British Microbiology Research Journal
11(2): 1-15, 2016, Article no.BMRJ.18750
ISSN: 2231-0886, NLM ID: 101608140

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Optimization of β-galactosidase Production from Yogurt and Dairy Soil Associated Bacteria Using Different Fermentation Media

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Authors' contributions

This work was carried out in collaboration between all authors. Author NMA designed the study. Authors BM and AUAK performed the experiments, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Author SA did the statistical analysis, managed and finalized the final draft. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/18750
Editor(s):
(1) Giuseppe Blaiotta, Department of Food Science, Via Università, Italy.
Reviewers:
(1) Anonymous, Manonmaniam Sundaranar University, Tamil Nadu, India.
(2) Luige Biciati Alvim, Federal University of Minas Gerais, Brazil.
(3) Sameh Awad, Alexandria University, Egypt.
(4) Anonymous, University of Milan, Italy.
(5) Junko Nishimura, Hachinohe Institute of Technology, Japan.
Complete Peer review History: http://sciencedomain.org/review-history/11834

Original Research Article

Received 8th May 2015
Accepted 2nd August 2015
Published 16th October 2015

ABSTRACT

Aims: The aim of current research was the production of β-galactosidase enzyme from *Lactobacillus delbruekii subsp. bulgaricus* isolated form yogurt and soil samples.
Study Design: Production of β-galactosidase.
Place and Duration of Study: Microbiology laboratory, Department of Zoology, GC University, Lahore, Pakistan, between 14 Nov, 2013 and 29 Nov, 2014.
Methodology: Bacterial isolates were isolated from yogurt and soil samples. Morphological, biochemical characterization, and 16S rRNA sequencing was used for the identification of the bacterial strains. The optimization and maximum production of β-galactosidase was carried out by

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using various temperatures, pH, carbon sources (corn flour, wheat flour, rice flour), nitrogen sources (Trypton, Peptone, Yeast extract), and incubation periods.
Results: National Centre for Biotechnology Information (NCBI) Blast analyzed that A11, A13 and A14 strains belongs to *Lactobacillus delbruekii* subsp. *bulgaricus* with accession numbers KP256199, KP264120, and KP264121. Maximum enzyme production was obtained after 48 h of incubation at 40-45°C temperature at pH 6.0-7.0. The highest enzyme production was observed in the presence of rice flour and yeast extract. The maximum enzyme activity was shown by A11, A13 and A25 (7.69 U/mL, 7.25 U/mL, and 7.28 U/mL).
Conclusion: Rice flour could be the potential carbon source for the production of β-galactosidase from *Lactobacillus delbruekii subsp. bulgaricus* whereas nitrogen sources reduced its production. Temperature and pH was important physical parameter for the production of β-galactosidase from yogurt and soil associated bacteria.

Keywords: β-galactosidase; lactic acid bacteria; fermentation medium; optimization.

1. INTRODUCTION

Enzymes are the most essential biomolecules obtained from different microbial sources such as bacteria and fungi for human needs [1]. They control and speed up the chemical reactions that's take place inside the living organism. Enzymes are used to make foodstuffs, to pre-digest some baby foods, to extract fruit juices, in biological detergent and in biosensors as well. Currently the field of biotechnology developing new applications for enzymes.

β-galactosidase is known as Beta-gal, an extracellular and hydrolytic enzyme that catalyses lactose (disaccharide in milk) into galactose and glucose which are monosacharides. β-galactosidase also involved in transglycosylation reaction. β-galactosidase is widely distributed in animals, plants and numerous microorganisms i.e., bacteria, fungi, archaea and yeast [2]. Among microorganisms a large number of Lactic Acid Bacteria (LAB) are most suitable because they are Generally Regard as Safe (GRAS), have been considered being excellent sources of β-galactosidases, particularly for purposeful food applications such as digestibility of dairy products, involved in to improve sweetness, solubility, and flavour [3]. β-galactosidase has two main uses; the production of galactosylated products and the lactose removal from milk products for lactose intolerant people. On the basis of its importance so, our work was mainly on the production of β-galactosidases (enzyme production) from bacterial sources.

Number of Lactic Acid Bacteria were isolated from various sources like whey, yogurt, soil sample from dairy processing plants, cattle area, cheese, and milk to produce β-galactosidase [4]. *Bacillus subtilis, B. licheniformis* ATCC 12759, *Streptococcus thermophiles*, and *Lactobacillus sp.* were used for the production of β-galactosidase by using different carbon, nitrogen and amino acid sources [5-8]. Among LAB, yogurt bacteria such as *Lactobacillus delbrueckii, Lactococcus lactis* and *Streptococcus thermophilus* are the highest β-galactosidase producers [9,10].

The aim of current research was the screening of β-galactosidase producing LAB from yogurt, and soil from dairy farm. The second object was the enhancement of β-galactosidase production at commercial level by using these bacterial isolates by using different fermentation media.

2. MATERIALS AND METHODS

2.1 Sample Collection

Ten yogurt samples were purchased from different shops from north Lahore, Pakistan in sterilized containers as well as ten soil samples were also collected from different dairy farms from Lahore, Pakistan in sterilized sealed bags or containers with the help of sterilized spoon for the isolation of β-galactosidase producing bacteria.

2.2 Culture Media Preparation

HYA (hyaluronidase in FertiCult™ Flushing) agar and MRS (de Man, Rogosa and Sharpe) broth media were used for isolation and culturing of *Lactobacillus bulgaricus* from collected yogurt and soil samples. For the preparation of HYA agar following ingredients were used as w/v (15 g agar, 10 g peptone, 1 g beef extract) was used to dissolve in 970 mL of DH_2O (double distilled water), autoclave at 121°C for 15 min at 15 psi.

After sterilization 10 mL galactose solution (2.5%), 10 mL lactose solution (5%) was added. Then pour the media in sterilized petri plates and allowed these plates to solidify. For the preparation of MRS broth, ingredients were used as w/v (10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g mangenase sulphate and 2 g dipotassium hydrogen phosphate) was used to dissolve in 1 L of DH_2O, autoclave at 121°C for 15 min at 15 psi.

2.3 Isolation of Bacteria from Soil Samples

Dissolved 1 g of soil sample in autoclaved 100 mL of DH_2O and prepared ten serial dilutions. Then take 100 µL from dilution no. 1, dilution no. 5 and dilution no. 10 and spread on sterilized HYA agar plates and incubate anaerobically at 37°C for 24 h. After incubation all the plates were observed for the formation of white small and large colonies. After that pick these small and large colonies and activate these colonies by successive transfer in MRS broth for 24 h at 37°C, glycerol stocks were prepared and stored at -80°C used for further studies.

2.4 Isolation of Bacteria from Yogurt Samples

To obtained pure colonies, a loop full of yogurt samples directly from container streaked on the HYA agar and incubated these plates at 37°C for 24 h. After incubation all the plates were observed for the formation of white small and large colonies. After that pick these small and large colonies and activate these colonies by successive transfer in MRS broth for 24 h at 37°C, glycerol stocks were prepared and stored at -80°C used for further studies.

2.5 Characterization of Bacterial Isolates

The obtained bacterial colonies were characterized by employing different test. Both morphological and biochemical tests were performed for the characterization of obtained bacterial isolates. Bacterial isolates were observed for colony morphology including shape of bacterial colonies, color of bacterial colonies, texture of colonies and studied microscopically by Gram's staining, endospore staining and motility test. Biochemical tests such as citrate test, catalase test, starch hydrolysis test, gelatin test, VP test, carbohydrate tests were also performed.

2.6 Determination of Optimal Growth Conditions

2.6.1 Growth curves

To obtain the growth curves of pure bacterial isolates, 20 mL of MRS broth was taken as a starter culture in 100 mL conical flasks; flasks were cotton plugged and wrapped with aluminium foil. The medium was autoclaved for 15 min at 121°C and 15 psi pressure. Triplicates were made to perform this experiment. After autoclaving, medium was inoculated with pure bacterial culture 10^7 colony forming units (CFU/mL) and placed in shaking incubator for overnight. After 24 h by using micropipette about 400 µL of this culture was drawn in cuvette from starter culture under sterile condition and optical density (OD_{590}) as taken by spectrophotometer at 590 nm. This practice is repeated after every 2 h. Growth curve was plotted by taking incubation time on X-axis and optical density was taken on Y-axis.

2.6.2 Determination of optimum Ph

To determine optimum pH for each bacterial strain, first of all of medium was prepared by taking 5 mL of MRS broth in test tubes in five containers, each of 5 containers were used for separate strain and medium pH was set up to 2, 4, 6, 8, and 10. The medium was autoclaved after pH adjustment. Then inoculation was given with 100 µL of freshly prepared culture of every isolated bacterial culture and incubation was given in shaking incubator at 37°C. After 20 h, OD was taken by spectrophotometer at 590 nm. Take these readings in triplicates for each strain. Finally the optimum pH of every strain was resolute by plotting a graph between pH on X-axis and OD along Y-axis. The graph found out the optimum pH for every bacterial isolate.

2.6.3 Determination of optimum temperature

To determine optimum temperature for each isolated strain, medium was prepared by taking 5 mL of MRS broth in test tubes in five sets, five test tubes were used for each strain. This test was conducted in triplicates. The medium was autoclaved at 15 psi pressure for 15 min. Then medium was inoculated with 100 µL of fresh culture of each isolated strain and incubated in at, 25°C, refrigerator temperature, 37°C, 45°C

and 60°C. Optical density was taken by spectrophotometer after 20 h at 590 nm. Lastly the optimum temperature of each strain was determined by plotting a graph between temperature on X-axis and optical density along Y-axis. The graph ascertained the optimum temperature for each bacterial isolate.

2.7 Molecular Characterization of Bacterial Isolates

The molecular characterization of bacterial isolates genomic DNA (gDNA) was isolated by phenol chloroform method [11]. Agarose gel electrophoresis (1 %) was done to assure that the samples contain isolated genomic DNA. After running gel, DNA bands in the gel were visualized using short wave ultraviolet light provided by a trans-illuminator and photographed was taken through Stratagene Eagle Eye still video system.

2.7.1 Ribotyping and sequencing

Ribotyping is aimed at molecular characterization of pathogenic isolates, so their 16S rRNA 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3') Universal primers were used to partially amplified 1500 bps DNA fragment through polymerase chain reaction [12]. PCR was performed in a thermocycler for thirty five reaction cycles. Total amount of reaction mixture taken for this was 50 µL. First denaturation took place at 94°C for five min, annealing was done at 52°C and elongation at 72°C for 30 sec, 40 sec and 30 sec respectively. Final extension was given at 72°C for 10 min. To confirm the amplification, PCR products were loaded in 1% agarose gel. Gel was run and the bands of amplified DNA were visualized under the UV light by using the trans-illuminator. After amplification anticipated band were eluted/cut and kept in eppendrofs. Then using GF-1 DNA recovery kit by Vivantis, gene was cleaned. The partial sequence of 16S rRNA fragment was delivered to the Molecular Biology Lab Malaysia for sequencing.

2.8 Enzyme Production

In order to produce enzyme 1% freshly prepared bacterial culture was inoculated in the flask containing production media and incubated these flasks at 40°C for 24 h. After incubation take 10 mL of fermentation broth and centrifuge at 6000 rpm for 15 min at 4°C .Then pellet was washed with 10 mL of 0.05 M sodium phosphate buffer having pH 7.0 and centrifuge again this solution at 6000 rpm for 15 min at 4°C. Again discard the supernatant and washed the pellets with 5 mL sodium phosphate buffer of pH 7.0 then vortex this solution and add 5 mg of glass beads of 1 mm size to this solution and centrifuge it at 2700 rpm in 8 operating cycles (1 operating cycle = 1 minute operation and 30 seconds cooling on ice). At the end of this operation centrifuge the solution at 6000 rpm for 15 min at 4°C and supernatant was used as enzyme assays and protein determination.

2.9 β-galactosidase Assay

β-galactosidase activity was determined by adding proper quantity of enzyme (100 µL) in reaction mixture that act as a substrate containing: 1 mL of lactose (2% w/v) or 1 mL of ortho-Nitrophenyl-β-galactoside (OPNG; 2% w/v) and 1 mL of 25 mM Sodium acetate buffer (pH 5.0) prepared in distilled water. This reaction mixture was incubated for 30 min at 30°C in incubator and then placed these test tubes in boiling water bath for 5 min. After that these tubes were placed in ice cold water for cooling. The amount of glucose produce was determined by adding 100 µL of reaction mixture to 1 mL of glucose measuring kit, which was based on glucose oxidase (Biocons, made in Germany) and was incubated at 37°C for 10 min. The OD was taken at 500 nm. One unit of β-galactosidase activity was equal to µmole of glucose equivalent liberated per minute under defined conditions. In 10 µL of glucose standard, 90 µL of distilled water was added and incubated at 37°C for 15 min. After incubation OD was taken at 500 nm on spectrophotometer. Take 100 µL of distilled water (blank control) was added to 1 mL of glucose kit and incubated at 37°C for 10 min and absorbance was adjusted to zero [8].

2.10 Culture Medium Selection for Optimum Production of β-galactosidase

In order to determine the optimum culture medium for the production of β-galactosidase, three production medium were used. Medium-1 (M-1) contains w/v (Lactose 10 g/l, peptone 1.5 g/l, yeast extract 1 g/l, KH_2PO_4 1 g/l, $NH_4H_2PO_4$ 7 g/l, $MgSO_4$. $7H_2O$ 1 g/l, $CaCl_2$ 0.3 g/l); Medium–2 (M-2) contains w/v (Lactose 10%, peptone 10%, yeast extract 10%, NaH_2PO_4 0.1%, $(NH_4)_2SO_4$

0.5%, $MgSO_4$. $7H_2O$ 0.05%) and Medium–3 (M-3) contains w/v (Lactose 4%, yeast extract 3.5%, KH_2PO_4 0.1%, K_2HPO_4 0.3%, $MgSO_4$. $7H_2O$ 0.05%, L-cysteine 0.03%). These media were inoculated with freshly bacterial culture and were then placed in incubator at 40°C for overnight. After 24 h enzymes were extracted for assays. The results of assays were recorded and then were compared to determine which medium having more production of enzyme.

2.11 Determination of Optimum Condition for β-galactosidase Production

2.11.1 Effect of pH on β-galactosidase production

In order to determine the effect of pH on β-galactosidase production, the sterilized medium with pH ranging from 4.0 – 9.0 was taken in flask and then inoculated with the freshly grown bacterial culture. It was then incubated for 24 h. After 24 h, the enzyme was extracted for performing enzyme assay.

2.11.2 Effect of temperature on β-galactosidase production

To investigate the optimum temperature, the bacterial isolates were grown in (Luria-Bertani) LB broth at temperature i.e., 25°C, 30°C, 37°C, 40°C and 45°C. After 24 h, the enzyme was extracted for performing enzyme assay.

2.11.3 Effect of various carbon sources

To determine the effect of different carbon source on the production of β-galactosidase, the freshly grown isolates inoculated in production media containing different carbon sources (1% m/v) including wheat flour, rice flour and corn flour and then incubated at 45°C for 24h. After 24h, the enzyme was extracted for performing enzyme assay.

2.11.4 Effect of various nitrogen sources

To determine the effect of different nitrogen source on the production of β-galactosidase, the freshly grown isolates inoculated in production media containing different nitrogen sources (1% m/v) including peptone, tryptone and yeast extract and then incubated at 45°C for 24h. After 24 h, the enzyme was extracted for performing enzyme assay.

2.11.5 Effect of various metal ions

To determine the effect of different metal ions on the production of β-galactosidase, the freshly grown isolates inoculated in production media containing different metal ions (1mM concentration) including $MnSO_4.7H_2O$, $ZnSO_4$ and $FeSO_4$ and then incubated at 45°C for 24 h. After 24 h, the enzyme was extracted for performing enzyme assay.

2.12 Protein Estimation

The standard curve for protein estimation was prepared using Bradford method [13]. Initially, a stock solution of BSA ranging from 10-100 µg protein/µL was prepared as control. For determining the concentration of the enzyme 0.1 mL of the enzyme extract was added into the test tube while the control contained the extract of the control media since the media had its own color. 5 mL of Bradford was added to both of them which were then incubated for 5 min and then their absorbance checked using Spectrophotometer at 595 nm. The absorbance reading was used to calculate the amount of protein present in the enzyme extract. The specific activity of the enzyme is then calculated using the following formula:

$$\text{Specific activity (U/mg of protein)} = \frac{\text{Enzyme activity (U)}}{\text{Protein content (mg)}}$$

2.13 Statistical Analysis

Each experiment was repeated in triplicates and mean ± standard deviation (M±SD) from absolute data were measured through on line Standard deviation calculator http://easycalculation.com/statistics/standard-deviation

3. RESULTS

3.1 Sampling

For the isolation of β-galactosidase producing bacteria, 20 yogurt and soil samples were collected from different shops and dairy farms around North Lahore, Pakistan in sterilized containers and sealed bags during November, 2013 (Table 1). The physiological parameters such as pH of the sample and temperature of sampling sites were also recorded (Table 1).

3.2 Bacterial Isolation

Thirty bacterial isolates were obtained from 20 collected samples. They represented as A1, A2,

A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A23, A24, A25, A26, A27, A28, A29, A30. After morphological and biochemical characterization only five bacterial isolates were selected for further study because of their higher production. Two bacterial strains isolated from yogurt and three from soil samples.

3.3 Morphological and Biochemical Characterization

Through Gram's staining it had been found that all the isolated bacterial cultures were purple and rod shaped with rounded ends. This showed that all the isolated bacterial cultures were gram positive bacilli. Through endospore staining it had been found that all the isolated species were non-spore forming. Motility test indicated that all the strains were non-motile. All biochemical and morphological characteristics of isolated bacteria were recorded and shown in Table 2.

3.4 Determination of Optimal Growth Conditions

3.4.1 Bacterial growth curves

In order to determine the incubation time at which the bacterial isolates showed maximum growth, the isolated bacterial cultures were grown at 37°C in shaking incubator and their growth was determined after every 2 h (Fig. 1A). In case of bacterial isolates A11 and A14, it was found that these isolates gave maximum growth at 22nd h of incubation and growth rate started decline after this period. In case of A21, the isolate showed maximum growth at 12 h and after that the growth of isolates was declining. In case of A13, the isolate showed highest growth rate at 18 h of incubation and after that after that its growth rate started declining. In case of A25, the isolate showed best growth at 20 h of incubation and after that its growth started declining.

3.4.2 Determination of optimum Ph

It was found that the optimum pH for the bacterial culture A11 and A21 was pH 5, in case of A13 the optimum pH was 6 while in case of A14 and A25 the optimum pH was pH 7 (Fig. 1B).

3.4.3 Determination of optimum temperature

It was found that optimum temperature for A11, A13 and A14 was 45°C while the optimum temperature for A21 and A25 was 40°C. After these temperatures their growth was started to decline (Fig. 1C).

Table 1. The physiological parameters (pH and temperature) of sampling sites

S. no.	Site name	Nature of sample	Temperature °C	pH
1	Iqbal Road, Shad Bagh	Yogurt	27	4.89
2	Iqbal Road, Shad Bagh	Yogurt	27	4.66
3	Goal Bagh	Yogurt	27	4.74
4	Wassan Pura	Yogurt	27	4.96
5	Wassan Pura	Yogurt	27	4.83
6	Nabi Baksh Park	Yogurt	26	4.98
7	Faiz Bagh	Yogurt	26	4.72
8	Faiz Bagh	Yogurt	26	4.81
9	Old Anarkali	Yogurt	26	4.57
10	Taj Pura, Shad Bagh	Yogurt	25	4.92
11	Ahlu Pind, Kahna	Soil	26	6.01
12	Bund Road, Shad Bagh	Soil	25	6.21
13	Bhama Juggian, Bund Road	Soil	27	5.96
14	Bhama Juggian, Bund Road	Soil	27	5.92
15	China Scheme, Kot Khawaja Saeed	Soil	26	6.12
16	China Scheme, Kot Khawaja Saeed	Soil	26	6.00
17	Bhagat Pura, Shad Bagh	Soil	26	6.23
18	Bhagat Pura, Shad Bagh	Soil	26	6.18
19	Badami Bagh	Soil	25	6.39
20	Badami Bagh	Soil	25	6.26

Table 2. Morphological and biochemical characterization of isolated bacterial strains

S. no	Gram staining	Endospore staining	Motility test	Catalase test	Urease test	Indole test	MR-VP test	Citrate test	Starch hydrolysis test	Gelatin hydrolysis	Litmus milk test	Carbohydrate fermentation		
												Glucose	Sucrose	Lactose
A1	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A2	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A3	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A4	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A5	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A6	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A7	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A8	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A9	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A10	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A11	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A12	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A13	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A14	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A15	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A16	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A17	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A18	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A19	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A20	+ve	-ve	-ve	-	-	-	+	-	+	+	+	+	+	+
A21	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A22	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A23	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A24	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A25	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A26	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A27	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A28	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A29	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+
A30	+ve	-ve	-ve	-	+	+	+	-	+	+	+	+	+	+

(+) indicates positive and (-) indicates negative results

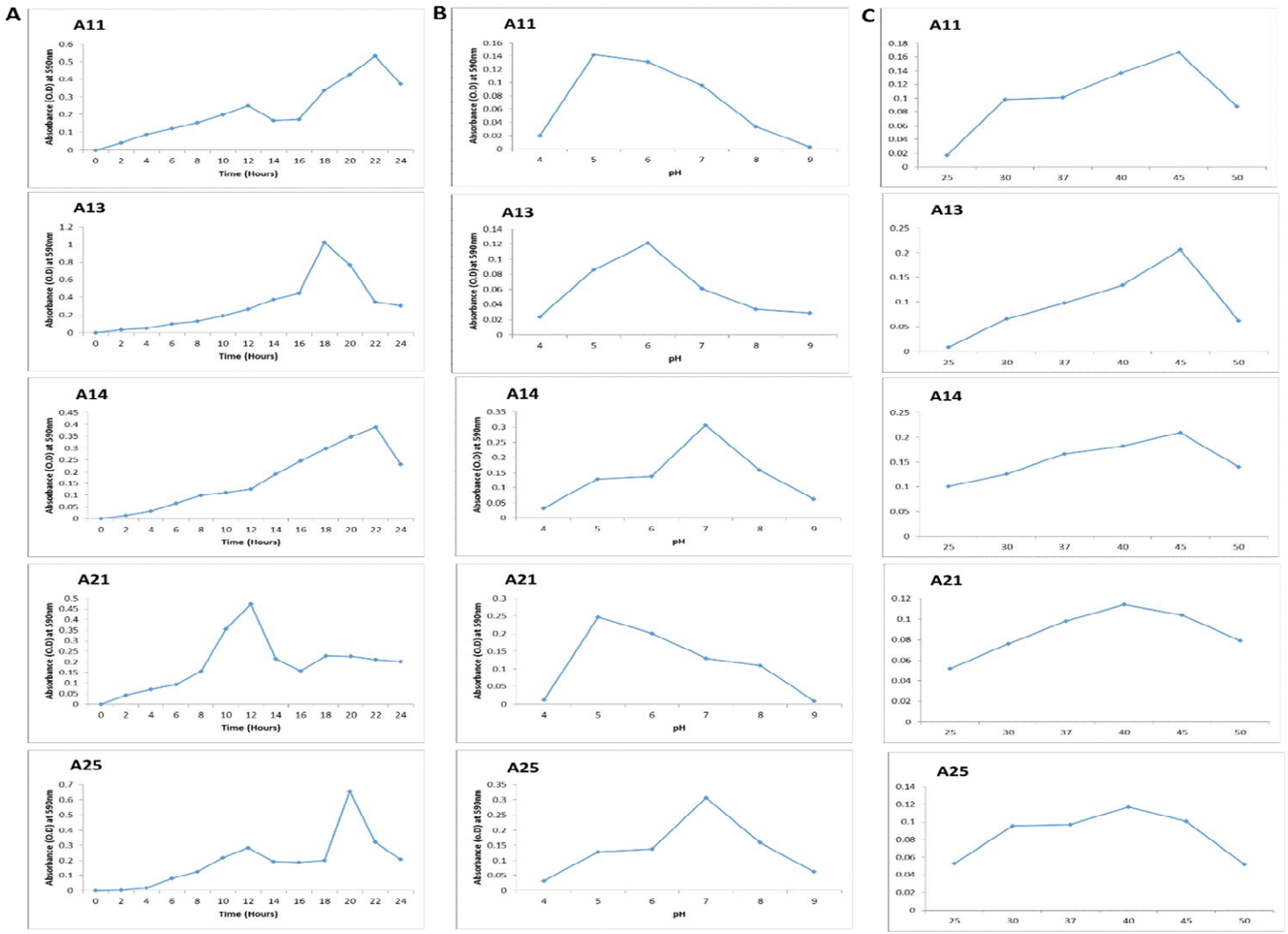

Fig. 1. Growth pattern and effect of pH and temperature on the bacterial isolates in MRS medium incubating at 37°C

A) Growth curve for 24h of time period, B) Effect of pH on the growth of bacterial isolates in MRS medium at different pH, C) Effect of temperature on the growth bacterial isolates in MRS medium

3.5 Molecular Characterization of Bacterial Isolates

About 5 µl genomic DNA was loaded for gel electrophoresis. As a result of gel electrophoresis sharp bands of genomic DNA of A11, A21, A13, A14 and A25 were visualized (Fig. 2A). By using the forward and reverse primers, the conserved region of 16S rRNA fragment was amplified from the genomic DNA of the bacterial isolates. The PCR product was then visualized on 1.0% agarose gel containing DNA ladder (Fig. 2B). From 1.0 % agarose gel, the amplified product was cut and sent to Malaysia for sequencing. After sequencing it was found that 16S rRNA fragment of A11 contains 608 bp and (NCBI) Blast analyze revealed that it belongs to *Lactobacillus delbruekii subsp. bulgaricus*, A13 contains 600 bp and belongs to *Lactobacillus delbruekii subsp. bulgaricus* and A14 contains 596 bp and also belongs to *Lactobacillus delbruekii subsp. bulgaricus* (Table 3).

3.6 Enzyme Assay

3.6.1 Selection of culture medium for optimum production of β-galactosidase

Three cultural media were used for the production of β-galactosidase from bacterial isolates. Out of three culture media examined, M-1 was considered to be the best medium for the production of β-galactosidase by bacterial isolates (Fig. 3A)

3.6.2 Effect of pH on β galactosidase production

Different pH was used to check the productivity of enzyme by bacterial isolates in the shaking incubators. pH of the medium was adjusted between 4 and 10 (Fig. 3B). 50 mL of 18 h old inoculum (2%) was inoculated from the starter culture and it was found that A11 was showed the maximum activity at pH 6. In case of bacterial isolate A21, the optimum pH was 7. A13 and A14 was showed maximum activity at pH 6 and bacterial isolates A25 was showed maximum activity at pH 7.

3.6.3 Effect of temperature on β galactosidase production

To investigate the effect of temperature on enzyme production by bacterial isolates in M-1 medium, the flasks containing 50 mL M-1 medium and bacterial culture were placed in shaking incubator at different temperatures (Fig. 3C). It was found that the isolates A11, A13 and A14 showed maximum production at 45°C while isolates A21 and A25 showed maximum production at 40°C.

3.6.4 Effect of incubation time on β galactosidase activity

To determine the optimum incubation period for the production of β-galactosidase by bacterial isolates, the M-1 medium was inoculated with the bacterial cultures and the number of enzyme units produced were calculated after every 24 h i.e., from 24-78 h (Fig. 3D). It was found that all the bacterial isolates produced maximum enzyme units at 48 h of incubation. Therefore the maximum incubation period for the maximum production of β-galactosidase was found to be 48 h.

3.6.5 Effect of carbon and nitrogen sources

To find the suitable carbon source for the production of β-galactosidase production three different carbon source were used i.e., wheat

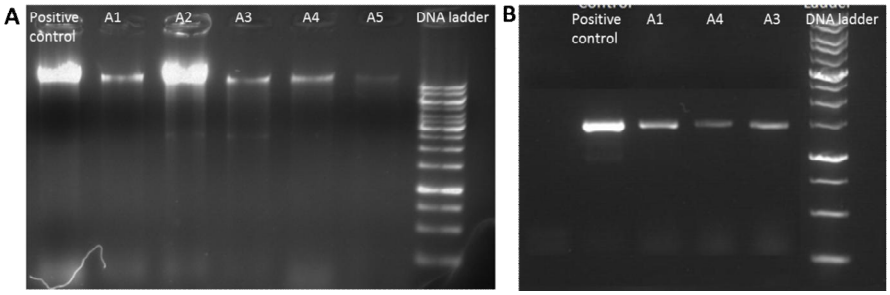

Fig. 2. 1% agarose gel electrophoresis of the genomic DNA and amplified PCR product isolated from β -galactosidase producing bacterial isolates

A) Genomic DNA isolation; B) Amplification of PCR product using universal primers A1(A11), A2(A21), A3(A13), A4(A14) and A5(A25)

flour, rice flour and corn flour (Fig. 4A). Among these three carbon sources rice flour showed the maximum enzyme production. To find the suitable nitrogen source for the production of β-galactosidase production three different nitrogen source were used i.e., Yeast extract, Peptone and tryptone (Fig. 4B). These three nitrogen sources showed the significant decrease in the enzyme production.

3.7 Enzyme Kinetics

3.7.1 Effect of temperature and pH on β galactosidase activity

To determine the temperature on the activity of β-galactosidase, the enzyme was treated with temperature ranges from 25-50°C. The enzyme solution was incubated at respective temperatures in the shaking incubator. The maximum enzyme production of bacterial isolates i.e., A11, A21, A13, A14 and A25 was found at 40°, after this the activity was rapidly declined (Fig. 5A). To determine the effect of pH on the activity of β-galactosidase, enzyme was incubated by using different buffer solutions of various pH i.e., 4.0-9.0 and placed at 25°C for 5 min. The maximum activity was showed at neutral pH i.e., 7.0 (Fig. 5B).

3.8 Protein Estimation by Bradford Method

Protein estimation of enzyme was done by Bradford method to measure the specific activity of β-galactosidase. Absorbance was taken at 595 nm and compared with the standard BSA to find the protein content (mg). The maximum enzyme activity was shown by A11, A13 and A25 (7.69 U/mL, 7.25 U/mL, and 7.28 U/mL).

4. DISCUSSION

β-galactosidase was an intracellular enzyme isolated from *Lactobacillus delbruekii subsp. bulgaricus* grown in yogurt. Physico-chemical properties like temperature and pH played a significant role in the production of β-galactosidase. The optimum pH for the β-galactosidase production was 6.0 for isolates A11, A13 and A14 while for A21 and A25 the optimum pH was 7.0.

Under submerged fermentation condition the optimal conditions for β-galactosidase enzyme production were determined. According to the results obtained it was observed that the optimum temperature was found to be in range of 40-45°C for the maximum production of β-galactosidase enzyme. Similarly, for isolates A11, A13 and A14 the optimum temperature was 45°C while for the isolates A21 and A25 it was 40°C. 37-45°C has been demonstrated as the optimum temperature range for maximum enzyme activity with different organisms by many researchers [14,15].

Incubation time illustrate the characteristics of the culture which is based on the enzyme production and growth rate [16]. The maximum production of β-galactosidase enzyme by all the bacterial isolates was found after 48h. After this time the enzyme production started to decline. This may be due to the reduction of the quantity of nutrients.

Table 3. NCBI results of isolated bacterial strains

Strain	Identified as	Accession no.	Identity % age
A1 (A11)	*Lactobacillus delbruekii subsp. bulgaricus*	KP256199	99% Identical with KF149461.1 *Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU92068 16S ribosomal RNA gene, partial sequence
A3 (A13)	*Lactobacillus delbruekii subsp. bulgaricus*	KP264120	100% Identical with KF149789.1 *Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU62149 16S ribosomal RNA gene, partial sequence
A4 (A14)	*Lactobacillus delbruekii subsp. bulgaricus*	KP264121	99% Identical with KF149768.1 *Lactobacillus delbrueckii* subsp. *bulgaricus* strain IMAU62128 16S ribosomal RNA gene, partial sequence

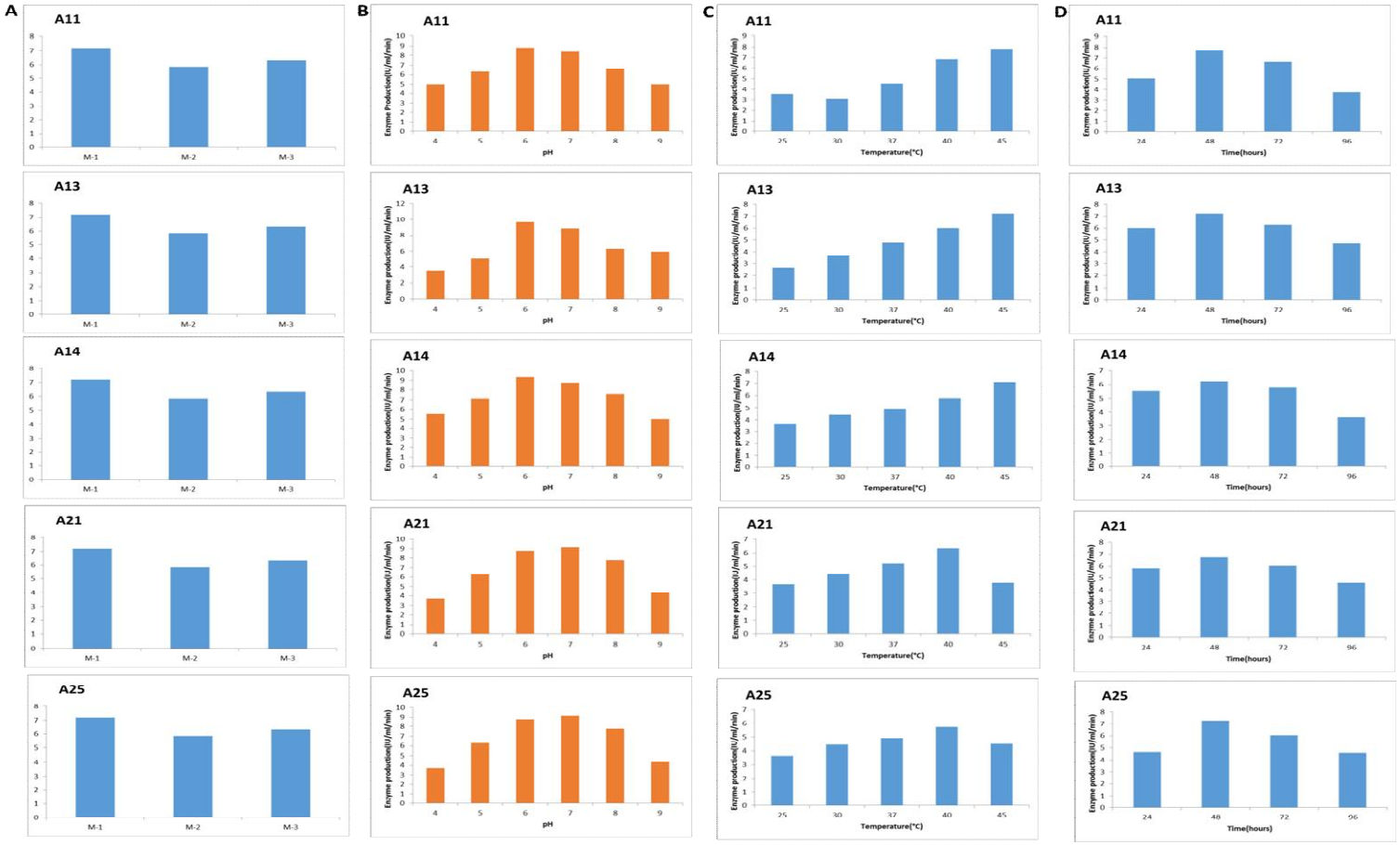

Fig. 3. Effect of various culture media, pH and temperature and incubation time period on the production of β-galactosidase by bacterial isolates

A) Effect of various culture media on β-galactosidase production by bacterial isolates, B) Effect of pH on β-galactosidase production by bacterial isolates growing in M-1 medium, C) Effect of temperature on β-galactosidase production by bacterial isolates growing in M-1 medium, D) Effect of incubation time on β-galactosidase production

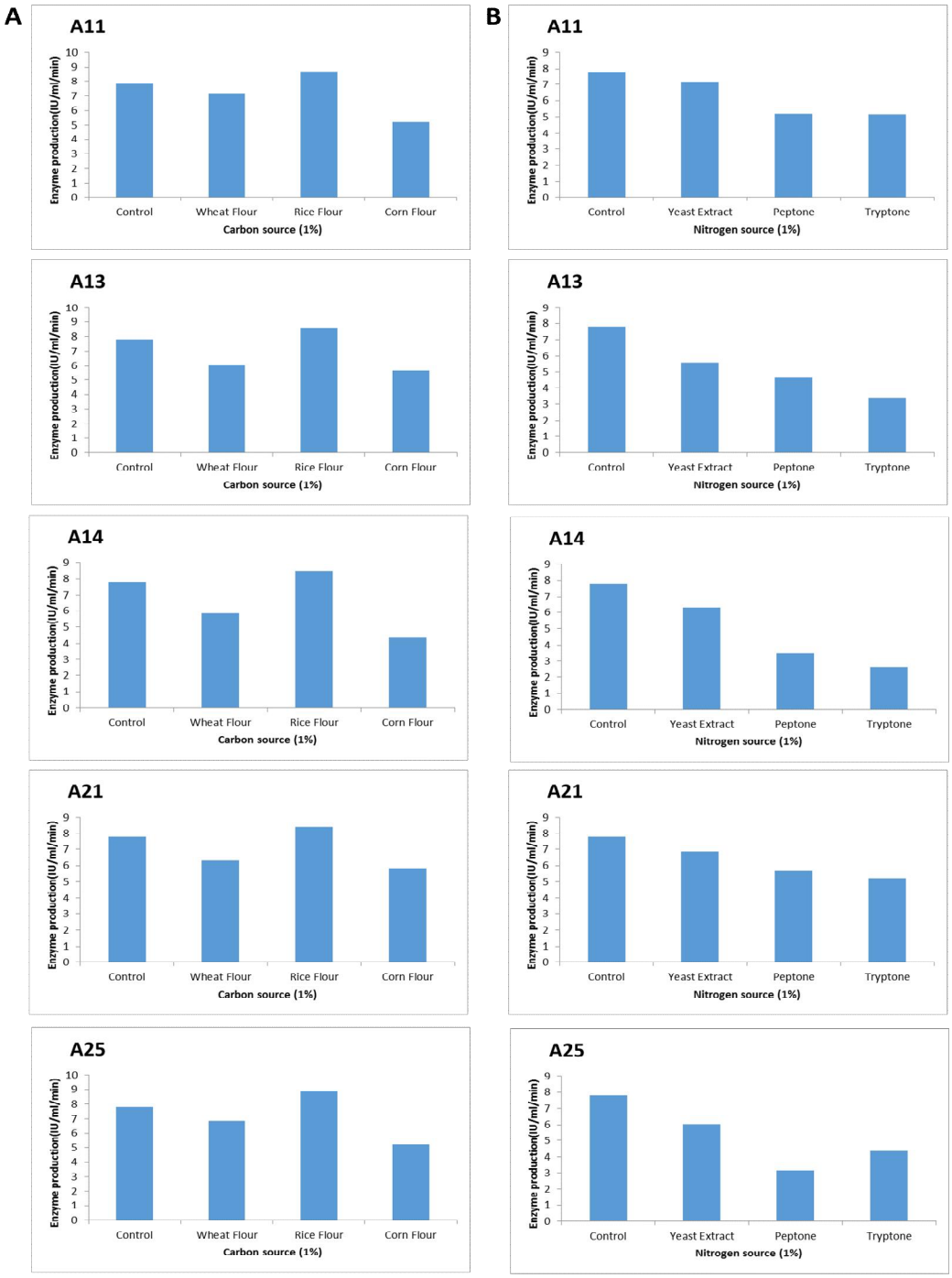

Fig. 4. Effect of carbon and nitrogen sources on the production of β-galactosidase by bacterial isolates growing in M-1 medium

A) Effect of carbon sources, B) Effect of nitrogen sources

Carbon source is very important for the production and growth of β-galactosidase enzyme from bacteria. Because the carbon control and regulates the biosynthesis of β-galactosidase enzyme in various microorganisms [17-20]. These all have showed that the role of carbon source may vary from one microorganism to another microorganism tested.

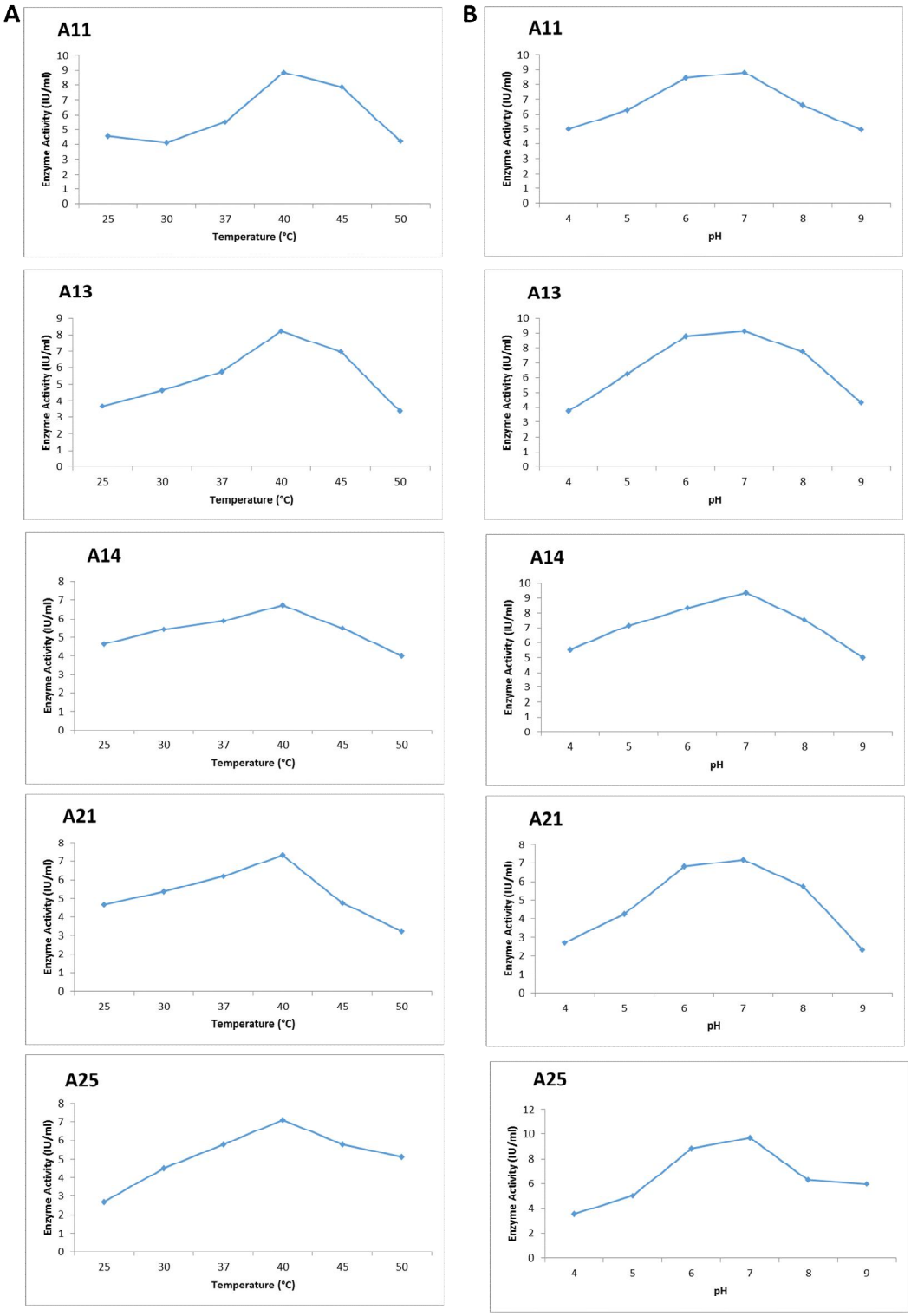

Fig. 5. Effect of temperature and pH on β-galactosidase kinetic activity by bacterial isolates growing in M-1 medium

A) Effect of temperature, B) Effect of pH

For commercial production purposes some cheaper carbon and nitrogen sources are used which is a key attraction for producers. To produce enzyme by using these sources through microbial agent is debatable point of interest [21]. In this study, rice flour enhanced the production of β-galactosidase enzyme while the other carbon sources like wheat flour and corn flour repressed the β-galactosidase enzyme production. It was obvious that some organic compounds may be necessary for the higher production of the enzymes.

Organic and inorganic source of nitrogen are metabolized in the cell of most organism to produce nucleic acid, amino acid, protein and cell wall components [16]. In this study three nitrogen sources were used i.e., yeast extract, tryptone and peptone. Among these nitrogen sources yeast extract showed more enzyme production as compared to tryptone and peptone but it was less than the control. This showed that these nitrogen sources have no much more effect on the production of β-galactosidase enzyme for these bacterial isolates.

It was observed that the optimum pH for the hydrolysis of ortho-Nitrophenyl-β-galactoside by the β-galactosidase enzyme was 7.2. β-galactosidase enzyme obtained from several bacteria and yeast have been reported that having similar results and have optimum pH in range of 6.5-7.5 [22,23]. The maintenance of about 70 percent of maximum enzyme activity between the range of pH 5.0 – 7.4 shows that enzyme is suitable for lactose hydrolysis in sweet whey and milk. In the present study all the bacterial isolates have showed that the maximum enzyme activity was at pH 7.0.

Similarly the effect of temperature ranging from 30-80°C on the enzyme activity was studied at pH 7.0. Because the thermostability of the β-galactosidase enzyme is very significant for the financial system of their food industry application and food consumption. The effect of temperature on the lactase was also noted and observe that the enzyme activity rise with the increase in temperature. It has been recognized that most of the enzymes that are used in food processing purposes becomes significant at high temperatures. In this study the maximum enzyme activity was observed at 40°C.

Different *Lactobacillus sp.* have shown different enzyme activity and specific activity i.e., *L. lactis, l. bulgaricus, L. delbrueckii, L. lactis and L. bulgaricus* have shown specific activity of 42.67, 50.04, 0.826, 17.612 and 22.18 respectively [4]. Similarly in the present study maximum enzyme activity was shown by A11, A13 and A25 (7.69 U/mL, 7.25 U/mL, and 7.28 U/mL).

5. CONCLUSION

The present study justifies the production of β-galactosidase from yogurt and soil associated *Lactobacillus delbruekii* subsp. *bulgaricus.* Therefore, it has been concluded that the rice flour could be potential carbon source for the production of β-galactosidase from *Lactobacillus delbruekii* subsp. *bulgaricus* whereas nitrogen sources reduced its production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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