IMPROVEMENT OF CYCLODEXTRIN GLUCANOTRANFERASE EXCRETION AND CELL VIABILITY OF RECOMBINANT *Escherichia coli* IMMOBILIZED ON HOLLOW FIBER MEMBRANE

ROHAIDA BINTI CHE MAN

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Universiti Teknologi Malaysia

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To my beloved parents (Hj. Che Man bin Mamat and Hjh. Hasmah binti Mohd Noh), my husband (Saiful Aflah bin Abdol Karim), daughter (Nur Alya Safiah), son (Muhammad Aiman Rafiqin), sisters (Fauziah, Rohaniah, Rohaniza and Rohaina) and brothers (Mohd Hatta, Mohd Lutffi, Mohd Asri, Mohd Nizam, Muhammad and Mohd Hafizullah). I dedicated this work in sincere gratitude for their patience, love and support.

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ABSTRACT

The excretion of a recombinant enzyme into culture medium presents significant advantages over cytoplasmic expression. However, cell lysis is one of the major drawbacks during the excretion of recombinant enzyme when using *Escherichia coli* (*E. coli*) as a host. Cell immobilization is a promising solution for the enhancement of enzyme excretion with reduction of cell lysis. In the present study, a recombinant *E. coli* was immobilized using hollow fiber membrane to improve the enzyme excretion, cell viability and plasmid stability. The effects of different polymers of hollow fiber membrane and culture conditions on the cyclodextrin glucanotransferase (CGTase) excretion, cell lysis and plasmid stability of immobilized *E. coli* were investigated. The immobilized cells on a polyvinylidene fluoride polymer exhibited a 2.0–4.5-fold increase in the CGTase excretion with 18-95% reduction of cell lysis and over 100% increment of plasmid stability compared to the free cells. The CGTase excretion was successfully optimized by response surface methodology. Under the optimized conditions [25 ºC of post- induction temperature, 0.011 mM isopropyl β-D-1-thiogalactopyronoside and pH 8.8], the CGTase excretion was 3.8-fold higher with 80% reduction of cell lysis compared to the value before optimization process. The use of low tryptone concentration (5 g/l) reduced the occurrence of cell lysis (90% reduction) and increased the plasmid stability (86% increment) without significant change in CGTase excretion in comparison with initial tryptone concentration (20 g/l). This approach (5 g/l) produced an approximately two times higher CGTase excretion (compared with 20 g/l during) recycle process. The membrane bioreactor also showed 2.5-fold increase in the CGTase excretion (473 x 10^3 U/ml) with 75% reduction of cell lysis compared to shake flask culture (190 x 10^3 U/ml of CGTase activity). Hence, the immobilization of *E. coli* on hollow fiber membrane proved to be valuable for the excretion of recombinant proteins in *E. coli* with high cell stability.

ABSTRAK

Perembesan enzim rekombinan ke dalam media kultur adalah pendekatan yang lebih baik berbanding pengungkapan sitoplasmik. Walau bagaimanapun, lisis sel adalah salah satu masalah utama dalam perembesan enzim rekombinan apabila menggunakan *Escherichia coli* (*E. coli*) sebagai perumah. Imobilisasi sel adalah penyelesaian yang baik untuk peningkatan perembesan enzim dengan pengurangan kadar lisis sel. Dalam kajian ini, *E. coli* rekombinan telah diimobilisasikan menggunakan polimer membran gentian berongga bertujuan untuk meningkatkan perembesan enzim, bilangan sel hidup dan kestabilan plasmid. Kesan polimer membran gentian berongga yang berbeza dan keadaan pertumbuhan untuk perembesan siklodekstrin glukanotransferase (CGTase), kadar lisis sel dan kestabilan plasmid bagi sel imobilisasi telah dikaji. Sel imobilisasi pada poliviniliden fluorida polimer mempamerkan 2.0-4.5 kali ganda peningkatan dalam perembesan CGTase dengan 18-95% pengurangan kadar lisis sel dan peningkatan kestabilan plasmid melebihi 100% berbanding dengan sel bebas. Perembesan CGTase berjaya dioptimakan dengan menggunakan kaedah gerak balas permukaan. Dengan menggunakan keadaan optimum [25 ºC suhu induksi, 0.011 mM isopropil β-D-1-thiogalaktopiranosida dan pH 8.8], perembesan CGTase adalah 3.8 kali ganda tinggi dengan pengurangan kadar lisis sel sebanyak 80% berbanding dengan nilai sebelum proses pengoptimuman. Penggunaan kepekatan tripton yang rendah (5 g/l) mengurangkan kadar lisis sel dengan 90% pengurangan dan meningkatkan kestabilan plasmid (86% peningkatan) tanpa perubahan perembesan CGTase yang ketara berbanding dengan kepekatan tripton yang asal (20 g/l). Pendekatan ini (5 g/l) membuktikan penghasilan perembesan CGTase dengan kira-kira dua kali ganda berbanding 20 g/l sepanjang proses berulang. Bioreaktor membran juga menunjukkan peningkatan perembesan CGTase (473 x 10^3 U/ml) sebanyak 2.5 kali ganda dengan pengurangan lisis sel sebanyak 75% berbanding kelalang kon (190 x 10^3 U/ml aktiviti CGTase). Oleh itu, sel *E. coli* imobilisasi pada membran gentian berongga berguna untuk tujuan perembesan protein rekombinan dengan kadar sel hidup yang tinggi.

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LIST OF SYMBOLS

g	-	gram
h	-	hour
l	-	liter
min	-	minute
ml	-	milliliter
sec	-	second
v/v	-	volume solute per volume solution
w/v	-	weight solute per volume solution

LIST OF ABBREVIATIONS

A_{600}	-	absorbance at wavelength 600 nm
ANOVA	-	analysis of variance
BRP	-	bacteriocin release protein
CCD	-	central composite design
CD		cyclodextrin
CGTase	-	cyclodextrin glucanotranferase
Da, kDa	-	dalton, kilodalton
DNA	-	deoxyribonucleic acid
E. coli	-	*Escherichia coli*
EDTA	-	ethylenediaminetetra-acetate
FESEM	-	field emission scanning electron microscopy
FFD	-	full factorial design
HCl	-	hydrochloric acid
IM	-	inner membrane
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
lac	-	lactose
LB	-	luria bertani
LPS	-	lipopolysaccharide
MgCl	-	magnesium chloride
MW	-	molecular weight
NMP	-	1-methyl-2-pyrolidon
OD	-	optical density
OFAT	-	one factor at one time
OM	-	outer membrane

ONPG	-	*ortho*-nitrophenyl-β-galactoside
PEG	-	polyethylene glycol
PES	-	polyethersulfone
rpm	-	revolution per minutes
RSM	-	response surface methodology
SDS-PAGE	-	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp.	-	species

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Protein excretion is important, as it enables active and stable enzyme production, which is essential for successful biocatalysis. In recombinant protein expression, excreted proteins are preferable to intracellular proteins production. Excretory production of recombinant proteins provides several advantages over intracellular protein production, including increased protein stability and solubility, correct formation of disulfide bonds and facilitation of downstream processing (Mergulhao *et al.*, 2005). However, studies have shown that excretion of enzymes in *Escherichia coli* (*E. coli*) may cause cell lysis because of pressure build-up through overproduction of the expressed recombinant protein (Fu *et al.*, 2005). Thus, the production of recombinant proteins by excretion without (or with less) cell lysis caused by overexpression of the recombinant protein in the host cell is difficult. Various attempts have been made to reduce cell lysis resulting from the excretion of proteins, such addition of glycine or Triton-X to the medium (Jang *et al.*, 1999), using bacteriocin release protein (BRP) (Fu *et al.*, 2003) and cultivating cells using favorable conditions (Shokri *et al.*, 2003).

Cell immobilization for the excretion of enzymes has various advantages over the conventional process using a free cell system. These advantages include the repeated and expanded use of cells, preservation of plasmid-bearing cells, simplicity of performing a continuous process for extended periods of time, decreased risk of contamination, protection of the cells from environmental stresses and easier downstream processing (Jamuna and Ramakrishna, 1992, Mamo and Gessesse, 1997, Moriwaki *et al.*, 2007). Using cell immobilization techniques, increased protein excretion and reduction in cell lysis can be achieved. Various techniques for cell immobilization, such as adsorption on surfaces (Mazzer *et al.*, 2008), covalent bonding to carriers (Atanasova *et al.*, 2009), entrapment in a polymer gel (Kunamneni *et al.*, 2007) and self-aggregation (Yang *et al.*, 2006), have been used to obtain cells with high levels of enzyme production and stability (Chen *et al.*, 2004). Therefore, the choice of immobilization technique and the mechanical properties of the matrix are significant factors affecting the enzyme activity and long-term stability of biocatalysts obtained from immobilized cells.

Calcium alginate, glass beads, polyacrylamide gel, alginate, silanized magnetite, agarose, polyurethane foam and carrageenan are the most commonly used matrices for the immobilization of cells. However, the use of alginate for gel entrapment is insufficient because the solidification of the gel requires the use of calcium chloride, which reduces the pH of the mixture and brings a negative effect on cell growth (Atanasova *et al.*, 2009). Furthermore, covalent binding to a matrix has the major disadvantages of high cost and low yield because of exposure of the cells to toxic reagents and rigorous reaction conditions. The development of cell immobilization techniques using hollow fiber membranes has attracted considerable interest because of the high surface-to-volume ratio that can be obtained with this type of membrane (Wang *et al.*, 1991). Several studies have been conducted using hollow fiber membranes as a matrix for the production of β-lactamase (Inloes *et al.*, 1983a), ammonia (Wang *et al.*, 1991), ethanol (Mehaia and Cheryan, 1984, Shukla *et al.*, 1989) and ethylene (Lloyd and Bunch, 1996).

In the present study, a hollow fiber membrane was employed as a matrix for cell immobilization to increase the excretion of cyclodextrin glucanotransferase (CGTase) with a low occurrence of cell lysis. *E. coli* were immobilized via adsorption and entrapment within the polymeric matrix based on the high porosity of the membrane. The major benefit of immobilization through adsorption is direct contact between nutrients and the matrix. Moreover, the limitation of diffusion imposed by cell immobilization through entrapment in a hollow fiber membrane is less severe because of the presence of liquid medium in the intra-capillary space of the membrane. In addition to being readily available and inexpensive, hollow fibers were selected as the matrix because of several other desirable properties, such as high mechanical strength, operational durability and lack of toxicity. Another advantage of hollow fiber membranes is the free exchange of nutrients and metabolic products, given the short diffusion distance between cells and the liquid medium (Bunch, 1988).

Most studies for CGTase production thus far have focused on attachment to cotton (Kriaa *et al.*, 2012); entrapment in different gels, such as polyacrylamide, agar, alginate and polyvinyl alcohol-cryogel gels (Abdel-Naby *et al.*, 2000, Martins *et al.*, 2003, Kunamneni *et al.*, 2007, Mazzer *et al.*, 2008); or covalent linking to a flat sheet membrane (Atanasova *et al.*, 2009). To the best of our knowledge, there have been no studies on the excretion of CGTase by *E. coli* that have been immobilized through a combination of adsorption and entrapment of the cells using a hollow fiber membrane. The results presented here suggest that immobilized cell is a promising method for CGTase excretion with less occurrences of cell lysis.

1.2 Objectives of the study

1. To improve CGTase excretion of immobilized recombinant *E. coli* on the hollow fiber membrane in comparison with free cell.
2. To increase cell viability with high plasmid stability of immobilized recombinant *E. coli* on the hollow fiber membrane.

1.3 Scopes of the study

Scopes of this study are:

a) Screening the effect of the culture conditions (polymer membrane, expression medium, time for cell immobilization, IPTG, post induction temperature, post induction time, agitation rate and pH) on enhancement of CGTase excretion and cell viability of the immobilized cell using one factor at one time method (OFAT).

b) Determination of significant factors of culture conditions (IPTG, post induction temperature, post induction time, agitation rate and pH) on the immobilized cell for the increment of CGTase excretion and cell viability using full factorial design (FFD).

c) Optimization of the culture conditions (IPTG, post induction temperature and pH) of the immobilized cell for the excretion of recombinant CGTase and reduction of cell lysis using response surface methodology (RSM).

d) Determination of the effect of expression medium formulation and cross-linkers on the immobilized cell for the improvement of CGTase excretion with less occurrence of cell lysis.

e) Production of high-level CGTase excretion of immobilized cell in the membrane bioreactor.

1.4 Problem statement and novelties of the study

In recent years, there has been great interest in the production of heterologous proteins by utilizing recombinant DNA technology. Some of these heterologous proteins have already been produced at the industrial level. However, the recombinant protein products accumulate in the microorganisms especially when using *Escherichia coli* as a host. Therefore, the excretion of these proteins to the external medium would be attractive with regard to further downstream processing and the stability of the protein product. However, the excretion of heterologous proteins remains problematic. In *E. coli,* excretion always correlates with non-specific leakage and cell lysis due to pressure build-up in the periplasmic space by high level production of the recombinant protein. Thus, it becomes a challenge when the recombinant protein needs to be excreted with low cell lysis. Using the cell immobilization method, increased protein excretion with reduction in cell lysis can be achieved.

There are three novelties of the study. The novelties of the study are as follows:

a) The cell immobilization method improves the excretion of recombinant protein with increase in cell viability and plasmid stability as compared to the conventional fermentation (free cell).

b) Cell immobilization on hollow fiber membrane involves adsorption technique with three interactions (two electrostatic interactions and one hydrophobic interaction).

c) High CGTase excretion with low cell lysis of immobilized cell using low tryptone concentration and non-treated hollow fiber membrane in reusability process.

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