>
<td>tomato</td>
<td>92.7±4.2</td>
<td>91.7±4.2</td>
<td></td>
</tr>
<tr>
<td>cucumber</td>
<td>95.8±4.2</td>
<td>95.8±4.2</td>
<td></td>
</tr>
<tr>
<td><strong>infested soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cucumber</td>
<td>0.0±0.0</td>
<td>76.1±5.1</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4-1  *P. syringae* pv. *syringae* strain G10 growing in ISM in a Winpact Bioreactor and Fermenter system with aeration and agitation produces SRE (×) and consumes glucose (○). Mannitol (■) is not consumed during growth.
Fig. 4-2 Chromatographic profile of a SRE extract using an ÄKTA avant 150 chromatography system. The solid line shows the 240 nm absorbance (A) profile and the dotted line the percentage of solvent B (0.1% formic acid in isopropanol) in solvent A (0.1% formic acid in water). The peak at 17.7 min is designated with an arrow and identified as SRE using HPLC.
Fig. 4-3  MALDI-TOF mass spectrum of the HPLC purified SRE material produced by *P. syringae* pv. *syringae* strain G10. The major ion detected at m/z = 1225.482 identified it as SRE.
Fig. 4-4  Oospore germination test. Germination rates of *P. ultimum* oospores were counted in PDB containing different concentrations of SRE (A-1), SYRA (B-1), and RL (C-1). Photographs (right panels) showing non-treated oospores and oospores treated with 250 µg·mL\(^{-1}\) SRE (A-2), 250/750 µg·mL\(^{-1}\) (SRE/RL) SYRA (B-2), 750 µg·mL\(^{-1}\) RL (C-2) treated oospores and non-treated (D) oospores were taken after 4 h incubation.
Fig. 4-5  DNA nucleotide sequence of the mycelial growth recovered from a cucumber seed planted in naturally infested soil. The sequence matches (highlighted, 99% identities) the partial sequence of the 18S ribosomal RNA gene of *P. ultimum* var. *ultimum* in the NCBI database.
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

Bacteria inhabit all ecological niches of the Earth and therefore they produce diverse bioactive metabolites as their survival tools for stress tolerances and interactions with other organisms. However, microbes often produce these valuable metabolites in tiny amounts just enough to satisfy their own needs. Recent advanced techniques in molecular biology, biological engineering, metabolic engineering, organic chemistry and other related fields of study focus on microorganisms as sources of natural products. Application of biological manipulations in bacteria and rapidly-growing unicellular organisms contribute to large-scale productions of natural products (3, 10). The aims of this study were scalable productions of two different natural products, potent cytoprotectant biliverdin IXα and potential organic-compatible agrofungicide syringomycin E (SRE) in *Escherichia coli* and *Pseudomonas syringae pv. syringae*, respectively.

Current commercially available biliverdin IXα is prepared by chemical oxidation of bilirubin IXα extracted from animal bile (6). This chemical process generates significant levels of biliverdin IIIα and XIIIα, and removal of these non-bioactive isomers is difficult (5, 7). In this study, biliverdin IXα production was conducted by gene recombination and fed-batch fermentation. A recombinant *E. coli* strain BL21(mHO1-GFP) transformed with a sequence-modified cyanobacterial heme oxygenase-1 (*mho1*) gene was reported to solely produce biliverdin IXα isomer (1). Enzymatic activity of HO is strongly dependent on electron donors and the electron flow from NADPH to
heterogeneous HO is considered to be NADPH $\rightarrow$ NADPH–flavodoxin/ferredoxin reductase (FNR) $\rightarrow$ flavodoxin (Fld) $\rightarrow$ mHO1 in recombinant E. coli. FNR and Fld were plasmid-overexpressed in E. coli strain BL21(mHO1-GFP) and two new strains BL21(mHO1-GFP+FNR) and BL21(mHO1-GFP+Fld) were obtained, respectively. The construct BL21(mHO1-GFP+Fld) yielded 17-40% more biliverdin IXα comparing to the strain BL21(mHO1-GFP) whereas BL21(mHO1-GFP+FNR) did not improve the yield. In addition, BL21(mHO1-GFP+Fld) achieved 13-26% higher cell density than BL21(mHO1-GFP) at the end of biliverdin IXα production growth. Overexpression of Fld in E. coli had been reported to confer resistance against oxidative stress (2). Further study will be required to clarify the mechanism how Fld contributes in high cell density cultivation of E. coli.

On the other hand, an enhanced SRE producer strain of P. syringae pv. syringae, G10, was isolated via UV radiation mutagenesis without artificial genetic modification to meet the USDA organic standards (8, 12). While SRE production in the past was done in still batch cultures, the strain G10 reported in this dissertation research allowed SRE production in agitated conditions. As a result of strain development, medium modification, and agitated growth conditions, SRE yield increased from 10-20 mg·l$^{-1}$ to 50 mg·l$^{-1}$ and the production period was shortened from 10 days to 40 h compared the conventional methods (4, 13). Then, SRE purification protocol was also improved from µg-levels to mg-levels with employment of a large scale chromatography system and organic-compatible solvents. The purified product SRE was examined as a seed protectant against a soil-borne pathogen P. ultimum. SRE suppressed germination of
oosporic *P. ultimum* which is resistant to fungicides (11). SRE (0.03% w·w⁻¹) - and non-coated vegetable seeds had the same germination rates under pathogen free environment while SRE-coated seed had higher germination rates in infested media. SRE behaved as a potential organic-compatible seed protectant.

Optimization of medium components was performed for the production of both biliverdin IXα and SRE. Different carbon sources were tested in media to maximize the yields. In the recombinant strain of *E. coli*, lactose was the key to induce heterogeneous mHO1 protein expression, whereas glycerol was the important factor for high cell density cultivation. In UV-mutated *P. syringae* pv. *syringae*, inclusion of mannitol in the culture medium facilitated SRE production under agitated conditions, while mannitol was not consumed throughout the fermentation period. Different carbon sources affect different levels of cell activities in different ways. Optimization of carbon sources is an essential process to increase yields of the desired metabolites.

In general industrial fermentation, foam causes undesirable consequences, reduced oxygen transfer rates, losses of fermentation culture, and contamination of culture by wetting the air filters (9). However, in this dissertation study, foam was desired for the separation of the products in fermenter systems. Foam formation was observed during biliverdin IXα and SRE production and the products were accumulated in the foam above the culture but not in the culture broth. The products were collected by siphoning foam materials, and foam floatation greatly facilitated extraction of products. In fed-batch production of biliverdin IXα, glycerol feed was used to boost biliverdin IXα production while the product was constantly removed from the fermenter system as foam.
Further growth optimization could be done to make continuous culture systems for extended production phases and thus enhanced yields.

In conclusion, bacteria are small organisms but have huge potentials to synthesize high-value bioactive compounds. Scalable production methods of biliverdin IXα and SRE were proved through strain development and fermentative growth of the resulting strains. Gene recombination and mutation were performed to achieve overproduction of the desired metabolites in bacteria. Growth parameters were modified to maximize the yields in fermenters. Despite the fact that production of biliverdin IXα and SRE was enhanced, there is ample scope for further improvement. For instance, additional manipulation of growth parameters may further enhance yields.

References


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Scalable production of biliverdin IXα by Escherichia coli
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Patent:

Meeting presentations: