Original Research Article   https://doi.org/10.20546/ijcmas.2017.608.176

Process Optimization for the Production of β-Galactosidase Using Potential *Lactobacillus* Cultures

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A B S T R A C T

Ten *Lactobacillus* cultures were considered for the study. Out of ten *Lactobacillus* cultures, V3 produced maximum β-galactosidase activity (3.381 O.D), followed by NK2 and NK9 up to 24h than other cultures during β-galactosidase activity. Extraction of β-galactosidase was carried out by disruption of *Lactobacillus* cells using sonication at 3, 4 and 5 minute (pulse 30 second on / 10 seconds off and 35 to 55 % amplitudes), lysozyme-EDTA (49 mg of lysozyme in 1.5 ml of TE buffer) treatment, toluene acetone (9:1 [toluene: acetone]) treatment, sodium dodecyl sulphate (SDS) - chloroform (250 µl chloroform and 100 ml of 0.1 % SDS) treatment. Out of 4 different treatments [sonication, lysozyme-EDTA, toluene-acetone and sodium dodecyl sulphate (SDS)-chloroform treatment] for β-galactosidase production, sonication treatment [V3 (1.093), NK2 (1.084) and NK9 (1.091)] was found to be best for production of β-galactosidase with optimized sonication period [5 min (pulse 15 sec off / 30 sec on at 55% amplitudes)]. Purification of β-galactosidase enzyme was carried out employing various t-butanol ratios (crude extract : t-butanol = 1:0.25, 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) with a different ammonium sulfate saturation i.e. 30, 60 and 90%.After purification by treating with ratio of crude enzyme: t-butanol (R) and ammonium sulphate saturation (AS), 2 % of R (0.396 O.D) and 60 % of AS (0.292 O.D) showed maximum β-galactosidase activity for three selected *Lactobacillus* cultures i.e. V3, NK2 and NK9. The molecular mass of partially purified β-galactosidase (60% Ammonium sulphate saturation) from selected *Lactobacillus* cultures i.e. V3, NK2 and NK9 as determined by SDS-PAGE was approx. 69 kDa. Therefore, the study entailed to conclude that production and purification of β-galactosidase enzyme from safe *Lactobacillus* cultures is an alternative source of biological enzyme for the preparation of lactose hydrolysed milk for lactose intolerant population.

Introduction

Lactose intolerance is due to lack of the enzyme lactase in the small intestines to break down the lactose into glucose and galactose (Deng *et al.,* 2015). There are four types: primary, secondary, developmental and congenital. Primary lactose intolerance is when the amount of lactase declines as people age. Secondary lactose intolerance is due to injury to the small intestine such as from infection, inflammatory bowel disease, celiac disease, or other diseases (Berni *et al.,* 2016). Developmental lactose intolerance may occur in the premature babies and usually improves over a short period of time. Congenital lactose intolerance is an extremely

rare genetic disorder in which no lactase is made from birth (NIDDK, 2016). In the family of β-galactosidase, lactase is one part of them which is a glycoside hydrolase. It is involved in the hydrolysis process of the disaccharide lactose into galactose and glucose monomers. Lactase is present in predominantly along with the brush border membrane of the differentiated enterocytes lining the villi of the small intestine (Skovbjerg *et al.,* 1981).

Jelen *et al.,* (1993) suggested that specific microbial strains that are high β-galactosidase producers could be used for lactose hydrolysis after cell disruption but with minimum additional purification. More recently, proposed a process for lactose hydrolysis in dairy systems using a crude enzymatic extract (CEE) from the Lactobacilli culture, with subsequent utilization of the fermentation medium used for the culture propagation (Vasiljevic and Jelen, 2001). Amongst lactic acid bacteria, yogurt bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) are the highest β- galactosidase producers. The β-galactosidase of these cultures has been characterized, showing high stability and activity at high temperatures. However, the β-galactosidase from thermophilic LAB is an intracellular enzyme. It is release from microorganisms, which is obtained either by mechanical disruption or by the chemical permeabilization of the cell membrane. The effectiveness of the various disruption methods differs for different microbial genera and strains. Generally, β-galactosidase activity in the medium can be greatly increased by rupturing cells using different cell disruption methods. The literature tends to focus on the disruption of yeasts with much less information being available on the disruption of lactobacilli. (Bury *et al.,* 2001). With this perspective, traditional yogurt samples obtained from Toros mountain region of Turkey with highly bio-diverse

environment were used as the source for the isolation of LAB cultures. Almost 136 isolated strains were screened for β-galactosidase activity based on their lactose consumption and lactic acid producing characteristics according to the method described by Bury *et al.,* (2001). Among these isolates only three *Lactobacillus delbrueckii subsp. bulgaricus* strains and three *Streptococcus thermophilus* strains showed a high potential. The activity and stability of β-galactosidase is strongly influenced by the enzyme origin and environmental conditions of the enzyme production and the hydrolysis process, such as temperature and pH optima, as well as the presence of activators or inhibitors. The use of CEEs instead of purified β-galactosidase preparation may complicate the final outcome of the hydrolysis due to the presence of other microbial enzymes, which may interfere with the lactose hydrolysis.

Prasad *et al.,* (2013) evaluated that the production of β-galactosidase by *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and the effect of 4 different extraction methods i.e. sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatment on enzyme activity from these organisms. Among the four methods used for β-galactosidase extraction, sonication exhibited the best result (6.80 Unit/mL) for *B. animalis* ssp. *lactis* Bb12 while lysozyme-EDTA treatment was also found to be the best (7.77 Unit/ mL) for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Duman and Kaya, (2013) evaluated the activity of β-galactosidase was completely decreased at 60 % ammonium sulfate saturation in aqueous phase, the best results of recovery and the purification fold of β-galactosidase (133 % and 10.1, respectively) was obtained in the interfacial phase. Higher salt concentrations from this point were

resulted with reducing of recovery and purification fold, which may be due to irreversible denaturation of protein (Narayan *et al.,* 2008).

Approximately 75 % of Earth's population is lactose intolerant and in India (particularly southern India) 70 % of the population are lactose intolerance and they feel uncomfortable whenever they consume milk and milk products. To meet this challenge, lactose hydrolysed milk is an alternative solution for proposed study. However the source of enzyme (β-galactosidase) from biological material like LAB is an added advantage from safety point of view as LAB are having GRAS status.

Materials and Methods

Ten LAB cultures used in the present study i.e. *L. helveticus* MTCC 5463 (V3), *L. rhamnosus* (NK2), *L. casei* (NK9), *L. rhamnosus* MTCC 25062 (NK10), *L. fermentum* (M5), *L. paracasei* (M16), *L. rhamnosus* (M31), *L. plantarum* (M38), *L. plantarum* (M10), *L. fermentum* TDS030603 (LBF) were obtained from the Culture Collection of Dairy Microbiology Department, SMC College of Dairy Science, Anand Agricultural University, Anand. The LAB cultures were propagated in sterilized reconstituted skim milk (10% TS) and stored at 5 ± 2°C.

The transfer was given every week during the course of the study. Most the bacteriological media, molecular biology grade chemicals and reagents were purchased either from Hi-Media (India), Sigma (USA), SDFCL (India), Chr. Hansen (Denmark) etc. During the entire study, glass wares of Borosil brand (Borosil Glass Works Ltd., Mumbai, India) and analytical grade and molecular biology grade chemicals were used. Glassware and other materials were sterilized by usual procedures

viz. 160-180°C for 2h in hot air oven, whenever required.

Evaluation of pH, acidity and *Lactobacillus* counts of fermented skim milk

All the cultures were activated by growing in sterilized skim milk. The activated cultures were added to 100 ml of skim milk flasks at the rate of 2%. After mixing them thoroughly, the cultures were incubated at 37°C for different intervals of 0, 6, 12, 18 and 24h. Samples were taken out for determination of pH, titratable acidity and viable counts after each interval. pH of fermented skim milk samples was measured using digital pH meter (OAKTON pH700, India).

The titratable acidity was estimated by the procedure described by (IS, 1960). *Lactobacilli* counts of fermented skim milk samples were determined as per the method described by IDF standards (146: 2003).Typical colonies were calculated and the counts were expressed as log cfu/ml.

Determination of β-galactosidase activity

All the cultures were activated by growing in MRS broth. The activated cultures were added to 100 ml of MRS broth at the rate of 2%. After mixing them thoroughly, the culture flasks were incubated at 37°C for different intervals of 0, 24, 48 and 72h. Samples were taken out for β-galactosidase activity at each interval.

Enzyme extraction

After 0, 24, 48 and 72h of incubation, the cells were harvested by centrifuging at 5000 rpm for 15 min at 4°C. The supernatant was considered to be containing extracellular enzymes. The cell pellet was crushed and washed twice with a 0.05 M sodium phosphate buffer (pH 6.8) and centrifuged at

5000 rpm for 15 min at 4°C. The washed pellets were resuspended in 10 mL of 0.05 M phosphate buffer (pH 6.8) for intracellular enzyme extraction using cell disintegration methods listed below (Prasad *et al.,* 2013):

Sonication treatment

The cell suspensions were sonicated for 30 min (pulse 30 seconds off / 30 seconds on and 61% amplitudes) in ice bath using sonicator (LABMAN, India), according to the method described by Prasad *et al.,* (2013).

The extract was then centrifuged at 5000 rpm at 4°C for 15 min and the supernatant containing the crude enzyme extract was stored at −20°C until used for enzyme assays.

Enzyme assay

The β-galactosidase was determined by the reaction mixture was composed of 0.5 mL of supernatant containing extracted enzyme and 2.0 mL of 15 mM O-nitrophenyl β-D-galactopyranoside (ONPG) in 0.05 M sodium phosphate buffer (pH 6.8). After incubation for 20 min at 37°C, 0.5 mL of 0.1 M sodium carbonate was added to the mixture to stop the reaction.

Absorbance was measured at 420 nm with a spectrophotometer (Systronics PC based double beam spectrophotometer 2202, India).

Production, extraction and purification of β-galactosidase from selected LAB

All the cultures were activated by growing in MRS broth. The activated cultures were added to 100 ml of MRS broth flasks at the rate of 2%. After mixing them thoroughly, the culture flasks were incubated at 37°C for 24h. Samples were taken out for β-galactosidase activity.

Enzyme extraction

After 24h of incubation, the cells were harvested by centrifuging at 5000 rpm for 15 min at 4°C. The supernatant was considered to be containing extracellular enzymes. The cell pellet was crushed and washed twice with a 0.05 M sodium phosphate buffer (pH 6.8) and centrifuged at 5000 rpm for 15 min at 4°C. The washed pellets were resuspended in 10 mL of 0.05 M phosphate buffer (pH 6.8) for intracellular enzyme extraction using four different cell disintegration methods listed below (Prasad *et al.,* 2013):

Sonication treatment

The cell suspensions were sonicated for 3, 4 and 5 minute intervals (pulse 15 seconds off / 30 seconds on as well as 35 and 55% amplitudes) in ice bath using sonicator (LABMAN, India), according to the method mentioned by Prasad *et al.,* (2013). The extract was then centrifuged at 5000 rpm and 4°C for 15 min and the supernatant containing the crude enzyme was stored at −20°C until used for enzyme assays.

Lysozyme EDTA treatment

Lysozyme solution was prepared by dissolving 49 mg of lysozyme (Hi-Media, India) in 1.5 mL of TE (Tris-EDTA; Ethylenediamine Tetraacetic Acid) buffer containing 1 mM EDTA and 10 mM Tris-HCl, adjusted to pH 8.0. The lysozyme preparation was added to the cell suspension at the rate of 150 μL per mL, incubated for 45 min at room temperature. Then supernatant obtained was kept at -20°C until enzyme activity measurement (Prasad *et al.,* 2013).

Toluene acetone treatment

10 ml cell suspension was ground for 10 min in a pestle and mortar with 2.0 g alumina

(SDFCL, India) and 0.2 mL of 9:1 mixture of toluene (LOBA Chemie, India with 99.5% purity) and acetone (LOBA Chemie, India with 99.8% purity) solvents. The suspension was extended in 8 mL phosphate buffer and centrifuged at 5000 rpm for 15 min at 4°C. The supernatant obtained was kept at -20°C until used for enzyme assay (Prasad *et al.,* 2013).

Sodium dodecyl sulphate (SDS) – Chloroform treatment

Permeabilization of cell membrane was carried out by vortexing 10 mL of the cell suspension in the presence of 250 μL chloroform and 100 μL 0.1% SDS solution for 30 min at room temperature. The suspension was centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was kept at -20°C until needed for the enzyme assay (Prasad *et al.,* 2013).

Quantitative analysis of β-galactosidase activity produced by selected *Lactobacillus* cultures through X-Gal assay

Sonication treatment

The cell suspensions were sonicated for 5 min (pulse 15 seconds off / 30 seconds on and 55% amplitudes) in ice bath using sonicator (LABMAN, India), according to the method described by Prasad *et al.,* (2013).

The extract was then centrifuged at 5000 rpm at 4°C for 15 min and the supernatant containing the crude enzyme extract was stored at −20°C until used for further study.

X-gal-IPTG treatment

2 ml of crude β-galactosidase enzyme was added in the mixture of 0.5 ml of X-gal (chromogenic substrate) and 0.5 ml of 0.1 [M] isopropyl β-D-1-thiogalactopyrinoside (IPTG)

and incubated for 3h at 37°C in water bath. After incubation, the β-galactosidase activity was measured using absorbance at 550 nm with a spectrophotometer (Systronics PC based double beam spectrophotometer 2202, India) depending on the intensity of the developed blue colour.

Purification of β-galactosidase enzyme

Purification of β-galactosidase enzyme was carried out employing various t-butanol ratios (crude extract: t-butanol = 1:0.25, 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) with a different ammonium sulfate saturation at 30, 60 and 90%. The mixture was mixed gently and then allowed to stand for 30 min at 37°C. afterward, the mixture was centrifuged at 5000 rpm for 15 min at 4°C to facilitate the separation of phases. The upper t-butanol phase was removed by the Pasteur pipette. The lower aqueous phase and the interfacial phase were separated carefully and each of phases were analyzed for enzyme activity. Different ammonium sulfate saturation effects (30, 60 and 90%) (w/v) were investigated at the best recovery activity of crude enzyme:t-butanol ratio that is 1.0:1.0. The bottom phase and interfacial phase were analyzed for enzyme activity (Duman and Kaya, 2013).

Purification of β-galactosidase enzyme through SDS-PAGE

The SDS-PAGE was performed following method of Laemmli (1970); Carrasco-Castilla *et al.,* (2012) with some modifications to identify the molecular weight of partially purified β-galactosidase enzyme.

Results and Discussion

Growth behavior of *Lactobacillus* cultures

During the evaluation of β-galactosidase activity, the *Lactobacillus* cultures were

inoculated at the rate of 2 % in sterilized reconstituted skim milk. Then pH, titratable acidity (% Lactic acid) and *Lactobacillus* counts were estimated at different time intervals (0, 6, 12, 18 and 24h) at 37°C. The titratable acidity (% Lactic acid), pH and *Lactobacillus* counts of individual *Lactobacillus* culture were evaluated and depicted in figures 1, 2 and 3 respectively. Titratable acidity was determined by calculating the amount of titratable acidity developed up to 24h of incubation. From the figure 1, it was observed that the titratable acidity of V3 was significantly (P<0.05) increased up to 24h. The titratable acidity was significantly higher at 24 h (1.109). Overall titratable acidity was ranged from 0.141 % LA (0h) to 1.109 % LA after 24h incubation at 37°C. During the growth in sterilized reconstituted skim milk, V3 produced highest titratable acidity (0.704 % LA), followed by M5 (0.691%) and NK9 (0.630%) after 24h at 37°C. From figure 2, it was also observed that the pH of V3 was significantly (P<0.05) decreased up to 24h of incubation period. The pH was significantly lowered at 24h (4.04). Overall pH was ranged from 6.56 (0h) to 4.04 after 24h at 37°C. During the growth in sterilized reconstituted skim milk, V3 produced highest reduction in pH (4.89), followed by M5 (4.97) and NK9 (5.00) after 24h at 37°C. From figure 3, it was observed that the *Lactobacillus* counts (log cfu/ml) of V3 was significantly (P<0.05) increased up to 24 h of incubation period. *Lactobacillus* counts (log cfu/ml) were significantly maximum at 24h (9.701). Overall *Lactobacillus* counts (log cfu/ml) were ranged from 6.486 log cfu/ml (0h) to 9.701 log cfu/ml after 24h incubation at 37°C. During the growth in sterilized reconstituted skim milk, V3 showed maximum *Lactobacillus* counts (8.892 log cfu/ml), followed by NK10 (8.481 log cfu/ml) and LBF (8.425 log cfu/ml) after 24h at 37 °C.

Hati *et al.,* (2015) studied the growth performance of *Lctobcillus rhamnosus* (NS4 and NS6), *Lactobacillus helveticus* MTCC 5463 (V3), *Lactobacillus delbruckii* (09), *Enterococcus feacalis* (ND3), *Enterococcus feacalis* (ND11) and *Lactobacillus rhamnosus* (SH8) by determining viable counts (log cfu/ml) and production of *Lactobacillus* acid measured by decline in pH in skim milk inoculated at the rate of 1 % and incubated at 37 °C for 12 h. It was observed that NS4 lowered down the pH at a maximum level compared to V3, ND3 and SH8. However, it was also observed that NS4 produced maximum acidity compared to V3, ND3, SH8 and I4.

Viable counts of all the cultures were measured after 12 h of incubation at 37 °C. From the study; it was found that NS4 gives highest viable cell counts 10.68 log cfu/ml than other bacterial isolates at this qrelatively exhibited lesser bacterial counts compared to other isolates in MRS agar medium. It was also concluded that, viable cell counts, pH and acidity varies due to the use of different strains (Hati *et al.,* 2015) as similar to our study. In another study, Hati *et al.,* (2017) evaluated the influence of whey protein concentrate on the production of antibacterial peptides derived from fermented milk by Lactic Acid Bacteria. They evaluated skim milk supplemented with WPC 70 (@ 1.0, 1.5, 2%) was inoculated with 2% lactic cultures i.e., *S. thermophilus* (MD2), *L. helveticus* (V3), *L. rhamnosus* (NS4) and *L. bulgaricus* (09). pH, titratable acidity, viable cell counts and proteolytic activity were evaluated after 3, 6, 9, 12 and 24 h of fermentation at 37°C. It has been previously reported that addition of WPC 70 to milk followed by heat treatment induced a decrease of fermentation time (Antunes *et al.,* 2005; Milanovic *et al.,* 2009). It has been also reported that addition of 1 or 2% WPC to a medium used for fermentation with *L. delbrueckii* ssp. *bulgaricus* or *S.*

thermophilus has produced significantly higher bacterial counts and much faster acidity (Burya *et al.,* 1998).

Determination of β-galactosidase activity

Out of ten *Lactobacillus* cultures three cultures were selected on the basis of β-galactosidase activity. β-galactosidase activity of *Lactobacillus* cultures were carried out in MRS medium using sonicator (LABMAN, India) for 30 min (30 seconds off / 30 seconds on and 61 % amplitudes) in ice bath, according to the method of Prasad *et al.,* (2013). Individually each *Lactobacillus* culture was statistically analyzed for the production of β-galactosidase enzyme at optimum incubation period. β-galactosidase enzyme production of ten *Lactobacillus* cultures in MRS medium was depicted in the figure 4.

From the figure 4, it was observed that the β-galactosidase activity of V3 was significantly (P<0.05) increased up to 24h of incubation. The β-galactosidase activity was significantly higher at 24h (1.444 O.D.) for all the *Lactobacillus* cultures compared to 48h (0.467 O.D.) and 72h (0.193 O.D.). V3 showed highest (1.023 O.D.) β-galactosidase activity at 24h. Overall β-galactosidase activity was ranged from 0.113 O.D. (LBF at 0h) to 3.381 O.D. (V3 up to 24h). V3 produced the maximum β-galactosidase activity at 24h (3.381 O.D.), followed by NK2 (2.502 O.D) and NK9 (2.417 O.D). Overall β-galactosidase activity was ranged from M16 (0.149 O.D) to V3 (1.023 O.D).

Vikas *et al.,* (2015) studied the efficiency of different methods for disruption of *Streptococcus thermophilus* cells, isolated from different dairy products, to release β-galactosidase and synthesis of Galacto oligosaccharides (GOS) by extracted enzyme using whey supplemented with different concentrations of lactose as a substrate was studied. Unlike most other studies on GOS synthesis which used only one method of cell disruption sonication (ultrasonics) and only few microbial strains, they compared five different cell disruption methods and used 30 strains of *S. thermophilus* in order to find out the most effective method is sonication (ultrasonics) and efficient strain for production of β-galactosidase. This report had a similar method from our study. In another study, Prasad *et al.,* (2013) studied the activity of β-galactosidase from *B. animalis* Bb12 and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and its extraction using various methods. *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 produced more (p<0.05) intracellular β-galactosidase than *B. animalis* ssp. *lactis* Bb12 with all extraction methods, except sonication. There were significant (p<0.05) differences in β-galactosidase levels extracted from each organism by the four extraction methods. Sonication method was found to be more effective for *B. animalis* Bb12 than the others methods. This report had a similar observation from our study. In another study, Kara (2004) evaluated the release and characterization of β-galactosidase from *Lactobacillus plantarum.* She optimized the lysozyme method, sonication method, and liquid nitrogen method for protein release from the cells of *Lactobacillus plantarum.* Sonication was found to be the most effective method. This report had a similar method from our study.

The aim of this study was to screen different *Lactobacillus* cultures on the basis of β-galactosidase activity and to optimize the growth conditions for the production of β-galactosidase enzyme of *Lactobacillus* cultures. Two important fermentation variables i.e., ten *Lactobacillus* cultures (*L. helveticus* MTCC 5463 (V3), *L. rhamnosus* (NK2), *L. casei* (NK9), *L. rhamnosus* MTCC

25062 (NK10), *L. fermentum* (M5), *L. paracasei* (M16), *L. rhamnosus* (M31), *L. plantarum* (M38), *L. plantarum* (M10), *L. fermentum* TDS030603 (LBF)) and incubation periods (0, 24, 48 and 72h) were studied. As per statistical analysis, V3, NK2 and NK9 were found to be strong β-galactosidase producer at 24h of incubation. As, the degree of β-galactosidase activity was found to depend on different strains of *Lactobacillus* cultures and incubation period i.e. 24 hours, V3, NK2 and NK9 *Lactobacillus* cultures were selected for the production of β-galactosidase enzyme. β-galactosidase activity being important factor for the β-galactosidase production, the variables studied were very relevant due to their significance in improving the β-galactosidase enzyme production from both the microorganisms and optimum growth temperature (24h).

Production, extraction and purification of β-galactosidase from selected LAB

Selected *Lactobacillus* cultures i.e. *L.helveticus* MTCC 5463 (V3), *L.rhamnosus* (NK2), *L.casei* (NK9) were considered to production, extraction and purification of β-galactosidase enzyme. This *Lactobacillus* cultures were activated by growing in 100 ml of MRS broth flasks at the rate of 2 % at 37°C for 24h. After 24h of incubation, the cells were harvested by centrifuging at 5000rpm for 15 min at 4°C.

The supernatant was considered to be containing extracellular enzymes. The cell pellet was crushed and washed twice with a 0.05 M sodium phosphate buffer (pH 6.8) and centrifuged at 5000 rpm for 15 min at 4°C. The washed pellets were resuspended in 10 mL of 0.05 M phosphate buffer (pH 6.8) for intracellular enzyme extraction using four different cell disintegration methods listed below:

Sonication treatment

The cell suspensions were sonicated for 3, 4 and 5 minutes intervals (pulse 15 seconds off/30 seconds on as well as 35 and 55% amplitudes) in ice bath using probe sonicator (LABMAN, India), according to the modified method of Prasad *et al.,* (2013). The extract was then centrifuged at 5000 rpm at 4°C for 15 min and the supernatant containing the crude enzyme was stored at –20°C for further used. *Lactobacillus* cultures i.e. V3, NK2 and NK9 were statistically analysed for the different time intervals of sonication along with different % of amplitudes of sonication for highest production of β-galactosidase enzyme. β-galactosidase production of *Lactobacillus* cultures i.e. V3, NK2 and NK9 were represented in figure 5.

It is evident from the figure 5 that the β-galactosidase production of V3 was significantly (P<0.05) increased with the % amplitude (from 35 to 55 %) and residence time interval (from 3 to 5 min) during the sonication. β-galactosidase activity was significantly maximum at 55 % amplitude for all the time intervals (3, 4 and 5 min) than 35 % amplitude.

Significantly maximum β-galactosidase activity was observed at 55 % amplitude and 5 min (1.442 O.D) followed by 4 min (1.076 O.D) and 3 min (0.943 O.D) compared to 35 % amplitude and 5 min (1.255 O.D) followed by 4 min (1.017 O.D) and 3 min (0.804 O.D). From figure 5, it was observed that the β-galactosidase activity of V3, NK2 and NK9 cultures were significantly (P<0.05) increased with the different amplitude (35 and 55 %) and time intervals (3, 4 and 5 min) between pulses in sonication treatment. Significantly maximum β-galactosidase activity was observed in V3 culture (1.093 O.D) followed by NK9 (1.091 O.D) and NK2 (1.084 O.D).

Lysozyme EDTA treatment

Lysozyme solution was prepared by dissolving 49 mg of lysozyme (Hi-Media, India) in 1.5 mL of TE (Tris-EDTA; Ethylenediamine Tetraacetic Acid) buffer containing 1.0 mM EDTA and 10 mM Tris-HCl, adjusted to pH 8.0. 150 µL lysozyme preparation was added to the cell suspension and incubated for 45 min at room temperature. Then supernatant obtained was kept at -20°C for further study (Prasad *et al.*, 2013). From the figure 6, it was observed that the β-galactosidase activity of V3, culture was significantly (P<0.05) higher than NK9 and NK2. V3 culture was also exhibited maximum β-galactosidase activity (0.124 O.D), followed by NK9 and NK2 (0.103 O.D).

Fig.1 Evaluation of lactic acid production by *Lactobacillus* cultures in Reconstituted Skim Milk
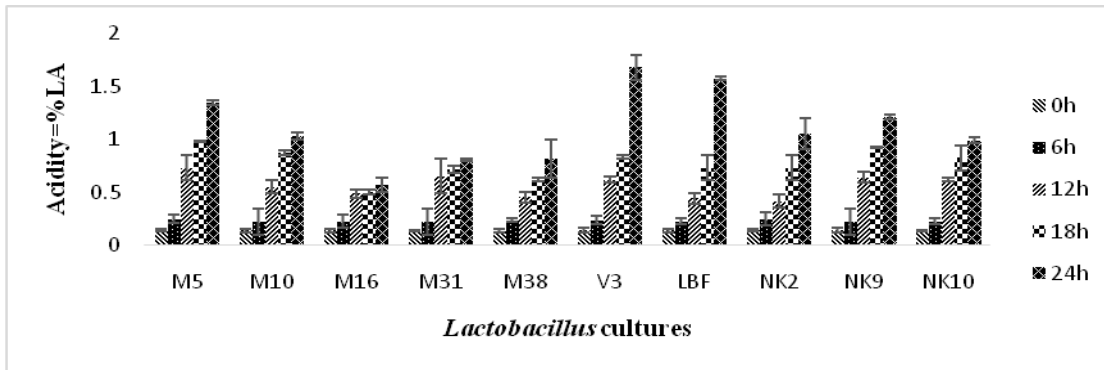
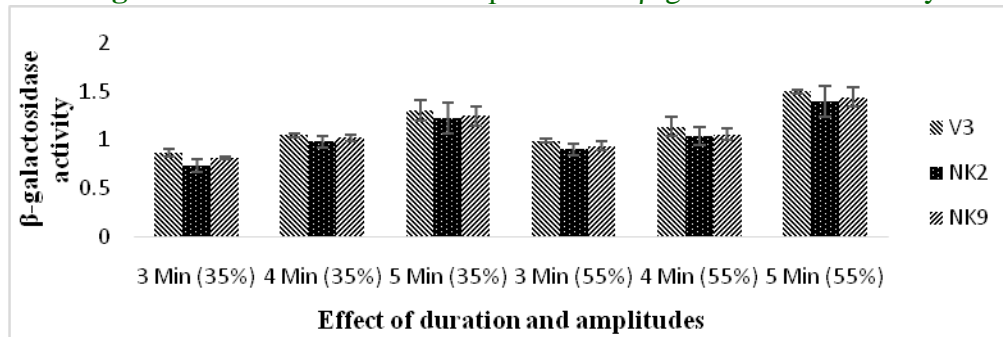**Fig.2** Changes in pH of Reconstituted Skim Milk produced by *Lactobacillus* cultures
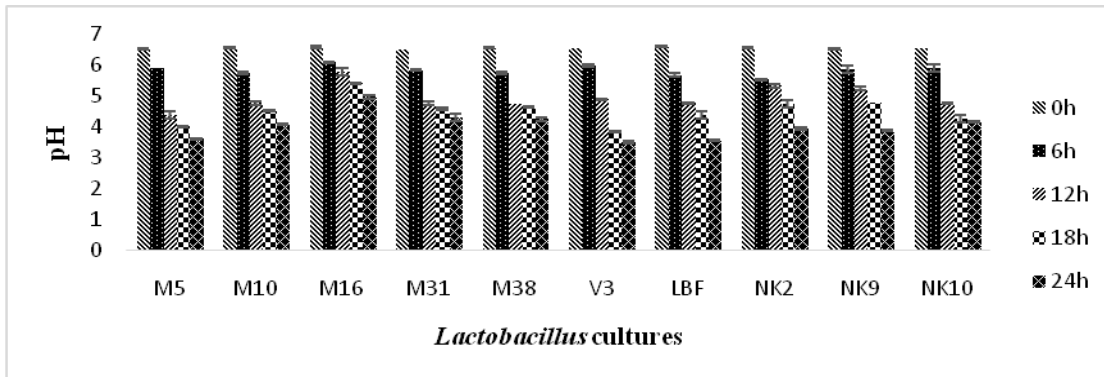
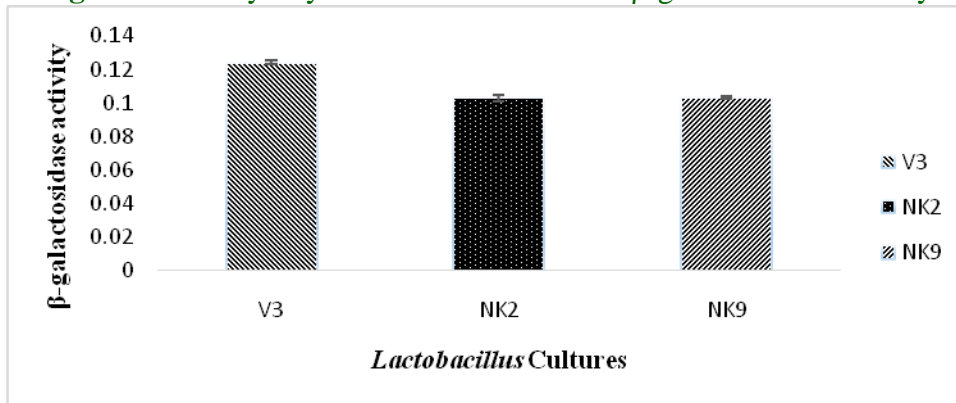**Fig.3** Evaluation of Lactic Counts of *Lactobacillus* cultures in Reconstituted Skim Milk
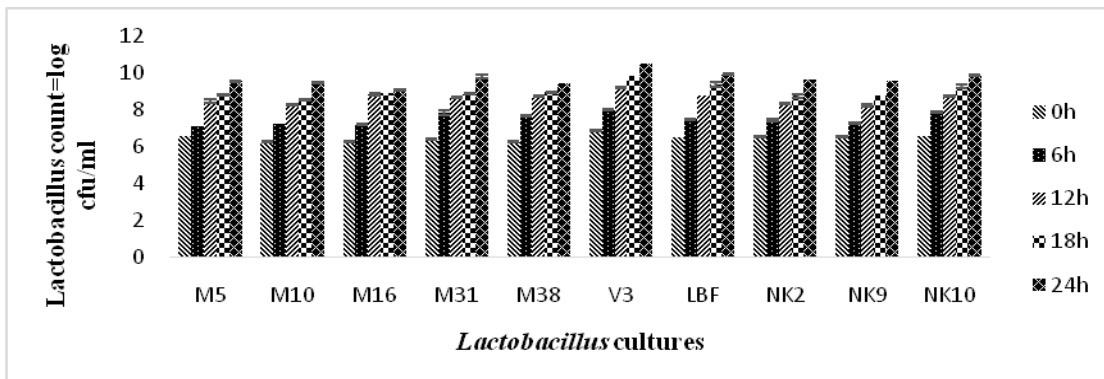
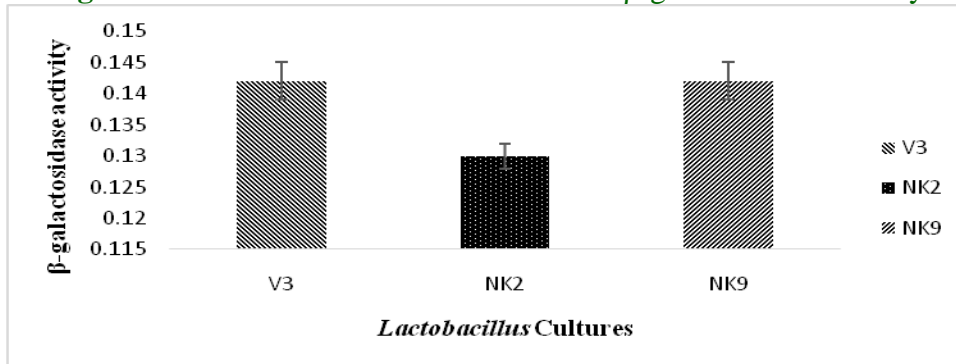**Fig.4** β-galactosidase activity of *Lactobacillus* cultures for different incubation periods
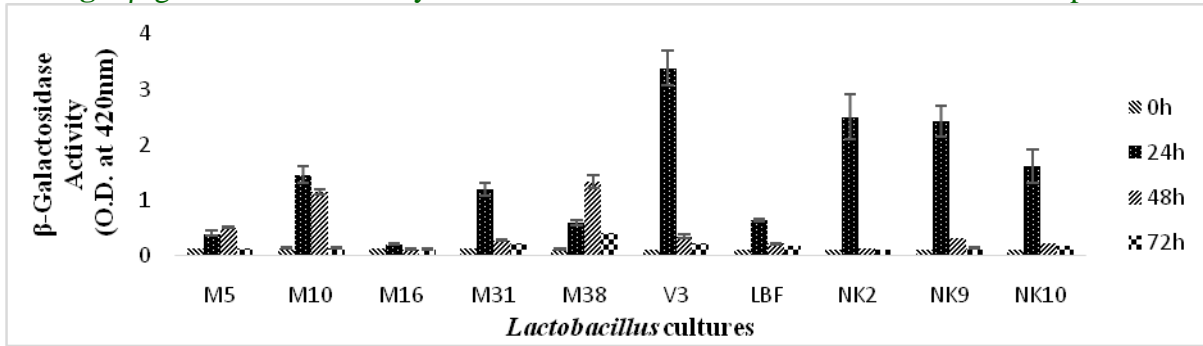
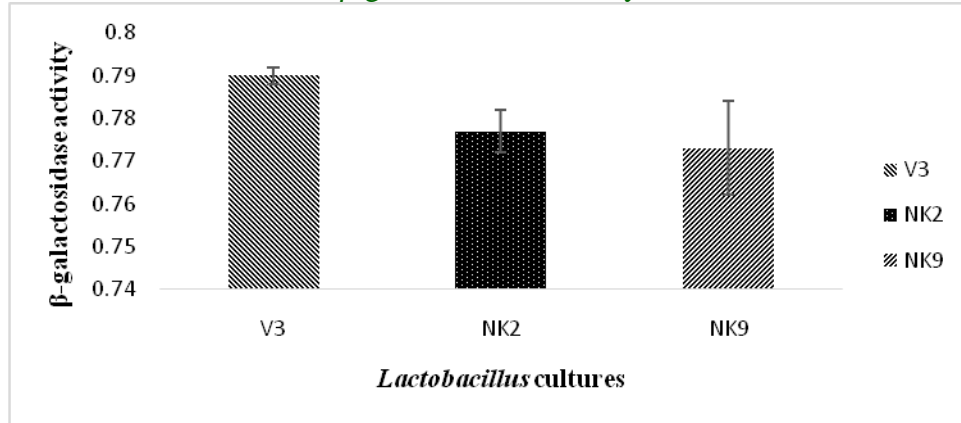**Fig.5** Effect of duration and amplitudes on β-galactosidase activity

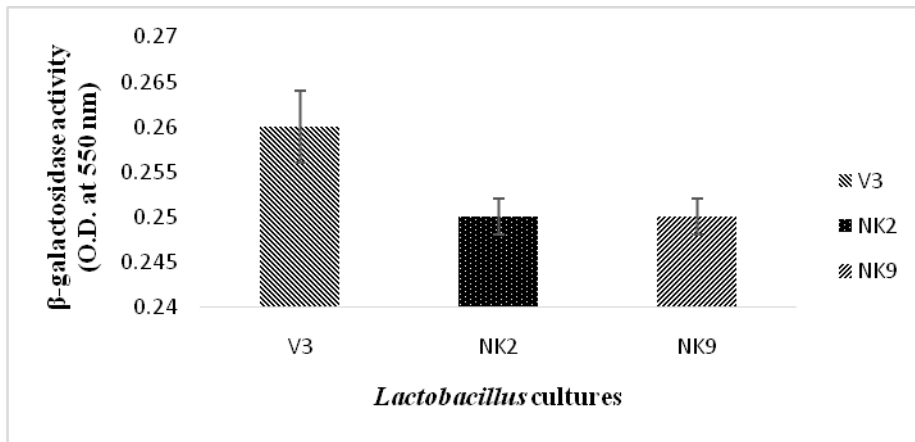**Fig.6** Effect of lysozyme EDTA treatment on β-galactosidase activity

**Fig.7** Effect of toluene acetone treatment on β-galactosidase activity

Fig.8 Effect of Sodium dodecyl sulphate (SDS) - chloroform treatment on
β-galactosidase activity
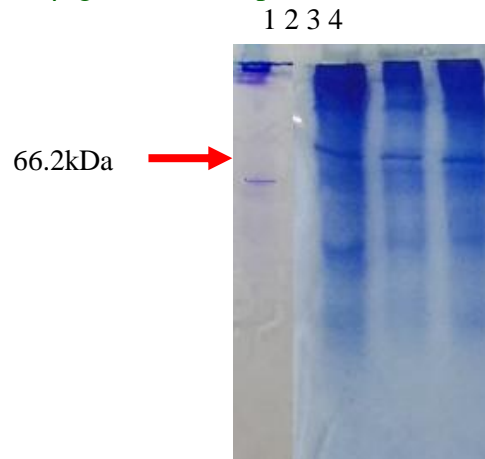

Fig.9 Evaluation of β-galactosidase activity of *Lactobacillus* cultures using X-gal

Fig.10 Effect of ammonium sulphate saturation (AS) and Ratio of crude enzyme: t-butanol (R)
on β-galactosidase activity of selected *Lactobacillus* cultures
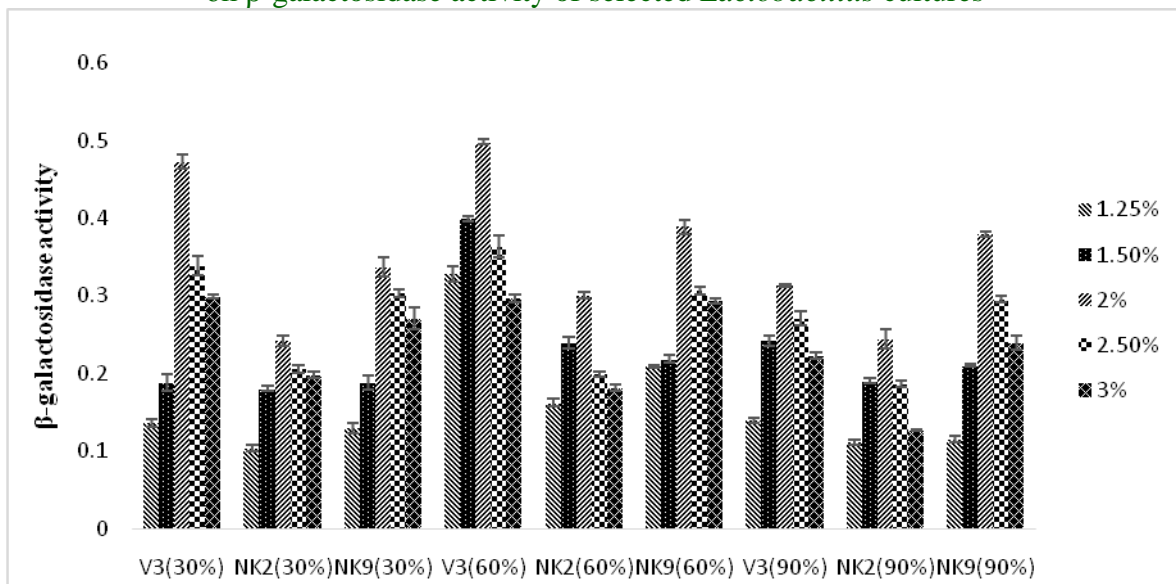
Fig.11 β-galactosidase profile of LAB revealed by SDS-PAGE

1 2 3 4

Lane 1, Bovine serum albumin (BSA- 66.2 kDa); Lane 2, V3; Lane 3, NK2; Lane 4, NK9.

Toluene acetone treatment

In Toluene acetone treatment,10 ml cell suspension was ground for 10 min in a pestle and mortar with 2.0 g alumina (SDFCL, India) and 0.2 mL of 9:1 mixture of toluene (LOBA Chemie, India with 99.5% purity) and acetone (LOBA Chemie, India with 99.8% purity) solvents. The suspension was extended in 8 mL phosphate buffer and centrifuged at 5000 rpm for 15 min at 4°C. The supernatant obtained was kept at -20°C for future enzyme assay (Prasad *et al.,* 2013).

From figure 7, it was observed that the β-galactosidase activity of V3 and NK9 cultures were significantly (P<0.05) higher than NK2. V3 and NK9 cultures were also exhibited maximum β-galactosidase activity (0.142 O.D) than NK2 (0.130 O.D).

Sodium dodecyl sulphate (SDS) – Chloroform treatment

In SDS-Cholorofom treatment, permeabilization of cell membrane was carried out by vortexing 10 mL of the cell suspension in the presence of 250 μL chloroform and 100 μL 0.1% SDS solution for 30 min at room temperature. The suspension was centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was kept at -20°C for further enzyme assay.

From the figure 8, it was observed that the β-galactosidase activity of V3 culture was significantly (P<0.05) higher than NK2 and NK9. V3 culture was also exhibited maximum β-galactosidase activity (0.790 O.D) followed by NK2 (0.777 O.D) and NK9 (0.773).

Out of 4 different treatments [Sonication, Lysozyme-EDTA, Toluene-Acetone and Sodium dodecyl sulphate (SDS) - Chloroform Treatment] for β-galactosidase production, sonication treatment {V3(1.093), NK2(1.084) and NK9(1.091)} was found to be best for production of β-galactosidase with optimized sonication period: 5 min (pulse 15 sec off / 30 sec on at 55% amplitudes) [Sonication treatment > Sodium dodecyl sulphate (SDS) - Chloroform Treatment) > Toluene-Acetone treatment > Lysozyme EDTA treatment].

Quantitative analysis of β-galactosidase activity produced by selected *Lactobacillus* cultures through X-Gal assay

β-galactosidase enzymes are lactose hydrolytic enzymes that are involved in catalytic process of lactose found in milk and make it as best industrial product for lactose intolerant population. The enzyme also regulates the transgalactosylation reactions in order to synthesize galactooligosaccharides. Considering their tremendous applications in different industries, various bacterial strains were

isolated and screened for β-galactosidase production (Kamaran *et al.,* 2016).

2 ml of crude β-galactosidase enzyme was added into the mixture of 0.5 ml of X-gal and 0.5 ml of 0.1 M IPTG and incubated for 3h at 37°C. After incubation it was measured at 550 nm with a spectrophotometer (Systronics PC based double beam spectrophotometer 2202, India) of β-galactosidase activity of V3, NK2 and NK9 and was represented in figure 9 using X-gal and IPTG. β-galactosidase activity produced by *Lactobacillus* cultures was determined in ranged from 0.25 O.D to 0.26 O.D. After 3h of incubation at 37°C. V3 showed maximum β-galactosidase activity (0.26 O.D) compared to NK2 and NK9 (0.25 O.D).

Kamran *et al.,* (2016) evaluating the qualitative screening of bacterial isolates for β-galactosidase production. Pure bacterial cultures were grown at 37°C for 48h onto the lactose agar medium, supplemented with 50 μg ml^{-1} X-gal (chromogenic substrate) and 1.0 mM of isopropyl β-d-1-thiogalactopyrinoside (IPTG) to examine the capability of isolates for β-galactosidase production (Jaturapiree *et al.,* 2012). Lactose agar medium contained (g L^{-1}): Lactose, 5.0; beef extract, 3.0; peptone, 5.0; Agar, 15.0 with pH-7.0. After 48 h of incubation period, blue colored colonies were examined. Selected isolates were stored on lactose medium slant and in 40% glycerol for short and long term preservation, respectively. The purified bacterial strains were screened for β-galactosidase production by growing on lactose agar medium (supplemented with X-gal and IPTG) at 37°C for 48h (Jaturapiree *et al.,* 2012). Among them, blue colored colonies of Bacillus strain B-2 were prominently observed on lactose agar medium that indicates the production of β-galactosidase into medium after cleaving the chromogenic substrate (X-gal) which was supplemented with lactose into culture medium plate. But in our study, we had developed a novel quantitative method to screen the isolates on β-galactosidase production using spectrophotometry method in X-Gal and IPTG solution.

Purification of β-galactosidase enzyme

Purification of β-galactosidase enzyme was carried out employing various t-butanol ratios (crude extract: t-butanol = 1:0.25, 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) with a different ammonium sulfate saturation at 30, 60 and 90%. The mixture was mixed gently and then allowed to stand for 30 min at 37°C. afterward, the mixture was centrifuged at 5000 rpm for 15 min at 4°C to facilitate the separation of phases. The upper t-butanol phase was removed by the Pasteur pipette. The lower aqueous phase and the interfacial phase were separated carefully and each of phases was analyzed for enzyme activity. Different ammonium sulfate saturation effects (30, 60 and 90%) (w/v) were investigated at the best recovery activity of crude enzyme: t-butanol ratio that is 1.0:1.0. The bottom phase and interfacial phase were analyzed for enzyme activity (Duman and Kaya, 2013).The enzyme assay evaluates the β-galactosidase enzyme, which was present in selected *Lactobacillus* cultures. *Lactobacillus* cultures i.e. V3, NK2 and NK9 were statistically analysed for the different ammonium sulphate saturation effects, different sample size of crude enzyme: t-butanol ratio and highest production of β-galactosidase enzyme. β-galactosidase enzyme production of *Lactobacillus* cultures i.e. V3, NK2 and NK9 were presented in figure 10. In figure 10, β-galactosidase activity of V3 culture was significantly (P<0.05) increased at 60% ammonium sulphate saturation and ratio of crude enzyme: t-butanol (1:1; 2%). β-galactosidase activity was significantly highest at 60 % ammonium sulphate saturation for all the three *Lactobacillus* cultures (V3, NK2 and NK9) followed by 30 and 90%. Significantly maximum β-galactosidase production was also observed at 60% ammonium sulphate saturation in case of V3 culture (0.377O.D), followed by NK9 (0.283 O.D) and NK2 (0.216 O.D) compared to 30% ammonium sulphate saturation of V3 (0.287 O.D) followed by NK9 (0.246 O.D) and NK2 (0.186 O.D) and subsequently also compared with 90 % ammonium sulphate saturation for NK9 (0.248

O.D), followed by V3 (0.238 O.D) and NK2 (0.172 O.D).From figure 10, it was observed that the β-galactosidase activity of V3, NK2 and NK9 cultures were significantly (P<0.05) increased with the different ammonium sulphate saturations (30 and 60 %) and various crude enzyme:t-butanol ratios (1:0.25;1.25, 1:0.5;1.5 and 1:1;2) during purification process. But β-galactosidase activity were significantly decreased at ammonium sulphate saturation effect (90 %) and various crude enzyme: t-butanol ratios (1:1.5; 2.5 and 1:2:3 %). Significantly maximum β-galactosidase activity was observed at (1:1:2 %) crude enzyme: t-butanol ratio (0.353 O.D), followed by 1:1.5; 2.5 % (0.274 O.D), 1:2:3 % (0.236 O.D), 1:0.5; 1.5 % (0.228 O.D) and 1:0.25; 1.25 % (0.159 O.D) during purification process.

Prasad *et al.,* (2013) evaluated that the production of β-galactosidase by *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in whey and the effect of 4 different extraction methods i.e. sonication, acetone-toluene, SDS-chloroform and lysozyme-EDTA treatment on enzyme activity from these organisms. Both organisms were grown in deproteinised whey containing yeast extract (3.0 g/L), peptone (5.0 g/L) and glucose (10.0 g/L) for 18 h, at 37ºC for *B. animalis* ssp. *lactis* Bb12 and at 45ºC for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. The optimum intracelluar β-galactosidase activity on 15 mM o-nitrophenyl β-D-galactopyranoside (ONPG) assay was at pH 6.8 for both organisms irrespective of the method of extraction used. Also, the effect of temperature on enzyme activity was studied at various temperatures (30, 35, 40, 45 and 50°C). At 35°C and 40°C, *B. animalis* ssp. *lactis* Bb12 evaluated more intracellular β-galactosidase activity extracted by sonication than other temperatures and methods. However, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 had more intracellular β-gal activity at 35°C and 45°C when extracted by lysozyme-EDTA treatment. Among the four methods used for β-galactosidase extraction, sonication exhibited the best result (6.80

Unit/mL) for *B. animalis* ssp. *lactis* Bb12 while lysozyme-EDTA treatment was also found to be the best (7.77 Unit/ mL) for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. These treatments are similar as our study. Duman and Kaya, (2013) evaluated the activity of β-galactosidase was completely decreased at 60% ammonium sulfate saturation in aqueous phase, the best results of recovery and the purification fold of β-galactosidase (133% and 10.1, respectively) was obtained in the interfacial phase. Probably, this saturation was caused by the precipitation of whole target protein molecule in the interfacial phase. Generally, researchers started with minimum salt concentration of 20% (w/v) to optimize the partitioning conditions (Dennison *et al.,* 1997). In that study also evaluated the minimum salt saturations for the beginning and the increasing of salt concentrations resulted with the increasing of recovery and purification fold in the interphase up to 60% (w/v) saturations. Higher salt concentrations from this point were resulted with reducing of recovery and purification fold, which may be due to irreversible denaturation of protein (Narayan *et al.,* 2008). This results obtained as similar data found in our study.

Purification of β-galactosidase enzyme through SDS-PAGE

β-galactosidase enzyme extracts from different *Lactobacillus* cultures were subjected to SDS-PAGE analysis along with Bovine serum albumin (BSA- 66.2 kDa) as a marker (Fig. 11). The molecular mass of partially purified β-galactosidase (60% Ammonium sulphate saturation) from selected *Lactobacillus* cultures i.e. V3, NK2 and NK9 as determined by SDS-PAGE was approx. 69 kDa (Fig. 11). Presence of high molecular weight protein band nearer to BSA in LAB showed Mol. Weight of β-galactosidase. The protein band of 62 kDa corresponds to β-galactosidase enzyme as reported by previous workers (Pal *et al.,* 2013). These results raised the expectation of finding β-galactosidase enzyme in LAB. Out of ten *Lactobacillus* cultures, three cultures i.e. V3, NK2 and NK9 showed highest production of β-

galactosidase after 24 h at 37ᵒC. Sonication treatment was also found best for the maximum production of β-galactosidase under the optimized growth condition. 60 % Ammonium sulphate and crude enzyme:t-butanol=1:1 also produced maximum partially purified β-galactosidase enzyme for further study. These three *Lactobacillus* cultures could be used as β-galactosidase producer.

Conflicts of interest

There is no conflict of interest in the study among the authors.

Authors' contributions

All the authors contributed equally to the writing of this paper. They were also involved in the overall work of experiments.

References

Antunes A. E. C., Cazetto T. F. and Bolini H. M. A., Viability of probiotic microorganisms during storage, post acidification and Fig. 12 MS/MS spectrum of fraction E1 inspected in MASCOT database (Identified 'ETVPYMFEN' amino acid sequence as 'lactoferrin')sensory analysis of fat-free yoghurts with added whey protein concentrate, *International Journal of Dairy Technology,* 58,169–173 (2005).

Berni Canani R., Pezzella V., Amoroso A. *et al.,* Diagnosing and treating intolerance to carbohydrates in children, *Nutrients,* 8, 155-157 (2016).

Bury, T. and Jelen, P., Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β-galactosidase extracts from *Lactobacillus bulgaricus* 11842, *Innovative Food Science and Emerging Technologies,* 3, 175-188 (2001).

Burya D., Jelena P., Kimura K., Whey protein concentrateas a nutrient supplement for lactic acid bacteria, *International Dairy Journal,* 8,149–151 (1998).

Carrasco-Castilla J., Hernandez A., Lvarez A. J.

et al., Antioxidant and metal chelating activities of Phaseolus vulgaris L. var. Jamapa protein isolaes, phaseolin and lectin hydrolysates. *Food chemistry,* 131, 1157-1164 (2012).

Deng Y., Misselwitz B., Dai N. and Fox M., Lactose intolerance in adults: biological mechanism and dietary management, *Nutrients,* 7, 8020–8035 (2015)

Dennison C. and Lovrein R., Three phase partitioning: concentration and purification of proteins, *Protein Expression and Purification,* 11, 149–161 (1997)

Duman Y. and Kaya E. Purification, recovery, and characterization of chick pea (*Cicer arietinum*) b-galactosidase in single step by three phase partitioning as a rapid and easy technique, *Journal of Dairy Science*, 91, 155–160 (2013)

Hati S., Patel N., Sakure A. and Mandal S. Influence of whey protein concentrate on the production of antibacterial peptides derived from fermented milk by lacticacid bacteria, International *Journal of Peptide Research and Therapeutics,* (2017)

Hati S., Sreeja V., Solank, J. and Prajapati J. B. Significance of proteolytic microorganisms on ACE-inhibitory activity and release of bioactive peptides during *fermentation of milk, Indian Journal of Dairy Science,* 68, 584-591 (2015)

IDF 146, Yogurt-Identification of characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*), International Standard, (2003)

Indian Standards., Methods of test for dairy industry part-Irapid examination of milk, Indian Standards Institution, New Delhi (1479) (1960)

Jaturapiree P., Phuengjayaeam S., Seangsawang S. *et al.,* Isolation and production of novel β-galactosidase from a newly isolated, moderate thermophile, *Bacillus* sp. *strain* B1, *Journal of Food Science and Engineering*, 2, 395–402 (2012)

Jelen P., Lactose hydrolysis using sonicated dairy cultures, In Lactose hydrolysis, IDF Brussels Belgium Bulletin, 289, 54-56 (1993)

Kamran A., Bibi Z., and Aman A. *et al.,* Lactose hydrolysis approach: Isolation and production of β-galactosidase from newly isolated *Bacillus* strain B-2, *Biocatalysis and Agricultural Biotechnology*, 5, 99–103 (2016)

Kara F., Release and characterization of beta-galactosidase from *Lactobacillus plantarum*, Biotechnology, METU (2004).

Laemmli U. K., Cleavage of structural proteins during assembly of the head of bacteriophage T4, *Nature,* 227, 680-685 (1970)

Milanovic S., Ilicic M., Duric M., Caric M., Effect of transglutaminase and whey protein concentrate on textural characteristicsof low fat probiotic yoghurt, *Milchwissenschaft,* 64, 388–392 (2009)

Narayan A. V., Madhusudhan M. C. and Raghavarao S. M., Extraction and purification of ipomea peroxidase employing three-phase partitioning, *Applied Biochemistry and Biotechnology*, 151, 263–272 (2008)

NIDDK, Lactose Intolerance, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), pp: 12-16 (2016)

Pal A., Lobo M. and Khanum F., Extraction, purification and thermodynamic characterization of almond (Amygdalus communis) β-galactosidase for the preparation of delactosed milk*, Food Technology and Biotechnology,* 51, 53–61 (2013)

Prasad L. N., Ghosh B. C., Sherkat F. *et al.,* Extraction and characterisation of β-galactosidase produced by *Bifidobacterium animalis* spp. *lactis* Bb12 and *Lactobacillus delbrueckii* spp. *bulgaricus* ATCC 11842 grown in whey, International Food Research Journal, 20, 487-494 (2013)

Skovbjerg H., Sjostrom H. and Noren O., Purification and characterization of amphiphilic lactase/phlorizin hydrolase from human small intestine, *European Journal of Biochemistry*, 114, 653–661 (1981)

Vasiljevic T. and Jelen P., Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria, *Innovative Food Science and Emerging Technologies,* 2, 75-85 (2001)

Vikas S., Sudhir K., Tomar B. A., Ram R. B. and Ashish K. S. Production of β-galactosidase from *Streptococcus thermophilus* for galactooligosaccharides synthesis, *Journal of Food Science and Technology,* 52, 4206–4215 (2015)