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Study on β-galactosidase enzyme produced by isolated lactobacilli from milk and cheese

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-galactosidases enzyme have been used in the dairy industry for the improvement of lactose intolerance. The aim of this study was to detect β-galactosidase enzyme produced by isolated lactobacilli from milk and cheese. Isolated lactobacilli were cultured on MRS agar. Lactobacilli were identified by Gram stain and standard bacteriological and biochemical methods. Their ability to hydrolyze 5-bromo-4-chloro-3-indolyl- -D- galactopyranoside (X-Gal) and O-nitrophenyl- -D-galactopyranoside (ONPG) was determined. A protein band of indicated β-galactosidase enzyme was also detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The colonies that produced green color on X-Gal plates were lactobacilli with β-galactosidase enzyme which had ONPG positive results. The highest enzymatic value (1,966 U/ml) was observed in one strain of *Lactobacillus delbrueckii*. A 116 kDa protein band was detected in some strains (37%) with highest enzyme value and in others (63%), protein band was weak by SDS-PAGE method. By adding *Lactobacilli* producing β-galactosidase enzyme as probiotic to dairy products, could help lactose intolerant people.

Key words: *Lactobacillus*, -galactosidase, X-Gal, ONPG, SDS-PAGE.

INTRODUCTION

Lactic acid bacteria (LAB) that used as starters for production of dairy products are the main factors of fermentation and protection of fermentative foods and also have a significant role in texture and flavour of food products (Chammas et al., 2006). One of the glycosi-dases, is -galactosidase enzyme that widely used in dairy industry and is produced by most lactobacilli (Karasova et al., 2002; Corral et al., 2006; Nguyen et al., 2007). This enzyme hydrolyzes lactose, the main carbo-hydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium (Troelsen, 2005; Vasiljevic and Jelen, 2001; Heyman, 2006). -galactosidase has two enzymatic activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to a certain extent cellulose and the other, splits -glycosides (Troelsen, 2005; Heyman, 2006). Low activity of -galactosidase causes digestive insufficiency, called lactose intolerance in most cases (Karasova et al., 2002; Vasiljevic and Jelen, 2001). The symptoms of lactose intolerance such as abdominal pain and diarrhea, nausea, flatulence, and or bloating after the ingestion of lactose or lactose containing food substances which can lead to decrease quality of life, and daily activities. Treatment is relatively simple by eliminating lactose from the diet or by using of supplemental -galactosidase enzyme replacement (Vasiljevic and Jelen, 2001). The bacterial species currently used by the dairy industry which produced -galactosidase enzyme belong to genera of *Lactobacillus* and *Bifidobacterium* and comprise a limited collection of strains (Fernandez et al., 1999; Xanthopoulos et al., 1999; He et al., 2008). These bacteria have become a focus of scientific studies for three particular reasons (Somkuti et al., 1998): a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects, b) These bacteria are generally regarded as safe (GRAS) so the -galactosidase enzyme derived

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from them might be used without extensive purification (Vasiljevic and Jelen, 2001). c) Some strains have probiotic activity such as improved digestion of lactose and a suitable strain selection must be carried out to manufacture probiotic dairy products (Vinderola and Reinheimer, 2003). The aim of this study was to compare the isolated lactobacilli with -galactosidase production in milk and cheese for selection of lactobacilli producing high -galactosidase enzyme as probiotic, by biochemical (X-gal and ONPG substrate) and molecular (SDS-PAGE) methods (Ausubel, 1994). Because, the existence or addition of lactobacilli producing high -galactosidase enzyme as probiotic can be use for more improvement of lactose digestion in dairy products especially in milk.

MATERIALS AND METHODS

Sampling

This cross -sectional study was conducted from 2006 to 2008 in Iran University of Medical Science. In this study, 50 samples of pasteurized and unpasteurized milk and cheese that bought from different shops throughout Tehran were studied.

Culture media and incubation conditions: For isolation of bacteria, 2 g of cheese and 2 ml of milk was added to 5 ml of MRS broth (Merck, Germany) and incubated anaerobically (24 h at 37^{o}C). Then, 50 µl of them were spread onto MRS agar (Merck, Darmstadt, Germany). Plates were incubated in anaerobic jars at 37^{o}C for 48 h. To determine -galactosidase activity, MRS broth without glucose (MRS-lac) but contained 1% of lactose was used (Vinderola and Reinheimer, 2003).

Identification of isolated bacteria: Bacteria were examined by Gram stain, and identified by standard bacteriological and biochemical methods (Sneath et al., 1984). Acid production from carbohydrates (glucose, galactose, maltose, mannitol, ribose, sucrose, arabinose, lactose, mannose, raffinose, rhamnose, xylose, sorbitol, salicin) in MRS broth base (without glucose and beef extract) was evaluated. Their ability to grow at various temperatures (4, 15, 25, 30, 37 and 40^{o}C) was examined after 3 - 10 days. Motility, production of indol and H_2S tests were carried out by using SIM medium (Merck, Germany).

Study of -galactosidase production

X-gal substrate: One colony of isolated bacteria were grown on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-- D-galactopyranoside, #R0401, fermentas, 20 mg/ml DMF) and 10 µl of IPTG (iso-propyl-thio- -D-galactopyranoside, dioxane free #R0391, fermentas) solution as an inducer. Plates were incubated at 37^{o}C for 24 h to 3 days. Colonies producing -galactosidase were green (Vinderola and Reinheimer, 2003).

ONPG substrate: All bacteria were inoculated into tubes containing ONPG (O-nitrophenyl- -D-galactopyranoside) (0.5 ml) and 0.01 M sodium phosphate buffer (pH 7.0) (5 ml) and peptone water. Production of yellow color was indicated positive ONPG results (Miller, 1998).

Value of -galactosidase

This test was performed according to the method of Miller (1998), Vinderola Reinheimer (2003). Briefly, all bacteria were harvested in MRS broth. After centrifugation at 12000 × g for 5 min. at 5^{o}C, washed twice in 60 mM $Na_2HPO_4 \times 7H_2O$, 40 mM NaH_2PO_4 buffer (pH 7.0) and inoculated in MRS-lac broth. Then, absorbance 560 nm was adjusted with the same buffer and recorded. One ml of the cell suspension was premeabilized with toluene/acetone (1:9 v/v) solution (50 µl), vortexed for 7 min. and immediately assayed for -galactosidase value. An aliquot (100 µl) of the permeabilized cell suspension was placed in a microtube and phosphate buffer (900 µl) and O-nitrophenyl- -D-galactopyranoside (200 µl of ONPG 4 mg/ml, Merck) were added. Microtubes were placed into a water bath (37^{o}C) for 15 min. Then, 1M Na_2CO_3 (0.5 ml) was added to each tube to stop the reaction. The contents of each microtube were centrifuged at 12000 × g for 5 min. to remove the cells. Absorbance values at both 420 and 560 nm were recorded for each microtubes and -galactosidase value was calculated in Miller units (Vinderola Reinheimer, 2003).

Protein extraction and SDS-PAGE

Lactobacillus strains were cultured in MRS broth (100 ml) without peptone and beef extract. Then, were centrifuged at 3500 × g for 20 min. After centrifugation, once, supernatant was added to Tris HCl and again to saccharose, Tris HCl and lysosyme. Then, EDTA was added. After centrifugation, solution was dissolved in SDS, Tris HCl, glycerol, mercaptoethanol, bromophenol blue and distilled water. After preparation, protein solution was precipitated with 40% of ammonium persulfate and then, centrifuged at 3500 × g at 5^{o}C for 20 min. Precipitate was dialyzed by dialysis tubes (0.2 µ) in distilled water for 72 h. Then, samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) according to Laemmli method on vertical slab gels (Laemmli, 1970). Resolving and stacking gel conditions were 12 and 4% acrylamide, respectively. Unstained protein molecular weight marker (#SM0431) was purchased from Fermentas. Gels were run for 4 h at 110 V. Protein bands were visualized by staining with Coomassie Brillint Blue (Merck) R-250 (Dunn et al., 1993).

RESULTS

Identification of isolated *lactobacillus* strains

Forty one *Lactobacillus* strains were isolated from 50 samples (Table 1). All bacteria did not grow at 4^{o}C but all of them grew at the maximum rate at 25^{o}C and weakly growth was detected at 40^{o}C.

-galactosidase screening with X-gal, ONPG

All bacteria produced green color colonies on X-Gal plates that indicating the presence of -galactosidase enzyme. All lactobacilli were produced a dark green colonies with or without halos (Figure 1). Some of them (37%) produced dark green colonies after 24 h incubation (rapid enzymatic activity) and others (63%) had delay (slow) enzymatic activity after 2 - 4 days of incubation.

All bacteria had positive ONPG results (production of yellow color). In ONPG method, values of - galactosidase enzyme were the range of 22.7 to 1,325 (U/ml) in milk isolates and of 103.1 to 1,966 (U/ml) in cheese isolates. High value of -galactosidase enzyme was detected in *L. delbrueckii* subsp. *bulgaricus* and *L. casei* subsp. *casei* (ranging from 867 to 1,966 U/ml) isolated from cheese and in *L. casei* subsp. *casei*, *L. casei* subsp.

Table 1. Isolated *Lactobacillus* from milk and cheese.

Isolate no.	Strains	Origin		Total	Isolated %
		Milk	Cheese		
1	*L. acidophilus*	1	2	3	7
2	*L. bifermentans*	1	1	2	5
3	*L. brevis*	---	1	1	2
4	*L. casei* subsp. *casei*	2	4	6	15
5	*L. casei* subsp *rhamnosus*	3	4	7	18
6	*L. casei* subsp. *tolerans*	2	2	4	10
7	*L. curvatus*	1	---	1	2
8	*L. delbrueckii* subsp. *bulgaricus*	---	2	2	5
9	*L. delbrueckii* subsp. *delbrueckii*	---	1	1	2
10	*L. delbrueckii* subsp. *lactis*	---	3	3	7
11	*L. fructosus*	---	1	1	2
12	*L. fermentum*	1	---	1	2
13	*L. helveticus*	---	2	2	5
14	*L. plantarum*	3	4	7	18
Total		14	27	41	100

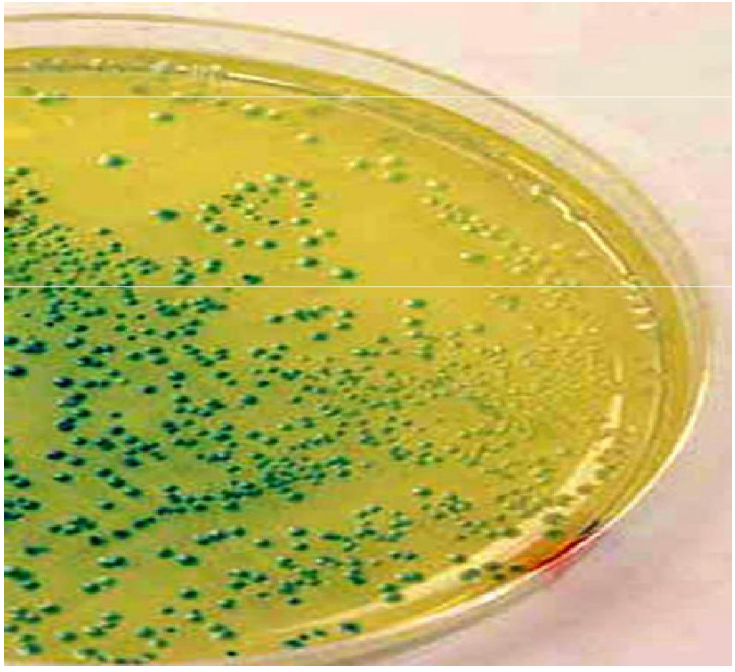

Figure 1. Green colonies of *Lactobacillus* strains on X-gal plates.

tolerans L. plantarum (ranging from 606 to 1,326 U/ml) isolated from milk. Low value of -galactosidase enzyme was detected in two strains of *L. casei* subsp. *rhamnosus* and in one strain of *L. plantarum* was 22.7, 44.4 and 64.4 (U/ml) in milk lactobacilli, respectively.

Our results showed that the addition of 1% lactose could increase -galactosidase values. In the presence of 1% glucose as sole of carbon source, a -galactosidase value was decreased. In this study X-gal and ONPG results were the same (positive results).

SDS-PAGE results

A protein band with a molecular weight of ~116 kDa was observed in some strains (37%) with the highest -galactosidase enzyme value and in many of them (63%), protein band was weak and could not detected very clearly (Figure 2).

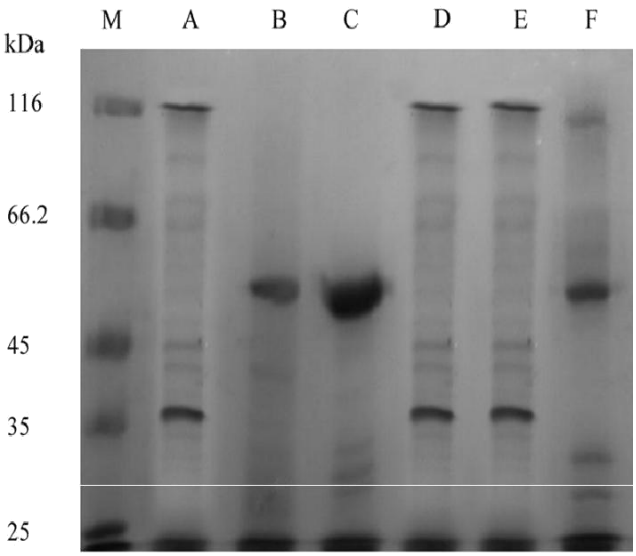

Figure 2. Protein bands of *Lactobacillus* strains in SDS-PAGE. A protein band with a molecular weight of ~116 kDa was observed in A: *L. delbruekii* subsp*. bulgaricus,* D: *L. delbruekii* subsp*. lactis* and E: *L. casei* subsp*. casei* which had the highest -galactosidase enzyme value. M: Molecular weight marker (left hand side of the pattern): -galactosidase (116 kDa).

DISCUSSION

Lactobacilli are the most abundant bacteria in gastrointestinal tract. They are the most important food fermentators and used as starters. Also these bacteria have the role in texture and flavour of dairy products, sausage and some other of fermentative foods. -galactosidase is an enzyme that produced by some of bacteria, especially lactobacilli in dairy products that is yoghurt, cheese and milk.[4] Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world (Heyman, 2006). Therefore, by addition of *Lactobacilli* producing -galactosidase as probiotic to milk and cheese and other dairy products could help lactose intolerance symptoms.

The fastidious nature of *Lactobacilli*, requiring rich medium for the growth and -galactosidase activity. It was reported that by using skim milk (it is a source of lactose), the production of -galactosidase enzyme value would increase (Vasiljevic and Jelen, 2001) and -galacto-sidase production in *Bacillus megaterium* is subject to catabolite repression by glucose and is lactose inducible (Show et al., 1998). The results of this study indicated that, enzymatic values were increased by adding of 1% lactose (because of lac operon stimulation), instead of 1% glucose into MRS broth. It was suggested that lactose is an inductive factor to increase -galactosidase enzyme. On the other hand, in present study, by adding of 1% glucose as sole of carbon source to MRS broth, -galactosidase values was decreased. So, our results confirmed the Vasiljevic and Jelen results and were similar to the results that they have reported about increase of -galactosidase enzyme by adding of lactose. Then it is better somehow glucose is eliminated and lactose is replaced it.

Thermophilc bacteria have become an object of interest for the commercial production of -galactosidase enzyme (Petzelbauer et al., 1999; Tabasco et al., 2007). The -galactosidase of yogurt mixed culture, consisting of *L. delbrueckii* sub sp*. Bulgaricus* has been characterized (Greenberg and Mahoney, 1982). Showing high activity and stability at temperatures above 50^{0}C. Such conditions can enhance the rate of lactose hydrolysis. In this study, no *Lactobacillus* species with the exception of one species (*L. casei*) was isolated from pasteurized milk, whereas different *Lactobacilli* was isolated from unpasteurized milk. From these observations, it can be concluded that *L. casei* was more stable at pasteurization temperatures and also probably lactobacilli in pasteurized milk were sensitive to high temperatures. These results in agreement with the various strategies, which have showed and proposed to improve the growth rate of bacterial probiotics in milk, mostly by adding of thermostable *Lactobacillus* strains to milk (Gaudreau et al., 2005).

Vinderola and Reinheimer reported that high value of -galactosidase enzyme in commercial strains of *L. delbrueckii* subsp. *Bulgaricus* isolated from cheese (Vinderola and Reinheimer, 2003). In our study, two strains of *L. delbrueckii* with high and rapid enzyme values from cheese were detected, which were in the range of values previously reported (Vinderola and Reinheimer, 2003) . So, it seems that the conditions in cheese for growth of *Lactobacilli* with production of -galactosidase enzyme is suitable. These results observed among *L. delbrueckii strains* further emphasize the importance of selecting appropriate strains for use as dietary adjuvants.

On the other hand, considering that milk avoidance has a negative effect on calcium and vitamin D intake in infants, children and adolescents which must provided by other dairy products (Heyman, 2006), therefore, consumption of cheese or modified milk (containing lactobacilli with high enzyme value) is recommended.

Favier et al. reported a method to detect bacteria with -galactosidase activity by X- gal. Colonies growing on X-gal medium with green color were regarded as bacteria containing -galactosidase enzyme (Favier et al., 1996). In this study, when the method of Favier et al. was applied to 41 *Lactobacilli*, all isolated lactobacilli containing -galactosidase enzyme had green colonies (some pro-ducing strong and rapid and many low and slow enzyme). Our results detected by different biochemical methods (ONPG, X-gal methods) confirmed each other which were similar to previous works (Favier et al., 1996).

It was reported that -galactosidase enzyme is tetrameric enzyme which consists of identical subunits with a molecular weight of 116 kDa.[21] In this study, by using of SDS-PAGE method, an intensive 116 kDa protein band

was observed in *Lactobacilli* with high values of -galactosidase enzyme (such as *L. delbruekii* subsp. *bulgaricus*, *L. delbruekii* subsp. *lactis*, *L. casei* subsp. *casei*). Our results were in accordance with results observed in previous studies (Nichtl et al., 1998). Therefore, the SDS-PAGE method is not suitable for all cases to detect -galactosidase activity. Since all of the isolated lactobacilli that produce high and low or weak -galactosidase, can be detected by X-gal and ONPG tests, so, biochemical tests, which are rapid, cheap and simple in all laboratory and do not need any expensive molecular materials and apparatus, is recommended.

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