UNIVERSITI PUTRA MALAYSIA

PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF HALOTOLERANT LIPASE FROM Leuconostoc mesenteroides SUBSP. mesenteroides ATCC 8293

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Chairman : Associate Professor Mehrnoush Amid, PhD
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Halotolerant lipases are essential in several industries, particularly food industry due to their ability to withstand different salt concentrations. Food industry usually involves food fermentation process, which requires the enzymes to be active and stable in the presence of high salt concentration in order to carry out the process efficiently. To date, only few halotolerant lipases have been discovered and characterized. Thus, the present work was conducted in order to produce, purify, and characterize halotolerant lipases from *L. mesenteroides* subsp. *mesenteroides* ATCC 8293.

Production of extracellular lipase from *L. mesenteroides* subsp. m*esenteroides* ATCC 8293 was optimized according to culture conditions (carbon sources, nitrogen sources, emulsifier, and surfactants) and physical parameters (temperature and pH) using the shake-flask fermentation system. It was observed that lipase production was optimized when the following culture composition was used: peptone, 0.2% (w/v), olive oil, 1.0% (v/v), monopotassium phosphate (KH_2PO_4) 0.5g, dipotassium phosphate (K_2HPO_4) 0.5g, sodium chloride (NaCl) 0.1g, calcium chloride ($CaCl_2$) 0.1g, magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) 0.5g, gum arabic, 0.1% (w/v) and Tween 80, 0.1% (v/v) per liter. The best physical parameters for maximum lipase production were at 30℃ and pH 6.

Purification of lipases produced from *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 was conducted using a novel aqueous two-phase system (ATPS) composed of Triton X-100 and maltitol. Although, the search for alternative components of ATPS has been widely investigated, little attention has been given to polyols as a component of a two-phase system. The ability of polyols to mimic the structure of water and

maintain an artificial sphere of hydration around the macromolecules makes them a potential candidate as a phase separating agent in ATPS. Initially, the phase diagram of this system was constructed using different concentrations of Triton X-100 and maltitol based on turbidimetric titration method. The partitioning of lipases was then optimized according to several parameters, which were pH, temperature, and crude load. It was observed that lipases preferentially migrated to the Triton X-100 rich phase. Optimum lipase partitioning was achieved in ATPS at tie-line length of 46.4%, crude load of 20% at a temperature of 30℃ and pH 8 respectively. Purification of lipases using this system resulted in high lipase purification factor of 17.28 and lipase yield of 94.7%. The purified lipase showed aprominent band on SDS-PAGE with an estimated molecular weight of 50 kDa. Hence, this study demonstrated that Triton X-100 and maltitol could be potentially used as an alternative ATPS in order to efficiently purify valuable enzymes.

The purified lipases were then characterized on the basis of temperature, pH, and the presence of surfactants, metal ions as well as different salt concentrations. Optimum lipase activity was observed at 37℃ and pH 8 respectively. The purified lipase was also observed to be stable at temperature range of 30-60℃ and pH range of 6-11. Lipase exhibited enhanced activity in the presence of non-ionic surfactants, which were Triton X-100, Tween 80 and Tween 20, with increased activity up to 40%. Conversely, the presence of SDS, an anionic surfactant, inhibited 78% of the lipase activity. Metals ions such as Na^{+}, Mg^{2+} K^{+} and Ca^{2+} stimulated lipase activity whereas divalent ions such as Zn^{+} and Cu^{2+} significantly reduced lipase activity by almost 50%. It was also observed that lipase activity was remarkably enhanced beyond 100% in the presence of different salt concentrations (0-10% w/v), thus confirming that lipase from *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 is a halotolerant enzyme.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN, PENULENAN DAN PENCIRIAN LIPASE HALOTOLERAN DARIPADA *Leuconostoc mesenteroides* SUBSP. *mesenteroides* ATCC 8293

Oleh

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Lipase halotoleran adalah penting dalam beberapa industri, terutama industri makanan disebabkan kemampuannya untuk bertahan daripada kepekatan garam yang berbeza. Industri makanan lazimnya melibatkan proses penapaian makanan yang memerlukan enzim menjadi aktif dan stabil dengan kehadiran kepekatan garam yang tinggi untuk menjalankan proses dengan efisien. Sehingga kini, hanya sedikit lipase halotoleran yang telah ditemui dan dicirikan. Oleh itu, kajian ini dijalankan untuk menghasil, menulen dan mencirikan lipase halotoleran dari *L. mesenteroides* subsp. *mesenteroides* ATCC 8293.

Penghasilan lipase ekstrasel dari *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 dioptimumkan mengikut keadaan kultur (sumber karbon, sumber nitrogen, pengemulsi, dan surfaktan) dan parameter fizikal (suhu dan pH) menggunakan sistem penapaian kelalang-goncang. Didapati bahawa penghasilan lipase adalah optimum apabila komposisi kultur berikut digunakan: pepton, 0.2% (w/v), minyak zaitun, 1.0% (v/v), monopotassium fosfat (KH_2PO_4) 0.5g, dipotassium fosfat (K_2HPO_4) 0.5g, natrium klorida (NaCl) 0.1g, kalsium klorida ($CaCl_2$) 0.1g, magnesium sulfat heptahidrat ($MgSO_4.7H_2O$) 0.5g, gum arabik, 0.1% (w/v) dan Tween 80, 0.1% (v/v) seliter. Parameter fizikal terbaik untuk pengeluaran lipase yang maksimum adalah pada suhu 30℃ dan pH 6.

Penulenan lipase yang dihasilkan dari *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 telah dijalankan menggunakan sistem dua-fasa akueus (SDFA) yang terdiri daripada Triton X-100 dan maltitol. Walaupun pencarian komponen alternatif SDFA telah dikaji secara meluas, perhatian yang sedikit telah diberikan kepada poliol sebagai

I certify that a Thesis Examination Committee has met on 3 April 2018 to conduct the final examination of Nurfadhilah Hidayah Eko Sukohidayat on her thesis entitled "Production, Purification and Characterization of Halotolerant Lipase from *Leuconostoc mesenteroides* Subsp. *mesenteroides* ATCC 8293" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

3-D	3- Dimensional
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ATCC	American Type Collection Culture
ATPS	Aqueous Two-Phase System
BSA	Bovine Serum Albumin
C	Carbon Chain
CTAB	Cetrimonium Bromide
DEAE	Diethylaminoethyl
DMSO	Dimethyl Sulfoxide
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetraacetic Acid
EMC	Enzyme-Modified Cheese
Exp	Exponential
FDA	Food and Drug Association
FF	Fast Flow
g	Gram
g	Gravitational Force
GRAS	Generally Regarded As Safe
HCl	Hydrochloric Acid
H_2O_2	Hydrogen Peroxide
LAB	Lactic Acid Bacteria

K_M	Michaelis Constant
kDa	Kilo Dalton
M	Molar
mg	Milligram
Min	Minute
mL	Millilitre
mM	MilliMolar
MRS	De Man, Rogosa and Sharpe
N	Normality
$NaOCl_2$	Sodium Chlorite
NaOH	Sodium Hydroxide
nm	Nano Metre
PEG	Polyethylene Glycol
pI	Isoelectric Point
PMSF	Phenylmethane Sulfonyl Fluoride
pNPP	Para-Nitrophenyl Palmitate
PUFA	Polyunsaturated Fatty Acid
Rpm	Revolution per Minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Sn	Stereospefically Numbered
TEMED	Tetramethylethylenediamine

TLL	Tie-Line Length
Tris	Trisaminomethane
U	Unit
μL	Micro Litre
μmol	Micro Mol
U/mg	Unit per Milligram
U/mL	Unit per Millilitre
USD	United State Dollar
V	Volt
V_{max}	Maximum Rate of Reaction
v/v	Volume per Volume
VOC	Volatile Organic Compound
w/v	Weight per Volume
w/w	Weight per Weight
Y_T	Top Phase Yield

CHAPTER 1

INTRODUCTION

Enzymes have become an important catalyst in the biotechnology industry as the demand for industrial enzymes are increasing throughout the years. The global market for industrial enzymes in 2015 was estimated at USD 4.94 billion and is expected to increase 6% annually to reach USD 6.99 billion by 2021 (Business Wire, 2016). Hydrolytic enzymes such as proteases, carbohydrases and lipases dominate the total sale of global enzymes. Recently, lipases have garnered particular attention in the biotechnology industry owing to their multifaceted properties. The global lipase market is expected to increase with an average of 6.5% annually between 2015 and 2020 to reach USD 590.5 million by 2020 (Markets and Markets, 2015).

Lipases (triacylglycerol acylhydrolase E.C. 3.1.1.3) are a group of enzymes that are able to catalyze the hydrolysis of triglycerides into glycerol and fatty acids at lipid-water interface (Emtenani *et al*., 2013). However, the catalytic reaction of lipases largely depends on the interfacial area, as lipases found in non-aqueous environment, are able to reverse the reaction, which leads to the synthesis of triglycerides from glycerol and free fatty acid (Papagora *et al*., 2013). Due to their versatile reactions in both aqueous and non-aqueous media, lipases have become one of the commonly used hydrolytic enzymes in the enzyme-related industries such as food, pharmaceutical, detergent, cosmetic and paper manufacturing (Ma *et al*., 2006; Sharma *et al*., 2002).

Lipases are ubiquitous as they can be found in plants, animals and microorganisms. However, lipases from microorganisms are found to be more suitable for biotechnology and commercial applications compared to animal and plant lipases due to the various advantages of microbial lipases such as short production time, large quantities production, simple extraction procedure and cost effectiveness (Rabbani *et al*., 2013). Although there are many microbial lipases currently available for the use of various industries, they are still insufficient to fulfill the operating requirements of most industries because lipases produced by different microbes will have different optimal reaction conditions. These conditions can greatly influence the applications of lipases in the industries.

Currently, limited researches regarding production and characterization of halotolerant lipases have become a common limitation for lipases used in industrial applications (Asoodeh & Ghanbari, 2013). Halotolerant enzymes are able to tolerate and remain active over a wide range of salt concentration. These enzymes are particularly essential in food industries. Lipases are commonly used in food fermentation, which is one of the common processes in food industry. This process involves salting step, in which high concentration of salt is present (Esteban-Torres *et al*., 2015). Hence, lipases used for this process are required to be halotolerant so that they can remain active and stable at high salt concentration in order to carry out the

fermentation process efficiently. Thus, it is important to find novel microbial enzyme sources for the production of lipases with suitable characteristics to meet the requirements of particular industries.

Leuconostoc mesenteroides is a gram positive and facultative anaerobe bacterium. It belongs to the group of lactic acid bacteria and widely used in the production of various fermented foods such as kimchi, sauerkraut, dairy and bread dough. Several lactic acid bacteria have been reported to produce lipase (Esteban-Torres *et al*., 2015; Ramyasree & Dutta, 2013). Although many studies have been done on the production of lipase from lactic acid bacteria, lipase production by *L. mesenteroides* has never been reported. Compared to other lactic acid bacteria, *L. mesenteroides* can tolerate fairly high concentration of salt and sugar. *L. mesenteroides* is also non-pathogenic and classified as generally regarded as safe (GRAS) organism by the U.S Food and Drug Administration (FDA). These characteristics are essential for the commercial applications of lipases in various industries.

Although the demand for enzymes is growing, the high cost of enzymes is a major concern for the enzyme-related industries. Enzymes are costly due to the complexity and difficulty of their downstream processing. The downstream processing of enzymes usually involves purification step, which accounts for more than 70% of total production cost of the enzymes (Raja *et al*., 2012). The purification methods traditionally used in the industries include several steps such as ultrafiltration, precipitation (salting out) and chromatography (ion exchange, hydrophobic interaction, size exclusion, and affinity), which resulted in the loss of product in each steps (Benavides *et al*., 2011). Furthermore, these conventional methods of purification are expensive, multistep, and discontinuous as well as labor and time extensive. Due to these characteristics, the conventional purification methods are found to be difficult to scale up, thus making them uneconomical at large-scale. Hence, there is a need to develop quick, cost-effective, efficient and scalable purification technique with a potential for continuous operations, which can improve the yield and purity of the biological product.

Aqueous two-phase system (ATPS) is an alternative purification technique that meets all the aforementioned criteria. ATPS was introduced during the mid-1950's by Albertsson (Srinivas, 2000), however its potential as an efficient purification method has been realized only recently. ATPS is conducted by mixing two different water-soluble compounds in water until the limiting concentrations of these two compounds have been exceeded, which then results in the formation of two immiscible aqueous two phases. When the mixture containing biomolecule of interest is added into this system, the target biomolecule will be partitioned and concentrated into one of the phases and the contaminants will be partitioned into the other phase (Raja *et al*., 2012) . Due to its water-rich environment, ATPS serves as a friendly and gentle environment for biological materials. Besides, the parameters of the system can be controlled and manipulated in order to achieve optimum partitioning (Amid *et al*., 2011).

Furthermore, this system is economical, continuous, simple, rapid, and easy to scale up.

The search for novel lipase-producing bacteria should be continuously pursued because currently there is limited availability of lipase possessing satisfactory operating characteristics. Besides, the high cost of lipases has become a significant concern for the biotechnological industries. Therefore, there is a need to develop inexpensive, simple, continuous and scalable purification strategy to cater to industries' needs of a fast and economic downstream process. Furthermore, this process should provide a biocompatible environment for lipase in order to improve the yield and purity of the lipase. Thus, this research was undertaken with the following objectives:

I. To optimize production of extracellular lipases from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293.
II. To purify extracellular lipases produced by *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 using novel aqueous system (ATPS) composed of Triton X-100 and maltitol.
III. To characterize the properties of the purified lipases.

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