Characterization of Thermally Stable β Galactosidase from *Anoxybacillus flavithermus* and *Bacillus licheniformis* Isolated from Tattapani Hotspring of North Western Himalayas, India

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A B S T R A C T

Nineteen thermophilic bacterial isolates were screened and only two (PW10 and PS7) produced extracellular, auto inducible β-galactosidase. PW10 and PS7 was Gram's positive, rod shaped and exhibit growth between 50-80 °C and pH 5-9. Optimum β-galactosidase activity of 32083.33 U/mg/min was observed at 60 °C and pH 7 for PS7, while 2666.66 U/mg/min at 60 °C and pH 9 for PW10. 16S rDNA sequencing of PW10 showed 99% similarity with *Anoxybacillus flavithermus* and PS7 with *Bacillus licheniformis* (GenBank accession no. KF039883 and KF039882). Lactose supplementation enhanced β-galactosidase production by 7.6 folds in PS7, while 2.5 folds in PW10. Ethanol and hydrogen peroxide does not affect growth of PS7 isolate, while ethanol decreased the growth by 7.3 folds. Hydrogen peroxide inhibited growth of PW10. β-galactosidase of PS7 was metal independent, while β-galactosidase was metal activated in PS10. Presence of lactose and glucose activated β-galactosidase, while glucose did not affect β-galactosidase activity in both isolates. Maximum β-galactosidase production was observed at ~ 72 h of incubation. Km value of 8.0 mM with ONPG (60° C) was determined for PS7 and 1.3 mM for PW10. β-galactosidase of both isolates was stable at 4 and 25 °C for 5-6 days.

Introduction

Thermophilic and thermostable β-galactosidase (EC 3.2.1.23) has applicable in food industry. β-galactosidase is a hydrolase enzyme which catalyzes the breakdown of substrate lactose, a disaccharide sugar found in milk into two monosaccharide galactose and glucose. β-galactosidase has tremendous potential in research and application in various fields like food, bioremediation, biosensor, diagnosis and treatment of disorders (Asraf, 2010). Lactose is a major problem in dairy and food industry. β-galactosidase deficiency or low level in intestine causes lactose intolerance and people face difficulty in consuming milk and dairy products. Lactose has a low relative sweetness and solubility, and excessive lactose in large intestine can lead to tissue dehydration, poor calcium

absorption, and fermentation of the lactose by microflora resulting in fermentative diarrhea, bloating, flatulence, blanching and cramps, and watery diarrhea (Shukla and Wierzbicki, 1975). Lactose gets crystallized, which is a major limitation of its application in the dairy industry. Cheese manufactured from lactose hydrolyzed milk ripens more quickly than that made from normal milk (Tweedie *et al.,* 1978; Pivarnik *et al.,* 1995). Furthermore, hydrolysis by β-galactosidase could make milk most suitable to a large number of adults and children that are lactose intolerant. Moreover, the hydrolysis of whey converts lactose into a very useful product like sweet syrup, which can be used in various processes of dairy, confectionary, baking, and soft drink industries (Shukla and Wierzbicki, 1975; Tweedie *et al.,* 1978). Therefore, lactose hydrolysis not only allows the milk consumption by lactose intolerant population, but can also solve the environmental problem of whey disposal (Martinez and Speckman, 1988; Gekas and Lopez-Leiva, 1985; Champluvier *et al.,* 1986). β-galactosidases are also very useful for the production of galactooligosaccharides (GOS). Galactooligosaccharides are used as prebiotic food ingredients and are produced simultaneously during lactose hydrolysis due to transgalactosylation activity of the β galactosidase (Rabiu *et al.,* 2001). Thermostable β-galactosidases are of particular interest, since they can be used to treat milk during pasteurization and boiling. Most effective β galactosidase would be extracellular in nature, not inhibited by sugars and metal ions present in milk and the β-galactosidase which can tolerate high temperature of pasteurization or boiling. An extremely thermostable β-galactosidase produced by a hyperthermophilic archaea of *Pyrococcus woesei* active up to 110 °C and optimally at 93 °C has been reported (Dabrowski *et al.,* 2000). Extracellular β-galactosidase was purified and isolated from

Bacillus sp. MTCC3088 (Chakraborti *et al.,* 2000). β-galactosidase of *Bacillus stearothermophilus* was cloned into *Bacillus subtilis*, and resulted into increase (50 folds) in β-galactosidase production (Hirata *et al.,* 1985). Thermophilic β-galactosidase from a thermophile B1.2 was isolated from Ta Pai hot spring, Maehongson, Thailand (Osiriphun and Jatrapire, 2009). β-galactosidase from thermophiles is of much interest because of their thermostability. Tattapani hotspring situated in North West Himalayas remained unexplored to identify thermophilic bacteria producing β-galactosidase. Therefore we decided to isolate thermophilic bacteria from Tattapani hotspring of Himachal Pradesh, situated in snowy mountains of North West Himalayas.

Materials and Methods

Screening of thermophiles for the production of β galactosidase

Nineteen thermophilic bacterial isolates named as PW1, PW2, PW3, PW4, PW5, PW6, PW7, PW8, PW9, PW10, PW11, PW12, PS2, PS3, PS4, PS5, PS7, PS9 and PS10 were isolated by Ms Parul Sharma, Ph.D. (Biotechnology) scholar, Shoolini University Solan, Himachal Pradesh, India These isolates were collected from Tattapani hotspring situated in Mandi District of Himachal Pradesh, India. All the isolates were screened for the production of β-galactosidase. The ability of the nineteen isolates to produce β-galactosidase was examined on nutrient agar medium containing 0.25 mM 5-bromo-4-chloro-3-idolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate and 6.25mM isopropyl β-D-1 thiogalactopyranoside (IPTG) as an inducer for the β-galactosidase. X gal acts as substrate for the β-galactosidase and is hydrolysed into blue colored compound named 5, 5'-dibromo-4, 4'-dichloro-indigo, which is formed by the dimerization and

oxidation of 5-bromo-4-chloro-3-hydroxyindole.

Quantitative Estimation of β-galactosidase enzyme

Bacterial cultures were grown at 60 °C and 250 rpm for 24 hours in nutrient broth medium. Cultures were centrifuged and cells were washed with 0.85% NaCl followed by 1 ml Z buffer. Cell pellet was resuspended in 1ml Z buffer containing 0.002 % SDS and 10 μl chloroform, followed by vortexing and incubation for 2 min at 30° C. The cell debris was separated by centrifugation at 4,000 rpm at 4 °C for 10 mins. The supernatant thus obtained served as intracellular source of crude β-galactosidase enzyme (Miller (1972)). For extracellular enzyme, cell free spent medium was used as enzyme source. The protein concentration was determined by the Bradford method (Bradford (1976)) using bovine serum albumin (BSA) as standard. For protein estimation, 1X Bradford dye was prepared from 5X stock solution. 50 μl of cell free spent medium or intracellular crude enzyme source was mixed with 3 ml of Bradford reagent (1X). This mixture was incubated at 25 °C for 5 mins and absorbance was taken at 595 nm. Standard graph of BSA was prepared by taking 2, 4, 6, 8 and 10 μg of BSA. Protein concentration was determined from the standard graph of BSA. β-galactosidase enzyme activity was quantitatively assayed at different temperatures of 4, 30, 40, 50, 60, 70 and 80 °C by incubating 5 μg total protein with 3.3 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in Z buffer for 1h. β-galactosidase activity was measured at different pH ranging from 3 – 11. Alkaline pH of Z buffer was adjusted by using disodium hydrogen phosphate (Na_2HPO_4) and acidic pH 3 and 5 by using dihydrogen sodium phosphate (NaH_2PO_4). The reaction was stopped by adding 500 μl of 1 M Na_2CO_3 and the amount of *o*-nitrophenol (ONP) released

was determined by measuring the absorbance at 420 nm (Miller, 1972). One unit of β-galactosidase activity (U) was defined as the amount of enzyme that releases 1 μmol of ONP from ONPG per minute.

Identification of PS7 and PW10 by Gram's staining and 16S rDNA amplification

Morphological (shape) characterization was performed by Gram's staining (15). For 16S rDNA amplification, strains PW10 and PS7 were grown in nutrient broth medium for 24 hours at 60 °C to A 600 of 1.5 – 2.0. For genomic DNA isolation, cultures were centrifuged at 8000 rpm for 5 minutes and cells were resuspended in extraction buffer (100 mM Tris HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl, 0.07% β mercaptethanol, 20 mg/ml lysozyme and 1% SDS). Reaction mixture was incubated at 65 °C for 30 mins and centrifuged at 12000 rpm for 15 min (Sambrook and Russell (2001)). Supernatant was collected and mixed with equal volume of phenol and chloroform (1:1), followed by vortexing and centrifugation at 12000 rpm for 5 min. Aqueous layer was collected and phenol chloroform step was repeated. To the aqueous phase, $1/10^{th}$ volume of 5M NaCl and 2.5 volumes of absolute ethanol was added and incubated at -20 °C for 2 hours, followed by centrifugation at 12000 rpm for 15 mins. Supernatant was discarded and pellet was washed with 70% ethanol, dried and resuspended in 30 μl TE buffer (1 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0). DNA quantification was performed by measuring absorbance at 260 and 280 nm in a UV-Visible spectrophotometer. The 16S rDNA was amplified using the universal primers 27F (3` AGAGTTTGATCCTGGCTCAG 5`) and 1492R (3` GGTTACCTTGTTACGACTT 5`) (Frank *et al.,* 2008). 50 ng of DNA was subjected to initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C (30 sec),

45 °C (30 sec), 72 °C (1:30 min), and final extension at 72 °C for 10 min. The amplified products were purified using Axygen gel elution kit. DNA sequencing of both the strands was done by 27F and 1492R primers at Xcelris Labs Ltd. Ahmedabad, India (http://www.xcelrislabs.com/). Overlapping of sequences obtained by forward (27F) and reverse (1492R) primers were remade manually. The DNA sequences thus obtained were subjected to nucleotide blast (nblast) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and results were analyzed for strain identification. A phylogenetic tree was constructed by taking 16S rDNA sequences of all related bacterial sp.

Effect of different solvents on the growth of thermophilic bacteria

Different solvents like phenol, cyclohexane, hydrogen peroxide, butanol, ethanol and toluene were supplemented in growth media (nutrient broth) at 0.05 and 0.1% concentration except ethanol, which was used at 0.5 and 1% concentration. Bacterial isolates (PS7 and PW10) were grown in the presence of these solvents for 24 hours at 60 °C and 250 rpm. Negative controls with no supplementation of solvents were used and absorbance was measured at 600 nm.

Effect of incubation time, temperature and pH on β-galactosidase activity

To optimize the time for the production of maximum β-galactosidase, bacterial isolates were grown at 60 °C and 250 rpm. Cultures were harvested at different time intervals (12, 24, 48, 72, 96, 120 and 144 hours) and β-galactosidase activity was determined by taking supernatant at different time intervals and performing ONPG assay at 60 °C for 1 hour. The optimal temperature and pH was determined over the range 30 – 80 °C and 4 °C temperature. The pH of the enzymatic assay varies from 3-11.

Effect of carbon and nitrogen sources on β-galactosidase activity

Different carbon sources such as glucose, fructose, galactose, raffinose, maltose, starch, sucrose, xylose, inositol, trehalose and sorbitol were employed to study their effect on β-galactosidase production by bacterial isolate PS7 and PW10. All the carbon sources were supplemented at 1% concentration in the nutrient broth medium. Similarly nitrogen sources like yeast extract and urea were supplemented in the nutrient broth medium to study the effect on β-galactosidase production by strains PS7 and PW10. The bacterial isolates PS7 and PW10 were grown in nutrient broth medium containing different carbon and nitrogen sources at 60 °C for 24 hours. Cell free spent medium was used to perform the β-galactosidase assay at 60 °C for 1 hour. The effect of carbon and nitrogen sources on the growth of isolates PS7 and PW10 was studied by measuring the absorbance at 600 nm and the correlation between enzyme activity and growth was studied by comparing the absorbance of the culture at 600 nm and specific activity.

Effect of glucose, galactose and lactose on β-galactosidase activity

Effect of sugars like glucose, galactose and lactose was studied on β-galactosidase activity by supplementing ONPG assay reaction with different concentrations (0.1, 0.5 and 1%) of sugars. In this assay, two substrates ONPG with glucose, ONPG with galactose and ONPG with lactose were used at the same time.

Effect of metal salts on β-galactosidase activity

The effect of metal ions (Na^+, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+}) on β-galactosidase activity was tested by adding different

concentrations of each different salts ranging from 1-5 mM into the ONPG assay. Effect of metal ions on growth was studied by growing strains PS7 and PW10 in the presence of metal ions (1 – 5 mM) at 60 °C and 250 rpm for 72 hours and measuring absorbance at 600 nm. Growth and β-galactosidase activity correlation was determined by comparing growth and β-galactosidase activity.

Kinetic parameters determination

Kinetic parameters like K_m and Vmax were determined by performing ONPG assay for bacterial isolate PS7 and PW10. ONPG assay was performed by varying the concentration of ONPG (0.15, 0.30, 0.45, 0.60, 0.75, 0.90, 1.05, 1.20, 1.35 and 1.50 mM) and keeping enzyme concentration constant (5 mg).

Reaction kinetics of β galactosidase

Reaction kinetics of β-galactosidase were studied for both PS7 and PW10 bacterial isoalte by varying the time period for ONPG assay from 10, 20, 30, 40, 50 and 60 minutes. After incubation, the reaction mixture was stopped by adding 500 μl of 1 M Na_2Co_3 and absorbance was measured at 420 nm.

Thermostability of β galactosidase

β galactosidase thermostability was studied by incubating enzyme source (supernatant) at 4, 25 and 60 °C for 1-6 days and performing ONPG assay at 60 °C for 1 h. ONPG assay was performed at different time intervals such as 0, 24, 48, 72, 96 and 120 hrs.

Results and Discussion

Screening of thermophiles for the production of β galactosidase

Nineteen thermophilic bacterial isolates (isolated from Tattapani hotspring, Mandi,

Himachal Pradesh, India) were screened for the production of β-galactosidase. All the isolates were creamish white in color, rod shaped and Gram's positive. All the bacterial isolates showed growth between 50 – 80° C. Figure 1 showed the growth of bacterial isolate PS7 and PW10 at different temperature. Both PS7 and PW10 did not show growth below 50° C. The optimum growth was observed at 70 ° C (Figure 1) and detectable growth was observed even at 80° C (data not shown). While screening for the production of β-galactosidase, quantitative and qualitative assays showed that only PS7 and PW10 showed β-galactosidase activity. Bacterial isolates PS7 and PW10 showed blue coloration when streaked on nutrient agar (NA) medium containing Xgal or IPTG and Xgal (Figure 2). β-galactosidase assay was also performed by using cell free spent medium and appearance of blue coloration was observed for PS7, PW10 and a mesophile bacterial isolate A5-2 isolate (control) at 30 °C (Figure 3). Interestingly, blue coloration was only observed in cell free spent medium of bacterial isolate PS7 and PW10 at 50 °C. Bacterial isolate A5-2 was a mesophilic strain and did not grow at 50 °C and hence no blue coloration due to β galactosidase production.

Nature (intracellular/extracellular) of β-galactosidase in PS7 and PW10 isolates

Bacterial isolates PS7 and PW10 were grown at 60 °C and 250 rpm for 24 hours. Cell free spent medium was assayed to test extracellular nature of β-galactosidase, while the cell lysate for intracellular form of β-galactosidase. Equal amount of proteins of cell extract and cell free spent medium was subjected to ONPG assay at different temperatures and pH. It was observed that cell free spent medium of PS7 isolate showed maximum activity at 60 °C (2700 U/mg). Similarly, maximum β galactosidase activity was observed at 60 °C (1200 U/mg) for PW10 isolate. The enzyme

activity was reduced to 69.3 % and 82.6 % for PS7 and PW10 isolate at 4 °C. interestingly, no β-galactosidase activity was observed in the cell extracts of both PS7 and PW10 isolate, which showed extracellular nature of β-galactosidase. In general, PS7 isolate showed 5.4 fold increase in the β-galactosidase activity as compared to the PW10 isolate in the cell free spent medium at 60 °C (Figure 4).

Identification of PS7 and PW10 isolates by 16S rDNA sequencing

For identification of PS7 and PW10 bacterial isolates, 16S rDNA amplification was performed. Total genomic DNA of PS7 and PW10 was isolated (Sambrook and Russell (2001)) as shown in Figure 5A. 16S rDNA was amplified by using universal primers 27F and 1492R (Frank *et al.,* 2008) The PCR product of approximately 1500 bps was observed (Figure 5B). PCR amplified DNA was sequenced on both the strands using 27F and 1492R primers. A complete nucleotide sequence of PS7 (1398 bps) and PW10 (1257 bps) was generated and subjected to nucleotide blast. Isolate PS7 showed 99% sequence similarity with *Bacillus licheniformis* (Accession no. NR_074923) (Ray *et al.,* 2004), while PW10 showed 99% sequence similarity with *Anoxybacillus flavithermus* (Accession no. NR_074667) (Saw *et al.,* 2008). Based on the nucleotide blast homology, PS7 was named as *Bacillus licheniformis* strain PS7 and PW10 as *Anoxybacillus flavithermus* strain PW10. Nucleotide sequences were submitted in the GenBank database, under the accession no. KF039882 for *Bacillus licheniformis* PS7 and KF039883 for *Anoxybacillus flavithermus* PW10. Extracellular β galactosidase of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 are the best among the reported thermophilic β galactosidases. In order to find out the lineage of PS7 and PW10

isolate, phylogenetic tree was constructed by selecting all the *Bacillus* spp. from the nblast results of 16S rDNA sequence. All the selected *Bacillus* spp showed four distinct groups. It was observed that *Bacillus licheniformis* PS7 evolved with *Bacillus licheniformis* DSM 13 (Genebank ID - KY174334), *Bacillus aerius* 24K and *Bacillus sonorensis* (Genbank ID - NR_042338 and KU922436) in a group but by an independent branch (Figure 6).

Unrooted phylogenetic tree in Figure 7 (supplementary material) was constructed by selecting all related *Anoxybacillus* spp from nucleotide blast results. It was observed that *Anoxybacillus flavithermus* PW10 evolved with *Anoxybacillus pushchinoensis* K-1 (Genbank ID - NR_037100). It is interesting that genus *Anoxybacillus* and *Geobacillus* formed a independent cluster. All the *Bacillus* spp formed four distinct groups as shown in phylogenetic tree (Figure 8). Among the four groups, there is only one group that contained *Anoxybacillus* spp. and *Geobacillus* spp, along with two *Bacillus* spp (*Bacillus abyssalis* SCSIO and *Bacillus stratosphericus*), except *Anoxybacillus rupiensis* R270, which has evolved with *Bacillus* spp. *Bacillus licheniformis* PS7 has evolved with *Bacillus lichiformis* DSM 13 and *Bacillus aerius*. Genus *Anoxybacillus* formed a group with *Aeribacillus pallidus* and evolved together, while genus *Geobacillus* also formed a group with *Saccharococcus thermophilus*.

Effect of physical parameters (temperature and pH) on β-galactosidase activity of PS7 and PW10 isolates

In order to validate thermophilic nature of β-galactosidase, β-galactosidase assays of cell free spent medium were performed at 4 °C and temperature ranging from 30 – 80 °C, with 10 °C rise in temperature for both PS7 and PW10 bacterial isolates. β-galactosidase activity was

maximum between 50 – 70 °C with 2600 – 2700 U/mg. The activity was reduced by 69, 59, 60 and 58 % at 4, 30, 40 and 80 °C respectively for PS7 isolate. On the other hand, maximum activity (1150 U/mg) of PW10 isolate was observed at 60 °C. β-galactosidase activity was inhibited by 57, 58, 5, 18, 40 and 82 % at 70, 80, 50, 40, 30 and 4 °C respectively for PW10 isolate.

To study effect of pH on β-galactosidase activity, assays were performed in an assay buffer adjusted to different pH (3-11) at 60 °C. Maximum β-galactosidase activity (2766.6 U/mg) was observed at pH 7 for PS7 isolate. There was 60% reduction in β-galactosidase activity at pH 5 and 9; which was further decreased to 29 % at pH 11. At pH 3, there was 62% inhibition of β-galactosidase activity of PS7 isolate. Maximum β-galactosidase activity (2199.99 U/mg) was observed at pH 9 for PW10 isolate and it was reduced by 62, 60, 51 and 54 % at pH 3, 5, 7 and 11 respectively (Figure 9). Optimum temperature and pH for β galactosidase activity was 60° C and pH 7 respectively for *Bacillus licheniformis* PS7. On the other hand, 60° C and pH 9 was optimum for β galactosidase of *Anoxybacillus flavithermus* PW10 indicating the thermophilic nature of β-galactosidase.

β-galactosidase production is maximum during decline phase of growth in thermophilic bacterial isolate PS7 and PW10

In order to find out whether the production of β-galactosidase is growth associated or not, PS7 and PW10 bacterial isolates were grown in NB medium supplemented with lactose. Cultures were withdrawn at different time intervals, cell density was measured at 600 nm and β-galactosidase activity was measured in the cell free spent medium as described under section 2. Both PS7 and PW10 bacterial isolates showed logarithmic growth till 24

hours of incubation. The growth was declined after 24 hours in PW10 isolate, but after 48 hours in PS7 isolate. In contrast, β-galactosidase activity was negligible (3000 U/mg for PS7 and 2500 U/mg for PW10 isolate), when the bacterial growth was maximum at 36 hours. There was a steep increase in β-galactosidase activity after 40 h of growth. Maximum β-galactosidase activity was observed at 72 hours of growth and declines after 72 hours (Figure 10). This data clearly indicate that β-galactosidase was produced as a seeding metabolite during death phase of PS7 and PW10 bacterial isolates. It was observed that β-galactosidase activity was 1.6 fold higher in PS7 isolate as compared to PW10 isolate. Supplementation of nutrient broth with lactose, not even enhanced the growth, but also increases β-galactosidase activity in PS7 and PW10 isolates. Lactose supplementation enhances β-galactosidase activity by 7.5 fold in PS7 and 2.5 fold in PW10 bacterial isolate as compared to nutrient broth (without lactose supplementation). This is the first report of its kind that β-galactosidase production is maximum during the declined phase of PS7 and PW10 bacterial isolates.

Effect of carbon and nitrogen sources on β-galactosidase activity

Effect of different carbon and nitrogen sources was studied to know the best carbon and nitrogen source for the production of β-galactosidase by *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10. Different sugars, like glucose, galactose, fructose, xylose, sucrose, maltose, sorbitol, starch, trehalose, raffinose, sorbitol, inositol and lactose were supplemented in the growth medium and β-galactosidase activity was measured. Among the sugars, galactose, starch, sucrose, inositol and lactose showed enhanced production of β-galactosidase by 5, 5, 1, 1, 3 and 7 folds respectively, as

compared to the un-supplemented (without carbon source) in *Bacillus licheniformis* PS7. In case of *Anoxybacillus flavithermus* PW10, galactose, sucrose, xylose, trehalose and lactose enhanced the β-galactosidase production by 1.5, 2, 1 and 2.5 folds respectively. As compared to control, medium containing lactose showed 32083 and 2666.66 U/mg/min β-galactosidase activity in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 respectively. β-galactosidase activity in *Bacillus licheniformis* PS7 was 12 folds higher as compared to the *Anoxybacillus flavithermus* PW10 in lactose containing medium (Figure 11A and B). β-galactosidase activity was inhibited by 75.7, 53, 71, 73.2, 46.9, 73 and 67 % when growth medium was supplemented with glucose, fructose, raffinose, maltose, xylose, trehalose and sorbitol respectively. Supplementation of glucose, raffinose, starch, inositol and sorbitol inhibited β-galactosidase activity by 67.1, 79.6, 31.2, 10.9 and 89 % respectively for PW10 isolate.

Yeast extract as a nitrogen source enhanced β-galactosidase activity by 3.5 folds in *Bacillus licheniformis* PS7 and by 1.4 folds in *Anoxybacillus flavithermus* PW10 (Figure 11 A and B). Galactose, starch, inositol and lactose supplementation enhanced the growth rate as compared to the nutrient broth (control) for *Bacillus licheniformis* PS7. In contrast, glucose, fructose, raffinose, maltose, sucrose, xylose, trehalose and sorbitol decreased the growth of *Bacillus licheniformis* PS7. Starch and lactose supplementation enhanced the growth of *Anoxybacillus flavithermus* PW10 as compared to the nutrient broth, while glucose, fructose, raffinose, maltose, sucrose, xylose, inositol, trehalose and sorbitol decreased the growth rate of *Anoxybacillus flavithermus* PW10. Supplementation of galactose, inositol and lactose enhanced the growth as well as β-galactosidase activity, while glucose, fructose, raffinose, maltose,

xylose, trehalose and sorbitol supplementation decreases growth as well as β-galactosidase activity of PS7 bacterial isolate. Lactose supplementation increases growth as well as β-galactosidase activity, while glucose, fructose, raffinose and maltose decreases growth as well as β-galactosidase activity of PW10 isolate.

Sugars like galactose, starch, sucrose, inositol and lactose enhanced β-galactosidase production in *Bacillus licheniformis* PS7. On the other hand, galactose, sucrose, xylose, trehalose and lactose were found to enhance β-galactosidase production in *Anoxybacillus flavithermus* PW10. Presence of lactose showed maximum β-galactosidase activity in both the isolates. However, catalytic activity of β galactosidase was not affected by the presence of glucose, maltose, lactose, sucrose, starch, xylose, inositol and sorbitol. This suggested that enzyme is not prone to substrate and product inhibition. In conclusion, β galactosidase of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 could be utilized for commercial production of lactose free dairy products and GOS.

Effect of different solvents on the growth of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10

PS7 and PW10 bacterial isolates were tested for their growth in the presence of solvents like ethanol, butanol, toluene, hydrogen peroxide, cyclohexane and phenol to study their application in bioremediation. It was observed that growth of *Bacillus licheniformis* PS7 in the presence of ethanol (0.5 and 1%), and hydrogen peroxide (0.05 and 0.1%) remains unaffected, while butanol, cyclohexane, phenol and toluene (0.05 and 0.1%) inhibited the growth by 8.8, 19.5, 4.7 and 1.2 fold respectively at 0.1% concentration. Ethanol was used in the higher

concentration (0.5 and 1%) as compared to the other solvents (0.05 and 0.1%), because bacteria are able to tolerate higher concentrations of ethanol than other solvents. Growth of *Bacillus licheniformis* PS7 was inhibited by cyclohexane, butanol, phenol and toluene by 1, 1.2, 9 and 1 fold at 0.05% concentration, while ethanol and hydrogen peroxide enhances the growth by 1 fold at 0.05% concentration. The growth of *Anoxybacillus flavithermus* PW10 was inhibited in the presence of ethanol (1% concentration), butanol, cyclohexane, phenol and toluene by 7.3 folds and 2.5, 1.4, 14.7 and 1.2 folds respectively at 0.1% concentration. Growth of *Bacillus licheniformis* PS7 was not inhibited by hydrogen peroxide (0.1%), while it was inhibitory for *Anoxybacillus flavithermus* PW10 (Figure 12). Growth of *Anoxybacillus flavithermus* PW10 was inhibited by ethanol, butanol, cyclohexane, phenol and toluene by 1.2, 1, 1.8, 2 and 1.2 fold at 0.05% concentration. Ethanol (1%) showed maximum inhibition (86.3 %) for *Anoxybacillus flavithermus* PW10 than *Bacillus licheniformis* PS7. Cyclohaxane at 0.1% concentration was inhibitory (94.9 %) for *Bacillus licheniformis* PS7, but not for *Anoxybacillus flavithermus* PW10. Therefore *Bacillus licheniformis* PS7 which can tolerate ethanol (0.1 – 1.0 %) can be utilized for bioremediation and production of bioethanol.

Effect of metal ions and EDTA on β-galactosidase activity

In order to investigate the effect of metal salts as cofactor for β-galactosidase activity, metal salts were individually supplemented in the β-galactosidase assay at the concentration of 1-5 mM. β-galactosidase activity was inhibited by 1.7, 1.3 and 11.3 folds at 5 mM concentration of Zn^{2+}, Ca^{2+} and Cu^{2+} respectively in *Bacillus licheniformis* PS7. On the other hand, β-galactosidase activity was enhanced by 1.6, 2.2, 2.8, 2.3 and 5.4 folds in the presence of Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+} and Mg^{2+} ions respectively in *Anoxybacillus flavithermus* PW10. In the presence of EDTA (25 mM), β-galactosidase activity was decreased by 1.7 fold in *Anoxybacillus flavithermus* PW10, while 1.1 fold for *Bacillus licheniformis* PS7. β-galactosidase activity of *Bacillus licheniformis* PS7 showed increase in activity in the presence of metal ions such as, Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Na^+ and Mg^{2+}. β galactosidase activity of *Anoxybacillus flavithermus* PW10 was increased in the presence of Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Na^+ and Mg^{2+} (Figure 13). Correlation between the growth and β-galactosidase activity was also determined by comparing the absorbance at 600 nm and specific activity of β-galactosidase. It was observed that growth was inhibited in the presence of Cu^{2+} and Zn^{2+} by 11.3 and 53.3 % respectively for *Bacillus licheniformis* PS7, while Ca^{2+}, Fe^{2+}, Mg^{2+} and Na^+ stimulated the growth by 1.3, 2.1, 1.2 and 0.3 folds respectively for *Anoxybacillus flavithermus* PW10. Growth of *Bacillus licheniformis* PS7 in the presence of Cu^{2+}, Na^+ and Zn^{2+} was decreased by 45.8, 21.1 and 75.4 % respectively.

β-galactosidase inhibition in the presence of metal ions present in milk and dairy products is an important aspect. Our data suggest that β-galactosidase of *Anoxybacillus flavithermus* PW10 is metal dependent, while β-galactosidase of *Bacillus licheniformis* PS7 is metal independent and could be utilized for commercial production of lactose free dairy products and GOS (Fig. 14).

Kinetic parameters (K_m and V_{max}) of β-galactosidase of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10

Kinetic parameters like maximum reaction velocity (V_{max}) and Michaelis–Menten' kinetics (K_m) were determined for β-galactosidase with respect to its artificial

substrate ONPG at 60°C and pH 7 by Lineweaver - Burk plots. Kinetic constant for β-galactosidase measured for ONPG was 8.0 mM and V_{max} was found to be 641.5 μg/mg/min for *Bacillus licheniformis* PS7. K_m of 1.3 mM and V_{max} of 3.233 U/mg/min was observed for β-galactosidase of *Anoxybacillus flavithermus* PW10 (Figure 15).

Reaction kinetics of β-galactosidase in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10

Kinetic parameters of β-galactosidase were studied for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 by performing ONPG assay and measuring the amount of ONP produced after 10, 20, 30, 40, 50 and 60 mins of reaction at 60 °C and pH 7. Maximum β-galactosidase activity was observed after 10 minutes of the reaction in *Bacillus licheniformis* PS7 as well as *Anoxybacillus flavithermus* PW10 (Figure 16 supplementary material). *Bacillus licheniformis* PS7 showed 2.5 folds higher β-galactosidase activity as compared to the *Anoxybacillus flavithermus* PW10. β galactosidase activity was reduced by 26.2, 42.3, 49.5, 47.4, 47.4 and 59.4 % at 20, 30, 40, 50 and 60 minutes for *Bacillus licheniformis* PS7. In contrast, β galactosidase activity was reduced by 30.2, 34.2, 44.4, 57.3 and 63.8 % at 20, 30, 40, 50 and 60 minutes respectively for *Anoxybacillus flavithermus* PW10. This data suggested that reaction rate was maximum within ten minutes for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10.

Effect of pre-incubation at different temperature on the β-galactosidase activity in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10

To study the thermostability of β galactosidase, enzyme preparation was pre incubated at 4, 25 and 60 °C for 0, 24, 48, 72, 96 and 120 hours and β galactosidase assay was performed at 60 °C and pH 7. Enzyme assay was performed for different time points (0, 24, 48, 72, 96 and 120 hours) at 60 °C and pH 7. β galactosidase was mostly stable at 4 and 25 °C (Figure 17). This result indicated that β galactosidase can be stored at room temperature for 4 – 5 days for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10. There was 65 % reduction in β-galactosidase activity after 24 h of incubation for *Bacillus licheniformis* PS7 at 60 °C, while 10 % reduction was observed between 24 – 120 h of incubation for *Anoxybacillus flavithermus* PW10 at 60 °C.

Stability of β-galactosidase was same when stored at 4° C or 25° C for 4 – 5 days in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10. This data suggested that β galactosidase for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 do not required low temperature for storage.

Effect of carbon sources on β-galactosidase activity of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10

Effect of substrates and reaction products like glucose, galactose and lactose (0.1 – 1 %) on the β-galactosidase activity of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 was studied at 0.1, 0.5 and 1% concentrations. Substrates and products were added to the standard enzyme assay and activity was determined. It was observed that glucose and lactose enhanced the β-galactosidase activity in *Bacillus licheniformis* PS7 by 2.1 and 1.1 folds respectively. β galactosidase activity was also enhanced by 1.6 and 2.0 folds in the presence of glucose and lactose respectively for the *Anoxybacillus flavithermus* PW10. Galactose decreases β-galactosidase activity by 2.5 folds in *Anoxybacillus flavithermus* PW10, while there was no effect of different concentrations (0.1,

0.5 and 1%) of galactose on *Bacillus licheniformis* PS7 (Figure 18).

The preference of substrates was studied in combination of different substrates such as ONPG combined with glucose, ONPG with galactose and ONPG with lactose. Glucose with ONPG increased enzyme activity in *Bacillus licheniformis* PS7 as well as in *Anoxybacillus flavithermus* PW10. Galactose and ONPG decreases β-galactosidase activity of *Anoxybacillus flavithermus* PW10, whereas β-galactosidase activity of *Bacillus licheniformis* PS7 was not affected.

Out of nineteen thermophilic bacterial isolates β-galactosidase production was shown by only PS7 and PW10 isolates, quantitatively as well as qualitatively. Tattapani hotspring has not been yet explored for thermophilic bacteria producing β galactosidase. Thermus thermophilus KNOUC114 (thermophile) is reported to produce β galactosidase and is isolated from a hot spring in the area of Golden springs in New Zealand (Ahn *et al.,* 2011). Lipase producing *Bacillus licheniformis* MTCC 10498 has been reported from Tattapani hotspring (Sharma *et al.,* 2012). More recently, thermophilic *Geobacillus* sp has been reported form Tattapani Hot spring, which secretes extracellular heat stable cellulose (Sharma *et al.,* (2015a)) and amylase (Sharma *et al.,* (2015b)). *Bacillus licheniformis* PS7 and *Anoybacillus flavithermus* PW10 both showed extracellular β galactosidase production. *Bacillus licheniformis* ATCC 12759 was reported to produce extracellular β galactosidase (Nurullah (2011)), while *Anoxybacillus* B1.2 (Osiriphun and Jaturapire (2009)) was reported to produce intracellular β galactosidase. Beside these, microorganisms like *Bacillus* sp. MTCC 3088 (Chakraborti *et al.,* (2000)), *Fusarium moniliforme* (Nurullah (2011)), *Bifidobacterium bifidum* and *Bifidobacterium infantis* (Moller *et al.,* 2001),

Rhizomucor sp. (Shaikh *et al.,* 1999) and *Bacillus* sp. (Sani *et al.,* 1999) have been reported to produce extracellular β galactosidase. Optimum temperature and pH for β galactosidase activity was 60° C and pH 7 respectively for *Bacillus licheniformis* PS7. On the other hand, 60° C and pH 9 was optimum for β galactosidase of *Anoxybacillus flavithermus* PW10 indicating the thermophilic nature of β-galactosidase. Optimum temperature and pH for the production of thermophilic β- galactosidase was reported to be 60° C and pH 8 for *Bacillus* sp. (Chakraborti *et al.,* 2000) and 60° C and pH 6.5 for *Anoxybacillus* B1.2. *Bacillus* sp. MTCC 3088 was isolated from the water samples of hotspring Manikaran, India. *Anoxybacillus* B1.2 was isolated from Ta Pai hot spring, Maehongson, Thailand. Lactose supplementation enhances β-galactosidase activity by 7.5 fold in *Bacillus licheniformis* PS7 and 2.5 fold in *Anoxybacillus flavithermus* PW10 as compared to nutrient broth (without lactose supplementation). This is the first report of its kind that β-galactosidase production is maximum during the declined phase of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10. Highest *β*-galactosidase activity was reported in *Thermus thermophilus* cells after 40 h of cultivation at 70°C in a medium containing 0.8% peptone, 0.4% yeast extract and 0.2% NaCl (Maciunska *et al.,* (1998)). β-Galactosidase specific activities of crude extracts obtained from bacterial cells (*Alicyclobacillus acidocaldarius*) grown in the presence and absence of lactose over a period of time (6–40 h) showed that β-galactosidase synthesis seems to be constitutive and increases by increasing time up to 40 h of cultivation (Guven *et al.,* 2007).

Sugars like galactose, starch, sucrose, inositol and lactose enhanced β-galactosidase production in *Bacillus licheniformis* PS7. On the other hand, galactose, sucrose, xylose,

trehalose and lactose enhanced β-galactosidase production in *Anoxybacillus flavithermus* PW10. Lactose presence showed maximum β-galactosidase activity in both the isolates. However, catalytic activity of β galactosidase was not affected by the presence of glucose, maltose, lactose, sucrose, starch, xylose, inositol and sorbitol. This suggested that enzyme is not prone to substrate and product inhibition. Enzyme activity was also reported to be strongly inhibited by galactose in *Bacillus* sp. (Chakrabotri *et al.,* (2000)). Decrease in β-galactosidase activity was reported in *Anoxybacillus* B1.2 strain in the presence of glucose, galactose and lactose (Osiriphun and Jaturapire (2009)). Among glucose, galactose and lactose, β-galactosidase production was enhanced in the presence of lactose in *Bacillus* sp. B 1.1 (Jaturapiree *et al.,* (2012)).

Growth of *Anoxybacillus flavithermus* PW10 was inhibited by ethanol (1% concentration), butanol, cyclohexane, phenol and toluene by 7.3 folds and 2.5, 1.4, 14.7 and 1.2 folds respectively at 0.1% concentration. Growth of *Bacillus licheniformis* PS7 was not inhibited by hydrogen peroxide (0.1%), while it was inhibitory for *Anoxybacillus flavithermus* PW10. Ethanol (1%) showed maximum inhibition (86.3 %) for *Anoxybacillus flavithermus* PW10 than *Bacillus licheniformis* PS7. Cyclohaxane at 0.1% concentration was inhibitory (94.9 %) for *Bacillus licheniformis* PS7, but not for *Anoxybacillus flavithermus* PW10. Therefore *Bacillus licheniformis* PS7 which can tolerate ethanol (0.1 – 1.0 %) can be utilized for bioremediation and production of bioethanol. There are various organisms such as, *Thermus brockianus*, *Bacillus* sp. and *Pedobacter cryoconitis* sp. which have been reported for the bioremediation of solvents (Gomes and Steiner, 2004). β-galactosidase inhibition in the presence of metal ions present in milk and dairy products is an important aspect. Our data suggest that β-galactosidase

of *Anoxybacillus flavithermus* PW10 is metal dependent, while β-galactosidase of *Bacillus licheniformis* PS7 is metal independent and could be utilized for commercial production of lactose free dairy products and GOS. El-Kader *et al.,* (2012), reported that β- galactosidase relative activity in *Bacillus subtilis* was found highest in the presence of 0.1 mM Mn^{2+}, 10 mM $Fe^{2+,}$ 0.1 and 1.0 mM Mg^{2+} and 0.1 mM Ca^{2+}. The presence of 1.0 mM Ca^{2+} decreased the relative activity of β-galactosidase of *Bacillus subtilis.* β-galactosidase enzyme activity was significantly inhibited by metal ions (Hg^{2+}, Cu^{2+} and Ag^+) in the 1–2.5 mM range. It has been reported that Mg^{2+} was a good activator of β-galactosidase from *Bacillus* sp MTCC3088 (Dabrowski *et al.,* (2000)). β-galactosidase activity of *Anoxybacillus flavithermus* PW10 was enhanced in the presence of Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} and Mg^{2+} ions. Effect of monovalent (Na^+ and K^+) cations was reported on β-galactosidase activity of *Anoxybacillus* sp. B1. Addition of monovalent cations (1 − 100 mm) had no effect on enzyme activity. The highest β galctosidase activity of *Anoxybacillus* sp. B1.2 was observed in the presence of 1 mM Fe^{2+} and 10 mM Mg^{2+}.

Kinetic constant for β-galactosidase measured for ONPG was 8.0 mM and V_{max} was found to be 641.5 μg/mg/min for *Bacillus licheniformis* PS7. K_m of 1.3 mM and V_{max} of 3.233 U/mg/min was observed for β-galactosidase of *Anoxybacillus flavithermus* PW10. The K_m values of β galactosidase for ONPG and lactose were 6.3 and 6.1 mM respectively for *Bacillus* sp MTCC 3088 (Chakraborti *et al.,* (2000)). Km of 5.9 mM with respect to ONPG and 19 mM with respect to lactose was reported for the β galactosidase of *Thermus* sp. A4 (Ohtsu *et al.,* 1998). Km of 28.85 mM with respect to ONPG was observed for the β galactosidase of *Anoxybacillus* sp. B1.2 (Osiriphun and Jaturapire, 2009).

Fig.1 Effect of temperature on the growth of PS7 and PW10 isolates: Bacterial isolate PS7 and PW10 were streaked on nutrient agar medium and incubated at different temperatures of 30, 40, 50, 60 and 70 °C for 24 h
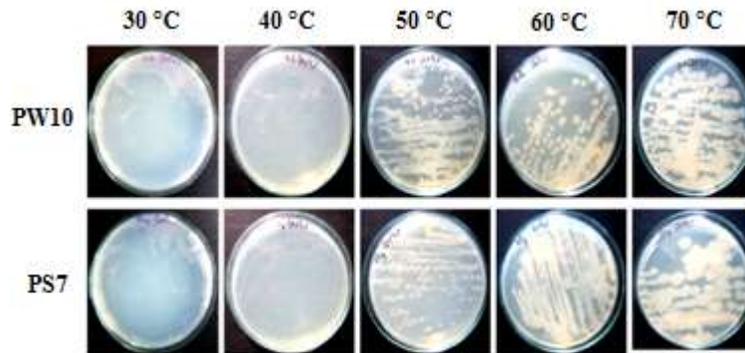

Fig.2 Qualitative test for the production of β-galactosidase by thermophilic bacterial isolates: Thermophilic isolates (PS7 and PW10) and DH5☐ as control were streaked on nutrient agar (NA) medium or NA medium supplemented with Xgal or Xgal and IPTG as indicated. Plates were incubated at 60 °C for 12 h
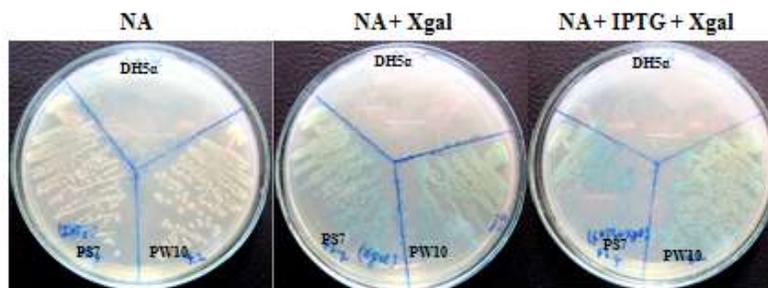
Fig.3 Qualitative assay for the production of extracelluar β galactosidase: Bacterial isolates were grown and cell free spent medium was tested for β-galactosidase activity at different temperature as indicated. Cell free spent medium (supernatant) of PS7 (tube no 1) and PW10 (tube no 2), mesophilic isolate A5-2 (tube no 3) as positive control, mesophilic DH5α and thermophilic strain PS1 (tube no 4 and 5 respectively) as negative control were incubated at 30, 40 and 50 °C in the presence of IPTG and Xgal
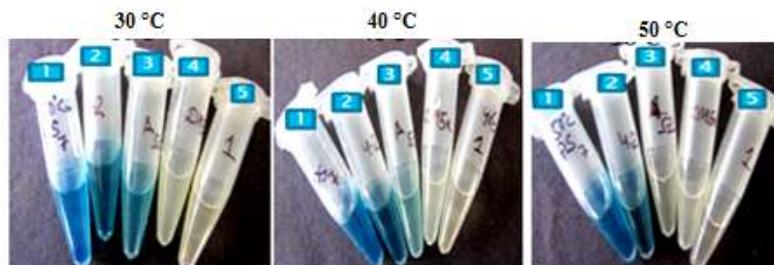
Fig.4 Effect of temperature and nature of β-galactosidase activity: ONPG assays were performed at different temperatures (4, 50 and 60 □ C) as indicated by using cell free spent medium as extracellular and whole cell extract and intracellular source of β-galactosidase
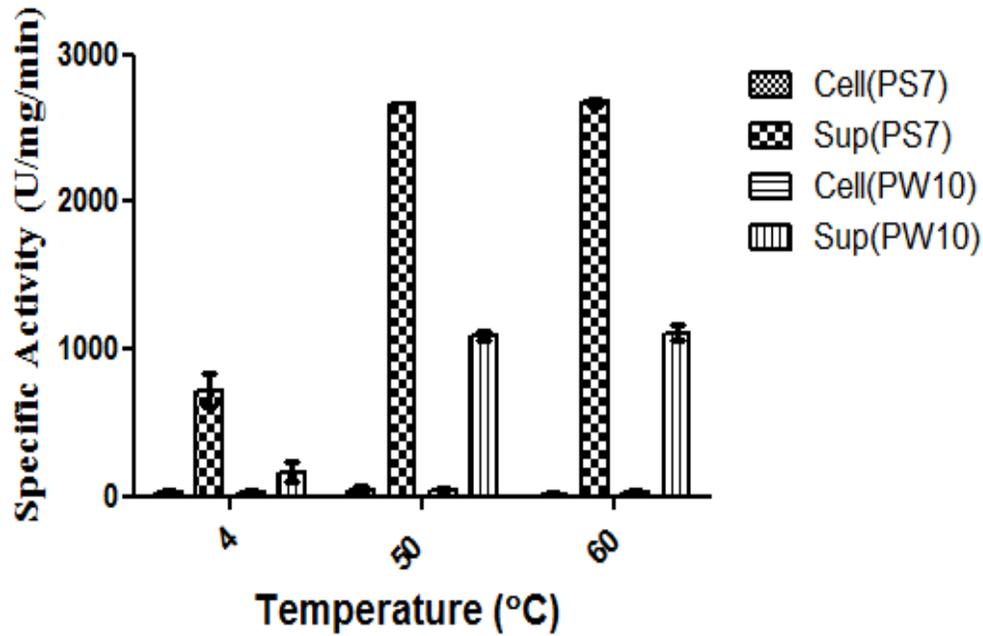
Fig.5 PCR amplification of 16S rDNA: Genomic DNA was isolated from PS7 and PW10 (A). 16S rDNA was PCR amplified by using 27F and 1492R primers. Reaction products were separated on 1% agarose gel (B). 'M' indicated molecular size marker (kb)
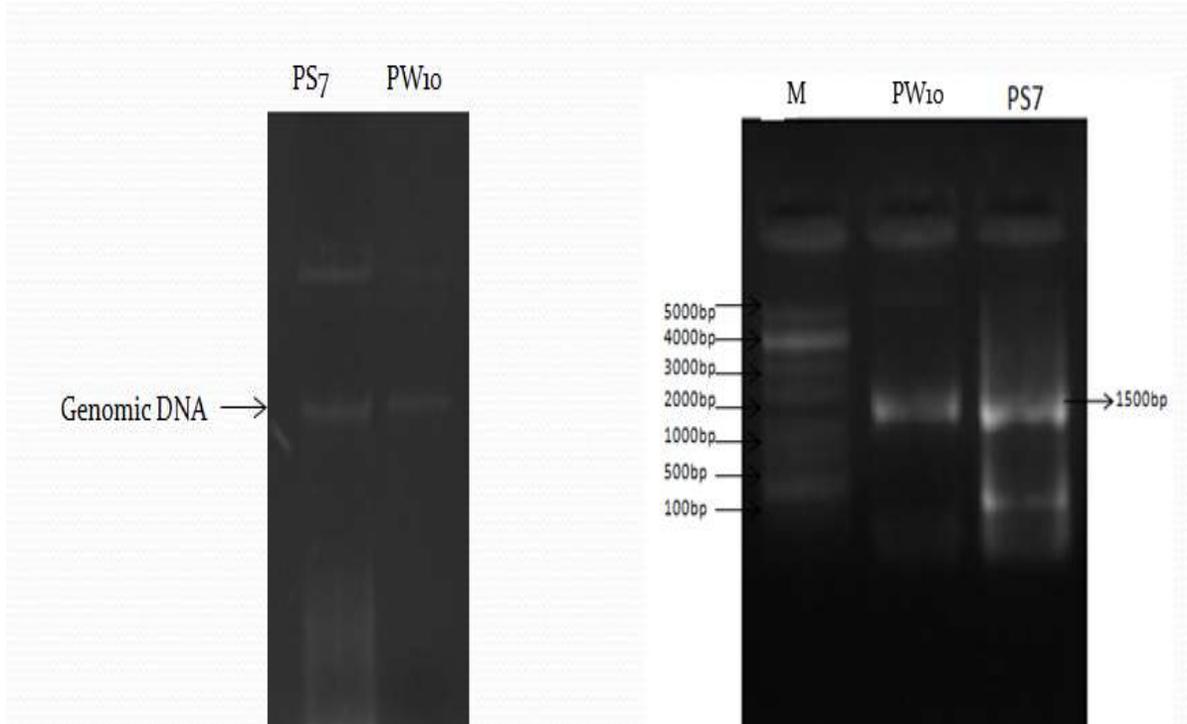
Fig.6 Phylogenetic evolution of *Bacillus licheniformis* PS7 based on 16S rDNA: 16S rDNA evolution and relatedness in *Bacillus licheniformis* PS7. Unrooted phylogenetic tree was constructed by selecting all the related bacillus spp. using phylip software
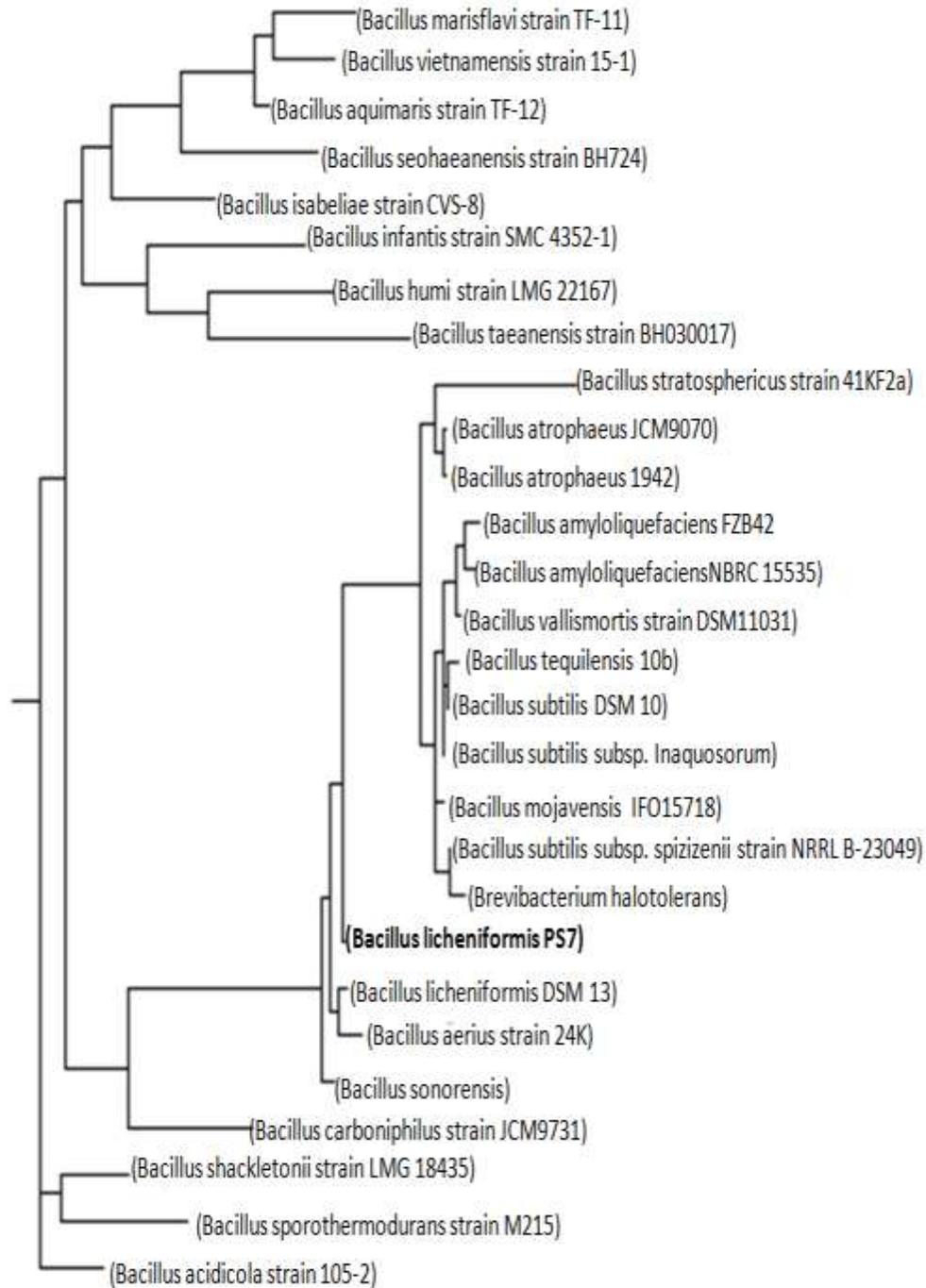
Fig.7 Phylogenetic evolution of *Anoxybacillus flavithermus* PW10 based on 16S rDNA:
Unrooted tree was constructed by selecting all the related *Anoxybacillus* sp. from nucleotide blast
results and 16S rDNA tree was constructed
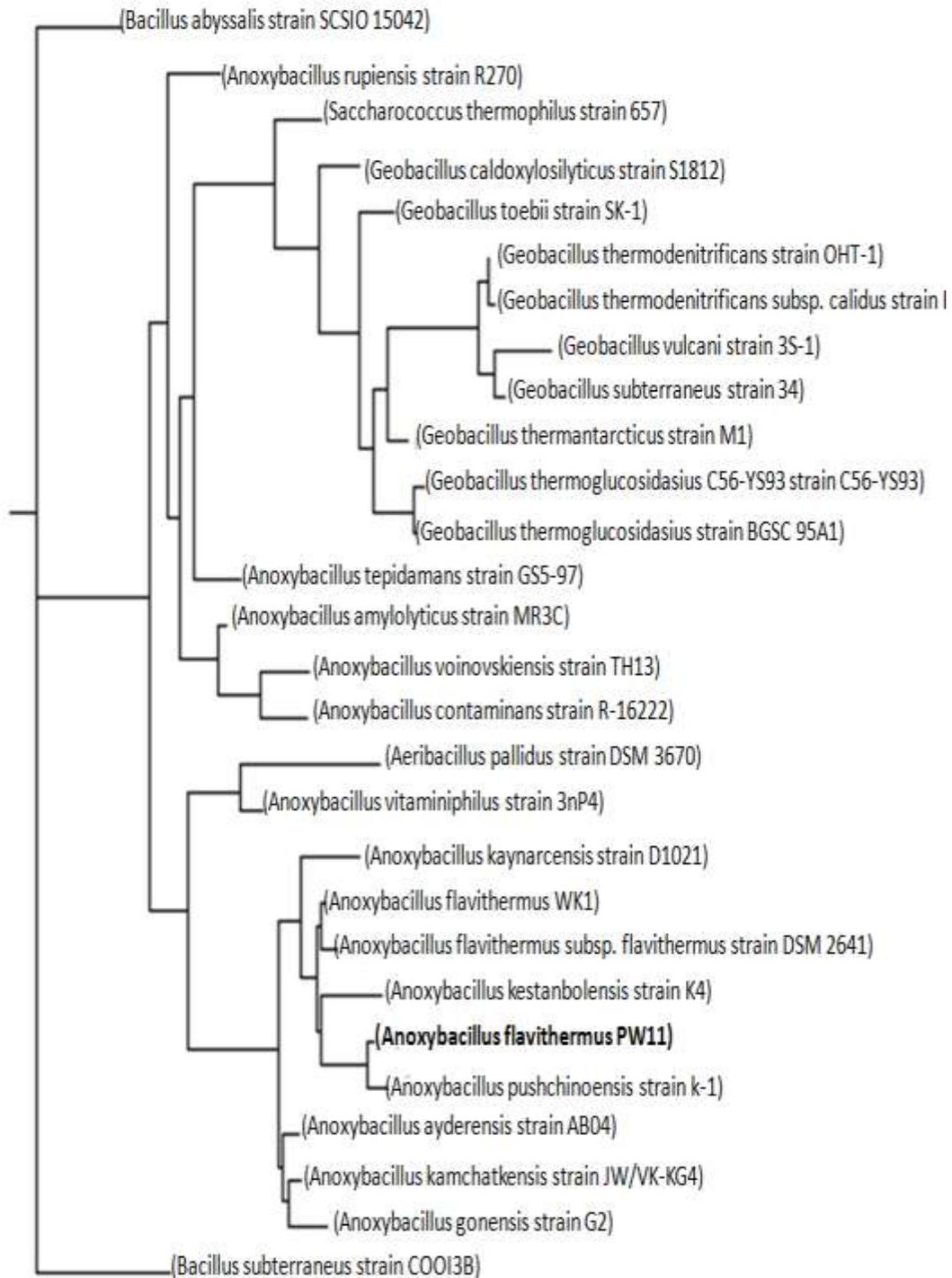
Fig.8 Phylogenetic evolution of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 with related species based on 16S rDNA: Unrooted tree was constructed by selecting all the related Bacillus and *Anoxybacillus* spp from nucleotide blast results of 16S rDNA
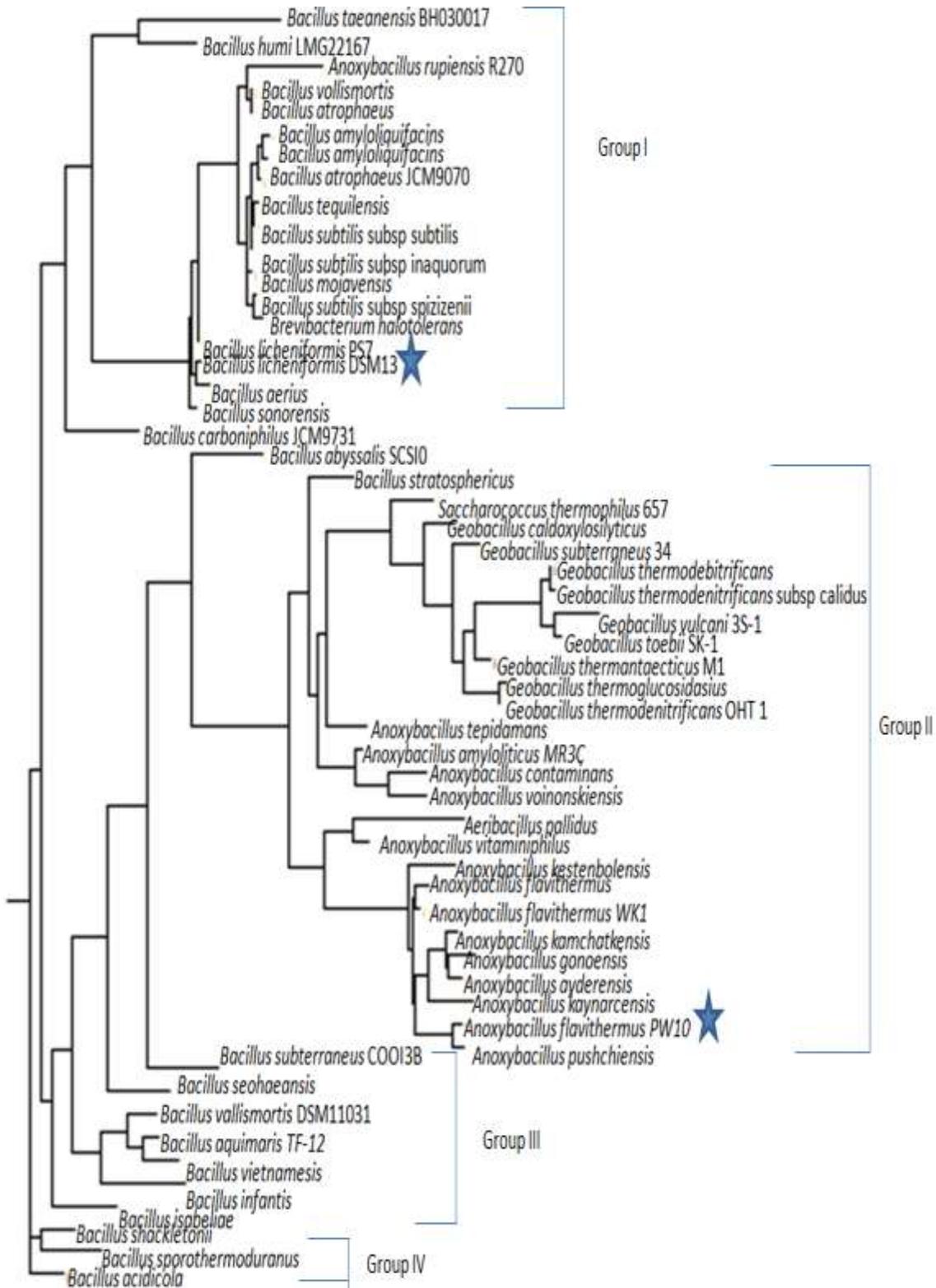
Fig.9 Effect of temperature and pH on the β galactosidase activity: ONPG assay was performed at different temperature (4, 30, 40, 50, 60, 70 and 80 ☐ C) and pH (3, 5, 7, 9 and 11). Specific activity was plotted for *Bacillus licheniformis* PS7 (A and C) and *Anoxybacillus flavithermus* PW10 (B and D)
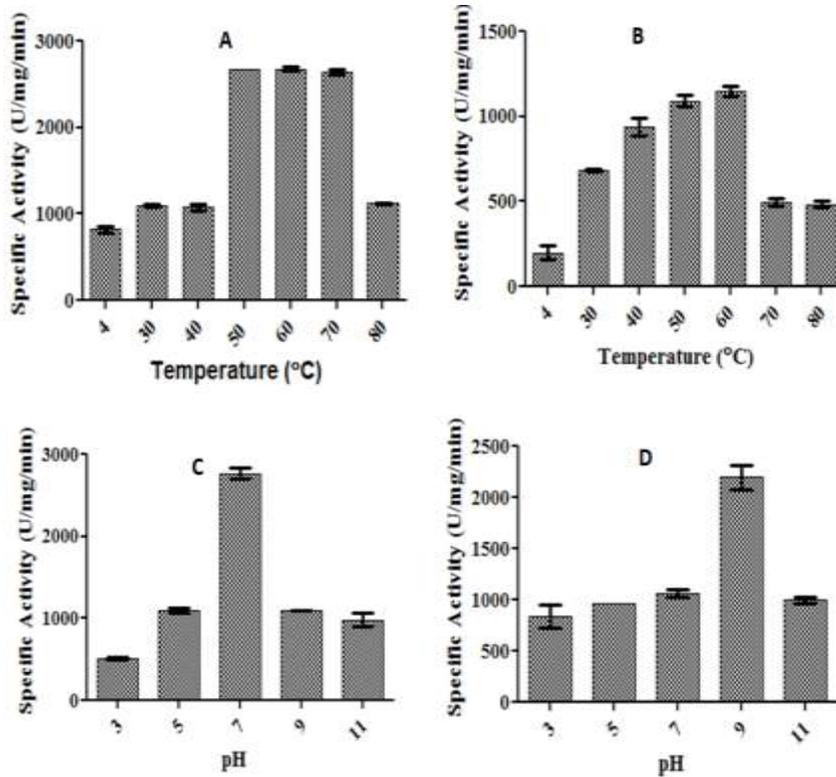
Fig.10 Correlation of β-galactosidase production with growth rate of thermophilic bacterial isolates: Microbial growth and β-galactosidase production was compared for *Bacillus licheniformis* PS7 (red) and *Anoxybacillus flavithermus* PW10 (blue) for different time as indicated
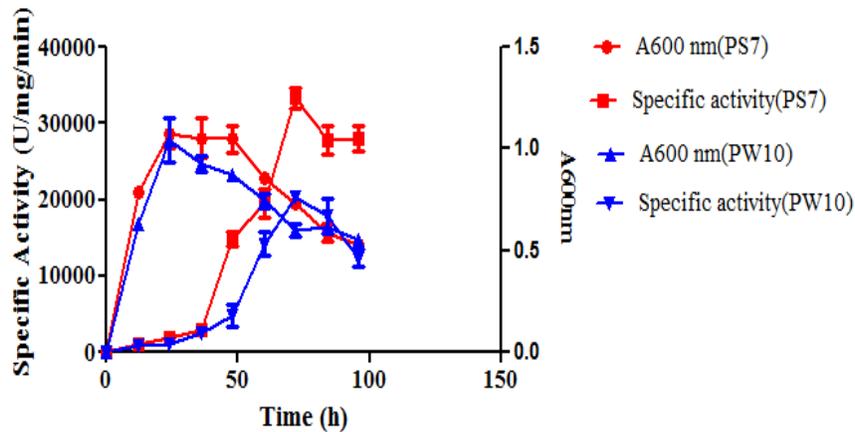
Fig.11 Effect of carbon and nitrogen sources on growth and □ galactosidase activity of*Bacillus licheniformis* PS7 (A and C) and *Anoxybacillus flavithermus* PW10 (B and D): PS7 and PW10 bacterial isolates were incubated in nutrient broth medium supplemented with different carbon sources like glucose, galactose, fructose, xylose, sucrose, maltose, sorbitol, starch, trehalose, raffinose, sorbitol, inositol and lactose (A and B) and nitrogen sources such as yeast extract or urea (B and C). Cultures were incubated at 60 □ C for 24 hours. □ galactosidase activity and growth was compared with nutrient broth alone
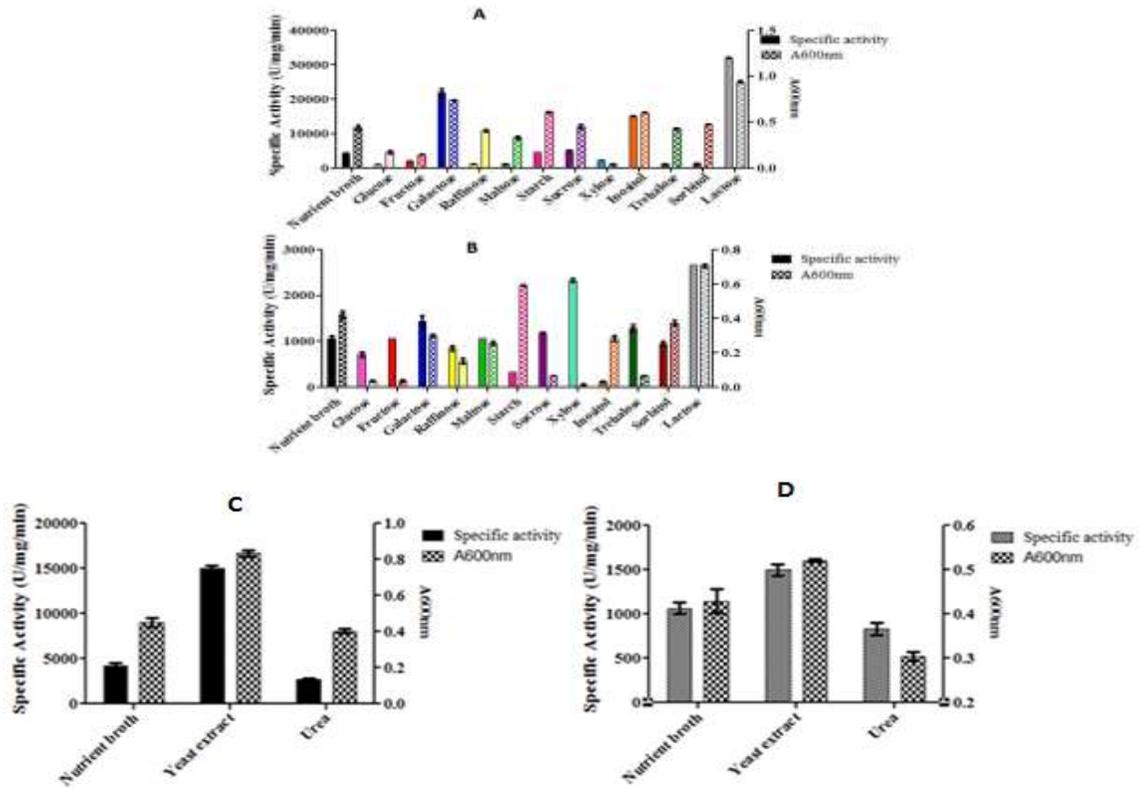
Fig.12 Effect of different solvents on the growth of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10: *Bacillus licheniformis* PS7 (A) and *Anoxybacillus flavithermus* PW10 (B) were grown in the presence of different solvents (ethanol, butanol, toluene, hydrogen peroxide, cyclohexane and phenol) as indicated and absorbance was measured at 600 nm after 24 h of growth
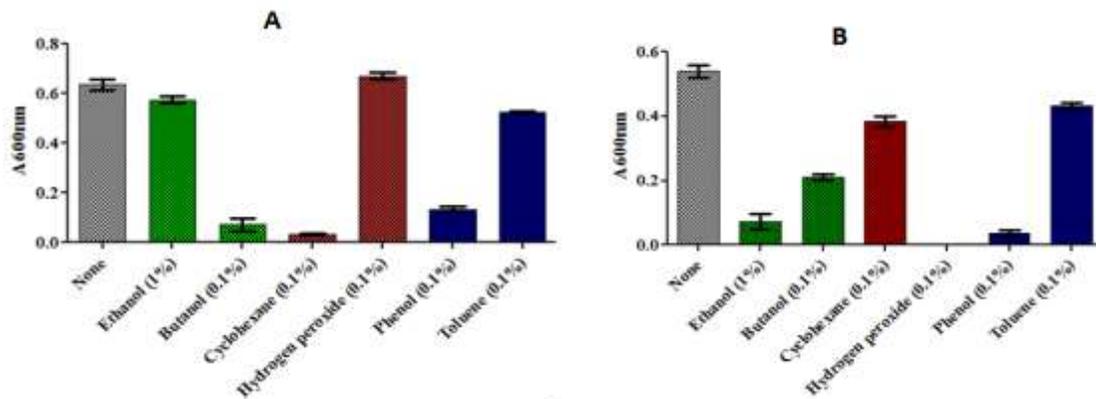
Fig.13 Effect of metal ions on growth and β-galactosidase activity of *Bacillus licheniformis* PS7: Salts of metal ions, like Na^+, Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+} as indicated were supplemented in nutrient broth medium and inoculated with equal number of bacterial cells. Cultures were incubated at 60 ☐ C for 24 h. Cell density was measured at 600 nm. To study the effect of metal ions on β-galactosidase activity, cultures were grown for 72 hours at 60 ☐ C and ONPG assays were performed using cell free spent medium
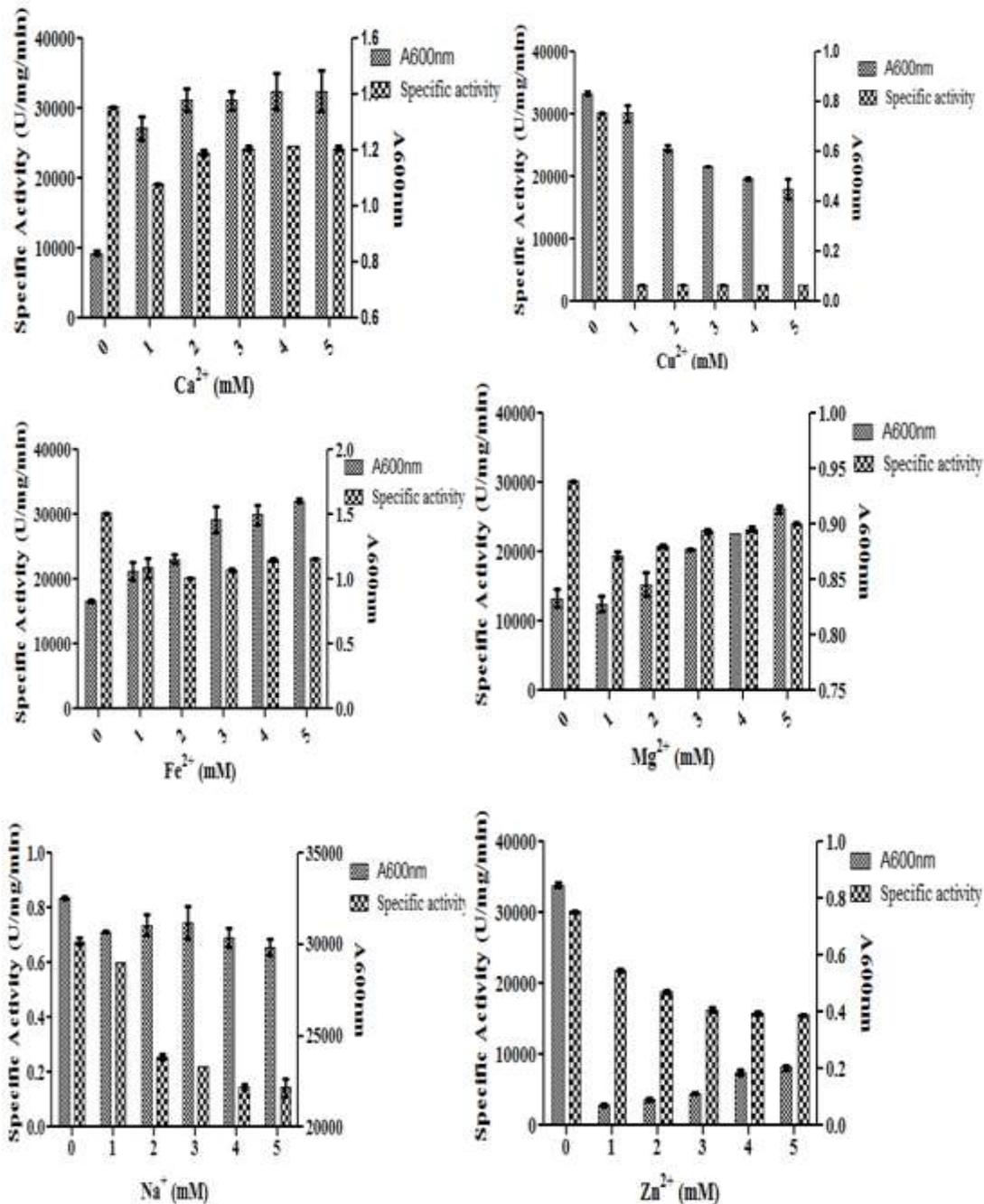
Fig.14 Effect of metal ions on growth and β-galactosidase activity of *Anoxybacillus flavithermus* PW10 Salts of metal ions, like Na$^+$, Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+} as indicated were supplemented in nutrient broth medium and inoculated with equal number of cells. Cultures were incubated at 60 □ C for 24 h. Cell density was measured at 600 nm. To study the effect of metal ions on β-galactosidase activity, cultures were grown for 72 hours at 60 □ C and ONPG assays were performed using cell free spent medium
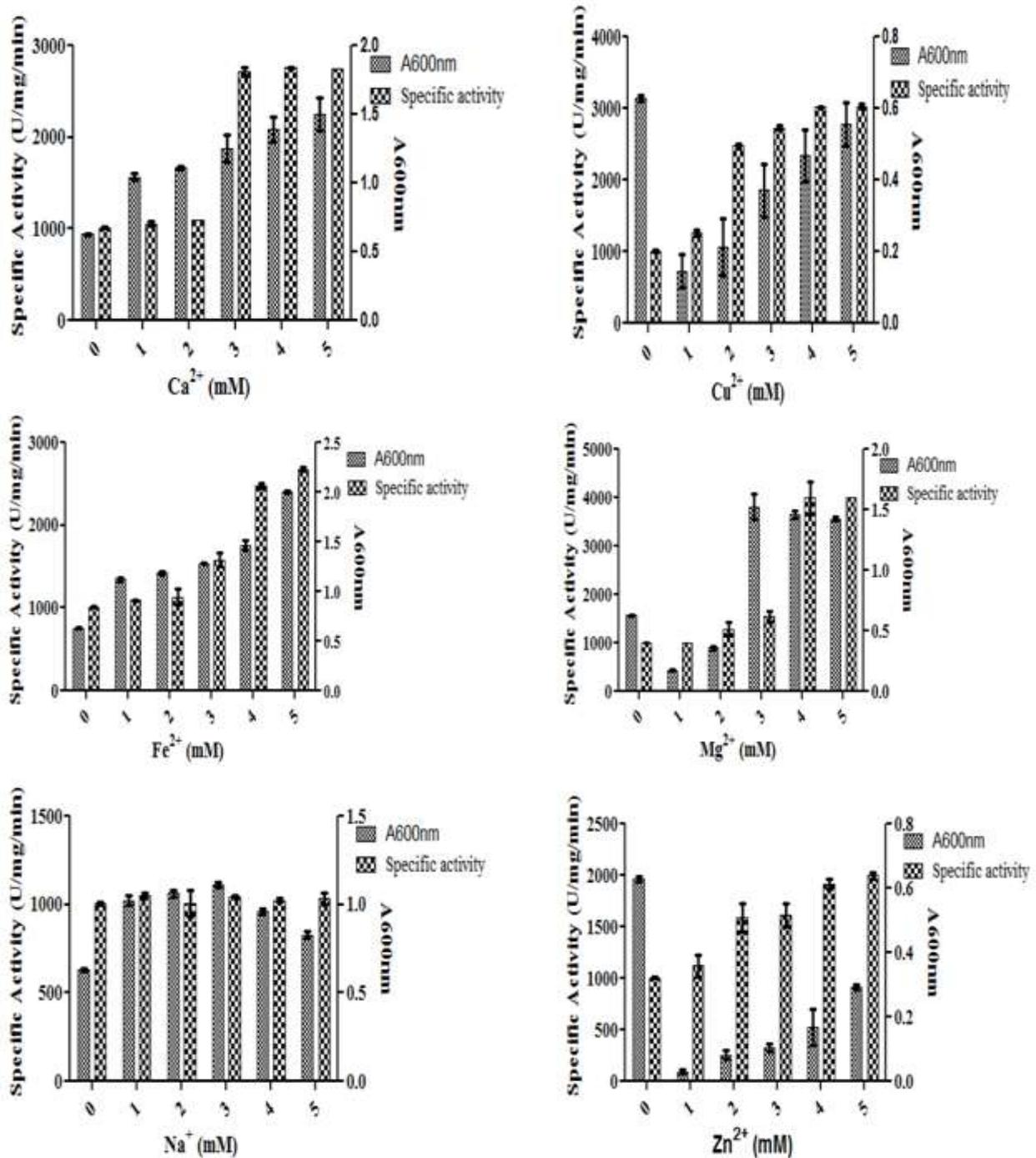
Fig.15 Kinetic constants of β-galactosidase activity for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10: β-galactosidase was assayed for the hydrolysis of ONPG at 60 °C and pH 7 for *Bacillus licheniformis* PS7 (A) and *Anoxybacillus flavithermus* PW10 (B) using different concentration of ONPG
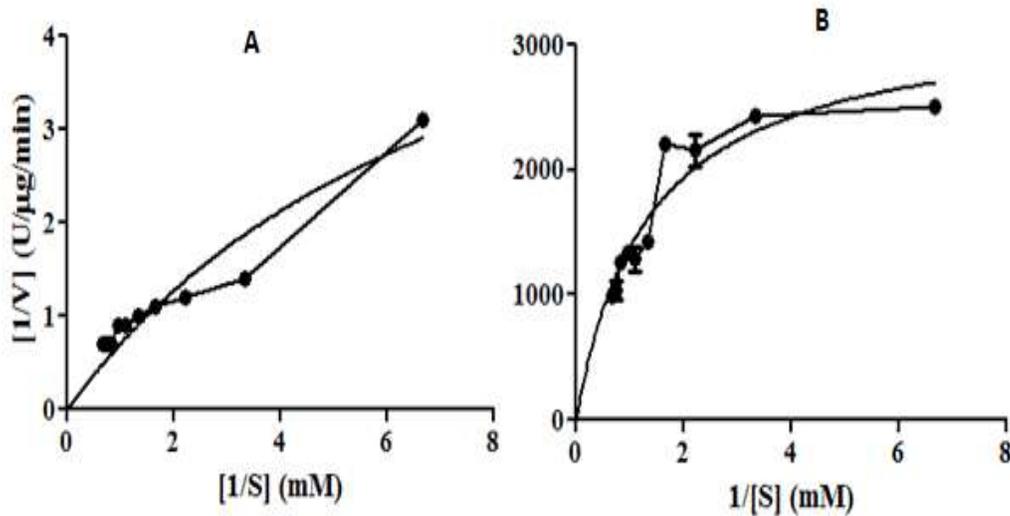

Fig.16 Reaction kinetics of β galactosidase for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10: Reaction rate was studied by performing ONPG assay and measuring the amount of ONP produced after 10, 20, 30, 40, 50 and 60 minutes in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 as indicated
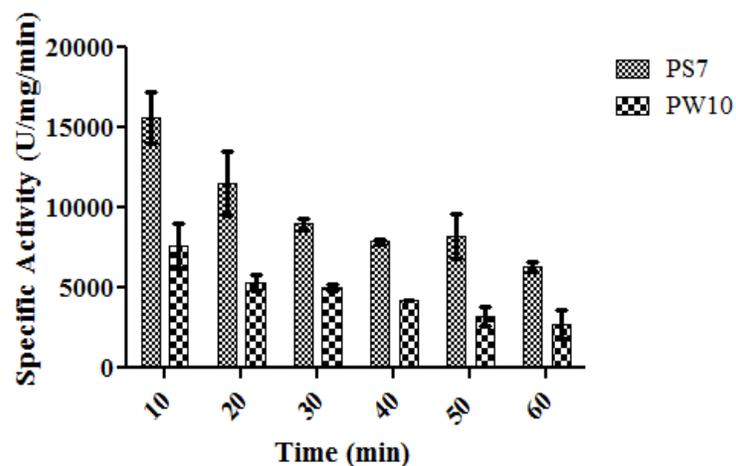
Fig.17 Effect of temperature on β-galactosidase activity of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10: ONPG assay was performed at 60 °C and pH 7 after incubating enzyme prepration at 4, 25 and 60 °C after 0, 24, 48, 72, 98 and 120 hours in *Bacillus licheniformis* PS7 (A, C and E) and *Anoxybacillus flavithermus* PW10 (B, D and F)
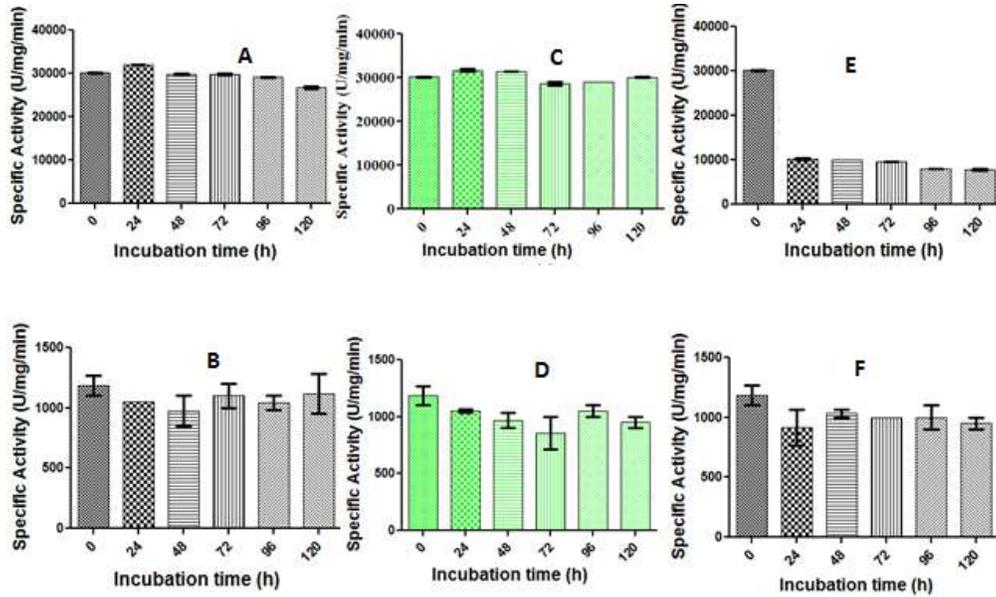
Fig.18 Effect of substrates and reaction products on the β-galactosidase activity of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10: substrates and reaction products like glucose, galactose and lactose were supplemented in the ONPG assay and activity was determined at 60 °C and pH 7 for *Bacillus licheniformis* PS7 (A) and *Anoxybacillus flavithermus* PW10 (B)
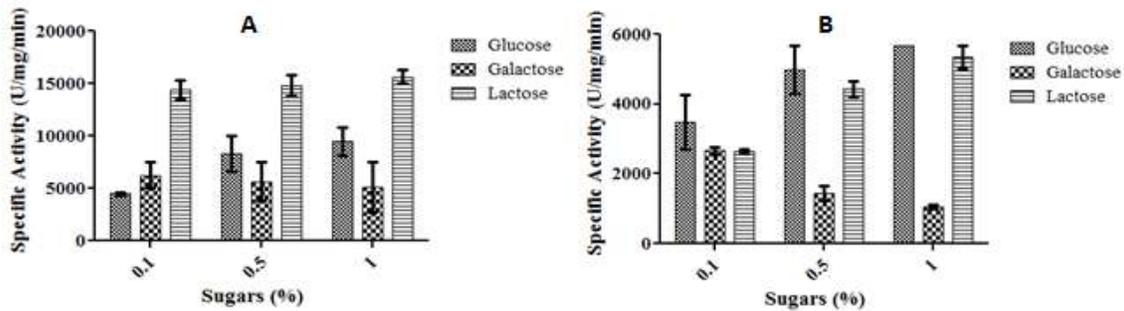
Stability of β-galactosidase was same when stored at 4° C or 25° C for 4 – 5 days in *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10. This data suggested that β galactosidase for *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 do not required low temperature for storage. The thermostability of the β-galactosidase enzyme in *Anoxybacillus* sp. B1.2 was in the range of 40 - 60° C, with the pH stability in the range of 6 - 10 (Osiriphun and Jaturapire, 2009). The preference of substrates was studied in combination of different substrates such as ONPG combined with glucose, ONPG with galactose and ONPG with lactose. Glucose with ONPG increased enzyme activity in

Bacillus licheniformis PS7 as well as in *Anoxybacillus flavithermus* PW10. Galactose and ONPG decreases β-galactosidase activity of *Anoxybacillus flavithermus* PW10, whereas β-galactosidase activity of *Bacillus licheniformis* PS7 was not affected. It is reported that β-galactosidase activity was moderately inhibited by its reaction products such as glucose and galactose in *Anoxybacillus* sp. B1.2 (Osiriphun and Jaturapire, 2009) and strongly inhibited by galactose in *Bacillus* sp. MTCC 3088 (Chakraborti *et al.,* 2000). In conclusion, β galactosidase of *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 could be utilized for commercial production of lactose free dairy products and for the production of Galactooligosaccharides.

In conclusion, the extracellular β-galactosidase present in *Bacillus licheniformis* PS7 as well as *Anoxybacillus flavithermus* PW10 could be useful for hydrolysis of lactose present in milk and its products and can be efficiently used in the dairy industry as well as for the production of galactooligosaccharides. Most importantly, β-galactosidase from *Bacillus licheniformis* PS7 and *Anoxybacillus flavithermus* PW10 are stable at room temperature (25 °C) for 6 days, and therefore does not require storage at lower temperatures. Further studies are required to purify the enzyme to homogeneity.

Acknowledgement

Conflict of Interest

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