Isolation and characterization of *Escherichia coli* producing β galactosidase from raw milk of dairy industry

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ABSTRACT

β galactosidase has been used in the dairy industry for the hydrolysis of lactose. The aim of this study was to isolate β galactosidase producing bacterial isolate from raw milk and curd sample of dairies of Punjab and Himachal Pradesh, India. Bacterial isolate designated as MH2 was selected on the basis of qualitative and quantitative screening analysis for β galactosidase activity. The isolation was performed by plating on nutrient agar medium containing the chromogenic substance X-gal and ONPG as substrate to determine the β galactosidase activity of bacterial isolate. Bacterial isolate MH2 showed highest activity of 186.2 (U/mg/ml) isolated from raw milk of Khurana sweets and dairy, Mohali, Punjab. The bacterial isolate MH2 was Gram's negative and creamish white, and rod shaped. On the basis of biochemical characterization, the bacterial isolate MH2 was positive for catalase test, MR test, motility test and negative for VP test, Simmon's citrate test, gelatin hydrolysis and urease test. On the basis of biochemical tests and 16S rDNA sequence analysis, the MH2 was identified as *Escherichia* species. A 1168 bp of 16S rDNA nucleotide sequence of MH2 isolate was submitted to NCBI under the accession number KX443778. From the phylogenetic analysis by MEGA 7, it has been found that isolated strain of *Escherichia coli* MH2 could be used as an ideal candidate for hydrolysis of lactose in milk, which can be consumed by lactose intolerant people. In future, this enzyme could be exploited at industrial scale for the development of low lactose dairy products.

Key words: β galactosidase, lactose intolerance, X-gal, ONPG, 16S rDNA, phylogeny.

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INTRODUCTION

Lactose is a disaccharide found in milk and dairy products. Lactose indigestion leads to lactose intolerance, the inability of adults and children to digest lactose and causes various side effects [1]. Lactose intolerant individuals have insufficient levels of lactase (β galactosidase) (E.C 3.2.1.23), an enzyme which is a glycoside hydrolase involved in the hydrolysis of the disaccharide lactose into constituents galactose and glucose monomers. Lactose indigestion by the microflora causes symptoms which include abdominal bloating, cramps, diarrhea, nausea or vomiting. About 70% of human adults downregulate and decrease the production of intestinal lactase after weaning [2]. β galactosidase is an important enzyme and has wide application in the food and pharmaceutical industry. Low lactose milk, dairy products and yogurt consumption generally decrease the intolerance symptoms arising due to lactose consumption. In industrial applications, this enzyme has been used to prevent crystallization of lactose, to improve the sweetness and to increase the solubility of the milk products [3]. The enzyme β galactosidase occurs widely in nature and has been isolated from animals, plants as well as microorganisms [4, 5]. As compared to animal and plant sources, the microbial enzyme is produced at higher yields and is more technologically advantageous [6].

β galactosidases are commercially produced from yeast such *as Kluyveromyces lactis* and *Kluyveromyces marxianus*, and moulds such as *Aspergillus niger and Aspergillus oryzae* [7]. Bacterial species such as *Bacillus coagulans, Bacillus circulans*, *Escherichia coli*, *Lactobacillus bulgaricus*, *Lactobacillus thermophile* also produce β galactosidase [8]. It is important to select a microorganism which produce β galactosidase with high catalytic efficiency [9]. The lactose reduced ingredients in the food and dairy products are commercially produced for lactose intolerant persons [10]. Microorganisms offer various advantages over other available sources such as easy handling, higher multiplication rate, high activities of the enzyme, good stability and high production yield. As a result of commercial interest in β galactosidase, a large number of microorganisms have been assessed as potential sources of this enzyme [11].

Though several studies on microbial β galactosidase have been carried out throughout the world, yet there appear few studies on β galactosidase enzyme produced by *Escherichia coli* isolated from dairy products of Punjab and Himachal Pradesh. An attempt, therefore has been

made for the isolation of β galactosidase producing bacterial isolate from raw milk and curd samples.

Keeping in view the above, the present study was designed to isolate and screen different bacterial strains from raw milk and curd samples and to identify most active bacterial isolate for production of β galactosidase. Biochemical characterization and their 16S identification would shed light on its phylogenetic status.

MATERIALS AND METHODS

Sample collection

Milk and curd samples were collected from different dairies of Punjab and Himachal Pradesh, India. Sample collection sites of Punjab were Khurana sweets and dairy, Mohali; Sharma dairy, Mohali; Satpal dairy, Ludhiana and sample collection site of Himachal Pradesh was Dudharu kender, Solan and Gangotri dairy, Dharampur. The samples were brought to the laboratory under aseptic conditions in a sterile container.

Isolation and purification of bacterial isolates

The dairy samples were subjected to serial dilution, where one ml of each sample was added to 9 ml of sterile distilled water and shaken gently. The sample was serially diluted upto 10^{-5} dilution and spread on nutrient agar (NA) medium. The plates were incubated for 24 h at 37° C. Isolated colonies were further purified by three successive streaking on NA medium and were preserved at 4° C on NA slants.

Screening of bacterial isolates for the production of β galactosidase

The ability of the bacterial isolate to produce β galactosidase was examined on nutrient agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate and isopropyl β-D-1 thiogalactosidase (IPTG) as an inducer for the β galactosidase. X-gal acts as substrate and is hydrolysed into blue colored compound, namely 5, 5'-dibromo-4, 4'-dichloro-indigo, which is formed by the dimerization and oxidation of 5-bromo-4-chloro-3-hydroxyindole [12]. The bacterial isolates were plated on nutrient agar medium supplemented with 0.25 mM X-Gal (5-bromo-4-chloro-3-indole-D-galactopyranoside; 20 mg/ml dissolved in DMSO) and 6.25 mM IPTG (0.1 mM). The plates were incubated at 37° C for 48 h. Blue colored colonies were observed on the plates indicating the presence of β galactosidase producing bacteria.

Quantitative estimation of β galactosidase enzyme

The cultures used for enzyme assay were grown at 37° C at 250 rpm for 24 h in nutrient broth medium. The cultures were centrifuged and cells were washed with 0.85 % NaCl followed by 1 ml Z buffer [13]. The cell pellet was resuspended in 1 ml Z buffer containing 0.1 % SDS and 2 drops of chloroform, followed by vortexing and incubation for 2 min at 37° C. The cell debris was separated by centrifugation at 4000 rpm at 4° C for 15 min. β galactosidase enzyme activity was quantitatively assayed by addition of 0.2 ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and initiate the reaction for 15 min. When a yellow color developed, the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 and the amount of *o*-nitrophenol (ONP) released was determined by measuring the absorbance at 420 nm and the value of standard curve of ONP [14]. One unit of β galactosidase activity (U) was defined as the amount of enzyme that releases 1 µmol of ONP from ONPG per minute.

Estimation of protein concentration

The protein concentration was determined by the Bradford's method [15] by using bovine serum albumin as a standard.

Identification of bacterial isolate by morphological and biochemical characterization

β galactosidase producing bacterial isolate was subjected to Gram's staining [16] and biochemical characterization according to Bergey's Manual of Systematic Bacteriology [17]. Morphological features (morphology of cell and colony) and biochemical tests such as [indole test (18), methyl red (MR) reaction test, Voges-Proskauer (VP) reaction test (19), Citrate utilization test (20), Catalase test (21), gelatin hydrolysis test (22), Urease test (23), Motility test (24)] were performed for β galactosidase producing bacterial isolate.

Identification of β galactosidase producing bacterial isolate by molecular approach

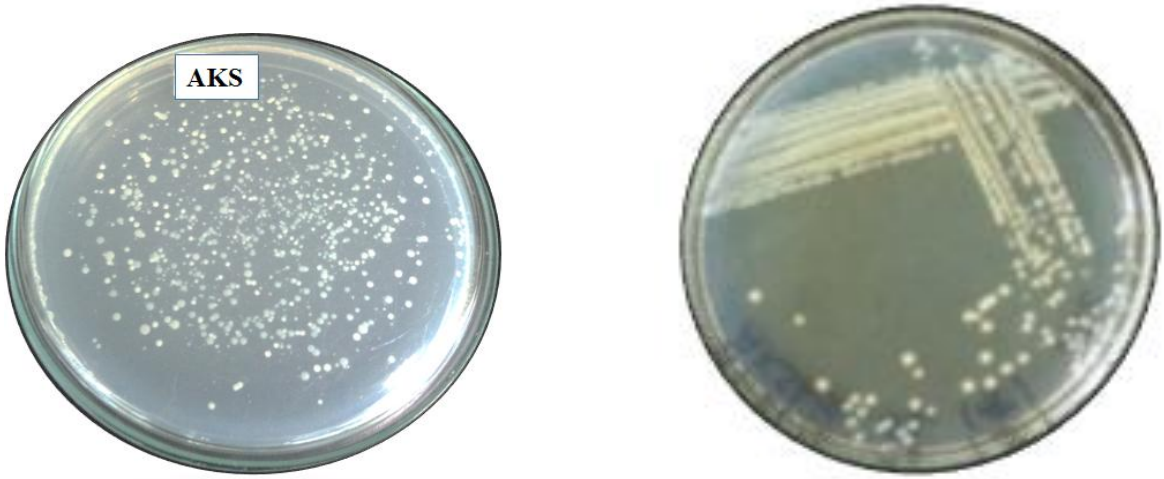
For 16S rDNA amplification, bacterial strain MH2 was grown in nutrient broth medium for 24 h at 37° C. For genomic DNA isolation, the culture was centrifuged at 4000 rpm for 5 min and then cells were resuspended in extraction buffer (100 mM Tris HCl, pH 8.0; 10 mM EDTA, pH 8.0; 500 mM NaCl, 0.07 % β mercaptoethanol, 20 mg/ml lysozyme and 1 % SDS). Reaction mixture was incubated at 37° C for 30 min and centrifuged at 12000 rpm for 15 min [25]. Supernatant was collected and mixed with equal volume of phenol/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 layer, $1/10^{th}$ volume of 5

M NaCl and 2.5 volumes of absolute ethanol was added and incubated at -20° C for 2 h, followed by centrifugation at 12000 rpm for 15 min. Supernatant was discarded and pellet was washed with 70 % ethanol, dried and resuspended in 30 µl TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0). DNA quantification was performed spectrophotometrically by measuring absorbance at 260 and 280 nm. The 16S rDNA was amplified using the universal primers 27F and 1492R [26]. The amplification was done by initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min. The amplified products were purified using Nucleospin gel elution kit (Invitrogen, Cat No. K1820-01). DNA sequencing of both the strands was done by 27F and 1492R primers at Europhins Labs Ltd. Bangalore, India (http://www.eurofins.com/). The DNA sequences thus obtained were subjected to nucleotide blast (nblast) search. A phylogenetic tree was constructed by taking 16S rDNA sequences of all related bacterial species showing > 99 % homology. The nucleotide sequences obtained were overlaid to remove the common region and the complete nucleotide sequence of 16S rDNA was subjected to nBLAST analysis [27] against the bacterial 16S rDNA database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete nucleotide sequences were submitted to GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The BLAST search matching > 99 % homology were selected to construct phylogenetic tree. Molecular Evolutionary Genetics Analysis 7 (MEGA 7) [28] was used to construct phylogenic tree using the neighbor joining method [29] to study the molecular evolution of bacterial isolate.

RESULTS AND DISCUSSION

Isolation of β galactosidase producing bacterial strains from raw milk and curd samples of dairy industries

The collected milk and curd samples were processed for isolation of bacteria. After 24 h of incubation, 200 – 250 bacterial colonies were observed in each plate [Fig 1a]. Nine different types of bacterial colonies were picked from the milk and curd samples [Table 1] and each bacterial colony was successively purified by streaking three times on nutrient agar medium [Fig 1b], a representative of one isolate.



(a) (b)

Fig 1: Isolation of β galactosidase producing bacteria from milk and curd samples: 100 µl of milk (10^{-5} dilution) was plated on nutrient agar (NA) medium (a). Purified bacterial isolates on nutrient agar medium (b) incubation was done at 37° C for 24 h.

Table 1: Source of sample for isolation of β galactosidase producing bacteria

S.No.	Sample collection site	Bacterial isolate	Source
1.	Sharma dairy, Mohali (Punjab)	SN1, SN2, SN3	Raw milk
2.	Satpal dairy, Ludhiana (Punjab)	LD2	Curd
3.	Khurana sweets and dairy, Mohali (Punjab)	MH2	Raw milk
4.	Dudharo kender, Solan (Himachal Pradesh)	PB1, PB3	Raw milk
5.	Gangotri dairy, Dharampur (Himachal Pradesh)	DH2, PH1	Curd

Total Nine bacterial isolates were qualitatively screened for β galactosidase activity. Among nine bacterial isolates, five bacterial isolates namely, SN1, MH2, SN3, PB1 and LD2 showed blue coloration when streaked on nutrient agar (NA) medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or IPTG (isopropyl β-D-1 thiogalactopyranoside)

[Fig 2]. Natrajan et al [30] studied the isolation of the serially diluted dairy effluent samples and screened bacteria of medium containing X-gal, which resulted in the formation of 18 blue colored colonies indicating the production of β galactosidase by the respective bacterial strains. Similarly, El Kader et al [31] has reported the similar observations, they isolated 52 bacterial strains from three different sources; 22 isolates from commercial cow milk, 9 isolates from commercial yogurt and 21 isolates from agricultural soil and assessed for the β galactosidase production. Maity et al [32] reported the isolation of two bacterial strains; BS1 and BS2 from the soils of cattle shed near Kolkata for the production of β galactosidase. They found that total 13 bacterial colonies able to grow on lactose medium supplemented with X-gal and ONPG was used as substrate and show the ability to produce β galactosidase.

Fig 2: Screening of bacterial isolates for production of β galactosidase. A representative bacterial isolates were screened on nutrient agar medium supplemented with X-gal and IPTG for β galactosidase production. Plates were incubated at 37 °C for 14 h.

Five bacterial isolates (SN1, MH2, SN3, PB1 and LD2) were further quantitatively screened to select the bacterial isolate, which produces maximum β galactosidase activity. β galactosidase activity of all five bacterial isolates is shown in [Table 2]. Except MH2, all other bacterial isolates showed very low level of the β galactosidaseactivity and MH2 bacterial isolate shows highest β galactosidase activity (186.2 U/mg/ml) as compared to other bacterial isolates. Maity et al [32] reported a bacterial isolate BS1, which showed at 39.17 (U/mg) β galactosidase activity and revealed that the isolated strain BS1 was found to exhibit the highest β galactosidase activity. Similarly, Mozumder et al [8] identified, β galactosidase

producing *Lactobacillus* bacteria isolated from yogurts. They found that highest activity was observed in *Lactobacillus bulgaricus* (50.04 U/mg) strain and summarizes the result of other *Lactobacillus* species such as *L. lactis* (42.67 U/mg) and *L. delbrueckii* (0.826 U/mg).

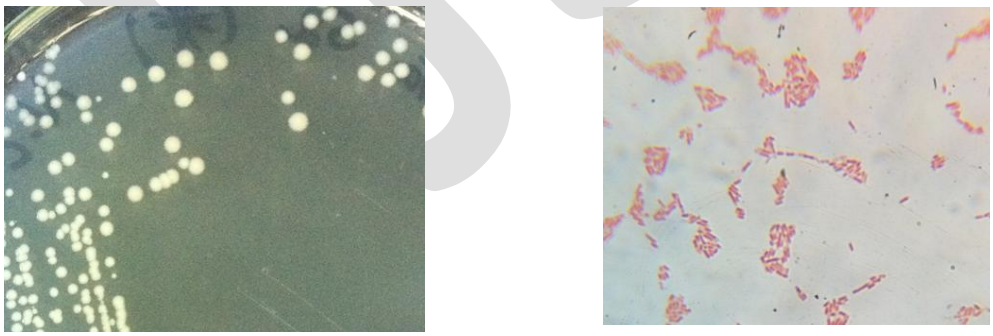
Table 2: Comparison of bacterial isolates for β galactosidase activity

S.No	Bacterial isolate	β galactosidase activity (U/mg)
1.	SN1	68.2
2.	SN3	27.31
3.	LD2	98.2
4.	MH2	186.2
5.	PB1	76.7

Hence, on the basis of both qualitative and quantitative analysis, MH2 bacterial strain from raw milk from Khurana sweets and dairy, Mohali, Punjab was selected for further characterization of bacterial isolate and β galactosidase and its phylogenetic evolution.

Morphological and Biochemical characterization

Bacterial isolate MH2 appeared singly or in chains as straight rods, Gram's negative organism under the microscope, creamish white, and circular colonies (Fig 3 a, b).



(a) (b)

Fig 3: Colony morphology and Gram's staining of MH2 bacterial isolate: Colony morphology of MH2 on nutrient agar medium (a) and microscopic examination of Gram's staining at 40X (b).

Various biochemical tests were performed to identify the bacterial isolate MH2. It was observed that MH2 isolate was negative for VP (Voges Proskauer) test, Simmon's citrate test, gelatin hydrolysis test and urease test, and positive for Catalase, MR (Methyl-Red) and Motility test (Table 3) [Figure 4]. On the basis of morphological and biochemical characterization as per the criteria of Bergey's Manual of Systematic Bacteriology, the bacterial isolate MH2 was identified as *Escherichia* species. Similarly, El-Kader et al [31] identified the bacterial isolate no. 12 according to the criteria described in Bergey's Manual of Systematic Bacteriology, using morphological identification and biochemical characteristics. The result showed that the isolate was identified as *Bacillus subtilis,* aerobic, Gram positive, and motile rods. On the basis of biochemical tests, it gives a positive reaction with catalase test, Voges-Proskauer test, utilization of citrate, hydrolysis of gelatin and gives a negative reaction with production of acid from starch and nitrate reduction.

Table 3: Biochemical tests of β galactosidase producing bacterial isolate MH2

S.No	BIOCHEMICAL TESTS	RESULTS
1	Catalase test	+
2	MR Test	+
3	VP Test	-
4	Simmon' s Citrate test	-
5	Gelatin hydrolysis test	-
6	Motility test	+
7	Urease test	-

(+) Sign indicates positive test, while (-) sign represents negative test results

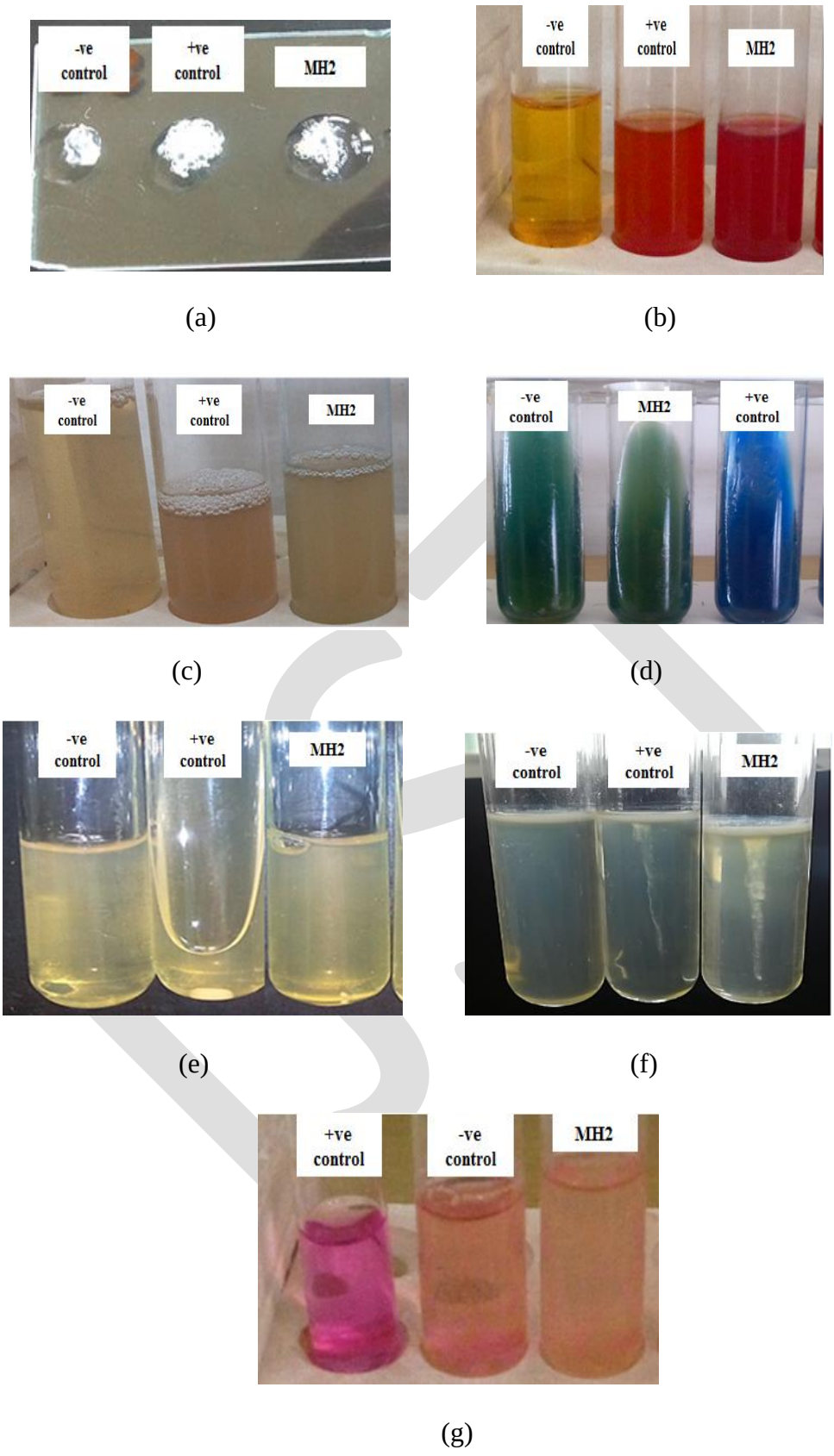
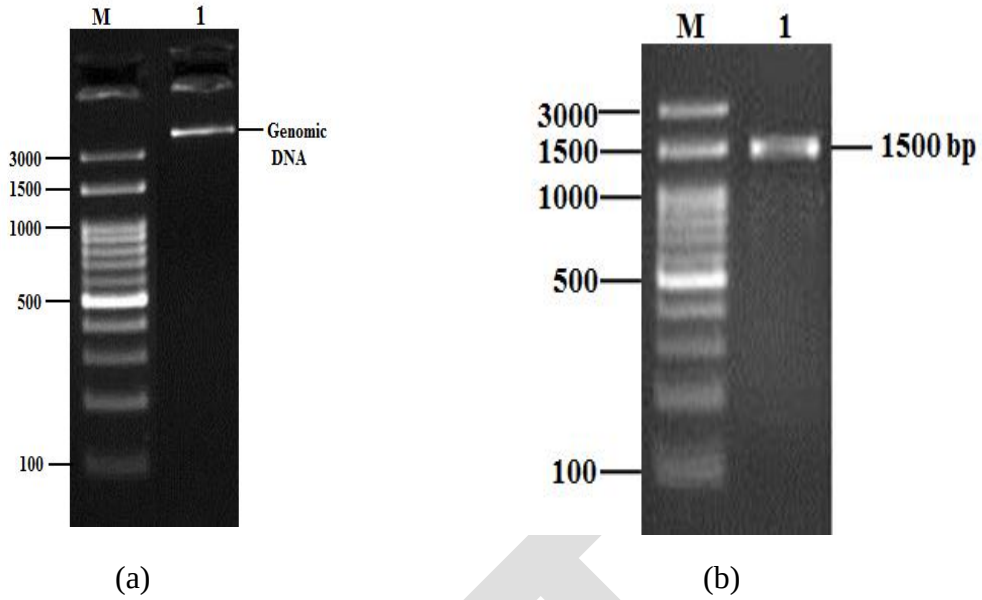


Fig 4: Biochemical tests of bacterial isolate producing β galactosidase. Data showing (a) Catalase test, MTCC 8515 was used as positive control whereas MTCC 8114 was used as negative control. (b) Methyl red (MR) test, *Escherichia coli* strain DH5α was used as positive

control whereas *Enterobacter aerogenes* was used as negative control. (c) Voges Proskauer (VP) test, *Enterobacter aerogenes* was used as positive control whereas *Escherichia coli* strain DH5α was used as negative control. (d) Simmon's citrate test, *Enterobacter aerogenes* was used as positive control whereas *Escherichia coli* strain DH5α was used as negative control. (e) Gelatin hydrolysis test, *Bacillus subtilis* was used as positive control whereas *Escherichia coli* strain DH5α was used as negative control. (f) Motility test, *Escherichia coli* strain DH5α was used as positive control and *staphylococcus aureus* was used as negative control. (g) Urease test, *Proteos vulgaris* was used as positive control and *Escherichia coli* strain DH5α was used as negative control.

Molecular identification of MH2 bacterial isolate

Genomic DNA [Fig 5a] of MH2 isolate was subjected to PCR amplification and separated on 1 % agarose gel. A PCR product of ~ 1500 bp was detected for MH2 isolate [Figure 5b]. The results of 16S rDNA sequencing revealed a nucleotide sequence of 1168 bp, 16S rDNA sequence of MH2 was subjected to nBLAST analysis against 16S rDNA bacterial database. On the basis of 16S rDNA studies, the MH2 isolate showed 99 % similarity with *Escherichia coli* (Accession number KX443778). Based on the nucleotide homology, MH2 was named as *Escherichia coli* strain MH2. From these results, the MH2 strain was identified as *Escherichia coli* and submitted under Genbank Accession number KX443778. Jayashree et al [30] isolated β galactosidase producing bacterial strain from dairy effluents. On the basis of various physiological tests and 16S rRNA sequence analysis, this bacterial strain was identified as *Bacillus* species BPTK 4. On the basis of molecular analysis, they reported that this β galactosidase producing isolate was *Bacillus subtilis* and revealed a close relatedness to *Bacillus subtilis* with 99-100 % similarity and the sequence was submitted to Genbank under accession number (JF749812). Similarly, Kumar et al [33] isolated bacterial strain producing β galactosidase from soil sample near dairy processing plant. On the basis of alignment of the 16S rDNA gene sequence of the isolated strain showed 100 % identity with various *Bacillus* species. Hence, the strain was confirmed as *Bacillus* species and sequence has been submitted to the Genbank (JQ6693396).



(a) (b)

Fig 5: Amplification of 16S rDNA of bacterial isolate producing β galactosidase. Total genomic DNA of bacterial isolate MH2 was subjected to 1% agarose gel electrophoresis (a). 16S rDNA was PCR amplified by using 27F and 1492 R primers (b). 'M' indicated molecular size marker (bp).

Phylogenetic analysis of MH2 bacterial isolate

For bacterial isolate MH2, phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis 7 (MEGA7) using the neighbor-joining method (Fig 6). In order to find out lineage of MH2 isolate, phylogenetic tree was constructed by selecting all the *Escherichia coli* strains from the nBLAST showing 99 % similarity of 16S rDNA sequence. It was observed that *Escherichia coli* MH2 was evolved together with *Escherichia coli* RCB749. Similarly, Ghosh et al [34] performed the molecular identification of a β galactosidase producing bacterial isolate from marine waters of Bay of Bengal. 16S rRNA nucleotide sequence of 1403 bp was obtained (Genbank no. JQ328041). Phylogenetic analysis of 3SC-21 based on 16S rRNA gene sequence has shown that the isolate was closely related to the genus of *Thalassospira* (MTCC 11253) and *Thalassospira* sp. MCCC 1A02041, *Thalassospira* sp. MCCC 1A02042 and *Thalassospira* sp. QDHT-16.

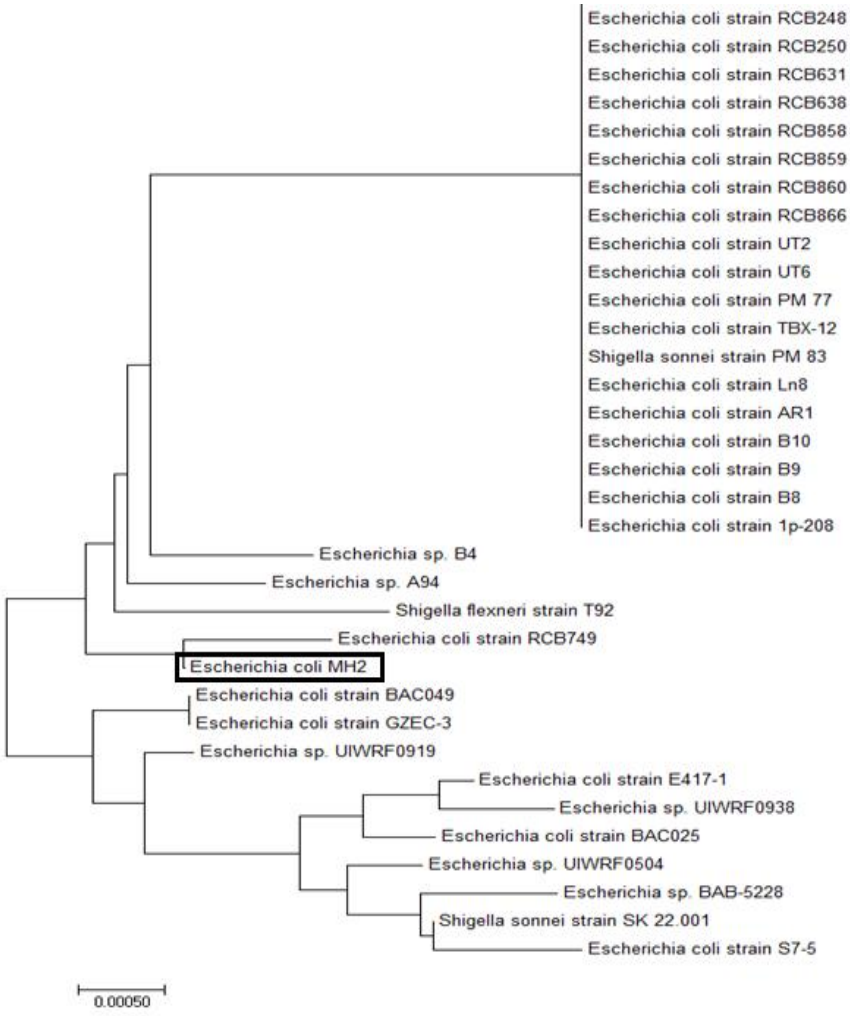


Fig 6: Phylogenetic analysis based on 16S rDNA sequences. Phylogenetic tree represents the relationship of MH2 *Escherichia coli* strain KX443778 and closely related species of *Escherichia coli* by MEGA 7 software.

CONCLUSION

This paper reports the isolation of bacterial isolate from raw milk of Khurana sweets and dairy, Mohali, Punjab. Once the bacterial strain named as *Escherichia coli* MH2 has been isolated and characterized, it was further characterized by various biochemical tests and identified for efficient β galactosidase production. Bacterial isolate MH2 was identified as *Escherichia coli* strain MH2 and showed highest activity at 186.2 (U/mg/ml). This suggests *that Escherichia coli* MH2 can be a potential producer of β galactosidase. This strain could be used as an ideal candidate for hydrolysis of lactose in milk, which can be used by lactose intolerant people.

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