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Optimization of Culture Conditions for Production of B-Galactosidase by *Bacillus Megaterium* NM56 Isolated from Raw Milk.

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ABSTRACT

β-galactosidase (E.C.3.2.1.23) has been widely used in dairy and pharmaceutical industries. In this study, *Bacillus megaterium* NM56 showing maximum production of β-galactosidase was isolated from raw milk. Effects of various culture conditions, namely, incubation period, temperature, pH, carbon source and nitrogen source were investigated. Results revealed that the highest level of β-galactosidase was produced after 48 h of incubation. Optimal temperature and pH for production was observed at 40°C and 7.5, respectively. Galactose, lactose, tryptone and yeast extract were the best carbon and nitrogen sources for enzyme production. Permeabilization efficiency of different solvents was assessed and results indicated that iso-propanol was the most potent permeabilizing agent.

Keywords: β-galactosidase, Screening, Optimization, Permeabilization.

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INTRODUCTION

β-galactosidase (EC 3.2.1.23), commonly known as lactase, is a commercially important enzyme that catalyzes the hydrolysis of β-D-galactopyranosides. It hydrolyzes lactose into monosaccharides, glucose and galactose. β-galactosidases are widely used in dairy industries for production of lactose-free milk for consumption by lactose intolerant people [1, 2]. The interest of the dairy industry in lactose hydrolysis has been raised because of the fact that more than 70% of the world's population suffer from the inability to digest lactose or lactose-containing products such as milk. This is due to the lactose intolerance symptoms caused by the lack of β-galactosidase activity in the mucosa of the small intestine [3, 4].

Furthermore, lactose hydrolysis in dairy industries gives rise to several advantages such as rapid fermentation of glucose, higher degree of sweetness and higher stability of frozen condensed milk compared to products whose milk is not treated with β-galactosidase [5]. In addition, high lactose content in non-fermented milk products such as sweetened condensed milk and ice-cream, can lead to excessive lactose crystallisation resulting in products with unfavorable characteristics as a sandy, mealy or gritty texture. Thus, lactose hydrolysis catalyzed by β-galactosidases is of important for the milk and dairy industries [6].

Recently, β-galactosidase gained much attention for its transgalactosylation activity. It was observed that β-galactosidase, at high lactose concentrations, can catalyze the production of galacto-oligosaccharides (GOS) [7, 8]. The latter are non-digestible oligosaccharides that considered as prebiotics food ingredients. When taken orally, galacto-oligosaccharides stimulate the growth of bifidobacteria in large intestine that improves microflora composition and has positive effects on the immune response [9]. β-galactosidase is widely distributed in nature, it has been found in different sources such as plants, animals, fungi, and bacteria [10]. Microbial sources of β-galactosidases are more preferred for industrial applications and have great biotechnological interest due to the ease of handling and high production rate, compared to those derived from other sources [11]. β-galactosidase can be produced from wide variety of microorganisms such as *Kluyveromyces marxianus, Aspergillus niger, Rhizopus oryzae, Bifidobacterium longum and Streptococcus thermophilus* [12-16]. However various sources of β-galactosidase are available, thermostability of the enzyme is an important issue in food manufacturing and other biotechnological applications because it decreases contamination and increases the shelf life of the enzyme. In this trend, several authors described thermostable β-galactosidases derived from different Bacillus spp. [17-19].

Since β-D-galactosidase is an interacellular enzyme, its industrial applications are being hampered due to the difficulty of releasing active enzyme from the cells in a good yield and in sufficient quantities [20-22]. So that various methods have been developed for cell disruption and releasing of intracellular enzymes such as glass bead mills, homogenization, sonication as well as enzymatic and chemical permeabilization of cell membrane [23-27, 21]. The effectiveness of different cell disruption methods varies by different microbial strains and genera [23, 24, 27].

Cell permeabilization may be carried out by using several organic solvents such as ethanol, methanol, iso-propanol, acetone, toluene and chloroform. Solvents mainly act on the cell membrane by denaturing its proteins and by solubilizing its phospholipids making cell more permeable and porous facilitating the passage of small molecules outside or inside cell [22, 28, 29]. The effectiveness of solvents depends on the incubation time and temperature as well as concentration of both solvents and cells. Several authors reported the permeabilization of microbial cells for β-galactosidase by organic solvents [11, 20, 22, 24, 30-32].

The present work aimed to isolate β-galactosidase-producing bacteria from raw milk and to optimize cultural factors for maximum production of enzyme and evaluation the efficacy of various solvent as permeabilizing agents.

MATERIALS AND METHODS

Isolation and primary screening of β-galactosidase-producing microorganisms

For isolation of β-galactosidase-producing microorganisms, raw cow's milk samples were serially diluted and plated on nutrient agar medium supplemented with 50µg/ml X-Gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside) as a chromogenic substrate. After 24h incubation at 37°C, blue colonies indicating the

presence of β-galactosidase activity were picked and maintained on tryptic soy agar slants at 4°C for further studies.

Secondary screening

β-galactosidase producing isolates selected by primary screening were subjected to secondary screening by quantitative estimation of β-galactosidase activity. Positive bacterial isolates were inoculated into 250 ml flasks containing 50 ml of modified LB broth (LB broth supplemented with 1% lactose) and incubated in shaking incubator for 24 h at 37 °C (200rpm). Cells were harvested by centrifugation at 6,000 rpm for 5 min at 4 °C, supernatants were discarded and pellets were resuspended in 0.1 M phosphate buffer (pH 7.0) and disrupted by sonication for 6 cycles (10 s bursts at 200 Watt with a 10 s pause period between each burst). The cell lysates were centrifuged at 20,000 ×g for 10 min at 4°C and the supernatants were assayed for β-galactosidase activity.

Enzyme assay

β-galactosidase activity was estimated by spectrophotometric determination of the o-nitrophenol (ONP) released from hydrolysis of O-nitrophenyl-β-D-galactopyrenoside (ONPG) as described by Miller [33]. Briefly, 0.2 ml of the crude enzyme solution was added to 0.5 ml of 6 mM ONPG in 0.1 M sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 40°C for 30 min. The reaction was terminated by adding 0.5 ml of 1 M Na_2CO_3 and the concentration of ONP released from ONPG was determined by measuring the absorbance at 420 nm, using a standard calibration curve. The enzyme activity was expressed as specific activity (U/mg protein) and one unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 µmol ONP per minute. Protein concentration was determined according to the method of Lowry et al. [34] using bovine serum albumin as a standard.

Identification of The most active isolate

To identify the most active isolate in β-galactosidase production, standard physiological and biochemical identification tests were carried out as described in Bergey's Manual of Systematic Bacteriology [35].

16S rRNA amplification and sequencing

The most active isolate was identified by sequencing of 16S rRNA gene. In brief, genomic DNA was extracted from NM56 cells using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, USA) according to manufacturer's protocol and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR was performed in a total volume of 50 µl containing 2.5 µl 10X DreamTaq buffer, 50 ng genomic DNA template, 0.4 µM of each primer, 0.2 mM of each dNTP, one unit DreamTaq DNA polymerase (Thermo Scientific, USA) and finally water was added to make volume up to 50 µl. The amplification reaction was done in GeneAmp 9700 themal cycler (Applied Biosystems, USA) with the following program: initial denaturation at 94°C for 4 min, denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 1.5 min for 30 cycles, and a final extension at 72°C for 8 min. The amplified PCR product was purified using the QIAquick PCR Purification kit (Qiagen, Germany) according to the supplier's instructions [60,61]. The purified DNA was sequenced with 27F and 1492R primers at GATC Biotech (Konstanz, Germany) using ABI 3730xI sequence analyzer (Applied Biosystems, USA). The forward and reverse DNA sequence reads were assembled to obtain the consensus sequence by using DNA Baser Sequence Assembler software v.3.5.3. Bacterial identification was conducted by comparing the obtained sequence against the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzTaxon-e server database [36].

Inoculum preparation and growth conditions

The primary inoculum (starter culture) was prepared by adding a loopful of freshly prepared pure culture into 50 ml of LB broth in a 250ml flask and incubated at 37ºC and 180 rpm in a shaking incubator for 24 h. Two percent (v/v) of this starter culture was inoculated into the production medium and incubated in orbital shaking incubator at 180 rpm.

Optimization of cultural conditions for β-galactosidase production

The production media were inoculated with *B. megaterium* NM56 starter culture. At the end of incubation period, cells were collected and disrupted by sonication and the specific activity of β-galactosidase was assayed. All experiments were conducted in triplicates and the mean values were calculated.

Effect of incubation period

To study the optimal incubation period for maximum β-galactosidase production, modified LB broth medium was inoculated with 2% starter culture of *B. megaterium* NM56 and incubated at 37°C. Samples were withdrawn periodically at every 6-h interval over 96 h and assayed for β-galactosidase activity.

Effect of incubation temperature

The effect of temperature on β-galactosidase production was studied by cultivating *B. megaterium* NM56 in modified LB broth for 48 h at different temperatures ranging from 20ºC to 45ºC (20, 25, 30, 35, 37, 40, 42 and 45ºC) as separate treatments.

Effect of initial pH

Modified LB broth media were adjusted to various levels of pH ranging from 4.5 to 11 before sterilization. After 48 h incubation at 37ºC, cells were harvested by centrifugation and analyzed for β-galactosidase activity.

Effect of carbon source

To test the effect of different types of carbon sources on β-galactosidase production, starch, sucrose, fructose, lactose, glucose or galactose were incorporated into LB broth at final concentration 1% (w/v). *B. megaterium* NM56 was inoculated and incubated at 37ºC for 48 h, then cells were collected and assayed for β-galactosidase activity.

Effect of nitrogen source

The impact of various nitrogen sources on β-galactosidase production was performed using M9 broth supplemented with 1% (w/v) casein, tryptone, peptone, beef extract, urea, yeast extract or $NaNO_3$ as nitrogen source, separately. *B. megaterium* NM56 inoculated and the cultures were incubated at 37ºC for 48 h.

Cell permeabilization

B. megaterium produces β-galactosidase intracellularly so different organic solvents were assessed for their capacity to permeabilize bacterial cells as described by Gobinath and Prapulla [37] with some modifications. n-butanol, ethanol, toluene, acetone, iso-propanol, and methanol were evaluated as permeabilization agents. Cells from 10 ml bacterial culture were harvested by centrifugation for 5 min at 10,000 rpm and 4°C, washed twice with 0.1M phosphate buffer (pH 7.0), and the optical density was adjusted to 5.5–6.0 at A_{600} with the same buffer. Cells were collected by centrifugation (10,000 rpm, 4°C) and the harvested cells were resuspended in 50% (v/v) n-butanol, iso-propanol, methanol, ethanol, toluene or acetone, stirred thoroughly for 5 min at 28±2 °C, washed twice, and resuspended in the same buffer. In control samples, cells were resuspended in 0.1M phosphate buffer (pH 7.0) instead of solvent.

Enzyme assay for permeabilized cells

To permeabilized cell suspension, 750 µl of 0.1 M sodium phosphate buffer (pH 7.0) was added, followed by 200 µl ONPG [10 mM in 0.1M sodium phosphate buffer (pH 7.0)]. After 10 min incubation in shaking water bath (100 rpm) at 37°C, reaction was stopped by adding 1 ml of 1M sodium carbonate and the released ONP was measured at A_{420} nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of ONP per min under the above assay conditions. The specific activity was

calculated in term of enzyme unit produced per gram dry weight. The cell biomass was determined by collecting known volume of samples at 10,000 rpm (4°C) and the pellet was used for biomass estimation. Pellets were dried on the pre-weighed Whatman No.1 filter paper and the difference in the weight was calculated as dry weight.

Statistical analysis

The measured data were subjected to the analysis of variance (ANOVA) appropriate to the design. The significant differences between treatments were compared with the critical difference at 5% level of probability by the Duncan's test using PASW 17.0 statistics software (SPSS Inc).

RESULTS AND DISCUSSION

Table 1: Results of the some characteristics and biochemical tests for identification of the isolate NM56

Characteristic or Test		Result
Cell diameter > 1.0 μm		+
Sporangium swollen		-
Parasporal crystals		-
Catalase		+
Anaerobic growth		-
Voges-Proskauer test		-
pH in V-P broth <6 >7		+ -
Acid from	D-Glucose	+
	D-Arabinose	-
	D-Xylose	+
	D-Mannitol	+
Gas from glucose		-
Hydrolysis of	Casien	+
	Gelatin	+
	Starch	+
Utilization of Citrate		+
Degradation of tyrosine		+
Deamination of phenylalanine		+
Egg-Yolk lecithinase		-
Nitrate reduced to nitrite		-
Formation of Indole		-
NaCl and KCl required		-
Allantion or urate required		-
Growth at pH	6.8, nutrient broth	+
	5.7	+
Growth in NaCl	2%	+
	5%	+
	7%	+
	10%	-
Growth at	5°C	+
	10°C	+
	20°C	+
	40°C	+
	50°C	-
	55°C	-
	60°C	-
Growth with lysozyme present		-
Autotrophic with H_2 +CO_2 or CO		-

Nutrient agar medium supplemented with X-Gal was used for isolation and primary screening of β-galactosidase producing bacteria. Incorporation of the chromogenic substrate (X-Gal) into screening media was reported for isolation of various β-galactosidase producing microorganisms [38-41]. In this study, 68 discrete blue colonies showing β-galactosidase activity were isolated and subjected to secondary screening by quantitative estimation of the enzyme. Based on the obtained results, the most active β-galactosidase-producing isolate designated NM56 was selected and identified.

Eextensive phenotypic characterization including morphological observation, microscopic examination and physiological characteristics was conducted to identify the bacterial isolate NM56 to the species level. Microscopic examination of stained smear indicated that isolate was Gram-positive, spore forming and rod-shaped. The isolate NM56 showed positive response to catalase test, citrate utilization, degradation of tyrosine, deamination of phenylalanine and degradation of starch, gelatin and casein. Negative results were observed for Voges-Proskauer test, indole production and nitrate reduction. Further characteristics for the isolate NM56 are detailed in Table 1. Depending on its characteristics, the isolate was identified as *Bacillus megaterium*. The obtained 16S rRNA gene sequence (1,391 bp) of the selected isolate was determined and submitted to the GenBank database under the accession number KT713740. Consistent with physiological traits, BLASTn analysis of the 16S rRNA sequence revealed that it was phylogenetically clustered with *Bacillus megaterium*. BLASTn analysis showed 100% similarity to *B. megaterium.* These results are in close agreement with the EzTaxon results. Various authors reported that β-galactosidase was produced by different strains of *Bacillus* spp. [5, 17, 42-44].

In an attempt to determine the optimum conditions for β-galactosidase production, the influence of five factors was investigated. The optimized factors were incubation period, temperature, initial pH, nitrogen sources and carbon sources.

To study the effect of incubation period on production of β-galactosidase, *B. megaterium* NM56 was grown in modified LB broth. Samples were withdrawn at periodic intervals; cells were collected and analyzed for enzyme activity. The time course of β-galactosidase production for a period of 96 h is shown in (Fig.1) indicating that β-galactosidase was produced in low level at 6 h incubation then the enzyme production increased gradually. Maximum production level was observed at 48th h of cultivation. Prolonged cultivation time, up to 96 h, did not show any significant influence on enzyme production. These findings are consistent with the previously reported results about the optimum incubation period for maximum production of β-galactosidase by *B. subtilis* that was 48 h [45]. In a similar study, maximum production of β-galactosidase by *Bacillus* Sp. MPTK 121 was observed at 48 h incubation [46]. Optimum incubation period for β-galactosidase production by *B. megaterium* NM56 was significantly shorter than that previously reported (72 h) for enzyme production by *B. licheniformis* ATCC 12759 [5]. These results give an advantage for potential application of *B. megaterium* NM56 isolate in β-galactosidase production.

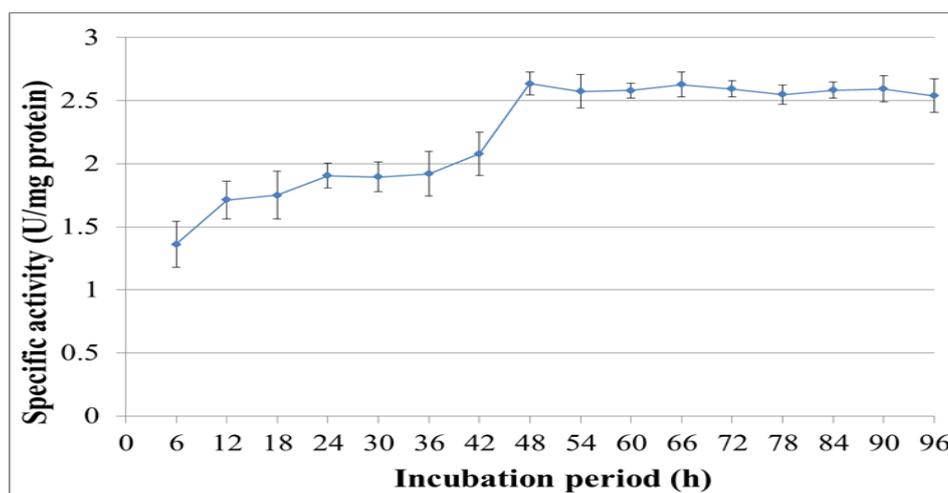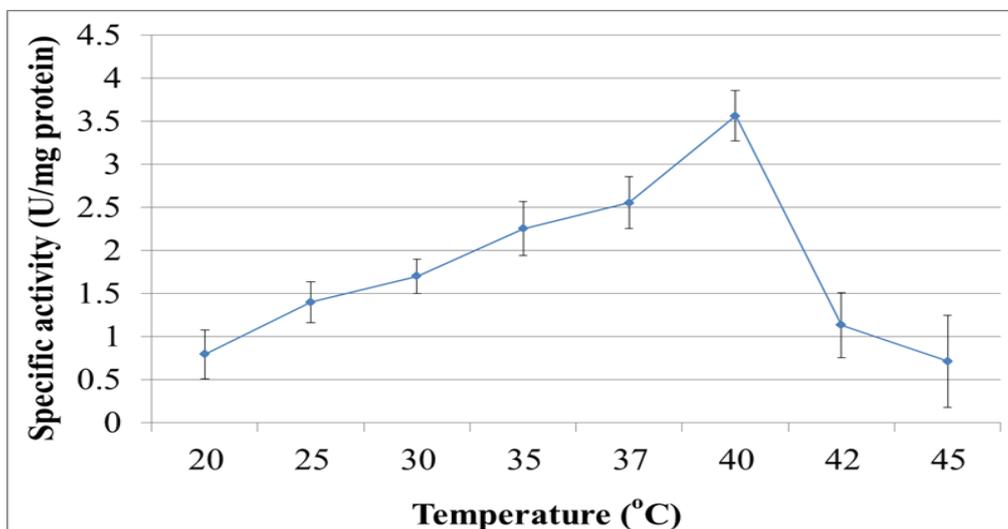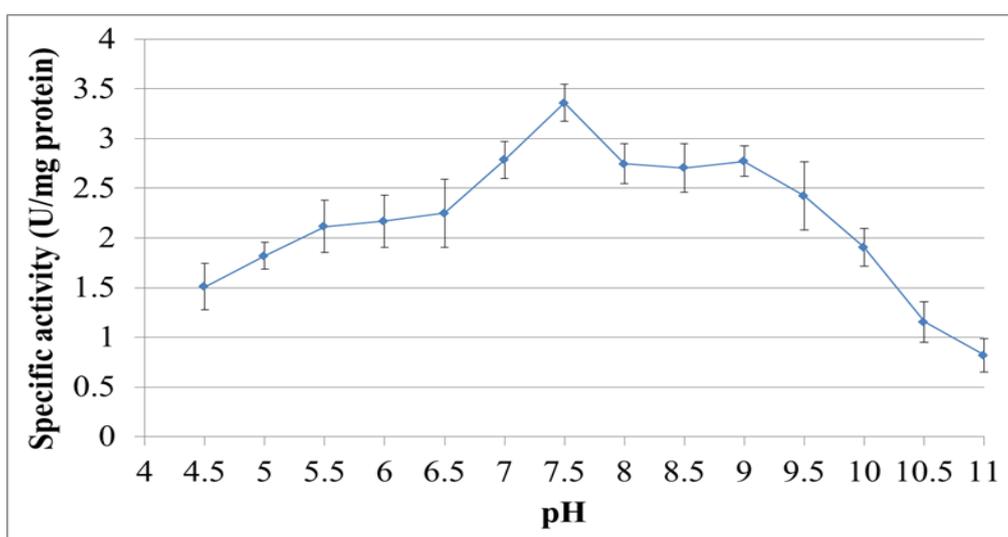**Figure 1: Effect of incubation period on β-galactosidase production. B. megaterium NM56 was grown in modified LB broth at 37°C. Samples were withdrawn periodically and analyzed for β-galactosidase production.**
- Error bars represent standard deviations (SD).

The effect of incubation temperature on the production of β-galactosidase was determined by incubating cultures at temperatures ranged from 20°C to 45°C. Results indicated that the production of β-galactosidase increases as incubation temperature increase. The optimal temperature for enzyme production was observed at 40°C **(Fig.2)**. Further increase in the temperature resulted in a sharp reduction in enzyme production. The obtained results are slightly higher than previously reported optimum temperature for β-galactosidase production by various *Bacillus* spp. that ranged from 30 to 37°C [44, 45, 47].

Figure 2: Effect of incubation temperature on β-galactosidase production. B. megaterium NM56 was cultured in modified LB broth at different incubation temperatures. Samples were withdrawn after 48 h incubation and analyzed for β-galactosidase production.
**- Error bars represent standard deviations (SD).**

To evaluate the effect of initial pH value on β-galactosidase production, the pH value of the medium was adjusted to various levels ranging from 4.5 to 11. It was found that *B. megaterium* NM56 produced β-galactosidase fairly over a wide pH ranging from 4.5 to 10.0 with maximum production at pH 7.5 **(Fig.3)**. Results showed a severe diminishing in β-galactosidase production at pH 10.5. Most of preceding investigations reported that maximum production of β-galactosidase ranging from pH 4.5 to 6.5 [15, 48, 49]. However there have been few reports on β-galactosidase production at range of pH observed in the current study [50, 51].

Figure 3. Effect of initial pH on β-galactosidase production. B. megaterium NM56 was grown in modified LB broth adjusted to different pH. Samples were withdrawn after 48 h incubation and analyzed for β-galactosidase production.
- Error bars represent standard deviations (SD).

In order to study the effect of different carbon sources on β-galactosidase production, LB broth was supplemented with 1% starch, sucrose, fructose, lactose, glucose or galactose. Results represented in (Fig.4) showed a clear variation in enzyme activity ranging from 0.37 to 3.18 U/mg protein when *B. megaterium* NM56 was grown on different carbon sources. Results revealed that maximum production of β-galactosidase was achieved when galactose or lactose was used as a carbon source. Statistical analysis clarified that galactose and lactose has the same stimulatory effect without any significant difference, when used separately. More than 7-fold enhancement in β-galactosidase activity was observed when galactose or lactose was used compared with that obtained by using glucose. These results agreed with findings of several authors who reported galactose is the best carbon source for production of β-galactosidase by *Bacillus licheniformis*, *Thermus thermophiles, Lactobacillus delbrueckii* ssp. *Bulgaricus* and *Lactobacillus crispatus* [5, 52-54]. Also in accordance with previous reports lactose was found to be the best inducer for β-galactosidase production by bifidobacteria, *Geobacillus stearothermophilus* and *Kluyveromyces marxianus* [48, 55, 56].

The results clearly showed that either sucrose or glucose significantly inhibited β-galactosidase synthesis when used as a carbon source (Fig.4). This suppressive effect was also recorded when fructose was used but to a lesser extent. In this trend, several authors documented the obvious inhibitory impact of glucose on production of β-galactosidase [44, 48, 57]. This decline in enzyme production may be due to catabolic repression or due the fact that bacteria primarily consume simpler sugars, thus does not induce significant β-galactosidase production.
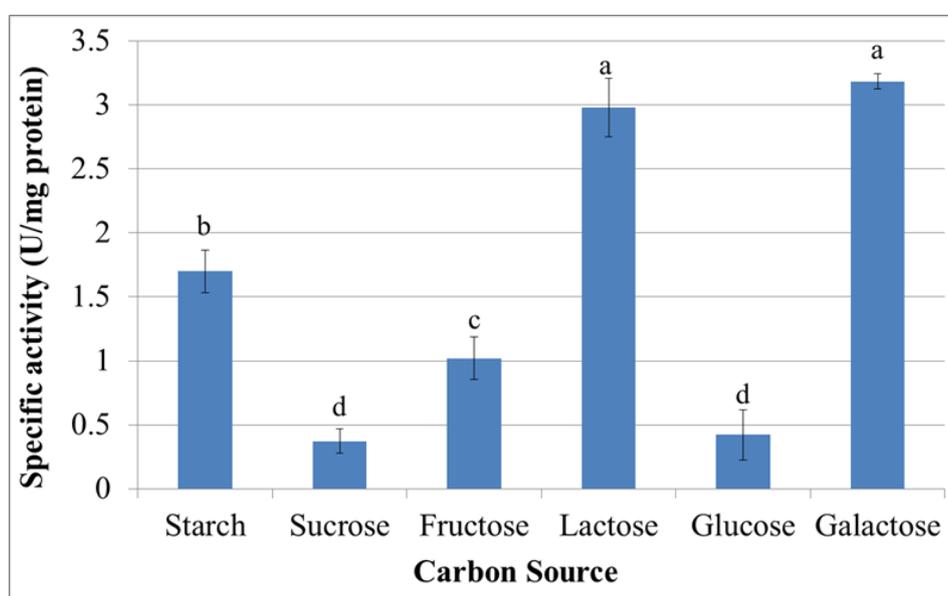

Figure 4: Effect of various carbon sources on β-galactosidase production. B. megaterium NM56 was grown in modified LB broth containing 1% starch, sucrose, fructose, lactose, glucose or galactose. Samples were withdrawn after 48 h incubation and analyzed for β-galactosidase production.
- Columns headed by the same letter were not significantly different according to Duncan's multiple range test (p < 0.05). Error bars represent standard deviations (SD).

The efficacy of different nitrogen sources on β-galactosidase production was studied using M9 broth. Casein, tryptone, peptone, beef extract, urea, yeast extract or NaNO$_3$ were assessed as nitrogen sources for the production of β-galactosidase at 37°C for 48 h. Results showed that tryptone and yeast extract induced maximum production of β-galactosidase when used separately (Fig.5). This followed by peptone and beef extract but with less proportion. These results are in line with previous studies that reported a stimulatory effect of yeast extract on β-galactosidase production by a wide range of microorganisms including *Lactobacillus plantarum*, *L. delbrueckii* ssp. *Bulgaricus, Thalassospira frigidphilosprofundus*, bifidobacteria [37, 53, 48, 58]. In a similar study investigated the effect of various nitrogen sources on β-galactosidase production by *B. subtilis*, yeast extract was the best nitrogen source followed by beef extract and peptone [45]. This stimulatory effect of yeast extract may be due to the presence of substances other than amino acids as co-factors, vitamins and growth factors that may have positive effects on microbial growth and metabolism.
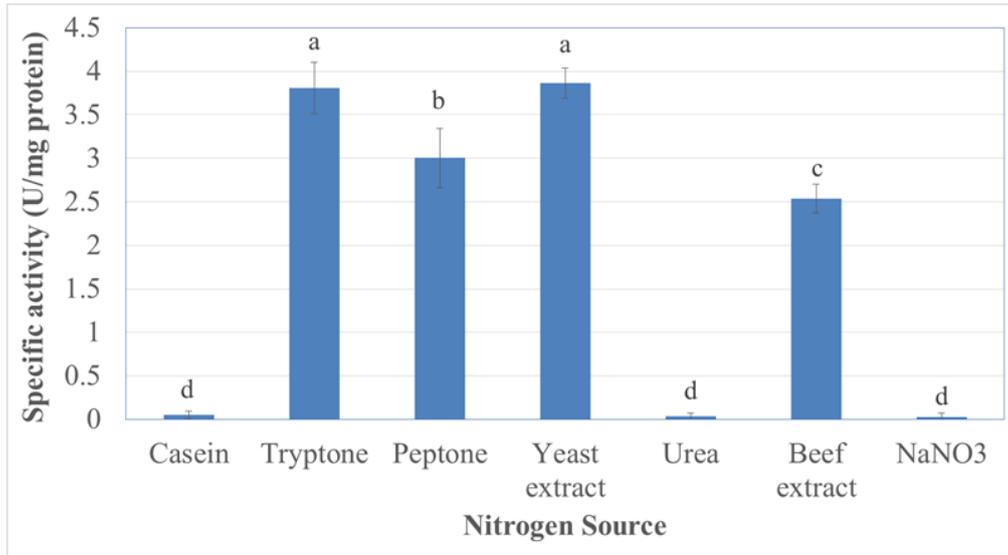
Figure 5: Effect of various nitrogen sources on β-galactosidase production. B. megaterium NM56 was grown in M9 broth supplemented with 1% (w/v) casein, tryptone, peptone, beef extract, urea, yeast extract or NaNO3. Samples were withdrawn after 48 h incubation and analyzed for β-galactosidase production.
- Columns headed by the same letter were not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bars represent standard deviations (SD).
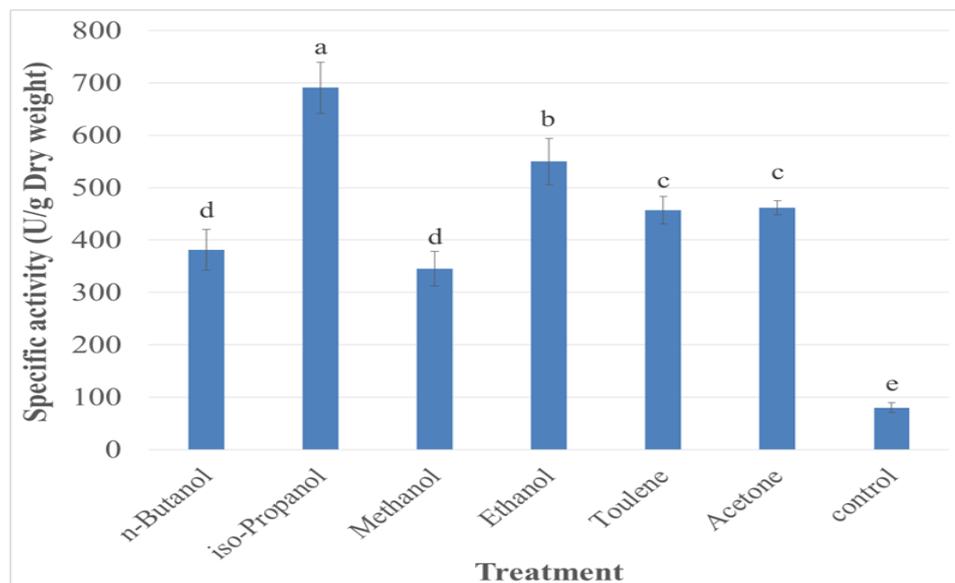
Figure 6: Effect of various solvents on permeabilization of B. megaterium NM56 cells to β-galactosidase. Cells were collected by centrifugation treated with 50% (v/v) n-butanol, iso-propanol, methanol, ethanol, toluene or acetone. Control samples were resuspended in 0.1M phosphate buffer (pH 7.0) instead of solvent. Cells analyzed for β-galactosidase activity.
- Columns headed by the same letter were not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bars represent standard deviations (SD).

Due to the fact that most of microbial β-galactosidases produced intracellularly, researchers devoted heavy effort to develop methods for releasing of this enzyme or making cells more permeable [21, 31, 32, 59]. To determine the most efficient solvent with respect to permeabilization, *B. megaterium* NM56 cells were treated with n-butanol, ethanol, toluene, acetone, iso-propanol, or methanol and β-galactosidase was assayed. Results disclosed that all the investigated solvents swimmingly permeabilized *B. megaterium* NM56 cells without any detrimental effect on β-galactosidase activity (Fig.6). Iso-propanol was found to be the most potent permeabilizing agent followed by ethanol while n-butanol and methanol were considered as the poorest permeabilizing agents. Cells treated with iso-propanol exhibited more than 8-fold increment in

enzyme activity as compared to untreated control. Also, it was observed that ethanol increased β-galactosidase activity by more than 6-fold over untreated control. Results indicated that *B. megaterium* NM56 cells treated with iso-propanol showed about 51% increase in enzyme activity compared to those treated with toluene. These results are contradictory regarding the permeabilization efficiency of different solvents. Most reports considered ethanol, toluene and n-butanol as the most active permeabilization agents towards β-galactosidase [22, 31, 37]. Despite there are no previous reports on this elevated capacity, the present investigation revealed that iso-propanol had the highest pemeabilizing power. These results encourage utilization of iso-propanol to permeabilize *B. megaterium* NM56 that improves its potential application as whole cell catalyst.

CONCLUSION

From above findings, it can be concluded that locally isolated *B. megaterium* NM56 is a potential source for production of significant amounts of β-galactosidase and can be applied as whole cell catalyst upon iso-propanol-mediated permeabilization.

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