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Isolation and Characterization of β-Galactosidase Producing *Bacillus* sp. from Dairy Effluent

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Abstract: β-galactosidase producing BPTK4 was isolated from the diary industry effluent in Chennai. Biochemical tests and 16S rRNA sequencing was used for the confirmation of the strain BPTK4 as *Bacillus subtilis*. The strain BPTK4 was assessed for its probiotic nature using antibiotic markers. The characterization of the enzyme and optimization of the production medium were carried out for the maximum production and activity of β-galactosidase. Maximum production of enzyme was obtained when the medium was incubated for 48 hours at the temperature of 35°C and maintained at pH 7. Various carbon (1% m/v) and nitrogen (0.015% m/v) sources, metal ions (1mM) and natural substrates (1% m/v) were introduced into the medium and their effects were studied. Xylose, Yeast extract, Mg^{2+} ion, Mn^{2+} ion and wheat bran increased the production of enzyme. The enzyme was purified by ion exchange chromatography using DEAE sephacel column and it was characterized for stability based on temperature and pH. The enzyme showed highest activity at the temperature of 55°C (0.350 U/ml) and at pH 7 (0.294 U/ml). The enzyme retained 100% of its activity at 45°C and retained 90% of its activity at pH 7.

Key words: *Bacillus* sp. · Probiotic · X-Gal · ONPG

INTRODUCTION

Lactose is a disaccharide found in milk and other dairy products. Lactose indigestion in the intestinal microflora leads to dizziness, headache and nausea. So milk and other dairy products are manufactured with less lactose content for lactose intolerance people which further adds the cost to about 80% of the normal unhydrolysed milk [1]. The lactose hydrolyzing enzyme, β-galactosidase (E.C 3.2.1.23) facilitates the reaction between the disaccharide molecules (Lactose) and water, thereby cleaving the oxygen bridge resulting in the production of two simple sugars (Glucose and Galactose). β-galactosidase has been widely used for industrial as well as medical application. In dairy industries β-galactosidase has been used to prevent crystallization of lactose, to improve sweetness and to increase the solubility of the milk product [2]. It is also important for the utilization of cheese whey, which would otherwise be an environmental pollutant. The transglycosylation activity has been used for the synthesis of galacto-oligosaccharide (GOS) and galactose containing chemicals (GCC) in recent years [3].

β-galactosidase can be obtained from various sources such as plants, animals and micro organisms. But micro organisms are considered as a suitable source for industrial applications. Among bacteria; yeast and fungi and a large number of bacteria are most suitable because they are Generally Regarded As Safe (GRAS). So our work was focused on bacterial production of enzyme. It is important to select a microorganism with high potentiality to produce β galactosidase [4]. *Bacillus species* is our area of interest because it is aerobic, thermostable, spore forming and it can resist high alkalinity. They are generally called as aerobic spore formers [5]. The species can be used as a probiotic source organism since it is an antagonist to fungal and bacterial pathogens [6]. In contrast, lactic acid bacteria (LAB) are not preferred as a digestion enhancer due to the inability to form spores and can't tolerate the acidic environment of the stomach and inviability [7].

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β-galactosidases produced from bacteria are used for the treatment of milk, whey and other dairy products of neutral pH because the enzyme is active in the pH range of 6.5-7. The organism was isolated and screened with X-gal and assayed to estimate the ONPG fermenting ability of β-galactosidase. Our main focus was on the optimization of the media based on the following factors: incubation time, temperature, pH, carbon and nitrogen sources, metal ions and natural substrates. Furthermore, the enzyme was purified by chromatographic technique and the highly purified enzyme was characterized based on the temperature and pH.

MATERIALS AND METHODS

Sample Collection: The dairy effluents were collected from the Heritage Foods India Ltd., Ambattur, Chennai. The samples were brought to the laboratory under aseptic conditions in a sterile container.

Isolation of β-Galactosidase Producing Bacteria: The diary effluent samples were serially diluted and plated on nutrient agar plates infused with 50 µl X-Gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside; 20mg/ml of DMSO) to select the colonies showing lactose fermenting ability. The plates were then incubated at room temperature for 48 hours. Blue colonies were observed on the plates indicating the presence of β-galactosidase producing bacteria [7].

Sporulation: Each blue colony was plated on nutrient agar medium and sub cultured periodically for maintaining pure culture. In order to isolate spore forming bacteria, one loopful of isolates were inoculated in 50 ml of Difco Sporulation Medium (DSM) [8]. The plates were incubated at 37°C for 48 hours and the spores obtained were purified as prescribed by Venkateswaran *et al*. [9] and the spore formations were checked microscopically using Schaeffer-Fulton staining technique [10]. Those spores which were stained green indicated the presence of endospores [11].

The bacterium which shown maximum enzyme activity was characterized based on Bergey's Manuel of Systematic Bacteriology [12].

16S rRNA Sequencing: The genomic DNA was isolated from the bacteria by the method described by Hosek *et al*. [13]. The highly purified DNA was then amplified in a thermocycler at conditions: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min which amplifies the 16S rRNA sequences specifically by using the primers [14].

Forward (16F27): 5′-AGAGTTTGATCCTGGCTCAG-3′
Reverse(16R1522):5′-AAGGAGGTGATCCAGCCGCA-3′

DNA sequencing was performed in a highly automated gene sequencer. These sequences were read in Genbank databases (BLAST) and compared with the other sequences to analyze the bacterial class and its phylogeny.

Antibiotic Susceptibility Marker to Detect the Probiotic Nature of BPTK4: The antibiotic susceptibility of BPTK4 was analyzed by using standard single disc-diffusion method [15, 6]. The overnight culture of the test organism was seeded on nutrient agar plate using a swab. The various antibiotic-impregnated discs containing amoxicillin (25 µg), penicillin (10 µg), ciprofloxacin (5 µg), gentamycin (10 µg), cotrimoxazole (25 µg), chloramphenicol (30 µg), bacitracin (8 µg), tetracycline (30 µg), kanamycin (30 µg), erythromycin (15 µg), vancomycin (30 µg), ampicillin (10 µg), streptomycin (10 µg), Clindamycin (2 µg) and Methicillin (5 µg) were placed on the seeded plate and the plates were incubated at 37°C. The zone of growth inhibition was determined after 24 hr.

Enzyme Production

Inoculum Preparation for the Production of β-Galactosidase: One loopful of overnight grown bacterial culture was transferred to the LB broth as prescribed by Robert *et al.*, 2006 [16].

The Production Medium: Lactose-10g/L, Peptone-1.5g/L, Yeast extract-1g/L, KH_2PO_4-1g/L, $NH_4H_2PO_4$-7g/L, $MgSO_4.7H_2O$-1g/L and $CaCl_2$-0.3g/L [17]. For effective production, it was carried out in a shaker with an aeration rate of 200 rpm under the volume 50/200 v/v at 37°C for 24 hours [18, 3]. The samples were centrifuged at 10,000 rpm for 10 min at 4°C and the culture filtrate was used for further assay procedures.

Enzyme Assay: The β-galactosidase activity was measured by the method of *Gumgumjee* and *Danial* [17]. The enzyme was assayed by using ONPG as a substrate prepared by dissolving 2.5 mg/ml of ONPG in 0.1M sodium acetate buffer (pH-5). 0.2 ml of the culture filtrate was added to 1 ml of the substrate solution and incubated at the temperature of 55°C for 20 min. The reaction was then stopped by adding 1 ml of 10% sodium carbonate.

The absorbance was read at 420 nm and the amount of ONP was calculated using standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µM of ONP per min at the temperature of 55°C.

The amount of protein was estimated by Bradford method using Bovine Serum Albumin (BSA) as a standard according to the instruction manual of Quick Start Bradford Protein Assay [19].

Optimization of Culture Conditions for Enzyme Production: The effects of time, temperature and pH on the production of the enzyme were studied. These were carried out by cultivating the isolate at different times (6-96 hrs) [3], different temperatures (20-60°C) [3] and different pH values (5-9) [20]. The β-galactosidase activity and the protein content were assayed [17, 19].

Effect of Various Carbon Sources: The isolate was grown in the production medium containing various carbon sources (1% m/v) including glucose, lactose, galactose, sucrose, starch, xylose, fructose to study their effect on enzyme production [3].

Effect of Various Nitrogen Sources: The production medium was supplemented with different nitrogen sources (0.015% m/v) including organic nitrogen sources such as yeast extract, tryptone, beef extract, peptone, casein and the inorganic nitrogen sources such as urea, $(NH_4)_2SO_4$, $NaNO_3$ to investigate their effect on enzyme production [3].

Effect of Various Metal Ions: The effect of various metal ions such as $MgCl_2.6H_2O$, $MnSO_4.7H_2O$, $FeSO_4$, $ZnSO_4$ and EDTA (at 1mM concentration) added to the culture medium on enzyme production was determined [20].

Effect of Natural Substrates: Natural substrates such as wheat bran, rice bran, wheat flour and potato starch were studied at 1% m/v and the medium was optimized for the maximum production of the enzyme [3].

Purification of the Enzyme: After 2 days of incubation period, cell free supernatant was subjected to precipitation by adding chilled acetone 1:1.5%*v/v* (culture broth to acetone) at 20°C, stirred well and the mixture was kept at 4°C for 12hrs. The precipitate was recovered by centrifugation at 8,850×*g* for 30min and dissolved in a minimal volume of 50mM acetate buffer, pH 5.0 and was dialyzed overnight against 5mM acetate buffer of pH 5.0. This enzyme solution was loaded onto a pre-equilibrated Sephadex-G-75 column (2□×□100cm) and was eluted with the same buffer at a flow rate of 0.5ml/min. Five-milliliter fractions were collected [21] and those showing β-galactosidase activity were pooled and collected fractions were analyzed for protein content and β-galactosidase activity [19, 17].

Electrophoretic Analysis: Protein homogeneity of purified β-galactosidase from the isolate was assessed by 10% SDS-PAGE [22]. The proteins were stained with 0.25% (*w/v*) Commassie Brilliant Blue R-250. Medium molecular weight (Sigma Chemicals Ltd, USA) protein markers were used.

Characterization of Purified Enzyme

pH and Temperature Dependence on the Activity of the Enzyme: The pH dependence was determined by using ONPG as a substrate in a series of buffers of different pH values ranging from 5-9. The different buffers used were 0.1 M sodium acetate buffer (pH 5-5.5), 0.1 M sodium phosphate buffer (pH 6-7.5) and 0.1M Tris HCL buffer (pH 8-9). The activity of the enzyme was measured at different pH after 30 min. The temperature dependence was determined by incubating the samples at different temperatures ranging from 35-95°C for 30 min and the activity of the enzyme was measured by standard ONPG conditions [17].

pH and Temperature Dependence on the Stability of the Enzyme: The activity of the enzyme was measured by incubating the enzyme solution in different pH values as mentioned above at 37°C for 30 min and then assayed under standard ONPG conditions. The influence of temperature on the stability of the enzyme was determined by incubating the enzyme solution in 0.1M sodium acetate buffer pH 5 over a broad range of temperature 35-95°C for 15-150 min. The tubes/aliquotes were then withdrawn and immediately kept in ice for few min and then assayed as described above [17].

RESULTS AND DISCUSSION

Microbial enzymes are of high industrial importance; can be used as a food supplement/dietary product and are available commercially [23]. β-galactosidase from fungal [24] and yeast [25] sources has been used widely and has dominated application in the industrial sector. Traces of beta galactosidases were also observed in *Penicillium* species [26]. The bacterial strains present in dairy industry effluents have been proved to be probiotic and has a greater ability to produce β-galactosidase [7].

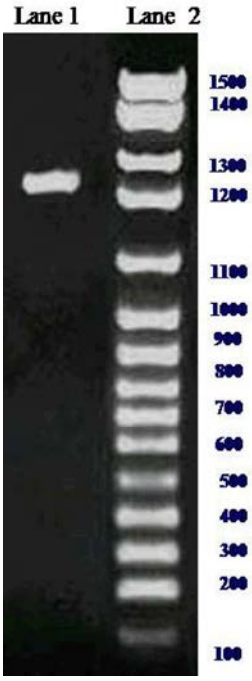

Fig. 1: Photographic representation of PCR amplified product in Agarose Gel (Lane 1, PCR amplified sample; Lane 2, 15 Kbp ladder)

The serially diluted dairy effluent samples cultivated into the screening medium incorporated with X-gal, which resulted in the formation of 18 blue colored colonies indicating the production of β-galactosidase by the respective bacterial strains. When the individual colonies were cultured in the sporulation media, it resulted with 6 strains with spore forming ability. Of the 6 spore forming strains, one best β-galactosidase producing strain (BPTK4) having maximum enzyme activity was selected and preceded for further investigations.

From microscopic appearance and the biochemical tests, the isolate was identified as *Bacillus* sp. BPTK4 and further confirmation was done by sequencing the 16S rDNA gene and compared with the GenBank databases using the BLASTN program (Fig. 1). The 16S rDNA sequence of the isolate revealed a close relatedness to *Bacillus subtilis* with 99-100% similarity. Hence the strain was confirmed as *Bacillus subtilis* and the sequence was submitted to Genbank (JF749812).

The isolate BPTK4 was inhibited by most of the antibiotics but showed resistance to penicillin. This makes the isolate to be used as a probiotic for lactose intolerance people and also in dairy industry. For a organism to act as probiotic, it should possess therapeutic and antimicrobial properties beside of their organoleptic characteristics [27].

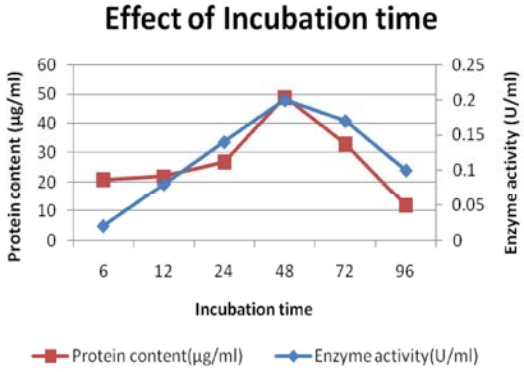

Fig. 2: The effect of incubation time on β-galactosidase production.

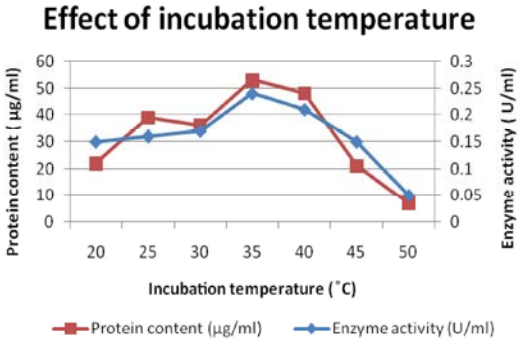

Fig. 3: The effect of temperature on β-galactosidase production.

David *et al.*, 1990 [28] reported the different strains of *Bacillus* sp. which are commercially available now like biosubtyl and Enterogemina as probiotic preparations by using antibiotic markers. On the other hand, Parvathi *et al.*, 2009 [6] reported that *Bacillus pumilus* causes skin infections in immunocompromised individuals. So, further studies must be performed to understand the effect of micro organisms on human health, their genetic basis and resistance to antimicrobials.

Incubation time depicts the characteristics of the culture and is also based on the growth rate and enzyme production [3]. There was a profound influence on the activity of enzyme (0.20 U/ml) at 48 hrs (Fig. 2). Similar results were obtained by Ismail *et al.*, 2010 [21] while working on the production of β-galactosidase from *L. acidophilus*. Maximum β-galactosidase production was also reported at 48 hrs by *Gumgumjee* and *Danial* [17]. The decrease in the enzyme activity after 48 hrs (Fig. 1) might be due to the decrease in the amount of nutrients in the medium or due to denaturation of the enzyme. Like other mesophilic bacteria, *Bacillus* sp. producing β-galactosidase was best grown when incubated at 35°C (0.24 U/ml) (Fig. 3). Chakraborti *et al.*, 2003 [29] showed

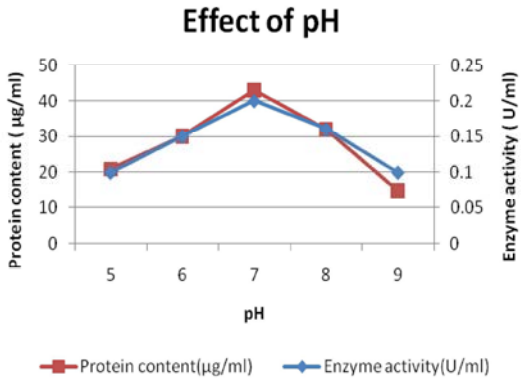

Fig 4: The effect of pH on β-galactosidase production.

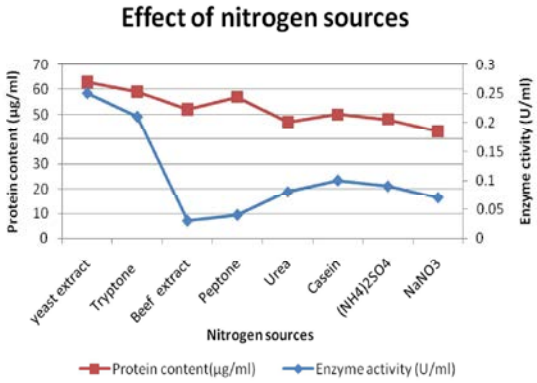

Fig. 6: The effect of various nitrogen sources on β-galactosidase production.

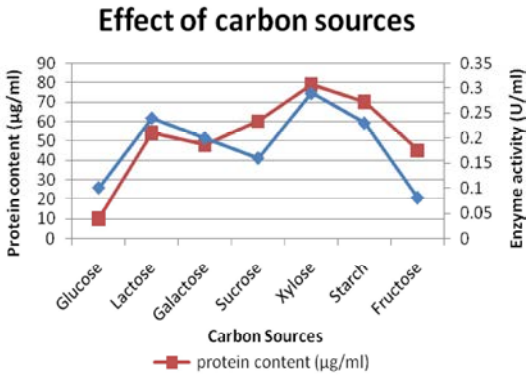

Fig 5: The effect of various carbon sources on β-galactosidase production.

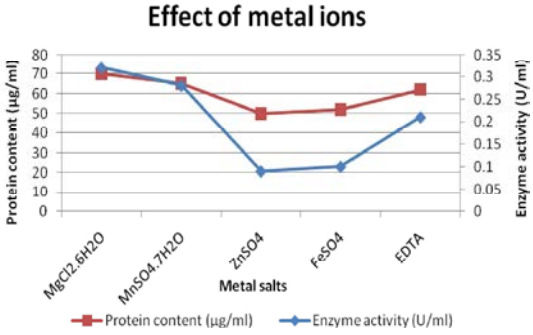

Fig. 7: The effect of addition of different metal ions on β-galactosidase production.

that β-galactosidase was produced at maximum level when maintained at temperatures of 37°C. The hydrogen ion concentration affects two aspects of the microbial cells: function of the cells and the transport of nutrients to the cells [24]. So maintaining the pH value is very important. The maximum production was observed at pH 7 (0.20 U/ml). At pH 5 and 9, the activity of βgalactosidase was 0.10 U/ml (Fig. 4). El-Shebawy *et al.*, 2007 [20], Chakraborti *et al.*, 2003 [29] and Yapi *et al.*, 2009 [30] reported that *Bacillus* sp. can be grown maximally when the medium is maintained at a pH of 7.

The carbon source is very essential for the effective production of the enzyme. Xylose (0.29 U/ml) (Fig. 5) was found to be the most effective carbon source for the maximum production of enzyme. This is correlated with the findings of Akcan [3]. In our study, lactose (0.24 U/ml) and galactose (0.20 U/ml) also seemed to enhance the enzyme production. Galactose was shown to be the effective carbon source in the biosynthesis of α-galactosidase by *B. circulans* [20].

In most of the organisms, both the organic and inorganic forms of nitrogen are metabolized within the cell to produce amino acids, nucleic acids, proteins and cell wall components [3]. Various nitrogen sources were used, of which, rganic sources such as yeast extract (0.25 U/ml) and tryptone (0.21 U/ml) had an influence over the enzyme production when compared to beef extract where the activity was reduced to 0.03 U/ml (Fig. 6). These findings are related to El-Shebawy *et al.* [20] who worked on the production of α-galactosidase from *B. circulans*. Ismail *et al*. [21] also showed that yeast extract had an influence on β-galactosidase activity. But the inorganic nitrogen sources such as ammonium sulfate (0.09 U/ml) and sodium nitrite (0.07 U/ml) did not contribute much to the good growth and the production when compared to organic sources. Similar results are shown by El-Shebawy *et al.* [20] and Ismail *et al*. [21].

When the culture was supplemented with Mn^{2+} (0.28 U/ml) and Mg^{2+} (0.32 U/ml) at 1 mM concentration, an increase in the enzyme activity was observed (Fig. 7), Different authors reported that Mn^{2+} and Mg^{2+} of different concentrations had an influence on the enzyme yield. Ismail *et al.*, 2010 [21] reported that Mn^{2+} and Mg^{2+} of 0.02 M increased the enzyme yield. Kumari *et al.*, 2011 [31] worked on the production of β-galactosidase

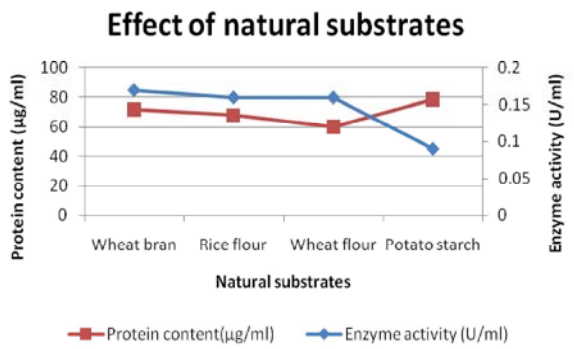

Fig. 8: The effect of natural substrates on β-galactosidase production.

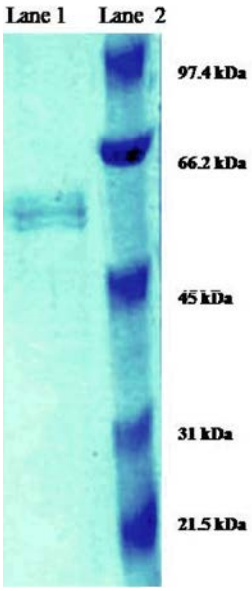

Fig. 9: Photographic representation of SDS-PAGE Gel (Lane 1, Marker Proteins; Lane 2 showing β-galactosidase single band after DEAE-Sephacel)

using yeast isolate from whey and reported that at concentration of 0.05%, Mg^{2+} increased the enzyme production.

Among the natural substrates used, wheat bran showed a relatively high production of enzyme (0.17 U/ml). The production rate was slightly decreased by other substrates (Fig. 8). On the other hand, Akcan [3]

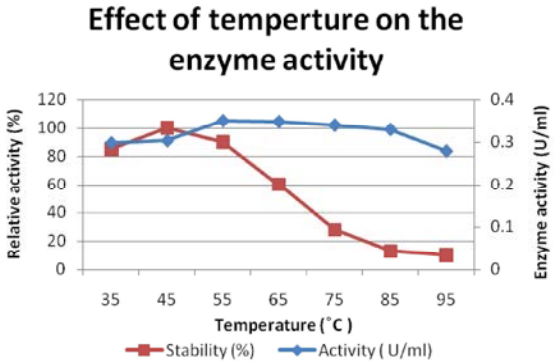

Fig. 10: The effect of temperature on the activity and stability of the enzyme.

reported that when the production medium was supplemented with rice flour, maximum production of β-galactosidase was observed.

The extracellular β-galactosidase activity was subjected to acetone precipitation at 1:1.5% *v/v* (culture filtrate/acetone) saturation. After solvent precipitation, approximately 24.98% yield was obtained. Sephedex-G 75 chromatography resulted to 10.14 % yield with increase in 9.5-fold of purification. After ion exchange chromatography using DEAE-Sephacel column, 15-fold of purification and overall yield of approximately 3.82% were observed (Table 1).

The molecular weight of the purified β-galactosidase was determined using SDS-PAGE analysis and was found to be 65 Kda (Fig. 9). Osiriphun and Jaturapiree [32] and Gote *et al*. [33] reported β galactosidase with the molecular weight of 75 and 79.9 Kda. The galactosidase activity of *Bacillus thuringiensis* (116 KDa) was also observed by El-Sadawy, 2008 [34].

The purified enzyme was characterized based on the temperature and pH and its activity was found to be maximum at the temperature of 55°C (0.350 U/ml) and pH 7 (0.294 U/ml) (Fig. 8, 9). Quyen *et al.* [35] and Fatma Isuk ÜSTOK, 2007 [36] also reported that the optimum temperature and pH for the better growth and activity of β-galactosidase was 55°C and pH 7. Osiriphun and Jaturapiree [31] reported that β-galactosidase activity increases at the temperature of 60°C with an optimum pH of 6.5.

Table 1: Purification of β-Galactosidase

Purification steps	Total volume (ml)	Enzyme activity (mg)	Protein activity (U/ml/mg)	Specific activity (U/ml/mg)	Purification fold	Yield %
Crude extract	100	0.490	70	0.007	1	100
Acetone precipitation	30	0.408	17.74	0.023	3.3	24.98
Sephadex-G75	12	0.414	1.89	0.219	9.5	10.14
DEAE-Sephacel	5	0.374	0.11	3.307	15.1	3.82

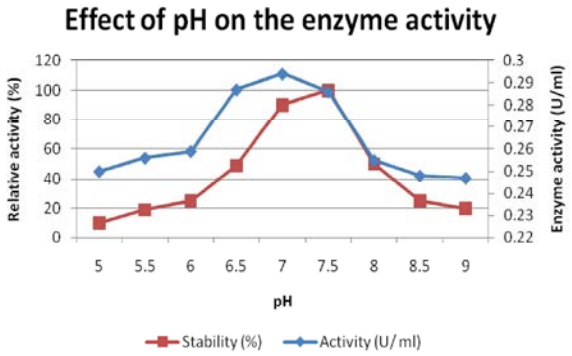

Fig. 11: The effect of pH on the activity and stability of the enzyme.

The stability of the purified enzyme was determined between the temperature ranges of 35-45°C where it retained 100% of its activity at the temperature of 45°C and at pH 7 (90%) (Fig. 10, 11). Quyen *et al.* [35] and Fatma Isuk ÜSTOK, 2007 [34] showed that the thermo stability of the enzyme was between the temperature range of 20-37°C when maintained at pH 7. A slight variation in the thermo stability range was observed at 27-37°C by Nagy *et al*. [25].

A thermostable *Bacillus subtilis* strain BPTK4 isolated from dairy effluent and characterized for β-galactosidase. This strain showed that it is an ideal candidate for hydrolysis of lactose in milk which can be used for lactose intolerant people.

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