

Full Length Research Paper

High level production of extracellular β -galactosidase from *Bacillus licheniformis* ATCC 12759 in submerged fermentation

Nurullah AKCAN

Health High School, Siirt University, TR-56100 Siirt – Turkey.
E-mail: nurullah.akcan@gmail.com. Tel: +90 484 2231056. Fax: +90 484 223 51 56.

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The aim of this study was various nutrients belonging to three categories, carbon, nitrogen and amino acid sources, were investigated in terms of their effect on the production of extracellular β -galactosidase by *Bacillus licheniformis* ATCC 12759. Amongst simple carbon sources, xylose and galactose supported maximum β -galactosidase production. Comparison with the control there was significant increase in enzyme yield in the case of the supplementation complex carbon source such as rice flour. Among the amino acid sources tested L-tryptophane, L-cysteine, L-phenylalanine, L-lysine, L-valine and L-tyrosine were favored the production respectively. In media containing the all organic and inorganic nitrogen sources resulted in a decrease production of β -galactosidase. FeSO_4 , ZnSO_4 and CuSO_4 suppressed β -galactosidase production. Maximum β -galactosidase production ($2.508.3 \pm 29.9$ U/mg) was obtained in a medium containing 0.01% L-tryptophane in 72 h 37°C.

Key words: *Bacillus licheniformis* ATCC 12759, β -galactosidase, submerged fermentation, enzyme production.

INTRODUCTION

β -galactosidase hydrolyze lactose and transfer galactose to the hydroxyl group of water, acceptor molecule, resulting in the liberation of galactose and glucose (Alliet et al., 2007; Juajun, 2009). The main industrial application of beta-galactosidase is converting lactose to glucose and galactose. There are several advantages embodied in lactose hydrolysis: (1) rapid fermentation of glucose, (2) a higher degree of sweetness of the liquid in which lactose has been hydrolyzed, (3) higher solubility of glucose and galactose, (4) higher stability of frozen condensed milk, in which lactose has been hydrolyzed, (5) application of lactose hydrolyzed milk in cheese making results in rapid fall of pH and as a consequence rapid development of cheese flavor and texture takes place, and (6) use of beta galactosidase in whey eliminates technological problems (such as sandiness in whey powder and ice cream) improving the nutritional quality of whey and whey powder. It also leads to the development of novel products and the production of new sweeteners (Wayne and Pitcher, 1980; Mahoney and Adamchuk, 1980; Sikyta, 1983; Jokar and Karbassi, 2009). On the other hand, the transglycosylation activity has been used to synthesize galacto-oligosaccharides

(GOS) and galactose containing chemicals (GCC) in recent years (Lu et al., 2009). GOSs have become established as functional components in beneficial physiological effects for human (Kunz and Rudloff, 1993). The prebiotic, GOS, have been used in human nutrition in significant quantities as active components or as side products of processed milk or milk products, and more side effects have been reported (Stahl et al., 2007).

β -galactosidase is present in a variety of sources, including plants, animals and microorganisms (Neri et al., 2009). Microorganisms are considered potentially to be the most suitable source of β -D-galactosidase for industrial applications. However, they differ in their optimum conditions for the production of enzyme. Recovery costs of the enzyme are primarily at the level of production and purification. β -Galactosidase has been identified in a wide variety of fungal, yeast and bacterial cultures. A large number of bacteria produce β -galactosidase but relatively few bacterial species are regarded as safe sources. However, *Streptococcus thermophilus* and *Bacillus stearothermophilus* can be considered as potential bacterial sources (Panesar et al., 2006).

Increased industrial demand for β -galactosidase requires good cost-effective production methods to ensure the economic viability of lactose hydrolysis at commercial scale (Nor et al., 2001; Manera et al., 2008). The overall cost of enzyme production and downstream processing is the major obstacle against the successful application of any technology in the enzyme industry (Gupta et al., 2002). Conventionally, commercial production of β -galactosidase has been carried out using submerged fermentation (SmF). Submerged fermentations are usually carried out with a substrate, which is either dissolved or remains suspended in an aqueous medium (Sumantha et al., 2006). Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling (Singhania et al., 2010). To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production (Gangadharan et al., 2008). The optimization of fermentation conditions, particularly physical and chemical parameters, are important in the development of fermentation processes due to their impact on the economy and practicability of the process (Francis et al., 2003). The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters is the primary task in a biological process (Dakhmouche et al., 2006).

Selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient and economic process (Konsoula and Liakopoulou-Kyriakides, 2007). The present study describes the effects of culture conditions on the production of extracellular β -galactosidase by *Bacillus licheniformis* ATCC 12759.

MATERIALS AND METHODS

Microorganism and growth medium

β -galactosidase producing *B. licheniformis* ATCC 12759 which was procured from MicroBioLogics, Inc. was used as biological material. *B. licheniformis* ATCC 12759 was grown on nutrient agar at 37°C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium (1% yeast extract, 0.5% peptone, 0.5% NaCl, (w/v), pH 7.0).

Enzyme production

The microorganism was grown at 37°C for 5 days in 25 ml of medium with shaking on a shaker (150 rpm). Samples were taken from 12 to 120 h. The supernatant of the culture after centrifugation (10,000 rpm, 10 min) at 4°C was used to determine extracellular β -galactosidase activity.

Enzyme assay

The reaction mixture containing 500 μ L of 6 mM o-Nitrophenyl β -D-

galactopyrynoside (ONPG) in 0.1 M phosphate buffer (pH 6.8) and 200 μ l of enzyme solution was incubated for 30 min at 37°C. The reaction was ended by adding 0.5 ml of 1 M Na₂CO₃ and the concentration of o-nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm, using a standard calibration curve.

One unit of β -galactosidase activity (U) was defined as the amount of enzyme that liberates 1 μ mole ONP per minute under assay conditions.

Results are represented as mean \pm S.D. of at least three experiments.

Assay of protein concentration

The protein concentration was determined by the Lowry method by using bovine serum albumin used as a standard (Lowry et al., 1951).

Effect of incubation period

The effect of incubation period was determined by incubating production medium for different incubation periods (12, 24, 48, 72, 96, 120 and 144 h) at 37°C taking other conditions into consideration.

Effect of carbon source

Different carbon sources such as soluble starch, wheat starch, potato starch, corn starch, wheat flour, rice flour, corn flour, soy flour, mannose, xylose, lactose, galactose, arabinose, glucose, sucrose, and fructose were employed to find the suitable carbon source for β -galactosidase production by *B. licheniformis* ATCC 12759. All these sources were studied at 1% (by mass per volume) initial concentration.

Effect of nitrogen sources

Two categories, viz. organic nitrogen sources and inorganic nitrogen sources were employed. The growth medium was initially supplemented with different organic nitrogen sources, i.e. yeast extract, tryptone, beef extract, peptone, casein, urea, each at 1% (by mass per volume). Among the inorganic nitrogen sources, ammonium nitrate (NH₄NO₃), ammonium chloride (NH₄Cl), ammonium sulphate ((NH₄)₂SO₄), and sodium nitrate (NaNO₃) tested at 1% concentration (by mass per volume).

Effect of amino acid sources

To study the effect of amino acid sources on production of β -galactosidase glycine, L-lysine, L-tyrosine, L-cysteine, glutamic acid, L-alanine, L-phenylalanine, L-isoleucine, L-valine, L-methionine and L-tryptophane selected as amino acid source. All these sources were studied at 0.01% (by mass per volume) initial concentration.

Effect of metal salts

The effect of metal salts on β -galactosidase production is determined by adding different metal salts in the fermentation medium. The metal salts selected for present study are FeSO₄, MgSO₄, CaCl₂, CuSO₄, and ZnSO₄, at 0.1% concentration.

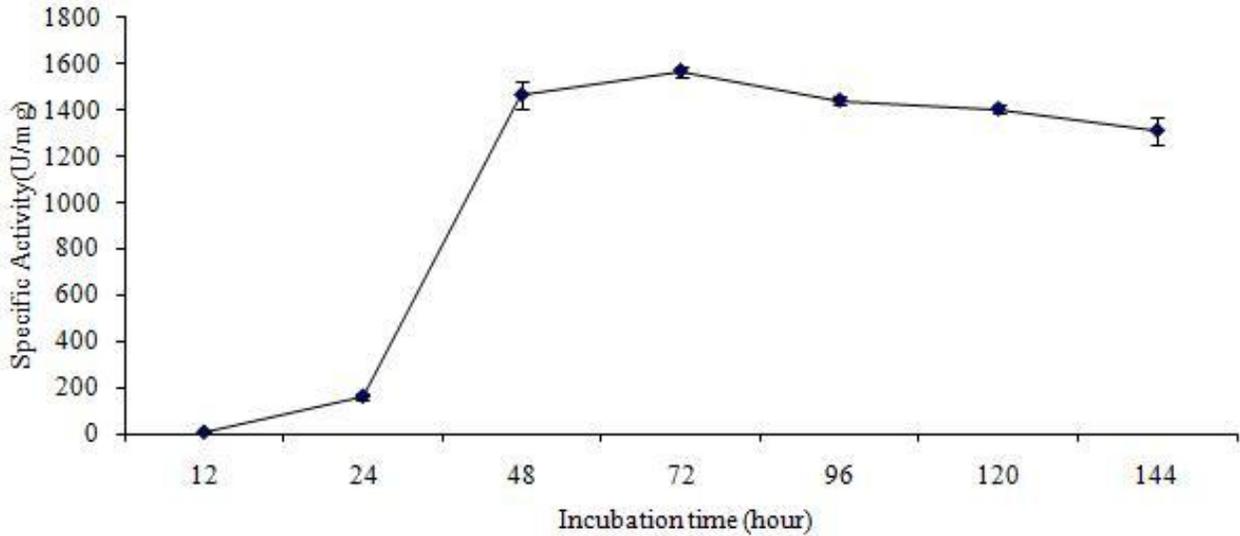


Figure 1. Effect of incubation time on the production of β -galactosidase from *B. licheniformis* ATCC 12759.

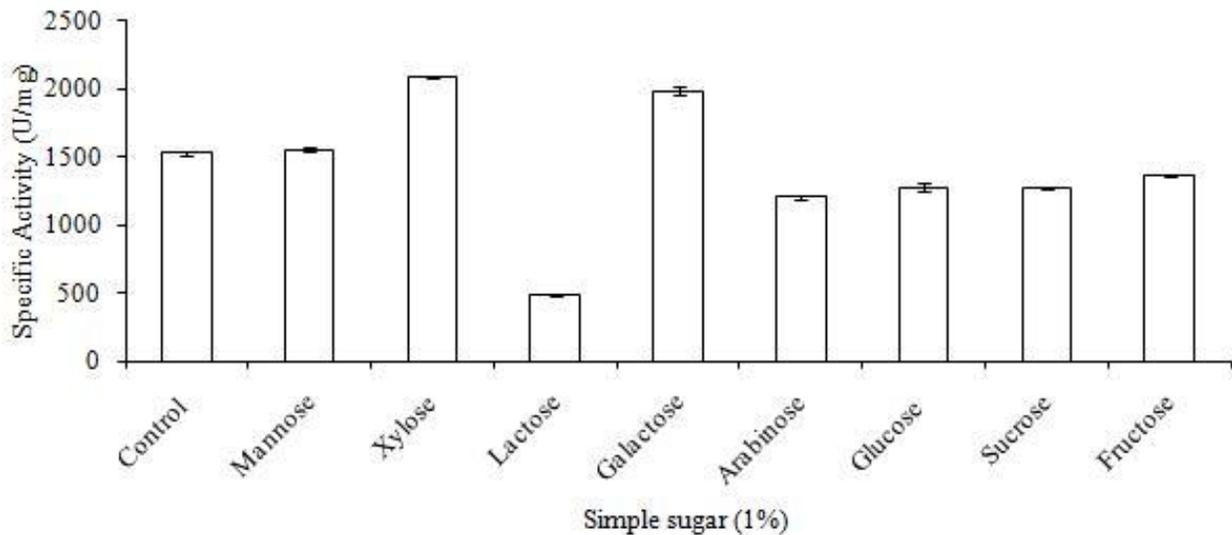


Figure 2. Effect of simple sugars on the production of β -galactosidase from *Bacillus licheniformis* ATCC 12759.

RESULTS

Data presented here show that *B. licheniformis* ATCC 12759 produces β -galactosidase. The optimal conditions for β -galactosidase production were determined under submerged fermentation conditions. According to the results taken at different time intervals, it was determined that the optimum incubation time for maximum production of β -galactosidase by *Bacillus licheniformis* ATCC 12759 was at 72 h (Figure 1). A prolonged incubation time beyond this period did not increase the enzyme yield.

Among the simple sugars, xylose and galactose enhanced the production of β -galactosidase while other

simple sugars especially, lactose, greatly repressed β -galactosidase production (Figure 2). Comparison with the control in media containing, xylose and galactose enhanced production of β -galactosidase range 36 and 30%, respectively. However, media containing lactose decreased β -galactosidase production range 69%.

In the medium containing rice flour, wheat flour and wheat starch enhanced the production of β -galactosidase. Comparison with the control (1419.5 ± 32.4 U/mg) in media containing rice flour, wheat flour and wheat starch enhanced of β -galactosidase range 56, 21 and 20%, respectively (Table 1). A marginal increase was noted with the addition of soy flour and potato starch

Table 1. Effect of complex carbon source on the production of β -galactosidase from *Bacillus licheniformis* ATCC 12759.

Carbon source 1% (w/v)	Specific activity (U/mg)
Control value	1419.5 \pm 32.4
Soluble starch	1190.6 \pm 36.4
Wheat starch	1713.2 \pm 29.9
Potato starch	1520.4 \pm 6.4
Corn starch	1038.0 \pm 50.8
Wheat flour	1725.9 \pm 9.3
Rice flour	2226.1 \pm 21.0
Soy flour	1638.9 \pm 6.7
Corn flour	1362.3 \pm 19.3

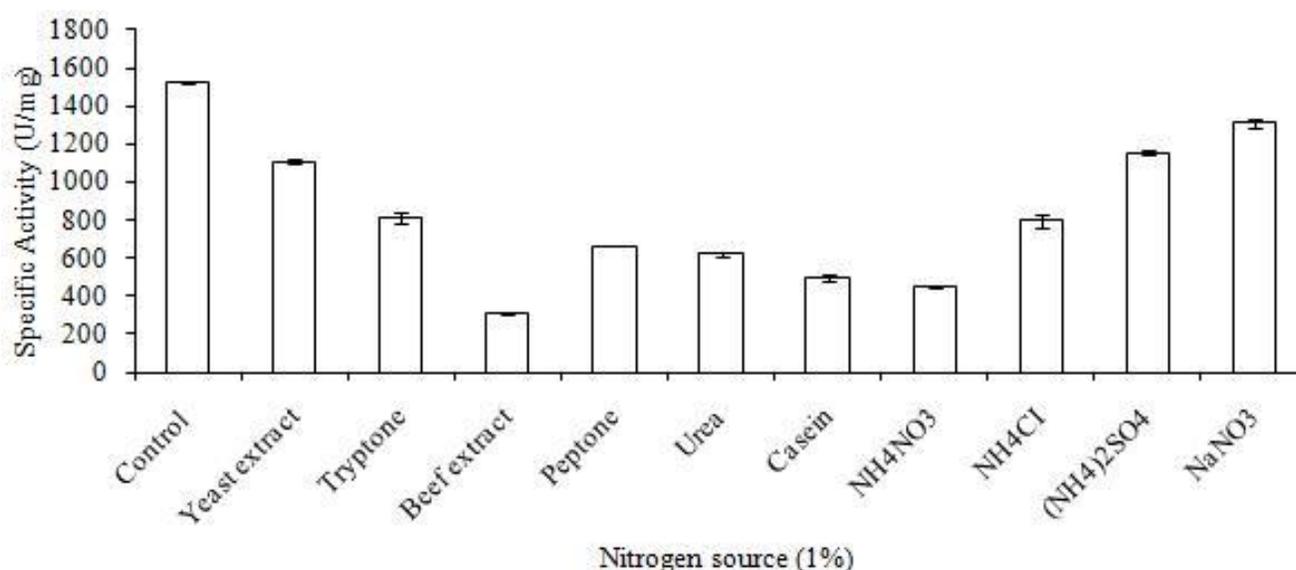


Figure 3. Effect of nitrogen sources on the production of β -galactosidase from *Bacillus licheniformis* ATCC 12759.

indicating that any of the sources can be alternatively used. The other complex carbon sources suppressed the enzyme production.

β -galactosidase production was tested in fermentation medium containing different nitrogen source at a concentration of 1%. As shown in Figure 3, comparison with the control (1527.3 \pm 8.4 U/mg) in media containing the all organic and inorganic nitrogen sources resulted in a decrease in β -galactosidase production. Enzyme production significantly reduced by media containing beef extract (310.7 \pm 4.5 U/mg).

The effect of amino acids supplementation of the production medium on enzyme production was studied. L-tryptophane, L-cysteine, L-phenylalanine, L-lysine, L-valine and L-methionine were found to be the ideal amino acids sources, respectively (Figure 4). Supplementation of the L-tryptophane (2.508 \pm 29.9 U/mg) in fermentation medium was enhanced 60% β -galactosidase production

comparison with the control (1532.6 \pm 12.2).

In order to investigate the effect of metal salts on β -galactosidase production, these salts were individually added to a main medium at 0.1%. The result of the impact of metal salts on enzyme production is shown in Figure 5. The β -galactosidase production increased when production medium was supplemented with MgSO₄ (1778.8 \pm 13.3 U/mg). The other metal salt CaCl₂ (720.3 \pm 35.2 U/mg) tested decreased enzyme production. However, FeSO₄, CuSO₄, and ZnSO₄ completely repressed β -galactosidase production.

DISCUSSION

The production of β -galactosidase by submerged fermentation (SmF) investigated and is affected by a variety of physicochemical factors. The incubation time is

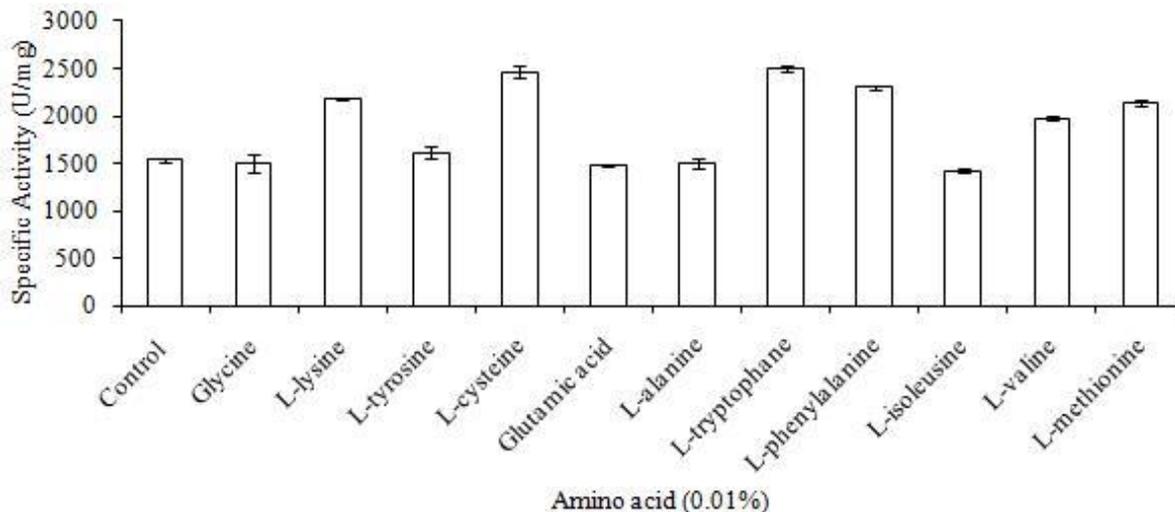


Figure 4. Effect of amino acid sources on the production of β -galactosidase from *Bacillus licheniformis* ATCC 12759.

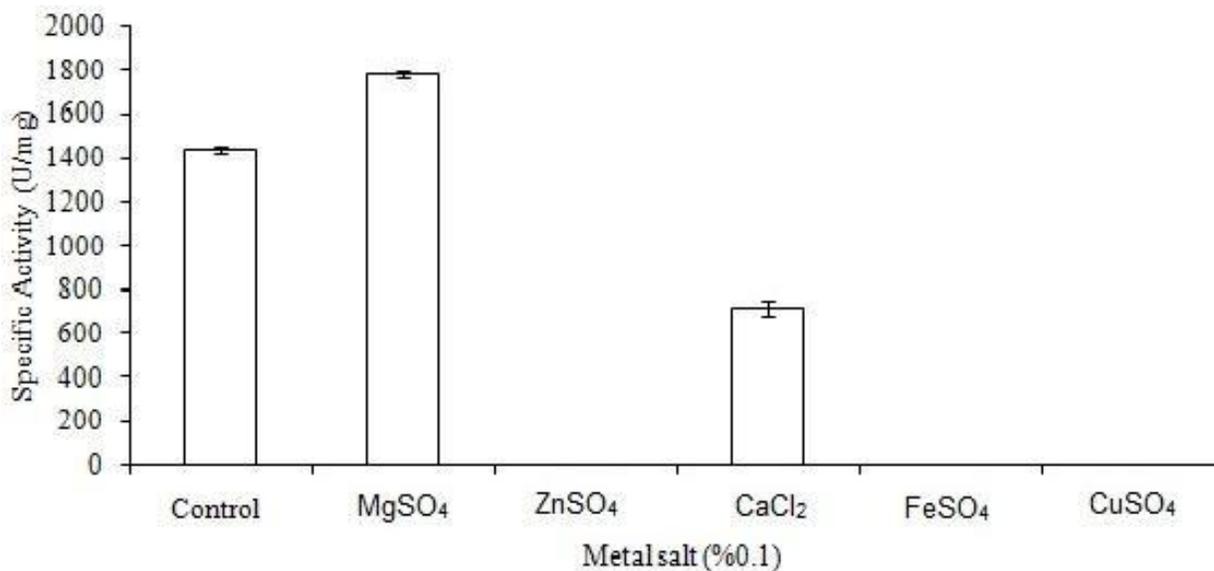


Figure 5. Effect of metal salts sources on the production of β -galactosidase from *Bacillus licheniformis* ATCC 12759.

governed by characteristics of the culture and also based on growth rate and enzyme production. Results indicated that the optimum incubation period for β -galactosidase production was found to 72 h. Comparing the results to the literature there is a broad incubation time reported for *Bacillus* strains (Chen et al., 2003; Clerck and Vos, 2004; Konsoula and Liakopoulou-Kyriakides, 2007). Based on these data our strains fit in this interval. A prolonged incubation time beyond this period did not increase the enzyme yield. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium and probably due to depletion of nutrients available to microorganism (Ramesh and Lonsane, 1987; Akcan et al., 2011).

The nature and amount of carbon source in culture media is important for the growth and production of extracellular β -galactosidase in bacteria. Carbon source regulates biosynthesis of β -galactosidase in various microorganisms (Nagy et al., 2001; Akolkar et al., 2005; Hsu et al., 2005; Konsoula and Liakopoulou-Kyriakides, 2007; Alazzeah et al., 2009). All indicated that the role of carbon source in the biosynthesis of β -galactosidase may vary and depend on the microorganisms tested. In our study, xylose and galactose enhanced the production of β -galactosidase while other simple sugars specially, lactose, greatly repressed β -galactosidase production (Figure 2). Kim and Rajagopal (2000) described that galactose was the best carbon source for the

biosynthesis of β -galactosidase by *L. crispatus*, while addition of glucose or lactose to the growth medium repressed the synthesis of β -galactosidase. However, Hsu et al. (2005) found that the final viable population of *B. longum* CCRC 15708 was higher in cultures containing either lactose or glucose as the sole carbon source with the highest β -galactosidase activity detected with lactose followed by galactose and the lowest activity with glucose as the carbon source. We observed these results suggest that β -galactosidase production from *B. licheniformis* ATCC 12759 is induced by some readily metabolisable sugars.

Cheaper sources of both carbon and nitrogen sources are the key attraction for commercialization of the production processes and thus, ability of the microbial agent to grow and produce enzymes using these sources has been arguably a point of interest (Patel et al., 2005). In the presence of rice flour β -galactosidase production was significantly enhanced. Konsoula and Liakopoulou-Kyriakides (2007) also found that the production of β -galactosidase was higher in the presence of corn flour along with the corn steep liquor, followed by wheat and rice flour. It was clear though that certain organic compounds may be necessary for the biosynthesis of this enzyme at high levels. The availability of higher amounts of nutrients, such as proteins, lipids and fibers in these flours may favor the biosynthesis of β -galactosidase.

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. Nitrogen sources may affect microbial biosynthesis of β -galactosidase (Rao and Dutta, 1977; Shaikh et al., 1997; Hsu et al., 2005). All organic and inorganic nitrogen sources resulted in a decrease in β -galactosidase production. These results show that the addition of organic and inorganic nitrogen sources in the medium was not sufficient to stimulate the β -galactosidase production from *B. licheniformis* ATCC 12759. Hsu et al. (2005) reported that yeast extract necessary for β -galactosidase production, while casein, peptone and beef extract repressed β -galactosidase formation. However, other works reported that better β -galactosidase synthesis in the presence of nitrogen sources (Konsoula and Liakopoulou-Kyriakides, 2007; Nizamuddin et al., 2008).

Supplementation of the L-tryptophane in fermentation medium was enhanced β -galactosidase production. Konsoula and Liakopoulou-Kyriakides (2007) reported that glycine was found to enhance the enzyme production from *Bacillus subtilis*. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of enzyme synthesis and excretion (Gupta et al., 2003). The enzyme synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in the growth medium. The differences in nutritional requirements of various

enzyme producing organisms or microbial strains could be attributed to the difference in their genetics (Rasooli et al., 2008).

The β -galactosidase production increased when production medium was supplemented with $MgSO_4$. This indicated that Mg^{2+} was necessary for enzyme induction and/or enzyme stabilisation. Positive effects of metal salts including Mg^{2+} and Mn^{2+} on β -galactosidase production have also been demonstrated by Rao and Dutta (1977).

The results obtained in this study show that there is appreciable high production of extracellular β -galactosidase. This suggests that *B. licheniformis* ATCC 12759 can be a potential producer of extracellular β -galactosidase which could find applications in industry and biotechnology. The enzyme thus produced is presently under optimization. Due to the importance of these findings, further studies will be carried on in order to commercialize the production process after necessary optimization for enhanced enzyme production.

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