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Optimization of B-Galactosidase Produced by a Potential Lactic Acid Bacteria *Lactobacillus casei* MB2 Isolated from Traditional Dairy Product of Himachal Pradesh

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ABSTRACT

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β -galactosidase enzyme is commercially important and is generally used in dairy industries for the improvement of lactose intolerance. In this study, different strains of isolated lactic acid bacteria were evaluated for their β -galactosidase productivity, but *Lactobacillus casei* MB2 resulted with the highest production. Therefore, optimal conditions were determined in submerged fermentation processes by using the “one factor at a time” approach. The optimal cultivation conditions predicated from this method were achieved by using MRS medium on 2nd day, pH 7.0, temperature 30°C with inoculum size of 4%, lactose as carbon source, peptone as nitrogen source with 3% substrate (lactose) concentration, Mg²⁺ as divalent ions and Tween 20 as surfactant added with an approximate yield of 139.65 IU/ml escalating the level of β -galactosidase production.

Introduction

β -galactosidase (β -D-galactosidegalactohydrolase, E.C.3.2.1.23, trivially lactase) is an enzyme that hydrolyze the lactose and it is an important enzyme that has been long accepted for dairy industry. One of the glycosidases, is β -galactosidase enzyme that are mostly produced by lactobacilli and widely used in dairy industry. The study of β -galactosidase production from different microorganisms is

not new but many of these microbes have not been approved for industrial use because not being recognized as food grade microorganisms by WHO.

Moreover, the yield from studied microorganisms is also low that limits their use for commercial purpose. Therefore, higher production of β -galactosidase from food grade probiotic microorganisms is highly cherished in industry and thus novel lactic

acid bacteria producing high level of β -galactosidase are most sought after globally by researchers.

Lactic acid bacteria (LAB) are considered as good source of enzyme because of their GRAS status. β -galactosidase from food grade probiotic microorganisms are safe for human use. LAB also plays a significant role in the food fermentation processes. Interest in LAB has increased because of their potential production of β -galactosidase. Capacity of LAB to produce β -galactosidase has widened their industrial applications. These β -galactosidase producing strains of LAB are used for the production of lactose intolerance yoghurt, cheese, acidophilus milk and milk based desserts.

More than 70% of the world's population suffer from the problem of lactose intolerance and it is caused by the low activity of β -galactosidase in the mucosa of the small intestine (Nogales and Lopez, 2006). The problem of lactose intolerance is prevalent and thus lactose free dairy products are in high demand in the market to cater the need of lactose intolerant patients. Enzymatic hydrolysis of lactose by β -galactosidase is one of the most popular technology to produce lactose reduced milk and related dairy products for consumption by lactose intolerant people. Consequently, there is a need for novel lactic acid bacterial isolates that are capable of producing economic quantities of β -galactosidases with the ability to function efficiently at high or low temperatures.

The objective of our study was to explore the potential of lactic acid bacteria isolated from traditional dairy products of Himachal Pradesh, India and to improve the yield of β -galactosidase produced from it by optimizing different environmental parameters affecting its activity.

Materials and Methods

Isolation of lactic acid bacteria

Different dairy products were collected from Himachal Pradesh for the isolation of β -galactosidase producing lactic acid bacteria. The serial dilution method was adopted for the isolation. Stock was made from these samples by adding 1 ml of sample in 9 ml of distilled water. All samples were serially diluted by serial dilution in the dilution range of 10^{-1} to 10^{-9} . The samples (0.1 ml each) from each dilution were mounted by spread plate method on sterilized petri plates containing solidified selected medium *viz.* de Man, Rogosa and Sharpe (MRS) agar medium (de Man *et al.*, 1960) for lactic acid bacteria. Plates were incubated at 37°C for 48 h under anaerobic conditions. The colonies so obtained were further sub-cultured and pure lines were established and maintained on the same medium.

Screening of isolates for β -galactosidase production

Qualitative screening of β -galactosidase

β -galactosidase production was evaluated as described by Lin *et al.*, (1989).

β -galactosidase activity assay (Miller, 1992)

1ml of bacterial cell pellets were harvested by centrifugation at 9000 rpm for 15 min and then washed twice with Z-Buffer. Resuspend the bacterial cell pellets in 1 ml of chilled Z-Buffer. Absorbance of cell suspension was measured at 600 nm against Z-buffer. For each reaction mixture, 1 ml of resuspended cells were permeabilized by adding 100 μ l chloroform and 50 μ l 0.1% SDS. The tubes were vortexed for 30 sec and equilibrated for 5 min in water bath at 37°C . The reaction was started by adding 0.2 ml ONPG (4 mg/ml)

substrate followed by incubation at 37 °C till the yellow color was appear. The reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃ and contents were centrifuged to remove debris and chloroform. OD was recorded at 420 nm and 550 nm. Miller units for β-galactosidase were calculated using following formula.

$$\text{Miller units} = 1000 * [\text{OD}_{420} - 1.75 * \text{OD}_{550}] / T * V * \text{OD}_{600}$$

where OD₄₂₀ and OD₅₅₀ are read from reaction mixture, OD₆₀₀ is cell density in washed cell suspension, T = reaction time (min), V = culture volume (ml) used in assay.

Protein content of culture filtrate was also determined by Folin-Ciocalteu reagent using Bovine Serum Albumin (BSA) standard (Lowry *et al.*, 1951). The best lactic acid bacterial strain showing maximum β-galactosidase activity without was selected for optimization studies.

Identification of hyper β-galactosidase producing isolate

Morphological studies

Characteristics of selected bacterial colonies were observed according to colony color, elevation, margin and by using differential staining method.

16S rRNA technique

Selected bacterial isolate was further identified at genomic level using 16S rRNA technique. PCR amplification was done from the genomic DNA by using forward and reverse primers i.e 16SF (5'AGAGTTTGATCCTGGTCA3') and 16SR(5'TACCTTGTTACGACTT3'). The translated nucleotide sequence was then analyzed for similarities by BLASTN tool (www.ncbi.nlm.nih.gov:80/BLAST).

Optimization of process parameters for hyper β-galactosidase production by one variable at a time (OVAT) approach

Various growth conditions *viz.* effect of incubation time, inoculum size, temperature, pH, carbon source, nitrogen, divalent cations, best carbon source concentration and surfactants were studied to monitor their effect on β-galactosidase production.

Effect of incubation time

Flask containing 50 ml of production medium (i.e MRS agar medium) were inoculated with 2% seed culture and incubated at 37°C with constant shaking. Following incubation for various time interval (24, 48, 72, ..., 144 h). The culture was centrifuged at 12,000 rpm for 15-20 min and bacterial pellets were collected and β-galactosidase activity was assayed by method of Miller, 1992.

Effect of inoculum size

Culture flasks each containing MRS agar medium were inoculated at a level of 1%, 2%, 3%, ..., 6% (v/v). The enzyme was extracted from each set following an incubation of 48 h at 37°C. β-galactosidase assay was performed to quantify the enzyme.

Effect of pH

The pH of optimized media was set at different levels such as 4, 5, 6, 7, 8, 9, 10 and activity of β-galactosidase was determined after incubation of 2 days at 37°C under constant shaking at 120 rpm.

Effect of temperature

Erlenmeyer flask each containing 50 ml of optimized medium was seeded and incubated at a temperature range varying from 25, 30, 35, 40, 45, 50°C for 2 days under optimized

pH condition. After incubation β -galactosidase was extracted and assayed.

Effect of different carbon sources

Various carbon sources including glucose, lactose, maltose, fructose, sucrose and xylose were added in each of the flask containing optimized medium (50 ml). Enzyme production was measured after 2 days at 30°C by Miller's method (Miller, 1992).

Effect of substrate concentration

Erlenmeyer flask each containing 50 ml of optimized medium was seeded with 2% bacterial culture. The flasks were incubated at 30°C at 120 rpm with different lactose concentration ranging from 1% to 6%. Enzyme production was measured after 2 days at 30°C by Miller's method (Miller, 1992).

Effect of different nitrogen sources

Different nitrogen sources i.e. peptone, beef extract, sodium chloride, ammonium nitrate, sodium nitrate and urea were used at a concentration of 1.0%. All the flasks were incubated at 30°C at 120 rpm. The enzymes was extracted and assayed for activity on 2nd day of incubation.

Effect of divalent ions

Various divalent ions including i.e. Ca^{2+} , Mg^{2+} , Cu^{2+} , Ba^{2+} , Fe^{2+} and Zn^{2+} were added in each of the flask containing optimized medium (50 ml). Enzyme production was measured after 2 days at 30°C by Miller's method.

Effect of surfactants

Various detergents like Tween 80, Tween 20, SDS, Glycerol, EDTA and Triton X100 were

added in optimized medium and their effect on β -galactosidase production was estimated after incubation at 30°C for 2 days under constant shaking conditions. The enzymes was then extracted by centrifugation at 12,000 rpm for 15-20min and assayed for activity by using Miller's Method.

Results and Discussion

The selected lactic acid bacterial strain MB2 was creamish in color having circular form, raised elevation and entire margin (Plate 1) It was gram positive in nature with bacilli in shape and had been identified as *Lactobacillus casei* MB2 using (16S rRNA) PCR technique (Plate2).

Lactobacillus casei MB2 showing maximum β -galactosidase activity i.e. 30.15 IU ml⁻¹

Optimization of β -galactosidase production of *Lactobacillus* sp.MK4 by using one variable at a time (OVAT) approach

Effect of incubation period

β -galactosidase activity was measured at regular intervals from 24 h to a period of 144 h. For *Lactobacillus casei* MB2, maximum β -galactosidase activity of 32.39 IU/ml was noticed at 48th h with a specific activity of 154.23 IU/mg (Fig 1). For *Lactobacillus casei* MB2, initial incubation period of 24 h have resulted in a lesser enzymatic activity of 25.52 IU/ml. Standard error of mean and critical difference has been given in the end of the table.

Effect of inoculum size

The size of inoculum plays an important role in the fermentation of enzymes. Different inoculum sizes studied for enhanced enzyme production were 1.0, 2.0, ..., 6.0% (v/v). Fig2 reveals the effect of inoculum size on enzyme

production by *Lactobacillus casei* MB2. Highest β -galactosidase titres of 63.25 IU/ml from *L. casei* MB2 were observed at 4% inoculum size. The β -galactosidase production was constantly increases from 2% to 4% inoculums size.

Maximal enzyme activity at optimal level was observed because at this point there is average number of cells which are highly active for maximal production of β -galactosidase. The decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks.

Effect of pH

Enzyme activity is markedly affected by pH. The effect of pH is crucial in terms of growth of the producing organism and the biosynthesis of β -galactosidase. The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006).

Keeping in view its importance, the effect of pH in the range of 5.0-10.0 was evaluated on β -galactosidase production. Fig 3 represents the effect of pH on the enzyme production potential from *Lactobacillus casei*MB2.

*L. casei*MB2 showed maximum enzyme activity of 63.15 IU/ml was observed at pH 7.0 with a specific activity of 203.70 IU/mg. At pH 5.0 and 6.0, β -galactosidase activity of 42.24 IU/ml and 54.37 IU/ml respectively was observed. Above optimum, pH 10.0 showed a dipped β -galactosidase activity of 32.28 IU/ml.

Changes in the external pH alter the ionization of nutrient molecules and reduce their availability to the organism thus

lowering their overall metabolic activity (Willey *et al.*, 2008).

Effect of temperature

The fermentation temperature appeared to have a dramatic effect on β -galactosidase MB2 production. *L. casei* produced maximum β -galactosidase activity (78.97IU/ml) at temperature of 30°C, while displaying minimum activity at 30°C (27.55 IU/ml) as is depicted in Fig 4.

Any temperature beyond the optimum range is found to have some adverse effects on the metabolic activities of the microorganisms and it is also reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperatures (Okolo *et al.*, 1995).

Effect of various carbon sources

Carbon source in the form of either monosaccharides or polysaccharides influence the production of β -galactosidase. β -galactosidase is an inducible enzyme and is generally induced in the presence of lactose. Perusal of the Fig5 revealed that *Lactobacillus casei* MB2 exhibited highest β -galactosidase activity of 102.52 IU/ml with lactose as a carbon source with a specific activity of 277.08 IU/mg. Other carbon sources such as glucose, maltose and sucrose failed to significantly induce β -galactosidase production.

On the basis of catabolic repression with intracellular accumulation of glucose by-products and the fact that bacteria primarily consume simple sugars, thus does not induce significant β -galactosidase production (Murad *et al.*, 2011). β -galactosidase production was induced by the presence of lactose as a carbon source yielding 102.52 IU/ml enzyme.

Effect of substrate concentration (%)

Besides the nature of carbon sources in the culture media, the amount of it proved to be equally important from the aspect of the β -galactosidase production (Ismail *et al.*, 2010). Varying concentrations of the best substrate i.e. lactose for *L. casei*MB2 ranging from 1.0 to 6.0% were explored for its effect on the enzyme yield. The results have been given in Fig6.

*L. casei*MB2, an induction in β -galactosidase production at 3% lactose concentration was observed with yield of 125.82 IU/ml and specific activity 314.55 IU/mg. β -galactosidase is lactose inducible enzyme and its activity yield represents a compromise between lactose induction and intracellular glucose repression.

There were statistical significant variations among the different substrate concentrations used for β -galactosidase production ranging from 1.0-6.0%. Very low substrate concentration fails to trigger enzyme production to desirable level because most of the inoculum remains without substrate and hence resulting in minimum secretion of enzymes. Optimum substrate concentration normally results in an increase in the yield and reaction rate of the hydrolysis (Regina *et al.*, 2008). However, high substrate concentration causes substrate inhibition, which substantially lowers enzyme production (Liu and Yang, 2007; Singhania *et al.*, 2007). An increase in substrate concentration from optimum upto 6.0% has resulted in decreased β -galactosidase activity.

Effect of various nitrogen sources

Nitrogen sources are the most important secondary energy compounds for the growth and metabolism of microorganisms. The nature of these compounds and the

concentration used may stimulate or down regulate the production of enzymes (Sharma and Singh, 2014). Fig 7 represents the effect of nitrogen sources on β -galactosidase production by *L. casei*MB2. *L. casei*MB2 enzyme production medium supplemented with peptone showed an increased enzyme activity of 126.24 IU/ml (specific activity of 315.60 IU/mg). The other nitrogen sources used *viz.* beef extract, sodium chloride, sodium nitrate, ammonium nitrate and urea however showed a decline in enzyme activity.

Effect of divalent ions

Enzyme action is specific to the presence or absence of a particular metal ion. Likewise, *L. casei*MB2 exhibited quite a variable behaviour towards β -galactosidase secretion in the presence of various metal ions in the production medium. As metal ions accept or donate electrons and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, bind enzyme and substrate by coordinate bonds, hold the reacting groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme (Palmer, 2001). Most of the enzymes are metalloenzymes by nature and exhibit an increased activity in the presence of metal ions. Fig8 represent the effect of various metal ions on β -galactosidase production by *L. casei* MB2

Effect of surfactants

Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability (Hashemi *et al.*, 2010). Therefore, addition of these surfactants is used for the production of enzymes (Sivaramakrishnan *et al.*, 2006). Most organisms are stimulated to increased enzyme production; however, enzyme production by some organisms is only slightly affected in the presence of surfactants.

The increase was less pronounced in the organisms which are normally good producers of the enzyme (Reese and Maguire, 1969). Fig-9 represent the effect of surfactants on β -galactosidase production by *L. casei*MB2. All the surfactants resulted in considerable by slightly enhancing the enzyme activity to varying extents. β -galactosidase production by *L. casei*MB2 also showed the same trend with the addition of surfactants such as Tween 20. With Tween 20 in the medium, enzyme activity was observed as 139.65 IU/ml with a specific activity of 332.50 IU/mg protein.

The maximum production of β -galactosidase was observed in the medium containing Tween 20 and minimum production was observed in the medium containing SDS and rest were found significantly different.

Classical approach i.e. one variable at a time (OVAT) used for optimization of β -galactosidase production from *L. casei*MB2 has resulted in statistically significant increase in the production of β -galactosidase.

Discussion

El Kader *et al.*, (2012) optimized the incubation time for maximum production of β -galactosidase by *B. subtilis* was at 48 h and observed that prolonged incubation time beyond this period did not increase the enzyme yield. They observed a maximum β -galactosidase activity of 440 IU/ml at stationary phase of *B. subtilis*. The β -galactosidase activity production exhibited a similar trend as the cell concentration curve and increased gradually as the fermentation started, reaching the maximum activity i.e. 0.781 IU/ml by *L. acidophilus* after incubation of 48 h (Carevic *et al.*, 2015).

In literature, inoculum size of 2% has been reported optimum for β -galactosidase production from *L. plantarum* (Khusniati *et*

al., 2015). Ahmed *et al.*, (2016) reported an enzyme activity of 91.49 IU/ml from *Lactobacillus sp.* KLSA 22 at an inoculum size of 1%. A higher inoculum size of 4% has been reported by Lu *et al.*, (2010) for β -galactosidase production by *Enterobacter cloacae* B5 yielding 14.964 IU/ml of enzyme.

There are several reports available in the literature regarding the production of β -galactosidase at neutral pH. Princely *et al.*, (2013) reported β -galactosidase production from *Streptococcus thermophilus* producing β -galactosidase at pH 7.2. Ahmed *et al.*, (2016) reported the maximum β -galactosidase production of 89.94 IU at pH 6.5 from *Lactobacillus sp.* KLSA 22.

Devi *et al.*, (2011) isolated *Lactobacillus sp.* from curd and yielded 86 IU/ml at 35°C. In accordance to our findings, Murad *et al.*, (2011) reported β -galactosidase production at 30°C by *Lactobacillus* strain isolated from different dairy sources. *Lactobacillus sp.* KLSA 22 isolated from milk by Ahmed *et al.*, (2016) yielded 90.05 IU/ml enzyme at 35°C. *B. subtilis* reported the maximum β -galactosidase at 35°C (Natarajan *et al.*, 2012).

Lactose has also been found to increase enzyme production in *L. acidophilus* ATCC 4356, *L. plantarum* B110 and *Lactobacillus sp.* as reported by Carevic *et al.*, (2015); Khusniati *et al.*, (2015) and Devi *et al.*, (2011) respectively. Other carbon sources viz. sucrose, maltose, fructose and xylose did not result in a considerable β -galactosidase induction. β -galactosidase production is subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes.

Several workers have optimized substrate concentrations for efficient β -galactosidase production. Devi *et al.*, (2011) however reported 7.0% lactose as optimum for β -

galactosidase production from *Lactobacillus* sp. Perusal of these results revealed that potential β -galactosidase producing strains *Lactobacillus* sp. MK4 and *L. casei*MB2 yielding 115.63 IU/ml and 125.82 IU/ml respectively of β -galactosidase at 5.0% and 3.0% substrate concentration are potential for hyper β -galactosidase production and for their effective use in dairy industries. Further increase of lactose concentration upto 10% dramatically decreased the β -galactosidase production.

The presence of more than one nitrogen source in the medium has been reported to give better results which are evident from the results of the experiment. Yeast extract has

been shown to induce β -galactosidase production by several workers Devi *et al.*, (2011); Nataranjan *et al.*, (2012); Ismail *et al.*, (2010) for *Lactobacillus* sp., *Bacillus* sp. and *L. acidophilus*, respectively.

Ca^{2+} and Mg^{2+} have been reported to enhance enzyme activity by Chanalia *et al.*, (2018); Carevic *et al.*, (2017) and Ustok *et al.*, (2010) from *Pediococcus acidilacti*, *L. acidophilus* and *Bacillus* sp. respectively. Catalytic activity and stability of β -galactosidase have been reported to be magnesium dependent. Ahmed *et al.*, (2016) reported maximum β -galactosidase production in the presence of 0.1% MgSO_4 from *Lactobacillus* sp. KLSA 22.



Plate.1 Morphological characteristics of MB2

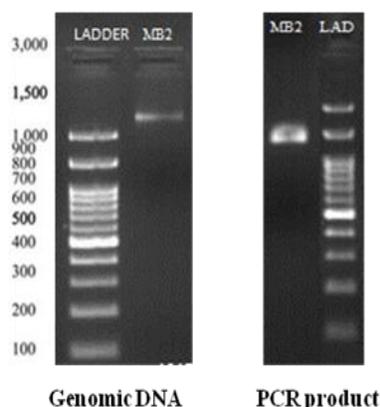


Plate.2 Molecular identification of hyper β -galactosidase producing lactic acid bacteria by 16S rRNA gene technique

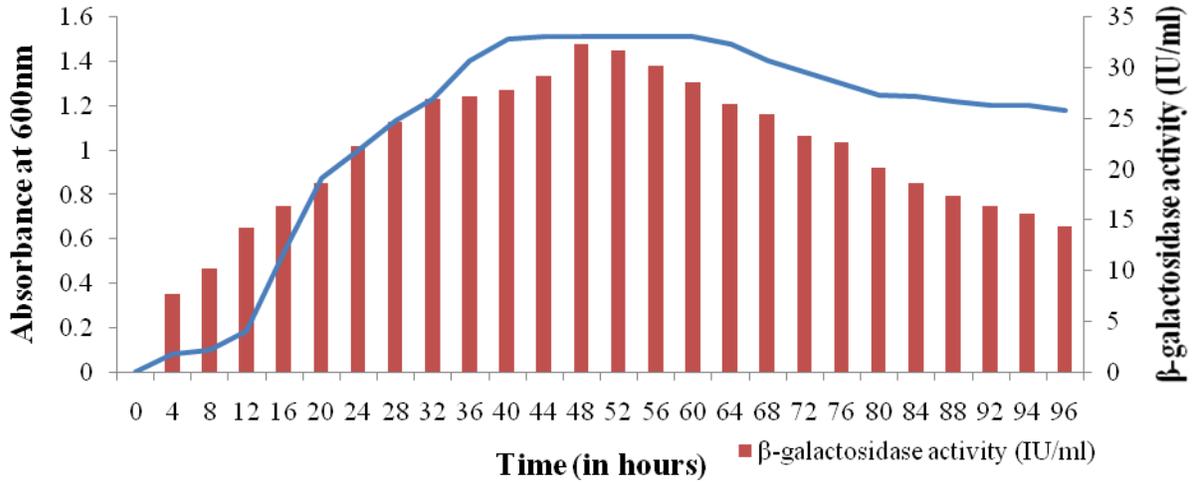


Fig.1 Growth cycle of hyper β -galactosidase producing lactic acid bacteria of *L. casei* MB2

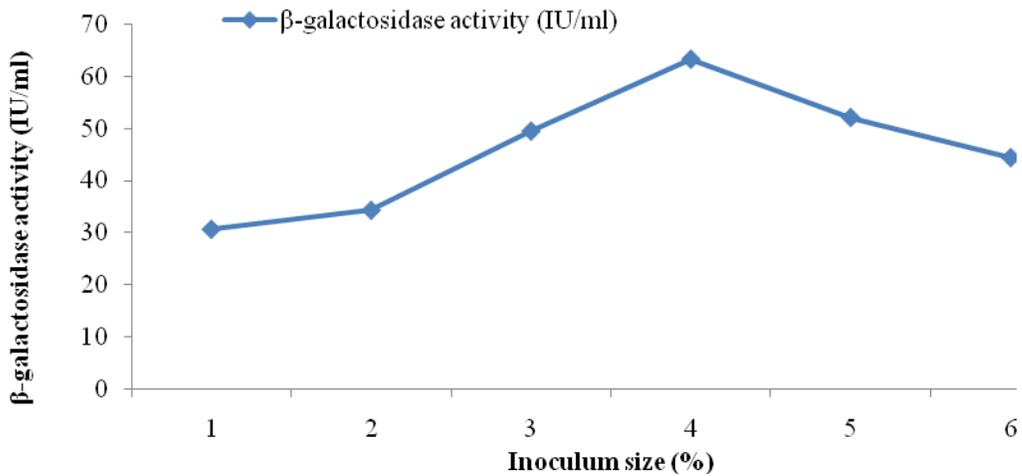


Fig.2 Effect of inoculum size on β -galactosidase production by *L. casei* MB2

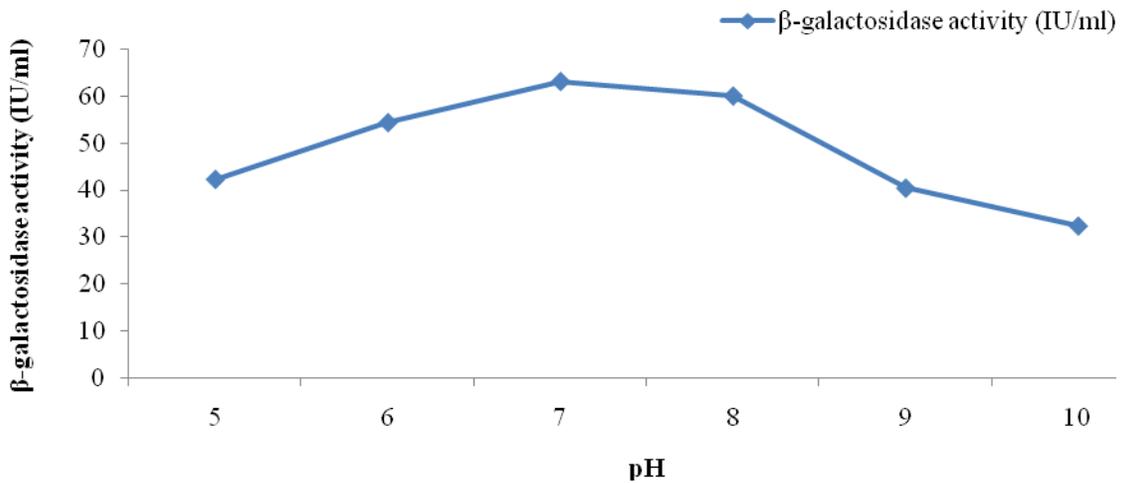


Fig.3 Effect of pH on β -galactosidase production by *L. casei* MB2

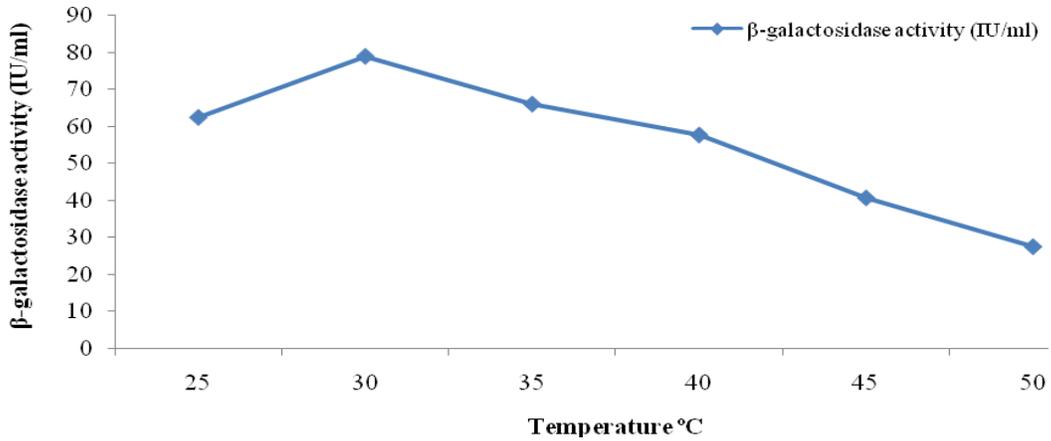


Fig.4 Effect of different temperature on β-galactosidase production by *L. casei* MB2

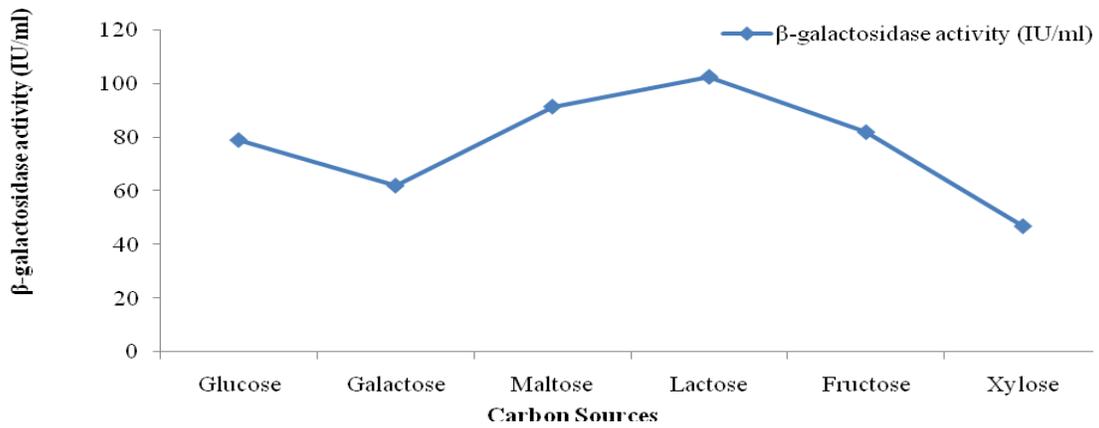


Fig.5 Effect of different carbon sources on β-galactosidase production by *L. casei* MB2

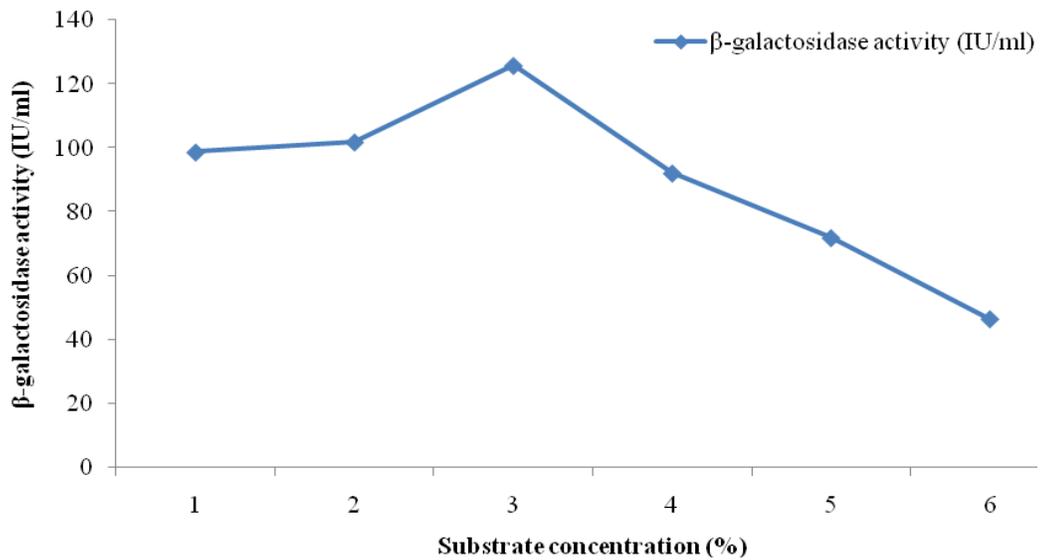


Fig.6 Effect of substrate concentration on β-galactosidase production by *L. casei* MB2

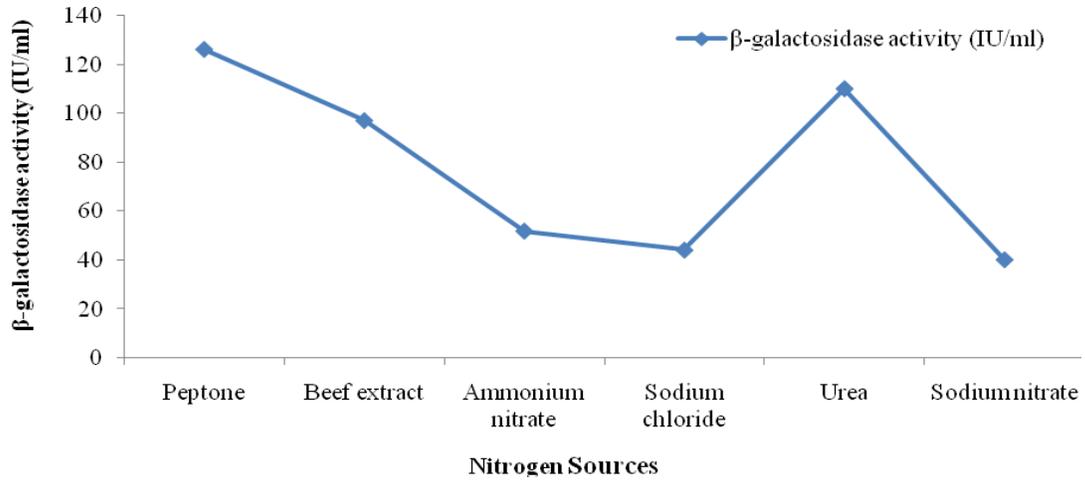


Fig.7 Effect of different nitrogen sources on β -galactosidase production by *L. casei* MB2

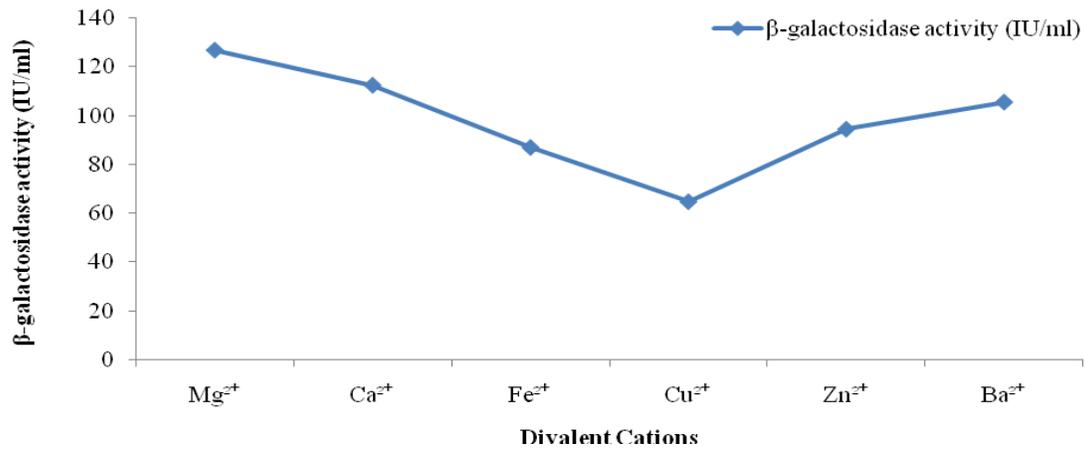


Fig.8 Effect of divalent ions on β -galactosidase production by *L. casei* MB2

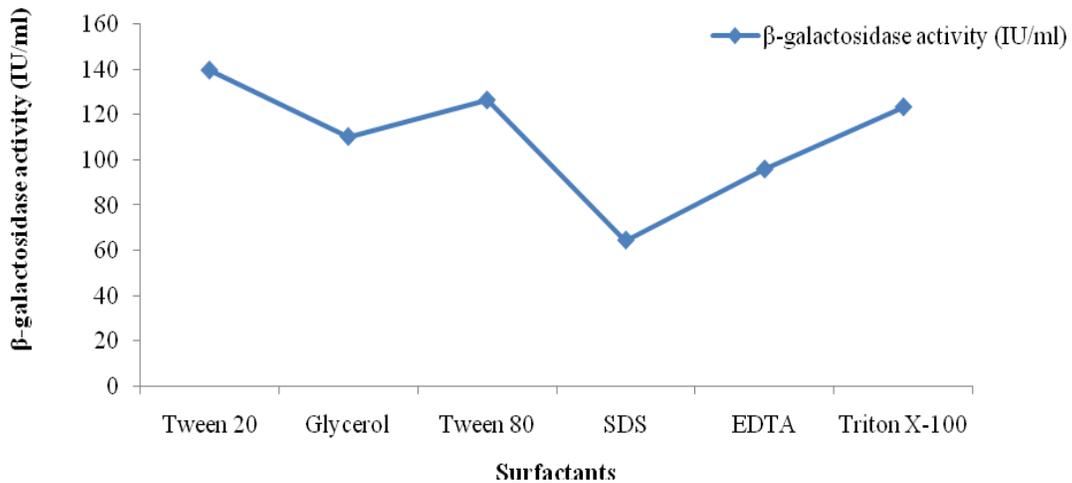


Fig.9 Effect of surfactants on β -galactosidase production by *L. casei* MB2

An increase in enzyme activity on addition of surfactants in the medium has been reported by Rao and Satyanarayana (2007) and Sodhi *et al.*, (2005). However the addition of SDS and EDTA in the medium resulted in inhibition of β -galactosidase. EDTA had no effect on enzyme activity. Contrarily EDTA inactivated the β -galactosidase of *Lactobacillus plantarum* (Iqbal *et al.*, 2010). Tween 20, Triton X-100 and glycerol slightly increases the enzyme activity considerably thus were added in the medium.

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