

PRODUCTION OF SYNBiotic PRODUCT CONTAINING GALACTO-OLIGOSACCHARIDES AND *SACCHAROMYCES BOULARDII* AND EVALUATION OF ITS IN-VITRO BIFIDOGENIC EFFECT

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ABSTRACT

The galacto-oligosaccharides and *Saccharomyces boulardii* are very useful for the intestinal microbiota. The yeast acts as probiotic and used for the reduction of monosaccharides from GOS mixture. The present study was concerned with the production of synbiotic product from GOS and *S. boulardii* through transgalactosylation process. The GOS was produced from β -galactosidase (156 U/1 ml of lactose solution) of *Kluyveromyces lactis* using lactose (250g/L) as substrate at 37°C. The GOS mixture was analyzed through thin layer chromatography and megazyme kit. The maximum production of GOS occurs after 5 hr at 37°C with phosphate buffer (pH 6.5). The GOS mixture was treated with probiotic *S. boulardii*. The monosaccharides were reduced at 37°C after 4 hrs using 300 μ l yeast/5 ml GOS mixture. Then synbiotic product was formed and undergoes lyophilization procedure. The total yeast count was 3.9×10^6 CFU/g in lyophilized product. The *in vitro* bifidogenic effect was determined. *Bifidobacterium* shows more positive effect towards probiotic and combined effect of prebiotic and probiotic as compared to *Lactobacillus*.

Keywords: GOS, *Saccharomyces boulardii*, Transgalactosylation, Synbiotic product

INTRODUCTION

The synergistic combination of prebiotic and probiotic is known as synbiotic. The prebiotic favors the probiotic and activates the host's beneficial intestinal microflora (Underwood *et al.*, 2009). The combination of oligofructose and bifidobacteria is the example of symbiotic (Schaafsma, 2008).

Prebiotics are classified as those ingredients that are fermented and permit changes in composition and activity in the intestinal microbes that are beneficial to host. The examples are fructooligosaccharides (FOS), galactooligosaccharides (GOS), and inulin. The health benefits of prebiotic are prevention of diarrhoea or constipation, modulation of the metabolism of the intestinal flora and cancer prevention. GOS are carbohydrates that are non-digestible and fermented by colon bacteria. They have great role in functional foods due to their benefits such as mineral absorption, role in lipid breakdown and anti-inflammatory (Schaafsma, 2008), show less potential to dental caries.

GOS can be produced using lactose through transgalactosylation process with the help of β -galactosidase enzyme. The transgalactosylation is a process in which transgalacto-oligosaccharides (TOS) are produced by enzyme using lactose as substrate. The mono and disaccharides are also produced that are not required in prebiotic production. They are undesirable because they increase the caloric value of product by absorbing in small intestine. The hydrolysis of glycosidic bonds is done by β -galactosidase enzyme. Therefore, purity of GOS is necessary from these byproducts. It helps to evaluate the functional properties of GOS such as *in vitro* prebiotic activity and determination of structures (Hernandez *et al.*, 2009). The GOS with less monosaccharides can be produced through several methods which include size exclusion chromatography, activated charcoal treatment, diafiltration and yeast treatments. Hernandez *et al.* (2009) purified GOS using *S. cerevisiae* and removed all monosaccharides from mixture (Hernandez *et al.*, 2009). Gaulas *et al.* (2007) examined the reduction of galactose with *S. cerevisiae* (Goulas *et al.*, 2007). Kunova *et al.* (2011) removed mono as well as disaccharides from GOS mixture through *Lactobacillus helveticus* (Kunova *et al.*, 2012). The chromatographic purification was done by (Rodriguez Colinas *et al.*, 2013).

The term probiotic is defined as microorganisms that are beneficial to host when sufficient amount of which enters into small intestine. The examples of probiotics used in food are *Lactobacillus reuteri*, *L. casei* and *L. acidophilus*. *Saccharomyces bulardii* is well known yeast which often used as probiotic and

dietary supplement. It reduces the diarrhea, alleviates inflammatory diseases related to intestine, helpful in prevention of infection of *Helicobacter pylori*, inhibits the growth of pathogens (Geyik *et al.*, 2006) and stimulates antibody production against diarrhea causing toxin (Qamar *et al.*, 2001).

In Pakistan there is no pharmaceutical and food industry which produces synbiotic such as combination of (GOS) and *S. bulardii*. Mostly GOS are imported from Yakult central institute for microbiological research, Kunitachi, Tokyo, Japan and Nissin sugar manufacturing Co Ltd. There is a need to produce synbiotic at commercial level in Pakistan due to its beneficial effects and increasing demands.

MATERIALS AND METHODS

All chemicals required in this research were of highest possible purity and were procured from (Merck, Germany) unless otherwise stated. The β -galactosidase from *Kluyveromyces lactis* enzyme was purchased from Sigma Aldrich, UK. Thymol (applichem, Germany), and Lactobacillus selective agar from (LAB, Germany).

Transgalactosylation

The transgalactosylation process was done with lactose (250g/L of phosphate buffer). Different pH (5-7), enzyme concentration (156U/1 ml of lactose solution) and time of incubation (0-5h) were optimized. The process was carried out at 37°C.

Analysis of GOS

GOS was analyzed by thin layer chromatography (TLC). The glucose (GOPOD Assay), galactose and lactose were measured using megazyme assay kits (Wicklow, Ireland) following standard procedure given in manual. The GOS mixture was analyzed through TLC using following method. The standards samples and samples obtained after transgalactosylation process (2 μ l) were applied on silica gel plate. The running buffer (n-butanol, n-propanol, ethanol, and distilled water) was used in ratio of 2:3:3:2, respectively. The staining buffer (0.6% thymol, 95ml ethanol and 5 ml H₂SO₄) was sprayed on dried plate. The sugar stains were visible after 110°C heating in hot air oven.

Saccharomyces boulardii

Biflor sachet (Bocodex) (250mg) was added in 50ml sabouraud dextrose broth aseptically and incubated at 37°C on shaking incubator for 3 days.

Removal of monosaccharides from GOS through *S. boulardii*

Different probiotic concentrations (100µl-400µl) were examined for the maximum removal of monosaccharides from GOS mixture. The effect of different time of incubation (0-48 hr) was evaluated for removal of monosaccharides. After optimization the following procedure was performed. *S. boulardii* (300µl) was added in 5ml of GOS solution. The reaction was carried on 37°C for 4 hr. The sample was heated at 90°C for 5 min and centrifuged at 10000 rpm for 5 min. The supernatants were separated in eppendorfs. The analysis was done through thin layer chromatography (TLC) and megazyme kit. After optimization conditions of transgalactosylation and monosaccharides removal, the synbiotic product was prepared. At the time of maximum amount of GOS, the product was frozen at -20°C followed by lyophilization.

Lyophilization

The product was frozen. After freezing, the product was placed under vacuum. Heat was applied to frozen product. Low temperature condenser plates were allowed to convert it into solid (Sharma et al., 2019).

Total count of *S. boulardii*

The synbiotic (1ml) was taken in 9ml normal saline solution. Tenfold serial dilutions were made in different seven test tubes. The sample (100µl) from each test tube was separated on sabouraud dextrose agar (SDA) and was incubated at 37°C for 3 days. The plate containing 30-300 colonies was considered for colony forming unit (CFU). The CFU/g was calculated as follows;

$$\text{CFU/g} = \frac{\text{No of colonies} \times \text{dilution factor}}{10}$$

In vitro bifidogenic effect of GOS

Bifidogenic effect of GOS was checked *in vitro* by comparing with other sugars. The assays were done for each sugar and 10% w/v of the particular sugar was added aseptically. MRS broth was taken in five (labeled as 1, 2, 3, 4 and 5) sterilized test tubes containing 4 ml broth in each. In test tube 1 no sugar was added. It was treated as control. In test tube 2, GOS (10% w/v) was added. In test tube 3, 4 and 5 glucoses (10% w/v), lactose (10% w/v) and GOS treated with *S. boulardii* (10% w/v) respectively.

Fecal sample of infant was used as a source of bifidogenic bacteria. For the isolation of *bifidobacterium* species, fresh infants stool specimens were suspended in sterile saline (9g of NaCl per liter), followed by tenfold dilutions in the same suspension medium. Sample (100µl) of each dilution (e.g. 10⁻¹ to 10⁻⁷) was pipetted onto MRS Agar + 0.05% cystiene (bifidus selective agar) and MRS Agar (lactobacillus selective Agar), which was then spread, inoculated and incubated anaerobically in anaerobic jar having anaerobe sachet at 37°C. A loop full of the isolated *bifidobacterium* and *lactobacillus* cultures were inoculated in MRS broth in different test tubes with different sugars as illustrated in above mentioned table. Cultivation was monitored at 37°C for 48hr anaerobically (Rossi et al., 2005). Growth of bacteria was checked by measuring optical density at OD₆₀₀.

Statistical Design

The comparison between GOS and treated GOS with yeast will be done using T-test. The data regarding growth of *bifidobacteria* and *lactobacilli* will be analyzed by one-way ANOVA using SPSS version 18.0. *P* values ≤ 0.05 will be considered significant.

RESULTS

Optimization conditions of transgalactosylation reaction

The effect of different pH of phosphate buffer on transgalactosylation process was recorded. The pH was adjusted from 5 to 7. The highest conversion of lactose occurs at pH 6.5 of phosphate buffer. At pH lower or higher than 6.5, there is no or very mild transgalactosylation occur. The various concentrations of enzyme were evaluated to check the optimal enzyme concentration at which maximum transgalactosylation process occur. The enzyme concentrations were 10µl-200µl. At equal concentration of lactose solution and enzyme concentration (1:1), there is no production of galactooligosaccharides (GOS). The maximum GOS was produced with 30µl enzyme. As enzyme concentration increases (50µl-200µl), the transgalactosylation process decreases. Hence, 30µl enzyme concentration was considered optimal for further whole experiments. Different

time of incubation (15 min-5h) was examined to obtain optimal time for the higher production of GOS. After 4hr maximum GOS was produced. At 2 or 3hr very less quantity of GOS was produced. Therefore, 4hr was considered optimal for further experiments.

Production of galacto-oligosaccharides

Lactose (250g/L) was used for the production of GOS. The high concentration of lactose facilitates the reaction of transgalactosylation. Lactose solution (10ml) was used to optimize the all conditions. After optimization, the GOS was prepared using 500ml lactose (250g/L), lactose was dissolved in 50mM sodium dihydrogen phosphate buffer (pH 6.5), and 1500µl enzyme. The reaction was carried on at 37°C for 4hr at 150rpm. Initially, the glucose and galactose were formed as the reaction proceeded. As the reaction catalyzed further by enzyme, the GOS were prepared. After the fourth hour the maximum GOS were produced, approximately 40% of total sugars present in the mixture. As the time was increased the concentration of GOS was decreased because these are not thermodynamically stable products. These are formed for very short interval of time.

Analysis of GOS

Thin layer chromatography

Thin layer chromatography (TLC) was used various time to check the composition of GOS mixture produced at the end of transgalactosylation procedure. TLC was performed using lactose at different interval of time and with different concentration of enzyme to optimize the conditions where maximum GOS was produced. Figure 1 shows the TLC (10ml lactose solution and 30µl enzyme concentrations).

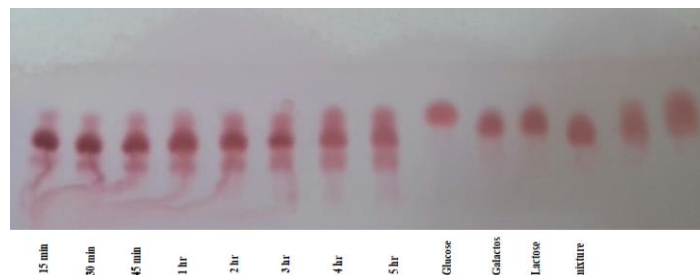


Figure 1 Thin layer chromatography (TLC) at 37°C at different interval of time (15min–5hr) with 30µl enzyme per 10ml of lactose solution (pH 6.5). Production of GOS is compared with standard solutions (20g/l glucose, 20g/l galactose, 20g/l lactose and 20g/l mixture of glucose, galactose and lactose

Quantification of GOS through megazyme kit

The maximum GOS production was at ~75% lactose conversion after 4hr of reaction. The final mixture contained 30% GOS, 30% D-glucose, 15% D-galactose and 25% untransgalactosylated lactose.

Saccharomyces boulardii

Saccharomyces boulardii was produced using biflor sachet (250mg) grown in 50ml sabouraud dextrose broth aseptically at 37°C on shaking incubator for 3 days. The simple and negative staining was performed for the identification of the yeast. The various concentrations of probiotic (*S. boulardii*) were examined to check the optimal reduction of monosaccharides from GOS mixture. The probiotic concentrations were 100µl-400µl. The maximum reduction was occurred with 300µl probiotic in 5ml prebiotic solution (GOS mixture). Therefore, 300µl culture of yeast was used for further experiments. Different time of incubation (15min-5h) was evaluated to obtain optimal time for the highest reduction of monosaccharides from GOS mixture. After 4hr maximum reduction was occurred. At 2 or 3hr very less quantity of glucose was reduced. Therefore, 4hr was considered optimum.

Removal of monosaccharides from GOS through *S. boulardii*

S. boulardii (300µl) was used for the removal of monosaccharides especially glucose. The conditions were optimized using 5ml of GOS mixture. The reaction was carried on 37°C for 4 hr. At the beginning of reaction, no glucose was removed. But with the passage of time, the glucose was reduced. The maximum reduction was occurred after 4 hr. The sample was heated at 90°C for 5 min and centrifuged at 10000rpm for 5 min. The supernatants were separated in eppendorfs. The analysis was done through thin layer chromatography (TLC) shown in. The synbiotic product containing GOS and *S. boulardii* was prepared. The product was lyophilized under the following conditions.

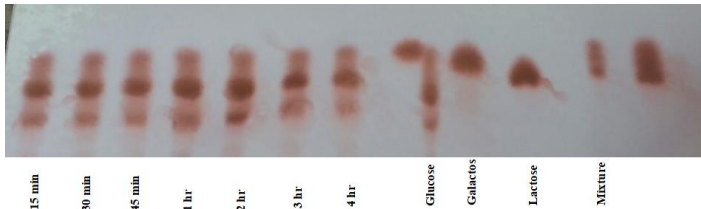


Figure 2 Thin layer chromatography (TLC) for the reduction of monosaccharides with time (15 min to 4 hr). Reduction of monosaccharides compared with standard solutions (20g/l glucose, 20g/l galactose, 20g/l lactose and 20g/l mixture of glucose, galactose and lactose

Colony forming unit of *S. boulardii* in lyophilized product

The colony count of yeast in lyophilized synbiotic product was identified using 10-fold dilution procedure. The plate containing 39 colonies was selected because it was within the standard range i.e.; 30–300 colonies. The CFU/g was calculated using following formula

$$CFU/g = \text{No of colonies} \times \text{dilution factor} \times 10$$

$$CFU/g = 39 \times 10^2 \times 10$$

$$CFU/g = 3.9 \times 10^4$$

In vitro bifidogenic effect

The *in vitro* bifidogenic effect was studied for maximum production. Different sugars glucose, lactose, GOS, GOS treated with *S. boulardii* were examined after inoculation of *Lactobacillus* and *Bifidobacterium*. The values of control were 0.337 in case of *Lactobacillus* and 0.357 in case of *Bifidobacterium*. The higher results in terms *Lactobacillus* growth ($p \leq 0.05$) was with glucose (0.567). The value of GOS treated with yeast (0.508) was closer to the value of glucose. It showed that the synbiotic product has good effect. The best results in terms *Bifidobacterium* growth was with GOS (0.433). In this case the value of glucose (0.402) was less than that of both GOS and GOS treated with yeast (0.432). It means that *Bifidobacterium* shows more positive effect towards prebiotic and combined effect of prebiotic and probiotic as compared to *Lactobacillus*.

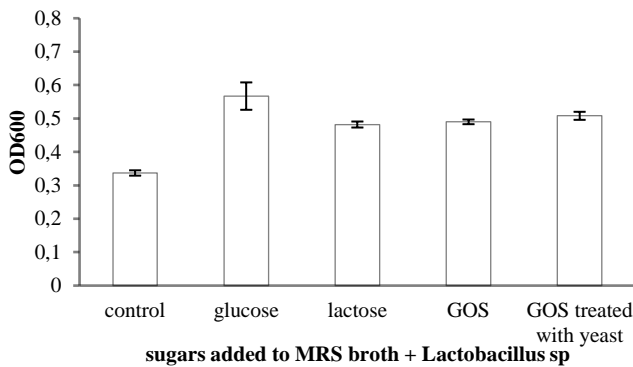


Figure 3 Behavior of *Lactobacillus* sp. with different sugars in MRS broth. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates. The values differ significantly at a level of $p \leq 0.05$

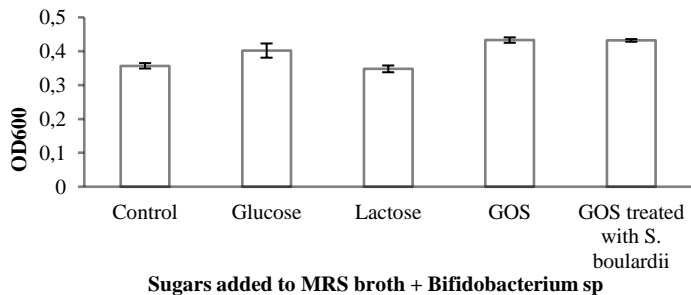


Figure 4 Behavior of *Bifidobacterium* sp. with different sugars in MRS broth. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates. The values differ significantly at a level of $p \leq 0.05$

DISCUSSION

The mixture of prebiotics and probiotics is known as Synbiotic product. The prebiotics enhance the growth of probiotic bacteria in the synbiotic product and also helpful for host’s microflora (Underwood et al., 2009). The present study is done to check either the combination containing galactooligosaccharide (GOS) and *S. boulardii* is more effective than the separate effect of prebiotics and probiotics. There is no review on the synbiotic at the clinical side as in formula fed term infants. Only the impact of prebiotics and probiotics has been examined in term of infants to prevent diseases (Osborn and Sinn, 2007).

The pronounced role of prebiotics has been determined through advanced research based on *in vitro* and *in vivo* studies. Now a day there is much awareness of prebiotics in nutritional and medical aspects. Prebiotics was first defined as non-digestible and fermented ingredients that allow specific changes in composition and activity in the intestinal microflora that give benefits to host health (Sanders et al., 2019). GOS are transparent in nature, high soluble in water and more viscous than high fructose syrups. The sweetness of GOS is third time less than sucrose and has low potential to cause dental cares as determined in *in vitro* studies (Kirsten et al., 2019). GOS can be added in several foods due to its stability, good taste, ability to enhance texture and mouth feels of foods. Therefore, GOS and other prebiotics such as FOS are widely used for commercial purposes in infant formulas, dairy products, sauces, soups, breakfast cereals, beverages, snack bars, ice creams, animal feeds, and as sugar replacements (Macfarlane et al., 2008).

The physicochemical and physiological properties of GOS mainly depend on the degree of polymerization and end products of transgalactosylation. The linkages formed in final products depend on the source of enzyme and conditions during the reaction. The galactosyl acceptor and active site of catalyst play a major role in formation of GOS mixture. β -galactosidase from microbial sources produces different linkages such as β -(1→3), β -(1→4), β -(1→6) and combination of these linkages. The β -galactosidase derived from *Bacillus circulans* and *Cryptococcus laurentii* has β -(1→4) linkage between two galactose units. While the β -galactosidase of *Aspergillus oryzae* and *Streptococcus thermophilus* produces product having β -(1→6) linkages (Tzortzis and Vulevic, 2009). In present research work β -galactosidase from *Kluyveromyces lactis* is used that containing β -(1→4) linkage (Kaplan and Hutkins, 2000). Due to these linkages GOS are resistant to adverse conditions of temperature and acidity. They remain stable at high temperature in acidic conditions even at 160°C for 10 min at pH 7. GOS are also stable at room temperature in acidic environment even for long time. Due to this property GOS can be used in beverages that stored at room temperature for several months (Leroy and De Vuyst, 2004). They are used as low caloric sweeteners in fermented milk products, bread and beverages. The products of transgalactosylation containing β -(1→6) linkage showed selectivity towards *Bifidobacteria* (Kaplan and Hutkins, 2000).

The production of GOS through β -galactosidase enzyme using lactose as substrate has been studied over the last 50 years because of its several functions as prebiotics (Yazdi et al., 2019). In present studies β -galactosidase was procured from Sigma, Aldrich. 10ml of lactose solution (250g/L) was taken and transgalactosylation process was performed to optimize the conditions. The sampling was done at different interval of time and subjected to heat for 5 min at 90°C to denature the enzyme. After that samples were analyzed through thin layer chromatography (TLC) and megazyme kit.

The results in fig 1 show that transgalactosylation was occurred maximum after 4th hours of reaction. The lactose is converted in to various products. The conversion of lactose can be easily seen in fig when compared to standards i.e.; glucose, galactose, lactose and mixture of these three sugars. The lactose was converted into GOS mixture by breaking β -linkages using 30 μ l enzyme/10ml lactose solution at 37°C for 4 hrs.

In another study GOS was produced from β -galactosidase of *Lactobacillus reuteri* L103 and *Lactobacillus plantarum*. The process was carried out at 37 °C using lactose (600mM in 50mM sodium phosphate buffer, pH 6.5). $MgCl_2$ (1mM) was also added in reaction mixture. The β -galactosidase activity was determined in term of lactose unit (U_{lac}). The yield of GOS was 41% w/w of total sugars in case of *L. plantarum* and 36% w/w of total sugars in case of *L. reuteri* L 103. The major components of GOS mixture were characterized using TLC, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (Splechtma et al., 2007a). The production of GOS from purified recombinant β -galactosidase of *Lactobacillus sakei* Lb790 was also examined. The process was carried out at 37°C using 1.2 lactase U per ml of reaction mixture at 300rpm. The initial concentration of lactose was 215g/L. The lactose was dissolved in sodium phosphate buffer (50mM, pH 6.5). After the reaction was completed, the GOS mixture was analyzed through TLC, (HPAEC-PAD) and capillary electrophoresis (CE) (Splechtma et al., 2007b).

The GOS mixture was produced by β -galactosidase of selected probiotic bacteria (*Bifidobacterium bifidum* BB-12, *Bifidobacterium infantis* DSM-20088, *Bifidobacterium pseudolongum* DSM-20099, *Bifidobacterium adolescentis* B-7, *Bifidobacterium angulatum*). When this mixture was analyzed through TLC, the different spectra were seen which was different from standard i.e. Oligomate 55.

In present study the GOS mixture was treated with probiotic (*S. boulardii*). The conditions were optimized using 5ml of GOS mixture. *S. boulardii* (300µl) was used. The reaction was carried on 37°C for 4 hr. At the beginning of reaction, no glucose was removed. But with the passage of time, the glucose was reduced. The maximum reduction was occurred after 4 hr. The sample was heated at 90°C for 5 min and centrifuged at 10000rpm for 5 min. The supernatants were separated in eppendorfs. The analysis was done through thin layer chromatography (TLC).

A study was showed by (Splechtna et al., 2007b) on the removal of glucose and galactose from GOS mixture. The separation was done using the Unibead UBK-530 strongly acidic cation-exchange resin (Mitsubishi Chemical Industries). The sample was subjected to freeze dried and desalted. After that it was dissolved in water. The sugars were approximately 70% (w/v) in solution. The 3.5ml of the solution was added to a column. The reaction was carried out at 70°C. The sampling was done and analyzed through TLC, CE and HPAEC-PAD. Various methods were used for the fractionation of oligosaccharides such as diafiltration, yeast treatment, activated charcoal adsorption. Many researchers used size exclusion chromatography for the separation of carbohydrates (Tzortzis et al., 2005). After reduction of monosaccharides, the synbiotic product containing *S. boulardii* and GOS was produced and lyophilized. The previous studies showed that *S. boulardii* was preserved after lyophilization and used as treatment for gastrointestinal disorders. But there is no study that shows the combination of *S. boulardii* and GOS in lyophilized form. This yeast is non-pathogenic and shows its beneficial effects through several modes of action i.e. competition with pathogens, inhibits pathogens from adhesion and neutralize the bacterial virulence factors and toxins (Sougioultzis et al., 2006).

The *in vitro* bifidogenic effect of sugars, GOS and synbiotic product was determined in present research work. *Lactobacillus* and *Bifidobacterium* were isolated from infant fecal sample on MRS agar through serial dilution method. After isolation, one colony of each bacterium was inoculated in MRS broth in two different set of test tubes containing glucose, lactose, GOS and GOS treated with *S. boulardii*. The incubation was occurred at 37°C anaerobically for 48 hr. Then, the growth of bacteria was checked spectrophotometrically at OD₆₀₀. In case of *Lactobacillus* the OD values were 0.567 for glucose, 0.490 for GOS and 0.508 for synbiotic product. It shows that synbiotic product is more effective as compared to GOS. The OD values for *Bifidobacterium* were 0.402 for glucose, 0.433 for GOS and 0.432 for synbiotic product. It shows that the effect of GOS and synbiotic product are almost same.

CONCLUSION

The GOS was produced from β-galactosidase enzyme using lactose through transgalactosylation. The maximum production of GOS occurs after 5 hr at 37°C with phosphate buffer (pH 6.5). The probiotic *S. boulardii* was grown and used for the treatment of GOS mixture. After that synbiotic product was developed and subjected to lyophilized procedure. Then, total yeast count was determined. The *in vitro* bifidogenic effect was determined. *Bifidobacterium* shows more positive effect towards prebiotic and combined effect of prebiotic and probiotic as compared to *Lactobacillus*.

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