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ORIGINAL ARTICLE

Experimental design of lactose hydrolsed milk and its evaluation after preparation into basundi

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ABSTRACT
Lactose intolerance is the most common disease today due to a lack of the lactase enzyme. This research includes a novel method of immobilizing β-D-galactosidase (4ml) on calcium alginate beads (2mm) to prepare lactose hydrolyzed milk (LHM) with incubation times ranging from 12 hours. Attempts have been made to re-use the beads to track the activity of the immobilized enzyme twice. The developed lactose hydrolyzed milk had a lower lactose content and a higher glucose and galactose content. Furthermore, LHM was used in the preparation of Basundi and evaluated the changes during storage period for 12 days. When compared to the control, LHFB and LHRB basundi had significantly higher HMF and acidity. However, free fatty acid was found to lower in LHFB and LHRB as compared to control.
Keywords: LHFB, LHRB, Lactose intolerance, β-D-galactosidase

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INTRODUCTION

Lactose is the primary carbohydrate found in milk, and it is a disaccharide molecule composed of glucose and galactose joined by a β-1,4 glycosidic bond (1). It has a low sweetness, solubility, and is difficult for lactose-intolerant people to digest. Lactose is the primary carbohydrate found in milk, as a result, direct utilization of this lactose is restricted. The intestinal brush border enzyme 'lactase' hydrolyzes lactose into absorbable sugars (glucose and galactose), which provide energy. The availability of lactase enzyme is higher during growing up, but its production decreases or even disappears with age (2). Many people are deficient in this enzyme, resulting in the disease known as "Lactose intolerance." Almost 75% of adult populations are unable to digest lactose due to a genetic deficiency of the lactase enzyme (3).

One of the enzymes with the greatest potential interest in the dairy industry is lactase. It is widely used in the pharmaceutical industry such as development of digestive supplements (prebiotics) and the treatment of disorders (lactose intolerance). Prebiotics are non-digestible food ingredients that have gained popularity because of providing the host by selectively stimulating the growth of indigenous microflora. Currently, β-galactosidases are used to synthesize galacto-oligosaccharides (GOS) or prebiotics in which galactose participates in trans glycosylation reactions after catalyzing lactose into glucose and galactose (4). Basically, this enzyme is found in a variety of sources, including microorganisms, plants, and animals (5). Enzymes derived from microbial sources are crucial for lactose hydrolysis in dairy (6). Kluyveromyces sp. yeasts, the preferred source from microbes, is a major commercial source to be used due to its optimum pH suitable for lactose hydrolysis in milk (7).

The immobilization method is highly efficient due to the mild temperature applied during its process, which reduces enzyme denaturation (8). Also the stabilizing effects of the support matrix improve the immobilized enzyme thermo stability and prevent extensive conformational changes (9). The main advantage of enzyme immobilization is that it can be used repeatedly for multiple batches with little loss of activity in the early stages. Therefore, enzymatic hydrolysis can be a new technology used to produce lactose-reduced milk and its derived dairy products for consumption by lactose-intolerant individuals whose metabolism exhibited a lack of β-galactosidase (10). Several techniques have been used to immobilize enzymes. Among all, Entrapment is the preferred method of enzyme immobilization because it prevents excessive loss of enzyme activity during immobilization, increases enzyme stability in the matrix microenvironment, protects the enzyme from microbial contamination, and is simple to apply (11).

Basundi is becoming increasingly popular and in demand (12). It is consumed directly as a delicious sweet dish. Hence its production and marketing is increasing in a few big cities of the country. Recently, attention has been focused on scaling up the operation by using lactose hydrolyzed milk. An attempt was thereby made to examine the reusability of β-D-galactosidase immobilized in beads.

MATERIAL AND METHODS

- Local farmers provided fresh cow milk.
- A 5200 neutral lactase unit (NLU) -D-galactosidase enzyme was obtained from Christian Hansen Pvt. Ltd. in New Delhi.
- Sodium alginate and calcium chloride were obtained from Sigma Aldrich in the United States.

Optimization of sodium alginate, calcium chloride and β-D-galactosidase concentration

The enzyme concentrations (3 and 4 ml) as well as the sodium alginate concentration (2%, 3%, and 4%) were measured. The enzyme was mixed with sodium alginate solution before being added to calcium alginate solutions of 2%, 3%, and 4% concentrations, respectively. The best quality of beads was optimized based on enzyme holding capacity into beads with spherical shape and stability for reutilization in subsequent cycles. The volume of enzyme (3ml and 4ml) was mixed with 2% sodium alginate and dripped into 3% calcium chloride solution, but stable beads to hold the enzyme were not found. When the beads were used for lactose hydrolysis in milk after the first cycle, they became too fragile and spongy. Because the beads were fragile and spongy, they could not be used in the next batch. Choi et al,(13) demonstrated that 2% alginate concentration decreased the loading efficiency of enzyme due to diffusion limitation. As a result, the sodium alginate concentration was increased to 3% to ensure that the beads could be reused in subsequent batches of milk. Sodium alginate at 4% was found to be too viscous, so a combination of enzyme and sodium alginate was added to a calcium chloride solution at 3% concentration. The beads formed were not stable and rigid at 3% calcium chloride solution concentration because calcium was unable to maintain the stability of beads. When the calcium chloride concentration was increased above 3%, the beads became brittle and hard, and the pH changed, causing milk to coagulate. Becerra et al., (14) reported that, 84.1% lactose hydrolysis was observed with immobilized cells reticulated in 3.0% CaCl2 while at higher concentration of CaCl2, a decrease in lactose hydrolysis was observed which might be due to the diffusion resistance with increase of CaCl2 concentration.

The best beads were optimized at 3% sodium alginate and 3% calcium chloride solution using both enzyme concentrations (3 and 4ml) and yielded spherical and rigid beads. Beads were reused in the next batch of fresh milk. However, more than 4 ml of enzyme concentration in 3% sodium alginate could not be sustained in Ca-alginate enzyme beads during reusability. Furthermore, the Ca-alginates beads were linked to the start of degeneration of entrapped biomass, as well as a decrease in its ability to carry out lactose hydrolysis.

Preparation of calcium alginate beads using immobilization

β-D-galactosidase was immobilized using calcium alginate beads, as described by (15). The combination of sodium alginate (3%) and β-D-galactosidase (4ml) were employed to produce calcium alginate beads. Using a peristaltic pump, the enzyme and alginate mixture was dropped into a 200 ml aqueous solution of calcium chloride (3%). Beads with immobilized enzyme were left in the calcium chloride solution for 30 minutes to harden appropriately. The calcium alginate beads were separated from the solution with a strainer and thoroughly washed three times with distilled water to remove excess CaCl2. Lactose hydrolysed milk was made by directly introducing -D-galactosidase beads into milk.

Preparation of lactose hydrolyzed milk

Lactose hydrolysed milk was made by directly introducing -D-galactosidase beads into milk. The experiment was carried out by placing pasteurized milk containing beads in a glass bottle (1000ml) and keeping it over a magnetic stirrer. To achieve the desired temperature and uniformity in the reaction mixture, the entire setup, including the glass bottle and magnetic stirrer, was refrigerated at 4-5°C for 12 hrs. After the first cycle, the beads were removed and washed with distilled water before proceeding to the next cycle for 12 hours, and the next fresh milk was hydrolyzed with the same amount of enzyme concentration beads that will provide reutilize milk. After that, the beads became too loose to be used in the next cycle, so they were discarded.

Preparation of Basundi

Basundi was prepared as displayed by (10). Fresh cow milk procured from local farmer and lactose hydrolysed milk using fresh beads (LHFB) and reutilization of same beads (LHRB). The optimum pH of milk was found to shift from 6.7 to 5.0 after the enzyme was immobilized. This pH change could be the result of milk coagulation. The addition of sodium phosphate at 0.03% improved the stability of lactose hydrolyzed milk. The milk started to boil almost to the partial concentration of 2.0X milk solid. The speed of stirring

and scraping was kept constant to evaporate the most moisture. At this point, sugar was added at a rate of 5% on a milk basis. However, less sugar was added @ 4% in lactose hydrolysed milk due to production of glucose and galactose during lactose hydrolysis. After adding sugar, the milk sample was continuously concentrated and vigorously stirred until 2.5X of the total solids was obtained. After final concentration, the product was allowed to cool at room temperature to achieve the desired body and texture. Following that, the product was placed in a polystyrene cup and stored at refrigerated (5-7 °C) for 12 days.

Analysis of Lactose, glucose and galactose by using RP-HPLC

Basundi preparations made with fresh milk and lactose hydrolyzed milk were withheld at four-day intervals. HPLC was used to determine lactose, glucose, and galactose using the procedure recommended by Chen et al., (16). 5 g of basundi was precisely weighed, and 5 ml of acetone (99%) solution was added for protein precipitation. After 15 minutes, the contents were filtered using a 0.45 m syringe filter. A 20l filtrate aliquot was taken for HPLC quantification of lactose, glucose, and galactose content in basundi. Flow rate was kept constant at 1 ml/min while using deionized HPLC grade water as mobile phase and column temperature was kept constant at 80°C. A refractive index detector measured lactose, glucose, and galactose concentrations. Standard lactose, glucose, and galactose concentrations ranging from 5- 25mg/ml were dissolved in distilled water. The solution was then filtered through a 0.45 m PTFE (Poly tetra fluoro ethylene) syringe filter. An aliquot of 20 µl of standard solutions was injected directly into the column via the injection port.

Proximate analysis

Basundi were analyzed for acidity as described by Indian Standards, (17) (SP:18). The Free fatty acid (FFA) levels of basundi were determined according to procedure given by (18). 10 g basundi was weighed accurately and 25ml of chloroform was added into sample and mixed properly. Then, 50 ml of neutralized ethyl alcohol was added and followed by addition of 3-4 drops of 0.5% phenolphthalein indicator. The contents of dish were mixed well and titrated against 0.1N NaOH with continuous stirring till a faint pink color appeared which persist for at least15 seconds. HMF content of basundi was determined by the method recommended by (19).

Statistical analysis

All determinations were carried out in triplicate and data was subjected to analysis of variance. In the experiments, one way and two ways analysis of variance (ANOVA) with a subsequent difference ($P > 0.05$) in the mean values was conducted as described by (20).

RESULTS AND DISCUSSION

Effect of Lactose hydrolysis on Lactose, glucose and galactose during storage period

Lactose, glucose, and galactose content of basundi prepared from fresh milk and lactose hydrolyzed milk (LHFB and LHRB) were analyzed, and figure 1 shows that significant ($p<0.05$) differences were observed in the residual lactose content of basundi prepared from fresh milk compared to basundi prepared from lactose hydrolyzed milk using fresh breads (LHFB) and reutilization of same beads (LHRB). Furthermore, similar changes were observed with increasing storage time in all basundi samples prepared from fresh milk and lactose hydrolyzed milk. The lactose content of basundi made from fresh milk and lactose hydrolyzed milk was significantly reduced during storage. A significant ($p<0.05$) decrease in lactose content was observed in basundi made from lactose hydrolyzed milk using fresh beads, followed by reutilization of same beads, and basundi prepared using fresh milk throughout the storage period. Over the course of storage, microbial fermentation resulting in the production of lactic acid and formic acid may cause a gradual decrease in the lactose content of basundi.

Similarly, significant ($P<0.05$) decrease in glucose and galactose were observed in all basundi samples prepared from lactose hydrolyzed milk using fresh beads and reutilization of the same beads (LHFB and LHRB) and fresh milk. All basundi samples showed non-significant ($P>0.05$) changes as the storage period progressed. The galactose content was found to be significantly lower ($P>0.05$) than the glucose content in basundi made from lactose hydrolyzed milk using fresh milk and reusing the same beads during storage. It could be because galactose is being converted into galacto-oligosaccharides. Our findings are consistent with those of Tossavainen and Kallioinen (21), galactose levels in lactose hydrolyzed milk were lower than glucose, and galactose was involved in the galacto-oligosaccharide process.

Effect of lactose hydrolysis on acidity development

It depicted from figure 2, On day zero, there was no significant difference in the acidity development of basundi prepared from lactose hydrolyzed milk using fresh beads (LHFB) and reusing the same beads (LHRB). In addition, there was little change in acidity in basundi made from fresh milk. It increased significantly ($p<0.05$) with the storage period after the fourth and eighth days. The acidity development was significantly ($P>0.05$) higher in basundi prepared from lactose hydrolyzed milk as the storage period

progressed, with more acidity development observed in basundi prepared from lactose hydrolyzed milk using fresh beads (LHFB) when compared to basundi prepared using reutilization of the same beads (LHRB). Furthermore, acidity development is significantly enhanced in lactose hydrolyzed milk using fresh beads, subsequently followed by reutilization of beads and control basundi when the sample is analyzed on the 12th day. It could be because monosaccharides such as glucose and galactose were liberated more in lactose hydrolyzed milk, contributing to an increase in acidity. Our findings are consistent with those of Gaikwad and Hembade, (22), who discovered that heat denaturation of proteins in lactose hydrolyzed milk occurs at temperatures above 80°C, and that the number of available amino groups with lactose caused a significant increase in acidity.

Effect of Lactose hydrolysis on free fatty acid during storage period

FFAs are formed by hydrolysis as well as cleavage and oxidation of double bonds in milk fat, and their measurement is used to determine the hydrolytic rancidity of fats and oils. Figure 3 depicts the outcomes. Initially, no significant difference was observed in basundi samples prepared from lactose hydrolyzed milk using fresh beads, followed by reutilization of the same beads, i.e., LHFB, LHRB, and control basundi. During the storage period, free fatty acids were found to be significantly ($p<0.05$) higher in basundi samples prepared from fresh milk compared to lactose hydrolyzed milk prepared from fresh beads and reusing the same beads. While observing sample on the 12th day of storage, a slight decrease in free fatty acid was observed in basundi made from lactose hydrolyzed milk using fresh beads and reusing the same beads. Furthermore, untreated basundi had significantly ($p<0.05$) higher free fatty acid levels than lactose hydrolyzed milk basundi. It could be because more reduced compounds, such as glucose and galactose, were formed in basundi made from lactose hydrolyzed milk using fresh beads (LHFB), followed by reusing the same beads (LHRB). Our findings are consistent with those of Tossavainen and Kallioinen, (21), who discovered that khoa prepared from lactose hydrolyzed buffalo milk produced less free fatty acid due to the monosaccharide produced during lactose hydrolysis of milk, which has a higher reducing property than the control. It is based on the fact that when lactose is hydrolyzed, the resulting sugar is both reducing and increases the reducing capacity of khoa prepared from lactose hydrolyzed milk.

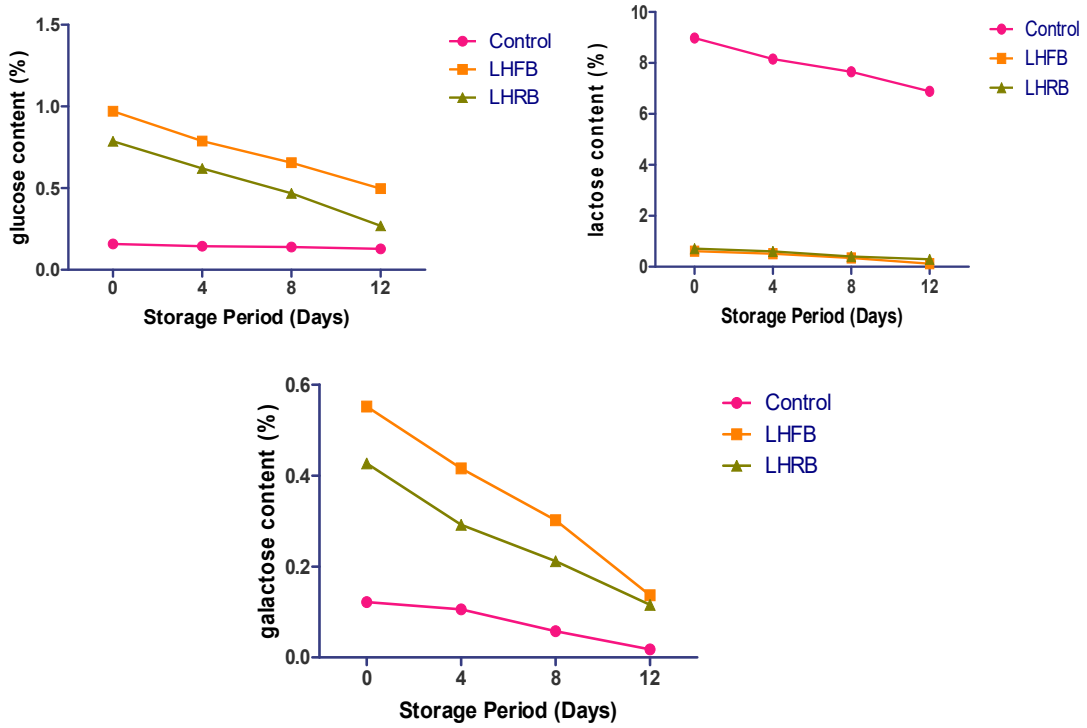

Figure 1: Graphical representation of lactose glucose and galactose content in basundi during storage period in days.

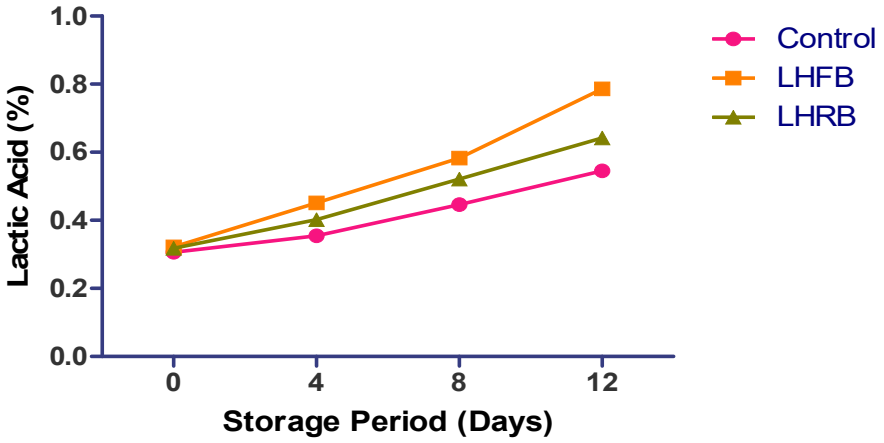

Figure 2: Total acidity in freshly prepared and lactose hydrolysed basundi during storage period in days

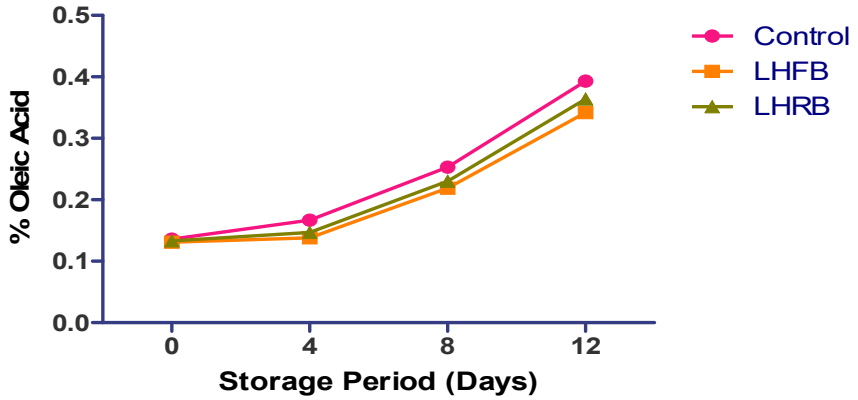

Figure 2: Total acidity in lactose hydrolysed basundi during storage period in days

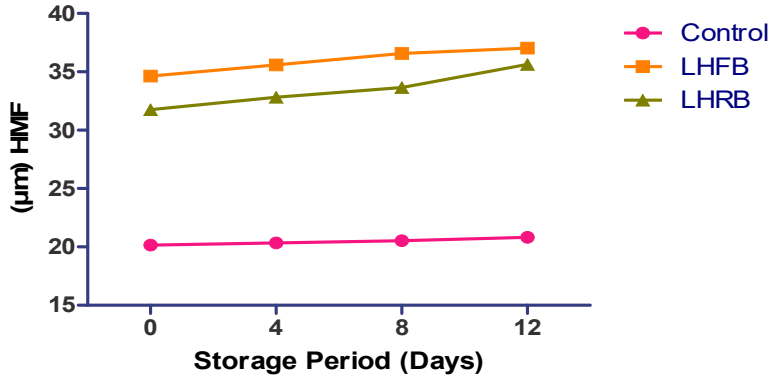

Figure 3: HMF content in freshly prepared and lactose hydrolysed basundi during storage period in days

Effect of Lactose hydrolysis on HMF content during storage period

According to Figure 4, basundi prepared from lactose hydrolyzed milk using fresh beads had a significantly ($P<0.05$) higher HMF content than basundi prepared with repurposed beads and control basundi. When basundi samples were withdrawn on the fourth and eighth days and analyzed for HMF content, it was discovered that there were no significant ($P>0.05$) differences in basundi samples prepared from lactose hydrolyzed milk using fresh beads followed by reutilization of beads and control basundi. When compared to control basundi, basundi prepared from lactose hydrolyzed milk had a significantly ($P>0.05$) higher HMF content. However, no statistically significant ($P>0.05$) differences were found in all basundi samples prepared from fresh milk and lactose hydrolyzed milk. Higher HMF in basundi prepared from lactose hydrolyzed milk was caused by higher monosaccharide (glucose and galactose) interactions with protein during lactose hydrolysis, and glucose and galactose actively participated in the Maillard reaction at higher

temperatures. Our findings are consistent with the earlier findings of Harini (23), who reported that higher HMF content was observed in lactose hydrolyzed gulabjamun, which is attributed to the release of monosaccharide formed during lactose hydrolysis as compared to control gulabjamun. Moreover, Messia (24) observed that the higher level of Maillard reaction products such as furosine and HMF were produced during the processing of the lactose-free milk as compared to control.

CONCLUSION

When it comes to the food industry, lactose hydrolysis holds great promise for creating lactose-free products that individuals with lactose intolerance can consume. Because immobilization technology allows for the repurposing of an enzyme for another cycle, its application considered as highly significant economically. The entrapment of β-D-galactosidase enzyme on calcium alginate beads reduced the lactose content in milk up to 95% and reusability of the beads into next fresh milk showed 82% reduction in lactose content of milk. Consequently, the dairy industry's use of immobilized β-galactosidase opens the door for the effective use of lactose hydrolyzed milk in basundi preparation.

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