SEQUENCE ANALYSIS OF STARCH DEGRADING ENZYMES FROM *Anoxybacillus* SPECIES

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My genuine dedications to,

My beloved mom and doting siblings,

Lulinna, Dorris, and William;

My enduring supervisor Dr. Goh Kian Mau;

My compassionate friends;

And the love ones;

Who are always there for me,

Every step of the way,

Thanks!

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ABSTRACT

The thermophilic *Anoxybacillus* sp. SK3-4 was formerly isolated from Sungai Klah hot spring, Malaysia. In a recent work, the genome of this bacterium was sequenced and the presence of several amylolytic enzymes was identified. Starch degrading enzymes have been greatly involved in several types of industrial applications typically for degradation and convertion to value-added sugars. Present work aimed to clone pullulanase type I, α-glucosidase, and pullulanase type II (amylopullulanase, Apu) and analyze the primary sequences through bioinformatic tools. The full length for pullulanase type I from *Anoxybacillus* was 2121bp (706 amino acids) with theoretical molecular weight of 82kDa while the α-glucosidase gene was 2343bp (780 amino acids, 90.5kDa). The Apu was 6102bp (2033 residues, 224.8kDa) and appeared to be huge in the size. Based on the analysis using SAPS software, the proteins were mainly constituted of hydrophobic amino acids. Pullulanase type I and α-glucosidase are most likely expressed inside the cell while Apu is an extracellular protein due to the presence of signal peptide. According to similarity search using Blastp, pullulanase and α-glucosidase shared higher similarities with counterpairs from *Geobacillus* and relative lower to *Bacillus* species. Most of these closest match sequences are deduced proteins obtained from genome sequencing data; hence the related information is thus limited. From conserved domain search, α-glucosidase is a member of Glycosyl Hydrolase (GH) 31 family, and was further proved by the evolutionary tree. The Apu exhibited a single active site for hydrolyzing α-1,4 and α-1,6 linkages in starch and is different to amylopullulanase from *Bacillus* sp. KSM1378 and *Bifidobacterium breve* UCC2003 that had dual catalytic properties. The *Anoxybacillus* Apu also has a surface-layer homology (SLH) domain at C-terminal which functions as an anchor that binds to the cell wall. In conclusion, based on the bioinformatic analysis, it is shown that the studied enzymes from *Anoxybacillus* sp. SK3-4 are novel and unexplored and future work should be done to study the biochemical properties of these enzymes.

ABSTRAK

Anoxybacillus sp. SK3-4 adalah bakteria termofilik yang telah dipencilkan dari sumber air panas di Sungai Klah, Malaysia. Baru-baru ini, genom bakteria tersebut telah dijujukkan dan beberapa enzim amilolitik telah dikenalpasti. Enzim amilolitik telah banyak digunakan dalam pelbagai industri terutamanya dalam proses yang melibatkan degradasi dan penukaran kanji kepada gula tambah nilai. Kajian ini bertujuan untuk mengklon pululanase jenis I, α-glukosida, dan pululanase jenis II (amilopululanase, Apu) serta menganalisis urutan protein bagi enzim amilolitik tersebut dengan menggunakan aplikasi bioinformatik. Gen bagi pululanase jenis I adalah sepanjang 2121bp (706 asid amino) dengan saiz teori 82kDa manakala gen α-glukosida terdiri daripada 2343bp (780 asid amino, 90.5kDa). Apu pula bersaiz besar dan sepanjang 6102bp (2033 asid amino, 224.8kDa). Berdasarkan analisi perisian SAPS, protein-protein tersebut terdiri daripada asid amino yang bersifat hidrofobik. Pululanase jenis I dan α-glukosida berkemungkinan besar dirembes di dalam sel manakala kehadiran isyarat peptide pada Apu menunjukkan protein tersebut dirembes di luar sel. Berdasarkan percarian kesamaan menggunakan aplikasi Blastp, pululanase dan α-glukosida mempunyai persamaan yang tinggi dengan protein daripada spesis *Geobacillus* berbanding dengan spesis *Bacillus*. Kebanyakkan urutan terdekat enzim amilolitik itu merupakan simpulan protein daripada data genom. Oleh itu, maklumat yang berkaitan dengannya adalah terhad. Pencarian menggunakan domain kesamaan menunjukkan α-glukosida tergolong dalam kumpulan *Glycosyl Hydrolase* 31 (GH31). Ia turut dibuktikan dengan menggunakan pokok evolusi. Apu mempunyai tapak aktif tunggal untuk menghidrolisis ikatan α-1,4 and α-1,6 pada kanji dan ini berbeza daripada Apu daripada *Bacillus* sp. KSM1378 and *Bifidobacterium breve* UCC2003 yang mempunyai dua tapak aktif. *Anoxybacillus* Apu turut mempunyai domain bagi homologi lapisan permukaan (SLH) pada C-terminal enzim tersebut. Ia berfungsi untuk memegang Apu pada dinding sel. Kesimpulannya, analisis bioinformatik yang dijalankan menunjukkan bahawa enzim amilolitik daripada *Anoxybacillus* sp. SK3-4 adalah baru, belum diterokai dan kajian mengenai ciri-ciri biokimia enzim-enzim tersebut boleh dijalankan pada masa hadapan.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	-	alpha
β	-	beta
°C	-	degree celcius
µg	-	microgram
µL	-	microliter
A	-	adenine
Å	-	angstrom
A.	-	*Anoxybacillus*
Ala	-	alanine
AMP	-	adenine monophosphate
Apu	-	amylopullulanase
Asp	-	aspartic acid
B.	-	*Bacillus*
BLAST	-	Basic Local Alignment Search Tool
Blastp	-	protein BLAST
bp	-	base pair
C	-	cytosine
'C'	-	cysteine
C.	-	*Caldalkalibacillus*
Ca^{2+}	-	calcium ion
CDART	-	Conserved Domain Architecture Retrieval Tool
CDs	-	cyclodextrins

CSID	-	congenital sucrose-isomaltase deficiency disease
Cys	-	cysteine
DNA	-	deoxyribonucleic acid
dNTPs	-	Deoxynucleotide Triphosphates
E. coli	-	*Escherichia coli*
E.C	-	enzyme commission number
EDTA	-	ethylenediaminetetra-acetate
G	-	guanine
G.	-	*Geobacillus*
g/L	-	gram/liter
GH	-	glycosyl hydrolase
Glu	-	glutamic acid
GOR	-	Garnier-Osguthorpe-Robson
HCl	-	hydrochloric acid
HFCS	-	high fructose corn syrup
Kb	-	kilobase pairs
kDa	-	kilodalton
kPa	-	kilopascal
LB	-	Luria broth
MEGA	-	Molecular Evolutionary Genetics Analysis
Met	-	methionine
mL	-	milliliter
mm	-	millimeter
mM	-	millimolar
MW	-	molecular weight
NaOH	-	caustic soda
NCBI	-	National Center for Biotechnology Information
O.	-	*Ornithinibacillus*
PCR	-	polymerase chain reaction
PGIs	-	phosphoglucose isomerases
pI	-	isoelectric point

RNA	-	Ribonucleic Acid
RNase	-	ribonuclease
rpm	-	revolutions per minute
SAPS	-	Statistical Analysis of Protein Sequences
SCWPs	-	secondary cell wall polymers
SignalP	-	signal peptide
SIS	-	sugar isomerase domain
SK	-	Sungai Klah
SLH	-	S-layer homology
sp.	-	species
SPW	-	starch processing wastewater
T	-	threonine
T.	-	*Thermoanaerobacter*
TAE	-	Tris-acetate-EDTA
Thr	-	threonine
Trp	-	tryptophan
U.S	-	United States
UV	-	ultraviolet
V	-	volts
v/v	-	volume/volume
W	-	watts
w/v	-	weight/volume
x g	-	times gravity
X-gal	-	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Sucrose from sugar cane, sugar beets, honey or fruits is widely used as a sweetener in food industries and it plays a vital role in human diet for centuries. However, there are some restrictions of using sucrose by the way that sucrose is easily hydrolyzed in acidic condition and this leads to the abrupt change of sweetness and flavor of foods and beverages (White, 2008). It is also easily crystalline under certain conditions and it needs to be eliminated from those having inherited congenital sucrose-isomaltase deficiency disease (CSID) as they lack the enzyme sucrase for starch digestion (Treem, 1996).

Therefore, high fructose corn syrup (HFCS) seems to be an alternative sweetener to sucrose and has gained commercial values to be applied in industries. HFCS is of interest over sugar because of its extra sweetness, easier for blending, and low cost of production. HFCS is classified as high quality liquid sweetener because it is stable over

acidic conditions, highly soluble and non-crystalline, has the ability to avoid microbial growth and increased shelf life (Johnson *et al.*, 2009).

In the Western countries, many manufacturers prefer corn starch as raw materials for the production of HFCS because of its abundance. Conventionally, starch is degraded in two steps namely liquefaction and saccharification which needs high energy expenses and caused processing to be ineconomical (Sun *et al.*, 2009). Nowadays, various enzymes produced by plants, animals, or even microorganisms are involved in the large scale production of HFCS. This makes the starch processing industry a distinctive field in industrial biotechnology (Crabb and Shetty, 1999). Enzymes from microbial sources have gained considerable interest because of their advantages such as cost-effectiveness, consistency, efficient in production by saving time and space (Chai *et al.*, 2012), and ease for process modification and optimization (Sun *et al*., 2009).

Previous studies showed that starch is better hydrolyzed when increased the incubation temperatures to 60˚C. Therefore, thermostable starch degrading enzymes are of interest without losing the activity during prolonged incubation (Goyal *et al.*, 2005). Starch is first partially degraded by amylolytic enzymes during liquefaction process by α-amylase into a mixture of oligosaccharides and dextrins. A second enzyme involved in saccharification process is amyloglucosidase or known as glucoamylase, responsible for degradation of oligosaccharides into glucose monomers. However, the presence of amylose (1-4 linkages) and amylopectin (1-6 linkages) in starch causes the need of the enzyme pullulanase. This is because the enzyme amyloglucosidase acts slower in degrading the 1-6 linkages and ends up with the product isomaltose. Pullulanase acts as a debranching enzyme to eliminate α-1,6-glycosidic linkages with fast hydrolysis. However, the presence of the enzyme amylopullulanase gained advantage in this process as it acts to degrade both α-1,4-linkages and α-1,6-linkages in starch into monomers (Crabb and Mitchinson, 1997; Klosowski *et al.*, 2009).

Many members in the genus *Anoxybacillus* are mainly isolated from hot springs. They are mostly alkaliphilic or alkalitolerant, neutrophilic, and moderately thermophilic with optimum temperature of 50˚C to 62˚C. It has been reported that *A. flavithermus* (Bolton *et al.*, 1997) and *A. amylolyticus* (Poli *et al.*, 2006) have thermostable α-amylase activity, *A. gonensis* has xylose utilizing ability (Belduz *et al.*, 2003), *A. mongoliensis* has proteinase activity (Namsaraev *et al.*, 2010), and *A. salavatliensis* has α-glucosidase activity (Cihan *et al.*, 2011).

1.2 Problem Statement

In comparison to *Bacillus* and *Geobacillus*, *Anoxybacillus* is a less studied genus (Goh *et al.*, 2013). At the time of this writing, the genome online system shows that more than 3,400 and 170 bacterial and archaeal genomes, respectively, are complete (http://www.genomesonline.org). Only one *Anoxybacillus* genome has been reported (Saw *et al.*, 2008). The draft genome of locally isolated *Anoxybacillus* sp. SK3-4 (Chai *et al.*, 2011; Chai *et al*., 2012), was lately sequenced using Ion-Torrent Next-Generation sequencer. The technology uses semiconductor concept to measure hydrogen ions that are released during the polymerization of DNA (www.iontorrent.com). The turnover rate and cost for this machine is relatively better than some other sequencers. Nevertheless, the accuracy of the approach has been questioned (Quail *et al*., 2012).

This work is intended to amplify three selected genes using conventional PCR amplification and validate with the Ion-Torrent data. The selected genes are encoding for pullulanase, α-glucosidase, and amylopullulanase. Besides, the reported sequences are assumed to have a potential market value in various starch degrading industries. Therefore, the bioinformatics analysis of the sequences was carried out in this study.

1.3 Objectives

(i) To design primers and conduct PCR amplification for pullulanase type I, α-glucosidase, and pullulanase type II genes

(ii) To compare the cloned gene sequences obtained from conventional PCR amplification to the sequences found in whole genome sequencing that encode for starch degrading enzymes

(iii) To analyze the deduced protein sequences from genome sequencing data using bioinformatics tools

1.4 Scope of the Research

Primers design was carried out to amplify the genes that encode for starch degrading enzymes (pullulanase, α-glucosidase, and amylopullulanase) using PCR method from *Anoxybacillus* sp. SK3-4. Ligation of the genes into pGEMT vector and transformation into competent cell *E.coli* JM109 was done and screening of the positive clones using blue white screening. Bioinformatics analysis was carried out for homology search and the phylogenetic analysis of the studied enzymes using ClustalW and Mega softwares, respectively.

1.5 Significance of the Study

Starch is abundantly found in plants as the storage polysaccharides and is cost-effective to be used for conversion into value-added sugars, which are widely used in food industries. Starch degrading enzymes from microbial sources have gained advantage to be utilized in various industries. Therefore, studies based on starch degrading enzymes (pullulanase, α-glucosidase, and amylopullulanase) from locally isolated *Anoxybacillus* sp. SK3-4 was carried out in this research.

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