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Original Article

Recovery and Screening of α-Galacotosidase Producing Lactic Acid Bacteria from Fermented Dairy Products

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Abstract: Lactic acid bacteria (LAB) present in fermented foods has long been consumed by humans without any obvious adverse effects. Therefore, they are potent candidates as vehicles for the delivery of digestive enzymes. Stachyose, a tetrasaccharide, is believed to contribute to flatulent properties of soyabeans that limit their use for human consumption. LAB including some *Lactobacillus plantarum, L.fermentum,L. buchneri and reuteri* hydrolyze α- galactosides or non-digestible carbohydrates into digestible carbohydrates during fermentation. These bacteria are therefore a source of α -galactosidase. If soy milk could be fermented with these microorganisms that utilize stachyose either to produce acid or to hydrolyze it to mono and disaccharides, the product thus prepared ought to be less flatulent and therefore, more acceptable. In present study, total 27 lactic acid bacteria were recovered selectively on MRS agar from the various milk and milk products. All the 27 isolates were characterized morphologically and the colonies were white to cream and gram positive. Out of 27 LAB only 5 isolates were found to be positive for α- galactosidase enzyme. α-galactosidase activities were determined by using p-NPG. All 5 α-galactosidase producer were further subjected for various biochemical characterization for partial identification and were catalase negative, and casein hydrolysis, sugar fermentation, nitrate reduction positive. Reduction of α-galactosides by the 5 selected isolates were evaluated. The isolate, RLAB α-4, CLAB α-14, CLAB, CLAB α-20 α-18 and WLAB α-25 degraded 67.56 %, 45.94%, 54.05%, 70.27%, and 64.86% α- galactosides respectively. CLAB α-20 degraded maximum concentration of α- galactosides and RLAB α-14 degraded least concentration of α- galactosides.

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INTRODUCTION

Microbial enzymes are widely used in different industries such as food, beverage, textile, leather, pharmaceutical and waste water treatment. Of these, glycosidases or carbohydrolases plays a pivotal role in hydrolysis of carbohydrates. The enzyme alpha galactosidase EC 3.2.1.22 is an exocarbohydrase that hydrolyzes the α-1, 6 galactosidic bonds present in melibiose, raffinose and stachyose (Ghazi et *al*., 2003). The major application of α-galactosidases is the hydrolysis of oligosaccharides present in the food substances. Since humans lack α-galactosidases in the intestinal mucosa, there is a chance for the formation of flatus due to the fermentation of such oligosaccharides by intestinal microflora and are thus responsible for intestinal disorders. Therefore it is necessary to remove the oligosaccharides from food with the help of α-galactosidases. Many attempts to reduce these anti-nutritional factors by soaking, germination and water extraction at different times have been made. Enzyme treatment of soymilk with alpha galactosidase from molds and yeast and lactic acid bacteria. Soy products have high protein content,essential amino acids. Soy derived foods contains polyunsaturated fatty acids , rich in iron zinc and magnesium and are devoid of lactose to which some individual are intolerant. But the consumption of soy food is limited due to the presence of 40% of alpha galactosides in them (Leblanc *et al*., 2004). The use of microorganisms expressing alpha galactosidase is promosing solution to eliminate non digestible oligosaccharides (NDO) before they reach large intestine. In the present study lactic acid bacteria engineered to degrade NDO have been constructed and are being used as tool to evaluate this solution. Lactic acid fermentations are believed to be oldest means of food preservation known to mankind (Benekerroum *et al*., 2007). LAB comprises a wide

range of genera and includes a considerable number of species. They are gram positive, usually catalase negative, grow under microaerophillic to strictly anaerobic conditions and produce lactic acid (Erdogul, *et al.,* 2006). Alpha galactosidase can be produced by controlled fermentation of yeast, mold, plants and bacteria. e.g. *Aspergillus niger ,Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis and Lactobacillus buchneri* are used in vegetable fermentations to hydrolyze alpha galactosides into digestible carbohydrates (Silverstroni *et al*., 2004). As LAB has great ability to convert sugars into lactic acid at higher levels so it is used in α galactosidase production. LABS are able to survive in the gastrointestinal tract (GIT) of man to exhibit various activites there (Connes *et al*., 2004). LAB are proved to be probiotic and beneficial for their host (Leblanc *et al*., 2008, 2004). Some reports showed that LAB feature the ability to produce kinds of bacterocin to inhibit growth of pathogens (Abrams *et al.,* 2011) and they are effective whether the LAB themselves are viable or dead(Angmo *et al*., 2016). Soymilk fermentation by LAB results in reduction of α-galactosides concentration in soymilk by producing α-galactosidases enzyme, thus eliminating possible undesirable physiological effects normally associated with its consumption (Leblanc *et al*., 2004).

MATERIAL AND METHODS

All the chemicals used were of analytical grade. The media used were procured from Hi-media and were used as per manufacture's direction. All the glass ware used was of borosilicate glass.

Collection of samples

30 samples were collected for isolation of lactic acid bacteria out of which 10 were of raw milk collected from nearby dairy and sweet shops; 10 were of homemade curd ; 5 samples of cheese were collected from nearby sweet shops, 5 samples of whey were used which procured from sweet shop were also used for isolation.

Isolation of Lactic Acid Bacteria

1ml of sample was homogenized with 9 ml of 0.85% sterile saline and then serially diluted 0.1 mL of the diluted sample was spreaded on MRS agar i.e Mann Rogosa Sharpe agar plates. The plates were incubated at 37±1°C for 24 hrs. Isolated colonies were taken from each plate. Tests were carried out on each isolate. At each step, useful isolates were selected, and others were not used for further study.

Maintenance of isolates

For the maintenance of isolated strains MRS agar was used. Slants of MRS agar were made and isolates were grown on these slants. After this the slant stored at 4°C with periodic interval of isolates after every 15 days.

Morphological characterization of isolates

For characterization of isolates, colony morphology was observed and recorded. Gram staining was also done for studying Gram reaction and characteristics cell arrangement of isolates.

Gram staining

All the isolates were Gram stained and cell morphology was observed. A smear was prepared from the isolated colony and then staining was performed according to procedure described by Christian Gram, (Aneja, 2003). Isolates were classified as Gram positive or Gram negative, rods or cocci, singular or in chains depending upon their characterstic appearance under the microscope (100 X).

Screening of the LAB for α galactosidase production

All the isolates were inoculated into tubes containing pNPG (0.5 ml) and 0.01M sodium phosphate buffer (pH 7.0) (5ml) and peptone water. Production of yellow colour indicated positive p-NPG results (Miller, 1959).

Biochemical characterization

Different biochemical tests viz. catalase test, motility test, casein hydrolysis, sugar fermentation test, nitrate reduction test, indole, MR-VP, Citrate, gelatin hydrolysis etc were done according to method described by (Aneja, 2003) for characterization and partial identification of the isolates.

Reduction of α-galactosides in soy milk by lactic acid bacteria

500ml of soymilk was inoculated with the selected isolates and incubated at 37±1°C for 24 hrs. Soymilk without any inoculation was taken as control. After, incubation the total sugar content and reducing sugar content of soy milk were determined by Anthrone method and DNS method respectively (Silverstroni *et al.,* 2004).

Estimation of sugar by Anthrone method

Concentrated sulphuric acid hydrolyzes glycosidic bonds to give the monosaccharides which are then dehydrated to furfural and its derivative. The furfural reacts with anthrone to give a blue green complex. 4 ml of anthrone reagent was added to 1 ml of cell free supernatant and was mixed rapidly. The tubes were placed in water bath for 10 minutes with a marble on a top to prevent loss of water evaporation. The tubes were cooled and absorbance was measured at 620 nm against reagent blank. Concentration of sugar in terms of glucose was

estimated by referring to standard curve of glucose range of 100-1000 µg/ml.

Estimation of reducing sugar by DNS method

To 3ml of reaction mixture, 3ml of distilled water and 3ml of DNS reagent was added. The tubes were were immersed in boiling water bath for 15 min. The mixture was cooled and then absorbance was read at 580 nm against reagent blank. The reducing sugars were quantitated from the standard curve, prepared by using glucose in the range of 100-1000 µg/ml.

RESULT

Isolation of Lactic acid bacteria

MRS agar is selective media for the growth of lactic acid bacteria. Therefore, MRS agar was used for the isolation of LAB. A total of 30 samples were taken from 5 sources and total of morphologically distinct 27 Lactic Acid Bacteria were recovered. The isolates were shown white, crème color colony after overnight incubation. All the distinct isolates further subculture on MRS and purified to obtained pure culture. The isolates obtained on MRS are shown in figure 4.1. The pure culture of all the isolates were preserved on MRS agar slants and used for further screening for α-galactosidase production.

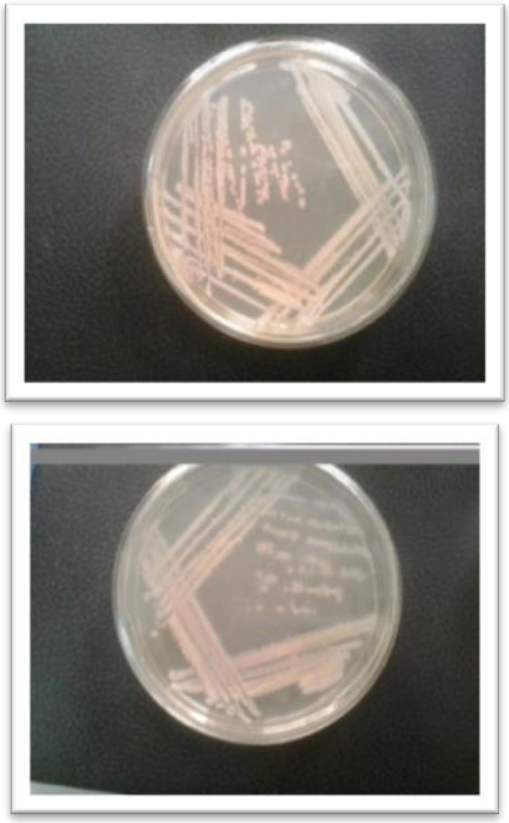

Fig4.1. Lactic Acid Bacteria on MRS agar

Characterization and identification of isolates

All the 27 isolates were further characterized for cell morphology, gram reaction and their cellular arrangements.

Morphological characterization

Cell morphology and Gram staining characteristics were determined by Gram staining. After performing the Gram staining the isolates were observed under light microscope (100 X objective). All the isolates were gram positive as they appeared violet in colour. However, cell morphology varies widely, from long, straight or slightly crescent shaped rods to coccobacilli. All the isolates were Gram positive. Some were rods and some were cocci.

Screening of LAB for α- galactosidase production

All the isolates were further subjected for the screening of production of α-galactosidase. The p-NPG i.e. para- nitro- phenyl α-D galactopyronoside was used as an indicator. The dye is colorless compound and turns yellow in the presence of α-galactosidase enzyme. The tubes are inoculated with the selected isolates and kept at 37±1°C for 24 hrs. The colour of which changed from colourless to yellow are considered positive for α-galactosidase. The positive results were demonstrated by appearance of yellow colour are shown in figure-4.2. Results are shown in table 4.1. Out of the 27 lactic acid bacteria only 5 isolates were able to produce yellow colour i.e. able to produce α-galactosidase enzymes.

Table 4.1- Screening of LAB for α-galactosidase production

S.No	Isolates	Designation	Colour change	Result
1	RLAB A-4	RLAB α-4	Yellow colour	Positive
2	CLAB A-14	CLAB α-14	Yellow colour	Positive
3	CLAB A-18	CLAB α-18	Yellow colour	Positive
4	CLAB A-20	CLAB α-20	Yellow colour	Positive
5	WLAB A-25	WLAB α-25	Yellow colour	Positive

Biochemical characterization

The isolates positive for α- galactosidase were further subjected for different biochemical tests like catalase test, nitrate reduction test, casein hydrolysis, gelatin hydrolysis and sugar fermentation for their partial identifiacation. Results are shown in table 4.2.

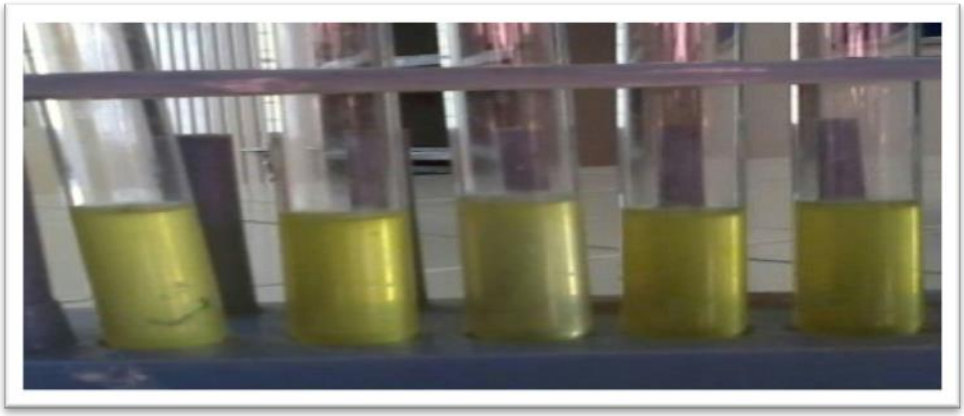

Fig 4.2: Isolates positive for α-galacotosidase production

Table 4.2: Biochemical tests for α-gal producing Lactic Acid Bacteria

Characteristics	R LAB α-4	C LAB α- 14	C LAB α -18	CLAB α -20	W LAB α -25
Motility	-ve	-ve	-ve	-ve	-ve
Catalase activity	-ve	-ve	-ve	-ve	-ve
Casein hydrolysis	+ve	+ve	+ve	+ve	+ve
Gelatin hydrolysis	-ve	-ve	-ve	-ve	-ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve
Methyl red	+ve	+ve	+ve	+ve	+ve
Voges proskauer	-ve	-ve	-ve	-ve	-ve
Glucose fermentation	+ve	+ve	+ve	+ve	+ve
Acid production	+ve	+ve	+ve	+ve	+ve

Reduction of α-galactosides in soy-milk by selected isolates

5 isolates were selected and found positive for α-galactosidase production were further subjected for the reduction of alpha galactosides/non reducing sugars in soy-milk. Out of them, RLAB α-4 degraded 67.56 %, CLAB α-14 degraded 45.94%, CLAB α-18 degraded 54.05%, CLAB α-20 degraded 70.27%, and WLAB α-25 degraded 64.86% α- galactosides/ non reducing sugar in soy milk. Therefore CLAB α-20 degraded maximum concentration of α- galactosides and RLAB α-14 degraded least concentration of α- galactosides. Results are shown in fig. 4.3.

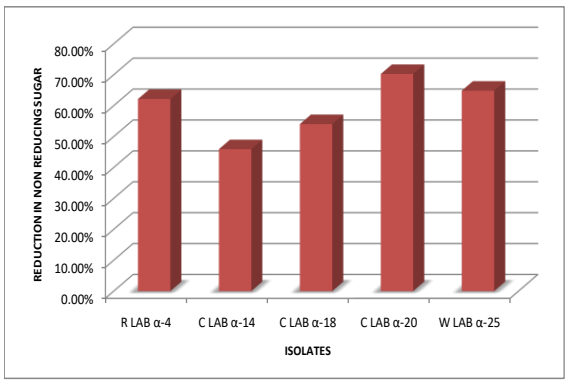

Fig. 4.3: Reduction of α-galactosides in soy milk by selected isolates

DISCUSSION

Lactic acid bacteria (LAB) occur naturally in raw milk and dairy products such as cheeses, yoghurts and fermented milks, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit. They are also found in fermented meats and vegetables, sourdough, silage, beverages and also in the intestinal and respiratory tract of man and animals (Savadogo *et al.*, 2006). LABS are used as natural or selected starters in food fermentations in which they perform acidification due to production of lactic and acetic acids flavor (Coeuret *et al*., 2003). Protection of food spoilage and pathogenic microorganisms by LAB is through production of organic acids, hydrogen peroxide, diacetyl and antifungal compounds such as fatty acids or phenyllactic acid and bactericins. LAB play important role in food fermentation because the products obtained from them is characterized by hygienic safety, storage stability and attractive sensory properties (Vuyst *et al*., 2007).

The 30 dairy samples were collected from local vender and inoculated on MRS agar. A total of 27 morphologically distinct colonies selected as LABs on MRS. All the isolated colonies were white, cream colour appearance ranging their size from small, medium to large. Similarly, Aspri *et al.* (2017) observed that Lactic acid bacteria produced white creamy colonies and they recovered from dairy fermented product and were Gram positive.

All the isolates were that morphologically characterized by staining procedure and found gram positive. In the present study, the shapes of cells of the isolates were cocci, short rod and long

rod and their cell arrangement was single, pair, clusters. Coeureet *et al*. (2003) have reported that lactic acid bacteria isolated from cheese were Gram positive. Patil *et al. (*2009) also reported that lactic acid bacteria isolated from curd were Gram positive.

Further, all 27 isolates were screened for α- gal production and only 5 isolates shown positive result. Similarly, Mital *et al. (*1974) for the first time reported the α-galactosidase activity in LAB. Connes *et al.,* 2003 demonstrated that α-galactosidase expressing LAB which efficiently degraded the α-galactosides. LeBlanc *et al.,* 2004 also reported the capacity of the LAB *Lactobacillus fermentum* to degrade alpha-galactosides by producing α-galactosidase enzyme.

The 5 isolates positive for α-galactosidase were further subject to different biochemical characterization for partial identification. All the 5 isolates shown negative test for catalase. All of them showed no bubble formation which indicated negative result. Coeuret *et al*., (2003) observed that all the LAB were catalase negative during the characterization and identification of LAB from dairy products. Similarly, Anukam *et al*. (2006) observed that all the LAB were catalase negative during the characterization of LAB from curd and cucumber.

Nitrate may be reducing to multiple compounds by two processes i.e. anaerobic respiration and denitrification. Sulfanilic acid and dimethyl 1-napthylamine are added to detect nitrite, which will complex with these molecules forming a red color. If no red color there are two possibilities; the nitrate has been reduced, or it has been reduced further than nitrite. To differentiate between these two possibilities, zinc powder is added, which will complex with nitrate forming a red color. Test was done on those 5 selected isolates.

In (2003), Staton et *al*. tested 23 strains of LAB for nitrate reduction and found all the strains were negative while in (2009), Zakpae reported that all the LAB strains isolated from fermented sausages were positive for this test. Thus it can be concluded that LAB strains show variable results for the reduction and in the present study also LAB strains showed variable results for the reduction of nitrate.
Casease is an exoenzyme that is produced by some bacteria in order to degrade casein. If the organism can produce casein, then there will be zone of clearing around bacterial growth. Among the 5 selected isolates two indicates positive result and rest all were negative. Patil *et al*. (2009), during the characterization of LAB from curd, observed that few strains of LAB were shown positive casein hydrolysis and some were not able to hydrolyze casein. Thus it can be concluded that LAB strains showed variable results for the casein hydrolysis and in the present study also LAB strains showed variable results for casein hydrolysis.

The most useful test for the determination of strain differences is carbohydrate fermentation. Test was done on selected 5 isolates all were glucose positive and acid positive results. As studied by Lilia *et al.,* (2002) out of 42 samples, 35 showed positive result for carbohydrate fermentation and 18 showed positive results for CO_2 gas production. Therefore according to Lilia LAB strains show variable results for sugar hydrolysis and in present study all LAB strains show variable results for the sugar hydrolysis.

α Galactosidases are used in the hydrolysis of raffinose and stachyose present in soy beans and other leguminous food and feed that cause intestinal discomfort, flatulence and low feed utilization in monogastrites. LAB including some *Lactobacillus plantarum, L.fermentum, L. buchneri and reuteri hydrolyze* α- galactosides or non-digestible carbohydrates into digestible carbohydrates during fermentation. These bacteria are therefore a source of α -galactosidase. In the present study the work was focused on the screening of α- galactosidase enzyme producing probiotic bacteria and their characterization. Connes *et al.* (2003) has demonstrated that the α-galactosidase expressing LAB can efficiently degrade the α-galactosides. In (2004) Leblanc *et al.* also observed that fermentation of soy-milk by LAB results in the reduction of α- galactosides concentrations in soy-milk, thereby eliminating possible undesirable physiological effects normally associated with its consumption.

CONCLUSION

As today ‘s consumers are increasingly getting aware of the processes that maybe necessary to maintain their health and nutrition , therefore scientific research has focused on roles that diet , stress and modern medical practices play in threatening human health. To combat these trends directly, alternative disease control strategies such as exploiting therapeutic potential of lactic acid bacteria can be implemented. This study was done to isolate potential lactic acid bacteria from various sources and for the production of α -galactosidase enzyme that helps to overcome health risks. The -galactosidase enzyme is applied in food and feed industries and basically used to decrease the level of raffinose in soy- milk. These sugars are not degraded by pancreatic enzyme of human and are manipulated by gas – producing bacteria of large intestine thus creating intestine disorder such as flatulence in sensitive individual. Fabry’s disease, diabetes can be controlled by α-galactosidase

enzyme. Therefore from this study and previous studies it is clear that LAB are capable of removing α -galactosides by producing α -galactosidase enzyme. The future potential of this study is that the α –galactosidase producing isolates can be exploit as probiotic drink to combat with the various health risk related with the consumption of soy/soy added product. But still there is lot of research scope for their molecular characterization as well as recovery of other value added product, economic aspect of production and safe for the consumption for humane being without any significant health risk.

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CONFLICT OF INTEREST

All the authors has no conflict of interest.

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