EXOPROTEOME ANALYSIS OF *Bacillus lehensis* G1 IN pH REGULATION AND STARCH UTILIZATION FOR SCREENING OF SIGNAL PEPTIDES FOR CYCLODEXTRIN GLUCANOTRANSFERASE SECRETION IN *Escherichia coli*

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To my beloved parents, brothers and sister in law

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ABSTRACT

Bacillus lehensis G1 is an alkaliphilic bacterium that is capable of surviving in environment as extreme as pH 11. Gram-positive *Bacillus* species are among the bacterial winners in the production of secreted enzymes. However, until recently, there has been no study related to exoproteome profiling of *B. lehensis* under different cultural conditions. The aim of this study was to analyze exoproteome profile of *B. lehensis* G1 in alkaline and starch-supplemented medium to elucidate adaptive mechanism and to identify potential secretion aid molecules (i.e. signal peptides). The exoproteome was extracted from the cell culture supernatant, separated by two-dimensional electrophoresis, excised, digested with trypsin and analyzed using MALDI-TOF/TOF approach. A total of 85 proteins were identified in the extracellular space of *B. lehensis* G1 cultured under different conditions (i.e. pH8, pH10, pH11 and starch-supplemented medium). Alkaline adaptation that occurred in *B. lehensis* G1 includes 33 differentially expressed proteins such as GlcNAc-binding protein A, chitinase, endopeptidase lytE, cell surface protein, flagellar hook-associated proteins and enolase. At pH 8, 5 proteins were up-regulated and 13 proteins were down-regulated, whereas at pH 11, 14 proteins were up-regulated and 8 were down-regulated. In response to medium pH changes, majority of the differentially expressed proteins were involved in cell wall, main glycolytic pathways, metabolism of amino acids and related molecules, and some proteins of unknown function. The bacteria exhibited thinner flagella at pH 11 compared to pH 10. Meanwhile, when the cells were grown in starch-supplemented medium, 23 proteins were specifically expressed including carbohydrate-degrading enzymes (e.g. cyclodextrin glucanotransferase (CGTase), glucanases and prokaryote trehalose catabolism protein ThuA). The identified extracellular proteins and their associated signal peptide could serve as an alternative to solve the problems encountered during the extracellular secretion in *E. coli* such as low secretion and cell lysis. Therefore, 14 signal peptides obtained through exoproteome analysis of *B. lehensis* G1 were selected and cloned together with CGTase in *E. coli* to assist its secretion across the cytoplasmic membrane. All clones were found to allow CGTase to be excreted into the medium as observed and measured by iodine plate assay and enzyme activity assay. Compared to native signal peptide of CGTase (G1), signal peptide of GlcNAc-binding protein A (GAP) significantly improved CGTase activities by 735% and 205% in extracellular and periplasmic compartment, respectively, with an increase of only ~1.7 fold the amount of β-galactosidase (cell lysis indicator) in the medium. Moreover, GAP has the highest secretion rate of 45.6 U/ml/hr among all clones, where physicochemical characteristics of signal peptide play significant role. GAP from this study secreted the highest amount of CGTase extracellularly (637.5 U/ml) in *E. coli* compared to *E. coli* signal peptides such as pelB and OmpA.

ABSTRAK

Bacillus lehensis G1 adalah bacteria alkalofilik yang mampu bertahan dalam persekitaran setinggi pH 11. Spesies *Bacillus* Gram-positif adalah antara bakteria terbaik dalam penghasilan enzim yang dirembes. Walaubagaimanapun, sehingga kini, tiada kajian dijalankan berkaitan profil exoproteome *B. lehensis* G1 dalam keadaan pengkulturan yang berbeza. Tujuan kajian ini adalah untuk menganalisa profil *B. lehensis* G1 dalam medium beralkali dan berkanji bagi menjelaskan mekanisme penyesuaian bakteria dalam persekitaran berbeza dan mengenal pasti molekul bantuan rembesan yang berpotensi (iaitu peptida isyarat). Exoproteome diekstrak daripada supernatan kultur sel, dipisahkan oleh elektroforesis dua dimensi, dicerna dengan trypsin dan akhirnya dianalisa menggunakan alat MALDI-TOF/TOF. Sebanyak 85 protein telah dikenalpasti di ruang ekstraselular *B. lehensis* G1 yang dikultur dalam keadaan yang berbeza (iaitu pH8, pH10, pH11 dan medium ditambah kanji). Penyesuaian alkali yang berlaku dalam *B. lehensis* G1 melibatkan 33 protein yang diekspres secara berbeza seperti protein GlcNAc-mengikat A, chitinase, endopeptidase lytE, protein permukaan sel, protein berkait flagelar dan enolase. Pada pH 8, 5 protein dikawal-naik dan 13 protein dikawal-turun, manakala pada pH 11, 14 protein dikawal naik dan 8 dikawal turun. Ekoran tindak balas kepada perubahan pH, majoriti protein yang diekspres secara berbeza terlibat dalam dinding sel, laluan glikolitik utama, metabolisme asid amino dan molekul yang berkaitan, dan beberapa protein yang fungsi tidak diketahui. Flagella yang dihasilkan pada pH 11 lebih nipis berbanding pH 10. Apabila bakteria ini dikultur dalam medium yang ditambah kanji, 23 protein diekspres secara khusus termasuk enzim yang menguraikan karbohidrat (seperti cyclodextrin glucanotransferase (CGTase), glucanases dan prokaryote trehalose protein katabolisme ThuA). Protein ekstraselular yang dikenalpasti dan peptida isyaratnya boleh bertindak sebagai suatu alternatif bagi mengatasi masalah yang dihadapi ketika rembesan ekstraselular di *E. coli* seperti rembesan yang sedikit dan lisis sel. Oleh itu, 14 peptida isyarat yang diperoleh melalui analisis exoproteome *B. lehensis* G1 dipilih dan diklon bersama CGTase dalam *E. coli* untuk membantu dalam rembesannya merentasi membran sitoplasma. Semua klon didapati membenarkan CGTase dirembes ke dalam medium, sebagaimana yang diperhatikan dan diukur melalui kaedah plat iodin dan aktiviti enzim. Berbanding dengan peptida isyarat asli CGTase (G1), isyarat peptida protein A yang mengikat GlcNAc (GAP) menunjukkan peningkatan aktiviti CGTase yang ketara sebanyak 735% dan 205% dalam bahagian ekstraselular dan periplasmik, disamping itu hanya ~ 1.7 kali ganda dalam peningkatan jumlah β-galactosidase (penunjuk lisis sel) dalam medium. GAP mempunyai kadar rembesan tertinggi sebanyak 45.6 U/ml/jam berbanding semua klon, di mana ciri-ciri fizikokimia peptida isyarat berperanan penting. GAP daripada kajian ini merembes jumlah CGTase yang tertinggi (637.5 U/ml) secara ekstraselular di *E. coli* berbanding dengan peptida isyarat *E. coli* seperti pelB dan OmpA.

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LIST OF SYMBOLS

cm	-	centimeter
g	-	gram
h	-	hour
L	-	liter
M	-	molar
ml	-	mililiter
mM	-	milimolar
ng	-	nanogram
nm	-	nanometer
ppm	-	parts-per notation
rpm	-	revolutions per minute
%	-	percentage
ºC	-	degree celsius
µg	-	microgram
µl	-	microliter
µm	-	micromolar

LIST OF ABBREVIATIONS

ABC transporter	-	ATP- binding cassette transporter
ACN	-	acetonitrile
Ala	-	alanine
ATP	-	adenosine-5-triphosphate
BGL	-	signal peptide of endo-beta-1,3-glucanase
BGL(4)	-	signal peptide of endo-1,3(4)-beta-glucanase 1
B. lehensis	-	*Bacillus lehensis*
bp	-	base pair
BSA	-	bovine serum albumin
$CaCl_2$	-	calcium chloride
CD	-	cyclodextrin
CGTase	-	cyclodextrin glucanotransferase
CHAPS	-	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chit	-	signal peptide of chitinase
CM	-	cell membrane
CSP	-	signal peptide of cell surface protein
CWB	-	cell wall binding repeats
Cyt	-	cytoplasm
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleotide triphosphate
DTT	-	dithiothreitol
ECDS	-	signal peptide of endonuclease/CDSuclease/phosphatase
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenedianetetra-acetate

Extr	-	extracellular
Ffh	-	fifty four homolog
G1	-	signal peptide of cyclomaltodextrin glucanotransferase
GAP	-	signal peptide of GlcNAc-binding protein A
GTP	-	guanosine triphosphate
H+	-	proton
HCCA	-	α-cyano-4-hydroxycinnamic acid
HCl	-	hydrochloride
hsp	-	heat shock protein
IAA	-	iodoacetamide
IEF	-	Isoelectric Focusing
IM	-	inner membrane
IPG	-	immobilized pH gradient
kDa	-	kilo dalton
lac	-	lactose
LB	-	Luria-Bertani
LPS	-	lipopolysaccharides
LytE	-	signal peptide of endopeptidase lytE
MALDI-TOF/TOF	-	matrix-assited laser desorption and ionization time-of-flight/time-of-flight
MEP	-	signal peptide of minor extracellular protease
min	-	minute
Na^+	-	sodium ion
NEB	-	New England Biolabs
OD_{600nm}	-	optical density at wavelength 600 nm
OM	-	outer membrane
ONPG	-	Ortho-Nitrophenyl-β-galactoside
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
pI	-	isolelectric point
PL	-	phospholipids
PLGS	-	ProteinLynx Global Server
pmf	-	proton motive force

PMF	-	peptide mass fingerprinting
PFF	-	peptide fragment fingerprinting
R	-	arginine
RNA	-	ribonucleic acid
SDS	-	sodium dodecyl sulfate
Sec	-	secretory
SEM	-	scanning electron microscope
SP	-	signal peptide
sp.	-	spesies
SPase	-	signal peptidase
SPPase	-	signal peptide peptidase
SRP	-	signal recognition particle
S-S	-	disulfide
T2SS	-	Type two secretion system
TAE	-	Tris-acetic acid-EDTA
Tat	-	twin-arginine translocase
TB	-	Terrific Broth
TCA	-	Trichloroacetic acid
TE	-	Tris-EDTA
TEM	-	transmission electron microscope
TEMED	-	N,N,N’,N’-tetramethylethylenediamine
TFA	-	Trifluoroacetic acid
TNPP	-	signal peptide of trifunctional nucleotide phosphoesterase protein
V	-	variety
v/v	-	volume per volume
w/v	-	weight per volume
1848	-	signal peptide of hypothetical protein AIC94426
1853	-	signal peptide of hypothetical protein AIC94431
3542	-	signal peptide of hypothetical protein AIC96089
3571	-	signal peptide of hypothetical protein AIC96118
β-CD	-	beta-cyclodextrin
2D	-	second dimension

2DE	-	second dimension electrophoresis
μ_{max}	-	maximum specific growth rate

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CHAPTER 1

INTRODUCTION

1.1 Background of study

In 1971, the first paper concerning an alkaline enzyme (protease) produced by *Bacillus* species was published (Horikoshi, 1971). The on-going research provides useful source of enzyme in industrial and medical applications (e.g. contact lens cleaning and bleach industry) (Joo *et al.*, 2004). *Bacillus* sp. can secrete numerous proteins such as amylase, xylanase and cellulase into their environment and the quantitites can reach gram per litre (Schallmey *et al.*, 2004). The high secretion capability of *Bacillus* sp. have attracted detailed investigation on their extracellular proteins or exoenzymes and the protein export mechanism (van Dijl and Hecker, 2013; Fujinami and Fujisawa, 2010; Harwood and Cranenburgh, 2008).

The variations in growth conditions (e.g. pH and nutrient availability) could affect microbial inhabitants leading to slow growth or cell death. Bacteria such as *Bacillus* sp. must have exhibited complex genetic and physiological changes to minimize, adapt to and repair damage caused by environmental disturbances. For example, under glucose-depleted medium, a few bacteria are able to secrete CGTase converting starch to cyclodextrins and intracellular conversion of cyclodextrins to glucose-6-phosphate (Labes and Schönheit, 2007; Fiedler *et al.*, 1996). Exoproteomic analyses of cold- and alkaline-adapted bacteria revealed a high abundance of six secreted proteases in the medium which are involved in complete degradation of extracellular protein (Lylloff *et al.*, 2016). However, a simultaneous exoproteome change to counteract alkaline stress is still unknown.

Proteomics has been widely used for large-scale protein analyses to understand the resulting physiological changes from stress-responsive proteomes and the identification of useful secretion aid molecules from membrane or extracellular proteomes. The proteins observed on the 2-DE can be used as potential targets to improve the production performance of recombinant cell factories. A good example of that was reported by Qian *et al.* (2008). The authors found that among the 22 potential fusion partners observed on the 2-DE gel, OsmY was identified as the most efficient excreting partner for extracellular production of recombinant proteins in *E. coli*. The strategy of employing the valuable information of naturally identified secreted proteins is useful for screening of secretion aid molecules (e.g. signal peptides) for recombinant protein secretion in *Escherichia coli* (*E. coli*). The existing known export components (e.g. signal peptides) of *Bacillus* sp. and *E. coli* proposed that gram-positive and gram-negative bacteria shared similar mechanism of protein translocation across the cytoplasmic membrane (Harwood and Cranenburgh, 2008).

E. coli has been the most widely used microorganism for recombinant proteins production (Baneyx, 1999) with a well studied genetics background, fast growth rate and high production levels which can be achieved in cost-effective way (Jong *et al.*, 2010). However, *E. coli* does not naturally secrete proteins into the extracellular medium under standard laboratory conditions (Pugsley and Francetic, 1998). Therefore, it is a challenge to engineer *E. coli* in producing high level of secreted proteins with minimum occurrence of cell lysis (Low *et al.*, 2012). Many attempts have been devised to improve secretion of recombinant proteins in engineered *E. coli* cells because extracellular proteins production offers simpler bioprocess and better product quality. One strategy is to use optimal signal peptide. A signal peptide contains information needed to direct desired protein to the translocation pathway thus has great influence in protein secretion (Gouridis *et al.*, 2009). There are several examples of the use of *Bacillus* sp. signal peptides in *E. coli* host such as secretion of human leptin (Jeong and Lee, 2000) were reported. Although signal peptides from *Bacillus* sp. were proven to work well in *E. coli* expression system, it is interesting to note that, there is a need for individually

optimal signal peptides for every recombinant protein secretion (Brockmeier *et al*. 2006).

Previously, CGTase from *Bacillus* sp. G1 fused with its native signal peptide was cloned into *E. coli* host and it was successfully secreted to the extracellular space (Ong *et al.*, 2008). Secretion of CGTase is highly desirable as it could leads to easier purification steps and improves product quality. CGTase is an enzyme which has enabled the extensive use of cyclodextrin in foodstuffs, pharmaceuticals and chemicals. Therefore, significant efforts in enhancing the enzyme secretion is required, for example, mutations of the native signal peptide enhanced CGTase secretion but still exhibited a high cell lysis rate (Jonet *et al.*, 2012). Hence, in this study, the high-throughput proteomic analysis of the naturally exported proteins of *Bacillus lehensis* G1 was conducted to allow identification of its associated signal peptides for the excretory protein production in *E. coli*.

1.2 Problem Statement and novelties of study

Alkaliphilic *Bacillus* sp. has been known to be an efficient microorganism in the production of enzymes. Its capability to secrete significant amount of enymes and other biochemicals into the extracellular culture medium make this organism as a good choice for industrial production. However, there is still much exploration required in research tapping the secretion mechanism and process that the bacteria have. Compare to *Bacillus* sp. which has been the best workhorse for industrial protein production, *E. coli* cannot express and secrete protein outside its cell. Moreover, extracellular secretion of recombinant proteins in engineered *E. coli* cells often encounters problems such as low secretion and cell lysis. Therefore, more exploration work is needed to improve its secretion system and one of them is by utilizing heterologous secretion system that *Bacillus* sp. have.

1.3 Objectives of study

1) To discover exoproteome of *B. lehensis* G1 under different medium pH conditions and carbon sources.
2) To identify new signal peptide for higher CGTase secretion potential

1.4 Scope of study

The scope of this study was:

1. Proteomic analysis of extracellular proteins expression of cell growth in different medium pH and carbon sources.
2. Computational analysis and mining of genomic data for secretion aid molecules (i.e. signal peptides).
3. Cloning of signal peptides in *E. coli* using CGTase as a reporter protein.
4. Analysis of expression and secretion efficiency through cell viability, cell integrity, protein secretion level and location and protein solubility.

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APPENDIX A: List of publications

1. Ling How Lie, Zaidah Rahmat, Low Kheng Oon, Nor Muhammad Mahadi, Rosli Md. Illias (2013). Computational analysis of signal peptide in *Bacillus lehensis* G1 genome database. *International Congress of the Malaysian Society for Microbiology (ICMSM)*. 12-15 Dec 2013, Langkawi, Kedah, Malaysia.

2. Ling How Lie, Zaidah Rahmat, Nor Muhammad Mahadi, Rosli Md. Illias (2014). Qualitative proteomic analysis of an alkaliphile *Bacillus lehensis* G1. *National Postgraduate Seminar (NPS)*. 10 Sept 2014, UPM Serdang, Selangor, Malaysia.

3. Ling How Lie, Zaidah Rahmat, Nor Muhammad Mahadi, Rosli Md. Illias (2014). Exploring secretome of an alkaliphile *Bacillus lehensis* G1 using gel-based approach. *32nd Symposium of the Malysian Society for Microbiology (MSM)*. 6-8 Dec 2014, Terengganu Equestrian Resort (TER), Kuala Terengganu, Malaysia.

4. How Lie Ling, Zaidah Rahmat, Nor Muhammad Mahadi, Rosli Md. Illias (2015). Proteome-based identification of potential fusion partner for extracellular production of recombinant proteins. *The 20th Biological Sciences Graduate Congress (20th BSGC)*. December 9-11, 2015. Bangkok, Thailand.

5. How Lie Ling, Zaidah Rahmat, Abdul Munir Abdul Murad, Nor Muhammad Mahadi, Rosli Md. Illias. (2017). Data for proteome analysis of *Bacillus lehensis* G1 in starch-containing medium. *Data in Brief*. 14: 35-40. https://doi.org/10.1016/j.dib.2017.07.026.

6. How Lie Ling, Zaidah Rahmat, Abdul Munir Abdul Murad, Nor Muhammad Mahadi, Rosli Md. Illias. (2017). Proteome-based identification of signal peptides for improved secretion of recombinant cyclomaltodextrin

glucanotransferase in *Escherichia coli*. *Process Biochemistry*. 61: 47-55. https://doi.org/10.1016/j.procbio.2017.06.018.

7. How Lie Ling, Zaidah Rahmat, Farah Diba Abu Bakar, Abdul Munir Abdul Murad, Rosli Md. Illias (2017). Secretome analysis of alkaliphilic bacterium *Bacillus lehensis* G1 in response to pH changes. *Microbiological Research*. (Submitted).