Final Report

Study of glutamate decarboxylase gene from GABA-producing lactic acid bacteria isolated from *Kung-Som*, the traditional Thai fermented shrimp

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ABSTRACT

Kung-Som is one of several traditional Thai fermented shrimp products, especially popular in the southern part of Thailand. This is the first report to reveal the bacterial communities in the finished product of *Kung-Som*. Ten *Kung-Som* samples were evaluated using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methodology combined with appropriated primers to study the dynamics of the bacterial population. Two primers sets (V3; 341f(GC)-518r and *rpo*B; *rpo*B1698f(GC)-*rpo*B2014r primers) were considered as a possible tool for study the communities of bacteria. PCR-DGGE analysis of both the V3-region and *rpo*B amplicon was successfully applied to discriminate between lactic acid bacteria (LAB) and other Gram positive strains in the bacterial communities of *Kung-Som* products. These preliminary results concluded that the main microbiology of finished product of *Kung-Som* was LAB and coagulase negative cocci (CNC).

Kung-Som presents a high glutamic acid which is a major substrate for biosynthesis of natural γ-aminobutyric acid (GABA) by LAB. The GABA-producing LAB from *Kung-Som* products were isolated, screened and identified. The strain CS3 showed the highest GABA-producing ability among the screened strains. Based on an API-CHL50 fermentation and phylogenetic trees of 16S rDNA sequence, the strain CS3 belonged to genus *Lactobacillus futsaii*. Newly *L. futsaii* CS3 with high GABA-synthesizing capacity was first discovered in the present report. The open reading frame (ORF) of glutamate decarboxylase (*gad*) gene was cloned by PCR. It was 1,410 bp encoding a polypeptide of 469 amino acids. In addition, the non-crystallization structure of *L. futsaii* CS3 glutamate decarboxylase enzyme (GAD) was also predicted by SWISS-MODEL server. The entire ORF sequence of *gad* gene was cloned into pColdI expression vector and expressed in *Escherichia coli* BL21 (DE3). The *gad* gene with His_6-Tag was expressed. The recombinant GAD was purified using a Ni-NTA column. SDS-PAGE analysis revealed that it had a molecular weight of approximately 53 kDa, which corresponded to the predicted size of the deduced protein (53.64 kDa). The results of these findings offer a way of replacing chemical GABA by natural GABA in fermented foods or functional foods. Moreover, it preliminary provides useful details for development of the molecular mechanism regulating GABA metabolism in valuable LAB.

บทคัดย่อ

กุ้งส้ม เป็นอาหารหมักชนิดหนึ่งที่นิยมบริโภคกันมากในภาคใต้ของประเทศไทย งานวิจัยครั้งนี้ศึกษาความหลากหลายของแบคทีเรียในผลิตภัณฑ์กุ้งส้ม โดยสุ่มเก็บตัวอย่างกุ้งส้มจากตลาดในจังหวัดสงขลา จำนวน 10 ตัวอย่าง นำมาวิเคราะห์โดยใช้เทคนิค PCR-DGGE ร่วมกับการใช้ primer 2 คู่ คือ ตำแหน่ง V3 ของ 16S rDNA และยีน *rpo*B เพื่อศึกษาประสิทธิภาพของ primer ทั้ง 2 คู่ ในการจำแนกชนิดของแบคทีเรียในกุ้งส้ม จากการตรวจวิเคราะห์พบว่า ตำแหน่ง V3 ของ 16S rDNA และยีน *rpo*B สามารถแยกแยะความแตกต่างระหว่างแบคทีเรียแลกติกและแบคทีเรียแกรมบวกอื่นๆในกุ้งส้มได้ และพบว่าผลิตภัณฑ์กุ้งส้มประกอบไปด้วย แบคทีเรียกลุ่มหลัก คือ แบคทีเรียกลุ่มแลกติกและกลุ่ม coagulase negative cocci เช่น *Staphylococcus piscifermentans* เป็นต้น

กุ้งส้มประกอบไปด้วยกรดอะมิโนกลูตามิกสูง ซึ่งแบคทีเรียแลกติกสามารถใช้กรดกลูตามิกเป็นสับสเตรทในการสังเคราะห์สารกาบาได้ ดังนั้นงานวิจัยชิ้นนี้ได้ทำการแยกและคัดเลือกแบคทีเรียแลกติกที่มีความสามารถในการผลิตสารกาบาจากกุ้งส้ม พบว่า แบคทีเรียแลกติกสายพันธุ์ CS3 ผลิตสารกาบาได้สูงที่สุด เมื่อนำมาจำแนกสายพันธุ์โดยใช้เทคนิคทางชีวเคมี (การหมักน้ำตาล) และ เทคนิคทางชีวโมเลกุล (หาลำดับเบสในส่วนของ 16S rDNA) พบว่า จัดเป็นสายพันธุ์ *Lactobacillus futsaii* ซึ่งยังไม่มีการรายงานมาก่อนว่าสายพันธุ์นี้มีความสามารถผลิตสารกาบา นอกจากนี้ยังได้ศึกษายีนที่ควบคุมการสร้างกาบา (glutamate decarboxylase gene) โดยเทคนิค PCR พบว่า ยีนนี้มี open reading frame ขนาด 1,410 คู่เบส และสามารถถอดรหัสได้ 469 อะมิโน อีกทั้งทำนายโครงสร้างสามมิติโดยใช้โปรแกรม SWISS-MODEL จากนั้นทำการโคลนยีนเข้าสู่เวกเตอร์ pColdI และศึกษาการแสดงออกของโปรตีนในแบคทีเรีย *Escherichia coli* BL21 (DE3) หลังจากการทำบริสุทธิ์รีคอมบิแนนซ์โปรตีนโดยใช้ โครมาโตกราฟีแบบจำเพาะ (Ni-NTA column) และวิเคราะห์ขนาดรีคอมบิแนนซ์โปรตีนโดยวิธี SDS-PAGE พบว่า รีคอมบิแนนซ์โปรตีนมีขนาดประมาณ 53 กิโลดาลตัล ซึ่งมีค่าใกล้เคียงตามที่ทำนายกับโปรแกรม ExPASy จากผลการทดลองดังกล่าว สามารถใช้เป็นข้อมูลพื้นฐานในการประยุกต์ใช้เชื้อแบคทีเรียแลกติกสายพันธุ์ CS3 เพื่อทดแทนการใช้สารกาบาสังเคราะห์ในอุตสาหกรรมอาหารได้

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ABBREVIATIONS

A.	=	*Aerococcus*
B.	=	*Bacillus*
E.	=	*Escherichia*
Ent.	=	*Enterococcus*
Lc.	=	*Lactococcus*
L.	=	*Lactobacillus*
Leu.	=	*Leuconostoc*
Ped.	=	*Pediococcus*
Ps.	=	*Pseudomonas*
S.	=	*Staphylococcus*
Sac.	=	*Saccharomyces*
Strep.	=	*Streptococcus*
Tetra.	=	*Tetragenococcus*
V.	=	*Vibrio*
W.	=	*Weissella*

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Nowadays, the consumers pay a lot of attention to the relation between food and health. As a consequence, the market for foods with health-promoting properties, so called functional foods, has shown a remarkable growth over the last few years. All of these, gamma-aminobutyric acid (GABA) is the one of substances that has several physiological functions and hence has a great application potential in functional foods. However, the direct addition of chemical GABA to food is regarded as unnatural and unsafe and is still illegal in Korea (Seok *et al*., 2008; Kim *et al*., 2009).

Kung-Som is one of Thai fermented foods that made from shrimp, sugar, salt and water (Hwanhlem *et al*., 2010) and is fermented with the natural microbial flora. The microbiology of *Kung-Som* is mainly lactic acid bacteria (LAB). Generally, LAB play a central role in fermentation processes, and have a long and safe history of application and consumption in the production of fermented foods and beverages (Leroy and Vuyst, 2004). In addition, LAB have been used as probiotics due to their properties and also protect foods by controlling the food spoilage pathogens by secreting acids, conjugated linoleic acid, vitamin, aroma compounds, bacteriocins, exopolysaccharides, GABA and enzymes (Djenane *et al*., 2005; Li and Cao, 2010; Dhakal *et al*., 2012). Therefore, the use of GABA-producing LAB strains as starter cultures in fermentation processes can help to achieve bio-synthetic production of the GABA, safety and many special physiological activities. This provides a way of replacing chemical GABA by natural GABA, at the same time offering the consumer with new, attractive food products. This reduces the production cost because of the omission the extra addition of GABA (Leroy and Vuyst, 2004; Li and Cao, 2010; Dhakal *et al*., 2012).

The biosynthesis of GABA is one step reaction of decarboxylating glutamate to GABA, catalyzed by glutamate decarboxylase enzyme (GAD). GAD acts as the essential enzyme for GABA production and needs a pyridoxal 5'-phosphate (PLP) as a cofactor. The glutamic acid or glutamate acts as a major substrate for biosynthesis of natural GABA by LAB. Fortunately, glutamic acid might be greatly found in *Kung-Som* products because shrimp contains a rich source of glutamic acid (Daul *et al*., 1994). Therefore, the development of *Kung-Som* containing GABA to contribute functional properties will be actively studied. However, the studies on GABA-producing LAB in *Kung-Som* have not been reported yet. Moreover, a few reports on the biochemical and molecular properties of

GAD have been published. Consequently, in this study we focused on the use of the hypervariable V3-region on the 16S rDNA and *rpo*B gene by using PCR-DGGE techniques as a tool to reveal the bacteria that commonly develop in the *Kung-Som* product. In addition, we isolated, screened and identified the newly GABA-producing LAB from *Kung-Som* products. Furthermore, the existence of *gad* gene from novel strain was also investigated for cloning and expression of the GAD protein combined with cold shock vector in *E. coli* system. These preliminary results may provide useful information for the potential expression of LAB *gad* gene in other microbes. It will further expand the application area of GABA-producing LAB to significantly enhance GABA production and value added of products in further studies.

Review of Literature

1. Bacterial community in a Thai traditional fermented foods

Fermentation is an ancient technique to preserve foods due to the inhibitory effect toward many microorganisms. It is undergoes relevant changes in the physico-chemical characteristics of raw material to product (Hutkins, 2006). *Kung-Som* is one of Thai fermented foods that made from shrimp, sugar, salt and water (Hwanhlem *et al*., 2010) and is fermented with the natural microbial flora at room temperature for 5-7 days according to family or local geographic preferences, especially in the southern parts of Thailand such as Nakhon Si Thammarat, Satun, Songkhla and Phatthalung Provinces. The microbiology of *Kung-Som* is various and complex. Many strains of microorganisms have already been isolated from *Kung-Som* by traditional methods. These microorganisms are mainly lactic acid bacteria (LAB).

Hwanhlem *et al*. (2010) isolated and screened LAB from *Kung-Som* at various fermentation periods. The results showed that only 274 isolates were identified as LAB using the criteria of being Gram positive and catalase negative. Of these 274 isolates, 120 isolates were cocci; 111 isolates were short rods and 43 isolates were rods. *L. plantarum* D10SM20, D8SM21, D10SM16 and D6SM3 as probiotics were screened from *Kung-Som*.

Miyashita *et al*. (2012) investigated the variety of LAB in fermented foods in Thailand. The 945 strains were isolated from 114 varieties of fermented foods from the northeastern, central and southern parts of the northern Thailand. The results showed that the isolates were divided into 50 groups of six genera: *Aerococcus, Enterococcus, Lactobacillus, Pediococcus, Tetragenococcus* and *Weissella*. Only one strain belonging to the genus *Aerococcus* showed sequence similarity to *A. viridans*. Forty-six strains were assigned to the genus *Enterococcus*. They were divided into six groups to which they were closely related, namely, *Ent. avium, Ent. faecalis, Ent. faecium, Ent. hirae, Ent. thailandicus*, *Ent. gilvus* and *Ent. raffinosus*. They sorted 309 strains belonging to the genus *Lactobacillus* showed sequence similarity to *L. acidipiscis, L. alimentarius, L. brevis, L. collinoides, L. crustorum, L. curvatus, L. farciminis, L. fermentum, L. futsaii, L. namurensis, L. nantensis, L. pantheris, L. paracasei, L. paralimentarius, L. plantarum, L. pobuzihii, L. saerimneri, L. senioris* and *L. versmoldensis*. Thirty-eight strains were classified in the genus *Pediococcus* (*Ped. acidilactici* and *Ped. pentosaceus*). Four isolates were classified in *Tetra. halophilus*. Twelve strains belonging to the genus *Weissella* were divided into five species, namely, *W. cibaria*, *W. confusa, W. paramesenteroides, W. thailandensis* and *W. viridescens*.

Additionally, in *Pla-som*, traditional Thai fermented fish, in early stages of the process were dominated by the presence of *Lc. garvieae*, *Strep. bovis* and *W. cibaria*. At 48 h of fermentation, *W. cibaria*, *Ped. pentosaceus*, *L. fermentum* and *L. plantarum* were prevalent, and gave way to a dominance of *L. plantarum* that completed the fermentation (Kopermsub and Yunchalard, 2010). Also, *Strep. salivarius*, *Ent. faecalis* (Hwanhlem *et al*., 2011), *L. plantarum*, *L. acidophilus*, *L. fermentum* and *L. pentosus* were found in these products (Panthavee *et al*., 2007).

In addition, *Tetra. halophilus*, *Ent. faecalis*, *Ent. hirae*, *Lactobacillus* sp., *L. farciminis*, *L. pentosus*, *L. plantarum* and *Leuconostoc* sp. were reported to be distributed in *Nam-pla*, *Budu*, *Tai-pla*, *Pla-ra*, *Pla-chom*, *Kung-chom*, and *Hoi-dong* products (Tanasupawat and Daengsubha, 1983; Tanasupawat *et al*., 1991; 1992a; b; c; 1998). Also, *Salinivibrio* species are generally isolated bacteria from fermented fish (*Pla-ra*) (Chamroensaksri *et al*., 2009).

Jaichumjai *et al*. (2010) also isolated and screened *L. plantarum* BCC9546 from *Nham* and used as the starter culture for *Nham* production. The type of microflora that develops is related to the fermentation time and raw materials. Mostly, lactobacillus species were the dominant LAB in the last stage of fermentation because they produce acid more slowly than cocci, but were more tolerant to acid than cocci (Moha Adnan and Tan, 2007; Kopermsub and Yunchalard, 2010; Hwanhlem *et al*., 2011). In addition, lactobacilli have been reported to exhibit probiotic properties (Papamanoli *et al*., 2003; Klingberg *et al*., 2005; Schillinger *et al*., 2005; Maragkoudakis *et al*., 2006). These varies between recipes depending on an initial food matrix, fermentation process, personal hygiene, local tradition or local geographic preferences, which are crucial factor to select for the growth of specific microbial communities.

In spontaneous fermented foods, LAB are widely distributed in nature and occur naturally as indigenous microflora. During the fermentation, LAB utilize carbohydrate substrates available in the fermentation system and produce organic acids, especially lactic acid as primary metabolites (Paludan-Müller *et al*., 2002) and also make a low pH value (4.0-5.9) (Hugas and Monfort, 1997; Riebroy *et al*., 2004). Moreover, many kinds of important products including conjugated linoleic acid, vitamins, aroma compounds, bacteriocins, exopolysaccharides, GABA and enzymes can be also produced as secondary metabolites by LAB. LAB is not only contributed to the taste, flavor, texture, the pleasant sensory profile and give a certain added value of the end product but also lower the product's pH which is one of the key factors to ensure quality and safety. Thus, LAB can

prolong the shelf life of foods. LAB possess special physiological activities and are generally regarded as safe (GRAS), and have been extensively utilized in food industries such as dairy products, cheeses, bread, fermented vegetables, meats, shrimp and fish, etc. (Cocolin *et al*., 2000; 2001c; 2004; Leroy and Vuyst 2004; Rantsiou *et al*., 2005a; c; Fontana *et al*., 2005a; b; Visessanguan *et al*., 2006; Lee *et al*. 2006; Yan *et al*., 2008; Randazzo *et al*., 2009; Karahan *et al*., 2010; Kopermsub and Yunchalard, 2010). Also, LAB have been used as probiotics due to their properties such as immunomodulation, inhibition of pathogenic bacteria, control of intestinal homeostasis, resistance to gastric acidity, bile acid resistance, and anti-allergic activity (Tuohy *et al*., 2003; Tannock, 2004; Nishida *et al*., 2008; Hwanhlem *et al*., 2010; Li and Cao, 2010).

On the other hand, many studies reported that not only LAB are important role in fermentation but also the coagulase-negative staphylococci (CNS) participate in the development of aroma, flavor, and color of fermented products, such as cassava fish (Anihouvi *et al*., 2007), *Pla-som* (Riebroy *et al*., 2004), *Pla-ra* (Tanasupawat *et al*., 1992b) and fermented meat or sausages (Cocolin *et al*., 2001b; 2004; Fontana *et al*., 2005a; b; Leroy and Vuyst 2004; Rantsiou *et al*., 2005b; c; Rantsiou and Cocolin, 2006). Also, the catalase positive cocci *S. carnosus* and *S. piscifermentans* were reported to be distributed in *Nam-pla*, *Budu*, *Tai-pla*, *Pla-ra*, *Pla-chom*, *Kung-chom* and *Hoi-dong* products (Tanasupawat and Daengsubha, 1983; Tanasupawat *et al*., 1991; 1992a; b; c; 1998).

The use of LAB and CNS as starter cultures are potential applied in fermented foods. Moreover, starter cultures LAB can produce some secondary metabolites as GABA which the potential as a bioactive component in foods and pharmaceuticals. Thus, the development of function foods containing GABA has been actively pursued. In recent years, many studies have been focused on the GABA production by using LAB (Kim *et al*., 2009; Coda *et al*., 2010; Di Cagno *et al*., 2010; Thwe *et al*., 2011; Cho *et al*., 2011; Kim and Kim, 2012). Therefore, the GABA-producing LAB is much interested in the field of fermented foods for a value added of products and help health benefits.

2. The GABA production by lactic acid bacteria in fermented foods

GABA is a four carbon, a non-protein amino acid (Fig.1) that is widely distributed in nature among microorganisms, plants and animals (Ueno, 2000). GABA is synthesized through the α-decarboxylation of L-glutamic acid in a reaction catalyzed by glutamate decarboxylase enzyme (GAD, EC 4.1.1.15) linked to the Kreb's cycle (Ueno, 2000; Komatsuzaki *et al*., 2008).

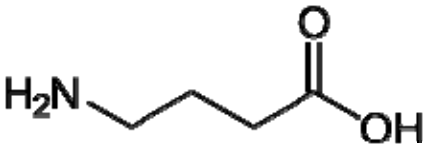


Figure 1 Structure of GABA

Source: Dhakal *et al*. (2012)

Nowadays, GABA is used considerably in functional foods and pharmaceuticals, and massively as a major active constitutes in several foods. GABA acts as the major inhibitory neurotransmitters in the central nervous system in mammalian brains. Clinical studies have related increased intake of GABA or analogues to several health benefits, including lowering of blood pressure of mildly hypertensive animals (Hayakawa *et al*., 2004) and humans (Inoue *et al*., 2003). GABA also improves the plasma concentration, growth hormones and the protein synthesis in the brain (Cho *et al*., 2007), but inhibits small airway-derived lung adenocarcinoma (Choi *et al*., 2006). Furthermore, GABA intake could help treat various neurological disorders such as seizures, Parkinson's disease, stiff-man syndrome, schizophrenia, tranquilizing, diuretic, sleeplessness, depression, autonomic disorders (Okada *et al*., 2000; Wong *et al*., 2003) and chronic alcohol-related symptoms (Oh *et al*., 2003). Hagiwara *et al*. (2004) reported that GABA strongly induced insulin secretion from the pancrease, therefore, effectively preventing diabetics (Adeghate and Ponery, 2002). In addition, the pancreatic β-cell can also produce GABA and exhibit high level of GAD activity (Okada *et al*., 1976; Gilon *et al*., 1991). GABA intake can regulate sensations of pain and anxiety and lipid levels in serum (Kono and Himeno, 2000; Miura *et al*., 2006). Furthermore, consumption of GABA-enriched foods can inhibit cancer cell proliferation (Park and Oh, 2007b) and improve memory and the learning abilities (Miura *et al*., 2006). Because of its physiological functions, GABA has been classified as a bioactive component in foods and pharmaceuticals. Generally, sufficient amounts of GABA can be produced in the human body. However, GABA production is sometimes inhibited by a lack of estrogen, zinc, or vitamins, or by an excess of salicylic acid and food additives (Oh *et al*., 2003; Cho *et al*., 2011).

2.1 Isolation source and GABA-producing LAB species

A number of microorganisms of bacteria, yeast and fungi have been reported to produce GABA (Komatsuzaki *et al*., 2005). Recent studies, the most interesting and practical group of bacteria for GABA production is LAB, which produce high levels of GABA (Kim and Kim, 2012; Seo and Lee, 2013; Ratanaburee *et al*., 2013). LAB possess

special physiological activities and are generally regarded as safe, and have been extensively utilized in food industries for a long time (Leroy and Vuyst, 2004; Yan *et al*., 2008; Karahan *et al*., 2010). It is clear that the GABA production by LAB is natural and safe. LAB also are some of the most commonly used microorganisms in food fermentation, and have been used not only in natural fermentation, but also often used as a starter culture to accelerate the fermentation time and consistency products. The primary function of LAB in fermentation system is to convert carbohydrates to several desired metabolites such as mainly lactic acid, acetic acid, alcohol and carbon dioxide. Moreover, LAB are capable of producing: amino acids and peptides as a result of proteolysis; lactate, bacteriocin and GABA as secondary metabolites. Some strains of LAB can catalyze the decarboxylation of glutamate, resulting in the release of the end products GABA and CO_2 (Fig.2) (Dhakal *et al*., 2012).

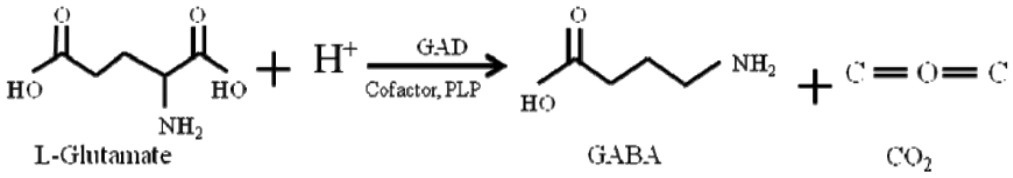


Figure 2 Decarboxylation of L-glutamate to GABA by glutamate decarboxylase (GAD). PLP: pyridoxal-5'-phosphate.
Source: Dhakal *et al*. (2012)

The GABA-producing LAB is mostly isolated from several fermented foods as previous reports. To date, *L. brevis* was isolated from many fermented foods, including, kimchi (Lee *et al*., 2010; Cho *et al*., 2011), Chinese traditional paocai (Li *et al*., 2008; Li *et al*., 2010b), fresh milk (Jiang *et al*., 2006; Huang *et al*., 2007a), cheeses (Siragusa *et al*., 2007) and fermented fish (Thwe *et al*., 2011). *L. farciminis* was also isolated from fermented fish (Thwe *et al*., 2011). *L. delbrueckii* subsp. *bulgaricus* was isolated from cheeses (Siragusa *et al*., 2007) and yoghurt (Watanabe *et al*., 2011). *Strep. thermophilus* was also isolated from yoghurt (Watanabe *et al*., 2011). *L. paracasei* was isolated from fermented fish (Komatsuzaki *et al*., 2005) and cheese (Siragusa *et al*., 2007). *L. plantarum* was isolated from cheeses (Siragusa *et al*., 2007; Coda *et al*., 2010; Di Cagno *et al*., 2010), fermented fish (Thwe *et al*., 2011) and fermented red seaweed beverage (Ratanaburee *et al*., 2011). *L. acidophilus* was also isolated from fermented red seaweed beverage (Ratanaburee *et al*., 2011). *L. buchneri* was isolated from kimchi (Cho *et al*., 2011). *L. helveticus* was isolated from fermented mare milk (*Koumiss*) (Sun *et al*., 2009). In addition, *Lc. lactis* was

isolated from cheeses (Siragusa *et al*., 2007; Rizzello *et al*., 2008), kimchi and yoghurt (Lu *et al*., 2008; 2009). Recently, the novel strain of *Leuconostoc* species (*Leuconostoc* NC5) was screened and selected with the GABA production from fermented shrimp (*Cincaluk*) (Farrah *et al*., 2009). In addition, two species of *Leuconostoc* (*Leuc. mesenteroides* and *Leuc. lactis*) and one species of *Weissella* (*W. viridescens*) were isolated and screened from kimchi, with the capacity to synthesize GABA under *in vitro* conditions (Kim and Kim, 2012). Currently, *Lactobacillus namurensis* was isolated from nham (Ratanaburee *et al*., 2013). Also, *Lactobacillus zymae* was isolated from kimchi (Park *et al*., 2014). Additionally, other GABA-producing LAB including *Pediococcus* (*Ped. acidilactici* and *Ped. pentosaceus*) and *Enterococcus* (*Ent. durans*, *Ent. faecalis* and *Ent. faecium*) have been slight reported (Chamba and Irlinger, 2004; Ratanaburee *et al*., 2013). Mostly, *Lactobacillus* species were reported as GABA-producing LAB which are the dominant LAB in the last stage of fermentation because they produce acid more slowly than cocci, but were more tolerant to acid than cocci (Mohd Adnan and Tan, 2007; Hwanhlem *et al*., 2010). Table 1 shows the production of GABA synthesized by different species of LAB isolated from various sources of fermented foods. Although, these LAB strains have already been isolated and identified, further research on isolation and characterization of the LAB is needed because various types of GABA-producing LAB are important for the food industry and also getting a huge attention (Komatsuzaki *et al*., 2005).

Table 1 Production of GABA by different microorganisms isolated from various sources

Strains	Isolation source	Culture medium	GABA production	References
Lc. lactis subsp. *lactis* 017	Cheese starter	Skim milk	2700 mg /l	Nomura *et al*. (1998)
L. brevis IFO-12005	Kimchi	Rice *shochu* distillery lees (*Kome shochu kusu*) + 10.5 mM MSG	1049.8 mg/ l	Yokoyama *et al*. (2002)
L. paracasei NFRI7415	Fermented fish (*funa-sushi*)	MRS medium + 500 mM MSG	31145.3 mg/l	Komatsuzaki *et al*. (2005)
L. brevis OPY-1 (KFCC 11337)	Kimchi	MRS medium + 5% MSG	825.3 mg/kg	Park and Oh (2005)
L. brevis OPK-3 (KFCC 11330)	Kimchi	MRS medium + 5% MSG	2023 mg/l	Park and Oh (2005)
L. brevis GABA057	NP	GYP medium + MSG	23381.0 mg/ l	Choi *et al*. (2006)
L. brevis hjxj-01	Fresh milk	GYP medium + MSG	7000 mg/l	Jiang *et al*. (2006)
L. brevis	Fresh milk	GYP medium + MSG	4599.2 mg/ l	Huang *et al*. (2007b)
L. buchneri MS	Kimchi	MRS broth + 5% MSG	25883.12 mg/l	Cho *et al*. (2007)
L. brevis BH2	Kimchi	MRS broth + MSG	19902.2 mg/l	Kim *et al*. (2007)

Table 1 (Cont.)

Strains	Isolation source	Culture medium	GABA production	References
L. plantarum C48	Cheeses	MRS medium + MSG	NP	Siragusa *et al*. (2007)
L. paracasei PF6	Cheeses	MRS medium + MSG	NP	Siragusa *et al*. (2007)
L. brevis PM17	Cheeses	MRS medium + MSG	NP	Siragusa *et al*. (2007)
L. delbrueckii subsp. *bulgaricus* PR1	Cheeses	MRS medium + MSG	NP	Siragusa *et al*. (2007)
Lc. lactis PU1	Cheeses	M17 medium + MSG	NP	Siragusa *et al*. (2007)
Strep. salivarius subsp. *thermophilus* Y2	NP	Nutrient medium + MSG	6000 mg/ l	Yang *et al*. (2006)
L. brevis	Paocai	MRS medium + MSG	15370.0 mg/ l	Li *et al*. (2008)
Lc. lactis subsp. *lactis* B	Kimchi and yoghurt	Brown rice juice, germinated soybean juice and skim milk (33:58:9, v/v/v)	6410 mg/l	Lu *et al*. (2008)
Lc. lactis subsp. *lactis* PU1	Cheese	Wheat flour	258.71 mg/kg	Rizzello *et al*. (2008)
Lactobacillus sp. OPK 2-59	Kimchi	Medium + MSG	180 mg/kg	Seok *et al*. (2008)

Table 1 (Cont.)

Strains	Isolation source	Culture medium	GABA production	References
Lc. lactis subsp. *lactis*	Chinese cabbage Kimchi	Brown rice juice, germinated soybean juice, and skim milk (33: 58: 9, v/ v/ v) and MSG	72 00 mg/ l	Lu *et al*. (2009)
L. brevis GABA 100	Kimchi	Black raspberry juice	13000 mg/l	Kim *et al*. (2009)
L. helveticus ND01	*Koumiss* (fermented mare milk drink)	Fermented skim milk	165.11 mg/l	Sun *et al*. (2009)
Leuconostoc NC5	Fermented shrimp (*Cincaluk*)	MRS medium + 50 mM MSG	292.86 mg/l	Farrah *et al*. (2009)
L. brevis NCL912	Paocai	Nutrient broth + 500 mM MSG	35662.0 mg/ l	Li *et al*. (2010b)
L. plantarum DSM19463	Cheeses	Grape must/whey milk/MRS	498.1 mg/ l	Di Cagno *et al*. (2010)
L. plantarum C48	Cheeses	Buckwheat, amaranth, chickpea and quinoa flours (1:1:5.3:1)	504 mg/kg	Coda *et al*. (2010)
L. brevis BJ20	Kimchi	MRS medium + 1% MSG	2,465 mg/ l	Lee *et al*. (2010)

Table 1 (Cont.)

Strains	Isolation source	Culture medium	GABA production	References
L. plantarum F311	Fermented fish	GYP medium + 5% MSG	82289.76 mg/l	Thwe *et al*. (2011)
L. farciminis D323	Fermented fish	GYP medium + 5% MSG	10054.2 mg/l	Thwe *et al*. (2011)
L. buchneri	*Mukeunjee* kimchi	MRS medium + 50 mM MSG	601.19 mg/l	Cho *et al*. (2011)
L. brevis	*Mukeunjee* kimchi	MRS medium + 50 mMMSG	202.12 mg/l	Cho *et al*. (2011)
L. plantarum DW12	Fermented red seaweed beverage	MRS medium + 0.5% MSG	4156 mg/l	Ratanaburee *et al*. (2011)
L. acidophilus YA031	Fermented red seaweed beverage	MRS medium + 0.5% MSG	4144 mg/l	Ratanaburee *et al*. (2011)
L. senmaizukei L-13	*Senmaizuke* (pickle)	GYP medium + MSG	80000 mg/l	Oda and Hiraga (2011)
L. delbrueckii subsp. *bulgaricus* IAM1120	Yoghurt	GYP medium + 1% MSG	less than 200 mg/l	Watanabe *et al*. (2011)
Strep. thermophilus IFO13957	Yoghurt	GYP medium + 1% MSG	less than 200 mg/l	Watanabe *et al*. (2011)

Table 1 (Cont.)

Strains	Isolation source	Culture medium	GABA production	References
Strep. thermophilus IFO13957	Yoghurt	GYP medium + 1% MSG	less than 200 mg/l	Watanabe *et al*. (2011)
Leu. mesenteroides	Kimchi	MRS medium + 10 mg MSG	NP	Kim and Kim (2012)
Leu. lactis	Kimchi	MRS medium + 10 mg MSG	NP	Kim and Kim (2012)
W. viridescens	Kimchi	MRS medium + 10 mg MSG	NP	Kim and Kim (2012)
L. brevis 340G	Kimchi	MRS medium + 1% MSG	1598.4 mg/l	Seo and Lee (2013)
L. namurensis NH2	Nham	MRS medium + 0.5% MSG	7339 mg/l	Ratanaburee *et al*. (2013)
Ped. pentosaceus HN8	Nham	MRS medium + 0.5% MSG	9096 mg/l	Ratanaburee *et al*. (2013)
L. zymae GU240	Kimchi	MRS medium + 3% MSG	NP	Park *et al*. (2014)

NP: Not provided; MRS medium: de Man, Rogosa and Sharpe medium; GYP medium: Glucose yeast extract poly-peptone medium; MSG: Monosodium glutamate.

2.2 Methods for screening GABA-producing LAB

Several methods are suitable for the detection of GABA in biological fluids, such as amino acid analyzer (Kono and Himeno, 2000; Komatsuzaki *et al*., 2005), gas chromatography (GC) (Kagan *et al*., 2008), high performance liquid chromatography (HPLC) (Cho *et al*., 2007; Kim *et al*., 2009), capillary liquid chromatographic/tandem mass spectrometric method (Song *et al*., 2005), and the flow-injection analysis (FIA) method based on GABase (Horie and Rechnitz, 1995). However, these methods require tedious sample preparation steps and are time consuming and can only analyze one sample each time. It is clear that they are not ideal methods in the screening work. Planar chromatography (Yokoyama *et al*., 2002; Li *et al*., 2008; 2009; Cho *et al*., 2007; 2011), pH indicator method (PIM) (Yang *et al*., 2006) and enzyme based microtiter plate assay (EBMPA) (Tsukatani *et al*., 2005) do not need expensive equipments, and are suitable for a parallel analysis of large numbers of samples, and therefore can be applied in high-throughput screening of GABA-producing strains.

For the PIM method, cells must be washed clean through several centrifugation and washing steps before they react with L-glutamic acid for a very long time (8-24 h). This method seems to be somewhat tedious and time-consuming. The EBMPA method needs the expensive GABase. In addition, components in culture medium may effect on the enzymatic reaction of GABase. There exists some difficulty to eliminate the interference factors. For planar chromatography, no any sample pretreatment and expensive chemical reagents are needed. Compared to the PIM and EBMPA methods, planar chromatography is a simple, convenient and inexpensive method for analysis of GABA. Many GABA-producing LAB strains have been isolated from some food samples by this method (Park and Oh, 2005; Cho *et al*., 2007; 2011; Li *et al*., 2008; Seok *et al*., 2008). The recently developed pre-staining planar chromatography has almost the same R_f values of the acids to those of the traditional method. On other hand, the pre-staining method is more clean, simple, convenient, inexpensive and reproducible (Li *et al*., 2009).

To reduce the workload and research cost, it is necessary to detect the content of GABA in samples to preliminarily determine whether GABA-producing LAB occur in the samples before screening (Siragusa *et al*., 2007; Li *et al*., 2008). The suspicious GABA-producing samples are then inoculated in the special medium (containing glutamate) for LAB isolation. After cultivation, the suspicious GABA producing cultures are selected from single colonies. The suspicious GABA-producing strains are further screened by HPLC. Finally, HPLC-MS should be used to confirm the results (Li *et al*., 2008).

2.3 Enzymatic properties of LAB GADs

Glutamate decarboxylase (L-glutamate-1-carboxylyase, EC 4.1.1.15, GAD) is a pyridoxal 5'-phosphate (PLP) dependent enzyme, which catalyzes the irreversible α-decarboxylation of L-glutamic acid to GABA and carbon dioxide (Fig. 2). This enzyme exists ubiquitously in eukaryotes and prokaryotes such as bacteria, plant, insect and mammalian (Ueno, 2000), but its presence varies in different types of different organisms.

LAB GAD is an intracellular enzyme (Huang *et al*., 2007a; Jun *et al*., 2007; Komatsuzaki *et al*., 2008) and induction of it is one of the acid stress responses in LAB (Sanders *et al*., 1998; Small and Waterman, 1998). GAD forms of LAB such as *L. brevis* IFO12005, *L. brevis* CGMCC1306 and *L. paracasei* NFRI7415 have indicated that is consisted of 2 subunits as a dimer (approximately 110-120 kD) (Jun *et al*., 2007; Komatsuzaki *et al*., 2008). Thus, GAD is produced as a mature form which consists of identical subunits with molecular mass ranging from 54 to 62 kD, not as a precursor protein, and has highly conserved catalytic amino acid residues containing a lysine residue (Lys 279) that is known to be essential to binding of PLP as well as to the active site residues (Thr215 and Asp246) that promote decarboxylation (Park and Oh, 2004; 2007a; Kim *et al*., 2007; Hiraga *et al*., 2008; Komatsuzaki *et al*., 2008; Fan *et al*., 2012).

The regulation of GABA synthesis

GAD activity is regulated by a cycle of activation and inactivation determined by the binding and release of its co-factor (PLP). The intact GAD-cofactor complex is called holoenzyme, decarboxylates glutamate to form GABA. Once the cofactor is removed, the remaining protein, called apoenzyme, will lose its enzyme activity. Apoenzyme can be activated by binding PLP to form holoGAD when additional GABA synthesis is required, thus completing the cycle (Martin and Rimvall, 1993; Battaglioli *et al*., 2003). The intermediate complex involves an ionic interaction between the phosphate group of PLP and the positive charged residues on the active site of GAD, to maintain the PLP in an appropriate position in the active centre (Chen *et al*., 1998). In *E. coli*, residue lysine (Lys 276) has been confirmed to the catalytic contribution in the active site of *E. coli* GAD. The evidence showing that mutation of Lys 276 could make the protein less flexible and its active site less accessible to the substrate and cofactor (Tramonti *et al*., 2002). It was demonstrated that *E. coli* GAD was naturally localized exclusively in the cell's cytoplasm, but tended to move to the cell's membrane when environmental pH fell (Capitani *et al*., 2003; Zhang *et al*., 2007). One molecule of *E. coli* GAD is composed of 6 subunits with molecular weight 50 kDa each. One of the unique properties of GAD is that it is activated by NaCl (Gut *et al*., 2006).

The apoenzyme is produced by an alternative-transamination reaction catalyzed by GAD that converts the normally tightly bound pyridoxal-P to pyridoxamine-5'-phosphate (pyridoxamine-P) which dissociates readily from the enzyme. The activation of apoGAD by pyridoxal-P is a two-step process involving a rapid, reversible association of the co-factor with the enzyme, followed by a subsequent slower conversion of the intermediate state to the fully active holoenzyme (Spink *et al*., 1985; Porter and Martin, 1988; Chen *et al*., 1998). The major reactions catalyzed by GAD are presented in Figure 3. During most catalytic events the holoenzyme (GAD containing bound pyridoxal-P) converts glutamate to GABA and is regenerated in the process; *i.e*. pyridoxal-P remains bound to GAD. During a small percentage of reactions, however, glutamate is decarboxylated and GAD then catalyzes an alternative transamination reaction that produces succinic semialdehyde (SSA) and pyridoxamine-P (PMP), which readily dissociates from the enzyme, leaving inactive apoGAD. ApoGAD can re-form holoGAD by combining with free pyridoxal-P, thus completing a cycle of inactivation and activation. When free pyridoxal-P is not present, activation cannot take place, and GAD inactivates as a function of time and substrate concentration (Battaglioli *et al*., 2003; Zhang *et al*., 2007).

The roles of various compounds in the apo-/holoenzyme cycle have been described in previous research (Martin, 1987; Martin and Rimvall, 1993). The concentration of glutamate regulates the cycle by affecting the rate of the reaction that forms apoGAD. Similarly, GABA, the usual end product of the GAD reaction, will also inactivate the enzyme if present at high concentrations (Porter and Martin, 1984), by reversing the last step of the GAD reaction to form the quinoid intermediate (EQ) (Fig. 3) from which apoenzyme can be formed. Inorganic phosphate (Pi) and nucleoside triphosphates (NTPs) also affect the cycle. To maintain active enzyme, apoGAD binds with pyridoxal-P to form holoGAD. ATP competes with pyridoxal-P for binding to apoGAD, thus slowing activation and shifting the balance of the cycle toward a higher level of apoGAD (Meeley and Martin, 1983). Thus, GAD is inactivated when incubated with glutamate and ATP, even when pyridoxal-P is present. Pi opposes the effect of ATP. Millimolar concentrations of Pi reversed the ATP-induced inhibition of activation and also increased activation under normal conditions without ATP (Meeley and Martin, 1983; Porter and Martin, 1988). In addition, GABA is a weak competitive inhibitor of GAD and also can inactivate GAD via the transamination reaction (Porter and Martin, 1984). The high concentration of GABA (50 mM) required to inactivate GAD is only achieved intracellularly within the synaptic vesicles of GABAergic neurons (Wood *et al*., 1976).

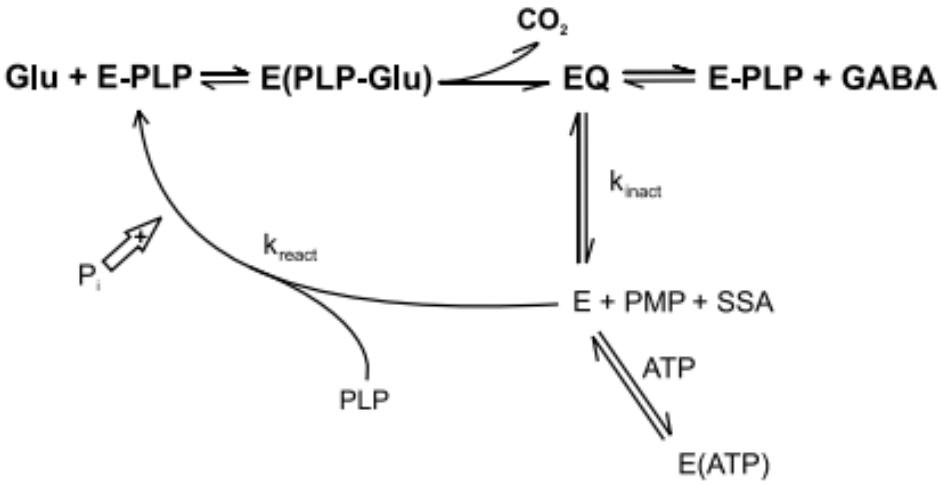


Figure 3 GAD activity cycle of interconversion of apoenzyme and holoenzyme. The primary reaction (bold face) produces GABA; holoGAD remains intact. The secondary reaction produces apoGAD which can be activated to holoGAD by reaction with pyridoxal-P. The primary reaction is more than 1000 times more frequent than the secondary reaction (Porter *et al*., 1985). E, apoGAD; E-PLP, holoGAD; EQ, quinoid intermediate; Pi, inorganic phosphate; PLP, pyridoxal-phosphate; PMP, pyridoxamine-phosphate; SSA, succinic semialdehyde; k_{inact}, inactivation rate constant; k_{act}, activation rate constant.
Source: Battaglioli *et al*. (2003)

2.4 Factors affecting on GABA synthesis

Different fermentation factors affect the rate of GABA production by microorganisms. Among them the most common and essential factors are pH, temperature, cultivation time and media additives of culture. The fermentation conditions can be optimized based on the biochemical characteristics of GAD of the fermenting microorganisms. Decarboxylation of glutamate occurred in LAB results in the stoichiometric release of the end product GABA and the consumption of a proton. The net effect of this reaction increases the alkalinity of the cytosol and environment. To maintain the optimum pH 5.0 at which the highest GABA production was obtained by *L. brevis*. H_2SO_4 was therefore supplemented into the fermenting broth in order to offset pH increase, arisen from the decarboxylation (Li *et al*., 2010b). Similarly, the glutamate content 500 mM in the culture medium was converted to 302 mM GABA by optimizing the fermentation condition of *L. paracasei* NFRI7415 at pH 5.0 with the addition of pyridoxal-5'-phosphate (PLP) (Komatsuzaki *et al*., 2005). The GABA production by *Strep. salivarius* subsp. *thermophilus* Y2 was also enhanced by optimizing fermentation condition at pH 4.5 and by the addition of PLP (Yang *et al*., 2006).

The optimum conditions vary among the fermenting microorganisms due to the different properties of the GADs. Accordingly, characterization of the biochemical properties of the GADs will be required in the interested LAB to achieve the highest GABA production. Optimal conditions for GABA-producing LAB are summarized below, especially on the effects of pH, temperature, cultivation time and media additives.

2.4.1 Effect of pH

The biosynthesis of GABA in microorganisms is mainly regulated by pH, which usually has the most pronounced effect for a fermentation process (Komatsuzaki *et al*., 2005; Tsai *et al*., 2006; Yang *et al*., 2006). The biochemical characteristics of GAD vary among different microorganisms, therefore, the effective pH value for the maximum GABA production is species-dependent (Yang *et al*., 2008; Li *et al*., 2010b). Small and Waterman (1998) and Yang *et al*. (2006) explained that the cytoplasmic decarboxylation results in the consumption of an intracellular proton after the uptake of glutamate by its specific transporter. The reaction product GABA is exported from cells by an antiporter, and the net result is an increase in the pH of the cytoplasm, due to the removal of hydrogen ions, and a slight increase in the extracellular pH, due to the exchange of extracellular glutamate for the more alkaline GABA. *L. plantarum* DSM19463 synthesized the maximum GABA (59 μM/h) at the pH 6.0 (Di Cagno *et al*., 2010). However, *L. paracasei* NFRI 7415 produced the highest GABA (210 mM) at pH 5.0 (Komatsuzaki *et al*., 2005). In *Strep. salivarius* subsp. *thermophilus*, the GABA production was highest (7984 mg/l) at pH 4.5 (Yang *et al*., 2006). *Strep. thermophilus* IFO13957 produced a large amount of GABA at pH 3.5 (Watanabe *et al*., 2011). *L. brevis* GABA057 converted total 10% of monosodium glutamate (MSG) to GABA at pH 4.2 (Soo *et al*., 2006).

In cheese, *L. paracasei* PF6, PF8, PF13, *L. plantarum* PF14, *Lactobacillus* sp. strain PF7 and *Ent. durans* PF15 produced high amounts of GABA (289-391 mg/kg) under the pH range of 4.68-5.70 (Siragusa *et al*., 2007). When GABA-producing *L. paracasei* was compared the GABA production capacity under different pH (4-6), GABA was produced significantly high (210 mM) at pH 5.0 (Komatsuzaki *et al*., 2005). *Lc. lactis* produced the highest amount of GABA (7.2 g/l) at pH ranged from 7.5 to 8.0. However, the GABA production was decreased when pH above 8.0, indicating that *Lc. lactis* has the optimum GABA production at weak alkaline pH, ranged from 7.5 to 8.0 (Lu *et al*., 2009). High GABA production of *L. buchneri* MS (above 200 mM) was maintained at pH range 5.0-8.0. The optimal initial pH for GABA production was found to be pH 5.0. GABA production rapidly decreased at pH values below 4.0 or above pH 8.0 (Cho *et al*., 2007). The optimum GABA-

producing pH 4.2 was found to be the optimum value for the production of GABA (5.83 mg/ml) in *L. buchneri* (Cho *et al*., 2011). The GABA production of *Leuconostoc* NC5 was synthesis when the pH of the culture medium was at acidic range (pH 3.5-5.5). Their synthesis started to decrease close to pH 6.0. These results indicated that pH 5.0 was the optimal pH for extracellular and intracellular GABA production at 2.2 mM and 2.3 mM, respectively (Farrah *et al*., 2009).

The pH in fermentation medium changes with time during fermentation, therefore, the initial pH affects final GABA yield and the pH of the medium should be adjusted timely to maintain the optimum pH (Kimura *et al*., 2002; Lu *et al*., 2009; Li *et al*., 2010b). The black raspberry juice fermented with *L. brevis* GABA 100 and monosodium glutamate (MSG) changed the initial pH of juice from 4, 4.5, 5, 5.5 and 6 to 3.9, 4.2, 4.4, 4.5 and 4.7 after 48 h, respectively (Kim *et al*., 2009). The MRS medium inoculated with *L. paracasei* changed the pH from 6.5 to about 4.5 within 50 h of fermentation (Jeng *et al*., 2007). The pH of culture MRS medium with and without 5% MSG with *L. buchneri* MS changed gradually from 6.5 to 7.06 and 4.5 culture after 48 h, respectively (Cho *et al*., 2007). A significant decrease in culture media pH with *L. buchneri*, from 6.5 to 4.8, was observed. Production of GABA by *L. buchneri* was associated with increased cell growth and decreased pH. The decreased pH apparently accelerated GABA production (Cho *et al*., 2011).

As reported, the optimum pH values for maintaining the activity of the bacterial GAD was in the range of 4-5 (Ueno *et al*., 2000). The optimum activity of purified GAD from *L. brevis* CGMCC1306 was observed at pH 4.4 (Jun *et al*., 2007). In high GABA-producing strains *L. paracasei* NFRI7415 (Komatsuzaki *et al*., 2008) and *L. brevis* IFO12005 (Ueno *et al*., 1997), the GAD activity was still observed at pH 4.0 or above pH 5.5, but very low levels of GAD activity were observed at pH 4.0 and no activity was detected above pH 5.5 in a low GABA-producing strain *Lc. lactis* (Nomura *et al*., 1998). These results suggest that low-pH GAD activity and broad-pH GAD activity might be important for producing high levels of GABA in LAB.

2.4.2 Effect of temperature

The incubation temperature is also a major factor affecting maximum GABA yield by fermentation. In addition to an effect on biocatalyst activity and stability, temperature has an effect on the thermodynamic equilibrium of a reaction. The high efficient conversion of glutamate to GABA needs the high cell density and also the appropriate culture temperature (Kim *et al*., 2009). GABA production in *L. brevis* NCL912 had a positive correlation with the cell density, which was dependent on the culture temperature (Li *et al*., 2010b). Production of

GABA by *L. buchneri* associated with an increased cell growth at 30 °C (Cho *et al*., 2011). *L. buchneri* in MRS broth also had the optimum temperature for GABA production at 30 °C (Cho *et al*., 2007). *L. brevis* NCL912 growth increased with higher temperature and peaked at 35 °C, then decreased over the temperature (Li *et al*., 2010b). *L. plantarum* DSM19463 synthesized the highest amount of GABA (59 μM/h) at temperatures between 30 °C and 35 °C (Di Cagno *et al*., 2010).

The optimum temperatures for *L. brevis* GAD and *L. brevis* CGMCC1306 were found to be as 30 °C and 37 °C, respectively (Ueno *et al*., 1997; Ueno, 2000; Jun *et al*., 2007). *L. brevis* GABA100 fermenting black raspberry juice produced maximum GABA (27.6 mg/ml) at pH 3.5 and 30 °C on 12 days of fermentation (Kim *et al*., 2009). Immobilized whole cells of *L. brevis* at 40 °C produced 92% of GABA after 8 h of fermentation (Huang *et al*., 2007b). The maximum GABA yields by *Lc. lactis* at the optimum temperature of 33 °C and 34 °C were found to be 310 mg/ml and 439 mg/ml, respectively (Lu *et al*., 2008; Li *et al*., 2010a). *Strep. salivarius* subsp. *thermophilus* had the optimum temperature for GABA production as 34 °C, at which, 12% of the total MSG was completely converted into GABA (Yang *et al*., 2006). *L. paracasei* NFRI7415 produced the highest GABA (302 mM) at 37 °C, however drastically decreased the GABA production and cell growth at 43 °C (Komatsuzaki *et al*., 2005). The optimal temperature of *Leuconostoc* NC5 for high GAD activity is at 37 °C with the highest GABA production at 5.9 mM (Farrah *et al*., 2009). Generally, fermenting temperatures ranged from 25 °C to 40 °C result in a high GABA yield within the temperatures. Also, the optimal temperatures of LAB GADs range from 25 to 50 °C (Jun *et al*., 2007).

2.4.3 Effect of the fermentation time

The time factor plays an important role in the fermentation and the production of GABA as temperature and pH do. *L. plantarum* DSM19463 and *L. paracasei* NFRI7415 required 72 h and 144 h of fermentation to reach the highest production of GABA at 4.83 mM and 60 mM, respectively (Komatsuzaki *et al*., 2005; Di Cagno *et al*., 2010). Black raspberry juice fermented with *L. brevis* GABA100 reached the highest production of GABA at 25.4 mg/ml and 26.5 mg/ml at the 15 days of the fermentation at 25 °C, pH 4.0 and 37°C, pH 5.5, respectively. It reached the highest level of GABA (27.6 mg/ml) at the 12 days when the samples were fermented at pH 3.5 and 30 °C (Kim *et al*., 2009). *Leuconostoc* NC5 showed the highest GABA production at 5.9 mM with cultivation time at 168 h (Farrah *et al*., 2009).

The addition time for the GABA substrate also affects the final GABA yield as well as the concentration of the substrate in the medium. A significant differences in GABA yield among various times of MSG addition was shown in the fermentation of *Lc. lactis*, as the highest GABA yield was obtained when MSG was added at the beginning of fermentation (0 h). However, the GABA yield lowered when MSG was added during 6 to 96 h of fermentation at 6 h interval of time (Lu *et al*., 2009). The addition of PLP in different time intervals also affected the production of GABA (Yang *et al*., 2006). The GABA production at 72 h reached 6272, 6570 and 7333 mg/l when PLP was added at 0, 24 and 48 h, respectively. The higher amount of GABA was produced by the addition of PLP at 48 h than at 0 and 24 h suggested that PLP could easily lose the role as cofactor due to the denaturalization in the culture broth during the fermentation. However, addition of PLP at 48 h could partly recover GAD activity (Yang *et al*., 2006). These results indicate that the highest GABA production by microorganisms can depend on the addition of appropriate medium additives and optimum additional time for the additives.

2.4.4 Effect of media additives

Nutrient composition and culture conditions affect the GABA production by microbe fermentation (Wang *et al*., 2003). Also, media additives including glutamate and PLP as the cofactor of GAD are the major factors affecting the production of GABA during the fermentation (Komutsuzaki *et al*., 2005; Cho *et al*., 2007; Lu *et al*., 2008; Yang *et al*., 2006; Li *et al*., 2010a). The medium composition, especially carbon and nitrogen sources and other components can influence the amount of GABA production. Furthermore, the concentrations of substrates are important for achieving high GABA yield (Yang *et al*., 2006). The substrate specificity of GAD from *L. brevis* was tested by using 22 kinds of amino acids (L-alanine, ε-aminocaproic acid, L-arginine, L-aspartic acid, L-citrulline, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-homoserine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-tyrosine and L-valine). The decarboxylated product was observed only for L-glutamic acid (Ueno *et al*., 1997). *Lc. lactis* GAD also reacted only with L-glutamate among the 20 α-amino acids (Nomura *et al*., 1999). These results indicate that GADs from LAB are specific for L-glutamic acid.

Glutamate addition increased GABA production in *L. paracasei* and *L. brevis* (Hayakawa *et al*., 1997; Komutsuzaki *et al*., 2005; Huang *et al*., 2007b; Li *et al*., 2010b). GABA concentration reached 161 mM after cultivation of 144 h in the medium containing 500 mM of glutamate by *L. paracasei* NFRI7415 (Komutsuzaki *et al*., 2005). *L. brevis* NCL912 and *L. brevis* also increased GABA production by the addition of glutamate

(Hayakawa *et al*., 1997; Huang *et al*., 2007b; Li *et al*., 2010b). The highest production of GABA by *Leuconostoc* NC5 was achieved at 20 mM in 200 mM of glutamate containing culture medium during the cultivation. However, when the concentration of glutamate exceeded 200 mM, GABA production decreased. These results suggested that the increment of GABA concentration depends on the addition of glutamate in the culture medium (Farrah *et al*., 2009). On the other hand, *Strep. salivarius* subsp. *thermophilus* Y2 did not increase GABA production significantly when glutamate was added 10-20 g/l of media, suggesting that these concentrations of glutamate are not appropriate for the synthesis of GABA in this species (Yang *et al*., 2006). The production of GABA by using glutamate as a substrate still remains with several problems, such as the high cost of the culture medium.

The addition of carbohydrates as carbon source increased the GABA production. *L. plantarum* DSM19463 produced 0.9 mM GABA by the fermentation of grape must diluted to 4% (w/v) of total carbohydrates (Di Cagno *et al*., 2010). Among different carbohydrates tested such as L-arabinose, ribose, D-xylose, galactose, glucose, fructose, maltose, melibiose, α-methyl D-glucoside, N-acetyl D-glucosamine and gluconate as carbon source, 1.25% glucose was the best carbon source for high production of GABA (Li and Cao, 2010). The mixed ratio (33:58:9) of brown rice juice, germinated soybean juice and enzymolyzed skim milk, a milk having deteriorated properties by the means of enzymatic action, as a source of carbon and nitrogen produced the highest GABA (6.41 g/l) by *Lc. lactis* subsp. *lactis* B (Lu *et al*., 2008).

The addition of carbon sources increased growth of *L. buchneri* MS, especially the addition of 2.0% arabinose. However, this high cell density did not result in increased GABA production. The addition of 2% arabinose caused the highest cell number ($A_{600} \simeq 7.26$), but the GABA concentration reached only 9.48 mM (3.5% conversion yield). The addition of over 1% of arabinose to culture media rapidly reduced the GABA conversion yield. However, GABA concentrations reached over 200 mM after adding fructose, galactose, glucose, lactose, or maltose (0.5-2.0%). The optimal carbon source was found to be glucose (at 1%), which resulted in a 230 mM of GABA production (87.9% conversion yield) (Cho *et al*., 2007). However, the addition of 1% glucose as a carbon source shows no increment in the GABA production (Farrah *et al*., 2009).

PLP is used as a cofactor of GAD for enhancing GAD activity (Sandmeier *et al*., 1994; Komutsuzaki *et al*., 2005). By the addition of PLP, GABA production increased and reached to 7333 mg/l, 200 mM and 504 mg/kg during the fermentation with *Strep. salivarius* subsp. *thermophilus* Y2, *L. paracasei* NFRI74150 and *L. plantarum* C48,

respectively (Komutsuzaki *et al*., 2005; Siragusa *et al*., 2007; Yang *et al*., 2006). The GABA production of *Leuconostoc* NC5 reached the highest level (35.7 mM), when 50 mM of PLP concentration were added under cultivation conditions at pH 5.0, 37 °C and incubated for 168 h in 200 mM of glutamate. The results of this study provided additional evidence on what was previously revealed that the activity of GAD increases by adding PLP and PLP might acts as a necessary cofactor of GAD (Farrah *et al*., 2009). The addition of 0.1 mM PLP to the diluted grape must, however, did not enhance the synthesis of GABA, which may be due to the presence of endogenous PLP in grape must (Di Cagno *et al*., 2010). The addition of PLP in the culture medium for the production of GABA by *L. brevis* NCL912 did not increase the amount of GABA, indicating that *L. brevis* NCL912 could synthesize the PLP by itself necessarily (Li *et al*., 2010a).

The addition of sulfate ions increased the GAD activity of *L. brevis* IFO 12005 in a dose-dependent manner, the order of effect was as follows: ammonium sulfate > sodium sulfate > magnesium sulfate, indicating that the increase of hydrophobic interaction between subunits causes the increase of GAD activity (Ueno *et al*., 1997; Hiraga *et al*., 2008). Hiraga *et al*. (2008) explained that hydrophobic interaction between the subunits under a high concentration of ammonium sulfate and proper binding of sodium glutamate to the GAD facilitates making an active tetramer from an inactive dimer. The addition of ammonium sulfate did not cause any significant structural changes, but did induce subtle structural changes at the active site, probably in the vicinity of the catalytic residues.

Total 5% of the MSG was converted into GABA within 48 h when 10 mM ammonium sulfate was added to the reaction medium of *L. brevis* GABA 057 (Soo *et al*., 2006). Five percent of glutamate converted to GABA, when 10 mM ammonium sulfate was added to the medium. The results showed that the addition of 10 mM ammonium sulfate significantly increased the GABA production to 12.3 mM, even though no dose dependent data was observed (Farrah *et al*., 2009). In glucose-yeast peptone medium, 7% of MSG as glucose concentration with 10 mM ammonium sulfate was the best combination for GABA production (Ueno *et al*., 1997). The addition of over 0.6% glucose without ammonium sulfate, however, did not increase the GABA conversion rate (Su *et al*., 2003). The cell viability and stability in the beads can be improved for the higher rate of GABA conversion by adjusting the concentrations of media additives, including skim milk, isomaltooligosaccharide, erythritol, and pectin in an optimum concentration (Soo *et al*., 2006). The beads with 0.6% isomaltooligosaccharide were the most effective combination for

GABA production and also improved probiotic survival in fermented milk (Chen *et al*., 2004; Soo *et al*., 2006).

The addition of other substrates such as the whole meal wheat sourdough and 50% of *tomokoji* enhanced the GABA production using *L. plantarum* C48 (Kono and Himeno, 2000; Rizzello *et al*., 2008). GABA could be produced by LAB using *shochu kasu* as a growth medium without addition of glutamate. The GABA concentration reached 10.05 mM or 10.18 mM after one or two day cultivation in *kome shochu kusu*, respectively (Yokoyama *et al*., 2002). Similarly, the addition of buckwheat and quinoa sourdough with *L. plantarum* C48 and amaranth and chickpea sourdoughs with *Lc. lactis* subsp. *lactis* PU1 enhanced the GABA production and reached to 643, 415, 816 and 1031 mg/kg, respectively (Coda *et al*., 2010). These processes have advantages over other fermentation processes due to the simplicity and low operation price.

2.6 Cloning of GAD genes in LAB

The full-length GAD genes from *L. paracasei* NFRI7415 (Komatsuzaki *et al*., 2008), *L. plantarum* KCTC3015 (Park and Oh, 2004), *L. brevis* OPK-3 (Park and Oh, 2007a), *L. brevis* IFO12005 (Hiraga *et al*., 2008), *L. brevis* BH2 (Kim *et al*., 2007), *L. brevis* CGMCC1306 (Fan *et al*., 2012) and *Lc. lactis* 01-7 (Nomura *et al*., 1999) and the core fragments of *gad*Bs from *L. paracasei* PF6 (accession number EF174473), *L. delbrueckii* subsp. *bulgaricus* PR1 (accession number EF174472), *Lc. lactis* PU1 (accession number EF174474) and *L. plantarum* C48 (accession number EF174475) were cloned and sequenced (Siragusa *et al*., 2007). In addition, the GAD genes from *L. plantarum* KCTC3015 (Park and Oh, 2004), *L. brevis* BH2 (Kim *et al*., 2007), *L. brevis* CGMCC1306 (Fan *et al*., 2012), *L. brevis* OPK-3 (Park and Oh, 2007a) and *L. zymae* GU240 (Park *et al*., 2014) were successfully expressed in *E. coli*. Moreover the GAD gene from *L. brevis* OPK-3 was successfully expressed in *B. subtilis* (Park and Oh, 2006). The properties of the reported GADs from LAB are shown in Table 2.

Table 2 The properties of lactic acid bacterial glutamate decarboxylases

Strains	Molecular weight of subunit (kDa)	Number of subunit	Optimal pH	Optimal temperature (°C)	K_m (mM)	pI	k_{cat} (s^{-1})	References
L. brevis IFO12005	60	2	4.2	30	9.3	-	6.5	Ueno *et al*. (1997)
Lc. lactis subsp. *lactis* 01-7	54	-	4.7	-	0.51	-	-	Nomura *et al*. (1999)
L. brevis OPK-3	53.4	-	-	-	-	5.65	-	Park and Oh (2007a)
L. brevis BH2	53.5	-	-	-	-	5.34	-	Kim *et al*. (2007)
L. brevis CGMCC1306	62	-	4.4	37	8.22	-	-	Huang *et al*. (2007a, b); Jun *et al*. (2007)
L. paracasei NFRI7415	57	2	5.0	50	5.0	-	-	Komatsuzaki *et al*. (2008)
L. senmaizukei L-13	57	-	4.5	50	0.85	-	78.8	Oda and Hiraga (2011)
L. brevis CGMCC1306	53.47	-	4.8	48	10.26	-	-	Fan *et al*. (2012)
L. zymae GU240	53	-	4.5	41	1.7	-	-	Park *et al*. (2014)

2 Method for monitoring of bacteria in a complex food environment

The fermentation is widely carried out at household level and is spontaneous. Then, a description of the microbial diversity of fermented foods is a prerequisite for the development of starter cultures to improve indigenous technologies. Sensitive and reliable methods for their detection and identification are of great importance for monitoring population changes during food fermentation when supported by natural microflora, as well as when promoted by the addition of starter and/or protective culture under controlled conditions (Ercolini *et al*., 2001; Ercolini, 2004). Methods for investigating microbiota from a given ecosystem of fermented foods can be either culture-dependent or culture-independent. The culture-dependent methods are based on growth of the bacterial species on synthetic media that resemble the conditions of the system from which the microorganisms are isolated. Culture-dependent methods present several disadvantages, above all for determining bacterial numbers; they are known to be biased because bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced *in vitro* (Nadkarni *et al*., 2009; Carraro *et al*., 2011). Problems with using culturing for community analysis arise from the fact that an artificial homogenous medium typically allows growth of only a small fraction of the organisms. In addition, when complex microbial communities are under investigation, enumerating bacteria by traditional microbial culturing techniques may produce erroneous results (Besnard *et al*., 2000; Carraro *et al*., 2011). Moreover, sugar fermentation profiles or other biochemical/physiological traits may sometimes be uncertain, complicated and time-consuming (Ercolini *et al*., 2001). For example, the identification of the *Lactobacillus* species by culture-dependent methods involved in meat fermentation resulted ambiguous. In particular, strains of *L. curvatus* and *L. sakei* isolated from meat products are not easy to differentiate (Reuter, 1981; Kandler, 1984). Identification at intraspecies level is also an important issue since it may help to distinguish groups of strains or single strains with peculiar technological properties. Moreover, in the last decade it was shown that culture-dependent methods do not accurately detect microbial diversity in environments such as traditional fermented foods in which culture methods not only under estimated biodiversity but failed to quantify precisely some dominant taxa (Hugenholtz *et al*., 1998; Fontana *et al*., 2005a). For these reasons, culture-independent methods or molecular methods have been used increasingly to simplify characterization procedures, to provide rapid and reliable identification, or validate phenotypically determined taxa (Ercolini *et al*., 2001).

Several molecular methods have been applied for the monitoring and identification of bacterial communities among the natural or inoculated flora, such as SDS-PAGE of whole cell proteins (Sanchez *et al*., 2003), restriction fragment length polymorphism

analysis of the 16S rRNA gene (Sato *et al*., 2000), hybridization with rRNA probes (Sakai *et al*., 2004), specie-specific PCR (Yost and Nattress, 2000), RAPD-PCR analysis (Andrighetto *et al*., 2001; Plengvidhya *et al*., 2004), PCR followed by temperature/denaturing gradient gel electrophoresis (TGGE/DGGE) (Cocolin *et al*., 2000; 2001b; 2001c; 2004) and reporter genes (Scott *et al*., 1998; Geoffroy *et al*., 2000; Gory *et al*., 2001; Phumkhachorn *et al*., 2007; Luxananil *et al*., 2009). In the recent decade, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been successfully applied to determine the microbiology of several fermented foods such as fermented sausages (Fontana *et al*., 2005a; b), fermented grains (Chao *et al*., 2008), fermented meat (Hu *et al*., 2009), and fermented dairy products (Liu *et al*., 2012) or other foods. This approach gives an alternative option free of separation and cultivation steps (Temmerman *et al*., 2004) and allows the resolution of complex microbial mixture or particular populations without further characterization of the individual inhabitants (Muyzer, 1999).

2.1 Determination of the bacterial populations using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique

2.1.1 Principle of PCR-DGGE

DGGE is an electrophoretic method capable of detecting differences between DNA fragments of the same size but different sequences by single bases. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile. The theoretical aspects of this separation were firstly described by Fisher and Lerman (1983). In an acrylamide gel, the denaturing conditions are provided by urea and formamide. A solution of 100% chemical denaturant consists of 7 M urea and 40% formamide in water. Low and high denaturing solutions are prepared, mixed with the acrylamide solution, and poured in a gel casting by using a gradient former in order to generate a linear denaturing gradient. Moreover, the electrophoresis is carried out at a constant temperature between 55 and 65 °C, mostly 60 °C. In a DGGE gel, DNA, which is negatively charged, is attracted by the positive electrode and forced to migrate through the pores of a polyacrylamide gel. A double-strand DNA fragments are subjected to an increasing denaturing environment as they encounter increasing concentrations of the denaturing agents and partially melt in discrete regions called "melting domains". The melting temperature (T_m) of these domains is sequence-specific. Once the T_m of the lowest melting domain is reached, that part of the fragment becomes partially melted, creating branched "breaking" molecules. This behavior reduces the DNA mobility in the acrylamide gel. Basically, what happens is that hydrogen bonding between the base pairs is broken by the temperature and the increasing gradient of denaturing chemicals (urea and

formamide) (Muyzer *et al*., 1993; Muyzer and Smalla, 1998). Therefore, DNA fragments of the same size but different base pair compositions will show a different response to the denaturing gradient. The different sequences of the DNA fragments will have melting domains with different T_m values that will run different distances in the DGGE gel. Briefly, DNA fragments of the same length but different sequences will be separated in DGGE. However, the fragments to be loaded on DGGE gels are usually PCR products. An optimal resolution is obtained when the molecules do not completely denature. The addition of a 30- to 40-bp GC clamp to one of the PCR primers insures that the fragment of DNA will remain partially double-stranded and that the region screened is in the lowest melting domain and to improve the sensitivity in the detection of mutations by DGGE (Myers *et al*., 1985; Sheffield *et al*., 1989; Cocolin *et al*., 2001b; 2004; Ercolini, 2004).

Bands in DGGE fingerprints can be revealed by ethidium bromide staining. The most sensitive procedure is silver staining (Felske *et al*., 1996), although silver-stained gels cannot be used for hybridization experiments and single-strand DNA fragments are also detected. SYBER Green I is also an alternative for visualizing DGGE gels (Muyzer *et al*., 1997). SYBER Green staining does not give background staining, thus allowing the detection of DNA fragments even at very low concentrations.

2.1.2 Application of PCR-DGGE techniques in fermented foods

PCR-DGGE is classified as part of the new discipline of molecular microbial ecology (Muyzer and Smalla, 1998). This approach has provided a new insight into microbial diversity without cultivation and allowed a more rapid, reliability, high-resolution description of microbial communities than did the traditional approaches because it allows the separation of DNA molecules that differ by single bases (Myers *et al*., 1987). Although, many studies clearly demonstrate the broad applicability of this method, the DGGE discriminating capabilities aimed to target bacteria are determined by the choice of the PCR primers. The use of appropriate consensus primers is a critical point to influence the resolution of DGGE analysis in mixed microbial systems, especially in LAB differentiation (Chen *et al*., 2008). The first step was to find suitable primers. The primers must be present in all the species and delimite variable sequences to separate each species. Then it was important to avoid interspecies differences: for each species only one band should appear in the electrophoresis gel. Duplex DNA problem is bypassed by an attachment of a GC rich DNA sequence (Myers *et al*., 1985). The last step is to find the most suitable and accurate gradient and the best DGGE condition (temperature, time) (Renouf *et al*., 2006b).

There are several published papers reviewed numerous PCR primers used for DGGE analysis to profile the microbial communities in several food systems without pre-cultivation steps as presented in Table 3. Obviously, the 16S rDNA seems to be by far the most widely used as a molecular marker to determine the phylogenetic relationships of bacteria. This is because it is a much conserved region of the genome that also includes variable regions which makes it possible to distinguish them by PCR-DGGE. Therefore, primers can be designed by hybridizing to conserved regions but spanning variable regions in order to obtain PCR amplicons with species-specific differences in base pair composition that can be separated by DGGE. Several primer pairs have been employed to amplify variable regions of the 16S rDNA for bacteria and 26S rDNA or 18S rDNA for eukaryote microorganisms. In fact, each species is theoretically supposed to yield a different DGGE profile after the amplification of variable regions of the rDNA.

However, the hypervariable V3-region on the 16S rDNA is the most popular objective domain to start the study of an unknown and complex bacterial community. In addition, the V3-region is considered to have a high grade of resolution and to be highly variable, and it is regarded as a good choice when it comes to length and specific-species heterogeneity (Coppola *et al*., 2001; Ercolini, 2004; Florez and Mayo, 2006; Hovda *et al*., 2007; Chen *et al*., 2008). Unfortunately, one problem related to the use of 16S rDNA in DGGE analysis is the complexity created by the existence of multiple heterogeneous copies within a genome due to its heterogeneity (Dahllof *et al*., 2000; Coenye and Vandamme, 2003; Crosby and Criddle, 2003; Rantsiou *et al*., 2004; Chen *et al*., 2008). Its heterogeneity reviewed by Fogel *et al*. (1999), several bands per species can be seen in high-resolution PCR-DGGE analysis. The amplified fragments of 16S rDNA will therefore appear as several bands on a DGGE gel, rather than a single band that would allow precise species identification. Consequently, a solution to the problem of 16S rDNA heterogeneity is provided by the analysis of a gene that exists in a single copy. The protein-coding gene, such as the gene encoding the beta-subunit of DNA-directed RNA polymerase, *rpo*B, has been proposed as the one to fulfill these criteria (Dahllof *et al*., 2000; Ko *et al*., 2002; Rantsiou *et al*., 2004; Renouf *et al*., 2006 a; b; Claisse *et al*., 2007).

The *rpo*B gene is used as a potential biomarker to overcome identification problems because it contains a region that represents all that is common to all bacteria that have the same key attributes as 16S rDNA and it has conserved as well as variable regions and that it functions as an evolutionary clock (Mollet *et al*., 1997; Dahllof *et al*., 2000; Ko *et al*., 2002). Targeting *rpo*B gene allowed a reliable discrimination of each species. This primer was able to avoid the interspecies heterogeneity problem caused by the use of the 16S rDNA, which appears

to exist in one copy only in bacteria. Moreover, this primer was widely used in many types of fermented foods (Table 3). However, the use of *rpo*B presents a taxonomic disavadantage: the database of the sequence is less well documented than that of the 16S rDNA (Rantsiou *et al*., 2004; Renouf *et al*., 2006a; b).

Table 3 PCR primers used for DGGE analysis of DNA from microbial communities directly extracted from foods

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
P1, V1f	GCGGCGTGCCTAATACATGC	41-60	16S V1	Bacterial 16S rDNA	Klijn *et al*.	Fermented sausages
P2, V1r	TTCCCCACGCGTTAC TCACC	111-130			(1991)	(Cocolin *et al*., 2001a;b; Fontana *et al*., 2005a; b)
63f	CAGGCCTAACACATGCAAGTC	63- 84	16S V1-V3	Bacterial 16S rDNA	El Fantroussi	Mineral water
518r	ATTACCGCGGCTGCTGG	518-534			*et al*. (1999)	(Dewettinck *et al*., 2001)
HDA1	ACTCCTACGGGAGGC AGCAG	338-357	16S V2-V3	Bacterial 16S rDNA	Walter *et al*.	Whisky
HDA2	GTATTACCGCGGCTG CTGGCAC	539-561			(2000)	(Van Beek and Priest, 2002)
						Fermented sausages
						(Cocolin *et al*., 2001c)
						Yoghurt and probiotic
						preparations (Fasoli *et al*., 2003)

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
338f	ACTCCTACGGGAGGCAGCAG	338-357	16S V3	Bacterial 16S rDNA	Ampe *et al*. (1999)	Cassava (Ampe *et al*., 2001; Miambi *et al*., 2003)
518r	ATTACCGCGGCTGCTGG	518-534			Muyzer *et al*. (1993)	Dairy products (Coppola *et al*., 2001; Ercolini *et al*., 2001; 2003; Mauriello *et al*., 2003)
						Mineral water (Dewettinck *et al*., 2001)
						Pozol (Ampe and Miambi, 2000)
						Vanilla beans (Roling *et al*., 2001)
						Fermented sausages (Cocolin *et al*., 2001c)

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
						Modified atmosphere packaged farmed Atlantic cod (Gadus morhua) (Hovda *et al*., 2007)
						Kefir grains (Chen *et al*., 2008)
						Cheese (Arcuri *et al*., 2012)
						Brazilian kefir grains (Leite *et al*., 2013)
357f	TACGGGAGGCAGCAG	357-372	16S V3	Bacterial 16S rDNA	Muyzer *et al*. (1993)	Yoghurts and probiotic preparations (Temmerman *et al*., 2003)
518r	ATTACCGCGGCTGCTGG	518-534				Tarag (Liu *et al*., 2012)
						Brazilian kefir grains (Leite *et al*., 2012)

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
339f	CTCCTACGGGAGGCAGCAG		16S V3	Bacterial 16S rDNA		Funazushi, fermented crucian carp (Fujii *et al.*, 2011)
53r	GTATTACCGCGGCTGCTGG					
P3	GGAATCTTCCACAATGGGCG	361-380	16S V3	Bacterial 16S rDNA	Klijn *et al*. (1991)	Fermented sausages (Cocolin *et al*., 2001c)
P4	ATCTACGCATTTCACCGCTAC	385-405				
Lac1r	AGCAGTAGGGAATCTTCCA	353-371	16S V3-V4	16S rDNA of a group of LAB (*Lactobacillus, Pediococcus, Leuconostoc* and *Weissella*)	Walter *et al*. (2001)	Sourdough (Meroth *et al*., 2003) Shochu (Endo and Okada, 2005) Brazilian kefir grains (Leite *et al*., 2012)
Lac2f	ATTYCACCGCTACACATG	651-679				

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
Lac3r	AGCAGTAGGGAATCTTCGG	352-370		16S rDNA of a group of LAB (*Lactococcus, Streptococcus, Enterococcus, Tetragenococcus* and *Vagococcus*)	Endo and Okada (2005)	
Ec1055	ATGGCTGTCGTCAGCT	1055-1070	16S V9	Bacterial 16S rDNA	Ferris *et al*. (1996)	Fermented sausages (Cocolin *et al*., 2001c)
Ec1392	ACGGGCGGTGTGTAC	1392-1406				
WBAC1 (r)	GTCGTCAGCTCGTGTCGTGAGA	1069-1090	16S V9	Bacterial 16S rDNA	Lopez *et al*. (2003)	Balsamic vinegar (De Vero *et al*., 2006)
WBAC2 (f)	CCCGGGAACGTATTCACCGCG	1374-1394				

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
Euk1427F	TCTGTGATGCCCTTAGATGTTCTGGG	1427-1452	18S	Eucarya 18S rDNA	Van Hannen *et al*. (1999)	Cassava (Ampe *et al*., 2001)
Euk1616r	GCGGTGTGTACAAAGGGCAGGG	1616-1637				Pozol (Ampe and Miambi, 2000)
NL1	GCCATATCAATAAGCGGAGGAAAAG	63-88	26S	Eucarya 26S rDNA	Cocolin *et al*. (2000; 2002a)	Milk (Cocolin *et al*., 2002a)
LS2	ATTCCCAAACAACTCGAC TC	266-285				Wine (Cocolin *et al*., 2000; 2001a; 2002b; Mills *et al*., 2002)
						Brazilian kefir grains (Leite *et al*., 2012; 2013)
*rpo*B1698f	AACATCGGTTTGATCAAC		*rpo*B	Bacterial *rpo*B gene	Dahllof *et al*. (2000)	Fermented sausage and cheese (Rantsiou *et al*., 2004)
*rpo*B2014r	CGTTGCATGTTGGTACCCAT					

Table 3 (Cont.)

Primer[a]	Sequence (5'-3')	Position[b]	Region	Target	References	Application to food products
*rpo*B1(r)	ATTGACCACTTGGGTA ACCGTCG		*rpo*B	Bacterial *rpo*B gene	Dahllof *et al*. (2000)	Wine (Renouf *et al*., 2006b; Claisse *et al*., 2007)
*rpo*B1o (r)	ATCGATCACTTAGGCA ATCGTCG					LAB cocci in fermented beverages (Renouf *et al*., 2006a)
*rpo*B2(f)	ACGATCACGGGTCAAA CCACC					
*rpo*B2491 f	AACCAATTCCGTATIGG TTT		*rpo*B	Staphylococci	Drancourt and Raoult (2002)	Fermented sausages (Ravyts *et al*., 2010)
*rpo*B3554 R	CCGTCCCAAGTCATGA AA					

[a]A CG clamp is added to each forward primer according to Muyzer *et al*. (1993).

[b]*E. coli* numbering for Bacteria and *Sac. cerevisiae* numbering for Eucarya

Primers 338f and HDA1 have the same sequence, but they were given different designations by the authors who described them.

However, PCR-DGGE techniques can also have disadvantages when applied to food product. Biases may already be introduced by sampling or sample handling such as aerobic or anaerobic storage, washing, transport, freezing, or refrigerating procedures may affect the development of the microbial species occurring in the food by increasing or reducing the number and the species to be detected. Further source of variability can be DNA extraction and purification step when it is applied to mixed bacterial cultures where it is very difficult to extract DNA from all the species with the same efficiency. The PCR amplification may be a source of bias because preferential amplification might be caused by reannealing of the template DNA, which compromizes the hybridization of primers (Reysenbach *et al*., 1992; Suzuki and Giovannoni, 1996). Additionally, the sequences of a primer, the annealing temperature and the number of PCR cycle have been also involved in the mistake of PCR (Hongoh *et al*., 2003). In fact, the number of species detected may not be real because of a lack of amplification by PCR of a specific DNA template. Therefore, the choice of the primer couple and the fragment to target is fundamental. It has been shown that, sometimes, targeting different 16S variable regions may lead to different results in species composition of the same sample (Ercolini *et al*., 2003). In addition, the incomplete extension of the GC clamp during PCR amplification, which may result in artifactual double bands in DGGE analysis, often complicates the interpretation of the profiles (Janse *et al*., 2004). However, the fragments to be resolved by DGGE cannot be longer than 500 bp. This represents a limiting factor for the sequence analysis and eventually probe design. This is strongly affected by the electrophoretic conditions and the amplicon-specific formation of melting domains. Moreover, other problems are the formation of chimeric (Liesack *et al*., 1991; Kopczynski *et al*., 1994) or heteroduplex molecules (Ferris *et al*., 1997), which can affect the distribution of the bands in the DGGE profile. Furthermore, co-migration of DNA fragments can be a problem for retrieving clean sequences from individual bands. In fact, even being different in sequences, the 16S rDNA fragments might have identical melting behavior and therefore they cannot be separated in DGGE. Another problem in the study of community diversity on the basis of 16S rRNA genes using DGGE is the presence of multiple copies of the 16S rDNA with sequence microheterogeneity (Cocolin *et al*., 2001b; Blaiotta *et al*., 2003; Ercolini, 2004).

Attempts have been carried out in order to establish the detection limit of the PCR-DGGE. Indeed, it is worthwhile to define the concentration of the microbial species, which is needed in the food matrix to reveal a band in a DGGE fingerprint. However, for only one or a few species, dilution series have been applied in PCR-DGGE after DNA

extraction. Detection limits have been indicated, ranging between 10^4 to 10^8 cfu/ml (Dewettinck *et al*., 2001; Fasoli *et al*., 2003; Temmerman *et al*., 2003; Fontana *et al*., 2005b; De Vero *et al*., 2006). As a matter of fact, the detection limit depends on the species and perhaps even the strain considered. Moreover, the number and the concentration of the other members of the microbial community, along with the nature of the food matrix, all represent variables influencing the detection limit of DGGE by affecting both the efficiency of DNA extraction and the PCR amplification due to the possible competition among templates (Ercolini, 2004).

Objectives of study

1. To evaluate the bacterial community in *Kung-Som* products using *rpo*B gene and V3 region of 16S rDNA analysis
2. To isolate, screen and identify the GABA-producing LAB isolated from *Kung-Som*
3. To clone the gene encoding GAD from the selected strain and expressed of the recombinant protein in *E. coli* system

CHAPTER 2
EVALUATION OF BACTERIAL COMMUNITY IN *KUNG-SOM* USING *rpo*B GENE AND V3 REGION OF 16S rDNA ANALYSIS BY PCR-DGGE TECHNIQUE

2.1 Abstract

Kung-Som is one of several traditional Thai fermented shrimp products, that is especially in the southern part of Thailand. This is the first report to reveal the bacterial communities in the finished product of *Kung-Som*. Ten *Kung-Som* samples were evaluated using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methodology combined with appropriated primers to study the dynamics of the bacterial population. Two primers sets (V3; 341f(GC)-518r and *rpo*B; *rpo*B1698f(GC)-*rpo*B2014r primers) were considered as a possible tool for the differentiation of bacteria and compared with respect to their efficiency of 16S rDNA and *rpo*B gene amplification. PCR-DGGE analysis of both the V3-region and *rpo*B amplicon was successfully applied to discriminate between lactic acid bacteria and Gram positive strains in the bacterial communities of *Kung-Som*. In conclusion, the application of these two primers sets using PCR-DGGE techniques is a useful tool for analyzing the bacterial diversity in *Kung-Som*. Moreover, these preliminary results provide useful information for further isolation of desired bacterial strains used as a starter culture in order to improve the quality of *Kung-Som*.

2.2 Introduction

Kung-Som is a traditional fermented shrimp product that is found widely distributed in the south of Thailand. It is made from shrimp, sugar, salt and water and is typically fermented with the natural, spontaneous microbial flora. The microbiology of *Kung-Som* is diverse and complex. The principal microorganisms found in *Kung-Som* are various lactic acid bacteria (LAB) (Tanasupawat *et al*., 1998; Hwanhlem *et al*., 2010). Species identification and population enumeration are critical in the study of bacterial communities. Due to the limitations of conventional microbiological methods, the identification of microorganisms that requires selective enrichment and subculturing is problematic or impossible. Moreover, classical microbial techniques used have not accurately analyzed the presence of the main bacterial species (Ben Omar and Ampe, 2000) and have not provided a completely accurate representation of these complex communities. On the other hand, culture-independent molecular techniques have provided better methods to give more information on the microbial diversity in complex food samples (Cocolin *et al*., 2001c; Ercolini, 2004). In addition, culture-independent molecular techniques based on specific nucleotide sequences are widely used for monitoring, detection, identification and classification of bacterial diversity.

In the recent decade, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been successfully applied to determine the microbiology of fermented food such as fermented sausages (Fontana *et al*., 2005a), fermented grains (Chao *et al*., 2008), fermented meat (Hu *et al*., 2009), and fermented dairy products (Liu *et al*., 2012), to name a few. This approach has provided new insight into the microbial diversity and allowed a more rapid, high-resolution description of microbial communities than did the traditional approaches since it allows the separation of DNA molecules that differ by single bases (Ercolini, 2004). The use of appropriate consensus primers is also a critical point in determining the resolution of DGGE analysis in mixed microbial systems, especially in LAB differentiation (Chen *et al*., 2008). From the evidence of several published papers, the 16S rDNA seems to be by far the most widely used as a molecular marker for the determination of the phylogenetic relationships of bacteria. The hypervariable V3-region on the 16S rDNA is the most frequently used to start the study of an unknown and complex bacterial community. In addition, the V3-region is considered to have a high grade of resolution and to be highly variable, and it is regarded as a good choice when it comes to length and inter-species heterogeneity (Coppola *et al*., 2001; Florez and Mayo, 2006; Hovda *et al*., 2007;

Chen *et al*., 2008). Unfortunately, one problem related to the use of 16S rDNA in DGGE analysis is the complexity created by the existence of multiple heterogeneous copies within a genome (Dahllof *et al*., 2000; Crosby and Criddle, 2003; Rantsiou *et al*., 2004).

Consequently, a solution to the problem of 16S rDNA heterogeneity is provided by the analysis of a gene that exists in only a single copy (Fogel *et al*., 1999). Certain protein-coding genes, such as the gene encoding the beta-subunit of DNA-directed RNA polymerase, *rpo*B, have been proposed to fulfill this criterion. *rpo*B is used as a potential biomarker to overcome identification problems because it is considered a housekeeping gene. Targeting the *rpo*B gene allowed the reliable discrimination of species. The use of this gene as a marker was able to avoid the intraspecies heterogeneity problem caused by the use of the 16S rDNA, which appears to exist in one copy only in bacteria (Dahllof *et al*., 2000; Ko *et al*., 2002). In addition, in some strains of bacteria, an internal region of *rpo*B is a more suitable sequence than 16S rDNA because of its higher nucleotide polymorphism (Khamis *et al*., 2005). However, the use of *rpo*B presents a taxonomic disavadantage: the database of the sequence is less well documented than that of the 16S rDNA (Rantsiou *et al*., 2004; Renouf *et al*., 2006b).

There are no data using the PCR-DGGE technique to characterize the dominant bacteria in *Kung-Som* product. Consequently, the aim of this present study was to focus on the use of the hypervariable V3-region on the 16S rDNA and *rpo*B gene by using PCR-DGGE techniques as a tool to reveal the bacteria that commonly develop in the *Kung-Som* product and to compare the efficiency of the 16S rDNA and *rpo*B gene sequences for species discrimination in such a complex food sample. The obtained results provide preliminary information for study to apply in the microbial starter cultures in *Kung-Som* fermentation.

2.3 Materials and Methods

2.3.1 Lactic acid determination in *Kung-Som*

Kung-Som finish product samples were purchased from different local markets in Songkhla Province, Thailand. The pH value was measured by a pH meter (420A ORION, USA). Total acidity as lactic acid was determined according to the AOAC (AOAC, 2005). Three independent measurements were made for each sample. Data presented are the means and standard deviations calculated.

2.3.2 DNA extraction from *Kung-Som*

DNA was extracted from the juice sample of *Kung-Som* by the method Cocolin *et al*. (2004), with slight modification. One millilitre of juice sample of each sample was

centrifuged at 14,000×g for 10 min at 4 °C to pellet the cells. The pellet was washed twice with 1 ml of sterile 0.85% (w/v) NaCl. The pellet was resuspended in 50 µl of 20 mg/ml lysozyme (Fluka, USA). After 30 min incubation at 37 °C, 30 µl of 25 mg/ml proteinase K (AMRESCO®, USA) and 150 µl proteinase K buffer were added. The tubes were incubated at 65 °C for 90 min before the addition of 400 µl breaking buffer and incubated futher at 65 °C for 15 min. Then, 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7) was added for extracting DNA, RNA and protein. The tubes were centrifuged at 12,000×g at 4 °C for 10 min, the aqueous phase was collected and the nucleic acid was precipitated with 1 ml of ice-cold absolute isopropanol. The DNA was obtained by centrifugation at 14,000×g at 4 °C for 10 min, washed briefly with 70% (v/v) ice-cold ethanol and centrifuged again. The DNA was dried at room temperature, resuspended in 20 µl of RNase-DNase-free sterile water, and treated with 5 µl of 10 mg/ml DNase-free Rnase (Vivantis, USA). After 5 min incubation at 37 °C, genomic DNA was stored at -20 °C.

2.3.3 The V3 region of 16S rDNA and *rpo*B gene amplification

Primers 341f (5′-CCTACGGGAGGCAGCAG-3′) and 518r (5′ATTACCGCGGCTGCTGG-3′) were used to amplify a region of approximately 200 bp of the V3 region of 16S rDNA (Muyzer *et al*., 1993). Primers *rpo*B1698f (5'-AACATCGGTTTGATCAAC-3') and *rpo*B2014r (5'-CGTTGCATGTTGGTACCCAT-3') were used to amplify a region of approximately 350 bp of the *rpo*B gene (Dahllof *et al*., 2000). Amplification reactions were carried out in volumes of 50 µl. In addition, a GC clamp was added to the forward primer to improve the sensitivity in the detection of mutations by DGGE (Sheffield *et al*., 1989). PCR products were examined by 2% (w/v) agarose gel electrophoresis. These were used to check the quality and size of PCR products before being subjected to DGGE analysis.

2.3.4 DGGE analysis

DGGE analysis was performed using the Dcode universal mutation detection system apparatus (Cleaver Scientific, UK) according to Fontana *et al*. (2005) with slight modification. Thirty millilitres of PCR product was mixed with loading dye and applied to 8% (w/v) polyacrylamide gels. A 28% to 55% denaturing gradient (100% of denaturant corresponded to 7 mol/l urea and 40% formamide) were used for both the 341f(GC)-518r and *rpo*B1698f(GC)-*rpo*B2014r primer sets. Electrophoresis was run in 1X TAE buffer at constant temperature (60 ºC) for 10 min at 20 V and subsequently for 16 h at 85 V. After electrophoresis, the gel was stained for 30 min with 1X (final concentration) SYBR Gold

(Invitrogen, USA) in 1X TAE buffer, rinsed in water, and then visualized and photographed under UV illumination with the Gel Documentation (UVI-TECH, England).

After running the DGGE analysis, relevant bands were punched from the gel with sterile pipette tips. Each piece was transferred into 20 µl of RNase-DNase-free sterile water and incubated overnight at 4 ºC to allow the diffusion of the DNA. Then, the eluted DNA was used as a template and re-amplification took place with primers without the GC clamp. The PCR products were purified by using the HiYieldGel/PCR DNA Fragments Extraction Kit (RBC, Taiwan), and sequenced by a DNA sequencer (Ward Medic Ltd., Malaysia).

2.3.5 Construction of phylogenetic tree

Searches in GenBank with the BLAST program on the NCBI website were performed to determine the closest known relatives of the determined 16S V3 region and *rpo*B gene sequences. Multiple sequence alignments were created by using the BioEdit version 3.3.19.0. The phylogenetic tree was obtained to compare similarities among the sequences by the neighbor-joining method (Saitou and Nei, 1987) using the MEGA software version 5. Kimura's method was followed and 1,000 repetitions were made for bootstrap (Tamura *et al*., 2011).

2.4 Results and Discussion

Kung-Som is a one of the traditional fermented food products from southern Thailand. The production process traditionally relies on a spontaneous fermentation initiated by natural and fortuitous microorganisms, mainly various LAB and coagulase-negative cocci (CNC).

Kung-Som is made from the main raw materials shrimp, sugar, salt and water. These raw materials and personal hygiene can also be the possible sources of pathogenic microorganisms or spoilage bacteria such as *E. coli*, *S. aureus*, *B. cereus*, *V. parahaemolyticus* and *Salmonella* sp. In all *Kung-Som* samples of this study, it was found that the pH and lactic acid concentration ranged from 3.58 to 4.04 and 1.78% to 3.12%, respectively (Fig. 4). The pH of all samples was below 4.5 because LAB utilizes the carbohydrate substrates available to produce organic acids, and especially lactic acid, as part of their metabolites. These acids not only contribute to the taste, aroma and texture of the product but also lower the pH of the product which is one of the important key factors to ensure quality and safety (Visessanguan *et al*., 2006; Kopermsub and Yunchalard, 2010). Generally, a pH lower than 4.4 can inhibit growth of *E. coli* (Alvarado *et al*., 2006) and *Salmonella* sp. (Sorrells and Speck, 1970); a pH lower than 3.7 can inhibit *S. aureus* (Alvarado *et al*., 2006); a pH lower than 4.0 inhibits *B. cereus* (Yang *et al*., 2008) and a pH of

4.5-5.0 has been demonstrated to inhibit *V. parahaemolyticus* (Adams and Moss, 2008). Consequently, the pathogenic or spoilage bacteria were inhibited by organic acids that affected the bacterial growth and extracted DNA concentration in our samples. Most finding reports are probably related to the PCR-DGGE detection limit (10^4 cfu/ml) (Fontana *et al*., 2005b; De Vero *et al*., 2006). In accordance with these results, no DNA bands corresponding to pathogenic and spoilage bacteria were detected in *Kung-Som* samples of our study.

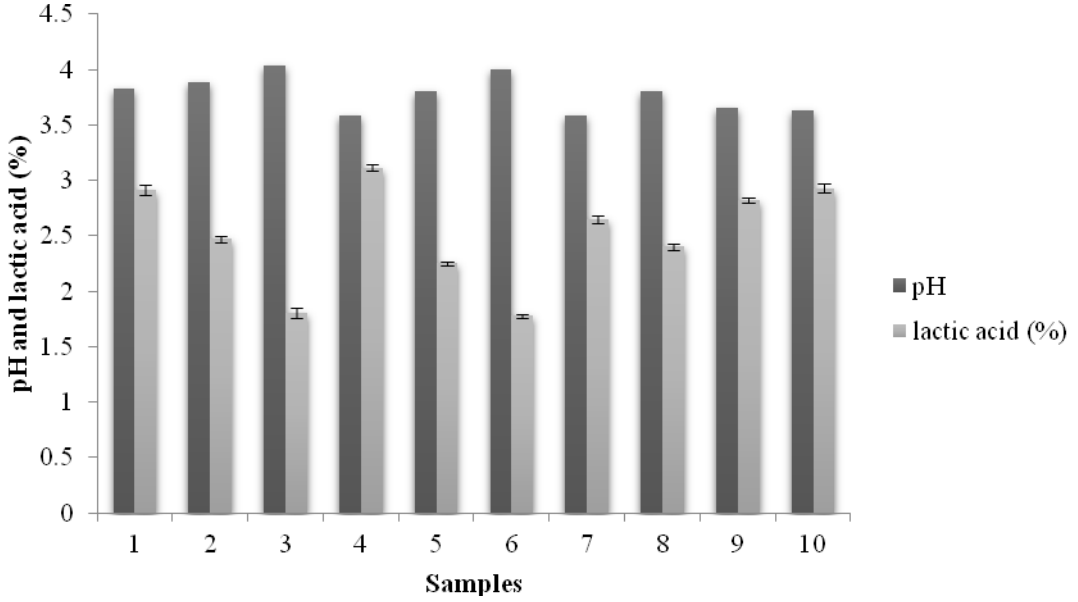
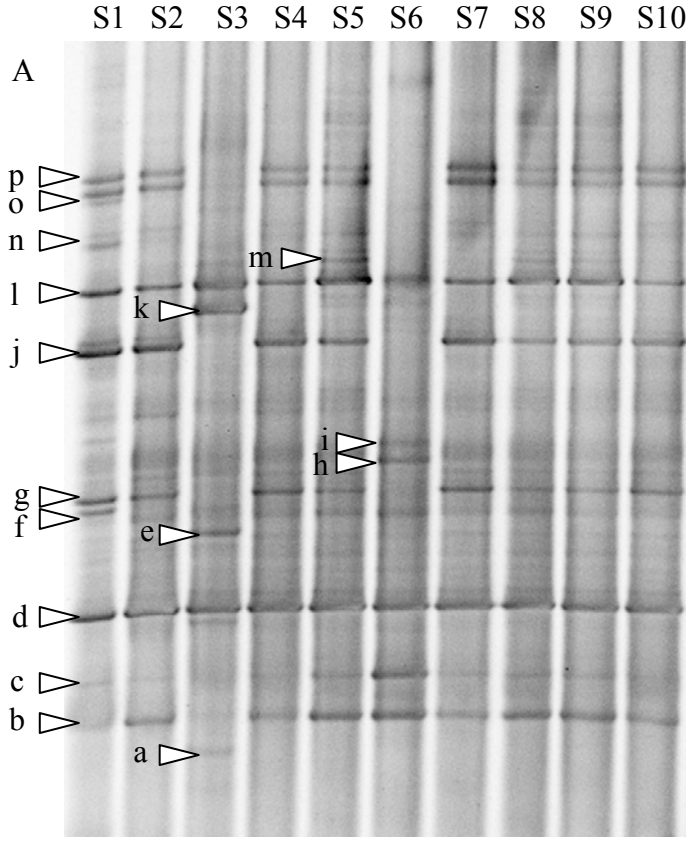


Figure 4 The pH value and titratable acidity of *Kung-Som* samples.

Renouf *et al*. (2006) reported that the first step for finding suitable primers is to assume that the primers must be present in all the species and delimit variable sequences to separate each species. The last step is to find the most suitable and accurate gradient and the best DGGE conditions (temperature, time). Two sets of primers (V3-region of 16S rDNA and *rpo*B gene) were considered suitable in this study since it is a housekeeping gene. Consequently, the bacterial diversity of the *Kung-Som* product was revealed by DGGE analysis. Figure 5 and 6 show the DGGE profiles obtained by the DNA directly extracted from *Kung-Som* in different regions in Songkhla Province, Thailand. Both the V3 region of 16S rDNA and the *rpo*B gene were amplified from these DNA templates. The V3 region of 16S rDNA and *rpo*B gene profiles displayed different patterns. The DGGE profile of the *rpo*B gene amplification showed that the numbers of the bands were lower than those of the V3 region of 16S rDNA amplification. For the DGGE profile and the phylogenetic relationship of the V3 region of 16S rDNA amplification (Fig. 5A and 5B), bands corresponding to *Tetra. halophilus* (band *b*), *L. farciminis* (band *d*) and *L. plantarum* (band *l*

and *k*) were prominent in all *Kung-Som* samples, which is in agreement with data published by Hwanhlem *et al*. (2010). Although band *k* was found in all samples, it was faint. *L. acetotolerans* (band *g*) and *L. rapi* (band *j, o*, *p*) were present in all samples except samples 3 and 6. *Salinivibrio sharmensis* (band *a*) and *Macrococcus* sp. (band *e*) appeared only in sample 3 and *L. crustorum* (band *h* and *i*) was only found in sample 6. *S. piscifermentans* (band *c*), *W. thailandensis* (band *m*) and *W. cibaria* (band *n*) were exhibited in some samples but with very weak intensity. *Salinivibrio sharmensis* (band *a*) was found only in sample 3. Several bands originating from a single species were observed on the DGGE gels, and these were from *L. crustorum*, *L. rapi* and *L. plantarum*. The reason for this is the sequence heterogeneity as described by Crosby and Criddle (2003).



B

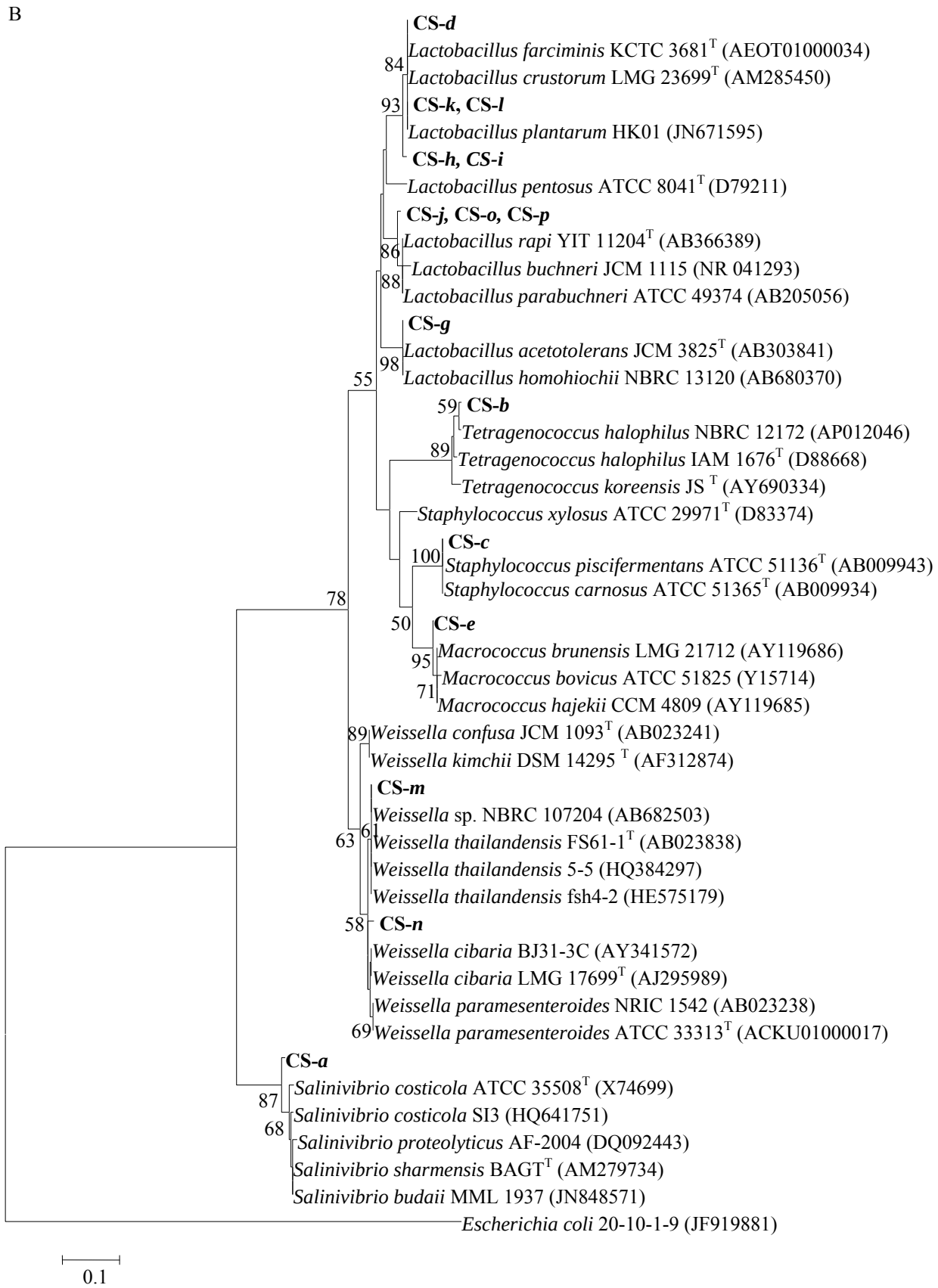

CS-d
Lactobacillus farciminis KCTC 3681T (AEOT01000034)
84
Lactobacillus crustorum LMG 23699T (AM285450)
93
CS-k, CS-l
Lactobacillus plantarum HK01 (JN671595)
CS-h, CS-i
Lactobacillus pentosus ATCC 8041T (D79211)
CS-j, CS-o, CS-p
Lactobacillus rapi YIT 11204T (AB366389)
86
Lactobacillus buchneri JCM 1115 (NR 041293)
88
Lactobacillus parabuchneri ATCC 49374 (AB205056)
CS-g
Lactobacillus acetotolerans JCM 3825T (AB303841)
98
Lactobacillus homohiochii NBRC 13120 (AB680370)
55
59
CS-b
Tetragenococcus halophilus NBRC 12172 (AP012046)
89
Tetragenococcus halophilus IAM 1676T (D88668)
Tetragenococcus koreensis JS T (AY690334)
Staphylococcus xylosus ATCC 29971T (D83374)
CS-c
100
Staphylococcus piscifermentans ATCC 51136T (AB009943)
Staphylococcus carnosus ATCC 51365T (AB009934)
78
50
CS-e
Macrococcus brunensis LMG 21712 (AY119686)
95
Macrococcus bovicus ATCC 51825 (Y15714)
71
Macrococcus hajekii CCM 4809 (AY119685)
89
Weissella confusa JCM 1093T (AB023241)
Weissella kimchii DSM 14295 T (AF312874)
CS-m
Weissella sp. NBRC 107204 (AB682503)
6
63
Weissella thailandensis FS61-1T (AB023838)
Weissella thailandensis 5-5 (HQ384297)
Weissella thailandensis fsh4-2 (HE575179)
58
CS-n
Weissella cibaria BJ31-3C (AY341572)
Weissella cibaria LMG 17699T (AJ295989)
Weissella paramesenteroides NRIC 1542 (AB023238)
69
Weissella paramesenteroides ATCC 33313T (ACKU01000017)
CS-a
Salinivibrio costicola ATCC 35508T (X74699)
87
Salinivibrio costicola SI3 (HQ641751)
68
Salinivibrio proteolyticus AF-2004 (DQ092443)
Salinivibrio sharmensis BAGT T (AM279734)
Salinivibrio budaii MML 1937 (JN848571)
Escherichia coli 20-10-1-9 (JF919881)
0.1

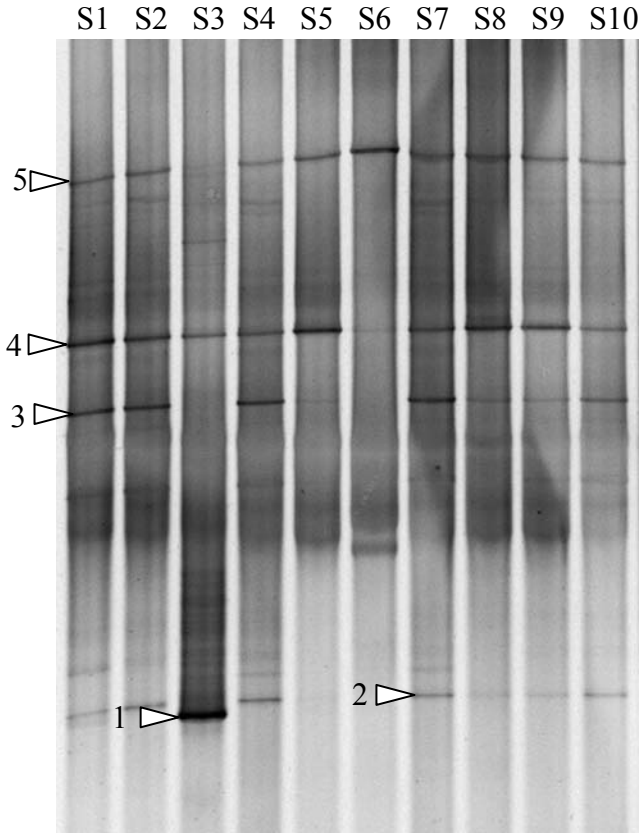
Figure 5 DGGE profiles (A) and the phylogenetic tree based on V3-region on 16S rDNA (B) of the bacteria community obtained from DNA directly extracted from *Kung-Som* samples. The scale bar represents the number of inferred substitutions per site.

For the DGGE profile and the phylogenetic relationship of *rpo*B gene amplification (Fig. 6A and 6B), bands 4 and 5, corresponding to *W. thailandensis* and *S. piscifermentans*, were predominating in every sample. Furthermore, *L. fermentum* (band 2) and *L. reuteri* (band 3) were present in all samples except sample 3 and 6. Band 1 was only detected in sample 3.

Species-specific DGGE bands from two sets of primers for the main members of LAB and CNC were exhibited. LAB (*Lactobacillus, Tetragenococcus* and *Weissella*), including CNC species (*Macrococcus* and *Staphylococcus*), are the most commonly isolated bacteria from fermented foods, especially meat and fish (Hu *et al*., 2008; Kopermsub and Yunchalard, 2010; Hwanhlem *et al*., 2011). In addition, the genus *Salinivibrio* was found to be the dominant species in sample 3 of the V3 region amplification. These species are generally isolated from fermented fish samples (Chamroensaksri *et al*., 2009). The differences in the results obtained, such as the DGGE pattern from the *rpo*B gene amplification, indicated the occurrence of very low bacterial diversity when compared with the V3-region amplification. These results depended on the specificity of the primers (Endo and Okada, 2005; Renouf *et al*., 2006b) and the PCR conditions (Hongoh *et al*., 2003).

Rantsiou *et al*. (2004) and Renouf *et al*. (2006b) reported that the use of *rpo*B gene amplification combined with PCR-DGGE can only reveal the predominant species in a sample. Moreover, Chen *et al*. (2008) suggested that the use of appropriate consensus primers is a critical point in influencing the resolution of DGGE analysis in mixed microbial systems, especially in LAB differentiation. Endo and Okada (2005) indicated that LAB could not be detected by universal bacterial PCR primer but were detected when groups of LAB-specific primer were used. This is because the DGGE profile can demonstrate only the diversity of bacteria present at more than 1% of the target bacteria. Therefore, the detection of numerous different species present at low concentrations appeared to be difficult using PCR-*rpo*B/DGGE. Thus, the *rpo*B gene pattern exhibited the different species which did not appear in the V3-region pattern.

A



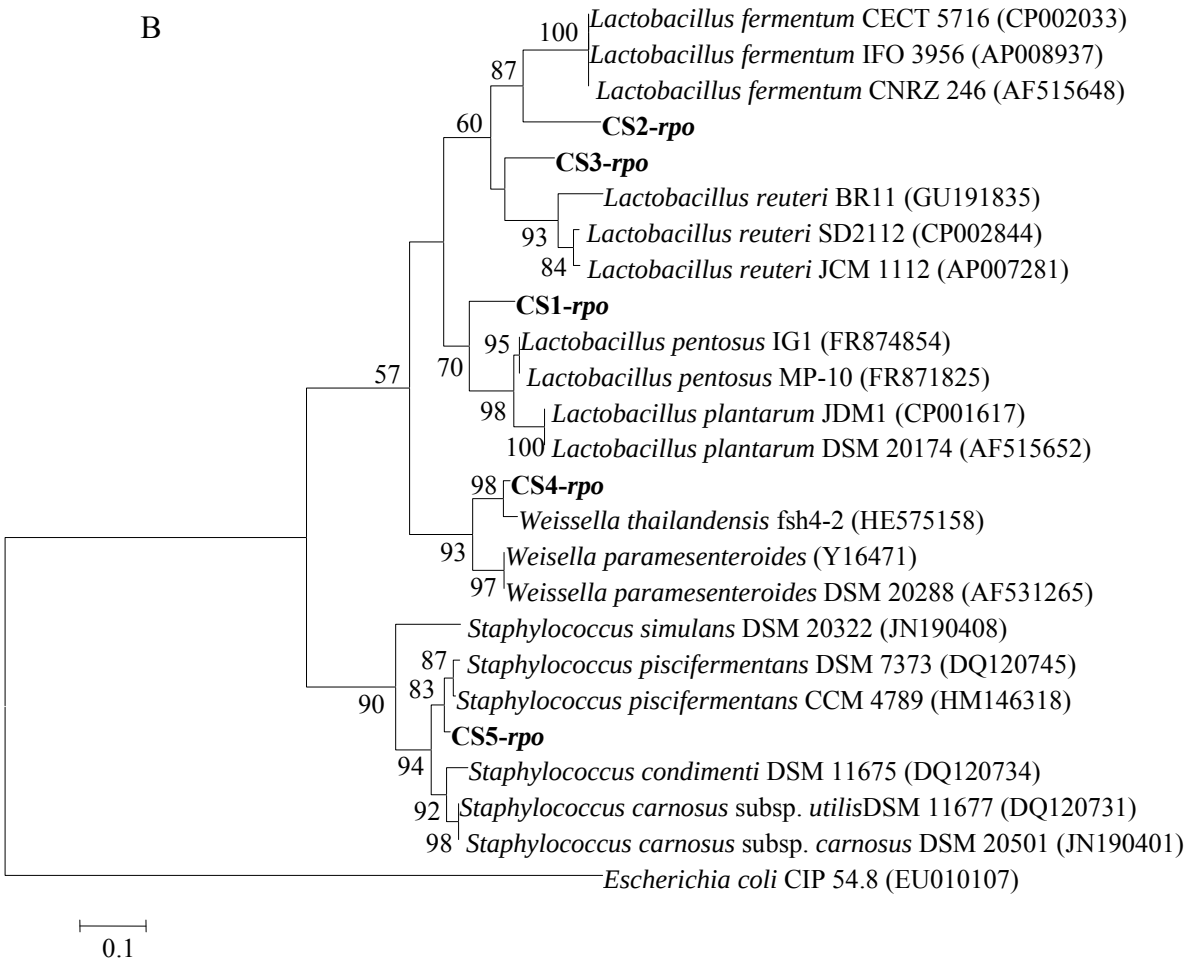


Figure 6 DGGE profiles (A) and the phylogenetic tree based on *rpo*B gene (B) of the bacteria community obtained from DNA directly extracted from *Kung-Som* samples. The scale bar represents the number of inferred substitutions per site.

In both the *rpo*B gene and V3-region patterns, samples 3 and 6 showed different DGGE patterns from those of compared to other samples. This is because these samples were originated from different recipes or processes of preparation which could vary the initial food matrix, fermentation process, personal hygiene, local tradition or local geographic preferences. All of these are crucial factors in determining the growth of specific microbial communities (Cocolin *et al*., 2004; Ercolini, 2004; Chen *et al*., 2008). This outcome was related to the higher pH and lower lactic acid content of samples 3 and 6 (Fig. 4). In accordance with this lower lactic acid concentration, a difference in the LAB groups of these samples was detected (Fig. 5 and 6).

A number of faint bands could not be identified because of their low content which might be related to the heterogeneous distribution of microorganism in the food matrix (Florez and Mayo, 2006). In a detection limit analysis, an individual species was identified by PCR-DGGE when its number was higher than 10^4 cfu/ml (Fontana *et al*., 2005b; De Vero *et al*., 2006). The detection limit of PCR-DGGE depends on the species or perhaps even the strain considered. Furthermore, the number and the concentration of the other members of the microbial community, along with the nature of the food matrix, all represent variables influencing the detection limit of DGGE. These factors affect both the efficiency of DNA extraction and the PCR amplification due to possible competition among templates (Ercolini, 2004; Temmerman *et al*., 2004; De Vero *et al*., 2006).

2.5 Conclusion

The suitability of the primers used was based on the discriminatory efficiency of the hypervariable V3-region and the *rpo*B gene that allowed species differentiation from the dominant groups of bacteria in *Kung-Som*. Although the applications of PCR-DGGE techniques combined with appropriate consensus primers to study complex microbial communities originating from food samples have been shown to be an efficient tool for detection of complex bacteria populations, we believe that our findings represent a preliminary analysis. The data cannot be considered sufficient to achieve a confident identification at species level, but should suggest that the relevant genes together with other target sequences such as other region of 16S rDNA, *gyr*A, *gyr*B, *rec*A or *rpo*C, should be used for unequivocal identification of individual species. Moreover, these preliminary results provide useful information for improving product quality. An improved understanding of the changing microflora in *Kung-Som* fermentations could be used to develop a starter culture in the future.

CHAPTER 3
ISOLATION AND CLONING OF A NOVEL GLUTAMATE DECARBOXYLASE GENE FROM A NEWLY GABA-PRODUCING LACTIC ACID BACTERIA, *LACTOBACILLUS FUTSAII* CS3

3.1 Abstract

Kung-Som is one of several traditional Thai fermented shrimp products, especially popular in the southern part of Thailand. *Kung-Som* presents a high glutamic acid which a major substrate for biosynthesis of natural γ-aminobutyric acid (GABA) by lactic acid bacteria (LAB). In the present study, the GABA-producing LAB from *Kung-Som* were isolated, screened and identified. The strain CS3 showed the highest GABA-producing ability among the screened strains. Based on an API-CHL50 fermentation and phylogenetic trees of 16S rDNA sequence, the strain CS3 belonged to genus *Lactobacillus futsaii*. Newly *Lactobacillus futsaii* CS3 with high GABA-synthesizing capacity was first discovered in the present report. The open reading frame (ORF) of glutamate decarboxylase (*gad*) gene was cloned by PCR. It was 1,410 bp encoding a polypeptide of 469 amino acids. In addition, the non-crystallization structure of *L. futsaii* CS3 GAD was also predicted by SWISS-MODEL server. The entire ORF sequence of *gad* gene was cloned into pColdI expression vector and expressed in *Escherichia coli* BL21 (DE3). The *gad* gene with His_6-Tag was expressed. The recombinant GAD was purified using a Ni-NTA column. SDS-PAGE analysis revealed that it had a molecular weight of approximately 53 kDa, which corresponded to the predicted size of the deduced protein (53.64 kDa). The results of these findings offer a way of replacing chemical GABA by natural GABA in fermented foods or functional foods. Moreover, it preliminary provides useful details for development of the molecular mechanism regulating GABA metabolism in valuable LAB.

3.2 Introduction

Nowadays, consumers are increasingly interested in the relation between food and health to improve the human health. As a consequence, many kinds of food with health-promoting properties have entered the global markets over the last few years. All of these, gamma-aminobutyric acid (GABA) is the one of bioactive substances that has several physiological functions and hence has a great application potential in functional foods and pharmaceuticals. However, the direct addition of chemical GABA to food is regarded as unnatural and unsafe and is still illegal in Korea (Seok *et al*., 2008; Kim *et al*., 2009).

GABA is a four carbons and non-protein amino acid that is widely distributed in nature among microorganisms, plants and animals. GABA is used considerably in functional foods and pharmaceuticals, and massively as a major active constitutes in several foods. GABA functions as the major inhibitory neurotransmitters in the central nervous system in mammalian brains (Huang *et al*., 2007b; Dhakal *et al*., 2012). Clinical studies have related increased intake of GABA or analogues to several health benefits, including lowering of blood pressure of mildly hypertensive animals and humans (Hayakawa *et al*., 2004; Diana *et al*., 2014). Hayakawa *et al*. (2004) reported that GABA strongly induced insulin secretion from the pancrease, therefore, effectively preventing diabetics. Furthermore, consumption of GABA-enriched foods can inhibit cancer cell proliferation (Park and Oh, 2007b) and improve memory and the learning abilities (Miura *et al*., 2006). Generally, sufficient amounts of GABA can be produced in the human body. However, GABA production is sometimes inhibited by a lack of estrogen, zinc, or vitamins, or by an excess of salicylic acid and food additives (Suk-Heung Oh, 2003; Cho *et al*., 2011).

Kung-Som is one of Thai fermented foods that made from shrimp, sugar, salt, and water (Hwanhlem *et al*., 2010) and is typically fermented with the natural, spontaneous microbial flora. The microbiology of *Kung-Som* is various lactic acid bacteria (LAB). Generally, LAB play a crucial role in fermentation processes, and have a long and safe history of application and consumption in the production of fermented foods and beverages (Leroy and De Vuyst, 2004). In addition, LAB have been used as probiotics due to their properties and also protect foods by controlling the food spoilage pathogens by secreting acids, conjugated linoleic acid, vitamin, aroma compounds, bacteriocins, exopolysaccharides, GABA and enzymes. Therefore, the use of GABA-producing LAB strains as starter cultures in fermentation processes can help to achieve bio-synthetic production of the GABA, safety and many special physiological activities. This provides a way of replacing chemical GABA by natural GABA, at the same time offering the consumer with new attractive food products.

This reduces the production cost because of the omission the extra addition of GABA (Leroy and De Vuyst, 2004; Li and Cao, 2010; Dhakal *et al*., 2012).

The biosynthesis of GABA is one step reaction of decarboxylating glutamate to GABA, catalyzed by glutamate decarboxylase enzyme (GAD). GAD acts as the essential enzyme for GABA production and needs a pyridoxal 5'-phosphate (PLP) as cofactor. The glutamic acid or glutamate acts as a major substrate for biosynthesis of natural GABA by LAB (Ueno, 2000). Fortunately, glutamic acid could be greatly found in *Kung-Som* products because the shrimp contains a rich source of glutamic acid (Daul *et al*., 1994). In the future, functional foods that presence of GABA has increased research interest since studies show that GABA may have significant health effects and is only found in low levels in natural substances. Then, much GABA research focuses on finding new ways to produce products high in GABA. However, the studies on GABA-producing LAB in *Kung-Som* have not been reported yet. Therefore, the development of *Kung-Som* containing GABA to contribute functional properties will be actively studied. In the present study, isolation and identification the newly GABA-producing LAB from *Kung-Som* was performed. In addition, the existence of *gad* gene from novel strain was also investigated for cloning and expression of the GAD protein combined with cold shock vector in *E. coli* system.

3.3 Materials and Methods

3.3.1 Isolation and screening of GABA-producing LAB

Kung-Som samples were purchased from local markets in Songkhla Province, Thailand. Samples were homogenized in sterile 0.1% (w/v) peptone and spread onto MRS agar supplemented with 0.02% (w/v) bromocresol purple and incubated at 37 °C for 24 h. Bacterial colonies exhibited yellow zone on the plates were tested for catalase and Gram-staining. The catalase-negative and Gram-positive isolates were cultivated in MRS broth containing 2% (w/v) monosodium glutamate (MSG) at 37 ºC for 24 h. Culture broth was centrifuged at 8,000×g for 5 min at 4 ºC. The GABA in the supernatant was presumptively detected by using thin-layer chromatography (TLC). Briefly, levels of GABA were determined qualitatively by TLC with Silicagel 60 F_{254} TLC plate (Merck, Germany). One microliter of supernatant was spotted onto TLC plates. TLC was conducted using an 1-butanol:acetic acid:distilled water (5:2:2) solvent mixture, and plates were subsequently sprayed with 0.5% (w/v) ninhydrin solution and then heated at 105 ºC for 5 min. R_f value was calculated as followed: R_f = distance travelled by component/distance travelled by

solvent. Bacterial strains having the same R_f value as the GABA standard (Sigma-Aldrich, UK) was selected for quantitation by HPLC.

3.3.2 GABA analysis

3.3.2.1 Sample preparation

Quantitative analysis of GABA in culture broth was derivatized with *o*-phthaldialdehyde (OPA) before detection using RP-HPLC according to Populin *et al*. (2007) with modification. The OPA solution (pH 9.3) was made by mixing 5.0 ml of a methanolic OPA, 20 ml of borate buffer (pH 9.9) and 50 µl of 2-mercaptoethanol. Methanolic OPA was made by dissolving 0.25 g of OPA in 50 ml of methanol. The borate buffer solution was prepared by mixing 0.2 M boric acid (dissolved in 0.2 M potassium chloride) and 0.2 M sodium hydroxide in a ratio of 50:50 (v/v). Fifteen microliters of sample and 75 µl of OPA solution were mixed and allowed to stand for 2 min before injection into the column.

3.3.2.2 RP-HPLC analysis

A RP-HPLC determination was performed with an Agilent Technologies 1200 series binary pump, autosampler and a fluorescence detector equipped with an automatic liquid sampler, injector program. The column was a Hypersil ODS C_{18} reverse phase column (4.6 x 250 mm, 5 µm) and thermostatted at 30 °C.

The separation of OPA-derivatives was performed with a mobile phase, consisting of 370 ml of water plus 90 ml of phosphate buffer at pH 7.0 as solvent A, while solvent B was acetonitrile. The gradient elution program was held at 13% of B for 15 min, ramped at 50% of B (40 min) and then at 85% of B (60 min) and held until the end of the run (62 min) with a flow rate of 1.0 ml/min. Detection was performed with a spectrofluorometer, model G1321A set at 330 nm (λ excitation) and 440 nm (λ emission).

3.3.3 Identification of GABA-producing LAB

3.3.3.1 Phenotypic identification

Morphological characterization of the superior GABA-producing isolates was done by examining colony growth, cell morphology, and Gram reaction and catalase test. Isolates that were found to be Gram positive and catalase negative were subjected to biochemical characterization using the API 50 CHL fermentation strips (Biomerieux SA, France) at 37 ºC according to the manufacturer's instructions. The cells were collected by centrifugation (8,000×g for 5 min at 4 ºC) and washed twice with phosphate buffer (pH 7.0). The washed cells were suspended in 10 ml of suspension medium (Bio Me´rieux) and inoculated into the API CHL strips. The strips were incubated at 37 ºC for 48 h, and then acid formation was determined by monitoring the change in color of bromocresol purple used as an indicator.

3.3.3.2 Genotypic identification

Genomic DNA from the superior GABA production was extracted and used as template for PCR to amplify the 16S rDNA by using the universal bacterial primers (approximately 1.5 kb) 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Shukla and Goyal, 2011). The amplification cycle was as follows: 30 repetitions of 95 °C for 1 min; 50 °C for 45 sec and 72 °C for 1 min 30 sec. An initial denaturation at 95 °C for 5 min and a final extension at 72 °C for 10 min were carried out. PCR products were purified and cloned into pGEM T-Easy vector (Promega, Madison, WI, USA). *E. coli* JM109 cells were transformed with the ligation mixture by a heat shock method (Chung *et al*., 1989). The sequencing results were compared with known sequences using the basic local alignment search tool (BLAST program, http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rDNA sequences from various species were downloaded from NCBI. A phylogenetic tree was constructed using the neighbour-joining method in the Molecular Evolutionary Genetics Analysis (MEGA 5) package (Tamura *et al*., 2011).

3.3.4 Growth profiles and GABA production of *L. futsaii* CS3

Lactobacillus futsaii CS3 was cultivated in MRS broth with 25 mg/ml of total MSG containing 1% (v/v) inoculums (OD_{600} ~ 0.4-0.5). Then, incubated at 37 ºC for 72 h. Growth of the isolate was determined by measuring culture turbidity at 600 nm every 3 h. Also, culture pH, lactic acid content (AOAC, 2005) and GABA concentration were measured at the same time intervals. The GABA conversion efficiency (%) was calculated from equation according to Ratababuree *et al*. (2011):

$$\text{GABA conversion efficiency (\%)} = \frac{[\text{GABA}]}{[\text{GABA}]_{max}} = \frac{[\text{GABA}]-[\text{GABA}]_0}{[\text{MSG}]_0 \times \frac{103.12}{169.13}} \times 100$$

[GABA] = concentration of GABA at time of sampling (g/l)

$[GABA]_{max}$ = concentration of GABA when all MSG converted to GABA by fermentation (g/l)

$[GABA]_0$ = concentration of GABA at the time of starting fermentation (g/l)

$[MSG]_0$ = concentration of MSG added (this study, 25 g/l)

169.13 and 103.12 are molecular weights of MSG and GABA, respectively

3.3.5 Cloning of *gad* gene

Based on the conserved sequences of *gad*B position of *Lactobacillus gad* genes available in GenBank (www.ncbi.nlm.nih.gov), a pair of primers, GAD_FT (5'-ATGGCAATGTTATACGGTAAACAC-3') and GAD_RT (5'-TCAGTGTGTGAATCCGTATTTCTT-3') was designed to amplify the ORF of *gad* gene. It was used to amplify the approximate 1.4 kb target sequence. The PCR was performed as follows: 94 ºC for 3 min; 35 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 1 min 30 sec; finally 72 ºC for 10 min. The amplified fragments were cloned into pGEM T-Easy vector. After being transformed into the competent cells of *E. coli* JM109, positive clones were sequenced in both directions with universal primers T7 and SP6. The similarity of *gad* gene with others was analyzed using the online BLAST program. The molecular weight and the predicted isoelectric point (pI) were obtained through ExPASy (http://web.expasy.org/compute_pi/).

3.3.6 Construction and expression of *gad* gene in *E. coli* BL21 (DE3)

Based on the ORF sequence of *gad* gene, a pair of primers, forward primers: 5′-GCG**CATATG**GCAATGTTATACGGTA-3′ and reverse primer 5′-GG**GAATTC**TCA GTGTGTGAATCCGT-3′ were designed to amplify the sequence encoding the mature peptide of GAD enzyme. The underlined portions in the primer sequences represent the recognition sites for the restriction enzymes *Nde*I and *Eco*RI, respectively. The amplified fragment was digested by *Nde*I and *Eco*RI and then inserted into the pColdI expression vector (Takara, Japan) to generate the expression vector pColdI-GAD. The recombinant plasmid pColdI-GAD was transformed and expressed in *E. coli* BL21 (DE3). Briefly, *E. coli* BL21 (DE3) cells carrying pColdI-GAD was inoculated into LB broth containing amplicilin (100 μg/ml) and grown at 37 ºC to reach an OD_{600} of approximately 0.3-0.5. After that, the culture solution was refrigerated at 15 ºC for 60 min to prepare the cells. The expression was induced by adding of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. It was cultivated with shaking (150 rpm) at 15 ºC for 24 h. The bacterial cells were harvested and the expression of recombinant protein was confirmed by 12.5% SDS-PAGE analysis.

3.3.7 Purification of the GAD-$(His)_6$ fusion protein

A single colony of the expressed cell was inoculated into LB medium containing 100 μg/ml of amplicilin. The cells were harvested by centrifugation (10,000×g, 4 °C, 10 min) after induction by IPTG at 15 ºC for 24 h. The recombinant protein was isolated from the pellet cells by lysis with lyzozyme followed by repeated cycles of freezing and thawing

(Johnson and Hecht, 1994). Briefly, the pellet cells was washed twice with 50 mM phosphate buffer (pH 8.0) and then resuspended in lysis buffer (50 mM sodium phosphate, 1 mM PMSF, 300 mM NaCl, 10 mM imidazole, pH 8.0). A final concentration of lysozyme 1 mg/ml was added in solution. Pellet cells were frozen by submerging the tube into liquid nitrogen for 5 min and then thawed by transferring it into water bath (37 ºC) for 10 min. This cycle was repeated 4 additional times. The cell debris was removed by centrifugation at 10,000×g at 4 °C for 20 min. The pellet was dissolved in 8M urea and purified by using the affinity chromatography which carried out with HisPur^{TM} Ni^{2+}-nitrilotriacetate (Ni-NTA) superflow agarose (Thermo Scientific, U.S.A.) under denaturing conditions. Briefly, the solution was first loaded onto an affinity column pre-equilibrated with equilibration buffer (20 mM NaH_2PO_4, 6M urea, 300 mM NaCl, 10 mM imidazole, pH 7.4). The contaminant proteins were removed with washing buffer (20 mM NaH_2PO_4, 6M urea, 300 mM NaCl, 20 mM imidazole, pH 7.4). Then, GAD protein was eluted with elution buffer (20 mM NaH_2PO_4, 6M urea, 300 mM NaCl, 300 mM imidazole, pH 7.4). The concentrations of purified protein were determined using Bradford approach with bovine serum albumin (BSA) as a standard (Bradford, 1976). The GAD molecular weight was determined by SDS-PAGE using 12.5% (w/v) acrylamide gel for separation and 5% (w/v) gel for stacking.

3.3.8 Computer modeling method

The molecular model of GAD from *L. futsaii* CS3 was obtained by homology modeling via the SWISS-MODEL server (Peitsch, 1995; Guex and Peitsch, 1997; Arnold *et al*., 2006). The *gad*B from *E. coli* at low pH (PDB ID: 1PMM) was selected as the template for model building. Sequence alignment was performed using ClustalX (Thompson *et al*., 1997). The resulting model was evaluated with SWISS-MODEL server.

3.4 Results

Screening and identification of GABA-producing LAB

The GABA-producing LAB was isolated from *Kung-Som*, a Thai traditional fermented shrimp. During the screening stage, 600 pure LAB isolates were cultivated in MRS medium containing 2% MSG. Then, the supernatant was qualitative tested for GABA-producing ability on TLC plates. Only two isolates (CS3 and CS5) exhibited a strong GABA spot when MSG was present (Fig. 7A). In addition, two GABA-producing LAB isolates were quantitatively confirmed using RP-HPLC (Fig.7B). GABA content of strain CS3 at 24 and 48 h was 3.25 and 6.30 mg/ml, respectively. Strain CS5 produced GABA content at 24 and 48 h was 2.55 and 6.0 mg/ml, respectively.

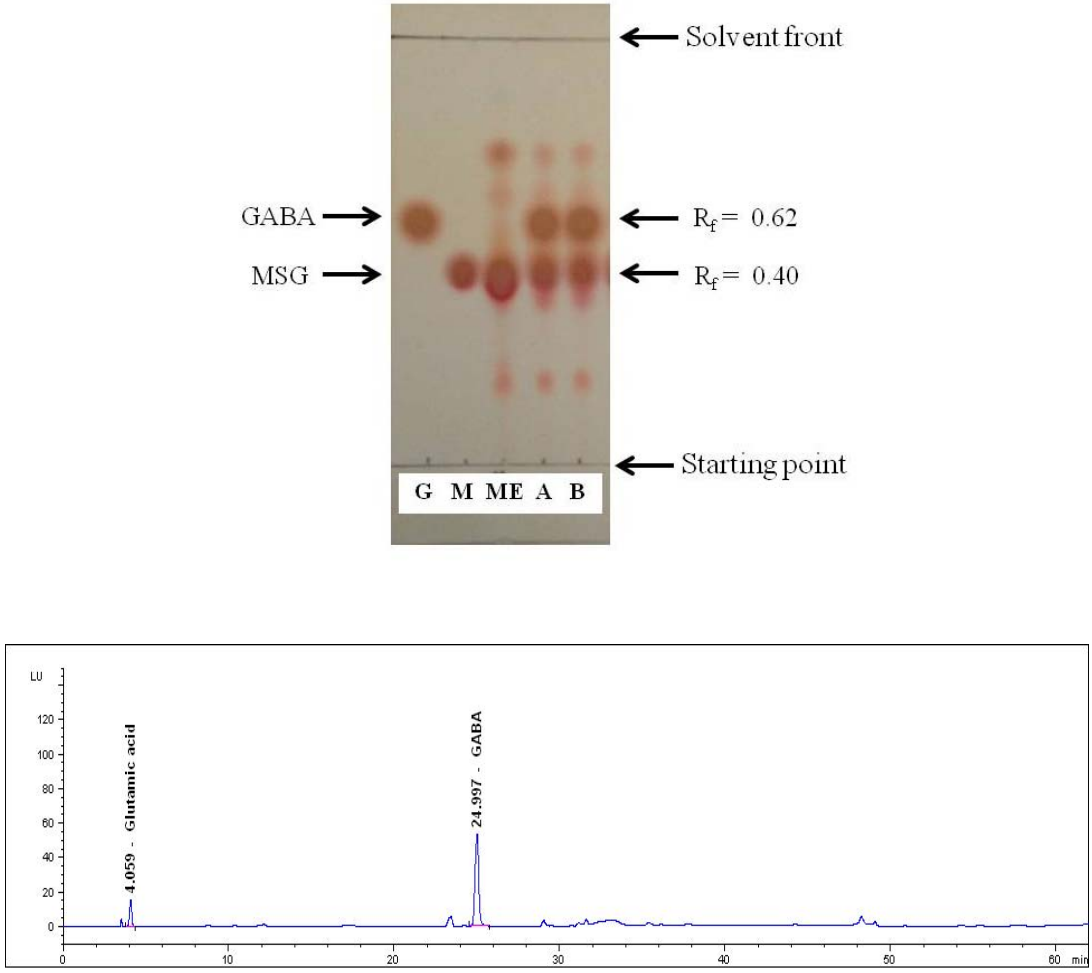


Figure 7 A; TLC chromatogram of GABA production by the isolated LAB. Lanes G, GABA standard; M, MSG standard; ME, MRS medium containing 2% MSG (control); A, isolate CS3; B, isolate CS5. B; HPLC chromatogram of MSG and GABA in the media.

The superior GABA-producing LAB strain CS3 and CS5 were a Gram-positive, rod shape, negative-catalase test and facultative anaerobic. To identify these strains, the biochemical tests using API-CHL50 fermentation strips and 16S rDNA using PCR amplification was performed. Based on the fermentation of 50 carbohydrates producing organic acids, monitoring the change in color of bromocresol purple used as an indicator was performed. This demonstrated the presence of a strong intra-strain fluctuation in carbohydrate metabolism patterns of *Lactobacillus* species. The phenotypic results obtained for two strains are shown in Table 4. Carbohydrate fermentations pattern of both strains were completely same. These results suggested that strain CS3 and CS5 correspond to the specie *L. futsaii* when comparing with type strains of *L. futsaii* YM0097^T or JCM 17355^T or BCRC 80278^T according to Chao et al. (2012).

Table 4 Biochemical characterization of the strain CS3 and CS5 using API 50 CHL fermentation strips

Parameter	Characteristics
Utilizable sugar	D-Galactose, D-Glucose, D-Fructose, D-Mannose, N-Acetylglucosamine, Amygdalin, Arbutin, Esculin ferric citrate, Salicin, D-Cellobiose, D-Maltose, D-Lactose (bovine origin), D-Saccharose (Sucrose), D-Trehalose, Gentiobiose, D-Tagatose
Non-utilizable sugar	Glycerol, Erythritol, D-Arabinose, L-Arabinose, D-Ribose, D-Xylose, L-Xylose, D-Adonitol, Methyl-βD-Xylopyranoside, L-Sorbose, L-Rhamnose, Dulcitol, Inositol, D-Mannitol, D-Sorbitol, Methyl-αD-Mannopyranoside, Methyl-αD-Glycopyranoside, D-Melibiose, Inulin, D-melezitose, D-Raffinose, Amidon (starch), Glycogen, Xylitol, D-Turanose, D-Lyxose, D-Fucose, L-Fucose, D-Arabitol, L-Arabitol, Potassium Gluconate, Potassium 2-Ketogluconate, Potassium 5-Ketogluconate

Moreover, based on the 16S rDNA sequence, phylogenetic tree analysis showed that the strain CS3 and CS5 belonged to the genus *L. futsaii* (Fig. 8) with 99.80% and 99.66% identity of *L. futsaii* YM0097^{T} (GenBank accession no. HQ322270), respectively. Therefore, the strain CS3 and CS5 was named as *L. futsaii* CS3 and *L. futsaii* CS5, respectively. The nucleotide sequence of 16S rDNA from *L. futsaii* CS3 and CS5 were deposited in DNA Data Bank of Japan (DDBJ) as the accession number AB839950 and LC019014, respectively.

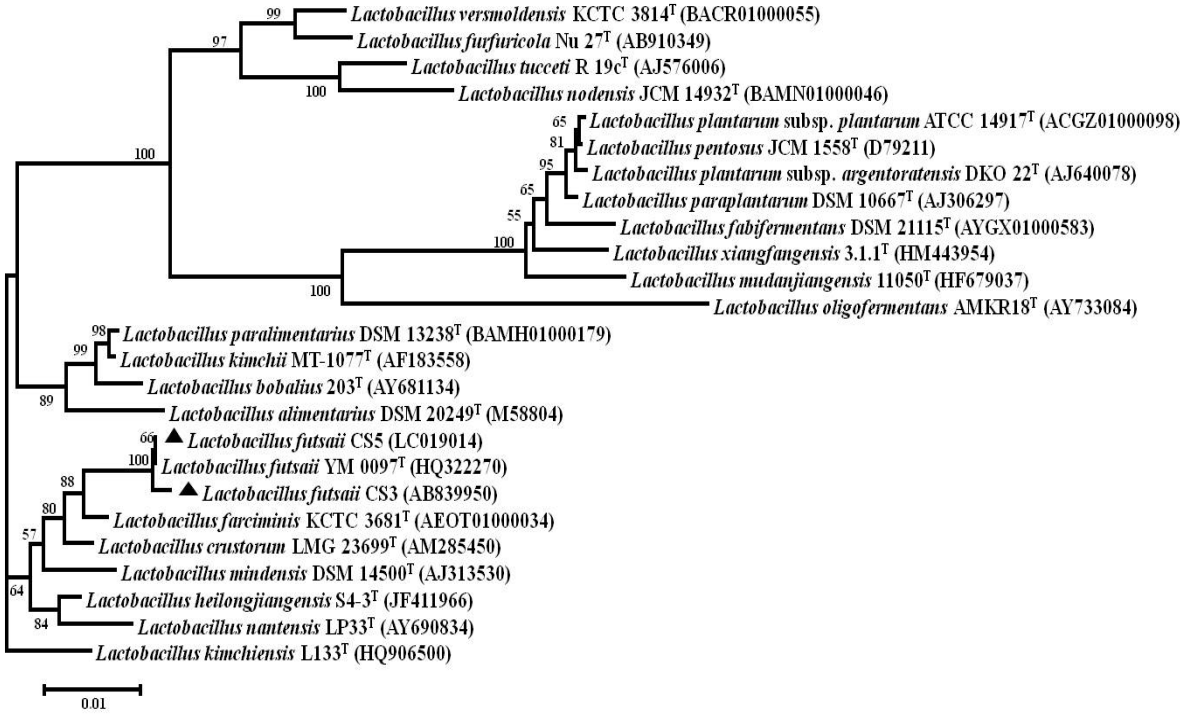


Figure 8 Neighbour-joining tree comprising 16S rDNA sequences of strain CS3 and CS5 with recognized *Lactobacillus* species. Bootstap values expressed as percentages of 1,000 replications (greater than 50% are shown at the branch points). Bar, 0.01 substitutions per nucleotide position

Growth profiles and GABA production of *L. futsaii* CS3

In order to investigate the cell growth association with GABA production pattern of *L. futsaii* CS3 strain, *L. futsaii* CS3 was cultivated in MRS broth supplemented with 25 MSG mg/ml at 37 °C for 72 h. As shown in Figure 9, the bacterium grew rapidly within the first 24 h (~9 log cfu/ml), which was correlated with the sharply decline in pH and rapidly increased in lactic acid concentration. After pH value of the medium dropped to lower than 4.5, the GABA was produced. Then, the GABA concentration was gradually increased until maximum at 72 h. The pH of the culture medium increased gradually to pH 5.0 at the end of fermentation. However, it was noted that *L. futsaii* CS3 could quite completely convert MSG to GABA at the maximum more than 99% of GABA conversion.

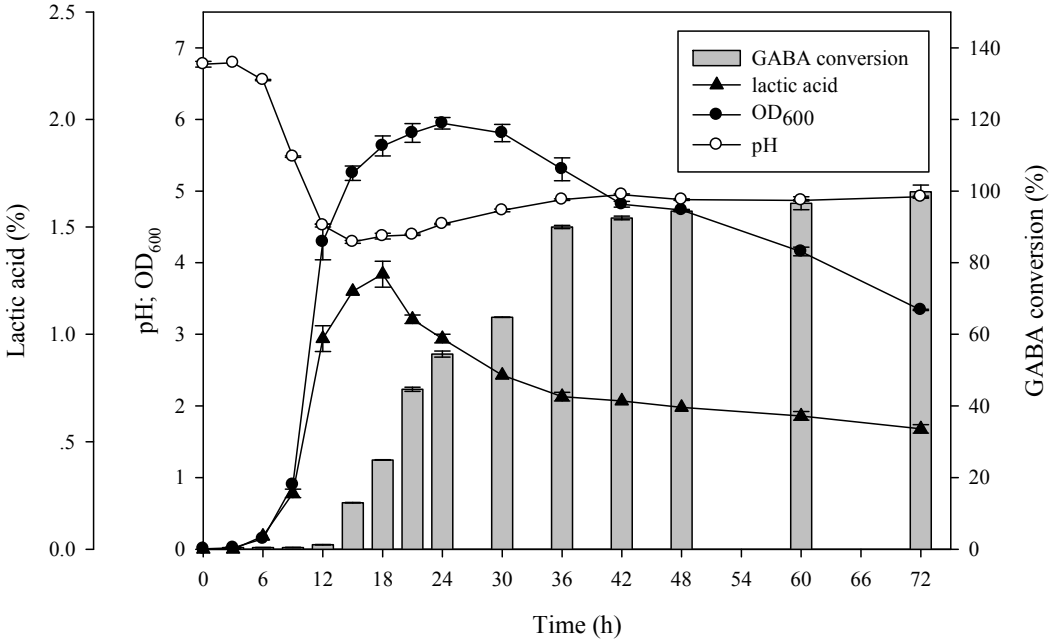


Figure 9 Growth of *L. futsaii* CS3 in MRS broth containing 25 mg/ml MSG, GABA production (% GABA conversion), and the changes in broth pH and lactic acid content

Molecular survey and cloning of *gad* gene

The ORF sequence of *L. futsaii* CS3 *gad* gene was amplified by using PCR with proof reading DNA polymerase enzyme. The result showed that 1.4 kb *gad* gene was successfully detected in *L. futsaii* CS3 (Fig. 10). Then, the *gad* gene was cloned into pGEM T-Easy vector and the nucleotide sequence was investigated. The entire open reading frame (ORF) was 1,410 bp in length that encoded a protein of 469 amino acids (Fig. 11) with a predicted molecular weight of 53.64 kDa and an estimated isoelectric point (pI) of 5.56 by ExPASy program. The *gad* sequence comparisons were carried out using BLAST program. The results indicated that *L. futsaii* CS3 *gad* gene showed 99% identity with *L. plantarum* (CP002222, AL935263 and CP001617), 98% with *L. reuteri* (KF751352), 97% with *Lc. lactis* subsp. *lactis* (KF751353) and *L. delbrueckii* subsp. *bulgaricus* (KF751355). The alignments of the deduced amino acid sequence of *gad* gene with others were also carried out. The results showed that *gad* sequence from *L. futsaii* CS3 showed similarity with *L. plantarum* CMPG5300 (KGH41583) (99%), *L. reuteri* ATCC 23272 (AHX56280) (97%), *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (AHX56283) (94%), *L. brevis* BH2 (ABU55419) (83%), and *L. brevis* OPK-3 (AAZ95185) (83%).

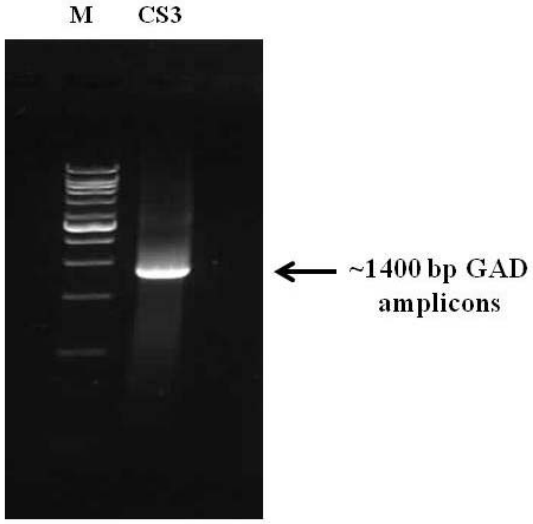


Figure 10 The presence of approximate 1.4 kb of the *gad* gene from *L. futsaii* CS3. Lane M = a 1 kb DNA ladder marker

```
   1 ATGGCAATGTTATACGGTAAACACAATCATGAAGCTGAAGAATACTTGGAACCAGTCTTTGGTGCGCCTTCTGAGCAACATGATCTTCCT
     M  A  M  L  Y  G  K  H  N  H  E  A  E  E  Y  L  E  P  V  F  G  A  P  S  E  Q  H  D  L  P
  91 AAGTATCGGTTACCAAAGCATTCATTATCCCCTCGAGAAGCCGATCGCTTAGTTCGTGATGAATTATTAGATGAAGGCAATTCACGACTG
     K  Y  R  L  P  K  H  S  L  S  P  R  E  A  D  R  L  V  R  D  E  L  L  D  E  G  N  S  R  L
 181 AACCTGGCAACTTTTTGTCAGACCTATATGGAACCCGAAGCCGTTGAATTGATGAAGGATACGCTGGCTAAGAATGCCATCGACAAATCT
     N  L  A  T  F  C  Q  T  Y  M  E  P  E  A  V  E  L  M  K  D  T  L  A  K  N  A  I  D  K  S
 271 GAGTACCCCCGCACGGCCGAGATTGAAAATCGGTGTGTGAACATTATTGCCAATCTGTGGCACGCACCTGATGACGAACACTTTACGGGT
     E  Y  P  R  T  A  E  I  E  N  R  C  V  N  I  I  A  N  L  W  H  A  P  D  D  E  H  F  T  G
 361 ACCTCTACGATTGGCTCCTCTGAAGCTTGTATGTTAGGCGGTTTAGCAATGAAATTCGCCTGGCGTAAACGCGCTCAAGCGGCAGGTTTA
     T  S  T  I  G  S  S  E  A  C  M  L  G  G  L  A  M  K  F  A  W  R  K  R  A  Q  A  A  G  L
 451 GATCTGAATGCCCATCGACCTAACCTCGTTATTTCGGCTGGCTATCAAGTTTGCTGGGAAAAGTTTTGTGTCTACTGGGACGTTGACATG
     D  L  N  A  H  R  P  N  L  V  I  S  A  G  Y  Q  V  C  W  E  K  F  C  V  Y  W  D  V  D  M
 541 CACGTGGTCCCAATGGATGAGCAACACATGGCCCTTGACGTTAACCACGTCTTAGACTACGTGGACGAATACACAATTGGTATCGTCGGT
     H  V  V  P  M  D  E  Q  H  M  A  L  D  V  N  H  V  L  D  Y  V  D  E  Y  T  I  G  I  V  G
 631 ATCATGGACATCACTTATACCGGTCAATATGACGACCTAGCCGCACTCGATAAGGTCGTTACTCACTACAATCATCAGCATCCCAAATTA
     I  M  D  I  T  Y  T  G  Q  Y  D  D  L  A  A  L  D  K  V  V  T  H  Y  N  H  Q  H  P  K  L
 721 CCAGTCTACATTCACGTTGACGCAGCGTCAGGTGGCTTCTATACCCCATTTATTGAGCCGCAACTCATCTGGGACTTCCGGTTGGCTAAC
     P  V  Y  I  H  V  D  A  A  S  G  G  F  Y  T  P  F  I  E  P  Q  L  I  W  D  F  R  L  A  N
 811 GTCGTTTCGATCAACGCCTCCGGGCACAAGTACGGTTTAGTTTATCCTGGGGTCGGCTGGGTCGTTTGGCGTGATCGTCAGTTTTTACCG
     V  V  S  I  N  A  S  G  H  K  Y  G  L  V  Y  P  G  V  G  W  V  V  W  R  D  R  Q  F  L  P
 901 CCAGAATTAGTCTTCAAAGTTAGTTATTTAGGTGGGGAGTTGCCGACAATGGCGATCAATTTCTCACATAGTGCAGCCCAGCTCATTGGA
     P  E  L  V  F  K  V  S  Y  L  G  G  E  L  P  T  M  A  I  N  F  S  H  S  A  A  Q  L  I  G
 991 CAATACTATAATTTCATTCGCTTTGGTATGGACGGTTACCGCGAGATTCAAACAAAGACTCACGATGTTGCCCGCTACCTGGCAGCCGCT
     Q  Y  Y  N  F  I  R  F  G  M  D  G  Y  R  E  I  Q  T  K  T  H  D  V  A  R  Y  L  A  A  A
1081 CTGGATAAAGTTGGTGAGTTTAAGATGATCAATAACGGACACCAACTCCCCCTGATTTGTTACCAACTAGCCCCGCGCGAAGATCGTGAA
     L  D  K  V  G  E  F  K  M  I  N  N  G  H  Q  L  P  L  I  C  Y  Q  L  A  P  R  E  D  R  E
1171 TGGACCCTTTATGATTTATCGGATCGCCTATTAATGAACGGTTGGCAAGTACCAACGTATCCTTTACCTGCTAATCTGGAACAACAAGTC
     W  T  L  Y  D  L  S  D  R  L  L  M  N  G  W  Q  V  P  T  Y  P  L  P  A  N  L  E  Q  Q  V
1261 ATCCAACGAATCGTCGTTCGGGCTGACTTTGGCATGAATATGGCCCACGATTTCATGGATGACCTGACCAAGGCTGTCCATGACTTAAAC
     I  Q  R  I  V  V  R  A  D  F  G  M  N  M  A  H  D  F  M  D  D  L  T  K  A  V  H  D  L  N
1351 CACGCCCACATTGTCTATCATCATGACGCGGCACCTAAGAAATACGGATTCACACACTGA
     H  A  H  I  V  Y  H  H  D  A  A  P  K  K  Y  G  F  T  H  *
```

Figure 11 Nucleotide and deduced amino acid sequence of the *L. futsaii* CS3 *gad*B gene. The predicted amino acids sequence (single-letter abbreviation) is shown above the nucleotide sequence. The presumed catalytic amino acid residues (Thr215 and Asp247, and Lys280) are shown in bold case.

In addition, the deduced amino acid sequence of CS3 *gad* gene belonged to the *gad*B family. It had both a highly conserved motif (HVDAASGG) that belongs to pyridoxal-5'-phosphate (PLP)-dependent decarboxylase and a PLP-binding domain (Fig. 12). Moreover, these positions in the non-crystallization structure are shown in Figure 13. The monomer homology model of recombinant GAD protein had almost the same fold as the subunit A of *gad*B from *E.coli*. The high degree of sequence identity was 46.23% similarity. Moreover, the Global Model Quality Estimation (GMQE) value of 0.74 also indicated fairly good quality of the constructed model. Correspondingly, the *gad*B of *L. futsaii* CS3 composed of three domains: N-terminal domain (residues 1-59), large (residues 60-350) and small (residues 351-469) domain. The *gad*B β-hairpin region spanning residues 304-316 was performed. Indeed, the nucleotide sequence of *gad*B gene from *L. futsaii* CS3 has been submitted to the DDBJ nucleotide sequence database under the accession no. AB986192.

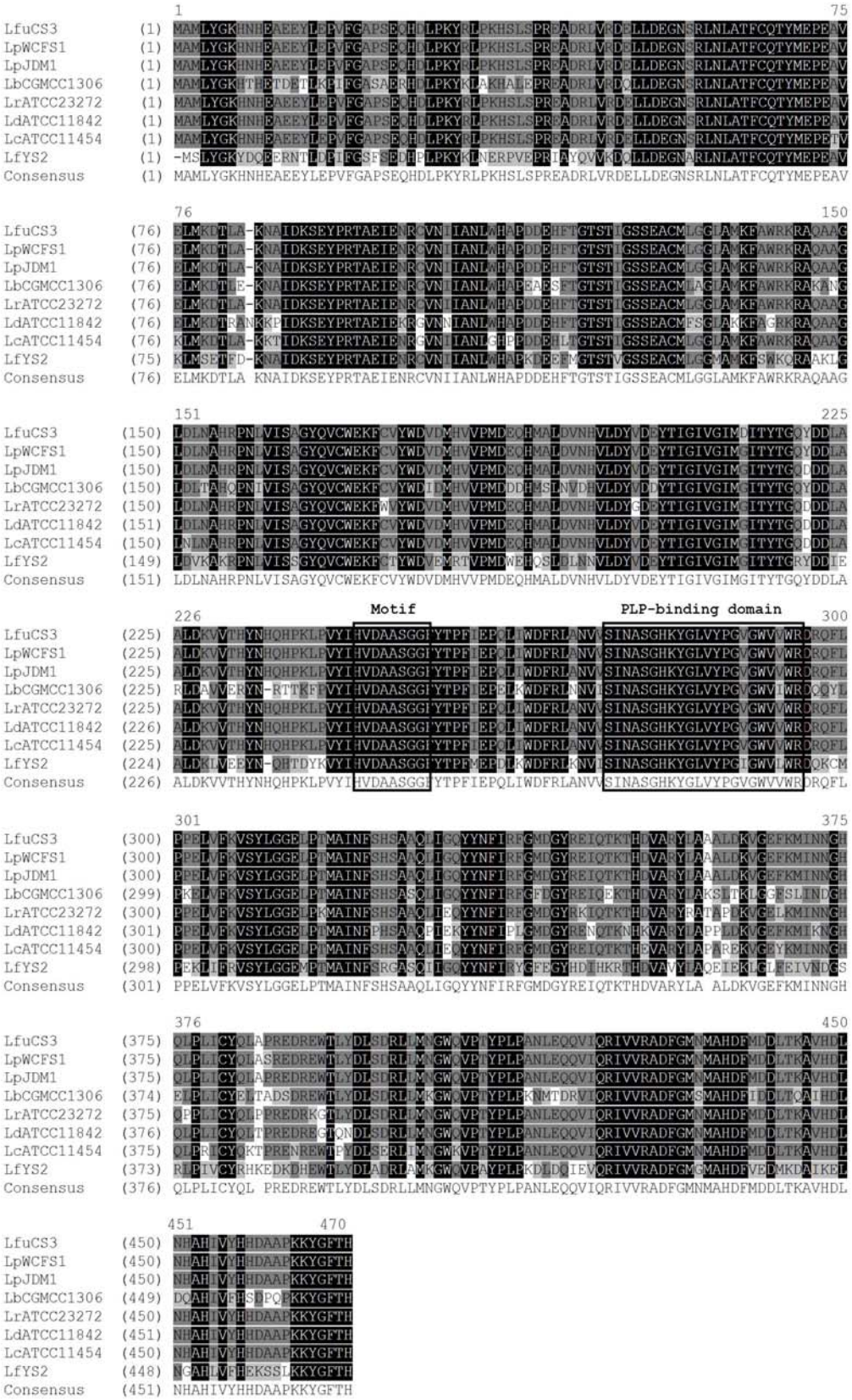


Figure 12 Alignment of the amino acid sequence of *L. futsaii* CS3 *gad*B with *gad*B proteins from other LAB. The amino acid residues (HVDAASGG) in the box are highly conserved in pyridoxal 5'-phosphate-dependent decarboxylase; the amino acid residues in other box are the pyridoxal 5'-phosphate binding domain.

Figure 13 Cartoon representation of the homology model of the *gad*B monomer at low pH. N-terminal domain is colored yellow, large domain pink color, small domain or C-terminal deep blue color and β-hairpin region green color. PLP-binding domain and motif sheets are colored light blue and white, respectively.

Expression of the recombinant GAD enzyme

The recombinant plasmid (pColdI-GAD) was transformed into *E. coli* BL21 (DE3) cells. After IPTG induction for 24 h at 15 ºC, the recombinant protein, mainly expressed as inclusion bodies, was analyzed using SDS-PAGE. Therefore, the inclusion bodies were dissolved in 8M urea and purified by Ni-NTA chromatography under denaturing conditions (Fig. 14). SDS-PAGE analysis showed that the molecular mass of the purified recombinant was about 53 kDa. In addition, the amount of proteins after purification was approximately 19.16 mg proteins/0.1 gram of pellet cell.

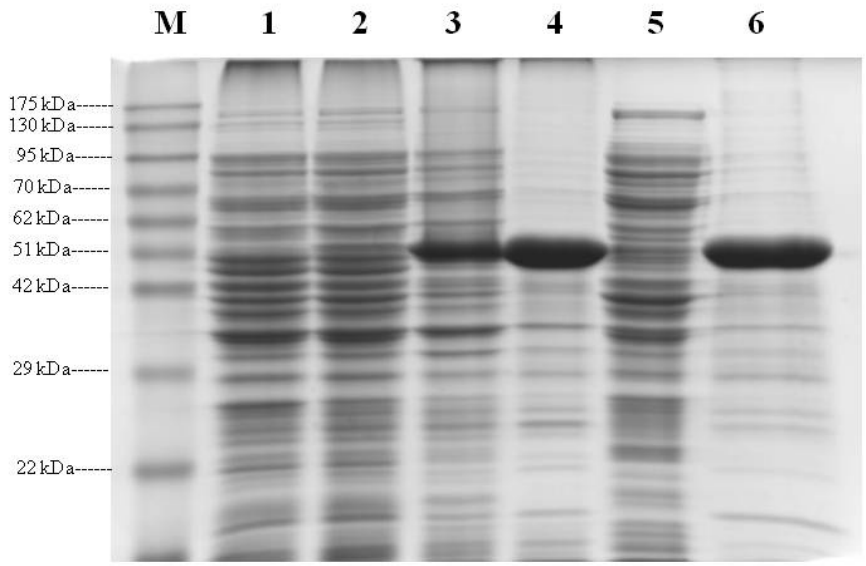


Figure 14 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the expression and purification of recombinant GAD protein in *E. coli* BL21 (DE3). The figure showed a 12.5% SDS-PAGE gel stained with Coomassie Blue. Lanes: M molecular weight marker, 1 soluble-insoluble fraction of *E.coli* BL21 with pColdI plasmid, 2 and 3 soluble-insoluble fraction of *E. coli* BL21 transformed with pColdI-GAD before and after 24h induction by IPTG, respectively. 4 and 5 insoluble and soluble fraction of *E. coli* BL21 transformed with pColdI-GAD after 24h induction by IPTG, respectively. 6 recombinant GAD purified by NI-NTA column kit

3.5 Discussion

The GABA-producing LAB have been isolated successfully from several fermented foods as previous reports such as fermented fish, fermented shrimp (*Cincaluk*) (Farrah *et al*., 2009) , fermented vegetable (*Kimchi*) (Kim and Kim, 2012), Chinese traditional paocai (Li *et al*., 2010b), yoghurt (Watanabe *et al*., 2011), fermented sausages (*Nham*) (Ratanaburee *et al*., 2013) or cheeses (Di Cagno *et al*., 2010). Mostly, *Lactobacillus* spp. were reported as GABA-producing LAB. They are the dominant LAB in the last stage of fermentation because they produce acid more slowly than cocci. However, they are more tolerant to acid than cocci (Mohd Adnan and Tan, 2007; Kopermsub and Yunchalard, 2010; Hwanhlem *et al*., 2011). In the present study, a promising *L. futsaii* CS3 was first reported in the finished products of *Kung-Som*, a traditional fermented shrimp of southern Thailand. It is a rich source of LAB and higher glutamic acid which a major substrate for biosynthesis of natural GABA by LAB. Actually, *L. futsaii* was first isolated from *fu-tsai*, the traditional fermented mustard products

of Taiwan by Chao *et al*. (2012). In addition, *L. futsaii* was isolated from several Thai fermented fish (*Pla-Som, Pla-ra, Pla-jom, Pla-jaw, Som-khai-pla, Mum-sai-pla*) (Miyashita *et al*., 2012). During cultivated *L. futsaii* CS3 in MRS medium containing 2% (w/v) MSG at 37 ºC for 24 h (Fig. 9), it produced a GABA content of 6,628.46 mg/l. It was remarkably higher than that of other strains such as *L. delbrueckii* subsp. *bulgaricus* IAM1120 and *Strep. thermophilus* IFO13957 (less than 200 mg/l) (Watanabe *et al*., 2011), *L. plantarum* DW12 (4,156 mg/l) (Ratanaburee *et al*., 2011), *Leuconostoc* NC5 (292.86 mg/l) (Farrah *et al*., 2009), and *L. brevis* 340G (1,598.4 mg/l) (Seo and Lee, 2013). Accordingly, *L. futsaii* CS3 could be a promising LAB strain for application as a starter culture in the food industrial production of GABA and the development of functional foods.

GABA is the product of the reaction catalyzed by glutamate decarboxylase enzyme which is the irreversible α-decarboxylation of L-glutamic acid to GABA and carbon dioxide (Ueno, 2000). The aim of growth characteristics observation was to obtain a better understanding about the mechanism of the GABA production in *L. futsaii* CS3. In the present study, a significant decrease and gradually increased in culture media pH was observed. This decrease could be due to the hetero-type lactic acid fermentation of *L. futsaii*, resulting in the formation of lactic acid and acetic acid (Cho *et al*., 2011). Production of GABA by *L. futsaii* was related to increase of cell growth and decrease of the culture media pH. The decreased pH apparently accelerated GABA production in the medium during periods of exponential cell growth. In other investigations using other species of GABA-producing LAB, the GABA and pH pattern during GABA production was found to be similar to the present study (Komatsuzaki *et al*., 2005; Cho *et al*., 2007; 2011). In addition, GABA production was also directly associated with its expression of *gad* genes. Physiologically, this is assumed to control the acidification of the cytosolic environment by consumption of an intracellular proton after the uptake of glutamate by its specific transporter. The decarboxylating reaction product GABA would then be exported from cells into the extracellular environment by an antiporter, The net result is an increase in the pH of the cytoplasm, due to the removal of hydrogen ions, and a slight increase in the extracellular pH, due to the exchange of extracellular glutamate for the more alkaline GABA (an acid-tolerance mechanism) (Cho *et al*., 2007; Siragusa *et al*., 2007; Hiraga *et al*., 2008).

Actually, the existence of *gad* gene in LAB has been described in many reports (Park and Oh, 2007a; Kim *et al*., 2007; Fan *et al*., 2012; Park *et al*., 2014). However, there is no information available on the *gad* gene sequences from *L. futsaii* strains. Fortunately, the sequences of *gad* gene of *L. futsaii* CS3 was discovered and first reported in the present

study. As discussed earlier, the presence of *gad* gene suggests an increased ability to produce GABA, since GAD enzyme must be present to produce GABA. In addition, sequence analysis showed that the fragment contained a complete ORF of 1,410 nucleotides that encoded a protein of 469 amino acids. Among the alignment of deduced amino acid with other *gad* sequence, the results revealed that the deduced amino acid sequence of the *L. futsaii* CS3 GAD contained a highly conserved catalytic domain that belong to the PLP-dependent decarboxylase superfamily. It possessed a conserved lysine residue (Lys280) that is known to be crucial for the binding of PLP as well as the active site residues (Thr215 and Asp247) that promote decarboxylation. Moreover, the motif [HVDAASGG] which is highly conserved in PLP-dependent decarboxylase was discovered in the *L. futsaii* CS3 *gad* sequence (Park and Oh, 2007a; Kim *et al*., 2007; Fan *et al*., 2012). In addition, non-crystallization structure of *L. futsaii* CS3 was predicted using SWISS-MODEL server. The structure of *L. futsaii* CS3 GAD was fairly good model. Because GAD enzyme is localized exclusively in the cytoplasm at neutral pH, it is recruited to the membrane when the pH falls (Capitani *et al*., 2003). Therefore, the subunit A of *gad*B from *E. coli* at low pH was used as the template for modeling evaluation. Also, bacterial GAD exhibits an acidic pH optimum (3.8-5.0) (Huang *et al*., 2007b; Komatsuzaki *et al*., 2008; Fan *et al*., 2012). In addition, the *gad* system is by far the most potent and is involved in conferring acid resistance to the bacteria in stationary phase, giving them survival capacity for at least 2 h in a strongly acidic environment (pH<2.5), such as that of the stomach (Capitani *et al*., 2003).

The expression of GAD enzyme was carried out with cold-shock expression vector, so called pColdI. It could be induced to create the cold-shock proteins base on the low-temperature by utilizing the promoter derived from *cspA* gene, which is one of the cold-shock genes. The *gad* gene was inserted to the multicloning site of pColdI vector to generate the recombinant expression vector construct pColdI-GAD. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) to express the GAD enzyme under the control of the *cspA* promoter. The *csp*A mRNA has a highly efficient structure for translation initiation. When the *csp*A mRNA is induced at 15 ºC using a multiple copy plasmid results in the trapping of most cellular ribosomes and the inhibition of other cellular protein synthesis (Mitta *et al*., 1997; Qing *et al*., 2004). Besides the pColdI vector is consisted of His-tag sequence, GAD protein was expressed as a His-tagged fusion protein to facilitate purification. The recombinant GAD proteins expressed from pColdI-GAD plasmid was constructed as inclusion bodied forms. However, approximate 70% of recombinant proteins are over-expressed as insoluble inclusion bodies. Although inclusion bodies cannot be directly used

for studies of protein activities, their insolubility provides an easy source of relatively pure protein (Yang *et al*., 2011). The inactive inclusion bodies forms are frequently occurred depending on many parameters such as the gene regulatory, characteristic of proteins, secretion mechanisms, types of plasmid, temperature induction, disulfide bond formations or whatever explained in Rudolph and Lilie (1996) and Singh and Panda (2005). As a result, a computer-based homology search program by NCBI revealed that the amino acid sequence deduced from *L. futsaii* CS3 GAD ORF showed different homology with recombinant GAD enzyme in microbes such as *L. brevis* BH2 (83%) (Park and Oh, 2007a) and *L. brevis* OPK-3 (83%) (Kim *et al*., 2007). Their results showed that the GAD recombinant proteins were expressed as the soluble protein forms. On the other hand, GAD proteins from *L. futsaii* CS3 showed 44.5% of hydrophobic amino acids. According to these results, it is generally assumed that highly hydrophobic protein and high-level expression is more prone to lead to accumulation as inclusion bodies in *E. coli* system (Mitraki *et al*., 1991). Indeed, this procedure was employed successfully with highly expressed proteins that formed inclusion bodies.

3.6 Conclusion

Kung-Som is the one of famous fermented shrimp that widely distributed in the south of Thailand. Because many kinds of fermented foods is mainly fermented by *Lactobacillus* sp., *L. futsaii* CS3 is a potential alternative to be extensively used in the functional foods and pharmaceuticals with contributing GABA. In addition, we have also first reported the ORF sequence of *gad*B gene from *L. futsaii* strain. In addition, the non-crystallization structure of *L. futsaii* CS3 GAD was predicted for additional basic knowledge. Furthermore, the GAD protein was successfully expressed in *E. coli* system. These preliminary results may provide useful information for the potential expression of LAB *gad* gene in other microbes. It will further expand the application area of GABA-producing LAB to significantly enhance GABA production and value added of products in further study.

CHAPTER 4
CONCLUSION AND SUGGESTION

4.1 Conclusion

Kung-Som is the one of famous fermented shrimp that widely distributed in the south of Thailand. From PCR-DGGE analysis combined with two primer sets, the dominant groups of bacteria in *Kung-Som* were LAB and CNC. *L. futsaii* was first discovered from *Kung-Som* products with contributing GABA production. In addition, we have also first reported the ORF sequence of *gad*B gene from *L. futsaii* strain. Moreover, the non-crystallization structure of *L. futsaii* CS3 GAD was predicted for additional basic knowledge. Furthermore, the GAD protein was successfully expressed in *E. coli* system. These preliminary results may provide useful information for the potential expression of LAB *gad* gene in other microbes. It will further expand the application area of GABA-producing LAB to significantly enhance GABA production and value added of products in further studies.

4.2 Suggestion

1. The data cannot be considered sufficient to achieve a confident identification at species level, but should suggest that the relevant genes together with other target sequences such as other region of 16S rDNA, *gyr*A, *gyr*B, *rec*A or *rpo*C, should be used for unequivocal identification of individual species.
2. According to molecular and genetic characterization of *gad* gene, the GAD protein was expressed as inclusion bodied form in *E.coli* system. Therefore, it should be improved the expression system. For example, it could be changed to other vectors or expression host. In our further studies, we will transform the *gad* gene into a food grade vector and expressed in *Lactobacillus* system.

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APPENDIX

BLAST results of nucleotide of 16S rDNA from *Lactobacillus futsaii* CS3

Lactobacillus futsaii strain YM 0097 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_117973.1| **Length:** 1542 **Number of Matches:** 1

See 1 more title(s)

Lactobacillus futsaii strain YM 0097 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|HQ322270.1|

Range 1: 1 to 1516 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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BLAST results of nucleotide of 16S rDNA from *Lactobacillus futsaii* CS5

Lactobacillus futsaii strain YM 0097 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_117973.1| **Length:** 1542 **Number of Matches:** 1

See 1 more title(s)

Lactobacillus futsaii strain YM 0097 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|HQ322270.1|

Range 1: 18 to 1486 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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Query  1201  CTTATGACCTGGGCTACACACGTGCTACAATGGTCGGTACAACGTGTTGCGAACTCGCGA  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1214  CTTATGACCTGGGCTACACACGTGCTACAATGGTCGGTACAACGTGTTGCGAACTCGCGA  1273

Query  1261  GGGCAAGCAAATCACTTAAAACCGATCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1274  GGGCAAGCAAATCACTTAAAACCGATCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCA  1333

Query  1321  TGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCC  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1334  TGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCC  1393

Query  1381  TTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACC  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1394  TTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACC  1453

Query  1441  CTTCGGGGAACTAGCCGCATAAGGTGA-ACAAA  1472
             ||||||||||||||||| |||||||  |||||
Sbjct  1454  CTTCGGGGAACTAGCCGCCTAAGGTGGGACAAA  1486
```

BLAST results of nucleotide of *gad*B gene from *Lactobacillus futsaii* CS3

Lactobacillus plantarum strain B21, complete genome

Sequence ID: gb|CP010528.1| **Length:** 3284260 **Number of Matches:** 1

Range 1: 3017629 to 3019038 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
2582 bits(1398)	0.0	1406/1410(99%)	0/1410(0%)	Plus/Plus

Features: glutamate decarboxylase

```
Query  1        ATGGCAATGTTATACGGTAAACACAATCATGAAGCTGAAGAATACTTGGAACCAGTCTTT  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017629  ATGGCAATGTTATACGGTAAACACAATCATGAAGCTGAAGAATACTTGGAACCAGTCTTT  3017688

Query  61       GGTGCGCCTTCTGAGCAACATGATCTTCCTAAGTATCGGTTACCAAAGCATTCATTATCC  120
               |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017689  GGTGCGCCTTCTGAACAACATGATCTTCCTAAGTATCGGTTACCAAAGCATTCATTATCC  3017748

Query  121      CCTCGAGAAGCCGATCGCTTAGTTCGTGATGAATTATTAGATGAAGGCAATTCACGACTG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017749  CCTCGAGAAGCCGATCGCTTAGTTCGTGATGAATTATTAGATGAAGGCAATTCACGACTG  3017808

Query  181      AACCTGGCAACTTTTTGTCAGACCTATATGGAACCCGAAGCCGTTGAATTGATGAAGGAT  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017809  AACCTGGCAACTTTTTGTCAGACCTATATGGAACCCGAAGCCGTTGAATTGATGAAGGAT  3017868

Query  241      ACGCTGGCTAAGAATGCCATCGACAAATCTGAGTACCCCCGCACGGCCGAGATTGAAAAT  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017869  ACGCTGGCTAAGAATGCCATCGACAAATCTGAGTACCCCCGCACGGCCGAGATTGAAAAT  3017928

Query  301      CGGTGTGTGAACATTATTGCCAATCTGTGGCACGCACCTGATGACGAACACTTTACGGGT  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017929  CGGTGTGTGAACATTATTGCCAATCTGTGGCACGCACCTGATGACGAACACTTTACGGGT  3017988

Query  361      ACCTCTACGATTGGCTCCTCTGAAGCTTGTATGTTAGGCGGTTTAGCAATGAAATTCGCC  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017989  ACCTCTACGATTGGCTCCTCTGAAGCTTGTATGTTAGGCGGTTTAGCAATGAAATTCGCC  3018048

Query  421      TGGCGTAAACGCGCTCAAGCGGCAGGTTTAGATCTGAATGCCCATCGACCTAACCTCGTT  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018049  TGGCGTAAACGCGCTCAAGCGGCAGGTTTAGATCTGAATGCCCATCGACCTAACCTCGTT  3018108

Query  481      ATTTCGGCTGGCTATCAAGTTTGCTGGGAAAAGTTTTGTGTCTACTGGGACGTTGACATG  540
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018109  ATTTCGGCTGGCTATCAAGTTTGCTGGGAAAAGTTTTGTGTCTACTGGGACGTTGACATG  3018168

Query  541      CACGTGGTCCCAATGGATGAGCAACACATGGCCCTTGACGTTAACCACGTCTTAGACTAC  600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018169  CACGTGGTCCCAATGGATGAGCAACACATGGCCCTTGACGTTAACCACGTCTTAGACTAC  3018228

Query  601      GTGGACGAATACACAATTGGTATCGTCGGTATCATGGACATCACTTATACCGGTCAATAT  660
               |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  3018229  GTGGACGAATACACAATTGGTATCGTCGGTATCATGGGCATCACTTATACCGGTCAATAT  3018288

Query  661      GACGACCTAGCCGCACTCGATAAGGTCGTTACTCACTACAATCATCAGCATCCCAAATTA  720
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018289  GACGACCTAGCCGCACTCGATAAGGTCGTTACTCACTACAATCATCAGCATCCCAAATTA  3018348

Query  721      CCAGTCTACATTCACGTTGACGCAGCGTCAGGTGGCTTCTATACCCCATTTATTGAGCCG  780
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018349  CCAGTCTACATTCACGTTGACGCAGCGTCAGGTGGCTTCTATACCCCATTTATTGAGCCG  3018408

Query  781      CAACTCATCTGGGACTTCCGGTTGGCTAACGTCGTTTCGATCAACGCCTCCGGGCACAAG  840
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018409  CAACTCATCTGGGACTTCCGGTTGGCTAACGTCGTTTCGATCAACGCCTCCGGGCACAAG  3018468

Query  841      TACGGTTTAGTTTATCCTGGGGTCGGCTGGGTCGTTTGGCGTGATCGTCAGTTTTTACCG  900
               ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018469  TACGGTTTAGTTTATCCCGGGGTCGGCTGGGTCGTTTGGCGTGATCGTCAGTTTTTACCG  3018528
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```
Query  901      CCAGAATTAGTCTTCAAAGTTAGTTATTTAGGTGGGGAGTTGCCGACAATGGCGATCAAT  960
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018529  CCAGAATTAGTCTTCAAAGTTAGTTATTTAGGTGGGGAGTTGCCGACAATGGCGATCAAC  3018588

Query  961      TTCTCACATAGTGCAGCCCAGCTCATTGGACAATACTATAATTTCATTCGCTTTGGTATG  1020
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018589  TTCTCACATAGTGCAGCCCAGCTCATTGGACAATACTATAATTTCATTCGCTTTGGTATG  3018648

Query  1021     GACGGTTACCGCGAGATTCAAACAAAGACTCACGATGTTGCCCGCTACCTGGCAGCCGCT  1080
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018649  GACGGTTACCGCGAGATTCAAACAAAGACTCACGATGTTGCCCGCTACCTGGCAGCCGCT  3018708

Query  1081     CTGGATAAAGTTGGTGAGTTTAAGATGATCAATAACGGACACCAACTCCCCCTGATTTGT  1140
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018709  CTGGATAAAGTTGGTGAGTTTAAGATGATCAATAACGGACACCAACTCCCCCTGATTTGT  3018768

Query  1141     TACCAACTAGCCCCGCGCGAAGATCGTGAATGGACCCTTTATGATTTATCGGATCGCCTA  1200
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018769  TACCAACTAGCCCCGCGCGAAGATCGTGAATGGACCCTTTATGATTTATCGGATCGCCTA  3018828

Query  1201     TTAATGAACGGTTGGCAAGTACCAACGTATCCTTTACCTGCTAATCTGGAACAACAAGTC  1260
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018829  TTAATGAACGGTTGGCAAGTACCAACGTATCCTTTACCTGCTAATCTGGAACAACAAGTC  3018888

Query  1261     ATCCAACGAATCGTCGTTCGGGCTGACTTTGGCATGAATATGGCCCACGATTTCATGGAT  1320
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018889  ATCCAACGAATCGTCGTTCGGGCTGACTTTGGCATGAATATGGCCCACGATTTCATGGAT  3018948

Query  1321     GACCTGACCAAGGCTGTCCATGACTTAAACCACGCCCACATTGTCTATCATCATGACGCG  1380
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3018949  GACCTGACCAAGGCTGTCCATGACTTAAACCACGCCCACATTGTCTATCATCATGACGCG  3019008

Query  1381     GCACCTAAGAAATACGGATTCACACACTGA  1410
                ||||||||||||||||||||||||||||||
Sbjct  3019009  GCACCTAAGAAATACGGATTCACACACTGA  3019038
```

BLAST results of amino acid sequences of *gad*B gene from *Lactobacillus futsaii* CS3

glutamate decarboxylase [Lactobacillus plantarum subsp. plantarum ATCC 14917 = JCM 1149 = CGMCC 1.2437]

Sequence ID: gb|EFK28268.1| **Length:** 469 **Number of Matches:** 1

See 1 more title(s)

glutamate decarboxylase [Lactobacillus plantarum 4_3]

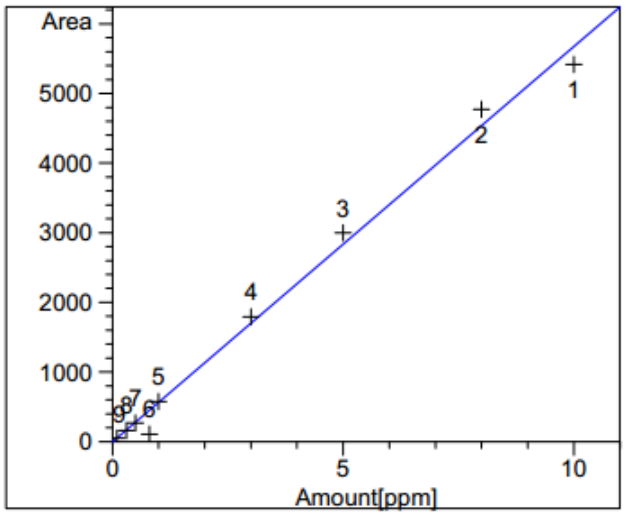
Sequence ID: gb|ETF12695.1|

Range 1: 1 to 469 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
980 bits(2534)	0.0	Compositional matrix adjust.	468/469(99%)	468/469(99%)	0/469(0%)

```
Query  1    MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL  60
            MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL
Sbjct  1    MAMLYGKHNHEAEEYLEPVFGAPSEQHDLPKYRLPKHSLSPREADRLVRDELLDEGNSRL  60

Query  61   NLATFCQTYMEPEAVELMKDTLAKNAIDKSEYPRTAEIENRCVNIIANLWHAPDDEHFTG  120
            NLATFCQTYMEPEAVELMKDTLAKNAIDKSEYPRTAEIENRCVNIIANLWHAPDDEHFTG
Sbjct  61   NLATFCQTYMEPEAVELMKDTLAKNAIDKSEYPRTAEIENRCVNIIANLWHAPDDEHFTG  120

Query  121  TSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVDM  180
            TSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVDM
Sbjct  121  TSTIGSSEACMLGGLAMKFAWRKRAQAAGLDLNAHRPNLVISAGYQVCWEKFCVYWDVDM  180

Query  181  HVVPMDEQHMALDVNHVLDYVDEYTIGIVGIMDITYTGQYDDLAALDKVVTHYNHQHPKL  240
            HVVPMDEQHMALDVNHVLDYVDEYTIGIVGIM ITYTGQYDDLAALDKVVTHYNHQHPKL
Sbjct  181  HVVPMDEQHMALDVNHVLDYVDEYTIGIVGIMGITYTGQYDDLAALDKVVTHYNHQHPKL  240

Query  241  PVYIHVDAASGGFYTPFIEPQLIWDFRLANVVSINASGHKYGLVYPGVGWVVWRDRQFLP  300
            PVYIHVDAASGGFYTPFIEPQLIWDFRLANVVSINASGHKYGLVYPGVGWVVWRDRQFLP
Sbjct  241  PVYIHVDAASGGFYTPFIEPQLIWDFRLANVVSINASGHKYGLVYPGVGWVVWRDRQFLP  300

Query  301  PELVFKVSYLGGELPTMAINFSHSAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLAAA  360
            PELVFKVSYLGGELPTMAINFSHSAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLAAA
Sbjct  301  PELVFKVSYLGGELPTMAINFSHSAAQLIGQYYNFIRFGMDGYREIQTKTHDVARYLAAA  360

Query  361  LDKVGEFKMINNGHQLPLICYQLAPREDREWTLYDLSDRLLMNGWQVPTYPLPANLEQQV  420
            LDKVGEFKMINNGHQLPLICYQLAPREDREWTLYDLSDRLLMNGWQVPTYPLPANLEQQV
Sbjct  361  LDKVGEFKMINNGHQLPLICYQLAPREDREWTLYDLSDRLLMNGWQVPTYPLPANLEQQV  420

Query  421  IQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH  469
            IQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH
Sbjct  421  IQRIVVRADFGMNMAHDFMDDLTKAVHDLNHAHIVYHHDAAPKKYGFTH  469
```

Standard curve of GABA standard



GABA at exp. RT: 18.628
FLD1 A, Ex=330, Em=440
Correlation: 0.99785
Residual Std. Dev.: 186.33612
Formula: y = mx
m: 567.80409
x: Amount
y: Area

RetTime (min)	Level	Amount (mg/l)	Area
24.230	1	0.01	2.56332
	2	0.05	16.31875
	3	0.1	35.35496
	4	0.5	210.45236
	5	1.0	423.29700
	6	5.0	2732.35229
	7	10.0	5676.29321