SURFACE DISPLAY OF ENDOGLUCANASE AND β–GLUCOSIDASE USING ICE NUCLEATION PROTEIN A FROM *Erwinia ananas* ON *Escherichia coli*

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DEDICATION

To my beloved father, mother, brother and sister

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ABSTRACT

Cells disruption for obtainment of targeted protein has increased the cost for expression of recombinant protein in *Escherichia coli*. Cell surface display system is one of the approaches to resolve this issue. The objective of this research is to study expression and characterization of surface display cellulases on *E. coli* using ice nucleation protein A (InA) anchor protein from *Erwinia ananas*. Cellulases such as endoglucanase Cel5A (EC 3.2.1.4), exoglucanase Cel9E (EC 3.2.1.91) and β-glucosidase BglC (EC 3.2.1.21) fused to the C-terminal of InA were expressed in *E. coli*. InA-Cel5A and InA-BglC were shown to be displayed on the cell surface by analyzing outer membrane protein on SDS-PAGE and Western blot. Enzyme assay and immunofluorescence microscopy analysis showed that InA-Cel5A and InA-BglC were functionally expressed on the cell membrane. InA-Cel9E was not successfully displayed on cell surface. The optimum temperature and pH of surface display InA-Cel5A and InA-BglC were 60 ºC and pH 7, respectively. Optimization of cultivation conditions of InA-Cel5A and InA-BglC were carried out at different post induction time, medium, post induction temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration. The optimized conditions obtained for expression of InA-Cel5A were M9 medium, 15 ºC induction temperature, 0.1 mM IPTG and 14 hours, which gave endoglucanase activity of 0.6537 U/mL. The optimized conditions obtained for expression of InA-BglC were M9 medium, 30 ºC induction temperature, 0.1 mM IPTG and 6 hours, which gave β-glucosidase activity of 198.439 U/mL. InA-Cel5A and InA-BglC were used for cellulose hydrolysis. Surface display InA-Cel5A and InA-BglC were used to degrade 4 % (w/v) Avicel at 50 ºC and 200 rpm, producing 0.204 mg/mL reducing sugars but with low glucose concentration. The optimum ratio of InA-Cel5A and InA-BglC used in the hydrolysis was found to be 4: 1. Results indicated that InA-Cel5A and InA-BglC were successfully displayed on *E. coli* using InA. Nevertheless, such recombinant *E. coli* showed a low hydrolytic activity and only low glucose concentration can be detected. The success of displaying enzyme on *E. coli* using ice nucleation protein showed great potential to be used in whole-cell biocatalysis.

ABSTRAK

Pemusnahan sel untuk mendapatkan protein sasaran telah menyebabkan kos penyataan protein rekombinan dalam *Escherichia coli* tinggi. Sistem paparan permukaan sel adalah salah satu pendekatan untuk menyelesaikan isu ini. Objektif kajian ini adalah untuk mengkaji penyataan dan pencirian selulase yang dipamerkan di permukaan *E. coli* dengan menggunakan protein A nukleasi ais (InA) sebagai protein penyangkut daripada *Erwinia ananas*. Selulase seperti endoglukanase Cel5A (EC 3.2.1.4), eksoglukanase Cel9E (EC 3.2.1.91) and β-glukosidase BglC (EC 3.2.1.21) yang digabung dengan terminal C InA dinyatakan dalam *E. coli.* Analisis membran protein luar pada SDS-PAGE dan Western blot menunjukkan InA-Cel5A dan InA-BglC berada di permukaan sel. Analisis aktiviti enzim dan mikroskopi immunofluoresen menunjukkan penyataan InA-Cel5A dan InA-BglC berfungsi di atas membran sel. InA-Cel9E tidak berjaya dipamerkan di permukaan sel. Suhu dan pH optimum bagi paparan permukaan InA-Cel5A dan InA-BglC adalah masing-masing pada 60 °C and pH 7. Pengoptimuman keadaan penanaman untuk InA-Cel5A and InA-BglC dilakukan pada masa selepas induksi, medium, suhu selepas induksi dan kepekatan isopropil β-D-1-thiogalaktopiranosida (IPTG) yang berbeza. Keadaan optimum bagi penyataan InA-Cel5A adalah medium M9, suhu induksi 15 °C, IPTG pada kepekatan 0.1 mM selama 14 jam dengan penghasilan aktiviti endoglukanase sebanyak 0.6537 U/mL. Keadaan optimum bagi penyataan InA-BglC adalah medium M9, suhu induksi 30 °C, 0.1 mM IPTG selama 6 jam dengan penghasilan aktiviti β-glukosidase sebanyak 198.439 U/mL. InA-Cel5A dan InA-BglC digunakan dalam hidrolisis selulosa. Paparan permukaan InA-Cel5A dan InA-BglC digunakan untuk menghidrolisis 4% (w/v) Avicel pada 50 °C dan 200 rpm, menghasilkan 0.204 mg/mL gula penurun dengan kepekatan glukosida yang sangat rendah. Nisbah optimum InA-Cel5A dan InA-BglC yang digunakan dalam hidrolisis adalah 4: 1. Keputusan ini menunjukkan bahawa InA-Cel5A dan InA-BglC berjaya dipamerkan di permukaan *E. coli* dengan menggunakan InA. Walaubagaimanpun, *E. coli* rekombinan tersebut menunjukkan aktiviti hidrolitik yang rendah dan hanya kepekatan glukosa yang rendah dapat dikesan. Kejayaan memaparkan enzim menggunakan protein nukleasi ais di permukaan *E. coli* menunjukkan potensinya untuk digunakan dalam pemangkinan-bio sel keseluruhan.

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LIST OF SYMBOLS AND ABBREVIATIONS

BSA	-	bovine serum albumin
BglC	-	β-glucosidase
CMC	-	carboxylmethylcellulose
Cel5A	-	endoglucanase
Cel9E	-	exoglucanase
cGTase	-	cyclodextrin glucanotransferase
C-terminal	-	the carboxyl-terminal (-COOH) of a polypeptide
C. cellulolyticum	-	*Clostridium cellulolyticum*
C. thermocellum	-	*Clostridium thermocellum*
Da	-	dalton
DNS	-	3,5-Dinitrosalicylic acid
DMSO	-	dimethyl sulphoxide
E. coli	-	*Escherichia coli*
EDTA	-	ethylenediamine tetraacetic acid
g	-	gram
h	-	hour
InA	-	ice nucleation protein A
INP	-	ice nucleation protein
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
kDa	-	kilodalton
l	-	liter
lac	-	lactose
LB	-	Luria-Bertani
lpp	-	lipoprotein
m	-	mili
mg	-	milligram
$MgSO_4$	-	magnesium sulphate
min	-	minute
mL	-	milliliter
mM	-	milimolar

MW	-	molecular weight
Na_2HPO_4	-	disodium hydrogen phosphate
NaCl	-	sodium chloride
NEB	-	New England Biolabs
N-terminal	-	the amino-terminal (NH_2) of a polypeptide
nm	-	nanometer
OD	-	optical density
OMP	-	outer membrane protein
PBS	-	phosphate buffered saline
pH	-	isoelectric point
PASC	-	Phosphoric acid swollen cellulose
PEG	-	polyethylene glycol
pnp	-	p-nitrophenol
pnpg	-	p-nitrophenyl glucopyranoside
P. putida	-	*Pseudomonas putida*
rpm	-	revolutions per minute
s	-	second
SecA	-	pre-protein translocase
SOB	-	Super Optimal broth
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TB	-	Terrific broth
TE	-	tris EDTA
TSS	-	transformation and storage solution
T. fusca	-	*Thermobifida fusca*
sp.	-	species
U	-	unit
UV	-	ultraviolet
UV-VIS	-	ultraviolet-visible spectrophotometry
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight

α	-	alpha
β	-	beta
°C	-	degree celcius
µ	-	micro
µm	-	micro meter
-	-	minus
%	-	percent

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CHAPTER 1

INTRODUCTION

1.1 Background

Microbes are favored sources for industrial enzymes because of several advantages such as the ease of product modification and optimization, fast growth rate and simpler cultivation on cheaper substrates (Gurung *et al.*, 2013). Development of genetic engineering and protein engineering has led to the more economical production of recombinant protein due to the producing organism could be easily engineered to produce desired amount of enzymes. To date, the availability of genetic tool has been developed for the high level production of proteins. Production of recombinant protein involves cloning of desired DNA into vector and the desired protein is amplified in appropriate expression system (Schumann and Ferreira, 2004). The ability to grow rapidly at high cell density, easy manipulation and low cost have made *Escherichia coli* to be one of the most frequently used microorganism for recombinant production (Jong *et al.*, 2010; Sivashanmugam *et al.*, 2009).

However, *E. coli* is incapable in secreting proteins into extracellular medium and it is unlikely to produce protein that require post-translational modification (Mergulhao *et al.*, 2005). Intracellular enzyme system protects enzyme from toxic and inhibitory reaction conditions. Despite of that, expression of intracellular protein in *E. coli* often results in the formation of insoluble aggregates or inclusion bodies (Mergulhao *et al.*, 2005). Intracellular system requires substrate or product to pass through the membrane for the reaction to occur inside the cell which increases downstream cost. Targeting the proteins into culture medium overcomes the problems of intracellular production and improves downstream processes, enabling

the secretion of folded protein at a lowered cost (Choi and Lee, 2004). However, cell free system requires freshly enzyme preparation and the preparation can only be used for once and then turns to waste, and thus leading to the increase of cost production (Schuurmann *et al.*, 2014).

To overcome these limitations, cell surface display is a technology that allow the protein of interest to be anchored on the outer surface of cell and freely access to the molecules or substrates in the exterior environment of the cell. This technology overcomes some drawbacks of using whole-cell biocatalysts that expressed enzyme intracellularly or secrete enzyme extracellularly such as laborious purification and transport limitations of substrate cross membrane for reaction to occur. Several different anchoring motifs available for Gram-negative bacteria including autotransporters (Sun *et al.*, 2015), outer membrane proteins (Lee *et al.*, 2013) and ice nucleation protein (INP) (Zhang *et al.*, 2016). Generally, surface display system consists of two main features such as a signal peptide to initiate the translocation of protein toward secretory pathway and a surface anchor protein that enables anchoring of the target protein on cell membrane.

Although surface display system have many advantages and a wide range of systems for display of heterologous protein on *E. coli* have been developed, size limitation of insert that can be expressed on cell membrane remains a considerable bottleneck (Han and Lee, 2015). Occasionally, large size of foreign protein has significant effect on cell membrane integrity which can result in growth inhibition and instability of outer membrane stability. Display efficiency of surface display system often related to the inefficient expression and secretion of target protein (Tanaka and Kondo, 2015).

Previously, ice nucleation protein A (InA) has been studied for its property of ice-nucleating when it was expressed in *E. coli* host (Abe *et al.*, 1989). Ice nucleation protein is an anchor protein consists of N- and C- terminal that has been used to display protein on cell surface. In this study, the truncated InA from *Erwinia ananas* was studied for its role as a surface display anchor. Cellulases were used as

the reporter proteins. The process of breaking down cellulose requires at least three cellulases including endoglucanase Cel5A (EC 3.2.1.4), exoglucanase Cel9E (EC 3.2.1.91), and β-glucosidase BglC (EC 3.2.1.21). The multi-step hydrolysis process has been hampered by the lack of efficient expression platforms in *E. coli* that allow a cost-effective production of cellulases. Therefore, in this study cellulases were displayed on *E. coli* using InA as the anchor protein, enabling direct application of whole-cell biocatalyst to the cellulose biomass, thereby reducing the high operation cost associated with cellulases production.

1.2 Problem Statement

Biocatalysts, especially enzymes are beneficial as they have high catalytic efficiency, high product selectivity and the ability to catalyze reactions under mild conditions. These characteristics make enzymes preferable to be used in industry compared to chemical catalysts. In the biocatalytic production process, recombinant enzyme preparation is time-consuming and often requires the additional process of cell disruption and purification, which causes enzyme loss, denaturation, and increased cost of production. *E. coli* has been known to be an efficient microorganism that has the ability to ferment sugars. However, *E. coli* cannot secrete saccharolytic enzymes outside its cell to hydrolyze polysaccharide. Therefore, it is crucial to conduct a study on developing whole-cell biocatalyst that can display enzymes on cell surface. Although surface display systems have many advantages and a wide range of systems for display of heterologous protein on *E. coli* have been developed, the stability of displayed enzyme and surface display efficiency of display system remains a considerable obstacle. Thus, it is interesting to study the surface display of saccharolytic enzymes such as cellulases on *E coli*.

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