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Production and optimization of β-galactosidase enzyme from probiotic *Lactobacillus sps.*

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ABSTRACT

β-galactosidase enzyme (EC 3.2.1.23) has been used in dairy industry for the improvement of lactose intolerance. The production of β-galactosidase enzyme by probiotic *Lactobacillus spp.*, was studied and their ability to hydrolyse 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and ONPG was determined. Crude enzyme extract was obtained by the lysis of the cell using lysis buffer. The β-galactosidase enzyme activity was assessed using Ortho-nitrophenyl-beta-D-galactopyranoside (ONPG) as an enzyme substrate. A protein band indicated β-galactosidase enzyme was detected by SDS-PAGE method. The β-galactosidase appeared as a single protein band in SDS-PAGE with a molecular mass of approximately 116 kDa. *Lactobacillus spp.*, produced blue colour colonies on X-Gal plates shows β-galactosidase positive results. The optimum pH and temperature for the β-galactosidase production were pH 5 (156 U/ml) and 35^{0}C (86 U/ml). The intracellular concentration of β-galactosidase enzyme increased linearly with increasing lactose concentration upto 7 % (172 U/ml) and by utilising yeast extract as nitrogen source (98 U/ml). Substitution of *Lactobacillus spp.*, producing β-galactosidase enzyme as probiotic to dairy products, could help lactose intolerant people. One variable at a time approach method was used for the optimization of medium components for the production of β-galactosidase by submerged fermentation in a semi-synthetic medium.

KEYWORDS

Lactobacillus;
β-galactosidase;
Optimization;
Fermentative production.

INTRODUCTION

Lactic acid bacteria (LAB) used as starters for production of dairy products are the main factors of fermentation and protection of fermentative foods and also have a significant role in texture and flavour of food products[3]. Lactic acid bacteria have become a focus of scientific studies for three particular reasons[23]: a) Lactose maldigesters may consume some fermented dairy products with little or no adverse effects, b) These bacteria are generally regarded as safe (GRAS) so the β-galactosidase enzyme derived from them might be used without extensive purification[26]. c) Lactic acid bacteria have probiotic activity, so suitable strain selec-

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tion must be carried out to manufacture probiotic dairy products[27]. Lactose is a disaccharide 4-O-β-D-galactopyranosyl-D-glucopyranose, found almost exclusively in milk. Therefore, lactose forms a main part of one's daily intake of carbohydrates. β- galactosidase hydrolyzes lactose into glucose and galactose, so it is commercially referred to as lactase[22]. One of the glycosidases, β-galactosidase is the enzyme widely used especially in dairy industry[4,16]. This enzyme provides two benefits that make its use attractive for dairy industry: Preparation of lactose-free milk and biosynthesis of galacto oligosaccharides that are interesting from the technological as well as health point of view. Low activity of β-galactosidase causes digestive insufficiency, called lactose intolerance in most cases[10]. Since lactose intolerance is affecting a large proportion of the people, a cheap source of β-galactosidase for the effective production of lactose-hydrolyzed dairy products is of a substantial potential[1].

The aim of this study was to select hyper producing strain of *Lactobacilli* for β-galactosidase production and optimization of process parameters for enzyme production using various sources.

MATERIALS AND METHODS

Collection of samples

Cultures of probiotic *Lactobacillus spp.,* isolated from buffalo curd maintained in the Department of virology, S. V. University, Tirupati used throughout the study. The culture was maintained on GYP agar slants.

Screening of β-galactosidase enzyme production with X-GAL

The probiotic *Lactobacillus* were grown on GYP agar medium containing 0.1% of X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) and the plates were incubated at 37^0c for 24-48 hrs[6].

Enzyme assay

Preparation of cell lysate

1.5ml of cultured broth was centrifuged at 4^0c for 5 minutes at 12000 rpm. The cells washed twice with 1 ml of PBS buffer and 200 µl of lysis buffer was added and incubated for 5 minutes at room temperature. The cells are pelleted at 12000 rpm for 5 minutes at 4^0c and the supernatant was removed. Resuspended the cell pellet with lysis buffer (200 µl) by mixing several times and centrifuged at 4^0c for 5 min at 12000rpm. The supernatant was transfered into a fresh eppendorf tube and the lysates are assayed for β-galactosidase enzyme[20].

β-galactosidase enzyme assay

β-galactosidase enzyme or hydrolytic activity was determined by measuring the release of O-nitrophenol from ONPG (o-nitrophenyl-β-D-galactopyranoside) at 420 nm. 100 µl of cell lysate was taken in an fresh tube and 900 µl of buffer A-β mercapto ethanol mixture was added and incubated in water bath at 37^0c for 10 minutes. The reaction was started by the addition of ONPG and incubated at 37^0c in water bath. After 20 min the reaction was stopped by adding 500 µl of Na_2Co_3. The O.D was recorded at 420 nm in UV spectro photometer. One unit of enzyme activity was equal to the release of 1µ.mol. of p-nitro phenol/min[15].

Biochemical assays

Sugar estimation

Several reagents have been employed to assay sugar by using their reducing properties. One such compound is 3, 5-dinitro salicylic acid (DNS reagent), in the alkaline solution, reduced to 3-amino, 5-nitro salicylic acid[14].

Protein estimation

The protein concentration was measured by Lowry's method using BSA as the standard[11].

Optimization of β-galactosidase enzyme activity using 'one variable at a time approach' method

β-galactosidase production is always depends upon the composition of the fermentation medium. Medium optimization for over production of the enzyme is an important step and involves a number of chemical and physical parameters such as the carbon, nitrogen sources, pH and temperature. The production of β-galactosidase enzyme was optimized by maintaining all parameters at a constant level, except the one being studied.

Effect of pH and temperature on β-galactosidase production

The effect of pH on the production of galactosi-

dase enzyme was determined by adjusting the medium with different pH values like 3,5,7,9,11. The fermentation broth was inoculated with 0.1% inoculum and incubated at 37°C in a rotatory shaker at 120 rpm[19]. The effect of temperature on the production of galactosidase enzyme was determined by incubating the cultured flasks at respective temperatures like 35^0c, 40^0c, 45^0c, 50^0c respectively. The inoculated cultured flasks were incubated in rotatory shaker at different temperatures for 24 hrs. After 24 hrs the culture flasks were withdrawn and microbial cells are harvested and enzyme activity was assayed[2].

Effect of lactose concentration on β-galactosidase production

The enzyme production with different concentrations of lactose was studied by supplementing 1%, 3%, 5%, 7% and 10% lactose into the medium. The sterilized medium flasks were inoculated with 0.1% inoculum of *Lactobacillus* and incubated in rotatory shaker at 120 rpm for 24 hrs. Samples were withdrawn after 24 hrs, microbial cells are harvested and β-galactosidase activity was assayed[12].

Effect of different nitrogen sources on β-galactosidase production

To study the enzyme production with various nitrogen sources the medium was supplemented with beef extract, peptone, yeast extract, and ammonium nitrate separately. The sterilized medium flasks with different nitrogen sources are inoculated with 0.1% inoculum of *Lactobacillus* isolate and incubated in rotatory shaker at 120 rpm for 24 hrs. Samples were withdrawn after 24 hrs and microbial cells are harvested and β-galactosidase activity was assayed[19].

β-galactosidase enzyme production in optimized medium

In the present study, all the optimized factors are assigned as indicated in TABLE 1. Submerged fermentation was carried out in 250 ml Erlenmeyer flask containing 100 ml of optimized production medium.

TABLE 1 : Selected factors assigned for β-galactosidase enzyme production

Lactose	7 g/100ml
Yeast extract	2 g/100ml
pH	5
Temperature	37^0c/100ml

Protein extraction and SDS-PAGE

The probiotic *Lactobacillus* were grown in GYP broth at 37^0c for 24 hrs. The cells were harvested by centrifugation at 10000 rpm for 5 minutes. The pellet was washed with 1 M Tris Hcl buffer (pH 6.8) and resuspended in 80 µl of the sample buffer (1 M Tris Hcl, pH 6.8, 20%SDS, 20%glycerol, 10%β-mercaptoethanol and 0.005% bromophenol blue) and boiled immediately in water bath at 100^0c for 5 min. After boiling, the samples were placed on ice immediately for 5 minutes and used for SDS-PAGE[13]. For electrophoresis 12.5% separating gel and 6.5% resolving gel were prepared. A volume of 15 µl of protein sample was loaded on gel and was run on mini gel electrophoresis at 100V for 2hrs and stained in a solution containing 0.1% coomassie blue, 10% acetic acid and 40% methanol. Destaining was performed in a solution containing 10% acetic acid and 4.5% methanol. The protein molecular mass marker (250-10 k Da) was used as standard[5].

RESULTS

Screening of β-galactosidase enzyme production

The *Lactobacillus* isolated from buffalo curd having thermo, osmo, acid tolerance and antimicrobial property was selected for the production of β-galactosidase enzyme. The isolate under study was found to produce β-galactosidase in considerable amount. The bacteria grown on agar medium containing 0.1% X-GAL produced blue color colonies. β-galactosidase when reacts with X-GAL gives blue colour chro-

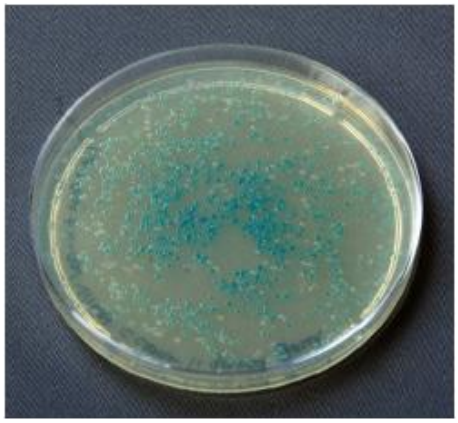

Figure 1 : *Lactobacillus* with blue colonies on X-gal contained medium

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mophore (Figure 1). This is the confirmation test for β-galactosidase enzyme producing bacteria.

Biochemical assays

Sugar estimation

Total reducing sugar concentration was determined by the di nitro salicylic acid method. The reducing sugar released from β-galactosidase per ml per minute at 540 nm was determined and calculated as 116 µg/ml. One unit of β-galactosidase activity was defined as the amount of enzyme releasing 1 u mole of reducing sugar per minute.

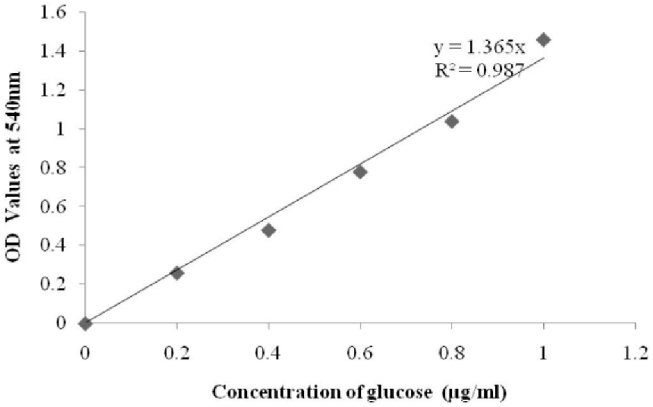


Figure 2 : Estimation of sugar

Protein estimation

The concentration of protein in the purified cell lysate was determined by Lowry's method by using bovine serum albumin as a standard. The protein concentration was calculated as 158 µg/ml.

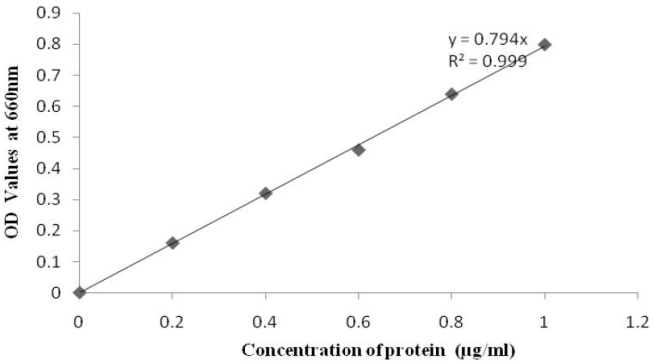


Figure 3 : Estimation of protein

Optimization of β-galactosidase enzyme activity using' one variable at a time approach' method

The *Lactobacillus* isolate which was screened for the β-gal enzyme production using X-Gal media was used for the hyper production of the β-galactosidase enzyme by optimizing the cultural conditions by varying the temperature, pH, carbon and nitrogen sources.

Effect of pH on β-galactosidase production

The β-galactosidase enzyme production was found maximum at pH 5 (156 U/ml). The β-galactosidase enzyme activity was decreased gradually when the pH increases (Figure 4).

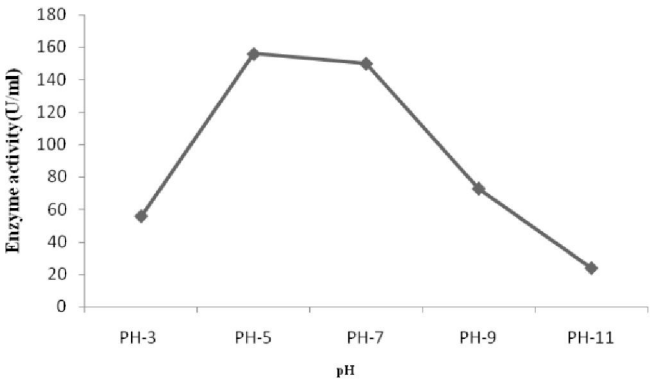


Figure 4 : Effect of pH on β-galactosidase enzyme production

Effect of temperature on β-galactosidase production

The enzyme production was stable at (35^0c-50^0c) range of temperatures and the maximum production was obtained at 35^0c of 86 U/ml and it was 78 U/ml at 40^0c and 71 U/ml at 45^0c. The enzyme drastically losts its activity at higher temp and showed only 39 U/ml of enzyme activity at 50^0C which is shown in the figure 5. β-gal with high catalytic activities might prove useful for removing lactose from milk, to be consumed by lactose intolerant population and converting the lactose present in whey into galactose and glucose to be used as carbon sources in fermentation processes.

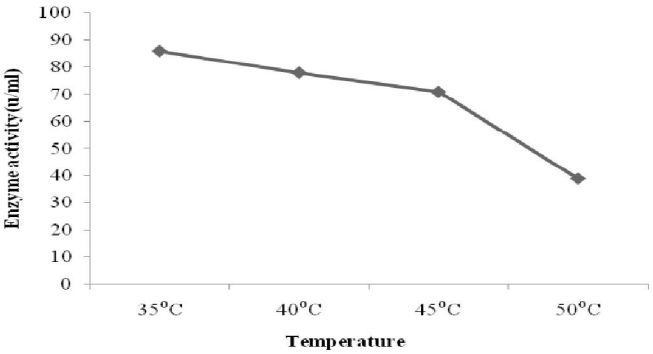


Figure 5 : Effect of temperature on β-galactosidase enzyme production

Effect of lactose concentration on β-galactosidase production

The *Lactobacillus* isolate showed maximum β-ga-

lactosidase enzyme production at 7% lactose concentration of 172 U/ml. As the incubation period extended, the enzyme production was also increased up to 72 hrs, later a decline in the enzyme production was noticed (148 U/ml). Simultaneously as the lactose concentration is increased, the enzyme production was increased up to 7% and gradual decrease in the enzyme production was observed when lactose concentration raised to 10 %.

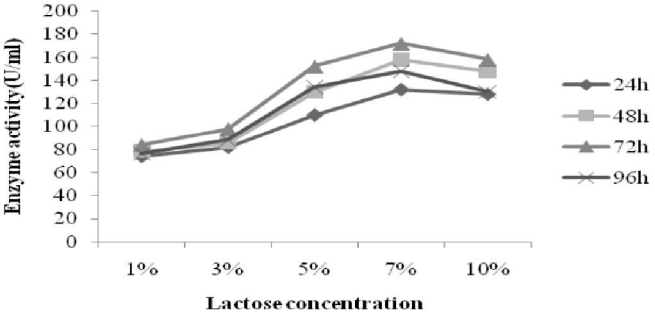


Figure 6 : Effect of lactose concentration on β-galactosidase enzyme production

Effect of different nitrogen sources on β-galactosidase production

Among the different nitrogen sources used for the production of β - galactosidase enzyme, the maximum enzyme production was obtained with yeast extract (98 U/ml) followed by tryptone (91 U/ml), beef extract (88 U/ml), peptone (83 U/ml) and an inorganic nitrogen source ammonium nitrate (24 U/ml).

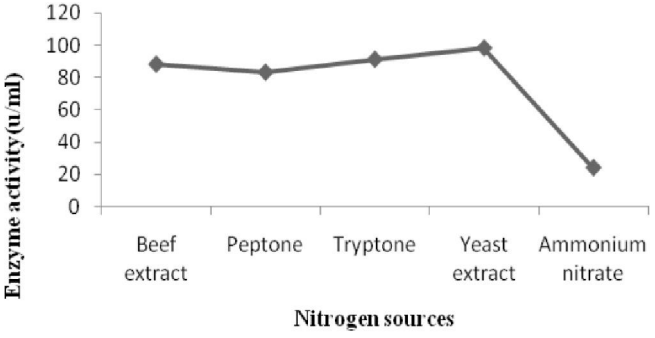


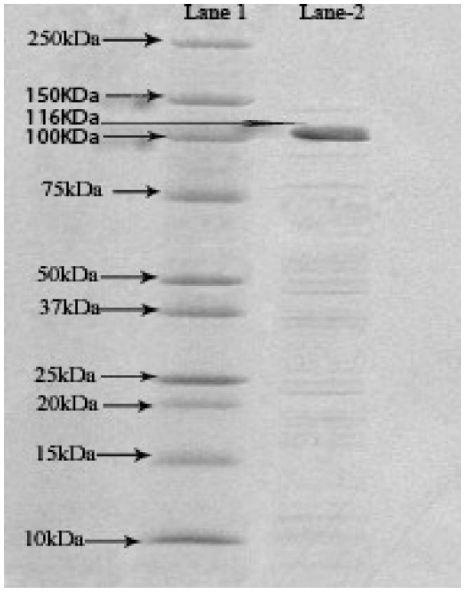
Figure 7 : Effect of different nitrogen sources on β-galactosidase enzyme production

β-galactosidase enzyme production in optimized medium

The designed optimized medium showed significant variation in β-galactosidase production by submerged fermentation. The production levels were found to be dependent on the culture condition and medium component. It could be observed that there is an increased expectation on production of β-galactosidase enzyme from 172 U/ml to 267 U/ml with the optimum conditions.

Protein extraction and SDS-PAGE

In order to determine tne molecular size of the protein isolated from *lactobacillus* the purified protein sample was subjected to SDS-PAGE analysis. As shown in (figure 8) the estimated molecular mass of the protein was found to be 116 kDa as evidenced in SDS-PAGE.



Lane 1 : Protein marker; Lane 2 : Sample protein.

Figure 8 : SDS-PAGE analysis of β-galactosidase from *Lactobacillus*.

DISCUSSION

Microorganisms which are used for production of β-galactosidase in the current study are probiotic *Lactobacillus*. Lactic acid bacteria are generally regarded as safe, so their enzyme\s can be used directly in foodstuff without any need for purification[26]. In addition to their nutritive value enzymes and other biochemical products from the microorganisms are obtained and are extremely useful for various purposes. A long term objective of the present work is to discover novel and potentially useful microorganisms or enzymes with high catalytic activities, β-galactosidases, which converts lactose in to galactose and glucose to be used as carbon sources

in fermentation processes. The ability to ferment lactose is critical to success of dairy fermentation involving milk or whey where lactose is the dominant carbon and energy substrate for growth and acid production. *Lactobacilli* are the most abundant bacteria in gastrointestinal tract. They are the most important food fermentators and used as starters. Also these bacteria have the role in flavor of dairy products, sausage and some other fermentative foods. β-galactosidase is an enzyme that produced by some of bacteria, especially *Lactobacilli* in dairy products that is yoghurt, cheese and milk. Lactose intolerance has been recognized for many years as a common problem in many children and most adults throughout the world[8]. Therefore, by addition of *Lactobacillus* producing β-galactosidase as probiotic to milk and cheese and other dairy products could help lactose intolerance symptoms. This enzyme hydrolyses lactose the main crbohydrate in milk into glucose and galactose, which can be absorbed across the intestinal epithelium[25].

A long term objective of the present work is to discover novel and potentially useful microorganisms or enzymes with high catalytic activities of β-galactosidases, which converts lactose in to galactose and glucose to be used as carbon sources in fermentation processes.

Favier *et al*.[7] reported a method to detect bacteria with β-galactosidase activity by X-gal. To screen the β-galactosidase producing bacteria X- gal method was used. Bacteria grown on X-gal medium, appearance of green coloured colonies shows the presence of β-galactosidase enzyme. In our study, the strain was found to produce β-galactosidase and the maximum enzyme production of 262 U/ml was obtained. The enzyme was characterized using various parameters, such as ONPG as substrate, X-gal methods and molecular weight and the results are comparable with the previous reports[7]. Similar findings were also reported by Vinderola and Reinheimer that high value of β-galactosidase enzyme in commercial strains of *L.delbrueckii Subsp. Bulgaricus* isolated from cheese[27]. In our study, strain of *Lactobacillus sps* screened from curd sample possessing maximum enzyme activity were detected.

According to[24], the selection of suitable strain of a microorganism can be regarded as the primary requirement for the fermentative production of enzyme. Strain was carefully selected for high growth and fermentation rate, low pH fermentation optimum and high temperature fermentation optimum, due to harsh environmental conditions (temperature, variation in pH and under which they have to survive in the gastro intestinal tract) for production of healthcare products. Hence the optimal conditions for β-galactosidase enzyme activity were determined at various temperature, pH, nitrogen sources and different lactose concentrations. The results of the present study, indicated that the *Lactobacillus* showed maximum enzyme activity at pH 5 (156 U/ml), 35^0c temperature (86 U/ml), 7% lactose concentration (172 U/ml) and by utilizing yeast extract (98 U/ml) as nitrogen source. Such conditions can enhance the rate of lactose hydrolysis. Nitrogen source is an important amendment that effects enzyme production. The optimized medium increased the production of β-gal of about 262 U/ml, when compared with initial production 172 U/ml. In this study, using SDS-PAGE method, an intensive 116 k Da protein band was observed in probiotic *Lactobacillus.* Biochemical tests which are rapid, cheap and simple in all laboratory and do not need any expensive molecular materials and apparatus, is recommended. So the probiotic *Lactobacillus* that produce β-galactosidase can be detected by X-gal and ONPG tests. Results are also in the agreement with the reports of previous studies[17].

CONCLUSION

Lactose intolerance is a physiological state in human beings where they lack the ability to produce an enzyme named lactase/β-galactosidase. Individuals lacking lactase will not be able to digest milk and is often possess a problem in new born infants. The avoidance of milk has a negative effect on calcium and vitamin-D intake in infants, children and adolescence which must provided by other dairy products, therefore consumption of cheese or modified milk (containing *Lactobacilli* with high enzyme value) is recommended. The isolated *Lactobacillus spp.* was found to produce β-galactosidase enzyme. This probiotic organism may be fed to lactose intolerance patients, milk lactose is hydrolysed by the probiotic *Lactobacillus* strain, where the lactose is assimilated and absorption of calcium also

favored and can prevent the problems like vomiting, diarrhea, nausea and calcium deficiency etc.

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