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ISOLATION OF ESTERASE PRODUCING *LACTOBACILLUS BREVIS* NJ13 FROM *KIM-CHI* AND INVESTIGATION OF EFFECTIVE MEDIUM INGREDIENTS FOR ESTERASE PRODUCTION USING STATISTICAL METHOD

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ABSTRACT

In this study, an esterase producing *Lactobacillus brevis* NJ13 was firstly isolated from *Kim-chi*, a Korean traditional fermented cabbage. The isolation was based on esterase screening method using tributyrin and 16S ribosomal RNA gene sequence analysis. Its esterase was characterized and the effects of medium ingredients on esterase production were investigated. *L. brevis* NJ13 esterase was highly specific toward *p*-nitrophenyl butyrate (C4 acyl group) and showed maximum activity at a temperature of 50°C and pH 8.0. The esterase was resistant to various organic solvents, especially *N,N*-dimethylformamide. Triton X 100, $MnCl_2$ and EDTA strongly inhibited the esterase, but $CaCl_2$ induced the activation of the esterase. Six medium ingredients were examined for their effects on esterase production using the Plackett-Burman design. Olive oil and glucose negatively affected the production of esterase, while soybean oil, tributyrin, sucrose and $CaCl_2$ displayed a positive effect. The effects were significant only for tributyrin and $CaCl_2$. Therefore, the present study is providing the primary information for the esterase from *L. brevis* NJ13 and its optimum esterase producing fermentation conditions.

Key words: Characterization, Esterase, *Lactobacillus brevis*, Medium ingredients, Plackett-Burman design.

INTRODUCTION

Lactic acid bacteria (LAB) has gained much interest for its health benefit properties and LAB is widely used as probiotics for animal and starter culture for fermented dairy products because of its GRAS status (Maragkoudakis *et al.* 2006). Particularly, LAB in fermented dairy product plays various roles during fermentation and the major benefit of its use is a preservative effect, suppressing harmful bacterial growth during fermentation or storage periods. Other feature of LAB is changing sensory properties of dairy products. For example, cheese flavour is mainly developed by LAB starter culture. It converts lactose in milk to lactic acid, diacetyl and acetaldehyde and these compounds are contributions to the flavour of cheese (Urbach 1995). The lipolytic activity of LAB was reported that it contributes to organoleptic characteristics in cheese produced by a ripening process (Bianchi-Salvadori *et al.* 1995). The lipolytic activity is performed by the action of esterase or lipase.

Carboxyl ester hydrolase (EC 3.1.1.x) is an enzyme that catalyzes the hydrolysis and/or formation of ester bond (Bornscheuer 2002). Esterases (EC 3.1.1.1 and EC 3.1.1.2) preferably catalyse the hydrolysis of carboxyl ester linkages composed of short-chain fatty acids only in aqueous solution, and they can also catalyse the ester synthesis and trans-esterification (Bornscheuer 2002, Holland *et al.* 2005). Esterases are ubiquitously distributed among organisms and have attracted interest concerning their broad industrial value such as chemical manufacture, agriculture and foods. Microbial esterases are resistant to organic solvents, which makes them important catalysts in organic syntheses, especially enantioselective and/or steroselective hydrolysis of esters (Choi *et al.* 2003). Other useful reactions performed by microbial esterases are the synthesis of short-chain esters, alcohol, lactones and phenolic compounds, which contribute to the flavour of food (Holland *et al.* 2005, Torres *et al.* 2009). An esterase from *Bacillus licheniformis* participates in the synthesis of the short chain ester isoamyl acetate that is an important flavour compound (Torres *et al.* 2009). Particularly, esterases from lactic acid bacteria (LAB) may be involved in the development of fruity flavours and the improvement of quality in dairy and meat products like cheese, cured bacon and fermented sausages (Gobbetti *et al.* 1997a). A number of esterases from several LAB have been

characterized and these LAB include *Lactobacillus casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, *Lactococcus lactis*, *Streptococcus thermophiles* and *Pediococcus pentosaceus* (Gobbetti *et al.* 1997a, Holland *et al.* 2005, Oliszewski *et al.* 2007), but there is little report about esterase producing *Lactobacillus brevis*. Generally, a variety of conditions including physicochemical and biochemical factors have been described; these factors can stimulate or repress the production of extracellular enzymes and are regarded as the most important conditions in biological process development (Cho *et al.* 2010, Hasan *et al.* 2009).

Several esterases from lactobacilli have been characterized, but esterase production by *L. brevis* has not been well-studied. So the present study firstly isolated esterase producing *L. brevis* from *Kim-chi* and we characterized its esterase enzyme and investigated the effects of medium ingredients on esterase production during fermentation using a statistical method to develop isolated strain as a starter culture for fermented dairy product.

MATERIALS AND METHODS

Reagents and source of isolation: General chemicals were purchased from Sigma-Aldrich (St. Louis, Mo, USA), unless otherwise stated. *Kim-chi* was prepared based on traditional recipe.

Isolation of esterase producing bacteria from *Kim-chi:* Bacterial isolate NJ13 was obtained from *Kim-chi* using an esterase screening method in solid medium (Kim *et al.* 2001). Ten gram of *Kim-chi* was suspended in 100 mL of sterilized 0.8% NaCl buffer solution and the suspension was serially diluted with the same buffer. The dilute suspensions were spread on CHO medium containing (per L): glucose 10 g, sodium acetate 10 g, K_2HPO_4 1 g, $MgSO_4$ 0.1 g, NaCl 10 g, yeast extract 3 g, tributyrin 10 mL and agar 15 g. Then the plate medium was incubated at 30°C until colonies were visible. Finally, colonies with clear zones were selected.

Culture conditions: Isolated NJ13 strain was maintained in Difco™ de Man, Rogosa, Sharpe (MRS) broth obtained from BD (Frnaklin Lakes, NJ, USA) at 30°C for 20 hours with agitation (150 rpm). The culture for enrichment and seed for main culture was prepared using test tube with 10 mL of medium volume. For main culture for enzyme production and investigation of effective medium ingredients, 250 mL erlenmeyer flask with 100 mL of medium volume was used and 1 mL of seed culture was inoculated. The culture was centrifuged (10,000 rpm, 10 min) and the enzyme containing supernatant was subsequently used for enzyme activity assay.

Taxonomic identification of NJ13: For the identification of isolate **NJ13**, the 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using two universal primers, 27F (5'-AGA GTT TGA TCC TGG CTC AG- 3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T- 3') (Weisburg *et al.* 1991), and then the sequences of 1,439 base pairs fragments obtained by PCR were determined using an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Franklin Lakes, NJ, USA). Comparison of the sequences was performed using the GenBank database using BLAST (Altschul *et al.* 1990). Multiple alignments were conducted using CLUSTAL_W program (Thompson *et al.* 1994) with 16S rRNA gene sequences of type strains involved in the same genus of the isolate NJ13 as a result of previous GenBank sequence comparisons. The evolutionary distance was determined by construction of a phylogenetic tree using the Maximum Composite Likelihood method in the MEGA 4 program (Tamura *et al.* 2007, Tamura *et al.* 2004).

Estimation of esterase activity: Estimation of esterase activity was carried out as described previously with slight modification (Cho *et al.* 2010). Esterase activity was determined using *p*-nitrophenyl (*p*-NP) esters (acetate, butyrate, palmitate, octanoate and mystrate) and the enzyme reaction was performed in 10 mM Tris-HCl (pH 8.0) buffer, unless otherwise stated. Briefly, a reaction mixture composed of 10 µL of 30 mM *p*-NP esters as substrate, 40 µL of ethanol, 950 µL of 100 mM Tri-HCl (pH 8.0) and 50 µL of NJ13 culture supernatant was incubated and the release of *p*-NP was measured at 405 nm using a spectrophotometer (OPTIZEN 2120UV, Daejeon, Korea). The spontaneously released *p*-NP was measured as a blank and it was subtracted from the amount of enzyme-mediated released *p*-NP to calculate enzyme activity. The concentration of liberated *p*-NP was determined by the standard regression of *p*-NP solution. One unit (U) of enzyme activity was defined as the amount of enzyme to produce 1 µM of *p*-NP per minute under the defined assay conditions. For the estimation of specific activity, protein content in supernatant was estimated using the Quick Start Bradford Protein Assay Kit 3 (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.

Effect of pH, temperature, organic solvents, detergents and metal ions: To determine the effect of pH and temperature, 30 mM *p*-NP butyrate was used as substrate and enzyme activities were investigated at temperatures between 25°C and 90°C and at pH 3.0 – 9.0 at the determined optimum temperature. Buffer solutions for different pH ranges were 100 mM glycine-HCl (pH 3.0), 100 mM acetate buffer (pH 4.0 – 5.0), 100 mM potassium phosphate buffer (pH 6.0 – 7.0) and 100 mM Tris-HCl buffer (pH 8.0 – 9.0). The effect of organic solvent, detergents and metal ions on esterase activity

was determined by reacting the enzyme at 50°C in 100 mM Tris-HCl (pH 8.0) buffer containing 10% organic solvents, 1% detergents and 10 mM of metal ions, respectively. All measurements were carried out in triplicate.

Effect of medium ingredients on esterase production: The effects of five carbon sources (olive oil, soybean oil, tributyrin, sucrose and glucose) and one mineral source ($CaCl_2$) as variables on esterase production during the fermentation of NJ13 strain were investigated. The present study screened for the main effects of ingredients and not the interaction effect between ingredients. Hence, a Plackett-Burman design (PBD), a two level fractional factorial design, was used (Plackett and Burman 1946). A total of six ingredients as variables were represented at high (+1) and low (-1) concentrations (Table 2). Each level of each variable was assigned to 12 trials according to PBD as shown in Table 3. The effect of each variable was estimated by the following equation:

$$E(x_i) = \frac{\sum R_i^{+1} - \sum R_i^{-1}}{N}$$

where $E(x_i)$ is the effect of tested variable and R_i^{+1} and R_i^{-1} are the esterase production from the trials where high concentration and low concentration of i^{th} variables were assigned, respectively.

Statistical analysis: The construction of 12 PBD trials with six variables, analysis of the effects of variables and their probability and analysis of variance were performed using MINITAB® (version 14.0, Minitab Inc., USA).

RESULTS AND DISCUSSION

Identification of isolate NJ13: Based on the appearance of the clear zone around colonies on tributyrin containing MRS agar, the NJ13 strain was isolated. The 16S rRNA gene was subsequently cloned. The NJ13 16S rRNA sequence was compared with GenBank sequences and a phylogenic tree was built based on the 16S rRNA gene sequences from 13 different *Lactobacillus* strains (Figure 1). Isolate NJ13 was closely related to *Lactobacillus brevis* ATCC 14869 [GenBank accession no. M58810] with 99.4% (1430/1438 bp) identity and was designated *Lactobacillus brevis* NJ13 [GenBank accession no. JQ023728]. *Lactobacillus brevis* is a heterofermentative Gram-positive organism frequently isolated from dairy products, silage, feces and the intestinal tract. This strain has the GRAS status and it has been typically used in probiotic products (Carballo *et al.* 2010, Rönkä *et al.* 2003) *L. brevis* was also isolated from Japanese *suguku*, a fermented vegetable (Yakabe *et al.* 2009). However, there is no report about an esterase production from *L. brevis.*

Substrate specificity: *L. brevis* NJ13 esterase activity towards *p*-NP esters was measured (Table 1). The highest activity (fixed as 100%) was towards *p*-NP butyrate (C4 acyl group). The activity of esterase towards *p*-NP acetate (C2 acyl group) was 59% of that towards *p*-NP butyrate. No activity was detected to *p*-NP octanoate (C8 acyl group), myristate (C14 acryl group) and palmitate (C16 acyl group). A lipolytic enzyme is often used interchangeably in terms of esterase and lipase, and both enzymes show activity toward *p*-NP esters (Quyen *et al.* 2007). But, these two enzymes differ from each other on the basis of the physicochemical nature of the substrate and the length of the substrate fatty acid carbon chain (Holland *et al.* 2005). An esterase prefers a water-soluble substrate and generally displays activity toward triglycerides composed of short carbon-chain fatty acids. On the contrary, a lipase prefers water-insoluble substrates composed of long carbon-chain fatty acids (Bornscheuer 2002). Most of the esterases from lactic acid bacteria are active towards short carbon chain fatty acid esters. Esterases from *L. lactis* subsp. *lactis* NCDO 763 (Chich *et al.* 1997), *L. fermentum* DT41 (Gobbetti *et al.* 1997b) and *L. plantarum* 2739 (Gobbetti *et al.* 1997a) show maximum activity towards *p*-NP butyrate, good activity towards *p*-NP caproate (C6 acyl group) and *p*-NP caprylate (C8 acyl group) and poor activity towards *p*-NP esters composed of long carbon chain fatty acids (C12 – C18). The archaea, *Aeropyrum pernix* K1, possesses an esterase that is active toward *p*-NP laurate (C12), palmitate (C16) and stearate (C18), although these activities are half of that towards *p*-NP butyrate (Gao *et al.* 2003).

Effect of temperature and pH on NJ13 esterase activity: To investigate the optimum temperature of *L. brevis* NJ13 esterase, enzyme activity was determined using *p*-NP butyrate at pH 8.0. The highest esterase activity was evident at 50°C (Figure 2). The optimal *L. brevis* NJ13 esterase activity was investigated at 50°C using 30 mM *p*-NP butyrate with a pH ranging from 3 – 9. The optimal activity occurred at pH 8.0 (Figure 3). When pH exceeded 9.0, the optical density from background reaction (blank) without enzyme did not differ with that of enzyme reaction because of pH induced auto-hydrolysis of the *p*-nitrophenyl ester. Hence, enzyme activity beyond pH 9.0 was not determined. The optimum temperature and pH for *L. brevis* NJ13 esterase were similar to those of *Lactococcus lactics* subsp. *lactis* NCDO 763 esterase, showing optimum temperature and pH of 55°C and 8.0, respectively (Chich *et al.* 1997). In contrast, the optimum temperature and pH of *L. fermentum* DT41 esterase is 30°C and 7.0, respectively (Gobbetti *et al.* 1997b). Above results about enzyme reaction conditions could be applied to laboratory enzyme work, optimizing reaction condition for precise analysis of its activity.

Effects of organic solvent, detergent and metal ions on esterase activity: The effect of 10% of organic solvents,

1% of detergents and 10 mM of metal ions on esterase activity was investigated by incubating esterase *L. brevis* NJ13 at 50°C in pH 8.0 with 30 mM *p*-NP butyrate as a substrate. All of the tested organic solvents decreased esterase activity in comparison with the activity in solvent-free control (Figure 4A). Especially, esterase activity was potently inhibited by dichloromethane, *tert*-butanol and methanol. All of the tested detergents decreased the esterase activity compared to the detergent-free control (Figure 4B). Complete depletion of activity was represented at Triton-X treatment. Concerning the metal ions, calcium chloride increased esterase activity compared to control while the other tested metal ions decreased activity, with complete inhibition being evident in the presence of $MnCl_2$ and EDTA (Figure 4C). Different effects of metal ions on the bacterial esterase activity have been reported for different genera of bacteria. For *L. fermentum* esterase, Ca^{2+}, Mg^{2+} and Mn^{2+} are activators (Gobbetti *et al.* 1997b). However, Mn^{2+} was inhibitory for *L. plantarum* in a previous study (Gobbetti *et al.* 1997a) and for *L. brevis* in this study. The information about the responses of esterase from *L. brevis* NJ13 to various detergents, organic solvents and metal ions could be applied to its industrial applications such as development of formulation conditions of enzyme products and optimizing catalysis reaction conditions.

Effects of medium ingredients on esterase production: Generally, the production of bacterial extracellular enzymes during fermentation is influenced by nutrients such as carbon, nitrogen and minerals (Cho *et al.* 2010). In this study, six medium ingredients as variables including three oil sources, two saccharides and one mineral source were selected, and two concentration levels of each variable was assigned to 12 trials according to a PBD. The esterase activity from each trial is summarized in Table 3. The probability of main effects in the analysis of variance for the PBD experiment was 0.03 and adjusted R square value was 74.51%. The effect of variables and their confidence levels are shown in Table

Table 1. Activity[1] and substrate specificity of esterase from *L. brevis* NJ13

Substrate	Specific activity (U/mg protein)	Relative activity (%)
p-nitrophenyl acetate	37.06	59.31
p-nitrophenyl butyrate	62.49	100.00
p-nitrophenyl octanoate	ND[2]	—
p-nitrophenyl myristate	ND	—
p-nitrophenyl palmitate	ND	—

[1]The esterase activity was measured by colorimetric assay with 30 mM p-NP ester as a substrate in 10 mM Tris-HCl buffer (pH 8.0) at 50°C and one unit (U) was defined as released μM p-NP per one minute.
[2]ND, not detected under specific conditions.

Table 2. Variables showing medium ingredients used in Plackett-Burman design and their concentrations assigned to coded levels

Variable	Concentrations (g L^{-1}) for coded levels	
	-1 values	1 values
Olive oil	0.1	1
Soybean oil	0.1	1
Tributyrin	0.1	1
Sucrose	1	10
Glucose	1	10
$CaCl_2$	0.1	1

Table 3. Plackett-Burman design matrix of six variables along with observed response of esterase production from *L. brevis* NJ13

Trials	Variables						Esterase activity[1] (U mL^{-1})
	Olive oil	Soybean oil	Tributyrin	Sucrose	Glucose	$CaCl_2$	
1	1	-1	1	-1	-1	-1	39.50±3.67[2]
2	1	1	-1	1	-1	-1	33.66±4.61
3	-1	1	1	-1	1	-1	38.74±6.53
4	1	-1	1	1	-1	1	41.11±6.81
5	1	1	-1	1	1	-1	27.83±5.69
6	1	1	1	-1	1	1	43.27±7.41
7	-1	1	1	1	-1	1	48.12±8.68
8	-1	-1	1	1	1	-1	39.50±7.60
9	-1	-1	-1	1	1	1	35.72±8.00
10	1	-1	-1	-1	1	1	36.56±3.83
11	-1	1	-1	-1	-1	1	35.72±4.20
12	-1	-1	-1	-1	-1	-1	27.04±4.50

[1]The esterase activity was measured by spectrophotometric assay with 30 mM *p*-NP-butyrate as a substrate in 10 mM Tris-HCl buffer (pH 8.0) at 50°C and one unit (U) was defined as released μM p-NP per one minute.
[2] Mean ± standard error (triplicates)

Table 4. Statistical analysis of medium ingredients in relation to esterase production from *L. brevis* NJ13 as per Plackett-Burman design

Variables	Effect	S.E.	*t*-value	*p*-value	Confidence level (%)
Olive oil	-0.485	0.871	-0.28	0.792	20.8
Soybean oil	1.318	0.871	0.76	0.483	51.7
Tributyrin	8.952	0.871	5.14	0.004	99.6
Sucrose	0.852	0.871	0.49	0.646	35.4
Glucose	-0.588	0.871	-0.34	0.749	25.1
$CaCl_2$	5.705	0.871	3.28	0.022	97.8

Adjusted R square value is 74.51%

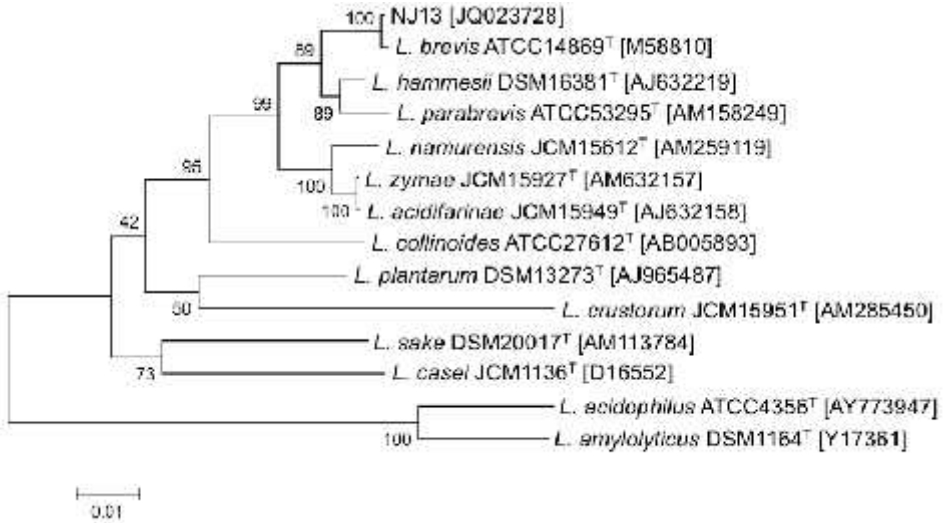

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence of isolate NJ13 and other type strains of lactobacilli. Bootstrap values (based on 1,000 trials and only values >50%) are shown at the nodes. Bar denotes 0.01 substitutions per nucleotide position.

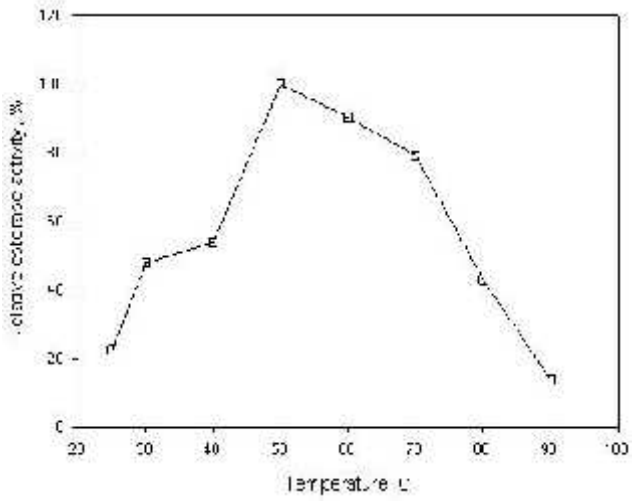

Fig. 2. Effect of temperature on the esterase activity. The activity was measured with 30 mM *p*-nitrophenyl butyrate at pH 8.0 and various temperatures ranging from 25 to 90°C.

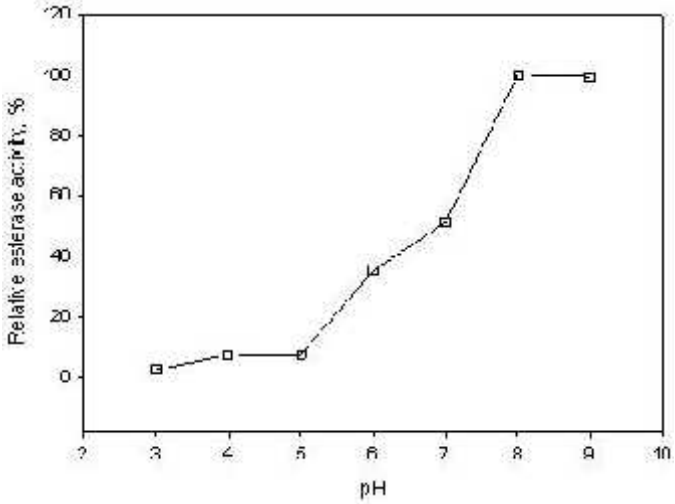

Fig. 3. Effect of pH on the esterase activity. The activity was measured with 30 mM *p*-nitrophenyl butyrate at 50°C and various pH from 3.0 to 9.0.

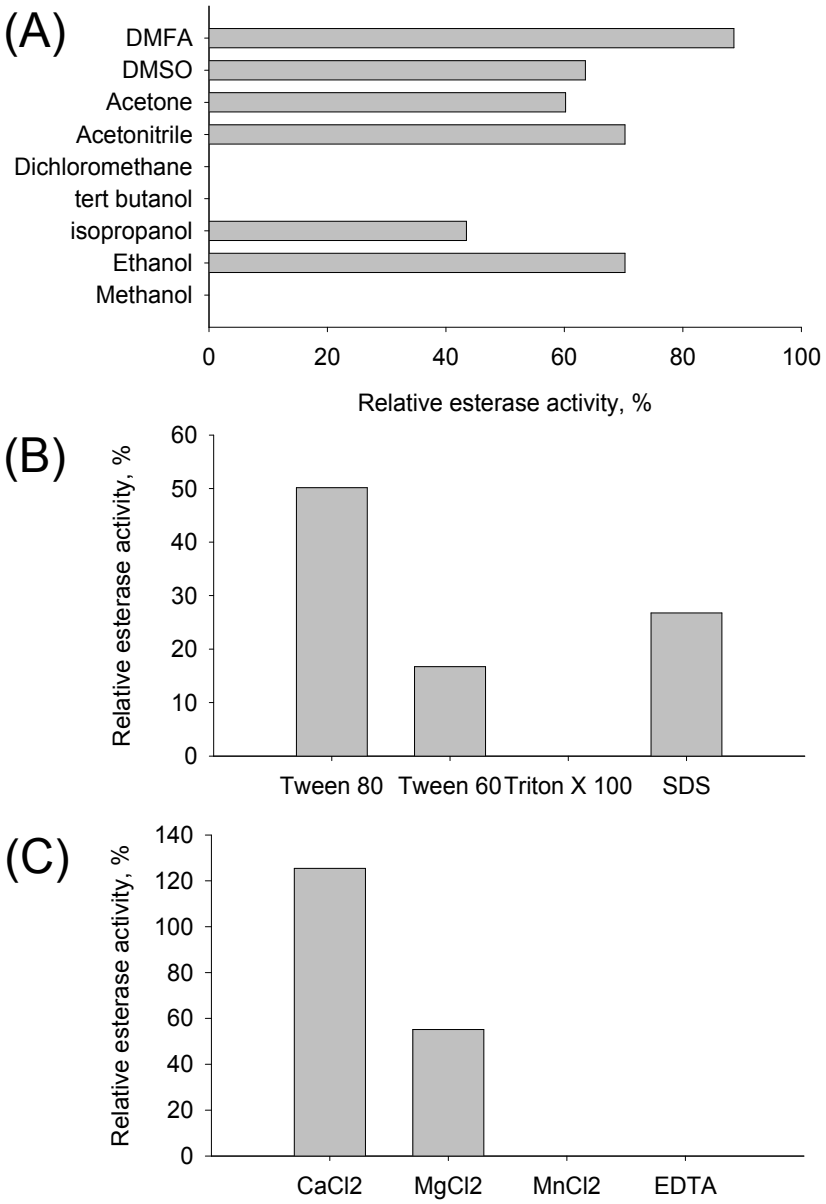

Fig. 4. Effects of organic solvents, detergents and metal ions on the esterase activity. The effect of 10% of organic solvents (A), 1% of detergents and 10 mM of metal ions on the esterase activity was measured with 30 mM *p*-nitrophenyl butyrate at pH 8.0 and 50°C. Relative activity is expressed as a percentage to the activity of control without treatment.

4. Soybean oil, tributyrin, sucrose and $CaCl_2$ displayed a positive effect (i.e., nutrient-related increase in esterase production). However, the effects were significant only for tributyrin and $CaCl_2$. $CaCl_2$ was an activator in both enzyme reaction and fermentation. The effective medium ingredients could be directly applied to development of optimum medium for esterase producing fermentation of *L. brevis* NJ13.

Conclusions: *L. brevis* is a representative LAB, a functional class of Gram-positive bacteria that primarily produces lactic acid from hexose sugar. LAB is associated with fermentation of dairy products and has also been studied as a source of probiotics. Esterase, a carboxylic ester hydrolase, is the most abundant enzyme widely distributed in all kingdoms of life, and has potential applications in a broad range of industrial fields. In this study, *L. brevis* producing esterase was isolated from *Kim-chi* and characterized. Effective medium ingredients for the esterase production from the

fermentation of *L. brevis* NJ13 were determined. The present results could be applied to optimize enzyme reaction conditions and the *L. brevis* NJ13 included fermentation such as fermented meat and dairy products. The study illuminates a previously unknown area, namely *L. brevis* esterase.

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