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Activities of amylase, proteinase, and lipase enzymes from *Lactococcus chungangensis* and its application in dairy products

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ABSTRACT

Several enzymes are involved in the process of converting milk to lactic acid and coagulated milk to curd and, therefore, are important in dairy fermented products. Amylase, proteinase, and lipase are enzymes that play an important role in degrading milk into monomeric molecules such as oligosaccharides, amino acids, and fatty acids, which are the main molecules responsible for flavors in cheese. In the current study, we determined the amylase, proteinase, and lipase activities of *Lactococcus chungangensis* CAU 28^T, a bacterial strain of nondairy origin, and compared them with those of the reference strain, *Lactococcus lactis* ssp. *lactis* KCTC 3769^T, which is commonly used in the dairy industry. *Lactococcus chungangensis* CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T were both found to have amylase, proteinase, and lipase activities in broth culture, cream cheese, and yogurt. Notably, the proteinase and lipase activities of *L. chungangensis* CAU 28^T were higher than those of *L. lactis* ssp. *lactis* KCTC 3769^T, with proteinase activity of 10.50 U/mL in tryptic soy broth and 8.64 U/mL in cream cheese, and lipase activity of 100 U/mL of tryptic soy broth, and 100 U/mL of cream cheese. In contrast, the amylase activity was low, with 5.28 U/mL in tryptic soy broth and 8.86 U/mL in cream cheese. These enzyme activities in *L. chungangensis* CAU 28^T suggest that this strain has potential to be used for manufacturing dairy fermented products, even though the strain is of nondairy origin.

Key words: *Lactococcus chungangensis*, amylase, proteinase, lipase, dairy product

INTRODUCTION

Lactic acid bacteria (**LAB**) have been widely used in the dairy industry to produce cheese, yogurt, butter, and fermented milk. The LAB often play an important role in the starter cultures of commercial dairy products, and ensure consistency of the process and product quality. *Lactococcus* is a genus of LAB that serves as a starter for several types of cheese, such as hard cheese without eyes or with small eyes (Leroy and De Vuyst, 2004), Cheddar (Cretenet et al., 2011), Moroccan soft white cheese (Ouadghiri et al., 2005), Domiati cheese (El-Baradei et al., 2007), and cream cheese (Konkit et al., 2015).

Lactococcus lactis ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Lactococcus raffinolactis* are well-known strains used as starters in cheese and fermented milk (Leroy and De Vuyst, 2004; Ouadghiri et al., 2005). For many fermented dairy products, the most important properties and functions of LAB are (1) fermentation, including the depletion of milk sugar and the production of acids in milk; (2) reduction of the redox potential; (3) citrate fermentation; and (4) casein degradation (Olson, 1990). The primary role of LAB, however, is to utilize milk as a substrate and convert it into monomeric molecules for use as nutrients for their metabolism and growth (Vihinen and Mantsiila, 1989). These changes during the fermentation process, including the secretion of nutritional and chemical substances, are associated with bacterial enzymes (Gurr, 1987).

Microbial enzymes are more valuable in manufacturing than animal and plant enzymes because of their variety of catalytic activities and the high yields possible (Seitz, 1990; Kunji et al., 1995). Furthermore, their rapid growth on inexpensive media and the stability of their enzyme products make bacteria the preferred enzyme source in the food industry (McSweeney and Sousa, 2000). Among the technical enzymes, amylase, proteinases, and lipases are the principal enzymes used in food and animal feed production. Amylase catalyzes the hydrolysis of starch, resulting in products such as glucose, maltose, and maltotriose units (Gupta et al., 2003; Kandra, 2003; Rajagopalan and Krishnan, 2008). Amylase was the one of the first indigenous enzymes to be identified in milk, with α-amylase being the principal enzyme and β-amylase the lesser (Sato, 1920). Milk starch is broken down by amylase and converted into primarily dextrin and then into maltose, to a lesser extent (Guzmán-Maldonado et al., 1995).

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The proteolytic systems of LAB are essential for their growth in milk and contribute significantly to the development of flavor in fermented milk products (Kunji et al., 1995, 1996). Proteinase is the one of the enzymes that converts milk casein to free AA and peptides necessary for growth and acid production (Law and Haandrikman, 1997). Some *Lactococcus* strains serve as starters for dairy fermentation and have proteolytic activity. Proteolysis is the first biochemical step in the process that determines the flavor and texture of dairy products (Fox et al., 1996; McSweeney and Fox, 1997). Specifically, lactococci possess a proteolytic system that, together with other protein-hydrolyzing enzymes, is responsible for the conversion of casein into peptides and AA.

Lipolytic enzymes are involved in the breakdown and mobilization of lipids within the cells of an individual organism as well as the transfer of lipids from one organism to another (Beisson et al., 2000). Milk fat is essential for the development of a desirable flavor in fermented dairy products. Lipase is the enzyme that hydrolyzes triglycerides to fatty acids and glycerol, and mono- or diglycerides that are the key to flavor development (McSweeney and Sousa, 2000). *Bacillus* species have been found to possess amylase, proteinase, and lipase enzymes that can be used in the food and household industries (Hasan et al., 2006). Among those, *Bacillus subtilis* is a well-known enzyme-producing species that plays an important role in the production of natto by solid-state fermentation of soybeans (Hara and Ueda, 1982). Their proteinase enzymes have been used in the production of household and heavy detergents (Schallmey et al., 2004). Moreover, *Bacillus* species have been used as probiotics (Ziaei-Nejad et al., 2006).

Lactococcus chungangensis CAU 28^T is a strain of nondairy origin, which was isolated from activated sludge in our laboratory (Cho et al., 2008). Transcriptomic analysis showed that the strain possessed genes such as cystathionine β-lyase (*MetC*) and O-acetylserine sulfhydrylase (*CysK*), which are important factors in the processing of cheese (Konkit et al., 2014). Moreover, the strain has been found to play a role in functional activities, such as alcohol dehydrogenase and aldehyde dehydrogenase, which can moderate the level of alcohol and aldehyde in vivo (Konkit et al., 2015, 2016). The objective of this study was to evaluate the potential amylase, proteinase, and lipase activities of *L. chungangensis* CAU 28^T in broth culture, yogurt, and cream cheese. A comparison was made with another culture, *L. lactis* ssp. *lactis* KCTC 3769^T, which is routinely used in the dairy industry.

MATERIALS AND METHODS

Bacteria Strains

Lactococcus chungangensis CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T were cultured in tryptic soy broth (Becton, Dickinson and Co., Sparks, MD) at 30°C for 24 h. *Lactococcus lactis* ssp. *lactis* KCTC 3769^T was obtained from the Korean Collection for Type Cultures (KCTC; Taejon, Korea).

Broth Culture with Each Enzyme Substrate

One percent (wt/vol) each of starch, casein, and olive oil was added to basal medium, as described by Zhang et al. (1983), which contained (g/L) $(NH_4)_2SO_4$ 1.0, K_2HPO_4 6.0, KH_2PO_4 3.0, $MgSO_4 \cdot 7H_2O$ 0.01, $CaCl_2 \cdot 2H_2O$ 0.05, $MnSO_4 \cdot 2H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.001, and $ZnSO_4 \cdot 7H_2O$ 0.001. An inoculum of each strain was added (1.0%, vol/vol) to each broth culture and then incubated at 30°C for 54 h.

Cream Cheese Making

According to a published method used for making cream cheese (Konkit et al., 2015), pasteurized milk (Pasteur Milk Co. Ltd., Seoul, Korea) was heated at 68°C for 30 min and cooled down, 5% (vol/vol) of a starter *Lactococcus* strain was added, and the mixture was incubated at 30°C for 48 h. During this period, the milk was acidified. It was then stirred and heated at 70°C for 5 min, and subsequently the whey was separated through a cloth bag. The curd was set and whey drained by adding 0.5% salt. Finally, each cream cheese sample was freeze-dried and stored in the dark at 4°C until further tests.

Yogurt Making

Pasteurized milk was heated to about 93°C and stirred gently to prevent it from boiling over and then cooled to around 44 to 46°C. The milk was stirred occasionally to prevent skin formation; then, a cup of warm milk was added to the inoculum strain (1% vol/vol) and allowed to set overnight.

Amylase Activity

Maltose (0.5 *M*) dilutions ranging from 0.3 to 0.5 μmol/mL, including a blank tube, were prepared. One milliliter of each dilution of maltose was pipetted into a series of corresponding numbered tubes, and 1 mL

of dinitrosalicylic acid color reagent was added to each tube. After this, the mixture was incubated in boiling water for 5 min, cooled to room temperature, and then 10 mL of distilled water was added to each tube and mixed well. Results (absorbance) were read at 540 nm. One milliliter of starch was pipetted into a test tube, along with 1.0 mL of enzyme solution, and incubated at 25°C for 3 min. Subsequently, 1 mL of dinitrosalicylic acid color reagent was added to each tube. All tubes were incubated in a boiling water bath for 15 min. The mixtures were cooled to room temperature and 9 mL of distilled water added. Samples were mixed well and read at 540 nm, with values for blank wells subtracted from the results. Maltose released (μmol) was determined from the standard curve (Hagberg, 1960).

Proteinase Activity

Tyrosine (1.1 m*M*) dilutions ranging from 0.1 to 0.5 μmol/mL, including a blank tube, were prepared. Two milliliters of each dilution of tyrosine was pipetted into a series of corresponding numbered tubes, and 5 mL of Na_2CO_3 and 1 mL of Folin-Ciocalteu phenol reagent were added to each tube. After this, the mixture was swirled and incubated at 37°C for 30 min, and then filtered using a 0.45-μm syringe filter. Results were read at 660 nm, with blank values subtracted. Five milliliters of casein was pipetted into a test tube, along with 1.0 mL of enzyme solution, and incubated at 37°C for 5 min. Following this, 5 mL of trichloroacetic acid reagent was added to each tube. All tubes were incubated at 37°C for 30 min, and then each solution was filtered using a 0.45-μm syringe filter. Two milliliters of test filtrate, 5.0 mL of Na_2CO_3, and 1 mL of 0.5 *M* Folin-Ciocalteu phenol reagent were added to each tube. The solutions were mixed well and incubated at 37°C. Samples were then read at 660 nm with blank values subtracted (McDonald and Chen, 1965).

Lipase Activity

Three milliliters of olive oil and 1.0 mL of 200 m*M* Tris-HCl were added to a test tube and incubated at 37°C for 10 min. After this, 1.0 mL of enzyme solution was added, and the mixture was incubated at 37°C for 30 min, followed by the addition of 3.0 mL of 95% ethanol. The solution was mixed well, and then 4 drops of thymolphthalein was added to each sample and to blank test tubes, followed by titration with 0.2 *M* NaOH to determine the activity levels (Stoytcheva et al., 2012).

RESULTS

Cream Cheese Making

Cream cheese was made using *L. chungangensis* CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T as starter cultures. The products were white and had slightly lactic acid and cultured diacetyl aromas, with off-flavors. The texture of the products was smooth without lumps or grittiness, and there was no cracking or wheying off.

Yogurt Making

Yogurt was made using *L. chungangensis* CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T as starter cultures. Each product was a cream color and had high tension and firmness with a sour smell. The texture of the product was soft and semi-liquid, and the pH of the yogurt decreased to 4.6 in 7 d.

Amylase Activity

In broth culture, basal medium, which was unsupplemented, was added to starch. *Lactococcus chungangensis* CAU 28^T was found to have the highest amylase activity, 5.28 U/mL, at 6 h. This level was similar to that seen with *L. lactis* ssp. *lactis* KCTC 3769^T, 5.30 U/mL (Figure 1A and 1B). In addition, in cream cheese made with each strain, *L. chungangensis* CAU 28^T had the highest activity on d 5 of incubation with 8.86 U/mL, whereas *L. lactis* ssp. *lactis* KCTC 3769^T had the highest activity on d 7 at 9.03 U/mL (Figure 2A). In yogurt, *L. chungangensis* CAU 28^T had the highest level of amylase activity on d 3 of incubation at 5.41 U/mL and a slight decrease from d 5 to 7, whereas *L. lactis* ssp. *lactis* KCTC 3769^T had the highest amylase activity on d 5 at 5.33 U/mL (Figure 2B).

Proteinase Activity

Casein was added to each culture broth to estimate the proteinase activity of *L. chungangensis* CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T. *Lactococcus chungangensis* CAU 28^T was found to have the highest proteinase activity, with 10.50 U/mL at 30 h. This result was the similar to that of the *L. lactis* ssp. *lactis* KCTC 3769^T culture that showed proteinase activity of 9.80 U/mL at 30 h (Figure 1A and 1B). In cream cheese made with each strain, *L. chungangensis* CAU 28^T had the highest proteinase level on d 7 with 0.54 U/mL, whereas *L. lactis* ssp. *lactis* KCTC 3769^T had the highest level on d 7 with 0.41 U/mL (Figure 3A).

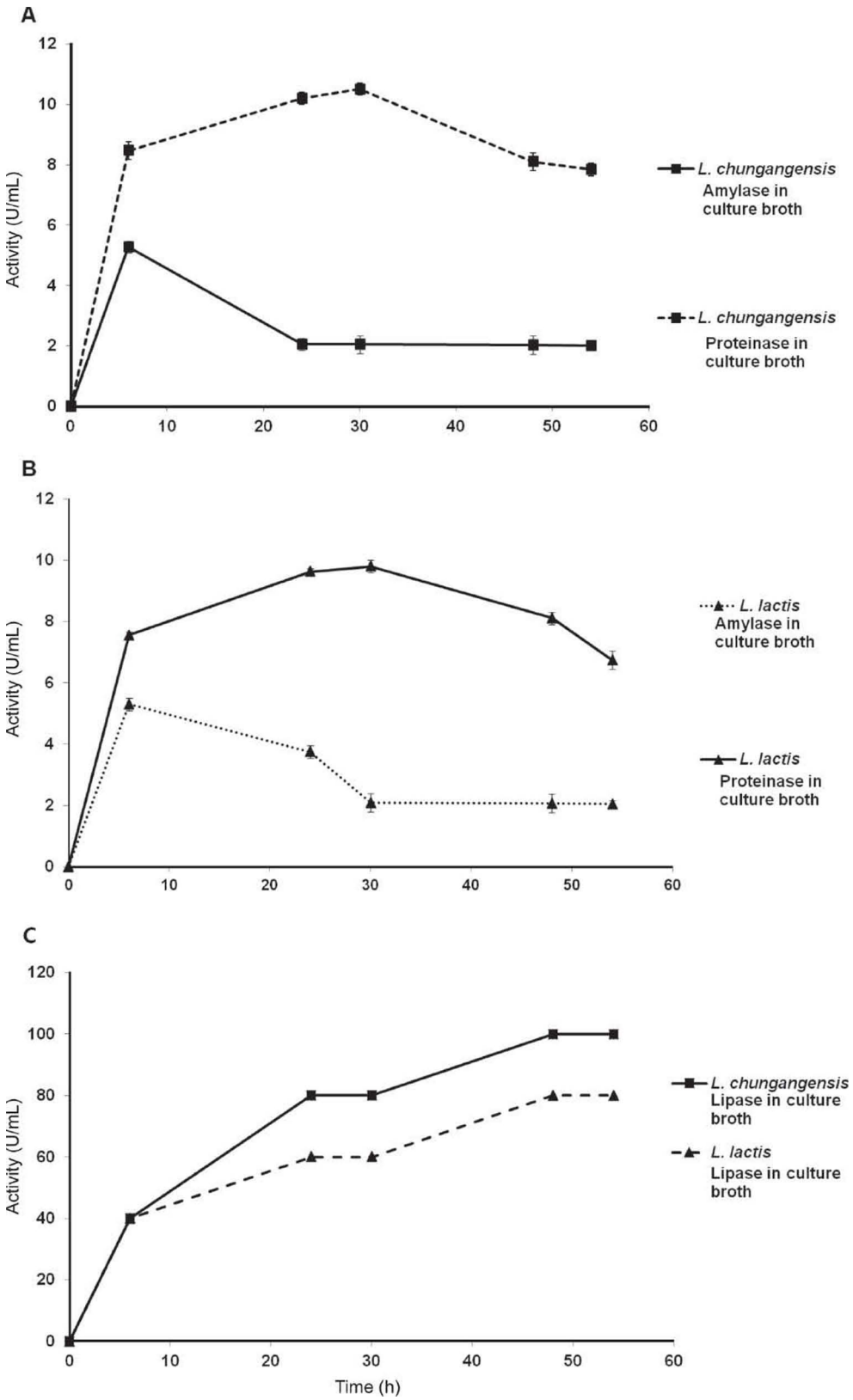

Figure 1. Amylase, proteinase, and lipase activities (U/mL) of *Lactococcus chungangensis* CAU 28^{T} and *Lactococcus lactis* ssp. *lactis* KCTC 3769^{T} in broth culture. Amylase and proteinase activities of (A) *L. chungangensis* CAU 28^{T} in culture broth; (B) amylase and proteinase activities of *L. lactis* ssp. *lactis* KCTC 3769^{T} in culture broth; and (C) lipase activities in culture broth. Error bars represent SD.

In yogurt, *L. chungangensis* CAU 28^T had the highest level of proteinase on d 3 with 8.64 U/mL. In contrast, *L. lactis* ssp. *lactis* had the highest level of proteinase on d 7 with 1.09 U/mL (Figure 3B).

Lipase Activity of L. chungangensis and L. lactis ssp. lactis

Olive oil was added to each culture broth to estimate the lipase activity of *L. chungangensis* CAU 28^T and *L. lactis* ssp. *lactis* KCTC 3769^T. *Lactococcus chungangensis* CAU 28^T was found to have the highest lipase activity, with 100 U/mL at 48 h, whereas *L. lactis* ssp. *lactis* KCTC 3769^T had lipase activity of 80 U/mL at the same time point (Figure 1C). In cream cheese made from each strain, *L. chungangensis* CAU 28^T had the highest lipase activity with 100 U/mL on d 7 (Figure 4A). This lipase activity was the same as that seen in the *L. lactis* ssp. *lactis* KCTC 3769^T culture, which showed lipase activity of 80 U/mL. In yogurt, *L. chungangensis* CAU 28^T had the highest level of lipase activity on d 7 at 100 U/mL, whereas *L. lactis* ssp. *lactis* KCTC 3769^T showed lipase activity of around 80 U/mL (Figure 4B).

DISCUSSION

For many fermented foods, but particularly milk-derived products, the characterization of microorgan-

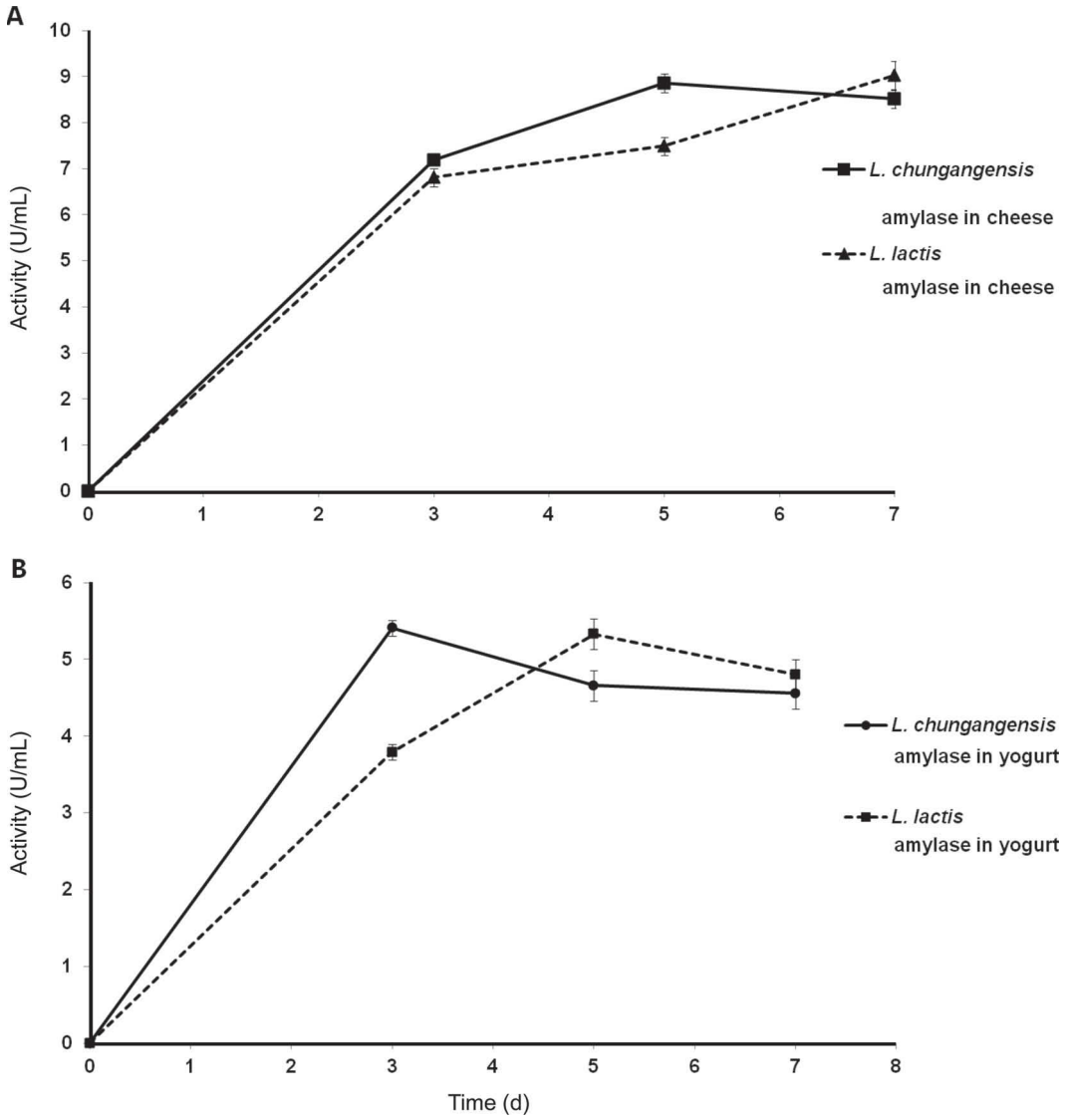

Figure 2. Amylase activity (U/mL) of *Lactococcus chungangensis* CAU 28^T and *Lactococcus lactis* ssp. *lactis* KCTC 3769^T in cream cheese and yogurt. Amylase activities in (A) cream cheese, and (B) yogurt. Error bars represent SD.

isms responsible for fermentation led to the isolation of starter cultures that could be produced on a large scale for the manufacture of these products (Caplice and Fitzgerald, 1999). Starter cultures are important to dairy manufacturing because they facilitate rapid acidification (Wouters et al., 2002).

The LAB are typically involved in the fermentation of food and dairy products; LAB belong primarily to the order *Lactobacillales* and are included in genera commonly used in fermented food production, such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* (Todar, 2006). These are important groups of microorganisms used in food fermentation. They contribute to the taste and texture of fermented products and inhibit food spoilage caused by other bacteria (Wouters et al., 2002) by producing growth-inhibiting substances and large amounts of lactic acid. As productive agents in fermentation, LAB are involved in making yogurt, cheese, cultured butter, sour cream, and sausage (Todar, 2006).

In fermented dairy products, LAB are naturally present in milk and produce lactic acid, which is required for the coagulation of milk. Lactococci are applied in dairy fermentation. These bacteria acidify the milk, and, consequently, the growth of other bacteria is largely inhibited. This process has been used for centuries to

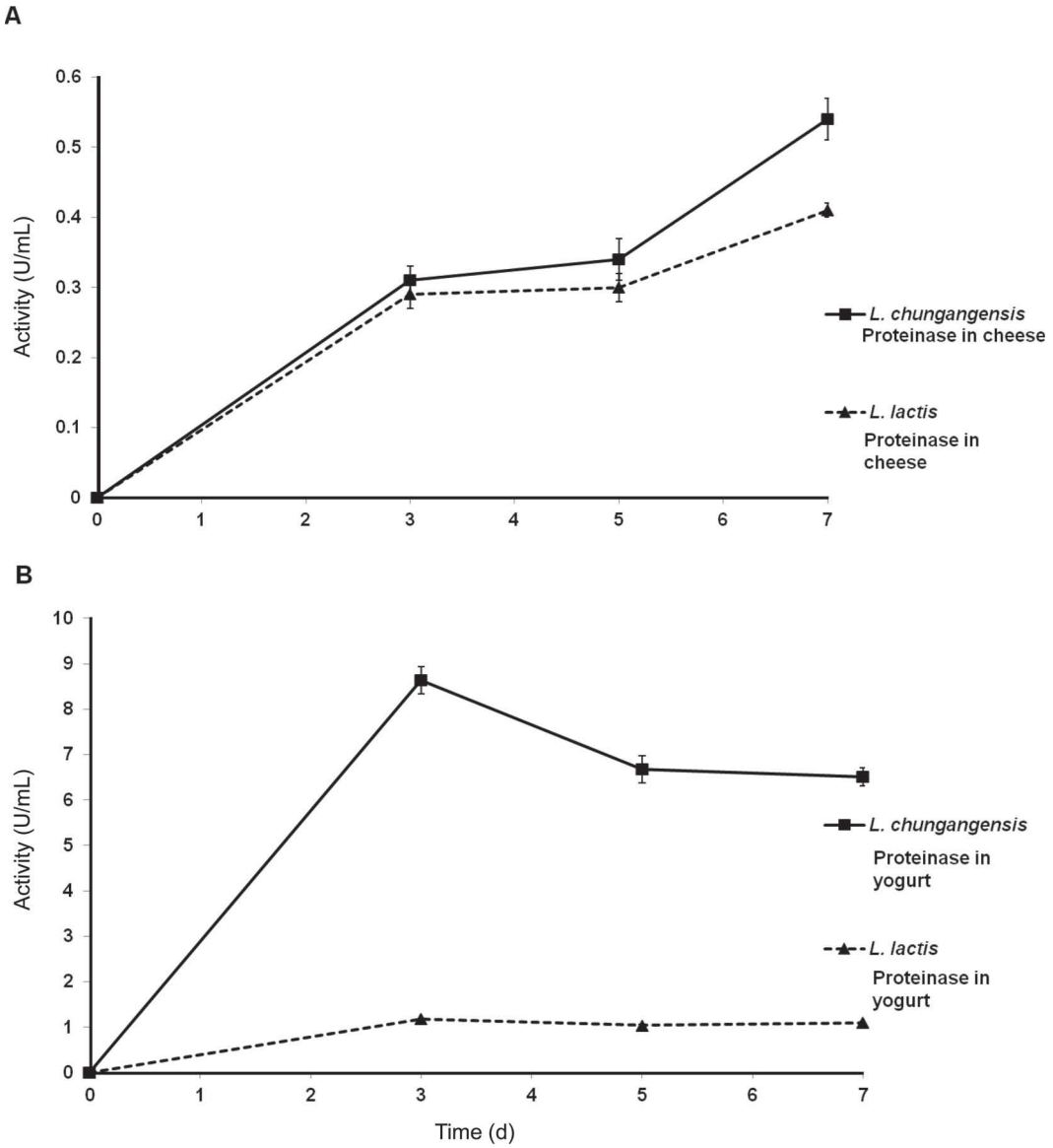

Figure 3. Proteinase activity (U/mL) of *Lactococcus chungangensis* CAU 28^{T} and *Lactococcus lactis* ssp. *lactis* KCTC 3769^{T} in cream cheese and yogurt. Proteinase activities in (A) cream cheese, and (B) yogurt. Error bars represent SD.

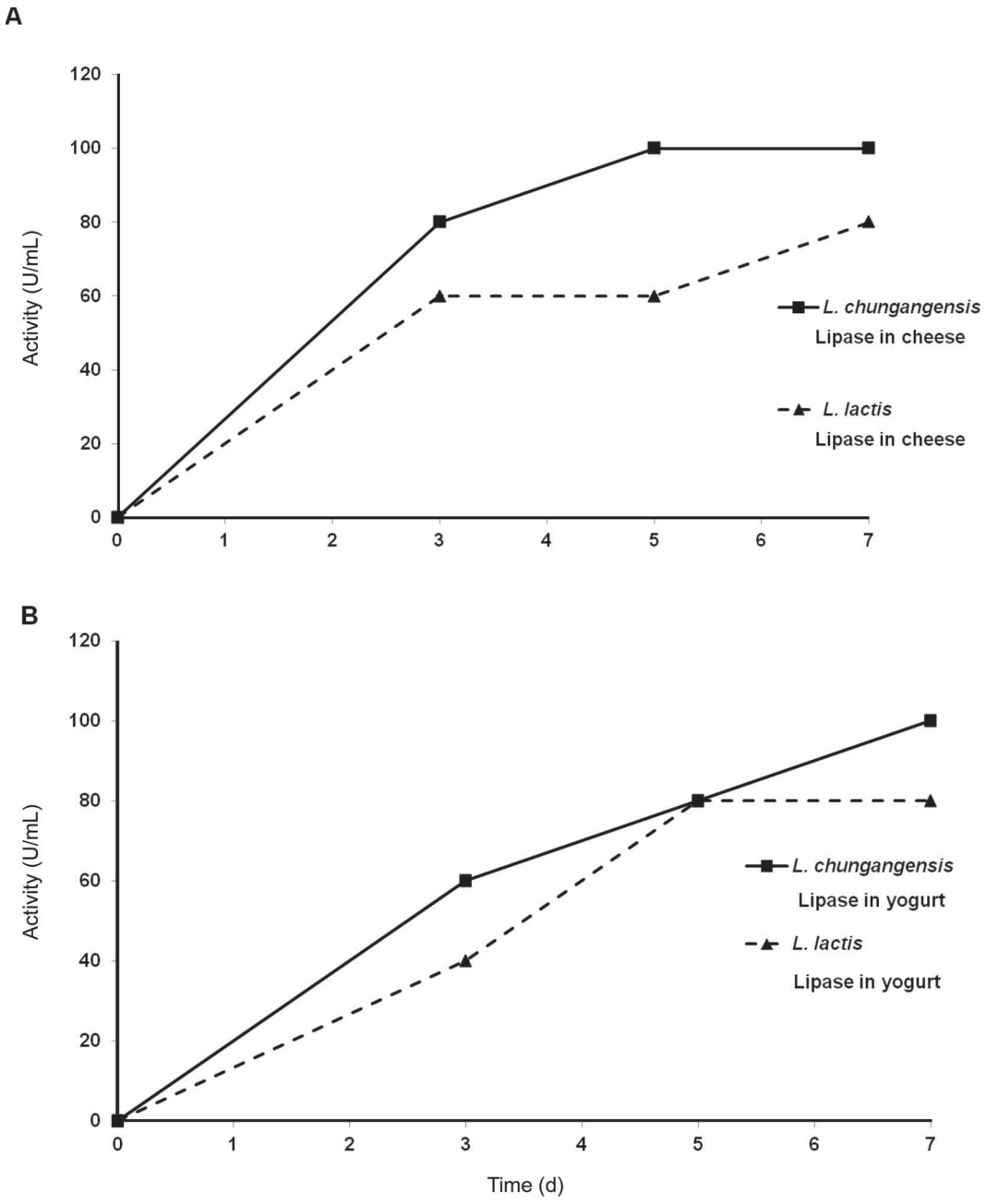

Figure 4. Lipase activity (U/mL) of *Lactococcus chungangensis* CAU 28^{T} and *Lactococcus lactis* ssp. *lactis* KCTC 3769^{T} in cream cheese and yogurt. Lipase activities in (A) cream cheese, and (B) yogurt.

manufacture fermented dairy products (Wouters et al., 2002).

Starter cultures of mesophilic lactococci are used in the manufacture of a broad array of cheese types, butters, and various fermented milk products. Lactococci have been used for lactic acid fermentation, casein breakdown, diacetyl production from citrate, and resistance to phage attack. The subspecies of *Lactococcus lactis* are of great economic importance and have been extensively studied for their biochemical and physiological characteristics and their effects on food (Teuber, 1995).

The most important properties of microorganisms as milk starters are their ability to produce acid in milk and to convert protein into flavor components (Wouters et al., 2002). Enzyme activity is critical to this process. By the early 20th century, many enzymes serving this function had been identified in milk, including lactoper-

oxidase, catalase, xanthine oxidase, proteinase, lipase, salolase (arylesterase), and amylase (Fox and Kelly, 2006).

The principle underlying the oxidation of carbohydrates and its derivatives is the generation of end products, which are generally acids, alcohol, and carbon dioxide. These end products control the growth of spoilage microorganisms. Amylase is an enzyme involved in milk production. Milk contains no starch and only low levels of oligosaccharides; however, these oligosaccharides are derived from lactose and contain unusual monosaccharides (e.g., fructose and *N*-acetylneuraminic acid) with glycosidic linkages. Therefore, it seems that α-amylase is highly specific for α(1→4) glycosidic bonds linking glucose molecules in the hydrolysis of oligosaccharides in milk (Gnoth et al., 2000).

In this study, *L. chungangensis* CAU 28^{T} was found to have amylase activity in culture broths of starch, cream cheese, and yogurt. It had the highest level of activity in cream cheese, with 8.86 U/mL, whereas *L. lactis* ssp. *lactis* KCTC 3769^{T} had 9.03 U/mL of amylase activity.

Lactococci require various AA for growth because of their limited biosynthetic capacity. The absence of functional genes for a specific biosynthetic reaction or specific regulatory mechanism results in the need for specific AA (Chopin, 1993). In milk, proteinase is important for the proteolysis of casein to amino acid and peptides. The proteolytic system of *Lactococcus* has been investigated in detail because it facilitates growth of these bacteria in milk and the development of flavor in cheeses (Kunji et al., 1996). Casein is degraded by a membrane-anchored serine proteinase (PrtP), with the resulting oligopeptides being small enough to allow them to be transported into the cell via an oligopeptide transport system (Opp) and further processed by a variety of intracellular peptidases (Kunji et al., 1995). *Lactococcus chungangensis* CAU 28^{T} had the highest proteinase activity in every condition compared with *L. lactis* ssp. *lactis* KCTC 3769^{T}. The highest activity was seen in yogurt with 8.64 U/mL.

Lipase has diverse enzymatic properties and substrate specificities. Lipase is part of a family of hydrolases that act on carboxylic ester bonds. The role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol (Hasan et al., 2006). Lipolysis is an important biochemical event occurring during the production of cheese. It is well established that milk fat is essential to the development of desirable flavors in cheese during ripening (Foda et al., 1974; El-Safty and Ismail, 1982; Wijesundera et al., 1998). The enzymatic hydrolysis of triglycerides to fatty acids and glycerol, and to mono- or diglycerides (lipolysis), is necessary to the development of some cheese varieties (McSweeney and Sousa, 2000). *Lactococcus chungangensis* CAU 28^{T} had the highest lipase activity in all conditions, showing the highest levels of activity (100 U/mL) in the culture broth with olive oil and in yogurt.

Our results show that *L. chungangensis* CAU 28^{T} has amylase, proteinase, and lipase enzyme activity; few studies have determined activity levels of these enzymes in LAB or related bacteria. Amylase, proteinase, and lipase have the ability to degrade substrates in broth culture, cream cheese, and yogurt. The amylase enzyme activity of this strain was not different compared with *L. lactic* ssp. *lactis*, a strain commonly used in the manufacture of dairy products. Interestingly, however, the proteinase and lipase activities of *L. chungangensis* CAU 28^{T} were higher than those of *L. lactic* ssp. *lactis*. In conclusion, *L. chungangensis* CAU 28^{T} has amylase, proteinase, and lipase activities that are essential to hydrolyzing substrates in milk to lactic acid, which is important in dairy milk fermentation.

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