PURIFICATION OF FUSION PEPTIDE BIOSURFACTANT USING COMBINED CHEMICAL EXTRACTION AND CROSS-FLOW MICROFILTRATION

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To my beloved parents, brothers and friends

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ABSTRACT

Biosurfactant becomes important nowadays as sustainable bio-derived surfactants emerged to replace the petroleum based surfactant. In this work, the fusion peptide biosurfactant (HSG) was successfully expressed when induced with 1 mM Isopropyl-ß-D-thiogalactopyranoside (IPTG). The combined process of chemical extraction and cross-flow microfiltration was conducted to disrupt the cell membrane and isolate the desired product, HSG. A combination of different concentration of Triton X-100 and Ethylenediaminetetraacetic Acid (EDTA) was investigated to obtain maximized protein extraction from the cells. The efficiency of the chemical extraction was compared with B-PER commercial bacterial extraction kit. Protein estimation was performed using Bicinchoninic Acid (BCA) Protein Assay method and Polyacrylamide Gel Electrophoresis (PAGE) analysis. The results showed that the combination of 1% vv^{-1} Triton X-100 and 1 mM EDTA released the highest amount of soluble protein and is comparable to the B-PER commercial bacterial extraction kit. The extraction broth is then applied to cross-flow microfiltration process with a 0.2 µm polysulfone hollow fiber membrane. The effect of rotor speed and transmembrane pressure (TMP) for peptide transmission were investigated. The rotor speed for the microfiltration test was varied at 150, 200 and 300 rpm which gave rise to the uncontrolled TMP of 2.5, 2.5 and 4 psig respectively. The highest overall permeate flux achieved at 300 rpm was selected for further investigation at two different TMP of 4 and 5 psig. It was found that the operating conditions at 300 rpm and 5 psig gave 36.33% more protein transmission as compared to operation at 300 rpm and 4 psig. Backpulsing was applied to the microfiltration system to minimize the fouling problem. An overall protein transmission of about 59.6% was achieved with the operating parameter of 300 rpm at constant transmembrane pressure of 5 psig.

ABSTRAK

Biosurfaktan menjadi penting pada masa kini kerana surfaktan yang dihasilkan secara biologi bakal menggantikan surfaktan yang dihasilkan daripada minyak petrol. Dalam kerja ini, fusi peptida biosurfaktan (HSG) berjaya dieksperasikan apabila diinduksi dengan 1 mM Isopropyl-ß-D-thiogalactopyranoside (IPTG). Kombinasi proses ekstraksi secara kimia dan penurasan mikro bersilang-aliran dijalankan untuk memecahkan membran sel dan mengasingkan produk yang dikehendaki, HSG. Kombinasi pelbagai kepekatan Triton X-100 dan Asid Ethylenediaminetetraasetik (EDTA) telah diselidik untuk mendapatkan ekstraksi protein yang optimum. Kecekapan ekstraksi secara kimia ini telah dibandingkan dengan hasil ekstraksi yang menggunakan kit ekstraksi bakteria protein komersil B-PER. Anggaran protein dilakukan dengan menggunakan kaedah ujian Protein Asid Bicinchoninic (BCA) dan dianalisa dengan Polyacrylamide Gel Electrophoresis (PAGE). Keputusan kajian menunjukkan bahawa kombinasi daripada 1% vv^{-1} Triton X-100 dan 1 mM EDTA menghasilkan protein terlarut yang paling tinggi di mana keputusan ini adalah setanding dengan keputusan daripada kit ekstraksi bakteria protein B-PER. Kaldu ekstraksi ini dituraskan secara penurasan mikro bersilang-aliran dengan menggunakan membran serat polisulfon berongga 0.2 μm. Pengaruh kelajuan rotor dan tekanan transmembran (TMP) terhadap protein transmisi telah diselidik. Ujian pengaruh kelajuan rotor dijalankan pada 150, 200 dan 300 rpm di mana TMP tidak terkawal yang tercapai adalah masing-masing pada 2.5, 2.5 dan 4 psig. Rembasan fluks keseluruhan yang tertinggi pada 300 rpm dipilih untuk ujikaji selanjutnya pada TMP 4 dan 5 psig. Didapati bahawa operasi pada 300 rpm dan 5 psig memberikan protein transmisi yang lebih tinggi iaitu sebanyak 36.33% berbanding operasi pada 300 rpm dan 4 psig. Penahan berbalik diaplikasikan pada sistem penurasan mikro bersilang-aliran untuk mengurangkan masalah membrane yang tersumbat. Sejumlah 59.6% protein transmisi keseluruhan telah tercapai dengan operasi parameter pada 300 rpm dan pada tekanan yang malar iaitu 5 psig.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xi
	LIST OF FIGURES	xii
	LIST OF ABBREVIATIONS	xiii
	LIST OF SYMBOLS	xvi
	LIST OF APPENDICES	xviii
1	**INTRODUCTION**	1
	1.1 General Review	1
	1.2 Background of Study	3
	1.3 Objectives of Study	4
	1.4 Scope of Study	5

2	**LITERATURE REVIEW**	6
	2.1 Biosurfactants	6
	2.2 The Recombinant Technology	8
	2.2.1 Fusion Protein	10
	2.2.2 The pET Plasmid System	12
	2.3 Chemical Extraction	14
	2.3.1 Cell Envelope of *E. coli*	15
	2.3.2 Characteristics of Common Chemical Reagents and Surfactants	17
	2.4 Membrane Separation	18
	2.4.1 Case Studies of Microfiltration	20
	2.4.2 Key Parameter of Microfiltration Unit	21
	2.4.2.1 Cross-flow Velocity	21
	2.4.2.2 Viscosity	22
	2.4.2.3 Feed Concentration	22
	2.4.2.4 Surfactant	22
	2.4.2.5 Transmembrane Pressure (TMP)	23
	2.4.2.6 pH	23
	2.4.2.7 Ionic Strength	24
	2.5 Problem Statement and Strategy to Overcome	24
	2.6 Experimental Design for Microfiltration Test	25
	2.6.1 Membrane Selection	25
	2.6.2 Operating Conditions	26
	2.6.3 Membrane Regeneration and Cleaning Method	28
	2.7 Membrane Efficiency	28
	2.7.1 Permeate Flux	28
	2.7.2 Protein Transmission	29

	2.8	Analytical Methods	29
		2.8.1 Total Protein Assay	29
		2.8.2 Polyacrylamide Gel Electrophoresis (PAGE)	30
3	**METHODOLOGY**		32
	3.1	Materials	32
	3.2	Instrumentation	33
	3.3	Bacterial Growth	34
	3.4	Methods for Chemical Extraction	35
		3.4.1 Chemical Extraction using B-PER Reagent as Control	35
		3.4.2 Evaluation of Chemical Extraction Protocols	35
	3.5	Total Protein Estimation	36
	3.6	PAGE Analysis	36
	3.7	Experimental Design for Microfiltration Runs	37
		3.7.1 Clean Water Flux Measurement and Membrane Regeneration	37
		3.7.2 Benzonase Treatment for Chemical Extraction Broth	37
		3.7.3 Protocols for Microfiltration of Extraction Broth	38
4	**RESULTS AND DISCUSSIONS**		40
	4.1	Chemical Extraction	40
	4.2	Microfiltration Test	43
		4.2.1 Membrane Performance	43
		4.2.2 Protein Transmission	45
	4.3	Limitations and Justification of Experimental Design	47

5	**CONCLUSIONS & RECOMMENDATIONS**	50
	5.1 Conclusions	50
	5.2 Recommendations	51
	REFERENCES	53
	Appendices	64-77

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Production of biosurfactants by various microbial strains	7
2.2	The advantages and disadvantages of different strategies for the production of recombinant proteins in *E. coli*	9
3.1	List of chemicals and reagents used	32
3.1	Summary of chemical extraction protocol performed	36
4.1	Results of one way ANOVA analysis for chemical extraction methods	42

LIST OF FIGURES

FIGURES NO.	TITLE	PAGE
2.1	The construct designated pET-HSG	12
2.2	Induction of pET system by IPTG	14
2.3	Schematic diagram of cell envelope for Gram-negative bacteria	16
2.4	Two main membrane filtration modes	20
2.5	Schematic diagram of a laboratory cross-flow microfiltration unit, Quixstand Benchtop System Model QSM-02SP	27
3.1	Picture of cross-flow microfiltration unit, Quixstand Benchtop System Model QSM-02SP	34
3.2	Schematic diagram of a laboratory cross-flow microfiltration unit, Quixstand Benchtop System Model QSM-02SP	38
4.1	Electrophoresis 4-12% Bis-Tris gradient gel for induced and uninduced total cell	41
4.2	Protein release for various chemical extraction protocols	42

4.3	Permeate flux as a function of time	44
4.4	Permeate flux profile as fixed rotor speed	45
4.5	Protein transmission profile for various operating conditions	46
4.6	Electrophoresis 4-12% Bis-Tris gradient gel for 300 rpm and 5 psig TMP profile	47

LIST OF ABBREVIATIONS

BCA	-	Bicinchoninic acid
Bis-Tris	-	(Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane)
BSA	-	Bovine Serum Albumin
CMC	-	Critical micelle concentration
DP	-	Designed peptide
DNA	-	Deoxyribonucleic acid
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetraacetic acid
GAM1	-	Peptide biosurfactant
GM-CSF	-	Granulocyte Macropharge-Colony Stimulating Factor
GST	-	Glutathione S-transferase
GuHCl	-	Guanidine hydrochloride
HCl	-	Hydrochlorid acid
HSG	-	Fusion peptide biosurfactant
HTLV-III	-	Human T-lymphotropic virus Type III
IBs	-	Inclusion bodies
IPTG	-	Isopropyl-β-D-thiogalactopyranoside
MBP	-	Maltose Binding Protein
$MgCl_2$	-	Magnesium chloride
MU	-	Microfiltration Unit
MWCO	-	Molecular weight cut off
NaCl	-	Sodium Chloride

NaOH	-	Sodium Hydroxide
NaOCl	-	Sodium Hypochlorite
NAM	-	N-acetyl muramic acid
PMP	-	Promegapoietin-1a
RNase	-	Ribonuclease
rIL-2IBs	-	Human interleukin-2
rpm	-	Rotation per minute
SDS	-	Sodium Dodecyl Sulphate
SDS-PAGE	-	SDS-Polyacrylamide Gel Electrophoresis
SUMO	-	Small ubiquitin-related modifier
TMP	-	Transmembrane pressure
TRX	-	Thioredoxin
Tris-HCl	-	Tris(hydroxymethyl)aminomethane
Ub	-	Ubiquitin
UV	-	Ultra-violet

LIST OF SYMBOLS

A_m	-	Filtration area
bar	-	Unit for pressure
bp	-	Base pairs
C_p, Cp	-	Concentration of the solute in the permeate
C_b, Cb	-	Concentration in the feed/bulk solution
cm	-	Centimeter
Ca^{2+}	-	Calcium (II) ion
Cu^{1+}	-	Copper (I) ion
Cu^{2+}	-	Copper (II) ion
g	-	Gram
J	-	Permeate Flux
kDa	-	Kilodalton
kPa	-	Unit for pressure, kilopascal
L min^{-1}	-	Litre per minute
L m^{-2} h^{-1}	-	Litre per meter square and hour
M	-	Molar
Mg^{2+}	-	Magnesium ion
min	-	minute
mg	-	Milligram
ml	-	Millilitre
nm	-	Nanometer
P	-	Pressure
Pi	-	Inlet pressure
Po	-	Outlet pressure
Pm	-	Pressure measured at permeate flow
psig	-	Unit for pressure
T	-	Transmission efficiency

t	-	Time
UmL^{-1}	-	Unit per millilitre
µm	-	Micrometer
µL	-	Microlitre
$µgmL^{-1}$		Microgram per millilitre
w/v, wv^{-1}	-	Weight per volume
v/v, vv^{-1}	-	Volume per volume
v	-	Volume
V	-	Voltage
%	-	Percentage
<	-	Less than
~	-	Approximately
4x	-	Four fold
^{o}C	-	Degree Celcius

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	Information of Model Protein (HSG)	64
B	List of Chemicals and Reagents Used	66
C	BCA Total Protein Assay Standard Curve	67
D	Experimental Results of Chemical Extraction	68
E	Colloidal Blue Staining Protocol	69
F	Membrane Regeneration Data	70
G	Optimal and Effective Condition for Benzonase nuclease	71
H	Experimental Data for Microfiltration Test	72

CHAPTER 1

INTRODUCTION

1.1 General Review

Biosurfactants are surface-active biomolecules which have both defined hydrophilic and hydrophobic groups. It becomes important nowadays as sustainable bio-derived surfactants emerged to replace the petroleum based surfactant and has gained importance in the fields of oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as biodegradability and lower toxicity (Healy *et al.*, 1996; Mukherjee *et al.*, 2006; Kaar *et al.*, 2008).

Recently, active designed peptide (DP) biosurfactants becomes more popular as its linear peptide sequences, without conjugated lipid, allowed more simplified bio-production compared with bacterial lipopeptides. In addition, peptide biosurfactants can be designed to allow stimuli-responsive control of interfacial elasticity (Dexter *et al.*, 2006; Dexter and Middelberg, 2007) and interfacial tension (Middelberg *et al.*, 2008), providing reversible control of emulsions (Dexter *et al.*, 2006) and foams (Malcolm *et al.*, 2006).

Conventionally, the method for protein processing is time consuming and labour-intensive because of the repeated cycles of mechanical disruption, enzymatic

and chemical treatment. Thus, scale up of the process can be problematic. However, the protein processing method can be simplified on scale-up by substituting the repeated homogenization and centrifugation unit operations with the chemical extraction and cross-flow microfiltration (Lee *et al.*, 2003).

In this work, the pET 26b system (Novagen) is engineered and designed to secrete the peptide biosurfactant GAM1 to the periplasm when induced with IPTG. The designed recombinant peptide surfactants thus produced have the advantages to enable the generation of improved variants by simple genetic manipulation compared to the production of naturally occurring biosurfactants which requires multi component enzymes for synthesis within the cell and are feed sources dependent during microbial growth rate (Peypoux *et al.*, 1999). To produce recombinant proteins, *Escherichia coli* were used commonly as its physiology and genetics are well documented and studied. However, peptides with short sequences of amino acids are either poorly expressed in *E. coli* or are rapidly degraded. Therefore, peptides are strategically produced together with the fusion partner known as fusion protein or chimeric protein. The reasons of using fusion protein are to facilitate purification, easy detection of the fusion protein from a complex mixture, and to avoid proteolytic degradation (Hammarstrom *et al.*, 2002; Yan *et al.*, 2005).

Recovery of the soluble fusion protein is usually done with a centrifuge. As an alternative, a cross-flow microfiltration unit is proposed to replace centrifugation for the separation of soluble protein from the complex mixture, after chemical extraction, since appropriate control of both the cross-flow filtration rate and the transmission of the solutes in the microfiltration unit (MU) process are of great interest. In this work, the plasmid construct designated pET-HSG, derived from modification of pET26b, consists of hexa-histidine affinity tag, fusion protein comprising SUMO, a protease cleavage site and the peptide GAM1. It was transformed into *E. Coli* BL21(DE3). After fermentation in the shake flask culture, the harvested cells undergo a non-solubilising chemical extraction procedure coupled with cross-flow microfiltration, to recover the soluble fusion peptide biosurfactant. Combination of EDTA and Triton X-100 has been reported to be capable of releasing

intracellular protein of interest to culture medium (Falconer *et al.*, 1996; Lee *et al.*, 2003). A commercial bacterial extraction kit, B-PER Bacterial Protein Extraction Reagent (Pierce), EDTA, Triton X-100 and GE QuixStand microfiltration unit with 0.2 μm pore size polysulfone membranes were used for this purpose.

1.2 Background of Study

Self-assembling peptide is a novel class of functional materials that has a great potential in superseding the performance of conventional materials like plastics and metals. Sustainability has become a key driver in achieving quality of life standards and peptide is expected to be more versatile in functionality and sustainable than conventional materials like plastic which is over-reliant on petroleum feed stock. Literature reported that the surfactant peptides are relatively inexpensive and chemically facile to modify, leading to potential tailoring of new materials for a broad spectrum of applications like serving as scaffolds to organize conducting and semiconducting nanocrystals into high-density ordered structures; incorporating other biomolecules on their surfaces; encapsulating molecules for molecular deliveries; and forming a scaffold for cell encapsulation (Vauthey *et al.*, 2002). Depending on the application, the physiochemical properties of such biomaterial can be designed to serve various applications, notably as biodegradable surfactant (Fairman and Akerfeldt, 2005).

The production of such novel functional biomaterials can become economically feasible if the process uses the technology that is approximately scale-invariant, easily automated for high-throughput processing, generic for a broad range of proteins, and economical (Middelberg, 2002). However, the conventional protein processing strategy using *E .coli* involves a series of processing steps that could account for 50-70% of production cost. These multiple steps may compromise production yield and thus economic feasibility. A range of research strategies is available, including intensification of the downstream processing to improve yield,

molecular manipulation such as fusion technology to simplify downstream processing and optimization of key unit operations (Wong *et al.*, 1996).

Process intensification by minimizing the number of unit operations without a loss of product purity is desirable to improve yield and reduce production cost. In this study, process intensification is employed as a key research strategy to minimize the number of unit operations for the purification of peptides from the *E. coli* host cells. The key is to make use of an innovative chemical extraction method and microfiltration unit to accomplish cell disruption and initial product recovery in place of the cumbersome multiple homogenization and centrifugation in the conventional process flow.

Cross-flow type microfiltration system is chosen as it can be used to concentrate the solution, clarify fermentation broth, as well as enhance diafiltration and fractionation. Besides, cross-flow microfiltration is fast, efficient and easy to scale up (Van Reis and Zydney, 2007). It is aimed that combined method of chemical extraction and cross-flow microfiltration would produce substantially pure peptide product in the permeate flow. This substantially pure peptide product (very low in solid contaminants like cellular debris) can subsequently be processed and polished to high purity using chromatography columns, in most cases by affinity capture (Kaar *et al.*, 2008) or further concentrate and purify the product with ultrafiltration.

1.3 Objectives of Study

Based on the background of study, there is a need to come up with an alternative downstream processing method which needs fewer steps and less manpower, consume less time and less cost to run. This will hence lead to substantial processing cost reduction and increase the production efficiency.

To alleviate some of the aforementioned problems, it is proposed to apply chemical extraction combined with a microfiltration unit. The purpose of this study is to produce recombinant peptide biosurfactant GAM1, as a fusion protein, using *E. coli* as host cell and to release fusion peptide biosurfactant, HSG from the host cells using non-solubilising chemical extraction method as a possible substitute for mechanical cell disruption. The extraction broth is then applied to cross-flow microfiltration process with a 0.2 μm polysulfone hollow fiber membrane. This research also aims to study the operation parameters of the cross-flow microfiltration unit such as rotor speed and transmembrane pressure for the purification of fusion peptide biosurfactant, HSG.

1.4 Scope of Study

In order to achieve the objectives, the scope of study is limited to the following.

a) To transform the plasmid containing the designated peptide biosurfactant, GAM1 using *E. Coli* BL21(DE3) host cells by chemical competent method.

b) To release soluble fusion peptide biosurfactant, HSG from the *E. Coli* BL21(DE3) host cells based on a combination of Triton X-100 and EDTA without solubilising the membrane of the host cell.

c) To remove the soluble contaminants with parameters of the cross-flow microfiltration process using 0.2 μm pore size polysulfone membranes by manipulating the rotor speed parameter (rpm) at constant transmembrane pressure (TMP).

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