การหาค่าเหมาะที่สุดสำหรับการผลิตร่วมและการทำให้บริสุทธิ์บางส่วนของโปรตีเอสและไลเพส
จาก *Bacillus licheniformis* 3C5 ในระบบน้ำสองวัฏภาค

นางสาวจิรภิญญา แสนสมัคร

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

OPTIMIZATION FOR CONCOMITANT PRODUCTION AND PARTIAL PURIFICATION OF PROTEASE AND LIPASE FROM *Bacillus licheniformis* 3C5 IN AQUEOUS TWO-PHASE SYSTEM

Miss Jirapinya Sansamak

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2015
Copyright of Chulalongkorn University

Thesis Title	OPTIMIZATION FOR CONCOMITANT PRODUCTION AND PARTIAL PURIFICATION OF PROTEASE AND LIPASE FROM *Bacillus licheniformis* 3C5 IN AQUEOUS TWO-PHASE SYSTEM
By	Miss Jirapinya Sansamak
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Jittra Piapukiew, Ph.D.
Thesis Co-Advisor	Associate Professor Alisa Vangnai, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)
.....Thesis Advisor
(Assistant Professor Jittra Piapukiew, Ph.D.)
.....Thesis Co-Advisor
(Associate Professor Alisa Vangnai, Ph.D.)
.....Examiner
(Assistant Professor Kittinan Komolpis, Ph.D.)
.....External Examiner
(Assistant Professor Ek Sangvichien, Ph.D.)

จิรภิญญา แสนสมัคร : การหาค่าเหมาะที่สุดสำหรับการผลิตร่วมและการทำให้บริสุทธิ์บางส่วนของโปรตีเอสและไลเพสจาก *Bacillus licheniformis* 3C5 ในระบบน้ำสองวัฏภาค (OPTIMIZATION FOR CONCOMITANT PRODUCTION AND PARTIAL PURIFICATION OF PROTEASE AND LIPASE FROM *Bacillus licheniformis* 3C5 IN AQUEOUS TWO-PHASE SYSTEM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. จิตรตรา เพียภูเขียว, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. อลิสา วังใน, 176 หน้า.

Bacillus licheniformis 3C5 เป็นแบคทีเรียที่เคยมีการรายงานการผลิตโปรตีเอสที่มีคุณสมบัติทนต่อสารอินทรีย์และสารซักล้าง ผลการศึกษานี้พบว่าแบคทีเรียสายพันธุ์นี้มีศักยภาพในการผลิตไลเพสซึ่งนอกจากจะทนต่อการถูกไฮโดรไลซิสจากโปรตีเอสที่ผลิตขึ้นเองแล้ว ยังมีคุณสมบัติที่ทนต่อสารอินทรีย์และสารซักล้างได้ดีเช่นเดียวกับโปรตีเอสอีกด้วย ลักษณะดังกล่าวนี้แสดงให้เห็นถึงศักยภาพของเอนไซม์ทั้งสองในการประยุกต์ใช้ร่วมกัน ดังนั้น การหาภาวะที่เหมาะสมในการผลิตเอนไซม์ทั้งสองพร้อมกันจึงเอื้ออำนวยต่อการนำเอนไซม์ไปประยุกต์ใช้ร่วมกันในอุตสาหกรรมต่างๆ อีกทั้งยังเป็นการลดต้นทุนและระยะเวลาในการผลิตเอนไซม์จากกระบวนการหมักอีกด้วย จากการหาภาวะการผลิตที่เหมาะสมด้วย Response surface methodology (RSM) แบคทีเรียสายพันธุ์นี้สามารถผลิตโปรตีเอสและไลเพสพร้อมกันสูงสุดที่เวลา 48 ชั่วโมง จากสูตรอาหารที่ปรับแล้วซึ่งประกอบด้วยส่วนประกอบในหน่วยร้อยละโดยน้ำหนักต่อปริมาตรดังนี้ กลูโคสร้อยละ 0.86 ยีสต์สกัดร้อยละ 0.6 โซเดียมคลอไรด์ร้อยละ 0.25 และน้ำมันมะกอกร้อยละ 2.5 ซึ่งผลิตเอนไซม์โปรตีเอสและไลเพสได้สูงกว่าสูตรอาหารตั้งต้นถึง 2.20 และ 12.57 เท่า ตามลำดับ จากการศึกษาเพิ่มเติมในการทำบริสุทธิ์บางส่วนของเอนไซม์ทั้งสองที่ผลิตได้ด้วยระบบน้ำสองวัฏภาค พบว่าระบบที่ประกอบด้วยพอลิเอธิลีนไกลคอลขนาดโมเลกุล 6000 และเกลือโซเดียมซิเตรท เป็นระบบที่ดีที่สุด โดยเกลือชนิดนี้มีคุณสมบัติที่สามารถย่อยสลายได้ด้วยวิธีการทางชีวภาพจึงเป็นระบบที่เป็นมิตรต่อสิ่งแวดล้อม ผลการทดลองหลังการออกแบบการทดลองด้วย RSM พบว่าระบบที่ดีที่สุดประกอบด้วยส่วนประกอบในหน่วยร้อยละโดยน้ำหนักดังนี้ พอลิเอธิลีนไกลคอลขนาดโมเลกุล 6000 ร้อยละ 18.58 โซเดียมซิเตรทร้อยละ 8.0 และ โพแทสเซียมคลอไรด์ร้อยละ 4.0 โดยโปรตีเอสแยกไปอยู่ชั้นบนของระบบให้ร้อยละของผลได้ (%yield) 93.18% และมีความบริสุทธิ์ (PF) สูงขึ้น 10.58 เท่า ในขณะที่ไลเพสแยกไปอินเตอร์เฟช ซึ่งให้ค่าผลได้และค่าความบริสุทธิ์เท่ากับ 92.59% และ 14.58 เท่า ตามลำดับ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5672191023 : MAJOR BIOTECHNOLOGY
KEYWORDS: BACILLUS LICHENIFORMIS / PROTEASE / LIPASE / RESPONSE SURFACE METHODOLOGY / AQUEOUS-TWO PHASE SYSTEM / CO-APPLICATION / CONCOMITANT PRODUCTION

JIRAPINYA SANSAMAK: OPTIMIZATION FOR CONCOMITANT PRODUCTION AND PARTIAL PURIFICATION OF PROTEASE AND LIPASE FROM *Bacillus licheniformis* 3C5 IN AQUEOUS TWO-PHASE SYSTEM. ADVISOR: ASST. PROF. JITTRA PIAPUKIEW, Ph.D., CO-ADVISOR: ASSOC. PROF. ALISA VANGNAI, Ph.D., 176 pp.

An organic solvent-, detergent-tolerant *Bacillus licheniformis* 3C5 has been recognized as protease-producing bacterium. Further tests revealed that this strain could also secrete proteolytic-resistant lipase with good stability in organic solvent and detergent and thus it is suitable for industrial co-application with protease. Further optimization studies for enhancing concomitant protease and lipase production were performed using response surface methodology (RSM). The maximum protease and lipase production was achieved using the optimized medium containing 0.86% glucose, 0.6% yeast extract, 0.25% NaCl and 2.5% olive oil (w/v), and led to an increase in yield by 2.20 and 12.57 folds. Finally, protease and lipase were partially purified by using an aqueous-two phase system (ATPS). The RSM was also applied to investigate the concentration effects of PEG6000, sodium citrate and KCl on the partition of both enzymes. It was found that protease was preferentially partitioned into PEG-rich top phase, whereas lipase was distributed to the interface. Accordingly, the best partitioning system was composed of 18.58% (w/w) PEG6000, 8.0% (w/w) sodium citrate and 4.0% (w/w) KCl. It provided 93.18% yield of protease and 92.59% yield of lipase with purification fold of 10.58 and 14.58, respectively.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Field of Study: Biotechnology Student's Signature .....
Academic Year: 2015 Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to Asst. Prof. Jittra Piapukiew, Ph.D., my advisor and also to my co-advisor, Assoc. Prof. Alisa Vangnai, Ph.D. for all valuable suggestions, support and encouragement throughout this research.

I am grateful to Asst. Prof. Tosak Seelanan, Ph.D., Asst. Prof. Kittinan Komolpis, Ph.D. and Asst. Prof. Ek Sangvichien, Ph.D. for serving as the committee and for their valuable suggestion to improve my thesis, completely.

My sincere thanks are also extended to all members of laboratory room 212, Botany Building, Department of Botany and Genetics, and laboratory room 604/1, Klum Vacharobol Building, Department of Biochemistry, Chulalongkorn University and to all my friends for their friendly help, suggestions and friendship all the time.

Finally, special thank for my family for their love, encouragement, kindly support, respect my decision and help to complete this entire thesis.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CONTENTS

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Table Page

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
BSA	Bovine serum albumin
Da	Dalton (g/mol)
g	Gram
h	Hour
Ke	Partition coefficient of enzyme
Kp	Partition coefficient of protein
L	Liter
M	Molar
mg	Milligram
ml	Milliliter
mM	Millimolar
mw	Molecular weight
OD	Optical density
PEG	Polyethylene glycol
PF	Purification fold
PPG	Polypropylene glycol
PVP	Polyvinyl pyrrolidone
rpm	Round per minute
SA	Specific activity (U/mg)
SDS	Sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
U	Unit
v	Volume
v/v	Volume by volume
Vr	Volume ratio
w	Weight
w/v	Weight by volume
w/w	Weight by weight

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I
INTRODUCTION

1.1 Statement of purposes

Nowadays, co-application of enzymes is interested and more valuable especially for laundry applications where cocktail of enzyme are added to detergent formulations for increasing efficiency to remove proteinaceous and fatty stains from cloths (Ruchi, Anshu and Khare, 2008). Protease and lipase are considered as the common applicable enzymes used together for many purposes mainly on hydrolysis of protein and triglyceride, respectively. Various processes of leather manufacture also insist the mixture of protease and lipase. For instance, Forezyme SK (La Forestal Tanica, Spain) or NovoLime (Novozyme), the commercial bioproduct comprising of a mixture of bacterial protease and lipase, are used in both soaking and liming processes for removing hairs, nails, hooves and other keratinous materials (Thanikaivelan *et al.*, 2004; Saxena, 2015). These enzymes are necessary for ripening processing for better flavor and texture (Ozcan and Kurdal, 2012). Also, both protease and lipase used together for cleaning of slaughter house equipment (Olsen, 2000). Moreover, a combination of protease and lipase further resulted in effective de-waxing and degumming, with positive effects on the wettability of silk in textile industry (Saxena, 2015).

Chulalongkorn University

Additionally, proteases are hydrolytic enzymes which are capable of auto-digestion and also digest other enzymes produced simultaneously (Aguilar *et al.*, 2002). Some literature cited that the production of lipase and protease are inter-related (Rajmohan, Dodd and Waites, 2002). The extracellular lipase level was regulated by an endogenous protease (Kok *et al.*, 1996). Also, Lipase production enhanced by increasing air pressure during fermentation for decline protease production (Lopes *et al.*, 2008). Furthermore, improvement in proteolytic resistance lipase by using genetic engineering was also perform by Ahmad *et al.* (2012). As mentioned thorough studies, the uses of lipase together with protease are limited, by which the proteolytic resistant lipase has a great potential especially for the co-applications. Therefore, the mixed enzyme preparation with both the influence activities and lipase not being

susceptible to hydrolyze by protease will have better applicability. Furthermore, since individual production of enzymes may face the problem of high cost and time consuming, hence, alternative single fermentation could resolve. For this reason, co-production protease and lipase bacterium have high potential for application in upstream proposes or subsequent downstream.

Generally, downstream processing provides 50-90% of enzyme production cost. Aqueous two-phase systems (ATPS) are the liquid-liquid extraction composing of water 70-90% in each phase corresponding to protect enzyme from denaturation. Moreover, the advantages of this method are easy, single step, fast and efficient to separate enzyme from other contaminated proteins in fermentation broth. Consequently, it deceased cost consuming of downstream processing and easy to use in up-scale industry which remain highly yield near to lab-scale. ATPS is used in extraction of protein, enzyme, biopharmaceuticals and fermentation broth. ATPS is divided into polymer-polymer, polymer-salt, ionic liquid-salt and alcohol-salt systems. The critical concentration of each combination causes two immiscible phases, which one-phase composes of predominant polymer and another phase composes of the others polymer or salt. The properties of each phase provide the condition for separation of protein by physio-chemical properties. Most recent ATPS system is polymer-salt systems which low viscosity, easily phase separation, shorten time, low cost and good sensitivity (Hatti-Kaul, 2001). Polyethylene glycol (PEG) is favorable polymer because of its cost and good phase forming polymer ability. Moreover, PEG supports proteins folding to protect activity of enzyme (Cleland and Wang, 1990). Although, ATPS method is low purity than conventional method, ion-exchange chromatography, ATPS are easy and short time consuming that suitable for detergent and leather processing, which is not necessary to use high purity.

Rachadech and coworker (2010) reported that the organic-solvent tolerant *Bacillus licheniformis* 3C5 produced an efficient protease, excellent stability to temperature, organic solvent and surfactant. As preliminary study, this strain also produced lipase and protease showed co-application potential. The aims of current study were to characterize of lipase, optimize of concomitant production and thereafter partially purified by ATPS method of protease and lipase from *B. licheniformis* 3C5.

1.2 Objectives of this research

The aim of the present study was to characterization of lipase, optimization of concomitant production and partial purification using ATPS technique of solvent-tolerant protease and lipase from *B. licheniformis* 3C5.

1.3 Scopes of study

1. To characterize the extracellular lipase produced by *B. licheniformis* 3C5 with respect to optimal temperature and pH as well as stability, and to assess the effect of several organic solvents and surfactants and detergents on lipolytic activity for its feasible applications.
2. To optimize the medium composition and condition for concomitant production of protease and lipase in the same medium and/or the same condition
3. To partially purify using polymer/salt ATPS of simultaneous production of protease and lipase

1.4 Expected outcome

Protease and lipase can be concomitantly produced in the same optimized medium and partially purified with more than 90%yield by ATPS

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER II

LITERATURE REVIEW

2.1 Proteases

Proteolytic enzymes (EC 3.4) are by far the most important of the commercially available industrial enzymes having found widely used for laundry, automatic dish washing, baking, dairy, textile manufacture and other applications. These enzymes catalyze the hydrolytic reaction of protein into oligo-peptide, peptide or amino acid as shown in Figure 1(Mótyán, Tóth and Tőzsér, 2013).

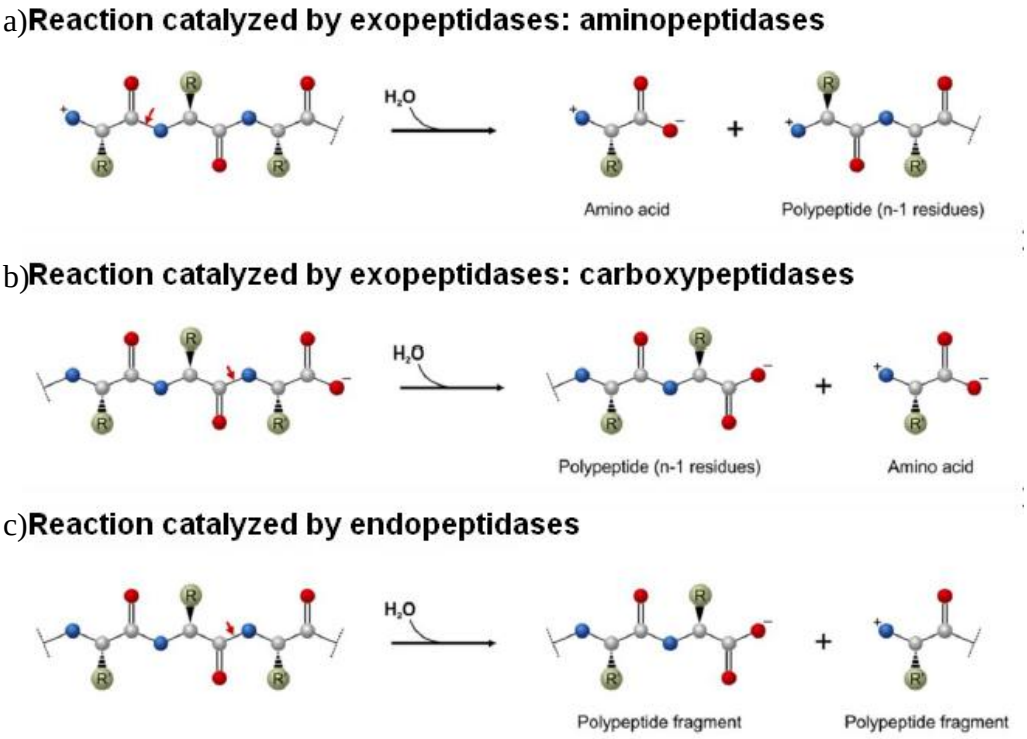


Figure 1 Action of exopeptidases and endopeptidases removing the terminal amino acid residues on a polypeptide substrate, a) reaction catalyzed by exopeptidases: aminopeptidase, b) reaction catalyzed by exopeptidases: carboxypeptidases and c) reaction catalyzed by endopeptidases. Arrows show the peptide bonds to be cleaved. (Mótyán *et al.*, 2013)

2.1.1 Classification of protease

Proteases can be divided into two groups depending on their site of action: exopeptidases (i.e., peptidases) that hydrolyze the peptide bonds near the *N*- or *C*-terminal ends of the substrate releasing free amino acids, and endopeptidases that hydrolyze internal peptides bonds and convert proteins into smaller peptidase (McAuliffe, 2012).

Exopeptidases can be categorized into a number group (Salleh *et al.*, 2006):

a. Aminopeptidase (3.4.11-19) which hydrolyze one residue from –N terminal
b. Carboxypeptidase (3.4.16-17) which hydrolyze one residue from –C terminal
c. Dipeptidases (3.4.13) which are specific for dipeptide, including enzyme that cleave dipeptide unit from either N terminal (3.4.14) or C terminal (3.4.15)
d. Omega peptidase (3.4.19) are the enzymes which hydrolyze amino acid residue substituted from N terminal (3.4.14) or C terminal (3.4.15)

As shown in Table 1, proteases are also classified according to their catalytic mechanism and the presence of amino acid residue(s) at the active site. The list of subtypes are as follows (Rao *et al.*, 1998):

- Serine proteases (EC 3.4.21)
- Cysteine proteases (EC 3.4.22)
- Aspartic protease (EC 3.4.23)
- Metalloproteases (EC 3.4.24)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 1 Classification and characteristic of proteases based on catalytic mechanism and the presence of amino acid residue(s) at the active site (summarized from Rao *et al.* (1998) and Salleh *et al.* (2006))

Protease	Catalytic site	Inhibitor	Molecular weight (kDa)	pH optimum	Example
Serine protease	Ser-His-Asp triad	▪ Diisopropyl fluorophosphate (DFP) ▪ Phenyl methylsulfonyl fluoride (PMSF) ▪ Tosyl-L-lysine chloromethyl ketone (TLCK)	18-35	neutral and alkaline pH (7-11)	▪ subtilisin Novo (BPN') ▪ subtilisin Carlsberg
Cysteine protease	Cys-His or His-Cys dyad	▪ Tosyl lysyl chloromethyl ketone (TLCK) ▪ Iodoacetic acid ▪ Sulfhydryl agents such as PCMB	21-30	neutral or acidic pH	▪ plant protease (papain, bromilain) ▪ mammalian lysomal cathepsin
Aspartic protease	Asp-Xaa-Gly, in which Xaa can be Ser or Thr	▪ Pepstatin ▪ Diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP)	30-45	pH 3-4	• digestive enzyme (pepsin and chymosin) • renin • fungal protease (penicillopepsin)
Metallo-protease	His, Asp, Glu or Lys	▪ Metal-chelating agent such as EDTA	34	pH 5-10	▪ thermolysin

2.1.2 Applications of protease

Alkaline proteases account for a major application in market all over the world. Alkaline protease produced from bacteria can be applied in numerous industrial sectors that are summarized in Table 2.

Table 2 Commercial microbial alkaline protease, sources, applications and their industrial suppliers (modified from (Gupta, Beg and Lorenz, 2002; Saxena, 2015)).

Supplier	Product trade name	Microbial source	Application
Novozymes (previously Novo Nordisk), Denmark	Alcalase	*Bacillus licheniformis*	Detergent, silk degumming
	Savinase	*Bacillus* sp.	Detergent, textile
	Esperase	*B. lentus*	Detergent, food, silk degumming
	Biofeed pro	*B. licheniformis*	Feed
	Durazym	*Bacillus* sp.	Detergent
	Novozyme 471MP	n.s.	Photographic gelatin hydrolysis
	Novozyme 243	*B. licheniformis*	Denture cleaners
	Nue	*Bacillus* sp.	Leather
	Purafact	*B. lentus*	Detergent
Genencor International, USA	Primatan	Bacterial source	Leather
	Subtilisin	*B. alcalophilus*	Detergent
	Opticlean	*B. alcalophilus*	Detergent
	Optimase	*B. licheniformis*	Detergent
Solvay Enzymes, Germany (part of Genencor Inc, USA)	Maxapem	Genetically modified *Bacillus* sp.	Detergent
	HT-proteolytic	*B. subtilis*	Alcohol, baking, brewing, feed, food, leather, photographic waste
	Protease	*B. licheniformis*	Food, waste
	Proleather	*B. subtilis*	Food
	Collagenase	*Clostridium* sp.	Technical
Amano Pharmaceticals, Japan	Amano protease S	*B. subtilis*	Food
	Enzeco alkaline protease	*B. licheniformis*	Industrial
	Enzeco alkaline protease-L FG	*B. licheniformis*	Food
Enzyme Development, USA	Enzeco high alkaline protease	*Bacillus* sp.	Industrial
	Bioprase concentrate	*B. subtilis*	Cosmetic, pharmaceuticals
	Ps. protease	*Pseudomonas aeruginosa*	Research
Nagase Biochemicals, Japan	Ps. elastase	*P. aeruginosa*	Research
	Cryst. protease	*B. subtilis* (K2)	Research
	Cryst. protease	*B. subtilis* (bioteus)	Research
	Bioprase	*B. subtilis*	Detergent, cleaning
	Bioprase SP-10	*B. subtilis*	Food
	Godo-Bap	*B. licheniformis*	Detergent, food
	Corolase 7089	*B. subtilis*	Food
Godo Shusei, Japan	Wuxi	*Bacillus* sp.	Detergent
Rohm, Germany	Protosol	*Bacillus* sp.	Detergent
Wuxi Synder Bioproducts, China	Wuxi	*Bacillus* sp.	Detergent
Advance Biochemicals, India	Protosol	*Bacillus* sp.	Detergent

n.s. Not specified

2.2 Lipase

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes that catalyze the hydrolystic cleavage of ester bonds present in long-chain water-insoluble triacylglycerols to liberate diglycerides, monoglycerides, fatty acids and glycerol (McAuliffe, 2012) (Figure 2).

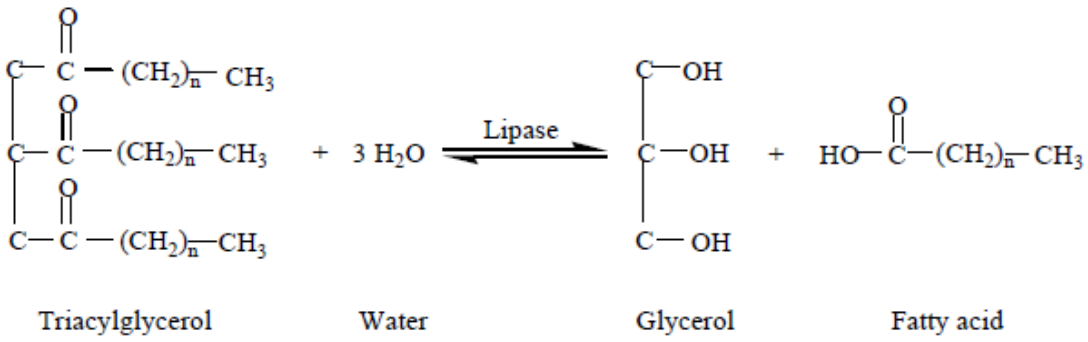


Figure 2 Hydrolytic and synthetic action of lipase (Rahman, Salleh and Basri, 2006)

Lipases (EC 3.1.1.3) and esterases (E.C. 3.1.1.1.) are enzyme catalyzing the hydrolysis of carboxyl ester bonds (carboxyl ester hydrolases). The difference between esterases and lipases are their substrate specificity and their capability to hydrolyze ester in emulsion and solution, respectively (Jaeger, Dijkstra and Reetz, 1999). In general, lipases are defined as enzymes display high activity against water-insoluble medium- and long-chain triacylglycerols (TAG) (Chahinian *et al.*, 2002). The long-chain acylglycerols can be regarded as an acyl chain length >10 carbon atoms, such as trioctanoin and triolein (Jaeger *et al.*, 1999). They are also perfectly capable of emulsions of water-soluble short-chain TAG, such as tripropionin and tributyrin (Chahinian *et al.*, 2002). Esterases act on solutions of short-chain acylglycerols such as methyl butyrate, ethyl butyrate and triacetin, which are poorly hydrolyzed by lipases (Chahinian *et al.*, 2002). The standard substrate of esterases are acylglycerols with an acyl chain length are less than 10 carbon atoms (Jaeger *et al.*, 1999). Moreover, esterases are inactive against emulsions of long-chain TAG (Chahinian *et al.*, 2002).

Structural characteristics of lipase generally have α/β hydrolase fold (β-strand surrounded by α-helix) containing a helical segment called the lid that covers the active site when the enzyme form in closed conformation. As the presence of lipid

aggregates, the lid opens, and the enzyme activity is increased, a phenomenon called interfacial activation (van Pouderoyen *et al.*, 2001). In addition, the active site residues are ordering in Ser-Asp/Glu-His. A catalytic triad consisting of a nucleophilic serine (Ser) situated in a highly conserved Gly-X-Ser-X-Gly pentapeptide, and an aspartate (Asp) or glutamate (Glu) residue that is hydrogen bonded to a histidine (His) (Jaeger *et al.*, 1999).

Lipases can be found in diverse source, such as plant, animal and microorganisms. More abundantly, they are found in variety of bacteria, yeasts, and filamentous fungi (Haki and Rakshit, 2003). Microbial extracellular lipases are usually more thermostable than animal and plant lipases. In addition, the majority of lipases currently available are derived from mesophilic sources and display optimum activity at 35 to 40 ºC (Sigurgísladóttir *et al.*, 1993).

2.2.1 Classification of lipase

Arpigny and Jaeger (1999) classified bacterial lipolytic enzymes to eight families on the basis of catalytic serine residue with highly conservative pentapeptide (Gly-Xaa-Ser-Xaa-Gly) and some basic properties of enzymes (Arpigny and Jaeger, 1999; Nthangeni *et al.*, 2001; Jaeger and Eggert, 2002). Although, these lipolytic enzymes have been identified as low sequence similarities, they share characteristics of α/β-hydrolase fold with a highly conserved catalytic triad (Ser-Asp/Glu-His). The eight families of lipolytic enzymes are described below (Table 3).

- **Family I**

  - *Pseudomonas* lipases

Family I is the largest family of lipase which is called true lipase. This family comprises 7 subfamilies (Table 3). The lipase in subfamilies I.1 and I.2 are secrete via the type II pathway, whereas those belonging to subfamily I.3 use the type I secretion pathway (Angkawidjaja and Kanaya, 2006). Lipases in subfamily I.1 have lower molecular mass (30-32 kDa) than those of subfamily I.2 (33kDa), due to an insertion in the amino acid sequence, which forms an anti-parallel double beta-strand at the surface of the molecule (Arpigny and Jaeger, 1999). Lipase producer from subfamily

I.3 secrete the enzyme with higher molecular mass (50-65 kDa) than those from subfamilies I.1 and I.2.

- *Bacillus* lipases

For lipase producing by *Bacillus*, an alanine residue replaces the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly (Arpigny and Jaeger, 1999). However, lipases from *B. subtilis* and *B. pumilus* and *B. licheniformis* were initially grouped in subfamily I.4 of true lipases. The lipases of this group have the conservative pentapeptide Ala-His-Ser-Met-Gly and smallest true lipase known (19-20 kDa) (Eggert *et al.*, 2002). This lipases subfamily is not thermostable because their activities decrease significantly at temperatures above 45 ºC. They exhibit extreme alkaline tolerance with maximum lipase activities at pH values between 9.5 and 12 (Guncheva and Zhiryakova, 2011).

Lipases in I.5 subfamily comprise mainly lipases from *Geobacillus* sp. They are larger protein with molecular weight of 40-45 kDa and higher. Their characteristic conserved pentapeptide is Ala-His-Ser-Gln-Gly. These lipases possess cysteine residues, participating in the formation of disulfide bonds that may stabilize the protein against thermal inactivation (Guncheva and Zhiryakova, 2011).

- Lipase from Gram-positive organisms

Lipases from subfamily I.6 such as lipase produced by *Staphylococcal haemolyticus* is a larger enzyme (~80 kDa) and secretes as a precursor that is cleaved in the extracellular medium by a specific protease and processed rapidly into a 45 kDa mature lipase (Arpigny and Jaeger, 1999; Oh *et al.*, 1999).

- Other lipases

The central region of lipases from *Propionibacterium acnes* and from *Streptomyces cinnamoneus* show significant similarity to lipases from *B. subtilis* and from subfamily I.2. The *Streptomyces cinnamoneus* lipase was no similar to other *Streptomyces* lipases known so far.

■ **Family II**

The new subclass of lipolytic enzymes has been known as the GDSL family. GDSL esterases and lipases are multifunctional properties such as broad substrate specificity and regiospecificity. These enzymes have little sequence homology to true lipases (Akoh *et al.*, 2004). They are separated from many lipases due to their Gly-Asp-Ser-(Leu)[GDSL] motif which differ from conventional pentapeptide Gly-Xaa-Ser-Xaa-Gly (GxSxG) motif found in many lipases (Arpigny and Jaeger, 1999). In addition, its serine-containing motif is closer to the N-terminus unlike other lipases where the GxSxG motif is near the center (Akoh *et al.*, 2004). The molecular weight appears in the range of ∼22–60 kDa (Akoh *et al.*, 2004). Lipases in this family have been identified as esterase located in the outer membrane of *P. aeruginosa.* These enzymes are an autotransporter which belonging to a previously identified family of channel-forming bacterial virulence factors (Loveless and Saier, 1997). The three-dimentional structure of the *Streptomyces scabies* enzymes showed the tertiary fold of esterases belonging to this family but different from the α/β hydrolase fold that found in most lipases (Jaeger *et al.*, 1999).

■ **Family III**

Enzymes from family III display the canonical fold of alpha/beta-hydrolases and contain a typical catalytic triad. The homolysin (HSL) family displays a striking amino acid sequence similarity to the mammalian HSL. A cold-adapted lipase of an Alaskan phychrotroph, *Pseudomonas* sp. Strain B11-1 has consensus motifs conserved in mammalian hormone-sensitive lipase (Rahman *et al.*, 2006).

■ **Family IV**

Member of family IV is described as human-sensitive lipases (HSL) originate from mesophilic bacteria, cold adapted or heat adapted organisms, due to high similarities to mammalian HSL (Kraemer and Shen, 2002). They exhibit the active-site serine residue in a consensus pentapeptide Gly-Asp-Ser-Ala-Gly, which is situated nearly to the N terminus of theprotein. In addition, they have another strictly conserved His-Gly-Gly-Gly motif of unknown function located immediately upstream of the active site consensus motif (Jaeger *et al.*, 1999).

■ **Family V**

It is noticed that families IV and V contain lipases originate from mesophilic bacteria, cold adapted or heat adapted organisms. The members of family V share significant amino acid sequence similarity (20-25%) with various bactria non-lipolytic enzymes, namely epoxide hydrolases, dehalogenases and haloperoxidases, which also possess the typical α/β-hydrolases fold and catalytic triad (Arpigny and Jaeger, 1999).

■ **Family VI**

Family VI contains carboxylesterase which are small protein with molecular mass around 23-26 kDa. They presumably located in the bacterial cytoplasm with similarity to mammalian lysophospholipases (Jaeger *et al.*, 1999). The subunit has the α/β-hydrolase fold and a classical Ser-Asp-His catalytic triad. This carboxylesterase hydrolyses small substrates with a broad specificity and displays no activity towards long-chain triglycerides (Arpigny and Jaeger, 1999). In addition, dimer formation is necessary to activate the enzyme from this family (Rahman *et al.*, 2006).

■ **Family VII**

Members of family VII comprise bacterial esterase with largely molecular weight about 55 kDa. They share significant amino acid sequence homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases (Arpigny and Jaeger, 1999).

■ **Family VIII**

The esterases in family VIII possess an active site more reminiscent of that found in class C β-lactamases, which involves a Ser-Xaa-Xaa-Lys motif conserved in the N-terminal part of both enzyme categories. In contrast, esterase/lipase consensus sequence Gly-Xaa-Ser-Xaa-Gly that appears in the *P. fluorescens* esterase shows esterase/lipase consensus sequence Gly-Xaa-Ser-Xaa-Gly which may be involved in the active site of the enzyme. Moreover, the motif lies near the C-terminus of the *P. fluorescens* and *Streptomyces chrysomallus* enzymes and no histidine residue follows it in the sequence (Arpigny and Jaeger, 1999). It indicates that the catalytic residues in

the sequence Ser-Asp-His that is conserved in superfamily of lipase would not be expected in family VIII (Rahman *et al.*, 2006).

2.2.2 Applications of lipase

Lipases from microbes represent the most widely used class of enzymes in innumerable application and industrial propose that lipids are modified or need to be removed (Table 4). In detergent industry, lipases are added to laundry detergent for the removal of fat-containing stains. In the paper industry, lipases remove fatty pitches from paper in the manufacturing process. These pitches stem from remaining resin of the wood chips. In the food industry, lipases are used in lipid modification processes, that used lipase-catalyzed the transesterification of lipids other than the original fatty acids for modifying the texture, digestibility or physical properties of natural lipids. In the baking industry, lipases are used to influence the quality of bread through modification of the wheat flour lipids. Finally, lipases are used for flavor enhancement of chees in the dairy industry. A new industrial application for lipases has been developed in the field of renewable energy. Lipases are used for methylation of plant oils resulting in methylated oils (biodiesel) that are used as a replacement or supplement for fossil fuel-derived diesel (McAuliffe, 2012).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 3 Families of lipolytic enzymes (modified from (Rahman *et al.*, 2006))

Family	Sub-family	Enzyme-producing strain	Properties
I	1	*Pseudomonas aeruginosa*	True lipase
		Pseudomonas fluorescens	
		Acinetobacter calcoaceticus	
	2	*Burkholderia glumae*	
		Chromobacterium viscosum	
		Bacillus cepacia	
		Pseudomonas luteola	
	3	*Pseudomonas fluorescens*	
		Serratia marcescens	
	4	*Bacillus subtilis*	
		Bacillus pumilus	
		Bacillus licheniformis	
	5	*Geobacillus stearothermophilus*	
		Geobacillus thermocatenulatus	
		Geobacillus thermoleovorans	
	6	*Staphylococcus aureus*	
		Staphylococcus hyicus	
	7	*Propionibacterium acnes*	
		Streptomyces cinnamoneus	
II (GDS)		*Aeromonas hydrophila*	Secreted acyltransferase
		Streptomyces scabies	Secreted esterase
		Pseudomonas aeruginosa	OM-bound esterase
		Photorhabdus luminescens	Secreted esterase
III		*Sreptomyces exfoliatus*	Extracellular lipase
		Sreptomyces albus	Extracellular lipase
		Moraxella sp.	Extracellular esterase 1
IV (HSL)		*Alicyclobacillus acidocaldarius*	Esterase
		Pseudomonas sp. B11-1	Lipase
		Escherichia coli	Carboxylesterase
		Alcaligenes eutrophus	Putative lipase
		Moraxella sp.	Extracellular esterase 2
V		*Pseudomonas oleovorans*	PHA-depolymerase
		Haemophilus influenzae	Putative esterase
		Psychrobacter immobilis	Extracellular esterase
		Moraxella sp.	Extracellular esterase 3
		Acetobacter pasteurianus	Esterase
VI		*Synechocystis* sp.	Carboxylesterases
		Spirulina platensis	
VII		*Arthrobacter oxydans*	Carbamate hydrolase
		Bacillus subtilis	p-Nitrobenzyl esterase
		Sreptomyces coelicolor	Putative carboxylesterase
VIII		*Arthrobacter globiformis*	Stereoselective esterase
		Streptomyces chrysomallus	Cell-bound esterase
		P. fluorescens	Esterase III

Table 4 Commercial microbial lipase, sources, applications and their industrial suppliers (Gupta, Gupta and Rathi, 2004).

Supplier	Product trade name	Microbial source	Application
Genencor International, USA	Lumafast	*P. menodocina*	Detergent
Gist-Brocades, The Netherlands; Genencor International, USA	Lipomax	*P. alcaligenes*	Detergent
Unilever, The Netherlands	n.s.	*P. glumae*	Detergent
Solvay, Belgium	n.s.	*B. pumilus*	Detergent
Altus Biologics, Manheim	Chiro CLEC-PC, Chirazyme L-1	*P. cepacia*	Organic synthesis
Amano Pharmaceuticals, Japan	Amano P, P-30, PS, LPL-80, LPL-200S	*P. cepacia*	Organic synthesis
	Lipase AH	*P. cepacia*	Organic synthesis
	Lipase AK, YS	*P. fluorescens*	Organic synthesis
Biocatalysts, UK	Lipase 56P	*P. fluorescens*	Biotransformations, chemicals
	Lipase K-10	*Pseudomonas* sp.	Amano Pharmaceuticals, Japan
	Lipase 50P	*C. viscosum*	Biotransformations, chemicals
	Lipase QL	*Alcaligenes* sp.	Meito Sankyo Co., Japan
Meito Sankyo Co., Japan	Lipoprotein lipase	*Alcaligenes* sp.	Research
	Lipase PL, QL/QLL, PLC/PLG, QLC/QLG	*Alcaligenes* sp.	Technical grade
	Alkaline lipase	*Achromobacter* sp.	Research
	Lipase AL, ALC/ALG	*Achromobacter sp.*	Technical grade
Biocatalysts, UK	Combizyme 23P (proteinase/lipase mix)	n.s.	Waste treatment
	Combizyme 61P (proteinase/lipase mix)	n.s.	Waste treatment
	Combizyme 209P (amylase/lipase/proteinase mix)	n.s.	Waste treatment, grease disposal
Novo Nordisk	Greasex (lipase)	n.s.	Leather
	Lipolase	*Thermomyces lanuginosus*	Detergent

n.s Not specified

2.3 Co-application of protease and lipase

The co-application of protease and lipase are more valuable especially for laundry applications where a cocktail of enzyme are added to detergent to improve washing capacity (Ruchi *et al.*, 2008). Various processes of leather manufacture also insist the mixture of protease and lipase. For instance, Forezyme SK (La Forestal Tanica, Spain) or NovoLime (Novozyme), the commercial bioproduct comprising of a mixture of bacteria protease and lipase, are used in both soaking and liming process for removing hairs, nails, hooves and other keratinous materials (Thanikaivelan *et al.*, 2004; Saxena, 2015). Olsen (2000) revealed protease and lipase also used in the cleaning of slaughter house equipment. Moreover, a combination of protease and lipase further resulted in effective de-waxing and degumming, with positive effects on the wettability of silk in textile industry (Saxena, 2015). Therefore, the preparation comprising both the influence activity and lipase not being susceptible to hydrolysis by protease will have better applicability.

Accordingly, the use of protease and lipase in combination required proteolytic-resistance enzyme, because proteases are hydrolytic enzymes which are capable of auto-digestion and also digest other enzymes produced simultaneously (Aguilar *et al.*, 2002). Kok *et al.* (1996) reported that proteolytic degradation of protease is important factor in regulating the level of lipase, due to rapid extracellular degradation of lipase by an endogenous protease. A thorough studying reveals several reports which state that the production of lipase and protease are inter-related (Rajmohan *et al.*, 2002). These studies showed that when the production of protease is affected either due to the influence of production parameter or due to genetic alterations, the production of lipase is enhanced. For instance, Lopes *et al.* (2008) proved that the increased air pressure during fermentation decreased protease production and thereby enhanced lipase production. Westers *et al.* (2005) investigated on the susceptibility of a lipase produced by *B. subtilis* to degradation by extracytoplasmic proteases located in the cell wall or in the growth medium. Furthermore, improvement in proteolytic resistance lipase by using genetic engineering are also perform by Ahmad *et al.* (2012).

A number of reports are available on the production of either lipases or proteases from various microorganisms including bacteria, fungi and actinomycetes bacteria. Nevertheless, there are few reports on proteolysis-resistant lipases are available on simultaneous production of protease and lipase that is only from bacteria using *Pseudomonas* sp. (Christen and Marshall, 1984; Ruchi *et al.*, 2008; Bisht *et al.*, 2013), *Serratia marcescens* (Henriette *et al.*, 1993; Kumar *et al.*, 2012) and *Bacillus* sp. (Sangeetha, Geetha and Arulpandi, 2010; 2010). Zhang *et al.* (2008) and Dutta and Ray (2009) produced lipase from *Streptomyces fradiae* and *B. cereus*, respectively, which was resistant to commercial neutral and alkaline proteases. Lipases produced by *P. aeruginosa, B. pumilus* and *B. licheniformis* were found to be low susceptibility toward its own co-produced native proteases (Ruchi *et al.*, 2008; Sangeetha *et al.*, 2010; 2010; Grbavčić *et al.*, 2011)

2.4 Microbial enzyme sources

2.4.1 Microbial producer

Industrial enzymes can be either extracted from natural sources (animal, plant, microbial) or large-scale microbial fermentation processes (McAuliffe, 2012). Microbial enzymes are favored over plant or animal source, due to their economic production, diverse in catalytic activities and properties, stable in applicable condition, ease to fermentation on a large scale, and ease of process modification and optimization. The microbial enzymes account for 90% of the total sales of enzymes (Illanes, 2008). The early enzyme production processes were based on microorganisms such as *Aspergillus niger*, *A. oryzae*, *Bacillus subtilis* and *B. licheniformis*, which produced significant amounts of enzyme that were found to be applicable in various processes and could be considered "Generally Recognized As Safe" (GRAS) based on FDA regulations (Hjort, 2007). Microbial enzymes are by far used in various industries and applications. Their microbial sources and commercial applications of the industrially important enzymes are listed in Table 5. Currently, the most frequently used enzymes in biotechnology are hydrolytic enzymes, being used for the degradation of various natural substances. In addition, enzymes have a wide range of applications in different industries including technical, feed, food or

chemical, pharmaceutical industry or any other (Table 6). Bacilli are well known for their ability to excrete many enzymes such as protease lipase and also amylase and are candidate for large-scale production of these enzymes.

2.4.2 *Bacillus licheniformis* 3C5

A Gram-positive bacteria, *Bacillus licheniformis* 3C5 has been recently reported, by Rachadech *et al.* (2010), as a slightly thermophilic and organic solvent-tolerant bacterium able to survive at 45 ºC in the presence of high concentrations of various organic solvents such as cyclohexane, toluene, etc. Moreover, its protease showed high activity and stability in the presence wide range of nonionic surfactants. The produced enzymes show excellent properties and good potential for biotechnological applications. Furthermore, *B. licheniformis* has been reported to produce an extracellular lipase (Gupta *et al.*, 2004; Guncheva and Zhiryakova, 2011). After preliminary study, this strain can also secrete lipase in addition to low activity of protease. Thus, *B. licheniformis* 3C5 has been selected for further studies of lipase characteristic, optimization of concomitantly production and partially purification of its protease and lipase.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 5 Applications of industrial enzymes (modified from (McAuliffe, 2012).

Enzyme function	Origin	Application area	Function
Class 1: Oxidoreductases			
Glucose oxidase	*A. niger*	baking	increase gluten strength
		brewing	shelf life improvement
		dairy	milk preservation
		textile	indirect enzymatic bleaching
		new industrial use	toothpaste
Hexose oxidase	*Chondrus crispus*	baking	increase gluten strength
Laccase	*Trametes vesicolor, Coprinus cinereus*	textile	prevent backstaining in enzymatic denim stone washing
		Pulp and paper	pulp bleaching
		new industrial use	cork treatment
Catalase	*A. niger*	brewing	shelf life improvement
		diary	milk preservation
		textile	hydrogen peroxide removal
Peroxidase, lactoperoxidase	*Phanerochaete chrysosporium*	baking	dough improvement
		dairy	milk preservation
Lipoxygenase	*Plants*	baking	whitening of breadcrumb
Class 2: Transferases			
Transglutaminase	*Bacillus circulans*	dairy	texture improvement in yoghurt and
Dextransucrase	*Leuconostoc mesenteroides*	brewing	production of isomalto-aligosaccharide beer
Class 3: Hydrolases			
α-Amylase	*Bacillus* sp.	laundry detergent	removal of starch-containing stains
		automatic dish washing	removal of starch-containing stains
		baking	antistaling in dough
		fruit juice	apple fruit production
		brewing	mashing, fermentation
		textile	textile desizing
Cellulase	*Aspergillus* sp., *Trichoderma reesei*	textile	cotton finishing and depilling
		animal feed	biomass processing
		Pulp and paper	enhancing the strength of fibers
		laundry detergent	promote fabric care

Table 5 Applications of industrial enzymes (modified from (McAuliffe, 2012). (Continued)

Enzyme function	Origin	Application area	Function
Xylanase	*Bacillus* sp., *Aspergillus* sp., *Trichoderma reesei*	baking	improved dough handling, dough stability
		animal feed	improve digestibility of cereals
		pulp and paper	pulp bleaching
Protease (subtilisin)	*Bacillus* sp.	laundry detergent	removal of proteous stains
		animal feed	improved digestibility of proteins in animal feeds
		Leather production	soaking, bating, dehairing process
Protease (other)	Animal, plant and microbial sources	textile	silk degumming, wool anti-shrinking
		new industrial use	artificial-denture cleaning
Pectin methylesterase	*Aspergillus* sp.	fruit juice	apple and red berry juice peeling
Phytase	*Aspergillus* sp.	brewing	mashing
		animal feed	releasing of phosphate from phytic acid in animal feed
Pullulanase	*Aspergillus* sp.	syrup production	increase fermentability of starch worts or syrups
β-Amylase	*B. cereus*	brewing	mashing
		fruit juice	apple juice production
Glucoamylase, amyloglucosidase	*A. niger*	brewing	mashing
		grain wet milling	Hydrolysisi of maltooligosaccharides
		new industrial use	toothpaste
Class 4: Lyases			
α-Acetolacetate decarboxylase	*B. brevis*	brewing	diacetyl removal for flavor enhancement
Pectate lyase	*Aspergillus* sp.	textile	cotton scouring
Pectin lyase	*Aspergillus* sp.	fruit juice	apple and red berry juice production
			citrus fruit peeling
Class 5: Isomerases			
Xylose isomerase	*Streptomyces* sp.	grain wet milling	High fructose corn syrup production
Class 6: Ligases			
Glutathione synthetase	*S. cerevisiae*	glutathione production	L-glutathione production

Table 6 Commercial application of enzymes from different microbes

Industrial Application	Industry	Enzyme	Source	Reference
Technical	Detergent	Protease	*B. licheniformis*	*Adrio and Demain (2014)*
			B. amyloliquefacien	*Gurung et al. (2013)*
		Lipase	*A. oryzae (Humicola lanuginose)*	*Adrio and Demain (2014)*
			P. mendocina	*Adrio and Demain (2014)*
			P. alcaligenes	*Saxena (2015)*
			Thermomyces lanuginosus	*Saxena (2015)*
		α-amylase	*B. licheniformis*	*Adrio and Demain (2014)*
			B. stearothermophilus	*Adrio and Demain (2014)*
			B. amyloliquefaciens	*Saxena (2015)*
		Cellulase	*Humicola insolens*	*Saxena (2015)*
		Mannanase	*Bacillus* sp.	*Adrio and Demain (2014)*
	Textile	Cellulase	*Hypocrea jecorina*	*Adrio and Demain (2014)*
			Humicola sp.	*Saxena (2015)*
			Streptomyces sp.	*Saxena (2015)*
			Melanocarpus albomyces	*Saxena (2015)*
		amylase	*Bacillus* sp.	*Gurung et al. (2013)*
			B. licheniformis	*Saxena (2015)*
			B. amyloliquefacien	*Saxena (2015)*
			B. stearothermophilus	*Saxena (2015)*
		cutinase	*P. mendocina*	*Saxena (2015)*
		Protease	*Streptomyces mobaraense*	*Saxena (2015)*
	Pulp and paper	Lipase	*Candida rugosa*	*Adrio and Demain (2014)*
		Xylanase	*Chiania* sp. NCL 82-5-1	*Adrio and Demain (2014)*
			S. roseiscleroticus	*Saxena (2015)*
			Saccharomonospora viridis	*Saxena (2015)*
			Thermomyces lanuginosus	*Saxena (2015)*
			Aureobasidium pullulans	*Saxena (2015)*
			T. reesei	*Saxena (2015)*
			B. subtilis	*Saxena (2015)*
			Streptomyces lividans	*Saxena (2015)*
		laccase	*S. cerevisiae*	*Adrio and Demain (2014)*
			T. reesei	*Saxena (2015)*
			A. oryzae	*Saxena (2015)*
			P. pastoris	*Saxena (2015)*
		Cellulase	*A. niger*	*Saxena (2015)*
			Trametes suaveolens	*Saxena (2015)*
	Leather	Lipase	*Bacillus* sp.	*Adrio and Demain (2014)*
		Protease	*A. flavus*	*Saxena (2015)*
			Bacillus sp.	*Saxena (2015)*
			Streptomyces sp.	*Saxena (2015)*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 6 Commercial application of enzymes from different microbes (Continued)

Industrial Application	Industry	Enzyme	Source	Reference
Feed	swine and poultry	Phytase	*B. subtilis*	*Selle and Ravindran (2007)*
			A. niger	*Selle and Ravindran (2007)*
			A. ficuum	*Saxena (2015)*
			Mucor piriformis	*Saxena (2015)*
			Cladosporium sp.	*Saxena (2015)*
		Protease		*Adrio and Demain (2014)*
		α-galactosidase		*Adrio and Demain (2014)*
		α-amylase		*Adrio and Demain (2014)*
Food	dairy	β-galactosidase (lactase)	*Kluyveromyces lactis*	*Adrio and Demain (2014)*
			K. fragilis	
			Candida pseudotropicalis	*Adrio and Demain (2014)*
	wine-making process	Pectinase	*A. niger*	*Saxena (2015)*
	dairy	Protease	*Mucor miehei*	*Adrio and Demain (2014)*
			B. subtilis	*Adrio and Demain (2014)*
			M. pusillus Lindt	*Adrio and Demain (2014)*
			Endothia parasitica	*Adrio and Demain (2014)*
	fructose corn syrup	glucose isomerase		*Adrio and Demain (2014)*
		α-amylase		*Adrio and Demain (2014)*
		glucoamylase		*Adrio and Demain (2014)*
	candy and jam	invertase	*Kluyveromyces fragilis*	*Adrio and Demain (2014)*
			S. calsbergensis	*Adrio and Demain (2014)*
			S. cerevisiae	*Adrio and Demain (2014)*
	cheese manufacturing process	Lipase	*Rhizomucor miehei*	*Saxena (2015)*
			A. oryzae	*Saxena (2015)*
			A. niger	*Saxena (2015)*
	starch	α-amylase	*B. stearothermophile*	*Adrio and Demain (2014)*
			B. licheniformis	*Adrio and Demain (2014)*
			B. amyloliquefacien	*Aunstrup et al. (1979)*
			A. oryzae	*Aunstrup et al. (1979)*
		Pullulanase	*K. aerogenes*	*Aunstrup et al. (1979)*
		glucoamylase	*A. niger (Rhizopus* sp.*)*	*Aunstrup et al. (1979)*
		glucose isomerase	*(Streptomyces sp.)*	*Aunstrup et al. (1979)*
			B. coagulans	*Aunstrup et al. (1979)*
			Actinoplanes sp.	*Aunstrup et al. (1979)*
Chemical and pharmaceutical	L-tyrosine	tyrosine phenol lyase	*Symbiobacterium toebii*	*Adrio and Demain (2014)*
	2-arylpropanoic acid	lipase	*Candida antarctica*	*Adrio and Demain (2014)*
			Pseudomonas sp.	*Adrio and Demain (2014)*

2.5 Factors affecting enzyme production

Most of the cell-produced products result from their response to environmental conditions, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation. Nutrients required by the cells can be classified into two categories (Liu, 2013):

- Macronutrients are required in concentration larger than 10^{-4}M, i.e. carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+} and K^{+} (Table 7).
- Micronutrients are needed in concentration of less than 10^{-4}M. Micronutrients are known as trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, vitamin, growth hormones and metabolic precursors.

Table 7 The major micronutrients and their physiological functions (Liu, 2013)

Element	Physiological Function	Required concentration (M)
Carbon	Constituents of organic Cellular material. Often the energy source.	$> 10^{-2}$
Nitrogen	Constituents of proteins, nucleic acids, and coenzymes.	10^{-3}
Hydrogen	Organic cellular material and water.	-
Oxygen	Organic cellular material and water. Required for aerobic respiration	-
Sulfur	Constituents of proteins and certain coenzyme.	10^{-4}
Phosphorus	Constituents of nucleic acids, phospholipids, nucleotides, and certain coenzyme.	10^{-4}-10^{-3}
Potassium	Principle inorganic cation in the cell and cofactor for some enzymes.	10^{-4}-10^{-3}
Magnesium	Cofactor for many enzymes and chlorophylls and present in cell wall and membrane.	10^{-4}-10^{-3}

Since, composition and concentration of media components significantly affect product concentration, yield and productivity. Therefore, the development of media composition and culture condition are required for optimum cell growth and product formation. It helps minimizing the amount of unutilized component at the end of fermentation. In addition, each strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999).

2.5.1 Biochemical factors

2.5.1.1 Carbon source

The carbon source is required for synthesis of building block for biomass and biomolecules, including protein, carbohydrates, lipids and nucleic acids. It also serves as a source of energy of the cell through the production of ATP either by substrate-level phosphorylation or oxidative phosphorylation (Vinci and Parekh, 2003). However, enzyme production is significantly influenced by carbon sources. As shown in Table 8, poly- and oligosaccharides like glucose, lactose, maltose and sucrose, also, polyols such as glycerol and mannitol are frequently used as carbon source in bioprocess for protease and lipase production (Guncheva and Zhiryakova, 2011; Bhunia, Basak and Dey, 2012). In addition, high carbohydrate concentration repressed enzyme production (Bhunia *et al.*, 2012).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.5.1.2 Nitrogen source

The type of nitrogen source in the medium serves as the synthesis of all nitrogen-containing compounds in the cell such as amino acids, nucleic acids, proteins and other cell wall components (Kumar and Takagi, 1999). The alkaline protease comprises 15.6% nitrogen and its production depends on the availability of both carbon and nitrogen sources (Kole, Draper and Gerson, 1988). Moreover, nitrogen source also influences the lipase titers in production broth (Guncheva and Zhiryakova, 2011). In generally, most microorganisms can utilize both inorganic and organic form of nitrogen. Peptone, tryptone, casamino acid and yeast extract are commonly used organic nitrogen sources for promote both protease and lipase secretion. However,

some inorganic nitrogen sources have also been revealed to be effective in some organisms (Gupta *et al.*, 2004). As shown in Table 9, the specific nitrogen requirement relies on individual organism.

2.5.1.3 Inducer

The lipolytic enzymes are inductive in the nature (Lotti *et al.*, 1998). Thus, substrate in the form of oil or any other lipidic substrate (triacyl glycerols, fatty acids, hydrolysable esters, tweens, bile salts and glycerol) must be present in the medium that enhances the production of lipase (Gupta *et al.*, 2004) (Table 10). Substrate specificity may depend on the chemical structure of substrate molecules and the physical properties of the emulsion or other surface (Hasan, Shah and Hameed, 2009).

2.5.1.4 Metal ion and salt

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese and molybdenum are required in the fermentation medium for optimum production of enzymes, while inorganic ions such as sodium ions, phosphate ions and calcium ions are essential for the growth of bacteria. Potassium phosphate was shown to be responsible for buffering of medium. The optimal phosphate concentration for protease production was 2 g/l. In contrast, excess concentration inhibit in cell growth and repression in protease production (Bhunia *et al.*, 2012).

2.5.2 Physical factors

2.5.2.1 pH and temperature

The important characteristic of most alkalophilic microorganisms is dependence on the extracellular pH for cell growth and enzyme production. The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). The cell growth and enzyme production of most alkalophilic microorganisms depends on extracellular pH, thus maintaining pH of fermentation medium above 7.5 increased the protease yields

(Kumar and Takagi, 1999). In the same way, maximum lipase production has been observed at pH more than 7 (Gupta *et al.*, 2004).

Temperature is another critical parameter that controlled growth rate and also control enzyme production. The mechanism of temperature control of enzyme production is not well understood (Chaloupka, 1985). However, Frankena *et al.* (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake. It has been observed that, in general, protease and lipase are produced in the temperature range 28-60 ºC (Kumar and Takagi, 1999) and 20-45 ºC (Gupta *et al.*, 2004), respectively.

2.5.2.2 Aeration and agitation

The aeration rate indirectly involve in dissolved oxygen level in the fermentation broth. The dissolved oxygen profiles can be controlled by: (i) adjust in aeration rate; (ii) adjust in the agitation rate; or (iii) use of oxygen-rich or oxygen-deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Moon and Parulekar, 1991). Moreover, the agitation rate also influences the nutrient availability for cells. Maximum yields of alkaline protease are produced at 200 rpm for *B. subtilis* ATCC 14416 (Chu, Lee and Li, 1992) and *B. licheniformis* (Sen and Satyanarayana, 1993). However, 150 rpm was optimal to sustain cell growth and lipase production (Shariff *et al.*, 2007).

Table 8 Some carbon sources utilized for lipase and protease production

Enzyme	Strain	Carbon sources	Concentration (%, w/v)	Reference
Lipase	*B. flexus* XJU-1	Soluble starch	1.50	(Niyonzima and More, 2014)
	B. safensis DVL-43	Glucose lactose	0.20 0.20	(Kumar, Parshad and Gupta,
	B. pumilus RK31	Glucose	2.00	(Kumar *et al.*, 2011)
	Bacillus sp.	Glucose Gum arabic	0.20 0.10	(Sharma *et al.*, 2014)
	B. coagulans	Starch	2.00	(Alkan *et al.*, 2007)
	Bacillus sp.	Glucose	1.00	(Dosanjh and Kaur, 2002)
	B. megaterium AKG-1	Mannitol Wheat bran	0.20 1.00	(Sekhon *et al.*, 2006)
	Bacillus sp.	Casamino acids	0.40	(Handelsman and Shoham,
	Bacillus sp. H-257	Glucose	0.20	(Imamura and Kitaura, 2000)
	B. cereus C7	Soluble starch	2.00	(Dutta and Ray, 2009)
	Bacillus spp.	Glucose	0.20	(Bradoo, Saxena and Gupta,
	B. alcalophilus	Maltose	1.00	(Ghanem, Al-Sayed and Saleh,
	G. stearothermophilus strain-5	Glycerol	0.20	(Berekaa *et al.*, 2009)
Protease	*B. licheniformis* N-2	Glucose	1.00	(Nadeem, Qazi and Baig, 2010)
	B. licheniformis	Xylose	1.00	(Suganthi *et al.*, 2013)
	Bacillus sp.	Maltose	1.50	(Prakasham, Rao and Sarma,
	B. subtilis	Galactose	1.00	(Pant *et al.*, 2015)
	Bacillus sp. SB12	Starch	2.00	(Briki, Hamdi and Landoulsi,
	B. koreensis (BK-P21A)	Sucrose	2.00	(Anbu, 2013)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 9 Some nitrogen sources utilized for lipase and protease production

Enzyme	Strain	Carbon sources	Concentration (%, w/v)	Reference
Lipase	*B. flexus* XJU-1	casein	1.50	(Niyonzima and More, 2014)
	B. safensis DVL-43	peptone	2.40	(Kumar *et al.*, 2014)
	Bacillus sp.	peptone	0.05	(Sharma *et al.*, 2014)
	B. coagulans	Ammonium nitrate	2.00	(Alkan *et al.*, 2007)
	Bacillus sp.	Yeast extract Peptone	0.20 0.50	(Dosanjh and Kaur, 2002)
	Bacillus sp. H-257	Yeast extract Peptone	1.00 0.50	(Imamura and Kitaura, 2000)
	B. cereus C7	Peptone	2.00	(Dutta and Ray, 2009)
	Bacillus spp.	Ammonium chloride	0.50	(Bradoo *et al.*, 1999)
	B. alcalophilus	Yeast extract Peptone	0.50 0.50	(Ghanem *et al.*, 2000)
	G. stearothermophilus strain-5	Yeast extract Peptone	0.50 1.00	(Berekaa *et al.*, 2009)
Protease	*B. licheniformis* N-2	Soybean meal	1.00	(Nadeem *et al.*, 2010)
	Bacillus sp.	Yeast extract	2.00	(Prakasham *et al.*, 2006)
	B. subtilis	Yeast extract	0.20	(Pant *et al.*, 2015)
	B. cereus strain CA15	Skim milk	1.00	(Uyar *et al.*, 2011)
	Bacillus sp. SB12	Yeast extract	1.00	(Briki *et al.*, 2016)
	B. koreensis (BK-P21A)	Yeast extract	0.20	(Anbu, 2013)
	Rhizopus oryzae	Sodium nitrate	0.25	(Banerjee and Bhattacharyya, 1992)
	Conidiobolus coronatus	Ammonium nitrate	3.00	(Phadatare, Deshpande and Srinivasan, 1993)

Table 10 Some inducers utilized for lipase and protease production

Enzyme	Strain	Carbon sources	Concentration (%, w/v)	Reference
Lipase	*B. flexus* XJU-1	Cottonseed oil	2.00	(Niyonzima and More, 2014)
	B. safensis DVL-43	Olive oil	0.74	(Kumar *et al.*, 2014)
	Bacillus sp.	Olive oil Tween 80	1.16 0.12	(Sharma *et al.*, 2014)
	B. coagulans	Olive oil	1.00	(Alkan *et al.*, 2007)
	B. megaterium AKG-1	Soybean oil	1.00	(Sekhon *et al.*, 2006)
	B. subtilis	Sesame oil	100	(Takaç and Marul, 2008)
	Bacillus sp. RSJ-1	cottonseed oil Tween 80	0.75 0.50	(Sharma *et al.*, 2002)
	B. licheniformis	Tween 80	1.00	(Khyami-Horani, 1996)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.6 Downstream process

2.6.1 Conventional downstream process

Downstream processing of biological products from fermentation broth is an important step of production which is usually composed of four steps namely, recovery, isolation, purification and polishing (RIPP). Among these four steps, purification steps account for 50–90% of the total downstream processes costs (Cunha *et al.*, 2003). Many unit operations involved in these four steps, the choice of which is defined not only by the properties of the product but also by the complexity of separations, scale of operation, economics, and desired purity of the product.

Downstream processing steps for the recovery and purification of enzymes usually divided into four stages as following describe and in Figure 3 (Lütz, 2013):

■ Recovery

A conventional sequence of recovery starts with separation of interesting enzymes from other bulk contaminants which depend on enzyme location either inside the cell membrane (intracellular), outside the cell membrane and inside the cell wall (periplasmic), or outside the cell wall (extracellular). In case the enzymes are intracellular, the cells must be disrupted to release the product followed by another solid-liquid separation step. For extracellular enzymes, those disrupted step should be avoided. The unit operations involved is normally centrifugation and filtration. The clarification step in Figure 3 can be replaced by using expanded bed adsorption and desorption (EBA) that is suitable for large-scale production of intracellular and periplasmic enzymes.

■ Isolation and concentration

Following the clarification step, the solution with intracellular, periplasmic, or extracellular enzymes should be concentrated using ultra- (or nano) filtration which is necessary to know the molecular weight of the target enzyme. The selection of appropriate filter should give a maximal activity yield. Thereafter, measured activity of the original solution and the concentrate must be compared to reach the desired concentrates. The cutoff of the filters should also be selected so that a >90% of the

subject enzyme is in the concentrate. After the concentration step, the contaminant proteins with lower molecular weight than the enzyme are reduced in the concentrate. Previously, protein precipitation using ammonium sulfate preferred for concentration step to precipitate target enzyme including other proteins. This technique can be avoided, especially in large-scale enzyme production due to a high salt content and N load in the wastewater. It can be replaced by using the ultrafiltration step.

In the concentration and filtration steps, the cells are practically completely removed from the concentrate. Moreover, the amount of low molecular weight impurities and ions that can interfere in the further purification steps reduced. The concentrate consist other biopolymers such as enzymes, proteins, nucleic acids, soluble polysaccharides, and endotoxins (Table 11). These impurities, especially the nucleic acid DNA and RNA can interfere in the further processing steps, mainly chromatographic which used for purification of target enzyme. Also, they increase the viscosity of the concentrates, and are adsorbed to anion exchangers. To avoid this, the nucleic acids can be removed by using specific nucleic acid adsorbents (that also can adsorb proteins), or hydrolyzing with nucleases (such as Benzonase™, Merck, Germany) before or after the concentration step. The important of impurities separation from desired enzyme depends on the final use and the required specific activity of the enzyme on a weight basis, whether the impurities disturb the process in which the enzyme is to be used as a catalyst.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

■ **Purification**

This purification steps are mainly chromatographic using suitable adsorbents. It is essential to know the following properties of the target enzyme:

- The isoelectric point (pI): It is important for selecting the separation conditions for ion-exchange or mixed-mode chromatography. This can be determined by isoelectric focusing (IEF).
- Molecular weight (MW): It can be determined by SDS gel electrophoresis under denaturating conditions.
- Cofators: The tightly bound FAD or PLP (pyridoxal-5'-phosphate) or metal ions required for optimal enzyme activity and stability, such as Ca^{2+} for penicillin amidase that may be lost during the purification procedures.

- The pH range: pH promotes enzyme stability, within which the purification must occur.

■ Polishing or formulation

After purification step the purified enzymes must be controlled the quality (enzyme activity and purity, other contents) and formulated which depends on enzyme proposes such as technique crystallization, drying, granulation, or immobilization. The latter applies mainly for technical enzymes.

Table 11 The composition of concentrates after isolation step (Lütz, 2013)

	Biopolymer (% of total biopolymer dry weight)			
	Prokaryoti cell, *E. coli,* penicillin amidase after disruption of whole cells[a)]	**Prokaryoti cell, *E. coli, periplasmic penicillin* amidase after isolation of the periplasmic cell fraction by osmotic shock[a)]**	**Eukaryotic yeast cell, *H. polymorpha* extracellular phase[a)]**	**Eukaryotic animal cell, CHO, extracellular monoclonal antibody[a)]**
Subject (enzyme/protein)	10	≤65	≤95	≤80
Other proteins	50	≥35	-5	≥10[b)]
DNA/RNA	<30	2	ND	≤10[b)]
Polysaccharides	<10	ND	ND	ND
Endotoxin[c)]	<1	<0.1		

a) Cell, strain and produced enzyme or mAb.

b) Estimated from the number of dead cells

c) Endotoxins, also known as pyrogen (cause fever), are lipopolysaccharides bound in the outer cell wall of gram-negative bacteria. They are toxic for animal and humans. Their contents are influence by the fermentation conditions and vary from strain to strain.

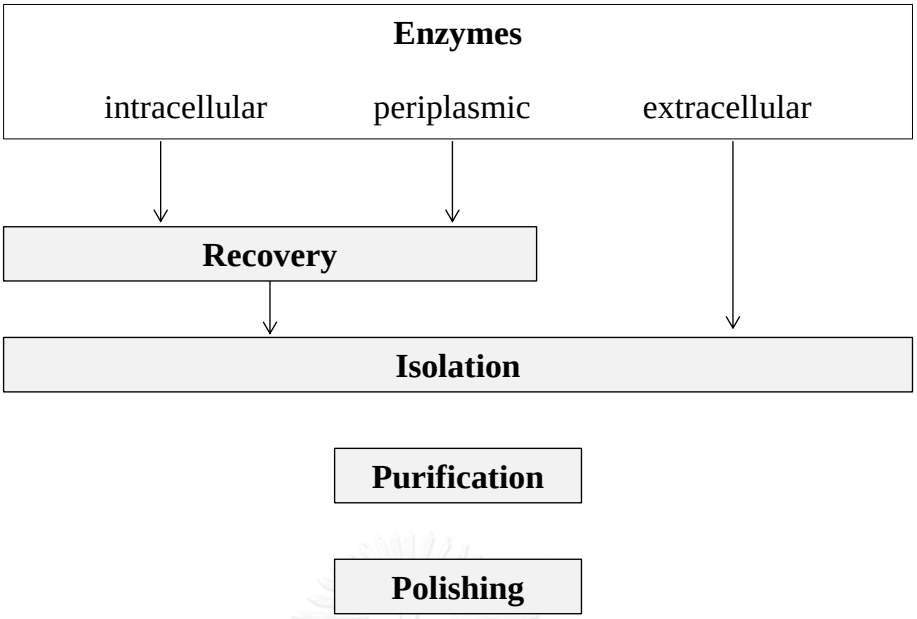


Figure 3 A schematic of downstream processing steps of enzymes

Additionally, biotechnology processes focus on the recovery and purification of the interesting product. The conventional techniques such as precipitation chromatography, or electrophoresis, have been widely employed. However, due to several steps of unit operations, considerable cost and low recovery yields as biological product loss in each step, the technique leads to limit their application in large-scale production of biological products (Raja *et al.*, 2011).

Extraction using aqueous two-phase system (ATPS) is an alternative technique which decreases number of steps and minimizes the overall cost. It has many advantages over conventional downstream processing like simple, rapid separation and low cost of phase forming materials. The ATPS also increases biological products recovered in highly purified form, due to the presence of high water content (70-90%) possibly reduced protein denaturation. Moreover, most of the polymer used have stabilizing effect on the protein tertiary structure and biological activity (Cleland and Wang, 1990; Hatti-Kaul, 2001). Moreover, the ATPS in lab scale has been successfully applied for large-scale enzyme separation and purification (Kula et al., 1982; Veide, Smeds and Enfors, 1983; Hustedt, Kroner and Papamichael, 1988).

2.6.2 Aqueous two-phase system (ATPS)

2.6.2.1 Introduction

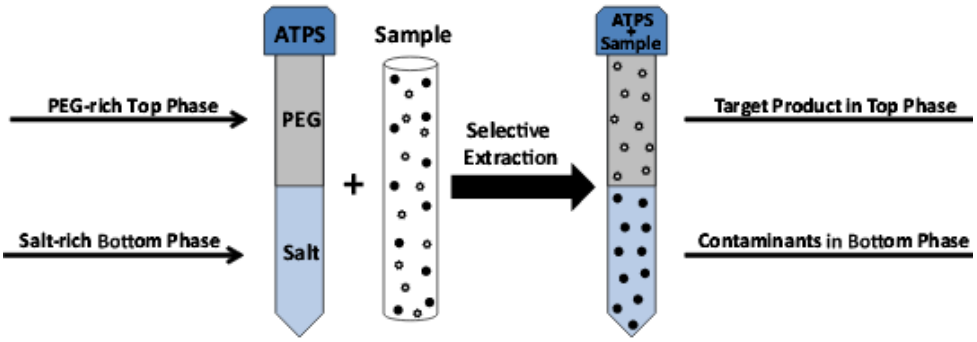


Figure 4 Extraction processes based on PEG/salt ATPS (Glyk, Scheper and Beutel, 2015)

ATPS has been firstly reported in the literature by Beijerinck in the 19th century, who discovered that agar and gelatin formed two phases when mixed at certain concentration (Diamond and Hsu, 1992). However, it was not until 1955 that Albertsson discovered that polyethylene glycol (PEG), potassium phosphate and water, and PEG, dextran and water formed two-phases and realized the potential use of these systems as an important separation technique in the downstream processing of biomolecules (Diamond and Hsu, 1992).

ATPS is a liquid-liquid extraction technique. As shown in Figure 4, the technique is based on incompatibility of two aqueous solutions. After mixing ATPS system with sample, phase separation is generated by centrifugation of settling under gravity, resulting in partitioning of target product to top phase and contaminants to bottom phase, or conversely. Thus, target product is recovered and concentrated in one phase.

In general, ATPS can be formed by two polymers, a polymer and a salt, an ionic liquid and a salt, or a low molecular weight alcohol and a salt (Rosa *et al.*, 2010). Furthermore, micellar and reverse micelle ATPS can also be formed with ionic and/or non-ionic surfactants solution (Ruiz-Ruiz *et al.*, 2012). ATPS can be modified with affinity ligands, which significantly increase the recovery yields and purification folds (Ruiz-Ruiz *et al.*, 2012).

2.6.2.2 Phase system and properties

The two immiscible aqueous phases are formed by mixing two components over the critical concentrations. The critical concentrations for generating two-phase area are specific in each ATPS which depends on the type of phase forming components and on the pH and temperature of the solution. The first component predominates in one phase and the second component predominates in the other phase, which the bulk of both phases consists mainly of water (Rosa *et al.*, 2010).

Figure 5 shows the example of phase diagram of polymer/salt system. Bottom phase (salt X% (w/w)) is plotted on the abscissa and top phase (polymer Y% (w/w)) is plotted on the ordinate. The binodal curve, at point TCpB as seen in Figure 5, separates the area of two immiscible aqueous phases (above the curve) from one phase (below the curve). The design of ATPS extraction is based on this binodal data. The Cp is critical point, while T and B are the final composition of the top and bottom phase, respectively. The three systems A1, A2 and A3 differ in their initial compositions and in volume ratios. However, they all have the same top phase equilibrium composition ($T_{Polymer}$,T_{Salt}) and the same bottom phase equilibrium composition ($B_{Polymer}$,B_{Salt}). This is because they are lying on the same tie-line (TL), whose end points determine the equilibrium phase composition and lie in the binodal curve. The ratio of the segments AB (top phase) and AT (bottom phase) can be approximated graphically by the volume ratio of the two phases. The critical of each TL is stated at the midpoint, which the volume ratio of the phase becomes equal.

The tie-line length (TLL) can be calculated from the equilibrium phase composition as follows:

$$TLL = \sqrt{(B_{salt}-T_{salt})^2+(T_{PEG}-B_{PEG})^2}$$

where T_{salt} and B_{salt} are final salt concentration in top and bottom phase, while $T_{Polymer}$ and $B_{Polymer}$ are final polymer concentration in top and bottom phase

or

$$TLL = \sqrt{(\Delta X)^2+(\Delta Y)^2}$$

where ΔY and ΔX are the difference in concentration of component X and Y between the two phases as shown in Figure 5.

This calculation generate based on Pythagoras' theorem. So, TLL has the same unit with the component concentrations (%, w/w). The length of TL is related to the mass of phases by equation:

$$\frac{V_t \rho_t}{V_b \rho_b} = \frac{AB}{AT}$$

where V and ρ are the volumes and densities of the top (t) and bottom (b) phase and AB and AT are segment lengths of the TL as shown in Figure 5.

As the TLL decrease in length, the critical point, where is determined by extrapolation (------) through the midpoints of a number of tie-lines as seen in Figure 5, alters to the the point that TLL=0. At this point the composition and volume of the two phases theorically become equal (Raja *et al.*, 2011).

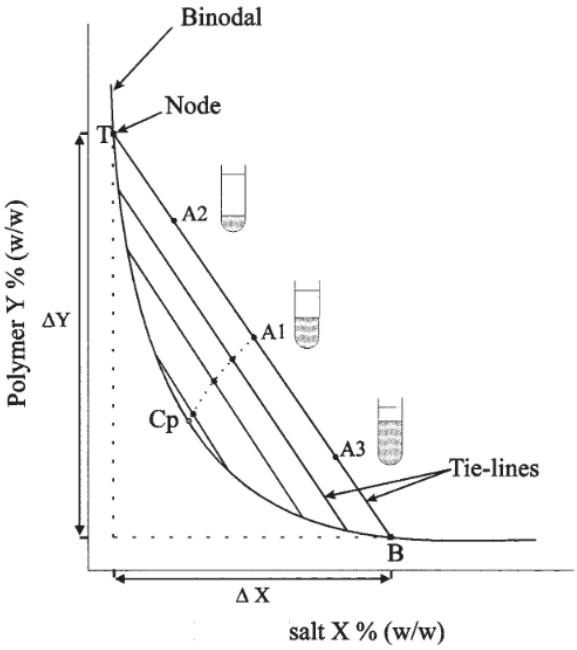


Figure 5 Binodal curve. TCpB represents binodal curve, which Cp (○) is critical point. The TB is tie-line, which, T and B (■) represent the final composition of the top and bottom phase, respectively. A1, A2, and A3 represent the total compositions (●) of three systems lying on the same tie-line (TB) with different volume ratios. The Cp is determined by extrapolation (------) through the midpoints of a number of tie-lines. The difference in concentration of component X and Y between the two phases is represented by ΔY and ΔX (Kaul, 2000).

2.6.2.3 Factors affecting partitioning of biomolecules in ATPS

The selective partitioning of the biomolecules in ATPS is depends on a number of parameters relating to the system properties (for example polymer type, molecular weight and concentration, salts type and concentration, pH value) and physicochemical properties of the solute (such as molecular weight, superficial electrochemical charge, hydrophobicity, etc.). Furthermore, the interaction between the system and physicochemical properties also control their partitioning behavior.

However, the complexity of the systems involved in the partitioning process can make these systems difficult to predict exactly behavior of target proteins in ATPS system. The presence of high concentration of polymers and salt affect the manipulating of protein characteristic (González-González *et al.*, 2011), corresponding to lack of information about the exact mechanism of partitioning and unpredictable (Mohamadi, Omidinia and Dinarvand, 2007). In addition, one major drawback concerning the use of ATPS is their lack of specificity. Although the manipulation of the system properties may prefer the selective partitioning of the interesting biomolecules towards a particular phase, specificity usually is not high enough to achieve complete separation between product and contaminants.

The role of several factors is discussed as following sections:

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.6.2.3.1 Polymer type

In polymer/salt systems, PEG was frequently used as polymer, because of high water solubility, rapid phase separation, inexpensive, non-toxic and biodegradable (Rosa et al., 2010). Furthermore, its stimulating ability to protein folding retained good enzyme activity (Cleland, Hedgepeth and Wang, 1992). Besides, alternative polymer either PPG or PVP are good water solubility similar to PEG, thereby they could be used as ATPS constituents. Some literatures reported that PVP has ability to prepare the ATPS (Zafarani-Moattar and Sadeghi, 2005; Sadeghi, Rafiei and Motamedi, 2006; Salabat *et al.*, 2006; Zafarani-Moattar and Seifi-Aghjekohal, 2007; Zafarani-Moattar and Zaferanloo, 2009; Zafarani-Moattar and Abdizadeh-Aliyar, 2014). Only one study of Mokhtarani *et al.* (2011) has been reported to using PVP/salt system for protein partitioning. Even though, PPG has a remarkable lower solubility

in water than PEG. Low molecular weight of PPG is completely water-soluble, while high molecular weight is only partly soluble. A few studies of PPG/salt system were cited (Sadeghi and Jamehbozorg, 2008; Salabat *et al.*, 2011; Xie *et al.*, 2011; Zhao, Xie and Yan, 2011). There have been only one report of Salabat, Abnosi and Motahari (2010) which revealed the protein partition using PPG/salt systems and also found higher efficiency over PEG/salt system.

2.6.2.3.2 Polymer molecular weight and concentration

In general, the higher the molecular weight of polymers, the lower concentration required for phase separation (Raja *et al.*, 2011). Additionally, Increase in polymer concentration alters properties between the phase such as density, refractive index and viscosity. Besides the high concentration of polymer, the high polymer molecular weight also increases viscosity of the phase. However, the high viscosity may impact further process (Ratanapongleka, 2010). The molecular weight of polymer has strong influent on the partitioning behavior of biomolecules. The increases in molecular weight of polymer results in an increase in the chain length of the polymer corresponding to decrease free volume of top phase which polymer effect is call exclusion effect. Subsequently, their partition efficient increases as molecular weight increase. The free volume in PEG phase decreased significantly with increasing PEG molecular weight from 600 to 20000 leading to alcohol dehydrogenase moves to the bottom phase (Madhusudhan, Raghavarao and Nene, 2008). Similar observation was found in partitioning of lipase and invertase (Zhang and Liu, 2010; Karkaş and Önal, 2012; Zhou *et al.*, 2013). Moreover, all biomolecules including undesirable molecules partition to top phase as using low molecular weight due to less selective partitioning of low polymer molecular weight (Bradoo, Saxena and Gupta, 1999). It allows the polymer to attract all the proteins to the upper phase.
Since, biomolecules partition into the PEG phase by hydrophobic interaction between biomolecules and PEG (Yavari *et al.*, 2013). As high concentration of PEG, high number of polymer units involve in the biomolecules partition also increases resulting in more biomolecules partitioning to PEG phase (Babu, Rastogi and Raghavarao, 2008). In contrast, the higher polymer concentration cause the larger amount of stayed

in the bottom phase (Ribeiro *et al.*, 2007). Therefore, the selective of appropriate intermediate polymer molecular weight including proper concentration is very important for increasing extraction efficiency of this technique.

2.6.2.3.3 Salt type and concentration

In polymer/salt system, the biomolecules partitioning depends on an exclusion effect of the polymer with a salting-out effect of salts. The partitioning of protein is influenced by the presence of salts. The addition of salts to the aqueous PEG solution leads to an arrangement of ordered water molecules around the PEG molecules. This is due to their water structure breaking effects (Farruggia *et al.*, 2004). The formation of a water layer around the cation results in a more compact structure with a minor volume of PEG molecules. This lead to the decrease in volume of the PEG rich phase (Rawdkuen *et al.*, 2011). This effect increases with the net charge of protein. In general, proteins with negatively charge favor to partition to the top phase in PEG/salt system, while the positive charged proteins normally move to the bottom phase (Bezerra *et al.*, 2006; Goja *et al.*, 2013). The role of salt affects the partitioning as describe follow: 1) the weakening or strengthening of the interactions, or 2) the interaction between ionized groups with the opposite net charge of proteins (Johasson, 1998). The salt effectiveness is determined by the nature of anion in order of SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^-, whereas, the order of cation is NH_4^+ > K^+ > Na^+ > Li^+ > Mg_2^+ > Ca_2^+ (Roe, 2000). Recently, the biodegradable salts like citrate and ammonium carbonate (volatile) are interested because of their high selectivity, less pollution, biocompartibility and easy to scale-up (Goja *et al.*, 2013; Lario *et al.*, 2016). Moreover, the raising of salt concentration results in an increase in the partition coefficients of bioproducts into top phase or interface (Barbosa *et al.*, 2011; Hemavathi and Raghavarao, 2011; Yücekan and Önal, 2011). However, the presence of high concentration of salt greatly decreases both the yield and the selectivity (Pan and Li, 2001; del-Val and Otero, 2003; Klomklao *et al.*, 2005). The less activity recovery may cause by denaturation of enzyme from salting out effect (Klomklao, Benjakul and Visessanguan, 2004). Therefore, the distribution of biomolecules is critical controlled by both type and concentration of salt.

2.6.2.3.4 pH value

Generally, pH affects either the charge of solute or ratio of the charge molecules. The partitioning of protein and enzymes to the phase in ATPS system depends on their isoelectric point (pI). The net charge of protein relies on whether the pH is greater than pI (negative), lesser than pI (positive) or equal to pI (zero). Furthermore, different types of proteins have different behavior to phases in ATPS depends on their pI. In PEG/salt systems, generally most proteins with acidic pI and consequently negatively charges prefer the top phase and positively charge proteins partition to bottom phase. Accordingly, the initial pH of the system must be above the pI of the interesting molecules. The higher the pH above pI, the higher yield observed in ATPS partitioning, whereas beyond the optimal pH related to reduce partition coefficient, yield and low stability of lipase (Zhang and Liu, 2010; Barbosa *et al.*, 2011). Moreover, inactivation of protease occurred at specific pH (Schmidt, Ventom and Asenjo, 1994; Nitsawang, Hatti-Kaul and Kanasawud, 2006). Enzyme stability slightly reduced in acidic area, but it was dramatically lost at pH above 9.0 (Ketnawa, Chaiwut and Rawdkuen, 2011). Most of the biomolecules, especially proteins and enzymes are stable at neutral pH, thus it is favorable condition to conduct the ATPS (Goja *et al.*, 2013).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.6.2.3.5 Presence of neutral salt

The addition of neutral salts, such as NaCl does not drastically affect the liquid-liquid equilibrium data of ATPS, while high salt concentration (more than 1M) alters the phase diagram (Raja *et al.*, 2011). In addition, an increase in hydrophobicity will decrease the amount of bound water, which keeps the final composition of the systems constantly (Rosa *et al.*, 2009). As presence of neutral salt type and concentration in ATPS, electrical potential difference between two phases was generated resulting in the preference of ions in the one phase relative to the other (Johansson, 1970). This electrical potential difference will strongly affect the partitioning of charge protein. The addition of NaCl or other neutral salts in ATPS can affect the water structure and hydrophobic interactions differently, in which the

interaction between hydrophobic chain (ethylene group) of PEG and hydrophobic surface are of protein will be facilitated (Ooi *et al.*, 2009). The hydrophobic ions force the partitioning of their counter ions to the more hydrophobic phase and viseversa, thus proteins prefer more partition to top phase (Saravanan *et al.*, 2006). The neutral salt has been used for enhancing selective partition of enzyme to top phase by many researchers (Su and Chiang, 2006; Mohamadi and Omidinia, 2007; Barbosa *et al.*, 2011; Yücekan and Önal, 2011).

2.6.2.3.6 Temperature

The effect of temperature is very different for each phase system depending on the type of polymer used. In PEG/salt systems are formed easily at high temperature, whereas in case of PEG/dextran systems, two phases will easily form at lower temperature (Walter and Johansson, 1994). In addition, the phase composition is different as increase in temperature. It enhances the concentration of PEG and salt in the top and bottom phase, respectively (Silva, Coimbra and Meirelles, 1997; Sivars and Tjerneld, 2000). This is due to the preferential adherence of the water molecules to the polar salt surface instead of the PEG at a higher temperature. Consequently, water molecules available for PEG solvation in the bottom phase, decreased as the temperature increased, thus, reducing the solubility of PEG in the bottom phase. It decreases protein solubility in each phase. The partition coefficient of enzymes in PEG/salt system increased with increasing temperature (25-55 ºC) that found in both α-galactosidase (Naganagouda and Mulimani, 2008) and β-glucosidase (Gautam and Simon, 2006). However, higher temperature may cause the loss of enzyme activity.

2.6.2.3.7 Molecular weight of protein

Since the protein have both size (molecular weight and hydrodynamic diameter) and geometry (tridimensional conformation). So, they are subjected to the steric effects imposed by the constituents of the system. These steric effects are generally related to the free volume in the phase. Andrews, Schmidt and Asenjo (2005) have been investigated the relationship between protein molecular weight and partition coefficient using both PEG/dextran and PEG/salt systems for a group of

proteins. They found that, in PEG/dextran systems, proteins with higher molecular weights partitioned more to the dextran-rich bottom phase. In PEG/salt systems no clear correlations between molecular weight and partition coefficient was found.

2.6.2.3.8 Protein concentration

Schmidt, Andrews and Asenjo (1996) have been studied the effect of protein concentration on partitioning in PEG/salt ATPS by using amyloglucosidase, subtilisin, and trypsin inhibitor. They found that the difference partitioning pattern appear depending on physico-chemical properties of proteins. In general, in the top phase, maximum protein concentration was determined mainly by a steric exclusion effect of PEG, and PEG-proteins hydrophobic interaction. Maximum protein concentration in the bottom phase was determined by salting-out effect of the salts. The higher the ionic strength in the system, the higher the protein concentration was occurred for all proteins. Furthermore, higher in ionic strength, higher in salting-out effect presented in the bottom phase. In case of amyloglucosidase, they concluded that its steric exclusion (large size) effect caused a very low maximum concentration in the PEG-rich top phase, its high hydrophilicity promoted a high concentration in the salt-rich bottom. In the case of subtilisin and trypsin inhibitor, their high concentrations in the top phase were due to their hydrophobic nature (hydrophobic interaction with PEG) and small size (negligible steric exclusion). Moreover, the maximum concentration in the bottom phase for trypsin inhibitor was lower than that of subtilisin which may cause by its higher hydrophobicity and, hence, a stronger salting-out effect. The actual concentration limit depends on the properties of the protein. When the concentration of a protein exceeds relatively low values, precipitation at the interface can be observed. The similar results were found in many literatures as increased in protein concentration or %loading of feedstock (Ooi *et al.*, 2010; Khayati and Alizadeh, 2013; Anvari, 2015).

2.6.2.4 Applications of ATPS

ATPS has been applied in several fields of biotechnology such as recovery of proteins, enzymes, nucleic acids, virus, antibodies, biopharmaceuticals and cell

organelles (Table 12). ATPS has important advantages over the currently established packed-bed chromatography, as it can combine a high biocompatibility with easy scale-up and continuous mode of operation(Rosa *et al.*, 2010). In addition, it can overcome some of the technical drawbacks currently encountered with most chromatographic supports, such as high cost, low productivities, scale-related packing problems and diffusional limitations (Shukla *et al.*, 2007; Azevedo *et al.*, 2009). Although, purification by using ATPS leads to lower purity than ion-exchange chromatography, but its high recovery yield makes ATPS as a practical method in industrial scale for protease partial purification which is used in some industries like detergent and tanning processes (Yavari et al., 2013).

2.6.2.4.1 Applications of ATPS to recovery of many proteins

The many reports used of ATPS in bioprocessing were mainly for separation and concentration of target protein from cell debris or from contaminating proteins in fermentation broth. A few studies have been reported to recover two or more partially purified proteins separately from both phases. Table 13 summarizes the use of liquid-liquid extraction in some reports with desired purposes.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 12 Aqueous two-phase system of biomolecules

Biomolecules	Production source	ATPS	Recovery yield (%)	Purification fold	Reference
Protease	*B. licheniformis* (ATCC 21424)	PEG-10000 /potassium phosphate	93.3	5.4	(Yavari *et al.*, 2013)
	tuna spleen	PEG/magnesium sulfate	69	6.61	(Klomklao *et al.*, 2005)
	Chicken intestine	PEG-6000/ sodium citrate	53.7	5	(Sarangi *et al.*, 2011)
	Mango peel	PEG-8000/ phosphate	97.3	14.37	(Mehrnoush *et al.*, 2012)
	Kesinai (*Streblus asper*)	PEG-6000/ magnesium sulfate	96.7	14.4	(Mehrnoush *et al.*, 2012)
	Tuna (*Thunnus alalunga*)	PEG-2000/ magnesium sulfate	89.1	2.43	(Nalinanon *et al.*, 2009)
	Latex (*Calotropis procera*)	PEG1000/ magnesium sulfate	74.6	n.s.	(Rawdkuen *et al.*, 2011)
Lipase	*Bacillus* sp. ITP-001	PEG 20000 /potassium phosphate	n.s.	201.53	(Barbosa *et al.*, 2011)
	Trichosporon laibacchii	PEG4000/ Potassium phosphate	80.4	5.84	(Zhang and Liu, 2010)
	Bacillus stearothermophilus SB-1	PEG6000/ phosphate	n.s.	5.21	(Bradoo *et al.*, 1999)
	Rhodotorula glutinis	PEG 4000/oxalate potassium	71.2	13.9	(Khayati and Alizadeh, 2013)
	Candida rugosa	PEG 2000/ phosphate	78.26	2.3	(Bassani *et al.*, 2010)
	Burkholderia pseudomallei	PEG6000/ Potassium phosphate	93	12.42	(Ooi *et al.*, 2009)
Human recombinant interferon α1 (rhIFN-α1)	*Escherichia coli*	PEG/phosphate ester	76	25	(Guan *et al.*, 1996)
Monoclonal antibody (human immunoglobulin G)	Chinese hamster ovary cells	PEG/phosphate	88	4.3	(Azevedo *et al.*, 2007)
Plasmid DNA	-	PEG/phosphate	80	-	(Luechau, Ling and Lyddiatt, 2009)
Glucoamylase	*Humicola grisea*	PEG/phosphate	85.81	2.68	(Ramesh and Murty, 2015)

n.s. Not specified

Table 13 A few reports on separation of proteins using liquid-liquid extraction

Purpose	System component	Results	Reference
Separation of β-1,3-1,4-glucanase from fermentation broth of *B. subtilis* ZJF-1A5, comprising with other contaminant proteins and enzyme like α-amylase and neutral proteases	PEG/$MgSO_4$	▪ Removed most of α-amylase and neutral proteases to different phase of β-glucanase ▪ Achieved 63.5% recovery with 6.6 purity fold of β-glucanase	He *et al.* (2005)
Removal β-lactoglobulin (β-lg, allergenic element), α-lactalbumin (α-la, food formulations, ingredients for) and glycomacroprotein (Gmp, diets for pateints) from whey protein	PEG/phosphate	▪ 97.3% yield for β-lg in bottom phase ▪ 81.1% and 97.8% for α-la and Gmp in the top phase, respectively	Alcântara *et al.* (2011)
Separation of α-galactosidase and invertase produced by *A. oryzae*	Ammonium sulfate/*tert*-butanol	▪ 15-and 12-fold purification with 50 and 54%yield of α-galactosidase and invertase, respectively	Dhananjay and Mulimani (2009)
Separation of amylase inhibitor and trypsin inhibitor from *Eleusine Coracana*	Ammonium sulfate/*tert*-butanol	▪ 20.1-and 16-fold purification with 39.5% and 32%yield of amylase inhibitor and trypsin inhibitor, respectively	Saxena, Iyer and Ananthanarayan (2007)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER III

MATERIALS AND METHODS

3.1 Laboratory equipment and chemicals

3.1.1 Laboratory equipments

Laboratory equipment	Company	Country
Autoclave HV-110	Hirayama	Japan
Autoclave NLS-3020	Sanyo Electric Co., Ltd	Japan
Balance (ME3002)	METTLER TOLEDO	USA
Balance (ME303)	METTLER TOLEDO	USA
Biological safety cabinet, forma class II A2	Termo eletron corporation	USA
Cabinet laminar flow (BBS-V1300-D)	BIOBASE	China
Centrifuge (5417C)	eppendorf	USA
Centrifuge (5804R)	eppendorf	USA
Chemical hood (FH1200X)	BIOBASE	China
Digital dry bath	Labnet International, Inc.	USA
Gel Doc™ XR + Image lab™ Software	Biored	USA
Incubator shaker (ZHWY-100B)	ZHCheng	China
Incubator shaker (ZHY-2102C)	ZHCheng	China
Micropipette 20, 100, 200, and 1000 µl Gilson	Gilson	France
Microplate reader (Multiskane GO)	Thermo Scientific	Germany
Mini Protean II cell electrophoresis unit	Bio-rad Labolatories	USA
pH meter (STARTER2100)	OHAUS	Japan
Vortex (VM-10)	DAIHAN SCIENTIFIC	China

3.1.2 Laboratory Chemicals

The symbols of chemical-quality grade were followed by AR: Analytical grade, BT: Biotechnology grade, FG: food grade, LAB: Laboratory grade and MB: Molecular biology grade.

Chemical	Quality grade	Company	Country
Acetone	AR	J.T.Baker	USA
Agar	BT	Scharlau Microbiology	Spain
Brij 35	AR	Merck	Germany
$CaCl_2.2H_2O$	AR	Ajax Finechem	Australia
Casein	AR	Sigma	Germany
Citric acid	AR	Ajax Finechem	Australia
$CoCl_2.6H_2O$	AR	Carlo Erba Reagenti	Italy
Coconut oil	FG	Ma-Praw	Thailand
$CuSO_4$	AR	Ajax Finechem	Australia
Cyclohexane	AR	Carlo erba	USA
Ethanol	AR	Merck	Germany
Folin–Ciocalteu reagent	AR	Sigma	Germany
Glucose	AR	Ajax Finechem	Australia
Glycerol	AR	Ajax Finechem	Australia
K_2HPO_4	AR	Riedel	Germany
KCl	AR	Ajax Finechem	Australia
KH_2PO_4	AR	Ajax Finechem	Australia
Lactose	AR	Ajax Finechem	Australia
Malt extract	LAB	BDH	England
Maltose	AR	Ajax Finechem	Australia
Mannitol	AR	Ajax Finechem	Australia
$MgCl_2.6H_2O$	AR	Ajax Finechem	Australia
$MnCl_2.4H_2O$	AR	Ajax Finechem	Australia
$Na_2MoO_4.2H_2O$	AR	BDH	England
NaCl	AR	Ajax Finechem	Australia
NaOH	AR	Ajax Finechem	Australia
n-decane	LAB	Sigma	Germany
n-decanol	LAB	Sigma	Germany
$(NH_4)_2SO_4$	LAB	Scharlau Microbiology	Spain
$NiSO_4.6H_2O$	AR	BDH Chemicals	New Zealand
Olive oil	FG	Unilever	Italy
Palm oil	FG	Morakot	Thailand
PEG4000	AR	Sigma	Germany
PEG6000	AR	Fluka	Switzerland

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Chemical	Quality grade	Company	Country
PEG8000	AR	Scharlau Microbiology	Spain
Peptone	LAB	Merck	Germany
PMSF	AR	Sigma	Germany
p-Nitrophenyl palmitate	AR	Sigma	Germany
PPG400	AR	Sigma	Germany
PVP10000	AR	Sigma	Germany
SDS	AR	Ajax Finechem	Australia
Sesame oil	FG	Oh Chin Hing	Thailand
Soluble starch	LAB	Scharlau Microbiology	Spain
Soybean oil	FG	TVO	Thailand
Sunflower oil	FG	Thanakorn	Thailand
TCA	LAB	QReC	China
Toluene	AR	Labscan	Thailand
Tributyrin	FG	Sigma	Germany
Tris-Base	AR	Sigma	Germany
Tri-Sodium Citrate	LAB	Ajax Finechem	Australia
Triton X-100	AR	Labscan	Thailand
Tryptone	LAB	Himedia	India
Tween 20	AR	Sigma	Germany
Tween 80	AR	Sigma	Germany
Xylose	LAB	Loba Chemie	India
Yeast extract	LAB	Scharlau Microbiology	Spain
$ZnCl_2.7H_2O$	AR	Merck	Germany

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.2 Microorganism, media and growth conditions

Bacillus licheniformis 3C5, a protease producing bacteria (Rachadech *et al.*, 2010) was used as a bacterium for protease and lipase production. The bacteria were cultivated in Luria-Bertani (LB) (APPENDIX A) agar plate at 37 °C for 12h and then maintained in 4°C. The strain was sub-cultured at monthly interval.

3.3 Determination of lipase activity

Lipase activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. The reaction mixture consisted of 0.16 ml of 0.4% Triton X-100 in 0.05 M Tris-HCl buffer (pH 9.0), 20 µl of enzyme and 20 µl of 0.01 M *p*-NPP. The mixture was incubated at 30 °C for 15 min, after which the release of

p-nitrophenol (*p*-NP) was measured at 410 nm. One unit of enzyme corresponds to the release of 1 µg of *p*-NP liberated per minute under the experimental conditions (APPENDIX B).

3.4 Determination of protease activity

Protease activity was determined as caseinolytic activity. The 20 µl of crude enzyme was mixed with 0.18 ml of 100mM Tris-HCl buffer (pH 9.0) containing 1% (w/v) casein and 0.35 ml of 100mM Tris-HCl buffer (pH 9.0), and then incubated for 30 min at 30 °C. The reaction was stop by addition of 0.45 ml 5% (w/v) trichloroacetic acid (TCA). The mixture was incubated at the same temperature for 15 min and then centrifuged at 1000×g for 5 min to remove the precipitate. The supernatant were taken and measured at 280 nm. A standard curve was prepared using solution of tyrosine. One unit of the proteolytic activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per ml in 1 min under standard conditions (APPENDIX B).

3.5 Determination of protein

Protein content for production process was measured following the method of Lowry et al. (1951) using Bovine Serum Albumin (BSA) as the standard (APPENDIX B).

Protein content for partially purify in downstream process was measured by the dye-binding method of Bradford (Bradford, 1976). Bovine serum albumin was used as the standard. For the determination of protein concentration from each phase in ATPS, samples withdrawn were diluted with a known amount of distilled water and its absorbance was measured at 595 nm. In order to avoid the interference of PEG and salts, the same solution of the corresponding phase without sample was used as a blank (APPENDIX B).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.6 Production of lipase

B. licheniformis 3C5 was grown in 250 ml Erlenmeyer flasks containing 50 ml YMO medium (APPENDIX A) pH 9.0. After 5% (v/v) inoculation, the culture was incubated at room temperature (33 °C) and agitated at 120 rpm. After 48h of incubation, the culture was harvested and crude enzyme was extracted by centrifugation at 3000 rpm for 20 min at 4 ºC.

3.7 Production of protease

The production medium for protease was according to Rachadech *et al*. (2010), minimal salt basal medium supplemented with yeast extract (MSBY) (APPENDIX A). *B. licheniformis* 3C5 was cultivated in 250 ml Erlenmeyer flasks containing 50 ml MSBY medium pH 7.0. After 5% (v/v) inoculation, the fermentation conditions were room temperature (33 °C) and stirred speed at 120 rpm. After 24h of incubation, the culture was harvested and crude enzyme was extracted by centrifugation at 3000 rpm for 20 min at 4 ºC.

3.8 Production of protease and lipase

Protease and lipase were obtained from *Bacillus licheniformis* 3C5 grown in 50 ml protease and lipase optimized production medium (PLO) (APPENDIX A) at temperature of 30 ºC in 250 ml shake flasks at 200 rpm, at pH 9. After 48h, the culture was centrifuged at 3000 rpm for 20 min at 4 ºC and the supernatant was used as crude enzyme.

3.9 Characterization of lipase

3.9.1 Effect of native protease on lipase stability

The aim of this study was to investigate the stability of lipase towards native protease, whether in the incubation period and cultivation process.

3.9.1.1 Effect of temperature and native protease on lipase stability

To investigate the stability of lipase towards protease, crude 3C5 lipase was mixed with crude 3C5 protease at volume ratio 1:1 (5 ml total volume) and then incubated at different temperatures (30 and 45 ºC). Determination of residual lipase activity compared activity to the mixture without protease (control) at 24h of incubation. The crude 3C5 lipase and protease were cultured from YMO (section 3.6) and MSBY (section 3.7) medium, respectively.

Additionally, other lipases such as Lipolase® 100L (Novo Nordisk), Lipozyme® CALB L (Novo Nordisk) and *Burkholderia* sp. CU-N1 were incubated with 3C5 protease. Furthermore, Alcalase® (Novo Nordisk), a commercial protease was also checked the effect to these lipases. These enzymes were used as appropriate dilution in 50mM Tris-HCl buffer. The tested proteases and lipases in this study were showed in Table 14.

Table 14 Proteases and lipases for testing the effect of temperature and native protease on lipase stability

Protease sources	**Lipase sources**			
B .licheniformis 3C5	Lipolase® 100L (Novo Nordisk) produced by *T. lanuginosus*	Lipozyme® CALB L (Novo Nordisk) produced by *A. niger*	*Burkholderia* sp. CU-N1	*B. licheniformis* 3C5
Alcalase® (Novo Nordisk) produced by *B .licheniformis*				

3.9.1.2 Effect of native protease on lipase stability during growth-dependent enzyme synthesis

To investigate the effect of native protease on lipase stability during growth-dependent enzymes synthesis, the experiments were performed by modifying the method of Pereira-Meirelles, Rocha-LeãO and Anna (1997). The protease inhibitors, 1mM PMSF was supplemented to the lipase production medium, YMO medium after 0h of cultivation as mentioned above in section 3.6. The growth (OD_{600}), protease and

lipase activities were measured at 6, 12, 24 and 48h of cultivation comparing to the cultivation without protease inhibitor.

3.9.2 Effect of temperature on lipase activity

The effect of temperature on the lipase activity was investigated by assaying the reaction mixture at various temperatures (30, 37 45 and 60 ºC). The effect of temperature on lipase stability was determined by pre-incubating the 5 ml of crude enzyme at various temperatures (30, 37 and 45 ºC) for 6 h. Thereafter, lipase activity was measured at standard condition (section 3.3) in each 3 h interval.

3.9.3 Effect of pH on lipase activity

The effect of pH on the lipase activity was determined by assaying the reaction mixture at different pH ranging from 4.0 to 12.0. Then the enzyme was assayed at different pH values at standard condition (section 3.3). For pH stability studies, 1.8 ml of crude enzyme was mixed with 0.1 ml of buffer at specific pH. The mixture was incubated at 30 ºC for 3 h, and the residual lipase activity was determined at 1 and 3 h using the standard condition (section 3.3). Buffer systems used at a final concentration of 100 mM: acetate buffer (pH 4.0-5.0) phosphate buffer (pH 6.0-7.0) Tris-HCl buffer (pH 7.0-9.0) and Tris-glycine buffer (pH 10.0-12.0).

3.9.4 Effect of organic solvent on lipase activity and stability

To investigate the effect of organic solvents on lipase activity, 3C5 lipase was analyzed by incubation the 1.395 ml of enzyme at 30 °C in 50 mM Tris-HCl buffer (pH 9.0) containing 1.050 ml (35% (v/v)) of various types of organic solvent (log P) such as *n*-decane (5.6), *n*-decanol (4.0), cyclohexane (3.2), toluene (2.5), dichloromethane (1.25) and acetone (-0.23) (Laane *et al.*, 2009). The activity was measured at the beginning and as the residual activity (%) at the end of incubation.

3.9.5 Effect of detergent on lipase activity

To study the effect of ionic and non-ionic surfactants to the enzyme, 1.9 ml of 3C5 lipase was incubated for 3 h at 30 °C in the presence of different concentration of (0.2 and 1% w/v) SDS and 1% (v/v) of Triton x-100 Tween-80 Tween-20 and Brij 35. These mixtures were adjusted to 2.0 ml total volume by in 50 mM Tris-HCl buffer

(pH 9.0). The enzyme stability was measured as the residual activity compared to activity at pH 9.

3.10 Medium optimization for concomitant production

B. licheniformis 3C5, a protease-producing bacterium was used for lipase production. The production medium for protease was according to Rachadech *et al*. (2010), while a modified MSBY medium (Rachadech *et al.*, 2010) with 1.5% (w/v) olive oil (pH9) was used as basal medium for concomitant production (MSBYO; APPENDIX A). The 50 ml-culture was incubated at room temperature (33 °C) for 96 h at agitation rate of 120 rpm. During the cultivation, bacterial growth, protease activity, lipase activity and protein content were measured. The growth supernatant or the crude enzyme was extracted by centrifugation at 3000 rpm for 20 min at 4 ºC.

3.10.1 Effect of carbon source

Effect of carbon source on both enzymes production was studied by adding different carbon sources such as citric acid, glucose, glycerol, lactose, maltose, mannitol, soluble starch and xylose to the basal medium. The concentration of each carbon source are 0.5 1.0 and 1.5% (v/v). The growth condition and crude enzyme preparation were performed according to section 3.10.

3.10.2 Effect of nitrogen source

Yeast extract present in the basal medium was replaced with different nitrogen sources such as casein peptone tryptone and yeast extract at the final concentration of 0.2 0.5 and 1% (w/v) to study the effect of nitrogen source on protease and lipase production by *B. licheniformis* 3C5. Also, the best carbon source from previous section (3.10.1) was supplemented to the basal medium. The growth condition and crude enzyme preparation were performed according to section 3.10.

3.10.3 Effect of inducer

The inducers used in this study were six commercial vegetable oils (such as coconut oil, palm oil, olive oil, soybean oil, sunflower oil and sesame oil), triglyceride (tributyrin) and surfactants (such as Tween 20, Tween 80 and Triton X-100). The concentration of inducer used (0.5 1.0 and 1.5% (v/v)) was specified in each experiment. These inducers were replaced with olive oil in basal medium that compose of selected carbon and nitrogen sources from previous section (3.10.1-3.10.2). The growth condition and crude enzyme preparation were performed according to section 3.10.

3.10.4 Effect of physical factor

The effect of physical factor on the enzyme production was performed over the pH ranging from of 7 to 9, temperature of 30, 37 and 45 ºC and agitation rate of 120 and 200 rpm. The ingredient of medium including appropriated inducers was similar to previous section 3.10.3. The crude enzyme preparation was performed according to section 3.10.

3.10.5 Effect of NaCl

NaCl at different concentrations (0.25, 0.5 and 1.0% (w/v)) was added to the medium to study the effect of NaCl on protease and lipase production. The medium used and growth condition were the best results from previous section (3.10.1-3.10.4). The crude enzyme preparation was performed according to section 3.10.

3.10.6 Central composite design and optimization by response surface methodology

Response surface methodology is a statistical method that is used to determine the optimum combination of the values of the selected factors for maximum concomitant production of protease and lipase by *B. licheniformis* 3C5. Central composite design (CCD) is one of RSM techniques usually utilized to obtain data that fits a full second-order polynomial model. The CCD experiments was designed using

Design Expert software, version 6.0.5 Stat-Ease inc.; USA, from 4 selected (independent) factors which were varied over five levels, coded -2, -1, 0, +1 and +2 for lowest , low middle, high and highest concentration, respectively (Table 15). These sets consisted of 2^4 factorial design (16 runs) with two axial points at a distance alpha = 1.682 from the design center (8 runs) and the replicates about the center point (6 runs) resulting in a total 30 runs (Table 16). Further 30 experimental runs were carried out in duplicate by keeping constant of all other factors (MSB medium concentration, temperature 37 ºC, agitation rate 200 rpm, inoculum size 5%) and the pH of the medium was adjusted to 9.0. Samples were taken at 48h of cultivation and then measured protease and lipase activity (U/ml) as response values. For predicting the optimal point, a second-order polynomial equation was fitted to correlate relationship between independent factors and the responses. The values of the independent factors that were analyzed via RSM were coded according to the following equation:

$$x = \frac{Xi - Xc}{\Delta Xi}$$

Where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_c the real value of an independent variable at the center point $((X_{-1} + X_{+1})/2)$ and ΔX_i is the step change value $((X_{+1}-X_{-1})/2)$.

The quadratic equation for predicting the response was expressed as

$$Y=\beta_0+\Sigma\beta_i x_i+\Sigma\beta_{ii}x^2_i+\Sigma\beta_{ij}x_i x_j$$

Where Y is dependent factors (U/ml); β0, βi, βii, และ βij are regression coefficients; xi and xj are coded independent factors. The quality of the polynomial model equation was expressed by the coefficient of determination R^2.

Table 15 Coded and actual levels of the factors in CCD for optimization of protease and lipase production by *B. licheniformis* 3C5

Symbols	Factors	Units	Coded and Actual levels				
			-2	-1	0	1	2
A	Olive oil	%, v/v	1	1.5	2	2.5	3
B	Glucose	%, w/v	0.4	0.6	0.8	1	1.2
C	Yeast extract	%, w/v	0	0.2	0.4	0.6	0.8
D	NaCl	%, w/v	0.125	0.25	0.375	0.5	0.675

Table 16 Experimental responses of the factors under different experimental conditions using CCD

Run	Olive oil (%)	Glucose (%)	Yeast extract (%)	NaCl (%)
1	2.5	0.6	0.6	0.5
2	2	0.8	0.4	0.375
3	2.5	0.6	0.6	0.25
4	2.5	0.6	0.2	0.5
5	2	0.8	0.4	0.375
6	2.5	1	0.2	0.5
7	1.5	1	0.2	0.5
8	2.5	1	0.2	0.25
9	2.5	1	0.6	0.5
10	2	0.8	0.4	0.375
11	2	0.8	0.4	0.375
12	1.5	0.6	0.6	0.5
13	1.5	1	0.6	0.25
14	1.5	1	0.2	0.25
15	2.5	0.6	0.2	0.25
16	1.5	0.6	0.2	0.5
17	1.5	1	0.6	0.5
18	2.5	1	0.6	0.25
19	1.5	0.6	0.2	0.25
20	1.5	0.6	0.6	0.25
21	2	0.8	0.4	0.375
22	2	0.4	0.4	0.375
23	2	0.8	0.4	0.125
24	2	1.2	0.4	0.375
25	2	0.8	0.0	0.375
26	2	0.8	0.4	0.625
27	2	0.8	0.4	0.375
28	3	0.8	0.4	0.375
29	2	0.8	0.8	0.375
30	1	0.8	0.4	0.375

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.11 Partial purification by using ATPS

Stock solutions of PEG (50%, w/w), PVP (50%, w/w), ammonium sulfate (40%, w/w), sodium citrate (40%, w/w) and potassium phosphate (50%, w/w) were prepared in deionized water, whereas liquid PPG from commercial product was directly used as stock solution. ATPS comprising of appropriate amounts (%, w/w) of polymer, salt and crude enzymes were prepared in water so as to obtain a total weight of 5.0 g in 15.0 ml graduated centrifugal tubes. The amount of crude enzyme added to the system was 1 g (20% w/w) and was the last adding component to the system. The pH of the phase system was adjusted with 2M NaOH or HCl. After vortex shaking, the two-phases allowed separation by centrifugation at 25 °C and 2500xg for 5 min (Kavakçıoğlu and Tarhan, 2013). Thereafter, the volumes were determined. The top and bottom phase were carefully separated for protease and lipase activity assay and protein determination using Bradford method (3.5). These data were analyzed as described in subsequent section (3.11.1). All experiments were performed in triplicate at room temperature (25±1 °C) and the result represented the mean values of three independent experiments.

3.11.1 Estimation of parameters and terms involved in ATPS

ATPS parameters were calculated as follows:

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The volume ratio (V_r) implies the ratio of volume of top to bottom phase. The lower in V_r, the higher in concentrate enzyme occurs. The V_r was defined as:

$$Vr = \frac{Vt}{Vb}$$

where V_t and V_b are top and bottom phase volume, respectively. The V_r

The partition coefficient of enzyme (K_e) represents the trend of enzyme distribution. The higher in K_e value leads to high partitioning of enzyme to the top phase. The K_e parameter was defined as:

$$Ke = \frac{At}{Ab}$$

where A_t and A_b are enzyme activity (U/ml) in top and bottom phase, respectively.

The partition coefficient of protein (K_p) was similar meaning to K_e parameter but it describes the distribution of proteins. The K_p value was defined as:

$$Kp = \frac{Ct}{Cb}$$

where C_t and C_b are protein content (mg/ml) in top and bottom phase, respectively.

The specific activity (SA) of extracted enzyme indicates the purity of enzyme in each phase of the ATPS. It was defined as:

$$SA = \frac{total\ enzyme\ activity\ (U)}{total\ protein\ content\ (mg)}; \text{ U/mg}$$

The purification fold (PF) as:

$$PF = \frac{SAt}{SAi}$$

where SA_t is the specific activity of top phase and SA_i is specific activity of initial enzyme (crude enzyme before partitioning).

The enzyme recovery (yield) refers to the yield of extracted enzyme in each phase comparing to initial enzyme adding to the ATPS. The yield (%) was defined as:

$$yield(\%) = \frac{At \times Vt}{Ai \times Vi} \times 100$$

where A_t and A_i are activity of top phase and initial enzyme activity (U/ml), respectively. V_t and V_i are volume of top phase and initial enzyme loaded (ml), respectively.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.11.2 Polymer selection

In order to select type of polymer, ATPS systems are performed at different polymer types e.g. PEG4000 PVP10000 and PPG400 with ammonium sulfate ($(NH_4)_2SO_4$), at different concentration ratios 7.5/15, 12/15 and 17.5/15 (%, w/w) and then contents were mixing with other composition and samples were measured as described in above section 3.11

3.11.3 Salt selection

Each ATPS system was composed of PEG4000, which was selected from previous section 3.11.2, and each salt such as ammonium sulfate ($(NH_4)_2SO_4$), potassium phosphate (K_2HPO_4) and sodium citrate ($Na_3C_6H_5O_7$). The system concentration ratios were 17.5/10, 17.5/12 and 17.5/15 (%, w/w). Thereafter, contents were performed as described in above section 3.11.

3.11.4 Polymer molecular weight selection

Different molecular weights of PEG (4000, 6000 and 8000) were mixed with sodium citrate ($Na_3C_6H_5O_7$) as the similar concentration of tie-line length (TLL) 34.99, 40.99 and 43.74 (%, w/w) that reported by Zafarani-Moattar, Emamian and Hamzehzadeh (2008). Thereafter, contents were performed as described in above section 3.11.

3.11.5 Volume ratio selection

Effect of volume ratio between top and bottom phase was studied by using optimized system from previous section (PEG6000/sodium citrate at TLL 34.99 (%, w/w)). The PEG6000 and sodium citrate was mixed with different concentration ratios which V_r were equal to 2, 0.8 and 0.5 as reported by Zafarani-Moattar *et al.* (2008). Thereafter, contents were performed as described in above section 3.11.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.11.6 pH selection

To investigate the effect of pH on the enzyme partitioning, the optimized system (PEG6000/sodium citrate at TLL 34.99 (%, w/w) with $V_r = 2$) was performed and adjusted the pH over the wide range of 6 to 10 by 2M NaOH or HCl. Thereafter, contents were performed as described in above section 3.11.

3.11.7 Neutral salt selection

The optimized system of 17.5/10 PEG6000-sodium citrate (%, w/w) was prepared and added neutral salts like NaCl and KCl at various concentrations (1, 3 and 5%, w/w). Thereafter, contents were performed as described in above section 3.11.

3.11.8 Central composite design and optimization by response surface methodology

A Central composite face-centered design (CCFD) was used to optimize the recovery of protease and lipase within system composed by PEG6000 and sodium citrate. CCFD was design by using Design Expert software (version 6.0.5 Stat-Ease inc; USA). Three independent factors were PEG concentration (A), sodium citrate concentration (B) and KCl concentration (C) were studied at two levels (+1 and -1) as shown in Table 17. These type of designs are easy to perform because they are based on two level factorials that have been augmented with a center point and 2k (k is the number of studied factors). Their general description is number of experiments = 2^{k-p} + 2k+cp, where k is the number of studied factors, p is the fractionalization element (full design, p=0), and cp is the number of central points (Shahbaz Mohammadi *et al.*, 2015). All the 20 runs (2^3+2×3+6) points which included six replication of the center point are descripted in Table 18. For the description of the analysis and a second-degree polynomial model was mentioned in section 3.10.6.

Table 17 Coded and actual levels of the factors in CCFD for partial purification in ATPS of protease and lipase produced by *B. licheniformis* 3C5

Factors	Symbol	Coded and actual levels		
		-1	0	1
PEG (%, w/w)	A	15.0	17.5	20.0
Citrate (%, w/w)	B	8.0	10.0	12.0
NaCl (%, w/w)	C	2.0	3.0	4.0

3.11.9 SDS-PAGE

SDS-PAGE was prepared according to the method of Laemmli (1970) using prescribed composition of 5.0% stacking and 12.0% separating gels. Samples were performed under reducing conditions and after electrophoresis at 15 mA per gel for 120 min using a Mini Protean II cell electrophoresis unit (Bio-rad Labolatories. Richmond, CA, USA), protein bands were visualized using silver staining (Damerval *et al.*, 1987). Molecular weights were based on the comparative migration of proteins

with respect to protein ladder (14.4-116 kDa, Fermentas protein Molecular marker SM0431).

Table 18 Experimental responses of the factors under different experimental conditions using CCFD for optimization of partial purification using ATPS of protease and lipase produced by *B. licheniformis* 3C5

Run	PEG6000 (%)	Citrate (%)	KCl (%)
1	20	12	4
2	17.5	10	4
3	20	8	4
4	15	12	4
5	17.5	8	3
6	17.5	12	3
7	17.5	10	3
8	15	12	2
9	15	10	3
10	17.5	10	2
11	20	12	2
12	15	8	2
13	17.5	10	3
14	17.5	10	3
15	20	8	2
16	15	8	4
17	17.5	10	3
18	17.5	10	3
19	20	10	3
20	17.5	10	3

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Characterization of lipase

4.1.1 Effect of native protease on lipase stability

The use of protease and lipase together requires proteolysis-resistant lipase, since lipase can be hydrolyzed by protease (Grbavčić *et al.*, 2011). The aim of this study is to determine the stability of lipase towards native protease, whether in incubation period and cultivation process.

4.1.1.1 Effect of temperature and native protease on lipase stability

Studies on proteolysis-resistant lipase have been performed by investigating the resistance of lipase to native protease. Moreover, commercial protease and lipase, e.g. protease from Alcalase® (Novo Nordisk)and lipase from Lipolase® 100L (Novo Nordisk) produced by *Thermomyces lanuginosus* and Lipozyme® CALB L (Novo Nordisk) produced by *Aspergillus niger* contained lipase gene of *Candida Antarctica* including lipase from *Bhurkholderia* sp. strain CU-N1, were also used as control. The results summarized in Table 19 showed that after 24h of incubation 3C5 lipase with 3C5 protease at 30 ºC (45 ºC), lipase retained 107.15±3.38% (88.77±8.72%) of its initial activity. Similarly, 3C5 lipase retained % (%) after incubation with Alcalase®. Besides, Lipolase® retained 56.19±11.73% (53.06±6.09%) of its initial activity after incubation with 3C5 protease, whereas the incubation between Lipolase® and Alcalase® remained lipase activity 8.39±5.66% (22.91±4.24%). In contrast, Lipozyme® and CU-N1 lipase showed a few retained lipase activity (Table 19). As shown in Figure 6, all of used lipases showed significantly different from its initial lipase activities. There was no significant residual lipase activity of 3C5 lipase comparing to its initial activity. Similar results have been reported by (Ruchi *et al.*, 2008; Sangeetha *et al.*, 2010; Grbavčić *et al.*, 2011), that lipase was also stable towards native proteases. Several reports implied that lipase can be degraded by endogenous protease (Kok *et al.*, 1996; Pereira-

Meirelles *et al.*, 1997; Lee *et al.*, 2003; Lopes *et al.*, 2008). This confirms low susceptibility of 3C5 lipase towards its own native protease and commercial protease (Alcalase®) validating its applicability together.

Table 19 Residual lipase activity after 24h of incubation with protease at 30 ºC and 45 ºC (n=3)

Protease sources / **Lipase sources**	%residual lipase activity (lipase activity, U/ml)						Temperature (ºC)
	no protease		*B. licheniformis* 3C5		Alcalase® (Novo Nordisk) produced by *B. licheniformis*		
Lipolase® 100L (Novo Nordisk) produced by *T. lanuginosus* (Novozyme)	100±11.92	(192.64±22.97)	56.27±11.75	(108.4±22.64)	9.46±2.04	(19.27±5.62)	30
	100±16.1	(155.1±24.97)	53±6.08	(82.21±9.43)	22.52±4.17	(35.5±6.58)	45
Lipozyme® CALB L (Novo Nordisk) produced by *A. niger* (Lipozyme)	100±6.48	(5.12±0.33)	45.09±12.31	(2.31±0.63)	65.12±1.42	(3.33±0.07)	30
	100±13.39	(6.27±0.84)	45.98±13.49	(2.88±0.85)	65.78±2.84	(4.12±0.18)	45
Burkholderia sp. CU-N1 (CU-N1)	100±6.61	(5.33±0.35)	10.54±0.32	(0.56±0.02)	69.62±0.9	(3.71±0.05)	30
	100±13.34	(1.4±0.19)	55.97±10.97	(0.78±0.15)	46.25±5.59	(0.65±0.08)	45
B. licheniformis 3C5 (3C5)	100±8.79	(2.63±0.23)	107.15±3.38	(2.82±0.09)	86.18±2.16	(2.26±0.06)	30
	100±30.83	(2.18±0.67)	73.21±7.19	(1.59±0.16)	114.82±3.92	(2.5±0.09)	45

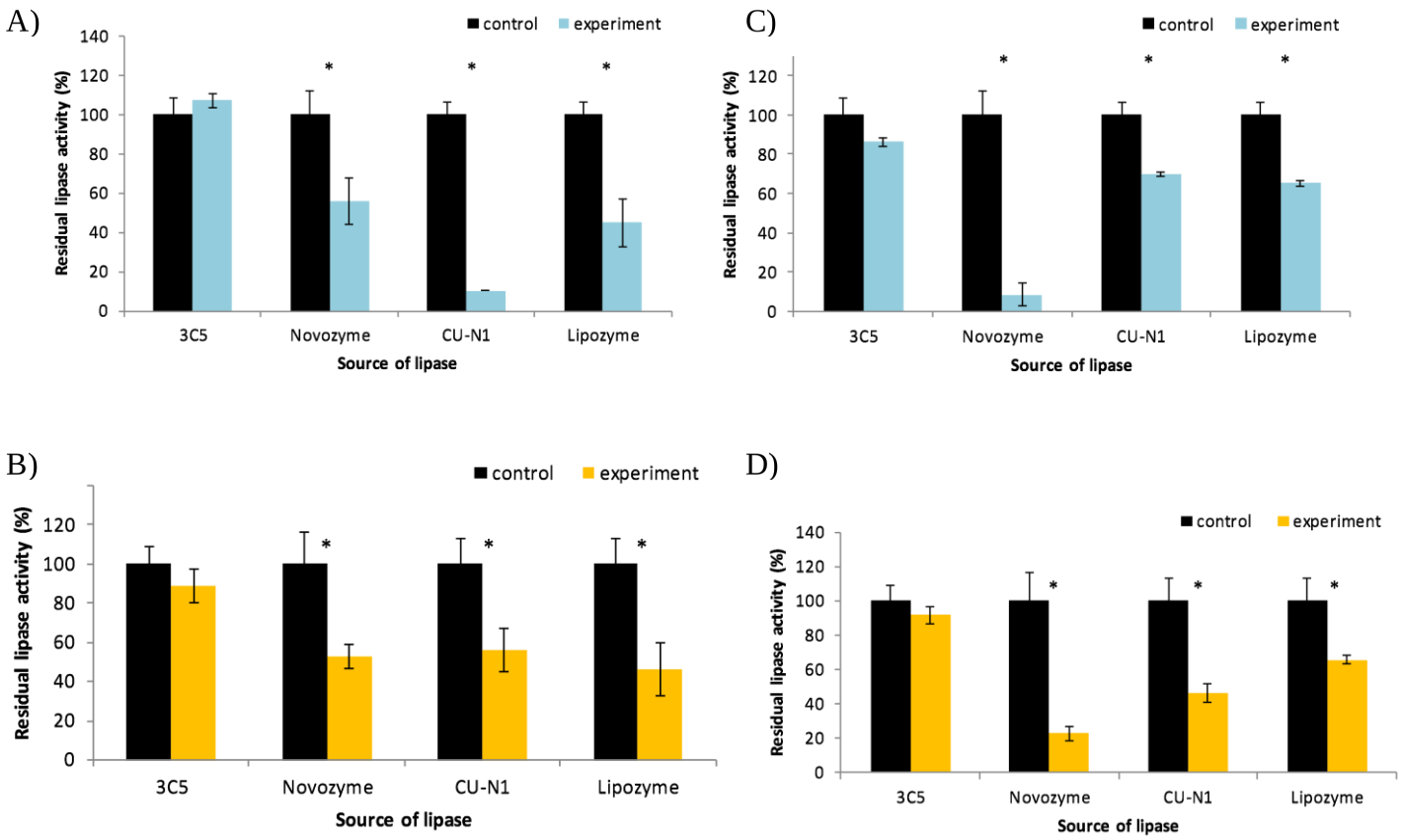
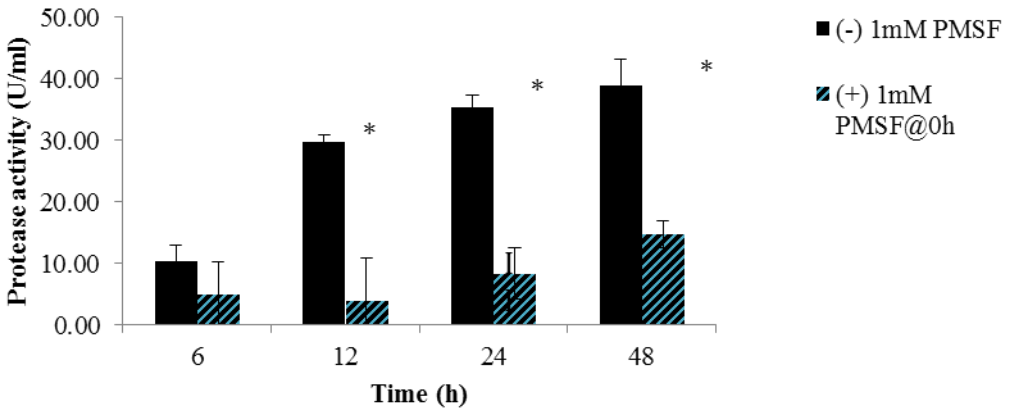


Figure 6 Proteolytic stability of several lipases (3C5: *B. licheniformis* 3C5, Novozyme: Lipolase® 100 L, CU-N1: *Burkholderia* sp. CU-N1 and Lipozyme: Lipozyme® CALB L). A) and B) indicate residual lipase activity after 24h of incubation between lipases and 3C5 protease at 30ºC and 45ºC, respectively, whereas C) and D) was lipase incubation with Alcalase® at 30ºC and 45ºC, respectively. The significant difference at $P_{0.05}$ is indicated by asterisk (n=3)

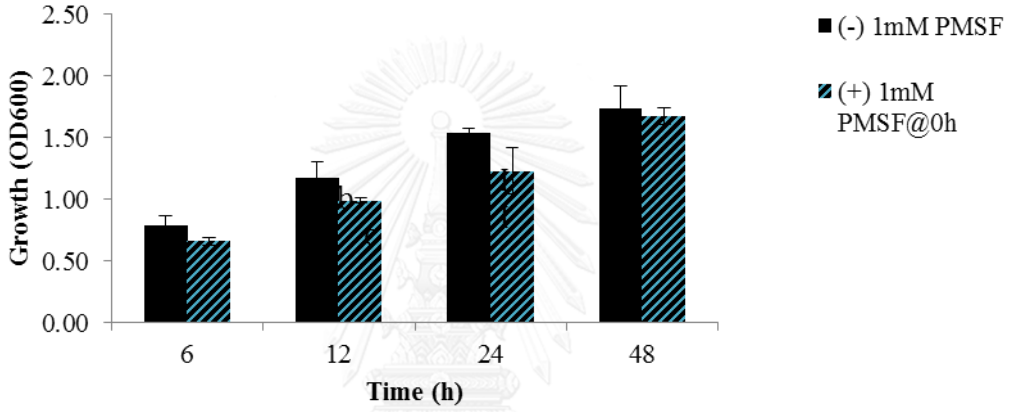
4.1.1.2 Effect of native protease on lipase stability during growth-dependent enzyme synthesis

The aim of this study was to proof the proteolytic-resistant ability of lipase coproduced with protease during growth-dependent enzyme synthesis in the same medium. The hypothesis is that if lipase had degraded by native protease, when protease was inhibited by protease inhibitor thereafter the yield of lipase would be increased when compare with the normal production process. This investigation was performed by modifying the method of Pereira-Meirelles et al. (1997). PMSF at 1mM was chosen as protease inhibitor (Lee *et al.*, 2003; Chakraborty and Paulraj, 2009). The results showed in Figure 7a demonstrated that protease activity was inhibited significantly by PMSF. Moreover, there was no significant difference of cell growth for this experiment comparing with the control (Figure 7b), which inhibitor was not added. From this result, it can be concluded that the inhibitor had no effect on cell growth. The results revealed that lipase activity was not significantly higher than that obtained from control (Figure 7c). However, protease increased slightly at 48h of cultivation. It may be caused by the instability of the PMSF inhibitor (James, 1978). The results were in contrast with Pereira-Meirelles *et al.* (1997) who observed the higher lipase activity from *C. lipolytica* after adding PMSF to the culture medium. It means that *C. lipolytica* lipase was degraded by native protease. The same results were obtained from previously section (4.1.1.1). Therefore, it can be concluded that 3C5 lipase has low susceptible toward its own protease.

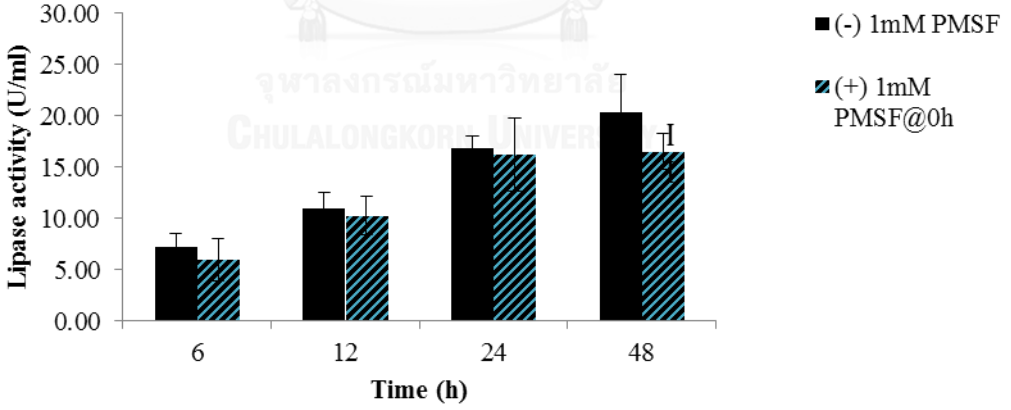
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY



a)



b)

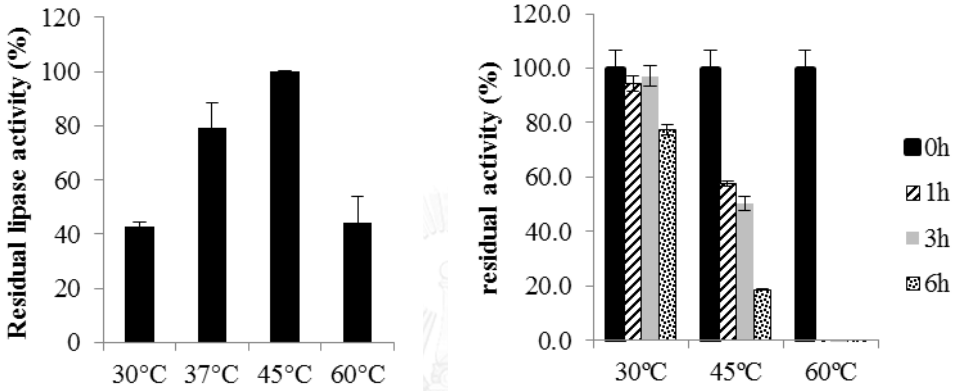


c)

Figure 7 Effect of native protease on lipase stability during growth-dependent enzyme synthesis of *B. licheniformis* 3C5 in YMO medium, a) Lipase activity, b) protease activity and c) growth in YMO medium ((-) 1mM PMSF), YMO medium with PMSF inhibitor (1mM) that as added at 0h of cultivation ((+) 1mM PMSF@0h). The significant difference at $P_{0.05}$ is indicated by star. (n=3)

4.1.2 Effect of temperature on lipase activity and stability

The crude lipase showed optimal reaction temperature at 45 ºC (Figure 8a). After incubated at temperature 30 ºC for 1 h, the lipase remained over 75% residual activity compared with the initial activity before incubation. The activity decreased sharply when the temperature was above 45 ºC (Figure 8b).

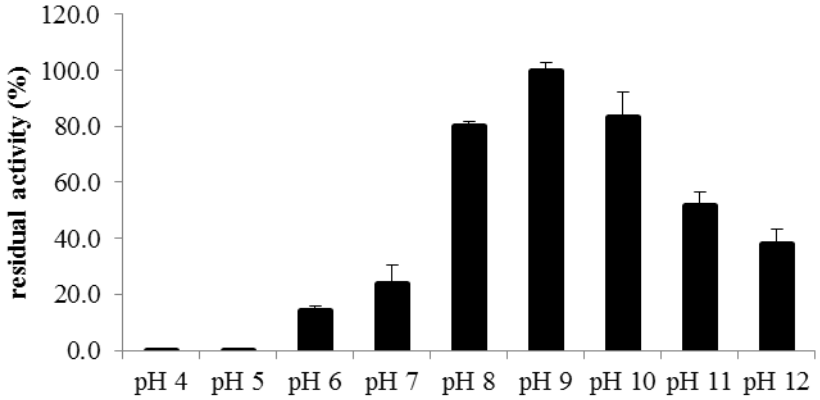


a) b)

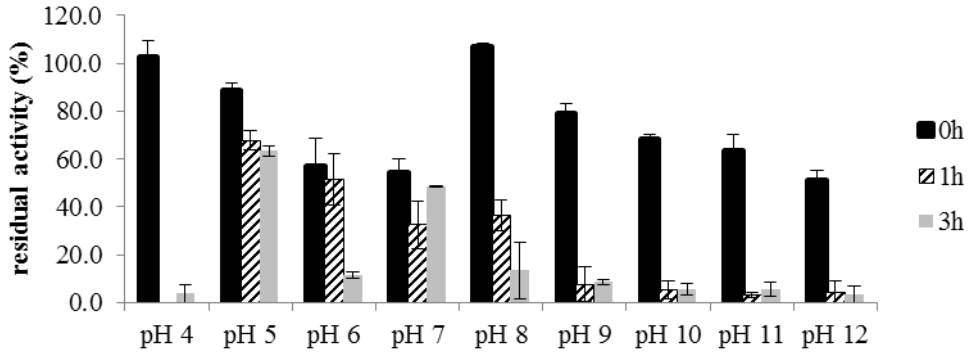
Figure 8 Effect of temperature on the a) activity and b) stability of lipase. (n=3)

4.1.3 Effect of pH on lipase activity

The crude lipase showed an optimum activity at pH 9 (Figure 9a). The pH stability of lipase showed that the enzyme was stable over pH 5-8 with more than 40% residual activity after incubated at 30 ºC for 1h, though maximum activity are presented in alkaline range (Figure 9b).



a)



b)

Figure 9 Effect of pH on the a) activity and b) stability of lipase. (n=3)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

4.1.4 Effect of organic solvent on lipase activity

The lipase activity from strain 3C5 in the solution containing *n*-decane, *n*-decanol and cyclohexane after incubation for 1h at 30 °C was found to retain about 80% (Figure 10). The most of enzyme lost their activity in organic solvents because of denaturation.

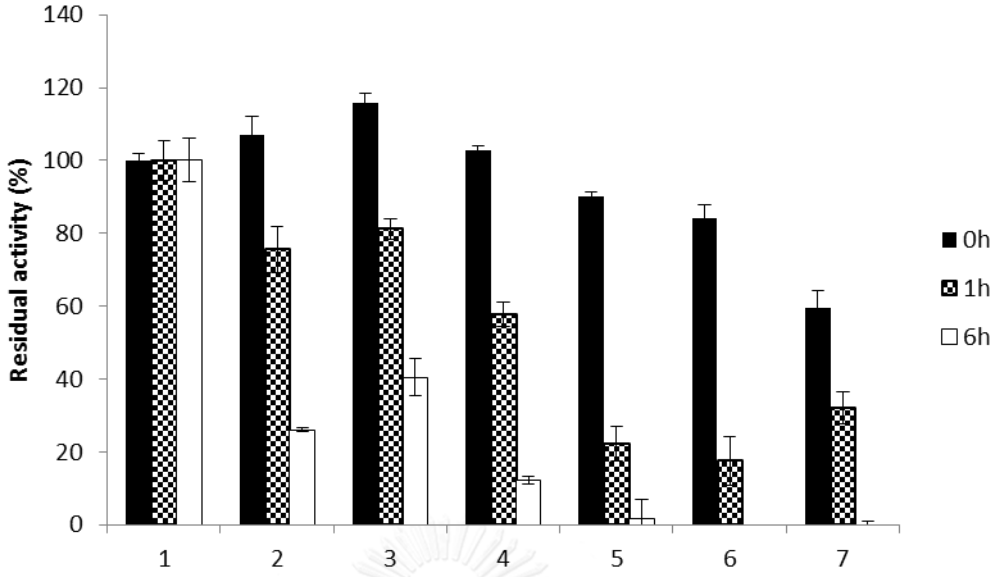


Figure 10 Effect of organic solvents on lipase activity and stability at 30 °C. The relative activity was determined after mixing (filled squares), and the residual activity was measured after 1-h (checked squares) and 6-h (blank squares) incubation and compared to that (100%) of non-solvent treatment control (1). The organic solvents were (2) *n*-decane, (3), *n*-decanol, (4) cyclohexane, (5) toluene, (6) dichloromethane and (7) acetone. (n=3)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

4.1.5 Effect of detergent on lipase activity

Lipase can be used as a major component in detergent formulations and industrial waste water treatment, the detergent stability was determined. The lipase from bacterial strain 3C5 were well stable toward several type of non-ionic surfactant at a relatively high temperature in that it retained about 100% of its activity after incubation with non-ionic surfactants, Triton X-100, Tween-80 and Tween-20, at 30 °C for 3 h (Figure 11).

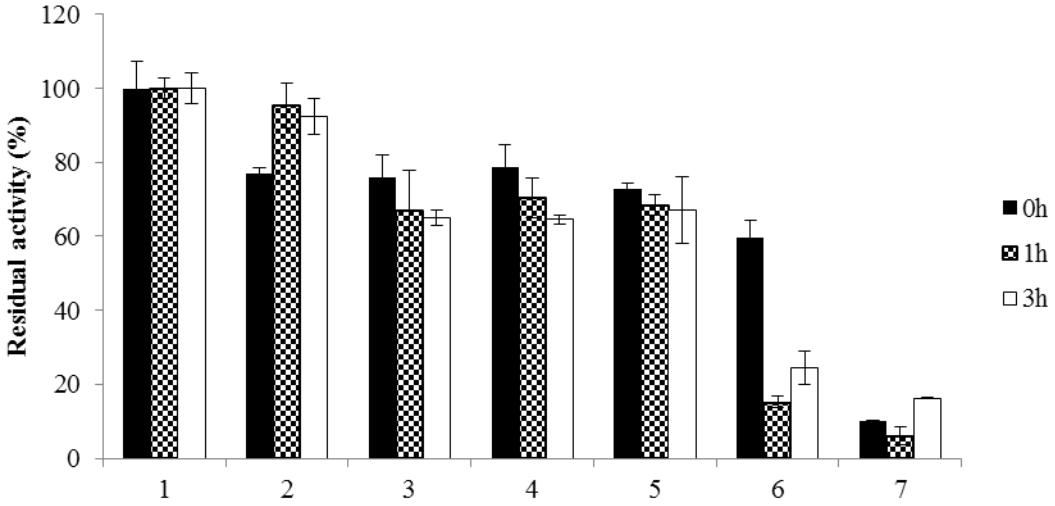


Figure 11 Effect of detergents on the activity and stability of lipase. The detergent was mixed with the crude enzyme and incubated at 30 °C. The relative activity was determined after mixing (filled squares), and residual activity was measured after 1-h (checked squares) and 3-h (blank squares) incubation and compared to that (100%) of the detergent containing control (1). The detergents were the non-ionic detergent (2) 1% (v/v) Triton x-100, (3) 1% (v/v) Tween-80, (4) 1% (v/v) Tween-20 and (5) 1% (w/v) Brij 35; as well as the anionic detergent (6) 0.2% (w/v) SDS and (7) 1% (w/v) SDS. (n=3)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.2 Medium optimization for concomitant production

4.2.1 Basal medium selection

In order to select the suitable basal medium, three media with different chemical composition were tested (MSBY, MSBYO and YMO). In accordance with the literature, our results revealed that medium component influenced on protease and lipase production. MSBY media has been reported by Rachadech *et al.* (2010) as the optimized protease production media, but lipase production has not been reported from it (Figure 12). Lipases are inducible enzyme; thereby the presence of inducer, such as vegetable oil or triglyceride, in the media is necessary to lipase expression (Guncheva and Zhiryakova, 2011). Consequently, lipase activity can be produced similarly in MSBYO or YMO, which olive oil was included as an inducer, but with different protease levels. The results illustrated, in Figure 13 and Figure 14, the maximum protease activity from MSBYO was 89.79±2.57 U/ml, while that from YMO was 42.12±6.25 U/ml. The excess of nitrogen source in YMO medium may cause low level protease production. There was in agreement with some reports on the inhibition role of an excessive of organic nitrogen sources in protease synthesis by *Bacillus* sp. (Cherdyntseva, Razin'kov and Egorov, 1982; Moon and Parulekar, 1991; Joo *et al.*, 2003; Chu, 2007). Although, MSBY medium composed a lot of many trace element, but its cost was lower than YMO medium which had three protein extract ingredients. Moreover, the ratio of protease and lipase combination applicable in industry was 50:1 or 100:1, thereby less activity of lipase in MSBYO was accepted. So, MSBYO medium was the best choice to use as the basal medium for further optimization of concomitantly protease and lipase production.

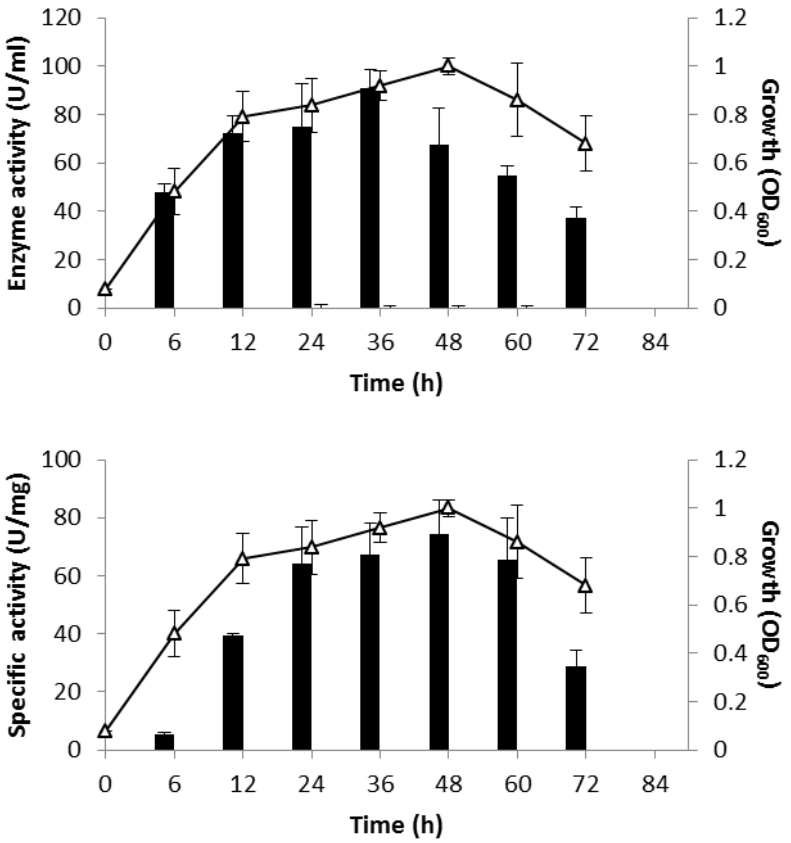


Figure 12 Time course of enzyme activities, protease production (filled squares), lipase production (striped squares) and cell growth curve (triangle) of a culture of *B. licheniformis* 3C5 in MSBY medium (n=3).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

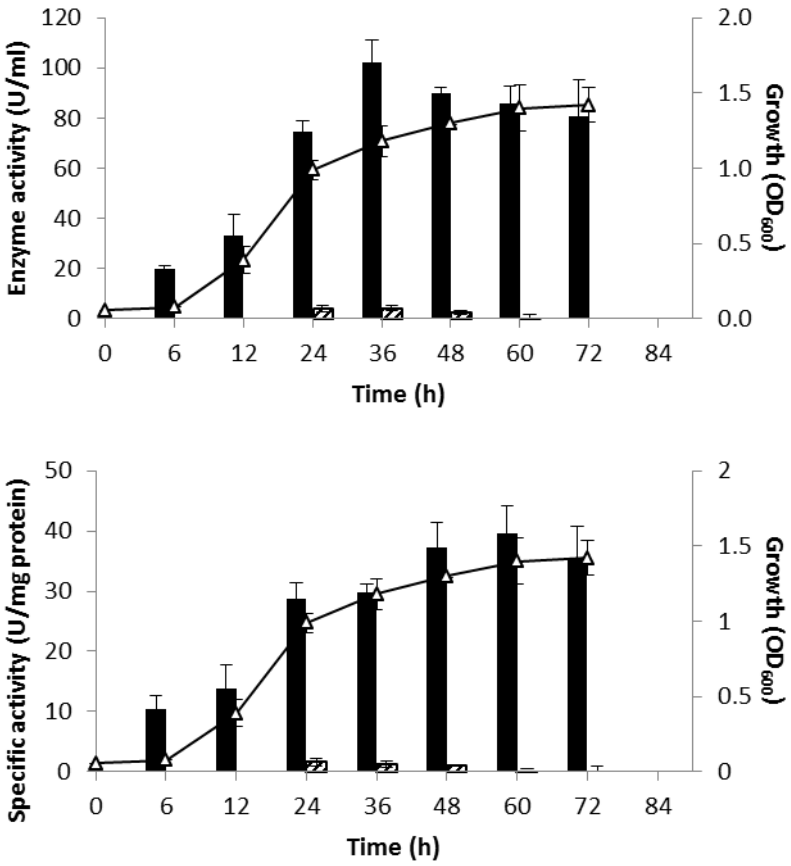


Figure 13 Time course of enzyme activities, protease production (filled squares), lipase production (striped squares) and cell growth curve (triangle) of a culture of *B. licheniformis* 3C5 in MSBYO medium (n=3)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

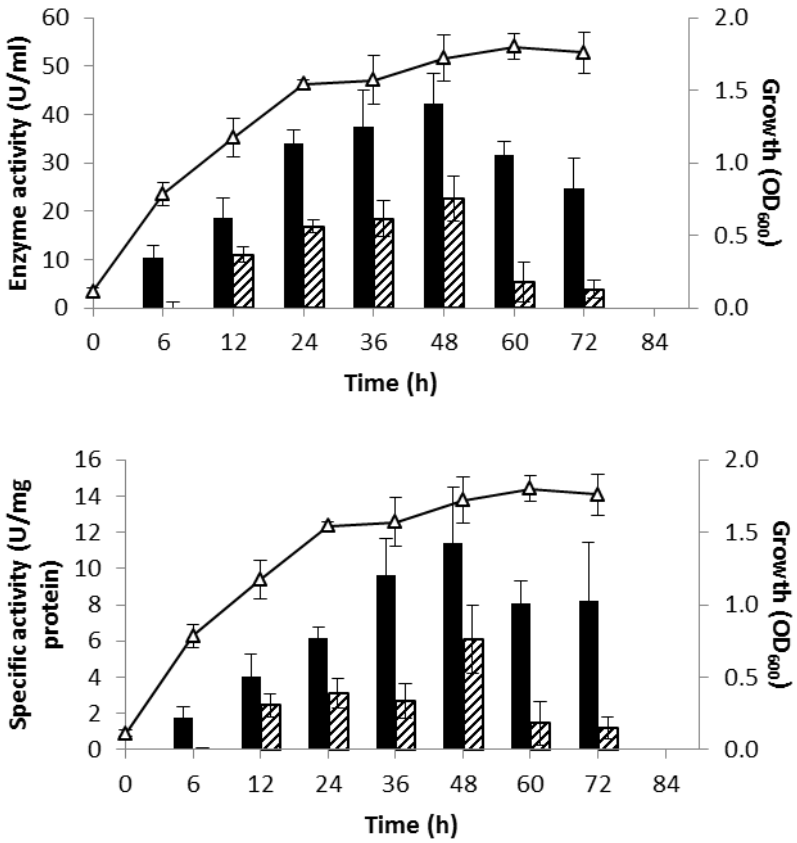


Figure 14 Time course of enzyme activities, protease production (filled squares), lipase production (striped squares) and cell growth curve (triangle) of a culture of *B. licheniformis* 3C5 in YMO medium (n=3)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

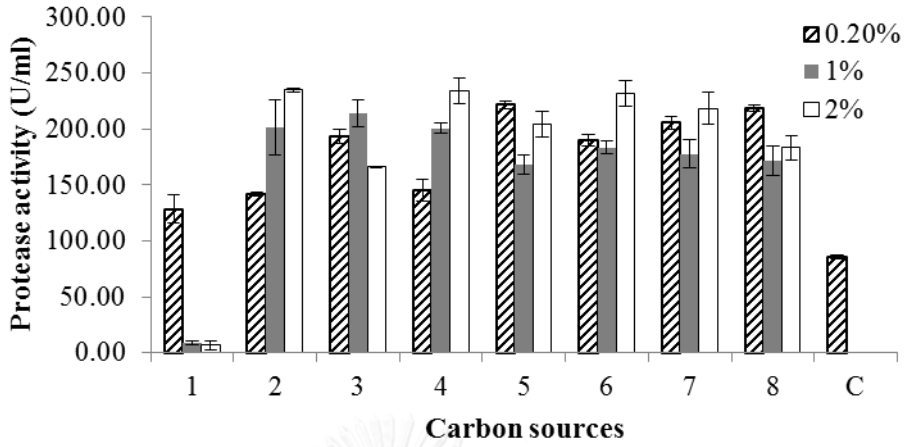
4.2.2 Effect of carbon source

In order to select the best carbon source, the basal medium was supplementation with the variety of carbon source, glucose, glycerol, soluble starch and xylose at various concentrations (0.2, 0.5 and 1.0% (w/v)). The results showed that these carbon sources increased both growth and enzyme production (Figure 15). The various carbon sources at 1% (w/v) clearly affected enzyme production. Figure 16 showed that the addition of all carbon sources, except for citric acid, increased 29-62% growth, 36-178% protease and 27-61%, lipase activity higher than that obtained from the basal medium. It suggested that carbon sources are important effect on the growth of bacteria and enzyme production in accordance with literature (Hasan *et al.*, 2009; Guncheva and Zhiryakova, 2011; Bhunia *et al.*, 2012). The enzyme production was found to reach maximum when the cell growth entered into the late exponential phase of growth culture indicating that protease and lipase production are growth associated (Gupta *et al.*, 2002; Guncheva and Zhiryakova, 2011). As shown in Figure 16, the higher growth of each added carbon source medium was higher both protease and lipase production than the basal medium. The present study was in contrast with the results of Takii, Kuriyama and Suzuki (1990) and Nashif and Nelson (1953) who showed that citric acid promoted protease and lipase production, correspondingly.

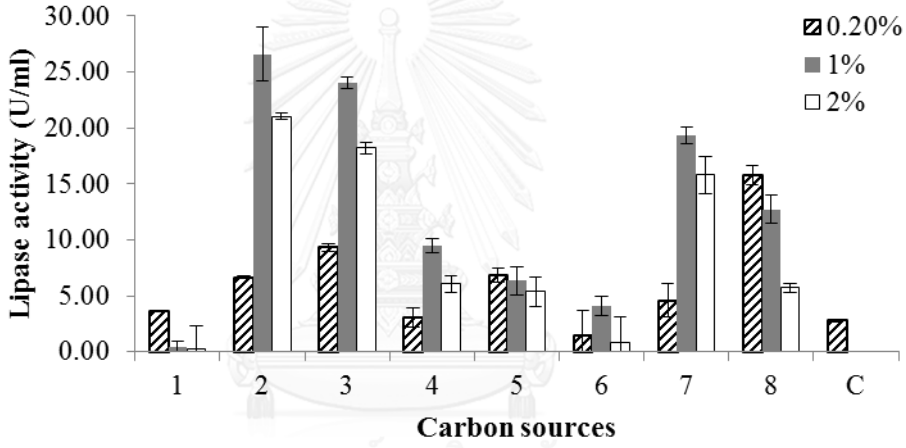
Among all carbon sources, glucose and glycerol showed the highest protease and lipase activity over other used carbon sources. Conversely, glucose as carbon source was preferred over glycerol to support a rapid growth rate. Commonly, glucose was considered as the preferred carbon source for *B. subtilis* (Meyer *et al.*, 2011).

As seen in Figure 17, though the maximum protease activity (234.65±1.70 U/ml) and lipase activity (26.60±2.44 U/ml) for glucose was observed at 2% and 1% (w/v), respectively, all paramerters were no significantly different over 1 and 2% (w/v). When increasing concentration of glucose to 2% caused reducing the yield of lipase production, due to catabolic repression where high glucose concentration repressed enzyme production (Gowland, Kernick and Sundaram, 1987; Eggert *et al.*, 2003; Mobarak-Qamsari, Kasra-Kermanshahi and Moosavi-nejad, 2011). This repression could also be found in protease synthesis (Patel, Dodia and Singh, 2005; Ibrahim *et al.*, 2015), but this study revealed that increasing glucose concentration

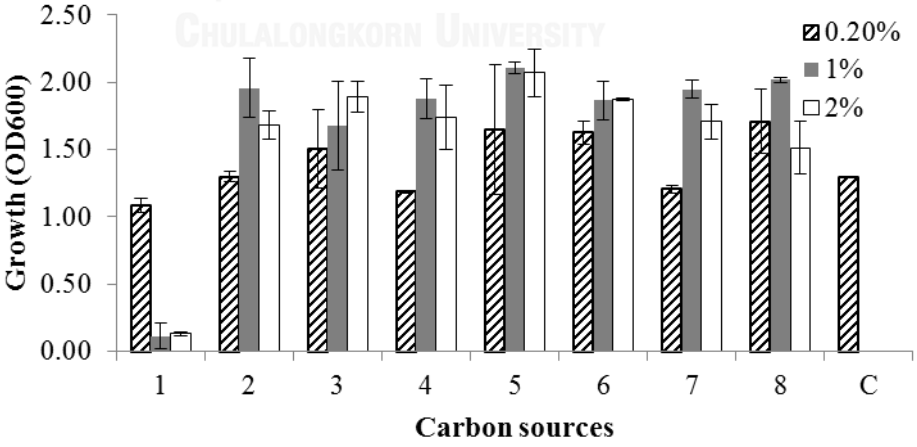
resulted in increasing protease production. Consequently, glucose 1% was selected for futher experiments.



a)



b)



c)

Figure 15 Effect of different carbon sources on the production of protease (a) and lipase (b), and cells growth (c) of *B. licheniformis* 3C5. The carbon sources were (1) citric acid, (2) glucose, (3) glycerol, (4) lactose, (5) maltose, (6) mannitol, (7) soluble starch, (8) xylose and (C) medium without carbon source (MSBYO). (n=3)

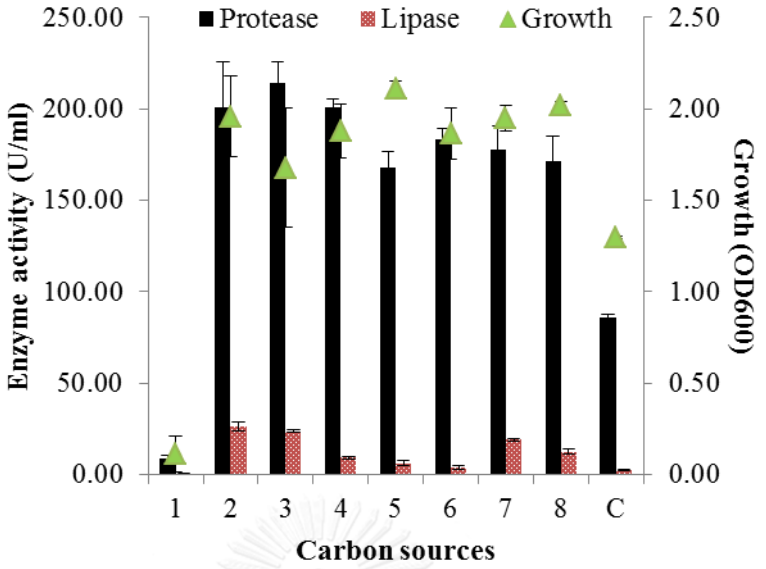
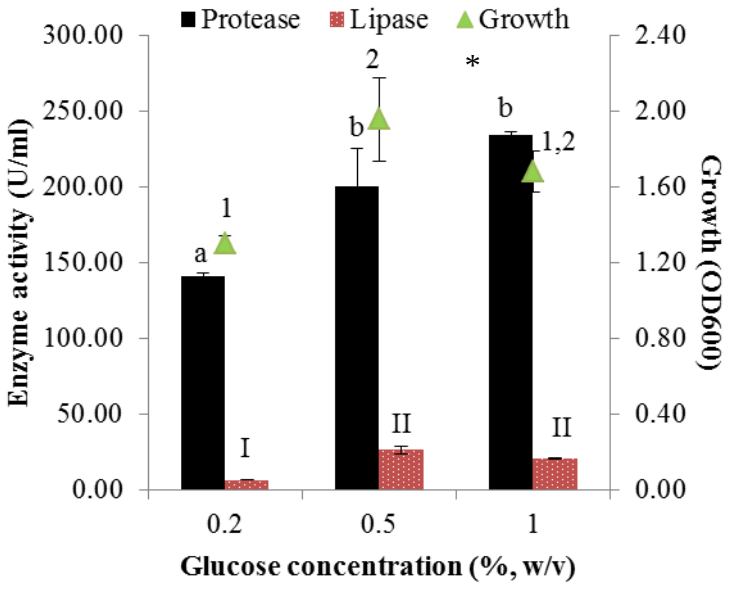


Figure 16 Effect of different carbon sources (1%, w/v) on the production of protease and lipase, and cells growth of *B. licheniformis* 3C5. The carbon sources were (1) citric acid, (2) glucose, (3) glycerol, (4) lactose, (5) maltose, (6) mannitol, (7) soluble starch, (8) xylose and (C) medium without carbon source (MSBYO). (n=3)



a.

Figure 17 Comparative production profile of *B. licheniformis* 3C5 in different concentrations of glucose. The significant at $P_{0.05}$ is indicated by different letters or numericals or Roman numericals on the error bars. The selected concentration is indicated by star. (n=3)

4.2.3 Effect of nitrogen source

Several reports have demonstrated that high enzyme production of *Bacillus* sp. was obtained from organic nitrogen sources rather than the inorganic nitrogen, as organic nitrogen sources plentiful in essential amino acids and short peptides (Gajju H, 1996; Gupta *et al.*, 2004; Mobarak-Qamsari *et al.*, 2011; Bhunia *et al.*, 2012). Therefore, organic nitrogen sources such as casein, peptone, tryptone and yeast extract were studied to identify the suitable nitrogen source, because the requirement for specific nitrogen source differs from organism to organism (Kumar and Takagi, 1999).

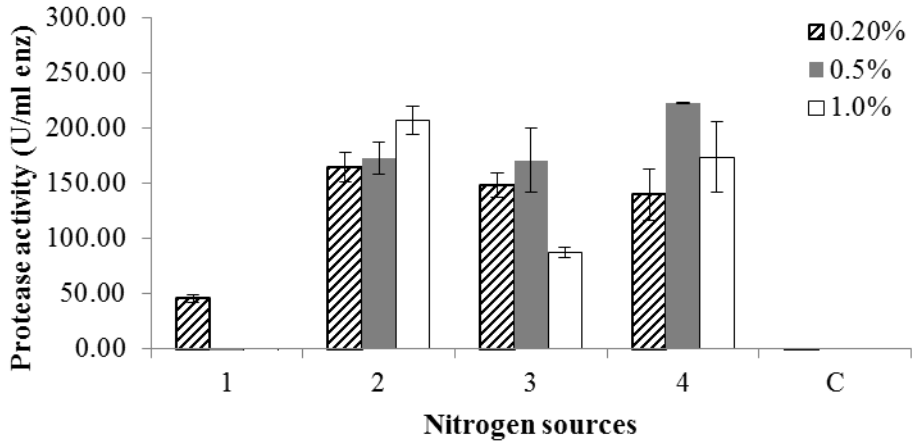
The results cited in Figure 18 showed that the peptone, tryptone and yeast extract generated no different in growth corresponding to enzyme productivity at 48h cultivation. Moreover, the absence of organic nitrogen source (control) was less protease production at 24h cultivation or no protease activity at 48h. Although the basal medium also has inorganic nitrogen source, it cannot support the protease production, as mentioned above. It is to be noted that nitrogen source necessary for protease production, which is constitutive or partially inducible in nature and thus are generally regulated by carbon and nitrogen stress (Gupta *et al.*, 2002).

In case of casein as nitrogen source, casein contained some essential amino acids as well as some carbohydrate and the inorganic elements calcium and phosphorus (Gomaa, 2013). It is clear from Figure 19 that the maximum protease yield of casein was found at 24h cultivation; meanwhile its protease activity drastically reduced at 48h cultivation. In contrast, maximum lipase activity was observed at 72h cultivation. On the other hand, peptone, tryptone and yeast extract gave highest protease and lipase activity at 48h cultivation. The decrease in protease activity is not clear whether nutrient depletion, or a complex mechanism of auto-digestion (Chu *et al.*, 1992).

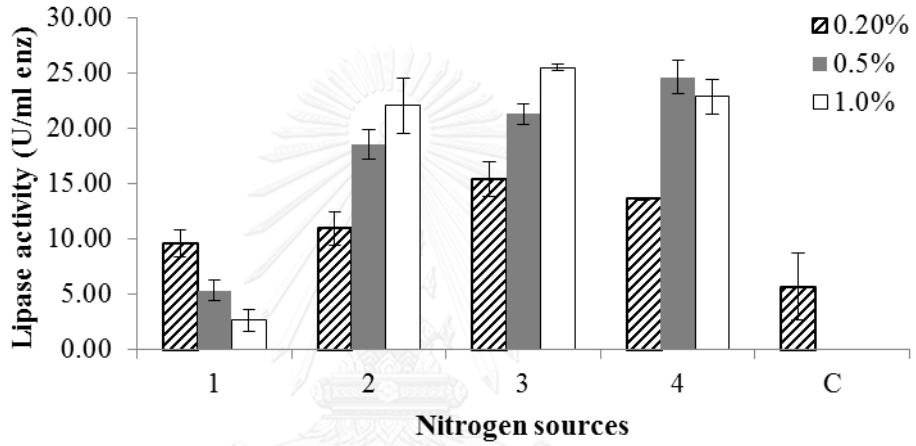
Among the different nitrogen sources used yeast extract was found to be the most suitable source (Figure 18). In yeast extract (0.5% (w/v)) maximum protease and lipase activity was observed (200.97±24.77 and 26.60±2.440.17 U/ml, respectively). Additionally, yeast extract is inexpensive organic source and contains abundant amino acids, proteins, vitamins and trace elements as well as many growth factors that enhancing enzyme production (Gupta, Sahai and Gupta, 2007; Gomaa,

2013). Yeast extract alone or in combination with other nitrogen sources is one of the most nitrogen sources for lipase production by different microorganisms (Pabai, Kermasha and Morin, 1996; Sidhu *et al.*, 1998; Sharma *et al.*, 2002; Ebrahimpour *et al.*, 2008; Bora and Bora, 2012). At various concentration of yeast extract, there was no significant different of protease activity, while lipase secreted significantly levels between 0.2 and 0.5% (w/v) but not observed in 0.5 and 1.0% (w/v) (Figure 21). A similar effect of increasing yeast extract concentration to lipase production by other *Bacillus* sp. have been reported by previous worker (Sidhu *et al.*, 1998). Therefore, yeast extract at 0.5% (w/v) was chosen as nitrogen source.

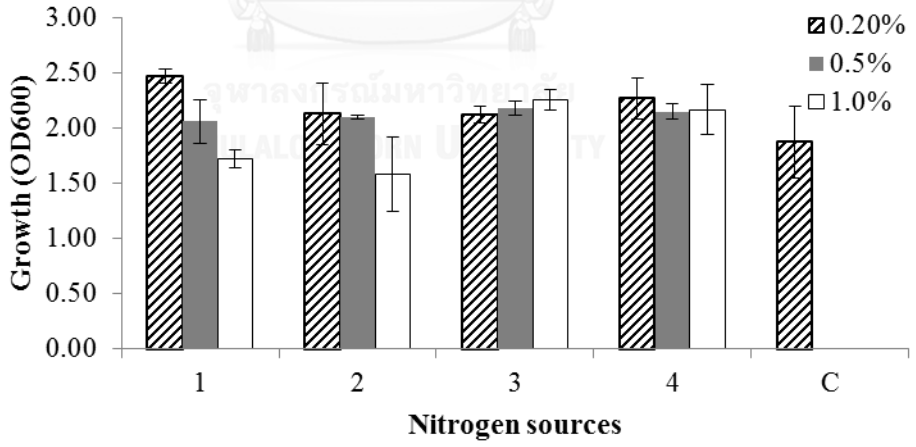
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University



a)



b)



c)

Figure 18 Effect of different nitrogen sources on the production of protease (a) and lipase (b), and cells growth (c) of *B. licheniformis* 3C5. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

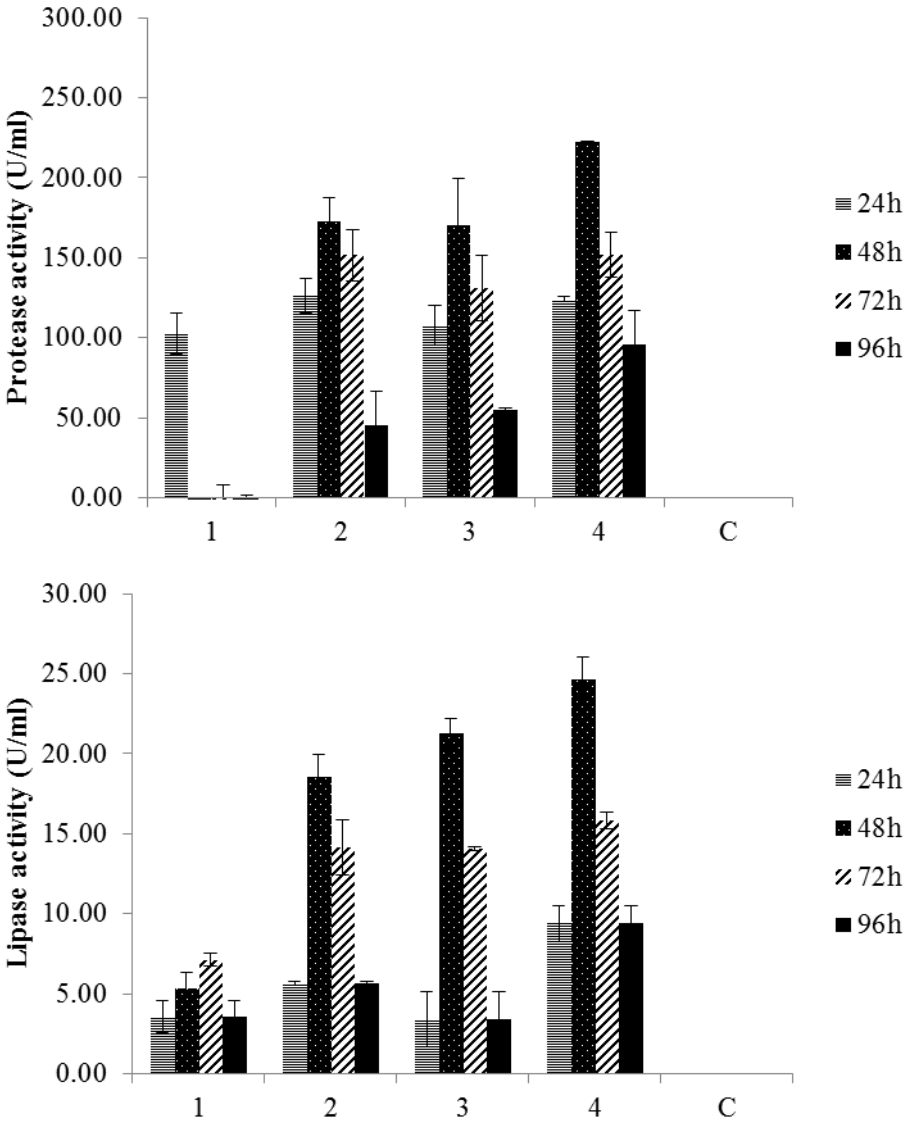


Figure 19 Effect of different nitrogen sources (0.5%, w/v) on the production of protease and lipase and cells growth of *B. licheniformis* 3C5 for 96h cultivation. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

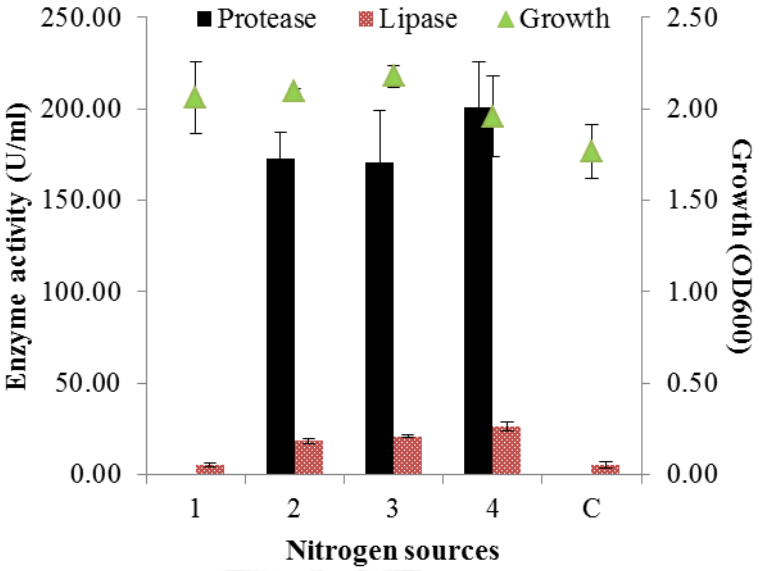


Figure 20 Effect of different nitrogen sources (0.5%, w/v) on the production of protease and lipase and cells growth of *B. licheniformis* 3C5. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

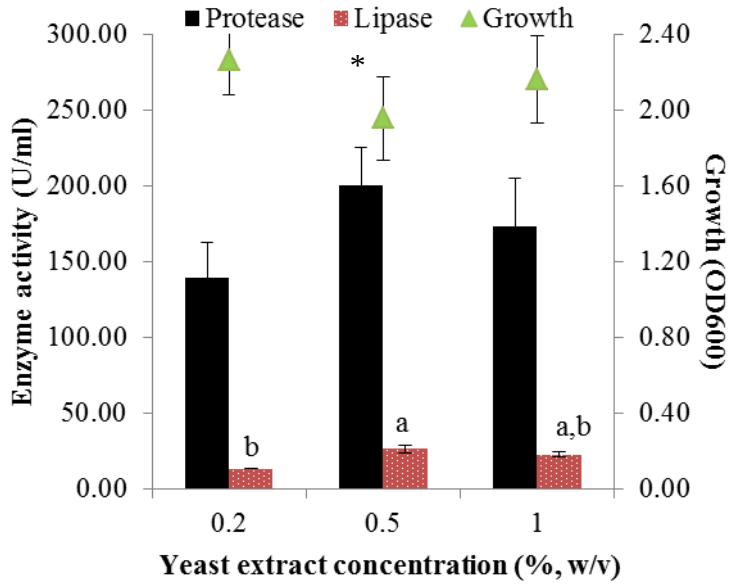


Figure 21Comparative production profile of *B. licheniformis* 3C5 in different concentrations of yeast extract. The significant at $P_{0.05}$ is indicated by different letters or numericals or Roman numericals on the error bars. The selected concentration is indicated by star. (n=3)

4.2.4 Effect of inducer

The influence of various inducers on the cell growth and lipase production were evaluated using the basal medium containing glucose as the sole carbon source and yeast extract as a nitrogen source. On an equivalent inducers basis, olive oil in the basal media of protease and lipase, respectively were replaced by different inducers (tributyrin, coconut oil, palm oil, olive oil, soybean oil, sunflower oil, sesame oil, Tween20, Tween80 and Triton X-100) at 0.5, 1.0 and 1.5% (v/v), one at a time. It was found that 48h cultivation gave the maximum lipase production. The results represented in Figure 23 show that there was no lipase activity in the absence of inducer. There was in accordance with the necessary of inducer in the medium to express of inducible enzyme like lipase (Lotti *et al.*, 1998). The order of inducers ability to stimulate lipase production was olive oil >sesame oil>sunflower oil>palm oil>soybean oil>>coconut oil>tributyrin>>Tween80=Tween20=Triton X-100, and the results are demonstrated in Table 20. Although, some reports recorded that the surfactants supported production and secretion of lipase from cells (Nthangeni *et al.*, 2001; Castro-Ochoa *et al.*, 2005). Conversely, no growth was observed in all used surfactants which this case caused to no lipase activity, though only Tween 80 showed good growth. The same result was found in case of tributyrin, which had four carbon atom of triglyceride. Consequently, high lipase production was found from various vegetable oils, including 12-18 carbon atoms of saturated and un-saturated dominant fatty acid (Table 21). Our results concluded that long-chain length fatty acid (including Olive oil, Sesame oil, Sunflower oil, Pal, oil, Soybean oil) was preferred to induce lipase production rather than short-chain length fatty acid (Coconut oil). In agreement with Ghosh *et al.* (1996) who founded that oleic acid (C18:1) and linoleic (C18:2) stimulated lipase production. Moreover, Takaç and Marul (2008) recorded that the presence of low amount of middle-chain length fatty acid like palmitic acid (C16:0) in vegetable oil increasing lipase production. Therefore, olive oil acted as the best inducer for lipase production by *B. licheniformis* 3C5, and also gave good protease production. In contrast, low level of protease production was founded in sesame oil and coconut oil. Further varying in the olive oil concentration had a consistent effect between growth and lipase production (Figure 24). Comparing these

results, the growth and lipase production were significantly induced by high olive oil concentration. It indicated that 1.5% (v/v) olive oil was suitable inducer.

Table 20 Effect of different inducers on the production of protease and lipase and cells growth of *B. licheniformis* 3C5 (n=3)

Inducers	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
Tributyrin	0.00 ± 0.00	0.12 ± 0.17	0.50 ± 0.01
Coconut oil (C12:0)	49.50 ± 0.02	0.72 ± 0.02	0.87 ± 0.09
Palm oil (C16:0)	150.20 ± 6.69	11.84 ± 1.40	1.91 ± 0.29
Olive oil (C18:1)	189.55 ± 26.37	24.42 ± 3.94	1.94 ± 0.19
Soybean oil (C18:2)	169.33 ± 1.65	10.38 ± 1.60	1.71 ± 0.13
Sunflower oil (C18:2)	71.06 ± 16.92	17.36 ± 5.17	2.05 ± 0.10
Sesame oil (C18:2)	90.53 ± 20.43	19.34 ± 4.49	2.15 ± 0.06
Tween 20	46.32 ± 10.28	0.00 ± 0.00	0.26 ± 0.04
Tween 80	59.12 ± 3.31	0.32 ± 0.45	1.13 ± 0.05
Triton X-100	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.01
Control	149.58 ± 4.50	0.00 ± 0.42	1.27 ± 0.00

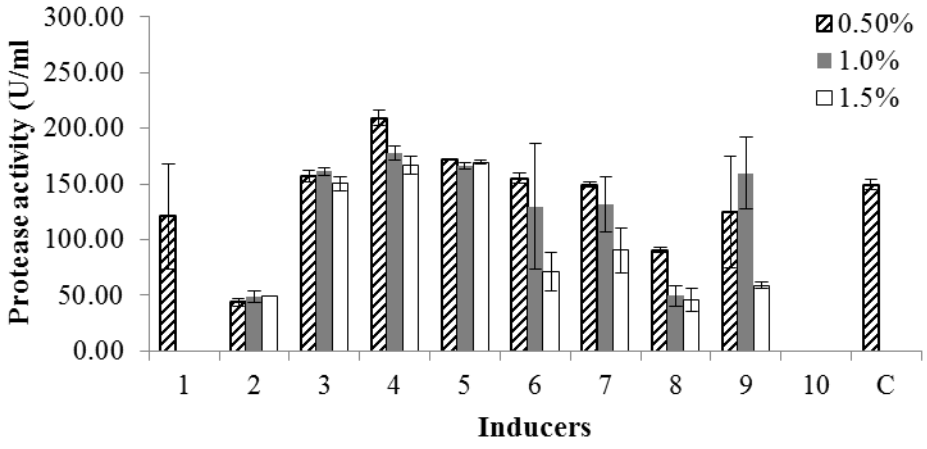
Control: the production medium with 1%glucose and 0.5%yeast extract without inducer

จุฬาลงกรณ์มหาวิทยาลัย

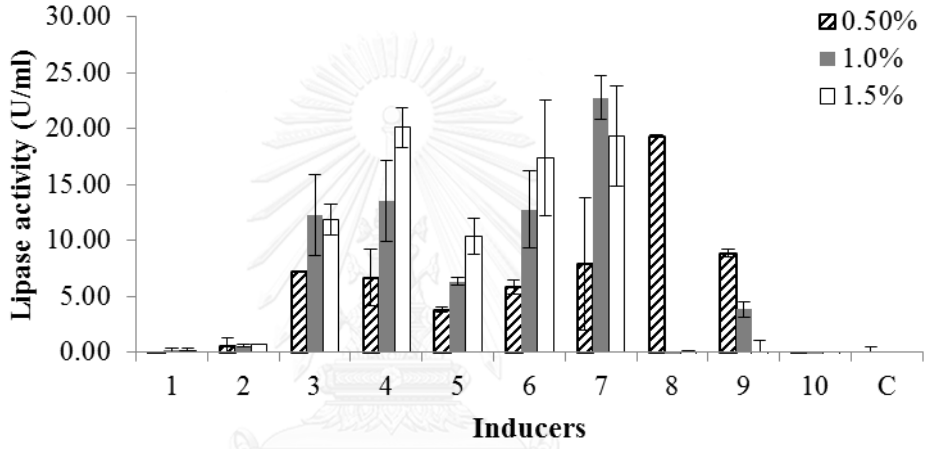
Table 21 Fatty acid composition of commercial vegetable oils

Vegetable oil	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C22:1	reference
Olive oil	ND	ND	ND	ND	12.3	0.8	2.7	73.4	9.2	0.6	0.5	0.3	0.2	ND	Takaç and Marul (2008)
Sesame	ND	ND	ND	ND	6.0	0.1	3.6	36.0	53.0	0.1	0.3	0.2	0.7	ND	Takaç and Marul (2008)
Sunflower	ND	ND	ND	ND	6.0	0.1	3.4	26.2	63.3	0.1	0.1	0.1	0.7	ND	Takaç and Marul (2008)
Palm oil	ND	ND	ND	1.7	43.3	ND	4.8	42.4	7.8	ND	ND	ND	ND	ND	Bhatnagar *et al.* (2009)
Soybean	ND	ND	ND	ND	13.8	0.1	3.4	25.1	51.2	5.6	0.3	0.2	0.2	ND	Takaç and Marul (2008)
Coconut oil	5.8	4.8	49.1	21.8	8.4	ND	2.8	6.1	1.2	ND	ND	ND	ND	ND	Bhatnagar *et al.* (2009)

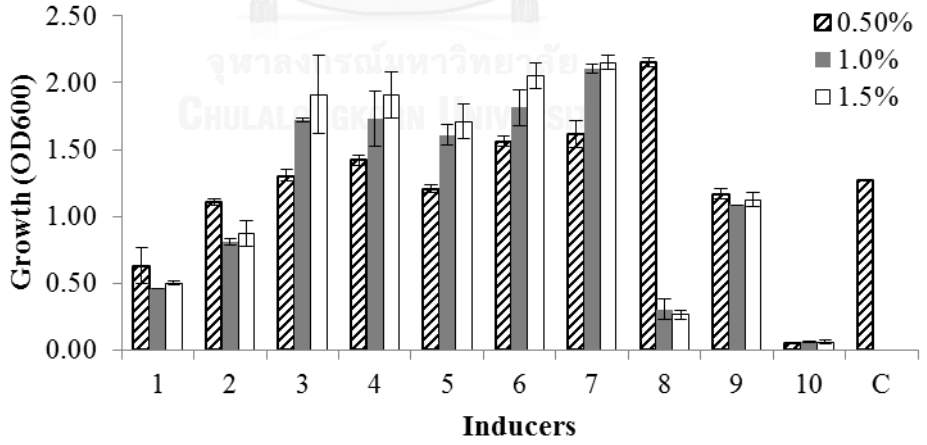
ND: not detected



a)



b)



c)

Figure 22 Effect of different inducers on the production of protease (a) and lipase (b), and cells growth (c) of *B. licheniformis* 3C5. The inducers were (1) Tributyrin, (2) Coconut oil, (3) Palm oil, (4) Olive oil, (5) Soybean oil, (6) Sunflower oil, (7) Sesame oil, (8) Tween 20, (9) Tween 80, (10) Triton X-100 and (C) the production medium with 1%glucose and 0.5%yeast extract without inducer. (n=3)

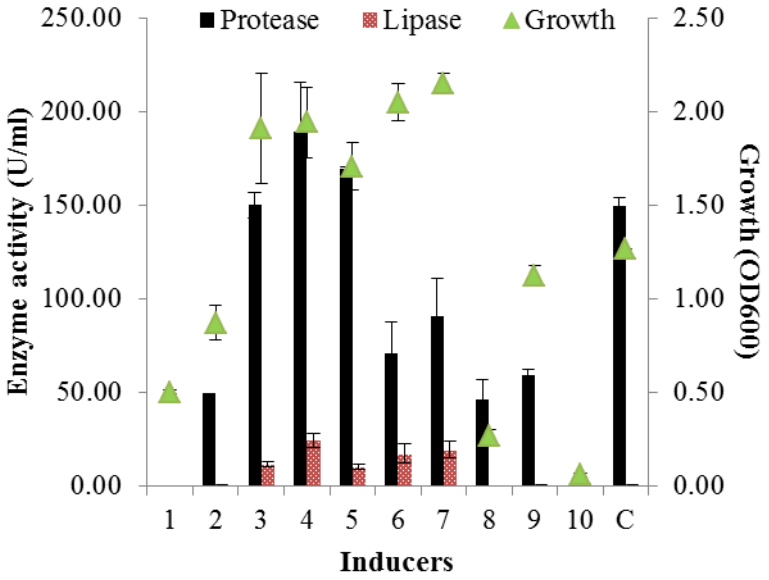


Figure 23 Effect of different inducers (1.5%, v/v) on the production of protease and lipase and cells growth of *B. licheniformis* 3C5. The inducers were (1) Tributyrin, (2) Coconut oil, (3) Palm oil, (4) Olive oil, (5) Soybean oil, (6) Sunflower oil, (7) Sesame oil, (8) Tween 20, (9) Tween 80, (10) Triton X-100 and (C) the production medium with 1%glucose and 0.5%yeast extract without inducer. (n=3)

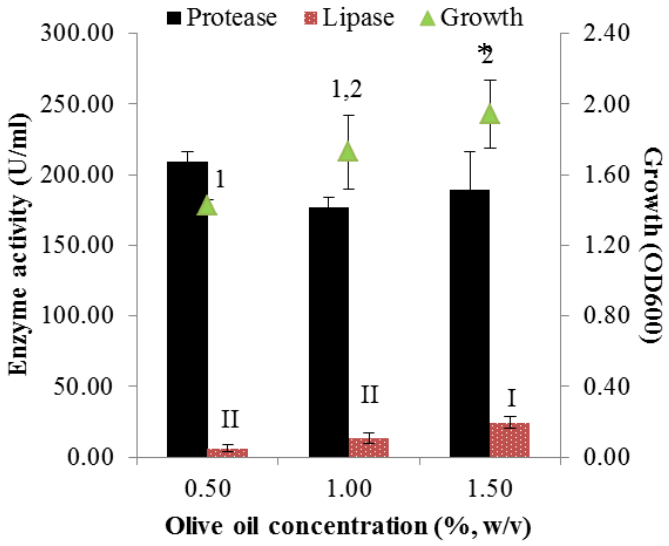


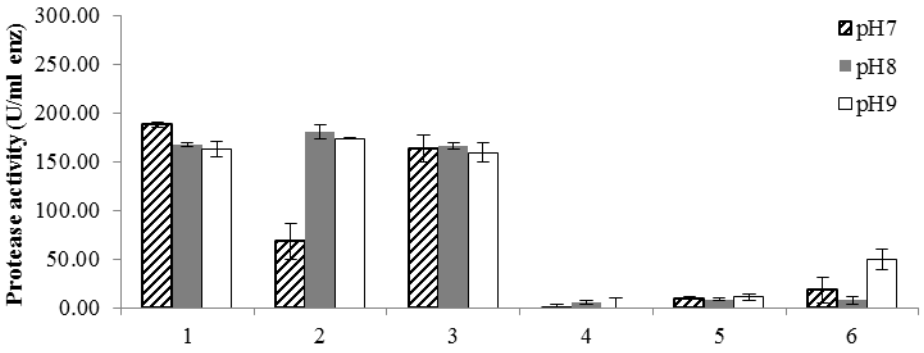
Figure 24Comparative production profile of *B. licheniformis* 3C5 in different concentrations of olive oil. The significant at $P_{0.05}$ is indicated by different letters or numericals or Roman numericals on the error bars. The selected concentration is indicated by star. (n=3)

4.2.5 Effect of physical factor

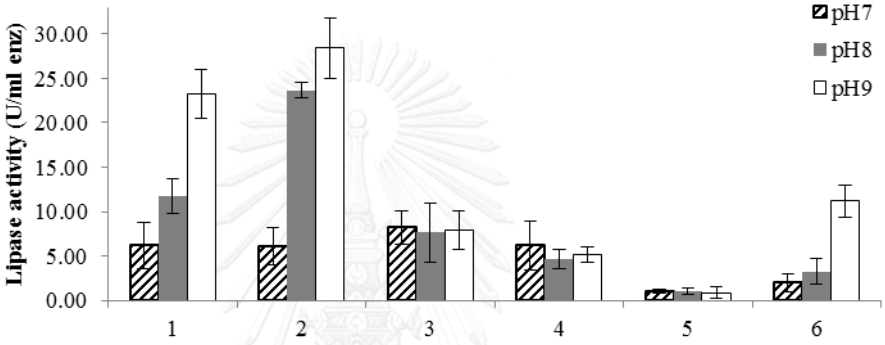
The results represented in Figure 25 showed that production of lipase was increased with increasing pH of the medium toward alkaline range from neutrality. Beside, protease production did not effect by pH of medium. The pH influences on cells growth and enzymes production. However, the growth of cell depended on enzyme ability, if cells were environed in pH-appropriated functional enzyme resulting in good growth rate (Villadsen, Nielsen and Lidén, 2011).

Furthermore, *B. licheniformis* 3C5 preferred moderate temperature (30 ºC) than high temperature (37 ºC and 45 ºC), while low agitation rate. However, at high temperature with low agitation rate was little cell growth and a few enzyme production. On the other hand, at high temperature with high agitation rate showed cell growth but was not suitable for enzyme production (Figure 25). The Bacilli cells favored the same temperature for growth and lipase production. Commonly, 20-45 ºC was proper for lipase production (Gupta *et al.*, 2004). The mechanism of influence of temperature on enzyme production are unclear (Chaloupka, 1985). Frankena *et al.* (1986) suggested that enzyme production and energy metabolism in *Bacilli* sp. were correlated with temperature and oxygen consumption.

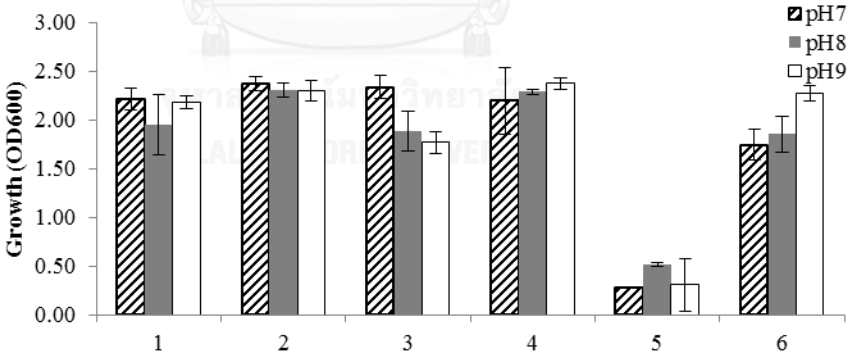
Bacilli cells commonly grow in aerobic environment, thus aeration rate involves in enhancing lipase production (Schmidt-Dannert *et al.*, 1994; Lee *et al.*, 1999; Sharma *et al.*, 2002; Hasan, Shah and Hameed, 2007). Aeration rate directly relates on agitation rate. Some literatures reported that growth rate and lipase production rate were directly involved in agitation (Sekhon *et al.*, 2006; Shariff *et al.*, 2007). Shariff *et al.* (2007) found that at static condition was not lipase production condition, while high agitation rate reduced lipase level by 50% which may cause from denaturation of enzyme in an oxidative environment. As mentioned above, *B. licheniformis* 3C5 cultured in the medium at pH 9, at 30 ºC with agitation rate 200 rpm were suitable for growth and also protease and lipase production.



a)



b)



c)

Figure 25 Effect of physical factors on the production of protease (a) and lipase (b), and cells growth (c) of *B. licheniformis* 3C5. (1) 30 ºC, 120 rpm, (2) 30 ºC, 200 rpm, (3) 37 ºC, 120 rpm, (4) 37 ºC, 200 rpm, (5) 45 ºC, 120 rpm and (6) 45 ºC, 200 rpm (n=3)

4.2.6 Effect of NaCl

It is clearly shown from Figure 26 that maximum lipase (31.20±2.40U/mL) and growth (2.48±0.02) were reported when NaCl was added in a concentration of 0.25% (w/v). These results concluded that NaCl significantly increased cells growth and lipase production by 1.6 and 1.5 folds. In agreement with some reports that NaCl stimulate lipase production (Ohkuro *et al.*, 1978; Kumar and Valsa, 2007). Besides, increasing NaCl concentration from 0.5 to 1.0% (w/v) showed reducing of lipase level (Figure 26). There was no significant effect of NaCl concentrations on the protease production. The growth of *Bacillus* sp. was controlled by sodium gradient, thereby sodium ion are important to bacterial energetic and metabolic processes, for example pH homeostasis and ATP synthesis (Horikoshi, 1999; Bhunia *et al.*, 2012). Furthermore, NaCl also achieved partially purify protease (Yavari *et al.*, 2013) and lipase (Barbosa *et al.*, 2011) by using ATPS. Therefore, the addition of 0.25% (w/v) NaCl in the production medium expects to both enzyme production and also further downstream process.

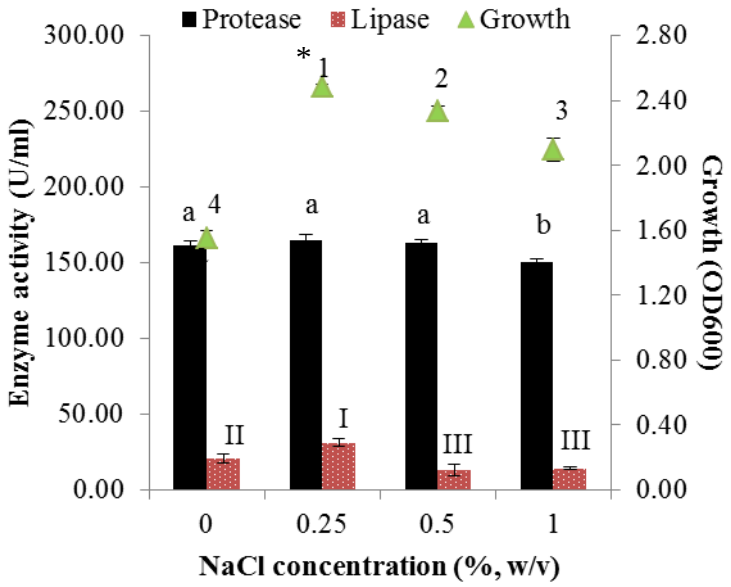


Figure 26 Comparative production profile of *B. licheniformis* 3C5 in different concentrations of NaCl. The significant at $P_{0.05}$ is indicated by different letters or numericals or Roman numericals on the error bars. The selected concentration is indicated by star. (n=3)

4.2.7 CCD and RSM

Four factors olive oil, glucose, yeast extract and NaCl, were found to affect the concomitant production of protease and lipase. The experimental plan to determine the optimum combination of medium components to enhance the enzymes production was done using CCD. The design of this experiment together with the experimental results is given in the Table 22.

The regression equation coefficients were calculated and the data were fitted into a second-order polynomial equation for the two responses. The quadratic response surface model represented in term of coded factors as following equation:

$$\text{Lipase activity} = 20.48 - 2.97A + 3.25B + 6.25C - 5.40D - 0.97A^2 - 6.52B^2 - 8.35C^2 - 4.24D^2 + 1.82AB + 5.68AC - 7.16AD - 9.55BC - 3.58BD - 4.09CD \quad (1)$$

$$\text{Protease activity} = 194.72 - 3.17A + 4.77B + 61.39C - 6.04D - 6.15A^2 - 21.21B^2 - 96.49C^2 - 9.48D^2 + 4.37AB + 26.44AC - 26.44AD - 0.56BC + 0.66BD - 15.63CD \quad (2)$$

Where A is olive oil concentration (%, v/v), B is glucose concentration (%, w/v), C is yeast extract concentration (%, w/v) and D is NaCl concentration (%, w/v)

The statistical significance was proved by Fisher's test value (F-value) for the analysis of variance (ANOVA). ANOVA analysis suggested that all of four factors, olive oil, glucose yeast extract and NaCl were significant ($P<0.05$) for lipase production that is shown in Table 35. Meanwhile, yeast extract was significant ($P<0.05$) for protease production as seen in Table 36. *P*-values, which denote the significance of coefficients, are pivotal in understanding the array of mutual interactions between different variables. The smaller is the magnitude of *P* more significant is the corresponding coefficient. Therefore, model terms with *P*-values less than 0.05 are considered significant (Rajendran and Thangavelu, 2007). The goodness of fit the model was determined by coefficient of determination (R^2). The closer the R^2 value to 1, the better is model fit to experimental data and less is the distance between predicted and experimental values. R^2 of lipase (protease) was 0.94 (0.92), which indicated that sample variation of more than 94% (92%) can be attributed to the variables and only less than 6% (8%) of the total variation could not be explained by the model.

The regression equation was solve and the optimal values of the tested factors in uncoded values were olive oil 2.5% (v/v), glucose 0.84% (w/v), yeast extract 0.6% (w/v) and NaCl 0.25% (w/v). To confirm the predicted optimized condition by RSM, an experiment was conducted at the optimum values of the test factors.

Under these optimized conditions, the lipase and protease activity were found to be 31.18±2.49 and 201.51±7.80 U/ml (predicted values 25.73±2.32 and 216.39±18.52 U/ml), respectively. The experimental and predicted values of enzymes activity show good agreement with one another, with a high degree of accuracy of the model of 94% and 92%. The optimization of fermentation conditions led to 12.57-fold increase of lipase production, while the production of protease was enhanced by 2.2-fold. The optimized lipase production was situated in the range of previously reported as seen in Table 23.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 22 CCD matrix of independent factors used in RSM with corresponding experimental results of protease and lipase activity (n=2)

Run	Olive oil (%)	Glucose (%)	Yeast extract (%)	NaCl (%)	Lipase activity (U/ml)	Protease activity (U/ml)
1	2.5	0.6	0.6	0.5	13.43±0.28	157.96±6.94
2	2	0.8	0.4	0.375	20.84±1.79	179.33±13.07
3	2.5	0.6	0.6	0.25	24.2±1.43	199.17±8.83
4	2.5	0.6	0.2	0.5	5.26±0.66	124.11±2.38
5	2	0.8	0.4	0.375	22.75±0.96	192.48±18.04
6	2.5	1	0.2	0.5	6.8±4.96	90.82±14.51
7	1.5	1	0.2	0.5	18.32±1.24	155.18±3.69
8	2.5	1	0.2	0.25	19.9±0.37	142.8±7.39
9	2.5	1	0.6	0.5	11.69±4.25	192.54±9
10	2	0.8	0.4	0.375	20.36±3.4	201.71±10.05
11	2	0.8	0.4	0.375	22.03±4.88	191.53±3.96
12	1.5	0.6	0.6	0.5	18.26±1.49	173.19±9.65
13	1.5	1	0.6	0.25	18.45±4.15	176.15±9.15
14	1.5	1	0.2	0.25	20.32±6.57	132.58±3.24
15	2.5	0.6	0.2	0.25	5.74±3.91	124.43±17.33
16	1.5	0.6	0.2	0.5	11.49±2.68	132.51±1.69
17	1.5	1	0.6	0.5	16.36±0.43	166.21±0.59
18	2.5	1	0.6	0.25	25.79±0.92	203.91±1.75
19	1.5	0.6	0.2	0.25	11.84±3.73	123.55±1.92
20	1.5	0.6	0.6	0.25	23.6±7	193.94±15.99
21	2	0.8	0.4	0.375	18.41±0.5	190.89±2.25
22	2	0.4	0.4	0.375	10.05±3.32	173.33±13.49
23	2	0.8	0.4	0.125	20.27±2.46	183.63±10.55
24	2	1.2	0.4	0.375	17.65±0.62	186.29±7.65
25	2	0.8	0.0	0.375	6.3±2.86	29.61±35.99
26	2	0.8	0.4	0.625	11.99±0.11	199.43±8.97
27	2	0.8	0.4	0.375	19.28±0.12	201.67±0.82
28	3	0.8	0.4	0.375	16.95±0.31	189.74±29.13
29	2	0.8	0.8	0.375	17.74±0.45	179.44±12
30	1	0.8	0.4	0.375	21.84±1.15	199.99±4.08

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 23 Previous reports of lipase production strain and its characteristics

Organism	Lipase Activity (ug p-NP /ml)	Properties	Reference
Pseudomonas monteilil TKU009	0.378	44 kDa The optimum pH, optimum temperature, and pH and thermal stabilities of F2 were 7, 40 °C, 8–11, and 50 °C; and of F1 were 6, 40 °C, 6–7, and 50 °C, respectively.	Wang *et al.* (2009)
Bacillus cereus C71	27.82	42 kDa The optimum pH was 9.0. The enzyme exhibited maximum activity at 33 °C and retained 92% of original activity after incubation at 35 °C for 3 h.	Shaoxin, Lili and Bingzhao (2007)
Bacillus megaterium AKG-1	45.21	35 kDa The enzyme showed optimum activity at 55 °C/pH 7.0. It retained 100% activity at 50 °C for 30 min with a half-life of 30 min at 70 °C	Sekhon *et al.* (2006)
Bacillus thermocatenulatus	69.56	34 kDa The enzyme showed optimum activity at 70-75 ºC and pH 7.5 and exhibited 50% of its original activity after 1 h incubation at 60 ºC and 30 min at 70 ºC	Lee *et al.* (1999)
Bacillus sp.	83.47	- The enzyme showed maximum activity at pH 10–11.5 and was remarkably stable at alkaline pH values up to 12. The	Nthangeni *et al.* (2001)
Bacillus coagulans BTS-3	93.20	31kDa The enzyme showed maximum activity at 55 °C and pH 8.5	Kumar *et al.* (2005)
Bacillus sp.	301.19	-	Sharma *et al.* (2014)

4.3 Partial purification by using ATPS

4.3.1 Polymer selection

The polymer/salt ATPS systems are often used PEG as a polymer because of high water solubility, rapid phase separation, inexpensive, non-toxic and biodegradable (Rosa et al., 2010). Furthermore, its stimulating ability to protein folding retained good enzyme activity (Cleland *et al.*, 1992). Besides, PPG and PVP is alternative polymer due to their good water solubility, thereby they could be used as ATPS constituents. Mokhtarani *et al.* (2011) used PVP/salt system for separation of α-lactalbumin and β-lactoglobulin from cheese whey protein. They reported that the PVP was efficient to form ATPS and to extraction of protein. Additioninally, there was only one report of Salabat et al. (2010) who studied protein partition using PPG/salt systems. The used PPG/salt system showed higher efficiency over PEG/salt system, due to its higher hydrophobicity.

In order to investigate the effects of phase-forming polymers on the partition of protease and lipase in polymer/salt ATPS, different phase-forming polymers including PEG4000, PPG400 and PVP10000 were studied. Among several salt types, ammonium sulfate was chosen as the salting-out agent because of its good solubility range and excellent protein-protein hydrophobic interactions promoting ability (Su and Chiang, 2006; Yücekan and Önal, 2011). These comparison show only the applicability of these polymers for protein separation without any consideration to the polymer molecular mass or its viscosity and hard handling during operation, because of its limitation of two-phase formation.

The results are shown in Figure 27. Table 24 represents all calculated parameters obtained from all tested systems. The partition coefficient of protease (Ke), which calculated by dividing the activity of protease at polymer-rich phase with its activity at salt-rich bottom phase, was lowest in PPG/salt system and high %yield of protease was almost found in bottom phase. It seems that protease preferred to move to the salt-rich bottom phase of PPG/salt system leading to high purification fold (PF) in bottom phase as appeared in Table 24. Conversely, the obtained K_e values of the PEG/salt system were similar to the Ke values of PVP/salt system. So, the relative ordering of the Ke value is PEG ≈ PVP > PPG. These Ke values are in

contrast with partition coefficient of protein (Kp) values which is PEG ≈ PVP << PPG making the maximum purification fold (PF) of protease found in PEG system (Table 24). The higher Ke and %yield of protease in the PVP system over the PEG system indicates that protease has good solubility in the hydrophobic polymer phase. Moreover, Ke values and %yield of protease of the PEG system are lower than the PVP system. This result may be explained by the lower hydrophobicity of PEG than PVP polymer. In fact, the PPG and PVP polymers are more hydrophobic structure than PEG, which is responsible for higher hydrophobicity of phases in the PPG and PVP systems than PEG system (Rogers and Zhang, 1996). Therefore, protease has higher tendency to interact to polymer through hydrophobic regions.

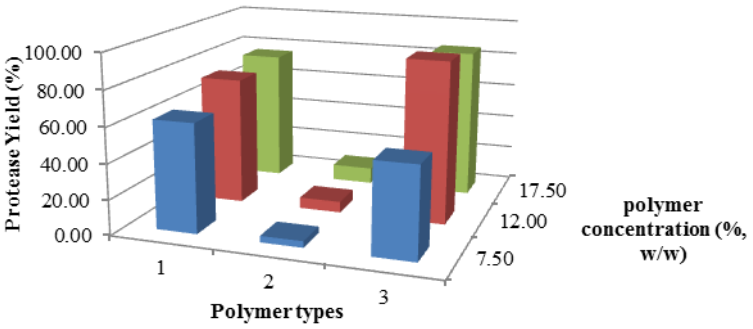
Because lipase was partitioned only into interface of PEG system, neither detected into polymer-rich top phase nor salt-rich bottom phase, thus partition coefficient (Ke) of lipase could not be calculated. As seen in Table 24, the maximum recovery of lipase yield about 20-30% in PEG system suggested that PEG system suited for ATPS system than the others. These results may cause by loss of activity due to its polymer properties or its ATPS. Usually, partially purification was aimed to partition target biomolecules into bulk phase (top or bottom). A few studies reported the partitioning of the biomolecules into interface, namely interfacial partitioning, for example plasmid DNA (pDNA) (Luechau, Ling and Lyddiatt, 2009; Luechau *et al.*, 2009), cells (Raymond and Fisher, 1995) and proteins (Dennison and Lovrien, 1997; Chiu *et al.*, 2015). Luechau, Ling and Lyddiatt (2010) reported that these aspects would cause the biomolecule partitioned to the interface, such as type of ATPS, feedstock composition and properties of biomolecules at interface. However, there are several advantages of interfacial partitioning. The biomolecules are concentrated at interface suitable for low concentration of biomolecules in feedstock (Luechau *et al.*, 2009; Chiu *et al.*, 2015), reducing process volume and operation time (Rito-Palomares and Lyddiatt, 1996; Hammar, 2000) and acceptable as alternative up-scale partial purification (Luechau *et al.*, 2010). The partitioning of protease and lipase into different phases were potential to the use of enzyme individually as different purposes as well as in the mixture form for co-application like detergent formulation or in bating processing in leather industry. Therefore, PEG was selected as polymer forming in ATPS.

Table 24 Effect of polymer type on the partitioning of protease and lipase in polymer/salts ATPS (n=3)

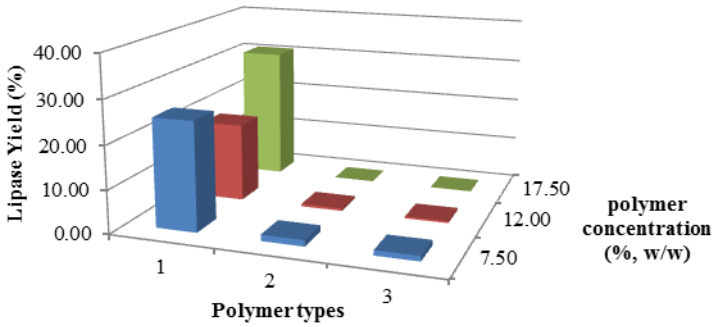
Polymer type	Polymer concentration (%, w/w)	Protease					Lipase		Kp
		polymer-rich phase		salt-rich phase		Ke	Interface		
		yield (%)	PF	yield (%)	PF		yield (%)	PF	
PEG	7.50	61.90 ± 1.33	3.59 ± 0.41	43.37 ± 5.26	1.19 ± 0.13	5.04 ± 0.72	25.16 ± 0.14	0.77 ± 0.06	1.67 ± 0.13
	12.00	72.79 ± 7.60	2.48 ± 0.34	33.49 ± 4.32	1.07 ± 0.09	4.35 ± 0.11	18.17 ± 2.47	0.65 ± 0.22	1.90 ± 0.37
	17.50	**75.74 ± 7.36**	**4.28 ± 0.65**	**17.94 ± 1.23**	**0.68 ± 0.04**	**5.27 ± 0.27**	**30.85 ± 2.15**	**1.39 ± 0.30**	**0.86 ± 0.12**
PPG	7.50	3.65 ± 0.88	0.14 ± 0.03	114.36 ± 0.07	4.53 ± 0.31	0.27 ± 0.07	1.47 ± 0.70	0.04 ± 0.02	9.00 ± 0.20
	12.00	6.27 ± 0.16	0.17 ± 0.04	133.14 ± 2.45	2.71 ± 0.28	0.25 ± 0.01	0.57 ± 0.12	0.01 ± 0.00	4.26 ± 1.68
	17.50	9.34 ± 2.90	0.13 ± 0.05	106.22 ± 9.02	2.62 ± 0.14	0.24 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	4.98 ± 0.88
PVP	7.50	51.40 ± 0.81	5.48 ± 2.59	45.89 ± 0.38	0.40 ± 0.02	8.96 ± 0.22	1.21 ± 0.31	0.16 ± 0.04	0.73 ± 0.36
	12.00	91.88 ± 4.99	7.92 ± 1.67	26.03 ± 12.41	0.25 ± 0.12	14.12 ± 7.40	0.44 ± 0.62	0.03 ± 0.04	0.40 ± 0.10
	17.50	85.47 ± 10.01	3.91 ± 0.52	30.43 ± 2.88	0.59 ± 0.14	5.61 ± 0.13	0.06 ± 0.08	0.01 ± 0.01	0.84 ± 0.11

PF: purification fold; K_e: partition coefficient of enzyme in the top phase; K_p: partition coefficient of protein in the top phase.

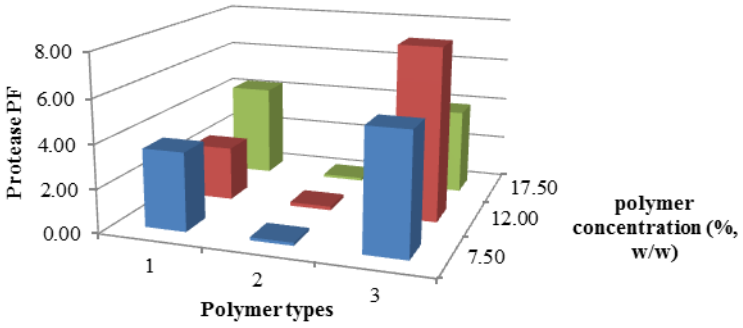
a)



b)



c)



d)

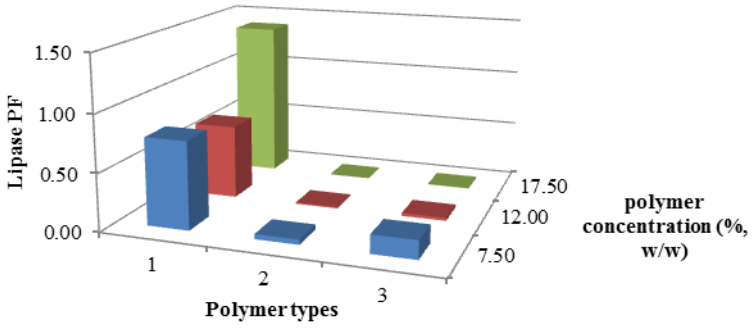


Figure 27 Effect of polymer type on the partitioning of protease and lipase in polymer/salts ATPS. a) yield of protease, b) yield of lipase, c) PF of protease and d) PF of lipase. The polymers were (1) PEG4000, (2) PPG400 and (3) PVP10000. (n=3)

4.3.2 Salt selection

To investigate the suitable phase forming salt in ATPS, the partitioning of enzymes was carried out using different inorganic salts including ammonium sulfate, potassium phosphate and sodium citrate. These salts commonly used in ATPS for promoting protein partitioning. Sulfate salt contributes to induce hydrophobic interaction between proteins. The PEG/phosphate system has ability to extract macromolecules from fermentation broth. Citrate is biodegradable and environmental friendly salt (Yang *et al.*, 2010).

For all the systems investigated, the enzymes partitioning was strongly depended on the types and concentrations of salt. The effectiveness of the salt is mainly determined by comparing its ability to reduce protein solubility, which is known as salting-out effect in salt-rich phase. In addition, the high salting-out ability of salts appears in the high Kp values leading to most of the proteins were partitioning more to the top phase. As shown in the Table 25, the order of salting-out effect followed: SO_4^{2-}>PO_4^{2-}>$Citrate^{3-}$, respectively. In general, the effectiveness of the salt is mainly determined by the nature of the anion. Our results were consistent with the order of salting-out effect based on Hofmeister series (Bauer and Stubbs, 1999). Furthermore, the proteins were also less solubility in bottom phase when increasing the concentration of salt and promoting protein moving to PEG-rich top phase. The salt ions decrease protein solubility by increasing either hydrophobic interaction between PEG and proteins or the hydration effect of salt molecules to proteins, so most proteins partition to the another phase which lower salt concentration than its (Yavari *et al.*, 2013). The higher amount of purification fold (PF) of protease was achieved in the top phases as comparing to the bottom phases. It represents the amount of enzyme purified as a measure of the total protein in the phase. Protease was found to preferentially partition to PEG-rich top phase. It indicates that protease is a negatively charge hydrophobic protein which are known to partition into polymer-rich top phase. However, all of salt systems showed the %yield of protease about 60-80% as presented in Table 25. The citrate system gave lowest yield, but its PF showed no difference comparing to the others. It seems that in case of sulfate and phosphate systems, both target and non-target proteins were mostly partitioned to the PEG-rich top phase. Thus, the higher Kp values were observed in both sulfate and phosphate

systems than that obtained from the citrate system, resulting in slightly similar specific activities as well as PF values.

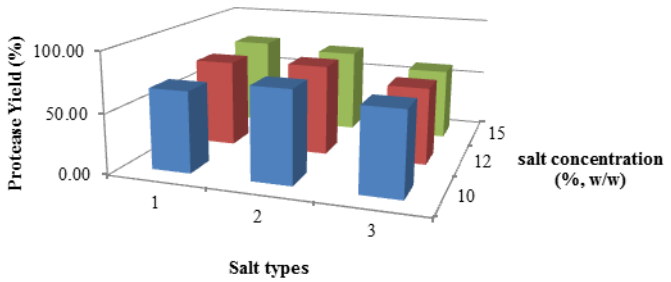
In contrast, lipase yields decreased in order of PO_4^{2-} > $Citrate^{3-}$ > SO_4^{2-} (Table 25). Ammonium sulfate showed the least %yield of lipase. It indicates that lipase activity is more loss in this system as comparing to the others. Protein denaturation by salting out or protein aggregation appears to be the most probable causes for these results (Amid *et al.*, 2012; Srivastava and Kapoor, 2013). Furthermore, the largest of interface layer and protein concentration in phosphate system resulted in low PF of lipase, though the yield of lipase was highest. However, citrate system showed lowest PF of protease, but %yield of lipase and PF were higher than the others. The high PF of citrate system is in accordance with several reports (Tubío, Nerli and Picó, 2007; Sarangi *et al.*, 2011). Thus, citrate salt was chosen as phase forming salt in ATPS.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

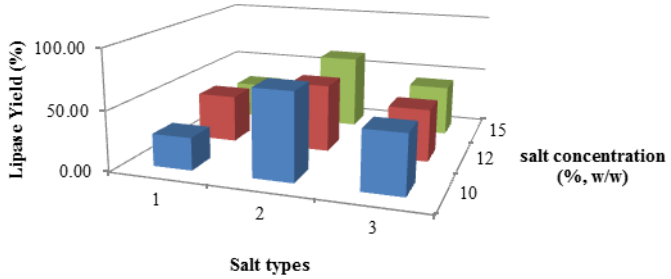
Table 25 Effect of salt on the partitioning of protease and lipase in PEG/salt ATPS (n=3)

Salt type	Salt concentration (%, w/w)	Protease					Lipase			
		polymer-rich phase		salt-rich phase		Ke	Interface		Kp
		yield (%)	PF	yield (%)	PF		yield (%)	PF	
$(NH4)_2SO_4$	10.0	67.40 ± 2.71	3.53 ± 0.49	19.54 ± 0.81	0.91 ± 0.11	0.76 ± 0.07	27.10 ± 2.61	0.83 ± 0.15	0.76 ± 0.07
	12.0	73.16 ± 8.87	3.57 ± 0.37	16.22 ± 8.40	0.65 ± 0.25	0.93 ± 0.10	39.96 ± 4.25	1.43 ± 0.45	0.93 ± 0.10
	15.0	75.74 ± 7.36	4.28 ± 0.65	17.94 ± 1.23	0.68 ± 0.04	0.86 ± 0.12	30.85 ± 2.15	1.39 ± 0.30	0.86 ± 0.12
K_2HPO_4	10.0	76.25 ± 12.94	4.01 ± 0.47	27.71 ± 1.13	1.26 ± 0.02	0.75 ± 0.08	72.44 ± 4.62	1.11 ± 0.08	0.75 ± 0.08
	12.0	75.87 ± 4.85	3.66 ± 0.50	23.24 ± 1.74	0.84 ± 0.07	0.77 ± 0.15	56.80 ± 5.10	0.91 ± 0.22	0.77 ± 0.15
	15.0	71.28 ± 6.52	3.63 ± 0.27	23.00 ± 4.65	0.76 ± 0.24	0.87 ± 0.15	63.10 ± 3.50	1.19 ± 0.02	0.87 ± 0.15
$Na_3C_6H_5O_7$	**10.0**	**68.78 ± 1.47**	**3.00 ± 0.24**	**36.49 ± 3.59**	**1.15 ± 0.18**	**0.37 ± 0.01**	**48.91 ± 13.67**	**3.15 ± 1.37**	**0.37 ± 0.01**
	12.0	64.19 ± 9.23	3.69 ± 0.19	42.71 ± 3.53	0.91 ± 0.20	0.33 ± 0.05	43.88 ± 4.90	2.10 ± 0.29	0.33 ± 0.05
	15.0	60.41 ± 1.41	3.48 ± 0.50	37.67 ± 4.99	0.56 ± 0.01	0.27 ± 0.07	42.77 ± 3.28	2.19 ± 0.38	0.27 ± 0.07

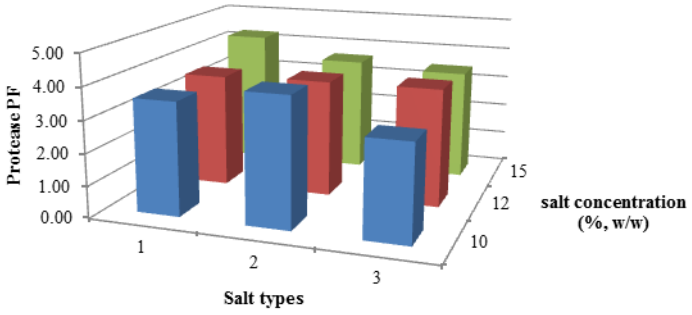
PF: purification fold; K_e: partition coefficient of enzyme in the top phase; K_p: partition coefficient of protein in the top phase.



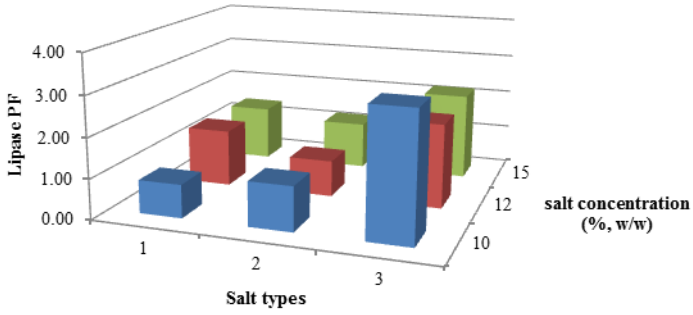
a)



b)



c)



d)

Figure 28 Effect of salt on the partitioning of protease and lipase in PEG/salts ATPS. a) yield of protease, b) yield of lipase, c) PF of protease and d) PF of lipase. The salt types were (1) $(NH_4)_2SO_4$, (2) K_2HPO_4 and (3) $Na_3C_6H_5O_7$. (n=3)

4.3.3 Polymer molecular weight selection

The behavior of total protein and enzymes depend on the PEG molecular mass in ATPS. The molecular mass of polymer influences the protein partition by changing the number of polymer-protein interactions. This is usually attributed to hydrophobic interactions between the chains of PEG and the hydrophobic area of protein. Therefore, the selection of the best mw of polymer is generally very important ATPS experiment (Anvari, 2015). In addition, the partition of a protein in PEG/salt ATPS depends on the hydrophobicity of the protein. Proteins with more apolar amino acid residues will show higher affinity for the PEG phase which is more hydrophobic than the lower salt phase (Yücekan and Önal, 2011).

The results are shown in Table 27 and Figure 29. The high %yield of protease (50-60%) observed nearly all systems, except for PEG8000 systems (20-30%). In PEG4000 system, protease showed the highest %yield and Ke values were more than 1 (Ke>1). This means that protease preferred to move to the top phase. Conversely, in others PEG systems, most proteases were partitioned into the lower phase (Ke<1). Similarly, contaminant proteins were also moved to PEG4000-top phase more than the others system. The ordering of Ke and Kp was similar to PEG4000 > PEG6000 > PEG8000. Among all ATPS tested, Figure 29 shows that the protease distribution to the top phase (%yield) declined, while its purity (PF) also reduced as the PEG molecular weight increase. As seen in Table 27, the Ke values drop with increasing the molecular weight of PEG which results in protease shifting to the bottom phase. With the increasing of PEG mw of 4000 to 6000 and 8000, the Ke values were decreased as seen in Table 27 at TLL 34.99% (w/w) from 0.95 to 0.53 and 0.25, respectively. Moreover, it was observed that volume ratio decreased with an increasung in PEG molecular weight (Table 27). The increasing in the chain length (with increasing molecular weight) could be also caused the reduction of the free volume, meaning less space available for the proteins, namely excluded volume effect. Subsequently, low Ke, Kp and %yield of protease presented in PEG8000 systems.

Furthermore, this experiment has also studied the effect of tie-line length (TLL) from 34.99 to 40.99 and 43.75% (w/w). The results cited in Table 26 shows that high TLL showed more Ke as well as selectivity. High selectivity indicated that

protease more preferred to top phase rather than bottom phase, meanwhile others contaminant protein also less partitioned to this top phase. Its high concentration of salt at high TLL resulting in hydrophobic area of protein are more exposed, corresponding to promoting hydrophobic interaction between protease and PEG (Bonomo *et al.*, 2006). Consequently, the high selectivity observed as increasing TLL.

The yield of lipase was decreased as PEG molecular weight increase. It may causes by enzyme loss activity. Moreover, this trend also found in protein yield at interface, resulting in higher PF of lipase was observed. However, maximum PF of lipase was obtained from PEG6000 system and no different in yield of lipase comparing to PEG4000 and PEG8000. The high viscosity complicated handling of PEG8000 system in protein purification. A PEG with a low molecular weight leads to a higher consumption of polymer and additional costs (Yücekan and Önal, 2011). Therefore, the intermediate molecular weight of PEG is the best choice for ATPS experiment. The molecular weight of PEG should be kept at 6000 for the subsequent studies.

Table 26 Effect of molecular weight of PEG and TLL on the partitioning of protease in PEG/sodium citrate ATPS (n=3)

Polymer molecular weight	**TLL (%, w/w)**	**Vr**	**Ke**	**Selectivity**
4000	34.99	2.00 ± 0.00	0.95 ± 0.07	2.58 ± 0.49
	40.99	1.25 ± 0.00	1.20 ± 0.09	3.64 ± 0.19
	43.75	1.00 ± 0.00	1.63 ± 0.26	6.08 ± 0.38
6000	**34.99**	**2.00 ± 0.00**	**0.53 ± 0.12**	**5.27 ± 0.59**
	40.99	1.25 ± 0.00	0.65 ± 0.20	5.37 ± 0.72
	43.75	0.87 ± 0.12	0.82 ± 0.29	5.29 ± 0.54
8000	34.99	1.25 ± 0.00	0.25 ± 0.07	1.90 ± 0.21
	40.99	1.00 ± 0.00	0.32 ± 0.04	2.34 ± 0.63
	43.75	0.80 ± 0.00	0.65 ± 0.03	4.39 ± 0.09

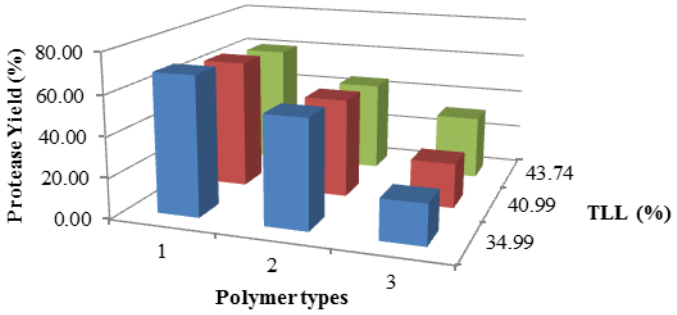
Vr: volume ratio (top/bottom); Ke: partition coefficient of enzyme in the top phase;
Kp: partition coefficient of protein in the top phase; Selectivity: enzyme selectivity (Ke/Kp).

Table 27 Effect of molecular weight of PEG and TLL on the partitioning of protease and lipase in PEG/sodium citrate ATPS (n=3)

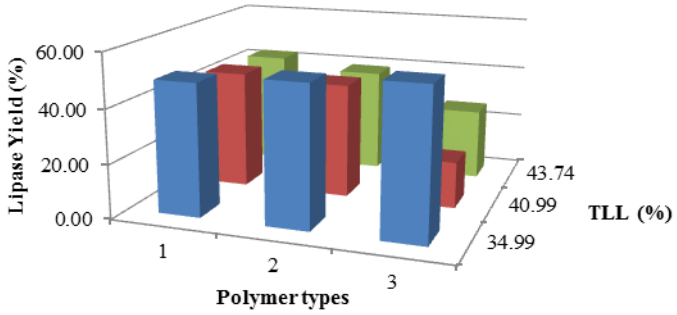
Polymer molecular weight	TLL (%, w/w)	Protease					Lipase		Kp
		polymer-rich phase		salt-rich phase		Ke	Interface		
		yield (%)	PF	yield (%)	PF		yield (%)	PF	
4000	34.99	68.78 ± 1.47	3.00 ± 0.24	36.49 ± 3.59	1.15 ± 0.18	0.95 ± 0.07	48.91 ± 13.67	3.15 ± 1.37	0.37 ± 0.01
	40.99	64.19 ± 9.23	3.69 ± 0.19	42.71 ± 3.53	0.91 ± 0.20	1.20 ± 0.09	43.88 ± 4.90	2.10 ± 0.29	0.33 ± 0.05
	43.75	60.41 ± 1.41	3.48 ± 0.50	37.67 ± 4.99	0.56 ± 0.01	1.63 ± 0.26	42.77 ± 3.28	2.19 ± 0.38	0.27 ± 0.07
6000	**34.99**	**53.53 ± 0.73**	**6.96 ± 1.08**	**51.96 ± 10.82**	**1.34 ± 0.35**	**0.53 ± 0.12**	**52.01 ± 2.70**	**6.07 ± 2.63**	**0.10 ± 0.02**
	40.99	49.24 ± 4.03	5.70 ± 1.38	62.97 ± 13.77	1.03 ± 0.35	0.65 ± 0.20	42.48 ± 1.72	6.03 ± 1.26	0.12 ± 0.03
	43.75	45.14 ± 4.26	5.89 ± 2.99	69.58 ± 24.52	1.15 ± 0.51	0.82 ± 0.29	39.04 ± 0.86	5.47 ± 1.25	0.15 ± 0.05
8000	34.99	19.94 ± 4.82	2.65 ± 1.03	64.11 ± 2.18	1.35 ± 0.17	0.25 ± 0.07	54.62 ± 0.73	5.75 ± 0.41	0.13 ± 0.03
	40.99	22.27 ± 3.14	2.71 ± 1.04	68.77 ± 0.59	1.13 ± 0.24	0.32 ± 0.04	17.13 ± 8.85	2.32 ± 1.04	0.14 ± 0.01
	43.75	31.62 ± 0.48	3.34 ± 0.57	60.53 ± 3.72	0.74 ± 0.02	0.65 ± 0.03	26.43 ± 11.82	4.44 ± 1.78	0.15 ± 0.04

PF: purification fold; K_e: partition coefficient of enzyme in the top phase; K_p: partition coefficient of protein in the top phase.

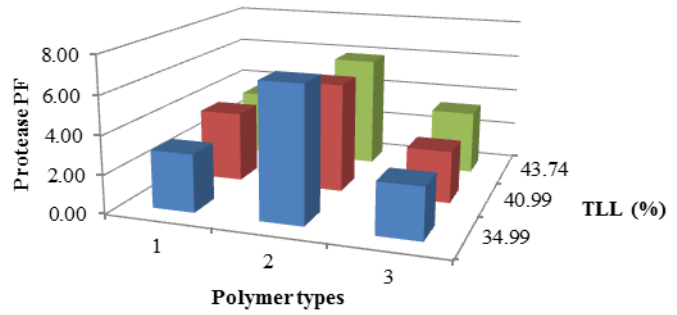
a)



b)



c)



d)

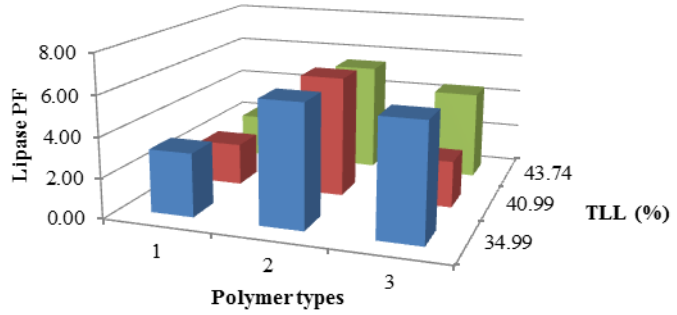


Figure 29 Effect of molecular weight of PEG and TLL on the partitioning of protease and lipase in PEG/sodium citrate ATPS. a) yield of protease, b) yield of lipase, c) PF of protease and d) PF of lipase. The molecular weights of PEG were (1) 4000, (2) 6000 and (3) 8000. (n=3)

4.3.4 Volume ratio selection

In principal, the protein partition behavior is not affected by changing the volume ratio (Vr), i.e. by moving along TLL because of the constant of relative partitioning of individual protein (Ooi *et al.*, 2009)In fact, as shown in Figure 30, our results recovered the decreasing of PF and yield of protease and lipase along TLL 34.99% (w/w) of PEG6000/citrate system at difference phase volume ratios (Zafarani-Moattar *et al.*, 2008). The Ke of protease sharply increased in accordance with the increasing Vr up 0.8 beyond which the values were found to decrease, while the recovery yield decreased as the Vr was increased. The similar trend of partitioning behavior was observed in other findings (Ooi *et al.*, 2009). It indicated that reducing of Vr affects to the purification by the presence of protein precipitation at the interface as the top phase volume decreases. This can be explained by the enzyme solubility in the PEG-rich top phase saturated as Vr decrease (Bauer and Stubbs, 1999). As a result, it was found that system with Vr of 2 showed the best partition efficiency as compared to the other Vr systems. Under these experimental conditions, the yield and purification fold of protease of Vr= 2 were 53.53 and 6.96, respectively (Figure 30).

Concomitant decline in yield and PF of lipase was registered with decreasing in Vr. It may cause by loss activity from higher concentration of citrate as Vr increase and also high protein at interface. The results indicate that the ATPS with Vr at 2 showed a higher purification of protease and lipase, and thus it is an optimum for partitioning.

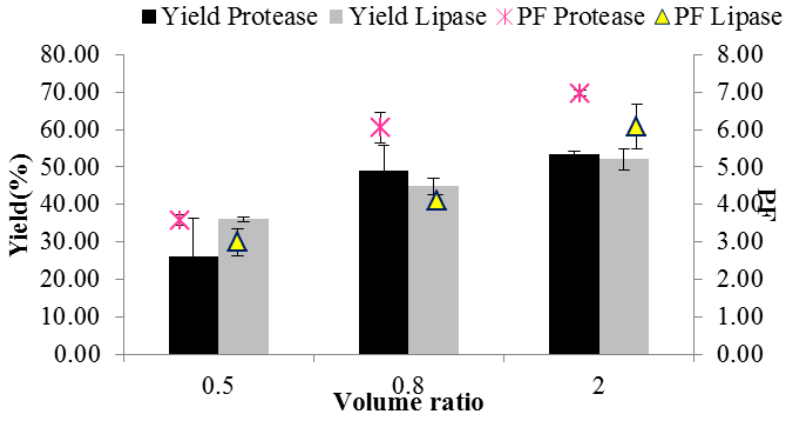


Figure 30 Effect of volume ratio on the partitioning of protease and lipase in PEG/sodium citrate ATPS (n=3)

4.3.5 pH selection

In order to study the effect of pH on partitioning, the system with different pH 6, 7, 8, 9 and 10 were carried out. The results showed that the maximal %yield of both protease and lipase were found at pH 10 and 7 correspondingly. The yield of protease increased consistent with increasing of pH, whereas lipase yield drastically decreased (Figure 31). Moreover, high Ke values were found as increasing pH of the system. These behaviors can be explained by isoelectric point (pI) of protein. Generally, at pH over pI of proteins subsequently to total negatively charge of protein, and conversely (Huddleston *et al.*, 1991). The proteins with positively charge favor distribution to bottom phase that is in contrast with negatively charge of protein (Bezerra *et al.*, 2006). In addition, pI of alkaline proteases were 4-6 (Rao *et al.*, 1998). Thus, higher yield of protease was observed beyond pH7. It indicates that the interaction between protease and PEG increasing. On the other hand, pI of protease are commonly 6 (Castro-Ochoa *et al.*, 2005). The yield reduced as increased pH may cause by low stability of lipase at higher pH. As all results above, pH of the system affects to the partition of proteins depending on their properties (pI). Therefore, the pH of the further systems was adjusted at pH7.

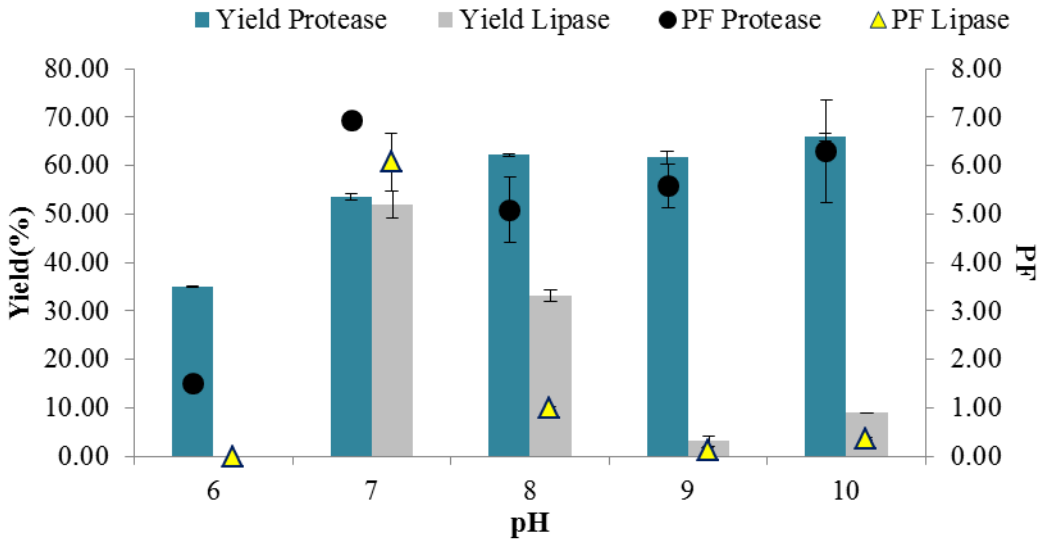


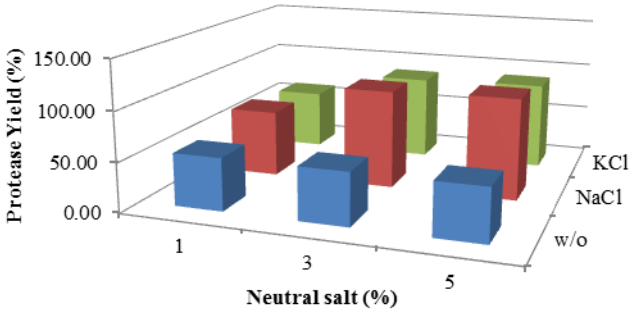
Figure 31 Effect of pH on the partitioning of protease and lipase in PEG/sodium citrate ATPS (n=3)

4.3.6 Neutral salt selection

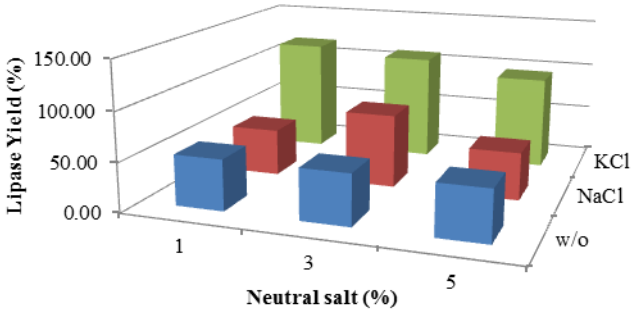
Neutral salts influenced on partitioning of enzymes in ATPS. The results presented in Figure 32 found that both NaCl and KCl as neutral salts promoted more protease to top phase reached to 100%. In the same way, the other contaminant proteins were also moving to top phase that tends to decline of PF. However, addition of NaCl at 3 or 5% was no different in PF comparing to the system without neutral salts that was in accordance with Yavari *et al.* (2013). NaCl is responsible for inducing phase hydrophobicity and promoting partition of hydrophobic protein to top phase (Asenjo *et al.*, 1994). This neutral salts decrease water activity of bottom phase as well as increasing of salt concentration (Lahore *et al.*, 1995). Furthermore, lipase was 117.38%yield when 1% of KCl was added. There were no difference in %yield compared between the system with (48.14%) or without 1%NaCl (52.01%) (Figure 32). Overall, the final ATPS condition was 17.5% PEG6000, 10% sodium citrate and 3% KCl with 86.79% yield and 5.65 PF of protease as well as 109.85% yield and 11.35 PF of lipase.

Neutral salts which are not the major constituent in ATPS promote partition of protein by increasing the distribution of salt ions along two phases. It changes the hydrophobic, hydrophilic and electrostatic interaction between protein and phase composition, depending on types and concentrations of used neutral salts (Hachem, Andrews and Asenjo, 1996). Moreover, neutral salt accelerates phase separation by affecting to phase potential and/or reducing apolar of proteins. The finding of higher selective partitioning after addition neutral salts is in agreement with many literatures (He *et al.*, 2005; Su and Chiang, 2006; Yue, Yuan and Wang, 2007; Karkaş and Önal, 2012).

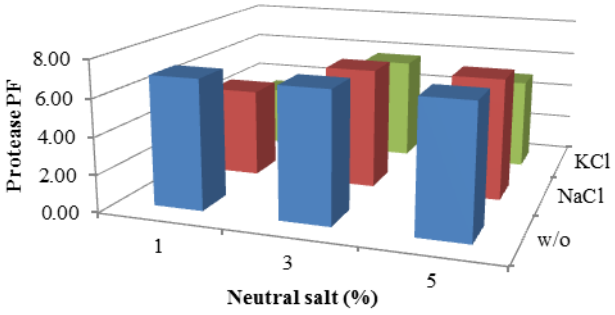
a)



b)



c)



d)

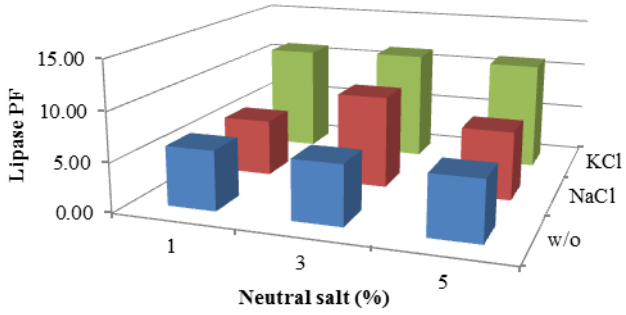


Figure 32 Effect of neutral salts on the partitioning of protease and lipase in PEG/sodium citrate ATPS. a) yield of protease, b) yield of lipase, c) PF of protease and d) PF of lipase (n=3)

4.3.7 CCFD and RSM

To achieve partially purification of interesting enzymes, RSM was applied to determine the optimal levels of factors. The optimal conditions for partially purify protease and lipase were obtained by identifying the experimental space around the conditions, which were selected from preliminary tests. The evaluation of the optimized condition was carried out by using previous three factors (PEG6000, sodium citrate and KCl concentration) effecting on partitioning of protease and lipase in ATPS, in which preselected experiments (17.5% PEG6000, 10% sodium citrate and 3% KCl), through a 2^3 CCFD with 3 central points (Table 28).

The regression analysis was performed by dividing into two parts, sequential sum of squares and model summary statistic. As shown in Table 37-Table 40, the linear and quadratic model showed p-value less than 0.05 which were significant model. However, its coefficient of determination (R^2) value was considered. It can be concluded that higher R^2 of quadratic model making the quadratic model suits for %yield of protease (Table 37), %yield of lipase (Table 39) and PF of lipase (Table 40). In contrast, two-factor interaction (2FI) was significant model for prediction the PF of protease (Table 38). The R^2 values for %yield of protease, PF of protease, % yield of lipase and PF of lipase were evaluated as 0.91, 0.84, 0.92 and 0.88, respectively. The R^2 value is always between 0 and 1, and all values closed to 1.0 indicates better accuracy of the models. The results cited in Table 5-8 showed that all models gave insignificant of the lack of fit implying that the second order models are satisfactory for estimating the responses.

The analysis of variance (ANOVA) for three factors (PEG6000, sodium citrate and KCl) was employed to identify the significant factors and to estimate the recovery of protease and lipase with ATPS. ANOVA results of all responses are shown in Table 41&Table 45-Table 44&Table 48. Model terms having p-value less than 0.05 are significant. Totally, the all achieved data confirmed that the three factors were very critical for partitioning and recovery of protease and lipase enzymes. Experimental data was analyzed by multiple regression analysis method and second-order polynomial equations are represented as follow:

$$\%\text{yield of protease} = 91.49 + 7.46A + 7.11B + 9.32C - 0.54A^2 + 6.31B^2 - 10.46C^2 - 0.49AB + 1.34AC - 3.16BC \quad (3)$$

$$\text{PF of protease} = 7.75 + 1.52A - 1.74B + 0.97C - 0.88AB + 0.17AC - 0.99BC \quad (4)$$

$$\%\text{yield of lipase} = 74.13 - 15.29A - 14.88B + 8.37C - 27.82A^2 - 6.33B^2 + 4.17C^2 + 4.01AB + 0.99AC - 7.65BC \quad (5)$$

$$\text{PF of lipase} = 5.93 - 1.24A - 1.90B + 1.08C - 4.00A^2 + 0.36B^2 + 1.26C^2 + 0.6AB - 0.23AC - 0.79BC \quad (6)$$

where A, B and C are coded values for PEG6000 concentration, sodium citrate concentration and KCl concentration, respectively.

According to the previous results, the optimized condition was predicted by the software for partially purification of protease and lipase with ATPS. The experiment was set by 18.58% (w/w) PEG-6000, 8.0% (w/w) sodium citrate and 4.0% (w/w) KCl for validation of empirical models predicted. Under the mentioned conditions, the PF of protease and lipase of 10.58 and 14.58, and the yield of protease and lipase of 93.18% and 92.59% were obtained experimentally. Consequently, the experimentally values show close similar to the predicted values as shown in Table 29, hence the validation of model is successful.

The high solid mass content and different enzymes produced at the same time cause difficulties in selectively purifying enzymes using conventional methods (He *et al.*, 2005). There are many references, which support the use of ATPS as a potentially attractive and efficient technique for resolving the difficulties in separation. He *et al.* (2005) separated β-glucanase from α-amylase and neutral protease presence in whole fermentation broth using PEG/$MgSO_4$ system**.** Alcântara *et al.* (2011) purified the β lactoglobulin, α-lactalbumin and glycomacropeptide from whey protein with high recovery yield.

This is the first report studying the concomitant partitioning behavior of protease and lipase in ATPS. As all RSM results above, the activity (%yield) and purity (PF) of the enzymes were increased in comparison with preliminary condition. Moreover, the obtained activity and purity of the recovery of protease in PEG/salt ATPS is similar to many reports (Klomklao *et al.*, 2005; Nalinanon *et al.*, 2009;

Sarangi *et al.*, 2011; Yavari *et al.*, 2013). Also, the recovered of lipase in this study is more effective than that in literatures (Bradoo *et al.*, 1999; Bassani *et al.*, 2010; Zhang and Liu, 2010; Barbosa *et al.*, 2011; Zhou *et al.*, 2013). Furthermore, citrate salts, at concentrations between 2.5-15% (w/w) were found to be easily biodegradable, not bio-accumulative and non-toxic to aquatic organisms (Lario *et al.*, 2016). Thus, ATPS studied in this work are interesting because of their low environmental impact and high biodegradability. Therefore, it can conclude that purification using ATPS is applicable methods for enzymes of interest.

Table 28 Factors and responses of CCFD carried out for partially purify of protease and lipase (n=3)

Run	PEG (%)	Citrate (%)	KCl (%)	Protease		Lipase	
				%yield	PF	%yield	PF
1	20	12	4	108.92 ± 3.80	6.76 ± 0.21	17.36 ± 1.69	1.67 ± 0.20
2	17.5	10	4	92.26 ± 3.46	8.89 ± 0.36	87.49 ± 0.00	9.05 ± 0.69
3	20	8	4	102.09 ± 1.51	14.99 ± 0.04	52.62 ± 1.46	4.06 ± 0.96
4	15	12	4	89.02 ± 0.31	5.23 ± 0.04	43.28 ± 0.15	2.34 ± 0.90
5	17.5	8	3	88.66 ± 0.57	8.45 ± 0.05	87.61 ± 1.88	9.08 ± 0.20
6	17.5	12	3	107.88 ± 1.90	7.72 ± 0.03	53.55 ± 1.32	3.27 ± 0.58
7	17.5	10	3	96.48 ± 2.08	7.77 ± 0.17	63.21 ± 3.80	6.54 ± 0.77
8	15	12	2	81.27 ± 0.76	5.00 ± 0.15	43.53 ± 4.01	3.00 ± 0.48
9	15	10	3	88.19 ± 0.35	6.18 ± 0.12	52.29 ± 3.71	2.65 ± 0.91
10	17.5	10	2	70.74 ± 0.93	8.05 ± 0.04	74.66 ± 1.12	5.12 ± 0.21
11	20	12	2	93.48 ± 2.81	6.49 ± 0.24	12.25 ± 1.00	0.70 ± 0.36
12	15	8	2	59.83 ± 0.99	5.76 ± 0.52	64.24 ± 2.34	4.64 ± 0.66
13	17.5	10	3	82.11 ± 2.96	7.45 ± 0.61	77.36 ± 2.71	5.95 ± 0.91
14	17.5	10	3	87.89 ± 0.39	8.47 ± 0.49	61.06 ± 3.00	3.85 ± 0.56
15	20	8	2	76.34 ± 0.52	10.14 ± 0.25	18.33 ± 0.00	2.51 ± 0.72
16	15	8	4	82.58 ± 5.06	9.30 ± 0.33	95.99 ± 3.59	9.69 ± 0.65
17	17.5	10	3	96.02 ± 1.69	7.29 ± 0.11	70.54 ± 6.85	7.88 ± 0.24
18	17.5	10	3	96.14 ± 4.76	5.29 ± 0.05	75.59 ± 5.57	5.79 ± 0.38
19	20	10	3	94.64 ± 0.26	8.30 ± 0.05	45.89 ± 2.14	0.99 ± 0.04
20	17.5	10	3	88.47 ± 3.46	7.53 ± 0.32	85.90 ± 3.88	5.96 ± 0.14

Table 29 Validation of quadratic model at optimized condition of 18.58%PEG6000, 8%sodium citrate and 4%KCl (w/w) ATPS (n=3)

	Yield of protease (%)	PF of protease (folds)	Yield of lipase (%)	PF of lipase (folds)
predicted value	96.62	12.56	89.85	9.68
actual value	93.18	10.58	92.59	14.58

4.3.8 SDS-PAGE

The purity of protease and lipase obtained from top phase and interface respectively of 18.58%PEG6000, 8%sodium citrate and 4%KCl (%, w/w) ATPS was evaluated by SDS-PAGE analysis. The SDS-PAGE profile of standard protein marker (lane 1), crude enzyme (lane 2), top phase (lane 3), interface (lane 4) and bottom phase (lane5) is shown in Figure 33. It can be observed that molecular weight of protease were 29.2 kDa which was nearly to report by Rachadech and coworker (2010). The interface appeared two bands, 43.1 and 30.7 kDa. The molecular weight of *B. licheniformis* lipase has been reported as 40kDa (Sangeetha *et al.*, 2010). The molecular weight of 3C5 lipase could be proved by further zymogram. However, the measured lipase yield and purity at interface were higher than that in the others phase. This result in bottom phase can be implied that high and low molecular weight of contaminating proteins present in the crude enzyme was removed to this phase.

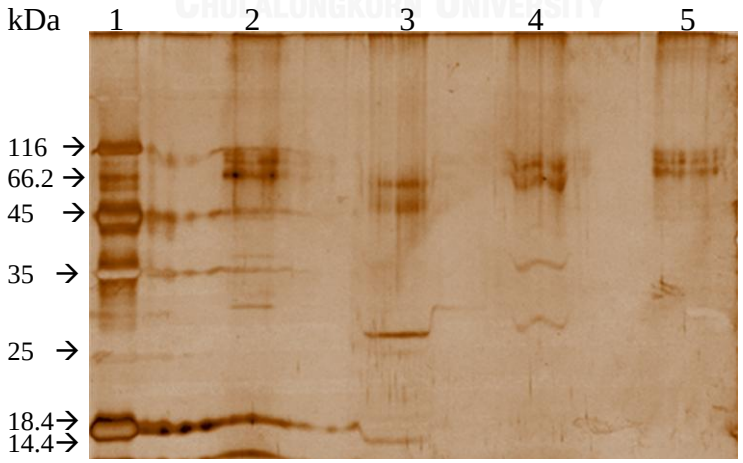


Figure 33 SDS-PAGE analysis of protease and lipase recovery from ATPS. Lane 1: protein molecular marker; Lane 2; control crude culture; Lane 3: top phase; Lane 4: interface; Lane 5: bottom phase

CHAPTER V
CONCLUSIONS

The protease and lipase are together required for application in various industry such as detergent, paper and pulp, leather, bread and food industry including in waste water treatment. Therefore, lipase is necessary to tolerance to the degradation by co-application protease. Previous reports recovered that co-production of protease and lipase was found in various organisms but the susceptible of lipase to its own protease are generally occurred. Furthermore, lipases from different sources were also sensitive to proteolytic activity, leading to improve stability of lipase to protease by another method like genetic engineering. Therefore, the single fermentation production comprising both of protease and lipase, and lipase not being susceptible to hydrolyze by protease should be better applicability.

B. licheniformis 3C5, a gram-positive organic-solvent tolerant bacterium, have been recognized as the predominant protease-producing bacterium, extensive studies on solvent-stable protease have been carried out (Rachadech *et al.*, 2010). Moreover, lipase production could be also produced by 3C5 strain. The results concluded that the 3C5 lipase showed relatively strong resistance towards native protease (3C5 protease) and other alkaline protease (Alcalase®). Therefore, these enzymes have great potentially applicable together in many industries. The proteolytic resistance character together with other attractive properties of lipase, including a strong tolerance in non-ionic surfactant, mild tolerance to n-decane or n-decanol and also toluene, may be helpful to potential applications in industrial processes, such as the production of detergents, leather manufacture and the paper industry.

The concomitant production of protease and lipase by *B. licheniformis* 3C5 by using four factors (olive oil, glucose, yeast extract and NaCl) obtained from one-variable-at-a-time preliminary method for developing the predicted polynomial equations to maximize protease and lipase production simultaneously. The statistical process was performed by using central composite design of RSM. Consequently, the optimized medium composed of olive oil 2.5% (v/v), glucose 0.84% (w/v), yeast extract 0.6% (w/v) and NaCl 0.25% (w/v). Totally, the concomitant production was

enhanced from 89.79±2.57 and 2.48±0.84 in basal medium to 201.51±7.80 and 31.18±2.49 U/ml giving a 2.2 and 12.57-fold increase in protease and lipase production, respectively. The production of both protease and lipase in single fermentation can be cost-effective, convenient and easy to scale-up than the conventional method of using blend of enzyme from different fermentation.

The partially purification of protease and lipase concomitantly produced by *B. licheniformis* 3C5 in aqueous two-phase system by using polymer/salt system was firstly studied to select the effective factors and finally studied by statistical analysis. The preliminary experiment found that PEG6000, sodium citrate and KCl are the most effective composition in ATPS. 17.5% (w/w) PEG6000, 10% (w/w) sodium citrate and 3% (w/w) KCl yielded 5.65 and 11.35 PF of protease and lipase with 86.79% and 109.85% yield of protease and lipase. Additionally, statistically models were used to find the suitable concentration of preselected condition by using central composite fractioned design of RSM for both %yield and PF of enzymes. The optimized composition consisted of 18.58% (w/w) PEG-6000, 8.0% (w/w) sodium citrate and 4.0% (w/w) KCl with PF of 10.58 and 14.58 and 93.18% and 92.59% yield as protease and lipase recovered, respectively. The close resemblance of experimental and predicted responses validated the RSM predicted model. Moreover, the higher PF and %yield obtained from RSM comparing to the preliminary conditions by a little raising PEG6000 and declining the sodium citrate concentration indicating that RSM are the powerful method for maximizing recovery including minimizing the material and cost consumption. The successful of partitioning, directly from culture supernatant containing the target enzymes, used a single step of ATPS. It suggested that ATPS is efficient method for recovery and purification of 3C5 protease and lipase. Yavari et al. (2013) revealed that ATPS are practical method in industrial scale for partially purification of enzymes which is used in detergent and tanning processes, while high purity is demanded for medicine and food applications. Although, ATPS leads to lower PF than ion-exchange chromatography, but its simplicity and high yield in addition to lower investments.

REFERENCES

Adrio, J. L. and Demain, A. L. (2014). "Microbial Enzymes: Tools for Biotechnological Processes." Biomolecules 4(1): 117-139.

Aguilar, C. N., Favela-Torres, E., Vinegra-González, G. and Augur, C. (2002). "Culture conditions dictate protease and tannase production in submerged and solid-state cultures of *Aspergillus niger* Aa-20." Applied Biochemistry and Biotechnology 102(1): 407-414.

Ahmad, S., Kumar, V., Ramanand, K. B. and Rao, N. M. (2012). "Probing protein stability and proteolytic resistance by loop scanning: A comprehensive mutational analysis." Protein Science 21(3): 433-446.

Akoh, C. C., Lee, G.-C., Liaw, Y.-C., Huang, T.-H. and Shaw, J.-F. (2004). "GDSL family of serine esterases/lipases." Progress in Lipid Research 43(6): 534-552.

Alcântara, L. A. P., Minim, L. A., Minim, V. P. R., Bonomo, R. C. F., da Silva, L. H. M. and da Silva, M. d. C. H. (2011). "Application of the response surface methodology for optimization of whey protein partitioning in PEG/phosphate aqueous two-phase system." Journal of Chromatography B 879(21): 1881-1885.

Alkan, H., Baysal, Z., Uyar, F. and Dogru, M. (2007). "Production of lipase by a newly isolated *Bacillus coagulans* under solid-state fermentation using melon wastes." Applied Biochemistry and Biotechnology 136(2): 183-192.

Amid, M., Shuhaimi, M., Islam Sarker, M. Z. and Abdul Manap, M. Y. (2012). "Purification of serine protease from mango (*Mangifera Indica Cv. Chokanan*) peel using an alcohol/salt aqueous two phase system." Food Chemistry 132(3): 1382-1386.

Anbu, P. (2013). "Characterization of solvent stable extracellular protease from *Bacillus koreensis* (BK-P21A)." International Journal of Biological Macromolecules 56: 162-168.

Andrews, B. A., Schmidt, A. S. and Asenjo, J. A. (2005). "Correlation for the partition behavior of proteins in aqueous two-phase systems: Effect of surface hydrophobicity and charge." Biotechnology and Bioengineering 90(3): 380-390.

Angkawidjaja, C. and Kanaya, S. (2006). "Family I.3 lipase: bacterial lipases secreted by the type I secretion system." Cellular and Molecular Life Sciences CMLS 63(23): 2804-2817.

Anvari, M. (2015). "Extraction of lipase from *Rhizopus microsporus* fermentation culture by aqueous two-phase partitioning." Biotechnology & Biotechnological Equipment 29(4): 723-731.

Arpigny, J. L. and Jaeger, K.-E. (1999). "Bacterial lipolytic enzymes: classification and properties." Biochemical Journal 343(1): 177-183.

Arpigny, J. L. and Jaeger, K. E. (1999). "Bacterial lipolytic enzymes: classification and properties." Biochemical Journal 343(Pt 1): 177-183.

Asenjo, J. A., Schmidt, A. S., Hachem, F. and Andrews, B. A. (1994). "8th International Conference on Partitioning in Aqueous Two-Phase Systems: Model for predicting the partition behaviour of proteins in aqueous two-phase systems." Journal of Chromatography A 668(1): 47-54.

Aunstrup, K., Andresen, O., Falch, E. A. and Nielsen, T. K. (1979). Chapter 9 - Production of Microbial Enzymes A2 - PEPPLER, H.J. Microbial Technology (Second Edition). Perlman, D., Academic Press**:** 281-309.

Azevedo, A. M., Rosa, P. A. J., Ferreira, I. F. and Aires-Barros, M. R. (2007). "Optimisation of aqueous two-phase extraction of human antibodies." Journal of Biotechnology 132(2): 209-217.

Azevedo, A. M., Rosa, P. A. J., Ferreira, I. F. and Aires-Barros, M. R. (2009). "Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing." Trends in Biotechnology 27(4): 240-247.

Babu, R. B., Rastogi, N. K. and Raghavarao, K. S. M. S. (2008). "Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system " Chemical Engineering and Processing 47: 83-89.

Banerjee, R. and Bhattacharyya, B. C. (1992). "Optimization of multiple inducers effect on protease biosynthesis by *Rhizopus oryzae*." Bioprocess Engineering 7(5): 225-228.

Barbosa, J. M. P., Souza, R. L., Fricks, A. T., Zanin, G. M., Soares, C. M. F. and Lima, Á. S. (2011). "Purification of lipase produced by a new source of Bacillus in submerged fermentation using an aqueous two-phase system." Journal of Chromatography B 879(32): 3853-3858.

Bassani, G., Fuciños, P., Picó, G. and Farruggia, B. (2010). "Candida rugosa lipase Lip1–polyethyleneglycol interaction and the relation with its partition in aqueous two-phase systems." Colloids and Surfaces B: Biointerfaces 75(2): 532-537.

Bauer, M. M. T. and Stubbs, M. T. (1999). Crystallization of Proteinases. Proteolytic Enzymes: Tools and Targets. Sterchi, E. E. and Stöcker, W. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 124-147.

Berekaa, M., Zaghloul, T., Abdel-Fattah, Y., Saeed, H. and Sifour, M. (2009). "Production of a novel glycerol-inducible lipase from thermophilic

Chulalongkorn University

Geobacillus stearothermophilus strain-5." World Journal of Microbiology and Biotechnology 25(2): 287-294.

Bezerra, R. P., Borba, F. K. S. L., Moreira, K. A., Lima-Filho, J. L., Porto, A. L. F. and Chaves, A. C. (2006). "Extraction of amylase from fermentation broth in poly (Ethylene Glycol) salt aqueous two-phase system." Brazilian Archives of Biology and Technology 49: 547-555.

Bhatnagar, A. S., Prasanth Kumar, P. K., Hemavathy, J. and Gopala Krishna, A. G. (2009). "Fatty Acid Composition, Oxidative Stability, and Radical Scavenging Activity of Vegetable Oil Blends with Coconut Oil." Journal of the American Oil Chemists' Society 86(10): 991-999.

Bhunia, B., Basak, B. and Dey, A. (2012). "A review on production of serine alkaline protease by *Bacillus spp.*" Journal of Biochemical Technology 3(4): 448-457.

Bisht, D., Yadav, S. K., Gautam, P. and Darmwal, N. S. (2013). "Simultaneous production of alkaline lipase and protease by antibiotic and heavy metal tolerant *Pseudomonas aeruginosa*." Journal of Basic Microbiology 53(9): 715-722.

Bonomo, R. C. F., Minim, L. A., Coimbra, J. S. R., Fontan, R. C. I., Mendes da Silva, L. H. and Minim, V. P. R. (2006). "Hydrophobic interaction adsorption of whey proteins: Effect of temperature and salt concentration and thermodynamic analysis." Journal of Chromatography B 844(1): 6-14.

Bora, L. and Bora, M. (2012). "Optimization of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* sp isolated from Hotspring of Arunachal Pradesh, India." Brazilian Journal of Microbiology 43: 30-42.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical Biochemistry 72(1): 248-254.

Bradoo, S., Saxena, R. K. and Gupta, R. (1999). "Partitioning and resolution of mixture of two lipases from Bacillus stearothermophilus SB-1 in aqueous two-phase system." Process Biochemistry 35(1–2): 57-62.

Bradoo, S., Saxena, R. K. and Gupta, R. (1999). "Two acidothermotolerant lipases from new variants of *Bacillus* spp." World Journal of Microbiology and Biotechnology 15(1): 87-91.

Briki, S., Hamdi, O. and Landoulsi, A. (2016). "Enzymatic dehairing of goat skins using alkaline protease from *Bacillus* sp. SB12." Protein Expression and Purification 121: 9-16.

Castro-Ochoa, L. D., Rodríguez-Gómez, C., Valerio-Alfaro, G. and Oliart Ros, R. (2005). "Screening, purification and characterization of the thermoalkalophilic lipase

produced by *Bacillus thermoleovorans* CCR11." Enzyme and Microbial Technology 37(6): 648-654.

Chahinian, H., Nini, L., Boitard, E., Dubès, J.-P., Comeau, L.-C. and Sarda, L. (2002). "Distinction between esterases and lipases: A kinetic study with vinyl esters and TAG." Lipids 37(7): 653-662.

Chakraborty, K. and Paulraj, R. (2009). "Purification and biochemical characterization of an extracellular lipase from *Pseudomonas fluorescens* MTCC 2421." Journal of Agricultural and Food Chemistry 57(9): 3859-3866.

Chaloupka, J. (1985). "Temperature as a factor regulating the synthesis of microbial enzymes." Microbiological Sciences 2(3): 86-90.

Cherdyntseva, T. A., Razin'kov, V. K. and Egorov, N. S. (1982). "[*Bacillus subtilis* var. amyloliquefaciens biosynthesis of extracellular protease possessing coagulase activity and formed under conditions of limiting the nitrogen sources in the medium]." Mikrobiologiia 51(3): 431-435.

Chiu, R. Y., Thach, A. V., Wu, C. M., Wu, B. M. and Kamei, D. T. (2015). "An aqueous two-phase system for the concentration and extraction of proteins from the interface for detection using the lateral-flow immunoassay." PLoS One 10(11): e0142654.

Christen, G. L. and Marshall, R. T. (1984). "Selected Properties of Lipase and Protease of *Pseudomonas fluorescens* 27 Produced in Four Media." Journal of Dairy Science 67(8): 1680-1687.

Chu, I. M., Lee, C. and Li, T.-S. (1992). "Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416." Enzyme and Microbial Technology 14(9): 755-761.

Chu, W. H. (2007). "Optimization of extracellular alkaline protease production from species of *Bacillus*." Journal of Industrial Microbiology and Biotechnology 34(3): 241-245.

Cleland, J. L., Hedgepeth, C. and Wang, D. I. (1992). "Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B. Reaction stoichiometry and refolding model." The Journal of Biological Chemistry 267(19): 13327-13334.

Cleland, J. L. and Wang, D. I. C. (1990). "Refolding and aggregation of bovine carbonic anhydrase B: Quasi-elastic light scattering analysis." Biochemistry 29(50): 11072-11078.

Cunha, M. T., Costa, M. J. L., Calado, C. R. C., Fonseca, L. P., Aires-Barros, M. R. and Cabral, J. M. S. (2003). "Integration of production and aqueous two-phase

systems extraction of extracellular Fusarium solani pisi cutinase fusion proteins." Journal of Biotechnology 100(1): 55-64.

Damerval, C., Guilloux, M. L., Blaisonneau, J. and de Vienne, D. (1987). "A simplification of Heukeshoven and Dernick's silver staining of proteins." Electrophoresis 8(3): 158-159.

del-Val, M. I. and Otero, C. (2003). "Biphasic aqueous media containing polyethylene glycol for the enzymatic synthesis of oligosaccharides from lactose." Enzyme and Microbial Technology 33(1): 118-126.

Dennison, C. and Lovrien, R. (1997). "Three phase partitioning: concentration and purification of proteins." Protein Expression and Purification 11(2): 149-161.

Dhananjay, S. K. and Mulimani, V. H. (2009). "Three-phase partitioning of α-galactosidase from fermented media of *Aspergillus oryzae* and comparison with conventional purification techniques." Journal of Industrial Microbiology & Biotechnology 36(1): 123-128.

Diamond, A. D. and Hsu, J. T. (1992). Aqueous two-phase systems for biomolecule separation. Bioseparation. Tsao, G. T. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 89-135.

Dosanjh, N. S. and Kaur, J. (2002). "Biochemical Analysis of a Native and Proteolytic Fragment of a High-Molecular-Weight Thermostable Lipase from a Mesophilic *Bacillus* sp." Protein Expression and Purification 24(1): 71-75.

Dutta, S. and Ray, L. (2009). "Production and Characterization of an Alkaline Thermostable Crude Lipase from an Isolated Strain of *Bacillus cereus* C7." Applied Biochemistry and Biotechnology 159(1): 142-154.

Ebrahimpour, A., Rahman, R. N. Z. R. A., Ean Ch'ng, D. H., Basri, M. and Salleh, A. B. (2008). "A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus* sp. strain ARM." BMC Biotechnology 8(1): 1-15.

Eggert, T., Brockmeier, U., Dröge, M. J., Quax, W. J. and Jaeger, K.-E. (2003). "Extracellular lipases from *Bacillus subtilis*: regulation of gene expression and enzyme activity by amino acid supply and external pH." FEMS Microbiology Letters 225(2): 319-324.

Eggert, T., van Pouderoyen, G., Pencreac'h, G., Douchet, I., Verger, R., Dijkstra, B. W. and Jaeger, K.-E. (2002). "Biochemical properties and three-dimensional structures of two extracellular lipolytic enzymes from *Bacillus subtilis*." Colloids and Surfaces B: Biointerfaces 26(1–2): 37-46.

Farruggia, B., Rigatuso, R., Capezio, L., Diez, V. and Picó, G. (2004). "Influence of high concentration monovalent cations on the protein partitioning in polyethyleneglycol 1500-phosphate aqueous two-phase systems." Journal of Chromatography B 809(2): 301-306.

Frankena, J., Koningstein, G. M., van Verseveld, H. W. and Stouthamer, A. H. (1986). "Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis*." Applied Microbiology and Biotechnology 24(2): 106-112.

Gajju H, B. T. C., Agarwal H O (1996). "Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77." Indian Journal of Microbiology 36: 153-155.

Gautam, S. and Simon, L. (2006). "Partitioning of β-glucosidase from *Trichoderma reesei* in poly(ethylene glycol) and potassium phosphate aqueous two-phase systems: Influence of pH and temperature." Biochemical Engineering Journal 30(1): 104-108.

Ghanem, E., Al-Sayed, H. and Saleh, K. (2000). "An alkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*." World Journal of Microbiology and Biotechnology 16(5): 459-464.

Ghosh, P. K., Saxena, R. K., Gupta, R., Yadav, R. P. and Davidson, S. (1996). "Microbial lipases: production and applications." Science Progress 79 (Pt 2): 119-157.

Glyk, A., Scheper, T. and Beutel, S. (2015). "PEG–salt aqueous two-phase systems: an attractive and versatile liquid–liquid extraction technology for the downstream processing of proteins and enzymes." Applied Microbiology and Biotechnology 99(16): 6599-6616.

Goja, A. M., Yang, H., Cui, M. and Li, C. (2013). "Aqueous two-phase extraction advances for bioseparation." Bioprocess and Biotechniques 4(1).

Gomaa, E. Z. (2013). "Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by *Bacillus pumillus* grown on *Ficus nitida* wastes." Brazilian Journal of Microbiology 44(2): 529-537.

González-González, M., Mayolo-Deloisa, K., Rito-Palomares, M. and Winkler, R. (2011). "Colorimetric protein quantification in aqueous two-phase systems." Process Biochemistry 46(1): 413-417.

Gowland, P., Kernick, M. and Sundaram, T. K. (1987). "Thermophilic bacterial isolates producing lipase." FEMS Microbiology Letters 48(3): 339-343.

Grbavčić, S., Bezbradica, D., Izrael-Živković, L., Avramović, N., Milosavić, N., Karadžić, I. and Knežević-Jugović, Z. (2011). "Production of lipase and protease from

an indigenous *Pseudomonas aeruginosa* strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance." Bioresource Technology 102(24): 11226-11233.

Guan, Y., Lilley, T. H., Treffry, T. E., Zhou, C.-L. and Wilkinson, P. B. (1996). "Use of aqueous two-phase systems in the purification of human interferon-α1 from recombinant *Escherichia coli*." Enzyme and Microbial Technology 19(6): 446-455.

Guncheva, M. and Zhiryakova, D. (2011). "Catalytic properties and potential applications of *Bacillus* lipases." Journal of Molecular Catalysis B: Enzymatic 68(1): 1-21.

Gupta, N., Sahai, V. and Gupta, R. (2007). "Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor." Process Biochemistry 42(4): 518-526.

Gupta, R., Beg, Q., Khan, S. and Chauhan, B. (2002). "An overview on fermentation, downstream processing and properties of microbial alkaline proteases." Applied Microbiology and Biotechnology 60(4): 381-395.

Gupta, R., Beg, Q. and Lorenz, P. (2002). "Bacterial alkaline proteases: molecular approaches and industrial applications." Applied Microbiology and Biotechnology 59(1): 15-32.

Gupta, R., Gupta, N. and Rathi, P. (2004). "Bacterial lipases: an overview of production, purification and biochemical properties." Applied Microbiology and Biotechnology 64(6): 763-781.

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: 18.

Hachem, F., Andrews, B. A. and Asenjo, J. A. (1996). "Hydrophobic partitioning of proteins in aqueous two-phase systems." Enzyme and Microbial Technology 19(7): 507-517.

Haki, G. D. and Rakshit, S. K. (2003). "Developments in industrially important thermostable enzymes: a review." Bioresource Technology 89(1): 17-34.

Hammar, L. (2000). Concentration and Purification of Viruses. Aqueous Two-Phase Systems: Methods and Protocols: Methods and Protocols. Hatti-Kaul, R. Totowa, NJ, Humana Press**:** 143-158.

Handelsman, T. A. L. and Shoham, Y. (1994). "Production and characterization of an extracellular thermostable lipase from a thermophilic *Bacillus* sp." The Journal of General and Applied Microbiology 40(5): 435-443.

Hasan, F., Shah, A. and Hameed, A. (2007). "Purification and characterization of a mesophilic lipase from *Bacillus subtilis* FH5 stable at high temperature and pH." Acta Biologica Hungarica 58(1): 115-132.

Hasan, F., Shah, A. A. and Hameed, A. (2009). "Methods for detection and characterization of lipases: A comprehensive review." Biotechnology Advances 27(6): 782-798.

Hatti-Kaul, R. (2001). "Aqueous two-phase systems." Molecular Biotechnology 19(3): 269-277.

He, G.-q., Zhang, X.-y., Tang, X. j., Chen, Q.-h. and Ruan, H. (2005). "Partitioning and purification of extracellular β-1,3-1,4-glucanase in aqueous two-phase systems." Journal of Zhejiang University SCIENCE 6B(8): 825-831.

Hemavathi, A. B. and Raghavarao, K. S. M. S. (2011). "Differential partitioning of β-galactosidase and β-glucosidase using aqueous two phase extraction." Process Biochemistry 46(3): 649-655.

Henriette, C., Zinebi, S., Aumaitre, M. F., Petitdemange, E. and Petitdemange, H. (1993). "Protease and lipase production by a strain of *Serratia marcescens* (532 S)." Journal of Industrial Microbiology 12(2): 129-135.

Hjort, C. (2007). 3 - Industrial enzyme production for food applications A2 - Rastall, Robert. Novel Enzyme Technology for Food Applications, Woodhead Publishing**:** 43-59.

Horikoshi, K. (1999). "Alkaliphiles: Some Applications of Their Products for Biotechnology." Microbiology and Molecular Biology Reviews 63(4): 735-750.

Huddleston, J. G., Ottomar, K. W., Ngonyani, D. M. and Lyddiatt, A. (1991). "Influence of system and molecular parameters upon fractionation of intracellular proteins from *Saccharomyces* by aqueous two-phase partition." Enzyme and Microbial Technology 13(1): 24-32.

Hustedt, H., Kroner, K. H. and Papamichael, N. (1988). "Continuous cross-current aqueous two-phase extraction of enzymes from biomass. Automated recovery in production scale." Process Biochem 23(5 , Oct. 1988): 129-137.

Ibrahim, A. S. S., Al-Salamah, A. A., Elbadawi, Y. B., El-Tayeb, M. A. and Ibrahim, S. S. S. (2015). "Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes." Electronic Journal of Biotechnology 18(3): 236-243.

Illanes, A. (2008). Enzyme Production. Enzyme Biocatalysis: Principles and Applications. Illanes, A. Dordrecht, Springer Netherlands**:** 57-106.

Imamura, S. and Kitaura, S. (2000). "Purification and Characterization of a Monoacylglycerol Lipase from the Moderately Thermophilic *Bacillus* sp. H-257." Journal of Biochemistry 127(3): 419-425.

Jaeger, K.-E. and Eggert, T. (2002). "Lipases for biotechnology." Current Opinion in Biotechnology 13(4): 390-397.

Jaeger, K. E., Dijkstra, B. W. and Reetz, M. T. (1999). "Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases." Annual Review of Microbiology 53: 315-351.

James, G. T. (1978). "Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers." Analytical Biochemistry 86(2): 574-579.

Johansson, G. (1970). "Partition of salts and their effects on partition of proteins in a dextran-poly (ethylene glycol)-water two-phase system." Biochim Biophys Acta 221(2): 387-390.

Johasson, G. (1998). Affinity partitioning of proteins using aqueous two-phase systems. Protein Purification: Principles, High-Resolution Methods, and Applications. Janson, J. C. and Ryden, L. Wiley-VCH, Inc, London.

Joo, H. S., Kumar, C. G., Park, G. C., Paik, S. R. and Chang, C. S. (2003). "Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: Production and some properties." Journal of Applied Microbiology 95(2): 267-272.

Karkaş, T. and Önal, S. (2012). "Characteristics of invertase partitioned in poly(ethylene glycol)/magnesium sulfate aqueous two-phase system." Biochemical Engineering Journal 60: 142-150.

Kaul, A. (2000). The Phase Diagram. Aqueous Two-Phase Systems: Methods and Protocols: Methods and Protocols. Hatti-Kaul, R. Totowa, NJ, Humana Press**:** 11-21.

Kavakçıoğlu, B. and Tarhan, L. (2013). "Initial purification of catalase from Phanerochaete chrysosporium by partitioning in poly(ethylene glycol)/salt aqueous two phase systems." Separation and Purification Technology 105: 8-14.

Ketnawa, S., Chaiwut, P. and Rawdkuen, S. (2011). "Aqueous two-phase extraction of bromelain from pineapple peels (‘Phu Lae’ cultv.) and its biochemical properties." Food Science and Biotechnology 20(5): 1219-1226.

Khayati, G. and Alizadeh, S. (2013). "Extraction of lipase from *Rhodotorula glutinis* fermentation culture by aqueous two-phase partitioning." Fluid Phase Equilibria 353: 132-134.

Khyami-Horani, H. (1996). "Thermotolerant strain of *Bacillus licheniformis* producing lipase." World Journal of Microbiology and Biotechnology 12(4): 399-401.

Klomklao, S., Benjakul, S. and Visessanguan, W. (2004). "Comparative studies on proteolytic activity of splenic extract from three tuna species commonly used in Thailand." Journal of Food Biochemistry 28(5): 355-372.

Klomklao, S., Benjakul, S., Visessanguan, W., Simpson, B. K. and Kishimura, H. (2005). "Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems." Process Biochemistry 40(9): 3061-3067.

Kok, R. G., Nudel, C. B., Gonzalez, R. H., Nugteren-Roodzant, I. M. and Hellingwerf, K. J. (1996). "Physiological factors affecting production of extracellular lipase (LipA) in *Acinetobacter calcoaceticus* BD413: Fatty acid repression of lipA expression and degradation of LipA." Journal of Bacteriology 178(20): 6025-6035.

Kole, M. M., Draper, I. and Gerson, D. (1988). "Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations." Journal of Chemical Technology and Biotechnology 41: 197-206.

Kraemer, F. B. and Shen, W.-J. (2002). "Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis." Journal of Lipid Research 43(10): 1585-1594.

Kula, M. R., Kroner, K. H., Hustedt, H. and Schutte, H. (1982). Scale-up of protein purification by liquid-liquid extraction. Enzyme Engineering: Proceedings of the International Enzyme Engineering Conference.

Kumar, C. G. and Takagi, H. (1999). "Microbial alkaline proteases: From a bioindustrial viewpoint." Biotechnology Advances 17(7): 561-594.

Kumar, D., Parshad, R. and Gupta, V. K. (2014). "Application of a statistically enhanced, novel, organic solvent stable lipase from *Bacillus safensis* DVL-43." International Journal of Biological Macromolecules 66(0): 97-107.

Kumar, D. J. M., Lawrence, L., Rajan, R., Priyadarshini, S., Sandhiyachittybabu and Kalaichelvan, P. T. (2012). "Characterization of lipase and protease from *serratia marcescens* DEPTK21 and its destaining capability." Asian Journal of Experimental Biological Sciences 3(3): 621-628.

Kumar, M. P. P. and Valsa, A. K. (2007). "Optimization of culture media and cultural conditions for the production of extracellular lipase by *Bacillus coagulans* " Indian Journal of Biotechnology 6: 114-117.

Kumar, R., Mahajan, S., Kumar, A. and Singh, D. (2011). "Identification of variables and value optimization for optimum lipase production by *Bacillus pumilus* RK31 using statistical methodology." New Biotechnology 28(1): 65-71.

Kumar, S., Kikon, K., Upadhyay, A., Kanwar, S. S. and Gupta, R. (2005). "Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3." Protein Expression and Purification 41(1): 38-44.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (2009). "Rules for Optimization of Biocatalysis in Organic Solvents." Biotechnology and Bioengineering 102(1): 1-8.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 227(5259): 680-685.

Lahore, H. M. F., Miranda, M. V., Fraile, E. R., Bonino, M. J. B. d. J. and Cascone, O. (1995). "Partition behaviour and purification of a Mucor bacilliformis acid protease in aqueous two-phase systems." Process Biochemistry 30(7): 615-621.

Lario, L. D., Malpiedi, L. P., Pereira, J. F. B., Sette, L. D. and Pessoa-Junior, A. (2016). "Liquid-liquid extraction of protease from cold-adapted yeast *Rhodotorula mucilaginosa* L7 using biocompatible and biodegradable aqueous two-phase systems." Separation Science and Technology 51(1): 57-67.

Lee, D.-W., Koh, Y.-S., Kim, K.-J., Kim, B.-C., Choi, H.-J., Kim, D.-S., Suhartono, M. T. and Pyun, Y.-R. (1999). "Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1." FEMS Microbiology Letters 179(2): 393-400.

Lee, D., Koh, Y., Kim, K., Kim, B., Choi, H., Kim, D., Suhartono, M. T. and Pyun, Y. (1999). "Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1." FEMS Microbiology Letters 179(2): 393-400.

Lee, M. H., Kim, H. K., Sung, M. H., Song, J. J., Rha, E. G., Lee, S. G., Poo, H. Y., Lee, S. C., Hong, S. P., Seu, Y. B., Kim, H. K., Sung, M. H., Hong, S. P. and Seu, Y. B. (2003). "High-level expression and secretion of *Bacillus pumilus* lipase B26 in *Bacillus subtilis* Chungkookjang." Journal of Microbiology and Biotechnology 13(6): 892-896.

Liu, S. (2013). Chapter 2 - An Overview of Biological Basics. Bioprocess Engineering. Amsterdam, Elsevier**:** 21-84.

Lopes, M., Gomes, N., Gonçalves, C., Coelho, M. A. Z., Mota, M. and Belo, I. (2008). "*Yarrowia lipolytica* lipase production enhanced by increased air pressure." Letters in Applied Microbiology 46(2): 255-260.

Lotti, M., Monticelli, S., Luis Montesinos, J., Brocca, S., Valero, F. and Lafuente, J. (1998). "Physiological control on the expression and secretion of *Candida rugosa* lipase." Chemistry and Physics of Lipids 93(1–2): 143-148.

Loveless, B. J. and Saier, M. H., Jr. (1997). "A novel family of channel-forming, autotransporting, bacterial virulence factors." Molecular Membrane Biology 14(3): 113-123.

Luechau, F., Ling, T. C. and Lyddiatt, A. (2009). "Interfacial partition of plasmid DNA in aqueous two-phase systems." Separation and Purification Technology 67(1): 64-70.

Luechau, F., Ling, T. C. and Lyddiatt, A. (2009). "Partition of plasmid DNA in polymer–salt aqueous two-phase systems." Separation and Purification Technology 66(2): 397-404.

Luechau, F., Ling, T. C. and Lyddiatt, A. (2010). "A descriptive model and methods for up-scaled process routes for interfacial partition of bioparticles in aqueous two-phase systems." Biochemical Engineering Journal 50(3): 122-130.

Lütz, S. (2013). "Biocatalysts and Enzyme Technology. 2nd Edition. By Klaus Buchholz, Volker Kasche and Uwe Theo Bornscheuer." Angewandte Chemie International Edition 52(28): 7073-7073.

Madhusudhan, M. C., Raghavarao, K. S. M. S. and Nene, S. (2008). "Integrated process for extraction and purification of alcohol dehydrogenase from Baker's yeast involving precipitation and aqueous two phase extraction." Biochemical Engineering Journal 38(3): 414-420.

McAuliffe, J. C. (2012). Industrial enzymes and biocatalysis. Handbook of industrial chemistry and biotechnology. Kent, J. A. New York, Springer Science+Business Media. 1&2**:** 1183-1228.

Mehrnoush, A., Mustafa, S., Sarker, M. Z. I. and Yazid, A. M. M. (2012). "Optimization of Serine Protease Purification from Mango (Mangifera indica cv. Chokanan) Peel in Polyethylene Glycol/Dextran Aqueous Two Phase System." International Journal of Molecular Sciences 13(3): 3636-3649.

Meyer, F. M., Jules, M., Mehne, F. M. P., Le Coq, D., Landmann, J. J., Görke, B., Aymerich, S. and Stülke, J. (2011). "Malate-Mediated Carbon Catabolite Repression in *Bacillus subtilis* Involves the HPrK/CcpA Pathway." Journal of Bacteriology 193(24): 6939-6949.

Mobarak-Qamsari, E., Kasra-Kermanshahi, R. and Moosavi-nejad, Z. (2011). "Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110." Iranian Journal of Microbiology 3(2): 92-98.

Mohamadi, H. S. and Omidinia, E. (2007). "Purification of recombinant phenylalanine dehydrogenase by partitioning in aqueous two-phase systems." J Chromatogr B 854.

Mohamadi, H. S., Omidinia, E. and Dinarvand, R. (2007). "Evaluation of recombinant phenylalanine dehydrogenase behavior in aqueous two-phase partitioning." Process Biochemistry 42(9): 1296-1301.

Mokhtarani, B., Mortaheb, H. R., Mafi, M. and Amini, M. H. (2011). "Partitioning of α-lactalbumin and β-lactoglobulin in aqueous two-phase systems of polyvinylpyrrolidone and potassium phosphate." Journal of Chromatography B 879(11–12): 721-726.

Moon, S.-H. and Parulekar, S. J. (1991). "A parametric study ot protease production in batch and fed-batch cultures of *Bacillus firmus*." Biotechnology and Bioengineering 37(5): 467-483.

Moon, S. H. and Parulekar, S. J. (1991). "A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*." Biotechnology and Bioengineering 37(5): 467-483.

Mótyán, J. A., Tóth, F. and Tőzsér, J. (2013). "Research Applications of Proteolytic Enzymes in Molecular Biology." Biomolecules 3(4): 923-942.

Nadeem, M., Qazi, J. I. and Baig, S. (2010). "Enhanced production of alkaline protease by a mutant of *Bacillus licheniformis* N-2 for dehairing." Brazilian Archives of Biology and Technology 53: 1015-1025.

Naganagouda, K. and Mulimani, V. H. (2008). "Aqueous two-phase extraction (ATPE): An attractive and economically viable technology for downstream processing of *Aspergillus oryzae* α-galactosidase." Process Biochemistry 43(11): 1293-1299.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. (2009). "Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems." Process Biochemistry 44(4): 471-476.

Nashif, S. A. and Nelson, F. E. (1953). "The Lipase of *Pseudomonas fragi1* II. Factors Affecting Lipase Production." Journal of Dairy Science 36(5): 471-480.

Nitsawang, S., Hatti-Kaul, R. and Kanasawud, P. (2006). "Purification of papain from *Carica papaya* latex: Aqueous two-phase extraction versus two-step salt precipitation." Enzyme and Microbial Technology 39(5): 1103-1107.

Niyonzima, F. N. and More, S. S. (2014). "Concomitant production of detergent compatible enzymes by *Bacillus flexus* XJU-1." Brazilian Journal of Microbiology 45(3): 903-910.

Nthangeni, M. B., Patterton, H.-G., van Tonder, A., Vergeer, W. P. and Litthauer, D. (2001). "Over-expression and properties of a purified recombinant Bacillus

licheniformis lipase: a comparative report on Bacillus lipases." Enzyme and Microbial Technology 28(7–8): 705-712.

Oh, B., Kim, H., Lee, J., Kang, S. and Oh, T. (1999). "*Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning." FEMS Microbiology Letters 179(2): 385-392.

Ohkuro, I., Komatsuzaki, T., Kawashima, M. and Kuriyama, S. (1978). "Influence of NaCl on colonies and lipase of *Natto bacilli*." Medicine and Biology 97: 171-174.

Olsen, H. S. (2000). Cleaning-in-place of soiled process equipment in dairy or slaughter house by circulating a solution containing a protease and a lipase., Novo Nordisk Als.

Ooi, C., Tey, B., Hii, S., Ariff, A., Wu, H., Lan, J., Juang, R., Kamal, S. and Ling, T. (2009). "Direct purification of *Burkholderia Pseudomallei* lipase from fermentation broth using aqueous two-phase systems." Biotechnology and Bioprocess Engineering 14(6): 811-818.

Ooi, C. W., Tey, B. T., Hii, S. L., Ariff, A., Wu, H. S., Lan, J. C. W., Juang, R. S., Kamal, S. M. M. and Ling, T. C. (2010). "Direct purification of *Burkholderia Pseudomallei* lipase from fermentation broth using aqueous two-phase systems." Biotechnology and Bioprocess Engineering 14(6): 811-818.

Ozcan, T. and Kurdal, E. (2012). "The effects of using a starter culture, lipase, and protease enzymes on ripening of Mihalic cheese." International Journal of Dairy Technology 65(4): 585-593.

Pabai, F., Kermasha, S. and Morin, A. (1996). "Use of continuous culture to screen for lipase-producing microorganisms and interesterification of butter fat by lipase isolates." Canadian Journal of Microbiology 42(5): 446-452.

Pan, I. H. and Li, Y. K. (2001). "Rapid process for purification of an extracellular beta-xylosidase by aqueous two-phase extraction." Journal of Chromatography B: Biomedical Sciences and Applications 754(1): 179-184.

Pant, G., Prakash, A., Pavani, J. V. P., Bera, S., Deviram, G. V. N. S., Kumar, A., Panchpuri, M. and Prasuna, R. G. (2015). "Production, optimization and partial purification of protease from *Bacillus subtilis*." Journal of Taibah University for Science 9(1): 50-55.

Patel, R., Dodia, M. and Singh, S. P. (2005). "Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization." Process Biochemistry 40(11): 3569-3575.

Pereira-Meirelles, F., Rocha-LeãO, M. and Anna, G. (1997). "A stable lipase from *Candida lipolytica*." Applied Biochemistry and Biotechnology 63-65(1): 73-85.

Phadatare, S. U., Deshpande, V. V. and Srinivasan, M. C. (1993). "High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents." Enzyme and Microbial Technology 15(1): 72-76.

Prakasham, R. S., Rao, C. S. and Sarma, P. N. (2006). "Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation." Bioresource Technology 97(13): 1449-1454.

Rachadech, W., Navacharoen, A., Ruangsit, W., Pongtharangkul, T. and Vangnai, A. S. (2010). "An organic solvent-, detergent-, and thermostable alkaline protease from the mesophilic, organic solvent-tolerant *Bacillus licheniformis* 3C5." Microbiology 79(5): 620-629.

Rahman, R. N. Z. R. A., Salleh, A. B. and Basri, M. (2006). Lipase: Introduction. New lipases and proteases. Salleh, A. B., Rahman, R. N. Z. R. A. and Basri, M. New York, Nova Science Publisher, Inc.**:** 1-22.

Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V. and Ramesh, V. (2011). "Aqueous two phase systems for the recovery of biomolecules—a review." Science Technol 1.

Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V. and Ramesh, V. (2011). "Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review." Science and Technology 1(1): 7-16.

Rajendran, A. and Thangavelu, V. (2007). "Sequential optimization of culture medium composition for extracellular lipase production by *Bacillus sphaericus* using statistical methods." Journal of Chemical Technology & Biotechnology 82(5): 460-470.

Rajmohan, S., Dodd, C. E. R. and Waites, W. M. (2002). "Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage." Journal of Applied Microbiology 93(2): 205-213.

Ramesh, V. and Murty, V. R. (2015). "Partitioning of thermostable glucoamylase in polyethyleneglycol/salt aqueous two-phase system." Bioresources and Bioprocessing 2(1): 1-8.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). "Molecular and Biotechnological Aspects of Microbial Proteases." Microbiology and Molecular Biology Reviews 62(3): 597-635.

Ratanapongleka, K. (2010). "Recovery of biological products in aqueous two phase systems." International Journal of Chemical Engineering and Applications 1(2): 191-198.

Rawdkuen, S., Pintathong, P., Chaiwut, P. and Benjakul, S. (2011). "The partitioning of protease from *Calotropis procera* latex by aqueous two-phase systems and its hydrolytic pattern on muscle proteins." Food and Bioproducts Processing 89(1): 73-80.

Raymond, F. D. and Fisher, D. (1995). Interfacial events in phase separation and cell partitioning in aqueous two-phase systems. Aqueous Biphasic Separations: Biomolecules to Metal Ions. Rogers, R. D. and Eiteman, M. A. Boston, MA, Springer US**:** 141-154.

Ribeiro, M. Z., Silva, D. P., Vitolo, M., Roberto, I. C. and Pessoa-Jr, A. (2007). "Partial purification of glucose-6-phosphate dehydrogenase by aqueous two-phase poly(ethyleneglycol)/phosphate systems." Brazilian Journal of Microbiology 38: 78-83.

Rito-Palomares, M. and Lyddiatt, A. (1996). "Impact of cell disruption and polymer recycling upon aqueous two-phase processes for protein recovery." Journal of Chromatography B: Biomedical Sciences and Applications 680(1–2): 81-89.

Roe, S. (2000). Protein purification techniques: A practical Approach. Oxford, University Press.

Rogers, R. D. and Zhang, J. (1996). "Effects of increasing polymer hydrophobicity on distribution ratios of TcO4- in polyethylene/poly(propylene glycol)-based aqueous biphasic systems." J Chromatogr B Biomed Appl 680(1-2): 231-236.

Rosa, P. A., Azevedo, A. M., Sommerfeld, S., Mutter, A., Aires-Barros, M. R. and Backer, W. (2009). "Application of aqueous two-phase systems to antibody purification: a multi-stage approach." Journal of Biotechnology 139(4): 306-313.

Rosa, P. A. J., Ferreira, I. F., Azevedo, A. M. and Aires-Barros, M. R. (2010). "Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals." Journal of Chromatography A 1217(16): 2296-2305.

Ruchi, G., Anshu, G. and Khare, S. K. (2008). "Lipase from solvent tolerant *Pseudomonas aeruginosa* strain: Production optimization by response surface methodology and application." Bioresource Technology 99(11): 4796-4802.

Ruiz-Ruiz, F., Benavides, J., Aguilar, O. and Rito-Palomares, M. (2012). "Aqueous two-phase affinity partitioning systems: Current applications and trends." Journal of Chromatography A 1244: 1-13.

Sadeghi, R. and Jamehbozorg, B. (2008). "Effect of temperature on the salting-out effect and phase separation in aqueous solutions of sodium di-hydrogen phosphate and poly(propylene glycol)." Fluid Phase Equilibria 271(1–2): 13-18.

Sadeghi, R., Rafiei, H. R. and Motamedi, M. (2006). "Phase equilibrium in aqueous two-phase systems containing poly(vinylpyrrolidone) and sodium citrate at different temperatures—Experimental and modeling." Thermochimica Acta 451(1–2): 163-167.

Salabat, A., Moghadasi, M. A., Zalaghi, P. and Sadeghi, R. (2006). "(Liquid + liquid) equilibria for ternary mixtures of (polyvinylpyrrolidone + MgSO4 + water) at different temperatures." The Journal of Chemical Thermodynamics 38(11): 1479-1483.

Salabat, A., Sadeghi, R., Moghadam, S. T. and Jamehbozorg, B. (2011). "Partitioning of l-methionine in aqueous two-phase systems containing poly(propylene glycol) and sodium phosphate salts." The Journal of Chemical Thermodynamics 43(10): 1525-1529.

Salabat, A. R., Abnosi, M. H. and Motahari, A. (2010). "Application of aqueous mixtures of polypropylene glycol or polyethylene glycol with salts in proteomic analysis." Journal of the Iranian Chemical Society 7(1): 142-149.

Salleh, A. B., Razak, C. N. A., Rahman, R. N. Z. R. A. and Basri, M. (2006). Protease: Introduction. New lipases and proteases. Salleh, A. B., Rahman, R. N. Z. R. A. and Basri, M. New York, Nova Science Publisher, Inc.**:** 23-40.

Sangeetha, R., Geetha, A. and Arulpandi, I. (2010). "Concomitant production of protease and lipase by *Bacillus licheniformis* VSG1: production, purification and characterization." Brazilian Journal of Microbiology 41: 179-185.

Sangeetha, R., Geetha, A. and Arulpandi, I. (2010). "Concomitant production, partial purification and characterization of a serine protease and a proteolysis-resistant metallolipase from *Bacillus pumilus* SG2." Z Naturforsch C 65(1-2): 61-65.

Sarangi, B. K., Pattanaik, D. P., Rathinaraj, K., Sachindra, N. M., Madhusudan, M. C. and Mahendrakar, N. S. (2011). "Purification of alkaline protease from chicken intestine by aqueous two phase system of polyethylene glycol and sodium citrate." Journal of food science and technology 48(1): 36-44.

Saravanan, S., Reena, J. A., Rao, J. R., Murugesan, T. and Nair, B. U. (2006). "Phase Equilibrium Compositions, Densities, and Viscosities of Aqueous Two-Phase Poly(ethylene glycol) + Poly(acrylic acid) System at Various Temperatures." Journal of Chemical & Engineering Data 51(4): 1246-1249.

Saxena, L., Iyer, B. K. and Ananthanarayan, L. (2007). "Three phase partitioning as a novel method for purification of ragi (*Eleusine coracana*) bifunctional amylase/protease inhibitor." Process Biochemistry 42(3): 491-495.

Saxena, S. (2015). Microbial enzymes and their industrial applications. Applied Microbiology. New Delhi, Springer India**:** 121-154.

Schmidt-Dannert, C., Sztajer, H., Stöcklein, W., Menge, U. and Schmid, R. D. (1994). "Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*." Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 1214(1): 43-53.

Schmidt, A. S., Andrews, B. A. and Asenjo, J. A. (1996). "Correlations for the partition behavior of proteins in aqueous two-phase systems: Effect of overall protein concentration." Biotechnology and Bioengineering 50(6): 617-626.

Schmidt, A. S., Ventom, A. M. and Asenjo, J. A. (1994). "Partitioning and purification of α-amylase in aqueous two-phase systems." Enzyme and Microbial Technology 16(2): 131-142.

Sekhon, A., Dahiya, N., Tewari, R. P. and Hoondal, G. S. (2006). "Production of extracellular lipase by *Bacillus megaterium* AKG-1 in submerged fermentation." Indian Journal of Biotechnology 5(2): 179-183.

Selle, P. H. and Ravindran, V. (2007). "Microbial phytase in poultry nutrition." Animal Feed Science and Technology 135(1–2): 1-41.

Sen, S. and Satyanarayana, T. (1993). "Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40." Indian Journal of Microbiology 33: 43-47.

Shahbaz Mohammadi, H., Mostafavi, S. S., Soleimani, S., Bozorgian, S., Pooraskari, M. and Kianmehr, A. (2015). "Response surface methodology to optimize partition and purification of two recombinant oxidoreductase enzymes, glucose dehydrogenase and d-galactose dehydrogenase in aqueous two-phase systems." Protein Expression and Purification 108: 41-47.

Shaoxin, C., Lili, Q. and Bingzhao, S. (2007). "Purification and properties of enantioselective lipase from a newly isolated *Bacillus cereus* C71." Process Biochemistry 42(6): 988-994.

Shariff, F. M., Leow, T. C., Mukred, A. D., Salleh, A. B., Basri, M. and Rahman, R. N. Z. R. A. (2007). "Production of L2 lipase by *Bacillus* sp. strain L2: nutritional and physical factors." Journal of Basic Microbiology 47(5): 406-412.

Sharma, D., Kumbhar, B. K., Verma, A. K. and Tewari, L. (2014). "Optimization of critical growth parameters for enhancing extracellular lipase production by alkalophilic *Bacillus* sp." Biocatalysis and Agricultural Biotechnology 3(4): 205-211.

Sharma, R., Soni, S. K., Vohra, R. M., Gupta, L. K. and Gupta, J. K. (2002). "Purification and characterisation of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1." Process Biochemistry 37(10): 1075-1084.

CHULALONGKORN UNIVERSITY

Sharma, R., Soni, S. K., Vohra, R. M., Jolly, R. S., Gupta, L. K. and Gupta, J. K. (2002). "Production of extracellular alkaline lipase from a *Bacillus* sp. RSJ1 and its application in ester hydrolysis." Indian Journal of Microbiology 42(1): 49-54.

Shukla, A. A., Hubbard, B., Tressel, T., Guhan, S. and Low, D. (2007). "Downstream processing of monoclonal antibodies—Application of platform approaches." Journal of Chromatography B 848(1): 28-39.

Sidhu, P., Sharma, R., Soni, S. K. and Gupta, J. K. (1998). "Effect of Cultural Conditions on Extracellular Alkaline Lipase Production by *Bacillus* sp. RS-12 and its Characterization." Indian Journal of Microbiology 38(1): 9-14.

Sidhu, P., Sharma, R., Soni, S. K. and Gupta, J. K. (1998). "Production of extracellular alkaline lipase by a new thermophilicBacillus sp." Folia Microbiologica 43(1): 51-54.

Sigurgísladóttir, S., Konráòsdóttir, M., Jónsson, Á., Kristjánsson, J. K. and Matthiasson, E. (1993). "Lipase activity of thermophilic bacteria from icelandic hot springs." Biotechnology Letters 15(4): 361-366.

Silva, L. H. M. d., Coimbra, J. S. R. and Meirelles, A. J. d. A. (1997). "Equilibrium Phase Behavior of Poly(ethylene glycol) + Potassium Phosphate + Water Two-Phase Systems at Various pH and Temperatures." Journal of Chemical & Engineering Data 42(2): 398-401.

Sivars, U. and Tjerneld, F. (2000). "Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins." Biochimica et Biophysica Acta - General Subjects 1474(2): 133-146.

Srivastava, P. K. and Kapoor, M. (2013). "Extracellular endo-mannanase from *Bacillus* sp. CFR1601: Economical production using response surface methodology and downstream processing using aqueous two phase system." Food and Bioproducts Processing 91(4): 672-681.

Su, C.-K. and Chiang, B. H. (2006). "Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system." Process Biochemistry 41(2): 257-263.

Suganthi, C., Mageswari, A., Karthikeyan, S., Anbalagan, M., Sivakumar, A. and Gothandam, K. M. (2013). "Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments." Journal of Genetic Engineering and Biotechnology 11(1): 47-52.

Takaç, S. and Marul, B. (2008). "Effects of lipidic carbon sources on the extracellular lipolytic activity of a newly isolated strain of *Bacillus subtilis*." Journal of Industrial Microbiology & Biotechnology 35(9): 1019-1025.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Takii, Y., Kuriyama, N. and Suzuki, Y. (1990). "Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. halodurans KP 1239." Applied Microbiology and Biotechnology 34(1): 57-62.

Thanikaivelan, P., Rao, J. R., Nair, B. U. and Ramasami, T. (2004). "Progress and recent trends in biotechnological methods for leather processing." Trends in Biotechnology 22(4): 181-188.

Tubío, G., Nerli, B. and Picó, G. (2007). "Partitioning features of bovine trypsin and α-chymotrypsin in polyethyleneglycol-sodium citrate aqueous two-phase systems." Journal of Chromatography B 852(1–2): 244-249.

Uyar, F., Porsuk, I., Kizil, G. and Yilmaz, E. I. (2011). "Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15." EurAsian Journal of BioSciences 5: 1-9.

van Pouderoyen, G., Eggert, T., Jaeger, K.-E. and Dijkstra, B. W. (2001). "The crystal structure of *Bacillus subtili* lipase: a minimal α/β hydrolase fold enzyme." Journal of Molecular Biology 309(1): 215-226.

Veide, A., Smeds, A.-L. and Enfors, S.-O. (1983). "A Process for large-scale isolation of β-galactosidase from E. coli in an aqueous two-phase system." Biotechnology and Bioengineering 25(7): 1789-1800.

Villadsen, J., Nielsen, J. and Lidén, G. (2011). Growth Kinetics of Cell Cultures. Bioreaction Engineering Principles. Boston, MA, Springer US**:** 271-357.

Vinci, V. A. and Parekh, S. R. (2003). Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells. New York, Humana public Inc.

Walter, H. and Johansson, G. (1994). Methods in Enzymology: Aqueous Two-Phase Systems. Methods in Enzymology: Aqueous Two-Phase Systems.

Wang, S.-L., Lin, Y.-T., Liang, T.-W., Chio, S.-H., Ming, L.-J. and Wu, P.-C. (2009). "Purification and characterization of extracellular lipases from *Pseudomonas monteilii* TKU009 by the use of soybeans as the substrate." Journal of Industrial Microbiology & Biotechnology 36(1): 65-73.

Westers, H., Braun, P. G., Westers, L., Antelmann, H., Hecker, M., Jongbloed, J. D. H., Yoshikawa, H., Tanaka, T., van Dijl, J. M. and Quax, W. J. (2005). "Genes Involved in SkfA Killing Factor Production Protect a *Bacillus subtilis* Lipase against Proteolysis." Applied and Environmental Microbiology 71(4): 1899-1908.

Xie, X., Wang, Y., Han, J. and Yan, Y. (2011). "Extraction mechanism of sulfamethoxazole in water samples using aqueous two-phase systems of poly(propylene glycol) and salt." Analytica Chimica Acta 687(1): 61-66.

Yang, L., Huo, D., Hou, C., He, K., Lv, F., Fa, H. and Luo, X. (2010). "Purification of plant-esterase in PEG1000/NaH2PO4 aqueous two-phase system by a two-step extraction." Process Biochemistry 45(10): 1664-1671.

Yavari, M., Pazuki, G. R., Vossoughi, M., Mirkhani, S. A. and Seifkordi, A. A. (2013). "Partitioning of alkaline protease from *Bacillus licheniformis* (ATCC 21424) using PEG–K_2HPO_4 aqueous two-phase system." Fluid Phase Equilibria 337(0): 1-5.

Yücekan, İ. and Önal, S. (2011). "Partitioning of invertase from tomato in poly(ethylene glycol)/sodium sulfate aqueous two-phase systems." Process Biochemistry 46(1): 226-232.

Yue, H., Yuan, Q. and Wang, W. (2007). "Purification of phenylalanine ammonia-lyase in PEG1000/Na2SO4 aqueous two-phase system by a two-step extraction." Biochemical Engineering Journal 37(3): 231-237.

Zafarani-Moattar, M. T. and Abdizadeh-Aliyar, V. (2014). "Phase diagrams for (liquid + liquid) and (liquid + solid) equilibrium of aqueous two-phase system containing {polyvinylpyrrolidone 3500 (PVP3500) + sodium sulfite (Na2SO3) + water} at different temperatures." The Journal of Chemical Thermodynamics 72(0): 125-133.

Zafarani-Moattar, M. T., Emamian, S. and Hamzehzadeh, S. (2008). "Effect of Temperature on the Phase Equilibrium of the Aqueous Two-Phase Poly(propylene glycol) + Tripotassium Citrate System." Journal of Chemical & Engineering Data 53(2): 456-461.

Zafarani-Moattar, M. T. and Sadeghi, R. (2005). "Effect of temperature on the phase equilibrium of aqueous two-phase systems containing polyvinylpyrrolidone and disodium hydrogen phosphate or trisodium phosphate." Fluid Phase Equilibria 238(1): 129-135.

Zafarani-Moattar, M. T. and Seifi-Aghjekohal, P. (2007). "Liquid–liquid equilibria of aqueous two-phase systems containing polyvinylpyrrolidone and tripotassium phosphate or dipotassium hydrogen phosphate: Experiment and correlation." Calphad 31(4): 553-559.

Zafarani-Moattar, M. T. and Zaferanloo, A. (2009). "Measurement and correlation of phase equilibria in aqueous two-phase systems containing polyvinylpyrrolidone and di-potassium tartrate or di-potassium oxalate at different temperatures." The Journal of Chemical Thermodynamics 41(7): 864-871.

Zhang, Y.-y. and Liu, J.-h. (2010). "Purification and in situ immobilization of lipase from of a mutant of *Trichosporon laibacchii* using aqueous two-phase systems." Journal of Chromatography B 878(11–12): 909-912.

Zhang, Y., Meng, K., Wang, Y., Luo, H., Yang, P., Shi, P., Wu, N., Fan, Y., Li, J. and Yao, B. (2008). "A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae* var. k11." Enzyme and Microbial Technology 42(4): 346-352.

Zhao, X., Xie, X. and Yan, Y. (2011). "Liquid–liquid equilibrium of aqueous two-phase systems containing poly(propylene glycol) and salt ((NH4)2SO4, MgSO4, KCl, and KAc): experiment and correlation." Thermochimica Acta 516(1–2): 46-51.

Zhou, Y.-J., Hu, C.-L., Wang, N., Zhang, W.-W. and Yu, X.-Q. (2013). "Purification of porcine pancreatic lipase by aqueous two-phase systems of polyethylene glycol and potassium phosphate." Journal of Chromatography B 926: 77-82.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX A

Media

LB medium

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

This solution was adjusted pH to 7.0 and then adjusted the volume to 1000 ml by distilled water. The 15 g of agar were added to preparation of LB agar.

MSBY (Rachadech *et al.*, 2010)

Yeast extract	5	g
K_2HPO_4	4.3	g
KH_2PO_4	3.4	g
$(NH_4)_2SO_4$	2.0	g
$MgCl_2.6H_2O$	0.34	g
$MnCl_2.4H_2O$	0.001	g
$CaCl_2.2H_2O$	0.026	g
$Na_2MoO_4.2H_2O$	0.002	g
$ZnCl_2.7H_2O$	0.0001	g
$CoCl_2.6H_2O$	0.00001	g
$CuSO_4$	0.0000156	g
$NiSO_4.6H_2O$	0.0000017	g
$NaSeO_4$	0.0000001	g
$FeSO_4.7H_2O$	0.006	g

This solution was adjusted pH to 7.0 and then adjusted the volume to 1000 ml by distilled water.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

YMO medium

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	10	g
Olive oil	16.35	ml

This solution was adjusted pH to 7.0 and then adjusted the volume to 1000 ml by distilled water.

MSBYO medium

Yeast extract	5	g
Olive oil	15	ml
K_2HPO_4	4.3	g
KH_2PO_4	3.4	g
$(NH_4)_2SO_4$	2.0	g
$MgCl_2.6H_2O$	0.34	g
$MnCl_2.4H_2O$	0.001	g
$CaCl_2.2H_2O$	0.026	g
$Na_2MoO_4.2H_2O$	0.002	g
$ZnCl_2.7H_2O$	0.0001	g
$CoCl_2.6H_2O$	0.00001	g
$CuSO_4$	0.0000156	g
$NiSO_4.6H_2O$	0.0000017	g
$NaSeO_4$	0.0000001	g
$FeSO_4.7H_2O$	0.006	g

This solution was adjusted pH to 9.0 and then adjusted the volume to 1000 ml by distilled water.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Protease and Lipase Optimized production medium (PLO)

Yeast extract	6.0	g
Olive oil	25.0	ml
Glucose	8.4	g
K_2HPO_4	4.3	g
KH_2PO_4	3.4	g
$(NH_4)_2SO_4$	2.0	g
$MgCl_2.6H_2O$	0.34	g
$MnCl_2.4H_2O$	0.001	g
$CaCl_2.2H_2O$	0.026	g
$Na_2MoO_4.2H_2O$	0.002	g
$ZnCl_2.7H_2O$	0.0001	g
$CoCl_2.6H_2O$	0.00001	g
$CuSO_4$	0.0000156	g
$NiSO_4.6H_2O$	0.0000017	g
$NaSeO_4$	0.0000001	g
$FeSO_4.7H_2O$	0.006	g

This solution was adjusted pH to 9.0 and then adjusted the volume to 1000 ml by distilled water

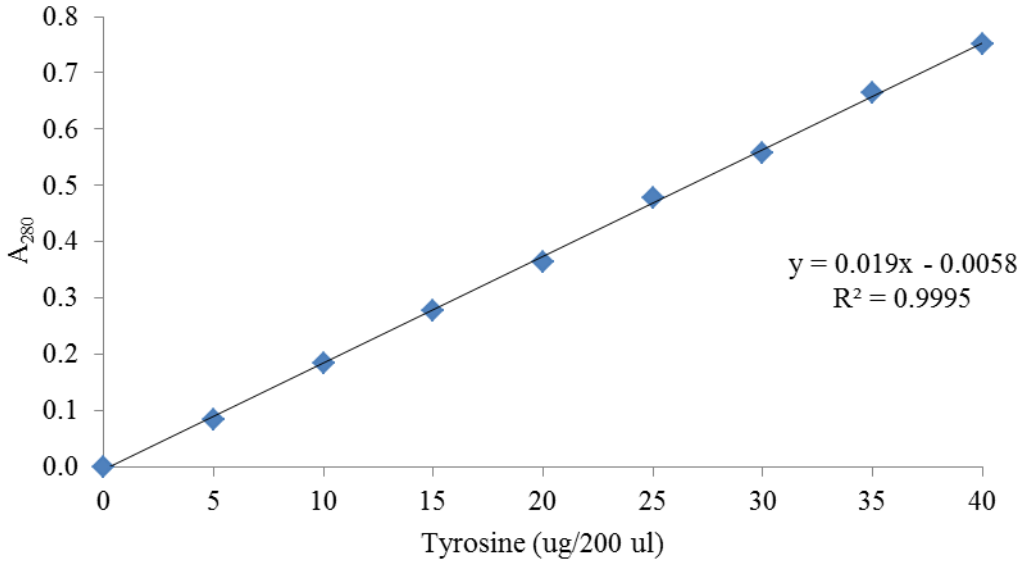
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX B

1. Calculation of the protease activity

1.1. Tyrosine as substrate

1.1.1. Calibration curve for determination of protease activity by using tyrosine as substrate



1.1.2. Calculation of protease activity

The absorbance value at 280 nm was calculated by:

$$y = 0.019x-0.005$$

where x is tyrosine content

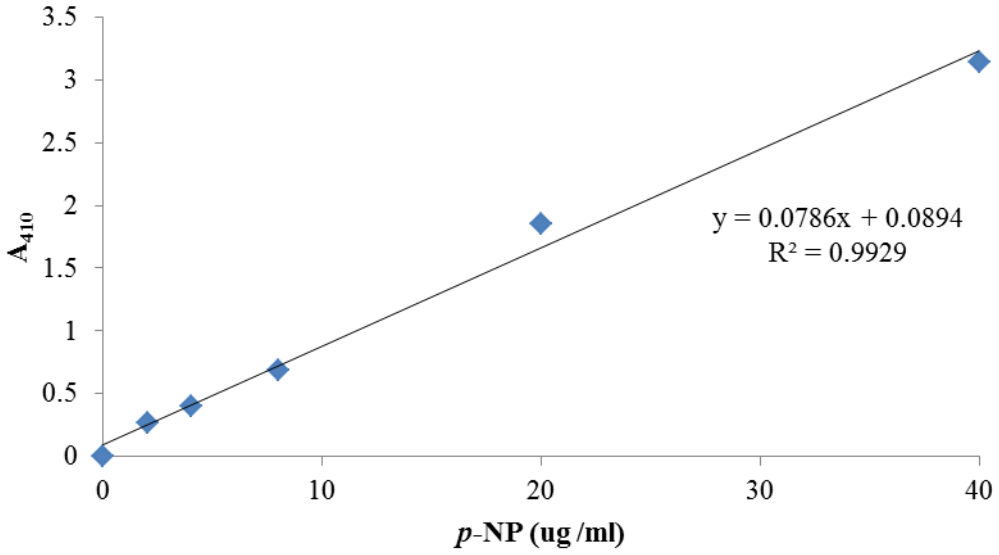
y is Absorbance at 280 nm

One unit of the proteolytic activity was defined as the amount of enzyme required to liberate 1 µg tyrosine per ml in 1 min under standard conditions

2. Calculation of the lipase activity

2.1. *p*-nitrophenyl palmitate (*p*-NPP) as substrate

2.1.1. Calibration curve for determination of lipase activity by using *p*-nitrophenol (*p*-NP) as product



2.1.2. Calculation of lipase activity

The absorbance value at 410 nm was calculated by:

$$y = 0.078x+0.089$$

where x is *p*-NP content

y is Absorbance at 410 nm

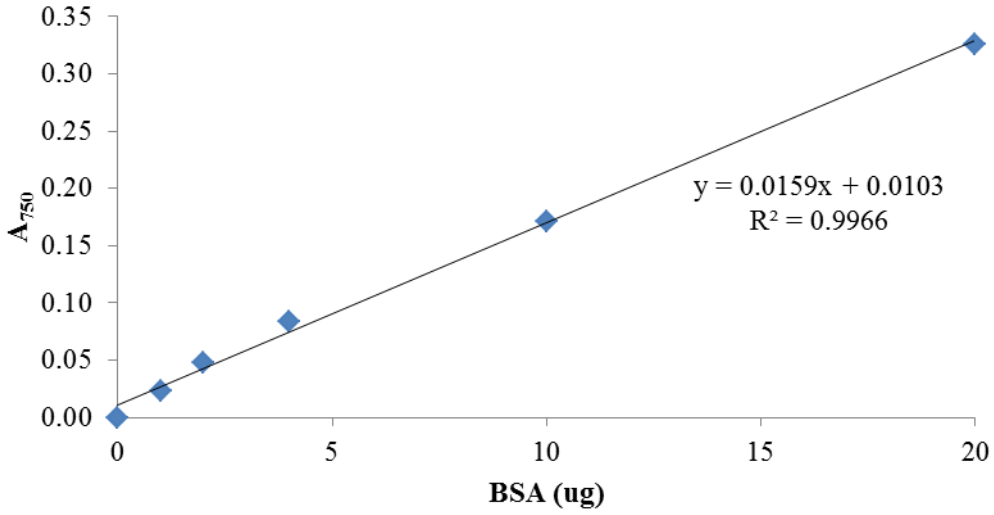
One unit of enzyme corresponds to the release of 1 µg of *p*-NP liberated per minute under the experimental conditions

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. Calculation of protein content

3.1. Protein determination by Lowry's method

3.1.1. Standard curve of BSA



3.1.2. Calculation of protein content

The absorbance value at 750 nm was calculated by:

$$y = 0.015x + 0.010$$

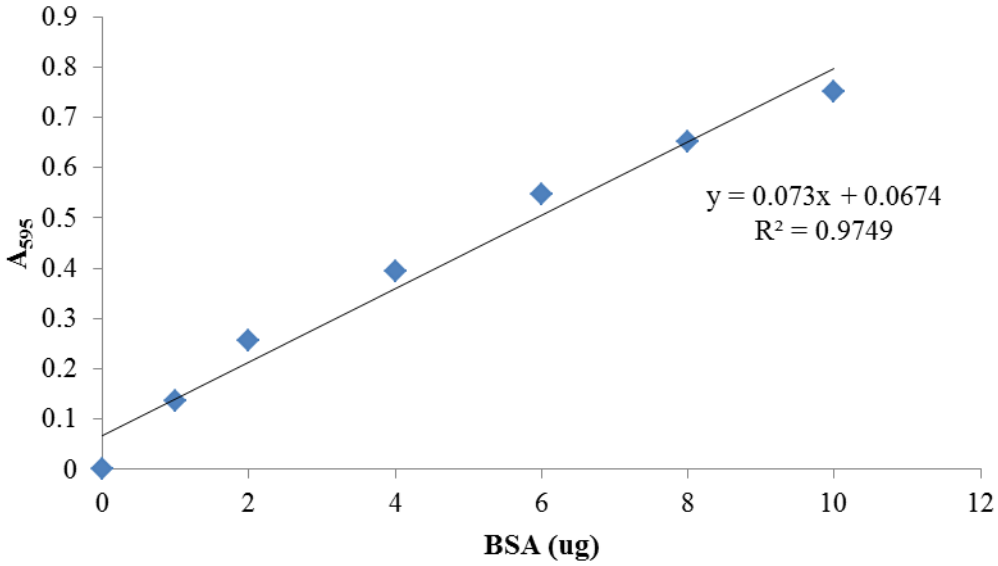
where x is standard protein content (ug)

y is Absorbance at 750 nm

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2. Protein determination by Bradford's method

3.2.1. Standard curve of BSA



3.2.2. Calculation of protein content

The absorbance value at 595 nm was calculated by:

$$y = 0.073x + 0.0674$$

where x is standard protein content (ug)

y is Absorbance at 595 nm

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX C

Reagents

1. Protease assay

1.1. Substrate solution

Casein	1	g
100mM Tris-HCl	100	ml

Heat this solution until dissolved and then add distilled water to a total volume of 100 ml

1.2. Stop solution

TCA	5	g

Add distilled water to a total volume of 100 ml

2. Lipase assay

2.1. Substrate solution

p-NPP	0.076	g
95% Ethanol	20	ml

2.2. Buffer solution

Triton X-100	0.4	ml

Add 50mM Tris-HCl pH9 to a total volume of 100 ml

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. Protein determination by Lowry's method

3.1. Reagent A

Na_2Co_3	20	g
SDS	5	g

Add 0.1M NaOH to a total volume of 1000 ml

3.2. Reagent B

$CuSO_4.5H_2O$	0.5	g
1% $KNaC_4H_4O_6$	100	ml

3.3. Reagent C

Folin-Ciocalteu reagent	10	ml
Distilled water	10	ml

4. Protein determination by Bradford's method

4.1. Bradford stock solution

Comassied Brilliant Blue G	350	mg
88% phosphoric acid	200	ml
95% ethanol	100	ml

4.2. Bradford working solution

Bradford stock solution	30	ml
88% phosphoric acid	30	ml
95% ethanol	15	ml
Distilled water	425	ml

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX D

Buffers and reagents for SDS-PAGE

1. Stock solutions

1.1. 2M Tris-HCl (pH 8.8), 100ml

Tris base	24.2	g
Distilled water	50	ml

Add concentrated HCl slowly to pH 8.8 and then add distilled water to a total volume of 100 ml

1.2. 1M Tris-HCl (pH 6.8), 100ml

Tris base	12.1	g
Distilled water	50	ml

Add concentrated HCl slowly to pH 6.8 and then add distilled water to a total volume of 100 ml

1.3. 10% SDS (w/v), 100 ml

SDS	10	g

Add distilled water to a total volume of 100 ml

1.4. 50% glycerol (v/v), 100 ml

Glycerol	50	ml
Distilled water	50	ml

1.5. 1% bromophenol blue (w/v), 10 ml

Bromophenol blue	100	mg

Bring to 10 ml with distilled water, stir until dissolved
Filtration will remove aggregated dye.

2. Working solutions

2.1. Solution A (acrylamide Stock solution), 100 ml

Acrylamide	29.2	g
Bis-acrylamide	0.8	g

Add distilled water to make 100 ml and stir until completely dissolved.
Working under hood and keep acrylamide solution covered with Parafilm until acrylamide powder is completely dissolved.

2.2. Solution B (4× separating gel buffer), 100 ml

2M Tris-HCl (pH8.8)	75	ml (1.5M)
10% SDS	4	ml (0.4%)
H_2O	21	ml

2.3. Solution C (4× stacking gel buffer), 100 ml

1M Tris-HCl (pH6.8)	50	ml (0.5M)
10% SDS	4	ml (0.4%)
H_2O	46	ml

2.4. 10% ammonium persulfate, 5 ml

Ammonium persulfate	0.5	g
H_2O	5	ml

2.5. Electrophoresis buffer, 1 L

Tris base	3	g (25mM)
Glycine	14.4	g (192mM)
SDS	1	g (0.1%)

H_2O to make 1 L

pH should be approximately 8.3.

2.6. 5× sample buffer, 10 ml

1M Tris-HCl (pH6.8)	0.6	ml (60mM)
50% glycerol	5	ml (25%)
10% SDS	2	ml (2%)
2-mercaptoethanol	0.5	ml (14.4mM)
1% bromophenol blue	1	ml (0.1%)
H_2O	0.9	ml

3. 5% stacking gel, 4 ml

Solution C	1	ml
H_2O	2.3	ml
Solution A	0.67	ml
10% ammonium persulfate	30	ul
TEMED	5	ul

4. 12% separating gel, 10 ml

Solution B	2.5	ml
H_2O	3.5	ml
Solution A	4	ml
10% ammonium persulfate	50	ul
TEMED	5	ul

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX E

1. Effect of carbon source on concomitant production

Table 30 Effect of different carbon sources (1.0%, w/v) on the production of protease and lipase, and cells growth by *B. licheniformis* 3C5 (data in Figure 16) (n=3)

Carbon sources	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
Citric acid	8.82 ± 1.97	0.39 ± 0.55	0.12 ± 0.10
Glucose	200.97 ± 24.77	26.60 ± 2.44	1.96 ± 0.22
Glycerol	214.00 ± 11.90	24.07 ± 0.51	1.68 ± 0.33
Lactose	200.66 ± 4.71	9.45 ± 0.64	1.88 ± 0.15
Maltose	168.04 ± 8.36	6.34 ± 1.22	2.11 ± 0.04
Mannitol	183.50 ± 6.06	4.12 ± 0.87	1.86 ± 0.14
Soluble starch	177.83 ± 12.97	19.34 ± 0.79	1.95 ± 0.07
Xylose	171.69 ± 13.57	12.73 ± 1.29	2.02 ± 0.02
Control	86.03 ± 1.83	2.78 ± 0.1	1.30 ± 0.01

Control: medium without carbon source (MSBYO)

Table 31 Comparative production profile of *B. licheniformis* 3C5 in different concentrations of glucose (data in Figure 17) (n=3)

Glucose concentration (%, w/v)	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
0.2	141.52 ± 1.89	6.67 ± 0.12	1.30 ± 0.04
1.0	200.97 ± 24.77	26.60 ± 2.44	1.96 ± 0.22
2.0	234.65 ± 1.70	21.05 ± 0.31	1.68 ± 0.11

Statistical analysis of effect of glucose concentration on enzymes production

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
G_protease	299.342	2	5	.000
G_lipase	7.819	2	5	.029
G_growth	13.270	2	5	.010

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
G_protease	Between Groups	9004.262	2	4502.131	12.187	.012
	Within Groups	1847.035	5	369.407		
	Total	10851.297	7			
G_lipase	Between Groups	530.940	2	265.470	73.755	.000
	Within Groups	17.997	5	3.599		
	Total	548.937	7			
G_growth	Between Groups	.584	2	.292	9.100	.022
	Within Groups	.160	5	.032		
	Total	.744	7			

Multiple Comparisons

Dependent Variable		(I) Glucose	(J) Glucose	Mean Difference (I-J)	Std. Error	95% Confidence Interval	
						Lower Bound	Upper Bound
G_protease	Dunnett C	0.2%	1.0	-59.44652*	12.45652	-113.6300	-5.2630
			2.0%	-93.12500*	1.79506	-127.3650	-58.8850
		1.0	0.2%	59.44652*	12.45652	5.2630	113.6300
			2.0%	-33.67848	12.44277	-87.3972	20.0403
		2.0%	0.2%	93.12500*	1.79506	58.8850	127.3650
			1.0	33.67848	12.44277	-20.0403	87.3972
G_lipase	Dunnett C	0.2%	1.0	-19.92162*	1.22395	-25.1241	-14.7191
			2.0%	-14.37000*	.23119	-18.7799	-9.9601
		1.0	0.2%	19.92162*	1.22395	14.7191	25.1241
			2.0%	5.55162	1.23978	-.1845	11.2877
		2.0%	0.2%	14.37000*	.23119	9.9601	18.7799
			1.0	-5.55162	1.23978	-11.2877	.1845
G_growth	Dunnett C	0.2%	1.0	-.65917*	.11458	-1.2465	-.0718
			2.0%	-.38385	.08042	-1.9179	1.1502
		1.0	0.2%	.65917*	.11458	.0718	1.2465
			2.0%	.27533	.13388	-.9108	1.4615
		2.0%	0.2%	.38385	.08042	-1.1502	1.9179
			1.0	-.27533	.13388	-1.4615	.9108

*. The mean difference is significant at the 0.05 level.

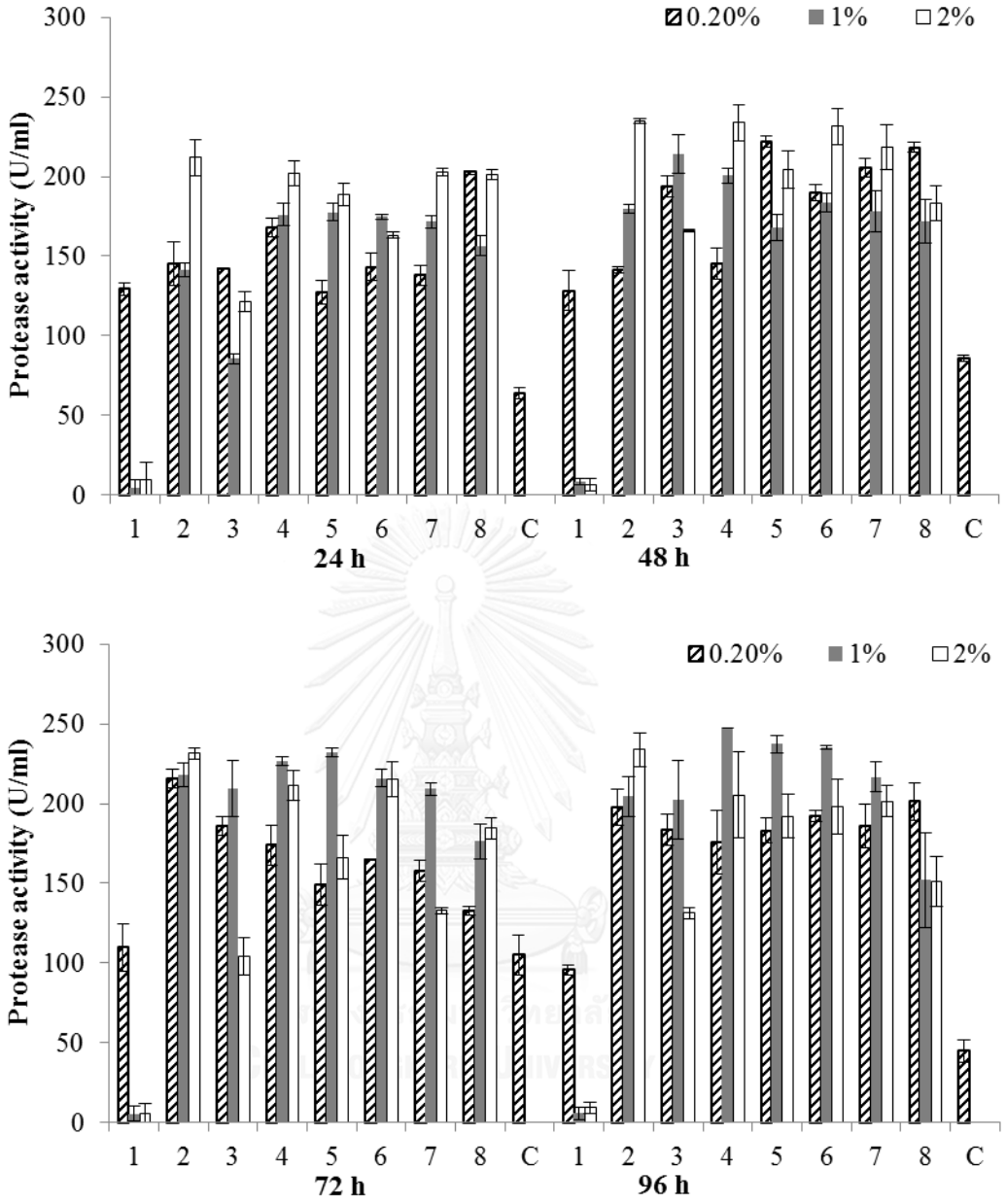


Figure 34 Effect of different carbon sources on the production of protease of *B. licheniformis* 3C5 in 96h. The carbon sources were (1) citric acid, (2) glucose, (3) glycerol, (4) lactose, (5) maltose, (6) mannitol, (7) soluble starch, (8) xylose and (C) medium without carbon source (MSBYO). (n=3)

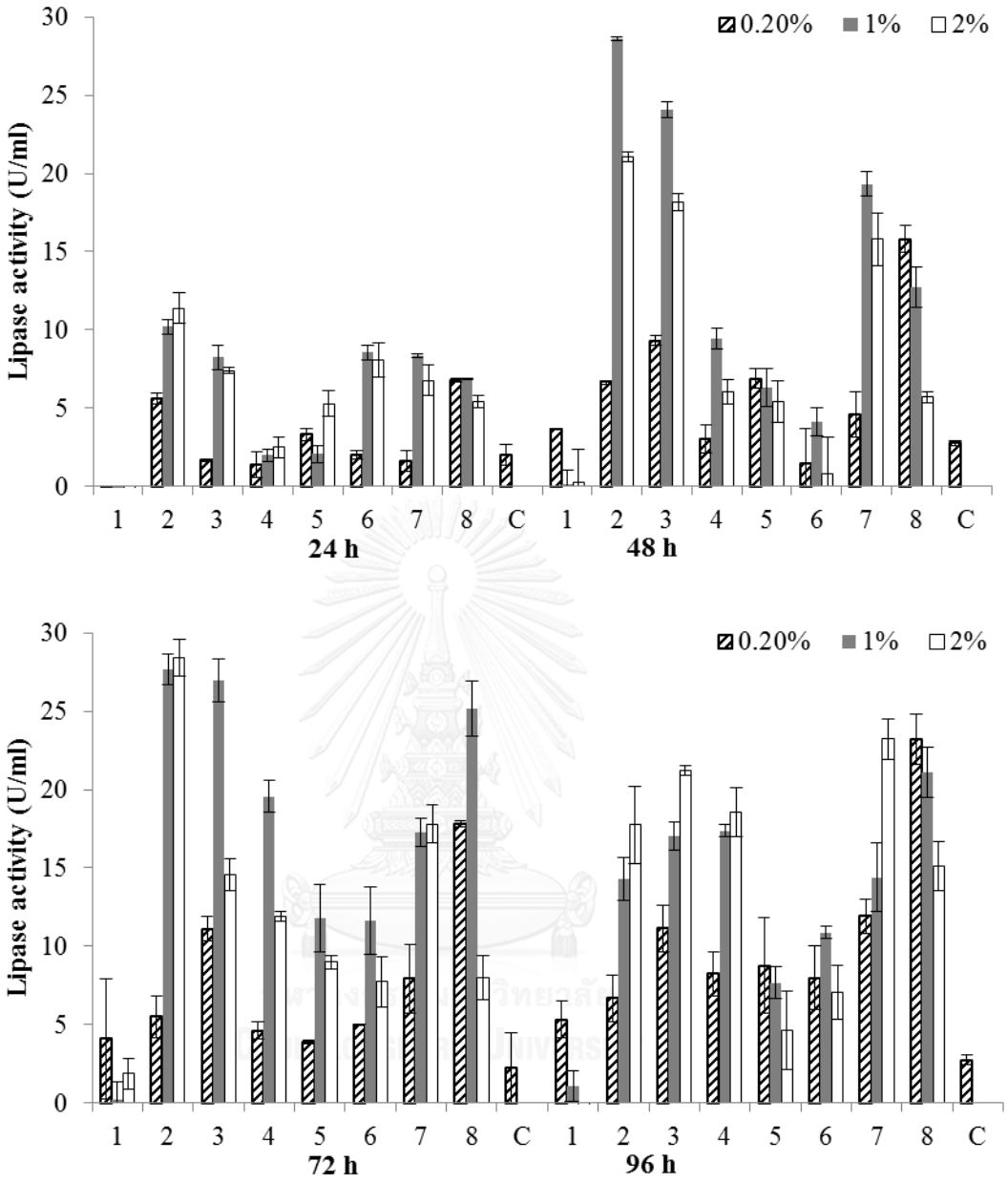


Figure 35 Effect of different carbon sources on the production of lipase of *B. licheniformis* 3C5 in 96h. The carbon sources were (1) citric acid, (2) glucose, (3) glycerol, (4) lactose, (5) maltose, (6) mannitol, (7) soluble starch, (8) xylose and (C) medium without carbon source (MSBYO). (n=3)

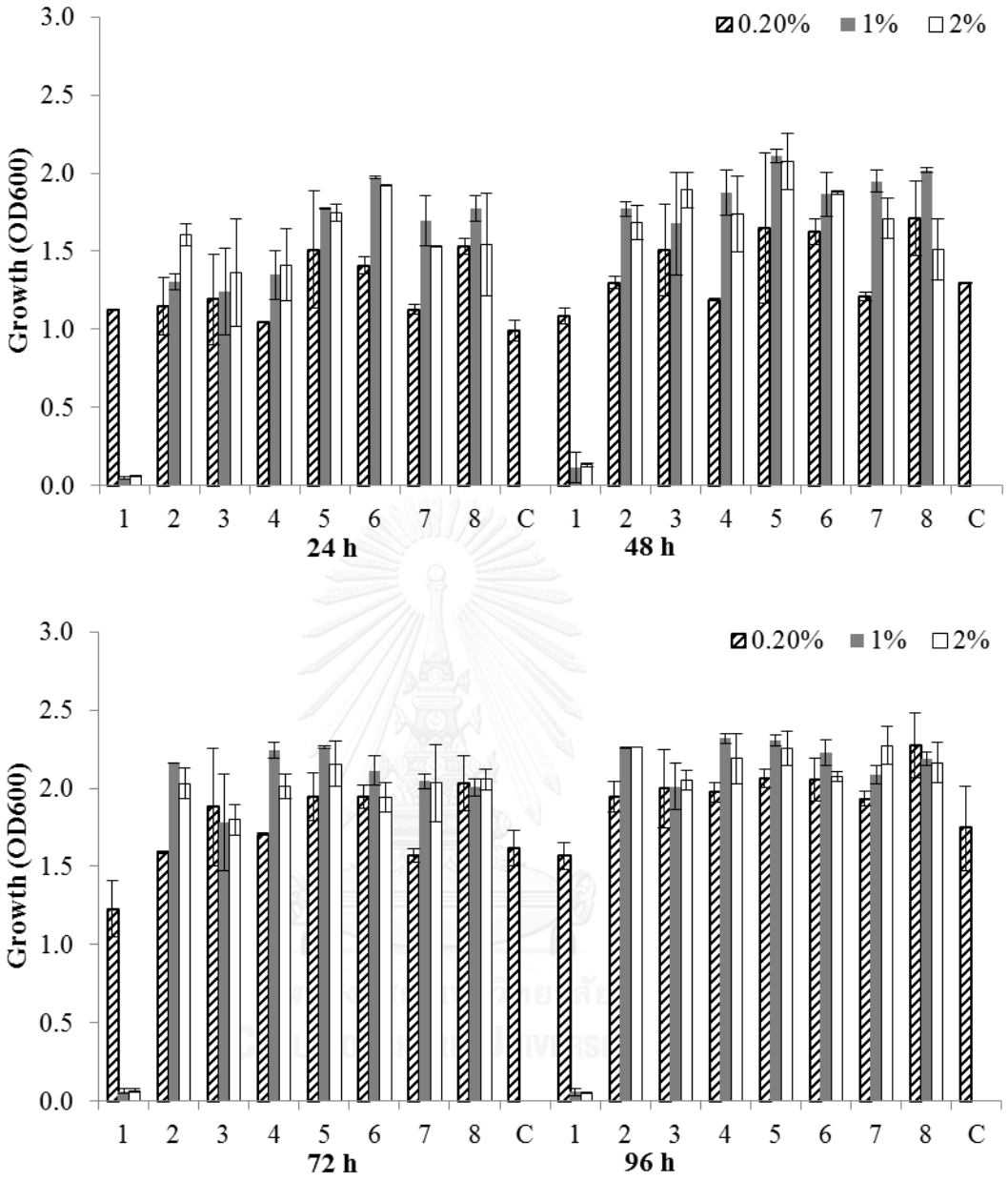


Figure 36 Effect of different carbon sources on cell growth of protease of *B. licheniformis* 3C5 in 96h. The carbon sources were (1) citric acid, (2) glucose, (3) glycerol, (4) lactose, (5) maltose, (6) mannitol, (7) soluble starch, (8) xylose and (C) medium without carbon source (MSBYO). (n=3)

2. Effect of nitrogen source on concomitant production

Table 32 Effect of different nitrogen sources (0.5%, w/v) on the production of protease and lipase and cells growth of *B. licheniformis* 3C5 (data in Figure 20) (n=3)

Nitrogen sources	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
Casein	0.00 ± 0.00	5.35 ± 0.97	2.06 ± 0.20
Peptone	172.78 ± 14.62	18.56 ± 1.37	2.10 ± 0.02
Tryptone	170.37 ± 28.89	21.30 ± 0.93	2.18 ± 0.06
Yeast extract	200.97 ± 24.77	26.60 ± 2.44	1.96 ± 0.22
Control	0.00 ± 0.00	5.68 ± 1.78	1.77 ± 0.15

Control: the production medium with glucose 1% (w/v) and without nitrogen source.

Table 33 Effect of different yeast extract concentration on the production of protease and lipase and cells growth of *B. licheniformis* 3C5 (data in Figure 21) (n=3)

Yeast extract concentration (%, w/v)	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
0.2	139.44 ± 23.40	13.60 ± 0.09	2.27 ± 0.18
0.5	200.97 ± 24.77	26.60 ± 2.44	1.96 ± 0.22
1.0	173.72 ± 31.35	22.85 ± 1.55	2.17 ± 0.23

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Statistical analysis of effect of yeast extract concentration on enzymes production

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
YE_p	14.723	2	5	.008
YE_l	5.821	2	5	.049
YE_g	1.530	2	5	.303

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
YE_p	Between Groups	5115.743	2	2557.871	3.794	.099
	Within Groups	3371.383	5	674.277		
	Total	8487.126	7			
YE_l	Between Groups	225.787	2	112.894	27.805	.002
	Within Groups	20.301	5	4.060		
	Total	246.089	7			
YE_g	Between Groups	.143	2	.072	1.529	.303
	Within Groups	.234	5	.047		
	Total	.377	7			

Multiple Comparisons

Dependent Variable		(I) YE	(J) YE	Mean Difference (I-J)	Std. Error	95% Confidence Interval	
						Lower Bound	Upper Bound
YE_p	Dunnett C	1.00	2.00	-61.52778	20.67043	-345.2811	222.2256
			3.00	-34.27632	27.66674	-562.0085	493.4559
		2.00	1.00	61.52778	20.67043	-222.2256	345.2811
			3.00	27.25146	25.39546	-367.1945	421.6974
		3.00	1.00	34.27632	27.66674	-493.4559	562.0085
			2.00	-27.25146	25.39546	-421.6974	367.1945
YE_l	Dunnett C	1.00	2.00	-12.99822*	1.22268	-18.1537	-7.8428
			3.00	-9.25178	1.09717	-30.1799	11.6764
		2.00	1.00	12.99822*	1.22268	7.8428	18.1537
			3.00	3.74644	1.64047	-14.0049	21.4977
		3.00	1.00	9.25178	1.09717	-11.6764	30.1799
			2.00	-3.74644	1.64047	-21.4977	14.0049
YE_g	Dunnett C	1.00	2.00	.30833	.17063	-1.8733	2.4899
			3.00	.10145	.20772	-3.8607	4.0636
		2.00	1.00	-.30833	.17063	-2.4899	1.8733
			3.00	-.20687	.19651	-3.0235	2.6098
		3.00	1.00	-.10145	.20772	-4.0636	3.8607
			2.00	.20687	.19651	-2.6098	3.0235

*. The mean difference is significant at the 0.05 level.

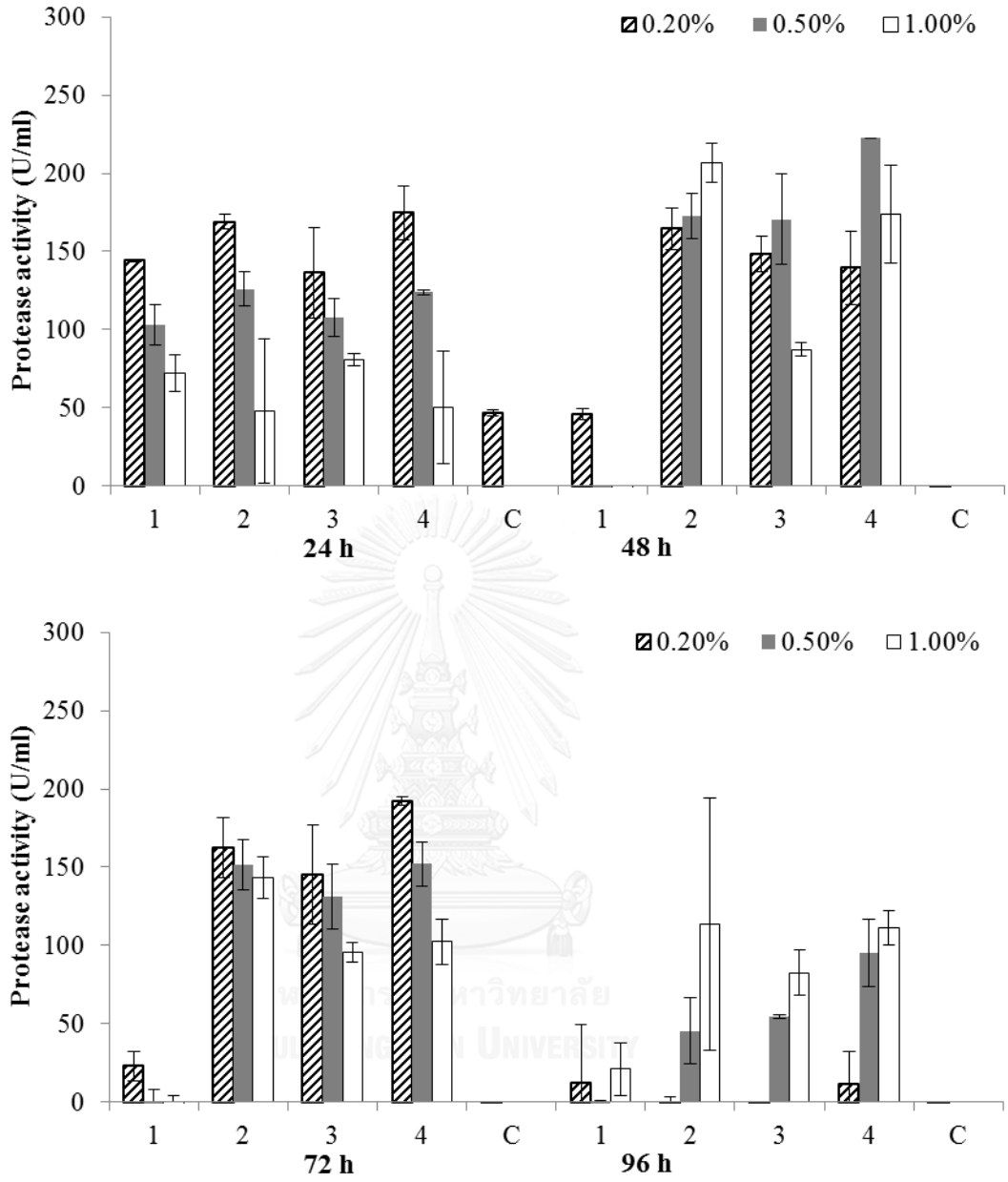


Figure 37 Effect of different nitrogen sources on the production of protease of *B. licheniformis* 3C5 in 96h. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

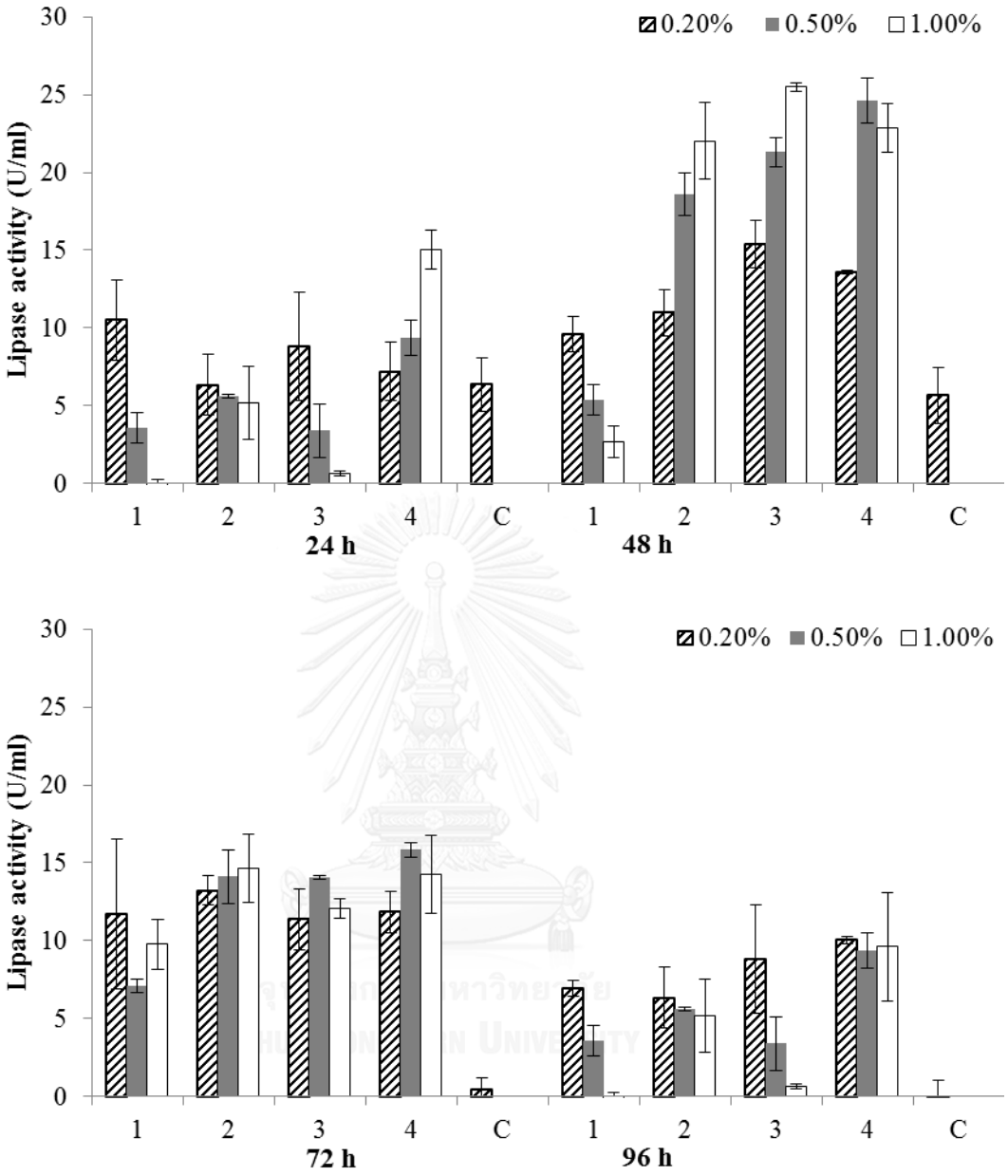


Figure 38 Effect of different nitrogen sources on the production of lipase of *B. licheniformis* 3C5 in 96h. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

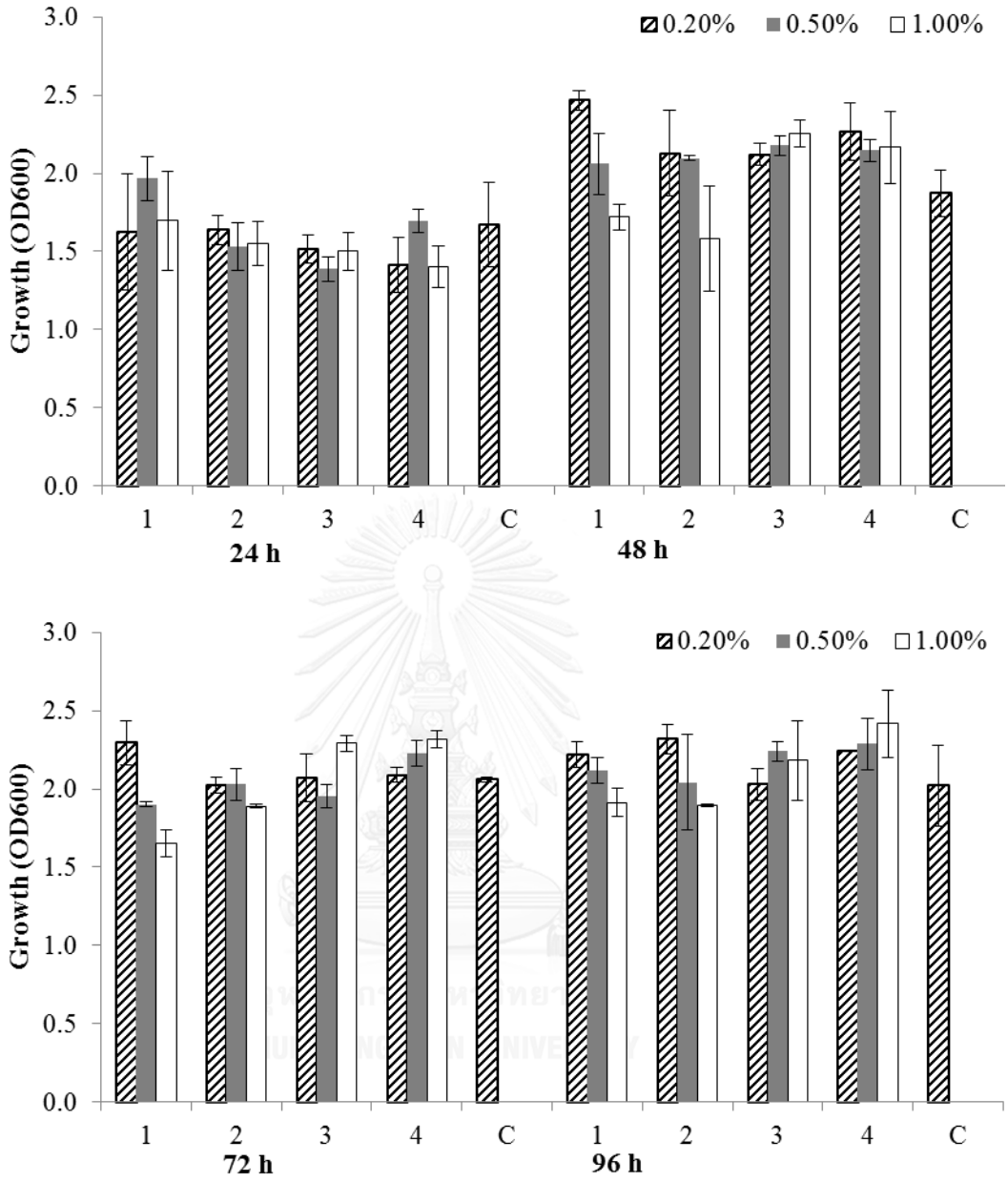


Figure 39 Effect of different nitrogen sources on cell growth of protease of *B. licheniformis* 3C5 in 96h. The nitrogen sources were (1) casein, (2) peptone, (3) tryptone, (4) yeast extract and (C) production medium with glucose 1% (w/v) and without nitrogen source. (n=3)

3. Effect of inducer on concomitant production

Table 34 Effect of different concentration of olive oil on cell growth and enzymes production by *B. licheniformis* 3C5 (data in Figure 24) (n=3)

Olive oil concentration (%, v/v)	Protease activity (U/ml)	Lipase activity (U/ml)	Growth (OD_{600})
0.50	209.27 ± 6.68	6.68 ± 2.54	1.42 ± 0.04
1.00	177.22 ± 6.33	13.52 ± 3.67	1.73 ± 0.21
1.50	189.55 ± 26.37	24.42 ± 3.94	1.94 ± 0.19

Statistical analysis of effect of olive oil concentration on enzymes production

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
O_p	4.159	2	7	.065
O_l	.612	2	7	.569
O_g	6.368	2	7	.027

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
O_p	Between Groups	1059.702	2	529.851	1.041	.402
	Within Groups	3562.335	7	508.905		
	Total	4622.037	9			
O_l	Between Groups	539.079	2	269.539	19.344	.001
	Within Groups	97.536	7	13.934		
	Total	636.615	9			
O_g	Between Groups	.419	2	.210	6.525	.025
	Within Groups	.225	7	.032		
	Total	.644	9			

Multiple Comparisons

Dependent Variable		(I) O	(J) O	Mean Difference (I-J)	Std. Error	95% Confidence Interval	
						Lower Bound	Upper Bound
O_p	Dunnett C	1.00	2.00	32.04678	6.50486	-92.0309	156.1245
			3.00	19.71491	11.75683	-48.5482	87.9780
		2.00	1.00	-32.04678	6.50486	-156.1245	92.0309
			3.00	-12.33187	11.65922	-77.4273	52.7635
		3.00	1.00	-19.71491	11.75683	-87.9780	48.5482
			2.00	12.33187	11.65922	-52.7635	77.4273
O_l	Dunnett C	1.00	2.00	-6.84224	3.15316	-66.9874	53.3030
			3.00	-17.74276	2.40985	-46.7170	11.2315
		2.00	1.00	6.84224	3.15316	-53.3030	66.9874
			3.00	-10.90052	3.05152	-55.6875	33.8865
		3.00	1.00	17.74276	2.40985	-11.2315	46.7170
			2.00	10.90052	3.05152	-33.8865	55.6875
O_g	Dunnett C	1.00	2.00	-.30680	.14945	-3.1575	2.5439
			3.00	-.52140*	.08231	-.9372	-.1056
		2.00	1.00	.30680	.14945	-2.5439	3.1575
			3.00	-.21460	.16604	-2.8096	2.3804
		3.00	1.00	.52140*	.08231	.1056	.9372
			2.00	.21460	.16604	-2.3804	2.8096

*. The mean difference is significant at the 0.05 level.

O_l

	O	N	Subset for alpha = 0.05	
			1	2
Duncan[a,b]	1.00	2	6.6809	
	2.00	2	13.5231	
	3.00	6		24.4237
	Sig.		.076	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

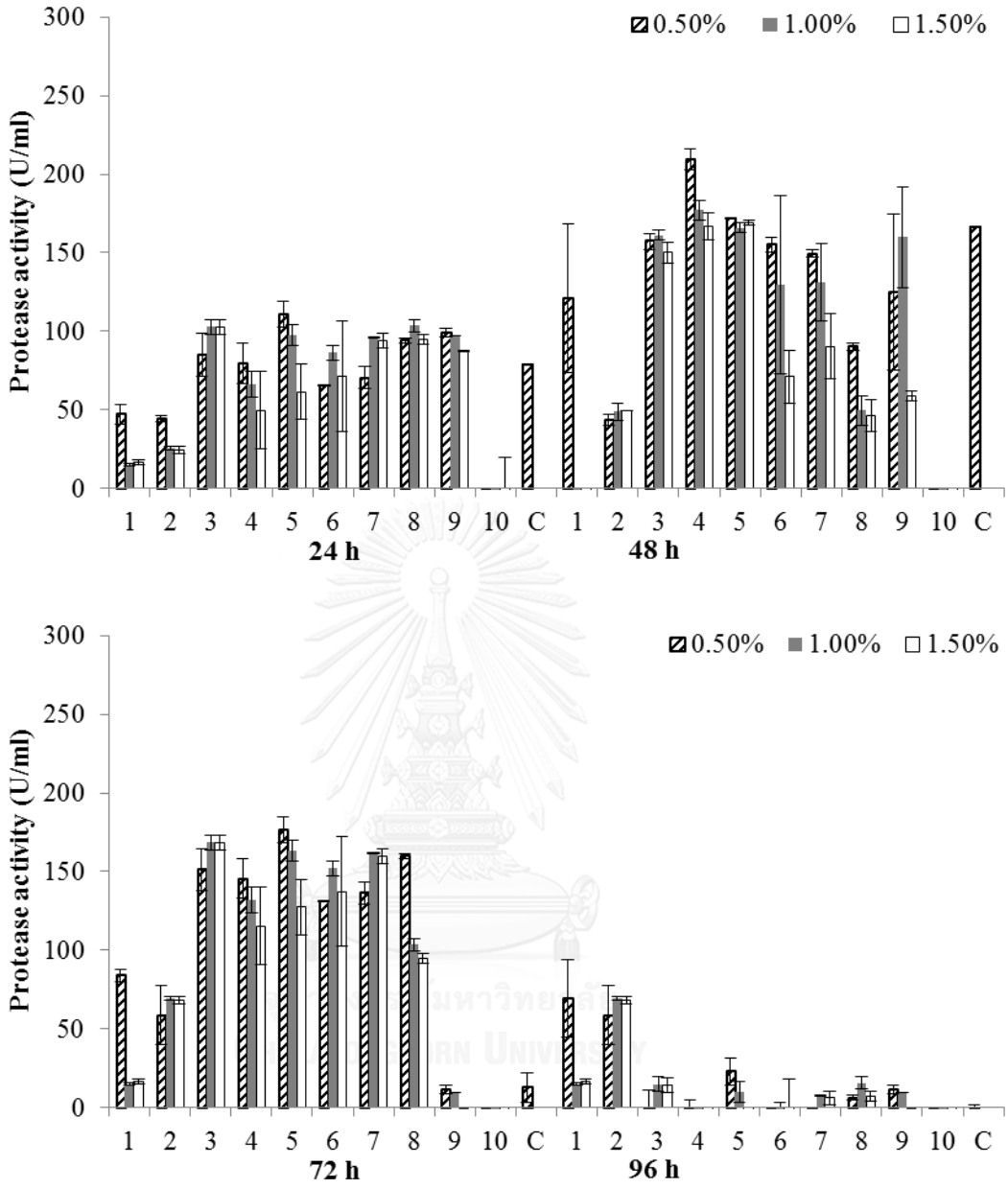


Figure 40 Effect of different inducers on the production of protease of *B. licheniformis* 3C5 in 96 h. The inducers were (1) Tributyrin, (2) Coconut oil, (3) Palm oil, (4) Olive oil, (5) Soybean oil, (6) Sunflower oil, (7) Sesame oil, (8) Tween 20, (9) Tween 80, (10) Triton X-100 and (C) the production medium with 1%glucose and 0.5%yeast extract without inducer. (n=3)

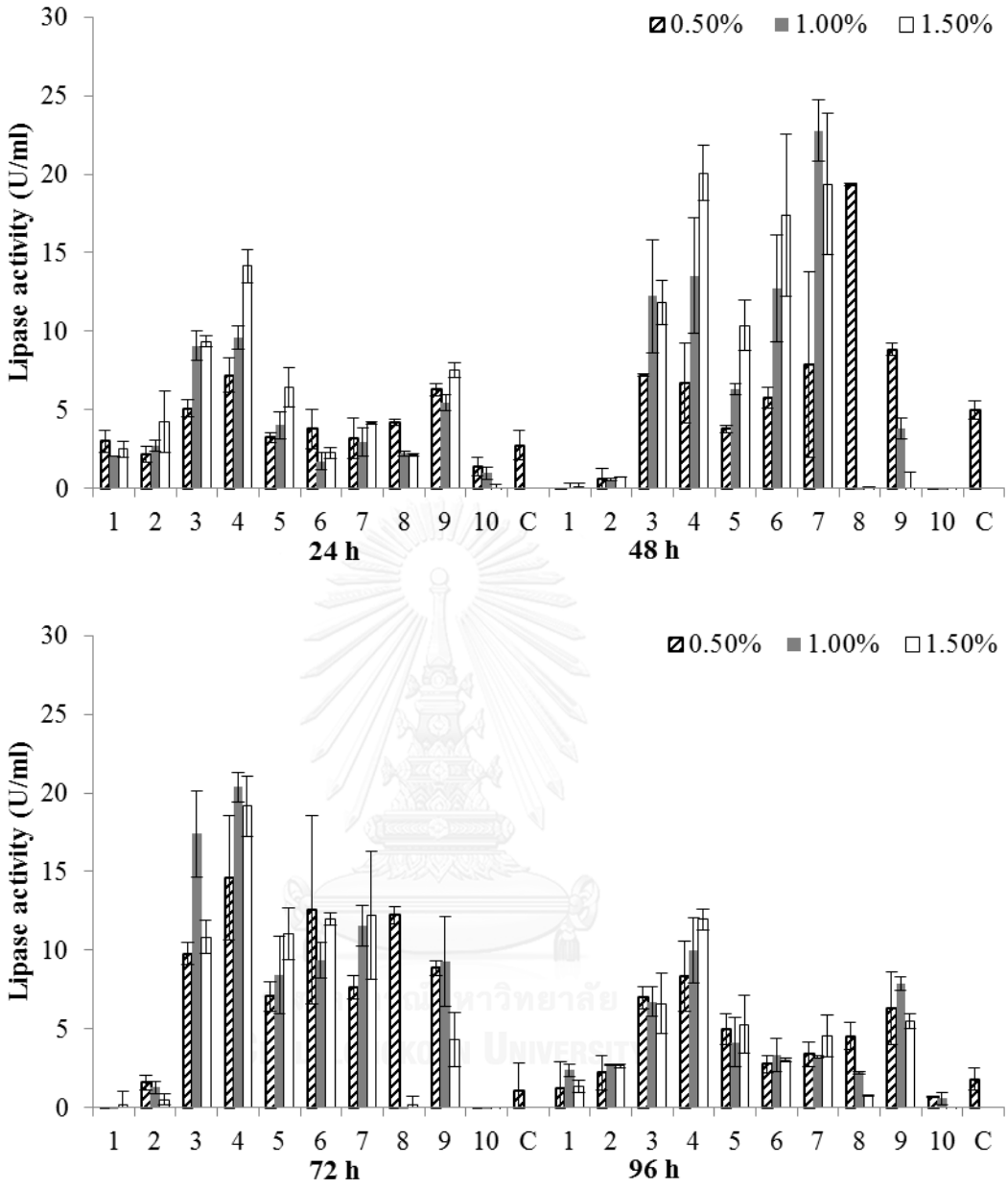


Figure 41 Effect of different inducers on the production of lipase of *B. licheniformis* 3C5 in 96 h. The inducers were (1) Tributyrin, (2) Coconut oil, (3) Palm oil, (4) Olive oil, (5) Soybean oil, (6) Sunflower oil, (7) Sesame oil, (8) Tween 20, (9) Tween 80, (10) Triton X-100 and (C) the production medium with 1%glucose and 0.5%yeast extract without inducer. (n=3)

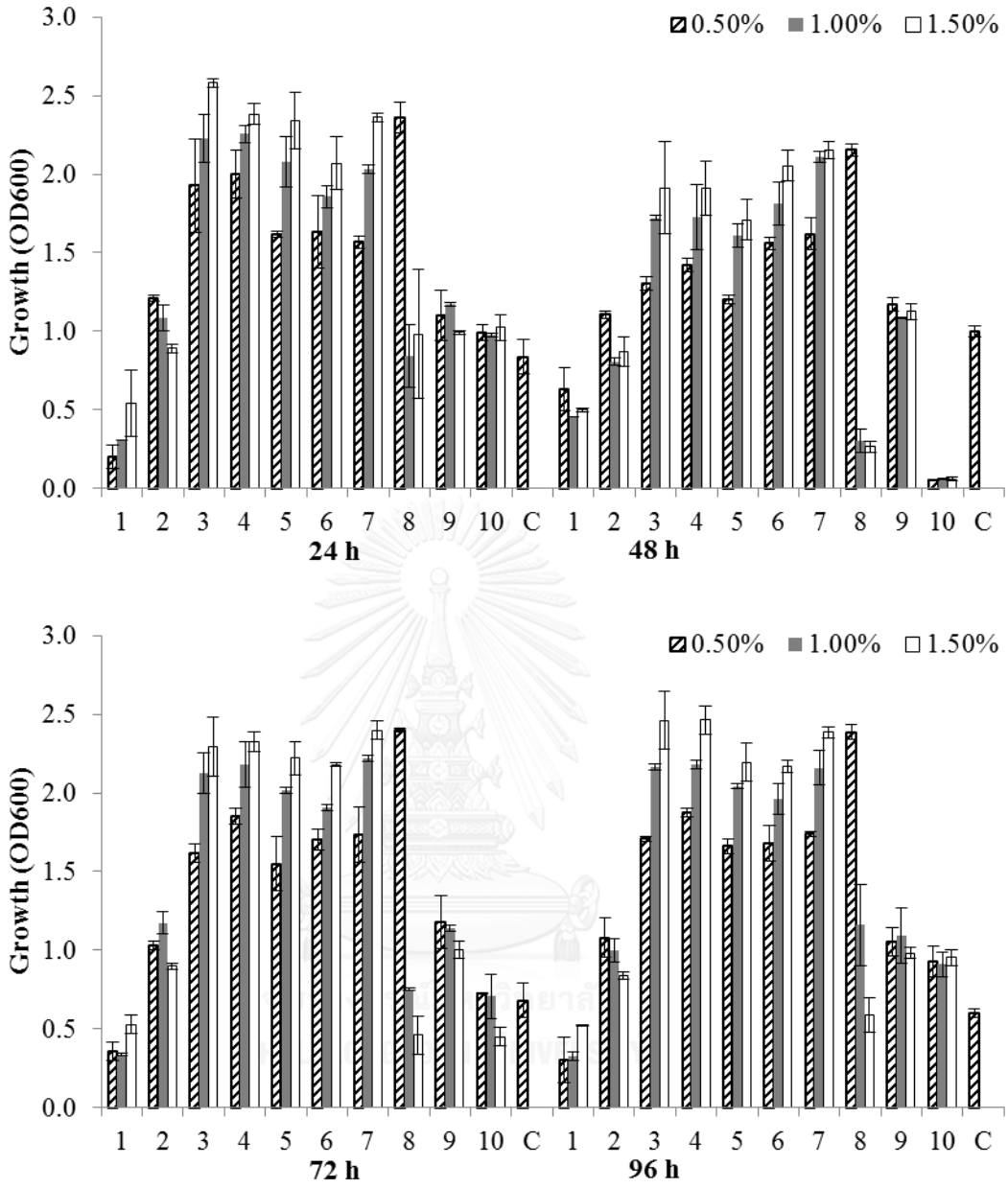
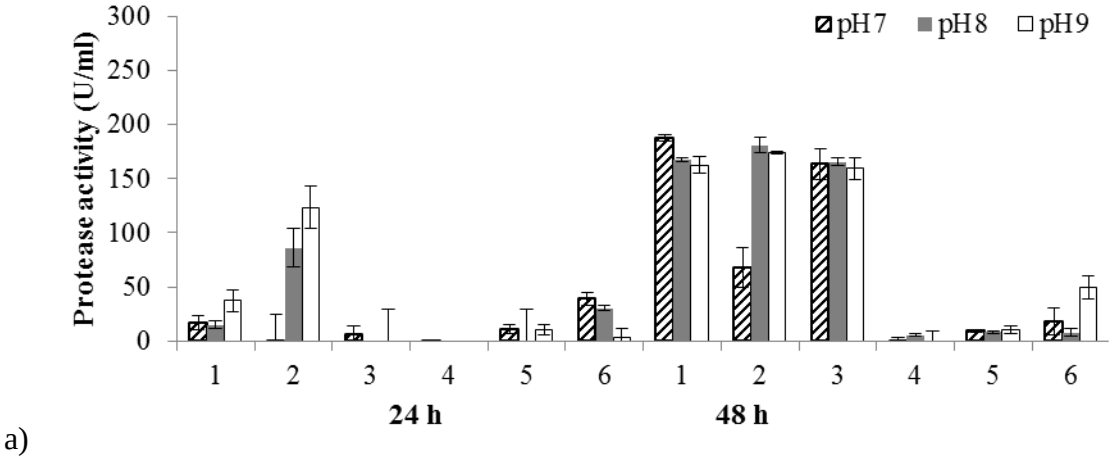
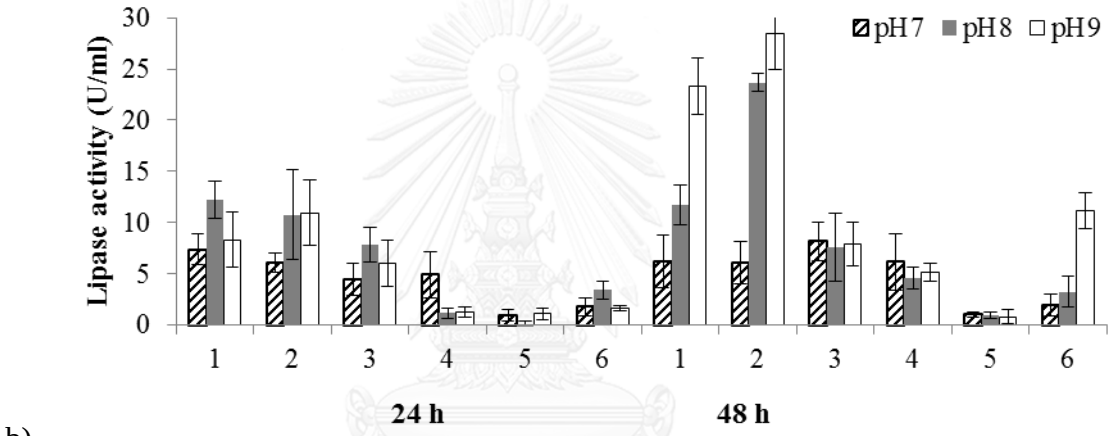


Figure 42 Effect of different inducers on cell growth of protease of *B. licheniformis* 3C5 in 96h. The inducers were (1) Tributyrin, (2) Coconut oil, (3) Palm oil, (4) Olive oil, (5) Soybean oil, (6) Sunflower oil, (7) Sesame oil, (8) Tween 20, (9) Tween 80, (10) Triton X-100 and (C) the production medium with 1%glucose and 0.5%yeast extract without inducer. (n=3)

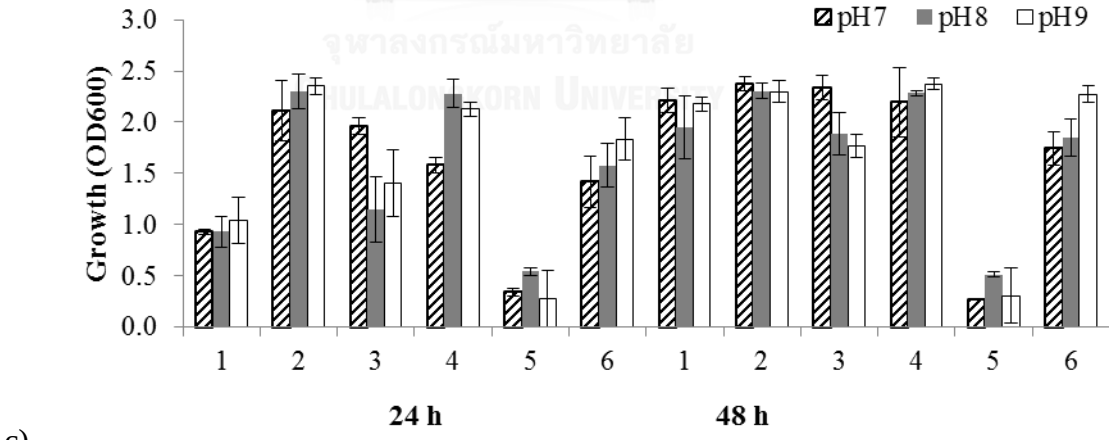
4. Effect of physical factor on concomitant production



a)



b)



c)

Figure 43 Effect of physical factors on the production of protease (a) and lipase (b), and cells growth (c) of *B. licheniformis* 3C5 in 48h. (1) 30 ºC, 120 rpm, (2) 30 ºC, 200 rpm, (3) 37 ºC, 120 rpm, (4) 37 ºC, 200 rpm, (5) 45 ºC, 120 rpm and (6) 45 ºC, 200 rpm (n=3)

5. Effect of NaCl on concomitant production

Statistical analysis of effect of NaCl concentration on enzymes production

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Na_p	.230	3	6	.873
Na_l	1.984	3	6	.218
Na_g	3.728	3	6	.080

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Na_p	Between Groups	279.901	3	93.300	8.714	.013
	Within Groups	64.242	6	10.707		
	Total	344.143	9			
Na_l	Between Groups	521.079	3	173.693	25.206	.001
	Within Groups	41.346	6	6.891		
	Total	562.426	9			
Na_g	Between Groups	1.454	3	.485	240.587	.000
	Within Groups	.012	6	.002		
	Total	1.466	9			

Na_p

	Na	N	Subset for alpha = 0.05	
			1	2
Duncan[a,b]	4.00	2	150.4250	
	1.00	3		161.5333
	3.00	2		163.1300
	2.00	3		164.9767
	Sig.		1.000	.307

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.400.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Na_l

	Na	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a,b]	3.00	2	13.2000		
	4.00	2	14.4050		
	1.00	3		20.7433	
	2.00	3			31.2033
	Sig.		.633	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.400.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

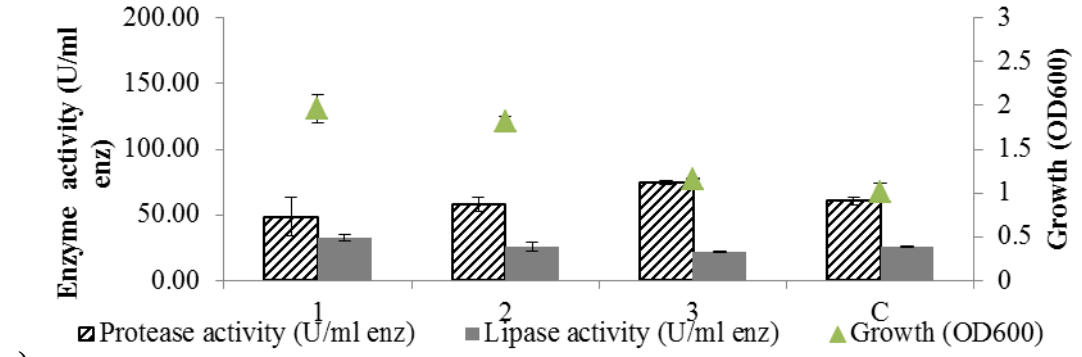
Na_g

	Na	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a,b]	1.00	3	1.5511			
	4.00	2		2.0989		
	3.00	2			2.3331	
	2.00	3				2.4835
	Sig.		1.000	1.000	1.000	1.000

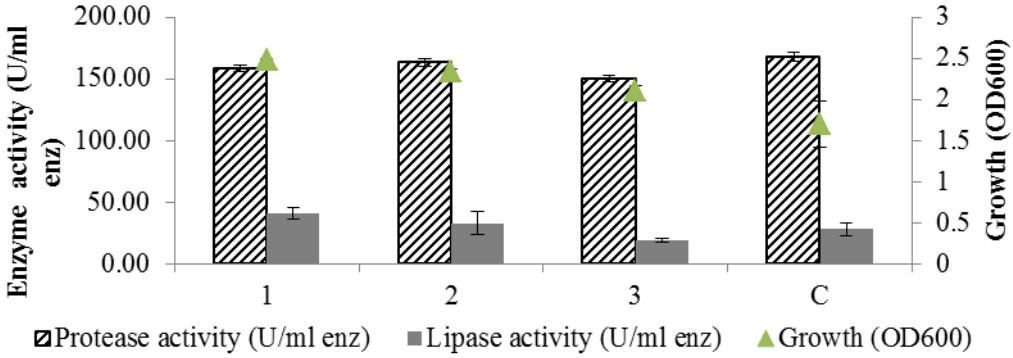
Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.400.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

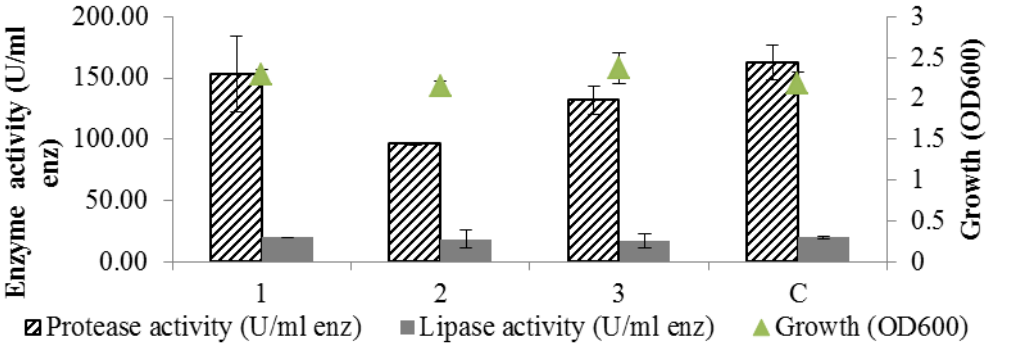
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University



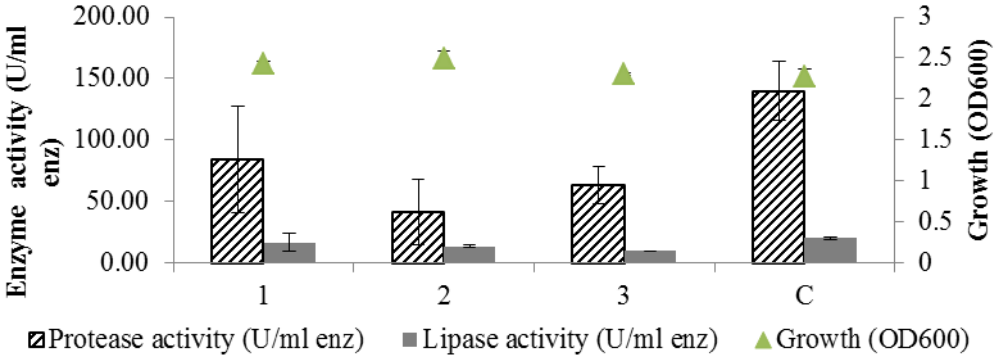
a)



b)



c)



d)

Figure 44 Effect of different concentration of NaCl on cell growth and enzymes production by *B. licheniformis* 3C5 in (a) 24h, (b) 48h, (c) 72h and (d) 96h. (1) 0.25% NaCl, (2) 0.5% NaCl, (3) 1.0% NaCl, (C) without NaCl (n=3)

CCD and RSM of medium optimization for concomitant production

Table 35 Analysis of variance of quadratic model for lipase production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Block	4.51	1	4.51		
Model	914.68	14	65.33	16.65	< 0.0001
A	52.86	1	52.86	13.47	0.0025
B	63.35	1	63.35	16.15	0.0013
C	234.29	1	234.29	59.71	< 0.0001
D	174.90	1	174.90	44.58	< 0.0001
A2	1.61	1	1.61	0.41	0.5321
B2	72.82	1	72.82	18.56	0.0007
C2	119.49	1	119.49	30.45	< 0.0001
D2	30.75	1	30.75	7.84	0.0142
AB	3.32	1	3.32	0.85	0.3730
AC	32.23	1	32.23	8.22	0.0124
AD	51.30	1	51.30	13.07	0.0028
BC	91.27	1	91.27	23.26	0.0003
BD	12.83	1	12.83	3.27	0.0921
CD	16.77	1	16.77	4.27	0.0577
Residual	54.93	14	3.92		
Lack of Fit	50.97	10	5.10	5.15	0.0640
Pure Error	3.96	4	0.99		
Cor Total	974.12	29			

R^2 = 0.94, adjusted R^2 = 0.89, predicted R^2 = 0.68, adequate precision = 15.37, C.V. = 11.93

Table 36 Analysis of variance of quadratic model for protease production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Block	762.94	1	762.94		
Model	40912.84	14	2922.35	11.73	< 0.0001
A	60.46	1	60.46	0.24	0.6299
B	136.54	1	136.54	0.55	0.4713
C	22615.61	1	22615.61	90.80	< 0.0001
D	218.52	1	218.52	0.88	0.3648
A2	64.80	1	64.80	0.26	0.6180
B2	770.94	1	770.94	3.10	0.1003
C2	15959.85	1	15959.85	64.08	< 0.0001
D2	154.11	1	154.11	0.62	0.4446
AB	19.09	1	19.09	0.08	0.7859
AC	698.98	1	698.98	2.81	0.1161
AD	698.98	1	698.98	2.81	0.1161
BC	0.31	1	0.31	0.00	0.9721
BD	0.43	1	0.43	0.00	0.9674
CD	244.31	1	244.31	0.98	0.3388
Residual	3487.01	14	249.07		
Lack of Fit	3175.73	10	317.57	4.08	0.0938
Pure Error	311.28	4	77.82		
Cor Total	45162.79	29			

R^2 = 0.92, adjusted R^2 = 0.84, predicted R^2 = 0.51, adequate precision = 14.43, C.V. = 9.49

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CCFD and RSM of partial purification by using ATPS

Table 37 The regression analysis for %yield of protease

Sequential Model Sum of Squares					
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**P Value**
Mean	158958.83	1.00	158958.83		
Linear	1930.04	3.00	643.35	14.53	< 0.0001
2FI	96.29	3.00	32.10	0.68	0.58
Quadratic	365.02	3.00	121.67	4.93	0.02
Cubic	68.38	4.00	17.09	0.57	0.69
Residual	178.54	6.00	29.76		
Total	161597.09	20.00	8079.85		
Model Summary Statistics					
Source	**SD**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**
Linear	6.65	0.73	0.68	0.59	1082.44
2FI	6.86	0.77	0.66	0.41	1552.19
Quadratic	4.97	0.91	0.82	0.69	808.99
Cubic	5.45	0.93	0.79	-0.01	2659.17

Table 38 The regression analysis for PF of protease

Sequential Model Sum of Squares					
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**P Value**
Mean	1202.01	1.00	1202.01		
Linear	63.06	3.00	21.02	11.39	0.0003
2FI	14.26	3.00	4.75	4.05	0.0310
Quadratic	2.67	3.00	0.89	0.71	0.5690
Cubic	6.25	4.00	1.56	1.47	0.3189
Residual	6.35	6.00	1.06		
Total	1294.61	20.00	64.73		
Model Summary Statistics					
Source	**SD**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**
Linear	1.36	0.68	0.62	0.41	54.71
2FI	1.08	0.84	0.76	0.57	39.94
Quadratic	1.12	0.86	0.74	0.33	61.68
Cubic	1.03	0.93	0.78	-7.85	819.16

Table 39 The regression analysis for %yield of lipase

Sequential Model Sum of Squares					
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**P Value**
Mean	69943.92	1.00	69943.92		
Linear	5252.99	3.00	1751.00	4.78	0.01
2FI	604.11	3.00	201.37	0.50	0.69
Quadratic	4360.48	3.00	1453.49	16.30	0.00
Cubic	387.37	4.00	96.84	1.15	0.42
Residual	504.08	6.00	84.01		
Total	81052.95	20.00	4052.65		
Model Summary Statistics					
Source	**SD**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**
Linear	19.13	0.47	0.37	0.07	10305.15
2FI	20.10	0.53	0.31	-1.80	31098.91
Quadratic	9.44	0.92	0.85	0.65	3875.14
Cubic	9.17	0.95	0.86	-6.88	87496.43

Table 40 The regression analysis for PF of lipase

Sequential Model Sum of Squares					
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**P Value**
Mean	448.89	1.00	448.89		
Linear	63.14	3.00	21.05	4.36	0.02
2FI	8.23	3.00	2.74	0.52	0.68
Quadratic	52.31	3.00	17.44	10.41	0.00
Cubic	8.16	4.00	2.04	1.42	0.33
Residual	8.59	6.00	1.43		
Total	589.33	20.00	29.47		
Model Summary Statistics					
Source	**SD**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**
Linear	2.20	0.45	0.35	0.11	124.91
2FI	2.31	0.51	0.28	-0.91	268.76
Quadratic	1.29	0.88	0.77	0.10	126.00
Cubic	1.20	0.94	0.81	0.03	136.48

Table 41 ANOVA for response surface quadratic model for %yield of protease

Source	Sum of Squares	DF	Mean Square	F Value	P Value
Model	2391.34	9.00	265.70	10.76	0.00
Residual	246.92	10.00	24.69		
Lack of Fit	70.36	5.00	14.07	0.40	0.83
Pure Error	176.56	5.00	35.31		
Corrected Total	2638.26	19.00			

Table 42 ANOVA for response surface quadratic model for PF of protease

Source	Sum of Squares	DF	Mean Square	F Value	P Value
Model	77.33	6.00	12.89	10.97	0.00
Residual	15.27	13.00	1.17		
Lack of Fit	9.58	8.00	1.20	1.05	0.50
Pure Error	5.69	5.00	1.14		
Corrected Total	92.60	19.00			

Table 43 ANOVA for response surface quadratic model for %yield of lipase

Source	Sum of Squares	DF	Mean Square	F Value	P Value
Model	10217.58	9.00	1135.29	12.74	0.00
Residual	891.45	10.00	89.14		
Lack of Fit	458.12	5.00	91.62	1.06	0.48
Pure Error	433.32	5.00	86.66		
Corrected Total	11109.03	19.00			

Table 44 ANOVA for response surface quadratic model for PF of lipase

Source	Sum of Squares	DF	Mean Square	F Value	P Value
Model	123.69	9.00	13.74	8.20	0.00
Residual	16.76	10.00	1.68		
Lack of Fit	8.27	5.00	1.65	0.97	0.51
Pure Error	8.49	5.00	1.70		
Corrected Total	140.44	19.00			

Table 45 Regression coefficient of the full polynomial model representing relationships between %yield of protease and independent factors

Source	Coefficient Estimate	Standard Error	P Value
Intercept	91.49	1.71	
A	7.46	1.57	0.001
B	7.11	1.57	0.001
C	9.32	1.57	0.000
A^2	-0.54	3.00	0.861
B^2	6.31	3.00	0.061
C^2	-10.46	3.00	0.006
AB	-0.49	1.76	0.786
AC	1.34	1.76	0.464
BC	-3.16	1.76	0.102

A: % PEG6000 (w/w); B: % sodium citrate (w/w); C: % KCl (w/w)

Table 46 Regression coefficient of the full polynomial model representing relationships between PF of protease and independent factors

Source	Coefficient Estimate	Standard Error	P Value
Intercept	7.75	0.24	
A	1.52	0.34	0.001
B	-1.74	0.34	0.000
C	0.97	0.34	0.014
AB	-0.88	0.38	0.038
AC	0.17	0.38	0.670
BC	-0.99	0.38	0.023

A: % PEG6000 (w/w); B: % sodium citrate (w/w); C: % KCl (w/w)

Table 47 Regression coefficient of the full polynomial model representing relationships between %yield of lipase and independent factors

Source	Coefficient Estimate	Standard Error	P Value
Intercept	74.13	3.25	
A	-15.29	2.99	0.001
B	-14.88	2.99	0.001
C	8.37	2.99	0.019
A^2	-27.82	5.69	0.001
B^2	-6.33	5.69	0.292
C^2	4.17	5.69	0.481
AB	4.01	3.34	0.257
AC	0.99	3.34	0.774
BC	-7.65	3.34	0.045

A: % PEG6000 (w/w); B: % sodium citrate (w/w); C: % KCl (w/w)

Table 48 Regression coefficient of the full polynomial model representing relationships between PF of lipase and independent factors

Source	Coefficient Estimate	Standard Error	P Value
Intercept	5.93	0.45	
A	-1.24	0.41	0.013
B	-1.90	0.41	0.001
C	1.08	0.41	0.025
A^2	-4.00	0.78	0.001
B^2	0.36	0.78	0.657
C^2	1.26	0.78	0.136
AB	0.60	0.46	0.220
AC	-0.23	0.46	0.621
BC	-0.79	0.46	0.117

A: % PEG6000 (w/w); B: % sodium citrate (w/w); C: % KCl (w/w)

VITA

Miss Jirapinya Sansamak was born on Aug 12, 1990 in Bangkok, Thailand. She graduated with Bachelor's degree of Science in field of Genetics from Faculty of Science, Chulalongkorn University in 2013. Then, she continues her study as a graduate student of Program in Biotechnology, Faculty of Science, Chulalongkorn University. Her present address is 23, Soi Prachasongkhro 24, Prachasongkhro road, Din daeng, Bangkok, Thailand, 10400. Contact number is 085-3296933 and e-mail: nongnooh-pc@hotmail.com.

Conference abstract:

Sansamak, J., Piapukiew, J. and Vangnai, A. S. (2015). Organic solvent-stable alkaline protease and lipase from organic solvent-tolerant Bacillus licheniformis 3C5. Industrial and Hazardous Waste Conference 2015, BITEC, Bangkok, Thailand. Jan 31, 2015. Poster no. P-08.

Conference proceeding:

Sansamak, J., Piapukiew, J. and Vangnai, A. S. (2015). Partitioning of protease from Bacillus licheniformis 3C5 in polyethylene glycol/salt aqueous two-phase systems. The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2015), Mandarin Hotel, Bangkok, Thailand. 2015 Nov 17-25, 2015. 286-291.

Tentative titles:

Sansamak, J., Piapukiew, J. and Vangnai, A. S. (2016). "Liquid-Liquid extraction of protease and lipase produced by Bacillus licheniformis 3C5 using aqueous two-phase systems." Applied Biochemistry and Biotechnology.

Sansamak, J., Piapukiew, J. and Vangnai, A. S. (2016). "Optimization for concomitant production of protease and lipase by Bacillus licheniformis 3C5." New Biotechnology.