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RESEARCH ARTICLE

Development of lactose hydrolyzed milk using micro fluidization assisted crude β-galactosidase enzyme of *Lactobacillus acidophilus*

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Abstract: The objectives of this study were to screen maximum β-galactosidase enzyme producing *Lactobacillus* strain out of seven commercial lactobacilli and development of lactose hydrolyzed milk using micro fluidization assisted crude β-galactosidase enzyme from selected maximum β-galactosidase enzyme producing *Lactobacillus* strain. From all the screening methods *L. acidophilus* ATCC 4356 culture was found to possess the maximum β-galactosidase enzyme, so this culture was selected for further studies. Crude β-galactosidase enzyme extract (CEE) was obtained from *L. acidophilus* ATCC 4356 using micro fluidization as cell disruption method. A total of 37.41 (µmol/ mL/ min) enzyme activity was obtained from crude extract. Lactose hydrolysis in milk was done using different concentrations (0.5, 1 and 1.5%) CEE of *L. acidophilus* ATCC 4356. CEE@1.5% showed highest 39.96% hydrolysis of lactose when compared with other CEE added milk after 8 h. There was significant difference found when sensory scores were recorded for lactose hydrolyzed milk obtained by using crude enzyme extracts concentrations @1.5% CEE of *L. acidophilus* ATCC 4356 and 1% commercial enzyme. Lactose hydrolyzed milk was successfully developed using crude enzyme extract and being an economical, innovative and therapeutic product, large scale production of the product can be taken up by large players of the field in future.

Keywords: β-galactosidase, Micro fluidization, Lactose hydrolyzed milk, Sensory analysis, Lactic acid bacteria

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Introduction

Lactose intolerance is a very common disease where any individual is unable to hydrolyze lactose (Vasiljevic and Jelen, 2001; Singroha et al. 2014; Singhroha et al. 2017; Szilagyi, and Ishayek, 2018). It is generally initiated by the deficiency of a specific enzyme β-galactosidase. β-galactosidase enzyme is also known for its various applications in dairy industry. β-galactosidase coverts lactose into glucose and galactose and it is also known for its ability to catalyze transglycosylation reactions (Oliveira et al. 2011).

In dairy industry, various applications of β-galactosidase have been reported like prevention of lactose crystallization, to increase the sweetness of the milk products, to produce low lactose food products and for cheese whey utilization by which water pollution can be controlled (Sani et al. 1999; Kaur et al. 2017; Joon et al. 2018). As we know, enzymes which are utilized for lactose free milk production are highly purified in nature. High purification of proteins generally makes the cost of enzymes higher. In this way, the expense of low lactose milk is nearly 80% greater than the standard un-hydrolyzed milk (Bury and Jelen, 2000).

One of the benefits of employing *Lactobacillus* strains as a source of β -galactosidase is to catalyze lactose hydrolysis. Since they are Generally Recognized As Safe (GRAS) organisms, so their enzymes can be utilized in milk products (Sani et al. 1999; Mishra et al. 2011).

β-galactosidase is an enzyme found inside the cell. For obtaining enzyme mechanical, enzyme disruption or chemical permeabilization of the cell membrane methods are generally utilized. The effectiveness of these methods for disruptions differs in terms of microorganism's genera and strains. In general, rupturing cells employing various cell disruption procedures can dramatically boost β-galactosidase activity in the media. In literature, disruption of yeasts is mainly focused; whereas less information on the disruption of lactobacilli. More lactose and lactose-containing dairy products will be manufactured, if a cost-effective lactose hydrolysis technology is developed and a suitable microbe source is discovered. Crude enzymatic extract

is one of the economical methods for lactose hydrolysis as enzyme purifying step is eliminated which is highly costly.

The objectives of the present study were to screen maximum β-galactosidase enzyme producing *Lactobacillus* strain out of seven commercial lactobacilli and development of lactose hydrolyzed milk using micro fluidization assisted crude β-galactosidase enzyme from selected lactobacillus strain.

Materials and Methods

Materials

Raw milk is the major ingredient used for the manufacturing of lactose hydrolyzed milk. Fresh, hygienic, good quality raw milk was procured from Experimental dairy plant, GADVASU Ludhiana. Commercial lactobacilli cultures for screening and extraction of β-galactosidase, were procured from ATCC through Hi media, Mumbai (Table 1.). Stock cultures were preserved at -80º C. Before any assay, strains were revived by transferring stock cultures into MRS medium and incubated at 37º C for 24 hours. Purity of each culture was ascertained by doing Gram staining and catalase test. The storage of cultures was done below 5ºC between transfers.

Screening of *Lactobacillus* isolates for their ability to produce β-galactosidase by Ortho-Nitrophenyl-β-galactoside (ONPG) Discs method

This test was used for the rapid detection of β-galactosidase activity by different lactobacilli cultures. Here, the lactose fermenters were identified. The 3 discs of ONPG were put in the sterile test tubes. To this, 5 ml of the 0.85% NaCl solution was added to these test tubes. One colony from each *Lactobacillus* strain was added to the respective tubes. Then tubes were maintained at temperature of 37±2°C in incubator. After every hour, the presence of yellow color was seen and observed till 6 hrs. Then color intensity was observed directly after 24 h of incubation.

Fermentation pattern of *Lactobacillus* isolates in milk

The curd formation ability of all 7 lactobacilli was observed. For this, 10 ml of milk was taken in centrifuge tube and 1% of each *Lactobacillus* strain was inoculated in the milk and incubated at 37°C till pH reach to 4.6 or below. Changes in acidity and pH were observed up to 6 hrs.

Titratable acidity and pH

The titratable acidity was observed by method of Indian Standards (1981). pH value of the fermented milk samples has been determined using laboratory pH meter (*Mettler Toledo)*. pH meter was previously calibrated using buffers 4.0, 7.0 and 10.0 and then used for subsequent samples.

Enzyme extraction by micro fluidization method

Method of Choi et al. (1997) was used with slight modifications. The microbe was fermented in 500ml of MRS-Lac broth. This cell biomass was centrifuged at 12000*g for 10 minutes at 4°C. Pellet was obtained and 5.0 ml of 0.05 M Na- phosphate buffer (pH 6.8) was added to this pellet and the suspension was vortexed vigorously. After the washing procedure the pellet was again centrifuged at 12,000*g for 10 minutes. Pellet was again suspended in 300ml of 0.05 M Na- phosphate buffer (pH 6.8). Cell disruption was done with the help of the micro fluidizer (Microfluidics M-110P, Newton, USA) pass for three times at 15,000 Pa. Supernatant was used for the enzyme assay after centrifugation at 12,000g for 10 minutes at 4 °C.

Enzyme assay

For analysis of activity, 300 µL of cell suspension was taken in a test tube. To this, 2.7 ml of phosphate buffer was added and 600 µL ONPG substrate was added. The tubes were immersed in a steam bath at 37°C for 15 minutes. After 15 min reaction was stopped by the addition of 2.25 ml of 1M Na_2CO_3 to the reaction mixture. Absorbance values were taken at 420 nm. One unit was described as the enzyme required to liberate 1 µmol of ONP from its substrate each minute under the same assay situations.

Manufacture of lactose hydrolyzed milk

For the research purpose, lactose hydrolyzed milk samples from raw milks were made under aseptic condition as shown in Figure 1.

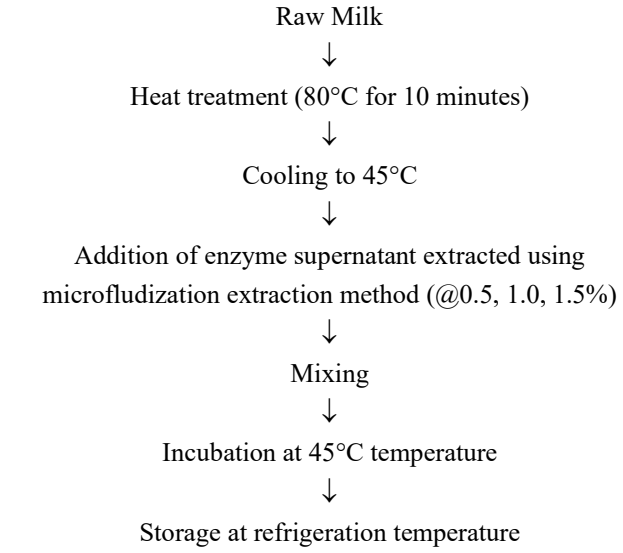

Figure 1. Flow chart for preparation of lactose hydrolyzed milk

Lactose estimation of milk

Lactose content of the samples was estimated as per Lane and Eynon method as described in Indian Standards (1981).

Glucose and galactose estimation of lactose hydrolyzed milk

Glucose and galactose content released after hydrolysis of lactose in the milk samples was estimated as per Nickerson et al. (1976).

Sensory evaluation of lactose hydrolyzed milk

The lactose hydrolyzed milk prepared by adding micro fluidization extracted crude enzyme @ 1.5% in previously boiled and cooled to 45°C milk and was placed in an incubator for a time period of 8 hours of incubation. After this, the hydrolyzed milk was stored in refrigeration condition i.e., 4±2°C. The product was then evaluated by a sensory trained panel for overall quality and acceptability. The product was served as coded (A-Control, B-1.5% CEE added, C-1% commercial enzyme added) and randomly arranged. The panel of seven judges evaluated the milk samples in terms of color and appearance, odor, flavor and taste and body (consistency) on the 100 point score card by BIS (IS: 7768, 1975). The sample for this purpose was brought to temperature condition of 10°C for sensory evaluation. The score given by evaluators were further considered for judging the final acceptability of milk samples (Makwana et al. 2019).

Statistical analysis

Under the supervision of a statistician, data gathered from numerous experiments during the screening and comparative analysis process were analyzed for two-way analysis of variance (ANOVA) and t-test using SAS 9.3 version. Microsoft excel was used to calculate the mean, standard error of l data, when needed.

Results and Discussion

Assessing purity of commercial *Lactobacillus* strains

During this study *L. acidophilus* ATCC 314, *L. plantarum* ATCC 8014, *L. sakei* ATCC 15521, *L. gasseri* ATCC 19992, *L. rhamnosus* ATCC 7469, *L. casei* ATCC 393 and *L. acidophilus* ATCC 4356 were used (Table 1.). All the lactobacilli were found to be Gram positive, rod shaped bacteria under microscope. All the lactobacilli were catalase negative.

Table 1: List of commercial lactobacilli purchased

S.No.	Name of Lactic acid bacteria
1	*Lactobacillus acidophilus* ATCC 314
2	*Lactiplantibacillus plantarum* ATCC 8014
3	*Lactobacillus sakei* ATCC 15521
4	*Lactobacillus gasseri* ATCC 19992
5	*Lacticaseibacillus rhamnosus* ATCC 7469
6	*Lacticaseibacillus casei* ATCC 393
7	*Lactobacillus acidophilus* ATCC 4356

Fig. 2 Ortho-Nitrophenyl-β-galactoside (ONPG) Disc Method for identification of β-galactosidase producing lactobacilli, where 1 tube: *L. acidophilus* ATCC 4356 and 2 tube: Negative control

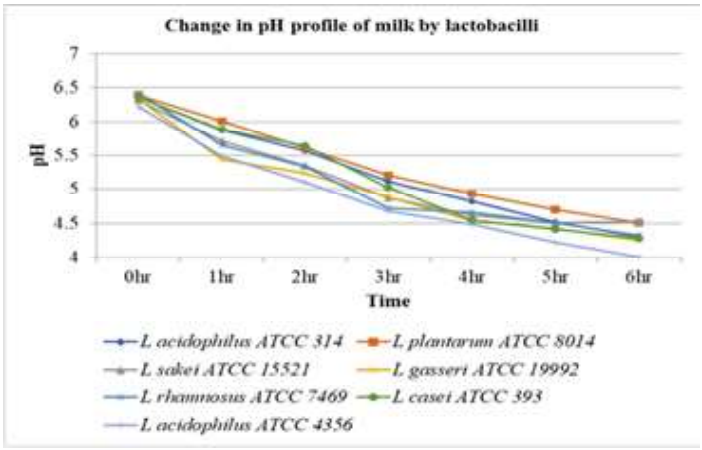

Fig. 3 Change in pH profile of milk by different lactobacilli

Screening by Ortho-Nitrophenyl-β-galactoside (ONPG) discs method

β-galactosidase enzyme not only acts on lactose but also on the other substrates like ONPG. When ONPG reacts with β-galactosidase it leads to the formation of substrates i.e., galactose and ONP. The ONP is a yellow chromogenic compound, thus, ONPG when used for screening it forms deep yellow color.

In our study all lactobacilli were screened by ONPG disc method and detected that the mix color changed to yellow color after some time of incubation. This indicated that all lactobacilli strains have the potential to hydrolyze ONPG into ONP (Figure 2). Favier et al. (1997) used a similar method and screened *Bifidobacteria* for β-galactosidase activity. In one of the study, *S. thermophilus* RD 102 *and S. thermophilus* RD 104 showed yellow color after incubation, thus confirmed β-galactosidase activity. The formation of color rapidly and slowly indicates its ability to form β-galactosidase production in terms of time (Iyer et al. 2010). *B. subtilis* VUVD001 showed similar results i.e., the formation of

deep yellow color in the Ortho-Nitrophenyl-β-galactoside (ONPG) disc method (Venkateswarulu et al. 2020).

Fermentation ability of *Lactobacillus* isolates

The curd formation ability of all 7 lactobacilli was observed by observing changes in acidity and pH after every hour up to 6 h duration. From the Figure 3, it can be observed that after 6 h of incubation maximum pH decrease in milk was observed by *L. acidophilus* ATCC 4356 (4.01), followed by *L. gasseri* ATCC 19992 (4.25), *L. casei* ATCC 393 (4.28), *L. acidophilus* ATCC 314 (4.3), *L. rhamnosus* ATCC 7469 (4.32), *L. plantarum* ATCC 8014 (4.51). The minimum pH decrease in milk was observed by *L. sakei* ATCC 15521 (4.53).

Similar pattern was observed for acidity increase (Figure 4) by all selected lactobacilli in milk medium except *L. acidophilus* ATCC 314 (0.723) which has shown the minimum increase in acidity after 6 h of incubation. After 6 h incubation, maximum acidity increase in milk was observed by *L. acidophilus* ATCC 4356 (0.881), followed by *L. gasseri* ATCC 19992 (0.871), *L. rhamnosus* ATCC 7469 (0.865), *L. casei* ATCC 393 (0.854), *L. plantarum* ATCC 8014 (0.822). The above pH and acid profile of all selected lactobacilli suggest that they all possess β-galactosidase enzyme as they all were able to ferment milk within 6 h of incubation time.

From all the above screening methods *L. acidophilus* ATCC 4356 culture was found to possess the maximum β-galactosidase enzyme, so this culture was selected for further studies.

β-galactosidase Enzyme activity after cell disruption using Micro fluidizer from *L. acidophilus* ATCC 4356

Cell disruption is necessary for the extraction of subcellular ingredients, and it has a considerable impact on subsequent extraction and purification processes. It is necessary to choose appropriate ways for breaking down cellular structures for the isolation of subcellular products. Here β-galactosidase extraction was done using micro fluidizer form previously selected *L. acidophilus* ATCC 4356 culture. Total 37.41+0.52 µmol/ mL/ min enzyme activity was obtained from crude extract

Comparison of addition of different crude β-galactosidase extract of *L. acidophilus* ATCC 4356 on glucose and galactose production in milk

Glucose and galactose production by crude enzyme extract (CEE) of *Lactobacillus acidophilus* ATCC 4356 in previously boiled and cooled milk to 37º C at different time intervals (0, 4, 6 and 8 h) with numerous concentrations of enzyme (0.5, 1, and 1.5%) were shown in the Table 2.

Comparison of addition of crude extract of β-galactosidase on hydrolysis of lactose in milk

Lactose hydrolysis by crude enzyme extract (CEE) of *Lactobacillus acidophilus* ATCC 4356 in previously boiled and cooled milk to 37º C at different time intervals (0, 4, 6 and 8 h) with different enzyme concentrations (0.5, 1, and 1.5%) were represented in the Table 3.

From the Table 3, it can be easily interpreted that the lactose hydrolysis (%) by commercial enzyme was significantly higher than other crude enzyme extract (CEE) used i.e., 80.64% after 8 h of incubation; whereas in the case of all 3 milk samples added with CEE of *L. acidophilus* ATCC 4356, CEE@1.5% showed highest 39.96% hydrolysis after 8 hrs. The hydrolysis rate by

Table 2: Comparison of addition of different crude enzyme extracts of *L. acidophilus* ATCC 4356 on Glucose and Galactose production in milk

Sample	Time			
	2 Hours	4 Hours	6 Hours	8 Hours
0.5% CEE of *L acidophilus* ATCC 4356	0.0857± 0.251[a]	1.918± 0.089[a]	2.54± 0.261[a]	3.31± 0.361[a]
1.0% CEE of *L acidophilus* ATCC 4356	0.114± 0.291[a]	2.661± 0.225[b]	3.15± 0.340[b]	3.9267± 0.197[b]
1.5% CEE of *L acidophilus* ATCC 4356	0.159± 0.341[a]	3.328± 0.169[c]	3.63± 0.232[c]	4.449± 0.170[c]
1% Commercial Enzyme	4.234± 0.105[b]	7.31± 0.225[d]	8.2312± 0.227[d]	9.11± 0.468[d]

Different alphabets ([a, b, c, d]) shows significant difference ($p \leq 0.01$) between the samples during the study

Table 3: Effect of crude extract of *L. acidophilus* ATCC 4356 β-galactosidase supernatant on hydrolysis of lactose

Sample	Time			
	2 Hours	4 Hours	6 Hours	8 Hours
0.5% CEE of *L. acidophilus* ATCC 4356	8.96± 0.251[a]	17.02± 0.089[a]	22.57± 0.261[a]	29.52± 0.361[a]
1.0% CEE of *L. acidophilus* ATCC 4356	10.24± 0.291[b]	23.44± 0.225[b]	26.59± 0.340[b]	33.56± 0.197[b]
1.5% CEE of *L. acidophilus* ATCC 4356	11.51± 0.341[c]	28.03± 0.169[c]	33.07± 0.231[c]	39.96± 0.170[c]
1% Commercial Enzyme	36.11± 0.105[d]	64.31± 0.225[d]	71.29± 0.227[d]	80.64± 0.468[d]

Different alphabets ([a, b, c, d]) shows significant difference ($p \leq 0.01$) between the samples during the study

CEE varies from 11.516 to 39.96%. The average rate of hydrolysis by the 1.5% CEE was observed 28.146% lactose hydrolysis. When the 8 h incubation was observed by 1.0% and 0.5% CEE the 33.56% and 29.52% lactose hydrolysis was observed. It was also observed that lactose reduction (%) by 0.5% CEE, 1.0% CEE and 1.5% CEE were significantly increased at different incubation periods of (2, 4, 6, and 8 hours). Statistically, time taken for hydrolysis was directly correlated with concentration of enzyme used (P d" 0.05) in milk.

Horner found the four times increase in the enzyme concentration lead to doubling of the hydroxylation of milk after 12 hours. This was examined by the commercial β-galactosidases of *Kluyveromyces* at 2ºC for 3 days (Horner et al. 2011). The hydrolysis of lactose was increased when enzyme concentrations increased (AKGUeL, 2012).

Sensory analysis of lactose hydrolyzed milk using crude enzyme extract of *L. acidophilus* ATCC 4356

When hydrolysis of milk occurs the lactose is fragmented into smaller molecules glucose and galactose is formed. This lead to an increase in glucose content in the milk and it tastes sweeter than raw milk. This is all because glucose is five degrees sweeter than lactose, whereas galactose is four times sweeter than lactose and both act as natural sweetener and thus provide sweet taste to milk.

Three combinations were decided (Control (A) Crude extract combinations (B), and commercial enzyme (C)) in this study. The hydrolyzed milk subjected to sensory should be free from unwanted flavor and other unwanted material. Lactose hydrolyzed milk samples were judged and graded on the basis of 100 point score card as per IS (IS:7768, 1975) for various sensory attributes: -

i) Color and Appearance,

ii) Odour (aroma),

iii) Flavour & taste and

iv) Body (consistency)

v) Overall acceptability

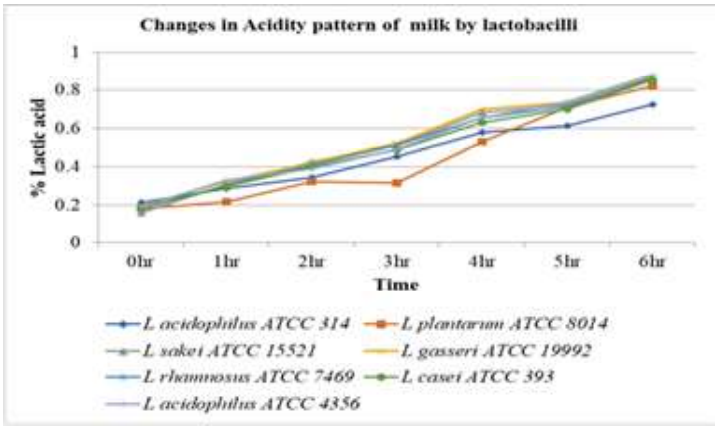

Fig. 4 Change in acidity pattern of milk by different lactobacilli

The alterations in the color and appearance scores of lactose hydrolyzed milk samples are shown in Table 4. Minimum score was recorded by the sample B (8.3) i.e., 1.5% CEE of *Lb. acidophilus* ATCC 4356. The mean color and appearance score was 8.433. Throughout, samples A and C showed the highest score and least by B.

The score card exhibited by the attribute odor of lactose hydrolyzed milk is shown in Table 4. The average odor score secured during analysis was 17.8. During sensory scoring by the penalists, Sample C (18.4) shown the highest score followed by B (18.0) and A (17.0). The mean odor score ranged from 17.0 to 18.4.

Attribute flavor and taste score results of lactose hydrolyzed milk were given in Table 4. During the sensory evaluation, Sample C (37.67) exhibited the highest score followed by B (35.7). Lowest score was observed by sample A (33) i.e., Control sample. The average flavor and taste score were 37.67.

The alterations in body (consistency) score results of milk samples were shown in Table 4. The mean body (consistency) score was 26.56. The sensory evaluators had analyzed Sample C having maximum score followed by B (26.8). The least score was recorded by sample A (25.5). The mean overall acceptability score was 86.89. During the sensory test, Samples C (90.67) had shown the maximum score followed by B (87.0). Lowest score was

Table 4: Sensory scores of lactose hydrolyzed milk using crude enzyme extract of *L. acidophilus* ATCC 4356

Sample	Attributes				
	Color and appearance (Max score 10)	Odor (Max score 20)	Flavor and taste (Max score 40)	Body (Max score 30)	Overall Acceptability (Max score 100)
A (Control)	8.5± 0.115^{a}	17± 0.311^{a}	33± 0.907^{a}	25.5± 0.208^{a}	83±0.371^{a}
B (1.5% CEE of *Lb. acidophilus* ATCC 4356)	8.3± 0.221^{b}	18± .260^{b}	35.7± 0.577^{b}	26.8±1.311^{b}	87±0.982^{b}
C (1% of Commercial)	8.5± 0.127^{a}	18.4± .508^{c}	37.67± 0.577^{c}	27.4± 0.585^{b}	90.67±0.692^{c}

Different alphabets ($^{a, b, c}$) shows significant difference ($p \leq 0.01$) between the samples during the study

observed in Sample A (83.0) i.e., Control sample. The overall acceptability score varied from 83.0 to 90.67.

Sensory attributes of ultra-pasteurized (UP) lactose-free milk having variable composition in term of fat and it was compared with normal milk. They also conducted the consumer survey. The low fat milk was regarded as lack of freshness and lower values on the sensory score. The ultra-pasteurized lactose-free milk had high cooked, processed and sweet flavor (Adhikari et al. 2010).

Also, Nielson with other scientists researched on variable storage condition effects on the shelf life of hydrolyzed-lactose UHT milk which was assessed with use of proteomics. Lactose was broken up to 40% with ultra and nano-filtration before the hydrolysis procedure. The stored milk was evaluated by the sensory characteristics of the product. The lactose-reduced milk was found to be bitter with increasing time period. This happened because of amount of peptides released with enzymatic or non-enzymatic pathway and heat and storage induction respectively. Here, the controlled sample taken was conventional boiled milk (Nielson et al. 2017).

Conclusions

Considering the fact that there is lack of scientific literature available with relevance to lactose hydrolyzed milk prepared from economical enzyme sources, current study was undertaken. In conclusions, technology for extraction of crude β-galactosidase extract from *L. acidophilus* ATCC 4356 by using micro fluidization extraction method had been optimized successfully. Also, lactose hydrolyzed milk was successfully developed using crude extracted enzyme and being an economical, innovative and therapeutic product, large scale production of the product can be taken up by large players of the field.

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Compliance with Ethical Standards

This article does not contain any studies with human or animal subjects.

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