PRODUCTION AND SECRETION OF *BACILLUS* CHITOSANASE IN *LACTOBACILLUS PLANTARUM* EXPRESSION SYSTEM

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การผลิตและการส่งออกไคโตซาเนสจากบาซิลัสโดยใช้ระบบการแสดงออกของเชื้อ *Lactobacillus plantarum*

นางสาวปีณิดา นามวิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2557

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ปีณิดา นามวิจิตร : การผลิตและการส่งออกไคโตซาเนสจากบาซิลัสโดยการใช้ระบบการแสดงออกของเชื้อ *Lactobacillus plantarum* (PRODUCTION AND SECRETION OF *BACILLUS* CHITOSANASE IN *LACTOBACILLUS PLANTARUM* EXPRESSION SYSTEM) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 91 หน้า.

วัตถุประสงค์ของวิทยานิพนธ์นี้คือการพัฒนาระบบการแสดงออกของยีนเพื่อการผลิตเอนไซม์ไคโตเนสจากเชื้อบาซิลัสที่เหมาะสมกับอุตสาหกรรมอาหาร โดยใช้เชื้อ *L. plantarum* เป็นเซลล์เจ้าบ้าน ซึ่งอาจจะสามารถนำไปประยุกต์ใช้ในการผลิตเอนไซม์ทางด้านอาหารในการผลิตเชิงพาณิชย์ต่อไปในอนาคต โดยในการศึกษาวิจัยนี้ เอนไซม์ไคโตซาเนสลูกผสม ถูกสร้างขึ้นด้วยการเชื่อมต่อกับเปบไทด์นำสัญญาณ 2 ชนิด คือ เปบไทด์นำสัญญาณตามธรรมชาติของเชื้อ *Bacillus subtilis* และเปบไทด์นำสัญญาณของโปรตีนเยื่อหุ้มชั้นนอกของเชื้อ *Escherichia coli* หรือ OmpA จากนั้นทำการโคลนยีนดังกล่าวเข้าสู่พาหะที่ใช้ในการแสดงออกของยีนชื่อเวคเตอร์ pSIP409 ด้วยวิธีการที่ต้องใช้ปฏิกิริยาลูกโซ่โพลิเมอเรสปลายเหนียว เพื่อเชื่อมต่อชิ้นของยีนเข้าไปในระหว่างตำแหน่งตัดจำเพาะของเอนไซม์ *NcoI* และ *XhoI* ของเวคเตอร์ pSIP409 ที่มียีนต้านยาปฏิชีวนะอีริ-โทรมัยซินเป็นเครื่องหมายในการคัดเลือกเวตเตอร์ที่ถูกต้อง ทำให้ได้เวคเตอร์ใหม่ 2 ชนิด คือ pSIP409CSN_nt และ pSIP409CSN_OmpA จากนั้นนำยีนเอนไซม์ไคโตซาเนสดังกล่าวไปแสดงออกในเชื้อ *L. plantarum* WCFS1 ซึ่งเป็นเชื้อที่สามารถใช้ในงานด้านอาหาร การแสดงออกของยีน *csn* จากเชื้อนี้ได้ถูกควบคุมโดยอาศัยตัวชักนำซึ่งมีคุณสมบัติเป็นเปบไทด์ฟีโรโมน จากนั้นจึงทำการวิเคราะห์เพื่อเปรียบเทียบระดับกิจกรรมเอนไซม์และประสิทธิภาพในการส่งออกของเอนไซม์ทั้งสองรูปแบบ

จากการวิเคราะห์พบว่า ผลผลิตของเอนไซม์ที่ได้จากการเลี้ยงเชื้อในปริมาตร 500 มิลลิลิตร ที่สภาวะเหมาะสม กิจกรรมเอนไซม์ที่อยู่ในน้ำเลี้ยงเชื้อมีประมาณ 10,700±25 ยูนิต และ 4,970±411 ยูนิต และมีประสิทธิภาพการส่งออกของเอนไซม์คิดเป็นร้อยละ 79.0 และ 89.0 สำหรับเอนไซม์ที่ใช้เปบไทด์นำสัญญาณจากธรรมชาติและเปบไทด์นำสัญญาณ OmpA ตามลำดับ ในขั้นตอนต่อไปได้ทำการสร้างเวคเตอร์สำหรับการผลิตเอนไซม์เพื่อสามารถใช้ในอุตสาหกรรมอาหาร โดยการแทนที่ตำแหน่งของยีนต่อต้านยาอิริโทรไมซินด้วยยีนอะลานีน ราซิเมส เรียกเวคเตอร์ใหม่ที่ได้สร้างขึ้นนี้ว่า pSIP609CSN_nt และ pSIP609CSN_OmpA ผลจากการวัดกิจกรรมของเอนไซม์ในเบื้องต้นโดยการเลี้ยงเชื้อในขวดรูปชมพู่ปริมาตร 100 มิลลิลิตร พบว่า ค่ากิจกรรมในส่วนน้ำเลี้ยงเชื้อวัดได้ประมาณ 12,600 ยูนิต (U) ต่อลิตร ผลสรุปจากการศึกษานี้แสดงว่า สามารถใช้เชื้อ *L. plantarum* ใน

การผลิตเอนไซม์ไคโตซาเนสจากเชื้อบาซิลัสได้ ซึ่งระบบการผลิตเอนไซม์ที่ได้พัฒนาขึ้นมานี้มีศักยภาพในการนำไปประยุกต์ใช้ในการผลิตโปรตีนชนิดอื่นๆ ได้ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PEENIDA NAMVIJITR : PRODUCTION AND SECRETION OF *BACILLUS* CHITOSANASE IN *LACTOBACILLUS PLANTARUM* EXPRESSION SYSTEM. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 91 PP.

RECOMBINAT CHITOSANASE/*LACTOBACILLUS PLANTARUM*/SECRETION/ SIGNAL PEPTIDE

The goal of this study was to develop a food-grade expression system for the production of *Bacillus* chitosanse in *Lactobacillus plantarum* that could be applied for commercial production of food-grade enzyme in the future. Two forms of recombinant chitosanase (Csn) fused with different signal peptides, i.e., *Bacillus subtilis* Csn native signal peptide (SP) and the signal peptide of the *Escherichia coli* outer membrane protein (OmpA) were cloned into *NcoI* and *XhoI* sites of pSIP409 expression vector, harboring erythromycin resistant gene (*erm*), by a sticky PCR-based method. The two constructs, designated pSIP409CSN_nt and pSIP409 CSN_OmpA, were generated and over-expressed from *L. plantarum* WCFS1, which is a food-grade expression host. The *csn* gene expression was controlled by using a peptide pheromone as inducer, and both enzyme activity and secretion efficiency of the two constructs were compared. The yields from 500 mL of culture volume at the optimal condition were approximately 10,700±25 U and 4,970±411 U/L, when using the native and OmpA SP, respectively. The secretion efficiencies were approximately 79.0% and 89.0% for the constructs containing the native and the OmpA SP, respectively. Subsequently, food grade vector for the expression of the recombinant chitosanase was constructed

by replacing the erythromycin resistance gene with the alanine racemase (*alr*) gene, resulting pSIP609CSN_nt and pSIP609CSN_OmpA constructs. Preliminary analysis of the yield of recombinant Csn containing native signal peptide from 100 mL of cultivation was approximately 12,600 U/L, when culture in a shake-flask. In conclusion, an efficient system for the production and secretion of recombinant chitosanase in *L. plantarum* was successfully developed. This system may potentially be applied for the production of other proteins.

School of Biotechnology Student's Signature_____

Academic Year 2014 Advisor's Signature_____

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CHAPTER I

INTRODUCTION

1.1 Significance of this study

Application of green/white biotechnology for the sustainable environment has become an important issue in this century. For food industry, commercial production of food enzymes and proteins for the production of commodities by green/white biotechnology has been growing tremendously in the past decade. To achieve an increasing in the demand and market expanding, recombinant DNA technology is necessary for the production of various food modifying enzymes and proteins. However, most of expression hosts and vectors that are commonly used for the expression of heterologous proteins do not comply with the "food-grade" definition (Peterbauer *et al.*, 2011). Therefore, food grade expression systems that based on food grade microorganisms or food-grade recombinant microorganisms must be developed and used as a cell factory for the production of recombinant proteins. The system is considered as safe and carries the "Generally recognized as safe" (GRAS) status for food applications purposes. In this system, the expression of the gene encoding heterologous proteins is controlled by quorum-sensing mechanism, which involves the production of antimicrobial peptide or bacteriocin (Sorvig *et al.*, 2003; Sorvig *et al.*, 2005).

Food- grade gene expression systems for lactic acid bacteria (LAB) are useful for applications in the food industry (Giraffa *et al.*, 2010). Recently, a new food-grade

host/vector system for *Lactobacillus plantarum* based on pSIP expression vectors and the use of the homologous alanine racemase gene (*alr*) as selection marker, without the use of antibiotics has been described by Nguyen and co-worker (2011).

The goal of this study was to develop a food-grade expression system based on the pSIP expression vectors for the production of *Bacillus* sp. chitosanase (Csn) in *L. plantarum* WCFS1. This expression system can be used for the bioconversion of chitin/chitosan wasters to generate value-added chitooligosaccharides (CHOS) for food, feed and pharmaceutical applications in the future.

1.2 Research objectives

1. To sub-clone *Bacillus* chitosanase (*csn*) genes into *Lactobacillus* expression vector (pSIP-based vector)
2. To compare the expression, secretion and activity of the different constructs in *L. plantarum*
3. To optimize the condition for the over-expression of selected recombinant enzymes
4. To construct food-grade expression vector using alanine racemase (*alr*) gene as a selection marker

CHAPTER II

LITERATURE REVIEW

2.1 Food-grade gene expression systems

Food grade systems are based on the integration and stable retention of a food grade expression vector in the host strain. The development of different selection markers is attended to specify the food-relevant definitions. Most importantly, these systems must not contain antibiotic resistance markers since their presence may increase the risk of transfer of antibiotic resistance to the human intestinal microbiota (Vos, 1999; Sybesma *et al.*, 2006).

Host strains used for genetic engineering must be safe, taxonomically well characterized, stable, and have the "Generally Recognized As Safe" (GRAS) status. Selection markers such as antibiotic resistance genes or markers that require the application of, for example, heavy metals for selection or plasmid maintenance, cannot be used, and no production of harmful (toxic or allergenic) compounds may occur (Peterbauer *et al.*, 2011). Molecular working techniques, modification tools and genetic elements should conform to the criteria of self-cloning. Finally, food-grade systems require stable processes in large-scale industrial applications (Konings *et al.*, 2000). Self-cloning limits the DNA that is to be introduced into a micro-organism to genes obtained from organisms of the same genus. A somewhat broader definition allows the introduction of genes from organisms that are more distantly related (beyond genus borders) provided that these donor organisms themselves have the

GRAS status. The introduction of DNA from non-GRAS organisms is acceptable during the construction process, provided that it is removed entirely in the final production strain (Sybesma *et al.*, 2006).

2.1.1 Food-grade expression hosts

Bacillus subtilis has a long history of application in enzyme production and food fermentation so that it is a GRAS microorganism (Meijer *et al.*, 1995; Kunst *et al.*, 1997). In order to develop an entirely food-grade recombinant *B. subtilis* expression system for food industry, a food-grade integrative vector was constructed by exploiting the endogenous *B. subtilis* alanine racemase (*dal*) gene as both a homologous sequence for integration and an efficient complementary marker for selection of recombinant *B. subtilis* strain (Xia *et al.*, 2005; Xia *et al.*, 2007). In addition to *Bacillus,* for a large-scale production of recombinant enzyme in food industry, the Gram-negative bacteria *E. coli* K-12, which has been approved for GRAS status by the US Food and Drug Administration (US-FDA), has also been used. This strain of *E. coli* has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion (Olempska-Beer *et al.*, 2006). Another attractive host for food grade expression is lactic acid bacteria (LAB). The genetic manipulation of LAB has many potential applications in food safety and in the development of improved food products. The safe use of genetically modified LAB requires the development of cloning systems that are composed solely of DNA from food-grade organisms. Different food-grade cloning systems published during the past decade have been reviewed (Vos, 1999).

2.1.2 Food-grade selection markers

2.1.2.1 Dominant selection markers

The highly strain-independent dominant selection is comparable to antibiotic resistance markers. They allow the direct selection of positive transformants and the stable integration of plasmids in host organisms as long as selection pressure is obtained. Dominant markers usually confer a new ability to the host strain such as bacteriocin immunity/resistance (Takala and Saris, 2002), heat-shock resistance (El Demerdash *et al.*, 2003), or sugar utilization abilities (Boucher *et al.*, 2002). Dominant markers have the advantage that they potentially may be used in a wide variety of strains, but the number of suitable food-grade markers is limited.

2.1.2.2 Complementation selection markers

The second main approach to obtain stable and selectable host/vector systems are based on specific mutations or deletions in a chromosomal gene of the host organism that are an essential step in a metabolic pathway or conferring certain properties to an organism or phenotype. To conduct the complementation, in the first step a feasible knockout mutant is constructed carrying the desired gene deletion in the host chromosome. The second step contains the construction of expression vectors carrying the compatible complementation. As a consequence, these markers can only be used in specific host-vector combinations (Dickely *et al.*, 1995; Peterbauer *et al.*, 2011).

Examples of such genes include the thymidylate synthase gene (thyA) (Sasaki *et al.*, 2004), and genes involved in lactose conversion, such as lactose phosphotransferase (lacF) (MacCormick *et al.*, 1995), or phospho-β-galactosidase (lacG) (Gosalbes *et al.*, 2000). The enzyme alanine racemase converts L-alanine to D-

alanine (Bron *et al.*, 2002), which is crucial for cell wall biosynthesis , and is thus an essential enzyme for growth of prokaryotic cells (Hols *et al.*, 1997). In lactococci and lactobacilli, alanine racemase activity is encoded by a single gene, *alr* (Hols et al., 1997; Palumbo *et al.,* 2004). D-Alanine is not a common ingredient of large-scale fermentation media, and previous studies have shown that the *alr* gene has considerable potential as a food-grade selection marker in lactic acid bacteria (Bron *et al.*, 2002).

2.2 Food-grade gene expression in lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are gram-positive, non-sporeforming cocci, coccibacilli or rod shape. LAB are facultative bacteria which are able to grow both aerobically or anaerobically. Most LAB are able to produce many enzymes, antimicrobial peptides, or metabolites that provide and improve the quality of food products (Schmidt, 2004). Many LAB considered to be non-pathogenic bacteria, are given "GRAS" status by US-FDA. The terms "long history" and "safe use" are, however, still somewhat arbitrary, and efforts are under way in the European Union to come up with a comparable regulatory term, "Qualified Presumption of Safety" (QPS), that should have more scientific precision without forcing extensive safety reviews for long established products (Sybesma *et al.*, 2006; Peterbauer *et al.*, 2011).

There are two well-known inducible expression systems developed for lactic acid bacteria (LAB), i.e. *Lactococcus* and *Lactobacillus* (Kleerebezemab *et al.*, 2000; Giraffa *et al.*, 2010). These systems are based on quorum-sensing mechanisms involved in regulation of bacteriocin production. These systems are derived from the genes involved in the production of nisin in *Lactococcus lactis* (Kuipers *et al.*, 1997)

or sakacin A and P (pSIP vectors) in *Lactobacillus sakei* (Sorvig *et al.*, 2003; Sorvig *et al.*, 2005)

2.2.1 NIsin Controller gene Expression system (NICE) for *Lactococcus lactis*

NICE system system has found widespread use as an inducible system for LAB. Nisin, an antimicrobial peptide with 34 amino acids, is used for the induction of a regulatory cascade starting with its binding to the membrane-bound histidine protein kinase (NisK), which upon binding autophosphorylates a conserved His residue in the cyctoplasmic domain of the NisK for its activation. In the next step the phosphate group from activated NisK is transferred to the intracellular response regulator (NisR), thereby activating this regulator. Activated NisR then induces the nisin operon at the nisin A promoter (PnisA) which controls the expression of the genes involved in nisin biosynthesis or a gene of interest when using the NICE expression system (Figure 1) (Maischberger *et al.*, 2010).

Recently, the first "true" heterologous food-grade overexpression of the enzyme *β*-galactosidase using the NICE system in *L. lactis* NZ9000 and NZ3900 has been reported. The construction of food-grade expression vectors was performed by replacing the chloramphenicol acetyltransferase (*cat*) gene in these plasmids with the promotorless lactococcal *lacF* gene, encoding the soluble carrier enzyme llA^{Lac}, so that *lacF* was then under control of the *repC* promotor (Figure 2). *L. lactis* NZ3900, carrying an in-frame deletion of the chromosomal *lacF* gene, was the host for these food-grade constructed vectors, which could easily be selected by complementation of this strain for growth on lactose (Maischberger *et al.*, 2010).

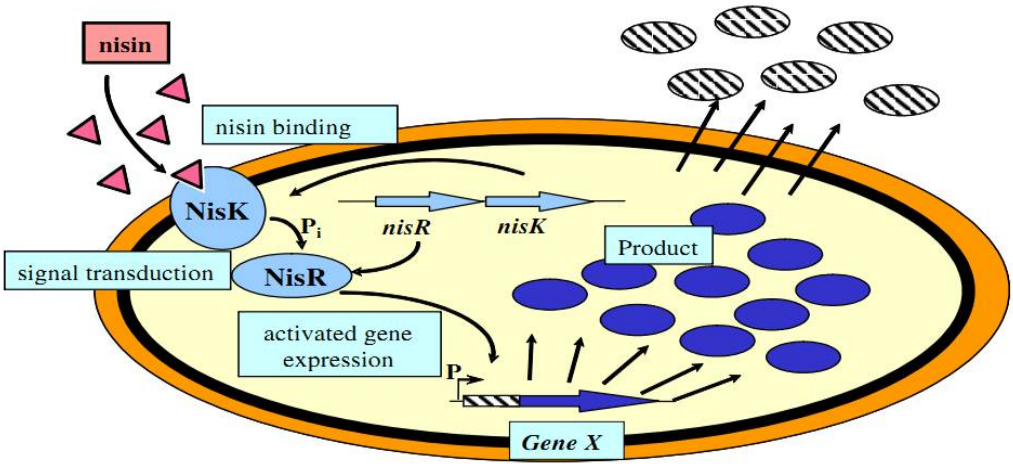

Figure 1. Schematic overview of the NICE system, its components and its function. NisK and NisR are the sensor protein and the response regulator, respectively. The product of the expressed gene can either accumulate in the cell or be secreted into the extracellular medium depending on the presence of a signal sequence in the construct (Mierau et al., 2005)

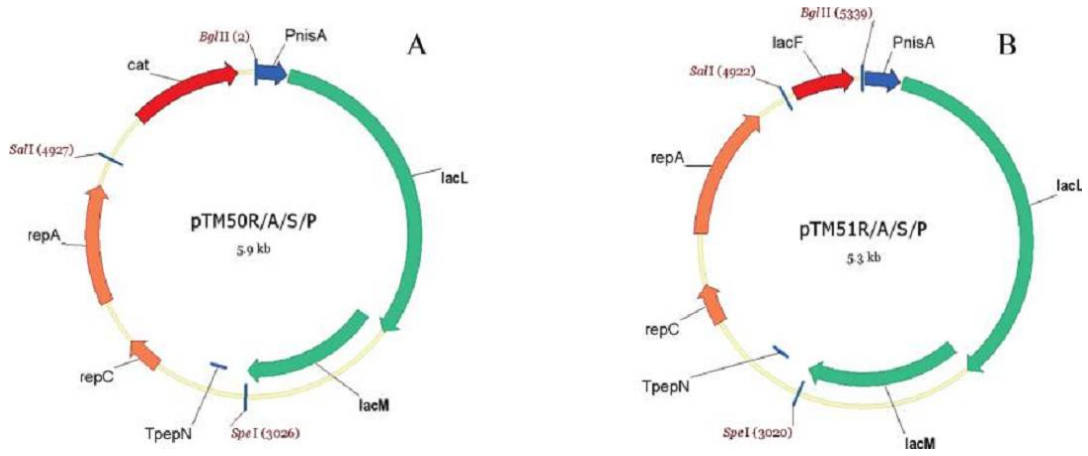

Figure 2. Vector map of pTM plasmids for the expression of *β*-galactosidase using NICE system. lacLM, structural gene; cat, chloramphenicol resistance marker; lacF, *lacF* gene for selection on lactose; *repA* and *repC*, replication determinants; P*nisA*,inducible promoter; *TpepN*, transcriptional terminator (Maischberger *et al.*, 2010).

2.2.2 Sakacin-based gene expression system (pSIP) for *Lactobacillus* strains

Many lactic acid bacteria (LAB) produce antimicrobial peptides, called "bacteriocins", to combat competing Gram-positive bacteria. The bacteriocin production is often regulated via quorum-sensing mechanisms based on a secreted peptide pheromone (Eijsink *et al.*, 2002). The bacteriocins from LAB have been classified into 3 classes: the lanthibiotics (class I), the non-modified heat-stable bacteriocins (class II) and the large heat labile bacteriocins (class III) (Nes *et al.*, 1996; Nes and Holo, 2000).

The production of some class II bacteriocins in lactobacilli is regulated by quorum-sensing mechanisms which differ from the mechanism employed for nisin regulation in *L. lactis*. These lactobacilli secrete a non-modified peptide whose primary function is to act as a pheromone and not as a bacteriocin. The regulatory operons in these systems consist of three genes, encoding the peptide pheromone precursor, a HK protein that senses the pheromone, and a cognate RR protein. Activation of the RR enhances transcription from regulated promoters that precede all operons involved in bacteriocin production, processing, secretion and immunity, as well as the regulatory operon itself (Figure 3) (Nes *et al.*, 1996).

Vectors for high-level expression of target genes, the so called pSIP-system, have been developed for inducible gene expression in *L. plantarum* and *Lactobacillus sakei* (Sorvig *et al.*, 2003; Sorvig *et al.*, 2005). A series of versatile expression vectors are based on the sap (pSIP300 series) and the *spp* (pSIP400 series) regulon. The vectors were constructed in a modular fashion, permitting easy exchange of different parts, such as the gene of interest, the promoter, the replicon, and the selection marker (Figure 4). In another series, such as in pSIP403, expression

was driven both by *ermB* read-through and by the original inducible promoter (P*sppIP*). In all variants, the structural gene for the IP, which naturally precedes the K-R genes, was inactivated by deletion. With respect to the choice of promoter, it must be noted that IP-regulated bacteriocin regulons contain several IP-controlled promoters, which in principle all can be used to drive expression of the gene of interest. Usually, the promoters driving expression of bacteriocin structural genes are the strongest and most tightly regulated (Brurberg *et al.*, 1997; Risøen *et al.*, 2000) Therefore, only such promoters were used in the pSIP series, i.e., PsapA in the pSIP300 series and P*sppA* or P*sppQ* in the pSIP400 series (P*sppQ* has been called P*orfX* in previous publications) (Mathiesen *et al.*, 2005; Diep *et al.*, 2009).

The lactobacillal expression vectors pSIP409 (figure 5) in this thesis is based on the sakacin P operon of *L. sakei* for expression in *L. plantarum* WCFS1. It has previously been shown that the pSIP-based expression system allows for production of high levels of intracellular proteins. The expression of the gene of interest is driven by strong, regulated promoters. The activity of these promoters is controlled via a two-component signal transduction system, which responds to an externally added peptide pheromone. The vectors have a modular design, permitting easy exchange of all essential elements including the inducible promoter, the cognate regulatory system, the gene of interest, the antibiotic resistance marker and the replicon (Sorvig *et al.*, 2003; Sorvig *et al.*, 2005).

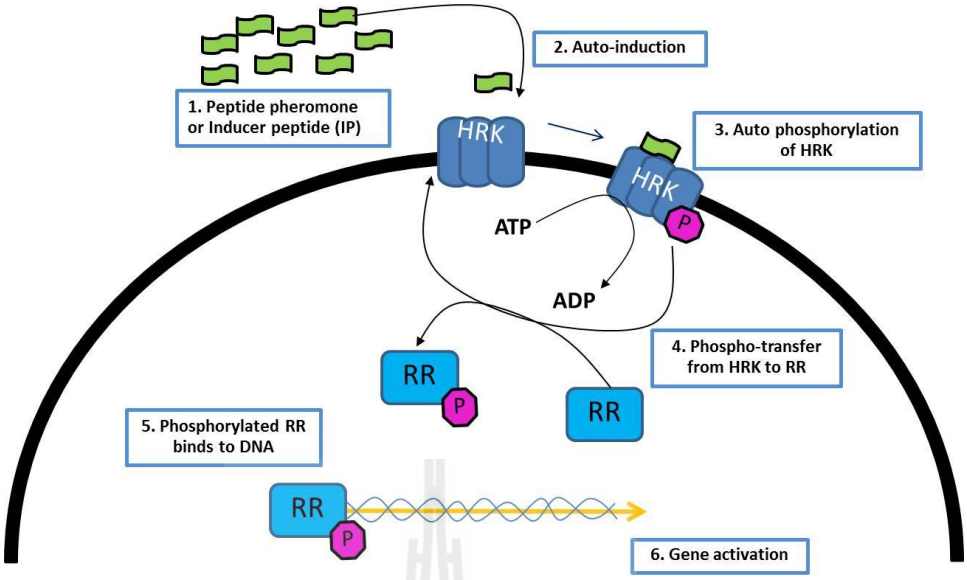

Figure 3. Schematic illustration of pheromone regulated bacteriocin production. (1) Low amounts of the constitutively induction peptide (IP) are produced in the cell. (2) The IP bind to the receptor histidine protein kinase (HPK). (3) This results in autophosporylation of a conserved histidine residue in the HPK. (4) The phosphoryl-group is transferred to the response regulator (RR) through interaction with the HPK. (5) The phosphorylated RR binds to DNA and activates transcription of all genes involved in bacteriocin biosynthesis (6). The figure is modified from Mathiesen (2004).

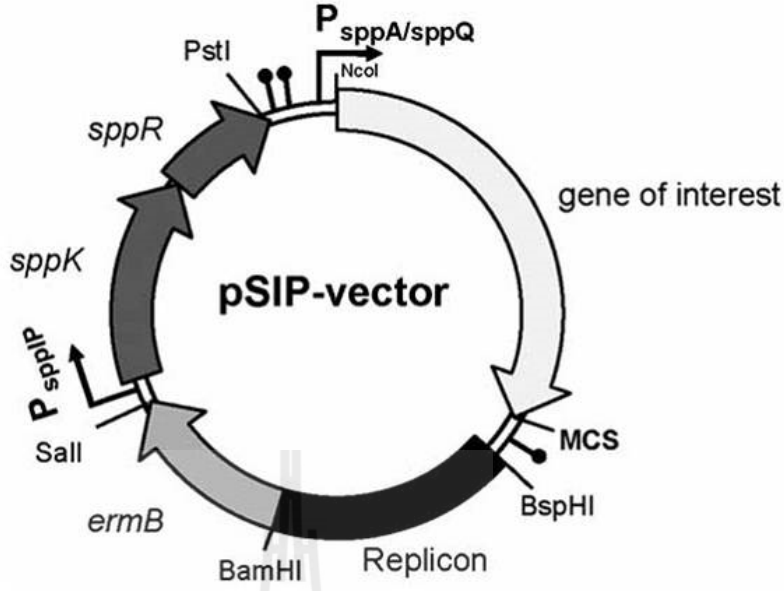

Figure 4. Schematic overview of the modular pSIP-vector expression system. The picture shows the main outline of the pSIP400 series, based on the *spp* regulon. The *sppK* and *sppR* genes encode the proteins in the two-component regulatory system. The gene of interest is under control of one of the strictly regulated bacteriocin promoters, P*sppA* (sakacin P) or P*sppQ* (sakacin Q) and is translation-ally coupled to the promoter via an NcoI restriction that incorpo-rates the ATG start codon. Unique restriction sites for easy re-placement of different modules are indicated. The replicon region consist of two determinants; pUC(pGEM)-ori for *E. coli* and 256rep for *L. sakei* and *L. plantarum*. Lollipops indicate transcription terminators. Note that the vectors vary with respect to the promoter driving the transcription of the *KR* operon (Diep *et al.*, 2009).

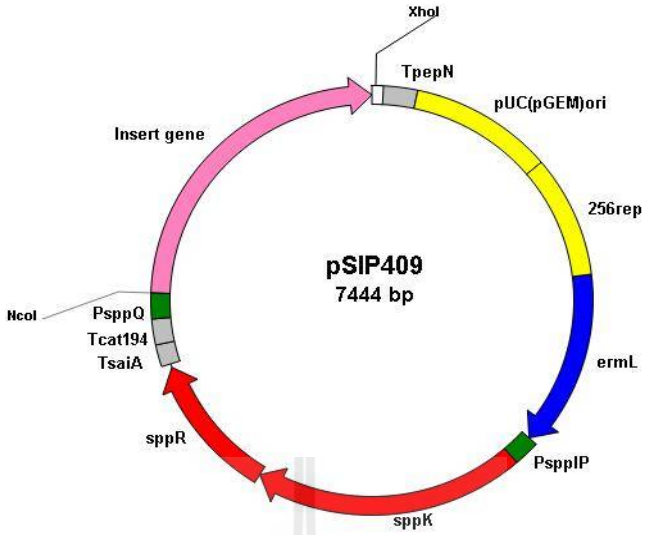

Figure 5. Schematic overview of pSIP409 plasmids that is used in this study. The picture shows the main outline of the pSIP409, based on the *spp* regulon. Yellow region represents replication determinants (pUC(pGEM)ori and 256rep); blue region represents erythromycin resistance marker; red regions, histidine protein kinase (*sppK*) and response regulator genes(*sppR*); green regions represents inducible bacteriocin promoters and inducible *sppIP* promoter; grey regions represent stranscriptional terminator; pink region represents insert gene (*gusA* for pSIP409). The figure was modified from Sorvig (2005).

Recently, Nguyen and co-workers (2011) described a new food-grade host/vector system for *L. plantarum* based on the complementation selection markers using the homologous alanine racemase gene (*alr*). A new series of expression vectors were constructed by exchanging the erythromycin resistance gene (*erm*) in pSIP vectors by the *L. plantarum* WCFS1 *alr* gene (Figure 5). The vectors were applied for the over-expression of desired genes from *Lactobacillus reuteri* L103 and *L. plantarum* WCFS1 in an *alr* deletion mutant of *L. plantarum* WCFS1 (named *L. plantarum* TLG02). The genome of *L. plantarum* contains a single *alr* gene encoding an alanine racemase which is enzyme that can catalyze the inter-conversion of D-alanine and L-alanine. The alanine racemase converts L-alanine to D-alanine that is involved in biosynthesis of cyclosporine locate in cell wall because D-alanine is cross-linkaging or apart of cell wall of *L. plantarum* that is essential for their growth (Palumbo *et al.*, 2004).

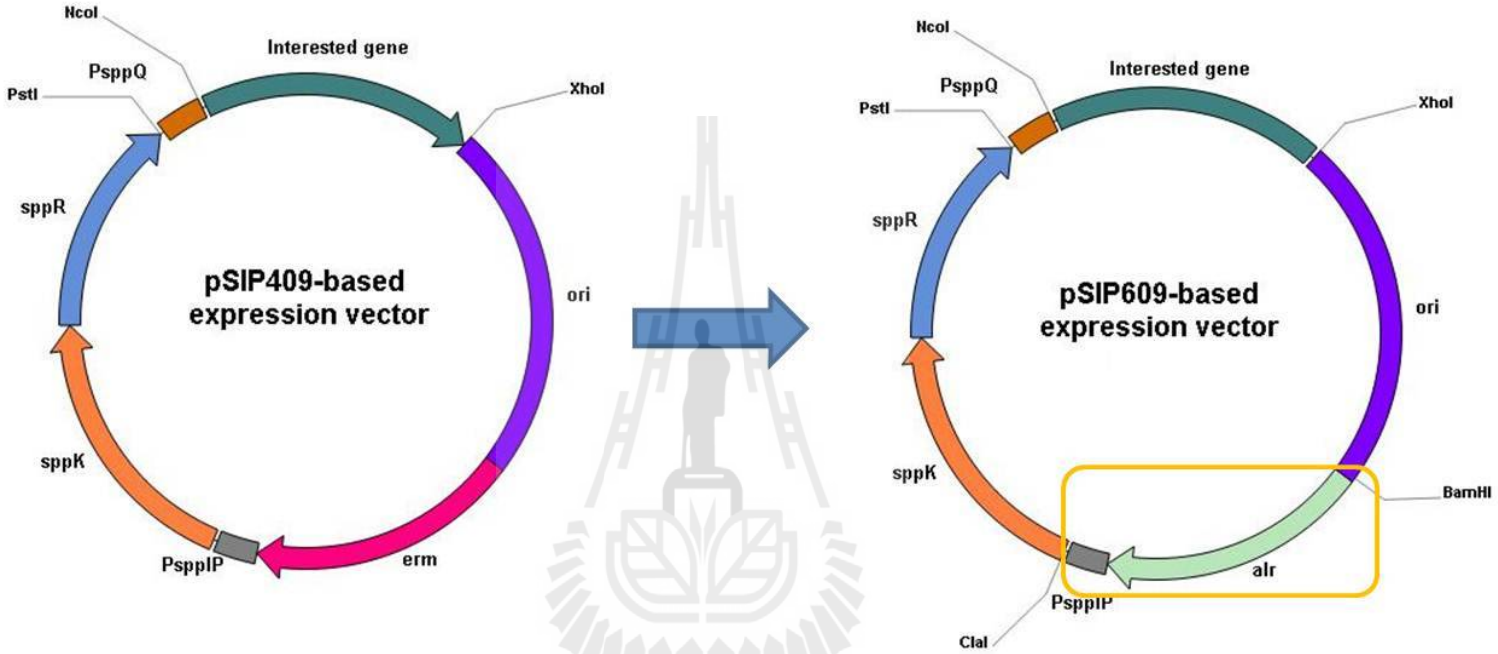

Figure 6. The construction of pSIP609-based expression vector, a food-grade expression vector from pSIP409 vector. The DNA fragment between *ClaI* and *BamHI* containing *erm* gene was replaced with the fragment containing *alr* gene that was obtained *L. plantarum* WCFS1 genome (Nguyen *et al.*, 2011)

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2.3 Chitosanases

Chitosan N-acetylglucosaminohydrolase or chitosanases (EC 3.2.1.132) are enzymes that catalyse the hydrolysis of the β-1,4 glycosidic bond of chitosan (Figure 7), a partially deacetylated derivative of chitin, which comprises N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues (Figure 8) (Dahiya *et al.*, 2006). These enzymes can be found in a wide variety of microorganisms including Gram-positive and Gram-negative bacteria, yeast and fungi (Eijsink *et al.*, 2010). The enzymes belong to glycoside hydrolase (GH) families 5, 7, 8, 46, 75 and 80, according to the carbohydrate-Active Enzymes database (CAZy) (Cantarel *et al.*, 2009). While families GH5, GH7 and GH8 contain a few chitosanases and other glycoside hydrolases, specifically, families GH46, GH75 and GH80 comprise exclusively chitosanases (Eijsink *et al.*, 2010). Chitosanase from family 46, especially those from *streptomyces* (Fukamizo and Brzezinski, 1997; Dubeau *et al.*, 2011), *Bacillus circulans* (Fukamizo *et al.*, 2005), and *Bacillus subtilis* 168 (Pechsrichuang *et al.*, 2013), are presently the best-studied enzymes.

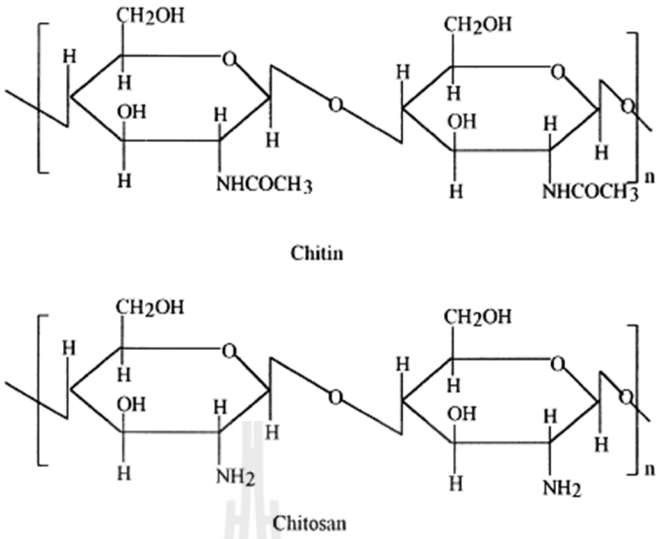


Figure 7. Structures of chitin and chitosan (Kim and Rajapakse, 2005).

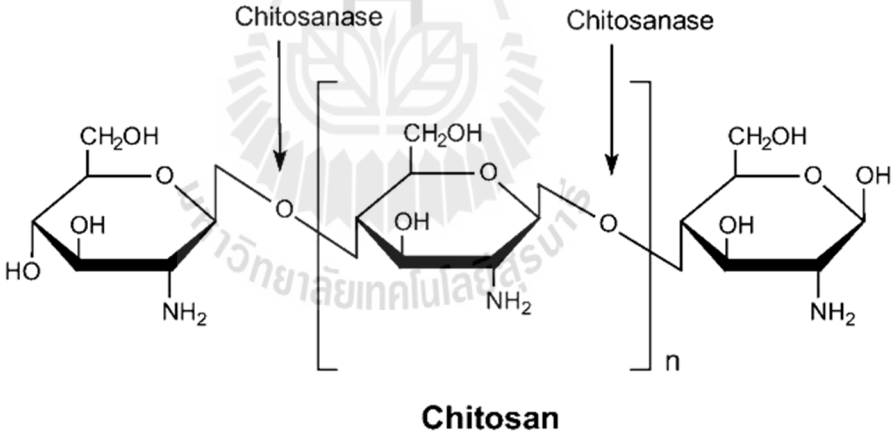


Figure 8. The chitosanase activity (Fukamizo *et al.*, 1995).

CHAPTER III

MATERIAL AND METHOD

3.1 Materials

3.1.1 Bacteria

Strain	Relevant characteristics/ Genotypes	Source of reference
Escherichia coli DH5αF'	Cloning host/ *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs (NEB)
Escherichia coli MB2159	Cloning host/ D-alanine auxotroph	(Strych *et al.*, 2001)
Escherichia coli TOP10	Cloning host/ F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen
Lactobacillus plantarum WCFS1	Expression host/ wild type	(Kleerebezem *et al.*, 2003)
Lactobacillus plantarum TLG02	Expression host/ Δ*alr* (D -alanine auxotroph)	(Nguyen *et al.*, 2011)

3.1.2 DNA Primers

Name	Sequence (5'-3')
B.subCsnfwNcoIlong	CATGAAAATCAGTATGCAAAAAGCAGATTTTTGG
B.subCsnfwNcoIshort	AAAATCAGTATGCAAAAAGCAGATTTTTGG
FlagNcoIfwlong	CATGAAAAAGACAGCTATCGCGATTG
FlagNcoIfwshort	AAAAAGACAGCTATCGCGATTG
6HisXhoIrvlong	TCGAGTCAATGGTGATGGTGATGGTG
6HisXhoIrvshort	GTCAATGGTGATGGTGATGGTG

3.1.3 Plasmids

Plasmid	Description	Source of reference
pSIP409gusA	*erm*, pSIP401 derivative, *gusA* controlled by P*sppQ*	(Sorvig *et al.*, 2005)
pSIP609gusA	pSIP409 derivative, *alr* replaced with *erm*	(Nguyen *et al.*, 2011)
pSIP409CSN_nt	*erm*, pSIP409 derivative, *csn_nt* controlled by P*sppQ*	This work
pSIP409CSN_OmpA	*erm*, pSIP409 derivative, *csn_OmpA* controlled by P*sppQ*	This work
pSIP609CSN_nt	*alr*, pSIP409 derivative, *csn_nt* controlled by P*sppQ*	This work
pSIP609CSN_OmpA	*alr*, pSIP409 derivative, *csn_OmpA* controlled by P*sppQ*	This work

3.1.4 Laboratory equipment

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Thermoscientific, Sorvall legend XTR centrifuge, USA
	Eppendrof centrifuge 5810 R, Eppendrof, USA
	Eppendrof minispin plus, wiswspin® feedback control digital timer function, Eppendrof, USA
Deep freezer -70 ^{o}C:	Thermosciencetific, forma 900 series, USA.
ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA

Freezer -20 ºC:	Heto, HLLF 370, Denmark.
	Haier, China.
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel dryer:	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA
Gel electrophoresis	Mini Protean® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA
	Innova® 42 incubator shaker series, USA
Incubator:	Memmert, BE 500, WTB Binder BD115,
Laminar hood:	Thermoscientific 1300 series A2, USA
	BH 2000 Series ClassII Biological Safety Cabinets
	BHA120 & BHA180, Clyde-Apac
Micro-centrifuge:	Mini spin plus, Eppendrof, USA
	Eppendorf 54154, Eppendorf, Germany
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
Rotator:	Certomat TCC, B. Braun Biotech International, Germany
Sequencing machine:	ABI prism model 310 Genetic Analyzer, Applied, Biosystems, USA

Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK
	Certomat TC2, B. Braun Biotech International, Germany
Sonicator:	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK
Stirrer:	Variomag Electronicrührer Poly 15, Germany
	Magnetic stirrer MSH300, USA
Thermomixer:	Thermomixer compact, Eppendrof, USA
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA

3.2 Methods

3.2.1 Culture of bacterial strains

Escherichia coli Top10 and *E.coli* DH5α were used as cloning hosts. They were grown in Luria- Bertani (LB) medium either on solid agar plates or in liquid medium, and incubated for overnight at 37ºC cabinet (for plates) or in a shaker incubator (for liquid cultures). The 800 μg/mL of erythromycin concentration was added to the medium when growing *E. coli* Top10 strains containing the plasmids.

E. coli MB2159 (D-alanine auxotroph) was used as cloning host for food-grade expression vectors. The bacterium was grown in LB medium supplemented with 200 μg/mL of D-alanine and incubated for overnight at 37ºC cabinet (for plates) or in a shaker incubator (for liquid cultures).

Lactobacillus plantarum WCFS1 (wild type) was grown either in MRS medium (Man-Rogosa-Sharpe) or on solid MRS-agar plates. *L. plantarum* cultures were incubated under facultative aerobic condition at 37 °C without agitation for 18-24 h. *L. plantarum* WCFS1 cells containing plasmids harboring an antibiotic resistance gene were grown in agar plates and liquid medium containing 5 μg/mL of erythromycin concentration.

L. plantarum TLG02 (Δ*alr*, D-alanine auxotroph) as used as expression host for food grade expression vectors. The cells were grown in MRS medium supplemented with 200 μg/mL D-alanine and incubated for overnight at 37ºC cabinet (for plates) or in a shaker incubator (for liquid cultures).

3.2.2 Molecular cloning of *Bacillus subtilis* chitosanase gene

The plasmids CsnNativepMY202 and CsnOmpApMY202 containing the complete chitosanase (*csn*) gene with native and OmpA signal peptide, and His-tag sequences (Pechsrichuang *et al.*, 2013) were used as templates for *csn* gene amplification. The oligonucleotides used for *csn* gene amplification were designed for cloning by a sticky PCR-based method (Yamabhai, 2009). The two forward primers [B.subCsnfwNcoIlong and B.subCsnfwNcoIshort] and two reward primers [6HisXhoI rvlong and 6HisXhoIrvshort] were used to amplify the *csn* gene containing *B. subtilis csn* native signal peptide (SP). The two forward primers [FlagNcoIfwlong and Flag-NcoIfwshort] and two reward primers [6HisXhoIrvlong and 6HisXhoIrvshort] were used to amplify the *csn* gene containing the *E. coli* outer membrane protein (OmpA) signal peptide. The lists of primers in this study are shown in Table 3.1.2, and were compatible with the *NcoI* and *XhoI* restriction sites. The vector pSIP409gusA, which is used for gene expression in *L. plantarum* WCFS1, as double digested with *NcoI* and *XhoI* restriction enzymes for cloning (Figure 9).

The PCR thermal profile (total volume of 50 µL) consisted of 10 µM of primers, 10 mM dNTP, 1.25 units of *Pfu* DNA Polymerase (Thermo scientific) and 10X *Pfu* Buffer with $MgSO_4$, provided by the manufacturer (MJ Research). The amplifications were done as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 45 s, annealing at 55 °C for 30 s extension at 72 °C for 2 min and followed by final extension step at 72 °C for 10 min. All PCR products and vectors were separated using 1% agarose gel in 1xTAE containing 0.2 µg/mL of ethidium bromide and visualized under a UV transilluminator. The PCR products and vectors were purified using the Wizard® SV gel and PCR Clean-Up system

(Promega). Approximately equal amounts of PCR products were mixed together in a PCR tube and heated at 95°C for 5 min, and then the denatured products were briefly mixed in a vortex mixer. The re-annealing was done in a thermal cycler machine by reducing the temperature slowly from 95 °C to 25°C. The total time for re-annealing is usually 2.35 h.

Ligation reactions were performed with the relevant restriction endonuclease used to digest the vector and a molar ratio of linearized vector to re-annealed insert of approximately 1:15. The amount of linearized vector used for each ligation was 100 ng. Ligations were performed for 16 h at 16°C in the presence of T4 DNA ligase in a final volume of 25 µL. T4 DNA ligase was heat-inactivated (65°C for 15 min) before transformation. The constructed was transformed into competent *E.coli* Top10 cell resulting in the plasmid pSIP409CSN_nt and pSIP409CSN_OmpA. The transformants were selected on LB agar containing 800 µg/mL erythromycin in the culture condition at 37 °C for 16 h.

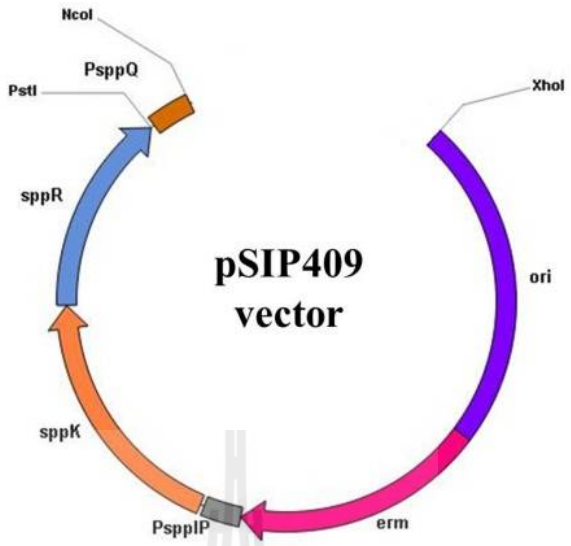


Figure 9. Schematic overview of pSIP409gusA plasmid used in this study. *NcoI* and *XhoI* restriction enzymes were used for cloning.

The plasmid was extracted using QIAGEN™ plasmid preparation kit. Then plasmids were digested with the *PstI* and *XhoI* to analyze the restriction pattern by agarose gel electrophoresis. The DNA sequence and the integrity of the constructs were confirmed by automated DNA sequencing (Macrogen, Korea). Assembly and analysis of DNA sequences were done by using Vector NTI. Afterwards, pSIP409-CSN_nt and pSIP409CSN_OmpA were transformed into competent *L. plantarum* WCFS1 cell which is an expression host by electroporation. Electroporation condition was performed at 25% amplitude, pulse 5 sec, 3 min. for 2 rounds on ice. The transformants were selected on MRS agar plate containing 5 µg/mL erythromycin. The colony PCR method was used for checking the positive clones. All strains were stored in sterilized 1.5 mL eppendorf tubes at -70 °C in MRS broth medium containing 20% (v/v) glycerol until used.

3.2.3 Construction of food grade expression vector

Food-grade expression vector of the recombinant chitosanase was constructed by replacing the erythromycin resistance gene with the alanine racemes gene (*alr*) from *L. plantarum* WCFS1 genome (Nguyen *et al.*, 2011). The erythromycin resistance (*erm*) genes in pSIP409CSN_nt and pSIP409CSN_OmpA were exchanged with alanice racemase *(alr)* gene between the restriction sites *BamHI* and *ClaI* (Figure 10), which are compatible with the same position for gene exchanging, resulting pSIP609CSN_nt and pSIP609CSN_OmpA, respectively.

The plasmids were extracted using QIAGEN™ plasmid preparation kit. Then, the plasmids were digested with the *BamHI* and *ClaI* to confirm the restriction site pattern, which indicated the *alr* gene fragment after digestion. Afterwards, pSIP609-CSN_nt and pSIP609CSN_OmpA were transformed into *L. plantarum* TLG02 (Δ*alr*/ D-alanine auxotroph) by electroporation. The transformants were selected on normal MRS agar plate. The colony PCR method was used to confirm the positive clones.

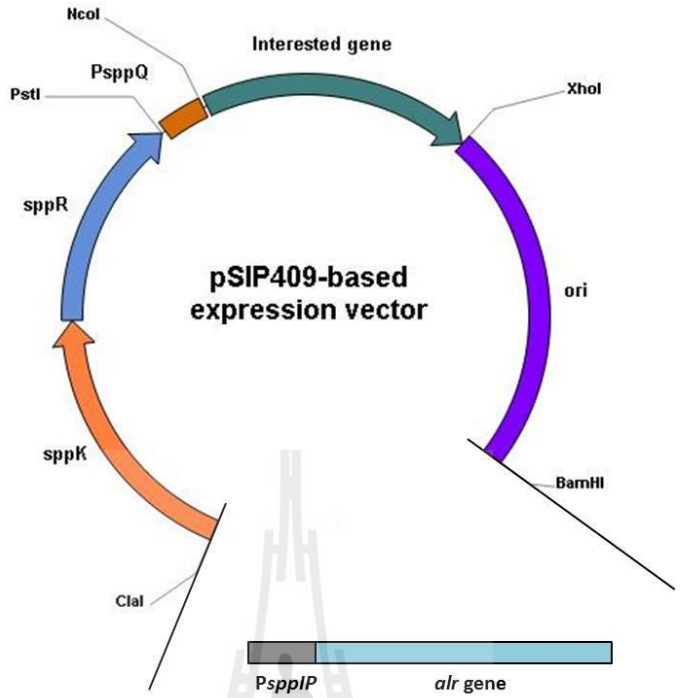


Figure 10. Schematic overview of pSIP409-based expression vector of which the *erm* gene was exchanged with *alr* gene.

3.2.4 Determination of optimal duration of induction

The transformed *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA in glycerol stock were taken from -80 ºC freezer and re-streaked and grown on MRS agar plate supplemented with erythromycin. Colonies of each constructs were picked and grown in 250 mL of MRS broth supplemented with 5 ug/mL erythromycin at 37 °C without shaking for 18-24 h. The overnight cultures were pooled together and mixed well and measured the cell density at the OD_{595} (ELISA reader, Sunrise, TECAN, Austria). The overnight cultures were added into MRS broth containing 5 µg/mL erythromycin at an OD_{595} of 0.1 and cultivated at 30 °C without agitation in anaerobic condition. The cells were induced for the expression of an enzyme at an OD_{595} of approximately 0.3 after by adding peptide

pheromone IP-673 at the concentration of 25 ng/mL (Amino acid sequence of IP-673 is Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg) (Brurberg *et al.*, 1997). The thirty milliliters of culture medium were sampling at 0, 6, 12, and 24 h to measure total enzyme activity. After induction, the cell and supernatant were separated by centrifugation at 4,000 rpm for 15 min at 4 °C (swing angle). Cells were washed twice with lysis buffer (50 mM Tris-HCl, pH 8.0), and re-suspended in lysis buffer supplemented with 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mg/ml lysozyme. The cell pallets were broken using sonicator at 25% amplitude, pulse 5 sec, 3 minutes for 2 rounds on ice. The cell lysate fraction was collected at 13,000 rpm, 4 °C for 45 min. to eliminate cellular debris.

To measure the enzyme activity in culture medium, 10-15 ml of super-natant containing secreted chitosanase was dialyzed with 10 mM Tris-HCl buffer, pH 8.0 at 250 rpm at 4 °C for 8-12 h, using snake skin dialysis tubing 10 kDa kit (Thermo scientific, USA). The dialysated fraction was collected and continuously kept on ice. The qualitative and quantitative analyses of recombinant chitosanase enzymes in cell extract and supernatant were assayed by SDS-PAGE and DNS method (Miller, 1959), respectively.

3.2.5 Determination of optimal inducer concentration

To determine the optimal inducer concentration, the recombinant cells were cultivated as described above. The cultures were inoculated into MRS broth containing 5 µg/mL of erythromycin to obtain an initial OD_{595} of 0.1. The culture was then incubated at 30 °C without agitation in anaerobic condition. Seventy milliliters of diluted cultures were induced in several inducer concentrations ranging from 0, 1.562, 3.125, 6.25, 12.5, 25, 50 and 75 ng/mL of IP-673. After 6 h. of induction time, the

crude enzymes in both fractions were prepared as previously described. The recombinant chitosanase enzymes in cell extract and supernatant were assayed.

3.2.6 Expression of recombinant *csn* in *L. plantarum* WCFS1 in 100-mL of culture volume

The recombinant *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA were grown in MRS broth containing 5 µg/ml of erythromycin overnight at 37°C. Then, the overnight cultures were diluted in 100 mL of MRS medium containing 5 µg/ml erythromycin to an O.D. at 595 nm of ~ 0.1 and incubated at 30 ºC, no agitation, in anaerobic condition to an O.D. of~0.3 (approx.2.30 h). Then, the cultures were induced with 12.5 ng/mL of IP-673. Cells were harvested at an OD_{595} of ~ 1.4-1.6 (around 6 hr.) by centrifugation (4,000 rpm for 15 min at 4 ºC), washed twice with lysis buffer (50 mM Tris-HCl, pH 8.0), and re-suspended in 6 mL of lysis buffer supplemented with 0.1 mM PMSF, 1 mg/ml lysozyme. Then, cell suspension was kept at -30 ºC for overnight. After that, cell suspension was thawed and disrupted using a sonicator at 25% amplitude, pulse 5 sec, 3 minutes for 2 rounds on ice. Cell debris was removed by centrifugation (13000 rpm, 45 min, 4 ºC) to obtain the crude enzyme extracts. To measure the enzyme activity in culture medium, 10-15 ml of supernatant was dialyzed with 10 mM Tris-HCl buffer, pH 8.0 at 250 rpm at 4 °C for 8-12 h, using snake skin dialysis tubing 10 kDa kit (Thermo scientific, USA). The dialysated fraction was collected and continuously kept on ice. The recombinant chitosanase enzymes in cell extract and supernatant were assayed.

3.2.7 Expression of recombinant *csn* in *L. plantarum* WCFS1 in 500-mL of culture volume

The recombinant *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA were grown in MRS broth containing 5 µg/ml of erythromycin overnight at 37°C. Then, the overnight cultures were diluted in 500 mL of MRS medium containing 5 µg/ml erythromycin to an O.D. at 595 nm of ~0.1 and incubated at 30 ºC, 150 rpm agitation, in anaerobic condition to an OD_{595} of ~ 0.3. Then, the cultures were induced with 12.5 ng/mL of IP-673. Cells were harvested at 0, 3, 6, 9, 12 and 20 hr. by centrifugation (4,000 rpm for 15 min at 4 ºC), washed twice with lysis buffer (50 mM Tris-HCl, pH 8.0) and re-suspended in 6 mL of lysis buffer supplemented with 0.1 mM PMSF, 1 mg/ml lysozyme. Then, cell suspension was kept at -30 ºC for overnight. Cell suspension was thaw and disrupted using a sonicator. Cell debris was removed by centrifugation (13000 rpm, 45 min, 4 ºC) to obtain the crude enzyme extracts. To measure the enzyme activity in culture medium, 10-15 ml of supernatant was dialyzed with 10 mM Tris-HCl buffer, pH 8.0 at 250 rpm at 4 °C for 8-12 h, using snake skin dialysis tubing 10 kDa kit (Thermo scientific, USA). The dialysated fraction was collected and continuously kept on ice. The recombinant chitosanase enzymes in cell extract and supernatant were assayed.

3.2.8 Enzyme assay of recombinant chitosanase

Gel electrophoresis analysis

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Protein bands were stained with Coomassie brilliant blue R-250 (Laemmli, 1970). A protein ladder (10–200 kDa) was purchased from Bio-Rad. The protein samples were heated at 100 °C for 10 min in the loading buffer.

Protein determination

Proteins concentration was determined by Quick start™ Bradford protein assay (Bio-Rad) using bovine serum albumin as standard. The dye reagent was mixed 1 to 4 with DI water and filtered. The properly diluted samples (60 µl) were mixed with 200 µl of dye reagent and stand at room temperature for 2 min. The absorbance at 595 nm was measured.

Chitosanase activity assay

Chitosanase activity were analyzed using the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixer consists of 40 µl of broth fraction or appropriate dilution of enzyme from cell fraction and 160 µl of 0.5% soluble chitosan in 200 mM sodium acetate buffer, pH 5.5 which was pre-incubated at 50 °C for 30 min. Then, the reaction was incubated at 50 °C for 5 min, at 900 rpm (Pechsrichuang *et al.*, 2013). The reaction was stopped by adding 200 µl of DNS solution, centrifuged at 13,000 rpm at for 5 min to precipitate the remaining chitosan. After that, the color was developed by heating the reaction at 100 °C for 20 min and cooled down on ice for 5 min. The reducing sugar was determined by measuring the OD at 540 nm using D-glucosamine as a standard. One unit of chitosanase was defined as the amount of

enzyme the released 1 µmol of D-glucosamine per minute under the standard assay condition.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) when normally distributed or as a median (range) when abnormally distributed. Statistical analyses were completed using the SPSS 16.0 system (SPSS Inc., Chicago, IL). Group differences from the experiments in 3.2.6 and 3.2.9 were analyzed by independent-samples t-test ($p < 0.05$). For the experiment in 3.2.7, One-Way ANOVA was applied to preparation about different between control and sample groups from sampling random of each treatment, and show mean ± standard deviation (X±S.D.) of each treatment by Turkey-Kramer post-hoc test at significant $p<0.05$.

3.2.9 Preliminary analysis of food-grade *csn* gene expression in *L. plantarum*

Freshly transformed *L. plantarum* TLG02 harboring pSIP609CSN_nt and pSIP609CSN_OmpA were grown on MRS agar plate. Colonies of each constructs were picked and grown in 5 mL of MRS broth at 37 °C without shaking for 18-24 h. Then, the overnight cultures were pooled together and mixed prior to measure OD at 595 nm before inoculation. The overnight cultures were inoculated into 100 mL of MRS broth to obtain an initial OD_{595} of 0.1. After that, the diluted cultures were grown at 30 °C without agitation in an anaerobic condition until OD_{595} value was 0.3. The culture was then collected after inducing with 25 ng/mL of IP-673 (Nguyen *et al.*, 2011) and incubated at 30 °C without agitation in anaerobic condition for 6 h or until the cell density reach an OD_{595} of ~ 1.4-1.6. The recombinant chitosanase enzymes in both fractions were assayed by SDS-PAGE and DNS method.

CHAPTER IV

RESULTS

4.1 Molecular cloning of *Bacillus subtilis* chitosanase gene

4.1.1 Cloning of chitosanse gene in pSIP(*erm*)-based expression vector

The special molecular cloning strategies for vector construction must be used because there were internal restriction sites in the *B. subtilis* csn gene. The optimal condition of sticky-PCR method was used to amplify and generate the *csn* genes containing either their native signal peptides or signal peptides from *E.coli* outer membrane protein, called OmpA, which were fused at N-terminal end of *csn* gene and histidine tag was linked at C-term. The sticky PCR strategy is also a powerful method for complex DNA inserts. Depending on the primers selected, it is possible to generate all four combinations of cohesive overhangs in the insert. Two pairs of oligo-nucleotide primers were required for each restriction site, one for the longer pro-truding end and the second one for the shorter end (Figure 11). PCR reactions were used to generate each type of the PCR products. All PCR products were performed at 55ºC of annealing temperature (Figure 12). For vector preparation, the pSIP409gusA vectors were double digested with the *NcoI* and *XhoI* to delete the *gusA* gene out before cloning (Figure 13). Then, the products from two sticky PCR reactions of each constructed were re-annealed to generate DNA fragments including the *csn* gene fusing with the native signal peptide was approximately 0.860 kbp, while the *csn* gene fusing with OmpA signal peptide was around 0.830 kbp (Figure 14(I)). Meanwhile,

linearized pSIP409 fragment without *gusA* gene was purified (Figure 14(II)). Next, each re-annealed PCR products were used for ligation into pSIP409-based expression vector at the *NcoI* and *XhoI* restriction sites to generate the new plasmids and *NcoI* site at 5' end of *csn* gene must be destroyed after ligation. The pSIP409CSN_nt was the vector containing the *csn* gene fused with the native SP. The pSIP409CSN_OmpA was the vector containing the *csn* gene fused with OmpA SP (Figure 15).

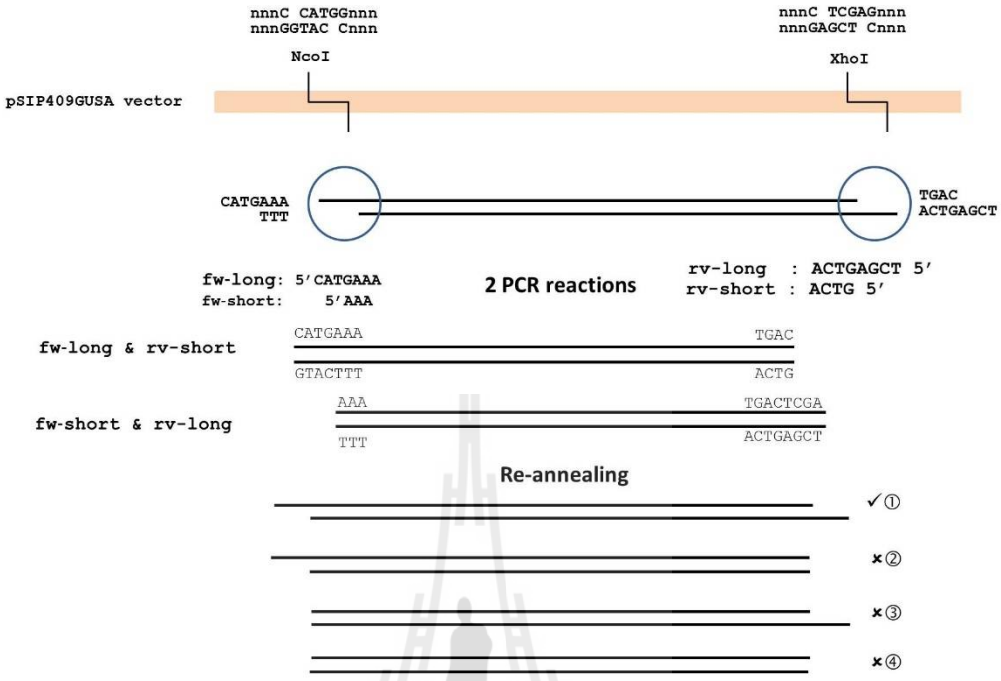


Figure 11. Schematic overview of the primer design for molecular cloning using sticky PCR method that demonstrates the primer design for permitting PCR cloning into *NcoI* and *XhoI* cloning sites of the linearized vector and illustrates the DNA sequences of the correct cohesive ends of the re-annealed products for the cloning of the *csn* gene.

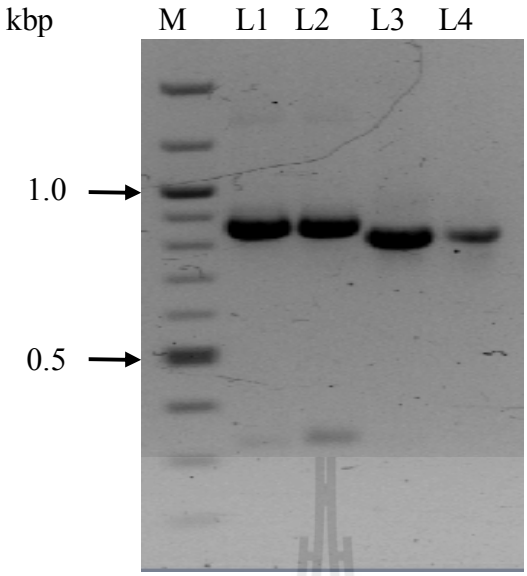


Figure 12. Agarose gel analysis of sticky PCR products after *csn* amplification. M; 100 bp DNA leader, L1; *csn* gene with native SP (primer: fwlong& rvshort). L2; *csn* gene with native SP (primer: fwshort&rvlong). L3; *csn* gene with OmpA (primer: fwlong&rvshort). L4; *csn* gene with OmpA (primer: fwshort&rvlong).

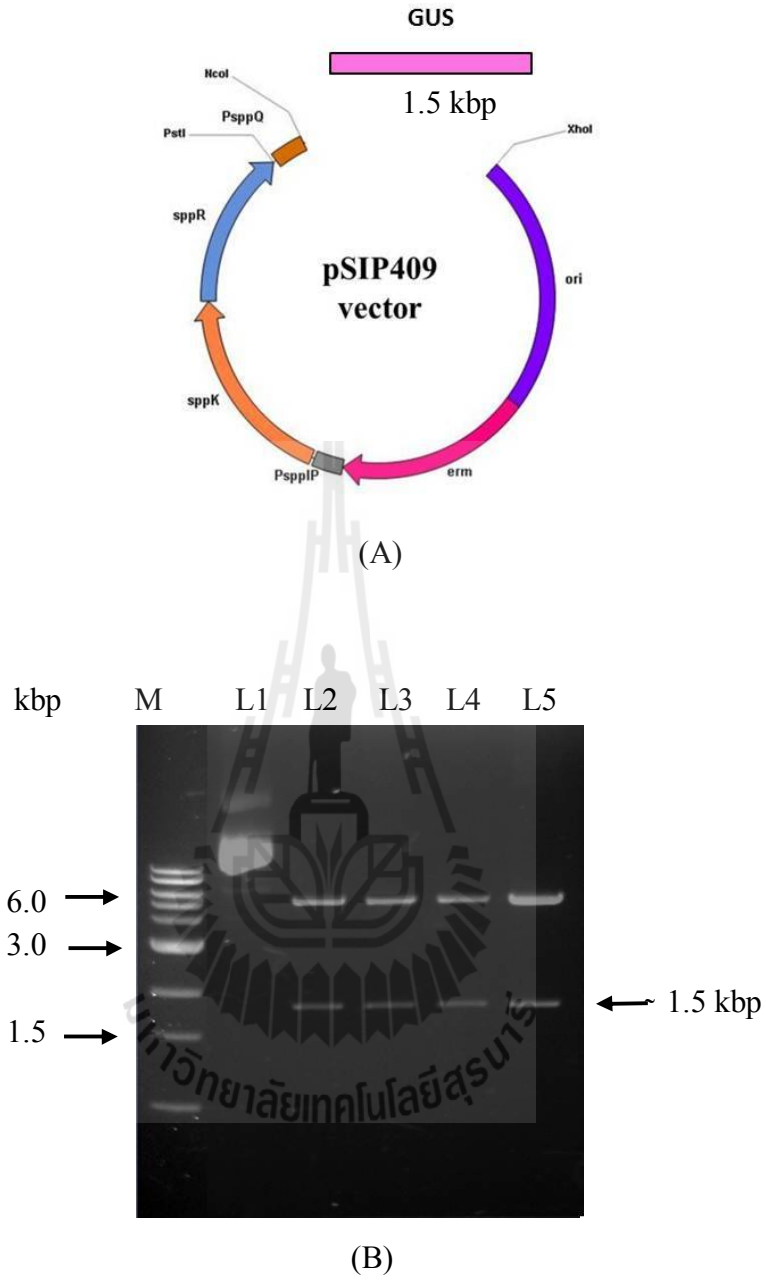


Figure 13. The Map of pSIP409gusA after cutting with *NcoI* and *XhoI* (A). Restriction fragment analysis of pSIP409gusA was shown (B). M ; 1 kb DNA leader. L1; uncut pSIP409gusA. L2-5; pSIP409gusA (Clone1-4) double digested with *NcoI* and *XhoI*.

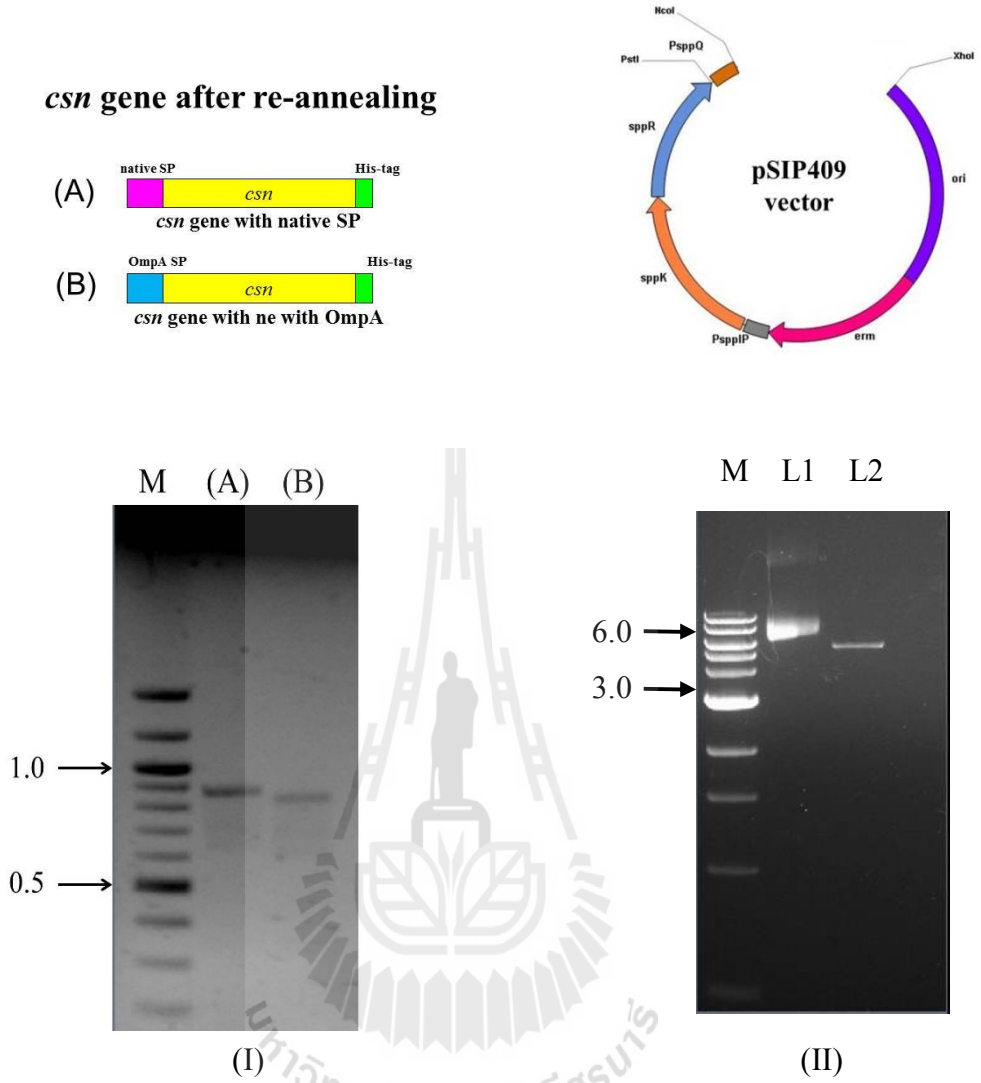
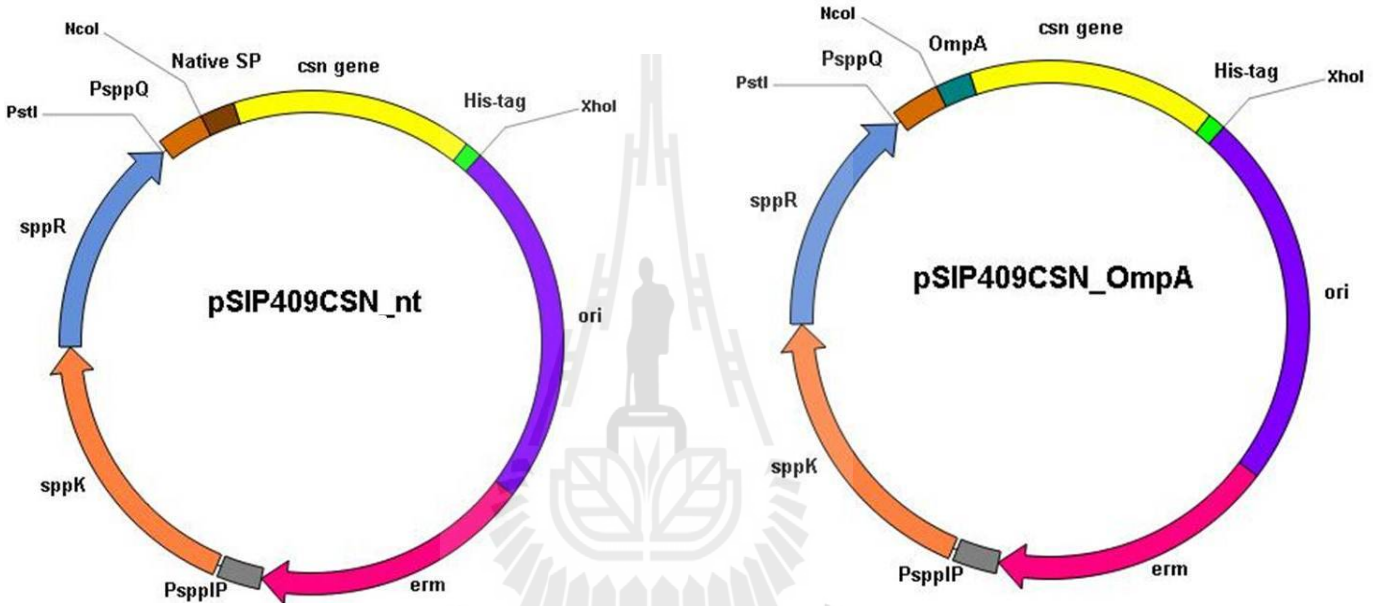


Figure 14. Agarose gel analysis of PCR products after re-annealing (I) and linearized pSIP409 vector after digesting with *NcoI* and *XhoI* restriction enzymes (II) were shown.. M; 100 bp DNA leader, M1; 1 kb DNA leader. L1; Uncut pSIP409 vector. L2 ; linearized pSIP409 after double digestion.

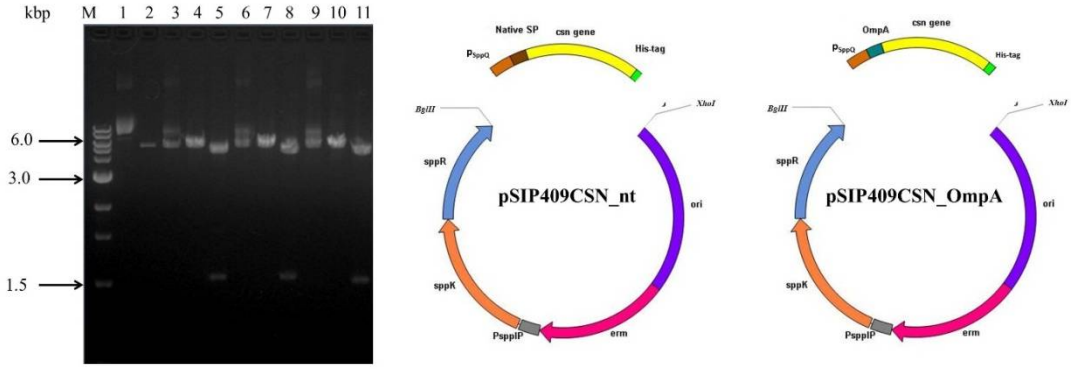


(A) (B)

Figure 15. The map of pSIP409CSN_nt (A) and pSIP409CSN_OmpA (B), a pSIP-based vector for the expression of Csn_nt and Csn_OmpA, respectively.

After transformation, plasmids from positive clones of recombinant *E.coli* habouring pSIP409CSN_nt and pSIP409CSN_OmpA were prepared and checked by restriction fragment analysis using the *BglII* and *XhoI* restriction enzymes. These enzymes digested at positions upstream and downstream of P_{sppQ} promoter, *csn* gene and his tag, respectively. The right band size of DNA fragments were shown approximately 1.5 kbp after gel electrophoresis analysis, indicating that the sticky-PCR based cloning was successful (Figure 16). The integrity of the construct was confirmed by automated DNA sequencing (Macrogen, Korean).

To express the enzyme, the recombinant vectors (pSIP409CSN_nt clone1 and pSIP409CSN_OmpA clone 1) were transformed into *L. plantarum* WCFS1 which is the expression host. The PCR products were the *csn* genes amplification from *L. plantarum* WCFS1 which harbored the different constructs using colony PCR method (Figure 17).



(A) (B) (C)

Figure 16. Restriction fragment analysis of positive clones was shown (A). M ; 1 kb DNA leader. Lane1; uncut pSIP409 vector. Lane2; purified pSIP409 vector. Lane3-5; pSIP409CSN_nt clone1. Lane6-8; pSIP409CSN_nt clone 2. Lane9-11; pSIPCSN_OmpA. Lane3, 6, and 9; Recombinant vectors were digested with *BglII*, Lane 4, 7, and 10; Recombinant vectors were digested with *XhoI*. Lane 5, 8, and 11; Recombinant vectors were double digested with *BglII* and *XhoI*. The map of pSIP409CSN_nt (B) and pSIP409CSN_OmpA (C), which are double digested with *BglII* and *XhoI*.

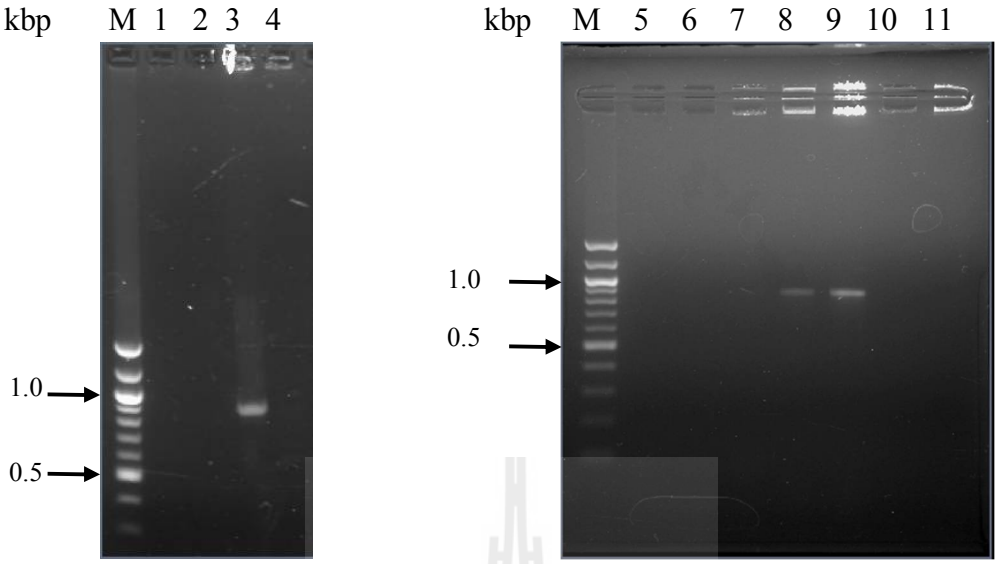


Figure 17. Confirmation of the presence of *csn* genes in *L. plantarum* by PCR. M; 100 bp DNA leader. Lane1 and 5; are PCR reactions when using *L. plantarum* WCFS1 (Negative control) as template. Lane2 and 6 are PCR products when using pSIP409LacZhis (Negative control) as templates. Lane3-4; PCR products when using *L. plantarum* WCFS1 habouring pSIP409CSN_nt clone 1 and 2. Lane7-11; PCR products when using *L. plantarum* WCFS1 habouring pSIP409CSN_OmpA clone 1-5 as templates.

4.1.2 Construction of food grade expression vectors for the expression of *Bacillus csn*

The food-grade expression vectors were constructed by replacing erythromycin resistant (*erm*) gene of the pSIP409CSN_nt and pSIP409CSN_OmpA vectors with alanine racemase (*alr*) gene. The pSIP609gusA was prepared and checked by restriction analysis. The DNA fragments of *gusA* gene (~2.2 kbp) and *alr* gene (~1.8 kbp) were shown after double digestion with *PstI-XhoI* and *BamHI-ClaI* restriction enzymes (Figure 18). The recombinant *csn* in these *alr*-based vectors are designed as pSIP609CSN_nt and pSIP609CSN_OmpA (Figure 19). The transcription of *csn* genes is controlled by the promoters P_{sppQ}. Selection of constructs harboring the food-grade expression vector were done by growing the *E. coli* MB2159 (D-alanine auxotroph) in LB media without using antibiotic. The integrity of the food-grade constructs were confirmed by digesting vectors with *BamHI* and *ClaI* restriction enzymes (Figure 20). The bands with the size of the *alr* gene of about 1.8 - 2.0 kbp are indicated by an arrow. After that, food-grade vectors were prepared to transform into *L. plantarum* TLG02 which is the expression host.

The recombinant *L. plantarum* TLG02 (D -alanine auxotroph) harboring the different constructs were confirmed by using colony PCR method. The PCR products showed the presence of the *csn* gene amplification from the positive clones of *L. plantarum* TLG2 harboring pSIP609CSN_nt and pSIP609CSN_OmpA (Figure 21).

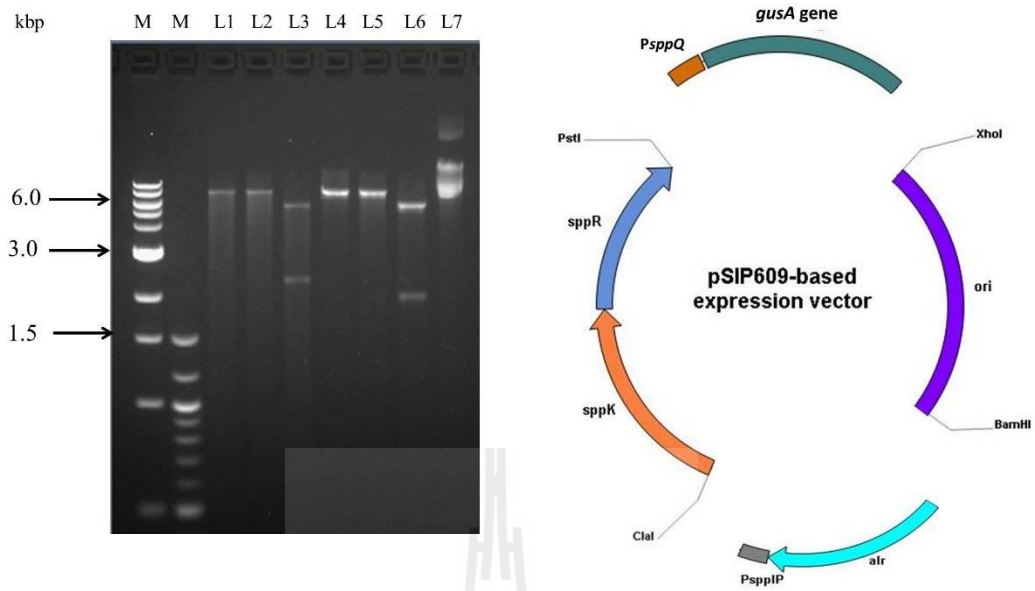


Figure 18. Restriction fragment analysis of pSIP609gusA. M;1 kbp and 100 bp DNA leader. L1; cut with *PstI*. L2; cut with *XhoI*. L3; double digest with *PstI* and *XhoI* showing *gus*A gene. L4; cut with *BamHI*. L5; cut with *ClaI*. L6; double digest with *BamHI*. and *ClaI* showing *alr* gene. L7; uncut vector.

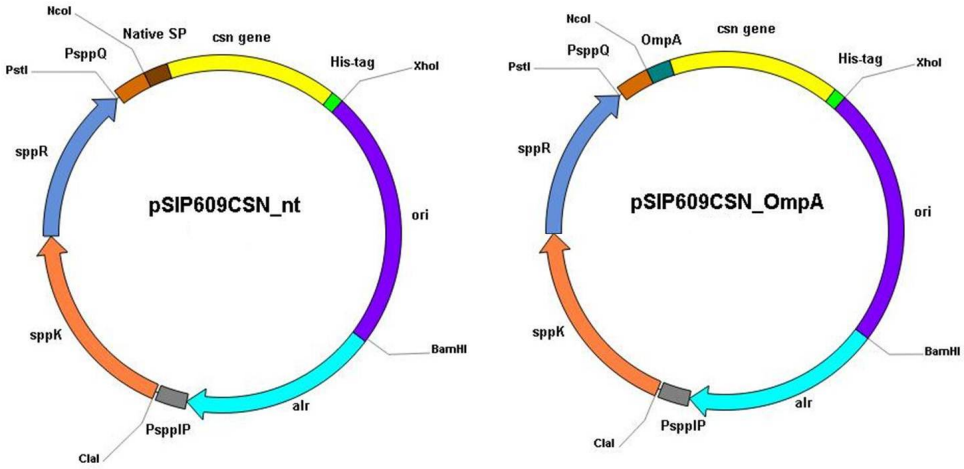


Figure 19. The map of pSIP609CSN_nt(A) and pSIP609CSN_OmpA (B), of which the *erm* genes were exchanged with *alr* gene.

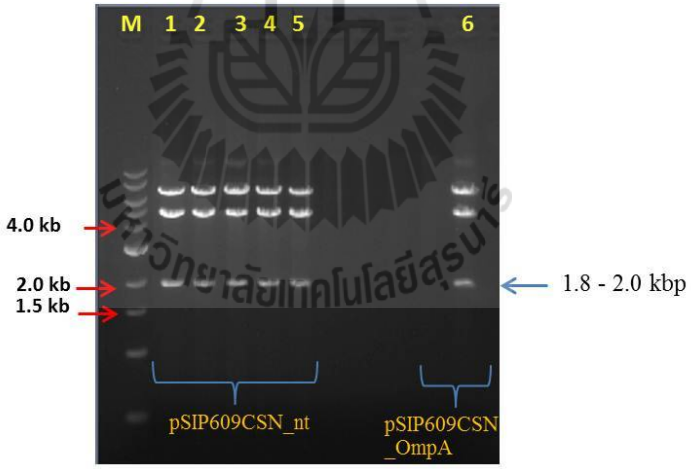


Figure 20. Restriction fragment analysis of recombinant constructs. M; 1 kb DNA leader. Lane 1-5; pSIP609CSN_nt clone1-5. Lane 6; pSIP609CSN_OmpA clone1. Recombinant vectors were double digested with *BamHI* and *ClaI*. The bands with the size of the *alr* gene of about 1.8 - 2.0 kbp.

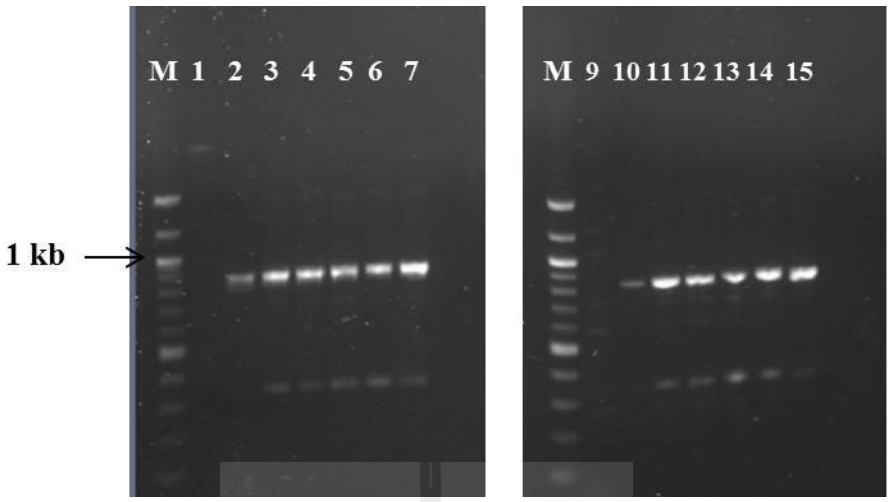


Figure 21. Colony PCR to confirm the presence of *csn* genes in *L. plantarum* TLG02. M; 1 kbp DNA leader. Lane1 and 9; Negative control (pSIP609GUSA). Lane 2 and 10; Positive control (pSIP409CSN_nt and pSIPCSN_OmpA. Lane 3-7; pSIP609CSN_nt clone 1-5. Lane 11-15; pSIP609CSN_OmpA clone 1-5.

4.2 Optimization of the expression of recombinant chitosanase gene in *L. plantarum* WCFS1

4.2.1 Determination of optimal duration of induction

SDS-PAGE analysis of Csn from recombinant *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA at different time point after induction is shown in Figure 22 (recombinant *csn* containing native signal peptide) and Figure 23 (recombinant *csn* containing OmpA peptide). The recombinant *csn* showed a molecular weight of approximately 30 kDa. These results correspond to the hypothetical molecular weight (MW) of the mature recombinant enzyme of 30.35 kDa, without the signal peptide. The enzymes could be found both in cell and culture supernatant as shown in Table 1. Our results indicated that the yield of the construct containing native signal peptide was much higher than those containing OmpA signal peptide. At 6 hr after induction, the chitosanase enzyme in the culture supernatant was the highest. Therefore, in the next experiment, the enzyme was harvested after a 6-h induction time.

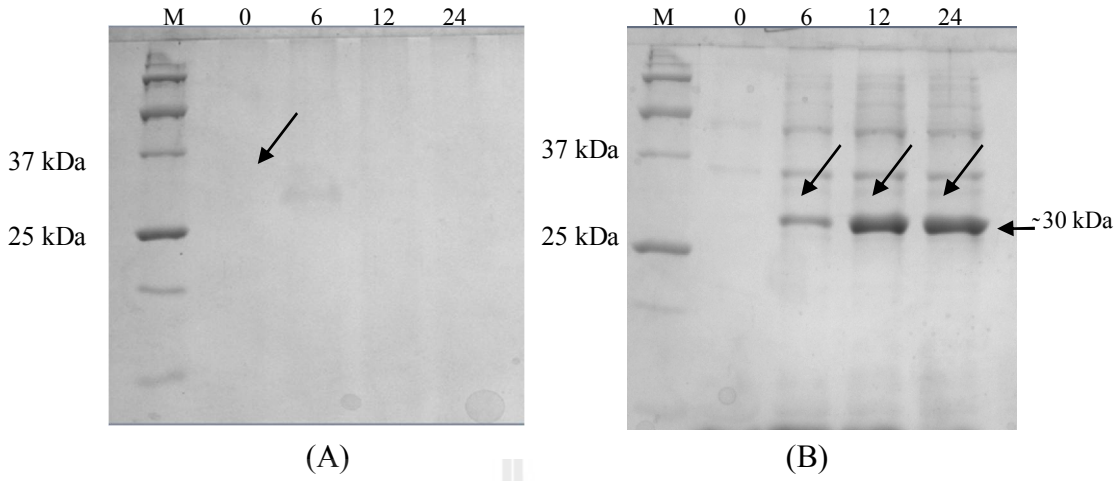


Figure 22. Expression and secretion of recombinant *B. subtilis* chitosanase containing *B. subtilis csn* native signal peptide. The enzyme were taken at 0, 6, 12, and 24 hr after induction with IP-673 and analyzed by SDS-PAGE (A) samples from supernatant, (B) samples from cell extract.

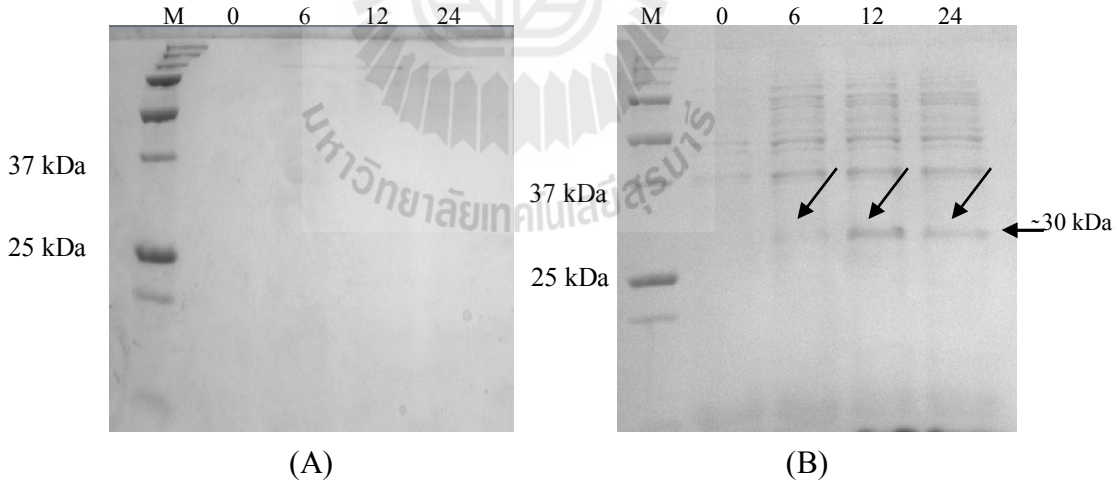


Figure 23. Expression and secretion of recombinant *B. subtilis* chitosanase containing OmpA signal peptide. The enzyme were taken at 0, 6, 12, and 24 hr after induction with IP-673 and analyzed by SDS-PAGE. (A) samples from culture supernatant, (B) samples from cell extract.

Table 1. Chitosanase activity of recombinant *csn* from different compartments at different time points after induction

Type of SP	Time (hr)	Enzyme activity (U/ml)		Volumetric activity (U/L)		
		Supernatant	Cell	Supernatant	Cell	Total
B. subtilis csn native SP	0	0.52	0.55	516	55	571
	6	4.25	36.8	4,250	3,670	7,920
	12	0.71	235	707	23,500	24,200
	24	0.72	166	721	16,600	17,300
OmpA	0	0.55	0.55	547	55	602
	6	1.42	8.65	1,420	865	2,280
	12	0.65	23.7	653	2,370	3,020
	24	0.59	11.8	594	1,180	1,770

The data represent average values obtained in three-replication from one-independent experiments.

4.2.2 Determination of the inducer concentration

Studies with different concentrations of peptide pheromone (IP-673) were performed on the sakacin P-based expression vectors, pSIP409CSN_nt, in *L. plantarum* WCFS1. In this strain, the *csn* activity was found to be IP dose-dependent. After induction at 6 hr, forty milliliters of culture were taken at OD_{595} ~ 1.4-1.6 (approx. 6 hr). Dose-response of the pSIP409CSN_nt system was investigated by adding different IP-673 concentrations in the range of 0, 1.563, 3.125, 6.25, 12.5, 25.0, 50.0, and 75.0 ng/mL (Figure 24). The optimum activity was achieved essentially at 12.5 ng/mL. The concentrations of IP-673 above 12.5 ng/mL up to 75 ng/mL showed essentially the same response as 12.5 ng/mL for csn activity from supernatant. There was very low activity in non-induced cultures. It can be concluded that an optimal concentration of inducer for *csn* gene expression was at 12.5 ng/mL of IP-673.

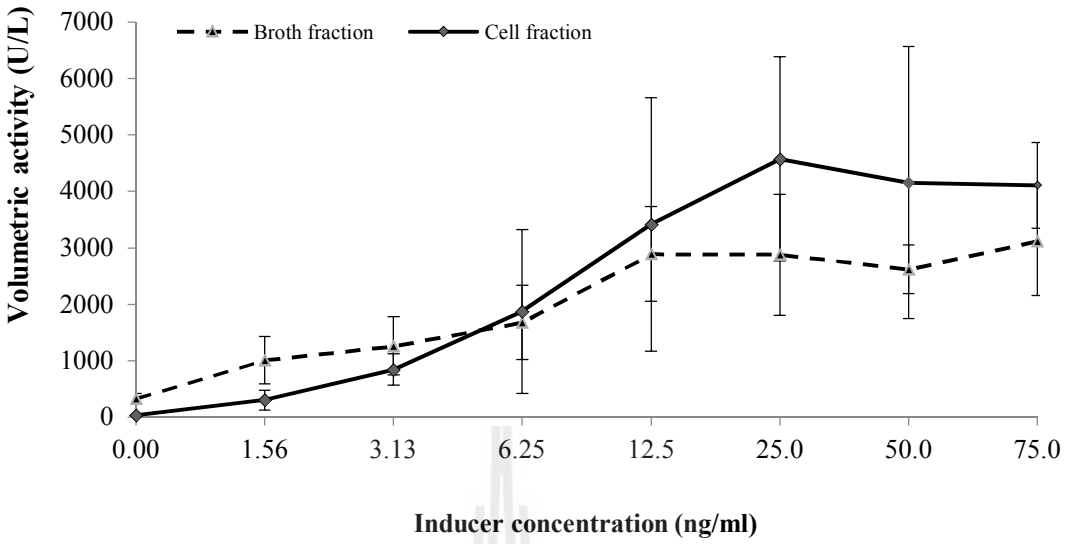


Figure 24. The expression of *csn* gene in *L. plantarum* WCFS1 harboring pSIP409 CSN_nt at various inducer concentration. Each data point shows *csn* activity after a 6-h induction at a given concentration of IP and is the average of three–replication from three-independent experiments.

4.2.3 Expression of recombinant *csn* in *L. plantarum* in 100-mL of culture volume

The *csn* enzymes from supernatant and cell were collected and analyzed. The result of the recombinant *B. subtilis* chitosanase from difference compartments was illustrated in figure 25 and the chitosanase protein bands were shown at approximately 30 kDa. The expression of recombinant *csn* obtained in laboratory cultivations as approximately 11,600±1,560 U and 3,340±269 U of chitosanase activity per liter of medium when using the native and the OmpA signal peptide, respectively. The secretion efficiencies (ratio of extracellular to intracellular activity) were approximately 50.9 % and 89.9% for the systems using the native and the OmpA SP (Table 2). These results suggested that both *B. subtilis* and *E. coli* signal peptides were able to direct the secretion of recombinant chitosanase in *L. plantarum*. The OmpA SP seemed to be more efficient in directing the secretion of the recombinant enzymes into the culture media, but the yield was lower

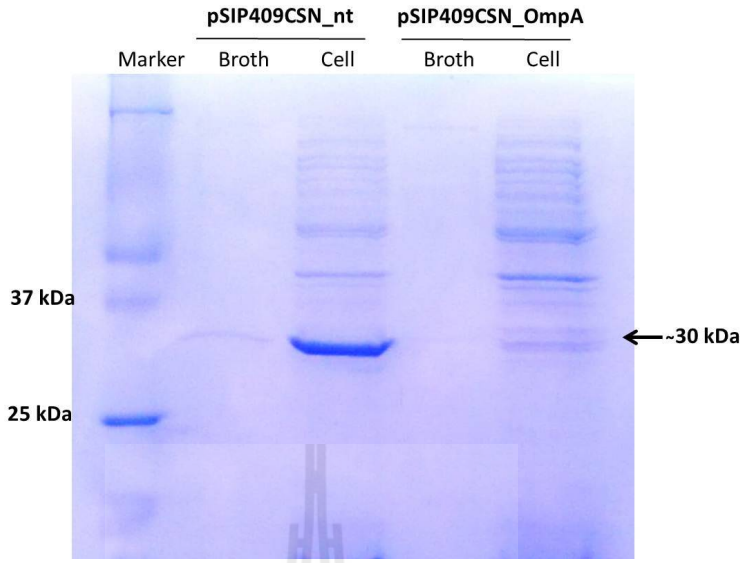


Figure 25. Expression of recombinant chitosanase in *L. plantarum* WCFS1. SDS-PAGE analysis illustrates the crude recombinant Csn_nt and Csn_OmpA from supernatant and cell extract.

Table 2. Chitosanase activity and secretion efficiency of recombinant *csn*

Type of SP	Volumetric activity (U/L)			% secretion efficiency
	supernatant	Cell fraction	Total	
B. subtilis csn native SP	5,900[a]	5700[b]	11,600[b]	50.9[a]
OmpA	3,000[a]	337[a]	3,340[a]	89.9[b]

The data represent average values obtained in three-replication of two-independent experiments. Unit of enzyme was performed from 100 ml of culture medium.

Note: Each value is mean ± SD. Means within columns and with different letter ([a, b]) are significantly different ($p < 0.05$).

4.2.4 Expression of recombinant *csn* in *L. plantarum* WCFS1 in 500-mL of culture volume

The recombinants *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA were induced with 12.5 ng/mL of IP-673 at O.D. values~0.3. Seventy milliliters of culture were harvest at 0, 3, 6, 9, 12, and 20 hr. after induction for approx. 2.0 h. The result showed that two types of chitosanase coud be secreted into broth medium and presented the maximum yield of chitosanase activity after induction for 3 hr. The maximum expressions of recombinant *csn* were approximately 10,700±25 U and 4,970±411 U of chitosanase activity per liter of medium when using the native and the OmpA signal peptide, respectively. The secretion efficiencies were approximately 79.0% and 89.0% for the systems using the native and the OmpA SP (Table 3). At 6 hr, the enzyme activities were approximately 10,100±1,450 U and 4,650±492 U of chitosanase activity per liter of medium when using the native and the OmpA signal peptide, respectively. The secretion efficiencies were approximately 74.9% and 89.7% for the systems using the native and the OmpA SP (Figure 26).

The pH of medium and the growth of recombinant strain harboring pSIP409CSN_nt and pSIP409CSN_OmpA and were checked at different time points of the cultivation at 30 °C, anaerobic condition and 150 rpm. The pH of medium was gradually reduced to final of pH 4. Our results indicated that after about 6 h of cultivation, these two strains reached the end of exponential phase (Figure 27).

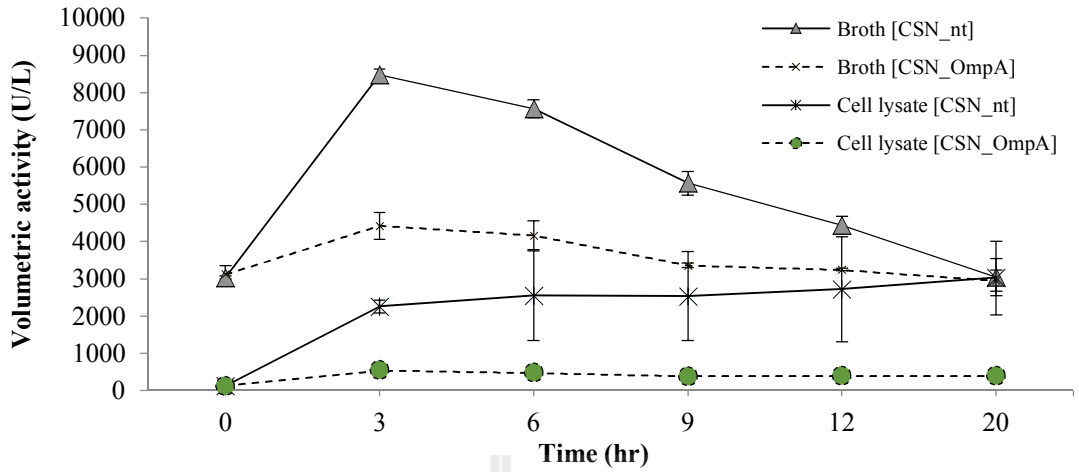


Figure 26. The *csn* activity of *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA at various time points. The cultures were induced at O.D.600~0.3. Each data point shows *csn* activity that was the average of two-independent experiments.

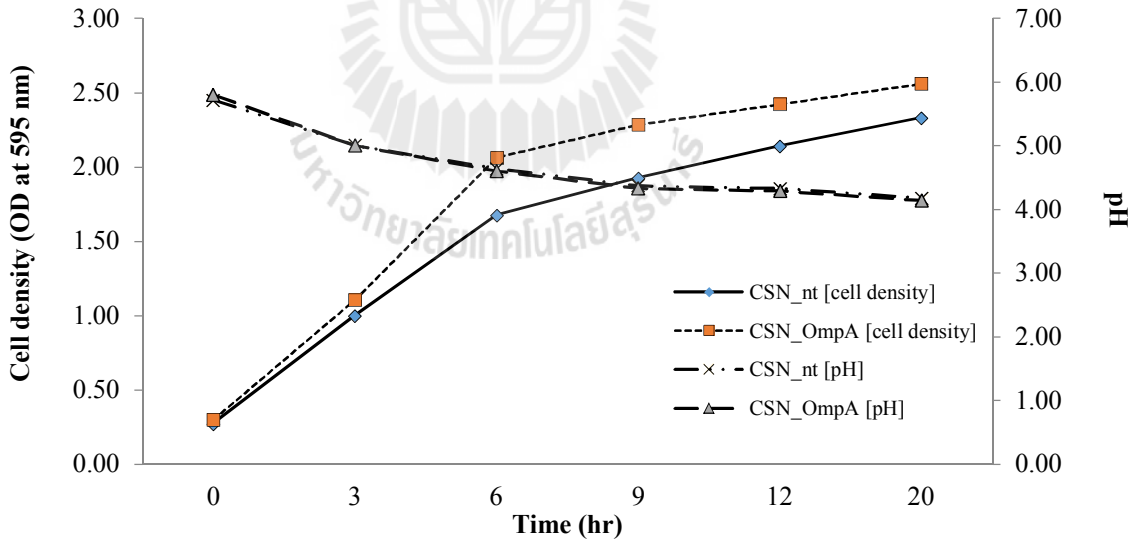


Figure 27. Growth and pH curve of the recombinant *L. plantarum* containing pSIP409CSN_nt and pSIP409CSN_OmpA. Each data point shows *csn* activity that was the average of two- independent experiments.

Table 3. Volumetric activity (U/L) of recombinant *B. subtilis* chitosanase fused to different signal peptides**,** in different compartments.

Time	The recombinant *csn* enzyme fused with *B. subtilis csn* native signal peptide				The recombinant *csn* enzyme fused with *E. coli* outer membrane protein (OmpA)			
	Supernatant	**Cell**	**Total**	**% secretion**	**Supernatant**	**Cell**	**Total**	**% secretion**
0	3,030±43^{a}	124±14^{a}	3,150±29^{a}	96.1^{b}	3,130±226^{ab}	128±18^{a}	3,250±207^{a}	96.1^{b}
3	8,480±145^{c}	2,260±170^{a}	10,700±25^{c}	79.0^{ab}	4,420±363^{c}	548±548^{b}	4,970±411^{c}	89.0^{a}
6	7,570±245^{c}	2,540±1,200^{a}	10,100±1,450^{c}	74.9^{ab}	4,170±382^{bc}	480±110^{b}	4,650±492^{bc}	89.7^{ab}
9	5,560±326^{b}	2,540±1,190^{a}	8,100±1,520^{bc}	68.7^{ab}	3,360±64^{ab}	386±69^{ab}	3,740±4^{ab}	89.7^{ab}
12	4,450±236^{b}	2,720±1,400^{a}	7,170±1,170^{bc}	62.0^{ab}	3,250±30^{ab}	391±20^{ab}	3,640±50^{ab}	89.3^{ab}
20	3,050±499^{a}	3,020±993^{a}	6,070±494^{ab}	50.2^{a}	2,950±281^{a}	396±100^{ab}	3,350±181^{a}	88.2^{a}

The data represent average values obtained from the average of the three-replication from two-independent experiments.

Unit of enzyme was performed from 500 ml of culture medium.

One-Way ANOVA was applied to preparation about different between control and sample groups from sampling random of each treatment, and show mean ± standard deviation (X±S.D.) of each treatment by Turkey-Kramer post-hoc test at significant $p<0.05$.

4.2.5 Preliminary analysis of food-grade expression of recombinant *csn* gene in *L. plantarum* in 100-mL of culture volume

Observation of the food-grade *csn* expression system was based on the optimal condition for pSIP-based expression system. Even though, secretion efficiencies are the same, the yield obtained from the construct containing native signal peptide was higher; therefore, the recombinant *L. plantarum* TLG02 harboring pSIP609CSN_nt was used in the assay for food-grade expression system. One hundred milliliters of culture were collected at O.D. value~1.4-1.6 (approximately 6 hr) after induction with 25 ng/mL of IP-673. The SDS-PAGE analysis of recombinant *csn* from difference fraction was shown in figure 28. The molecular weight of the recombinant enzyme was approximately 30 kDa. The result showed that the expression of recombinant *csn* in laboratory cultivation was approximately 12,600 U of total chitosanase activity per liter (Table 4). This food-grade expression system will be further optimized and investigated for the food-grade production of *csn* in the future.

Table 4. Volumetric activity (U/L) of recombinant food-grade chitosanase

Type of SP	Compartments	Volumetric activity (U/L)
B. subtilis csn native SP	supernatant	8,700[b]
	Cell extract	3,900[a]
	Total	12,600

Unit of enzyme was performed in the average of three-replication from 100 ml of culture medium.

Note: Each value is mean ± SD. Means within columns and with different letter ([a, b]) are significantly different ($p < 0.05$).

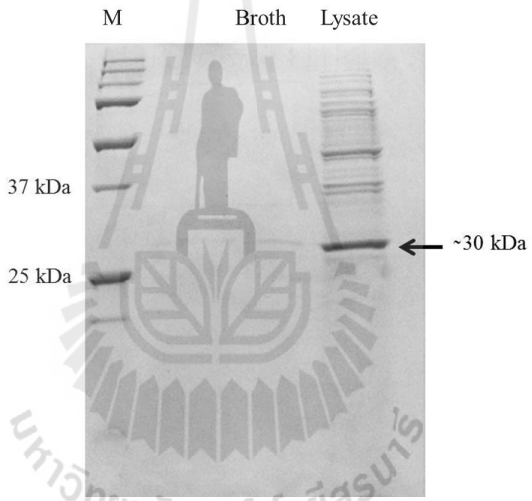


Figure 28. SDS-PAGE analysis of recombinant Csn from food-grade expression system.

CHAPTER V

DISCUSSION

The expression of *csn* gene, encoding GH46 chitosanase from *B. subtilis,* which is suitable for various biotechnological applications has been previously reported (Pechsrichuang *et al.*, 2013). However, the *E. coli* expression system is not suitable for food applications. The work in this thesis is the first report on expression and secretion of recombinant chitosanase (Csn) in *L. plantarum* WCFS1, which is a food-grade expression host. Two types of the recombinant chitosanase (*csn*) gene were sub-cloned into pSIP409-based expression vector by sticky PCR-based method. The first construct, designated pSIP409CSN_nt, contained the csn gene fused with *B. subtilis csn* native signal peptide (SP) sequence containing 35 amino acids (MKI SMQ KAD FWK KAA ISL LVF TMF FTL MMS ETV FA); while the second construct, pSIP409CSN_OmpA vector, the mature enzyme was fused with signal peptide of the *E. coli* outer membrane protein (OmpA) (MKK TAI AIA VAL AGF ATV AQA). After demonstrated that both of these constructs could be expressed and secreted in *L. plantarum*, a food-grade system for *csn* gene expression in *Lactobacillus* was generated by replacing erythromycin resistant gene (*erm*) in pSIP409-based vector with *alr* gene. The *alr* gene is a food-grade selection marker that encodes alanine racemase enzyme. This enzyme is essential in *L. plantarum* because it is used to convert L-alanine to D-alanine, which is involved in the bio-synthesis of cyclosporine in the cell wall of *L. plantarum* (Palumbo *et al.*, 2004).

Therefore, this expression system is applicable for enzyme production in food, feed or pharmaceutical applications as it can be used to produce recombinant Csn without the use of antibiotic form a food grade host.

Recently, it has been reported that both native and OmpA SP could be used to direct the secretion of *bacillus* extracellular enzymes using *E. coli* expression system. (Yamabhai *et al.*, 2008; Songsiriritthigul *et al.*, 2010a; Songsiriritthigul *et al.*, 2010b; Pechsrichuang *et al.*, 2013). In this research, it was found that the recombinant Csn could be expressed and secreted out of the cell of *L. planatarum*, when either the native or OmpA SP were used as signal peptide as well. The optimal expression of recombinant Csn could be achieved after induction with 12.5 ng/mL of peptide pheromone (IP-673) (Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg) (Brurberg *et al.*, 1997) , when the OD_{595} reached approximately 0.3. The maximum yields of Csn from *L. plantarum* system were approximately 10,700±25 U/L (Csn_nt) and 4,970±411 U/L (Csn_OmpA) from 500-ml shake flask culture. These yields were lower than previous report in the *E. coil* expression system (Pechsrichuang *et al.*, 2013), which showed routinely about 57,000 U of total Csn activity in 1-L shake flask culture. The study of secretion efficiencies of recombinant Csn from this research revealed that the *E. coil* OmpA signal peptide seemed to be more efficient in directing the secretion of the Csn to the supernatant (89.0%) when compare to the Csn with native signal peptide (79.0%). However, the total yield of Csn fusing OmpA SP was twice lower than Csn containing native SP. Interestingly, the amount of secreted chitosanase from *L. plantarum* WCFS1 were the same as the secretion efficiency of those obtained from *E.coil* expression system (78.9%) (Pechsrichuang *et al.*, 2013). The secretion efficiencies of both chitosanase seemed to

be more efficient than the secretion of *L. amylovorus* amylase (69%), of which Amy SP is coupled to its natural cognate amylase gene, from the same *L. plantarum* expression system (Mathiesen *et al.*, 2008). Since, *B. subtilis csn* native SP sequence belongs to Gram-positive bacteria, which is the same type of cell wall polymer as those of *L. plantarum*; therefore, it can be expected that the secretion machinery of *L. plantarum* can recognized *Bacillus* signal peptide as well. However, our results indicated that a signal peptide from Gram-negative bacteria (OmpA) could also be used to direct the secretion of Csn out of *L. plantarum* cell wall as well. Nevertheless, the yield obtained from this constructs was much lower. These results confirmed previous observation that it is very difficult to rationalize expression level and secretion efficiency (Brockmeier *et al.*, 2006). The difference in secretion efficiency was observed when different culture volume was used. The secretion efficincies of Csn_nt from 500-mL and 100-mL of culture volume after 6-h of induction were approximately 74.9%, 50.9%, respectively. These data indicated that the effect of agitation and the different culture volume may affect the secretion efficiency recombinant enzyme.

To create a food-grade expression system, the recombinant Csn construct contaning native signal peptide was used because the expression level was much higher. Preliminary study of the expression of Csn from food-grade expression system showed enzyme activity in both supernatant and cell extract. The food-grade *csn* gene expression levels obtained from 100-mL of cultivations was approximately 12,600 U of total chitosanase activity per liter. This result demonstrated that the food-grade expression system containing the recombinant *csn* gene could be used as an

alternative sources for the production of recombinant chitosanases for food, feed, or pharmaceutical applications in the future.

CHAPTER VI

CONCLUSION

1. The recombinant *Bacillus subtilis* chitosanase (*csn*) was successfully cloned into *Lactobacillus* expression vector (pSIP-based expression vector) using sticky-PCR based method. The recombinant constructs contained the *csn* gene fused with different signal peptides, i.e. *B. subtilis csn* native signal peptide (SP) and the signal peptide of the *E. coli* outer membrane protein (OmpA).
2. Production and secretion of the recombinant *B.subtilis* chitosanase from *L. plantarum* could be obtained using pSIP-based expression vector when either native or OmpA were used as a signal peptide.
3. The expression of the *csn* gene was controlled by using a peptide pheromone as inducer. Both enzyme activity and secretion efficiency were compared for the different constructs. Expression levels obtained from 500 mL of cultivation at the optimal condition were approximately 10,700±25 U and 4,970±411 U of chitosanase activity per liter of medium when using the native and the OmpA SP, respectively. The secretion efficiencies were approximately 79.0% and 89.0% for the systems using the native and the OmpA SP. The enzyme activity obtained from 100 mL of cultivation was approximately 11,600±1,560 U and 3,340±269 U of chitosanase activity per liter of medium and showed the secretion efficiencies 50.9% and 89.9% for the systems using the native and the OmpA SP.

4. The food-grade *csn* gene expression levels obtained from 100 mL of cultivations was approximately 12,600 U of total chitosanase activity per liter of medium, when the native signal peptide was used.
5. This is the first report on the expression and secretion of csn gene from *B.subtilis* from *L. plantarum* food-grade expression system.

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APPENDICES

APPENDIX A

REAGENT PREPERATION

10% Ammonium persulfate (APS)	**1**	**mL**
Ammonium persulfate	0.1	g
Add DI H_2O to	1	mL
Use frash solution and do not storage		
1% Chitosan solution	**1**	**L**
Chitosan low molecular weight	10	g.
1 M acetic acid	90	mL
Add DI H_2O	400	mL
Mix well and adjust pH 5.5 with 1 M Sodium acetate		
Adjust DI H_2O to	1	L
Remark: Chitosan low molecular weight [product number 448869 (75–85% DDA)] was purchased from Sigma–Aldrich.		
200 mg/mL D-alanine solution	**2**	**mL**
D-alanine	400	mg
Add DI H_2O	2	mL
Sterilize the solution using 0.22 μm filter and storage -30 °C		
Destaining solution	**1**	**L**
Methanol	400	mL
Glacial acetic acid	100	mL
Add DI H_2O to	1	L

3,5 Dinitrosalicylic acid (DNS solution)	**250**	**mL**
3,5 Dinitrosalicylic acid	2.5	g
2 N NaOH	50	mL
Potasium sodium tartrate	75	g
add DI H_2O to	250	mL
Stir for overnight		
100% Glycine solution		
Glycine	100	g
add DI H_2O to	100	mL
Autoclave the solution for 15 minutes at 121 °C		
Luria bertani (LB)	**1**	**L**
Bacto-Tryptone	10	g
Bacto-yeast extract	5	g
NaCl	5	g
Agar (for agar medium)	15	g
Adjust volume by deionized water (ddH_2O) to	1	L
Autoclave the solution for 15 minutes at 121 °C		
30% Polyethylene glycol 1500 (or 1450) solution	**100**	**mL**
Polyethylene glycol 1500 (or 1450)	10	g
add DI H_2O to	100	mL
Autoclave the solution for 15 minutes at 121 °C		

100 µg/mL Peptide pheromone (IP-673)

2 mg/mL peptide pheromone (IP-673)	50	µL
DNase,RNase-free H_2O	950	µL
Storage -30 °C		
4X Runing gel buffer	**200**	**mL**
Tris	36.3	g
Adjust pH 8.8 with HCL and add DI H_2O to	200	mL
Storage 4 °C in the dark		
4X Stacking gel buffer	**50 mL**	
Tris	3	g
Adjust pH 6.8 with HCL and add DI H_2O to	50	mL
Storage 4 °C in the dark		
10% SDS	**100**	**mL**
Sodium dodecyl sulfate	10	g
dd DI H_2O to	100	mL
Staining solution	**1**	**L**
Coomassie brilliant blue R-250	1.25	g
Methanol	400	mL
Glacial acetic acid	100	mL
Add DI H_2O to	1	L

10X Tank buffer	**1**	**L**
Tris	36.3	g
Glycine	144.13	g
Sodium dodecyl sulfate	10	g
Add DI H_2O to	1	L
12.5% Running gel SDS-PAGE (for 4 gel)		
40%acrylamine	4.7	mL
4X Running gel buffer	3.75	mL
10% SDS	0.15	mL
DI H_2O	6.35	mL
10% SDS	75	µL
TEMED	7	µL
4% Stacking gel SDS-PAGE (for 4 gel)		
40%acrylamine	0.499	µL
4X Stacking gel buffer	1.25	mL
10% SDS	50	µL
DI H_2O	3.166	mL
10% SDS	25	µL
TEMED	12	µL
200 mg/mL Erythromycin solution	**5**	**mL**
Erythromycin	500	mg
Ethanol	5	mL
Storage -30 °C in the dark		

APPENDIX B

COMPETENT CELL PREPARATION

1. **Preparation of chemically competent *Escherichia coli* TOP10 cells and chemical transformation**

Materials:

- Ca/Glycine buffer

0.6 M $CaCl_2$	100	mL
0.5 M Pipas	20	mL
Glycine [100% used]	100	mL
Add DI H_2O to	1000	mL

Stirilize by autoclaving

- LB medium

Procedure:

1. A single *E. coli* TOP 10 colony was picked and cultivated in 10 mL of LB broth, overnight in a shaking incubator at 37 º C, 250 rpm.
2. The culture was innoculated of 1.5 mL of culture into 250 mL of LB broth and grown at 37 º C, with shaking at 250 rpm, around 4 hr until OD_{600} ~ 0.5-0.6.
3. The culture was placed on ice for 10 min. The culture was transferred into 50 mL Nunc tubes and centrifuged at 4 º C at 4000 rpm for 10 min.

4. The supernatant was discarded and the pellet re-suspended in 40 mL of the ice-cold Ca/Glycine buffer. The cell suspension was centrifuged at 4 º C at 3000 rpm for 10 min.
5. The supernatant was discarded and the pellet re-suspended in 40 mL of the ice-cold Ca/Glycine buffer and incubated on ice for 30 min . The cell suspension was centrifuged at 4 º C at 3000 rpm for 10 min.
6. The supernatant was discarded and the pellet re-suspended in 6 mL of the ice-cold Ca/Glycine buffer. The 100 µL of re-suspension of culture were dispensed in eppendorf tubes and placed in liquid nitrogen. Then, the competent cells were stored at – 80 ºC.

Transforming chemically competent *E. coli* TOP10 cells

1. Thaw competent *E. coli* TOP10 cells on ice and add DNA, pipette gently to mix (1µl of prepped plasmid is more than enough).
2. Place for 30 minutes on ice.
3. Incubate cells for 90 seconds at 42^{o}C and incubate cells on ice for 5 min.
4. Add 900 µL of LB broth and incubate for 1 hour at 37^{o}C on shaker at 150 rpm.
5. Spread 30, 100 µl and spread all onto a plate made with 800 µg/mL of erythromycin and grow overnight at 37 °C.

2. Preparation of chemically competent *Escherichia coli* MB2159 cells and chemical transformation

Materials:

- Ca/Glycine buffer

0.6 M $CaCl_2$	100	mL
0.5 M Pipas	20	mL
Glycine [100% used]	100	mL
Add DI H_2O to	1000	mL
Stirilize by autoclaving		

- LB medium with D-alanine 200 µg/mL

Procedure:

1. A single *E. coli* MB2159 colony was picked and cultivated in 10 mL of LB broth with D-alanine 200 µg/mL, overnight in a shaking incubator at 37 º C, 250 rpm.
2. The culture was innoculated of 2 mL of culture into 200 mL of LB broth with D-alanine 200 µg/mL and grown at 37 º C, with shaking at 250 rpm until OD_{600} ~ 0.5-0.6 for approximately 2.30 h.
3. The culture was placed on ice for 10 min. The culture was transferred into 50 mL Nunc tubes and centrifuged at 4 º C at 4000 rpm for 10 min.
4. The supernatant was discarded and the pellet re-suspended in 40 mL of the ice-cold Ca/Glycine buffer. The cell suspension was centrifuged at 4 º C at 3000 rpm for 10 min.

5. The supernatant was discarded and the pellet re-suspended in 40 mL of the ice-cold Ca/Glycine buffer and incubated on ice for 30 min . The cell suspension was centrifuged at 4 ° C at 3000 rpm for 10 min.
6. The supernatant was discarded and the pellet re-suspended in 4.8 mL of the ice-cold Ca/Glycine buffer. The 100 µL of re-suspension of culture were dispensed in eppendorf tubes and placed in liquid nitrogen. Then, the competent cells were stored at – 80 ºC.

Transforming chemically competent *E. coli* MB2159 cells

1. Thaw competent *E. coli* MB2159 cells on ice and add DNA, pipette gently to mix (1µl of prepped plasmid is more than enough).
2. Place for 30 minutes on ice.
3. Incubate cells for 90 seconds at 42^{o}C and incubate cells on ice for 5 min.
4. Add 900 µL of normal LB broth and incubate for 1 hour at 37^{o}C on shaker at 150 rpm.
5. Spread 30, 100 µl and spread all onto a normal LB plate and grow overnight at 37 °C.

3. Preparation of electro-competent *L. plantarum* WCFS1 cells and electro-transformation

Materials:

- MRS (with 1% glycine)
- 30% PEG-1500 (or PEG 1450)
- MRS (with 0.5 M sucrose, 0.1 M $MgCl_2$)
- MRS medium with appropriate antibiotics
- Electro-competent *L. plantarum* WCFS1
- Electroporation cuvettes

Procedure:

1. Grow *L. plantarum* WCFS1 overnight in MRS at 37°C
2. Make a serial dilutions from 10^{-2} to 10^{-8} in MRS + 1% glycine, and grow these overnight at 37°C.
3. Stop the precultures with OD_{600} = 2.5 ± 0.5. Dilute 1 to 20 in fresh MRS + 1% glycine and incubate this culture at 37° until exponential phase (OD_{600} = 0.70 ± 0.05)
4. Keep the culture on ice for 10 minutes.
5. Centrifuge for 10 minutes at 4°C, 4500 rpm.
6. Re-suspend the pellet in 0.5 - 1 culture volume in ice-cold 30% PEG-1450
7. Keep the cell suspension on ice for 10 minutes.
8. Centrifuge for 10 minutes at 4°C, 4500 rpm.
9. Re-suspend the pellet in 1/50 - 1/100 culture volume in ice-cold 30% of PEG-1450.

10. The cells are kept on ice until use or pipette aliquots of 40 µl into pre-cool and sterile eppies and store these at -80 °C.

Electroporation of *L. plantarum* WCFS1 competent cells

1. Fill an ice-cold electroporation cuvet with 40 µl competent cells (freshly prepared or thawed out from -80°C deep freezer and kept on ice).
2. Add 1 - 5 µg DNA.
3. Set the electroporator as follows in voltage 1.5 kV
4. Place the dry electroporation cuvet in the gene pulser and give electric pulse.
5. Incubate 2 minutes on ice and
6. Transfer the contents of the cuvet into a sterile eppi with 500 µl MRS + 0.5M sucrose + 0.1 M $MgCl_2$ and incubate 1 - 2 hours at 37 °C.
7. Plate the dilutions on MRSA plates with a suitable antibiotic.
8. Incubate the plates 1 - 3 days at 37 °C until the colonies are visible.

4. Preparation of electro-competent *L. plantarum* TLG02 cells and electro-transformation

Materials:

- MRS (1% glycine, 200 mg/mL D-alanine)
- 30% PEG-1450
- MRS (0.5 M sucrose, 0.1 M $MgCl_2$)
- MRS medium with 200 mg/mL D-alanine
- Electro-competent *L. plantarum* WCFS1
- Electroporation cuvettes

Procedure:

1. Grow *L. plantarum* WCFS1 overnight in MRS at 37°C
2. Make a serial dilutions from 10^{-2} to 10^{-8} in MRS (1% glycine, 200 mg/mL D-alanine), and grow these overnight at 37°C.
3. Stop the precultures with OD_{600} = 2.5 ± 0.5. Dilute 1 to 20 in fresh MRS (1% glycine, 200 mg/mL D-alanine) and incubate this culture at 37° until exponential phase (OD_{600} = 0.70 ± 0.05)
4. Keep the culture on ice for 10 minutes.
5. Centrifuge for 10 minutes at 4°C, 4500 rpm.
6. Re-suspend the pellet in 0.5 - 1 culture volume in ice-cold 30% PEG-1450
7. Keep the cell suspension on ice for 10 minutes.
8. Centrifuge for 10 minutes at 4°C, 4500 rpm.
9. Re-suspend the pellet in 1/50 - 1/100 culture volume in ice-cold 30% of PEG-1450.

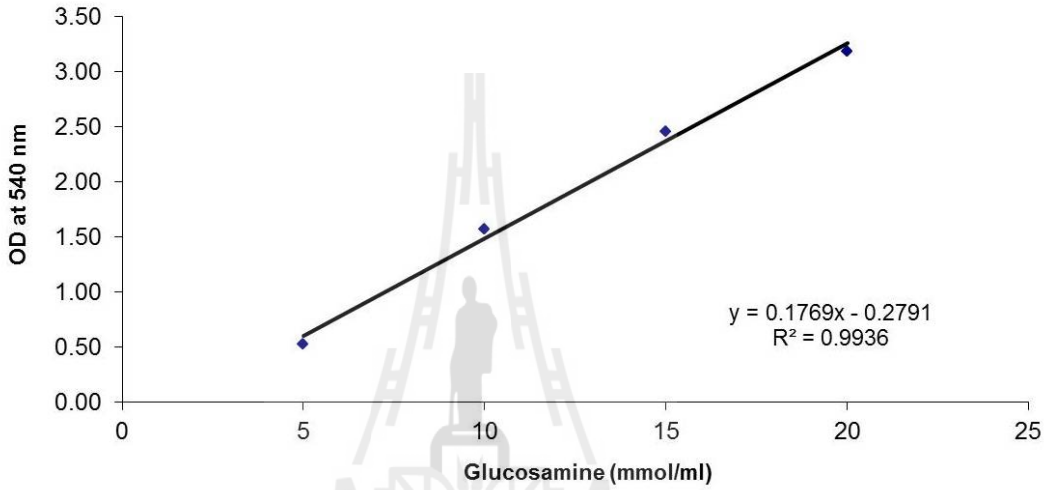
10. The cells are kept on ice until use or pipette aliquots of 40 µl into pre-cool and sterile eppies and store these at -80 °C.

Electroporation of *L. plantarum* TLG02 competent cells

1. Fill an ice-cold electroporation cuvet with 40 µl competent cells (freshly prepared or thawed out from -80°C deep freezer and kept on ice).
2. Add 1 - 5 µg DNA.
3. Set the electroporator as follows in voltage 1.5 kV
4. Place the dry electroporation cuvet in the gene pulser and give electric pulse.
5. Incubate 2 minutes on ice and
6. Transfer the contents of the cuvet into a sterile eppi with 500 µL of MRS (0.5 M sucrose, 0.1 M $MgCl_2$) and incubate 1 - 2 hours at 37 °C.
7. Plate the dilutions on MRS plates.
8. Incubate the plates 1 - 3 days at 37 °C until the colonies are visible.

APPENDIX C

STANDARD CURVE

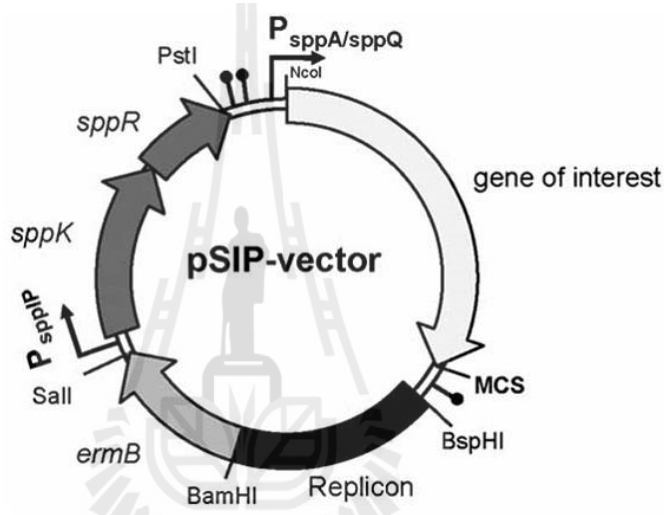


Glucose standard curve for determining chitosanase activity using DNS method.

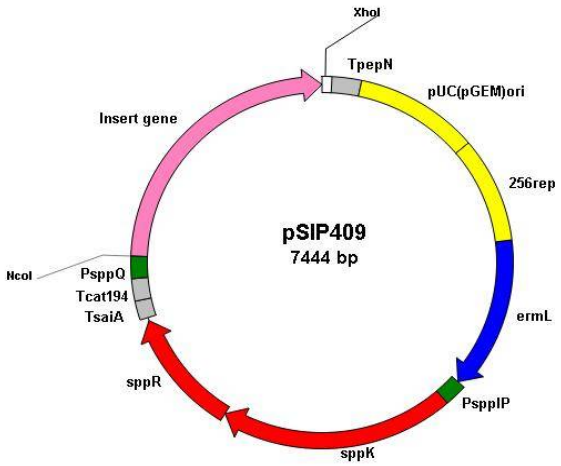
APPENDIX D

VECTOR MAPPING AND DNA SEQUENCE

1. Schematic overview of the modular pSIP-vector expression system.



2. Schematic overview of pSIP409 plasmids that is used in this study



3. Amino acids sequence alignment of pSIP409CSN_nt and pSIP409CSN_OmpA

```
pSIP409CSN_ntC1     MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFAAGLNKDQKRRAEQLTSIFENGTTEI 60
pSIP409CSN_ntC2     MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFAAGLNKDQKRRAEQLTSIFENGTTEI 60
pSIP409CSN_OmpA     ----------MKKTAIAIAVALAGFATVAQAS---AGLNKDQKRRAEQLTSIFENGTTEI 47
                              **:**:: *    *: : . :   *************************

pSIP409CSN_ntC1     QYGYVERLDDGRGYTCGRAGFTTATGDALEVVEVYTKAVPNNKLKKYLPELRRLAKEESD 120
pSIP409CSN_ntC2     QYGYVERLDDGRGYTCGRAGFTTATGDALEVVEVYTKAVPNNKLKKYLPELRRLAKEESD 120
pSIP409CSN_OmpA     QYGYVERLDDGRGYTCGRAGFTTATGDALEVVEVYTKAVPNNKLKKYLPELRRLAKEESD 107
                    ************************************************************

pSIP409CSN_ntC1     DTSNLKGFASAWKSLANDKEFRAAQDKVNDHLYYQPAMKRSDNAGLKTALARAVMYDTVI 180
pSIP409CSN_ntC2     DTSNLKGFASAWKSLANDKEFRAAQDKVNDHLYYQPAMKRSDNAGLKTALARAVMYDTVI 180
pSIP409CSN_OmpA     DTSNLKGFASAWKSLANDKEFRAAQDKVNDHLYYQPAMKRSDNAGLKTALARAVMYDTVI 167
                    ************************************************************

pSIP409CSN_ntC1     QHGDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDVRYDDLMNPANHDTRDEWR 240
pSIP409CSN_ntC2     QHGDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDVRYDDLMNPANHDTRDEWR 240
pSIP409CSN_OmpA     QHGDGDDPDSFYALIKRTNKKAGGSPKDGIDEKKWLNKFLDVRYDDLMNPANHDTRDEWR 227
                    ************************************************************

pSIP409CSN_ntC1     ESVARVDVLRSIAKENNYNLNGPIHVRSNEYGNFVIKGSVDHHHHHH-- 287
pSIP409CSN_ntC2     ESVARVDVLRSIAKENNYNLNGPIHVRSNEYGNFVIKGSVDHHHHHH-- 287
pSIP409CSN_OmpA     ESVARVDVLRSIAKENNYNLNGPIHVRSNEYGNFVIKGSVDHHHHHHHH 276
                    **************************************************
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APPENDIX E

POSTER PRESENTATION

Production and secretion of *Bacillus subtilis* chitosanase using a food-grade expression system

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[2] Laboratory of Food Biotechnology, BOKU University of Natural Resources and Life Sciences, Vienna, Austria
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Abstract :

Nowadays, food industry and commercial production have been growing extensively. Food-grade gene expression systems are alternative choices and useful for food applications, because they are considered as safe and carry the "Generally Recognized As Safe" (GRAS) status for lactic acid bacteria. We constructed expression vectors based on the pSIP system, containing the chitosanase (*csn*) gene from *Bacillus subtilis*, fused to the *B. subtilis csn* native signal peptide (SP) as well as the signal peptide of the *E. coli* outer membrane protein (OmpA) expressing in the food-grade host *Lactobacillus plantarum*. Expression of the *csn* gene was controlled by using a peptide pheromone as inducer, and both enzyme activity and secretion efficiency were compared for the different constructs. Expression levels obtained in laboratory cultivations were approximately 11600 U and 3340 U of chitosanase activity per liter of medium when using the native and the OmpA SP, respectively. The secretion efficiencies (ratio of extracellular to intracellular activity) were 51% and 90% for the systems using the native and the OmpA SP. In conclusion, these constructs resulted in efficient secretion of the recombinant target protein. Currently, we are changing the selection marker in these constructs by replacing the antibiotic resistance gene with the alanine racemase gene, thereby obtaining true food-grade expression systems.

The aim:

The aim of this research was to apply a food-grade expression system based on the pSIP expression vectors for the over-expression and secretion of the recombinant chitosanase from *B. subtilis* in *L. plantarum*. Our result demonstrated that this food-grade expression system could be used as an alternative sources for the production of recombinant chitosanases for food, feed, or pharmaceutical applications.

Methodology:

Molecular cloning **:** The *csn* genes encoding chitosanase from *Bacillus subtilis* 168, fused to different signal peptides including *B. subtilis csn* native signal peptide (SP) and the signal peptide of the *E. coli* outer membrane protein (OmpA) (Pechsrichuang et al, 2013), were cloned by a sticky PCR-based method according to a published protocol (Yamabhai, 2009). The PCR products were compatible with *NcoI* and *XhoI* restriction sites of pSIP *erm*-based expression vectors, resulting pSIP409CSN_nt and pSIP409CSN_OmpA (Fig 1). Subsequently, food-grade expression systems for recombinant *csn* genes were obtained by replacing the erythromycin resistance (*erm*) gene with the alanine racemase (*alr*) gene, resulting pSIP609CSN_nt and pSIP609CSN_OmpA (Fig 3B, 3C.)

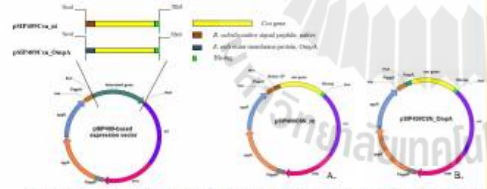

Figure 1. Map of pSIP409CSN_nt (A) and pSIP409CSN_OmpA (B), which is a pSIP-based vector for the expression of Csn_nt and Csn_OmpA, respectively.

Expression of recombinant csn: The overnight culture of *L. plantarum* WCFS1 harboring pSIP409CSN_nt and pSIP409CSN_OmpA were diluted in fresh MRS containing erythromycin to an OD_{600} of ~0.1 and incubated at 30 ºC without agitation and anaerobic condition. The cell were induced at an OD_{600} of ~0.3 by adding 12.5 ng/mL of peptide pheromone IP-673. Cells were harvested at an OD_{600} of 1.5-1.6 (around 6 h).

Chitosanase assay and protein determination : Chitosanase activity was analyzed by dinitrosalicylic acid (DNS) method (Miller, 1959). Chitosan was used to be substrate for analysis of chitosanase activity. Protein concentrations were determined by Bradford method (Bradford 1976), according to the manufacturer's protocol (Biorad), using bovine serum albumin (BSA) as standard.

Results and discussion :

***Expression of recombinant chitosanase enzymes in L. plantarum* WCFS1**

L. plantarum WCFS1 was used as a host for the expression of recombinant *B.subtilis* chitosanase containing native and OmpA signal peptide. Cultures were taken after induction at OD_{600} ~ 1.5-1.6 (around 6 h of expression time). SDS-PAGE analyses of crude enzyme obtained from culture supernatant and cell lysate were illustrated in Fig. 2, the chitosanase protein bands were shown at approximately 30 kDa.

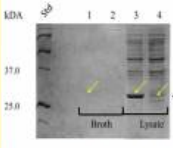

Figure 2. SDS-PAGE analysis illustrates these crude recombinant Csn_nt (lane 1) and Csn_OmpA (lane 2) from supernatant; and crude recombinant Csn_nt (lane 3) and Csn_OmpA (lane 4) from cell lysate

The expression of recombinant Csn obtained in laboratory cultivations were approximately 11600 U and 3340 U of chitosanase activity per liter of medium when using the native and the OmpA signal peptide, respectively. The secretion efficiencies (ratio of extracellular to intracellular activity) were 51% and 90% for the systems using the native and the OmpA SP (Table1). These results suggested that both *Bacillus* and *E. coli* signal peptides were able to direct the secretion of recombinant Csn in *L. plantarum*. The OmpA SP seemed to be more efficient in directing the secretion of the recombinant enzymes into the culture media.

However, recombinant *Csn* could be expressed at a much higher level when the native signal peptide was used. Therefore, it is very difficult to rationalize expression level and secretion efficiency, as has been previously investigated for other enzymes (Brockmeier et al, 2006).

Table 1 Chitosanase activity and secretion efficiency of recombinant *Csn*

Type of signal peptide	Fraction	Volumetric activity (U/L)	% Secretion efficiency
B. subtilis csn native SP	Broth supernatant	5900 ± 950	~51 %
	Cell lysate	5670 ± 610	
	Total	*11600 ± 1560*	
OmpA SP	Broth supernatant	3000 ± 215	~90 %
	Cell lysate	376 ± 55	
	Total	*3340 ± 269*	

Construction of food-grade expression vectors for the expression of Bacillus Csn: The food-grade expression vectors for the expression of *csn* were constructed by replacing erythromycin resistant gene of the pSIP409CSN_nt and pSIP409CSN_OmpA vectors with alanine racemase (*alr*) gene, resulting pSIP609CSN_nt and pSIP609CSN_OmpA, respectively. Selection of recombinant *L. plantarum* containing the expression vector can be done by growing appropriate strain of the bacteria (TLG02) without using antibiotic. The integrity of the food-grade constructs were confirmed by digesting vectors with *BamHI* and *ClaI* restriction enzymes (Fig. 3). The bands with the size of the *alr* gene of about 1.8 - 2.0 kbp are indicated by an arrow. These expression vectors will be further investigated for the food-grade production of *Csn* in the future.

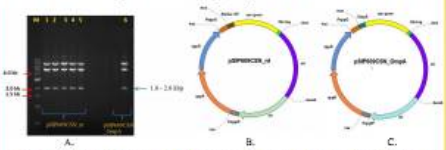

Figure 3. Construction of food-grade expression vectors. (A) Restriction fragment analysis of recombinant constructs. The map of pSIP609CSN_nt (B) and pSIP609CSN_OmpA (C), of which the *erm* genes were exchanged with *alr* gene, are shown.

Conclusion:

Production and secretion of the recombinant *B.subtillis* chitosanase from *L.plantarum* could be obtained using pSIP-based expression vector when either native or OmpA were used as a signal peptide.

Acknowledgement : This is research was supported by National Research Council of Thailand (NRCT), Suranaree University of Technology (SUT) and by the Higher Education Research Promotion and National Research University Project of Thailand (NRU), Office of the Higher Education Commission.

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Production and secretion of *Bacillus subtilis* chitosanase using a food-grade expression system

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Abstract

The applications of green and white biotechnology for sustainable environment have become an important issue. Nowadays, food industry and commercial production have been growing extensively. To achieve an increase in the demand and market expanding, recombinant DNA technology is necessary for the production of various food modifying enzymes and proteins. Food-grade gene expression systems are alternative choices and useful for food applications, because they are considered as safe and carry the "Generally Recognized As Safe" (GRAS) status for lactic acid bacteria. We constructed expression vectors based on the pSIP system, containing the chitosanase (*csn*) gene from *Bacillus subtilis*, fused to different signal peptides. These included the *B. subtilis csn* native signal peptide (SP) as well as the signal peptide of the *E. coli* outer membrane protein (OmpA). These expression vectors were constructed for expression in the food-grade host *Lactobacillus plantarum*. Expression of the *csn* gene was controlled by using a peptide pheromone as inducer, and both enzyme activity and secretion efficiency were compared for the different constructs. Expression levels obtained in laboratory cultivations were approximately 12000 U and 3400 U of chitosanase activity per liter of medium when using the native and the OmpA SP, respectively. The secretion efficiencies (ratio of extracellular to intracellular activity) were 51% and 90% for the systems using the native and the OmpA SP. In conclusion, these constructs resulted in efficient secretion of the recombinant target protein. Currently, we are changing the selection marker in these constructs by replacing the antibiotic resistance gene with the alanine racemase gene, thereby obtaining true food-grade expression systems.

Keywords: Chitosanase, Lactobacillus plantarum, secretion, signal peptide.

BIOGRAPHY

Miss Peenida Namvijitr was born on December 18, 1983 in Khonkhan Province, Thailand. She received hes Bachelor's Degree in Agricultural Technology (Food Technology) form Suranaree University of Technology in 2006. After graduation, she has been employed under the position of research and development supervisor by Surapon Foods Public Co.,Ltd. In 2011, she decided to further study for master degree in the field of Molecular Biology at school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her research topic was production and secretion of *Bacillus* chitosanase in *L. plantarum* food-grade expression system. The results from part of her study have been poster presented and published in FOOD INNOVATION ASIA CONFERENCE 2014 "Science and Innovation for Quality of Life" 12-13 June, 2014 BITEC, Bangkok, Thailand.