ลักษณะสมบัติของเอนโดกลูแคเนสและการวิเคราะห์จีโนมของ *Lysobacter enzymogenes* ที่คัดแยกจากดินเขตร้อน

นางสาวศิรประภา สร่ายหอม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

CHARACTERIZATION OF ENDOGLUCANASE AND GENOME ANALYSIS OF *Lysobacter enzymogenes* ISOLATED FROM TROPICAL SOIL

Miss Siraprapa Saraihom

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
Faculty of Science
Chulalongkorn University
Academic Year 2015

Thesis Title	CHARACTERIZATION OF ENDOGLUCANASE AND GENOME ANALYSIS OF *Lysobacter enzymogenes* ISOLATED FROM TROPICAL SOIL
By	Miss Siraprapa Saraihom
Field of Study	Biological Sciences
Thesis Advisor	Associate Professor Hunsa Punnapayak, Ph.D.
Thesis Co-Advisor	Professor Donald Kobayashi, Ph.D.
	Assistant Professor Pongtharin Lotrakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

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

THESIS COMMITTEE

.....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)

.....Thesis Advisor
(Associate Professor Hunsa Punnapayak, Ph.D.)

.....Thesis Co-Advisor
(Professor Donald Kobayashi, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Pongtharin Lotrakul, Ph.D.)

.....Examiner
(Assistant Professor Sehanat Prasongsuk, Ph.D.)

.....Examiner
(Assistant Professor Pongchai Harnyuttanakorn, Ph.D.)

.....External Examiner
(Associate Professor Sanha Panichajakul, Ph.D.)

ศิรประภา สร่ายหอม : ลักษณะสมบัติของเอนโดกลูแคเนสและการวิเคราะห์จีโนมของ *Lysobacter enzymogenes* ที่คัดแยกจากดินเขตร้อน (CHARACTERIZATION OF ENDOGLUCANASE AND GENOME ANALYSIS OF *Lysobacter enzymogenes* ISOLATED FROM TROPICAL SOIL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. หรรษา ปุณณะพยัคฆ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. โดนัลด์ โคบายาชิ, ผศ. ดร. พงศ์ธาริน โล่ห์ตระกูล, 158 หน้า.

Lysobacter สายพันธุ์เขตร้อนที่ผลิตเอนไซม์ย่อยสลายหลายชนิดถูกคัดแยกได้ในประเทศไทย โดยอาศัยการเสริมไคตินในกระบวนการคัดแยก จากแบคทีเรียจำนวน 9 สายพันธุ์ที่สามารถย่อยสลายคาร์บอกซีเมทิลเซลลูโลสได้ มีเพียงสายพันธุ์ 521 ที่สามารถจัดจำแนกชนิดได้เป็น *Lysobacter enzymogenes* บนพื้นฐานของลักษณะทางสัณฐานวิทยาและสรีรวิทยา ร่วมกับการเปรียบเทียบและวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการของลำดับเบสของยีน 16S rRNA เมื่อเปรียบเทียบกับแบคทีเรีย *L. enzymogenes* C3 สายพันธุ์จากเขตอบอุ่น พบความแตกต่างกันของฟีโนไทป์บางประการ การเติบโตและการผลิตเอนโดกลูแคเนสในสายพันธุ์ *L. enzymogenes* 521 เอนโดกลูแคเนสถูกผลิตขึ้นโดย *L. enzymogenes* 521 ภายใต้ภาวะที่เหมาะสม และเมื่อทำให้บริสุทธิ์เพื่อศึกษาสมบัติทางชีวเคมี เอนโดกลูแคเนสบริสุทธิ์ที่มีขนาดมวลโมเลกุล 41 กิโลดาลตัน มีไบฟังก์ชั่นแอคติวิตีของคาร์บอกซีเมทิลเซลลูเลสและไคโตซาเนสโดยพบจากการวิเคราะห์ทางไซโมแกรม ภาวะที่เหมาะสมที่สุดในการทำงานของคาร์บอกซีเมทิลเซลลูเลสอยู่ที่ค่าความเป็นกรดด่าง 5 และอุณหภูมิ 40 องศาเซลเซียส และไคโตซาเนสอยู่ที่ค่าความเป็นกรดด่าง 5 และอุณหภูมิ 50 องศาเซลเซียส เอนไซม์บริสุทธิ์เสถียรที่อุณหภูมิ 40 องศาเซลเซียส และช่วงค่าความเป็นกรดด่างระหว่าง 4 ถึง 11 แอลฟา-เซลลูโลสและสารแขวนลอยไคโตซานเป็นสารตั้งต้นที่จำเพาะที่สุดของเอนไซม์นี้ โดยมีค่าแอคติวิตีสัมพัทธ์ในการย่อยสลายอยู่ที่ 49% และ 45% ตามลำดับ เมื่อเทียบกับค่าแอคติวิตีสัมพัทธ์ (100%) ในการย่อยสลายซีเอ็มซี จีโนมของ *L. enzymogenes* 521 มีขนาด 5.8 ล้านคู่เบส ประกอบด้วยยีนที่กำหนดรหัสโปรตีน 5,008 ยีน และอาร์เอ็นเอ 94 ยีน จีโนมของ *L. enzymogenes* 521 แสดงถึงยีนร่วมบรรพบุรุษ ที่คล้ายกับแบคทีเรียสายพันธุ์อื่นๆ ในวงศ์ Xanthomonadaceae จากการวิเคราะห์ยีนที่กำหนดรหัสโปรตีนที่อยู่ในกลุ่มเอนไซม์ไกลโคไซด์ไฮโดรเลส 33 ชนิด พบว่ามี 7 ยีนที่สร้างโปรตีนที่เกี่ยวข้องการการย่อยสลายเซลลูโลส การวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการของยีนเอนโดกลูแคเนสและการเปรียบเทียบลำดับกรดอะมิโนอนุมาน แสดงให้เห็นว่าเอนโดกลูแคเนส (Cel8A) จาก *L. enzymogenes* 521 ถูกจัดอยู่ร่วมกับไกลโคไซด์ไฮโดรเลส แฟมิลี 8 (GHF-8) และพบว่ามีความคล้ายคลึงกับไบฟังก์ชั่นเอนโดกลูแคเนสจากแบคทีเรีย *Lysobacter* sp. สายพันธุ์ IB 9374 (98.3% amino acid sequence similarity)

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5273857123 : MAJOR BIOLOGICAL SCIENCES
KEYWORDS: CHITINOLYTIC BACTERIA / TROPICAL LYSOBACTER ENZYMOGENES / BIFUNCTIONAL ENDOGLUCANASE-CHITOSANASE / GLYCOSIDE HYDROLASE FAMILY 8 / DRAFT GENOME SEQUENCE

SIRAPRAPA SARAIHOM: CHARACTERIZATION OF ENDOGLUCANASE AND GENOME ANALYSIS OF *Lysobacter enzymogenes* ISOLATED FROM TROPICAL SOIL. ADVISOR: ASSOC. PROF. HUNSA PUNNAPAYAK, Ph.D., CO-ADVISOR: PROF. DONALD KOBAYASHI, Ph.D., ASST. PROF. PONGTHARIN LOTRAKUL, Ph.D., 158 pp.

A tropical *Lysobacter* sp. with lytic enzyme activities was isolated in Thailand, using chitin enrichment procedure. Among nine isolates capable of carboxymethylcellulose degradation, only the strain 521 was identified as *Lysobacter enzymogenes* based on morphological and physiological traits, together with 16S rRNA sequence comparison and phylogenetic analysis. Variation of phenotypic characters, growth and endoglucanase production of *L. enzymogenes* 521 with respect to its counterpart from the temperate, *L. enzymogenes* C3, were observed. Endoglucanase produced by *L. enzymogenes* 521 under the optimal condition was purified in order to study its biochemical properties. The purified endoglucanase, with a molecular mass of 41 kDa, exhibited bifunctional hydrolytic activities of carboxymethylcellulase (CMCase) and chitosanase as revealed by zymogram analysis. The optimal pHs and temperatures for CMCase and chitosanase activity were 5.0, 40ºC and 5.0, 50ºC, respectively. The purified enzyme was stable at 40°C, and between pH 4 and 11. Alpha-cellulose and colloidal chitosan were found to be its most specific substrates with about 49 and 45% relative activity, respectively, with respect to that of CMC (100%). The 5.8-Mbp genome of *L. enzymogenes* 521 consisted of 5,008 protein coding genes and 94 RNA genes. Comparative genomic analysis indicated that *L. enzymogenes* 521 genome exhibited highly conserved orthologs to bacteria belonging to Xanthomonadaceae. From analysis of 33 genes encoding proteins in the glycoside hydrolase class, seven genes encoded proteins involving in cellulolytic degradation. Phylogenetic analysis of the endoglucanase gene and multiple alignment of its deduced amino acid sequence suggested that the endoglucanase Y (Cel8A) from *L. enzymogenes* 521 belong to the Family 8 glycoside hydrolases (GHF-8), with high similarity to the bifunctional endoglucanase (Cel8A) from *Lysobacter* sp. IB-9374 (98.3% amino acid sequence similarity).

Field of Study: Biological Sciences
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

For seven years of my doctoral thesis compilation, I have worked and met with a great number of wonderful people who made this thesis possible. It is my pleasure to convey my gratitude to them all in my humble acknowledgement.

First of all, I would like to show my gratitude to my advisor, Associate Prof. Dr. Hunsa Punnapayak who has been very kind providing me an opportunity to receive a great scholarship, Chulalongkorn University Dutsadi Phiphat Scholarship, to start my Ph.D. work in Thailand and abroad laboratories. With his excellent supervision, great works have been done and succeed.

I would like to express the deepest appreciation to my co-advisors, Professor Dr. Donald Y. Kobayashi, and Assistant Prof. Dr. Pongtharin Lotrakul. They have made available their supports in a number of ways from the beginning stage of this thesis and throughout the work. It is an honor for me to work with them. Without their contribution and supervision, this part of my thesis and publication would not have been accomplished.

My gratitude is also extended to Professor Dr. Douglas E. Eveleigh, Department of Biochemistry and Microbiology, Rutgers, The State University of New Jersey, for his generousness to provide academic support, and financial support of Eveleigh-Fenton Fund of Rutgers, the State University of New Jersey.

I am grateful to acknowledge Assistant. Prof. Dr. Tosak Seelanan who has instructed basic knowledge on Phylogenetic analysis, for molecular technical support, and for his kindness to be thesis chairman. I would like to thank Assistant Prof. Dr. Sehanat Prasongsuk, Assistant Prof. Dr. Pongchai Harnyuttanakorn and Associate Prof. Dr. Sanha Panichajakul for their valuable suggestions, useful comments and serving as thesis committee.

I am indebted to many of my colleagues both from the Plant Biomass Utilization Research Unit (PBURU), members in Department of Botany and friends from the Biological Sciences Program at Chulalongkorn University to support my research and private life. I adore my deepest gratitude to Dr. Nicola Lanata, who has lighted up my life, for his academic and moral supports. Finally, my parents deserve special dearest mention for their greatest supports throughout my life.

CONTENTS

Page

Page

Page

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter I

Introduction

1.1 Rationale

The interest in beneficial microorganisms as alternative control agents for plant diseases has increased due to concerns for the environmental impact of pollution and the potential health risks associated to the use of synthetic chemicals. Known members of the genera *Lysobacter* and *Stenotrophomonas* (*Lysobacter*-type species) belonging to the family Xanthomonadaceae are reportedly biotechnological important because of their properties as biological control agents (Hayward et al. 2010; Kobayashi et al., 1996; Kobayashi et al., 2002; Kobayashi and Yuen, 2007). Furthermore, they are a valuable source of lytic enzymes. *Lysobacter enzymogenes* representing the most studied member of the genus *Lysobacter* for biological control applications has also attracted considerable attention because of its rich stock of lytic enzymes (protease, chitinase, β-1, 3-glucanase and lipase) (Kobayashi and Yuen 2007). Besides the traits involving biological control mechanisms, cellulolytic enzymes produced by *L. enzymogenes*, such as endoglucanase have been purified and investigated for their bifunctional activities on carboxymethylcellulose (CMC) and chitosan (Hedges and Wolfe, 1974; Ogura et al., 2006).

Cellulases are enzymes produced by various microorganisms (including fungi, bacteria and actinomycetes) to degrade cellulosic materials in order to derive energy and carbon sources. Both fungi and bacteria have been intensively investigated for their abilities to produce a wide variety of cellulases. In particular, bacterial cellulase-producing strains have been investigated as a potential producer because they have the following advantages with respect to fungi: (i) They generally grow faster than fungi. (ii) Bacteria are resistant to environmental stresses, and consequently they are often able to produce enzymes that are stable even under extreme conditions. (iii) Bacterial cellulases are less inhibited by end-product (feedback inhibition), and have often multiple activity modes. (iv) They can be easily modified by genetic engineering in order to enhance their cellulase production (Maki et al. 2009).

Well-known cellulases are endoglucanase (carboxymethylcellulase; CM-cellulase), exocellobiohydrolase (exoglucanase) and β-glucosidase. Bacterial cellulases are usually classified as either exoglucanase or endoglucanases. The cellulase systems produced by bacteria are usually complex, comprising a multiplicity of activities providing increased function and synergy. In particular, multiple activity is displayed by bifunctional enzymes. A bifunctional endoglucanase

with hydrolytic activity on both CMC and chitosan has been reported in several bacterial species, including *Bacillus circulans* WL-12 (Mitsutomi et al. 1998), *B. cereus* S1 (Kurakake et al. 2000), *B. megaterium* P1 (Pelletier and Sygusch 1990), *Streptomyces griseus* HUT 6037 (Tanabe et al. 2003), *Paenibacillus fukuinensis* D2 (Kimoto et al. 2002), *Lysobacter sp.* AL-1 (Myxobacter AL-1) (Hedges and Wolfe 1974), and *Lysobacter* sp. IB-9374 (Ogura et al. 2006). Interestingly, endoglucanase produced by *Lysobacter* species exhibited higher activity on CMC than chitosan even when cultured in media without using CMC or chitosan as a carbon substrate. At present, endoglucanase from *Lysobacter* spp. has not been well studied, and the ecological role of the bifunctional endoglucanase in these bacteria is still unclear. The fact that the bifunctional endoglucanase is an extracellular enzyme suggests that it may have an advantage in biotechnological applications over the usual intracellular and periplasmic enzymes. Although bifunctional endoglucanases have been used as cellulase in cellulose-based textile industry (Kuhad et al. 2011) and as chitosanase in chitooligosaccharides production (Xia et al. 2008), their dual mode of activity has not been exploited in any application.

The genus *Lysobacter* has been found mainly in temperate habitats such as the America, Europe, Japan, Korea and in a few sub-tropical areas in China (Reichenbach 2006, Wei et al. 2012). Taxonomic methodology based on DNA sequence analysis has resulted in greater precision, and polyphasic approaches have led to the description of 15 new *Lysobacter* species in the last ten years (Srinivasan *et al*. 2010; Ten et al. 2009; Wang et al. 2009; Weon et al. 2006; Zhang et al. 2011). The discovery of fifteen new species is remarkable, and supports the importance of this taxonomic methodology. The knowledge of the distribution of the genus *Lysobacter* has also rapidly improved. These bacteria live in soil and fresh water and diverse environments, including rhizosphere soil (Aslam et al. 2009), ginseng field soil (Jung et al. 2008), gamma-irradiated sand soil (Liu et al. 2011), iron-mined soil (Luo et al., 2012), marine sponge (Romanenko et al. 2008), stream sediment (Ten et al. 2008) and geothermal soil (Wei et al. 2012). Yin (2010) has performed extensive studies in diverse ecosystem survey of the genus *Lysobacter* and, in particular, of the species *L. enzymogenes*, in which it was demonstrated that *Lysobacter* is present more often in soil samples isolated from areas covered by perennial grass rather than from non-grass areas. However, in tropical countries such as Thailand, where a high diversity of microorganisms and plant pathogenic fungi has been observed, only a related species, *Stenotrophomonas malthophilia* has been reported (Kongsaengdao et al., 2005; Tongarun et al., 2008). As shown in a study concerning hydrolytic enzyme degradation in grassland soils (Trasar-Cepeda et al. 2006), increasing the incubation temperature generally increases the rate of the enzyme catalysis. Therefore, extending the search of these bacteria to tropical habitats would be of interest, as it would open up the

opportunity to discover new potential lytic-enzyme producers with interesting properties such as thermophile and thermotolerance.

Currently, whole genome sequencing technology has advanced considerably. In fact, this technique enable us not only to identify simultaneously all of the cellylolytic enzyme-encoding genes contained in a bacterial genome, but also to understand extensively biochemistry, genetics, and molecular biology of bacteria. Because of the importance of *L. enzymogenes* as a potential biological control agent and its various types of extracellular lytic enzymes, it would be important to study the genome of this bacteria.

The goal of this study are to (i) isolate and identify the tropical *Lysobacter*-type species from different soils in Thailand based on morphological and physiological characteristics together with molecular data, (ii) purify and characterize of endoglucanase produced by a selected isolate, (iii) sequence genome of a selected isolate and analyze genes encoding proteins involved in cellulolytic activity, and (iv) clone and express of a recombinant cellulolytic enzyme in the selected isolate. Results obtained from this study will provide a useful basis to understand the *Lysobacter*-type bacteria and also to investigate possible future applications of its produced enzymes.

1.2 Objectives of this study

1) To isolate, identify and characterize *Lysobacter*-type bacteria from soils in Thailand
2) To purify and characterize cellulolytic enzymes produced from *Lysobacter*-type bacterium
3) To sequence a draft genome and analyze genes which encode proteins involved in cellulolytic activity from a selected *Lysobacter*-type bacterium

1.3 Key words

Chitinolytic bacteria, Tropical *Lysobacter enzymogenes*, Bifunctional endoglucanase-chitosanase, Glycoside hydrolase family 8, Draft genome sequence

1.4 Anticipated benefits

Knowledge gained from this study will lead to a better understanding in diversity and distribution of *Lysobater enzymogenes* including *Lysobacter*-type bacteria and a range of industrial applications of its bifunctional endoglucanase.

Chapter II

LITERATURE REVIEWS

2.1 Cellulose

2.1.1 Cellulose composition

Cellulose is an unbranched polymer of anhydro- of D-glucopyranose joined together by β-1, 4-glycosidic bonds with degrees of polymerization ranging approximately between 100 to and 10,000. Cellulose is produced by algae and higher plants but also by a number of bacteria, fungi, marine invertebrates, slime moulds and amoebae (Richmond, 1991). Cellulose is an important structural component of the primary cell wall of plants, many forms of algae and the oomycetes. In green plants, primary plant cell walls consist of cellulose, hemicelluloses that are associated with pectins and proteins. In the secondary cell walls of plants, cellulose and hemicellulose are associated with lignin. Despite the great differences in composition and in the anatomical structure of cell walls across plant species, a high cellulose content lies typically within a range between approximately 35% and 50% of plant dry weight. In nature, pure cellulose is obtained from a few sources such as bacterial cellulose, cotton etc. However, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30% of plant dry weight (Lynd et al., 1999). The composition constitutes a complex named “lignocellulose”.

The exact composition of cellulose, hemicellulose and lignin in plant cell wall varies between plant taxa and with cell differentiation (Tomme et al., 1995). For example, the woody feedstocks, such as beech and spruce, consist of high percentage of cellulose, hemicellulose and lignin in their dry matter but eucalyptus has markedly higher percentage of organic extractives. The herbaceous crops, including cereal straws, share significantly higher organic extractives and ashes, especially switchgrass and miscanthus (Fig. 2.1) (Piotrowski and Carus, 2011).

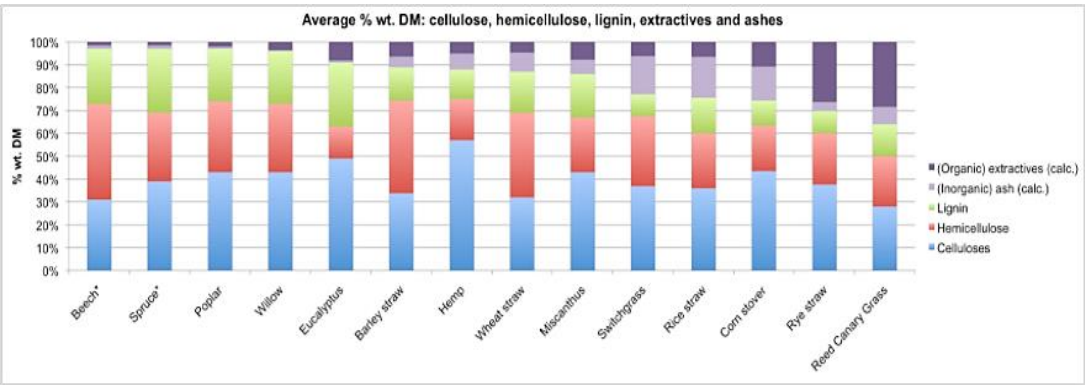


Figure 2.1 Average composition of cellulose, hemicellulose, lignin, extractives and ashes of plant (Piotrowski and Carus, 2011).
*Without bark and needles.

2.1.2 Cellulose type

Based on enzymatic hydrolysis considerations on the classification and on the mode of action of cellulases, native cellulose can be divided into the following forms: crystalline and amorphous.

2.1.2.1 Cellulose I (crystalline cellulose)

Native cellulose occurs in nature in the form of a crystal lattice structure (cellulose I), where the orientation (direction of reducing end) of the individual cellulose chains are parallel to the long axis of the microfibril (Fig. 2.2). A crystalline structure is a structure where all atoms in the molecule are present in fixed positions relative to each other. Due to its extremely tight structure, cellulose prevents entry not only of the enzymes but also of smaller molecules. Purified preparations of insoluble unsubstituted plant celluloses are used for various purposes as close approximations to native crystalline cellulose. Cotton and cellulose produced by bacteria in the genus *Acetobacter* are good examples of highly crystalline cellulose (Tomme et al., 1995).

2.1.2.2. Cellulose II (amorphous cellulose)

Cellulose II, produced by alkali treatment (mercerization) of cellulose I, has a different lattice structure with respect to Cellulose I. The orientation of the molecule of cellulose II is opposite to long axis of the microfibril (reducing ends are opposite). Since amorphous regions have large number of hydrogen bonding sites available, cellulose II can absorb large amounts of water. The amorphous regions of its structure allow the penetration of large molecules, including cellulolytic enzymes. Carboxymethyl cellulose swollen in Chloroacetic acid is considered as amorphous. Avicel is a blend of these crystalline and amorphous forms, which is a common

analytical substrates derived from bleached commercial wood pulps. Cotton or Avicel swollen in concentrated phosphoric acid (Walseth, 1971) is often considered as a convenient source of amorphous cellulose. However, its structure is unclear and recent reports described it as a low crystallinity form of cellulose II amorphous cellulose. α-cellulose, which is the major component of wood and paper pulp, is also considered as amorphous, and it can be separated from the other components by soaking the pulp in a 17.5% solution of sodium hydroxide.

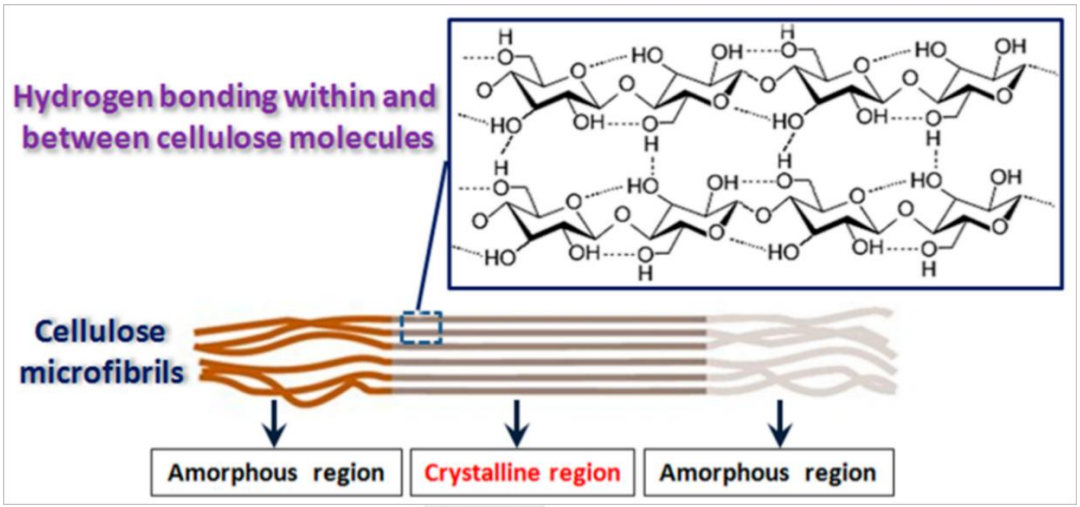


Figure 2.2 Interaction scheme between cellulose molecular chains within the crystalline region of cellulose microfibrils (Zhou and Wu, 2012).

2.2 Degradation of cellulose

The majority of cellulose is produced as a component of plant cell walls, which is the common substrate which provides cellulolytic microorganisms with energy and carbon in natural environments (Tomme et al., 1995). Therefore, cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, which is the most abundant carbohydrate produced by plants. Cellulose biodegradation by cellulases and cellulosomes is performed by numerous microorganisms. The complete enzymatic hydrolysis of cellulosic materials as indicated in Figure 2.3 needs to process simultaneously at least three different the following types of cellulases (Yi et al., 1999; Wood, 1989; Sengupta and Dasgupta, 2006):

(I) Endoglucanase (1,4-β-D-glucan-4-glucanohydrolase, carboxymethyl cellulase or CMCase; EC 3.2.1.4)

(II) Exoglucanase (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.74 or 1, 4-β-D-glucan cellobiohydrolase; E.C.3.2.1.91)

(III) β-glucosidase (β-D-glucosideglucohydrolase; EC 3.2.1.21).

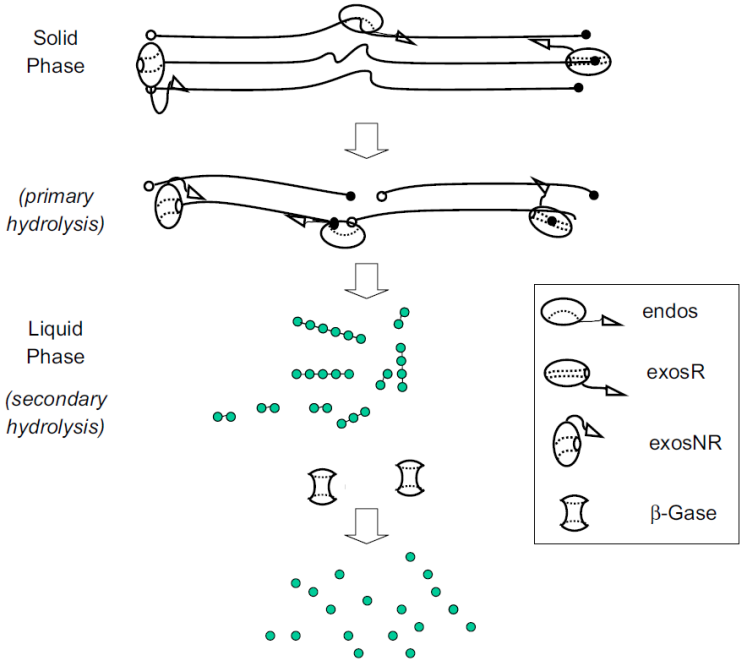


Figure 2.3 Synergistic scheme of enzymatic cellulose hydrolysis by fungal cellulases (Zhang et al., 2006). Acronym: edos, endoglucanase; exosR, exoglucanase-reducing end; exosNR, exoglucanase-nonreducing end, β-Gas, β-glucosidase.

Endoglucanases are believed to be primarily responsible for decreasing the degree of polymerization of cellulose structure by internally cleaving randomly intramolecular β-1,4-glucosidic bonds of amorphous cellulose, thereby generating new cellulose chain ends susceptible to the action of exoglucanase. Exoglucanases processively cleave cellulose chains at the ends to release soluble cellobiose or glucose. β-glucosidases subsequently hydrolyzes cellobiose and small oligosaccharides to glucose. Primary hydrolysis by endoglucanases and exoglucanases occurs on the surface of solid substrates. Soluble sugars with a degree of polymerization (DP) up to 6 are released into the liquid phase upon hydrolysis. The enzymatic depolymerization step performed by endoglucanases and exoglucanases is the rate-limiting step for the whole cellulose hydrolysis process. The secondary hydrolysis occurring in the liquid phase involves primarily the hydrolysis of cellobiose to glucose by β-glucosidases, although some β-glucosidases hydrolyzes also longer cellodextrins (Lynd et al., 2002; Zhang and Lynd, 2004).

2.3 Cellulase producing microorganisms

The ability to digest cellulose is widely distributed among many genera of bacteria, actinomycetes and fungi. They produce cellulases as inducible enzymes during their growth on cellulosic materials (Samira et al., 2011). Fungi are well-known agents of decomposition of organic matter and, in particular, of cellulosic substrates. Members of genera whose cellulolytic enzymes and/or wood-degrading capability have been studied extensively include: *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); *Bulgaria, Chaetomium*, and *Helotium* (Ascomycetes); and *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium*, and *Trichoderma* (Deuteromycetes) (Lynd et al., 2002).

There exist a fundamental difference between the cellulolytic strategies of the aerobic and anaerobic groups of bacteria. Anaerobes, especially thermophilic bacterium *Clostridium thermocellum*, degrade cellulose primarily via complexed cellulase system exemplified by the well-characterized cellulosome organelles. This protein complex, with a high-molecular-weight aggregate of 2.1 million daltons and ca. 18 nm in diameter, binds to and is active on crystalline cellulose (Lamed et al., 1983). Cellulosome in *C. thermocellum* cultures are mostly distributed on the surface of the cells (membrane-bound enzymes) rather than in the liquid phase (extracellular enzymes) (Schwarz, 2001). Cellulosome is also produced by the other anaerobic cellulolytic bacteria, including *C. cellulolyticum, C. cellulovorans, C. josui*, rumen bacteria *Ruminococcus,* and bacteria in the genera *Anaerocellum, Fibrobacter, Spicochaeta* etc. (Lynd et al., 2002).

A non-complexed cellulase system was found in aerobic cellulolytic bacteria. They utilize cellulose by producing a substantial amounts of extracellular cellulase enzymes that are freely recoverable from culture supernatants (Rapp and Beerman, 1991). It is also notable that most aerobic cellulolytic bacterial species common in soil are classified within genera that are well known for secondary metabolites (non-growth-associated metabolites) production, including the formation of distinct resting states (*Bacillus, Micromonospora*, and *Thermobifida*) and/or production of antibiotics (*Bacillus* and *Micromonospora*) and other secondary metabolites (Lynd et al., 2002).

Table 2.1 Major morphological characters of cellulolytic bacteria (Lynd et al., 2002).

Oxygen relationship	Genus	Representative species[a]	Gram reaction	Morphology	Growth temp.[b]	Resting state	Motility	Features of cellulase system
Aerobic	*Acidothermus*	*A. cellulolyticus*	+	Rod	Thermo			Noncomplexed, cell free
	Bacillus	*B. pumilis*	+	Rod	Meso	Endospore	Flagellar	Noncomplexed, cell free
	Caldibacillus	*C. cellovorans*	+	Rod	Thermo	Endospore		Noncomplexed, cell free
	Cellulomonas[c]	*C. flavigena, C. uda*	+	Rod	Thermo	None	Flagellar	Noncomplexed, cell free
	Cellvibrio	*C. fulvus, C. gilvus*	−	Curved rod	Meso	None	Flagellar	Noncomplexed, cell free
	Cytophaga	*C. hutchinsonii*	−	Rod	Meso	None	Gliding	Noncomplexed?, cell free?
	Erwinia	*C. carotovora*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free
	Micromonospora	*M. chalcae*	+	Filamentous rod	Meso	Spore[d]	Nonmotile	Noncomplexed, cell free
	Pseudomonas	*P. fluorescens* var. *cellulosa*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free
	Sporocytophaga	*S. myxococcoides*	−	Rod	Meso	Spore[d]	Gliding	Noncomplexed, cell free
	Rhodothermus	*R. marinus*		Rod	Thermo			
	Streptomyces	*S. reticuli*	+	Filamentous rod	Meso	Spore[d]	Nonmotile	Noncomplexed, cell free
	Thermobifida	*T. fusca*	+	Filamentous rod	Thermo	Spore[d]	Nonmotile	Noncomplexed, cell free
Anaerobic	*Acetivibrio*	*D. cellulolyticus*	−	Curved rod	Meso	None	Nonmotile	Complexed
	Anaerocellum	*D. thermophilum*	+	Rod	Thermo	None	Flagellar	Noncomplexed, cell free
	Butyrivibrio	*B. fibrisolvens*	+	Curved rod	Meso	None	Flagellar	Noncomplexed
	Caldicellulosiruptor	*C. saccharolyticum*	−	Rod	Thermo	None	Flagellar	Noncomplexed, cell free
	Clostridium	*C. thermocellum, C. cellulolyticum*	+	Rod	Thermo, meso	Endospore	Flagellar	Complexed, mostly cell bound[e]
	Eubacterium	*E. cellulosolvens*	+	Rod	Meso	None	Nonmotile	
	Fervidobacterium	*F. islandicum*	−	Rod	Thermo	None	Flagellar	
	Fibrobacter	*F. succinogenes*	−	Rod	Meso	None	Nonmotile	Complexed, cell bound
	Halocella	*H. cellulolytica*	−	Rod	Meso	None	Flagellar	Noncomplexed, cell free
	Ruminococcus	*R. albus, R. flavefaciens*	+	Coccus	Meso	None	Nonmotile	Complexed, cell bound
	Spirochaeta	*S. thermophila*	+	Spiral	Thermo	None		Noncomplexed, cell free
	Thermotoga	*T. neapolitana*	−	Rod	Thermo			

[a] Not all strains of the indicated species are cellulolytic, and some less active or less studied cellulolytic species within these genera are not listed.
[b] Meso, mesophilic; Thermo, themophilic.
[c] Most strains can also grow anaerobically.
[d] Unlike true endospores, these spores have only moderate resistance to environmental stress.
[e] Except for *C. stercorarium* (81, 83, 606).

2.4 Enzymatic hydrolysis of cellulases on chitosan

2.4.1 Cellulase–chitosanase producing microorganisms

Characterization of cellulases on degrading chitosan have been reported in many microorganisms (Table 2.2). In order to study further the hydrolysis mechanism of cellulases on chitosan, chitosanlytic cellulases from various sources have been purified, and action modes of a number of cellulases on chitosan hydrolysis have also been characterized. Cellulases from different sources all show chitosanase activity, although their biological properties differ (Xia et al., 2008). Cellulase showing bifunctional activity was first reported by Hedges and Wolfe (1974). Later, cellulases with chitosanase activity purified were reported for: *Streptomyces griseus* (Tanabe et al., 2003), *Bacillus circulans* (Mitsutomi et al., 1998), *B. cereus* S1 (Kurakake et al., 2000), and *Bacillus* sp. 0377BP (Choi et al., 2004), *B. cereus* D-11 (Gao et al., 2007), *T. viride* (Liu and Xia, 2006), *T. reesei* (Ike et al., 2007). This confirmed that most cellulases have a bifunctional cellulase–chitosanase activity. Bifunctional enzymes that have both cellulase and chitosanase activities can be divided into two groups based on their hydrolytic activity towards CM-cellulose and chitosan substrates. The first group exhibits higher activity on CM-cellulose than chitosan and, while the second group exhibits higher activity on chitosan than CM-cellulose. For the first group, the enzymes from *B. cereus* D-11 (Gao et al., 2007), *Myxobacter* sp. AL-1 (Pedraza-Reyes, 1997), *B. circulans* (Mitsutomi et al., 1998), *T. viride* (Liu and Xia, 2006) and *Lysobacter* sp. IB-9374 (Ogura et al., 2006) exhibited a chitosanase activity equal to 15–40% of its cellulase activity. Instead, bifunctional cellulase–

chitosanases from *B. cereus* S1 (Kurakake et al., 2000), *Bacillus* sp. 0377BP (Choi et al., 2004) and *T. reesei* (Ike et al., 2007) exhibited a CMCase activity equal to 3–30% of its chitosanase activity. These results indicated that one enzyme with two different hydrolytic activities was involved in the process causing the hydrolysis of chitosan by cellulase non-specifically, and that the action modes of cellulase may differ between the two substrates: chitosan and cellulose.

Table 2.2 Comparison of cellulolytic and chitosanolytic activities of cellulases from different microorganisms

Sources	Cellulolytic activity[b]	Chitosanolytic activity[b]	References
Bacillus cereus D-11 (U/mg)	264.5	347.8	Gao et al. (2007)
T. reesei (mU/mg)	34.9	350	Ike et al. (2007)
T. viride (U/mg)	0.358	0.402	Liu and Xia (2006)
Lysobacter sp. IB-9374 (U/mg)	484	128	Ogura et al. (2006)
Bacillus sp. KCTC 0377BP (U/mg)	51	1700	Choi et al. (2004)
T. viride (U/mg)	>15	0.92	Zhou et al. (2003)
Streptomyces griseus HUT 6037[a] (U/mg)	25.8	86.2	Tanabe et al. (2003)
Bacillus cereus S1 (U/mg)	40	196	Kurakake et al. (2000)
B. curculan WL-12 (U/mg)	24	22	Mitsutomi et al. (1998)
Myxobacter sp. AL-1 (U/mg)	48	61	Pedraza-Reyes (1997)

[a] Indicates ChoII purified from *Streptomyces griseus* HUT 6037.

[b] Each activity is tested under their optimum conditions.

2.4.2 Action modes of bifunctional cellulase on chitosan

Bifunctional cellulases from different sources showed different substrate specificity and hydrolytic action patterns (Table 2.3), which were also dependent on the degree of polymerization and of acetylation of chitosan. The level of acetylation in the substrate chitosan seems to influence the rate of hydrolysis by different cellulases, and fungus and bacteria originated cellulases show distinct action modes to chitosan. In bacteria, bifunctional chitosanase–cellulases from *B. cereus* D-11, *Bacillus* sp. S65, B. cereus S1, *Bacillus* sp. KCTC 0377BP, *Bacillus* sp. MET 1299 showed higher degrading activity with increasing deacetylation (DA) of chitosan. They showed the maximum activity on chitosan that have been deacetylated more than 90%. The enzyme retained 72% of its relative activity toward the 65–75% DA chitosan, compared to the activity of the 94% DA of chitosan. The enzymes from the above *Bacillus* sp. required substrates with three or more

glucosamine or N-acetyl-glucosamine residues for the expression of activity. They were capable of cleaving bonds between glucosamine or N-acetyl-glucosamine and glucosamine in an endo-type, belonging to chitosanase I.

Table 2.3 Purification and action modes of chitosanase from cellulase producing microorganisms (Xia et al., 2008)

Source	Methods of purification	Mode of enzyme action on chitosan	Substrate specificity	Reference
Bacillus cereus D-11	Sephadex G-150 and CM-Sephadex chromatography	GlcN–GlcNAc, GlcNAc–GlcN, GlcN–GlcN	CMC, chitosan	Gao et al. (2007)
T. reesei	Ammonium sulfate precipitation (80%), DEAE-Sepharose FF, butyl-Toyopearl 650M	GlcN–GlcN	PNPG2, CMC, Avcel, chitosan	Ike et al. (2007)
Bacillus sp. S65	DEAE Sepharose FF, Superdex 75	GlcN–GlcN	CMC, chitosan	Su et al. (2006)
Trichoderma viride	DEAE-Sepharose CL-6B, Phenyl-Sepharose CL-4B, Sephadex G-75	GlcN–GlcNAc, GlcNAc–GlcN, GlcN–GlcN cleavage from the non-reducing end	Chitosan, CMC	Liu and Xia (2005, 2006)
Pseudomona sp. H3	Ammonium sulfate precipitation, Sephadex G-25 desaltion, Sepharose Q-XL and Superdex G-75	GlcN–GlcN	Chitosan, CMC	Qiu et al. (2004)
Bacillus sp. KCTC 0377BP	CM-Toyopearl, Superose 12HR FPLC	GlcN–GlcN	Chitosan, chitin, glucan	Choi et al. (2004)
Bacillus sp. MET 1299	SP-Sephadex G-50	GlcN–GlcN, cleavage at random	Colloidal chitosan, β-glucan	Kim et al. (2004)
Bacillus cereus H-1	Ammonium sulfate precipitation, CM-Sephadex	GlcN–GlcN	CMC, colloidal chitosan, soluble chitosan	Jang et al. (2003)
Streptomyces griseus HUT 6037	Ammonium sulfate fractionation, CM-Sephadex C-25, Sephadex G-75	GlcN–GlcN, GlcN–GlcNAc, GlcNAc–Glc, transglycosidation	CMC, chitosan	Tanabe et al. (2003)
Bacillus cereus S1	Ammonium sulfate fractionation,70% acetone precipitation, Sephadex G-25 and Super Q Toyopearl	GlcN–GlcN	CMC, colloidal chitosan, soluble chitosan	Kurakake et al. (2000)
Bacillus circulans WL-12	Ammonium sulfate precipitation, Mono-Scation exchange and Superdex 75	GlcN–GlcN, GlcN–GlcNAc	β-1,3-/β-1,4-Glucan, chitosan	Mitsutomi et al. (1998)
Myxobacter sp. AL-1	ZnCl2 precipitate, CM-BioGel-A, BioGel P-60	GlcN–GlcN	CMC and cellobioside-derivatives, chitosan	Pedraza-Reyes (1997)

The cleavage patterns of the chitosan oligosaccharides presented in Table 2.3, suggested that the substrate-binding cleft of the bifunctional enzyme accommodated at least six GlcN residues for the superior hydrolysis. The cleavage of the glycosidic linkage occurred preferably at the center of the bound hexameric unit, yielding $(GlcN)_3$ as a main product. The fact that the enzyme showed higher activity toward chitosan than that of the short-chained oligosaccharides implied that the extra bindings of chitosan, other than six GlcN residues bound to the cleft, can enhance the hydrolysis rate of the enzyme for chitosan (Kurakake et al., 2000). Kim et al. (2004) found that N-acetylglucosamine residues in the chitosan play an important role in the recognition of the substrate by the enzyme, which suggested that the bifunctional enzyme has some differences in recognition sites of chitosan and cellulose.

2.5 Industrial application of cellulase

2.5.1 Bioethanol industry

Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, switch grass, saw dust, and forest residues by cellulases for biofuel production are perhaps the most important applications currently investigated (Sukumaran et al., 2005; Kuhad et al., 2010; Gupta et al., 2011). Bioconversion of lignocellulosic materials into useful and higher value products requires usually multistep processes (Kuhad et al., 2010; Ghosh and Singh, 1993; Wyman et al., 2005). These processes include: pretreatment (mechanical, chemical, or biological), hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars), bioconversion of these smaller molecules to support microbial growth and/or produce chemical products, and the separation and purification of desired products.

2.5.2 Pulp and paper industry

Most applications proposed so far concern the use cellulases and hemicellulases for the release of ink from the fiber surface by partial hydrolysis of carbohydrate molecules (Kuhad et al., 2010). It has been suggested that improvements in dewatering and deinking of various pulps result in the peeling of the individual fibrils and bundles, which have high affinity for the surrounding water and ink particles (Kibblewhite et al., 1995). Mixtures of cellulases (endoglucanases I and II) and hemicellulases have also been used for biomodification of fiber properties with the aim of improving drainage and beatability in the paper mills before or after beating of pulp (Dienes et al., 2004). These enzymes are also used in preparation of easily biodegradable cardboard (Buchert et al., 1998) and removal of adhered paper (Sharyo et al., 1978).

2.5.3 Textile industry

Cellulases are the most successful enzymes used in textile wet processing, especially finishing of cellulose-based textiles, with the goal of improved hand and appearance. Endoglucanase activity-rich cellulase is also proved useful for biofinishing. Most cotton or cottonblended garments, during repeated washing, tend to become fluffy and dull, which is mainly due to the presence of partially detached microfibrils on the surface of garments. The use of cellulases can remove these microfibrils and restore a smooth surface and original color to the garments. The use of cellulase also helps in softening the garments and in removal of dirt particles trapped within the microfibril network (Hebeish and Ibrahim, 2007; Ibrahim at al., 2011).

2.5.4 Detergent industry

The use of cellulases along with protease and lipase in detergents is a more recent industrial innovation (Singh at al., 2007). Cellulase preparations capable of modifying cellulose fibrils can improve color brightness, feel, and dirt removal from the cotton blend garments. The industrial application of alkaline cellulases as a potential detergent additive is being actively pursued. This property benefit to selectively contact the cellulose within the interior of fibers and remove soil in the interfibril spaces in the presence of the more conventional detergent ingredients (Sukumaran et al., 2005; Singh at al., 2007).

2.5.5 Wine and brewery industry

Microbial glucanases and related polysaccharides play important roles in fermentation processes to produce alcoholic beverages including beers and wines (Sukumaran et al., 2005). In wine production, enzymes such as pectinases, glucanases, and hemicellulases can be utilized to improve color extraction, skin maceration, must clarification, filtration, and finally the wine quality and stability (Singh at al., 2007). β-glucosidases can improve the aroma of wines by modifying glycosylated precursors. Macerating enzymes also improve pressability, settling, and juice yields of grapes used for wine fermentation (Galante et al., 1998). Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α- and β-amylases, carboxypeptidase, and β-glucanase that hydrolyze the seed reserves (Bamforth, 2009).

2.5.6 Chitosan industry

Cellulase is used for the hydrolysis of chitosan to produce chitooligomers because it is economically less costly than chitosanase. Cellulase is used to hydrolyze chitosan, to produce chitooligosaccharides with a degree of polymerization (DP) 1–6, and to produce low-molecular-weight chitosan (12 kDa), which has high antibacterial activity (Tsai et al., 2004). Chitooligosaccharides of DP 3–10 can be produced using the cellulase complex with membrane separation technology (Zhang et al., 1999). Hong and Kim (1998) investigated also the ability to degrade chitosan into oligosaccharides of cellulases produced by *T. viride* and *T. reesei*, as well as the ability of commercial cellulose Celluclast from *T. reesei*. Furthermore, they proposed possible applications of Celluclast for the production of various types of chitooligomers.

2.6 Description of *Lysobacter*

2.6.1 Characteristic of *Lysobacter*

Description of morphological and physiological of the gunus *Lysobacter* was intensively pursued by Christensen and Cook (1978). The morphological characters of various *Lysobacter* species resemble one another closely. The cells are in cylindrical shape with rounded ends. The cell size is typically 0.3–0.6 × 2–6 μm, but much longer threads of incompletely divided cells and cell chains, up to 70 μm, are also observed (Fig. 2.4a-2.4b). A mixed population of short and long cells is a peculiar feature of the lysobacters, which distinguish them with respect to any other myxobacterium. The lysobacters sometimes move by gliding (Fig. 2.4c-2.4d). Usually, their movements are rather slow, but sufficient to be observed under the microscope. Only *L. gummosus* appears not to spread on any medium, and its gummy colonies always have an entire edge. In general, the spreading of lysobacter swarms is relatively slow, and it may take 2–3 weeks before they cover the entire plate. On rich media like PC agar, the colonies tend to remain compact, small, with a smooth convex surface and an entire edge. Those colonies often are highly mucoid. *Lysobacter*, depending on the species and on the medium, may have colony colors of offwhite; cream; pale to deep yellow, sometimes with a greenish hue; pinkish; salmon; or orange-brown (Reichenbach, 2006).

The DNA of *Lysobacter* has a high %GC content of 65-71 mol% (Christensen and Cook, 1978; Daft and Stewart, 1971; Mitchell et al., 1969; Shilo, 1970; Stewart and Brown, 1971). The *Lysobacter* are aerobic organisms, although many strains appear to grow best at a reduced oxygen level (10% oxygen). They are positive for catalase and oxidase, but the catalase reaction is very sensitive and very weak sometimes, depending on the culture conditions. The suitable pH for their growth is between 4.5 and 10, and the optimal pH is between 7 and 9. The ability to grow at acid pH is generally different for different strains. Many of them do not grow below pH 6. The temperature optimum is usually around 30 °C, even for the freshwater organisms, but it varies substantially from isolate to isolate. It can be as low as 25 °C or as high as 40 °C, and a few strains can grow at 2 °C and at 50 °C. The salt tolerance is usually limited to 1% NaCl or less, and no strain was found to grow in the presence of 3% NaCl (Reichenbach, 2006).

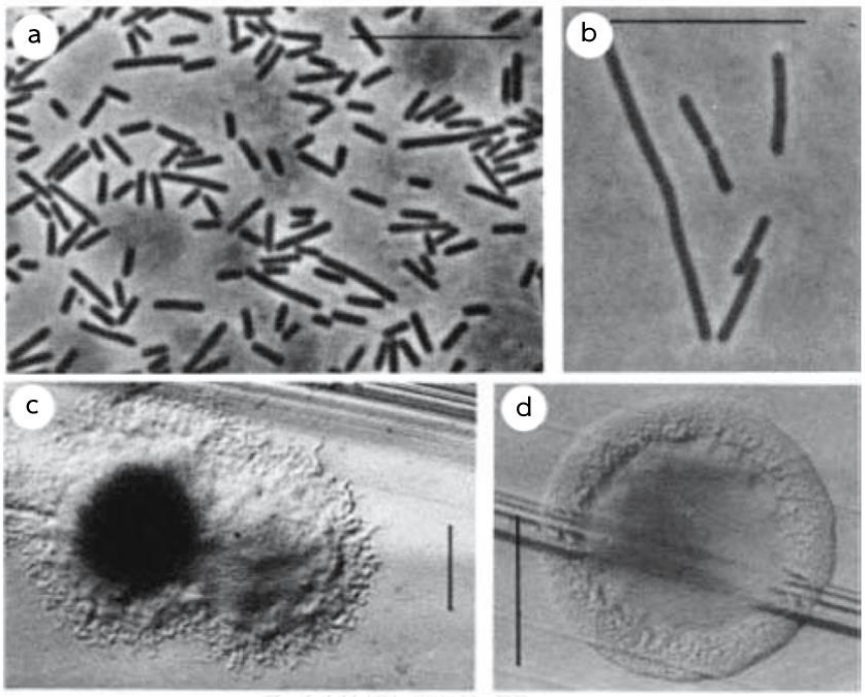


Figure 2.4 Colony morphology of *Lysobacter* sp. (a), (b) Phase contrast photomicrographs of *L. antibioticus* UASM L17 (= ATCC 29480) grown on CY agar. Typical cell is a regular cylindrical cell shape and a variation in cell length. (c), (d) Gliding colonies of *L. antibioticus* UASM L17 grown on different media for 7 days at 20 °C: (c), on CY agar; (d), on CA2 agar (Reichenbach, 2006).

2.6.2 Taxonomy and identification

In the past, strains which classified as *Lysobacter* spp. were firstly considered myxobacteria, as they share some of their distinctive traits, including gliding motility and micropredatory behavior. Christensen and Cook introduced the name genus *"Lysobacter"* in 1978. They distinguished *Lysobacter* from other myxobacteria because of its properties of being non-fruit forming and having high G+C content. *Lysobacter* is now grouped in Gamma-proteobacteria, and belongs to the family Xanthomonadaceae. The family Xanthomonadaceae is comprised of phylogenetically related bacteria of diverse origins and significance, including the genera *Xylella*, *Xanthomonas*, *Pseudoxanthomonas*, *Lysobacter* and *Stenotrophomonas* (Hayward et al., 2010). In the past, routine identification of Gram-negative bacteria placed reliance on metabolic profiles, fatty acid analyses, and more recently the sequence of nucleotides in the 16S rRNA genes. Due to the deficiency of representation in the databases of all cultivable Gram-negative bacteria and some similarity in metabolic profile and fatty acid content, *Lysobacter* has been confused with *Xanthomonas* and *Stenotrophomonas* (Hayward et al., 2010). A polyphasic taxonomic study has been later used to correctly distinguish these closer-relationships (Sullivan et al., 2003; Islam et al., 2005). In addition, *L. enzymogenes* and *S. maltophilia* have been referred to as '*Lysobacter*-type

species' (Dempsey et al., 2007), a designation which may not reflect the true-relationship between two species.

Taking advantage of a polyphasic approach to taxonomy together with the advent of databases more representative of the diversity among *Lysobacter* and related bacteria (Kobayashi and Yuen 2007), the number of new species have been increased dramatically since 2005. Up to date, more than 39 species of *Lysobacter* have been identified (Table 2.4).

2.6.3 Habitat and distribution

Bacteria in the the genus *Lysobacter* have been previously isolated from a number of distinct habitats, mainly in temperate zones such as the Americas, Canada, Europe, and also Asia (such as Japan and Korea). They have been later isolated also in sub-tropical zones such as China and Taiwan. However, *Lysobacter defluvii* is the only species that have been discovered in a tropical area. A number of publications reported that species of genus *Lysobacter* are abundant in diverse ecosystems including many soil sources (rhizosphere soil, chlorothalonil-contaminated soil, iron-mined soil, gamma-irradiated sand soil, and geothermal soil), fresh water, sea water, deep-sea sponge, air, anaerobic granules, stream sediment, activated sludge, abandoned lead-zinc ore, and also tomato stems. Other *Lysobacter* spp., sources and countries where they have been discovered are listed in Table 2.4.

Table 2.4 *Lysobacter* species described between 1978 and 2016

Species	Source of the type strain	References
Lysobacter antibioticus	Soil (Canadian provinces)	Christensen and Cook 1978
Lysobacter brunescens	Fresh water (Canadian provinces)	Christensen and Cook 1978
Lysobacter enzymogenes	Soil (Canadian provinces)	Christensen and Cook 1978
Lysobacter gummosus	Soil (Ontario, Canada)	Christensen and Cook 1978
Lysobacter concretionis	Anaerobic granules in an upflow anaerobic sludge blanket reactor	Bae et al. 2005
Lysobacter daejeonensis	Greenhouse soil (Korea)	Weon et al. 2006
Lysobacter koreensis	Ginseng field soil (Korea)	Lee et al. 2006
Lysobacter yangpyeongensis	Greenhouse soil (Korea)	Weon et al. 2006
Lysobacter defluvii	Soil from municipal landfill (India)	Yassin et al. 2007
Lysobacter niabensis	Greenhouse soil (Korea)	Weon et al. 2007

Table 2.4 *Lysobacter* species described between 1978 and 2016 (Continue)

Species	Source of the type strain	References
Lysobacter niastensis	Greenhouse soil (Korea)	Weon et al. 2007
Lysobacter capsici	Rhizosphere of pepper (Korea)	Park et al. 2008
Lysobacter daecheongensis	Stream sediment (Korea)	Ten et al. 2008
Lysobacter ginsengisoli	Soil (Korea)	Jung et al. 2008
Lysobacter spongiicola	Deep-sea sponge (Philippine sea)	Romanenko et al. 2008
Lysobacter oryzae	Rhizosphere of rice (Korea)	Aslam et al. 2009
Lysobacter panaciterrae	Ginseng field soil (Korea)	Ten et al. 2009
Lysobacter ximonensis	Soil (China)	Wang et al. 2009
Lysobacter soli	Ginseng field soil (Korea)	Srinivasan et al. 2010
Lysobacter dokdonensis	Soil (Korea)	Oh et al. 2011
Lysobacter korlensis	Soil (China)	Zhang et al. 2011
Lysobacter bugurensis	Soil (China)	Zhang et al. 2011
Lysobacter ruishenii	Chlorothalonil-contaminated soil (China)	Wang et al. 2011
Lysobacter xinjiangensis	gamma-irradiated sand soil (China)	Liu et al. 2011
Lysobacter arseniciresistens	Iron-mined soil (China)	Luo et al. 2012
Lysobacter thermophilus	Geothermal soil (China)	Wei et al. 2012
Lysobacter oligotrophicus	Antarctic freshwater (Antarctica)	Fukuda et al. 2013
Lysobacter panacisoli	Ginseng field soil (Korea)	Choi et al. 2014
Lysobacter agri	Soil (Korea)	Singh et al. 2015
Lysobacter caeni	Activated sludge (China)	Ye et al. 2015
Lysobacter fragariae	Rhizosphere of strawberry (Korea)	Singh et al. 2015
Lysobacter rhizosphaerae	Rhizosphere of strawberry (Korea)	Singh et al. 2015
Lysobacter hymeniacidonis	Crude Oil-contaminated marine sponge (China)	Xin et al. 2015
Lysobacter lycopersici	Tomato stems	Lin et al. 2015
Lysobacter mobilis	Abandoned lead-zinc ore (China)	Yang et al. 2015
Lysobacter terrae	Rhizosphere soil (Korea)	Ngo et al. 2015
Lysobacter tyrosinelyticus	Soil (Korea)	Du et al. 2015
Lysobacter chengduensis	Air of Giant Panda enclosures (China)	Wen et al. 2016
Lysobacter maris	Seawater (Korea)	Yoon 2016

2.6.4 Applications of *Lysobacter*

Research on *Lysobacter* focused mostly on the following applications: (i) use as biological control agents for plant diseases, (ii) production of antibiotics for human medicine, and (iii) enzymes for commercial applications. These research areas are briefly described below:

(i) Biological control agents for plant diseases

Strains of *Lysobacter* spp. were reported to have broad spectrum antagonism in vitro against bacteria, fungi, unicellular algae and nematodes. *L. enzymogenes* has been reported to be the most effective strain for the control of a number of diseases, among the species known so far. For example, *L. enzymogenes* strain C3 was reported to control diseases caused by fungal pathogens, including *Rhizoctonia solani* (Giesler and Yuen 1998), *Bipolaris sorokiniana* (Zhang and Yuen 1999 and 2000, Kilic-Ekici and Yuen 2004.), *Uromyces appendiculatus* (Yuen et al., 2001), *Fusarium graminearum* (Yuen, et al., 2003), *Magnaporthe poae* (Kobayashi and Yuen, 2005), and *Phytophthora capsici* (in combination of *L. enzymogenes* C3 with *Serratia plymuthica* C-1 and *Chromobacterium* sp. C-61) (Kim et al., 2008). Strain C3 and others also were inhibitory displayed inhibitory effects towards nematodes (Chen et al., 2006; Katznelson et al., 1964) and oomycetous pathogens in the genera *Aphanomyces* and *Pythium* (Kobayashi et al., 2005; Palumbo et al., 2005; Islam, 2009; Postma et al., 2009). This broad spectrum of biocontrol activity can be attributed to a wide variety of possible mechanisms: hyperparasitism involving types III, IV and VI secretion systems (Reedy et al., 2003; Blackmoore et al., 2009; Patel et al., 2009), extracellular enzymes (Palumbo et al., 2003; Ahmed et al., 2003; Chohnan et al., 2004), secondary metabolites (Yuen et al., 2005), and induced resistance (Kilic-Ekici and Yuen, 2003).

(ii) Production of antibiotics

Antibiotics are microbial toxins that can poison or kill other microorganisms even at low concentrations. The ability of *Lysobacter* spp. to produce powerful antimicrobial compounds is one of the main reasons why this bacteria is regarded as a potential agent for biological control of plant diseases. *Lysobacter* strain was found to produce myxin as a broadspectrum phenazine antibiotic inhibiting bacteria and fungi already many years ago (Peterson et al., 1966). Christensen (2001) reported an antibiotic with a wide spectrum produced by *L. antibioticus* identified as 1-hydroxy-6-methoxyphenazine. A family of antibiotics consisting of dihydromaltophilin, called heat-stable antifungal factor (HSAF), is produced by *L. enzymogenes* strain C3 and was proved to be responsible for control of fungi and oomycetes by disruption of the fungal polarized growth (Yu et al., 2007, Li et al., 2006). A similar compound produced by *L. enzymogenes* strain 3.1T8 was found having an inhibitory activity against oospores and cyst germination (Folman et al., 2004). At the

same time, xanthobaccins A produced by *Lysobacter* sp. SB-K88 was identified as a macrocyclic lactam and a structural analogue of dihydromaltophilin (Nakayama et al., 1999, Yu et al., 2007). Broad production of maltophilin-related antibiotics is another characteristics trait of *Lysobacter* spp., which have important potential ecological applications, as these antibiotics are effective inhibitors of fungi.

(iii) Enzymes for commercial applications

Diverse microorganisms secrete and excrete metabolites that can interfere with pathogen growth and/or activities. In particular, a number of microorganisms produce and release lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Proteases, chitinases, glucanases, lipases and phospholipases produced by *Lysobacter* can degrade the cell walls of all groups of plant pathogens. Proteases were the earliest enzyme group studied for biocontrol activity and were first thought to be involved in against nematodes (Katznelson et al., 1964). Later, proteases were reported to be active also against several gram-positive and gram-negative bacteria (Ensign and Wolfe, 1965 and 1966). Chitinase activity of *L. enzymogenes* strain C3 has been reported to be involved in biological control activity for *Bipolaris sorokiniana* (Zhang and Yuen, 2000). β-1,3-Glucans, comprising over 80% of the cell wall polysaccharides, are critical components of cell wall structure in yeast, fungi, and oomycetes (Blaschek et al., 1992). β-1, 3-glucanase produced by *L. enzymogenes* strains C3 and N47 can decompose the cell wall of these microotganisms (Palumbo et al., 2005).

The activity of cellulolytic enzymes such as carboxymethyl cellulase; CMase (acting endoglucanase) produced by *L. enzymogenes* has been also investigated. Bifunctional cellulase produced from *L. enzymogens* AL-1 and *Lysobacter* sp. IB-9374 have been purified and characterized (Hedges and Wolfe, 1974; Ogura et al., 2006). They exhibited higher activity of β-1,4-glucanase on CM-cellulose than chitosanase on chitosan. At present, the ecological function of the enzymes in these bacteria is still unclear. In fact, it has been reported that members of Gammaproteobacteria were predominant in the rhizospheres of many plants (Debette and Blondeau, 1980; Lambert et al., 1987; Wolf et al., 2002; Lee et al., 2006; Someya et al., 2011). They may play a role in enzymatic colonization into host-plant root. However, a research on postharvest disease control revealed that chitosan can stimulate microbial degradation of pathogens similar to that of an applied hyperparasite (Benhamou, 2004). Improvement of plant growth substratum with chitosan suppressed the root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato (Lafontaine and Benhamou, 1996). Although the mechanism of action of chitosan is not still well understood, it has been observed that treatment with chitosan could increase resistance to several pathogens (Deepmala et al., 2014).

2.7 Whole genome sequencing

2.7.1 Genome sequencing technology

Whole genome sequencing (complete genome sequencing) is a method to determine the complete DNA sequences containing in an organism. Besides chromosomal DNA, this also includes plasmids for single cell microorganisms, mitochondria for mammals, and chloroplast for plants (Markowitz et al., 2010). The sequencing techniques can be categorized into four main strategies, including amplification and mass spectrometry, *in vitro* cloning, *in vivo* cloning, and single-molecule approaches. In the older methods, each reaction has a different base as a terminator (one for each base: G, A, T and C), while the size of each fragment can be identified by running it on a gel. The modern method is based on the use of different colored fluorescent dyes to label the terminators (Prober et al., 1987), so that all of them are incorporated in a single reaction, and the size of each fragment is separated on capillaries, giving the average length of a sequencing read at 850 bp (Madabhushi, 1998).

Genomic DNA is firstly fragmented by restriction nuclease digestion or mechanical shearing or a combination of these approaches. Subsequently, DNA fragments are conventionally cloned, transformed. Subsequently, a number of randomly selected transformants are subjected to sequencing (Anderson, 1981; Roach et al., 1995). However, this approach could not be applied to complicated genomes (such as the human genome) as it was too laborious and time consuming for this purpose. In addition, one of the main drawbacks this approach is that there are inherent biases against certain stretches of DNA, for example, some DNA fragments contain regions that do not replicate well in *E. coli* or that code for toxic compounds (Hall, 2007). Therefore, the genome sequencing by *in vivo* technique is currently replaced by *in vitro* cloning, or called also "Next-generation sequencing" (NGS) technologies.

The NGS technologies are high-throughput sequencing techniques that parallelize the sequencing process, producing thousands or millions of sequences at once (Hall, 2007). Well known examples of NGS promising technologies are: 454 pyrosequencing (Margulies et al., 2005), the Solexa system (Bennett et al., 2005), and the SOLiD system (Shendure et al., 2005). These sequencing platforms, which are different in terms of engineering configurations and sequencing chemistries, are briefly described below.

(i) 454 pyrosequencing

This technique is derived from the combination of single-molecule emulsion PCR and pyrosequencing (Margulies et al., 2005). The pyrosequencing is a sequencing approach based on chemiluminescent detection of pyrophosphate released during polymerase mediated deoxynucleoside triphosphate (dNTP) incorporation. For sequencing, genomic DNA is fragmented

in order to generate a library of DNA templates. Fragments in the library are sequentially ligated to adapter oligonucleotides, and hybridized to individual beads containing sequences complementary to adapter oligonucleotides (Fig 2.5). The DNA library fragments captured on beads, enzyme, and PCR reagents in a water mixture are injected into small cylindrical plastic containers containing synthetic oil. The water mixture includes an enzyme that causes the single and isolated DNA fragment in each droplet to be amplified into millions of copies of DNA. After amplification, the beads are separated and deposited into individual "picotiter-plate" wells and combined with sequencing enzymes. As a nucleotide incorporates in a clonally amplified template in a well, it releases pyrophosphate which produces localized luminescence. The luminescent signal is transmitted through the fiber-optic plate and recorded on a charge-coupled device camera. With the flow of each dNTP reagent, wells are sequentially imaged, analyzed for their signal-to-noise ratio, filtered according to quality criteria, and subsequently algorithmically translated into a linear sequence output (Tawfik and Griffiths, 1998; Voelkerding et al., 2009).

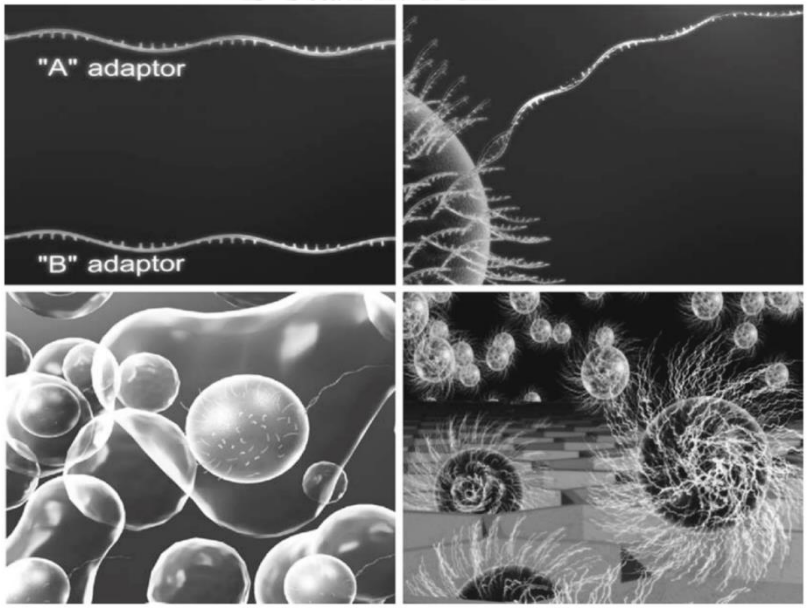


Figure 2.5 The 454 pyrosequencing steps: ligation of adapters, capture of DNA fragments on beads, and clonal amplification by PCR in emulsion microreactors (Tettelin and Feldblyum, 2009).

(ii) Solexa/Illumina

The main idea of this approach is to sequence single DNA molecules attached to microspheres (Bennett et al., 2005). An overview of Illumina sequencing technology is presented in Figure 2.6. A flow cell consists of an optically transparent slide with 8 individual lanes on the surfaces, which are bound oligonucleotide anchors. Template genomic DNA is fragmented into several hundred base pairs in length and subsequently end-repaired to generate blunt ends. The

fragment is used to add a single A base to the blunt phosphorylated DNA fragments. Then the fragment is ligated with oligonucleotide adapters overhanging of a single T base. The flow-cell anchors are complementary to the adapter oligonucleotides. The single-stranded template DNA is added to the flow cell and immobilized by hybridization to the anchors. Unlike emPCR, DNA templates are amplified in the flow cell in a distinct geometry called "bridge" amplification. The single-molecule DNA template is converted to a clonally amplified arching "cluster" via multiple amplification cycles, wherein each cluster contains approximately 1000 clonal molecules (Voelkerding et al., 2009). For sequencing, the clusters are denatured in order to leave only forward strands for single-end sequencing. The forward strands are sequenced by hybridizing a primer complementary to the adapter sequences, followed by addition of polymerase and a mixture of 4 differently colored fluorescent reversible dye terminators. The terminators are incorporated into in each strand in a clonal cluster if their sequences are complementary. After incorporation, excess are washed away and the fluorescence is recorded from the clusters. Before to begin the next round, the reversible dye terminators are unblocked, the fluorescent labels are cleaved and washed away, and the next iterative sequencing cycle is performed.

(iii) SOLiD/Applied Biosystems

The SOLiD (Supported Oligonucleotide Ligation and Detection) system is based on ligation of short-read sequencing (Shendure et al., 2005). An overview of SOLiD platform technology is presented in Figure 2.7. The preparation of the library of the DNA template shares similarities with the 454 technology, where DNA fragments are ligated to oligonucleotide adapters, attached to beads, and clonally amplified by emPCR. After the clonal amplification, beads with template are immobilized onto a derivitized-glass flow-cell surface. Sequencing is initiated by annealing a primer oligonucleotide complementary to the adapter at the adapter template junction. During the first "ligation sequencing" step, the primer is oriented to provide a 5′ phosphate group for ligation to interrogation probes instead of providing a 3′ hydroxyl group. The interrogation probe is an octamer consisting of (in the 3′-to-5′ direction) 2 probe specific bases linked with one of 4 fluorescent labels. In the first ligation-sequencing step, interrogation probes compete for annealing to the template sequences. Optical intensity of fluorescence signals are collected upon the cleavage of the ligated probes and another washing step is employed to remove the fluor and regenerate the 5′ phosphate group. One round of reaction is referred to seven cycles of ligation performed to extend the first primer. The synthesized strand is then denatured, and a new sequencing primer offset by 1 base in the adapter sequence is annealed. Five rounds total are performed, each time with a new primer with a successive offset.

These techniques are a high-throughput and cost-effective sequencing approach. Since the first NGS platform was commercially lunched in 2005 (Voelkerding et al., 2009), the number of successfully sequenced genomes has dramatically gone up ever. This is a promising technology for genetic-based research in the near future and could be also a compliment technology towards any other sequencing technologies *e.g.* DNA array (Hurd and Nelson, 2009; Teng and Xiao, 2009).

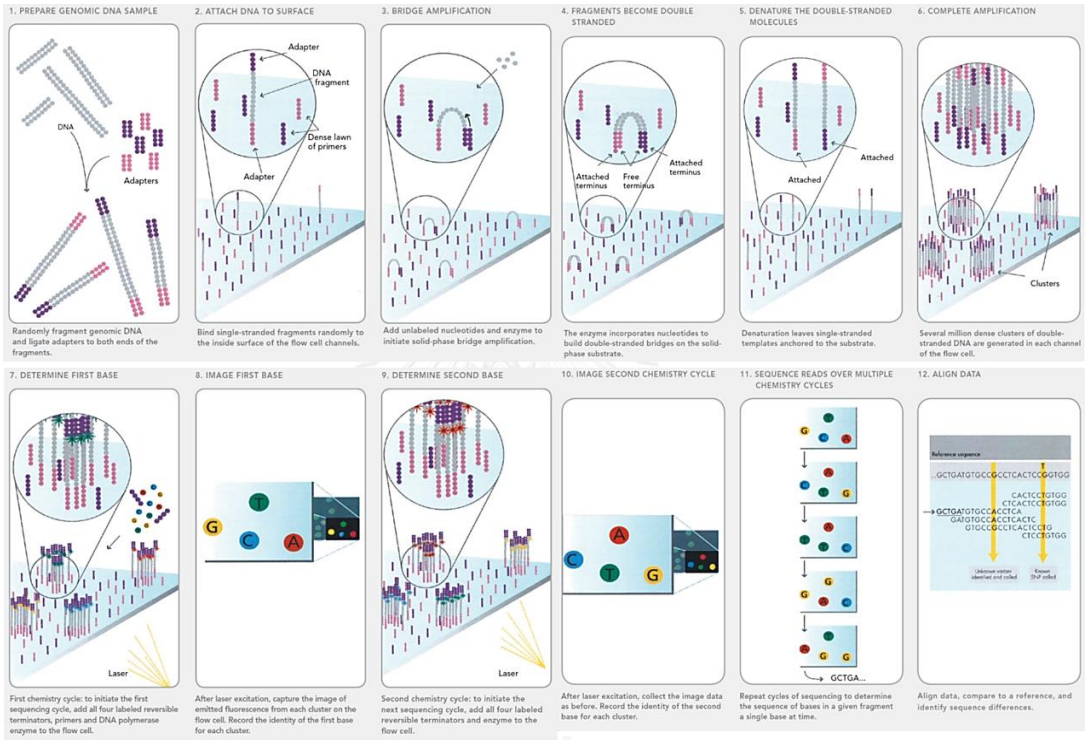


Figure 2.6 Illumina sequencing technology overview (www.illumina.com)

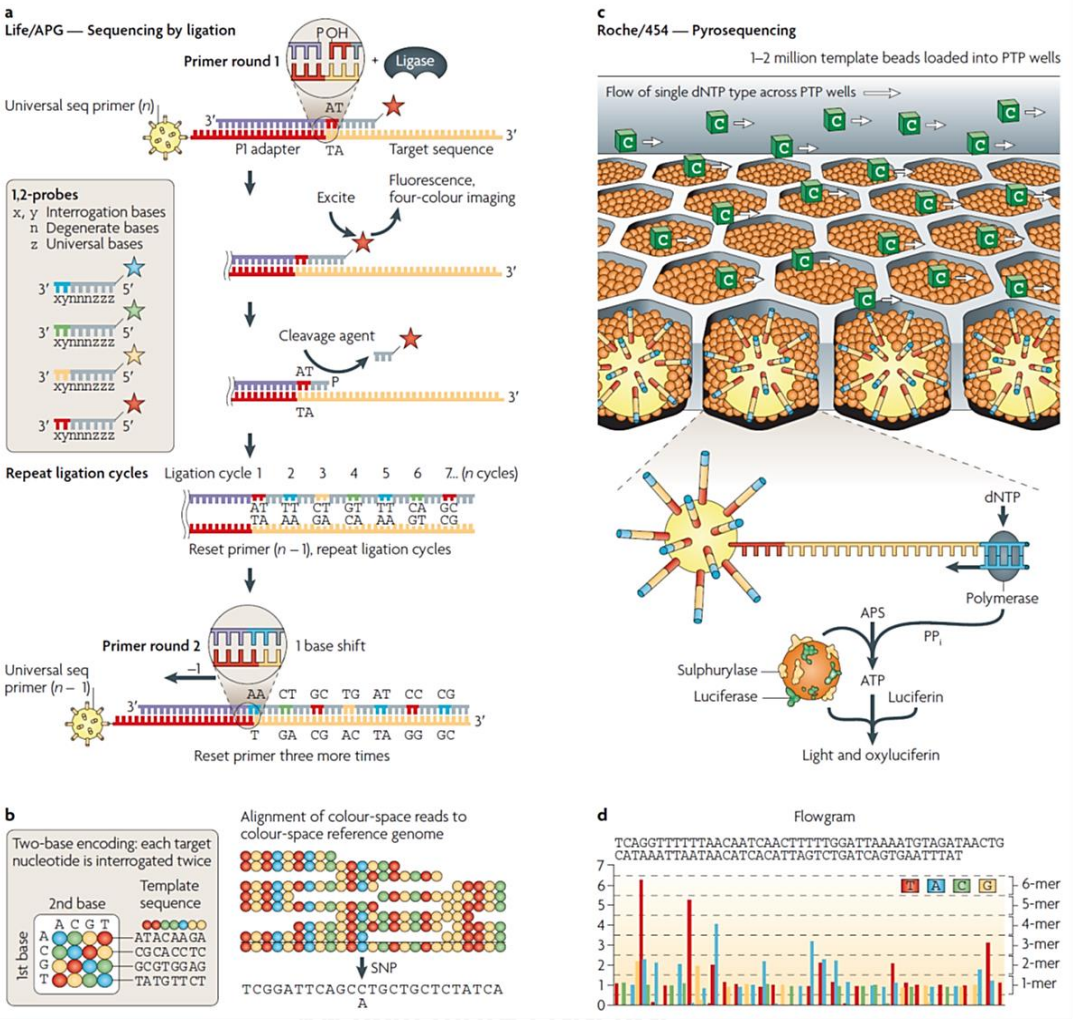


Figure 2.7 SOLiD platform technology overview (Metzker, 2010)

2.7.2 Genomic overview of bacteria in the genus *Lysobacter*

Due to the significance in term of clinical and biotechnological uses, viruses and bacteria are the most studied groups of microorganisms. Two decades have passed since the first bacterial genome was completely sequenced, and the number of sequenced genomes has continued to increase dramatically in the last 10 years (Fig. 2.8A). The six phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes* together represent 96% of the data collected until now. Other phyla have been investigated less than 1 % of total genomes (Fig. 2.8B). Note that currently almost half of all the genomes sequenced are from the *Proteobacteria* phylum. Thus, the knowledge of bacterial genomes, in particular *Proteobacteria* genomes, and their coding

products are currently most comprehensively established in several aspects, such as gene, genome, and function, along with the expanding of sequencing technologies.

Based on NCBI database submission, 25 genomes of bacteria in the genus *Lysobacter* have been determined. Table 2.5 shows genomic features of several selected finished genomes. These bacteria were isolated from different sources including soil, iron-mined soil, grass leave, wastewater and solid waste. As shown in Table 2.5, the genomic sizes were found in between 3.0 and 6.4 Mbp, with average %G+C of 67.6. Maximal number of protein coding sequences (CDSs) was found in *L. mcapsici* 55 (5685 CDSs), while the average was 4156 CDSs. Bacteria in *Lysobacter* species contain average rRNA operon (5S, 16S, and 23S rRNA subunits) and tRNAs of 6.3 and 53.8 genes, respectively. At present, there are no reports of plasmid detection in these species. It should be noted that about 35.41% of the average CDSs from all genomes are proteins with unknown functions and biological significance, whose study might lead to novel perspectives and applications.

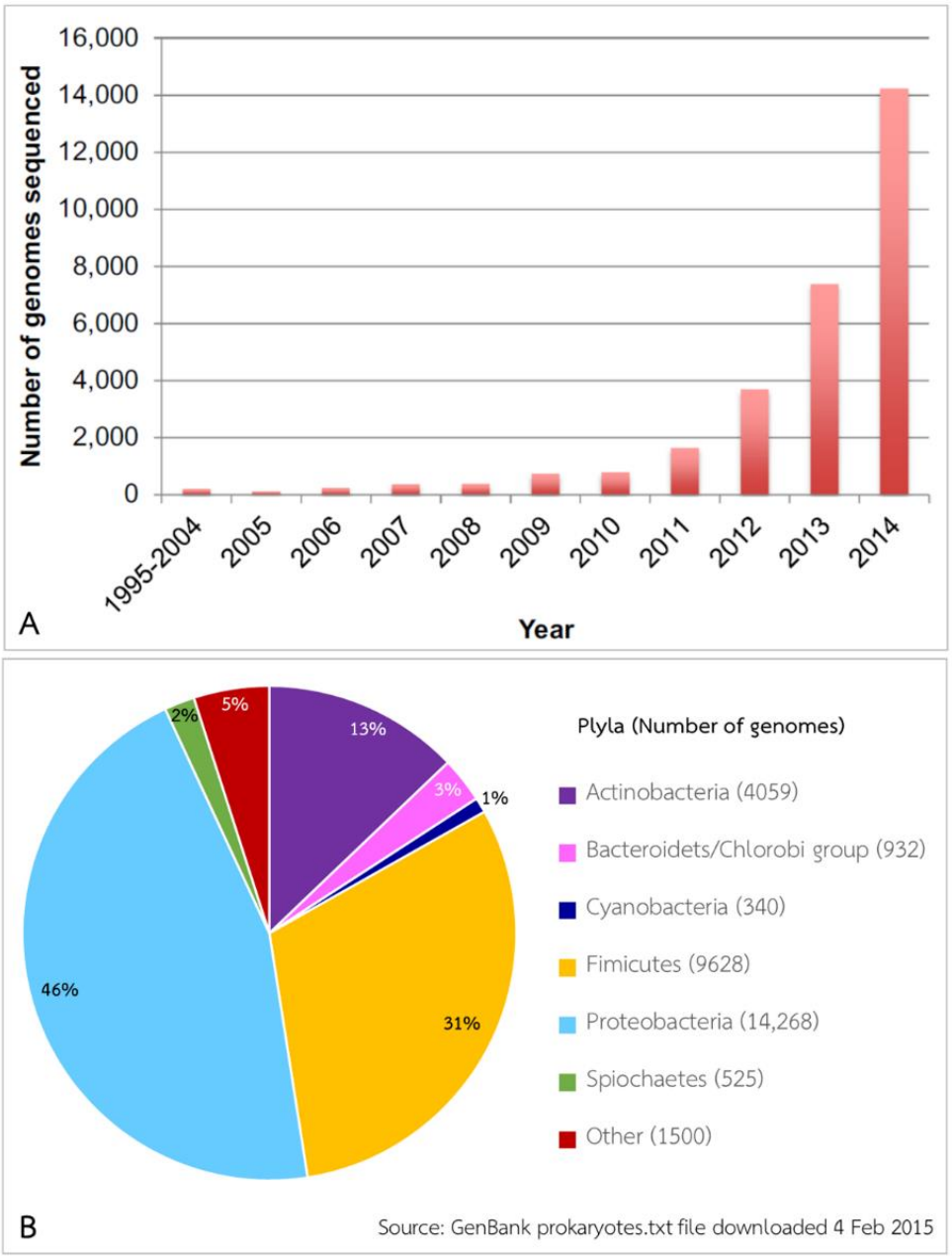


Figure 2.8 Number of bacterial and archaeal genomes sequenced each year and submitted to NCBI (A), and number of sequenced genomes for 6 selected phyla and the percent of all genomes found in the phyla (B) (Land et al., 2015).

Table 2.5 Genome statisitics and features of the *Lysobacter* genome sequences

Taxa: 1, *L. antibioticus* ATCC29479; 2, *L. antibioticus* 76; 3, *L. antibioticus* 13-6; 4, *L. capsici* 55; 5, *L. capsici* AZ78; 6, *L. capsici* X2-3; 7, *L. gummosus* 3.2.11; 8, *L. enzymogenes* C3; 9, *L. dokdonensis* DS-58T; 10, *L. arseniciresistens* ZS79; 11, *L. conceretionis* Ko07; 12, *L. daejeonensis* GH1-9; 13, *L. defluvii* IMMIB APB-9

	1	2	3	4	5	6	7	8	9	10	11	12	13
Geographic isolation	soil	bulk soil	rhizosphere soil	bulk soil	tobacco rhizosphere	wheat rhizosphere soil	bulk soil	Kentucky bluegrass leave	wastewater-treatment reactor	Iron-mined soil	Anaerobic granules	Green house soils	Municipal solid waste
Genome size (Mb)	5.8	5.9	5.5	6.4	6.3	5.9	6.1	6.2	3.3	3.1	3.0	3.3	3.27
G+C content (%)	67.0	66.8	67.1	66.6	66.4	66.8	66.5	69.8	67.2	69.56	67.25	67.29	70.22
CDSs number	5178	5143	4056	5685	5448	4880	5322	5547	3155	2363	2232	2570	2443
Hypothetical proteins	1338 (25.84)[a]	1832 (35.62)	-	2123 (37.34)	-	2019 (41.37)	1845 (34.67)	2088 (37.64)	-	-	-	-	-
rRNA clusters	9	6	-	6	7	-	6	6	-	3	3	4	13
tRNAs	55	51	-	52	85	-	51	60	-	46	46	48	44
Reference	Bruijn et al., 2015	Bruijn et al., 2015	Bruijn et al., 2015	Bruijn et al., 2015	Bruijn et al., 2015	Yi et al., 2015	Bruijn et al., 2015	Bruijn et al., 2015	Kwak et al., 2015	Liu et al., 2015	Liu et al., 2015	Liu et al., 2015	Liu et al., 2015

[a] Numbers in parenthesis are percentage of total protein codding genes presenting in respective genomes

- indicates unknown

Chapter III

MATERIALS AND METHODS

3.1 Materials and chemicals

- 3, 5-dinitrosalicylic acid (DNS): Sigma-Aldrich Inc., MO, USA
- Acetonitrile: Fisher Scientific, Leicestershire, UK
- Ammonium Chloride: Merck, Darmstadt, Germany
- API 20NE kit: bioMe´rieux, France
- Baker's yeast: Bruggeman, Ghent, Belgium
- BLUeye Prestained Protein Ladder: GeneDireX, Inc., NV, USA
- Calcium chloride: Ajex Finechem, Auckland, New Zealand
- Carboxymethylcellulose (CMC; dp: 400, ds: 0.65-0.90): Sigma-Aldrich, MO, USA
- Casein: Himedia, Mumbai, India
- Cesium chloride: Sigma-Aldrich Inc., MO, USA
- Chitin (from shrimp shells): Sigma-Aldrich Inc., MO, USA
- Chitosan (75-85% deacetylated, low MW): Sigma-Aldrich Inc., MO, USA
- Congo Red: Ajex Finechem, Auckland, New Zealand
- Ethylene diamine tetra-acetic acid (EDTA): Vivantis, CA, USA
- Folin's Phenol Reagent: Fisher Scientific, Leicestershire, UK
- Glucosamine: Sigma-Aldrich Inc., MO, USA
- Glucose: Sigma-Aldrich Inc., MO, USA
- Glycine: Vivantis, CA, USA
- Hydrochloric acid: Fisher Scientific, Leicestershire, UK
- Isopropanol: Fisher Scientific, Leicestershire, UK
- Kanamycin sulfate: Sigma-Aldrich Inc., MO, USA
- McFarland standard scale: bioMe´rieux, France
- Magnesium sulfate: Carlo Erba, MI, Italy
- Monopotassium phosphate: Carlo Erba, MI, Italy
- MyTaq™ DNA Polymerases: Bioline, London, UK
- N-acetylglucosamine: Sigma-Aldrich Inc., MO, USA
- Penicillin G sodium salt: Sigma-Aldrich Inc., MO, USA
- Peptone: Himedia, Mumbai, India
- Phenol: Merck, Darmstadt, Germany

- *p*-nitrophenol (*p*NP): Sigma-Aldrich Inc., MO, USA
- *p*-nitrophenylpalmitate (*p*NPP): Sigma-Aldrich Inc., MO, USA
- Potassium phosphate: Carlo Erba, MI, Italy
- Potassium sodium tartrate: Ajex Finechem, Auckland, New Zealand
- Rose Bengal: Sigma-Aldrich Inc., MO, USA
- Skim milk powder: Difco, KS, USA
- Sodium acetate: Merck, Darmstadt, Germany
- Sodium carbonate: Carlo Erba, MI, Italy
- Sodium chloride: Ajex Finechem, Auckland, New Zealand
- Sodium dodecyl sulphate (SDS): Vivantis, CA, USA
- Sodium hydroxide: Ajex Finechem, Auckland, New Zealand
- Sodium phosphate: Ajex Finechem, Auckland, New Zealand
- Sodium metabisulfite: Carlo Erba, MI, Italy
- Standard Gram's staining reagents: Fisher Scientific, Leicestershire, UK
- Trichloroacetic acid: Merck, Darmstadt, Germany
- Tris-base: Vivantis, CA, USA
- Trisodium citrate: Merck, Darmstadt, Germany
- Triton X-100: Sigma-Aldrich Inc., MO, USA
- Tryptone: Difco, KS, USA
- Tyrosine: Sigma-Aldrich Inc., MO, USA
- Yeast extract: Himedia, Mumbai, India

3.2 Equipment

- Autoclave: Model SA-500K, Sturdy Industrial, Taipei, Taiwan
- Autopipette: LioPette, Bangkok, Thailand
- Bright-field microscope: OLYMPUS Optical, Tokyo, Japan
- Electrophoresis unit: Model Mini-Protean II Cell, Bio-Rad, CA, USA
- Gel documentation: Gel Doc 2000, Bio-Rad, CA, USA
- Genome sequencer: MiSeq sequencer, Illumina, CA, USA
- HiTrap Phenyl HP: GE Healthcare, Uppsala, Sweden
- Hitrap SP HP: GE Healthcare, Uppsala, Sweden
- Hot plate boiler and stirrer: Model C-MAC HS10, IKA, NC, USA
- Incubator: Model KT115, Binder, Tuttlingen, Germany
- Incubator shaker: Model VS-8480SFN, Vision Scientific, Seoul, Korea
- Laminar air flow: Model BVT 123, ISSOC, Bangkok, Thailand
- Microcentrifuge: Model Denville 260D, Denville Scientific, NJ, USA
- Nanodrop spectrometry: Model ND-1000, Thermo Fisher Scientific, DE, USA
- PCR machine: Peltier PTC-100^{TM} Thermal Cycler, MA, USA
- Peristaltic pump: Model EP-1 Econo Pump, Bio-Rad, CA, USA
- Peristaltic pump: Model Easy-load, MasterFlex, IL, USA
- pH meter: Model PP-50, Sartorius AG, Goettingen, Germany
- Power supply: Model EC-250-90, E-C Apparatus, MA, USA
- QIAprep Spin Miniprep kit: Qiagen, CA, USA
- QIAquick PCR Purification kit: Qiagen, CA, USA
- Refrigerated centrifuge: Model Universal 32R, Hettich, Tuttlingen, Germany
- Solid phase microextraction (SPME) GC-MS: Model
  GC6890N and 7694 Headspace sampler, Agilent Technology, CA, USA
- Spectrophotometer, microplate reader: Model Opsys MR, Dynex technology, VA, USA
- Spectrophotometer: Model UV-2800, UNICO, NJ, USA
- Tube sealer: Beckman Tube Sealer, Beckman, CA, USA
- Ultracentrifuge: Model Optima XL-100K, Beckman, CA, USA
- Vivaflow 50: Sartorius AG, Goettingen, Germany
- Vortex mixture: Model Genie 2, Scientific industries, NY, USA
- Water bath: Model Clifton NE5-28D, Fisher Scientific, Leicestershire, UK
- Weight balance, 2 digits: Model BL610 Sartorius, Goettingen, Germany
- Weight balance, 4 digits: Model AB104-S, Mettler Toledo, Greifensee, Switzerland

3.3 Primers use for the 16s rRNA gene analysis

- 27F (5'-AGA GTT TGA TCM TGG CTC AG-3' (M=C: A))
  : Bio Basic, Ontario, Canada
- 1525R (5'-AAG GAG GTG WTC CAR CC-3' (W=A: T; R=A: G))
  : Bio Basic, Ontario, Canada

3.4 Microbial cultures and nucleotide sequence accession numbers

Rhizopus oligosporus used as mycelia bait in isolation procedures was provided by the Deprtment of Microbiology, Faculty of Science, Chulalongkorn University.

Lysobacter enzymogenes C3 (Sullivan et al., 2003) used as a reference strain for bacterial indentification was provided by the Department of Plant Biology & Pathology, Rutgers, The State University of New Jersey, USA.

The DNA sequences of the 16S rRNA and *cel*8A genes of *L. enzymogenes* 521 were deposited in the GenBank database under accession numbers KR445659 and KR445660, respectively. The draft genome sequence of *L. enzymogenes* 521 was available in GenBank database under accession number LXSD00000000.

3.5 Procedures

3.5.1 Sample collection and isolation of *Lysobacter*-like bacteria

3.5.1.1 Sample collection

Soil samples were collected by pushing hand spade into the soil near plant rhizoid to a depth of approximately 10 cm. A 100 grams of each soil sample was obtained from mixing of 20 grams of random 5 nearby sites. The soil samples were put in a clean plastic zip-lock bag and kept in a container. The samples were immediately transferred to laboratory or kept below 4 °C until used.

3.5.1.2 Isolation of *Lysobacter*-like bacteria

Each of the soil samples collected throughout Thailand were used as an inoculum for bacterial isolation using the following procedures: fungal mycelial baiting, enrichment culture with fungal mycelia, and enrichment culture with colloidal chitin supplemented with antimicrobials (Kobayashi et al., 1995; 1996).

Fungal mycelial baiting procedure was conducted by growing *Rhizopus oligosporus* on cellophane-paper covered potato dextrose agar (PDA) plate at room temperature (28 ± 2 °C) for three days. The mycelia were separated from the medium, folded into 1.5 cm × 2.5 cm square, and buried under a soil sample contained within an incubating box. The samples were incubated at room temperature for three days. After incubation, the mycelia were recovered and washed with sterile dH_2O to remove loosely adhering soil particles by mixing with vortex mixture, then rinsed the mycelia with sterile dH_2O for 20 min. The washed mycelia were subsequently placed on yeast cell agar (YCA) consisting of 0.5% (w/v) autoclaved Baker's yeast cell wall and 1.5% (w/v) agar (Appendix A). Colonies capable of lysing autoclaved yeast cell wall were detected by a surrounding clear zone and selected. The so-obtained colonies were further purified by streaking them on fresh YCA repeatedly in order to yield a single colony.

Enrichment cultures were conducted in 100 mL M9 mineral salt medium (Appendix A) supplemented with 1 week growth on PDA of 10% (wet w/v) *R. oligosporus* mycelia, grown for one week on PDA, or 0.1% (w/v) colloidal shrimp chitin (Appendix B) as a substrate. The following antimicrobials were added to the colloidal chitin enrichment medium: 50 mg/L kanamycin, 50 mg/L penicillin and 100 mg/L Rose Bengal. Enrichment cultures were initiated by adding one gram of soil sample to the medium followed by 150-rpm shaking at room temperature, for seven days. The cultured medium solutions were collected, serially diluted and spread on YCA. Colonies capable of lysing autoclaved yeast cell wall were selected and purified by streaking them on the fresh YCA repeatedly in order to yield a single colony as previously described.

3.5.2. Screening and identification of *Lysobacter*-like bacteria

3.5.2.1 Screening of *Lysobacter*-like bacteria

3.5.2.1.1 Morphological observation

Gram's staining was conducted by first growing cells on Luria-Bertani (LB) agar (Appendix A) at 25 °C for three days. After staining, cells were observed using an Olympus BX51 bright-field light microscope. Gliding motility and colonial characteristics were observed by growing cells on Cook's *Cytophaga* Agar (CCA) and casein yeast extract agar (CYA) at 20 °C for 5 days (Appendix A). Colony morphology was observed under the bright-field light microscope.

3.5.2.1.2 Biochemical observation

3.5.2.1.2.1 Production of lytic enzymes on agar plates

Production of distinguish lytic enzymes including CMCase, chitinase, β-1,3-glucanase, protease and lipase were performed following the methods described by Kobayashi and El-Barrad (1996) and Reichenbach (2006). Colloidal chitin was prepared as described in Appendix B. Bacteria were grown in LB until the exponential growth phase was reached (18-24 h). Then, one mL of the cell culture was spun at 10,000 ×g for 2 min, supernatant was discarded and the cells were resuspended in 100 µl of saline solution. Ten µl of the so-obtained cell suspension were placed onto five different enzyme secretion agar media for specific, qualitative enzyme assay and incubated at 30 °C.

Production of CMCase was indicated by Congo Red color change caused by the degraded CMC after culturing the cell for three days on M813 (Palumbo et al., 2003) agar supplemented with 1% (w/v) CMC. After cultivation, the media were stained with 0.5% (w/v) Congo Red for 15 min and washed with 1 M NaCl for 15-30 min. A yellow halo around the colony indicated the CMCase activity.

Production of chitinase was detected by culturing the cell for five days on M813 agar containing 1% (w/v) colloidal chitin as the substrate. Appearance of a clear zone surrounding the bacterial colony indicated chitin degradation.

Production of β-1,3-glucanase was detected by culturing the cell on YCA for three days. Appearance of a clear zone around the colony indicated the degradation of β-1,3-glucan from yeast cell wall.

Activity of protease was detected by culturing the bacteria on skim milk agar medium for 24 h. Appearance of a clear zone around the colony indicated the digestion of milk proteins by the protease.

Secretion of lipase was detected by culturing the cell for five days on M813 supplemented with 1% (v/v) olive oil. Free fatty acid generated by lipid hydrolysis in the medium was indicated by color change of Phenol Red from red to be yellow of Phenol Red around the bacterial colony.

3.5.2.1.2.1 Catalase and oxidase test

Catalase and oxidase tests were performed using standard methods (Hitchins et al., 1998). Catalase test was conducted by using sterile toothpick or plastic tip to pick a bacterial colony and mixing into a drop of 3% (v/v) H_2O_2 on a glass slide. The presence of catalase was indicated by the bubbles of oxygen released. Oxidase test was conducted by mixing the picked bacterial cells into a drop of 1% (w/v) tetramethyl-p-phenylenediamine on a filter paper. The presence of cytochrome c oxidase was indicated by the purple color resulting from oxidation of the reagent.

3.5.2.2 Identification of *Lysobacter*-like bacteria by molecular technique

Species-level identification of *Lysobacter*-like bacteria was performed by the 16S rRNA gene sequence analysis. Genomic DNA was isolated following the protocol described by Jones and Bartlet (1990) (Appendix D). The 16S rRNA gene was amplified using the universal primer set 27F and 1525R (Lane, 1991). DNA amplification was conducted following the procedure described in Appendix D. The PCR product was purified using the QIAquick PCR Purification kit and was subsequently sequenced by Macrogen Inc., Seoul, Korea. The so-obtained assembled sequences were compared with other closely-related bacterial 16S rRNA sequences in the GenBank database searched by Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pairwise alignments were conducted with closely-related bacterial 16S rRNA gene sequences in the GenBank using Clustal X program (ftp://ftp.ebi.ac.uk/pub/ software/clustalw 2/2.0.11/, UK).

3.5.3 Phylogenetic analysis

Phylogenetic tree of the 16S rRNA sequences was constructed using maximum-likelihood analysis by Bayesian phylogenetic inference using the program MrBayes version 3.2.2 (Ronquist et al., 2012). In order to construct the tree of the 16S rRNA gene, the almost-complete DNA sequence (~1500 bp) of bacterial isolates were aligned with the sequences found in GenBank database and the ambiguous regions were subsequently excluded. Evolutionary distances were calculated using the Kimura two-parameter model. Analysis of 550,000 generations was done with tree diagnosing every 500 generations. The final 155 likely trees were obtained and the most consensus likely tree is presented with 10^3 bootstraps data set.

3.5.4 Characterization of *Lysobacter* sp.

3.5.4.1 Morphological characterization

Gram's stain and cell morphology was observed using cells grown in LB agar and skim milk acetate agar (Appendix A), respectively, in order to investigate variation in cell size within population by an Olympus BX51 bright-field light microscope. Gliding motility and colonial characteristics were observed as previously described in section 3.5.2.1.1.

3.5.4.2 Physiological and biochemical characterization

3.5.4.2.1 Growth temperature determination

Bacterial growth was observed at various temperatures including 20 °C, 25 °C, 30 °C, 35 °C, 37 °C and 40 °C. Bacterial seed cultures were prepared by growing them in LB at 200 rpm, 30 °C until the exponential growth phase was reached (18-24 h). The seed cultures were diluted with fresh LB until the optical density at 600 nm (OD_{600}) was 0.1. One mL of each diluted cell suspension was subsequently added into nine mL of fresh LB (10% (v/v) inoculum size). The cultures were incubated at different temperatures on a shaking incubator at 200 rpm for 24 h. OD_{600} of the cultures was measured and the corresponding levels of growth were determined by using the McFarland standard scale as a reference (McFarland, 1907).

3.5.4.2.2 Culture odor characterization

Solid phase microextraction (SPME) GC-MS analysis was performed at Central Instrument Facility, Mahidol University, Bangkok, Thailand in order to provide a volatile compound profile of the bacterial culture odors. A brief description of the SPME process was given in Table 3.1.

Table 3.1 Solid phase microextraction (SPME) GC-MS analysis condition for volatile compound profile produced in the bacterial culture.

Condition	Description
Sample:	5 mL of bacterial culture filtrate in a 20 mL vial, allowed to equilibrate at 40 °C for 30 min. with magnetic stirring
SPME fiber:	A 50/30 μm DVB/CarboxenTM /PDMS StableFlex™ (SUPELCO-5728-U)
Extraction:	headspace, 40 °C for 10 min. with magnetic stirring
Desorption process:	230 °C for 5 min
Column:	HP-INNOWax, 30 m x 0.25 mm x 0.25 μm
Oven:	Initial temp: 50 °C (2 min), Final temp: 220 °C (2 min)
Inlet temperature:	230 °C
Gas flow:	1 mL/min
Split mode:	Splitless
Scan mass:	50-550

3.5.4.2.3 Biochemical tests

Biochemical tests for non-fastidious gram negative bacteria were performed using API 20NE kit according to manufactory instruction. Catalase and oxidase test were conducted as described in section 3.5.2.1.2.1.

3.5.4.2.4 Production of lytic enzymes on agar plates

Production of CMCase, chitinase, protease and lipase were performed following the methods described in section 3.5.2.1.2.1. Furthermore, chitosanase assay was performed to investigate the dual mode of a bifunctional endoglucanase produced by *Lysobacter* sp.

Colloidal chitosan was prepared from 75-85% deacetylated chitosan as described in Appendix B. Secretion of chitosanase was detected after culturing the bacteria for 3 days on M813 agar supplemented with 1% (w/v) colloidal chitosan based on Congo Red color change as described previously.

3.5.4.2.5 Quantitative enzyme assays

Production of CMCase, chitosanase, chitinase, protease and lipase were carried out in M813 supplemented with 1% (w/v) CMC, 1% (w/v) colloidal chitosan, 1% (w/v) colloidal chitin, 0.5% (w/v) casein and 1% (v/v) olive oil emulsified in 1% (w/v) Na_2HPO_4, respectively. Bacterial seed cultures were prepared by growing them in LB at 30 °C for 18-20 h. Bacterial seed cultures were prepared

as in section 3.5.4.2.1. Ten mL of diluted cell suspensions were subsequently added to 90 mL of each enzymatic production medium (10% (v/v) inoculum size). The cultures were incubated under constant shaking at 200 rpm for 48 h at 30 °C, and subsequently collected and centrifuged at 4 °C (10,000 ×g, for 5 min). The supernatants were stored at 4 °C until used.

CMCase, chitosanase and chitinase activities were assayed using DNS method (see Appendix B). The reaction mixture included: (1) 50 µl of crude enzyme (0.028 Unit) and (2) 0.45 mL of either 1.0% (w/v) CMC, 1.0% (w/v) colloidal chitosan or 1.0% (w/v) colloidal chitin in 50 mM sodium acetate buffer, pH 5.0. The mixtures were incubated at 40 °C for 15 min and the reducing sugars released in the reaction mixtures were determined using 0-1500 µg/mL glucose (CMCase), glucosamine (chitosanase) and N-acetylglucosamine (chitinase) as standards (Appendix B). The reaction mixtures were added with 1.5 mL of DNS reagent and subsequently boiled for 5 min. Absorbance of the mixture was measured at 540 nm in order to quantify the amount of reducing sugar released in the reaction.

Protease activity was assayed following a procedure described by Cupp-Enyard (2008). The reaction mixture included: (1) 50 µl of crude enzyme and (2) 0.45 mL of 0.65% (w/v) casein in 50 mM potassium phosphate buffer, pH 7.5. After incubation at 40 °C for 15 min, the reaction was stopped by adding 0.5 mL of 110 mM Trichloroacetic acid. The amino acids released in the reaction mixture were determined using 0-100 µg/mL L-tyrosine as a standard (Appendix B). The 0.4 mL of reaction mixture and the standard were mixed with 1 mL of 0.5 M sodium carbonate and 0.2 mL of 0.5 mM Folin-Cioaclteu's Phenol Reagent, and incubated at 40 °C for 30 min. Absorbance of the mixture was measured at 660 nm.

Lipase activity was assayed following a procedure described by Glogauer et al. (2011) using *p*-nitrophenylpalmitate (*p*NPP) as a substrate. The substrate solution was prepared by mixing 50 mM Tris-HCl buffer (pH 7.5), 1 mM $CaCl_2$, 0.3% (v/v) Triton X-100, 4% (v/v) isopropanol, 1% (v/v) acetonitrile and 1 mM *p*NPP, in shaking water bath at 60 °C. The reaction mixture included: (1) 50 µl of crude enzyme and (2) 0.45 mL of the substrate solution. The mixtures were incubated at 40 °C for 15 min, and their absorbance were measured at 410 nm by using 0-500 µg/mL *p*-nitrophenol as a standard (Appendix B).

3.5.4.2.6 Protein determination

Protein concentration was determined by the Lowry method (Lowry et al., 1951) using 0-300 µg/mL bovine serum albumin (BSA) as a standard (Appendix C). The reaction mixture, including: (1) 0.5 mL of either crude enzyme or appropriately diluted purified enzyme and (2) 2.5 mL Lowry reagent, was incubated for 10 min at room temperature. Then, 0.25 mL Folin-Cioaclteu's Phenol reagent was added to the reaction mixture and incubated for 30 min at room temperature.

Absorbance of the reaction was measured at 750 nm and the protein concentration was calculated by using the standard BSA as a reference.

3.5.5. Optimization of endoglucanase production

Bacterial seed cultures were prepared as described in section 3.5.4.2.5. The cultures were incubated at 30 °C in 250 mL flask with a working volume of 100 mL M813 under constant shaking at 200 rpm for 24 h. The cultures were collected and centrifuged at 4 °C (10,000 ×g, for 5 min) for further endoglucanase activity assay using CMC as described previously.

3.5.5.1 Selection of carbon and nitrogen sources

Effects of different carbon and nitrogen sources on the endoglucanase production of *Lysobacter* sp. were investigated. A number of simple and complex carbon sources were examined along with different nitrogen sources for endoglucanase production in M813 (Appendix A). Combinations of carbon and nitrogen sources used in this experiment are shown in Table 3.2. Carbon and nitrogen sources were added into the medium at the final concentrations of 1% (w/v) and 0.25% (w/v), respectively, with a carbon:nitrogen ratio at 4:1. Combination of carbon and nitrogen sources that yielded the highest endoglucanase activity was selected for further optimization.

Table 3.2 Run number of carbon and nitrogen sources combinations for optimization of endoglucanase production from *L. enzymogenes* 521

Carbon	Nitrogen				
	Casein	Yeast extract	Peptone	$(NH_4)_2SO_2$	NH_4Cl
CMC	1	2	3	4	5
α-cellulose	6	7	8	9	10
Filter paper	11	12	13	14	15
Avicel	16	17	18	19	20
Sucrose	21	22	23	24	25
Glucose	26	27	28	29	30
Galactose	31	32	33	34	35

3.5.5.2 Experimental design for optimization of carbon and nitrogen concentrations

Response Surface Methodology (RSM) was used to investigate the optimal range of carbon and nitrogen concentrations. A set of experimental design (Box-Behnken design; BBD) was employed using different combinations of two independent variables: the carbon concentration (X_1) and the nitrogen concentration (X_2). The endoglucanase activity was the corresponding output

variable to be maximized. The ranges of the variables investigated (three levels including three replicates at the center point) are listed in Table 3.3. The measured BBD data were fitted with the following second-order polynomial equation (Box and Behnken, 1960).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_{11}^2 + \beta_{12} X_{12} + \beta_{22} X_{22}^2$$

where Y is the measured response, β_0 is the intercept term, β_1 and β_2 are linear coefficients, β_{12} is the logarithmic coefficient, β_{11} and β_{22} are quadratic coefficients, and X_1 and X_2 were coded independent variables.

Table 3.3 Box-Behnken design (BBD) variable codes and concentrations used in this study.

Run	Code		Concentration (% w/v)	
	carbon (X_1)	nitrogen (X_2)	carbon (X_1)	nitrogen (X_2)
1	-1	-1	0.25	0.2
2	-1	+1	0.25	0.6
3	+1	-1	1.25	0.2
4	+1	+1	1.25	0.6
5	-1	0	0.25	0.4
6	+1	0	1.25	0.4
7	0	-1	0.75	0.2
8	0	+1	0.75	0.6
9	0	0	0.75	0.4
10	0	0	0.75	0.4
11	0	0	0.75	0.4
12	0	0	0.75	0.4

The Statistica version 12 (StatSoft Inc., USA) was used to analyze statistically the regression coefficient, variable of the model (ANOVA) and to construct the three-dimensional response surface model. The optimal concentrations of carbon and nitrogen sources predicted by the model were verified in a repeated experiment and used in the following optimization.

3.5.5.3 Effect of glucan supplementation

Effect of glucan supplementation on endoglucanase production was investigated. The following glucans were tested: CMC, α-cellulose, filter paper and Avicel. The bacterium was cultured in M813 supplemented with each of the glucans at a number of concentrations between

0.2% (w/v) and 0.8% (w/v). Glucan that yielded the highest endoglucanase activity was added to the optimized medium for the following enzyme production.

3.5.5.4 Effect of temperature on growth rate and endoglucanase activity

The seed culture of *Lysobacter* was incubated at 30 ℃ for 18-20 h in the optimized M813 before it was diluted in saline solution until OD_{600} reached 0.1. Ten mL of the diluted cell suspension were subsequently added to 90 mL of the optimized M813 (10% (v/v) inoculum size). The cultures were incubated for 96 h under constant shaking at 200 rpm at different temperatures between 30 ℃ and 39 ℃. The cultures were aerobically incubated for 96 h under constant shaking at 200 rpm at different temperatures between 30 ℃ and 39 ℃. One mL of the bacterial cultures were sampled every 24 h and centrifuged at 4 ℃ (10,000 ×g, for 5 min). The supernatants were assayed for endoglucanase activity using the DNS method (Miller et al., 1960) as described in section 3.5.4.2.5.

Bacterial cell density was measured using a standard direct-plate count method. Cells were sampled from the cultures grown for different incubation times between 24 h and 96 h and temperatures between 33 ℃ and 39 ℃ before they were serially diluted with 0.85% NaCl. Ten µL of the diluted cell suspension was spread on LB agar plate and incubated for 48 h. The cell density and bacterial growth rate were calculated using the following equations:

$$\text{Colony-forming units per milliliter (CFU/mL)} = \frac{\text{Number of colony} \times \text{Dilution factor}}{\text{Diluted mixture volume}}$$

3.5.6. Purification of endoglucanase

3.5.6.1 Preparation of the crude endoglucanase

Endoglucanase production was conducted in the optimized medium under the optimal condition obtained from section 3.5.5.4. The bacterium was cultured in 250 mL flask with 100 ml working volume at 30 ℃, 200 rpm, for 48 h. After incubation, the cultured broth (3.5 L) was harvested by centrifugation at 10,000 ×g, 4 ℃, for 20 min. The supernatant was further concentrated by ultrafiltration using 10 kDa MW membrane cut-off Vivaflow 50 (Sartorius AG, Germany). Endoglucanase activity was assayed as previously described in section 3.5.4.2.5. Protein content of the initial and concentrated enzymes were determined as described in section 3.5.4.2.6. Enzyme solutions were stored at 4 ℃ or on ice before being used in the following experiments.

3.5.6.2 Ammonium sulfate precipitation

To selectively precipitate the enzyme from the concentrated supernatant (750 mL), ammonium sulfate fractionation was conducted from 0-20%, 20-40%, 40-60%, 60-80%, and 80-90% saturations, respectively. The required amount of ammonium sulfate of each fraction was obtained from the table 1C in appendix C. The concentrated supernatant was stirred on an ice box on top of a stirrer, and ammonium sulfate was slowly added to the solution until 20% saturation was achieved. The solution was kept stirring for 6 h and the precipitate was then recovered by centrifugation at 10,000 ×g for 20 min. The protein pellet was completely dissolved in 500 mL 50 mM sodium acetate buffer, pH 5.0. Ammonium salt remaining in the solution was removed using 10 kDa MW ultrafiltration membrane cut-off Vivaflow 50 until a final volume of 30 mL was achieved. Assay of endoglucanase activity and protein concentration of the solution (0-20% saturation) were determined as described in section 3.5.4.2.5 and 3.5.4.2.6, respectively. This procedure was repeated until the precipitated proteins from all fractions were obtained. Fractions with high specific enzyme activity were pooled and stored at 4 °C until used.

3.5.6.3 Column chromatography

3.5.6.3.1 Hydrophobic interaction chromatography

HiTrap Phenyl HP column (GE Healthcare, Sweden) was used to purify the endoglucanase by separating it from proteins with different hydrophobicity. The column was prepared according to manufacturer's instructions. The start buffer used for HiTrap Phenyl HP was 1 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 (Appendix C). The pooled protein fraction obtained from ammonium sulfate precipitation was dialyzed with the start buffer of HiTrap Phenyl HP using ultrafiltration membrane, and subsequently loaded onto the equilibrated column. After the start buffer passed through, the bound proteins were eluted with a linear gradient from 1 M to 0 M ammonium sulfate at a flow rate of 1.0 mL/min. Five-mL fractions were collected and assayed for endoglucanase activity as described in section 3.5.4.2.5. Protein concentration of each fraction was determined using the Lowry method as described in section 3.5.4.2.6. Fractions containing endoglucanase activity were pooled, salt was removed by dialysis in 50 mM sodium acetate buffer (pH 4.0) and the enzyme was stored at 4 °C until used.

3.5.6.3.2 Ion exchange chromatography

Hitrap SP HP column (GE Healthcare, Sweden) was used to further purify the enzyme by separating it from proteins with different surface charge distribution. The column was prepared according to manufacturer's instructions. The start buffer was 50 mM sodium acetate buffer, pH 4.0 (Appendix C). The pooled fraction obtained from HiTrap Phenyl HP column was dialyzed in the

start buffer of Hitrap SP HP using ultrafiltration membrane and was loaded onto the equilibrated column. Five-mL fractions were collected at a flow rate of 5.0 mL/min and assayed for the endoglucanase activity and protein concentration. Fractions containing enzyme activity were pooled, salt was removed by dialysis with into 50 mM sodium acetate buffer (pH 5.0) using the ultrafiltration membrane. The purified enzyme was concentrated and stored at 4 °C until used.

3.5.6.4 SDS-PAGE and zymographic analysis

SDS-PAGE was performed in 5% stacking and 10% resolving gel, respectively. Proteins were detected by Coomassie Brilliant Blue G250 (Neuhoff et al., 1985). Standard molecular weight marker, BLUeye Prestained Protein Ladder (MW from 10 kDa to 245 kDa) (GeneDireX, Inc., Nevada, USA), was used. Electrophoresis was conducted at room temperature at a constant current of 30 mA for 2 h.

For zymographic analysis, CMC and colloidal chitosan were separately added at 0.2% (w/v) final concentration into a native PAGE of 5% stacking and 10% resolving gel (Sambrook et al., 1989). The purified endoglucanase (10 µg protein) mixed with the sample buffer (2% (w/v) SDS, 5% (v/v) β-mercaptoethanol) was applied into each native-gel. Electrophoresis was operated on ice at a constant current of 30 mA for two h. The CMCase and chitosanase zymograms were prepared using a method of Bischoff et al. (2006) with slight modifications. After electrophoresis, the gels were washed with 20% (v/v) isopropanol in 50 mM sodium acetate buffer (pH 5.0) for 20 min. The gels were then washed three times (20 min each) in the same buffer without isopropanol. The gel was incubated at 40 °C in the endoglucanase assay buffer, 50 mM sodium acetate buffer (pH 5.0), for one h, stained with 0.1% (w/v) Congo Red for 30 min, and destained with 1 M NaCl for 30 min. The CMCase and chitosanase activities were visualized on the gel as a yellow band resulting from the hydrolysis of the CMC and colloidal chitosan, respectively.

3.5.7. Characterization of the purified endoglucanase

Endoglucanase activity assay for each biochemical property was carried out according to the method previously described, unless otherwise specified.

3.5.7.1 Optimal pH and temperature of endoglucanase and chitosanase activity

The optimal conditions for the endoglucanase and chitosanase activity were determined as different combinations between pH and temperature. Assays were conducted at various temperatures ranging from 30 °C to 80 °C and various pH values ranging of from 3.0 to 11.0 in three buffers (Table 3.4). Reaction was conducted by adding 50 µl of the diluted, purified endoglucanase into 450 µl of 1% (w/v) CMC or colloidal chitosan dissolved in buffer at each specific pH and

incubated separately at certain temperatures for 15 min. The endoglucanase and chitosanase activity were calculated as a relative activity (%) of the maximum activity.

Table 3.4 Buffers used for the purified endoglucanase and chitosanase activity assays

pH	Buffer	pH	Buffer
3.0	50 mM sodium acetate	8.0	50 mM phosphate
4.0	50 mM sodium acetate	9.0	50 mM glycine
5.0	50 mM sodium acetate	10.0	50 mM glycine
6.0	50 mM phosphate	11.0	50 mM glycine
7.0	50 mM phosphate		

3.5.7.2 Thermal stability

To investigate thermal stability, the purified endoglucanase was incubated in the absence of CMC or colloidal chitosan at various temperatures (30, 40, 50, 60, 70 and 80 °C) for 180 min (three h). Aliquots of the purified enzyme were collected every 30 min and immediately chilled on ice for 10 min. Residual activities were measured in 50 mM sodium acetate buffers (pH 5.0) at the optimal temperatures for both activities. Initial activities prior to incubation were defined as 100% relative activity.

3.5.7.3 pH stability

For pH stability test, 700 µL of purified endoglucanase was added to 2.1 mL of buffer at various pHs (Table 3.4) and incubated both at room temperature (30 ± 2 °C) and 4 °C for seven days. Aliquots of the enzyme were collected every 12 h and enzyme activity assay was initiated by adding 50 µl of the incubated endoglucanase into 450 µl of 1% (w/v) CMC or colloidal chitosan in 50 mM sodium acetate buffer (pH 5.0) and further incubated for 15 min at the optimal temperatures. Residual activity was calculated as a percentage of initial activity before incubation.

3.5.7.4 Substrate specificity

The substrate specificity of the purified endoglucanase was tested with final concentration at 1% (w/v) each of the following substrates; CMC, α-cellulose, colloidal chitosan, filter paper, Avicel, fibrous-cellulose, cellobiose, cotton, and 0.1% (w/v) each of the following substrates; colloidal chitin and laminarin. Colloidal chitosan and colloidal chitosan were prepared according to the method described by Yabuki et al. (1988) (Appendix B). Endoglucanase activity was assayed under the optimal condition as described in section 3.5.7.3. The enzyme activity toward CMC was defined as 100% relative activity.

3.5.7.5 Effect of metal ions, chelator and salts

Effect of metal ions including Ba^{2+}, Ca^{2+}, Co^{+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Rb^{+}, Zn^{2+}, together with a metal chelator, EDTA, and NaCl were investigated. An equal amount of the purified endoglucanase was separately incubated at 4°C for 30 min in 50 mM sodium acetate (pH 5.0) containing each metal/chelator at two different final concentrations of 1 and 10 mM. For effect of salt, NaCl was tested at 100 mM and 1000 mM. The enzyme reaction was assayed at 40°C for 10 min and the residual activity was calculated using a control reaction without metals/chelator/salt as 100% relative activity.

3.5.7.6 Effect of substrate concentration

Enzyme kinetic of the purified endoglucanase was determined by assaying the enzymatic activity toward CMC at various concentrations ranging from 1.0 to 6.0% (w/v) in 50 mM sodium acetate buffers (pH 5.0) under the optimal condition for 15 min as previously described. The Michaelis-Menten kinetic constants (*K*m, *V*max) were calculated by fitting the initial velocity data to the Michaelis–Menten equation and linear regression of the Lineweaver and Burk double-reciprocal plot.

3.5.8 Statistical analysis

One-way analysis of variance (ANOVA) and/or Duncan's Multiple Range Test (DMRT), when appropriate, were applied to calculate statistical differences among the means of data using SPSS 19.0 software package (SPSS Inc., Chicago, U.S.A.). Experiments were performed in triplicate (N=3). Differences at $P<0.05$ were considered significant.

3.5.9. Draft genome sequencing and molecular characterization of the endoglucanase gene

3.5.9.1 Genome sequencing

Lysobacter chromosomal DNA isolation was carried out using a modified method by Staskawicz et al. (1987) (Appendix D). DNA quality was determined by nanodrop spectrometry and agarose gel electrophoresis. Genome sequencing and genome assembly were conducted at Waksman Institute of Microbiology, Rutgers, The State University of New Jersey using the Illumina's desktop sequencing system and about 800 contigs with largest contig size of 60 Kbps have been assembled with default assembly parameters using Velvet version 1.0.3 (Zerbino and Birney, 2008).

3.5.9.2 Draft genome analysis

The draft genome sequence was simultaneously annotated by RAST (http://rast.nmpdr.org/) (Aziz et al., 2008) and MicroScope platform (Vallenet et al., 2012) pipeline servers without manual curation. All gene product names used in this study are derived from the

homologs of *Lysobacter enzymogenes* C3 as presented in the GenBank database, Accession number NZ_CP013140 (http://www.ncbi.nlm.nih.gov/genome). General genome analysis and visualization were conducted in Unipro UGENE (http://ugene.unipro.ru/index.html), RAST (http://rast.nmpdr.org/) and CGView (Grant and Stothard, 2008).

3.5.9.3 Analysis of genes encoding proteins belong to glycoside hydrolase class

Proteins belong to glycoside hydrolase class were searched from the annotated data derived from the draft genome sequences. Comparative analysis of the glycoside hydrolase gene abundant was conducted based on protein sequences derived from the complete genome sequences of *L. enzymogenes* C3 and other related bacteria which have been submitted in Carbohydrate-Active enZYmes database (CAZy database; http://www.cazy.org/b5226.html).

3.5.9.4 Analysis of the full-length endoglucanase (cel8A) gene

The annotated data were searched for endoglucanases, and those sequences were compared with the endoglucanase gene sequences found in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple amino acid sequence alignment of Cel8A with related family 8 glycoside hydolases (GHF-8 proteins) was performed using Mega 5.0 package (Tamura at al., 2011). Amino acid sequences of Cel8A from *Clostridium thermocellum* ATCC 27405 (Alzari and Dominguez 1996) and chitosanase from *Bacillus* sp. K17 (Adachi et al., 2004) were analyzed with the Cel8A for substrate binding sites and substrate recognition sites.

3.5.9.5 Phylogenetic trees of the endoglucanase (*cel*8A) gene

The full-length *cel*8A gene sequence was aligned with the ralated GHF-8 protein sequences found in the GenBank database and the ambiguous regions were subsequently excluded. The evolutionary distances was calculated using General Time Reversible (GTR) model. The final 79 likely trees were obtained from analysis of 380,000 generations. The most consensus likely tree is shown with 10^3 bootstraps data set at the branch points.

Chapter IV

RESULTS

4.1. Sample collection and isolation of *Lysobacter*-like bacteria

4.1.1 Sample collection

Sixty-five samples of soils, including rhizosphere soil, forest soil, grass covered soil, and other types of soil (listed in Table 1F, Appendix F), were collected between 2010 and 2013 from 12 provinces throughout Thailand (Fig. 1). Part of the samples were collected during the rainy season (June, August and November) while the others were collected during the dry season (February to May).

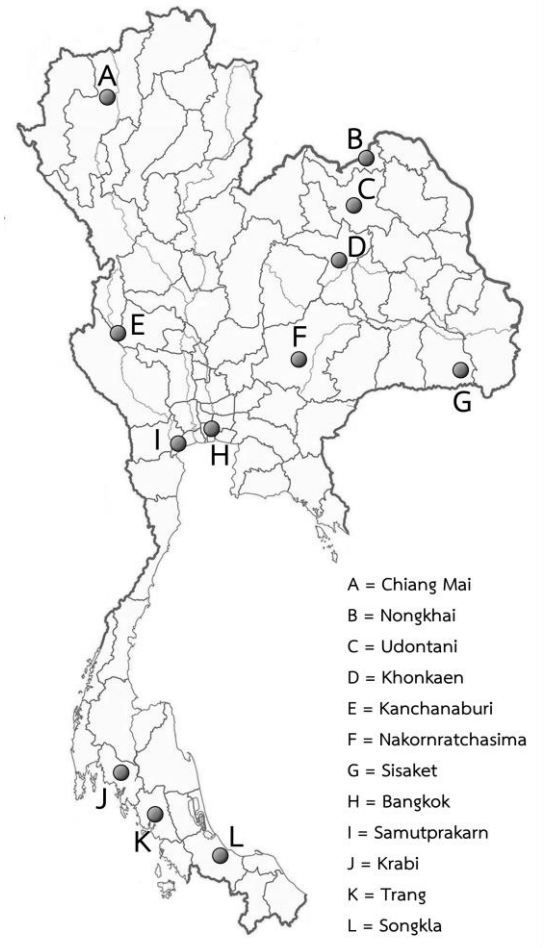


Figure 4.1 Collection sites of soil samples from 12 provinces in Thailand

4.1.2 Isolation of *Lysobacter*-like bacteria

The mycelia bating using *Rhizopus oligosporus* was a difficult procedure to recover *Lysobacter*-like bacteria from soil samples due to the contamination of *R. oligosporus* mycelia with the desired group of bacteria (Fig. 4.2A). On the other hand, the enrichment culture using fungal mycelia as a selected substrate yielded a number of bacteria that can utilize fungal cell wall component as energy source. The colloidal chitin-enrichment culture with the aid of antibiotics was the most effective procedure that yield more number of chitinolytic bacteria, which have been visually distinguished based on the presence of clear zones around the colonies on the YCA plate (Fig. 4.2, B). Consequently, this method enabled us to successfully isolate the desired group of bacteria.

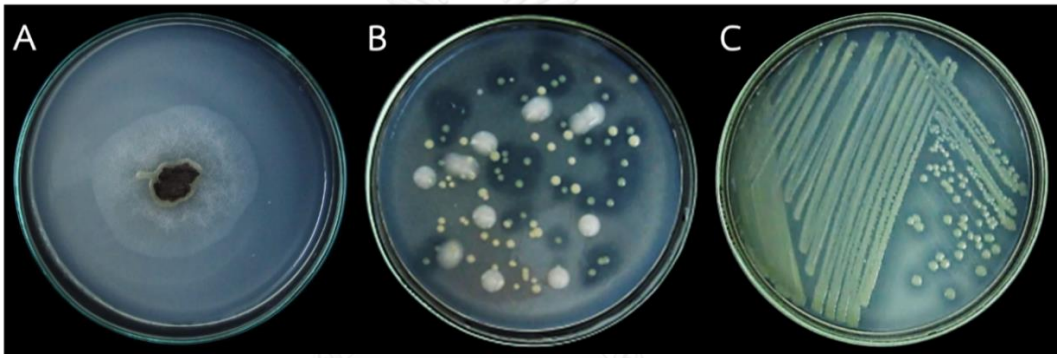


Figure 4.2 *Lysobacter*-like bacteria isolated from the fungal mycelium baiting method (A), the colloidal chitin enrichment method (B), and the purified bacterial colony capable of degrading yeast cell wall (C).

Lysobacter-like bacteria seem to be broadly distributed in Thailand as they have been isolated from many regions throughout the country. In particular, *Lysobacter*-like bacteria have been frequently found in cereals rhizosphere soils (57%) and grass covered soils (52%). The occurrences in the other samples were between 42% and 25% (Table 4.1).

Table 4.1 Source of soil samples and occurrence of *Lysobacter*-like bacteria based on isolation method

Source	Sample Name*	Number of samples	Number of *Lysobacter*-like bacteria obtained from using each isolation method		
			A[a]	B[b]	C[c]
Cereals rhizosphere soil (corn, rice)	1, 2, 3, 4, 5, 6, 57	7	-	3	1
Other plant rhizosphere soil (cassava, sugar cane, bamboo, chilli, bean)	7, 14, 18, 19, 23, 24, 25, 28	8	-	-	2
Forest soil	13, 29, 30, 31, 32, 33	7	-	1	2
Grasses cover soil	34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 48, 49, 50, 52, 53, 54, 55, 61, 64, 65	23	-	-	12
Others (non-plant cover)	8, 9, 10, 11, 12, 15, 16, 17, 20, 21, 22, 26, 27, 47, 51, 56, 58, 59, 60, 62, 63	21	-		7

* Details of the soil samples were given in Table 1F (Appendix F)

[a] A = Fungal mycelial baiting method

[b] B = Fungal mycelial enrichment method

[c] C = Colloidal chitin enrichment with antimicrobial supplementation method

4.2 Screening and identification of *Lysobacter*-like bacteria

4.2.1 Screening of *Lysobacter*-like bacteria

In order to distinguish the *Lysobacter*-like bacteria from other taxonomic groups, morphological and physiological traits were compared between the purified bacterial cultures obtained from single colonies (Fig. 4.2, C) and the reference strain, *Lysobacter enzymogenes* C3

(Sullivan et al., 2003). A total of 237 single-colony bacterial cultures isolated by chitin-based isolation procedures were obtained from a number of soil samples. All bacterial isolates were categorized into three groups based on the appearance of several characters (Table 4.2). Among these bacteria, 44 chitinolytic isolates (*Lysobacter*-like bacteria) formed clear zones on YCA (β-1,3-glucanase positive) and colloidal chitin agar (chitinase positive). Within these 44 isolates, nine expressed hydrolytic activities on CMC agar (endoglucanase positive), skim milk agar (protease positive) and olive oil agar (lipase positive) (Fig. 4.3). As shown in Table 4.3, most of the endoglucanase-producing bacterial isolates were recovered from grass-covered soil collected from shady lowlands and open highlands in Thailand.

Table 4.2 Gram's stain and biochemical tests of distinguishable characters standardized by *Lysobacter enzymogenes* C3 characters. The number of bacterial isolates categorized in each group is shown below the table.

Test	*Lysobacter enzymogenes* C3	*Lysobacter enzymogenes*-like bacteria	*Lysobacter*-like bacteria	Other groups of bacteria
Gram's stain	-	-	-	+/-
Gliding motility	+	+	+/-	+/-
Enzyme degradation:				
Endoglucanase	+	+	+/-	+/-
Chitinase	+	+	+	-
β-1,3-glucanase	+	+	+	-
Protease	+	+	+	+/-
Lipase	+	+	+	+/-
Oxidase test	+	+	+/-	+/-
Catalase test	+	+	+/-	+/-
Number of bacterial isolate		1	43	193
Number of endoglucanase positive isolate		1	8	13

Table 4.3 Soil source and locations where the endoglucanase-producing bacterial isolates were collected. The bacteria were all recovered by enrichment culture supplemented with colloidal chitin and certain antimicrobials.

Isolate	Province	Collection Date	Area	Source
346	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
385	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
391	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
425	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil under mushroom mycelia
455	Nakornratchasima	May, 2013	Open highland	Grasses cover soil under coconut trees
513	Udontani	May, 2013	Open highland	Grasses cover soil under coconut trees
521	Nongkhai	May, 2013	Open highland	Grasses cover soil near Makhong river
561	Songkla	Jun, 2013	Shady lowland	Grasses cover soil
573	Songkla	Jun, 2013	Shady lowland	Rice field soil

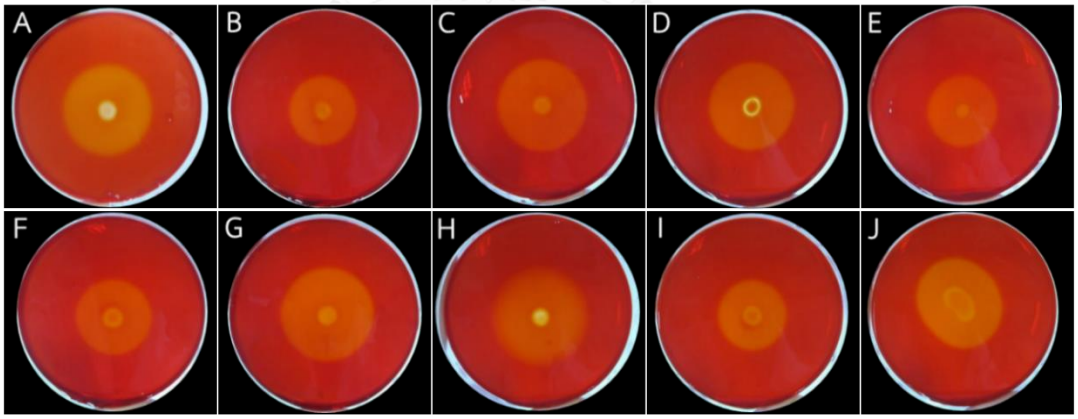


Figure 4.3 Endoglucanase activity towards CMC of the chitinolytic bacterial isolates as evidenced by clear zones on CMC agar stained with 0.5% (w/v) Congo Red. (A) *Lysobacter enzymogenes* C3, and the isolates (B) 346, (C) 385, (D) 391, (E) 425, (F) 455, (G) 513, (H) 521, (I) 561, (J) 573

The nine endoglucanase-producing isolates, 346, 385, 391, 425, 455, 513, 521, 561, and 573, were Gram negative an average cell size of 0.7-1.5 × 3.0-8.5 µm. Six of them, including 346, 391, 425, 521, 561 and 573, displayed gliding motility on CCA and CYA. Colony color of these bacteria varied from pale yellow to creamy yellow when cultured in different media. They were all positive for catalase activity, while some of them were negative for oxidase activity. All isolates produced distinguishable lytic enzymes endoglucanase, chitinase, β-1,3-glucanase, protease, and lipase (Table 4.4).

4.2.2 Identification of *Lysobacter*-like bacteria by molecular technique

Genomic DNAs of the nine endoglucanase-producing *Lysobacter*-like bacteria were extracted and analyzed by agarose gel electrophoresis (Appendix D). The genomic DNAs were used as DNA templates to amplify 16S rRNA gene using the primers 27F and 1525R. The PCR products were purified and visualized by agarose gel electrophoresis (Fig. 4.4). The amplified product sizes of were about 1.5 Kbp.

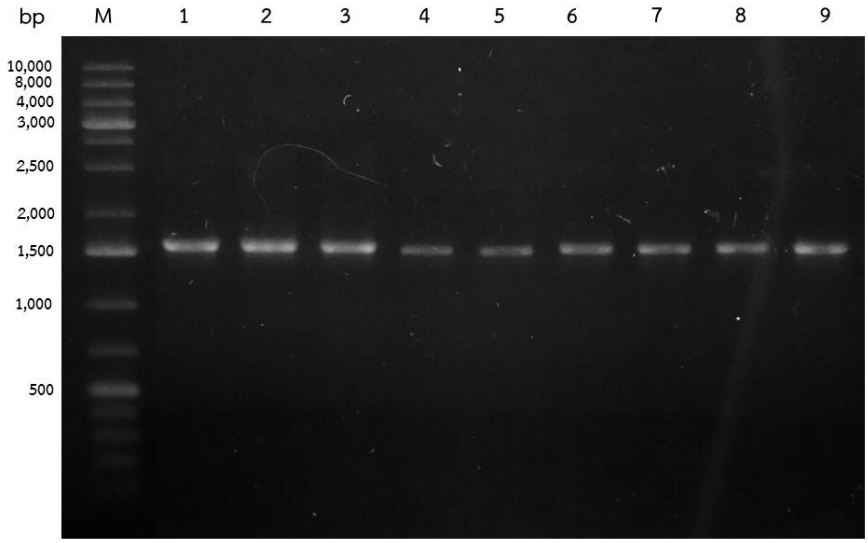
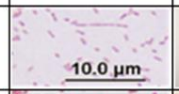
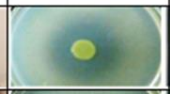
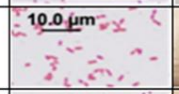
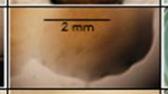
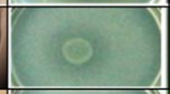
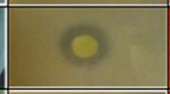
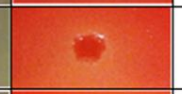
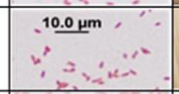
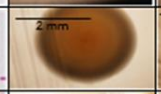
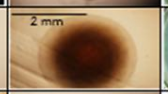
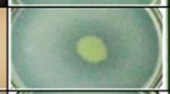
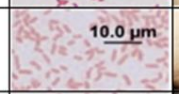
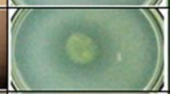
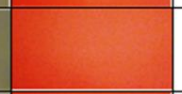
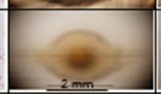
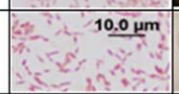
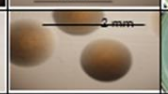


Figure 4.4 Purified amplification product of 16S rRNA gene from nine endoglucanase-positive isolates. Lanes: M = Molecular marker; 1 Kb ladder, 1 to 9 = PCR products of the isolates 346, 385, 391, 425, 455, 513, 521, 561 and 573, respectively.

Table 4.4 Characteristics of endoglucanase-producing *Lysobacter*-like bacterial isolates recovered from enrichment cultures in comparison with the reference strain, *Lysobacter enzymogenes* strain C3.

Isolate	Cell morphology	Gliding motility		Enzyme activity on agar plate				Catalase test	Oxidase test
		on CCA	on CYA	Chitinase	β-1,3-glucanase	Protease	Lipase		
L. enzymogenes C3								+	+
346								+	-
385								+	-
391								+	+
425								+	-
455								+	+
513								+	+
521								+	+
561								+	+
573								+	+

Based on 16S rRNA gene sequence analysis, the nine endoglucanase-producing isolates were identified as Gammaproteobacteria, belonging to the family Xanthomonadaceae with nucleotide sequence identity between 99.1% and 99.6% (Table 4.5). Among the identified isolates, the isolate 521 was the only one belonging to the genus *Lysobacter*, and was identified as *Lysobacter enzymogenes*, with 99.4% nucleotide sequence identity to *Lysobacter enzymogenes* strain 495.

Table 4.5 Taxonomic identification of endoglucanase-producing bacterial isolates and endoglucanase activity. The bacteria were all recovered by enrichment culture supplemented with colloidal chitin and antimicrobials.

Isolate	Closest species[a]	GenBank Accession No.	% Identity	Endoglucanase activity (U/mL)
346	*Xanthomonas translucens*	NR 036968	99.1	0.12 ± 0.001
385	*Xanthomonas albilineans*	NR 074403	99.4	0.28 ± 0.005
391	*Xanthomonas sacchari*	NR 026392	99.3	0.22 ± 0.002
425	*Xanthomonas translucens*	NR 036968	99.4	0.12 ± 0.003
455	*Xanthomonas sacchari*	NR 026392	99.2	0.14 ± 0.005
513	*Xanthomonas sacchari*	NR 026392	99.4	0.26 ± 0.006
521	*Lysobacter enzymogenes*	NR 036925	99.4	0.11 ± 0.004
561	*Xanthomonas sacchari*	NR 026392	99.6	0.13 ± 0.002
573	*Xanthomonas sacchari*	NR 026392	99.3	0.13 ± 0.004

[a] Based on 16S rRNA gene sequence of the isolate (Appendix F) with the sequences in the GenBank database

4.2.3 Phylogenetic analysis of the 16S rRNA gene sequence

Phylogenetic relationship of the obtained chitinolytic bacteria and other related species of Gammaproteobacteria is shown in Figure 4.5. From the tree, all nine endoglucanase-producing *Lysobacter*-like bacteria isolated from grass-covered soils in Thailand belong to the family Xanthomonadaceae. Most of the isolates belong to *Xanthomonas* spp. lineages with percentage of bootstrap at 100%. The isolate 521, which was identified as *Lysobacter enzymogenes,* was the only one belonging to *Lysobacter* spp. lineage with percentage of bootstrap at 100%. Based on percent nucleotide identity, the most similar bacteria to the isolate 521 were: *L. enzymogenes* strain 495 (99.4%), *L. enzymogenes* strain C3 (99.3%) and *L. oryzae* strain YC6269 (97.5%). The isolates 346 and 425 were similar to *Xanthomonas translucens* LMG 876 (99.1 and 99.4%) whereas the isolate 385 was similar to GPE PC73 (99.4%). The rest were similar to *X. sacchari* strain LMG 471

(99.3%-99.6%). From the phylogenetic tree, there was no clear relationship between subgroups, sources of sample, and geographic locations where the bacteria were isolated. Results from phylogenetic analysis were in accordance with their morphological and biochemical characteristics, indicating that these bacteria do belong to the family Xanthomonadaceae and that the isolate 521 is *L. enzymogenes*.

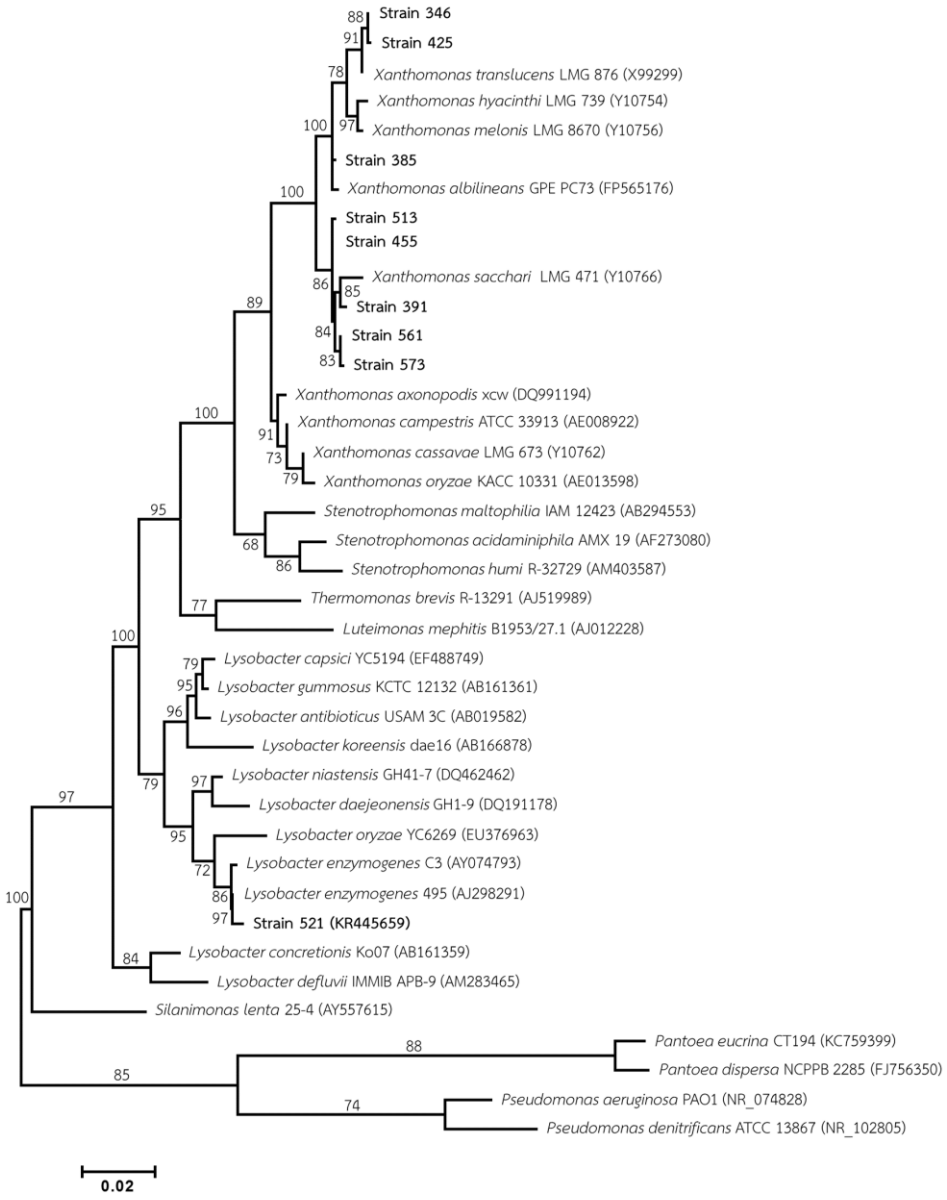


Figure 4.5 Phylogenetic relationship of the isolates 346, 385, 391, 425, 455, 513, 521, 561 and 573 with *Lysobacter* spp. and other species of Gammaproteobacteria (GenBank accession numbers in parentheses). The tree was constructed by maximum-likelihood analysis on the basis of 16S rRNA gene sequence analysis using MrBayes version 3.2.2. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points.

4.3 Characterization of *Lysobacter enzymogenes* 521

4.3.1 Morphological characterization

The tropical *L. enzymogenes* 521 was characterized in comparison to its counterpart from the temperate, *L. enzymogenes* C3. Generally, the tropical *L. enzymogenes* 521 is a Gram-negative, rod-shaped bacterium displaying gliding motility on CCA and CYA. Typical cells are rod-shaped (0.5-1 × 3.5-5 µm) (Fig. 4.6A), but they are present as a morphologically diverse population with the cell length up to 20 µm when cultured in skim milk acetate medium for 24 hours (Fig. 4.6B). The great variation in cell length is a distinguishable character of *Lysobacter* (Reichenbach 2006). The morphology of the colony was creamy-yellow irregular, with flat to convex shape with undulated edges. Morphological variations between the tropical *L. enzymogenes* 521 and the temperate *L. enzymogenes* C3 were observed. Slight differences were found in cell size, colony color and shape (Fig. 4.6A-B) and gliding features on CCA and CYA (Fig. 4.6C-D). Strain 521 had cell size between 0.5-1 × 3.5-5 µm, while strain C3 had longer cells between 0.5-1 × 7.2-8.4 µm and 50-60 µm when cultured in skim milk acetate medium. Colony color of the strain 521 was creamy yellow, while that of the strain C3 was pale yellow when cultured on LB medium.

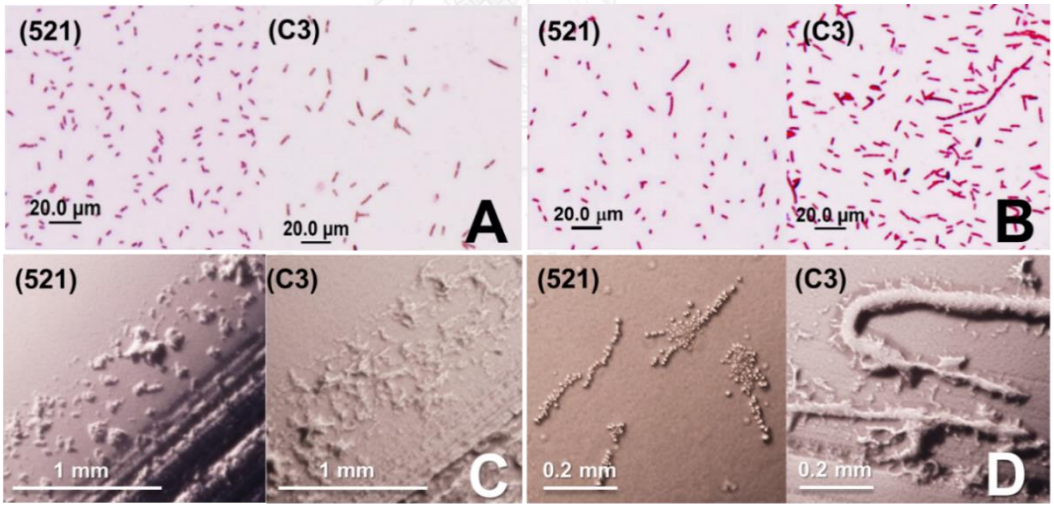


Figure 4.6 Distinguishable characters of *Lysobacter enzymogenes* 521 (Left) compared with the reference strain, *Lysobacter enzymogenes* C3 (Right). (A) cell morphology on LB agar, (B) cell morphology on skim milk acetate agar, (C) gliding motility on CC agar, (D) gliding motility on CY agar

4.3.2 Physiological and biochemical characterization

4.3.2.1 Determination of optimal growth temperature

Both strains could grow within a range of temperatures between 20 °C and 40 °C (Table 4.6). However, the temperate *L. enzymogenes* C3 grew at a slower rate with respect to the tropical 521 within the entire range of temperatures. The optimal temperature for growth was 30 °C for both strains. However, *L. enzymogenes* 521 displayed the ability to grow rapidly also at higher temperatures (37 °C and 40 °C).

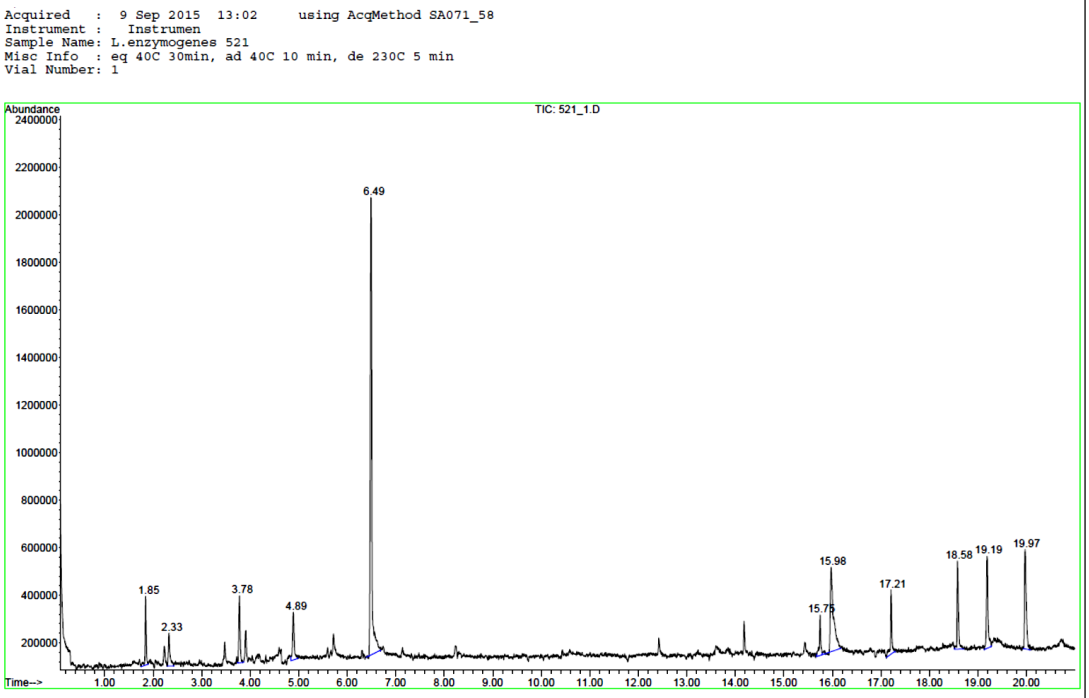
Table 4.6 Differential phenotypic characteristics of *L. enzymogenes* 521 and *L. enzymogenes* C3

Characteristic		*L. enzymogenes* 521	*L. enzymogenes* C3
Growth at	20 °C	+[a]	+
	25 °C	+	+
	30 °C	++	++
	35 °C	+	+
	37 °C	+	w
	40 °C	+	w

[a] Based on McFarland standard No.: ++; rapidly grew (No.5), +; grew (No.2-4), w; slowly grew (No.0.5-1), -; not grew

4.3.2.2 Culture odor characterization

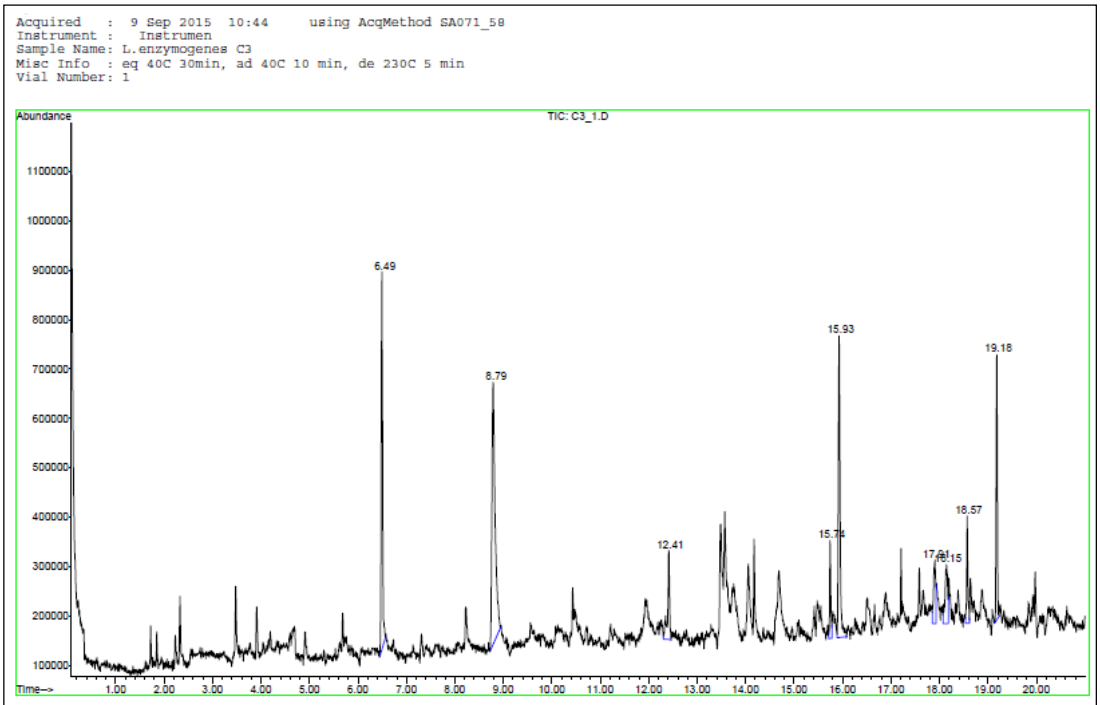
The strains 521 and C3 had clearly different odors generated by different volatile compounds in the culture medium. The strain 521 had a smell of fermented sugar, while the strain C3 had a peculiar medicinal smell. Based on Solid phase microextraction (SPME) GC-MS analysis of the cultured medium, both strains had different volatile compound profiles. Some compounds were produced by both strains although in different amounts (Fig. 4.7 and Fig. 4.8). The major odorous volatile compounds produced by the strain C3 included 2-Methylbutanol, 2-ethyl-1-hexanol, nonanoic acid and 4-morpholineethanamine, while in the strain 521 only 2-Methylbutan-1-ol was detected. There were minor odorous volatile compounds detected in the strain 521, but not presented in the strain C3, including (Fig. 4.7-4.8).



Peak No.	Retention time	Compound[a]	% area
1	1.85	Heptane	3.61
2	2.33	Hexamethyl-cyclotrisiloxane	2.81
3	3.78	Methyl 2-methylbutyrate	4.55
4	6.49	2-Methylbutanol	40.13
5	15.75	Eicosamethyl-cyclodecasiloxane	2.84
6	15.97	Nonyl-Cyclopropane	14.24
7	18.58	BISTRIMETHYLSILYL N-ACETYL EICOSASPHINGA-4,11-DIENINE	6.28
8	19.19	2,4-Bis(1,1-dimethylethyl)-Phenol	7.95

[a] Compared with Wiley Database

Figure 4.7 Mass spectrum of volatile products produced by *L. enzymogenes* 521 cultured in liquid LB at 30 °C, for 48 h. Identified volatile compounds are shown below the graph.



Peak No.	Retention time	Compound[a]	% area
1	6.49	2-Methylbutanol	18.52
2	8.79	2-Ethyl-1-hexanol	28.87
3	12.41	Tetracosamethyl-cyclodecasiloxane	5.76
4	15.75	Eicosamethyl-cyclodecasiloxane	3.74
5	15.93	Nonyl-Cyclopropane	17.95
6	17.91	Nonanoic acid	4.55
7	18.15	4-Morpholine Ethanamine	5.24
8	18.57	BISTRIMETHYLSILYL N-ACETYL EICOSASPHINGA-4,11-DIENINE	4.58
9	19.18	2,4-Bis(1,1-dimethylethyl)-Phenol	10.78

[a] Compared with Wiley Database

Figure 4.8 Mass spectrum of volatile products produced by *L. enzymogenes* C3 cultured in LB at 30 ℃, for 48 h. Identified volatile compounds are shown below the graph.

4.3.2.3 Biochemical tests

The biochemical tests showed that both strains displayed a number of similar biochemical traits. They were positive for catalase, oxidase, β-glucosidase, gelatinase and β-galactosidase and negative for arginine dihydrolase, urease, indole production, nitrate reduction, and glucose fermentation. Both strains were able to utilize D-glucose, D-mannose, D-maltose and N-acetylglucosamine, but not L-arabinose, D-mannitol, capric acid, adipic acid, phenylacetic acid and potassium gluconate. On the other hand, the strain 521 could utilize malic acid and trisodium citrate, while the strain C3 could utilize weakly malic acid, but not trisodium citrate (Table 4.7).

4.3.2.4 Production of lytic enzymes on agar plates and enzyme activities

From agar plate assays, both 521 and C3 were positive for CMCase, chitosanase, chitinase, protease and lipase (Fig. 4.9). The strain 521 visibly secreted all enzymes to a wider area with respect to that of C3.

Quantitative assay of the lytic enzymes clearly showed that the strain 521 produced higher specific activities in all enzymes than C3 under the same conditions. The maximum CMCase, chitosanase and chitinase specific activities from the culture supernatants of *L. enzymogenes* 521 were higher than those observed for *L. enzymogenes* C3 by 1.7, 1.9 and 1.4 times, respectively. Moreover, the maximum protease and lipase specific activities of 521 were much higher than those observed for C3 by 2.6 and 3.3 times, respectively (Fig. 4.9).

Table 4.7 Biochemical characteristics of *L. enzymogenes* 521 and *L. enzymogenes* C3

Characteristic	*L. enzymogenes* 521	*L. enzymogenes* C3
Catalase test	+[a]	+
Oxidase test	+	+
Reduction of NO_3 to NO_2/ Reduction of NO_2 to N_2	-/-	-/-
Indole production	-	-
Glucose fermentation	-	-
Arginine dihydrolase	-	-
Urease	-	-
β-Glucosidase	+	+
Gelatinase	+	+
β-Galactosidase	+	+
Assimilation:		
D-glucose	+	+
L-arabinose	-	-
D-mannose	+	+
D-maltose	+	+
D-mannitol	-	-
N-acetyl-Glucosamine	+	+
Capric acid	-	-
Adipic acid	-	-
Malic acid	+	w
Phenylacetic acid	-	-
Potassium gluconate	-	-
Trisodium citrate	+	-

[a] + = positive/assimilated, - = negative/not assimilated, w = weakly assimilated

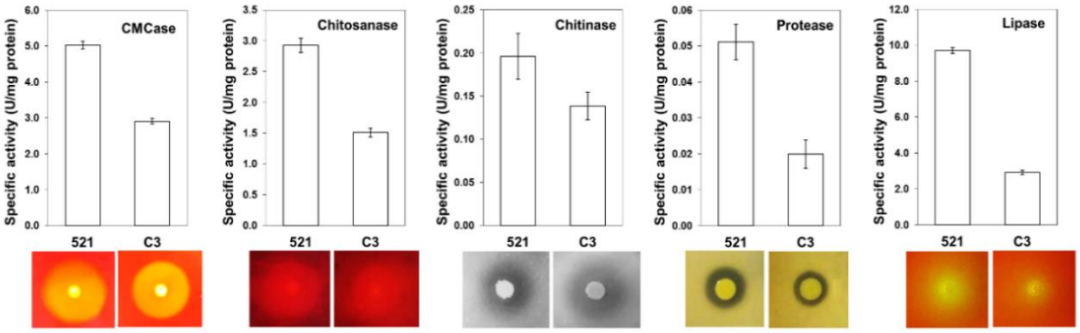


Figure 4.9 Specific lytic enzyme activities of *L. enzymogenes* 521 and *L. enzymogenes* C3 grown in different media at 30 °C for 48 h. The activities are presented as means and one standard deviations derived from three replicates. Below the graphs, the enzyme activities are indicated by haloes around colonies grown on M813 agar supplemented with 1% CMC (CMCase), 1% colloidal chitosan (chitosanase), 2% colloidal chitin (chitinase), 0.5% casein (protease), or 1% olive oil (lipase) (all w/v).

4.4 Optimization of endoglucanase production

4.4.1 Selection of carbon and nitrogen sources

In order to evaluate the optimal combination of carbon and nitrogen sources for endoglucanase production of *L. enzymogenes* 521, a number of simple and complex carbon sources were examined in combination with different nitrogen sources (Table 4.8). All organic nitrogen sources (casein, yeast extract and peptone) enhanced significantly higher endoglucanase activity compared to inorganic nitrogen sources ($(NH_4)_2SO_2$ and NH_4Cl). The strain had no apparent endoglucanase activity when cultured with inorganic nitrogen unless a monosaccharide, ie. glucose or galactose, was present. For carbon sources, simple monosaccharides (glucose and galactose) strongly enhance endoglucanase production, while more complex carbons (CMC, α-cellulose, filter paper, Avicel and sucrose) had significantly lower enzyme production. High production of endoglucanase was achieved by combinations of a monosaccharide and an organic nitrogen. No endoglucanase activity was observed when CMC, α-cellulose, filter paper, Avicel and sucrose were used in combination with neither inorganic nitrogen.

The maximum endoglucanase activity (1.07 U/mL) was achieved when glucose was used as carbon source and casein was used as nitrogen source after 24 h of incubation. Therefore, glucose and casein were selected for further studies.

Table 4.8 Optimization of carbon and nitrogen sources for endoglucanase production of *L. enzymogenes* 521 grown for 24 h at 30 °C. Culture samples were collected and filtrates were evaluated for release of reducing sugar from CMC using the DNS assay.

	Endoglucanase activity (U/mL)*				
Combination	Casein	Yeast extract	Peptone	$(NH_4)_2SO_2$	NH_4Cl
CMC	0.45 ± 0.008 $^{a, C}$	0.27 ± 0.004 $^{c, C}$	0.32 ± 0.015 $^{b, C}$	0.00 ± 0.000 $^{d, B}$	0.00 ± 0.000 $^{d, B}$
α-cellulose	0.35 ± 0.010 $^{a, D}$	0.25 ± 0.015 $^{c, C}$	0.28 ± 0.010 $^{b, C}$	0.01 ± 0.002 $^{d, B}$	0.00 ± 0.000 $^{d, B}$
Filter paper	0.38 ± 0.010 $^{a, CD}$	0.27 ± 0.011 $^{b, C}$	0.29 ± 0.014 $^{b, C}$	0.00 ± 0.000 $^{c, B}$	0.00 ± 0.000 $^{c, B}$
Avicel	0.36 ± 0.007 $^{a, D}$	0.25 ± 0.015 $^{c, C}$	0.28 ± 0.010 $^{b, C}$	0.00 ± 0.000 $^{d, B}$	0.01 ± 0.000 $^{d, B}$
Sucrose	0.45 ± 0.005 $^{a, C}$	0.33 ± 0.020 $^{b, B}$	0.33 ± 0.018 $^{b, C}$	0.00 ± 0.000 $^{c, B}$	0.01 ± 0.000 $^{c, B}$
Glucose	1.07 ± 0.094 $^{a, A}$	0.48 ± 0.054 $^{b, A}$	0.53 ± 0.040 $^{b, A}$	0.07 ± 0.068 $^{c, A}$	0.11 ± 0.068 $^{c, A}$
Galactose	0.86 ± 0.061 $^{a, B}$	0.52 ± 0.055 $^{ab, A}$	0.41 ± 0.066 $^{b, B}$	0.11 ± 0.081 $^{c, A}$	0.07 ± 0.068 $^{c, AB}$

* The endoglucanase activity was present as means±one standard deviation derived from triplicates (N=3).

Different superscript lowercase letters in the same column and different uppercase letters in the same row indicate that the values are significantly different (ANOVA and DMRT, $P < 0.05$).

4.4.2 Experimental design for optimization of carbon and nitrogen concentrations

In order to evaluate the effects of glucose and casein, different combinations of glucose and casein concentrations were designed using BBD. The predicted and observed responses obtained from experimental design are shown in Table 4.9. The experimental maximum endoglucanase activity from crude endoglucanase was achieved at 1.46 U/mL after incubation for 24 h. The statistical significance of the second-order polynomial equation was evaluated by the analysis of variance (ANOVA) shown in Table 4.10. ANOVA of total 12 experiments for endoglucanase production indicates that the model was statistically significant. The computed *F*-value is 7.5 times higher than the tabulated *F*-value ($F_{model} = 100.397 > F_{0.01, (4,7)} = 7.85$), and model terms values of *P* are less than 0.0009. The high value coefficient of determination of maximum endoglucanase activity ($R^2 = 0.983$) indicates that the regression model is significant at the 98.3% confidence level. This result indicates that the model had a good prediction accuracy for the analysis using the following second-order polynomial equation;

$$Y = -1.5026+3.9073X_1+6.8819X_2-2.508X_1X_1-0.4225X_1X_2-7.4875X_2X_2$$

where, *Y* represents response value or endoglucanase activity (U/mL). X_1 and X_2 were glucose (% w/v) and casein (% w/v), respectively.

Table 4.9 Design and results of Box-Behnken design (BBD) in this study

Run	Code value		Concentration value		Observed endoglucanase activity (U/mL)	Predicted endoglucanase activity (U/mL)
	X1	X2	Glucose (% w/v)	Casein (% w/v)		
1	-1	-1	0.25	0.2	0.332	0.373
2	-1	+1	0.25	0.6	0.837	0.688
3	+1	-1	1.25	0.2	0.306	0.434
4	+1	+1	1.25	0.6	0.642	0.580
5	-1	0	0.25	0.4	1.200	0.830
6	+1	0	1.25	0.4	1.049	0.806
7	0	-1	0.75	0.2	0.722	1.031
8	0	+1	0.75	0.6	0.872	1.261
9	0	0	0.75	0.4	1.466	1.445
10	0	0	0.75	0.4	1.461	1.445
11	0	0	0.75	0.4	1.452	1.445
12	0	0	0.75	0.4	1.444	1.445

Table 4.10 Analysis of variance (ANOVA) of the Box-Behnken experimental model developed for endoglucanase production by *L. enzymogenes* 521.

Source	Sum of Squares	df	Mean Square	F	Sig. (*P*-value)
Corrected Model	2.011[a]	4	0.503	100.397	0.000
Intercept	7.043	1	7.043	1406.632	0.000
Glucose (X_1)	0.262	2	0.131	26.189	0.001
Casein (X_2)	01.212	2	0.606	121.030	0.000
Error	0.035	7	0.005		
Total	13.616	12			
Corrected Total	2.046	11			

[a] R Squared = 0.983 (Adjusted R Squared = 0.973); Significant at $P < 0.01$

The response surface based on independent variables glucose (X_1) and casein (X_2) in Figure 4.10 indicated that the maximum endoglucanase activity was 1.45 U/mL, which would corresponds to the concentrations of 0.75% (w/v) glucose and 0.45% (w/v) casein. The so-obtained optimal conditions of glucose and casein concentrations were selected for the subsequent optimization study with respect to the concentration of glucan, which is discussed in the next section.

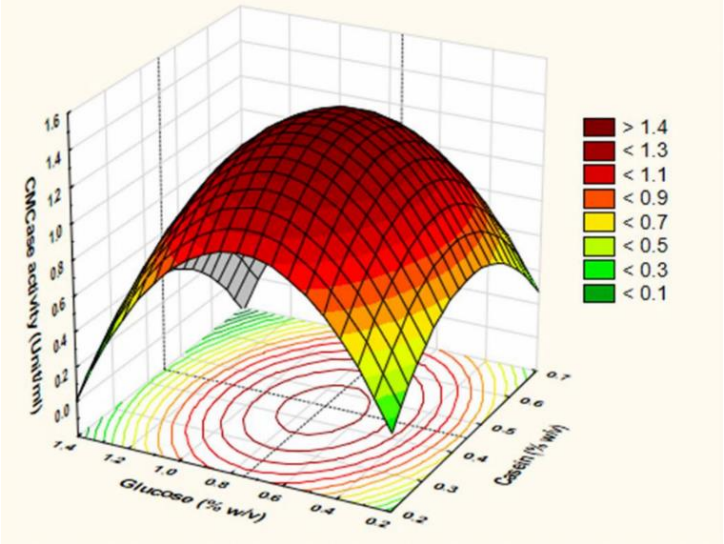


Figure 4.10 Response surface for extracellular endoglucanase activity describing effect of glucose and casein at the curtained concentrations. Culture samples were collected and filtrates were evaluated for release of reducing sugar from CMC using the DNS assay

4.4.3 Effect of glucan supplementation

The effect of glucan on the optimal production medium was also investigated in order to enhance the endoglucanase production by *L. enzymogenes* 521. The effect of supplementation of glucan at various concentrations is shown in Figure 4.11. We found that small concentrations of glucan (0.2-0.6% w/v) slightly increased the endoglucanase activity, while at the high concentrations of alpha-cellulose decreased it. The highest endoglucanase activity (1.94 ± 0.027 U/mL) was obtained when 0.2% (w/v) of CMC were added into the culture medium. Under these conditions, the endoglucanase activity increased by approximately 1.4 times with respect to the sample with no glucan added.

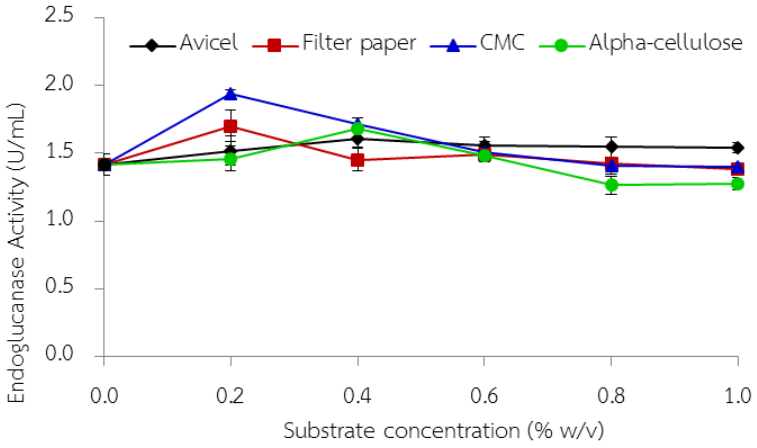


Figure 4.11 Effect of supplementation of glucan for endoglucanase production of *L. enzymogenes* 521. The bacterium was cultured in the optimal M813 (0.75% (w/v) glucose and 0.45% (w/v) casein) at room temperature (30±0.5 °C) for 24 h and then quantitatively determined by DNS assay.

4.4.4 Effect of temperature on growth and endoglucanase activity

The effect of temperature on growth and endoglucanase activity of the tropical *L. enzymogenes* 521 in comparison with the temperate *L. enzymogenes* C3 was investigated at different temperatures for 96 h in M813 prepared at the optimal conditions obtained from experiments discussed in the previous sections. The profiles of the growth and endoglucanase production of *L. enzymogenes* 521 and *L. enzymogenes* C3 are illustrated in Figure 4.12. Endoglucanase was produced both during growth phase and in the stationary phase. After 48 hours of incubation in the optimal M813, endoglucanase activity of *L. enzymogenes* 521 reached the maximum at 3.29 U/mL, while the maximum endoglucanase activity of *L. enzymogenes* C3 was achieved at 2.48±0.039 U/mL at 30 °C. At temperatures higher than 30 °C, the growth was delayed for 24 h in comparison with the optimal temperature (30 °C), and the endoglucanase activity decreased substantially. Interestingly, our results showed that *L. enzymogenes* 521 had higher growth and endoglucanase activity than those of *L. enzymogenes* C3 at all incubation temperatures, and especially between 33 °C and 39 °C. The maximum endoglucanase activity obtained from endoglucanase of *L. enzymogenes* 521 was 2.46 ± 0.19 U/mL, which was about 1.5 times higher than the one of *L. enzymogenes* C3 at the optimal incubation temperature.

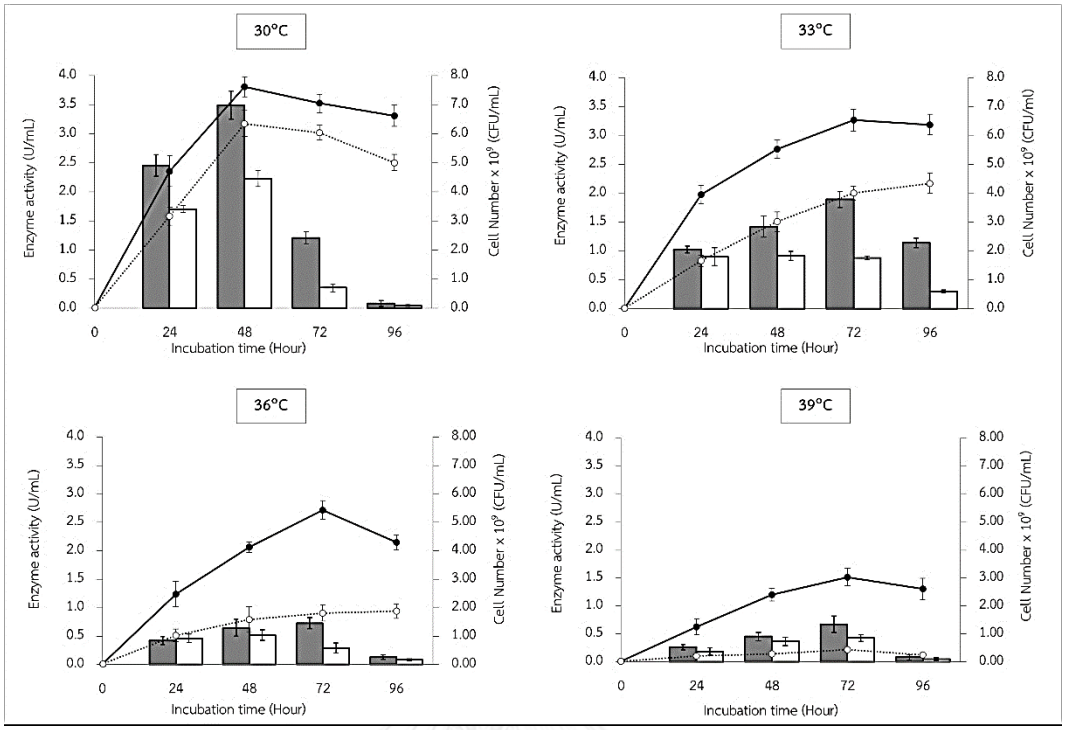


Figure 4.12 Endoglucanase activity and growth profiles of *L. enzymogenes* 521 and *L. enzymogenes* C3 cultured in modified M813 (M813 supplemented with 0.75% (w/v) glucose, 0.45% (w/v) casein and 0.2% (w/v) CMC). The cultures were aerobically incubated for 96 h, at 30 °C, 33 °C, 36 °C, and 39 °C. Endoglucanase activity was determined every 24 h. Symbols: (-●-) cell number and (■) endoglucanase activity of *L. enzymogenes* 521, (-○-) cell number and (□) endoglucanase activity of *L. enzymogenes* C3. Experiments were performed in triplicate (N = 3). The error bars indicate the standard deviations.

4.5 Purification of endoglucanase

4.5.1 Preparation of the crude endoglucanase

Crude endoglucannase was prepared from 3.5 liters of the optimal M813 as described in section 3.5.5.4. The culture was spun at 4 °C in order to remove cells. The culture supernatant (3000 mL) had total 3,348.0 U of endoglucanase activity and produced 3,177.0 mg of proteins. Thus, the specific activity of the enzyme in the crude preparation was 1.05 U/mg proteins. The crude enzyme was then concentrated by ultrafiltration to obtain 750 ml with 2,454.0 U of endoglucanase activity, 930.75 mg protein and 2.64 U/mg proteins of endoglucanase specific activity. The so obtained concentrated crude enzyme was further purified by ammonium sulfate precipitation.

4.5.2 Ammonium sulfate precipitation

An aliquot of 300 mL concentrated crude enzyme was purified by a stepwise increase of 20% saturations with ammonium sulfate ($(NH_4)_2SO_4$) from 0% to 80%, and additional increase of 10% saturations from 80% to 90%. The endoglucanase activity was mainly detected in 40-60% and 60-80% saturation fractions with the highest enzyme activity in 60-80% fraction (Table 4.11). In order to optimize enzyme harvest and purity, the protein fractionation of the remained concentrated crude enzyme was carried out in the range of 50-80% saturation with ammonium sulfate. After 50-80% saturation, the 122.43 mg of proteins were harvested with endoglucanase activity at 712.47 U in total. The endoglucanase with specific activity at 5.82 U/mg protein was obtained from the precipitation (table 4.12).

Table 4.11 Ammonium sulfate precipitation of endoglucanase from *L. enzymogenes* 521

Percent saturation range of $(NH_4)_2SO_4$	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor
Crude extract	300	184.20	282.88	1.54	1.00
0-20	30	16.25	1.45	0.09	0.06
20-40	30	24.30	2.00	0.08	0.05
40-60	30	25.02	76.65	3.06	1.99
60-80	30	26.80	200.25	7.47	4.87
80-90	30	13.86	0.67	0.05	0.03
90% supernatant	384	46.85	0.38	0.01	0.01

4.5.3 Column chromatography

4.5.3.1 Hydrophobic interaction chromatography

The enzyme prepared from ammonium sulfate precipitation was applied onto HiTrap Phenyl HP column as described in section 3.5.6.3.1. The chromatographic separation of the endoglucanase from other proteins is shown in Figure 4.13. Unbound proteins were firstly washed from the column with start buffer (1 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate, pH 7.0). The bound proteins were then eluted with linear salt gradient of 1.0 to 0 M in the elution buffer (50 mM sodium phosphate, pH 7.0). The protein with endoglucanase activity was eluted at fraction 32–39. The fractions were then pooled, the salt was removed and the enzyme was concentrated for a further purification step. The 18.99 mg protein was harvested with endoglucanase activity at 371.61 U/mL. The endoglucanase with specific activity at 19.57 U/mg protein was obtained from this step (table 4.12). The enzyme was purified about 18.6 fold and the yield was 11% with respect to the crude enzyme.

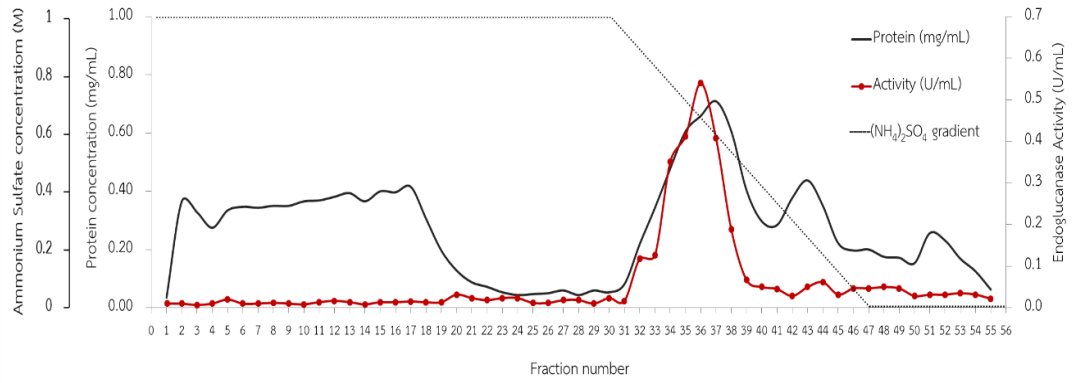


Figure 4.13 Chromatographic separation of extracellular enzymes from 50–80% ammonium sulfate fraction using HiTrap Phenyl FF column (1.6×4.5 cm column - fast flow at 1.0 mL/min. 5 mL fractions).

4.5.3.2 Ion exchange chromatography

The enzyme fraction obtained from HiTrap Phenyl HP column was applied onto Hitrap SP HP as discussed in section 3.5.6.3.2. The chromatographic separation of the endoglucanase from other proteins is illustrated in Figure 4.14. Unbound proteins were not detected when washed the column with start buffer (50 mM sodium acetate buffer, pH 4.0). The bound protein was eluted with linear salt gradient of 0 to 1.0 M in the elution buffer (1 M NaCl in 50 mM sodium phosphate, pH 7.0). The protein with endoglucanase activity was eluted at fraction 14. The fraction was then mixed 50 mM sodium acetate buffer pH 7.0 and the salt was removed. The enzyme was concentrated and stored at 4 °C for further characterization. The 1.70 mg protein was harvested with endoglucanase activity at 73.53 U/mL. Therefore, the endoglucanase with specific activity at 43.75 U/mg protein was obtained from this step (table 4.12). The enzyme was about 41 fold purified and the yield was 2.2% compared with crude enzyme.

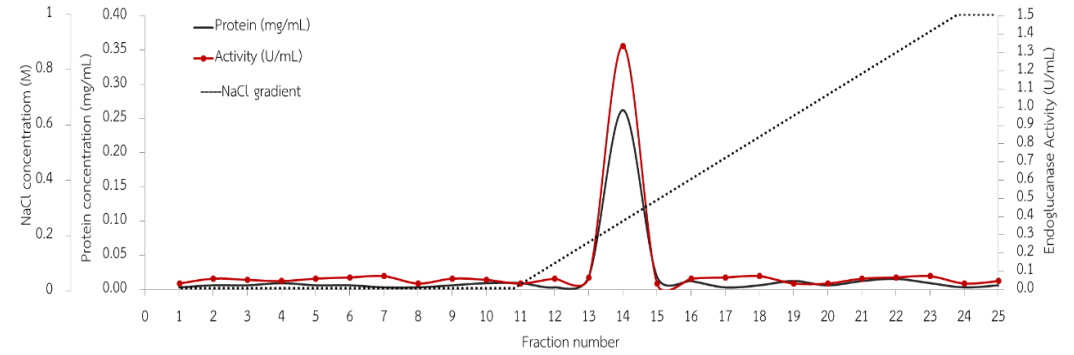


Figure 4.14 Chromatographic separation of extracellular enzymes from HiTrap Phenyl FF column fraction using Hitrap SP HP (1.6×4.5 cm column - fast flow at 5.0 mL/min. 5 mL fractions).

Table 4.12 Purification of endoglucanase produced from *L. enzymogenes* 521

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)[a]		Specific activity (U/mg)		Purification (fold)		Yield (%)	
			Endo[b]	Chit[c]	Endo	Chit	Endo	Chit	Endo	Chit
Culture supernatant	3000	3177.00	3348.00	1780.85	1.05	0.56	1.0	1.0	100	100
Ultrafiltration	750	930.75	2454.00	1333.70	2.64	1.43	2.5	2.6	73	75
$(NH_4)_2SO_4$ precipitation (50–80% saturation)	110	122.43	712.47	391.47	5.82	3.20	5.5	5.7	21	22
HiTrap Phenyl HP	30	18.99	371.61	194.56	19.57	10.25	18.6	18.3	11	11
Hitrap SP HP	10	1.70	73.53	39.75	43.25	23.38	41.0	41.7	2.2	2.2

[a] 1U = Amount of endoglucanase required to release 1 µmol glucose equivalent per min or amount of chtosanase required to release 1 µmol glucosamine equivalent per min under the assay conditions

[b] Endo = Endoglucanase

[c] Chit = Chitosanase

4.5.4 SDS-PAGE and zymographic analysis

It has been previously reported that *L. enzymogenes* has dual endoglucanase activities of CMCase and chitosanase (Hedges and Wolfe 1974; Ogura et al., 2006). In order to investigate the dual mode of activities of the endoglucanase from *L. enzymogenes* 521, SDS-PAGE and zymographic analysis were performed. Purity and mass of the purified endoglucanase was analyzed by SDS-PAGE, and enzyme activity was illustrated by native-PAGE as described in section 3.5.6.4. High protein purity with a single band was observed on the Coomassie-stained SDS-PAGE. The 41 kDa of protein mass was calculated using BLUeye Prestained Protein Ladder standard curve as a reference (Appendix E). Purified protein containing endoglucanase activity from *L. enzymogenes* 521 clearly exhibited an activity band on both the CMC-zymogram gel and the chitosan-zymogram gel, both corresponding to a single protein band observed on the Coomassie-stained SDS-PAGE gel (Fig. 4.15). This result demonstrated that the endoglucanase from *L. emzymogenes* 521 exhibited also CMCase-chitosanase bifunctional activity.

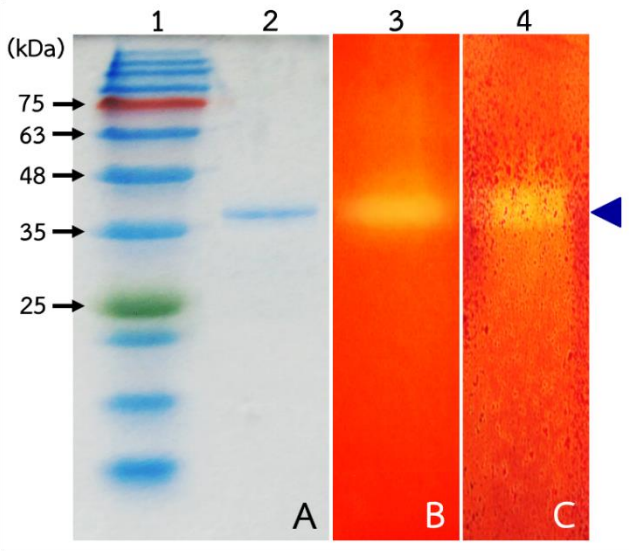


Figure 4.15 SDS-PAGE (a) and zymogram (b, c) analysis of the purified endoglucanase from *L. enzymogenes* 521. Protein mass and purity were determined by SDS-PAGE stained with Coomassie Brilliant Blue (a). endoglucanase activity of the protein was determined in a native PAGE containing 0.2 % (w/v) CMC (b) and chitosanase in a native PAGE containing 0.2 % (w/v) colloidal chitosan (c). The native-PAGE gels were stained with 0.1 % (w/v) Congo Red. Lanes: 1 Molecular weight markers (BLUeye Prestained Protein Ladder, molecular size markers in kDa), 2-4 purified endoglucanase (10 µg)

4.6 Characterization of the purified endoglucanase

4.6.1 Optimal pH and temperature of endoglucanase and chitosanase activity

The optimal condition for endoglucanase and chitosanase activity were determined as described in Section 3.5.7.1. The effect of pH and temperature on the activities of the purified endoglucanase is illustrated in Figure 4.16. The purified endoglucanase displayed endoglucanase activity within a wide pH range at 5–9, with maximal activity at pH 5 (Fig 4.16A). More than 70% of endoglucanase activity was observed at pH 5 and 6. The enzyme activity decreased dramatically at pH lower than 4 and higher than 9. For what concerns the effect of temperature on the endoglucanase activity, the enzyme activity was optimal at 40 °C, and it decreased significantly for all pH as the incubation temperature was raised from 50 °C to 80 °C.

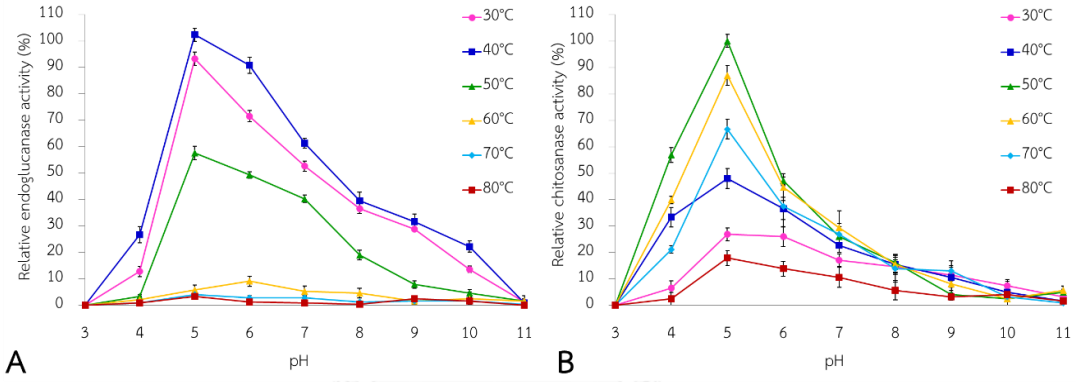


Figure 4.16 Effect of and pH temperature on endoglucanase activity (A) and chitosanase activity (B) of purified endoglucanase from *L. enzymogenes* 521 cultivated on optimal modified M813 (contained 0.75% glucose, 0.2% CMC and 0.45% casein). Reactions were conducted for 30 min with 0.028 Unit of purified enzyme and 1% (w/v) CMC/colloidal chitosan in 50 mM of sodium acetate buffers (pH 3.0–5.0), phosphate buffers (pH 6.0–8.0) or glycine buffers (pH 9.0-11.0) at various temperatures. The relative activity was calculated as the percentage of enzyme activity assayed in 50 mM sodium acetate buffers (pH 5.0) at 40 °C. Experiments were performed in triplicate (N=3) and error bars indicated standard deviations.

For the chitosanase activity, the purified enzyme showed chitosanase activity in a wide pH range at 5–9 with maximal activity at pH 5 (Fig 4.16B). The chitosanase activity was higher than 60% only at pH 5, and the enzyme activity dramatically decreased at pH higher than 8. The chitosanase activity was optimal at 50 °C, and it was decreased significantly for all pH as the incubation temperature was raised up to 80 °C. The optimal conditions for endoglucanase activity (pH 5.0, 40 °C) and for chitosanase activity (pH 5.0, 50 °C) of the purified endoglucanase were similar to those obtained from the crude enzyme (data not shown).

4.6.2 Thermal stability

The effect of temperature on the stability of the purified enzyme for endoglucanase and chitosanase activities was assessed at 30-80 ℃ as described in section 3.5.7.2. After 180 min remaining in the incubator, the purified enzyme was found to be relatively stable more than 80% at 30 ℃ and more than 60% at 40 ℃ of its original endoglucanase activity (Fig. 4.17A). The purified enzyme was inactivated after heating it up to 50^{o}C for 30 min, which reduced the endoglucanase activity to less than 50% residual activity. The endoglucanase activity was decreased by more than 80% after heating over 60^{o}C for 30 min. The thermal stability of chitosanase activity and endoglucanase activity were similar (Fig. 4.17B).

4.6.3 pH stability

The effect of pH on stability of the purified endoglucanase on endoglucanase and chitosanase activities was assessed by incubating it at room temperature (30 ± 2 ℃) and 4 ℃ for 7 days in buffers ranging between pH 4 and 11. After incubating the enzyme at room temperature, more than 80% of the endoglucanase activity persisted over a pH range of 6.0-9.0 (sodium acetate buffer pH 6.0, sodium phosphate buffer pH 6.0-9.0) (Fig. 4.18A). The effect of pH on stability for chitosanase activity showed similar trend to the endoglucanase activity. More than 80% of chitosanase activity persisted over a pH range of 6.0-9.0 (sodium acetate buffer pH 6.0, sodium phosphate buffer pH 6.0-9.0) (Fig. 4.18B). The enzyme stability was dramatically decreased when incubated at pH lower than 4.0 and higher than 10.0. On the other hand, more than 85% of endoglucanase and chitosanase activities persisted over the entire pH range (4.0 to 11.0) for up to 7 days of incubation at 4 ℃ (data not shown).

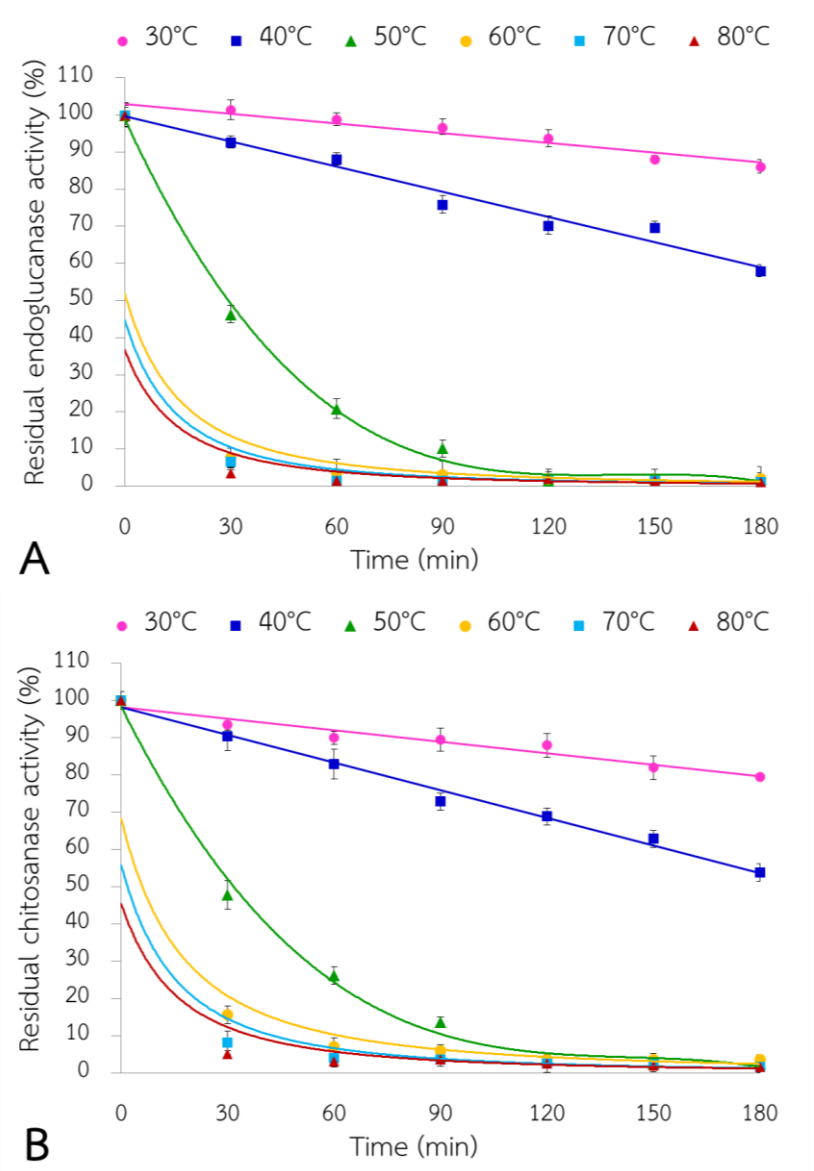


Figure 4.17 Effect of temperature on endoglucanase stability (A) and chitosanase stability (B) of purified endoglucanase from *L. enzymogenes* 521 cultivated on optimal modified M813 (contained 0.75% glucose, 0.2% CMC and 0.45% casein). The purified enzyme was separately pre-incubated in 50 mM sodium acetate buffers (pH 5.0) for 30 to 180 min at various temperatures. Reactions were conducted for 30 min with 0.028 Unit of purified enzyme and 1% (w/v) CMC/colloidal chitosan in 50 mM sodium acetate buffers (pH 5.0) at 40°C and the residual activity was then calculated as the percentage of enzyme activity. Experiments were performed in triplicate (N=3) and error bars indicated standard deviations.

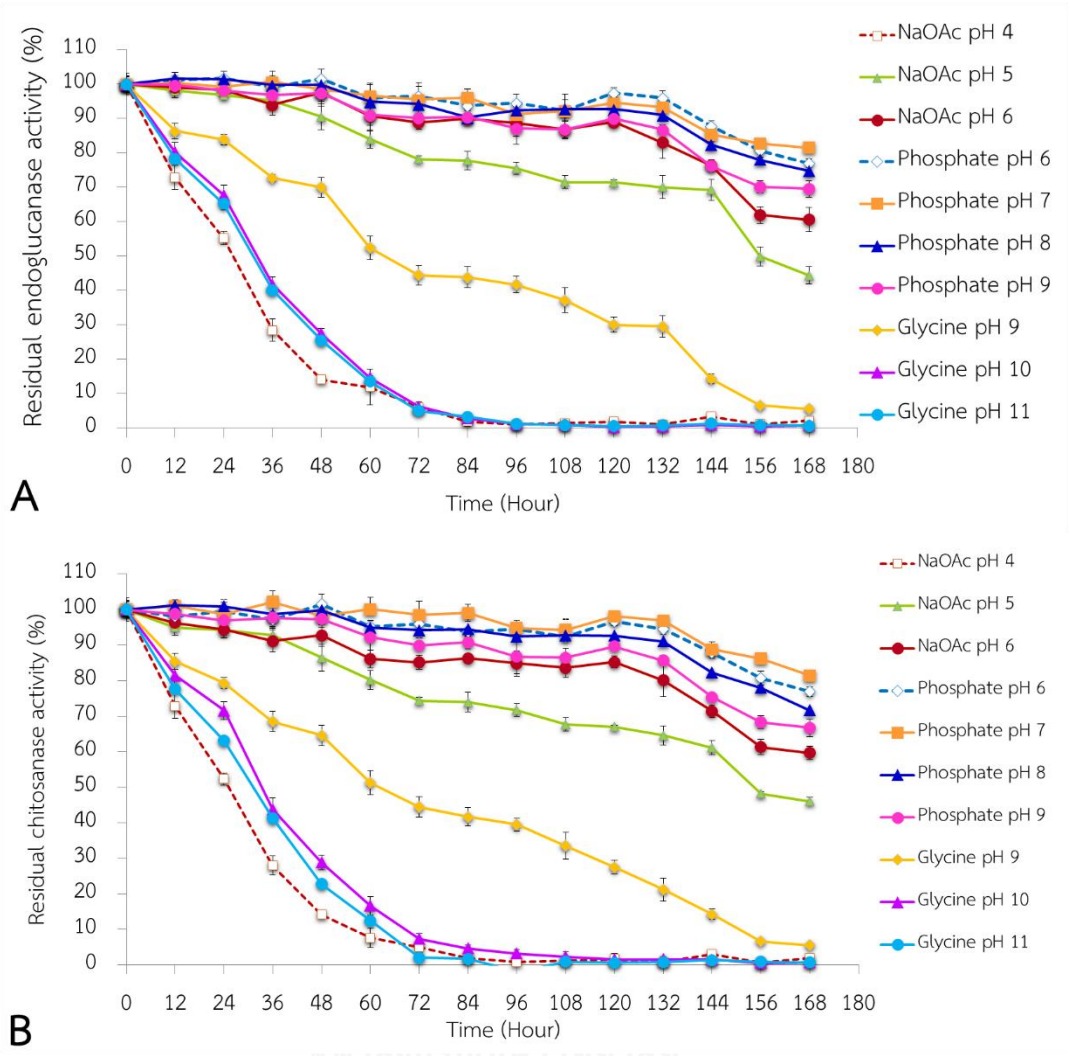


Figure 4.18 Effect of pH on endoglucanase stability (A) and chitosanase stability (B) of purified endoglucanase from *L.enzymogenes* strain 521 cultivated on optimal modified M813 (contained 0.75% glucose, 0.2% CMC and 0.45% casein). The purified enzyme was separately pre-incubated at room temperature (30±2 °C) in various pH including 50 mM of sodium acetate buffers (pH 3.0-6.0), phosphate buffers (pH 6.0-9.0) or glycine buffers (pH 9.0-11.0) for 168 h. Reactions were conducted for 30 min with 0.028 Unit of purified enzyme and 1% (w/v) CMC/colloidal chitosan in 50 mM sodium acetate buffers (pH 5.0) at 40°C and the residual activity was then calculated as the percentage of enzyme activity. Experiments were performed in triplicate (N=3) and error bars indicated standard deviations.

4.6.4 Substrate specificity

The purified endoglucanase showed different degrees of specificity towards glucans from different sources (Table 4.13). CMC was the best substrate among those tested (5.08 U/mg specific activity). α-cellulose and colloidal chitosan were hydrolyzed with relative activity at 48.8% (2.48 U/mg) and 44.9% (2.28 U/mg) of that of CMC, respectively. The purified endoglucanase hydrolyzed the following substrates: filter paper, Avicel, fibrous-cellulose, cellobiose and cotton only 11-28% of that of CMC. These results showed that the action mode of the purified enzyme was endocleavage type in that it could hydrolyze amorphous substrates more efficiently than crystalline substrates. In contrast, the enzyme activity towards colloidal chitin and laminarin was detected in a small range. Resistance of colloidal chitin and laminarin suggest that the purified enzyme had no chitinase and β-1, 3-glucanase, respectively.

Table 4.13 Substrate specificity of purified endoglucanase from *L. enzymogenes* 521.

Substrate	Specific activity (U/mg)	Relative activity
CMC	5.08 ± 0.204	100.0 ± 4.03^{a}
α-cellulose	2.48 ± 0.264	48.8 ± 5.20^{b}
Colloidal chitosan (80% deacetylation)	2.28 ± 0.226	44.9 ± 4.45^{b}
Fibrous-cellulose	1.44 ± 0.264	28.4 ± 5.20^{c}
Avicel	0.98 ± 0.264	19.3 ± 5.20^{d}
Cotton	0.97 ± 0.108	19.1 ± 2.13^{d}
Cellobiose	0.69 ± 0.173	13.6 ± 3.41^{de}
Filter paper	0.58 ± 0.264	11.4 ± 5.20^{ef}
Colloidal chitin	0.33 ± 0.109	6.5 ± 2.14^{f}
Liminarin	0.26 ± 0.041	5.1 ± 0.81^{f}

Relative activity expressed as rates relative to the activity of enzyme on CMC.

Mean ± one standard deviation derived from three replicates (N = 3).

Different superscript letter in the same column and row indicate that the values are significantly different (ANOVA and DMRT, $P < 0.05$).

4.6.5 Effect of metal ions, chelator and salts

The effect of cations, EDTA and NaCl on the purified endoglucanase is shown in Table 4.14. The enzyme activity was slightly inhibited by the presence of Ba^{2+}, Ca^{2+}, Li^{+} , $Mg^{2+}$and Zn^{2+} at 1 mM, and it was strongly inhibited by the presence of these cations at 10 mM. The enzyme activity was slightly suppressed by the presence of Rb^{+} at 1 mM, while it was completely suppressed at 10

mM. Cations Cu^{2+}, Fe^{2+}, Hg^{2+} and Mn^{2+} acted as strong inhibitors for the purified endoglucanase. In fact, 1 mM of these cations suppressed the enzyme activity by 22-85%.

Table 4.14 Effect of metal ions, chelator and salt on activity of purified endoglucanase from *L. enzymogenes* 521. Reactions were conducted for 15 min at 40°C with 0.028 Unit of purified enzyme, 1% (w/v) CMC in 50 mM of sodium acetate buffers (pH 5.0) and different concentrations of salts or metal ions. The residual activity was defined as the percentage of enzyme activity.

Substance	Relative activity (%) at 1 mM	Relative activity (%) at 10 mM
Ba^{2+}	88.0 ± 2.58^{c}	64.2 ± 6.84^{E}
Ca^{2+}	$95.6 \pm 5.34a^{bc}$	86.6 ± 6.25^{D}
Co^{+}	48.5 ± 0.76^{f}	105.1 ± 2.02^{BC}
Cu^{2+}	65.2 ± 1.53^{e}	21.8 ± 5.34^{F}
Fe^{2+}	78.0 ± 6.61^{d}	7.3 ± 2.02^{G}
Hg^{2+}	52.2 ± 9.32^{f}	29.1 ± 9.32^{F}
Li^{+}	91.2 ± 5.76^{bc}	87.9 ± 6.78^{D}
Mg^{2+}	99.1 ± 1.53^{ab}	92.5 ± 2.29^{CD}
Mn^{2+}	15.4 ± 1.53^{g}	11.8 ± 2.64^{G}
Rb^{+}	95.2 ± 5.76^{abc}	11.2 ± 4.25^{G}
Zn^{2+}	97.8 ± 4.77^{ab}	93.2 ± 6.25^{CD}
EDTA	90.8 ± 2.75^{bc}	111.0 ± 1.53^{B}
NaCl*	109.7 ± 1.61^{a}	121.4 ± 2.69^{A}
None	100.0 ± 2.75^{a}	100.0 ± 2.75^{C}

* The two different concentrations of NaCl were tested at 100 mM and 1000 mM.

Mean ± one standard deviation derived from three replicates (N = 3).

Different superscript letter in the same column and row indicate that the values are significantly different (ANOVA and DMRT, $P < 0.05$).

In contrast, the presence of Co^{+} at a small concentration (1 mM) showed inhibitory effects on the enzyme activity, while it showed enhancing effects at a high concentration (10 mM). This result was similar to the effect of EDTA, which showed that the enzyme activity was slightly inhibited by presence of EDTA at 1 mM, but enhanced by the presence of EDTA at 10 mM. The presence of NaCl showed a dose-dependent enhancing effect on the enzyme activity. These results showed that the metal ions were not essential for the catalytic action of the enzyme.

4.6.6 Effect of substrate concentration

Purified endoglucanase from *L. enzymogenes* 521 was assayed in the presence of different CMC concentrations at the optimal conditions (40 °C and pH 5.0). The endoglucanase specific activity increased exponentially until reaching the limiting rate at 5% (w/v) of substrate concentration (Fig. 4.19). The coefficients (R^2) of the Lineweaver–Burk plot (Fig. 4.20), which were calculated to be 0.99 for substrate concentrations ranging from 1.0 to 6.0% (x/v) CMC, could be used to indentify the model as first order kinetics. From the X-intercept value, the Michaelis constant (K_m) was calculated to be 8.40 mg/mL, while the V_{max} determined from Y-intercept value was calculated to be 11.45 µmol/min.mg protein. The Specificity constant (V_{max}/K_m) was 1.36, while the enzyme concentration used was 72 µg/ml.

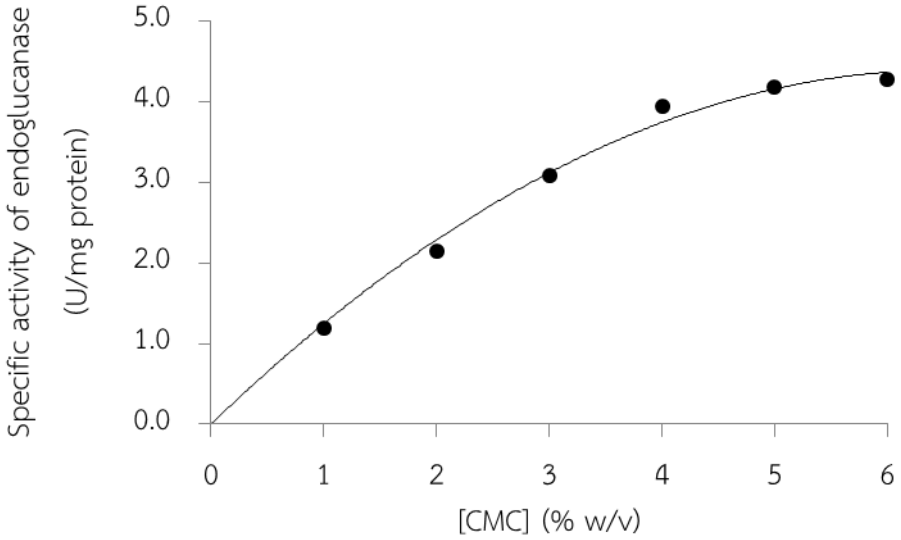


Figure 4.19 Effect of CMC at various concentrations on endoglucanase specific activity of purified enduglucanase from *L. enzymogenes* 521

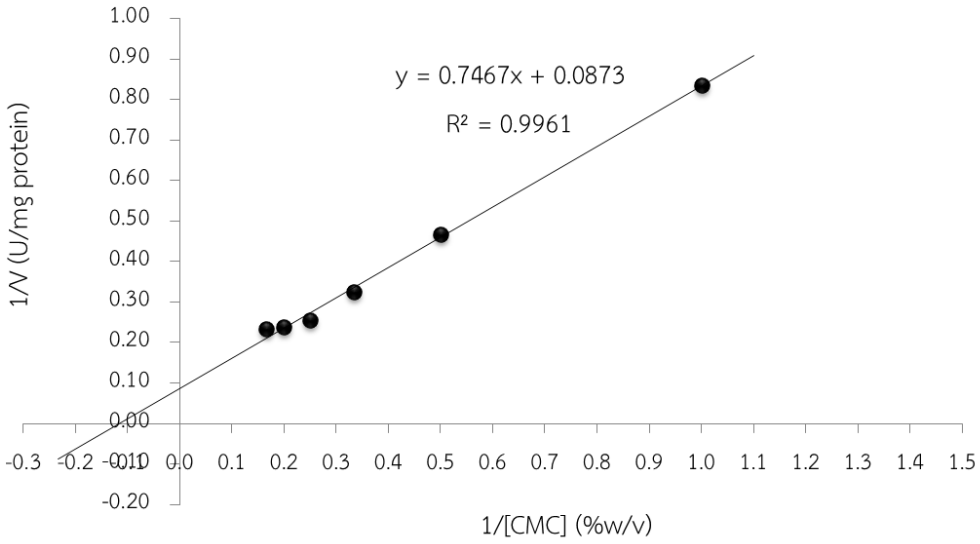


Figure 4.20 Lineweaver–Burk plot of specific activity versus [CMC] for the determination of Michaelis kinetic constants (V_{max}, K_m) of purified endoglucanase of from *L. enzymogenes* 521. Velocity (V) = U/mg protein and S = [CMC % (w/v)]. The intercept on X-axis = $1/K_m$ = 0.119 whereas intercept on Y-axis = $1/V_{max}$ = 0.0873. Data presented are average values ± SD of N = 3 experiments.

4.7 Draft genome sequencing and molecular characterization of the endoglucanase gene

4.7.1 General feature and annotation of draft genome

An overview of general features of *L. enzymogenes* 521 draft genome and *L. enzymogenes* C3 complete genome is presented in Table 4.15, and the graphical representation of *L. enzymogenes* 521 genome is presented in Figure 4.21. The draft genome of *L. enzymogenes* 521 is a single circular chromosome containing 5,851,805 bp. The genome had 69.8% G+C content, which was related with the *L. enzymogenes* C3 genome and those of *Lysobacter* sp. previously reported (Reichenbach 2006). In the chromosome, CDSs are transcribed on the positive and negative strands at 47.77 and 52.23%, respectively. A total of 5,102 genes in the genome were functionally annotated by RAST server. 5,008 protein coding sequences (CDSs) covering 98.16% and 94 RNA genes covering 1.84% were identified in the genome. The *L. enzymogenes* 521 genome contained a high number of sequence polymorphisms of 35 rRNA genes. For the *L. enzymogenes* C3 genome, only 6 rRNA genes were found (Table 4.15). In the meanwhile, the same number of tRNA genes (in total of 59 genes), representing all of the 20 amino acids, were identified in both strains. In *L. enzymogenes* 521 genome, tRNAs are organized into 12 clusters and 30 single genes. Out of the 12 tRNA cluster, 5 are associated with rRNA operons. The patterns of gene organization found in *L. enzymogenes* 521 genome were tRNA-16S, 5S-23S-tRNA-16S and 16S-tRNA 23S-5S.

Instead, the pattern of gene organization found only in *L. enzymogenes* C3 genome was 16S-tRNA-23S-5S.

Table 4.15 General feathers of the *L. enzymogenes* 521 and *L. enzymogenes* C3 genomes

Feathers	*L. enzymogenes* 521	*L. enzymogenes* C3[a]
Genome size (bp)	5,851,805	6,157,384
G+C content (bp)	4,074,888 (69.77%[b])	4,298,470 (69.81%)
Total genes	5,102	5,180
Protein-coding genes (CDS)	5,008 (98.16%)	5,115 (98.75%)
Subsystem genes	449 (8.80%)	448 (8.65%)
Total RNA genes:		
RNA operons		
16S	22	-
tRNA-16S	1	-
5S-23S-tRNA-16S	1	-
16S-tRNA-23S-5S	3	2
tRNA	59	59

[a] Genome sequence of *L. enzymogenes* C3 was derived from NCBI database (GenBank Accession Number: NZ_CP013140).

[b] The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.

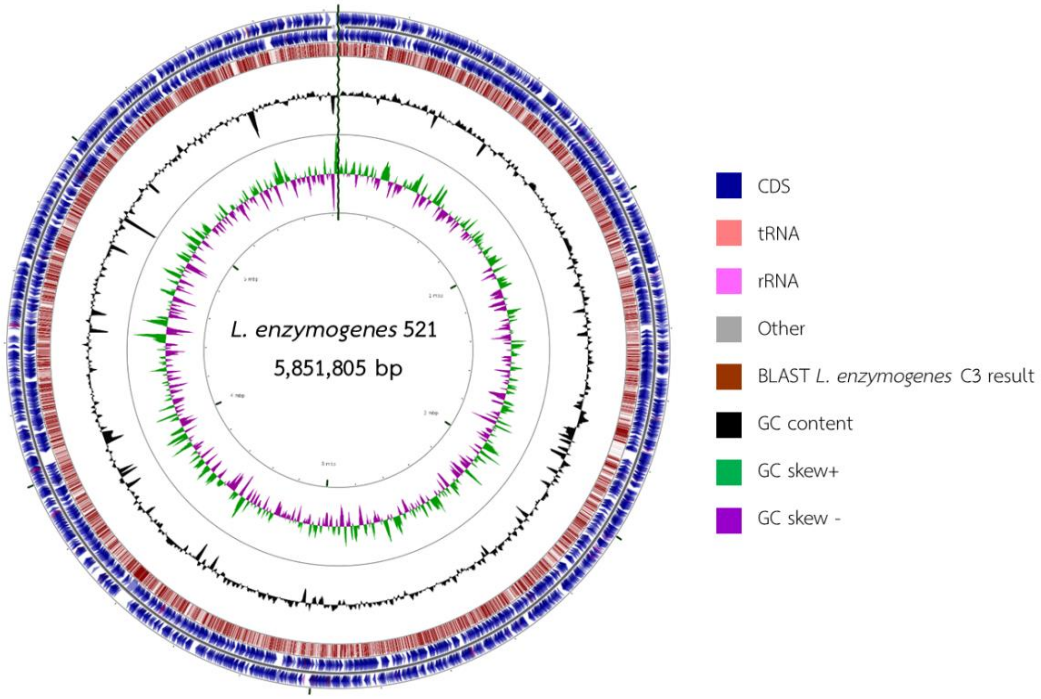


Figure 4.21 Genome atlas of *L. enzymogenes* 521. The color coding of the genomic feature represents different categories given on the right hand.

4.7.2 Comparative genome analysis

The closest neighbors to *L. enzymogenes* 521 genome revealed by functional comparison of genome sequences in the RAST server are shown in Table 4.16. The most related genus was *Xanthomonas* (with a score between 524 and 323) followed by the genus *Stenotrophomonas* (with a score between 316 and 256). *L. enzymogenes* 521 genome was mostly related to *Xanthomonas campestris* pv. campestris strains ATCC 33913, 8004 and B100, followed by *X. axonopodis* pv. citri str. 306, *X. campestris* pv. vasculorum NCPPB702, and *X. oryzae* pv. oryzae KACC10331. Interestingly, all of the closest groups of bacteria listed in Table 4.16 are phytopathogenic bacteria, which cause diseases on several plant species.

Pairwise analysis of the genome of *L. enzymogenes* 521 against *L. enzymogenes* C3 and the selected bacterial complete-genome sequences listed in RAST server were constructed (Fig 4.22). The genome of *L. enzymogenes* 521 was mostly similar to the genome of genome of *L. enzymogenes* C3 as indicated by a high average identity of orthologous sequences at 90-95%. Average sequence identity of orthologous sequences was found at 50-70% for the other bacterial genome in the genera *Xanthomonas* and *Stenotrophomonas*, and at 40-60% for *E. coli* ATCC 8739. About 45-51 genes of the 521 showed more than 90% sequence identity with respect to all

bacterial strain of the genera *Xanthomonas* and *Stenotrophomonas*, and 131 genes showed more than 99% identity to *L. enzymogenes* C3. Most of the functions of these genes are related to ribosomal proteins, motility proteins, cell division proteins, and other proteins in DNA transcription and translation clustered. Instead, 54 genes of 521 showed more than 70% sequence identity to *E. coli* ATCC 8739, where their major functions are related to ribosomal proteins, proteins in DNA transcription and translation clustered, and protein metabolism.

Table 4.16 Closest neighbor of *L. enzymogenes* 521 genome suggested by functional comparison of genome sequences in the RAST server

Genome ID	Score	Genome name
190485.1	524	*Xanthomonas campestris* pv. *campestris* ATCC 33913
314565.3	500	*Xanthomonas campestris* pv. *campestris* str. 8004
509169.3	451	*Xanthomonas campestris* pv. *campestris* str. B100
190486.1	398	*Xanthomonas axonopodis* pv. *citri* str. 306
559736.3	345	*Xanthomonas campestris* pv. *vasculorum* NCPPB702
291331.3	340	*Xanthomonas oryzae* pv. *oryzae* KACC10331
427081.3	323	*Xanthomonas fuscans* subsp. *aurantifolii* str. ICPB 11122
391601.3	316	*Stenotrophomonas* sp. SKA14
360094.4	304	*Xanthomonas oryzae* pv. *oryzae* PXO99A
383407.3	294	*Xanthomonas oryzae* pv. *oryzicola* BLS256
391008.3	283	*Stenotrophomonas maltophilia* R551-3
1185656.3	281	*Xanthomonas axonopodis* pv. *manihotis* str. Xam672
342109.7	273	*Xanthomonas oryzae* pv. *oryzae* MAFF 311018
559737.3	270	*Xanthomonas campestris* pv. *musacearum* NCPPB4381
40324.1	256	*Stenotrophomonas maltophilia* K279a
316273.10	252	*Xanthomonas campestris* pv. *vesicatoria* str. 85-10

There were at least 4 small genomic islands on the *L. enzymogenes* 521 genome (Figure 4.22). The genomic island I appeared in considerable size (about 9 kb), while the islands II, III, and IV had sizes between 5.6 and 6.1 kb. All defined island contained hypothetical proteins more than 80% of all CDSs in the genomic islands. The island I contained also minor genes of phage-related proteins, while the island IV contained also genes associated with cell wall and membrane

transport. Noted that total genes which presented in all of these genomic islands account to 3.6% (about 179 genes) of total CDSs in the *L. enzymogenes* 521 genome.

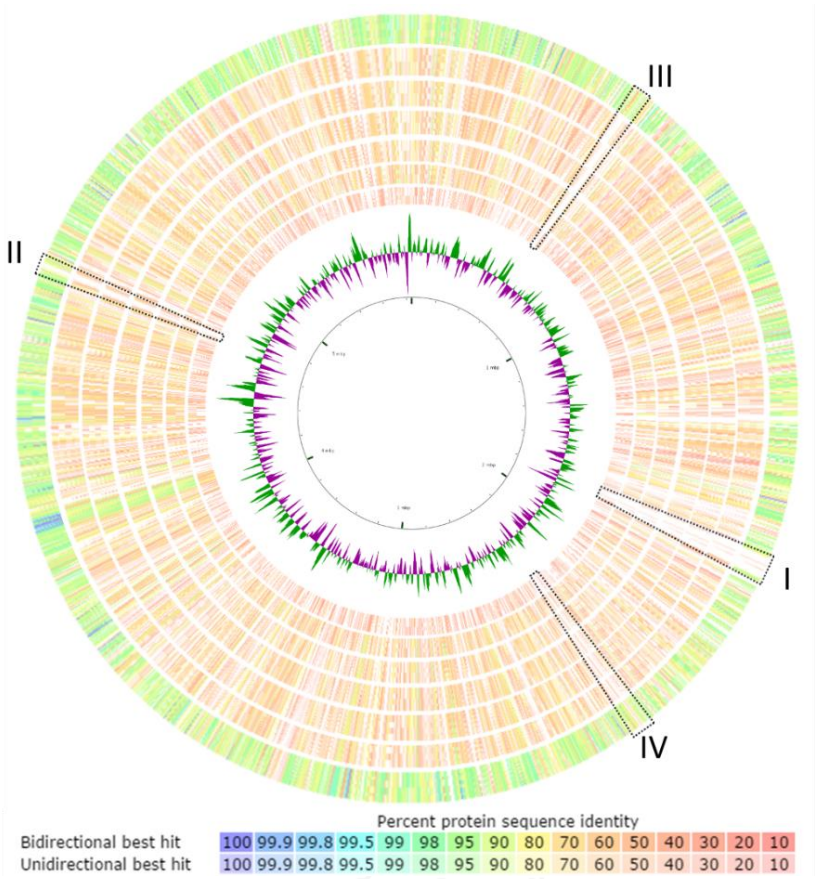


Figure 4.22 Pairwise analysis of orthologous sequences between *L. enzymogenes* 521 and other related bacteria found in RAST server database. From outer to inner cycles: *L. enzymogenes* C3, *X. campestris* pv. *campestris* ATCC 33913, *X. campestris* pv. *vesicatoria* 85-10, *X. axonopodis* pv. *citri* str. 306, *X. oryzae* pv. *oryzae* KACC10331, *S. maltophilia* R551-3, and *E. coli* ATCC 8739. The %protein sequence identity are indicated by color scales. Genomic islands (I to IV), which are the regions on the strain 521 genome that did not show orthologous sequences to other compared bacteria are marked. Percent G+C content of the strain 521 genome are indicated in the inner cycle.

4.7.3 Function based comparison

Function based comparison between the *L. enzymogenes* 521 genome and the closest strain, *L. enzymogenes* C3 genome, was also performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) available in the RAST server (Fig. 4.23). A total 1,920 genes in 449 subsystems, which covered 39% of total CDSs, were functionally annotated from the *L. enzymogenes* 521 genome. Similarly, a total 1,902 genes in 448 subsystems, which covered 38% of total CDSs, were

functionally annotated from the *L. enzymogenes* C3 genome. The comparison of metabolic reconstruction revealed that both strains genomes contained various gene clusters (over than 250 genes in a subsystem) for biotin synthesis, protein metabolism, amino acids and derivatives, and

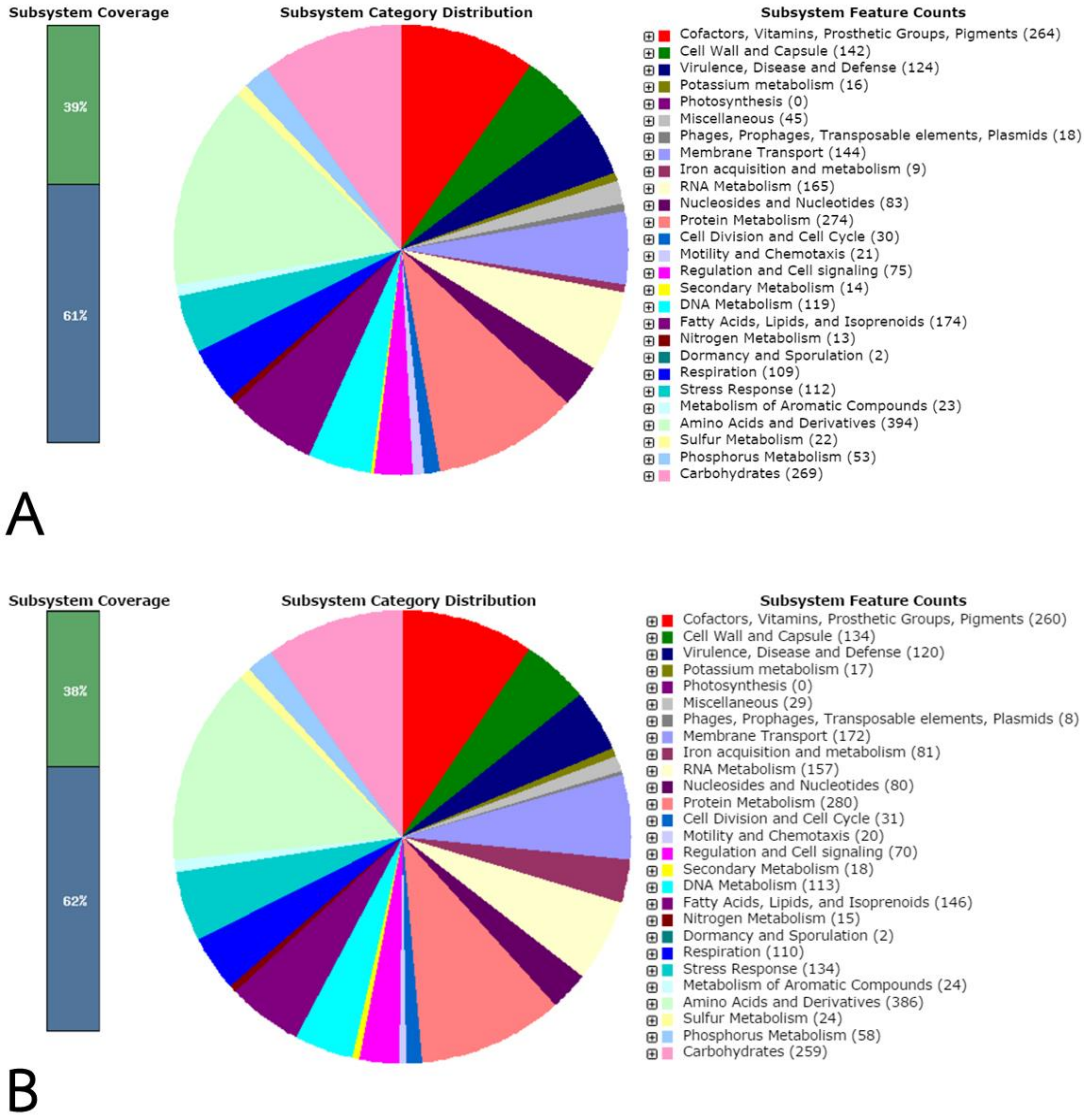


Figure 4.23 Subsystem category distribution of *L. enzymogenes* 521 (A) and *L. enzymogenes* C3 (B) based on RAST annotation server. The color coding represents different subsystem categories given on the right hand and subsystem feature counts in each category given in the parentheses.

central carbohydrate metabolism. Both strains shared about 95% of all categorized genes. There were 2.21% and 2.53% of all categorized genes that were unique to *L. enzymogenes* 521 and *L. enzymogenes* C3 genome, respectively. Variation of gene number between the two strains genome were mostly found in membrane transport, iron acquisition and metabolism, and phospholipid

metabolism subsystems. According to subsystem analysis, 48 genes associated with fatty acid biosynthesis FASII categorized in phospholipid metabolism subsystems were identified in the *L. enzymogenes* 521 genome, while only 20 genes in this category were found. In contrast, 28 genes involved in protein secretion system-Type VI (categorized in membrane transport subsystem), and 68 genes involved in iron acquisition and metabolism subsystem have been found in *L. enzymogenes* C3 genome, but they have not been found in *L. enzymogenes* 521 genome. Slightly differences of gene number in each subsystem between the two strains were also observed throughout the genomes.

4.7.4 Carbohydrate metabolism in *Lysobacter enzymogenes* 521

Genes encoding proteins involved in carbohydrate metabolism of the *L. enzymogenes* 521 genome were identified based on KEGG metabolic pathway annotation. The KEGG metabolic pathway analysis revealed that all of the central pathways for carbohydrate metabolisms, including starch and sucrose metabolism and amino sugar and nucleotide sugar metabolism, are present (Fig 4.24A-B). The genes encoding enzymes involved in starch and glycogen metabolism were absent (Fig. 4.24A). However, the gene encoding α-glucosidase (EC 3.2.1.20) for degrading starch and maltose was detected, as well as the enzymes which are required to convert the monomers to derivatives capable of entering to metabolic pathways, glucokinase (EC 2.7.1.2) and fructokinase (EC 2.7.1.4), were detected.

Glycan hydrolysis enzyme-encoding genes were also identified in the *L. enzymogenes* 521 genome. This analysis resulted in the identification of 33 genes encoding putative glycoside hydrolases (Fig. 4.25). The annotation displayed multiple enzyme genes encoding β-glucosidase (GHF 3), chitinase (GHF 18), and lysozyme type G (GHF 23). A gene sequence putatively encoding glycosyl hydrolase family 5, two copies of endoglucanase (EC 3.2.1.4) gene, as well as four copies of β-glucosidase (EC 3.2.1.21) gene sequences were identified in the starch and sucrose metabolism, except for the exoglucanase (EC 3.2.1.91) which was not found (Fig. 4.24C). These genes may be responsible for the observed cellulose-degrading capability in strain 521, and it indicated a possible narrow preference for amorphous cellulose as a carbon source. For what concerns the hemicellulose-degrading enzyme, neither endoxylanase; EC 3.2.1.8 nor β-xylosidase; EC 3.2.1.37 genes were identified in the genome. However, only a single copy of the sequence of β-L-arabinofuranosidase (EC 3.2.1.185) was identified. Other gene sequences for hydrolytic enzymes identified include those for β-mannosidase (EC 3.2.1.25), α-1,2-mannosidase (EC 3.2.1.-), endo-1,3-β-glucanase (EC 3.2.1.39), β-hexosaminidase and β-N-acetylhexosaminidase (EC 3.2.1.52).

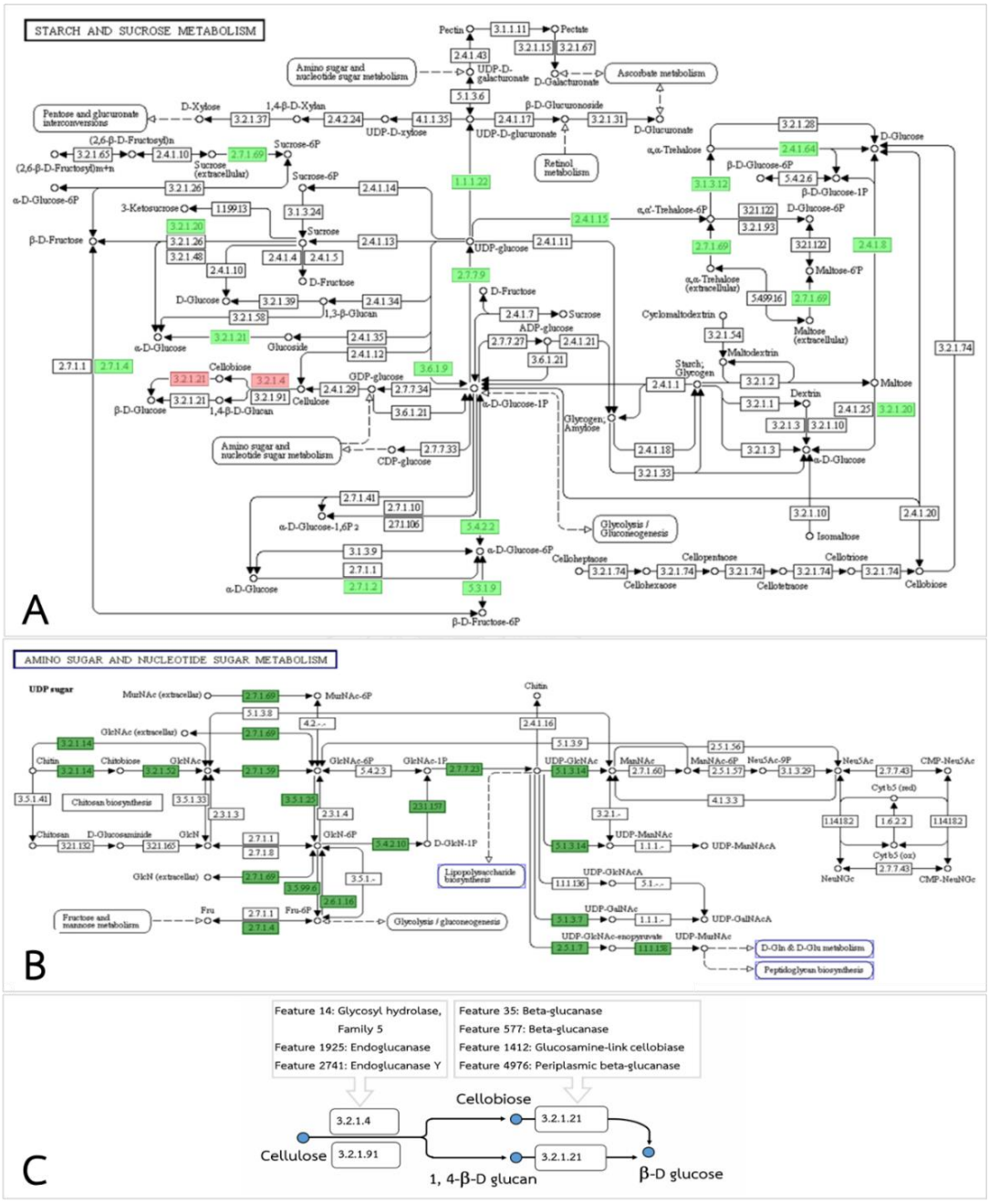


Figure 4.24 Starch and sucrose metabolism (A), amino sugar and nucleotide sugar metabolism (B) found in bacteria, and genes encoding proteins involved in cellulose degradation (C). Above, the enzymes involved in each pathway found in *L. enzymogenes* 521 genome were highlight in green color, and the enzymes involved in cellulose degradation found in *L. enzymogenes* 521 genome were highlight in red color. The EC number and each enzyme name are given in Table 3F (Appendix F).

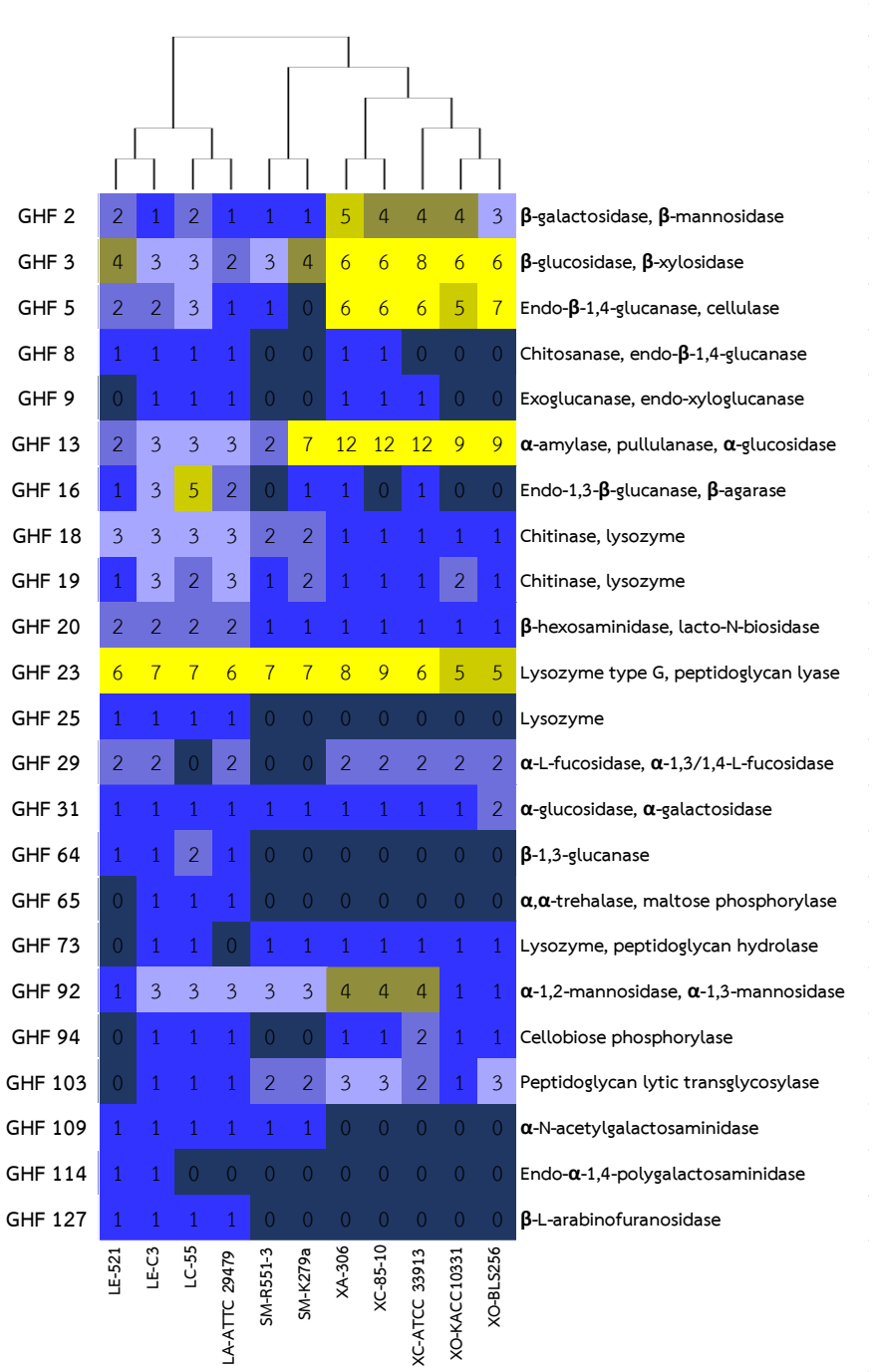


Figure 4.25 Gene abundant profile of glycoside hydrolases found in genome of 10 species of bacteria in family Xanthomonadaceae and *L. enzymogenes* 521. Acronym: LE-521 (*L. enzymogenes* 521), LE-C3 (*L. enzymogenes* C3), LC-55 (*L. capsici* 55), LA-ATCC 29479 (*L. antibioticus* ATCC 29479), SM-R551-3 (*S. maltophilia* R551-3), SM-K279a (*S. maltophilia* K279a), XA-306 (*X. axonopodis* pv. *citri* str. 306), XC-85-10 (*X. campestris* pv. *vesicatoria* str. 85-10), XC-ATCC 33913 (*X. campestris* pv. *campestris* ATCC 33913), XO-KACC10331 (*X. oryzae* pv. *oryzae* KACC10331), XO-BLS256 (*X. oryzae* pv. *oryzicola* BLS256).

4.7.5 Analysis of the full-length endoglucanase Y gene (*cel*8A)

The molecular characteristics as well as the evolutionary relationship between the endoglucanase from *Lysobacter* strains and other related proteins are relatively little known. Based on the KEGG metabolic pathway analysis, three genes putatively encoding endoglucanase (EC 3.2.1.4) were identified. The gene encoding protein endoglucanase Y, found in the feature 2741 of the *L. enzymogenes* 521 genome, was the most similar to those bifunctional endoglucanases found in GenBank database (Hedges and Wolfe 1974; Ogura et al., 2006). Therefore, this gene was considered for further studies.

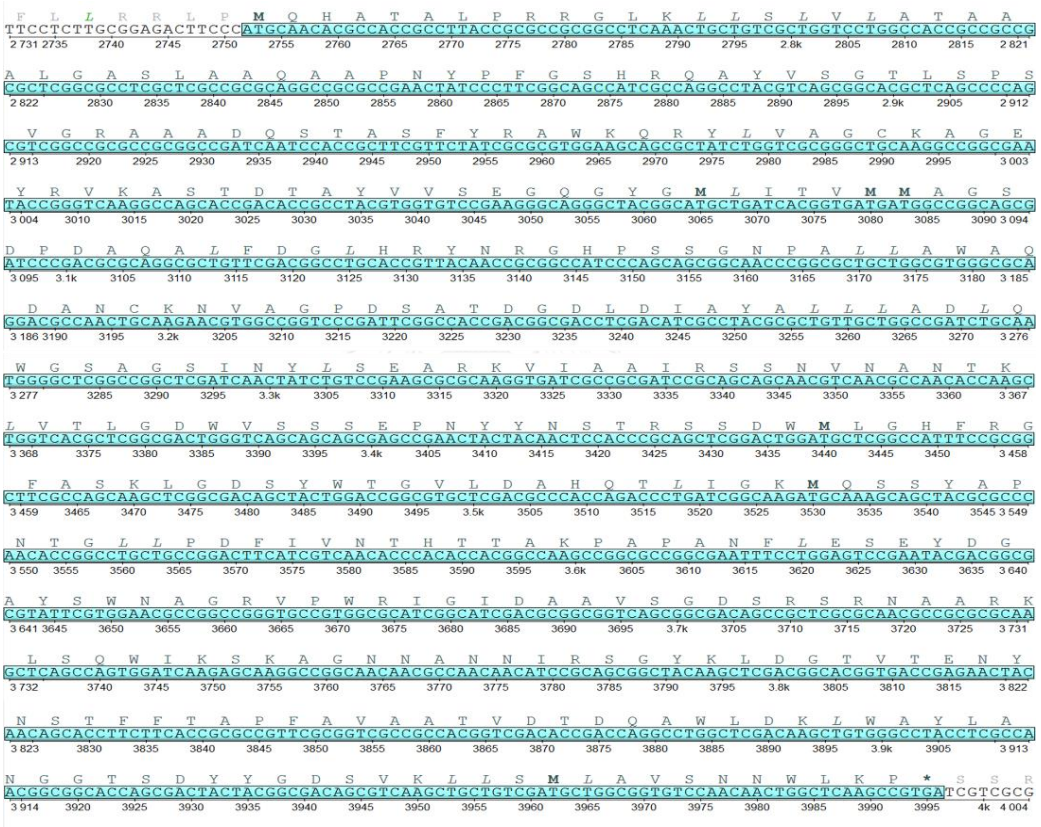


Figure 4.26 Nucleotide and deduced amino acid sequences of endoglucanase Y (cel8A) gene from *L. enzymogenes* 521. Translation starts at position 2752. Stop codon is indicated by an asterisk.

The nucleotide and deduced amino acid sequences of the *cel*8A gene are shown in Figure 4.26. The *cel*8A gene comprises a 1,242 bp open reading frame (ORF) encoding 414 amino acid residues, indicated by single-letters above the nucleotide sequence. The ORF begins with ATG start codon at nucleotide position 2752 and terminates with TGA termination codon at nucleotide position 3954. According to the description by Ogura et al., (2006), residues 1-33 of the polypeptide were assigned to be the signal peptide. The remaining of 381 amino acids (residues 34-414) was

calculated molecular mass of 41,241 Da, which is consist with the molecular mass experimentally calculated by SDS-PAGE in Section 4.5.4.

The endoglucanase Y (EC 3.2.1.4, Cel8A homologue) sequence from *L. enzymogenes* 521 was compared phylogenetically with related sequences of the glycoside hydrolase proteins found in GenBank database. The phylogenetic tree showed that the sequence of the endoglucanase Y gene was mostly similar to endoglucanase (Cel8A, bifunctional enzyme) from *Lysobacter* sp. IB-9374 (97.0%), β-glucanase from *L. enzymogenes* C3 (89.8%), β-1,4-glucanase from *L. capsici* AZ78 (82.5%) and the others related proteins with similarity between 56.1% and 46.6% (Fig. 4.27). All of the related proteins in the same clade showed homology to the family 8 glycoside hydrolases (GHF-8), which includes chitosanase (EC 3.2.1.132); endoglucanase (EC 3.2.1.4); licheninase (EC 3.2.1.73); endo-1,4-β-xylanase (EC 3.2.1.8); reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156)

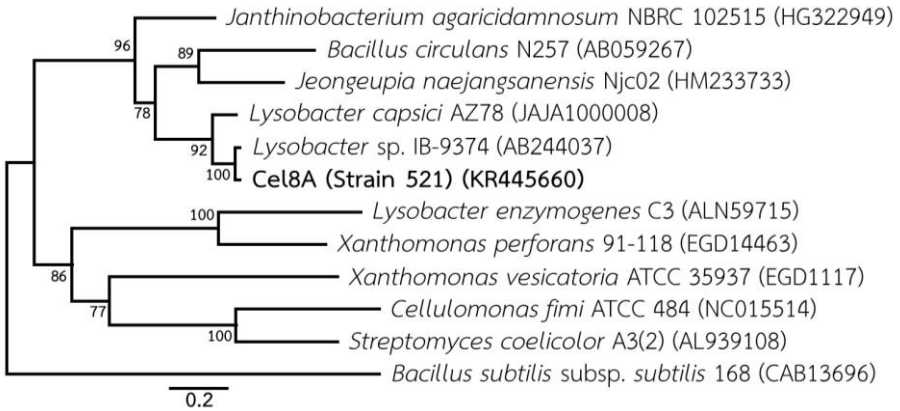


Figure 4.27 Maximum Likelihood tree of the cel8A gene sequence of *L. enzymogenes* 521 and other related glycoside hydrolase proteins found in the GenBank database (GenBank accession numbers in parentheses). The tree was constructed employing the maximum-likelihood analysis using the program MrBayes version 3.2.2. The evolutionary distances were calculated using the General Time Reversible (GTR) model. The bootstrap values (expressed as percentages of 10^3 replications) greater than 50% are shown at the branch points. Sequences aligned: endoglucanase from *Janthinobacterium agaricidamnosum* NBRC 102515; glycosyl hydrolase 8 superfamily protein from *Jeongeupia naejangsanensis* Njc02; endoglucanase (Egl257) from *Bacillus circulans* N257; β-glucanase from *Lysobacter capsici* AZ78; β-glucanase from *Lysobacter enzymogenes* C3; Cel8A from *Lysobacter enzymogenes* 521; endoglucanase (Cel8A) from *Lysobacter* sp. IB-9374; endoglucanase Y (Cel8A) from *Xanthomonas vesicatoria* ATCC 35937; cellulase (Celf0376) from *Cellulomonas fimi* ATCC 484; cellulase (CelB) from *Streptomyces coelicolor* A3(2); endoglucanase Y (Cel8A) from *Xanthomonas perforans* 91-118; endo-1,4-beta-glucanase (EglS) from *Bacillus subtilis* subsp. *subtilis* 168.

The multiple sequence alignment of the deduced amino acid sequence of the Cel8A from *L. enzymogenes* 521 was analyzed with glycoside hydrolase proteins (Fig. 4.28). The enzyme showed homology to endoglucanase (Cel8A, bifunctional enzyme) from *Lysobacter* sp. IB-9374 (98.3%), β-glucanase (Bgc) from *L. enzymogenes* C3 (90.3%), β-1,4-glucanase from *L. capsici* AZ78 (78.1%), endoglucanase (Egl257) from *B. circulans* N257 (43.9%), chitosanase (ChoK) from *Bacillus* sp. K17 (38.2%), endoglucanase A (Cel8A) from *C. thermocellum* ATCC 27405 (25.4%), endoglucanase Y (Cel8A) from *X. vesicatoria* ATCC 35937 (23.0%) and endoglucanase Y (Cel8A) from *X. perforans* 91-118 (22.1%).

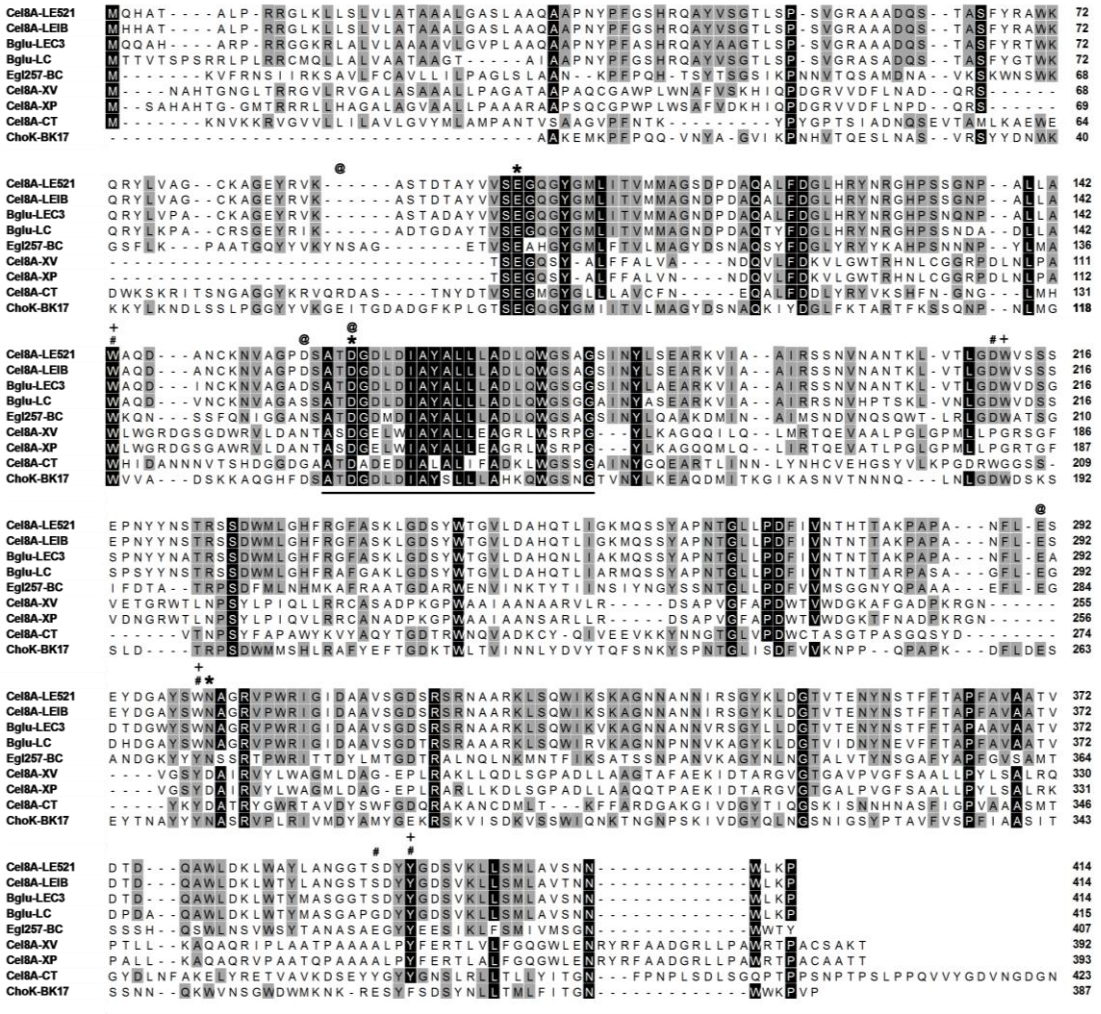


Figure 4.28 (see next page)

Figure 4.28 Multiple deduced amino acid sequence alignment of Cel8A with related cellulases. Sequences aligned: Cel8A (Cel8A-LE521) from *Lysobacter enzymogenes* 521 (GenBank accession number: KR445660); endoglucanase (Cel8A-LEIB) from *Lysobacter* sp. IB-9374 (AB244037); β-glucanase (Bglu-LEC3) from *Lysobacter enzymogenes* C3 (ALN59715); β-glucanase (Bglu-LC) from *Lysobacter capsici* AZ78 (EYR68409); endoglucanase (Egl257-BC) from *Bacillus circulans* N257 (AB059267); endoglucanase Y (Cel8A-XV) from *Xanthomonas vesicatoria* ATCC 35937 (EGD1117); endoglucanase Y (Cel8A-XP) from *Xanthomonas perforans* 91-118 (EGD14463); endoglucanase A (Cel8A-CT) from *Clostridium thermocellum* ATCC 27405 (A3DC29), chitosanase (ChoK-BK17) from *Bacillus* sp. K17 (1V5C_A). Identical and similar (>60%) amino acid residues among all enzymes are shaded in black and gray, respectively. The conserved amino acid residues are marked by a black line below the alignment. The asterisks indicate the conserved catalytic residues in GHF-8 proteins and the hash tags indicate the substrate recognition sites in Cel8A from *C. thermocellum* ATCC 27405. The @ and + signs indicate the conserved substrate binding and substrate recognition sites in chitosanase from *Bacillus* sp. K17, respectively. The gaps in the sequence alignment are indicated by dashed lines. The alignment was performed using Clustal X program.

Chapter V

DISCUSSION AND CONCLUSION

5.1 Isolation of *Lysobacter*-like bacteria

Since chitinolysis is a distinguishable character of *Lysobacter* spp. (Christensen and Cook, 1978), several isolation procedures were performed using chitin-based selective media. Following chitinolytic section, in order to isolate further *Lysobacter*-like bacteria from the shrimp chitin enrichment broth, crude yeast cell wall preparations containing a small amount of chitin, high concentration of proteins and other β-glucans were used as a further selective carbon source in the agar medium. Since kanamycin and penicillin inhibited the growth of bacteria, while Rose Bengal reduced fungal growth, the enrichment culture using colloidal chitin as a selected substrate with the aid of antibiotics yielded to a higher concentration of desired bacteria. However, the isolation in this study was based on the chitinolytic activity, and many species in the genus *Lysobacter* do not display this property. Therefore, the use of richer nutrients than chitin (such as yeast cell wall) in the enrichment procedure would have enhanced the species diversity of *Lysobacter* by differential enhancement of growth.

Most of the *Lysobacter*-like bacteria considered in this study were isolated from cereal rhizosphere soil and grass-covered soil in Thailand (Table 4.1). The finding of cellulolytic enzyme producing *Lysobacter*-like bacteria in these areas was consistent with the report by Yin (2010) that *Lysobacter* is found frequenctly in perennial grass covered areas. In addition, Thompson et al. (1996) reported that *Lysobacter* spp. population were increased up to fivefold in soil after cropping with grass and clover. A possible explanation is that it would be due to a greater root mass of the plants might favor a better colonization of *Lysobacter*-like bacteria. Furthermore, living under grass roots over years provides a stable environment for microorganisms. On the other hand, the changes of rhizosphere environment in agricultural soil is occurred each year so that the growth of *Lysobacter* population could be disturbed and they might not be able to maintain low population and to compete with other dominant species in soil, such as bacteria in the genera *Pseudomonas*, *Bacillus* and *Actinomyces* (Araragi, 1978; Lyngwi et al., 2013).

5.2 Screening and identification of *Lysobacter*-like bacteria

5.2.1 Screening of *Lysobacter*-like bacteria

Bacteria in the genus *Lysobacter* were grouped primarily with myxobacteria, as they shared properties of micropredatory activity and gliding motility, and they were known also for production

of several extracellular enzymes (Kobayashi and Yuen 2007). Therefore, these characters have been used to distinguish *Lysobacter*-like bacteria from other groups of bacteria. More than two hundred bacteria enriched using shrimp shell-colloidal chitin as a sole energy source were differentiated based on the phenotypic characters detected from the well-known strain, *Lysobacter enzymogenes* C3 (Table 4.2). Remarkably, most of so-obtained bacteria could be discarded based on their lack of chitinase and β-1,3-glucanase activity.

5.2.2 Identification of *Lysobacter*-like and phylogenetic analysis of the 16S rRNA gene sequence

Lysobacter are known to be a gliding, Gram-negative bacterium with high GC content, aerobic and unicellular. However, these characteristics are common also to myxobacteria and the *Cytophaga* group (Reichenbach, 2006). Consequently, molecular identification was necessary in order to clarify the taxonomic position of this group of bacteria. Since the enrichment culture method is biased towards *L. enzymogenes* characteristics, most isolates were found to be related with *L. enzymogenes.* Most of the endoglucanase-producing isolates belong to the genus *Xanthomonas* with sequence similarity greater than 99%. All of the endoglucanase-producing isolates grew rapidly in M813 supplemented with CMC as a sole carbon source and produced endoglucanase with activities between 0.235 U/mL (*X. sacchari* 513) and 0.112 U/mL (*L. enzymogenes* 521) (Table 4.5). All of the strains in the genus *Xanthomonas* had higher endoglucanase activity than *L. enzymogenes* 521. This might be related with the fact that cellulolytic enzymes have been reported to crucially involve in penetration into plant hosts and in degradation of plant cell wall to be used for nutrition in phytophatogens (Hu et al., 2007, Gough et al., 1988), while the significant role of endoglucanase in *L. enzymogenes* has not been investigated.

The phylogenetic analysis of Gammaproteobacteria constructed from 16S rRNA gene sequnces revealed that the genus *Lysobacter* were clustered together with members in the genera *Xanthomonas, Stenotrophomonas*, *Thermomonas*, *Luteimonas* and *Silanimonas* (Fig. 4.5). They fall clearly into separate cluster of other genera in family *Entorobacteriaceae*, including *Pantoea* and *Pseudomonas.* This analysis was consistent with previous reports that *Lysobacter* spp. fall into a related cluster together with *Xanthomonas* spp. and *Stenotrophomonas* spp. (Hayward et al., 2009; Lee et al., 2006; Romanenko et al., 2008; Ten et al., 2009). The high bootstrap value (97 %) provided strong support for the inclusion of strain 521 in the genus *Lysobacter*, which was selected for further characterizations.

5.3 Characterization of *Lysobacter enzymogenes* 521

5.3.1 Morphological characterization

The bacteria classified in the genus *Lysobacter* is known to be slender and cylindrical Gram-negative rods, and move by gliding. The cells size are typically within the range of 0.4–0.6 × 2-5 μm. The presence of the population of long cells (up to 70 μm) are also always characteristic for the genus (Reichenbach, 2006). Both of these characters emerged clearly from the morphological observation of the strain 521 (Fig. 4.6). Two different colony types in strains of *L. enzymogenes* have been observed by Christensen and Cook (1978): a yellowish nonmucoid colony and a dirty-white mucoid colony. Color of the colonies observed in the tropical strain 521 and the temperate strain C3 were slightly different, but they were both yellow-based and nonmucoid.

5.3.2 Physiological and biochemical characterization

Most of species members in the genus *Lysobacter* are mesophilic bacteria, and the optimal temperature for their growth is about 30 °C according to previous reports. However, the exact value of this temperature is different for different isolates, and depends, in particular, on their respective origin habitats. In general, they can grow within a range of temperatures between 2 °C and at 50 °C (Reichenbach, 2006). The highest optimal temperature for growth ever observed were these of the spercies *L. brunescens* (Christensen and Cook, 1978) and *L. thermophilus* (Wei et al., 2012), which were 40 °C and 50 °C, respectively. For the species *L. enzymogenes*, many strains of *L. enzymogenes* were investigated for the temperature range for growth. It was reported a temperature range for growth, between 4 °C and 40 °C, and optimal values for growth between 30 °C and 40 °C (Christensen and Cook, 1978). The optimal temperature for growth observed in *L. enzymogenes* 521 and *L. enzymogenes* C3 was consistent with previous reports. However, the growth of these strains was not investigated for temperatures higher than 40 °C.

The fact some bacteria produce volatile compounds is well known. In fact, many of these volatile compounds have been used as aroma components for production of wine, cheese, and other fermented food (Schulz and Dickschat, 2007). Moreover, bacterial volatile compounds have been applied for biocontrol purposes. Several strains of *Pseudomonas* sp. could produce volatile compounds, including benzothiazole, cyclohexanol, *n*-decanal, dimethyl trisulfide, 2-ethyl 1-hexanol, and nonanal, which completely inhibited growth and germination of several plant pathogens (Fernando et al., 2004). One of those compounds produced by *Pseudomonas* sp., 2-ethyl 1-hexanol, was produced also by *L. enzymogenes* C3 (Fig. 4.8) but not by *L. enzymogenes* 521 (Fig. 4.7). 2-methylbutanol was the most volatile compound produced by *L. enzymogenes* 521, and it was detected in high amount in *L. enzymogenes* C3. It has been reported that this compound

is weakly active against wood-decaying fungi in many bacteria, especially actinomycetes (Schulz and Dickschat, 2007). An anti-pathogenic agent, identified as 2,4-Bis(1,1-dimethylethyl)-Phenol, has been characterized for its biofilm inhibition and for increasing the susceptibility of *S. marcescens* to gentamycin (Padmavathi et al., 2014). This compound was also detected in both *L. enzymogenes* 521 and *L. enzymogenes* C3. These results indicated that both strains produced a number of volatile compounds involved in defensive activities against pathogens, especially *L. enzymogenes* C3, which could produce larger amounts of active volatile compounds than *L. enzymogenes* 521.

The phenotypic characteristic comparisons showed clearly that strain 521 is a member of the *L. enzymogenes* species. Essentially all observed hydrolytic activities do not contradict to that of *L. enzymogenes* C3 indicated in Table 4.7, and those of *L. enzymogenes* described by Christensen and Cook (1978). Furthermore, the assimilation of the substrates listed in Table 4.7 are consistent with the *L. enzymogenes* identification. An exception was observed for assimilation of malic acid and trisodium citrate in both strains. Strain 521 was found positive for utilizing both of the substrates malic acid and trisodium citrate, while strain C3 utilized weakly malic acid, and was found negative for utilizing trisodium citrate. However, this finding is in contrast with previous publications concerning the strain *L. enzymogenes* DMS 2043 (ATCC 29487) (Yassin et al., 2007; Weon et al., 2007; Romanenko et al., 2008, Wang et al., 2009; Zhang et al., 2011; Wei et al., 2012). It might be confirmed whether the result of assimilation of malic acid and trisodium citrate were reproducible for *L. enzymogenes*. Likewise, it is unclear whether the variation in morphological and physiological features between strains was related to the geographical locations where the stains were isolated.

The ability of *Lysobacter* spp. to secrete a wide range of extracellular lytic enzymes capable of degrading cell wall components of other microbes and nematodes, combined with their gliding motility, enabled their development in rich organic-matter habitats (Aslam et al., 2009; Jung et al., 2008; Kobayashi and El-Barrad, 1996; Kobayashi and Yuen, 2007; Srinivasan et al., 2010; Staskawicz et al., 1987; Wang et al., 2009; Weon et al., 2006; Yin, 2010; Zhang et al., 2011). The notable lytic capabilities of *Lysobacter* spp. suggest that they may play an important environmental role in the control of microbial populations. It may be possible to eliminate pathogenic or otherwise undesirable bacteria in sewage plants, or blooming organisms in a limited area, by introducing strains of *Lysobacter* with strong lytic enzymes (Reichenbach, 2006). The presence of lipase activity in both strains was consistent with a previous work by von Tigerstrom and Stelmaschuk (1989), which reported that one of the two esterases produced by *L. enzymogenes* was secreted into culture medium supplemented by olive oil. Both strains displayed strong proteolytic activity —— a trait typical of *Lysobacter* strains that have been previously characterized for their antagonistic activity against nematodes and bacteria (Kobayashi and Yuen 2007). It was

reported that the lytic enzymes responsible for chitin degradation were often found in *Lysobater* (Christensen and Cook, 1978). The presence of chitosanase activitiy in both strains might be reflected with CMCase activity. In fact, an extracellular enzyme with both β-1,4-glucanase and chitosanase activity has been reported in *L. enzymogenes* (Hedges and Wolfe, 1974; Ogura, 2006). Remarkably, the lytic enzyme assays performed in this study showed that the strain 521 had higher specific activities for all types of lytic enzymes compared with strain C3 (Fig. 4.9). It would be interesting to investigate further the relationship between growth and lytic enzyme production in this bacterium.

5.4 Optimization of endoglucanase production of *Lysobacter enzymogenes* 521

5.4.1 Selection of carbon and nitrogen sources

Optimization of enzyme production is nowadays very important for a number of industrial processes. In particular, it is convenient to use crude enzyme from culture supernatants, which has small economical and technical costs. The effect of carbon and nitrogen sources have been investigated for endoglucanase production in many Gram-positive and Gram-negative bacteria, including *Bacillus*, *Streptomyces*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* (Amat et al., 2014; Bano et al., 2013; Kamoun and Kado, 1990; Kazemi-Pour et al., 2004). However, in most of the reports it was employed a one-factor-at-a-time methodology. In this study, the combined effect of carbon and nitrogen sources were investigated at the same time. A specific combination of glucose and casein was found to be the best condition for endoglucanase production by *L. enzymogenes* 521. Glucose, which has been reported to be a cellulase activity repressor which influences the production of endoglucanase of many strains of fungi and bacteria, such as *Bacillus subtilis* (Bano et al., 2013), *Trichoderma reesei* (Muthuvelayudham & Viruthagiri, 2006), *Stachybotrys microspore* (Hmad et al., 2014), *Aspergillus fumigatus* (Ximenes et al., 1996). Interestingly, glucose gave the highest levels of endoglucanase activity by *L. enzymogenes* 521. This result was consistent with the case of *Streptomyces* sp., where glucose was reported to give highest levels of endoglucanases, which indicate its constitutive nature (Jaradat et al., 2008). For the nitrogen source, organic nitrogen sources seem to be suitable for endoglucanase production by *X. axonopodis* pv. *punicae* (Amat et al., 2014), *B. subtilis* CY5 and *B. circulans* TP3 (Ray et al., 2007), In contrast, inorganic nitrogen sources induced a higher endoglucanase production by *B. subtilis* KIBGE HAS (Bano et al., 2013) and *Streptomyces* sp. J2 (Jadarat et al., 2008). This study suggests that carbon source and nitrogen sources are key factors that influence the endoglucanse production of *L. enzymogenes* 521 by increasing the endoglucanase activity from 0.11 U/mL (Table 4.5) to 1.07 U/mL, which is about 10-fold enhancement.

5.4.2 Experimental design for optimization of carbon and nitrogen concentration

Statistical prediction using the RSM regression model confirmed the experimental optimization. The model for endoglucanase production in this study was significant, as indicated by a high value of determination coefficient (R^2 = 0.983), which means that the regression model is significant at the 98.3% confidence level. The agreement bewteen predicted (1.47 U/mL) and experimental (1.45 U/mL) values was good, indicating that this model derived from RSM can be used to describe the effect of the concentration of glucose and casein on the endoglucanase production by *L. enzymogenes* 521.

The RSM counter plots showed that endoglucanase production tended to increase and had a maximum at center-point concentrations between 0.6-0.95% (w/v) glucose and 0.35-0.55% (w/v) casein. This result was similar to a previous report of the effect of mono-saccharide and organic nitrogen on endoglucanase production by *B. pumilus* EWBCM1 (Shankar and Isaiarasu, 2012), where it was found that galactose and malt extract could enhance endoglucanase activity, which reached its maximum at particular concentrations of galactose and malt extract. As glucose has been reported to be repressor of the endoglucanase produced by many strains of bacteria and fungi, a 20-fold-higher enzyme yield was obtained in continuous cultures when not more than 0.8 gram of glucose was present per liter of medium (Andrews and Asenjo, 1987; Asenjo and Dunnill, 1981; Hunter and Asenjo, 1987). This report might explain the limit for supplementation of glucose in the production medium. Supplementation of the other substrate, such as glucan polymers, will be useful to investigate for endoglucanase activity enhancement.

5.4.3 Effect of supplementation of glucan

Among glucan polymer evaluated, CMC was the most effective in enhancing of endoglucanase activity by *L. enzymogenes* 521. This was consistent with previous studies, which reported that cellulose have been recognized as one of the most effective inducers (Krishna et al., 2000; Mandels and Reese, 1957; Muthuvelayudham and Viruthagiri, 2006; Persson et al., 1991; Sheng et al., 2012). In this study, it was found that a small concentration of CMC (0.2% w/v) was optimal for the endoglucanase induction (Fig 4.11). A similar trend was observed in *Aspergillus nidulans*, where cellulase activity was found to increase by increasing of CMC concentration up to 0.12% (w/v), and it started to decrease significantly after all (Jabasingh and Nachiyar, 2010). Moreover, it was found that the production of CMCase, FPase and β-glucosidase by *Aspergillus fumigatus* was enhanced by CMC for concentrations up to 1.5% (w/v) under submerged fermentation, while the production of these enzymes declined significantly at higher CMC concentrations (Das et al., 2013). It is important to note that the endoglucanase production from this experiment was 1.4-fold higher with respect to the value obtained in the previous section.

5.4.4 Effect of temperature on growth rate and endoglucanase activity

Temperature and incubation time are important factors that influence the cullulolytic-enzyme production in many bacterial strains (Sethi et al., 2013; Sheng et al, 2012; Karmakar and Ray, 2013). In this experiment, the growth and endoglucanse production of both strains reached the maximum when the cultures were incubated at 30 °C for 48 h. This result was consistent with those of Christensen and Cook (1978) and Reichenbach (2006), which reported that the optimal temperature for growth of several *L. enzymogenes* strains was between 25 °C and 35 °C. Enzyme production started during the growth period, and the increasing of enzyme activity along with bacterial growth was observed in both strains. High temperatures could influence the growth rate, coincidently, the enzyme production was delayed along with the growth. These results confirmed that the endoglucanase production by the strains 521 and C3 were greatly dependent on the cell concentrations and their growth rates. This phenomenon were observed also in several other bacteria and fungi (Das et al., 2013; Sheng et al, 2012; Jabasingh and Nachiyar, 2010).

Endoglucanase activity of several aerobic bacteria was reported to be between 0.43 U/ml and 2.00 U/ml (Da Vinha et al., 2011; Deka et al., 2011; Macedo et al., 2013; Shankar and Isaiarasu, 2011). After completion of the optimization processes, the maximum endoglucanase activity obtained from endoglucanase of *L. enzymogenes* 521 was 2.46 U/mL, which is higher than the values reported in previous works, and it was about 22-fold higher with respect to the original M813, when the bacterium was quantified for endoglucanse activity.

5.5 Purification of endoglucanase produced from *Lysobacter enzymogenes* 521

General properties of enzymes, which differ from the other undesired proteins, can be exploited in order to devise a protein purification scheme. The following important properties will be considered: charge, solubility, size, specific binding, and other special properties such as thermostability or alkaline/acidic stability (Doonan and Cutler, 2004). In this study, preparation of a crude extract containing the protein was firstly performed in an optimal production medium. An extralcellular enzyme was then subjected to the following three steps of purification processes: ammonium sulfate precipitation, hydrophobic interaction chromatography on Hitrap phenyl HP followed cation exchange chromatography on Hitrap SP HP. Since proteins tend to precipitate differentially depending on the concentration of neutral salts in the solution, the proteins in a crude extract was precipitated by increasing stepwise the concentration of ammonium sulfate. Proteins with endoglucanase activity produced by *L. enzymogenes* 521 were mostly precipitated at 60 to 80% saturation of ammonium sulfate, while a small endoglucanse activity remained within the 40-60% saturation fraction. Therefore, 50-80% saturation with ammonium sulfate was performed in order to keep the highest recovery yield of the desired protein. Similarly, precipitation

of endoglucanase from other strains of *Lysobacter* sp. and *Bacillus* sp. was found within the range of 40 to 90 % saturation of ammonium sulfate (Ogura et al., 2006; Sadhu and Maiti, 2013). A total of 5.5-fold increasing of specific endoglucanase activity, which is equivalent to 5.7-fold increasing of specific chitosanase activity, was obtained from this process (Table 4.12).

Conventional column chromatography is a commonly used powerful method to purify a number of bacterial endoglucanses and other related enzymes (Anu et al., 2014; Bischoff et al., 2006; Choi et al., 2004; Hedges and Wolfe 1974; Lui and Xia, 2006; Ogura et al., 2006; Sadhu et al., 2013; Woo et al., 2004). Among the chromatrographic methods, hydrophobic interaction chromatography (HIC) has been used effectively as an intermediate step to purify hydrolytic enzymes belonging to the glycoside hydrolase family 5 and 8 (Bischoff et al., 2006; Lui and Xia, 2006). Based on hydrophobicity, HIC separates the proteins that interact differently with hydrophobic ligands immobilized on the medium. In this study, after the protein sample dissolved in high ionic strength of ammonium sulphate (1 M) was loaded onto the column HiTrap phenyl HP, proteins contained in the sample were eluted differentially during a gradient decreasing of ionic strength from 1M to 0 M. Proteins with low hydrophobicity that could not bind with hydrophobic ligands were eliminated after the sample were applied onto the column, and the proteins with endoglucanase activity and stronger hydrophobicity was subsequently eluted (Fig.4.13). As HIC gives only moderate resolution for separation of eluted proteins, collection of fractions containing impurities has been avoided. This process led to a total of 18.6-fold increasing of specific endoglucanase activity, which equivalent to 18.3-fold increasing of specific chitosanase activity (Table 4.12).

Ion-exchange chromatography has been widely used to purify cellulases (Anu et al., 2014; Bischoff et al., 2006; Lui and Xia, 2006; Ogura et al., 2006; Sadhu et al., 2013). This chromatographic technique separates proteins based on their magnitude of net electric charge. A strong cation exchange chromatography column, HiTrap SP HP, was used for the last step of purification in this study. A cation exchanger (Sulphopropyl groups; SP) immobilized onto the dextran-based sepharose matrix separates proteins containing net positive charges. In this study, the protein fractionated from the previous chromatography was then prepared in 50 mM sodium acetate buffer at pH 4.0 and applied onto the column. The protein with endoglucanse activity was eluted with high resolution during a gradient increasing of ionic strength from 0 M to 1 M of NaCl. This result indicated that the active protein was purified in a high resolution by displaying an eluted-single peak of protein. In principle, a protein will carry a positive net charge and will bind to a cation exchanger (SP) when it is exposed to a pH below its isoelectric point (pI). The net charge of *L. enzymogenes* 521 endoglucanase in the working buffer, 50 mM sodium acetate buffer (pH 4.0), was positive. Therefore, this indicated that its pI was more than pH 4.0. Most of the pI of bacterial

endoglucanses have been reported between pH 3.5 and 6. The pI of endoglucanases from *Clostridium thermocellum* LQRI is 6.72, *Thermomonospora fusca* is 4.5, and *Erwinia chrysanthemi* 3665 is 4.3 (Robson and Chambliss, 1989). The overall level of endoglucanase recovery from this step was approximately 2.2%, with a 41-fold enrichment of specific endoglucanase activity (equivalent to 41.7-fold increasing of specific chitosanase activity).

The single band of 41 kDa mass protein was detected on SDS-PAGE, indicating that the protein purification processes successful purified the endoglucanase produced by *L. enzymogenes* 521. After the zymogram analysis performed in native PAGE-gels supplemented with CMC and chitosan, a single band corresponded to the protein band on SDS-PAGE exhibited a clear hydrolysis activity towards CMC and chitosan (Fig. 4.15). This result was consistent with previous studies, which reported that a purified protein with bifunctional activities of CMCase and chitosanase produced by bacteria have molecular mass in the range of 32 to 45 kDa, except for *Paenibacillus illinoisensis* KJA-424, whose molecular mass is about 54 kDa (Table 5.1). Dual mode of activity has been reported to be a key character of enzymes belong to glycoside hydrolase family 8 (Gao et al., 2008).

5.6 Characterization of purified endoglucanase

5.6.1 Optimal pH and temperature of endoglucanase and chitosanase activity

The purified endoglucanase from *L. enzymogenes* 521 had the optimal pH and temperature (pH 5.0, 40 °C) for CMCase activity correspond to that of the purified bifunctional endoglucanase from *Lysobacter* sp. IB-9374 (Ogura et al., 2006). The *L. enzymogenes* 521 endoglucanase had the optimal pH and temperature slightly different with respect to the purified endoglucanases of *Lysobacter* sp. AL-1 (pH 5.0, 38 °C) (Hedges and Wolfe, 1974) and *Bacillus cereus* S1 (pH 6.0, 40 °C) (Kurakake et al., 2000). For the chitosanase activity detected in the *L. enzymogenes* 521 endoglucanase, it was found that the optimal conditions of pH and temperature (pH 5.0, 50 °C) were different from previous reports. Chitosanase detected in bifunctional enzymes from the other bacteria listed in Table 5.1 had optimal pH within the range of 6.0-7.0, while these enzymes showed higher optimal temperature within the range of 60-70 °C (Gao et al., 2006; Jung et al., 2005; Kurakake et al., 2000; Ogura et al., 2006; Hedges and Wolfe, 1974; Tanabe et al., 2003).

5.6.2 Thermal stability

The *L. enzymogenes* 521 endoglucanase was stable up to 40 °C for 3 hours when it was assayed for CMCase and chitosanase activity. This result was similar to those reported for other bifunctional enzymes in previous works (Ogura et al., 2006; Hedges and Wolfe, 1974; Kurakake et al., 2000; Tanabe et al., 2003; Gao et al., 2006). However, it was different with respect to a result

reported for a fungal enzyme (a bifunction endoglucanase from *Trichoderma viride*) which was stable at temperatures between 30 and 70 °C for 2 hours (Liu and Xia, 2006).

5.6.3 pH stability

After incubation at room temperature, the *L. enzymogenes* 521 endoglucanase was stable in a mildly acidic to alkaline condition (pH 6.0-9.0) when it was assayed for CMCase and chitosanase activity. While it was stable at pH 4.0-11.0 when it was incubated at 4 °C. This was similar to the pH stability of enzymes from the following microorganisms; *Lysobacter* sp. IB-9374 (pH 5.0-8.0) (Ogura et al., 2006), *Bacillus cereus* S1 (pH 6.0-11.0) (Kurakake et al., 2000), *Streptomyces griseus* HUT 6037 (6.0-9.0) (Tanabe et al., 2003), *Bacillus cereus* D-11 (pH 6.0-11.0) (Gao et al., 2006). Instead, this was different with respect to a previous result reported for a bifunction endoglucanase from a fungus *Trichoderma viride*, which was stable in acidic to neutral conditions (pH 3.0-7.0) (Liu and Xia, 2006).

5.6.4 Substrate specificity

Since CMC it is a standard substrate for endoglucanase (Lynd et al., 2002; Zhang et al., 2006) and it was the substrate that gave the highest activity in this study, the enzyme activity towards CMC was set as 100% relative activity by definition. The *L. enzymogenes* 521 endoglucanase could hydrolyze several glucan substrates, including colloidal chitin, α-cellulose, filter paper, Avicel, fibrous-cellulose, cellobiose and cotton. Taking into account the differential behavior of these bifunctional enzymes in attacking β-1,4 linked polysaccharides with different chemical structures, it was reasonable to assume that these enzymes possess different catalytic sites for cleaving the glycosidic linkages of those polysaccharides (Liu and Xia, 2006).

Concerning to enzyme activity towards chitosan, the relative chitosanase activity was 54.1% of the CMCase activity detected in *L. enzymogenes* 521 endoglucanase. This result indicated that the endoglucanase is bifunctional enzyme that displays chitosanase activity in a comparable quantity to the CMCase activity. Similar results were found in the previous reports of *Lysobacter* sp. (Table 5.1). The bifunctional endoglucanase of *Lysobacter* sp. IB-9374 and *Lysobacter* sp. AL-1 displayed 39.3% and 61.3% relative activities, respectively. On the other hand, many bifunctional enzymes from the other microorganisms showed the opposite effect on hydrolysis towards these two substrates. The relative CMCase activities within the range of 21.6-75.6% with respect to chitosanase activity were detected in *Streptomyces griseus* HUT 6037, *Bacillus cereus* S1, *Bacillus cereus* D-11, *Paenibacillus illinoisensis* KJA-424, *Trichoderma viride* (Table 5.1).

Bueno et al. (1990) reported that a 1,3-1,4-,B-D-glucanase with molecular mass of 40.5 kDa was produced by *B. circulans* WL-12, which had relative CMCase activity of 9.2% with respect to the 1,3-1,4-,B-D-glucanase activity. In a later work, *B. circulans* WL-12 chitosanase with the same

molecular mass of 1,3-1,4-,B-D-glucanase was produced in an inducing medium using chitosan as a carbon source (Mitsutomi et al., 1998). It was found that relative chitosanase activity of the induced enzyme was 87.2% with respect to the 1,3-1,4-,B-D-glucanase activity, while it had relative CMCase activity of 8.2% to that of 1,3-1,4-,B-D-glucanase activity. The differences between the relative activities of these bifunctional enzymes might be due to the fact that different inducers were used in each individual study of this group of bacteria.

5.6.5 Effect of metal ions, chelator and salts

A number of metal ions, chelator and salts have been reported to have different effects on the endoglucanase activity in several microorganisms. Inhibitory effect by the presence of Cu^{2+} and Hg^{2+} for endoglucanse activity produced by *L. enzymogenes* 521 are consistent with previous reports of *Lysobacter* sp. IB-9374 (Ogura et al., 2006), *Lysobacter* sp. AL-1 (Hedges and Wolfe, 1974), *Streptomyces griseus* HUT 6037 (Tanabe et al., 2003), *Bacillus cereus* D-11 (Gao et al., 2006), *Paenibacillus illinoisensis* KJA-424 (Jung et al., 2005), *Trichoderma viride* (Liu and Xia, 2006) and *Aspergillus niger* ANL301 (Chinedu et al., 2010). In contrast, it has been reported that Ba^{+} which which acted as an inhibitor for *L. enzymogenes* 521 endoglucanase acted as an activator for *Lysobacter* sp. IB-9374 endoglucanase. Similarity, Mn^{+}, which acted as an inhibitor for *L. enzymogenes* 521 endoglucanase, acted as an activator for *Trichoderma viride* and *Aspergillus niger* ANL301 endoglucanases. An inhibitory effect by presence of EDTA indicates a metal dependent character of metallo-protein. For example, 10 mM of EDTA inhibited 71.3% the endoglucanase activity of *Aspergillus niger* ANL301 (Chinedu et al., 2010). However, EDTA had no such an inhibitory effect on *L. enzymogenes* 521 endoglucanase. These observations are consistent with previous reports concerning the *Lysobacter* sp. IB-9374 endoglucanase (Ogura et al., 2006). These results indicated that the enzyme was not metallo-protein, and did not require any specific ions in order to perform its catalytic action. On the other hand, NaCl displayed an enhancing effect on the *L. enzymogenes* 521 endoglucanase. Because of this fact, it indicates an advantage as several steps of enzyme purification were performed at high ionic strength of NaCl.

5.6.6 Effect of substrate concentration

A hyperbolic curve was obtained for the endoglucanase activity towards CMC at different concentrations. The endoglucanase increased as the concentration of CMC increased, indicating a small effect on the rate of endoglucanase activity. From the Lineweaver–Burk plot, Michaelis-Menten constant (K_m) of 8.40 mg/mL and maximal reaction velocity (V_{max}) of 11.45 μmol/min.mg protein were obtained for *L. enzymogenes* 521 endoglucanase. The *L. enzymogenes* 521 endoglucanase had K_m and V_{max} higher with respect to the *Lysobacter* sp. AL-1 endoglucanase (Hedges and Wolfe, 1974). In comparison with bifunctional enzymes dominant for chitosanase

activity, the *L. enzymogenes* 521 endoglucanase had K_m and V_{max} higher with respect to *Bacillus cereus* D-11 (Gao et al., 2006), *Paenibacillus illinoisensis* KJA-424 (Jung et al., 2005) and *Trichoderma viride* (Liu and Xia, 2006) (Table 5.1). Endoglucanase activity with high K_m of 7.33 mg/mL and V_{max} of 833 μmol/min.mg was detected from a thermophilic Gram-positive bacterium, *Thermomonospora curcata* (Lin and Stutzenberger, 1995).

Table 5.1 Biochemical properties of *L. enzymogenes* 521 endoglucanase in comparison with other enzymes produced by microorganisms

Source/name	Molecular mass (kDa)[a]	CMCase[b]		Chitosanase[c]		Relative activity (CMC/Chitosan) (%)	K_m	V_{max}	Inhibitor	Activator	Reference
		Specific activity (U/mg)	Optimum pH / Temp (°C)	Specific activity (U/mg)	Optimum pH / Temp (°C)						
Lysobacter enzymogenes 521	41	43.25	5.0./ 40	23.38	5.0/ 50	(100/54.1)	8.4	11.45	Ba^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}	-	This study
Lysobacter sp. IB-9374	41	484	5.0/ 40	190	7.0/ 70	(100/39.3)	NR	NR	Hg^{2+}, NBS, EDC	Ca^{2+}, Mg^{2+}, Zn^{2+}, Ba^{2+}	Ogura et al. (2006)
Lysobacter sp. AL-1	32	1,060	5.0/ 38	650	7.0/ 70	(100/61.3)	1.68	2.20	Hg^{2+}, Ag^{2+}, Zn^{2+}, Cd^{2+}	-	Hedges and Wolfe (1974)
Streptomyces griseus HUT 6037	34	29.9	NR	86.2	6.0/ 60	(34.7/100)	NR	NR	Hg^{2+}	-	Tanabe et al. (2003)
Bacillus cereus S1	45	42.3	6.0/ 40	196	6.0/ 60	(21.6/100)	NR	NR	NR	NR	Kurakake et al. (2000)
Bacillus cereus D-11	41	199.9	NR	264.5	6.0/ 60	(75.6/100)	7.5	0.013	Hg^{2+}, Pb^{2+}, Cu^{2+}, DEPC,	-	Gao et al. (2008)
Paenibacillus illinoisensis KJA-424	54	4.5	NR	17.50	5.0/ 60	(25.9/100)	1.12	1.48	Ag^{2+}, Hg^{2+}	-	Jung et al. (2005)
Trichoderma viride	66	0.258	4.2/ 50	0.402	5.2/ 60	(64.2/100)	0.88	3.50	Ag^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+}	Mn^{2+}, Mg^{2+}	Liu and Xia (2006)

[a] Determined by SDS-PAGE analysis with purified enzyme.

[b,c] The enzyme activities were tested under their optimal conditions.

NR = not reported

5.7 Draft genome sequencing and molecular characterization of endoglucanase gene

5.7.1 General feature and annotation of draft genome

The size of *L. enzymogenes* 521 genome was 5.85 Mbp, which is similar to the genome size between 5.5 and 6.4 Mbp reported for *Lysobacter* by Bruijn et al. (2015). The average G + C content of *L. enzymogenes* 521 genome was 69.77%, which is slightly lower than that of the *L. enzymogenes* C3 genome (69.81%), but is higher with respect to the genomes of the other members in the genus *Lysobacter* (Bruijn et al., 2015) and those of the following bacterial genomes in the other genera: *Xanthomonas oryzae* pv. *oryzae* KACC10331 (63.7%) (Lee et al., 2005), *X. oryzae* pv. *oryzae* PXO99 (63.6%) (Salzberg et al., 2008), *X. campestris* pv. *campestris* ATCC33913 (65.0%) and *X. axonopodis* pv. *citri* 306 (64.7%). The *L. enzymogenes* 521 genome contained 35 copies of rRNA genes, which had a high number of sequence polymorphisms. While, the other *Lysobacter* genomes contain generally about 6-9 copies of rRNA genes. For what concerns the tRNA gene, the number of genes found in *L. enzymogenes* 521 genome was similar to the other *Lysobacter* genomes, which were reported to be in the range of 51-60 tRNA genes (Bruijn et al., 2015). A large number of sequence polymorphisms of 85 tRNA genes were reported in *L. capsici* AZ78 (Puopolo et al., 2014).

5.7.2 Comparative genome analysis

Recently, the genomes of several phytopathogenic bacteria, such as *Xanthomonas* spp., have been intensively studied in order to investigate the mechanisms of pathogenesis and the processes to limit the host range of the pathogens (Lee et al, 2015; Salzberg et al., 2008; Silva et al., 2002). Based on limit of genome representatives existed in the RAST server, the most related genomes to the strain 521 genome were those of phytopathogenic bacterial in the genus *Xanthomonas* genomes. This result was in disagreement with the report of Bruijn et al. (2015) stating that the phylogenetic analysis showed a distinct clade of the *Lysobacter* genomes from the *Xanthomonas* and *Stenotrophomonas* genomes. However, pairwise analysis between genome of *L. enzymogenes* 521 and *L. enzymogenes* C3 showed a high average identity of orthologous sequences (90-95%).

5.7.3 Function based comparison

Metabolic profiles among strains of *Lysobacter* has been reveled based on Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation (Bruijn et al., 2015). CDSs unique for each strain was found in the range of 7.5-22.4% as follows: *L. antibioticus* ATCC29479 (9.5%), *L. antibioticus* 76 (7.5%), *L. capsici* 55 (12.5%), *L. capsici* AZ78 (8.8%), *L. gummosus* 3.2.11 (17.2%) and *L. enzymogenes* C3 (22.4%). In this study, approximately 95% of all categorized genes in metabolic

reconstruction were shared between the *L. enzymogenes* 521 and *L. enzymogenes* C3 genomes, and only 2.5% of all categorized genes were unique between the two strains. This result indicated that there is a high level of similarity between these two strains.

Secretion systems (especially of type III), have been identified as a key characteristic of many Gram-negative bacteria, which enable them to establish pathogenic interactions with their hosts (Kobayashi and Yuen, 2007). The comparative genome ananlysis revealed that genes encoding type I, II, III, IV, V secretion systems and type IV pili were highly conserved in *Lysobacter* genomes, while type VI secretion systems were only found in *L. enzymogenes* C3 and *L. gummosus* 3.2.11 (Bruijn et al., 2015). Instead, genes involved in encoding type VI secretion systems were not found in *L. enzymogenes* 521.

Several bacteria have been reported to be animal pathogens, including *Escherichia coli, Shigella dysenteriae, Haemophilus influenza* and *Neisseria meningitides.* It has been reported that the availability of iron acquisition and metabolism in bacteria is a key of pathogenesis in animal hosts (Janecky, 2013). In this study, the genes encoding proteins in hemin transport system, uptake and utilization which is categorized in iron acquisition and metabolism subsystem were present in *L. enzymogenes* C3 genome, but absent in *L. enzymogenes* 521. It is still not clear whether availability of iron acquisition and metabolism in *L. enzymogenes* C3 would impact the virulence on nematode hosts. In addition, it would be interesting to inquire whether the differences between the genes of these two strains are due to the evolutionary adaption process to their respective environments.

5.7.4 Carbohydrate metabolism in *Lysobacter enzymogenes* 521

The KEGG metabolic pathway annotation of *L. enzymogenes* 521 genome revealed that the genome of *L. enzymogenes* 521 contained various gene clusters of 10.1%, 9.9%, and 6.4% of total subsystem genes for protein metabolism, carbohydrate metabolism, and fatty acid, lipid, and isoprenoid metabolism, respectively. For what concerns the carbohydrate metabolism, the genes encoding enzymes involved in starch and glycogen metabolism were absent. This result was consistent with the assimilation test using biochemical test kit in Table 4.7 and with the previous report that starch and agar could not be utilized by strains of *L. enzymogenes* (Sullivan et al., 2003).

There were 33 genes encoding proteins involved in glycan hydrolysis in the *L. enzymogenes* 521 genome identified based on their annotation (Fig. 4.25). The genomes of *L. enzymogenes* 521 and related bacteria in family Xanthomonadaceae contain variety of genes encoding proteins which are responsible for β-glucan and β-xylan hydrolysis (GHF3 and GHF5), α-glucan hydrolysis (GHF13), and peptidoglycan hydrolysis (GHF23). Many plant pathogenic bacteria secrete a variety of plant cell wall degrading enzymes, such as cellulases and xylanases. Since the

cellulose and hemicellulose degrading enzymes are believed to play crucial roles in virulence and in bacterial nutrition in plant pathogenic *Xanthomonas* species (Salzberg et al., 2008), the gene abundant analysis suggested that the *Xanthomonas* genomes contained several of these enzymes (in GHF3, GHF5, GHF13) (more than those identified in the *Lysobacter* and *Stenotrophomonas* genomes). On the other hand, secretion of extracelllar enzymes, especially chitinases and β-1,3-glucanases acts as an important agent against various fungal pathogens by degrading their cell wall components (Kobayashi and Yuen, 2007). This report supports the gene abundant analysis that the *Lysobacter* genomes contained the genes encoding proteins responsible for chitin degradation (GHF18 and GHF19) and β-1,3 glucan degradation (GHF64) much more than that of identified in the *Xanthomonas* genomes.

The importance of the enzymes involved in lignocellulose hydrolysis in the ecology and physiology of *Lysobacter* is not still clear. However, we expect that there are many other genes encoding proteins involved in carbohydrate metabolic pathways in *Lysobacter* awaiting for investigation and characterization.

5.7.5 Analysis of the full-length endoglucanase Y gene (*cel*8A)

The 414 amino acid residues encoded from ORF of *L. enzymogenes* 521 *cel*8A matches with that of the *Lysobacter* sp. IB-9374 *cel*8A (Ogura et al., 2006). The predicted molecular mass (MM) of the endoglucanase Y is similar to the molecular mass of family 8 bifunctional endoglucanase genes from *B. circulans* KSM-N257 (MM of 41.84 kDa) (Hakamada et al., 2002) and *Paenibacillus cookii* SS-24 (MM of 41.68 kDa) (Shinoda et al., 2012). On the other hand, family 8 bifunctional chitosanase gene from *Bacillus* sp. KCTC 0377BP had molecular mass 50.69 kDa (Choi et al., 2004), which is larger with respect to the *L. enzymogenes* 521 endoglucanase Y. The phylogenic analysis of the *L. enzymogenes* 521 *cel*8A sequence with the other glycoside hydrolase gene sequences revealed that the *L. enzymogenes* 521 *cel*8A fall into family 8 endoglucanases clade with a high bootstrap value (77 %) (Fig. 4.27). Therefore, the endoglucanase of this study is assigned to the glycoside hydrolase family 8.

The deduced protein sequence of endoglucanase Y contains a typical conserved signature amino acid sequence, ATDGDLDIAYSLLLAHDLQW, of glycoside hydrolase family 8 (Ogura et al., 2006; Shinoda et al., 2012). Furthermore, the enzyme showed a high sequence similarity (98-44% identity) with family 8 glycoside hydrolases, such as bifunctional endoglucanase from *Lysobacter* sp. IB-9374, β-1,4-glucanase from *L. capsici* AZ78, and endoglucanase from *B. circulans* N257. The catalytic amino acid residues glutamic acid (Glu95) and the two aspartic acid (Asp152 and Asp278), which have also been reported in all members of GHF-8 (Ogura et al., 2006, Yasutake et al., 2006), were conserved in Cel8A at corresponding positions 99 and 160 for Glu95 and Asp152 respectively,

but was not conserved for Asp278. Based on the crystal structure and the ultra-high resolution analysis of the Cel8A from the well-defined cellulase-producer *C. thermocellum* ATCC 27405, the following five aromatic residues have been reported to involve in substrate recognition: Trp132, Trp205, Tyr277, Tyr369 and Tyr372 (Alzari and Dominguez, 1996). The alignment analysis showed that the Cel8A had two conserved corresponding aromatic residues, Trp143 and Try395, but the other three residues of Trp205, Tyr277 and Tyr369 reported in the Cel8A of *C. thermocellum* ATCC 27405 were not present. In addition, the crystal structure analysis of chitosanase from *Bacillus* sp. K17 suggested that the following four aromatic residues were conserved as the substrate binding sites: Trp119, Trp188, Tyr271 and Phe366 (Adachi et al., 2004). Only two corresponding residues at Trp143 and Trp212 were present in the Cel8A, but not the Tyr271 and Phe366 in the chitosanase. For the substrate recognition sites, four acidic residues (Glu60, Asp132, Asp136 and Glu262) were conserved in the Cel8A at the three corresponding residues at Asp156, Asp160 and Glu291, but were not conserved at the residue Glu60 in chitosanase from *Bacillus* sp. K17 (Adachi et al., 2004). These data suggested that the substrate recognition and binding of the bifunctional CMCase-chitosanase from *L. enzymogenes* 521 might be different from those of *C. thermocellum* ATCC 27405 and *Bacillus* sp. K17. However, in order to prove whether the different amino acid elements are important for the bifunctional CMCase-chitosanase properties, it will be necessary to investigate further the structure-function relationship of the bifunctional enzyme requires more research.

5.8 Conclusion and prospects

Bacterial strain 521 producing endoglucanase activity identified as *Lysobacter enzymogenes* was isolated from soil in Thailand. This finding extends our knowledge of the geographical habitats of *Lysobacter* spp. to tropical environments. The phenotypic characteristic differences between the two strains of *L. enzymogenes*, tropical strain 521 and temperate strain C3, suggested that there might be some interest properties between them to be investigate. This study showed, in particular, that the growth and enzyme production in both strains of the temperate strain C3 was effected considerably more at high temperatures compared with the tropical strain 521. Production of endoglucanase by tropical *L. enzymogenes* 521 was successfully optimized by enhancing 22-fold of endoglucanase activity with respect to the activity obtained from the screening medium. The endoglucanase was successfully purified, and its biochemical properties were characterized as similar to those of bifunctional enzymes belonging to the glycoside hydrolase family 8 (GHF8). Draft genome sequence analysis provided us with a better understanding of the tropical *L. enzymogenes* 521 genome and of its differences with respect to the temperate *L. enzymogenes* C3 and other related bacterial genomes. Particularly, the power of

genome sequence allowed us to effectively study the *cel*8A gene. Zymogram analysis of the purified endoglucanase from *L. enzymogenes* 521, along with nucleotide sequence and deduced amino acid sequence analysis of the Cel8A gene obtained from draft genome sequence, suggested that the endoglucanase contained an enzyme with bifuntional activities of CMCase and chitosanase, and showed a high homology to GHF8 proteins. The study of the CMCase from the strain 521 accomplished in this work will be further pursued in order to understand its function at molecular level and to explore its potential as a useful CMCase/chitosnase producer for biotechnological applications based on its interesting bifunctional property.

REFERENCES

Adachi, W., Y. Sakihama, S. Shimizu, T. Sunami, T. Fukazawa, M. Suzuki, R. Yatsunami, S. Nakamura and A. Takénaka (2004). Crystal structure of family GH-8 chitosanase with subclass II specificity from *Bacillus* sp. K17. *Journal of Molecular Biology* 343: 785-795.

Ahmed, K., S. Chohnan, H. Ohashi, T. Hirata, T. Masaki and F. Sakiyama (2003). Purification, bacteriolytic activity, and specificity of β-lytic protease from *Lysobacter* sp. IB-9374. *Journal of Bioscience and Bioengineering* 95(1): 27-34.

Alzari, P. M. and R. Dominguez (1996). The crystal structure of endoglucanase CelA, a family 8 glycosyl hydrolase from *Clostridium thermocellum*. *Structure* 4: 265-275.

Amat, D., A. Arora, L. Nain and A. K. Saxena (2014). Biomass hydrolyzing enzymes from plant pathogen *Xanthomonas axonopodis* pv. punicae: optimizing production and characterization. *Annals of Microbiology* 64(1): 267-274.

Anderson, S. (1981). Shotgun DNA sequencing using cloned DNase I-generated fragments. *Nucleic Acids Research* 9(13): 3015-3027.

Andrews, B. and J. Asenjo (1987). Enzymatic lysis and disruption of microbial cells. *Trends in Biotechnology* 5(10): 273-277.

Araragi, M., S. Motomura, T. Koyama, T. Matsuguchi, C. Chammek, N. Niamsrichand, B. Tangcham, S. Patiyuth and A. Seiayosakol (1978). Dynamic behaviour of soil nitrogen in paddy soils of Thailand. *Japan Agricultural Research Quarterly* 12(2): 79-85.

Asenjo, J. and P. Dunnill (1981). The isolation of lytic enzymes from Cytophaga and their application to the rupture of yeast cells. *Biotechnology and bioengineering* 23(5): 1045-1056.

Aslam, Z., M. Yasir, C. O. Jeon and Y. R. Chung (2009). *Lysobacter oryzae* sp. nov., isolated from the rhizosphere of rice (*Oryza sativa* L.). *International Journal of Systematic and Evolutionary Microbiology* 59: 675-680.

Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass and M. Kubal (2008). The RAST Server: rapid annotations using subsystems technology. *BMC genomics* 9(1): 75.

Bae, H.-S., W.-T. Im and S.-T. Lee (2005). *Lysobacter concretionis* sp. nov., isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor. *International journal of systematic and evolutionary microbiology* 55(3): 1155-1161.

Bamforth, C. (2009). Current perspectives on the role of enzymes in brewing. *Journal of Cereal Science* 50(3): 353-357.

Bano, S., S. A. U. Qader, A. Aman, M. N. Syed and K. Durrani (2013). High production of cellulose degrading endo-1, 4-β-D-glucanase using bagasse as a substrate from *Bacillus subtilis* KIBGE HAS. *Carbohydrate Polymers* 91(1): 300-304.

Benhamou, N. (2004). Potential of the mycoparasite, *Verticillium lecanii*, to protect citrus fruit against *Penicillium digitatum*, the causal agent of green mold: A comparison with the effect of chitosan. *Phytopathology* 94(7): 693-705.

Bischoff, K. M., A. P. Rooney, X. L. Li, S. Liu and S. R. Hughes (2006). Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. *Biotechnology Letter* 28: 1761-1765.

Blackmoore, M., N. Patel, B. Hillman and D. Kobayashi (2009). Involvement of type IV secretion in *Lysobacter enzymogenes* pathogenesis of fungal and algal hosts. Phytopathology, AMER PHYTOPATHOLOGICAL SOC, MN , USA.

Blaschek, W., J. Käsbauer, J. Kraus and G. Franz (1992). Pythium aphanidermatum: culture, cell-wall composition, and isolation and structure of antitumour storage and solubilised cell-wall (1⟶ 3),(1⟶ 6)-β-d-glucans. *Carbohydrate research* 231: 293-307.

Box, G. E. and D. W. Behnken (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2(4): 455-475.

Box, G. E. P., W. G. Hunter and J. S. Hunter (1978). *Statistics for experimenters*. New York, Wiley Inc.

Bruijn, I., X. Cheng, V. de Jager, R. G. Expósito, J. Watrous, N. Patel, J. Postma, P. C. Dorrestein, D. Kobayashi and J. M. Raaijmakers (2015). Comparative genomics and metabolic profiling of the genus *Lysobacter*. *BMC genomics* 16(1): 991.

Buchert, J., T. Oksanen, J. Pere, M. Siika-Aho, A. Suurnäkki and L. Viikari (1998). Applications of *Trichoderma reesei* enzymes in the pulp and paper industry. *Trichoderma and gliocladium* 2: 343-363.

Bueno, A., C. V. de Aldana, J. Correa, T. G. Villa and F. Del Rey (1990). Synthesis and secretion of a *Bacillus circulans* WL-12 1, 3-1, 4-beta-D-glucanase in *Escherichia coli*. *Journal of Bacteriology* 172(4): 2160-2167.

Burnett, T. J., G. Shankweiler and J. H. Hageman (1986). Activation of intracellular serine proteinase in *Bacillus subtilis* cells during sporulation. *Journal of Bacteriology* 165(1): 139-145.

Chen, J., W. Moore, G. Yuen, D. Kobayashi and E. Caswell-Chen (2006). Influence of *Lysobacter enzymogenes* strain C3 on nematodes. *Journal of nematology* 38(2): 233.

Chinedu, S. N., A. O. Eni, A. I. Adeniyi and J. A. Ayangbemi (2010). Assessment of growth and cellulase production of wild-type microfungi isolated from Ota, Nigeria. *Asian Journal of Plant Sciences* 9(3): 118-125.

Chohnan, S., K. Shiraki, K. Yokota, M. Ohshima, N. Kuroiwa, K. Ahmed, T. Masaki and F. Sakiyama (2004). A second lysine-specific serine protease from *Lysobacter* sp. strain IB-9374. *Journal of Bacteriology* 186(15): 5093-5100.

Choi, J.-H., J.-H. Seok, J.-H. Cha and C.-J. Cha (2014). *Lysobacter panacisoli* sp. nov., isolated from ginseng soil. *International journal of systematic and evolutionary microbiology* 64(7): 2193-2197.

Choi, Y. J., E. J. Kim, Z. Piao, Y. C. Yun and Y. C. Shin (2004). Purification and characterization of chitosanase from *Bacillus* sp. strain KCTC 0377BP and its application for the production of chitosan oligosaccharides. *Applied and Environmental Microbiology* 70(8): 4522-4531.

Christensen, P. (2001). Genus IV *Lysobacter* Christensen and Cook 1978. *Bergey's manual of systematic bacteriology. Springer, New York*: 95-101.

Christensen, P. and F. D. Cook (1972). The isolation and enumeration of cytophagas. *Canadian Journal of Microbiology* 18: 1933-1940.

Christensen, P. and F. D. Cook (1978). *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio. *International journal of systematic bacteriology* 28: 367-393.

Cupp-Enyard, C. (2008). Sigma's Non-specific Protease Activity Assay-Casein as a Substrate. JoVE. 19, Citeseer.

Da Vinha, F. N. M., M. P. Gravina-Oliveira, M. N. Franco, A. Macrae, E. P. da Silva Bon, R. P. Nascimento and R. R. R. Coelho (2011). Cellulase production by Streptomyces viridobrunneus SCPE-09 using lignocellulosic biomass as inducer substrate. *Applied biochemistry and biotechnology* 164(3): 256-267.

Daft, M. and W. Stewart (1971). Bacterial pathogens of freshwater blue-green algae. *New Phytologist* 70(5): 819-829.

Das, A., T. Paul, S. K. Halder, A. Jana, C. Maity, P. K. D. Mohapatra, B. R. Pati and K. C. Mondal (2013). Production of cellulolytic enzymes by *Aspergillus fumigatus* ABK9 in wheat bran-rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp. *Bioresource technology* 128: 290-296.

Dawson, A. and C. Thorne (1969). Preparation and some properties of L-3-glycerophosphate dehydrogenase from pig brain mitochondria. *Biochemical Journal* 111(1): 27-34.

Debette, J. and R. Blondeau (1980). Presence of *Pseudomonas maltophilia* in the rhizosphere of several cultivated plants. *Canadian journal of microbiology* 26(4): 460-463.

Deepmala, A. (2014). Study on Fixed Point Theorems for Nonlinear Contractions and its Applications, Ph. D. Thesis.

Deka, D., P. Bhargavi, A. Sharma, D. Goyal, M. Jawed and A. Goyal (2011). Enhancement of cellulase activity from a new strain of *Bacillus subtilis* by medium optimization and analysis with various cellulosic substrates. *Enzyme research* 2011.

Dempsey, K. E., M. P. Riggio, A. Lennon, V. E. Hannah, G. Ramage, D. Allan and J. Bagg (2007). Identification of bacteria on the surface of clinically infected and non-infected prosthetic hip joints removed during revision arthroplasties by 16S rRNA gene sequencing and by microbiological culture. *Arthritis Research and Therapy* 9(3): R46.

Dienes, D., A. Egyhazi and K. Reczey (2004). Treatment of recycled fiber with *Trichoderma* cellulases. *Industrial Crops and Products* 20(1): 11-21.

Doonan, S. and P. Cutler (2004). General strategies. *Protein purification protocols*: 1-13.

Du, J., H. Singh, H. T. Ngo, K. Won, K.-Y. Kim and T.-H. Yi (2015). *Lysobacter tyrosinelyticus* sp. nov. isolated from gyeryongsan national park soil. *Journal of Microbiology* 53(6): 365-370.

Ensign, J. and R. Wolfe (1965). Lysis of bacterial cell walls by an enzyme isolated from a *myxobacter*. *Journal of Bacteriology* 90(2): 395-402.

Ensign, J. and R. Wolfe (1966). Characterization of a small proteolytic enzyme which lyses bacterial cell walls. *Journal of Bacteriology* 91(2): 524-534.

Folman, L., M. De Klein, J. Postma and J. Van Veen (2004). Production of antifungal compounds by *Lysobacter enzymogenes* isolate 3.1 T8 under different conditions in relation to its efficacy as a biocontrol agent of Pythium aphanidermatum in cucumber. *Biological Control* 31(2): 145-154.

Fukuda, W., T. Kimura, S. Araki, Y. Miyoshi, H. Atomi and T. Imanaka (2013). *Lysobacter oligotrophicus* sp. nov., isolated from an Antarctic freshwater lake in Antarctica. *International journal of systematic and evolutionary microbiology* 63(9): 3313-3318.

Galante, Y., A. De Conti and R. Monteverdi (1998). Application of *Trichoderma* enzymes in the food and feed industries. *Trichoderma and gliocladium* 2: 327-342.

Gao, X.-A., W.-T. Ju, W.-J. Jung and R.-D. Park (2008). Purification and characterization of chitosanase from *Bacillus cereus* D-11. *Carbohydrate Polymers* 72(3): 513-520.

Ghosh, P. and A. Singh (1993). Physiochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass. *Advances in Applied Microbiology* 39: 295-333.

Giesler, L. J. and G. Y. Yuen (1998). Evaluation of *Stenotrophomonas maltophilia* strain C3 for biocontrol of brown patch disease. *Crop Protection* 17: 509-513.

Glogauer, A., V. P. Martini, H. Faoro, G. H. Couto, M. Müller-Santos, R. A. Monteiro, D. A. Mitchell, E. M. de Souza, F. O. Pedrosa and N. Krieger (2011). Identification and characterization of a new true lipase isolated through metagenomic approach. *Microbial Cell Factories* 10(1): 1.

Gough, C. L., J. M. Dow, C. E. Barber and M. J. Daniels (1988). Cloning of two endoglucanase genes of *Xanthomonas campestris* pv. campestris: analysis of the role of the major endoglucanase in pathogenesis. *Molecular Plant-Microbe Interactions* 1: 275-281.

Grant, J. R. and P. Stothard (2008). The CGView Server: a comparative genomics tool for circular genomes. *Nucleic Acids Research* 36(suppl 2): W181-W184.

Gupta, R., Y. P. Khasa and R. C. Kuhad (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. *Carbohydrate Polymers* 84(3): 1103-1109.

Hakamada, Y., K. Endo, S. Takizawa, T. Kobayashi, T. Shirai, T. Yamane and S. Ito (2002). Enzymatic properties, crystallization, and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. *Biochimica et Biophysica Acta (BBA)-General Subjects* 1570(3): 174-180.

Hall, N. (2007). Advanced sequencing technologies and their wider impact in microbiology. *Journal of Experimental Biology* 210(9): 1518-1525.

Han, L., J. Feng, C. Zhu and X. Zhang (2009). Optimizing cellulase production of *Penicillium waksmanii* F10-2 with response surface methodology. *African Journal of Biotechnology* 8: 3879-3886.

Hayward, A., N. Fegan, M. Fegan and G. Stirling (2010). *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. *Journal of Applied Microbiology* 108: 756-770.

He, Y. W., A. Y. J. Ng, M. Xu, K. Lin, L. H. Wang, Y. H. Dong and L. H. Zhang (2007). *Xanthomonas campestris* cell–cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Molecular microbiology* 64(2): 281-292.

Hebeish, A. and N. Ibrahim (2007). The impact of frontier sciences on textile industry. *Colourage* 54(4): 41-55.

Hedges, A. and R. S. Wolfe (1974). Extracellular enzyme from *Myxobacter* AL-1 that exhibits both β-1, 4-glucanase and chitosanase activities. *Journal of Bacteriology* 120: 844-853.

Hitchins, A. D., P. Feng, W. D. Watkins, S. R. Rippey, L. A. Chandler, Food and D. Administration (1998). *Bacteriological analytical manual*.

Hmad, I. B., S. Abdeljalil, W. Saibi, B. Amouri and A. Gargouri (2014). Medium initial pH and carbon source stimulate differential alkaline cellulase time course production in Stachybotrys microspora. *Applied biochemistry and biotechnology* 172(5): 2640-2649.

Hong, S.-P. and D.-S. Kim (1998). Chitosanolytic characteristics of cellulases from *Trichoderma viride* and *Trichoderma reesei*. *Korean Journal of Food Science and Technology* 30(2): 245-252.

Hsu, S. and J. Lockwood (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology* 29(3): 422-426.

Hsu, S. C. and J. L. Lockwood (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology* 29: 422-426.

Hunter, J. and J. Asenjo (1987). Kinetics of enzymatic lysis and disruption of yeast cells: II. A simple model of lysis kinetics. *Biotechnology and bioengineering* 30(4): 481-490.

Hurd, P. J. and C. J. Nelson (2009). Advantages of next-generation sequencing versus the microarray in epigenetic research. *Briefings in Functional Genomics*: elp013.

Ibrahim, N., K. El-Badry, B. Eid and T. Hassan (2011). A new approach for biofinishing of cellulose-containing fabrics using acid cellulases. *Carbohydrate Polymers* 83(1): 116-121.

Ike, M., Y. Ko, K. Yokoyama, J.-I. Sumitani, T. Kawaguchi, W. Ogasawara, H. Okada and Y. Morikawa (2007). Cellobiohydrolase I (Cel7A) from *Trichoderma reesei* has chitosanase activity. *Journal of Molecular Catalysis B: Enzymatic* 47(3): 159-163.

Islam, M. T., Y. Hashidoko, A. Deora, T. Ito and S. Tahara (2005). Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne peronosporomycetes. *Applied and Environmental Microbiology* 71(7): 3786-3796.

Jabasingh, S. A. and C. V. Nachiyar (2010). A new combinational statistical approach for cellulase optimization in *Aspergillus nidulans*. *Indian Journal of Science and Technology* 3(8): 871-878.

Jaradat, O. K., A. Al-Omari and S. Momani (2008). Existence of the mild solution for fractional semilinear initial value problems. *Nonlinear Analysis: Theory, Methods & Applications* 69(9): 3153-3159.

Jung, H. M., L. N. Ten, W. T. Im, S. A. Yoo and S. T. Lee (2008). *Lysobacter ginsengisoli* sp. nov., a novel species isolated from soil in Pocheon Province, South Korea. *Journal of Microbiology and Biotechnology* 18: 1496-1499.

Kamoun, S. and C. I. Kado (1990). A plant-inducible gene of *Xanthomonas campestris* pv. *campestris* encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. *Journal of Bacteriology* 172(9): 5165-5172.

Karmakar, M. and R. R. Ray (2013). Endoglucanase Synthesis from Submerged Fermentation of Dried Grass by *Streptomyces* sp. *American Journal of Bioengineering and Biotechnology* 1: 1-10.

Kasana, R. C., R. Salwan, H. Dhar, S. Dutt and A. Gulati (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology* 57(5): 503-507.

Katznelson, H., D. Gillespie and F. Cook (1964). Studies on the relationships between nematodes and other soil microorganisms: III. Lytic action of soil *Myxobacters* on certain species of nematodes. *Canadian journal of microbiology* 10(5): 699-704.

Kazemi-Pour, N., G. Condemine and N. Hugouvieux-Cotte-Pattat (2004). The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. *Proteomics* 4(10): 3177-3186.

Kibblewhite, R., A. Bawden and C. Brindley (1995). TMP fibre and fines qualities of thirteen radiata pine wood types. *Appita journal* 48(5): 367-377.

Kilic-Ekici, O. and G. Y. Yuen (2003). Induced resistance as a mechanism of biological control by *Lysobacter enzymogenes* strain C3. *Phytopathology* 93(9): 1103-1110.

Kim, S.-K. and N. Rajapakse (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers* 62(4): 357-368.

Kim, Y. C., H. Jung, K. Y. Kim and S. K. Park (2008). An effective biocontrol bioformulation against Phytophthora blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions. *European Journal of Plant Pathology* 120(4): 373-382.

Kimoto, H., H. Kusaoke, I. Yamamoto, Y. Fujii, T. Onodera and A. Taketo (2002). Biochemical and Genetic Properties of *Paenibacillus* Glycosyl Hydrolase Having Chitosanase Activity and Discoidin Domain. *Journal of Biological chemistry* 277(17): 14695-14702.

Kobayashi, D. Y. and N. El-Barrad (1996). Selection of bacterial antagonists using enrichment cultures for the control of summer patch disease in Kentucky bluegrass. *Curr Microbiol* 32: 106-110.

Kobayashi, D. Y., M. Guglielmoni and B. B. Clarke (1995). Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biology and Biochemistry* 27(11): 1479-1487.

Kobayashi, D. Y., R. M. Reedy, J. D. Palumbo, J. M. Zhou and G. Y. Yuen (2005). A *clp* gene homologue belonging to the Crp gene family globally regulates lytic enzyme production, antimicrobial activity, and biological control activity expressed by *Lysobacter enzymogenes* strain C3. *Applied and Environmental Microbiology* 71: 261-269.

Kobayashi, D. Y. and G. Y. Yuen (2007). The potential of *Lysobacter* spp. as bacterial biological control agents for plant diseases. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 2: 1-11.

Kongsaengdao, S., S. Bunnag and N. Siriwiwattnakul (2005). Treatment of survivors after the tsunami. *New England Journal of Medicine* 352(25): 2654-2655.

Kuhad, R., R. Gupta and Y. Khasa (2010). Bioethanol production from lignocellulosic biomass: an overview, Teri Press, New Delhi, India.

Kuhad, R. C., R. Gupta and A. Singh (2011). Microbial cellulases and their industrial applications. *Enzyme research* 2011.

Kuhad, R. C., G. Mehta, R. Gupta and K. K. Sharma (2010). Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. *Biomass and Bioenergy* 34(8): 1189-1194.

Kurakake, M., S. Yo-u, K. Nakagawa, M. Sugihara and T. Komaki (2000). Properties of chitosanase from *Bacillus cereus* S1. *Current microbiology* 40(1): 6-9.

Kwak, M.-J., S.-K. Kwon, J.-H. Yoon and J. F. Kim (2015). Genome sequence of *Lysobacter dokdonensis* DS-58 T, a gliding bacterium isolated from soil in Dokdo, Korea. *Standards in genomic sciences* 10(1): 1.

Lafontaine, P. J. and N. Benhamou (1996). Chitosan treatment: an emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Biocontrol Science and Technology* 6(1): 111-124.

Lambert, B., F. Leyns, L. Van Rooyen, F. Gosselé, Y. Papon and J. Swings (1987). Rhizobacteria of maize and their antifungal activities. *Applied and Environmental Microbiology* 53(8): 1866-1871.

Lamed, R., E. Setter and E. A. Bayer (1983). Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. *Journal of Bacteriology* 156(2): 828-836.

Land, M., L. Hauser, S.-R. Jun, I. Nookaew, M. R. Leuze, T.-H. Ahn, T. Karpinets, O. Lund, G. Kora and T. Wassenaar (2015). Insights from 20 years of bacterial genome sequencing. *Functional & integrative genomics* 15(2): 141-161.

Lane, D. (1991). 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*: 125-175.

Lee, B.-M., Y.-J. Park, D.-S. Park, H.-W. Kang, J.-G. Kim, E.-S. Song, I.-C. Park, U.-H. Yoon, J.-H. Hahn and B.-S. Koo (2005). The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Research* 33(2): 577-586.

Lee, J. W., W. T. Im, M. K. Kim and D. C. Yang (2006). *Lysobacter koreensis* sp. nov., isolated from a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 56: 231-235.

Lee, M., J. Do, M. Park, S. Jung, K. Lee, K. Bae, S. Park and S. Kim (2006). Dominance of *Lysobacter* sp. in the rhizosphere of two coastal sand dune plant species, *Calystegia soldanella* and *Elymus mollis*. *Antonie van Leeuwenhoek* 90: 19-27.

Li, S., L. Du, G. Yuen and S. D. Harris (2006). Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. *Molecular biology of the cell* 17(3): 1218-1227.

Lin, S.-Y., A. Hameed, C.-Z. Wen, Y.-C. Liu, Y.-H. Hsu, W.-A. Lai and C.-C. Young (2015). *Lysobacter lycopersici* sp. nov., isolated from tomato plant Solanum lycopersicum. *Antonie van Leeuwenhoek* 107(5): 1261-1270.

Lin, S. B. and F. Stutzenberger (1995). Purification and characterization of the major β-1, 4-endoglucanase from *Thermomonospora curvata*. *Journal of Applied Bacteriology* 79(4): 447-453.

Lin, Y., S. Taylor, H. Li, K. S. Fernando, L. Qu, W. Wang, L. Gu, B. Zhou and Y.-P. Sun (2004). Advances toward bioapplications of carbon nanotubes. *Journal of Materials Chemistry* 14(4): 527-541.

Liu, J. and W. Xia (2006). Purification and characterization of a bifunctional enzyme with chitosanase and cellulase activity from commercial cellulase. *Biochemical Engineering Journal* 30(1): 82-87.

Liu, L., S. Zhang, M. Luo and G. Wang (2015). Genomic information of the arsenic-resistant bacterium *Lysobacter arseniciresistens* type strain ZS79T and comparison of *Lysobacter* draft genomes. *tandards in genomic sciences* 10(1): 1-7.

Liu, M., Y. Liu, Y. Wang, X. Luo, J. Dai and C. Fang (2011). *Lysobacter xinjiangensis* sp. nov., a moderately thermotolerant and alkalitolerant bacterium isolated from a gamma-irradiated sand soil sample. *International Journal of Systematic and Evolutionary Microbiology* 61: 433-437.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193(1): 265-275.

Luo, G., Z. Shi and G. Wang (2012). *Lysobacter arseniciresistens* sp. nov., an arsenite-resistant bacterium isolated from iron-mined soil. *International Journal of Systematic and Evolutionary Microbiology* 62: 1659-1665.

Lynd, L. R., P. J. Weimer, W. H. Van Zyl and I. S. Pretorius (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews* 66(3): 506-577.

Lynd, L. R., C. E. Wyman and T. U. Gerngross (1999). Biocommodity engineering. *Biotechnology progress* 15(5): 777-793.

Lyngwi, N. A., K. Koijam, D. Sharma and S. Joshi (2013). Cultivable bacterial diversity along the altitudinal zonation and vegetation range of tropical Eastern Himalaya. *Revista de Biología Tropical* 61(1): 467-490.

Macedo, E., C. Cerqueira, D. Souza, A. Bispo, R. Coelho and R. Nascimento (2013). Production of cellulose-degrading enzyme on sisal and other agro-industrial residues using a new Brazilian actinobacteria strain *Streptomyces* sp. SLBA-08. *Brazilian Journal of Chemical Engineering* 30(4): 729-735.

Maki, M., K. T. Leung and W. Qin (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International journal of biological sciences* 5: 500.

Mandabhushi, R. S. (1998). Separation of 4-color DNA sequencing extension products in noncovalently coated capillaries using low viscosity polymer solutions. *Electrophoresis* 19(2): 224-230.

Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y.-J. Chen and Z. Chen (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437(7057): 376-380.

Markowitz, V. M., I.-M. A. Chen, K. Palaniappan, K. Chu, E. Szeto, Y. Grechkin, A. Ratner, I. Anderson, A. Lykidis and K. Mavromatis (2009). The integrated microbial genomes system: an expanding comparative analysis resource. *Nucleic Acids Research*: gkp887.

McFarland, J. (1907). The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association* 49(14): 1176-1178.

Metzker, M. L. (2010). Sequencing technologies—the next generation. *Nature reviews genetics* 11(1): 31-46.

Miller, G. L., R. Blum, W. E. Glennon and A. L. Burton (1960). Measurement of carboxymethylcellulase activity. *Analytical Biochemistry* 1: 127-132.

Mitchell, T., M. S. Hendrie and J. M. Shewan (1969). Symposium on Myxobacteria and Flavobacteria: Paper VIII). The Taxonomy, Differentiation and Identification of Cytophaga Species. *Journal of Applied Bacteriology* 32(1): 40-50.

Mitsutomi, M., M. Isono, A. Uchiyama, N. Nikaidou, T. Ikegami and T. Watanabe (1998). Chitosanase activity of the enzyme previously reported as β-1, 3-1, 4-glucanase from *Bacillus circulans* WL-12. *Bioscience, biotechnology, and biochemistry* 62(11): 2107-2114.

Muthuvelayudham, R. and T. Viruthagiri (2006). Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw. *African Journal of Biotechnology* 5(20).

Nakayama, T., Y. Homma, Y. Hashidoko, J. Mizutani and S. Tahara (1999). Possible Role of Xanthobaccins Produced by *Stenotrophomonas* sp. Strain SB-K88 in Suppression of Sugar Beet Damping-Off Disease. *Applied and Environmental Microbiology* 65(10): 4334-4339.

Neuhoff, V., R. Stamm and H. Eibl (1985). Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis. *Electrophoresis* 6(9): 427-448.

Ngo, H. T., K. Won, J. Du, H.-M. Son, Y. Park, M. Kook, K.-Y. Kim, F.-X. Jin and T.-H. Yi (2015). *Lysobacter terrae* sp. nov. isolated from Aglaia odorata rhizosphere soil. *International journal of systematic and evolutionary microbiology* 65(2): 587-592.

Nwe, N., T. Furuike and H. Tamura (2010). Chitin and chitosan from terrestrial organisms and microorganisms: production, properties and applications *Biodegradable Materials*. J. B.M. and B. Z.E., Nova Science Publishers, Inc.: 29-50.

Ogura, J., A. Toyoda, T. Kurosawa, A. L. Chong, S. Chohnan and T. Masaki (2006). Purification, characterization, and gene analysis of cellulase (Cel8A) from *Lysobacter* sp. IB-9374. *Bioscience, Biotechnology, and Biochemistry* 70: 2420-2428.

Oh, K.-H., S.-J. Kang, Y.-T. Jung, T.-K. Oh and J.-H. Yoon (2011). *Lysobacter dokdonensis* sp. nov., isolated from soil. *International journal of systematic and evolutionary microbiology* 61(5): 1089-1093.

Padmavathi, A. R., B. Abinaya and S. K. Pandian (2014). Phenol, 2, 4-bis (1, 1-dimethylethyl) of marine bacterial origin inhibits quorum sensing mediated biofilm formation in the uropathogen Serratia marcescens. *Biofouling* 30(9): 1111-1122.

Palumbo, J. D., R. F. Sullivan and D. Y. Kobayashi (2003). Molecular characterization and expression in *Escherichia coli* of three β-1, 3-glucanase genes from *Lysobacter enzymogenes* strain N4-7. *Journal of Bacteriology* 185: 4362-4370.

Park, J. H., R. Kim, Z. Aslam, C. O. Jeon and Y. R. Chung (2008). *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. *International journal of systematic and evolutionary microbiology* 58(2): 387-392.

Patel, N., M. Blackmoore, B. Hillman and D. Kobayashi (2009). *Evidence for the role of Type VI secretion during Lysobacter enzymogenes pathogenesis of fungal hosts*. Phytopathology, AMER PHYTOPATHOLOGICAL SOC 3340 PILOT KNOB ROAD, ST PAUL, MN 55121 USA.

Pedraza-Reyes, M. and F. Gutiérrez-Corona (1997). The bifunctional enzyme chitosanase-cellulase produced by the gram-negative microorganism *Myxobacter* sp. AL-1 is highly similar to Bacillus subtilis endoglucanases. *Archives of microbiology* 168(4): 321-327.

Pelletier, A. and J. Sygusch (1990). Purification and characterization of three chitosanase activities from *Bacillus megaterium* P1. *Applied and Environmental Microbiology* 56(4): 844-848.

Peterson, E., D. Gillespie and F. Cook (1966). A wide-spectrum antibiotic produced by a species of *Sorangium*. *Canadian journal of microbiology* 12(2): 221-230.

Phiarais, B. P., B. D. Schehl, J. C. Oliveira and E. K. Arendt (2006). Use of response surface methodology to investigate the effectiveness of commercial enzymes on buckwheat malt for brewing purposes. *Journal of The Institute of Brewing* 112: 324-332.

Pio, T. F. and G. A. Macedo (2008). Cutinase production by *Fusarium oxysporum* in liquid medium using central composite design. *Journal of Industrial Microbiology and Biotechnology* 35: 59-67.

Piotrowski, S. and M. Carus (2011). Ecological benefits of hemp and flax cultivation and products. *Nova Institute* 5: 1-6.

Postma, J., L. H. Stevens, G. L. Wiegers, E. Davelaar and E. H. Nijhuis (2009). Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1 T8 and chitosan. *Biological Control* 48(3): 301-309.

Prober, J. M., G. L. Trainor, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen and K. Baumeister (1987). A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 238(4825): 336-341.

Puopolo, G., P. Sonego, K. Engelen and I. Pertot (2014). Draft genome sequence of *Lysobacter capsici* AZ78, a bacterium antagonistic to plant-pathogenic oomycetes. *Genome Announc* 2: e00325-00314.

Puopolo, G., P. Sonego, K. Engelen and I. Pertot (2014). Draft genome sequence of *Lysobacter capsici* AZ78, a bacterium antagonistic to plant-pathogenic oomycetes. *Genome announcements* 2(2): e00325-00314.

Rapp, P., A. Beermann, C. Haigler and P. Weimer (1991). Bacterial cellulases. *Biosynthesis and biodegradation of cellulose.*: 535-597.

Ray, A., A. Bairagi, K. S. Ghosh and S. Sen (2007). Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and Bacillus circulans TP3 isolated from fish gut. *Acta Ichthyologica et Piscatoria* 1(37): 47-53.

Reichenbach, H. (2006). The genus *Lysobacter*. *The Prokaryotes, A Handbook on the Biology of Bacteria*. D. M, F. S, R. E, S. K. H and S. E. New York, USA, Springer Science + Business Media LLC. 6: 939-957.

Richmond, V. P., J. C. McCroskey and S. K. Payne (1991). *Nonverbal behavior in interpersonal relations*, Prentice Hall Englewood Cliffs, NJ.

Roach, J. C., C. Boysen, K. Wang and L. Hood (1995). Pairwise end sequencing: a unified approach to genomic mapping and sequencing. *Genomics* 26(2): 345-353.

Robson, L. M. and G. H. Chambliss (1989). Cellulases of bacterial origin. *Enzyme and Microbial Technology* 11(10): 626-644.

Romanenko, L. A., M. Uchino, N. Tanaka, G. M. Frolova and V. V. Mikhailov (2008). *Lysobacter spongiicola* sp. nov., isolated from a deep-sea sponge. *International Journal of Systematic and Evolutionary Microbiology* 58: 370-374.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard and J. P. Huelsenbeck (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Sadhu, S. and T. K. Maiti (2013). Cellulase production by bacteria: a review. *British Microbiology Research Journal* 3(3): 235.

Salzberg, S. L., D. D. Sommer, M. C. Schatz, A. M. Phillippy, P. D. Rabinowicz, S. Tsuge, A. Furutani, H. Ochiai, A. L. Delcher and D. Kelley (2008). Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. oryzae PXO99A. *BMC genomics* 9(1): 204.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). *Molecular cloning, A Laboratory Manual*. New York, USA, Cold Spring Harbor Laboratory Press Cold Spring Harbor.

Sambrook, J. and D. W. Russell (2001). *Molecular cloning: a laboratory manual (3-volume set)*, Cold Spring Harbor Laboratory Press Cold Spring Harbor, New York.

Samira, M., R. Mohammad and G. Gholamreza (2011). Carboxymethyl-cellulase and filter-paperase activity of new strains isolated from Persian Gulf. *Microbiology Journal* 1(1): 8-16.

Schulz, S. and J. S. Dickschat (2007). Bacterial volatiles: the smell of small organisms. *Natural product reports* 24(4): 814-842.

Schwarz, W. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Applied microbiology and biotechnology* 56(5-6): 634-649.

Sengupta, S. and M. Dasgupta (2008). Industrial and clinical applications excluding Diagnostic clinical enzymology. *Enzymology* : 1-25.

Sethi, S., A. Datta, B. L. Gupta and S. Gupta (2013). Optimization of cellulase production from bacteria isolated from soil. *ISRN biotechnology* 2013.

Shankar, T. and L. Isaiarasu (2012). Statistical optimization for cellulase production by *Bacillus pumilus* EWBCM1 Using Response Surface Methodology. *Global Journal of Biotechnology & Biochemistry* 7(1): 01-06.

Sharyo, M., H. Sakaguchi, M. Ohishi, M. Takahashi, K. Kida, H. Tamagawa, M. Schulein and N. Franks (2002). Method of making sanitary paper from chemical pulp using a single component cellulase that does not contain cellulose-building domain, Google Patents.

Shendure, J., G. J. Porreca, N. B. Reppas, X. Lin, J. P. McCutcheon, A. M. Rosenbaum, M. D. Wang, K. Zhang, R. D. Mitra and G. M. Church (2005). Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* 309(5741): 1728-1732.

Sheng, P., S. Huang, Q. Wang, A. Wang and H. Zhang (2012). Isolation, screening, and optimization of the fermentation conditions of highly cellulolytic bacteria from the hindgut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Applied biochemistry and biotechnology* 167(2): 270-284.

Shilo, M. (1967). Formation and mode of action of algal toxins. *Bacteriological reviews* 31(3): 180.

Shinoda, S., S. Kanamasa and M. Arai (2012). Cloning of an endoglycanase gene from *Paenibacillus cookii* and characterization of the recombinant enzyme. *Biotechnology letters* 34(2): 281-286.

Silva, A. R., J. A. Ferro, F. Reinach, C. Farah, L. Furlan, R. Quaggio, C. Monteiro-Vitorello, M. Van Sluys, N. a. Almeida and L. Alves (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* 417(6887): 459-463.

Singh, A., R. C. Kuhad and O. P. Ward (2007). Industrial application of microbial cellulases. *Lignocellulose Biotechnologgy: Future Prospects*: 345-358.

Singh, H., J. Du, H. T. Ngo, K. Won, J.-E. Yang, K.-Y. Kim and T.-H. Yi (2015). *Lysobacter fragariae* sp. nov. and *Lysobacter rhizosphaerae* sp. nov. isolated from rhizosphere of strawberry plant. *Antonie van Leeuwenhoek* 107(6): 1437-1444.

Singh, H., K. Won, J. Du, J.-E. Yang, S. Akter, K.-Y. Kim and T.-H. Yi (2015). *Lysobacter agri* sp. nov., a bacterium isolated from soil. *Antonie van Leeuwenhoek* 108(3): 553-561.

Someya, N., S. Ikeda, T. Morohoshi, N. TM, T. Yoshida, H. Sawada, T. Ikeda and K. Tsuchiya (2011). Diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan. *Microbes Environ* 26: 7-14.

Srinivasan, S., M. K. Kim, G. Sathiyaraj, H. B. Kim, Y. J. Kim and D. C. Yang (2010). *Lysobacter soli* sp. nov., isolated from soil of a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 60: 1543-1547.

Staskawicz, B., D. Dahlbeck, N. Keen and C. Napoli (1987). Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *Journal of Bacteriology* 169: 5789-5794.

Staskawicz, B. J., D. Dahlbeck and N. T. Keen (1984). Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on Glycine max (L.) Merr. *Proceedings of the National Academy of Sciences* 81(19): 6024-6028.

Stewart, J. R. and R. M. Brown (1969). Cytophaga that kills or lyses algae. *Science* 164(3887): 1523-1524.

Sukumaran, R. K., R. R. Singhania and A. Pandey (2005). Microbial cellulases-production, applications and challenges. *Journal of Scientific and Industrial Research* 64(11): 832.

Sullivan, R. F., M. A. Holtman, G. J. Zylstra, J. F. White and D. Y. Kobayashi (2003). Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. *Journal of Applied Microbiology* 94: 1079-1086.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* 28(10): 2731-2739.

Tanabe, T., K. Morinaga, T. Fukamizo and M. Mitsutomi (2003). Novel chitosanase from *Streptomyces griseus* HUT 6037 with transglycosylation activity. *Bioscience, biotechnology, and biochemistry* 67(2): 354-364.

Tavvfik, D. S. and A. D. Griffiths (1998). Man-made cell-like compartments for. *Nature biotechnology* 16.

Ten, L. N., H. M. Jung, W. T. Im, S. A. Yoo, H. M. Oh and S. T. Lee (2009). *Lysobacter panaciterrae* sp. nov., isolated from soil of a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 59: 958-963.

Ten, L. N., H. M. Jung, W. T. Im, S. N. Yoo and S. T. Lee (2008). *Lysobacter daecheongensis* sp. nov., isolated from sediment of stream near the Daechung dam in South Korea. *Journal of Microbiology* 46: 519-524.

Teng, X. and H. Xiao (2009). Perspectives of DNA microarray and next-generation DNA sequencing technologies. *Science in China Series C: Life Sciences* 52(1): 7-16.

Tomme, P., R. Warren and N. Gilkes (1995). Cellulose hydrolysis by bacteria and fungi. *Advances in microbial physiology* 37(1): 1-81.

Thompson, D., B. Clarke and D. Kobayashi (1996). Evaluation of bacterial antagonists for reduction of summer patch symptoms in Kentucky bluegrass. *Plant Disease* 80(8): 856-862.

Tongarun, R., E. Luepromchai and A. S. Vangnai (2008). Natural attenuation, biostimulation, and bioaugmentation in 4-chloroaniline-contaminated soil. *Current microbiology* 56(2): 182-188.

Trasar-Cepeda, C., F. Gil-Sotres and M. Leirós (2007). Thermodynamic parameters of enzymes in grassland soils from Galicia, NW Spain. *Soil Biology and Biochemistry* 39(1): 311-319.

Tsai, G.-J., S.-L. Zhang and P.-L. Shieh (2004). Antimicrobial activity of a low-molecular-weight chitosan obtained from cellulase digestion of chitosan. *Journal of Food Protection®* 67(2): 396-398.

Vallenet, D., E. Belda, A. Calteau, S. Cruveiller, S. Engelen, A. Lajus, F. Le Fèvre, C. Longin, D. Mornico and D. Roche (2012). MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Research*: gks1194.

Voelkerding, K. V., S. A. Dames and J. D. Durtschi (2009). Next-generation sequencing: from basic research to diagnostics. *Clinical chemistry* 55(4): 641-658.

Von Tigerstrom, R. G. and S. Stelmaschuk (1989). Localization and characterization of lipolytic enzymes produced by *Lysobacter enzymogenes*. *Microbiology* 135(4): 1027-1035.

Wang, G.-L., L. Wang, H.-H. Chen, B. Shen, S.-P. Li and J.-D. Jiang (2011). *Lysobacter ruishenii* sp. nov., a chlorothalonil-degrading bacterium isolated from a long-term chlorothalonil-contaminated soil. *International journal of systematic and evolutionary microbiology* 61(3): 674-679.

Wang, Y., J. Dai, L. Zhang, X. Luo, Y. Li, G. Chen, Y. Tang, Y. Meng and C. Fang (2009). *Lysobacter ximonensis* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 59: 786-789.

Wei, D. Q., T. T. Yu, J. C. Yao, E. M. Zhou, Z. Q. Song, Y. R. Yin, H. Ming, S. K. Tang and W. J. Li (2012). *Lysobacter thermophilus* sp. nov., isolated from a geothermal soil sample in Tengchong, south-west China. *Antonie van Leeuwenhoek* 102: 643-651.

Wen, C., L. Xi, R. She, S. Zhao, Z. Hao, L. Luo, H. Liao, Z. Chen, G. Han and S. Cao (2016). *Lysobacter chengduensis* sp. nov. Isolated from the Air of Captive Ailuropoda melanoleuca Enclosures in Chengdu, China. *Current microbiology* 72(1): 88-93.

Weon, H.-Y., B.-Y. Kim, M.-K. Kim, S.-H. Yoo, S.-W. Kwon, S.-J. Go and E. Stackebrandt (2007). *Lysobacter niabensis* sp. nov. and Lysobacter niastensis sp. nov., isolated from greenhouse soils in Korea. *International journal of systematic and evolutionary microbiology* 57(3): 548-551.

Weon, H. Y., B. Y. Kim, Y. K. Baek, S. H. Yoo, S. W. Kwon, E. Stackebrandt and S. J. Go (2006). Two novel species, *Lysobacter daejeonensis* sp. nov. and *Lysobacter yangpyeongensis* sp. nov., isolated from Korean greenhouse soils. *International Journal of Systematic and Evolutionary Microbiology* 56: 947-951.

Wolf, A., A. Fritze, M. Hagemann and G. Berg (2002). *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *International journal of systematic and evolutionary microbiology* 52(6): 1937-1944.

Wood, T. (1971). The cellulase of Fusarium solani. Purification and specificity of the β-(1$\longrightarrow$ 4)-glucanase and the β-d-glucosidase components. *Biochemical Journal* 121(3): 353-362.

Wood, T. (1989). Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi. *Enzyme Systems for Lignocellulose Degradation*: 17-35.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. Lee (2005). Coordinated development of leading biomass pretreatment technologies. *Bioresource technology* 96(18): 1959-1966.

Xia, W., P. Liu and J. Liu (2008). Advance in chitosan hydrolysis by non-specific cellulases. *Bioresource Technology* 99(15): 6751-6762.

Ximenes, E. A., C. R. Felix and C. J. Ulhoa (1996). Production of cellulases by *Aspergillus fumigatus* and characterization of one β-glucosidase. *Current microbiology* 32(3): 119-123.

Xin, Y., J. Qu, J. Xu, P. Wu, X. Cao and S. Xue (2015). *Lysobacter hymeniacidonis* sp. nov., isolated from a crude oil-contaminated marine sponge. *Journal of Ocean University of China* 14(6): 1019-1024.

Yabuki, M., A. Uchiyama, K. Suzuki, A. Ando and T. Fujii (1988). Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *Journal of General and Applied Microbiology* 34: 255-270.

Yang, S.-Z., G.-D. Feng, H.-H. Zhu and Y.-H. Wang (2015). *Lysobacter mobilis* sp. nov., isolated from abandoned lead-zinc ore. *International journal of systematic and evolutionary microbiology* 65(3): 833-837.

Yassin, A., W.-M. Chen, H. Hupfer, C. Siering, R. Kroppenstedt, A. Arun, W.-A. Lai, F.-T. Shen, P. Rekha and C. C. Young (2007). *Lysobacter defluvii* sp. nov., isolated from municipal solid waste. *International journal of systematic and evolutionary microbiology* 57(5): 1131-1136.

Yasutake, Y., S. Kawano, K. Tajima, M. Yao, Y. Satoh, M. Munekata and I. Tanaka (2006). Structural characterization of the *Acetobacter xylinum* endo-β-1, 4-glucanase CMCax required for cellulose biosynthesis. *Proteins: Structure, Function, and Bioinformatics* 64: 1069-1077.

Ye, X.-M., C.-W. Chu, C. Shi, J.-C. Zhu, Q. He and J. He (2015). *Lysobacter caeni* sp. nov., isolated from the sludge of a pesticide manufacturing factory. *International journal of systematic and evolutionary microbiology* 65(3): 845-850.

Yi, J.-L., J. Wang, Q. Li, Z.-x. Liu, L. Zhang, A.-X. Liu and J.-F. Yu (2015). Draft genome sequence of the bacterium *Lysobacter capsici* X2-3, with a broad spectrum of antimicrobial activity against multiple plant-pathogenic microbes. *Genome announcements* 3(3): e00589-00515.

Yin, H. (2010). Detection Methods for the Genus *Lysobacter* and the species *Lysobacter enzymogenes*. *Biological Sciences*, University of Nebraska. Master of Science: 54.

Yoon, J. (2015). Polyphasic Characterization of *Lysobacter maris* sp. nov., a Bacterium Isolated from Seawater. *Current microbiology*: 1-6.

Yu, F., K. Zaleta-Rivera, X. Zhu, J. Huffman, J. C. Millet, S. D. Harris, G. Yuen, X.-C. Li and L. Du (2007). Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action. *Antimicrobial agents and chemotherapy* 51(1): 64-72.

Yuen, G., C. Jochum, L. Osborne and Y. Jin (2003). Biocontrol of Fusarium head blight in wheat by *Lysobacter enzymogenes* C3. *Phytopathology* 93: S93.

Yuen, G., S. Li, S. Harris, F. Yu and L. Du (2005). An antibiotic produced by the biocontrol agent *Lysobacter enzymogenes* C 3 inhibits fungal growth by blocking ceramide synthesis. *Phytopathology* 95(6).

Yuen, G., J. Steadman, D. Lindgren, D. Schaff and C. Jochum (2001). Bean rust biological control using bacterial agents. *Crop Protection* 20(5): 395-402.

Zerbino, D. R. and E. Birney (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome research* 18(5): 821-829.

Zhang, H., Y. Du, X. Yu, M. Mitsutomi and S.-i. Aiba (1999). Preparation of chitooligosaccharides from chitosan by a complex enzyme. *Carbohydrate research* 320(3): 257-260.

Zhang, L., J. Bai, Y. Wang, G. L. Wu, J. Dai and C. X. Fang (2011). *Lysobacter korlensis* sp. nov. and *Lysobacter bugurensis* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 61: 2259-2265.

Zhang, Y.-H. P., M. E. Himmel and J. R. Mielenz (2006). Outlook for cellulase improvement: screening and selection strategies. *Biotechnology advances* 24(5): 452-481.

Zhang, Y. H. P. and L. R. Lynd (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnology and bioengineering* 88(7): 797-824.

Zhang, Z. and G. Y. Yuen (2000). The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of Bipolaris sorokiniana. *Phytopathology* 90(4): 384-389.

Zhou, C. and Q. Wu (2012). *Recent development in applications of cellulose nanocrystals for advanced polymer-based nanocomposites by novel fabrication strategies*, INTECH Open Access Publisher.

Zhou, J., Y.-H. Wang, J. Chu, Y.-P. Zhuang, S.-L. Zhang and P. Yin (2008). Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14. *Bioresource technology* 99(15): 6826-6833.

APPENDIX

APPENDIX A

Culture medium

M9 minimal salt medium (Sambrook et al., 1989)

Per one liter of M9 mineral medium was prepared by mixing solutions given below:

1. 5X M9 salt	use	200 mL

5X M9 salt was made by dissolving the following salts in deionized water:

$Na_2HPO_4 \cdot 7H_2O$	12.8 g
KH_2HPO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g

2. 20% (w/v) glucose	use	20 mL
3. 10% (w/v) of colloidal	use	100 mL
4. Deionized H_2O	use	680 mL

The compositions were separately sterilized by autoclaving for 15 min at 15 lb/sq.in.

Baker's yeast cell wall preparation (Palumbo et al., 2003)

Yeast cell walls were prepared by autoclaving 50 g of fresh baker's yeast (*Saccharomyces cerevisiae*) dissolved in 500 mL distilled H_2O. The solution was removed from cell debris by centrifuging it at 5,000 ×g for 10 min. Pelleted cell solids were then washed three times with distilled H_2O, and sterilized by autoclaving. Aliquot of the sterilized yeast cell wall were taken in triplicate, dried in a hot oven, and the cell dry weight were calculated before using.

Potato Dextrose Agar (PDA)

Fresh potato	200 g
D-glucose	20.0 g
Agar	20 g
Distilled H_2O to	1.0 L

Yeast cell wall agar (YA)

Autoclaved yeast cell wall	5.0 g
Agar	15.0 g
Distilled H_2O	1.0 L

Luria-Bertani (LB) broth

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Distilled H_2O	1.0 L

* LB agar was prepared by adding 15.0 g of agar

Skim milk acetate agar (Reichenbach 2006)

Skim milk powder	5.0 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Agar	15.0 g
Distilled H_2O	1.0 L

Cook's Cytophaga Agar (CCA) (Christensen and Cook 1972)

Tryptone	2.0 g
Agar	10.0 g
Distilled H_2O	1.0 L

Casein Yeast extract Agar (CYA) (Christensen and Cook 1972)

Casitone	3.0 g
Yeast extract	1.0 g
Agar	15.0 g
Distilled H_2O	1.0 L

5X M813 solution (Palumbo et al., 2003)

K_2HPO_4	15.0 g
NaH_2PO_4	6.0 g
NH_4Cl	5.0 g
$MgSO_4 \cdot 7H_2O$	1.5 g
KCl	0.75 g

$CaCl_2$	50.0 mg
$FeSO_4$	12.5 mg
Deionized H_2O	1.0 L

CMCase production medium (Kobayashi and El-Barrad, 1996)

5X M813 solution	200.0 mL
CMC	10.0 g

Dissolved in distilled water to final volume 1 liter.

* CMCase plate agar was prepared by adding 15.0 g of agar

Chitosanase production medium

5X M813 solution	200.0 mL
Colloidal chitosan	10.0 g

Dissolved in distilled water to final volume 1 liter.

* Chitosanase plate agar was prepared by adding 15.0 g of agar

Chitinase production medium (Kobayashi and El-Barrad, 1996)

5X M813 solution	200.0 mL
Colloidal chitin	10.0 g

Dissolved in distilled water to final volume 1 liter.

* Chitinase plate agar was prepared by adding 15.0 g of agar

Protease production medium (Kobayashi and El-Barrad, 1996)

5X M813 solution	200.0 mL
Casein	5.0 g

Dissolved in distilled water to final volume 1 liter.

Protease plate agar

5X M813 solution	200.0 mL
Skim milk	10.0 g
Agar	15.0 g

Dissolved in distilled water to final volume 1 liter.

Lipase production medium

5X M813 solution	200.0 mL
Olive oil	10.0 mL
Na_2HPO_4	10.0 g

Dissolved in distilled water to final volume 1 liter.

* Lipase plate agar was prepared by adding 15.0 g of agar and 0.1 g of phenol red

APPENDIX B

Enzymatic assay

1. CMCase, chitosanase and chitinase activity assay

1.1 Material, buffer and reagent preparation

(1) Colloidal chitin preparation (Hsu and Lockwood 1975)

1.1) Add 400 mL of 16 M HCl into 1000 mL flask and put on ice on top of the stirrer.

1.2) Add 20 grams of ground shrimp shell chitin into the cold HCl solution, mix until the solution become clear for 1 to 2 h.

1.3) Mix the solution on shaking water bath at 37 °C for 10 to 15 min.

1.4) Filter the solution mixture through cotton fiber into 4 liter of cold distilled H_2O, chitin will be re-crystallized.

1.5) Centrifuge the solution mixture at 5000 ×g for 5 min, discard aqueous away.

1.6) Wash the chitin precipitate with distilled H_2O, repeatedly centrifuge and discard aqueous until reach to pH ~7.0.

1.7) Add 200 mL of sterilized distilled H_2O to the chitin precipitate and store it at 4 °C until use.

1.8) Take aliquot of colloidal chitin solution in triplicate, dry in hot oven, and the dry weight of chitin is calculated before using.

(2) Colloidal chitosan preparation (Yabuki et al., 1988)

2.1) Dissolve 15 g of 75%-85% deacetylated chitosan in 1 L of 0.2 N HCl.

2.2) Neutralize the solution to alkaline, pH 9.0, with a 0.1 N NaOH solution on stirrer.

2.3) Wash the resulting precipitates with water by centrifugation for 20 min at 1,000 x g until the pH of the supernatant became neutral.

2.4) Resuspend the washed precipitates in distilled H_2O and adjust the pH of the suspension to 6.2.

2.5) Take aliquot colloidal chitosan solution in triplicate, dry in hot oven, and the dry weight of chitosan is calculated before using.

(3) 50 mM sodium acetate buffer, pH 5.0

Sodium acetate ($CH_3COONa \cdot 3H_2O$)	6.8 g
Distilled H_2O to	1.0 L
*pH was adjusted by glacial acetic acid	

(4) DNS reagent (Miller et al., 1960)

3,5-Dinitrosalicylic acid	7.49 g
NaOH	13.98 g
Rachelle salt (Na-K tartrate)	216.10 g
Na-metabisulfite	5.86 g
Phenol	5.37 mL
Distilled H_2O to	1.00 L

1.2 Standard curve

(1) Glucose standard curve

Six different concentrations (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL) of D-glucose were prepared in distilled water. The amount of released glucose in an enzyme reaction was calculated by using the following standard glucose curve as a reference.

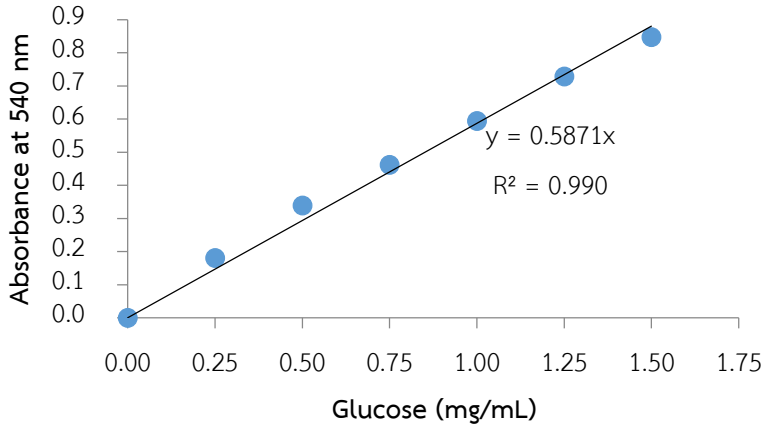


Figure 1B Glucose standard curve for CMCase activity assay

(2) Glucosamine standard curve

Six different concentrations (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL) of D-glucosamine were prepared in distilled water. The amount of released glucosamine in an enzyme reaction was calculated by using the following standard glucosamine curve as a reference.

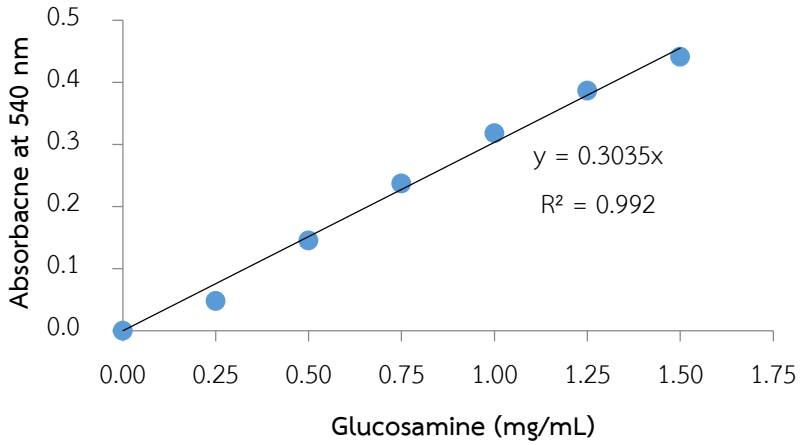


Figure 2B Glucosamine standard curve for chitosanase activity assay

(3) N-acetylglucosamine standard curve

Six different concentrations (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL) of N-acetyl-D-glucosamine were prepared in distilled water. The amount of released N-acetylglucosamine in an enzyme reaction was calculated by using the following standard N-acetylglucosamine curve as a reference.

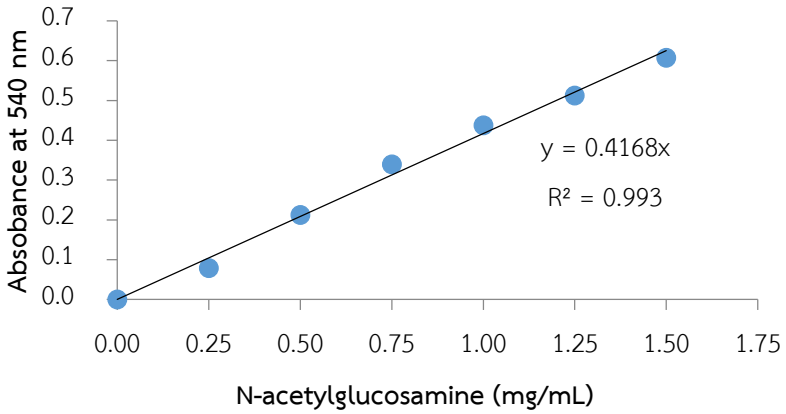


Figure 3B N-acetylglucosamine standard curve for chitinase activity assay

1.3 Enzyme activity calculation

$$\text{Enzyme activity (Unit/mL)} = \frac{\text{Product concentration (mg/mL)} \times 1000 \times \text{enzyme dilution factor}}{\text{MW of product (g/mol)} \times \text{incubation time (minute)} \times \text{enzyme volume (mL)}}$$

Note

1 Unit of CMCase = Amount of enzyme which releases 1 µmole of glucose per minute at 40 °C, at pH 5.0

1 Unit of chitosanase = Amount of enzyme which releases 1 µmole of glucosamine per minute at 40 °C, at pH 5.0

1 Unit of chitinase = Amount of enzyme which releases 1 µmole of N-acetylglucosamine per minute at 40 °C, at pH 5.0

Specific activity = Unit per mg protein (U/mg)

2. Protease activity assay (Cupp-Enyard 2008)

2.1 Material, buffer and reagent preparation

(1) 50 mM potassium phosphate buffer, pH 7.5

$K_2HPO_4 \cdot 3H_2O$	1.14 g
Distilled H_2O	100 mL

*pH was adjusted with 1 M HCl. This solution is placed at 37 °C prior to use.

(2) 0.65% (w/v) casein solution

Casein	0.65 g
50 mM potassium phosphate buffer	100 mL

Gentle stir the solution at 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. The pH was then adjusted if necessary with NaOH and HCl.

(3) 110 mM Trichloroacetic acid solution

Trichloroacetic acid	1.8 g
Distilled H_2O	100 mL

(4) 0.5 mM Folin - ciolcaltea phenol reagent

2 N Folin - ciolcaltea phenol	25 mL
Distilled H_2O	75 mL

(5) 500 mM Sodium Carbonate solution

Na_2CO_3 anyhydrous	5.3 g
Distilled H_2O	100 mL

(6) L-tyrosine standard stock solution (1 mg/ml)

L-tyrosine	20 mg
Distilled H_2O	20 mL

*As with the casein, do not boil this solution. Allow the L-tyrosine standard to cool to room temperature. This solution will be diluted further to make our standard curve.

2.2 Standard tyrosine curve

Six different concentrations (10, 20, 40, 60, 80 and 100 µg/mL) of L-tyrosine were prepared in distilled water. The amount of released tyrosine in an enzyme reaction was calculated by using the following standard tyrosine curve as a reference.

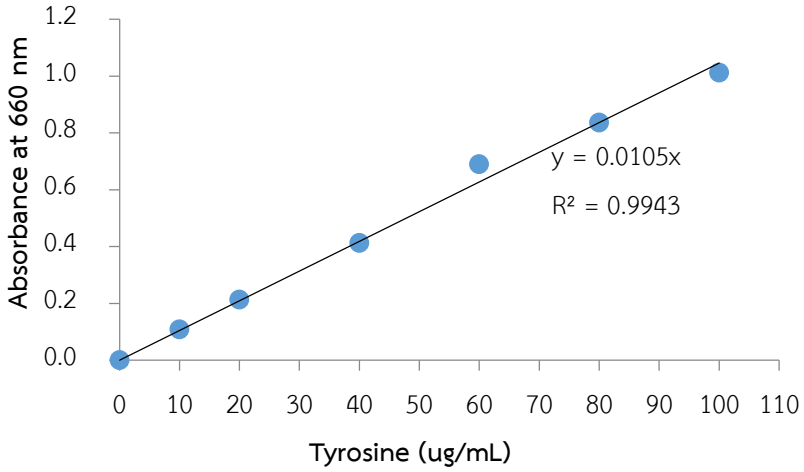


Figure 4B Tyrosine standard curve for pretease activity assay

2.3 Enzyme activity calculation

$$\text{Protease activity (Unit/ml)} = \frac{\text{Tyrosine concentration (ug/mL)} \times \text{enzyme dilution factor}}{\text{MW of tyrosine (g/mol)} \times \text{incubation time (minute)} \times \text{enzyme volume (mL)}}$$

Note

1 Unit of protease = Amount of enzyme which releases 1 µmole of tyrosine per minute at 40 ℃, at pH 7.5

Specific activity = Unit per mg protein (U/mg)

3. Lipase activity assay (Glogauer et al., 2011)

3.1 Material, buffer and reagent preparation

(1) 5X 50 mM Tris-HCl buffer, pH 7.5

$C_4H_{11}NO_3$ (Tris-base)	3.03 g
$CaCl_2$	55.50 mg
Distilled H_2O to	100 mL

*pH was adjusted with 1 M HCl.

(2) *p*-nitrophenyl-palmitate (*p*NPP) solution

5x 50 mM Tris-HCl buffer	20 mL
Triton – x (0.3% w/v)	300 µL
Isopropanol (4% w/v)	4.0 mL
Acetonitrile (1% w/v)	1.0 mL
*p*NPP	37.8 mg
Distilled H_2O to	74.7 mL

*The substrate solution was mixed under agitation in a water bath at 60 ℃, until the solution become transparent.

3.2 *p*-nitrophenol standard curve

Six different concentrations (10, 100, 200, 300, 400 and 500 µg/mL) of *p*-nitrophenol were prepared in distilled water. The amount of released *p*-nitrophenol in an enzyme reaction was calculated by using the following standard *p*-nitrophenol curve as a reference.

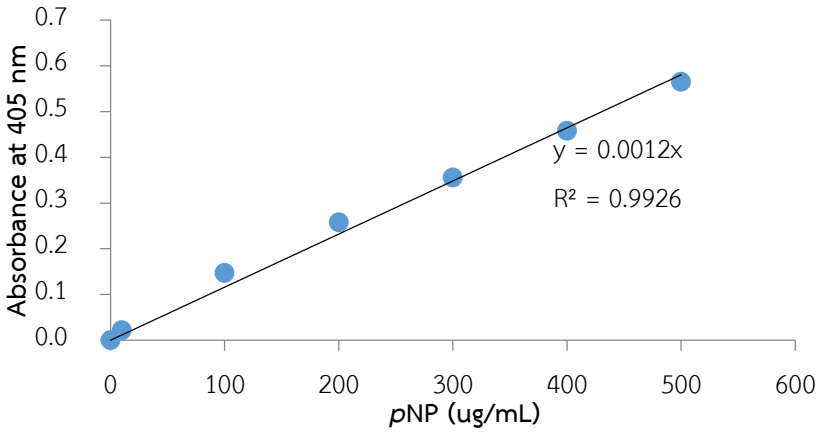


Figure 5B *p*-nitrophenol standard curve for lipase activity assay

3.3 Enzyme activity calculation

$$\text{Lipase activity (Unit/mL)} = \frac{\text{p-nitrophenol concentration (ug/mL)} \times \text{enzyme dilution factor}}{\text{MW of p-nitrophenol (g/mol)} \times \text{incubation time (minute)} \times \text{enzyme volume (mL)}}$$

Note

1 Unit of lipase = Amount of enzyme which releases 1 µmole of *p*-nitrophenol per minute at 40 °C, at pH 7.5

Specific activity = Unit per mg protein (U/mg)

APPENDIX C

Protein determination and enzyme purification

1. Lowry assay reagent (Lowry et al., 1951)

1.1) Solution A

$CuSO_4$	0.5 g
$Na_3C_6H_5O_7 \cdot 2H_2O$	1.0 g
Distilled H_2O to	100 mL

Solution may be stored indefinitely at room temperature.

1.2) Solution B

Na_2CO_3	20.0 g
NaOH	4.0 g
Distilled H_2O to	1.0 L

Solution may be stored indefinitely at room temperature.

1.3) Solution C

Solution A	10 mL
Solution B	500 mL

1.4) Solution D

Folin-Ciocalteu phenol reagent	10 mL
Distilled H_2O	10 mL

2. Assay

(1) Add 0.5 mL sample solution to test tube.

(2) Add 2.5 mL Solution C, vortex and let stand at room temperature for 10 min.

(3) Add 0.25 mL Solution D and vortex

(4) Incubate for 20 – 30 min, measure the absorbance at 750 nm

3. Bovine serum albumin (BSA) standard curve

Six different concentrations (0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/mL) of BSA were prepared in distilled water. The amount of released amino acid was calculated by using the following standard BSA curve as a reference.

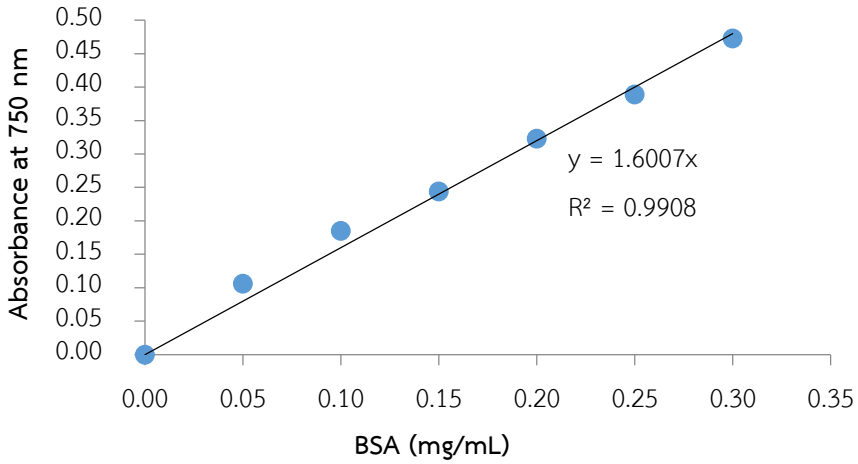


Figure 1C BSA standard curve for protein concentration determination

4. Protein concentration calculation

$$\text{Protein concentration (mg/mL)} = \frac{\text{BSA concentration (mg/mL)} \times \text{dilution factor}}{\text{sample volume (mL)}}$$

5. Ammonium sulfate precipitation table (Dawson et al., 1969)

Table 1C Solid ammonium sulfate to be used for ammonium sulfate precipitation

Initial concentration of ammonium sulfate	Final concentration of ammonium sulfate -% saturation at 0 °C								
	20	30	40	50	60	70	80	90	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution								
0	106	164	226	291	361	436	516	603	697
10	53	109	169	233	301	374	452	536	627
20	0	55	113	175	241	312	387	469	557
30		0	56	117	181	249	323	402	488
40			0	58	120	187	258	335	418
50				0	60	125	194	268	348
60					0	62	129	201	279
70						0	65	134	209
80							0	67	139
90								0	70
100									0

6. Buffer systems for enzyme purification

6.1 HiTrap Phenyl HP column

(1) Start buffer: 50 mM sodium phosphate, 1.5 M ammonium sulphate, pH 7.0

Na_2HPO_4	7.1 g
$(NH_4)_2SO_4$	198.2 g
Distilled H_2O to	1.0 L

*pH was adjusted with 1 N HCl.

(2) Elution buffer: 50 mM sodium phosphate, pH 7.0

Na_2HPO_4	7.1 g
Distilled H_2O to	1.0 L

*pH was adjusted with 1 N HCl.

6.2 HiTrap SP HP column

(1) Start buffer: 50 mM sodium acetate, pH 4.0

Sodium acetate ($CH_3COONa \cdot 3H_2O$)	6.8 g
Distilled H_2O to	1.0 L

*pH was adjusted by glacial acetic acid

(2) Elution buffer: 50 mM sodium acetate, pH 4.0, 1 M NaCl

Sodium acetate ($CH_3COONa \cdot 3H_2O$)	6.8 g
NaCl	58.4 g
Distilled H_2O to	1.0 L

*pH was adjusted by glacial acetic acid

APPENDIX D

DNA isolation and molecular biology technique

1. Reagent and buffer

TE Buffer (pH 8.0)

Tris-base (10 mM)	1.21 g
EDTA (1 mM)	0.29 g
Distilled H_2O to	1.0 L

*pH was adjusted by 1 N HCl

10% Sodium dodesyl sulphate (SDS)

SDS	10.0 g
Distilled H_2O to	100 mL

3 M Sodium acetate buffer, pH 7.0

Sodium acetate ($CH_3COONa \cdot 3H_2O$)	6.8 g
Distilled H_2O to	1.0 L

*pH was adjusted by glacial acetic acid

2. Genomic DNA isolation (Jones and Bartlet 1990)

1) Transfer 1.5 mL media to a microcentrifuge tube and spin at 10000 ×g, for 2 min. Decant the supernatant and drain well onto a sterilized tissue paper.

2) Resuspend the pellet in 567 µL TE buffer by repeated pipetting

3) Add 100 µL of 10% SDS and 3 µL of proteinase K, mix, and incubate 1 h at 37 °C

4) Add an equal volume of phenol/chloroform and mix well by inverting the tube until the phase are completely mixed.

5) Spin for 2 min and transfer the upper aqueous phase to a new tube

6) Add an equal volume of phenol/chloroform and mix well and spin for 2 min

7) Transfer the upper aqueous phase to a new tube.

8) Add 1/10 volume of 3 M sodium acetate.

9) Add 0.6 volume of isopropanol and mix gently until the DNA precipitates.

10) Centrifuge 10 min to pellet DNA.

11) Wash with 70% EtOH, dry, and resuspend DNA in 100 µL TE buffer.

12) After DNA has dissolved, measure the concentration by diluting 10 a µL of DNA into 1 mL of TE (1: 100 dilution) and measure absorbance at 260 nm.

13) Concentration of original DNA solution in µg/mL = Abs × 100 × 50 µg/mL.

2. Polymerase chain reaction (PCR) condition

5x PCR buffer	10 µL
50 mM $MgCl_2$	1.5 µL
10 mM dNTPs	1.0 µL
Forward primer (10 mM)	2.0 µL
Reward primer (10 mM)	2.0 µL
Taq DNA polymerase	1.0 µL
5% (v/v) DMSO	2.5 µL
DNA template (~20 ng)	4.0 µL
dI H_2O (to 50 µl)	26 µL

PCR cycle

Initial denaturation	94 °C	3 min
Denaturation	94 °C	1 min
Annealing	55 °C	1 min
Extension	72 °C	2 min
Final extension	72 °C	15 min

*Run for 30 to 35 cycles

3. Chromosomal DNA isolation (Staskawicz et al. 1987)

1) Inoculate 100 mL LB with cells and grow at 30 °C to turbidity (18-24 h)

2) Spin cells at 10,000 ×g for 5 min

3) Resuspend cells in 100 mL sterile distilled H_2O (a wash step can be added, but has not been necessary)

4) Add 8 ml 20% (w/v) SDS and incubate at 37 °C, 10-15 min. (cell suspension should become viscous and clear—if not, continue to incubate)

5) Add 12 mL 3 M sodium acetate buffer, mixing thoroughly

6) Add 64 mL isopropanol, mixing thoroughly

7) Spool out precipitated DNA with a Pasteur pipet, avoiding any clear mucoidal material

8) Resuspend DNA in 15 mL TE buffer or sterile distilled H_2O at 65 °C, for 2 h. (Vigorously shake samples periodically, every 15 min, to resuspend DNA)

9) Spin DNA suspension at 20,000 ×g for 10 min to pellet any undissolved DNA

10) Weight 8.63 g Cesium chloride (CsCl) into glass tube, add 7 mL of DNA solution into the tube, mix thoroughly until CsCl become dissolved

11) Load the DNA solution into uncapped heat-seal tube and add 400 µL Ethedium bromide (EtBr), fulfill the tube with distilled H_2O, and seal the tube with heater apparatus.

12) Put the tube into a vacuum ultracentrifuge and spin overnight at 240,000 ×g, 20 °C

13) Transfer DNA with a syringe into new tube, and fulfill the tube with saturated-CsCl TE buffer and seal with heater

14) Transfer DNA a syringe into a clean tube, extract EtBr with isopropanol for several times. The DNA is dissolved in aqueous phase (lower phase) while the EtBr is dissolved in organic phase (upper phase).

15) Transfer DNA (lower fraction) into dialysis bag and remove CsCl by soaking DNA bag into TE buffer for several times

16) The dialyzed DNA is kept at 20 °C until use

APPENDIX E

SDS-PAGE and zymogram analysis

1. Reagent and buffer

(1.1) Resolving gel Buffer (1.5 M Tris-HCl, pH 8.8), 100 mL

Dissolved 18.2 g of Tris-base in 70 mL dI H_2O, adjust pH to be 8.8 with 1N HCl and add dI H_2O to 100 mL.

(1.2) Stacking gel Buffer (0.5 M Tris-HCl, pH 6.8), 100 mL

Dissolved 6.1 g of Tris-base in 70 mL dI H_2O, adjust pH to be 6.8 with 1N HCl and add dI H_2O to 100 mL.

(1.3) Sample Buffer, 10 mL

Stacking gel buffer	2.5 mL
Glycerol	1 mL
10% SDS	2 mL
0.5% Bromphenol blue	0.5 mL
dI H_2O	3.5 mL

Before using, sample buffer was mixed freshly with mercaptoethanol at the ratio 190:10 (example; 950 µL sample buffer: 50 µL mercaptoethanol).

(1.4) Electrode Buffer (Tris-glycine buffer, pH 8.3), 1000 mL

Tris-base	15 g
Glycine	72 g
SDS	5 g
dI H_2O	1 L

(1.5) Staining reagent, 500 mL

Methanol	200 mL
Acetic acid	50 mL
Coomassie brilliant blue	0.5 g
dI H_2O	250 mL

(1.6) Destaining reagent, 500 mL

Methanol	200 mL
Acetic acid	50 mL
dI H_2O	250 mL

(1.7) 10% Ammonium persulfate, 1 mL

Dissolved 100 mg Ammonium persulfate in 1 mL dI H_2O

(1.8) 0.1% (w/v) Congo Red, 100 mL

Dissolved 1 g Congo Red in 100 mL dI H_2O

(1.9) 1M NaCl, 1 L

Dissolved 58.44 g sodium chloride in 1000 mL dI H_2O

2. Preparation of SDS-PAGE and zymogram (native-PAGE) gel

The SDS-PAGE for Cel8A purity and native-PAGE gels zymogram analysis of CMCase and chitosanase activity were made up by mixing the following ingredients listed in Table 1E.

Table 1E Components of gel used for SDS-PAGE and zymogram analysis in this study

Chemical component	Volume required (µL)	
	SDS-PAGE	Native-PAGE
Resolving gel (10%)		
Resolving buffer	1,250	1,250
10% (w/v) SDS	50	-
40% (w/v) Polyacrylamide-bis acrylamide	1,250	1,250
10% (w/v) Ammonium persulfate	25	25
TEMED	2.5	2.5
1% (w/v) CMC / colloidal chitosan	-	500
dI H_2O	2,420	1,970
Total volume	5,000	5,000

Table 1E Components of gel used for SDS-PAGE and zymogram analysis in this study (continue)

Chemical component	Volume required (µL)	
	SDS-PAGE	Native-PAGE
Stacking gel (5%)		
Stacking buffer	500	500
10% (w/v) SDS	20	-
40% (w/v) Polyacrylamide-bis acrylamide	250	250
10% (w/v) Ammonium persulfate	10	10
TEMED	1	1
dI H_2O	1,220	1,240
Total volume	2,000	2,000

3. Protein marker (BLUeye Prestained Protein Ladder) standard curve

Table 3E Weight of molecular markers used in SDS-PAGE plus distance migrated down the gel.

Distance migrated (cm)	Weight of molecular marker (kDa)	Log MW
0.3	245	2.39
0.46	180	2.26
0.6	135	2.13
0.83	100	2.00
1.05	75	1.88
1.43	63	1.80
2.04	48	1.68
2.82	35	1.54
3.9	25	1.40
4.4	20	1.30
5.3	17	1.23
6.16	11	1.04

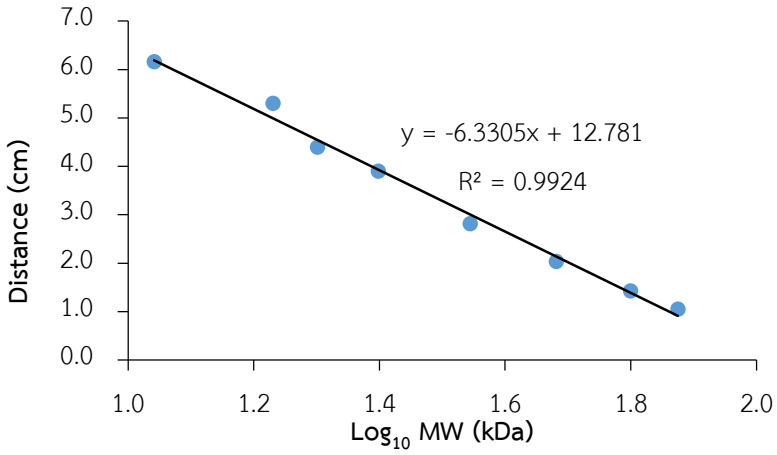


Figure 1E Standard curve of the distance travelled by each molecular marker protein down the SDS-PAGE gel against log MW to calculate the molecular weight of the protein sample.

APPENDIX F

Supporting data

Table 1F Soil samples collected throughout Thailand

Sample No.	Province	Collection date	Area	Source
1	Bangkok	Sep, 2010	Shady lowland	Soil nearby corn tree
2	Bangkok	Sep, 2010	Shady lowland	Rhizosphere soil from corn field
3	Bangkok	Sep, 2010	Shady lowland	Rhizosphere soil from corn field
4	Sisaket	Oct, 2010	Plain land	Rhizosphere soil from rice field
5	Sisaket	Oct, 2010	Plain land	Rhizosphere soil from corn field
6	Sisaket	Oct, 2010	Plain land	Rhizosphere soil from corn field
7	Sisaket	Oct, 2010	Plain land	Rhizosphere soil from casava field
8	Trang	Oct, 2010	Coastal land	Soil from Peninsular Botanical Garden (Thung Khai)
9	Nakornratchasima	Feb, 2011	Open highland	Soil nearby tree
10	Bangkok	Mar, 2011	Shady lowland	Soil nearby corn tree
11	Samut songkhram	Mar, 2011	Muddy shore	Soil nearby banyan tree
12	Samut songkhram	Mar, 2011	Muddy shore	Soil nearby brackish water
13	Kanchanaburi	May, 2011	Hill	Soil nearby cork tree
14	Kanchanaburi	May, 2011	Hill	Soil nearby fungal mycelia
15	Kanchanaburi	May, 2011	Hill	Rhizosphere soil of bamboo
16	Kanchanaburi	May, 2011	Hill	Soil nearby clump of bamboo
17	Kanchanaburi	May, 2011	Hill	Soil nearby clump of bamboo
18	Saraburi	Nov, 2011	Shady lowland	Soil nearby stream
19	Saraburi	Nov, 2011	Shady lowland	Cassava field soil
20	Saraburi	Nov, 2011	Shady lowland	Sugarcane field soil
21	Chiang Mai	July, 2012	Open highland	mud
22	Chiang Mai	July, 2012	Open highland	Soil nearby tree
23	Sisaket	Aug, 2012	Plain land	Rhizosphere soil from casava field
24	Sisaket	Aug, 2012	Plain land	Rhizosphere soil from chilli field

Table 1F Soil samples collected throughout Thailand (continue)

Sample No.	Province	Collection date	Area	Source
25	Sisaket	Aug, 2012	Plain land	Rhizosphere soil from long bean field
26	Chiang Mai	Nov, 2012	Open highland	Garden soil
27	Sisaket	Feb, 2013	Plain land	Garden soil
28	Sisaket	Feb, 2013	Plain land	Garden soil
29	Nakornratchasima	Mar, 2013	Open highland	Soil in Khao Yai National Park
30	Nakornratchasima	Mar, 2013	Open highland	Soil in Khao Yai National Park
31	Nakornratchasima	Mar, 2013	Open highland	Soil in Khao Yai National Park
32	Kanchanaburi	Mar, 2013	Hill	Soil nearby water fall
33	Kanchanaburi	Mar, 2013	Hill	Soil in forest
34	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
35	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
36	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
37	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
38	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
39	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil
40	Bangkok	Apr, 2013	Shady lowland	Garden soil
41	Bangkok	Apr, 2013	Shady lowland	Garden soil
42	Bangkok	Apr, 2013	Shady lowland	Grasses cover soil under mushroom mycelia
43	Bangkok	Apr, 2013	Shady lowland	Garden soil
44	Nakornratchasima	May, 2013	Open highland	Grasses cover soil nearby pond
45	Nakornratchasima	May, 2013	Open highland	Grasses cover soil under coconut trees
46	Nakornratchasima	May, 2013	Open highland	Garden soil
47	Nakornratchasima	May, 2013	Open highland	Soil nearby cassava plant
48	Nakornratchasima	May, 2013	Open highland	Grasses cover soil nearby cassava plant
49	Khonkean	May, 2013	Open highland	Vegetable garden soil
50	Khonkean	May, 2013	Open highland	Vegetable garden soil
51	Udontani	May, 2013	Open highland	Grasses cover soil under coconut trees

Table 1F Soil samples collected throughout Thailand (continue)

Sample No.	Province	Collection date	Area	Source
52	Nongkhai	May, 2013	Open highland	Grasses cover soil near Makhong river
53	Khonkean	May, 2013	Open highland	Soil nearby damn
54	Khonkean	May, 2013	Open highland	Garden soil
55	Nakornratchasima	May, 2013	Open highland	Garden soil near corn field
56	Songkla	Jun, 2013	Shady lowland	Rice field soil
57	Songkla	Jun, 2013	Shady lowland	Grasses cover soil
58	Songkla	Jun, 2013	Shady lowland	Grasses cover soil
59	Songkla	Jun, 2013	Shady lowland	Grasses cover soil
60	Krabi	Jun, 2013	Coastal land	Soil nearby freshwater pond
61	Krabi	Jun, 2013	Coastal land	Grasses cover soil nearby freshwater pond
62	Krabi	Jun, 2013	Coastal land	Soil under Cannonball Tree
63	Songkla	Jun, 2013	Shady lowland	Grasses cover soil in zoo
64	Songkla	Jun, 2013	Shady lowland	Grasses cover soil
65	Songkla	Jun, 2013	Shady lowland	Grasses cover soil

Table 2F Nucleotide sequence of 16s rRNA gene of endoglucanase-producing *Lysobacter*-like bacteria

Identical strain	Nucleotide sequence (5' to 3')
Xanthomonas translucens 346	GCAAGTTCGAACGGCAGCACAGTAAGAAGCTTGCTCTTACGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGG GGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTC GGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTC CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTG TAAAGCCCTTTTGTTGGGAAAGAAAAGCAGCCGGTTAATACCCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCA GCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGG CTCAACCTGGGAATTGCAGTGGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGG AGGAACATCTGTGGCGAAGGCGGCCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCG CAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTG ACATCCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTCAGGACAGAGGGCTGCAAGCTCGCGAGAGTAA GCCAATCCCAGAAACCTGATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGC GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTT GCCACGGTGTGGCCGATGACTGGG
Xanthomonas albilineans 385	CAAGTTCGAACGGCAGCACAGGAAGAAGCTTGCTCCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGG GATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCG GATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGT AAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAG CCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCT CAACCTGGGAATTGCAGTGGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAG GAACATCTGTGGCGAAGGCGGCCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA CGCCCTAAACGATGCGAACTGGATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTACCGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCCGC AAGACTGAAACTCAAAGGAATTGACGGGGCCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCACCGCGAAGAACCTTACCTGGTCTTGA CATCCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAG GTGGGGAGGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTAAGGACAGAGGGTTGCAAACTCGGGAGAGTAA GCCAATCCCAGAACCCTTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGC GGTGAATACGTTCCCGGCCCTTGTCCACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTACCCTTCGGGAGGGCGCTT GCCACGGTTGGCCGATGACTGGGGTGAAGTCGGA
Xanthomonas sacchari 391	CAAGTTCGAACGGCAGCACAGGAAGAACTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGA TAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGA TTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAA AGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCC GCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCA ACCTGGGAATTGCAGTGGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGA ACATCTGTGGCGAAGGCGGCCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG CCCTAAACGATGCGAACTGGATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAG ACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACAT CCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA AGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGG GGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTGGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCA ATCCCAGAAACCCCATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTG AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCA CGGTGTGGCCGATGA

Table 2F Nucleotide sequence of 16s rRNA gene of endoglucanase-producing *Lysobacter*-like bacteria (continue)

Identical strain	Nucleotide sequence (5' to 3')
Xanthomonas translucens 425	GCAAGTCCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGAT AACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTA GCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCC TTTTGTTGGGAAAGAAAAGCAGCCGGTTAATACCCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGG AATTGCAGTGGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCTGT GGCGAAGGCGGCCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCTTAAACG ATGCGAACTGGATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAACTTT CCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA GCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT CATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTCAGGACAGAGGGCTGCAAGCTCGCGAGAGTAAGCCAATCCCAGAAACCTGAT CTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGCCCT TGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCACGGTGGGCCGATG
Xanthomonas sacchari 455	TACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA GGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG GACACTGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTA ATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAAT TACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGGCAACTAGAGT ATGGTACAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCTGTGGCGAACGCGGCCATCTGGACCGAGACTG ACACTGAGGCACAAATGCGTTGGCCTGCCGGTACCCCAAAGAATAAGCCCCCGGCTAACTTCGTGCCAGCAGCCCGCGGTAATACGAAGGGTGCA AGCGTTACTCGGAATTTACTGGGCGTCAGATCCAGTCTAGGATGTCGTATAAGTCCGATCAGAAAGCCTTGCGCTCAACCTGGGAATTGCAGTGGA TACTGGGCAACTAGAGTATGTAAGAGAATGACAGAATTCCCCGATGTAGCAATGAAAGGCGTAGAGATCGGGAGGATCATCTGTGGCGAAGGCGG CCATCTGGACCAACGCTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGG ATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC GGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAACTTTCCAGAGATGGA TTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT GTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC TTACGACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGCCAATCCCAGAAACCCTATCTCAGTCCGG ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAG
Xanthomonas sacchari 513	GGCAAGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGAT AACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTA GCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCC TTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGG AATTGCAGTGGATACTGGGCAACTAGAGTGTGGTACAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCTGT GGCGAAGGCGGCCATCTGGACCGAGACTGACACTGAGGCACAAATGCGTTGGCCTGCCGGTACCCCAAAGAATAAGCCCCCGGCTAACTTCGTGC CAGCAGCCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTTACTGGGCGTCAGATCCAGTCTAGGATGTCGTATAAGTCCGATCAGAAAGC CTTGCGCTCAACCTGGGAATTGCAGTGGATACTGGGCAACTAGAGTATGTAAGAGAATGACAGAATTCCCCGATGTAGCAATGAAAGGCGTAGAGA TCGGGAGGATCATCTGTGGCGAAGGCGGCCATCTGGACCAACGCTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT AGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGT CGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTT GACATCCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGG TGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGC CAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGT GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCA CGGTGGGCCGATGA

Table 2F Nucleotide sequence of 16s rRNA gene of endoglucanase-producing *Lysobacter*-like bacteria (continue)

Identical strain	Nucleotide sequence (5' to 3')
Lysobacter enzymogenes 521	CGAACGGCAGCACAGAGGAAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACGTCGGAATCTGCCTATTTGTGGGGGATAACGTAG GGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGTGGGGGACCGCAAGGCCTCACGCAGATAGATGAGCCGACGTCGGATTAGCTAGTTG GCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG GCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGA AAGAAAAGCTTAGGGTTAATAACCCTGAGTCATGACGGTACCGGAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTG CAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTTGTTAAGTCTGATGTGAAAGCCCTGGGCTCAACCTGGGAATGGCATTGGAA ACTGGCTTACTAGAGTGCGGTAGAGGGTAGCGGAATTCCCGGGGTAGCAGTGAAATGCGTAGATATCGGGAGGAACATCCGTGGCGAAGGCGGCTAC CTGGACCAGCACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTG GGGGCAACTTGGCCCTCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGAAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC CGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGCAGAACCTTACCTGGCCTTGACATGTCGAGAACTTGGCAGAGATGCCTTGGTGCC TTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGT TGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAG GGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAACCCGCGAGGGCAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGC AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA TGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCACGGTGGG
Xanthomonas sacchari 561	GCAAGTCCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAA CGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTAGCT AGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTT GGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG GGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGT GGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCTGTGGCGAAGGCG GCCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGA TGTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGG GGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAACTTTCCAGAGATGGATTG GTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC TTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA CCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGT CTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC ACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCC
Xanthomonas sacchari 573	GCCAGTCCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAA CGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTAGCT AGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG GGAGGCACCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTT GGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACTTGGGAATTGCAGTG GATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCTGTGGCGAAGGCGG CCATCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGAT GTTGGGTGCAACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAGGACTGAAACTCAAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGACCCTTACCTGGTCTTGACATCCACGGAACTTTCCAGAGATGGATTGG TGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCT TAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAC CAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTC TGCAACTGGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGCCCTTGTACACACCGCCCGTCACA CCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCACGGTGGGGCG

Table 3F Glycoside Hydrolase Family and the known activity enzyme in each family

Family	Known activity
2	β-galactosidase (EC 3.2.1.23) ; β-mannosidase (EC 3.2.1.25); β-glucuronidase (EC 3.2.1.31); α-L-arabinofuranosidase (EC 3.2.1.55); mannosylglycoprotein endo- β-mannosidase (EC 3.2.1.152); exo- β-glucosaminidase (EC 3.2.1.165)
3	β-glucosidase (EC 3.2.1.21); xylan 1,4- β-xylosidase (EC 3.2.1.37); β-glucosylceramidase (EC 3.2.1.45); β-N-acetylhexosaminidase (EC 3.2.1.52); α-L-arabinofuranosidase (EC3.2.1.55); glucan 1,3-β-glucosidase (EC 3.2.1.58); glucan 1,4-β-glucosidase (EC 3.2.1.74); isoprimeverose-producing oligoxyloglucan hydrolase (EC 3.2.1.120); coniferin β-glucosidase (EC 3.2.1.126); exo-1,3-1,4-glucanase (EC 3.2.1.-); β-N-acetylglucosaminide phosphorylases (EC 2.4.1.-)
5	endo-β-1,4-glucanase / cellulase (EC 3.2.1.4); endo-β-1,4-xylanase (EC 3.2.1.8); β-glucosidase (EC 3.2.1.21); β-mannosidase (EC 3.2.1.25); β-glucosylceramidase (EC 3.2.1.45); glucan β-1,3-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); exo-β-1,4-glucanase / cellodextrinase (EC 3.2.1.74); glucan endo-1,6-β-glucosidase (EC 3.2.1.75); mannan endo-β-1,4-mannosidase (EC 3.2.1.78); cellulose β-1,4-cellobiosidase (EC 3.2.1.91); steryl β-glucosidase (EC 3.2.1.104); endoglycoceramidase (EC 3.2.1.123); chitosanase (EC3.2.1.132); β-primeverosidase (EC 3.2.1.149); xyloglucan-specific endo-β-1,4-glucanase (EC 3.2.1.151); endo-β-1,6-galactanase (EC 3.2.1.164); hesperidin 6-O-α-L-rhamnosyl-β-glucosidase (EC 3.2.1.168); β-1,3-mannanase (EC 3.2.1.-); arabinoxylan-specific endo-β-1,4-xylanase (EC 3.2.1.-); mannan transglycosylase (EC 2.4.1.-)
8	chitosanase (EC 3.2.1.132); endoglucanase (EC 3.2.1.4); licheninase (EC 3.2.1.73); endo-1,4-β-xylanase (EC 3.2.1.8); reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156)
9	endoglucanase (EC 3.2.1.4); endo-β-1,3(4)-glucanase / lichenase-laminarinase (EC 3.2.1.6); β-glucosidase (EC 3.2.1.21); lichenase / endo-β-1,3-1,4-glucanase (EC 3.2.1.73); exo-β-1,4-glucanase / cellodextrinase (EC 3.2.1.74); cellobiohydrolase (EC 3.2.1.91); xyloglucan-specific endo-β-1,4-glucanase / endo-xyloglucanase (EC 3.2.1.151); exo-β-glucosaminidase (EC 3.2.1.165)

Table 3F Glycoside Hydrolase Family and the known activity enzyme in each family (continue)

Family	Known activity
13	α-amylase (EC 3.2.1.1); pullulanase (EC 3.2.1.41); cyclomaltodextrin glucanotransferase (EC 2.4.1.19); cyclomaltodextrinase (EC 3.2.1.54); trehalose-6-phosphate hydrolase (EC3.2.1.93); oligo-α-glucosidase (EC 3.2.1.10); maltogenic amylase (EC 3.2.1.133); neopullulanase (EC 3.2.1.135); α-glucosidase (EC 3.2.1.20); maltotetraose-forming α-amylase (EC 3.2.1.60); isoamylase (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); maltohexaose-forming α-amylase (EC 3.2.1.98); maltotriose-forming α-amylase (EC 3.2.1.116); branching enzyme (EC 2.4.1.18); trehalose synthase (EC 5.4.99.16); 4-α-glucanotransferase (EC 2.4.1.25); maltopentaose-forming α-amylase (EC 3.2.1.-) ; amylosucrase (EC2.4.1.4) ; sucrose phosphorylase (EC 2.4.1.7); malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141); isomaltulose synthase (EC 5.4.99.11); malto-oligosyltrehalose synthase (EC 5.4.99.15); amylo-α-1,6-glucosidase (EC 3.2.1.33); α-1,4-glucan: phosphate α-maltosyltransferase (EC 2.4.99.16); 6?-P-sucrose phosphorylase (EC 2.4.1.-)
16	xyloglucan:xyloglucosyltransferase (EC 2.4.1.207); keratan-sulfate endo-1,4-β-galactosidase (EC 3.2.1.103); endo-1,3-β-glucanase (EC 3.2.1.39); endo-1,3(4)-β-glucanase (EC3.2.1.6); licheninase (EC 3.2.1.73); β-agarase (EC 3.2.1.81); κ-carrageenase (EC 3.2.1.83); xyloglucanase (EC 3.2.1.151); endo-β-1,3-galactanase (EC 3.2.1.181); β-porphyranase (EC 3.2.1.178); hyaluronidase (EC 3.2.1.35); endo-β-1,4-galactosidase (EC 3.2.1.-); chitin β-1,6-glucanosyltransferase (EC 2.4.1.-); endo-β-1,4-galactosidase (EC3.2.1.-)
18	chitinase (EC 3.2.1.14); lysozyme (EC 3.2.1.17); endo-β-N-acetylglucosaminidase (EC 3.2.1.96); peptidoglycan hydrolase with endo-β-N-acetylglucosaminidase specificity (EC3.2.1.-); Nod factor hydrolase (EC 3.2.1.-); xylanase inhibitor; narbonin
19	chitinase (EC 3.2.1.14); lysozyme (EC 3.2.1.17)
20	β-hexosaminidase (EC 3.2.1.52); lacto-N-biosidase (EC 3.2.1.140); β-1,6-N-acetylglucosaminidase) (EC 3.2.1.-); β-6-SO3-N-acetylglucosaminidase (EC 3.2.1.-)
23	lysozyme type G (EC 3.2.1.17); peptidoglycan lyase (EC 4.2.2.n1) also known in the literature as peptidoglycan lytic transglycosylase; chitinase (EC 3.2.1.14)
25	lysozyme (EC 3.2.1.17)
29	α-L-fucosidase (EC 3.2.1.51); α-1,3/1,4-L-fucosidase (EC 3.2.1.111)

Table 3F Glycoside Hydrolase Family and the known activity enzyme in each family (continue)

Family	Known activity
31	α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22); α-mannosidase (EC 3.2.1.24); α-1,3-glucosidase (EC 3.2.1.84); sucrase-isomaltase (EC 3.2.1.48) (EC 3.2.1.10); α-xylosidase (EC 3.2.1.177); α-glucan lyase (EC 4.2.2.13); isomaltosyltransferase (EC 2.4.1.-); oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161)
64	β-1,3-glucanase (EC 3.2.1.39)
65	α,α-trehalase (EC 3.2.1.28); maltose phosphorylase (EC 2.4.1.8); trehalose phosphorylase (EC 2.4.1.64); kojibiose phosphorylase (EC 2.4.1.230); trehalose-6-phosphate phosphorylase (EC 2.4.1.216); nigerose phosphorylase (EC 2.4.1.279); 3-O-α-glucopyranosyl-L-rhamnose phosphorylase (EC 2.4.1.282); 2-O-α-glucopyranosylglycerol: phosphate β-glucosyltransferase (EC 2.4.1.-); α-glucosyl-1,2-β-galactosyl-L-hydroxylysine α-glucosidase (EC 3.2.1.107)
73	lysozyme (EC 3.2.1.17); mannosyl-glycoprotein endo-β-N-acetylglucosaminidase (EC 3.2.1.96); peptidoglycan hydrolase with endo-β-N-acetylglucosaminidase specificity (EC3.2.1.-)
92	mannosyl-oligosaccharide α-1,2-mannosidase (EC 3.2.1.113); mannosyl-oligosaccharide α-1,3-mannosidase (EC 3.2.1.-); mannosyl-oligosaccharide α-1,6-mannosidase (EC3.2.1.-); α-mannosidase (EC 3.2.1.24); α-1,2-mannosidase (EC 3.2.1.-); α-1,3-mannosidase (EC 3.2.1.-); α-1,4-mannosidase (EC 3.2.1.-); mannosyl-1-phosphodiester α-1,P-mannosidase (EC 3.2.1.-)
94	cellobiose phosphorylase (EC 2.4.1.20); laminaribiose phosphorylase (EC 2.4.1.31); cellodextrin phosphorylase (EC 2.4.1.49); chitobiose phosphorylase (EC 2.4.1.-); cyclic β-1,2-glucan synthase (EC 2.4.1.-); cellobionic acid phosphorylase (EC 2.4.1.321); β-1,2-oligoglucan phosphorylase (EC 2.4.1.-)
103	peptidoglycan lytic transglycosylase (EC 3.2.1.-)
109	α-N-acetylgalactosaminidase (EC 3.2.1.49)
114	endo-α-1,4-polygalactosaminidase (EC 3.2.1.109)
127	β-L-arabinofuranosidase (EC 3.2.1.185)

VITA

Miss Siraprapa Saraihom was born on March 9th, 1985 in Si Sa Ket province, Thailand. She studied for her high school at Kanthalak Wittaya School (2001-2004). In December 2008, she received the degree of Bachelor of Science with a major in Microbiology from Khonkean University. Since June 2009, she received the Chulalongkorn University Dutsadi Phiphat Scholarship to study for the degree of Doctor of Philosophy in Biological Sciences Program, at the Faculty of Science, Chulalongkorn University. During her graduate study, she published her work under the title of "First report of a tropical Lysobacter enzymogenes producing bifunctional endoglucanase activity towards carboxymethylcellulose and chitosan" in Annals of Microbiology in 2016.

Oral and poster presentations:

- Siraprapa Saraihom, Donald Y. Kobayashi, Pongtharin Lotrakul, Sehanat Prasongsuk, Douglas E. Eveleigh and Hunsa Punnapayak. Characterization of glycoside hydrolase-producing bacteria isolated from Thailand soils. 2012 American Phytopathological Society (APS) Annual Meeting, August 4th-8th, 2012, Providence RI, USA

- Siraprapa Saraihom, Donald Y. Kobayashi, Pongtharin Lotrakul, Sehanat Prasongsuk, Douglas E. Eveleigh and Hunsa Punnapayak. Characterization of a cellulase-producing Lysobacter enzymogenes strain isolated from soils in Thailand. 2014 Meeting in Miniature and 61th Selman A. Waksman Honorary Lectureship (New Jersey Branch of the American Society for Microbiology), 3rd April 2014, Rutgers, the State University of New Jersey, NJ, USA.

- Siraprapa Saraihom, Donald Y. Kobayashi, Pongtharin Lotrakul, Sehanat Prasongsuk, Douglas E. Eveleigh and Hunsa Punnapayak. First Report of the CMCase-producing tropical Lysobacter enzymogenes isolated from Thai soil. The 8th Korea-ASEAN Joint Symposium 2014: Biomass Utilization and Renewable Energy, 18th-22nd August 2014, Korea University, Seoul, Republic of Korea.

- Siraprapa Saraihom, Donald Y. Kobayashi, Pongtharin Lotrakul, Sehanat Prasongsuk, Douglas E. Eveleigh and Hunsa Punnapayak. First report of the tropical cellulase-producing Lysobacter enzymogenes isolated from soils in Thailand. 19th Biological Sciences Graduate Congress (BSGC), 12th-14th December 2014, National University of Singapore, Singapore.