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Production and partial purification of β-galactosidase enzyme from probiotic *Bacillus subtilis* SK09

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In the present study, lactose hydrolyzing enzyme β-galactosidase was produced from a novel probiotic *Bacillus subtilis* SK09 and it was compared with commercially obtained, *B. subtilis* MTCC 2413. The probiotic Bacilli was initially screened for its ability to hydrolyze the X-gal followed by production of blue colonies. The production medium was formulated using varied nutrients including carbon, nitrogen, mineral salts and amino acid sources. The crude enzyme with an activity of 76.2 U/ml was achieved from our novel strain *B. subtilis* SK09, and subjected for partial purification by solvent precipitation, dialysis and ion exchange chromatography using DEAE cellulose (DEAE-C) column. Partially purified β-galactosidase enzyme showed 11.6 folds increase in specific activity of 137.02 U/ml. This partially purified enzyme was further characterized and it was observed that it is homogeneous in nature, with molecular weight of 43 kDa. When compared, the β-galactosidase activity of our novel strain was found five times higher than that of commercially obtained strain (27.6 U/ml). Hence, this characteristic of *B. subtilis* SK09 of increase enzymatic activity can make it a promising candidate for various industrial as well as biotechnological applications.

Keywords: *Bacillus* sp, probiotic, β-galactosidase, X-gal, DEAE cellulose.

Introduction

β-galactosidases are metal-activated group of enzymes which are able to cleave lactose into galactose and glucose[1-3]. It gains industrially importance as, the enzyme have been used to prevent crystallization of lactose, to improve sweetness, to increase stability of frozen condensed milk, to improve nutritional quality of whey and also leads to production of new sweeteners and novel products[4-6]. On the other hand, the trans-glycosylation activity has been used to synthesize galacto-oligosaccharides (GOS) and galactose containing chemicals (GCC) in recent years[7-8]. GOS is widely use as a prebiotic food reserve for a range of nutritional foods for its ability to promote the growth and establishment of *Bifidobacteria* species in the human intestine and also for inhibiting the potentially harmful gut bacteria such as *Clostridia* and *Bacteriodes* species[9-10]. It was estimated that over 70% of the world's adult human population reported to have lactose intolerance or lactose malabsorption as a major health problem[11]. Fermentation of indigested lactose by colonic microflora present in the large intestine can cause the abdominal pain, gas formation, nausea and diarrhoea[12].

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β-galactosidases are found in microorganisms (bacteria fungi, yeasts), plants (almonds, peaches, apricots, apples) and animal organs[13-15]. β-galactosidases from bacterial sources have been widely used for lactose hydrolysis, as it shows the ease of fermentation, high enzymatic activity and good stability[16-17]. Increased industrial demand for β-galactosidase requires a good cost-effective production method to ensure the economic viability of lactose hydrolysis at commercial scale[18-19]. Selection of appropriate carbon and nitrogen sources or other nutrients is one of the most critical stages in the development of an efficient economic process[20]. Amongst all the β-galactosidase thus far studied, the *Escherichia coli* enzyme has been well explored following the discovery of the lactose operon for its use as recombinant genetic tool[21.] The industrial use of β-galactosidase enzyme from *E. coli* is hampered by the fact that it is not considered safe for food applications. Nevertheless it is commercially available for analytical purposes[22]. Therefore the production of β-galactosidase from probiotic microorganisms which are safer for human use becomes the need of the hour.

In this regard, lactic acid bacteria (include a diverse group of *Lactococci, Streptococci, Bacillus subtilis, Lactobacillus* and *Bifidobacterium*) which are generally recognized as safe (GRAS) organisms, have been regarded as good sources of β-galactosidase,

especially for functional food applications[23]. The objective of this study was the production β-galactosidase from novel probiotic *B. subtilis* and partial purification and characterization of the crude enzyme extract.

Materials and Methods

Microorganisms

The novel probiotic strain *B. subtilis* SK09 that was isolated and characterized previously[24] and a commercially obtained strain *B. subtilis* MTCC 2413 were revived using *Lactobacillus* MRS broth deMan (1960) *et al*, obtained from Hi-Media.

Screening of Enzyme

The strains were platted on nutrient agar, with 0.5% lactose infused with 50 µl 2% X-gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside was prepared using dimethyl sulfoxide (DMSO) as solvent) and incubated at room temperature for 24 hrs.

Culture Propagation and Extraction of Enzyme

Using 500 ml conical flask, 100 ml of defined medium was prepared in duplicates containing: lactose 1 g; beef extract 1.5 g; peptone 0.5 g; yeast extract 0.05 g; and sodium chloride 0.15 g; pH was adjusted to 6.9 and autoclaved at 121°C for 20 min as mentioned in earlier study[25-26]. Inoculation was done using seed culture and kept in incubator shaker (Scigenics Biotech, Orbitek) at 32°C for 48 hours at 150 rpm. After incubation, the culture broth was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and analysed for the presence of the desired enzyme which is extracellular in nature.

Enzyme Assay

The amount of protein present in the supernatant was estimated by Bradford's method with standard protein 1 mg/ml of bovine serum albumin (BSA)[27]. The β-galactosidase enzyme assay was done using ONPG (O-nitrophenyl-β-D-galactopyranoside) as a substrate. The ONPG (2.5 mg) was suspended in 1 ml of 0.1 M sodium acetate buffer (pH 5.0) with which 200 µl of cell free supernatant was added and incubated at 55°C for 20 min and the reaction was stopped by adding 1 ml of 10% sodium carbonate solution. The absorbance was read at 420 nm, and the amount of ONPG released was thus ascertained[28]. One unit of β-galactosidase enzyme activity was defined as the amount of enzyme that liberates 1 µM of ONPG per min at the temperature of 55°C.

Effect of Incubation Time and pH on Enzyme Production

The production parameters such as effect of incubation time (24, 48, 72, 96 hrs) and pH range (5.0, 5.5, 6.0, 6.5, 6.9, 7.0, 7.5, 8.0) were studied on enzyme productivity. For each trial the enzyme activity and protein content were assayed.

Effect of Media Nutrients on Enzyme Production

Carbon Sources

The production strain was subjected for growth using 1% of various carbon sources such as galactose, glucose, sucrose and fructose and the resulting protein content and enzyme activity were studied.

Nitrogen Sources

The culture isolates were grown with the medium containing 1% of following nitrogen sources (organic: yeast extract, beef extract, peptone) and (inorganic: ammonium chloride and ammonium sulphate) and corresponding yield in terms of protein content and β-galactosidase enzyme activity were studied.

Metal Salts

To study the effect of metal salts on the enzyme production, the medium was supplemented with 0.1% concentration of $FeSO_4$, $CaCl_2$, $CuSO_4$, $ZnSO_4$ and $MgSO_4$.

Amino Acid Sources

The effect of amino acid sources on the production of enzyme was studied by adding l-alanine, glutamic acid and tryptophan at a concentration of 0.01%.

Statistical Analysis of Enzyme Activity

The statistical analysis such as analysis of variance (ANOVA) test and Kruskal-Wallis rank sum test was carried out using the R language (R studio 1.1.423.0) to analyze the non-parametric study of the enzyme activity with independent variables (incubation time, pH, carbon source, nitrogen source, metal salts and amino acids) are given in Table 1.

Table 1 — Statistical analysis of β-galactosidase enzyme activity

S. No.	Optimized parameters	Enzyme activity	p-value (Kruskal-Wallis rank sum test)
1	Incubation time (48 hrs)	11.8 U/ml	0.3916
2	pH (6.9)	11 U/ml	0.4289
3	Carbon source (Glucose)	76.2 U/ml	0.3916
4	Nitrogen source (Beef extract)	1.2 U/ml	0.406
5	Metal salts ($MgSO_4$)	9.8 U/ml	0.406
6	Amino acid (Glutamic acid)	5.2 U/ml	0.3679

Partial Purification of Enzyme

The cell free supernatant containing β-galactosidase enzyme was subjected to organic solvent precipitation using chilled acetone in the ratio of 1:1.5% at 20°C. After thorough stirring the mixture was incubated at 4°C for 12 hrs. The resulting solution was centrifuged at 8500 rpm for 30 min at 4°C. The pellet containing desired enzyme was dissolved in minimal volume of 50 mM acetate buffer (pH 5.0) and was further dialyzed for 24 hrs using a dialysis membrane of pore size 1.0 kDa. The dialysed sample was run using an anion exchange chromatography (DEAE cellulose column) and the purified fractions were eluted using 50 mM acetate buffer at a flow rate of 1 ml/min[29-30]. These fractions were analyzed for protein concentration and β-galactosidase activity.

Characterization of Enzyme

The partial purity and homogeneity of the enzyme was analyzed by SDS-PAGE using medium range molecular weight protein markers[31]. After electrophoresis, the proteins were stained by Coomassie brilliant blue and distained using deionised water and gel was observed for bands under transillumination.

Results and Discussion

The probiotic derived enzymes gains high industrial importance and hence can be used as a food supplement/dietary product and are available commercially[32]. The bacterial strains which were obtained from the dairy industry effluents have been proved to be probiotic with the ability to produce the enzyme β-galactosidase[33]. Blue colonies were produced on the nutrient agar plates infused with X-gal which proves the presence of the hydrolyzing enzyme β-galactosidase. On comparing with the enzyme activities of *B. subtilis* SK09 and MTCC 2413, the former showed 5 times more activity and hence the same was selected for further investigations. The incubation time depicts the characteristics of the culture is based on the growth rate and enzyme production[25,34-35].

Based on the samples withdrawn at regular intervals (24-96 hrs), a significant increase of the enzyme activity (11.8 U/ml) was observed at 48 hrs (Fig. 1). The subsequent decrease of enzyme activity might be due the depletion of medium nutrients and catabolic repression of enzyme. The hydrogen ion concentration affects two aspects of the microbial cells: function of the cells and the transport of nutrients to the cells[36]. The optimal incubation time of 48 hrs had been fixed for further studies of effect of pH on β-galactosidase production. The pH range 5.0 to 8.0 with an increment of 0.5 and its effect on enzyme production was analyzed (Fig. 2) and the maximum activity found at pH of 6.9, which is also coincides with reference to earlier studies[37-39].

Cheaper carbon and nitrogen sources are the key attraction for commercialization and the ability of microorganisms to grow and produce enzymes using these sources has been arguably a point of interest[40]. Selection of suitable carbon and nitrogen sources were the critical step during the enzyme production process[41]. Among the carbon sources investigated, it was found that glucose (1.0%) yielded more enzyme activity of 76.2 U/ml than other carbon sources such as sucrose, fructose and galactose. Sucrose and galactose showed a moderate yield, but in case of fructose a

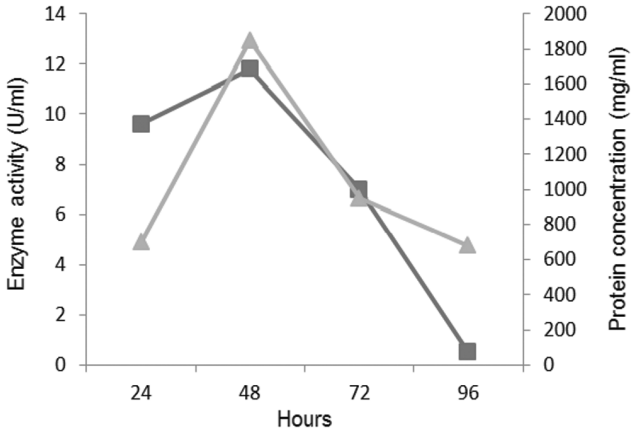

Fig. 1— Effect of incubation time on β-galactosidase enzyme production (red colour indicates enzyme activity and green colour indicates protein concentration).

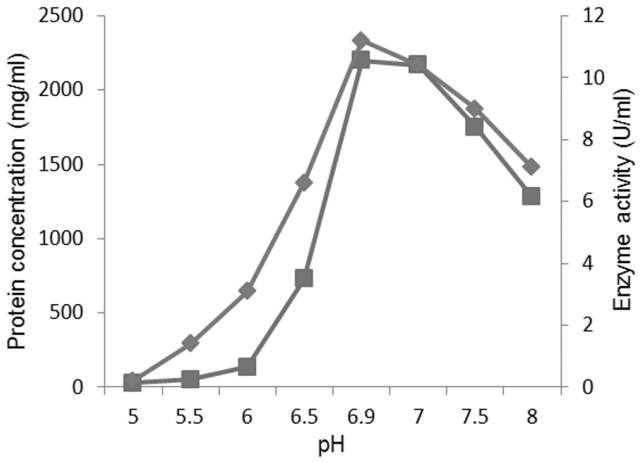

Fig. 2 — Effect of pH on β-galactosidase enzyme production (blue colour indicates enzyme activity and brown colour indicates protein concentration).

significant yield depletion was observed. These yield differences could be due to the presence of the functional groups: aldehyde (galactose and sucrose) and ketone (fructose and sucrose). The presence of former functional group favours the production of enzyme when compared to the later. Since the sucrose contains both the functional groups, a moderate dip in production has been observed (Fig. 3).

The production of β-galactosidase was tested in fermentation medium containing various nitrogen sources at a concentration of 1% (organic: yeast extract, beef extract, peptone) and (inorganic: ammonium chloride and ammonium sulphate). Among these sources, beef extract and peptone contains amino acids and short chained peptides which may promote better utilisation of the nitrogen sources and hence resulted in high enzyme productivity. The rest of nitrogen sources had shown a moderate activity when compared to the former (Fig. 4). The availability of nitrogen source in the form of complex proteins (yeast extract) and inorganic salts (ammonium chloride and ammonium sulphate) had resulted in decreased enzyme activity which is also observed in the case of *B. licheniformis*[42].

From the experiments with the addition of metal salts (0.1% of $FeSO_4$, $CaCl_2$, $CuSO_4$, $ZnSO_4$ and $MgSO_4$), we could infer a depleted enzyme activity (Fig. 5) which might be due to the inhibition of enzyme in the presence of divalent metal ions. The synthesis of a particular enzyme by a microorganism could be correlated to the presence or absence of a particular amino acid in the growth medium and it could be attributed to genetics[43]. The supplementation of amino acids on productivity had been analyzed by adding (0.01%) polar and non-polar amino acids such as glutamic acid, L-alanine and tryptophan, respectively (Fig. 6). The results proved that the

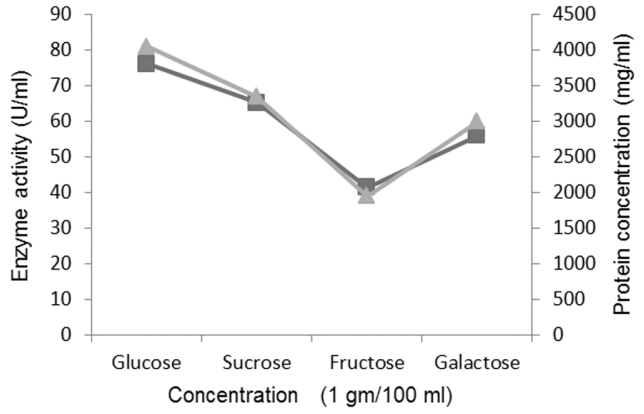

Fig. 3 — Effect of carbon sources on β-galactosidase enzyme production (red colour indicates enzyme activity and green colour indicates protein concentration).

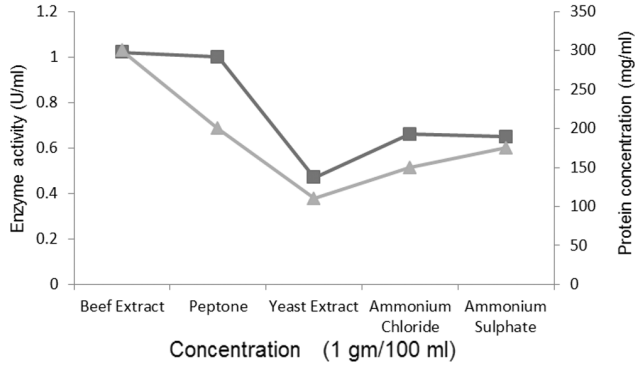

Fig. 4 — Effect of nitrogen sources on β-galactosidase enzyme production (brown colour indicates enzyme activity and green colour indicates protein concentration).

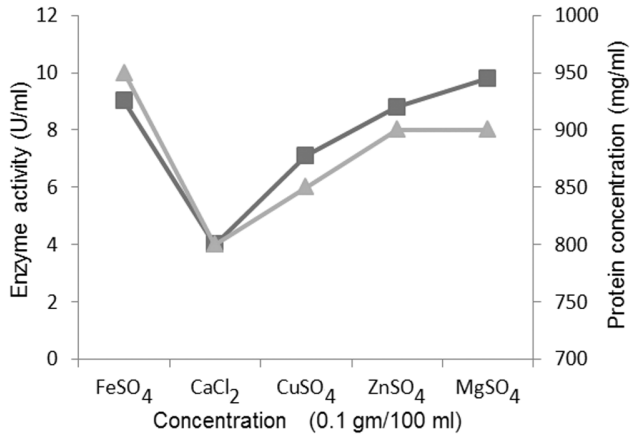

Fig. 5 — Effect of metal salts on β-galactosidase enzyme production (brown colour indicates enzyme activity and green colour indicates protein concentration).

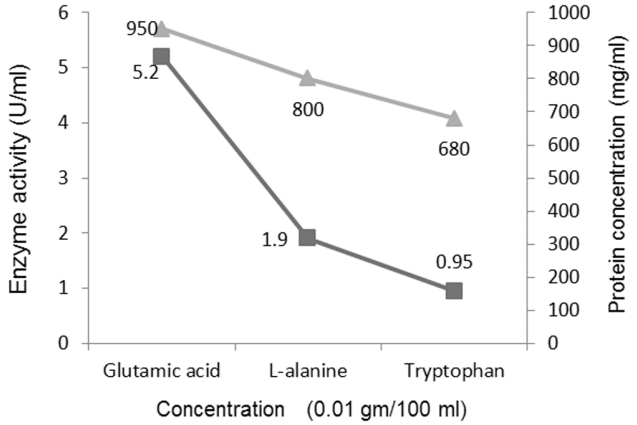

Fig. 6 — Effect of amino acids on β-galactosidase enzyme production (brown colour indicates enzyme activity and green colour indicates protein concentration).

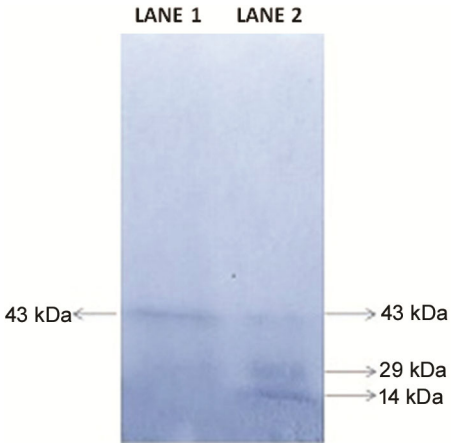

Fig. 7 — SDS PAGE analysis of purified β-galactosidase enzyme.

presence of hydrophilic amino acid (glutamic acid) favoured the productivity when compared to the hydrophobic amino acids (L-alanine, tryptophan). This could be due the ready solubility and utilization of former in the aqueous medium.

The downstream processing is considered as the obstacle in the total production cost and hence needed a cheaper extraction and purification methods[44]. The crude extract of β-galactosidase was initially subjected to acetone precipitation (1:1.5% v/v culture filtrate/acetone) resulted an initial activity to 69.7 U/ml. The subsequent dialysis of the crude extract results in an increased activity up to 105.57 U/ml. This partially purified active principle was further subjected to ion-exchange chromatography using DEAE cellulose column. The final partially purified compound showed 11.6 folds of increased activity (137.02 U/ml) and the efficiency of stepwise purification is listed in Table 2. Characterization of the purified enzyme was carried out using SDS-PAGE analysis and clear distinct bands were observed with molecular weight of 43 kDa (Fig. 7). This result confirms that the β-galactosidase enzyme is a monomer (active form) which also coincides with the findings of Leng *et al*[45].

Conclusion

The strong industrial interest on β-galactosidase arises not only from their ability to hydrolyze lactose, but also its trans-glycosylation activity. In this study, we have reported the production, partial purification and characterization of β-galactosidase enzyme from the probiotic *B. subtilis* SK09 strain, which is recognized safe, thus promoting its application in the dairy and confectionary industries. On comparing with the commercially strain of *B. subtilis* MTCC 2413, our strain of interest had five times higher enzyme activity. Thus the production process appears highly attractive due to the cost effective nutritive sources and downstream processes. Further studies also proved that the purified enzyme showed better activity over a broad range of temperature and pH, which makes it an interesting candidate for various industrial, biotechnological, medical, analytical and other applications.

Table 2 — Purification profile of β-galactosidase enzyme from *Bacillus subtilis* SK09

S. No.	Level of purification	Enzyme activity	Efficiency of purification
1	Cell free culture broth	11.8 U/ml	-
2	Organic solvent precipitation	69.7 U/ml	5.8 folds
3	Dialysis membrane of pore size 1.0 kDa	105.57U/ml	8.9 folds
4	DEAE column chromatography	137.02U/ml	11.6 folds

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