RESEARCH ARTICLE

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β-Galactosidase from strains isolated from breast milk and infant saliva: characterization and participation in absorption of different oligosaccharides

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ABSTRACT
Lactobacillus are Gram-positive anaerobic bacteria that naturally occur in the human intestinal tract. They are considered to be beneficial commensals for human health because they prevent the growth of pathogenic bacteria by lowering the intestinal pH and stimulate the host's immune system to enhance antipathogenic and anticarcinogenic activities. The intestines of breastfed infants are colonized by *Lactobacillus* within a week after birth, in comparison with those of bottle-fed infants. In our study, we have isolated bacterial strains of breast milk and infants saliva. The isolates were identified by 16S RNA and API identification. This report details the ability of the isolated strain to metabolized human milk oligosaccharides and we clarified that HMOs was sequentially degraded by different glycosidases. We studied the potential of β-galactosidases from studied strains to metabolize carbohydrates from breast milk. We investigated the β-galactosidase activity after cultivation of medium containing different concentration HMOs, lactose and some oligosaccharides.

Key words: β-galactosidase, probiotic, oligosaccharides, breast milk, microbiome, *Lactobacillus*

Introduction

Lactobacillus are Gram-positive anaerobic bacteria that naturally occur in the human intestinal tract. They are considered to be beneficial commensals for human health because they prevent the growth of pathogenic bacteria by lowering the intestinal pH and stimulate the host's immune system to enhance antipathogenic and anticarcinogenic activities. The intestines of breastfed infants are colonized by *Lactobacillus* within a week after birth, in comparison with those of bottle-fed infants (Dogra et al., 2015). Several beneficial functions such as vitamin production, production of digestive enzymes, and stimulation of the immune system have been suggested for the members of this genus as well as for other probiotic strains. The concept of prebiotics has been employed in achieving an increased number of beneficial microorganisms in the intestines. A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Urashima et al., 2014). Certain oligosaccharides, specific types of dietary carbohydrates, are nondigestible for humans and monogastric animals, promote selectively the growth of desirable bacteria in the colon at the expense of less desirable bacteria and thus are classified as prebiotics (Urashima et al., 2014). The physiological importance and health benefits of nondigestible oligosaccharides have been reported (Rastall et al., 2001 Asakuma et al. 2008). Because of these benefits, prebiotic oligosaccharides are of great interest for both human and animal nutrition. To be utilized by the target group of microorganisms, these oligosaccharides have to be cleaved by suitable glycosidases. The enzyme of particular interest in this work is β-galactosidase (β-gal, EC 3.2.1.23). β-galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) (Nakayama et al., 1999) and are widespread in nature.

The human milk oligosaccharides (HMOs) are defined as the oligosaccharides in human milk excluding lactose. They seem to be indigestible for the host and most intestinal bacteria but are thought to be assimilated by bifidobacteria and thus to promote the growth of those bacteria (Ward et al. 2006; LoCascio et al. 2007). HMOs are present at a concentration of 10–20 g/L in human milk and are characterized by their highly complex structures, more than 130 types of HMOs have so far been isolated (Kunz et al. 2000; Asakuma et al. 2008). HMOs are composed of the five monosaccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia), with N-acetylneuraminic acid (Neu5Ac). Lactose or the elongated oligosaccharide chain can be fucosylated in α1-2, α1-3 or α1-4 linkage and/or sialylated in α2-3 or α2-6 linkage. Some HMOs occur in several isomeric

forms, e.g. lacto-N-fucopentaose (LNFP) or sialyllacto-N-tetraose (LST). More than a hundred different HMOs have been identified so far, but not every woman synthesizes the same set of oligo- saccharides (Kobata et al., 2010).

In the past decade, some culture-dependent studies revealed that colostrum and milk from healthy women contain bacteria, including *staphylococci*, *streptococci*, *corynebacteria*, lactic acid bacteria, *propionibacteria*, and *bifidobacteria*. Later, the application of culture-independent techniques, including microbiome approaches, confirmed the presence of DNA from these and other bacterial genera (Fernandez et al., 2013).

Although the human salivary microbiome is still widely unknown, *Streptococcus* species seem to be dominant both in adults (Nasidze et al., 2009; Yang et al., 2012) and in edentulous infants. *Streptococci* are also among the dominant phylotypes in human milk (Hunt et al., 2011), suggesting a potential role in the shaping of the salivary microbiota. The origin of the oral microbiota is far from elucidated and deserves research attention because of its relevant implications for human health.

In our study, we have isolated bacterial strains of breast milk and infants' saliva. The isolates were identified by 16S RNA and API identification. This report details the ability of isolated strains to metabolized human milk oligosaccharides and we clarified that HMOs was sequentially degraded by different glycosidases. We studied the potential of β-galactosidases from studied strains to metabolize carbohydrates from breast milk.

We investigated the β-galactosidase activity after cultivation of medium containing different concentration HMOs, lactose and some oligosaccharides.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) and were of the highest quality available unless otherwise stated. MRS broth powder (for *Lactobacillus* broth according to De Man, Rogosa, and Sharpe), lactulose and galacto-oligosaccharides were obtained from Merck (Darmstadt, Germany). HMO was isolated from breast milk of 20 healthy women, aged between 23 and 35 years, who were breastfeeding children from birth to 1 year old. Fucosyllactose (2-FL) is provided from Frisland Campina, Netherlands. Gamma rays ^{60}Co and EMS treatments

Identification using the API 20 NE system

The API 20 NE system covers 61 non-enterobacterial gram-negative taxa. Testing was performed according to the instructions of the manufacturer (bioMérieux, Marcy l'Etoile, France).

Identification using Sequencing of 16S rRNA gene

Following nucleic acid isolation and purification, the 5′ part of the 16S rRNA gene was amplified using specific primers. 16S rRNA gene sequences were compared with those available in the GenBank. (BLAST).

Strains and Culture Conditions for Screening

Eight strains of *Lactobacillus spp.* were isolated from breast milk and saliva samples. The strains were stored in sterile vials at - 80 ° C in MRS broth medium containing glycerol (15%, v/v) and activated by three successive transfers every 24 h in MRS broth medium. They were grown on MRS broth medium (10 g/L peptone, 2 g/L dipotassium hydrogen phosphate, 8 g/L meat extract, 2 g/L diammonium hydrogen citrate, 4 g/L yeast extract, 5 g/L sodium acetate, 0.2 g/L magnesium sulfate, 1 g/L Tween 80, and 0.04 g/L manganese sulfate), in which galacto-oligosaccharides, HMOs, lactulose and 2-fucosyllactose (2-FL) served as the carbon source and incubated anaerobically at 37 ° C for 24 h. The optical densities (OD 600) of the cultures were measured with spectrophotometer. To screen for β-galactosidase activity, cells were harvested from a liquid culture by centrifugation (10 000 rpm for 10 min at 4°C) using an Eppendorf centrifuge and resuspended in 50 mM sodium phosphate buffer (pH 6.8) containing 20% (w/v) glycerol and 1 mM dithiothreitol. The cells were disrupted by ultrasonication, and debris was removed by centrifugation (10 000 rpm for 15 min at 4°C) to obtain the cell-free extract.

Enzyme Assays

β-Galactosidase activity was determined using o-nitrophenyl β-D-galactopyranoside (ONPG) as the substrates. (Bahl et al., 1969) When chromogenic ONPG was used as the substrate, the determination of β-galactosidase activity was carried out at 37 ° C with 20 mM ONPG in 50 mM sodium phosphate buffer (pH 6.5). The reaction was initiated by adding 100 µL of enzyme solution to 500 µL of the substrate solution, and then the mixture was incubated for 10 min. The reaction was stopped by adding 3 mL of 1 M Na_2CO_3. The release of o-nitrophenol (ONP) was assessed by determining the absorbance at 410 nm. One unit of oNPG activity was defined as the amount of enzyme releasing 1µmol of ONP per minute under the described conditions. Ammonium sulfate was slowly added to the crude extract to 60% saturation. The precipitate was obtained by centrifugation as described above. The supernatant was discarded; the pellet was dissolved in 50 mM sodium phosphate buffer containing 1 M ammonium sulfate. After that, the samples were lyophilized with Labcombo, USA. The protein concentration was determined by the method of Bradford.

Enzyme Purification

Approximately 50 g of biomass (wet weight) was suspended in 100 mL of buffer. The cells were disrupted by ultrasonification, and debris was removed by centrifugation (25000g for 15 min at 4°C) to obtain the crude extract.

In situ analysis and active staining

Native polyacrylamide gel electrophoresis (PAGE) and denaturing sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE) were performed on a PhastSystem unit (Amersham) using precast polyacrylamide gels (PhastGel 8-25) (Manchenko et al., 1994).For SDS - PAGE, the enzyme was pre-incubated with SDS buffer mM Tris-HCl (pH 6.8) containing 34 mg/mL SDS, 0.1 mg/mL bromophenol blue, 5% (v/v) mercaptoethanol, and 15% (v/v) glycerol] at 60°C for 5 min. Coomassie blue staining was used for the visualization of the protein bands. Active staining for the visualization of the bands with β-galactosidase activity was carried out by applying filter paper soaked with the staining solution 50 mM sodium phosphate buffer (pH 6.5) and 3.5 mg/mL 4-methylumbelliferyl β-D-galactoside onto the gel and incubation at 37°C for 30 min. After application of 1 M carbonate -bicarbonate buffer (pH 10.0) onto the gel using a filter paper, the protein bands displaying enzyme activity were visualized under UV light, thus detecting the release of 4-methylumbelliferone (Manchenko et al., 1994).

Hydrolysis of HMOs, lactulose and galactooligosaccharides from resting cells

Cells, after cultivation for 6, 12, 18, and 24 hours in mMRS with different carbon sources, were separated by centrifugation at 9000 rpm. for 15 minutes at 4 ° C and washed twice with 100 mM sodium acetate buffer pH 6.0. The cells are resuspended in a thirty-fold reduced volume (relative to the culture) of solutions of different carbohydrates. The hydrolysis was run at 37°C, samples were taken at different time intervals and the various enzyme activities analyzed. All analyzes for hydrolysis of HMOs, lactulose and galactooligosaccharides and enzyme activity from resting cells were performed with a minimum of three times repeatability.

Results

Bacterial identification

Bacterial strains were isolated from the samples of breast milk and infant saliva. After API identification these strains were defined as *Lactobacillus fermentum*. Bacterial genomic DNA was extracted and the DNA was used for PCR amplification of 16S ribosomal DNA. The purified PCR product of each isolate were sequenced. The most common isolated microorganisms were Gram-negative from *L. fermentum.*

For our research we used 8 strains *Lactobacillus fermentum* like 6 of them *Lactobacillus fermentum* st 5, *Lactobacillus fermentum* st 6, *Lactobacillus fermentum* st 8, *Lactobacillus fermentum* st 10, *Lactobacillus fermentum* st 22, and *Lactobacillus fermentum* st24 was isolated from breast milk and the resting 2 strains *Lactobacillus fermentum* ss5 and *Lactobacillus fermentum* ss8 was isolated from infant saliva. (Table 1).

Metabolising of galactooligosaccharides (GalOs), lactulose and human milk oligosaccharides (HMOS) from the isolated from breast milk and infant saliva L. fermentum strains.

Eight *Lactobacillus fermentum* strains were screened for the ability to grow on 2% galactooligosaccharides, 2% lactulose and 2% HMO and was detected for β-galactosidase activity. The ability of studied strains to grow on the following substrates was judged on the basis of the optical densities of the cultures (OD_{600}) measured during the course of their cultivation and acidification of the media (Table 2).

The largest amount of biomass (1.92 mg/mL) was detected on 24 hours after the start of cultivation in *L. fermentum* St5 strain culturing 2% breast milk oligosaccharides, in acidification of the media to pH of 4.6.

In addition, as a result of the metabolism of the tested strains, we also monitored their β-galactosidase activity (Figure 1 and Figure 2).

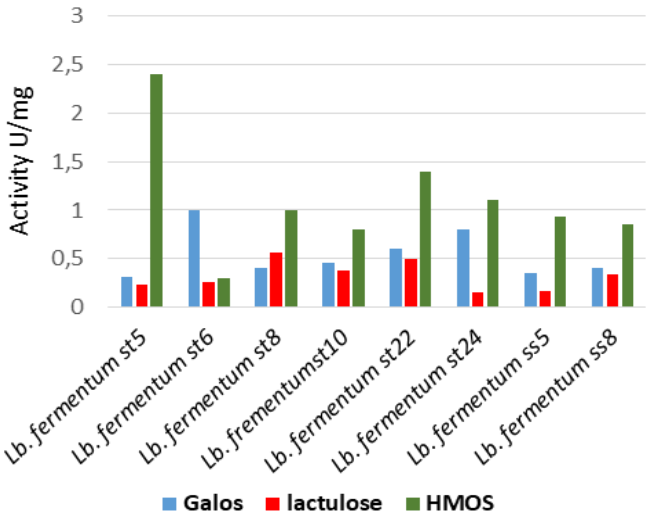

Figure 1. *β - galactosidase activity on 12 h after cultivation on lactulose, galactooligosaccharides and Human milk oligosaccharides (HMOS)*

The highest specific activity was detected in the *L. fermentum* St5 strain (2.4 U/mg) and the *L. fermentum* strain St22 (2 U/mg) in the culture medium containing HMOs. Thus, HMOs are the strongest inducer for the transcription of β-galactosidase encoding genes, subsequent translation and secretion of the enzyme in active form.

Table 1. *Strains with significant coincidence after BLAST.*

Strain	Sequences showed significant coincidence	E-value	% Identicality
St 5	*Lactobacillus fermentum* Strain CBA 16S ribosomal RNA gene, partial sequence	0.0	98
St 6	*Lactobacillus fermentum* Strain CIP 102980 16S ribosomal RNA, partial sequence	0.0	98
St 8	*Lactobacillus fermentum* Strain NBRC 16S ribosomal RNA, partial sequence	0.0	98
St 10	*Lactobacillus fermentum* Strain CIP 102980 16S ribosomal RNA gene, partial sequence	0.0	98
St 22	*Lactobacillus fermentum* Strain CIP 102980 16S ribosomal RNA, partial sequence	0.0	98
St 24	*Lactobacillus fermentum* Strain CIP 102980 16S ribosomal RNA gene, partial sequence	0.0	97
Ss 5	*Lactobacillus fermentum* Strain 2-4 16S ribosomal RNA gene, partial sequence	0.0	98
Ss 8	*Lactobacillus fermentum* Strain CAU:216 16S ribosomal RNA gene, partial sequence	0.0	98

Table 2. *Biomass growth (mg/ml) on after cultivation on 2% lactulose, 2% galactooligosaccharides(GalOs) and 2% Human milk oligosaccharides (HMOS).*

Strain	GalOs 2%		lactulose 2%		HMOS 2%	
	12h	24h	12h	24h	12h	24h
L. fermentum st5	1.23	1.23	0.9	1	1.25	1.92
L. fermentum st6	0.922	0.99	0.4	0.5	1.13	1.43
L. fermentum st8	1.43	1.54	1	1.1	1.6	1.7
L. fermentum st10	1.3	1.37	1	1.2	1.34	1.7
L. fermentum st22	1	1.5	0.5	1.2	1.24	1.7
L. fermentum st24	0.72	0.9	0.4	0.6	1	1.2
L. fermentum ss5	1	1.4	1	1.26	1.5	1.7
L. fermentum ss8	1.3	1.2	0.98	1.2	1.5	1.8

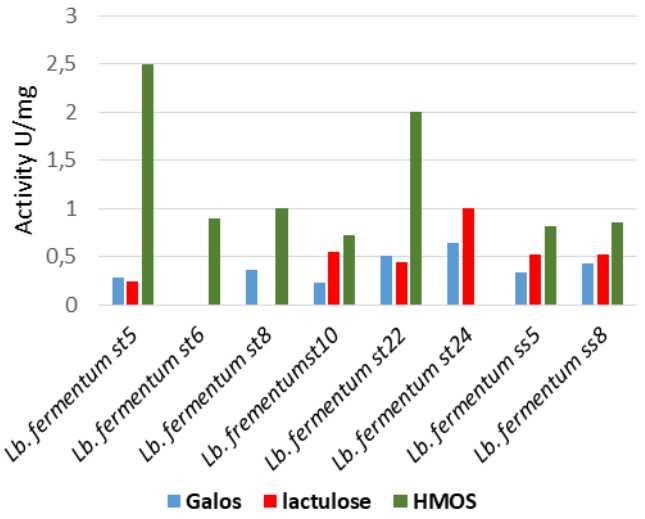

Figure 2. *β - galactosidase activity on 24 h after cultivation on lactulose, galactooligosaccharides and Human milk oligosaccharides (HMOS).*

We have studied also the enzymatic activities in selected strains using the so-called resting cells from them to study induction of the enzyme secretion. The cells from studied strains were separated after centrifugation and removed in 2% solutions of lactulose, galactooligosaccharides and HMOs. The hydrolysis of the using carbohydrates by the activities of β-galactosidases were monitored. The results are presented in Figures 3, 4 and 5.

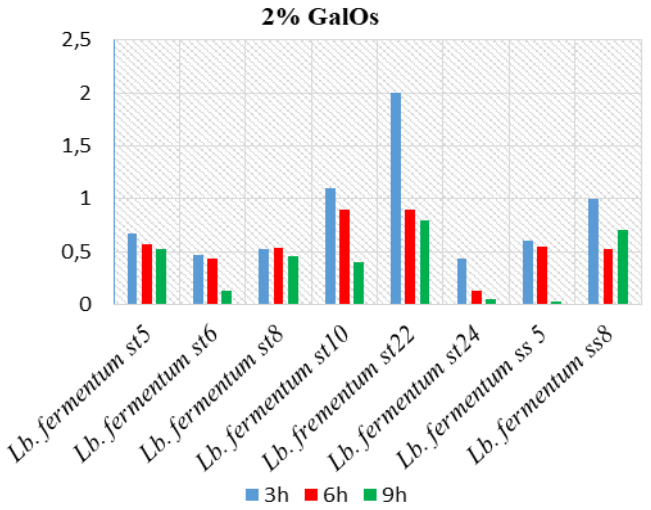

Figure 3. *β-Galactosidase activity (U/mg) from resting cells cultivated on 2% GalOs).*

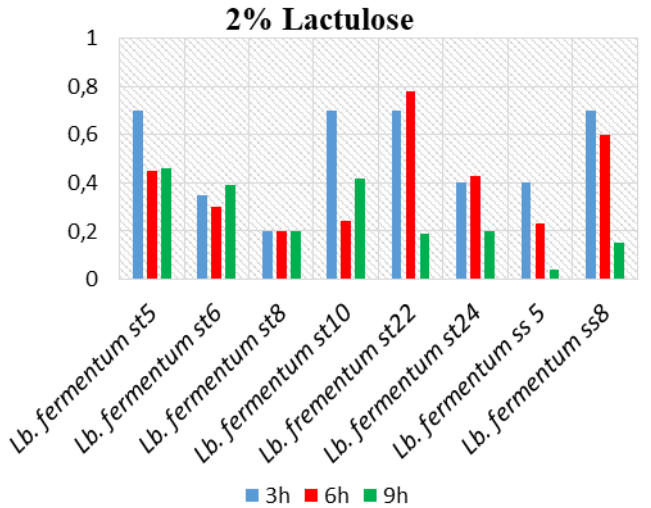

Figure 4. *β-Galactosidase activity (U/mg) from resting cells cultivated on 2% lactulose.*

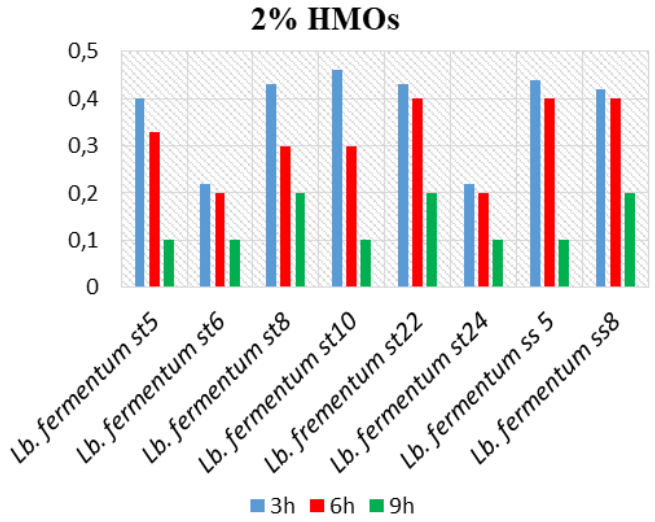

Figure 5. *β-Galactosidase activity (U/mg) from resting cells cultivated on 2% HMOs.*

However, in the presence of lactulose, β-galactosidase activity vary strongly in the studied strains. Highest β-galactosidase activity was recorded in *L. fermentum* St22 strain in the presence of 2% lactulose at the 3 -hour post-start time, 2 U/mg.

In the presence of 2% galactooligosaccharides, the trend for the highest β-galactosidase activity at third hours from the beginning of the reaction and subsequent reduction to the 9th hour of cultivation was maintained in all strains tested. Comparison with activity in 2% lactulose, it is between 2 and 3 times lower. In the presence of 2% HMOs, the highest β-galactosidase activity was reported at 3 hours from the onset of hydrolysis (0.4 - 0.46 U/mg of protein) and a decrease in activity over time. However, it is 4-5 times lower than the measured lactulose activity.

β-galactosidase activity in the presens of fucose, mucine and fucolyllactose (2-FL)

In comparison we tested the β-galactosidase activity in the presence of 2% fucose, 2% mucine and 2% fucosyllactose. Overall, the highest β-galactosidase levels were reported in the tested strains after incubation in of 2 % mucin (*Lactobacillus fermentum* ss5 – 2.8 U/mg) and 2% oligosaccharides (*Lactobacullis fermentum* st 24 – 1.7 U/mg) isolated from breast milk. (Table 3) The enzyme activity is approximately 5 times higher compared with the enzyme activity to some strains when they are grown in presens of 2% fucose and 2% fucosyllactose.

Table 3. *β-galactosidase activity (U/mg) on 24h after beginning of cultivation on fucose, mucin and 2-FL.*

Strain	media containing			
	Fucose 2%	Mucin 2%	2-FL 2%	Lactose 2%
L. fermentum st5	0.1	1.6	0.14	2.9
L. fermentum st6	0.23	2.5	0.13	2.4
L. fermentum st8	0.5	1.5	0.43	3.6
L. fermentum st10	0.11	3	0.11	2
L. fermentum st22	0.3	2	1.6	2.5
L.fermentum st24	0.5	1.7	1.7	2.6
L. fermentum ss5	0.6	2.8	0.4	2.4
L. fermentum ss8	0.3	1.3	0.3	2.3

In situ analysis for detection of activity of partially purified β-galactosidase from Lactobacillus fermentum st5 and Lactobacillus fermentum st22.

As judged by SDS-PAGE (Figure 6A), the molecular mass of β-galactosidases are defined as approximately 105 kDa. Active staining of the partially purified β-galactosidases from *Lactobacillus fermentum* st5 and *Lactobacillus fermentum* st22 strains using 4-methylumbelliferyl α-D-galactoside as the substrate showed that the band corresponding to band of the reference β-galactosidase (Figure 6B).

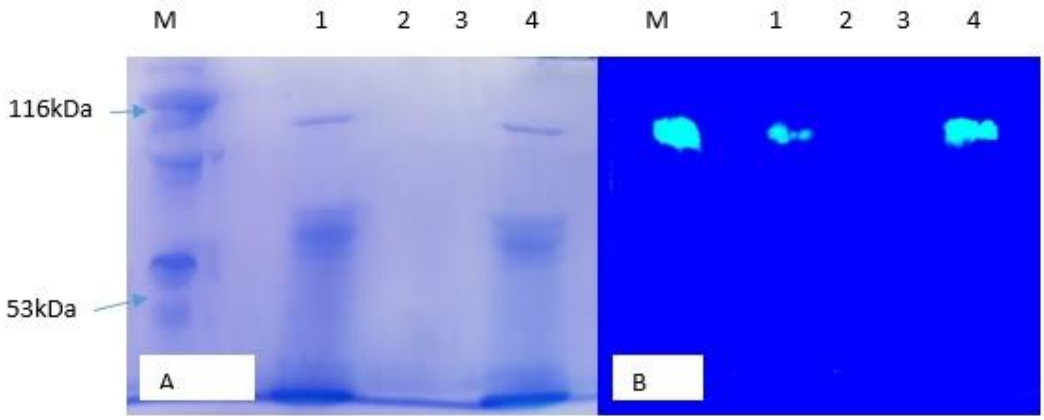

Figure 6. *SDS-PAGE and native-PAGE analysis for detection of activity of partially purified β-galactosidase from Lactobacillus fermentum st5 and Lactobacillus fermentum st22. A – SDS PAGE, B- native PAGE, β -galactosidase activity was detected using MUG as the substrate. M, range marker; 1, concentrated fraction from Lactobacillus fermentum st5; 4, concentrated fraction from Lactobacillus fermentum st22.*

Conclusion

The most of the isolated Lactic acid bacteria from breast milk and infant saliva were identified as *Lactobacillus fermentum*. On the other sand, infant microflora of the intestine is formed as a result of a tripartite connection between maternal milk, newborn and bacteria. (Toscano et al., 2007). Our results show that the importance of *L. fermentum* as a one of the first lactic acid bacteria that metabolize lactose and HMOs from breast milk.

Genus *Lactobacillus* is an important part of the gastrointestinal microbe in healthy babies (Toscano et al., 2007). Consumption of breast milk promotes the proliferation of lactobacilli in the gastrointestinal tract as it provides nutrients such as prebiotic HMOs. HMOs are good inducers of β-galactosidase secretion and the enzyme itself is one of the major contributors to the metabolism of these sugars. The studied enzyme is important to factor for metabolization of other prebiotic oligosaccharides as galactooligosaccharides. For the first time, we demonstrated that L-fucose, mucine and fucosyllactose can induce β-galactosidase secretion in *L. fermentum* strains isolated from breast milk.

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