PRODUCTION AND CHARACTERIZATION OF PROTEASE FROM HALOPHILIC *VIRGIBACILLUS* SPECIES CD6

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

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

JANUARY 2017

Specially dedicated to *my beloved family, future life partner, soulmates and friends*

ACKNOWLEDGEMENT

First and foremost, I would also like to express my deepest gratitude towards my supervisor, Dr. Chong Chun Shiong who had guided me in every step taken to accomplish my master dissertation throughout these 1.5 years. Even though he was at his post-doctoral position at remote country for 1 year, however, he still concerned about my progress and always kept in touch to update the latest situation. Besides that, I would like to thank my co-supervisor, Dr. Haryati Jamaluddin for being so supportive in term of giving advices and resources, especially during the period when my main supervisor was not around.

Not forgetting fellow seniors in the Enzyme Research Laboratory including Ms. Suganthi Thevarajoo, Ms. Chitra Selvaratnam and Mr. Lim Jia Chun, assistant science officers (Mrs. Fatimah Harun and Ms. Norsyuhada Jaafar), administrative assistant (Mrs. Zulbaidah Muhammad), coursemates (Ms. Tan Ee Yau, Ms. Sonia Nair P. Kreshnan and Mrs. Zetty Amirah Zulkifli), other seniors, juniors and staffs in Faculty of Biosciences and Medical Engineering. Thank you for all of your help during problem solving, listening ears and experiences that you have shared to me.

Apart from that, I would like to acknowledge MyBrain15 scholarship (MyMaster) from Ministry of Higher Education Malaysia, Ministry of Education Malaysia (Project number: 4F265) and Universiti Teknologi Malaysia RU grant (Project number: 07H43) as my financial support for master degree. Last but not least, I would like to take this opportunity to sincerely thank my parents (Lam Poy Leng and Lye Chou Ngo), siblings, relatives, soulmates and friends who have supported me always so that I am able to accomplish what I have in life so far either mentally or physically.

ABSTRACT

In enzyme production industries, the major challenges that hinder the efficient and economic commercial scale application of proteases are their stability in broad range of pH, temperature, salinity, as well as their optimal activity in the presence of metal ions, organic solvents and detergents. Moreover, the enzyme purification steps also contribute to the cost of production. To overcome this problem, characterization and production of crude protease with attractive properties from wild bacterial isolate could be an alternative as it is a more cost-effective way compared to production of protease that involves purification steps and protein engineering approach. Therefore, crude protease of *Virgibacillus* sp. CD6 isolated from salted-fish was characterized in this study using azocasein assay and bioinformatics tools. Protease production was found to be highest when using soybean meal and yeast extract as nitrogen source compared to other organic nitrogen sources. The protease exhibited vast range of stability with optimum activity at 10.0 % (w/v) NaCl, 60ºC, pH 7 and 10, indicating its polyextremophilicity. The enzyme activity was enhanced by Mg^{2+}, Mn^{2+}, Cd^{2+} and Al^{3+}. Both PMSF and EDTA hindered protease activity, denoting the presence of serine protease and metalloprotease properties respectively. High protease stability (>80%) was demonstrated in presence of organic solvents and detergent constituents investigated, and surprisingly it is exceptionally compatible with commercial detergents. Phylogenetic analyses revealed that proteases of *Virgibacillus* sp. demonstrated far distance relationship with other species, which worth for further exploration. Attributes of this protease can actualize necessity of searching superlative enzymes from extremophiles for diverse applications, particularly in detergent industry.

ABSTRAK

Dalam industri penghasilan enzim, cabaran utama yang menghalang aplikasi komersial protease yang cekap dan ekonomi adalah ciri-ciri protease yang stabil dalam pelbagai pH, suhu, kadar garam serta aktiviti optimum dalam ion logam, pelarut organik, dan unsur detergen. Selain itu, proses penulenan enzim juga menyumbang kepada kos penghasilan. Bagi mengatasi masalah ini, pencirian dan penghasilan protease dari bakteria tanpa melibatkan proses penulenan boleh menjadi alternatif kerana ia adalah cara yang kos efektif berbanding dengan penghasilan protease yang melibatkan penulenan enzim dan kejuruteraan protein. Oleh itu, protease daripada *Virgibacillus* sp. CD6 yang dipencilkan daripada ikan masin telah dicirikan dalam kajian ini dengan penggunaan azocasein assay dan alat bioinformatik. Penghasilan protease didapati paling tinggi apabila menggunakan kacang soya dan ekstrak yis sebagai sumber nitrogen berbanding dengan sumber nitrogen organik yang lain. Protease tersebut mempamerkan luas kestabilan dengan aktiviti optimum pada 10.0% (w/v) NaCl, 60ºC, pH 7 dan 10, menunjukkan ciri poli-ekstremofi. Aktiviti enzim telah dipertingkatkan oleh Mg^{2+}, Mn^{2+}, Cd^{2+} dan Al^{3+}. Kedua-dua PMSF dan EDTA didapati menghalang aktiviti protease, menandakan ciri protease serine dan metalloprotease masing-masing. Kestabilan protease yang tinggi (>80%) telah ditunjukkan dalam pelarut organik dan unsur detergen, serta amat serasi dengan bahan pencuci komersial. Analisis filogenetik menunjukkan bahawa protease daripada *Virgibacillus* sp. mempunyai hubungan yang jauh dengan spesies lain, bernilai untuk penerokaan selanjutnya. Sifat-sifat protease ini boleh merealisasi keperluan mencari enzim cemerlang dari esktremofi untuk pelbagai aplikasi, terutamanya dalam industri detergen.

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LIST OF SYMBOLS

A_{420}	-	Absorbance at 420 nm
A_{750}	-	Absorbance at 750 nm
α	-	Alpha
≈	-	Approximately
β	-	Beta
^{o}C	-	Degree celcius
D	-	Diameter
=	-	Equal
γ	-	Gamma
g	-	Gram
g/L	-	Gram per liter
>	-	Greater than
h	-	Hour
kPa	-	Kilo Pascal
<	-	Less than
L	-	Liter
log_{10}	-	Logarithm to base 10
mg/ml	-	Milligram per milliliter
µl	-	Microliter
mg	-	Milligram
mg/L	-	Milligram per liter
ml	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
M	-	Molar mass
nm	-	Nanometer

-	-	Negative
n	-	Number
OD_{600}	-	Optical density at 600 nm
/	-	Or
%	-	Percent
cm^{-1}	-	Per centimeter
M^{-1}	-	Per molar
π	-	Pi
±	-	Plus-minus
+	-	Positive
®	-	Registered trademark
^{2}	-	Square
×	-	Times
™	-	Trademark
U/mg	-	Units per milligram
U/ml	-	Units per volume
v/v	-	Volume per volume
w/v	-	Weight per volume

LIST OF ABBREVIATIONS

A	-	Alanine
Al^{3+}	-	Aluminum ion
$Al_2(SO_4)_3$	-	Aluminum sulfate
APC	-	Activated protein C
ATP	-	Adenosine triphosphate
BLASTp	-	Protein-protein Basic Local Alignment Search Tool
BSA	-	Bovine serum albumin
C	-	Cysteine
$C_6H_5Na_3O_7$	-	Trisodium citrate
$C_6H_5Na_3O_7.2H_2O$	-	Trisodium citrate dihydrate
Ca^{2+}	-	Calcium ion
$CaCl_2$	-	Calcium chloride
Cd^{2+}	-	Cadmium ion
$Cd(NO_3)_2$	-	Cadmium nitrate
Cl^-	-	Chloride ion
Co^{2+}	-	Cobalt ion
$CoCl_2$	-	Cobalt chloride
Cu^{2+}	-	Copper (II) ion
$CuSO_4$	-	Copper (II) sulfate
$CuSO_4.5H_2O$	-	Copper (II) sulfate pentahydrate
D	-	Aspartic acid
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic Acid
DTT	-	Dithiothreitol
EC	-	Enzyme commission
EDTA	-	Ethylene Diamine Tetraacetic Acid

et al.	-	And friends
F	-	Phenylalanine
FDA	-	Food and Drug Administration
Fe^{3+}	-	Ferum (III) ion
$FeCl_3$	-	Ferum (III) chloride
G	-	Glycine
Glu, E	-	Glutamic acid
H-bond	-	Hydrogen bond
H^+	-	Hydrogen ion
H_2O_2	-	Hydrogen peroxide
HCl	-	Hydrochloric acid
His, H	-	Histidine
I	-	Isoleucine
IAA	-	Iodoacetic acid
ID	-	Identifier
K	-	Lysine
K^+	-	Potassium ion
K_2HPO_4	-	Dipotassium hydrogen phosphate
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
KNO_3	-	Potassium nitrate
L	-	Leucine
M	-	Methionine
MEGA 7.0	-	Molecular Evolutionary Genetic Analysis version 7.0
Mg^{2+}	-	Magnesium ion
$MgCl_2$	-	Magnesium chloride
$MgSO_4.7H_2O$	-	Magnesium sulfate heptahydrate
Mn^{2+}	-	Manganese ion
$MnCl_2$	-	Manganese chloride
N	-	Asparagine
Na^+	-	Sodium ion
Na_2CO_3	-	Sodium carbonate
NaCl	-	Sodium chloride

$NaHCO_3$	-	Sodium bicarbonate
$NaNO_2$	-	Sodium nitrite
NaOH	-	Sodium hydroxide
NH_4Cl	-	Ammonium chloride
Ni^{2+}	-	Nickel ion
$NiSO_4$	-	Nickel sulfate
OH^-	-	Hydroxide ion
P	-	Proline
PHB	-	Polyhydroxybutyrate
PMSF	-	Phenylmethylsulfonyl fluoride
pI	-	Isoelectric point
PSI-BLAST	-	Position-Specific Iterated Basic Local Alignment Search Tool
Q	-	Glutamine
R	-	Arginine
rcf	-	Relative centrifugal force
rpm	-	Rotary per minute
rRNA	-	Ribosomal ribonucleic acid
SAPS	-	Statistical Analysis of Protein Sequences
SD	-	Standard deviation
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser, S	-	Serine
sp.	-	Species (singular)
spp.	-	Species (plural)
T	-	Threonine
t-PA	-	Tissue plasminogen activator
TCA	-	Trichloroacetic acid
Tris	-	2-Amino-2-(hydroxymethyl)propane-1,3-diol
u-PA	-	Urokinase type plasminogen activator
USA	-	United States of America
USD	-	United States dollar

UV	-	Ultraviolet
V	-	Valine
W	-	Tryptophan
X, Xaa	-	Unknown amino acid
Y	-	Tyrosine
Zn^{2+}	-	Zinc ion
$ZnSO_4$	-	Zinc sulfate

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Halophilic bacteria has been recognized as one of the extremophiles that has valuable applications in industry and environment (Oren, 2010; Edbeib *et al.,* 2016; Yin *et al.,* 2015). They are found in natural saline and hypersaline habitats such as seawater, salt marshes and lagoon. Occurrence of halophiles can be from seawater to brines (Brock, 1979), some habitats include Dead Sea between Israel and Jordan and also Great Salt Lake in Utah (Oren, 2006). Besides that, salty environments inhabited by halophilic and halotolerant bacteria include food products such as salted fish and fermented food (Enache *et al.,* 2012), and these type of foods are commonly found in Malaysia.

Well-adapted strategies in saline environments utilized by halophilic bacteria made them useful in industrial applications. These halophilic bacteria has been used for production of valuable metabolites and solutes such as stress protectants (DasSarma and DasSarma, 2006), saline wastewater treatments (Shivanand and Mugeraya, 2011) and biodegradation of organic pollutants in environmental biotechnology (Le Borgne *et al.*, 2008). Halophilic bacteria can be classified under different phyla. Under different phylum, halophilic bacteria have different physiological requirements such as compatible solute used and salt concentration required. This diversity makes the halophilic bacteria as one of the source of opportunity and abundance, including industrial enzymes.

One of the enzymes produced by halophilic bacteria is protease, which is a type of hydrolase. Protease can be produced from animal, plant and microbial source. Protease from microbial source has been extensively used in various application especially in detergent industry since 1960 (Rao *et al.*, 1998) due their effectiveness in removing protein stains (Karn and Kumar, 2015). Until today, proteases contributed approximately 60% of the global industrial enzymes market (Anithajothi *et al.*, 2014). While from this amount, microbial proteases constitute 40% of total enzyme production (Raval *et al.*, 2014) which applied in various industries. The largest market undeniable is detergent industry, as this industry contributed to production of 13.5 billion tons per year (Adrio and Demain, 2014).

Apart from that, use of eco-friendly protease recovered from industrial sludge for bio-conversion of proteinaceous waste material into value-added products has become an increasingly concern due to it is a cost effective process (Karn and Kumar, 2015). And also, protease has been engineered using rational design and directed evolution approach to improve its properties and functions to be applied as therapeutic agents and in food processing (Li *et al.*, 2013). Based on huge demand of protease market and its application, new candidate of protease remained a worth for further discovery.

1.2 Problem statement / significance of study

Halophilic bacteria produce polyextremophilic enzymes that may have useful application in various biotechnological field. For instance, protease can act as fibrinolytic agent and also removing protein based stains such as blood and sweat effectively (Karn and Kumar, 2015). Most of the commercial bacterial proteases used in detergent industry are produced from *Bacillus* sp. (Gupta *et al.*, 2002b), lesser investigation on protease from *Virgibacillus* sp., and until today, no commercial protease is originated from genus *Virgibacillus* as well. Furthermore, expenditure cost in detergent industry such as purification, production (Niyonzima and More, 2015b) and protein engineering to increase protease efficiency (Li *et al.*, 2013) are expensive. To sort out these problems, a single step of production with the use of crude enzyme is required (Niyonzima and More, 2015a), a more cost effective way compared to purification. Moreover, exploration on novel enzymes with extraordinary properties from extremophiles is always in demand and continuously in research field. Therefore, this study was conducted to characterize extracellular protease produced from a halophilic bacterium, *Virgibacillus* sp. strain CD6 that is potentially to be applied in various industries, especially in detergent formulation.

1.3 Objectives of study

The objectives of this research are:

i. To select the best nitrogen source for protease production.
ii. To assess the effect of physico-chemical factors on the activity and stability of protease from *Virgibacillus* sp. CD6.
iii. To analyze extracellular protease sequences encoded for *Virgibacillus* sp.

1.4 Scope of study

The previously isolated halophilic bacteria, *Virgibacillus* sp. strain CD6 was initially screened for extracellular protease activity by using qualitative approaches, (skim milk agar and gelatin liquefaction). After that, medium for protease production was formulated and effect of nitrogen sources on protease production was investigated. The optimum conditions of protease activity and its stability in terms of pH, temperature and salt concentration were determined. Then, protease stability in presence of metal ions, inhibitors, detergent constituents and organic solvent was assessed. Compatibility of protease with commercial detergents and substrate specificity of protease were also investigated. Lastly, annotated protein sequences of extracellular proteases of *Virgibacillus* sp. were analyzed using bioinformatics approach and phylogenetic protein tree was constructed.

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