IN SILICO ANALYSIS AND TRUNCATION STUDIES OF THE C TERMINAL ALPHA AMYLASE FROM *ANOXYBACILLUS* SPECIES

LILLIAN SEA SHUN YI

A dissertation submitted in partial fulfillment of the
requirements for the award of the degree of
Master of Science (Biotechnology)

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

FEBRUARY 2015

To my beloved mother and father.

ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude to my supervisor, Dr. Goh Kian Mau, for his invaluable guidance, and insightful comments throughout the completion of this project. I am heartily appreciated all her encouragement, advices, supervision and patience, enabling me to accomplish the project with better understanding

Special thanks to all the labmates, Ummirul, Chai Kian Piaw, Chan Chia Sing, Amira and Lee Li Sin for their patience, guidance, support, encouragements and friendship. Additional thanks to laboratory assistance for providing needs and supplies throughout the journey of my project.

Last but not least, I would like to extend my deepest and sincere appreciation to my beloved family and friends for their love, support, tolerance and understanding in my life. Without their kindness, the progress of the project would difficult to accomplish.

ABSTRACT

Thermostable and alkalitolerant α-amylase from *Anoxybacillus* sp. SK3-4 (ASKA) yields high level of maltose (69%) from starch hydrolysis. A putative raw starch binding domain was found in the C-terminal domain of ASKA. Starch binding domain (SBD) plays a role in hydrolyzing insoluble starch which enhances the amylolytic rate. The gene that encodes the ASKA is 1,518 bp. The present study involved *in silico* analyses and protein mutagenesis of two C-terminal truncated variants, namely mutant Δ50 and Δ100. The respective mutants had an elimination of 81 and 112 amino acids from C-terminal end of ASKA. Firstly mutagenesis was made in *in silico* structure. Bioinformatics analysis revealed that the eight β-strand forms as an open-sided distorted β-sandwich fold which of putative domain C. Using Neighbor-Joining (NJ) method, the phylogenetic analysis of the domain C had confirmed that starch binding region of ASKA is the non-catalytic domain C and not the typical SBD of CBM families. Actual gene truncation was constructed via protein engineering approach and the truncated genes were expressed in *Escherichia coli*, under the promoter of T7. However, both recombinant proteins exhibited no activity with soluble starch as a result of inclusion bodies aggregation; despite several optimization on expression conditions were made.

ABSTRAK

α-amilase daripada *Anoxybacillus* sp. SK3-4 (ASKA) adalah enzim yang tahan haba, toleransi dalam keadaan beralkali dan mampu menghasilkan maltose dalam kadar yang tinggi (69%) daripada hidrolisis kanji. ASKA mempunyai domain kanji tidak terlarut pada domain terminal-C. Domain pengikat kanji (SBD) memainkan peranan yang penting untuk meningkatkan kadar amilolitik dalam proses menghidrolisis kanji. Gen ASKA adalah sepanjang 1,518 bp. Kajian ini melibatkan analisis in silico dan mutasi protein yang melibatkan pemotongan terminal-C, menghasilkan dua variasi berbeza iaitu mutan Δ50 dan Δ100. Mutan Δ50 dan Δ100 masing-masing kehilangan amino asid sebanyak 81 dan 112 daripada terminal-C ASKA. Peringkat pertama kajian ini melibatkan mutasi menggunakan pengstrukturan *in silico*. Analisis bioinformatik menunjukkan mutasi menyebabkan lapan lipatan-β pada terminal-C ASKA terganggu lalu membentuk kawasan terbuka pada lipatan-β tersebut. Analisis filogenetik menggunakan teknik Neighbor-Joining (NJ) mengesahkan bahawa domain C pada ASKA adalah domain bukan-kata litik yang lazimnya adalah SBD daripada keluarga modul pengikat karbohidrat (CBM). Peringkat kedua kajian ini adalah melibatkan pemotongan gen (Δ50 dan Δ100) menggunakan kaedah kejuruteraan protein dan kedua-dua mutan tersebut telah diekspresi dalam *Escherichia coli* yang mengandungi promoter T7. Namun, kedua-dua rekombinan enzim Δ50 dan Δ100 diekspresi dalam bentuk aggregasi gumpalan dan tidak menunjukan sebarang akiviti terhadap kanji terlarut walaupun beberapa optimasi terhadap parameter ekspresi telah dilakukan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3D	-	Three-dimensional
Arg	-	Arginine
ASKA	-	*Anoxybacillus* species SK3-4 alpha-amylase
Asp	-	Aspartate
BSA	-	Bovine serum albumin
C	-	Carbon
Ca^{2+}	-	Calcium ion
CAZy	-	Carbohydrate-Active enZymes
CBM	-	Carbohydrates-binding modules
CDART	-	Conserved Domain Architecture Retrieval Tool
Cys	-	Cysteine
DNA	-	Deoxyribonucleic acid
DNS	-	3,5-dinitrosalicyclic acid
DT	-	Dusun Tua
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	Ethylene glycol tetraacetic acid
GH(s)	-	Glycoside hydrolase(s)
Gln	-	Glutamine
Glu	-	Glutamate
GST	-	Glutathione S-transferase
Gt-amyII	-	*Geobacillus thermoleovorans* alpha-amylase
HCl	-	Hydrochloric acid
HFCS	-	High Fructose Corn Syrup
Ile	-	Isoleucine
IPTG	-	Isopropyl-β-D-thiogalactopyranoside

lac	-	Lactose
LB	-	Luria Bertani
Lys	-	Lysine
Met	-	Methionine
N	-	Nitrogen
NCBI	-	National Center for Biotechnology Information
NJ	-	Neighbor-joining
O	-	Oxygen
OD	-	Optical density
ORF	-	Open readinng frame
PCR	-	Polymerase Chain Reaction
PDB	-	Protein Database
pI	-	Isoelectric point
Pizzo	-	*Geobacillus thermoleovorans* subsp. *stromboliensis* alpha-amylase
RSDE	-	Raw Starch Degrading Enzymes
SBD	-	Starch-binding domain
SDS	-	Sodium Dodecyl Sulfate
SDS-PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	-	Scanning electron microscope
Ser	-	Serine
SK	-	Sungai Klah
SOC	-	Super Optimal broth with Catabolite repression
sp.	-	Species
subsp.	-	Subspecies
TIM	-	Triosephophate isomerase
TNTC	-	Too numerous to count
Tris	-	Tris(hydroxymethyl)methylamine
Trp	-	Tryptophan
Tyr	-	Tyrosine
Ub	-	Ubiquitin
UniProt	-	Universal Protein Resource
Val	-	Valine
NaCl	-	Sodium chloride

LIST OF SYMBOLS

%	-	Percentage
<	-	Less than
℃	-	Degree Celsius
∞	-	Infinity
bp	-	Base pair
Ca^{2+}	-	Calcium ion
g	-	Gravity
g/L	-	Gram per liter
h	-	Hour
kDa	-	Kilodalton
L	-	Liter
mg/mL	-	Microgram per microliter
min	-	Minute
mL	-	Milliliter
mM	-	Millimolar
ng	-	Nanogram
ng/µL	-	Nanogram per microliter
nm	-	Nanometer
pH	-	Power of hydrogen
rpm	-	Revolutions per minute
sec	-	Second
T_m	-	Melting temperature
w/v	-	Weight per volume
α	-	Alpha
β	-	Beta
µg/mL	-	Microgram per milliliter
µL	-	Microliter

μM	-	Micromolar
μmol	-	Micromoles per litre
φ	-	phi
ψ	-	psi

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of study

Starch is an essential carbohydrate source of food in human diet, which constituents a large proportion of food consumed. In plant, starch serves as an energy reservoir by storing in granule forms. The starch is densely packed with amylose and amylopectin, connecting by α-1,4- and α-1,6-glycosidic linkages. To manufacture starch-based products, starch is chemically or enzymatically processed into a variety of products such as starch hydrolysates, glucose syrups, fructose and starch or maltodextrin-based derivatives (El-Fallal *et al.*, 2012). Crops like maize, tapioca, potato and wheat are mainly used as an inexpensive source in various starch-processing industries (Uthumporn *et al.*, 2010). The typical starch hydrolysis requires two energy-intensive steps called liquefactions which involved partial hydrolysis and loss in viscosity and saccharification (the production of glucose and maltose via further hydrolysis). As starch is insoluble in cold water, a primary step, gelatinization, is needed and achieved by heating granular starch with water at high temperature (60-80 ℃) (Jœzef, 2007; Alvani *et al.*, 2012). The elevated temperature of the starch hydrolysis will lead to enzyme denaturation enzymes as some enzyme usually lose their activities at temperature above 50-60 ℃.

Raw starch degrading enzymes are mainly belonging to glycoside hydrolases (GH) 13, 14 and 15, which cleave glycosidic bonds either from the non-reducing end or in the interior of starch chemical structure (Puspasari *et al.*, 2013). These enzymes are multi-domain proteins that consist of a catalytic domain and a non-catalytic domain, namely carbohydrates-binding modules (CBM). CBMs with the affinity for insoluble raw starch are known as starch-binding domain (SBD) which localized frequently at C-terminal or scarcely at N-terminal in some amylolytic enzymes (Peng *et al.*, 2014). Based on sequence-based classification, SBDs belong to ten CBMs families: CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, CBM45, CBM48, CBM53 and CBM58 (Janecek *et al.*, 2011). The presence of SBD can be known as a prerequisite for the hydrolysis of insoluble starch as it improves the efficiency of the enzyme by hydrolyzing raw starch or disrupting the surface of the raw starch granule (Guillen *et al.*, 2007; Sun *et al.*, 2010; Rodriguez-Sanoja *et al.*, 2000; Hayashida *et al.*, 1990). A recent study conducted by Mehta and Satyanarayana (2014) elucidated that C-terminal domain of thermostable α-amylase from *Geobacillus thermoleovorans* (Gt-amyII) plays a role in raw starch adsorption and thermostability.

Alpha-amylases (1,4-α-D-glucan-glucanhydrolase, E.C. 3.2.1.1) are endo-acting enzymes that catalyses the hydrolysis of internal α-1,4 glycosidic, liberating various length of oligosaccharides (Gurung *et al.*, 2013). For decades, raw starch degrading α-amylases had been widely used in different processes in food, textile, brewing, paper industry and in starch liquefaction as these enzymes efficiently react at sub-gelatinisation temperatures (Park *et al.*, 2014). Therefore, the energy intensive starch gelatinization step is eliminated. Thermostable α-amylase from microbial sources *Bacillus amyloliquefaciens, B.subtilis* and *B. licheniformis* are preferable to employ commercially in the industries due to advantages such as improves starch gelatinization, decrease media viscosity, accelerate catalytic reaction and decrease contamination risk and energy saving (Pandey *et al.*, 2000; Haki, 2003; Jœzef, 2007). In recent advances, the study of enzymes (extremozymes) from extremophile had drawn special interest as they are able to withstand harsh industrial processes (Demirjian *et al.*, 2001). Therefore, thermostable enzyme isolated from thermophilic microorganisms that is *Anoxybacillus* sp. SK3-4 was selected in the study.

The genus *Anoxybacillus* was proposed under *Bacillaceae* cluster in the *Firmicutes* phylum by Pikuta *et al.* (2000). The name of *Anoxybacillus* refers to a mild thermophile of rod-shaped microorganism that can survive in the environment where the oxygen is depleted (Goh *et al.*, 2013). The *Anoxybacillus* sp. strain SK3-4 is a moderate thermophile that was isolated from Malaysian hot spring with an optimal growth temperature of 55 ℃ (Goh *et al.*, 2013). A full-length of α-amylases gene from *Anoxybacillus* sp. strain SK3-4 is named as ASKA which shares 69% similarity with the α-amylase from *Geobacillus thermoleovorans* subsp. *stromboliensis* Pizzo and *Geobacillus thermoleovorans* Gt-amyII. Previous studies (Chai *et al.*, 2012) had reported that ASKA belongs to the main representative family of glycoside hydrolase GH13 and it might become industries preference in future as it has higher maltose production than those commonly used α-amylases. However, the role of domain C of α-amylase from *Anoxybacillus* sp. strain SK3-4 has not been documented yet.

In this study, two truncated forms of C-terminal domain of α-amylase from *Anoxybacillus* sp. strain SK3-4, namely mutant ΔC100 and mutant ΔC50 were undergone bioinformatics analysis. Subsequently, primers used for PCR amplification of truncated ΔC100 and ΔC50 genes were designed. The amplified mutagenized-genes were subcloned into pET-28a vector and transformed into *Escherichia coli* BL21 (DE3). The successfully transformed cells were used for protein expression by induced with isopropyl-β-ᴅ-thiogalactopyranoside (IPTG). The optimization for the production of truncated proteins was carried out. The molecular weight of the proteins and their enzymatic activity was determined by SDS-PAGE and DNS assay, respectively.

1.2 Objectives

The objectives of this study were to:

i. Analyse and compare the primary, secondary and tertiary structure of the two truncated form of genes.
ii. Identify the putative functional domain, conserved region and phylogenetic analysis of domain C.
iii. Construct two C-terminal truncation of α-amylase gene from *Anoxybacillus* sp. SK3-4 (mutant ΔC100 and mutant ΔC50).

1.3 Scope of study

i. Bioinformatics analysis of α-amylase (ASKA) genes with its truncated C-terminal.
ii. Construction of C-terminal truncation of α-amylase from *Anoxybacillus* sp. SK3-4, namely mutant ΔC100 and mutant ΔC50).
iii. Transformation of recombinant pET-28a into *E. coli* BL21 (DE3).
iv. DNA sequencing of the successfully transformed mutant ΔC100 and mutant ΔC50.
v. Protein expression of mutant ΔC100 and mutant ΔC50 by IPTG induction.
vi. Optimization of the protein expression mutant ΔC100 and mutant ΔC50.
vii. Determination of TASKA ΔC100 and TASKA ΔC50 activity by enzyme assay using DNS method.

REFERENCES

Abbas, K. A., Khalil, S. K., and Hussin, A. S. M. (2010). Modified starches and their usages in selected food products: A review study. *Journal of Agricultural Science*. 2, 90-100.

Alvani, K., Qi, X., and Tester, R. F. (2012). Gelatinisation properties of native and annealed potato starches. *Starch - Stärke*. 64, 297-303.

Álvarez, M. S., Moscoso, F., Rodríguez, A., Sanromán, M. A., and Deive, F. J. (2013). Novel physico-biological treatment for the remediation of textile dyes-containing industrial effluents. *Bioresource Technology*. 146, 689-695.

Bennett, J. W. (2002). In search of Dr. Jokichi Takamine and the origins of industrial mycology. *Inoculum*. 53, 6-10.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T. G., Bertoni, M., Bordoli, L., and Schwede, T. (2014). SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research*. 42, W252-W258.

Bolhuis, A., Matzen, A., Hyyryläinen, H.-L., Kontinen, V. P., Meima, R., Chapuis, J., Venema, G., Bron, S., Freudl, R., and van Dijl, J. M. (1999). Signal peptide peptidase- and ClpP-like proteins of *Bacillus subtilis* required for efficient translocation and processing of secretory proteins. *Journal of Biological Chemistry*. 274, 24585-24592.

Brendel, V., Bucher, P., Nourbakhsh, I. R., Blaisdell, B. E., and Karlin, S. (1992). Methods and algorithms for statistical analysis of protein sequences. *Proceedings of the National Academy of Sciences of the United States of America*. 89, 2002-2006.

Brinkman, F. S. L., and Leipe, D. D. (2001). Phylogenetic analysis. *Methods of Biochemical Analysis*. 43, 323-358.

Canakci, S., Kacagan, M., Inan, K., Belduz, A., and Saha, B. (2008). Cloning, purification, and characterization of a thermostable α-l-arabinofuranosidase from

Anoxybacillus kestanbolensis AC26 *Sari*. *Applied Microbiology and Biotechnology*. 81, 61-68.

Chai, Y. Y., Rahman, R. N., Illias, R. M., and Goh, K. M. (2012). Cloning and characterization of two new thermostable and alkalitolerant alpha-amylases from the *Anoxybacillus* species that produce high levels of maltose. *Journal of Industrial Microbiology and Biotechnology*. 39, 731-741.

Chakravarty, S., and Varadarajan, R. (2000). Elucidation of determinants of protein stability through genome sequence analysis. *FEBS Letters*. 470, 65-69.

Chen, C., Zhou, X., Tian, Y., Zou, X., and Cai, P. (2006). Predicting protein structural class with pseudo-amino acid composition and support vector machine fusion network. *Analytical Biochemistry*. 357, 116-121.

Chen, K., Kurgan, L., and Rahbari, M. (2007). Prediction of protein crystallization using collocation of amino acid pairs. *Biochemical and Biophysical Research Communications*. 355, 764-769.

Choo, K. H., Tan, T. W., and Ranganathan, S. (2005). SPdb - A signal peptide database. *BMC Bioinformatics*. 6, 249-249.

Colovos, C., and Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Science : A Publication of the Protein Society*. 2, 1511-1519.

de Groot, N. S., Espargaró, A., Morell, M., and Ventura, S. (2008). Studies on bacterial inclusion bodies. *Future Microbiology*. 3, 423-435.

Demirjian, D. C., Morís-Varas, F., and Cassidy, C. S. (2001). Enzymes from extremophiles. *Current Opinion in Chemical Biology*. 5, 144-151.

El-Fallal, A., Dobara, M. A., El-Sayed, A., and Omar, N. (2012). *Starch and microbial α-amylases: From concepts to biotechnological applications*. Croatia: InTech.

Ellis, J. T., and Magnuson, T. S. (2012). Thermostable and alkalistable xylanases produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3. *ISRN Microbiology*. 2012.

Fasnacht, M., Zhu, J., and Honig, B. (2007). Local quality assessment in homology models using statistical potentials and support vector machines. *Protein Science : A Publication of the Protein Society*. 16, 1557-1568.

Flickinger, M. C., and Drew, S. W. (1999). *Encyclopedia of bioprocess technology : Fermentation, biocatalysis, and bioseparation*. New York: John Wiley.

Gao, Y., Dai, J., Peng, H., Liu, Y., and Xu, T. (2011). Isolation and characterization of a novel organic solvent-tolerant *Anoxybacillus* sp. PGDY12, a thermophilic gram-positive bacterium. *Journal of Applied Microbiology*. 110, 472-478.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., and, and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy server. *In:* Walker, J. M. (ed.) *The Proteomics Protocols Handbook.* Portland: Ringgold Inc.

Goh, K. M., Gan, H. M., Chan, K. G., Chan, G. F., Shahar, S., Chong, C. S., Kahar, U. M., and Chai, K. P. (2014). Analysis of *Anoxybacillus* genomes from the aspects of lifestyle adaptations, prophage diversity, and carbohydrate metabolism. *PLoS One*. 9, 1-15.

Goh, K. M., Kahar, U. M., Chai, Y. Y., Chong, C. S., Chai, K. P., Ranjani, V., Illias, R., and Chan, K. G. (2013). Recent discoveries and applications of *Anoxybacillus*. *Applied Microbiology and Biotechnology*. 97, 1475-1488.

Gromiha, M. M., and Suresh, M. X. (2008). Discrimination of mesophilic and thermophilic proteins using machine learning algorithms. *Proteins: Structure, Function, and Bioinformatics*. 70, 1274-1279.

Guillen, D., Santiago, M., Linares, L., Perez, R., Morlon, J., Ruiz, B., Sanchez, S., and Rodriguez-Sanoja, R. (2007). Alpha-amylase starch binding domains: Cooperative effects of binding to starch granules of multiple tandemly arranged domains. *Applied and Environmental Microbiology*. 73, 3833-3837.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*. 38, 1599-1616.

Gursahani, Y. H., and Gupta, S. G. (2011). Decolourization of textile effluent by a thermophilic bacteria *Anoxybacillus rupiensis*. *Journal of Petroleum and Environmental Biotechnology*. 2, 108.

Gurung, N., Ray, S., Bose, S., and Rai, V. (2013). A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Research International*. 2013, 329121.

Haki, G. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresource Technology*. 89, 17-34.

Hayashida, S., Teramoto, Y., Inoue, T., and Mitsuiki, S. (1990). Occurrence of an affinity site apart from the active site on the raw-starch-digesting but non-raw-starch-adsorbable *Bacillus subtilis* 65 α-amylase. *Applied and Environmental Microbiology*. 56, 2584-2586.

Horton, H. R., Moran, L. A., Scrimgeour, K. G., Perry, M. D., and Rawn, J. D. (2006). *Principles of biochemistry*. (4th ed.). New Jersey: John Wiley & Sons Inc.

Horváthováí, V., Janeček, S., and Ŏturdík, E. (2000). Amylolytic enzymes: Their specificities, origins and properties. *Biologia*. 55, 605-615.

Jain, C. K., Gupta, M., Prasad, Y., Wadhwa, G., and Sharma, S. K. (2014). Homology modeling and protein engineering of alkane monooxygenase in *Burkholderia thailandensis* MSMB121: *In silico* insights. *Journal of Molecular Modeling*. 20, 1-11.

Jane, J.-l. (2009). Chapter 6 - Structural features of starch granules II. In BeMiller, J., and Whistler, R. (Ed.) *Starch (3rd ed.).* San Diego: Academic Press.

Janeček, Š. (2002). How many conserved sequence regions are there in the α-amylase family? *Biologia - Section Cellular and Molecular Biology*. 57, 29-41.

Janecek, S., Svensson, B., and MacGregor, E. A. (2011). Structural and evolutionary aspects of two families of non-catalytic domains present in starch and glycogen binding proteins from microbes, plants and animals. *Enzyme and Microbial Technology*. 49, 429-440.

Janecek, S., Svensson, B., and MacGregor, E. A. (2014). Alpha-amylase: An enzyme specificity found in various families of glycoside hydrolases. *Cellular and Molecular Life Sciences*. 71, 1149-1170.

Jordan, J., and Yeates, T. O. (2011). Widespread disulfide bonding in proteins from thermophilic archaea. *Archaea*. 2011.

Jòzef, S. (2007). The use of starch processing enzymes in the food industry. In Polaina, J., and MacCabe, A. (Ed.) *Industrial Enzymes* (pp. 19-34). Springer Netherlands.

Kahar, U. M., Chan, K. G., Salleh, M. M., Hii, S. M., and Goh, K. M. (2013). A high molecular-mass *Anoxybacillus* sp. SK3-4 amylopullulanase: Characterization and its relationship in carbohydrate utilization. *International Journal of Molecular Sciences*. 14, 11302-11318.

Kapp, K., Schrempf, S., Lemberg, M., and, and Dobberstein, B. 2000. Post-targeting functions of signal peptides. *In:* Zimmermann, R. (ed.) *Protein Transport into the Endoplasmic Reticulum.* Austin (TX): Landes Bioscience.

Karaoglu, H., Yanmis, D., Sal, F. A., Celik, A., Canakci, S., and Belduz, A. O. (2013). Biochemical characterization of a novel glucose isomerase from *Anoxybacillus gonensis* G2T that displays a high level of activity and thermal stability. *Journal of Molecular Catalysis B: Enzymatic*. 97, 215-224.

Karmakar, R., Ban, D. K., and Ghosh, U. (2014). Comparative study of native and modified starches isolated from conventional and nonconventional sources. *International Food Research Journal*. 21, 597-602.

Kiefhaber, T., Rudolph, R., Kohler, H.-H., and Buchner, J. (1991). Protein aggregation in vitro and in vivo: A quantitative model of the kinetic competition between folding and aggregation. *Nature Biotechnology*. 9, 825-829.

Kirk, O., Borchert, T. V., and Fuglsang, C. C. (2002). Industrial enzyme applications. *Current Opinion in Biotechnology*. 13, 345-351.

Koropatkin, N. M., and Smith, T. J. (2010). SusG: A unique cell-membrane-associated α-amylase from a prominent human gut symbiont targets complex starch molecules. *Structure*. 18, 200-215.

Kumar, S., Tsai, C.-J., and Nussinov, R. (2000). Factors enhancing protein thermostability. *Protein Engineering*. 13, 179-191.

Laskowski, R. A., Macarthur, M. W., Moss, D. S., and Thornton, J. M. (1993). PROCHECK: A program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography*. 26, 283-291.

Lo, H.-F., Lin, L.-L., Chiang, W.-Y., Chie, M.-C., Hsu, W.-H., and Chang, C.-T. (2002). Deletion analysis of the C-terminal region of the α-amylase of *Bacillus* sp. strain TS-23. *Archives of Microbiology*. 178, 115-123.

Machovic, M., and Janecek, S. (2006). Starch-binding domains in the post-genome era. *Cellular and Molecular Life Sciences* 63, 2710-2724.

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Lu, F., Marchler, G. H., Mullokandov, M., Omelchenko, M. V., Robertson, C. L., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D., Zhang, N., Zheng, C., and Bryant, S. H. (2011). CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Research*. 39, D225-D229.

Martoglio, B. (2003). Intramembrane proteolysis and post-targeting functions of signal peptides. *Biochemical Society Transactions*. 31, 1243-1247.

Matzke, J., Schwermann, B., and Bakker, E. P. (1997). Acidostable and acidophilic proteins: The example of the α-amylase from *Alicyclobacillus acidocaldarius*. *Comparative Biochemistry and Physiology Part A: Physiology*. 118, 475-479.

Mehta, D., and Satyanarayana, T. (2014). Domain C of thermostable alpha-amylase of *Geobacillus thermoleovorans* mediates raw starch adsorption. *Applied Microbiology and Biotechnology*. 98, 4503-4519.

Mereghetti, P., Ganadu, M. L., Papaleo, E., Fantucci, P., and De Gioia, L. (2008). Validation of protein models by a neural network approach. *BMC Bioinformatics*. 9, 66-66.

Meruelo, A. D., Han, S. K., Kim, S., and Bowie, J. U. (2012). Structural differences between thermophilic and mesophilic membrane proteins. *Protein Science : A Publication of the Protein Society*. 21, 1746-1753.

Miguel, Â. S. M., Lobo, B. W. P., da Costa Figueiredo, É. V., Dellamora-Ortiz, G. M., and Martins-Meyer, T. S. (2013). *Enzymes in Bakery: Current and Future Trends*. INTECH Open Access Publisher.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31, 426-428.

Mitraki, A., Fane, B., Haase-Pettingell, C., Sturtevant, J., and King, J. (1991). Global suppression of protein folding defects and inclusion body formation. *Science*. 253, 54-58.

Mok, S. C., Teh, A. H., Saito, J. A., Najimudin, N., and Alam, M. (2013). Crystal structure of a compact α-amylase from *Geobacillus thermoleovorans*. *Enzyme and Microbial Technology*. 53, 46-54.

Morris, A. L., MacArthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992). Stereochemical quality of protein structure coordinates. *Proteins: Structure, Function and Genetics*. 12, 345-364.

Mülhardt, C., and Beese, E. W. (2007). Chapter 2 - Fundamental Methods. *Molecular Biology and Genomics* (pp. 11-36). Burlington: Academic Press.

Nwagu, T. (2012). Adsorption and stabilization of a raw starch digesting amylase on micro bead silica gel 300 A. *British Biotechnology Journal*. 2, 85-101.

Ōsawa, S., Osawa, S., Su, Z.-H., and Inmura., Y. 2004. Chapter 3: Molecular phylogenetic tree. *Molecular Phylogeny and Evolution of Carabid Ground Beetles.* Middletown: American Library Association dba CHOICE.

Pack, S. P., and Yoo, Y. J. (2004). Protein thermostability: structure-based difference of amino acid between thermophilic and mesophilic proteins. *Journal of Biotechnology*. 111, 269-277.

Panchenko, A., and Madej, T. (2005). Structural similarity of loops in protein families: Toward the understanding of protein evolution. *BMC Evolutionary Biology*. 5, 10.

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., and Mohan, R. (2000). Review: Advances in microbial amylases. *Biotechnology Applied Biochemistry*. 31, 135-152.

Park, J.-T., Suwanto, A., Tan, I., Nuryanto, T., Lukman, R., Wang, K., and Jane, J.-l. (2014). Molecular cloning and characterization of a thermostable α-amylase exhibiting an unusually high activity. *Food Science and Biotechnology*. 23, 125-132.

Peng, H., Zheng, Y., Chen, M., Wang, Y., Xiao, Y., and Gao, Y. (2014). A starch-binding domain identified in alpha-amylase (AmyP) represents a new family of carbohydrate-binding modules that contribute to enzymatic hydrolysis of soluble starch. *FEBS Letters*. 588, 1161-1167.

Peterson, M. E., Chen, F., Saven, J. G., Roos, D. S., Babbitt, P. C., and Sali, A. (2009). Evolutionary constraints on structural similarity in orthologs and paralogs. *Protein Science : A Publication of the Protein Society*. 18, 1306-1315.

Peterson, T. N., Brunak, S., Von Heijne, G., and, and Nielsen, H. (2011). SignalP 4.0: Discriminating signal petides from transmembrane regions. *Nature Methods*. 8, 785-786.

Pikuta, E., Cleland, D., and Tang, J. (2003). Aerobic growth of *Anoxybacillus pushchinoensis* K1T: Emended descriptions of *A. pushchinoensis* and the genus *Anoxybacillus*. *International Journal of Systematic and Evolutionary Microbiology*. 53, 1561-1562.

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G., and Laurinavichius, K. (2000). *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*. 50, 2109-2117.

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G., and Laurinavichius, K. (2009). Genus IV. *Anoxybacillus*. In De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K.-H., and Whitman, W. B. (Ed.) *Bergery's Manual of Systematic Bacteriology* (pp. New York: Springer.

Puspasari, F., Radjasa, O. K., Noer, A. S., Nurachman, Z., Syah, Y. M., van der Maarel, M., Dijkhuizen, L., Janecek, S., and Natalia, D. (2013). Raw starch-degrading alpha-amylase from *Bacillus aquimaris* MKSC 6.2: Isolation and expression of the gene, bioinformatics and biochemical characterization of the recombinant enzyme. *Journal of Applied Microbiology*. 114, 108-20.

Raghunathan, G., Soundrarajan, N., Sokalingam, S., Yun, H., and Lee, S.-G. (2012). Deletional protein engineering based on stable fold. *PLoS ONE*. 7.

Robertson, G. H., Wong, D. W. S., Lee, C. C., Wagschal, K., Smith, M. R., and Orts, W. J. (2006). Native or raw starch digestion: A key step in energy efficient biorefining of grain. *Journal of Agricultural and Food Chemistry*. 54, 353-365.

Rodriguez-Sanoja, R., Morlon-G, J., Jore, J., Pintado, J., Juge, N., and Guyot, J. P. (2000). Comparative characterization of complete and truncated forms of *Lactobacillus amylovorus* α-amylase and role of the C-terminal direct repeats in raw-starch binding. *Applied and Environmental Microbiology*. 66, 3350-3356.

Rodriguez-Sanoja, R., Oviedo, N., and Sanchez, S. (2005). Microbial starch-binding domain. *Current Opinion in Microbiology*. 8, 260-267.

Roy, S., Martinez, D., Platero, H., Lane, T., and Werner-Washburne, M. (2009). Exploiting Amino Acid Composition for Predicting Protein-Protein Interactions. *PLoS ONE*. 4, e7813.

Saboury, A. A. (2002). Stability, activity and binding properties study of α-amylase upon interaction with Ca2+ and Co2+. *Biologia - Section Cellular and Molecular Biology*. 57, 221-228.

Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Research*. 31, 3381-3385.

Shabnam, G., Abbas, A. S., Mohammad, R. R., Fereydon, M., and Hossein, H. (2011). Effectiveness of inoculation with isolated *Anoxybacillus* sp. MGA110 on municipal solid waste composting process. *African Journal of Microbiology Research*. 5, 5373-5378.

Sharma, A., and Satyanarayana, T. (2013). Microbial acid-stable α-amylases: Characteristics, genetic engineering and applications. *Process Biochemistry*. 48, 201-211.

Shen, H., and Chou, K.-C. (2005). Using optimized evidence-theoretic K-nearest neighbor classifier and pseudo-amino acid composition to predict membrane protein types. *Biochemical and Biophysical Research Communications*. 334, 288-292.

Singh, S. M., and Panda, A. K. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*. 99, 303-310.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., and Soccol, C. R. (2006). Alpha-amylases from microbial sources - An overview on recent developments. *Food Technology and Biotechnology*. 44, 173-184.

Souza, P. M. d., and Magalhães, P. d. O. e. (2010). Application of microbial α-amylase in industry - A review. *Brazilian Journal of Microbiology*. 41, 850-861.

Stanger, H. E., Syud, F. A., Espinosa, J. F., Giriat, I., Muir, T., and Gellman, S. H. (2001). Length-dependent stability and strand length limits in antiparallel beta - sheet secondary structure. *Proceedings National Academy Sciences of U S A*. 98, 12015-20.

Sumitani, J. I., Tottori, T., Kawaguchi, T., and Arai, M. (2000). New type of starch-binding domain: The direct repeat motif in the C-terminal region of *Bacillus* sp. no. 195 α-amylase contributes to starch binding and raw starch degrading. *Biochemical Journal*. 350, 477-484.

Sun, H., Zhao, P., Ge, X., Xia, Y., Hao, Z., Liu, J., and Peng, M. (2010). Recent advances in microbial raw starch degrading enzymes. *Applied Biochemistry and Biotechnology*. 160, 988-1003.

Tako, M., Tamaki, Y., Teruya, T., and Takeda, Y. (2014). The principles of starch gelatinization and retrogradation. *Food and Nutrition Sciences*. 5, 280-291.

Tomazic, S. J., and Klibanov, A. M. (1988). Mechanisms of irreversible thermal inactivation of Bacillus alpha-amylases. *Journal of Biological Chemistry*. 263, 3086-3091.

Tonie Wright, H., and Urry, D. W. (1991). Nonenzymatic Deamidation of Asparaginyl and Glutaminyl Residues in Protein. *Critical Reviews in Biochemistry and Molecular Biology*. 26, 1-52.

Ukkonen, K., Veijola, J., Vasala, A., and Neubauer, P. (2013). Effect of culture medium, host strain and oxygen transfer on recombinant Fab antibody fragment yield and leakage to medium in shaken *E. coli* cultures. *Microbial Cell Factories*. 12, 73-87.

Uma Maheswar Rao, J. L., and Satyanarayana, T. (2008). Biophysical and biochemical characterization of a hyperthermostable and Ca2+-independent α-amylase of an extreme thermophile *Geobacillus thermoleovorans*. *Applied Biochemistry and Biotechnology*. 150, 205-219.

Uthumporn, U., Zaidul, I. S. M., and Karim, A. A. (2010). Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. *Food and Bioproducts Processing*. 88, 47-54.

Vallee, B. L., Stein, E. A., Sumerwell, W. N., and Fischer, E. H. (1959). Metal content of alpha-amylases of various origins. *The Journal of Biological Chemistry*. 234, 2901-2905.

Van Der Maarel, M. J. E. C., Van Der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., and Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology*. 94, 137-155.

von Heijne, G. (1990). The signal peptide. *The Journal of Membrane Biology*. 115, 195-201.

Yokota, K., Satou, K., and Ohki, S. y. (2006). Comparative analysis of protein thermostability: Differences in amino acid content and substitution at the surfaces and in the core regions of thermophilic and mesophilic proteins. *Science and Technology of Advanced Materials*. 7, 255-262.

Yuan, T. Z., Yao, B., Luo, H. Y., Wang, Y. R., Wu, N. F., and Fan, Y. L. (2005). Expression of acidophilic alpha-amylase from *Alicyclobacillus acidocaldarius*. *Chinese Journal of Biotechnology*. 21, 78-83.

Zuo, X., Li, S., Hall, J., Mattern, M., Tran, H., Shoo, J., Tan, R., Weiss, S., and Butt, T. (2005). Enhanced expression and purification of membrane proteins by SUMO Fusion in *Escherichia coli*. *Journal of Structural and Functional Genomics*. 6, 103-111.