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Screening of β-galactosidase enzyme production by probiotic lactic acid bacteria isolated from raw and fermented milk

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Abstract

β-Galactosidase is a glycoside hydrolase enzyme that catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. This study aimed to isolate β-galactosidase-producing bacteria from different types of milk. Focus on isolating lactic acid bacteria. Sixty-eight bacterial isolates were isolated from eight fermented and nonfermented camel and cow milk samples. Gram staining revealed that there were 68 gram-positive bacterial isolates (30 bacilli and 38 cocci), and all of them were catalase-negative and non-spore-forming. The rapid plate assay for the β-galactosidase enzyme showed that ten isolates are β-galactosidase-producing bacteria. The isolate MH-010 was the most potent β-galactosidase producer; phenol concentrations ranging from 0.4% to 0.6% significantly inhibited the growth of the bacterial isolate MH-010, resulting in a decrease in $\log_{10}$ CFU/ml. The bacterial isolates showed 96% auto-aggregation activity after 2 hours of incubation at 37 °C, a 42.8% co-aggregation ratio with *Klebsiella* sp., and hydrophobicity over 80%. The MH-010 isolate was identified as *Enterococcus lactis* and registered in GenBank under accession number (PP803411). According to the findings, the *Enterococcus lactis* strain can be used to produce the β-galactosidase enzyme for use in different industrial fields.

Keywords: *Enterococcus lactis*, lactic acid bacteria, probiotic, β-Galactosidase enzyme.

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1. Introduction

β-Galactosidase, or lactase, is an enzyme that breaks down the β-glycosidic bond between galactose and glucose (Juers *et al*., 2012). This enzyme is produced by some lactic acid bacteria strains (LABs) commonly found in dairy products (Kolev *et al*., 2022). It's very important for the production of energy through the breakdown of lactose into galactose and glucose (Cohn and Monod, 1951; Schaafsma, 2008). In human beings, consuming the galactooligosaccharide enzyme provides notable health advantages, including anti-cancer characteristics in the colon, mineral absorption, lipid metabolism, anti-inflammatory effects, immune effects, and overall well-being (Macfarlane *et al*., 2008). LABs are gram-positive, non-spore-forming bacteria used as starter cultures and sugar fermentative bacteria (Singh and Sharma, 2009). They are anaerobic or facultative aerobic rods or cocci that have a broad distribution in the natural environment and are normally present as indigenous microorganisms in unpasteurized milk, which plays a crucial role in many food and feed fermentations (Edalati *et al*., 2019). The ability of the LABs to create lactic acid and a variety of metabolites, including organic acids, antioxidants, and antibacterial agents, is well documented, and this supports the improvement and regulation of the gut microbial balance (Aswathy *et al*., 2008). Additionally, LAB is crucial to the fermentation processes involved in food. The rising interest in LAB can be attributed to their possible ability to produce β-galactosidase. The ability of LAB to generate β-galactosidase has expanded their industrial uses. These LAB strains that produce β-galactosidase are utilized in the manufacturing of lactose intolerant yoghurt, cheese, acidophilus milk, and sweets that contain milk. β-galactosidase Since it separates milk's lactose into its two main components, glucose and galactose, which eventually fermented to form lactic acid (Chandel and Sharma, 2020). The most common bacterial genera of LAB found in commercially available probiotic supplements are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* (Selvaraj and Gurumurthy, 2023; Wang *et al*., 2023). Probiotics are live organisms that, when consumed in appropriate amounts, provide health benefits to the host by restoring microbial balance in the system (Somashekaraiah *et al*. 2019). An effective probiotic isolate must demonstrate specific characteristics, such as the ability to survive and the colonies' varied environmental circumstances (Palachum *et al*., 2018). The isolates must be able to endure the acidic conditions of gastric juice, show resistance to bile salts, and stick to epithelial cells (Somashekaraiah *et al*., 2019). The objective of this investigation was to isolate and screen β-galactosidase enzyme production by LABs isolated from different milk samples, as well as evaluate the probiotic features and 16S-rRNA identification of the most potent enzyme-producing strain.

2. Materials and methods

2.1 Sample collection

Eight milk samples (cow milk and camel

milk) were collected from different regions (Sohag and Assiut) in Egypt. Each sample was gathered in a container and sent in a refrigerated container to the laboratory, where it was refrigerated at 4 °C for the isolation of lactic acid bacteria.

2.2 Isolation and purification of LAB

The bacterial isolates were grown on MRS agar medium using the spread plate method and incubated at 37 °C for 72 hours anaerobically. After incubation, all pin colonies that appeared on the plates were selected for purification using the streak plate technique on MRS agar medium provided with 0.5% (w/v) CaCO3. The unobstructed area surrounding the growth on the chalked agar plates signifies the presence of the lactic acid bacterium isolates (*Kurniati et al.*, 2021).

2.3 Confirmation tests of LAB

2.3.1 Gram staining

All purified bacterial isolates were subjected to gram-staining and subsequently examined under a light microscope with an oil immersion lens to ascertain the morphology of the bacterial cells and validate their purity.

2.3.2 Catalase test

A loopful of a 24-hour culture grown on MRS agar was mixed with drops of 3% (v/v) H_2O_2 on a glass slide. The release of oxygen in the form of gas bubbles serves as an indication of the existence of the catalase enzyme (Cowan, 1974).

2.3.3 Spore formation test

A tube of MRS broth medium was inoculated with growth from the agar medium, in which sporulation was suspected to occur. The tube was immersed in a water bath set at a temperature of 80 ºC, together with a broth that had not been inoculated. A thermometer was also included in the soup. The water level in the bath was greater than the level of the broth. Then, inoculated tubes were incubated for 10 minutes, starting when the thermometer reached 80 ºC, cooled, and incubated at 37 °C for 48 h. After incubation, the growth (turbidity) tubes indicated endospore formation (Sneath and Holt, 2001).

2.4 Screening for the β-galactosidase enzyme

2.4.1 Rapid plate assay method

To screen the initial β-galactosidase activity of LABs, tryptic soy agar plates were prepared, and 40 mL of 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-gal 20 mg/mL) was added to each plate as a substrate and distributed uniformly (β-d-galactopyranoside test). The plates were allowed to dry for 30 minutes, after which they were inoculated with the MRS broth cultures. The presence of a blue tint surrounding the growing colonies suggested a positive enzymatic outcome, which is caused by cleavage of the β-d-galactopyranoside bond by β-galactosidase,

releasing the blue color of the indole (Kolev *et al*., 2022).

2.4.2 Quantitative assay method

The activity of β-galactosidase enzyme production by 10 LABs was measured as described in (Khusniati *et al*., 2015). Briefly, 1000 µl buffer phosphate 0,1 M pH 7 and 100 µl enzyme were poured into a reaction tube, and it was incubated at a temperature of 37 ºC for 5 minutes. Then 200 µl (4 mg/ml) of o-Nitrofenil-β-D-galaktopiranosida (ONPG) was incubated at a temperature of 37 ºC for 15 minutes. At minute 15, it was added 1000 µl Na_2CO_3 1 M. The solution was analyzed by using a spectrophotometer (JENWAY 7315) at 420 nm. The enzyme activity (U/ml) was defined as the amount of µM o-nitrophenol (ONP) formed per minute per milliliter of an enzyme at treatment conditions.

2.5 Characterization of isolate MH-010

The probiotic features of MH-010, which exhibited the highest activity of the β-galactosidase enzyme, were assessed using the following methods.

2.5.1 Acid and bile salt tolerance tests

The isolate's resistance to both acidic pH levels and bile salt was evaluated based on (Somashekaraiah *et al*., 2019), with some slight alterations (Salem *et al*., 2023). The bacterial strain MH-010 was cultured overnight and then inoculated with 1% (v/v) in an MRS broth medium that had been adjusted to a pH value of 2.0 and 0.3% bile salt, separately. The pH of the MRS broth medium was adjusted to 6.5, and another without bile salt was considered the control for pH condition and bile salt, respectively. The specimen was subjected to incubation at a temperature of 37 °C for specific time intervals (0, 1, 2, and 3 h). The biomass concentration measured in colony-forming units per milliliter (CFU/ml) of the culture was assessed by incubating it on an MRS agar plate at a temperature of 37 °C for 24 hours. The survival rate (%) was calculated using the following formula:

$$\text{Survival rate } (\%) = \frac{Biomass\ at\ a\ time\ (t)}{Biomass\ at\ the\ initial\ time\ (0)} \times 100$$

2.5.2 Phenol tolerance test

The bacterial strain MH-010 phenol tolerance was assessed according to (Somashekaraiah *et al*., 2019). The overnight-cultured isolate was introduced into the MRS broth medium, which was enriched with 0.4% and 0.6% phenol, respectively, in terms of volume/volume. After being incubated at a temperature of 37 °C for 24 hours, the cultures were diluted in a series and then evenly distributed on MRS agar plates. The plate count method calculated the cell viability (log CFU/ml).

2.5.3 Auto-aggregation ability test

The auto-aggregation test was performed by (Zuo *et al*., 2016). The bacterial strain MH-010 was grown in the MRS broth

medium for 16 hours at a temperature of 37 °C. After centrifuging the cells at 6000 g for 10 min, the pellets were washed twice and mixed with phosphate-buffered saline (PBS, pH 7.4) until the optical density (OD_{600}) reached 1.0. Following a two-hour incubation period at 37 °C, 100 µl of the upper suspension was moved to a different tube containing 1.9 ml of PBS, and the OD_{600} was determined. The auto-aggregation (%) was calculated using the following formula:

$$\text{Auto aggregation (\%)} = 1 - \left(\frac{\text{OD}_{600} \text{ of upper suspension}}{\text{OD}_{600} \text{ of total bacterial suspension}}\right) \times 100$$

2.5.4 Co-aggregation ability test

Cell suspensions were prepared for co-aggregation, following a procedure similar to the auto-aggregation test. A cuvette was used to combine 1 ml of cell solution from bacterial strain MH-010 with the pathogen strain *Klebsiella* sp. The OD_{600} was immediately measured. The mixture was placed in an incubator at a temperature of 37 °C for two hours. Subsequently, the optical density at a wavelength of 600 (OD_{600}) was measured once more. The co-aggregation percentage was determined using the following equation provided in the study (Nagaoka *et al*., 2008):

$$\text{Co} - \text{aggregation (\%)} = \frac{(\text{A0} - \text{At})}{\text{A0}} \times 100$$

2.5.5 Hydrophobicity test

The hydrophobicity of the isolate was assessed using xylene extraction, following the procedure outlined in (Zuo *et al*., 2016). The bacterial strain was cultured in MRS broth for 24 hours. The cells were then separated by centrifuging at 6000 g for 5 minutes and washed twice with 50 mM K_2HPO_4 buffer (pH 6.5). The absorbance at 600 nm (A_{600}) was set to 0.5± 0.05, and then 3 ml of bacterial suspension and 0.6 ml of hydrocarbon (xylene) were mixed and stirred for 180 seconds. Following incubation at ambient temperature for one hour, the liquid phase was extracted, and its A_{600} was measured. The hydrophobicity percentage (%) was determined using the following formula:

$$\text{Hydrophobicity (\%)} = \frac{(\text{A0} - \text{A})}{\text{A0}} \times 100$$

Where A0 = initial absorbance and A = final absorbance.

2.6 Molecular identification of the most potent isolate

The bacterial isolate was cultivated in a test tube containing 10 ml of autoclaved nutrient broth medium (Zimbro *et al*., 2015) and incubated at 28 °C for 48 hours. The culture was sent to the molecular biology research unit at Assiut University for DNA extraction using a path-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The extracted DNA samples were shipped to SolGent Company, Daejeon, South Korea, for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using two universal primers, namely 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTT ACGACTT-3').

The PCR product was confirmed using a nucleotide marker (100 base pairs) by electrophoresis on a 1% agarose gel. The purified PCR product was sequenced in the sense and antisense directions using 27F and 1492R primers with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture (White *et al.*, 1990). The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website and submitted to the GenBank sequence database for accession numbers. Phylogenetic analysis of sequences was done using MegAlign (DNA Star) software version 5.05.

2.7 Data analysis

All tests and measurements were repeated three times, and the values were expressed as the mean ± SD using Microsoft Excel 2010.

3. Results

3.1 Isolation, purification, and confirmation tests of LAB

D We isolated sixty-eight LABs from eight fermented and non-fermented milk samples (cow and camel). Fermented camel milk is the most abundant source in isolate number with 25 isolates (36.76%), followed by fermented cow milk with 21 isolates (30.88%), and in both fresh milk (cow and camel) with 11 isolates (16.18%), as shown in Table (1). After the completion of the isolation and purification processes, all isolates were stained with gram staining, and all bacterial isolates were gram-positive.

Table (1): Characteristics of lactic acid bacterial isolates.

Isolation source	Region	No. of isolates	% of total isolates	Gram stain		Morphological shape	
				+ve	-ve	Bacilli	Cocci
Raw cow milk	Assiut	4	5.88	4	0	2	2
	Sohag	7	10.29	7	0	4	3
	Total	11	16.18	11	0	6	5
Raw camel milk	Assiut	5	7.35	5	0	3	2
	Sohag	6	8.82	6	0	2	4
	Total	11	16.18	11	0	5	6
Fermented cow milk	Assiut	11	16.18	11	0	5	6
	Sohag	10	14.71	10	0	6	4
	Total	21	30.88	21	0	11	10
Fermented camel milk	Assiut	12	17.65	12	0	5	7
	Sohag	13	19.12	13	0	6	7
	Total	25	36.76	25	0	11	14
Total isolates count		68	100.00	68	0	33	35

(-Ve): negative; (+Ve): positive.

Also, the morphological shape of bacterial cells was determined by examination under a light microscope, indicating that 33 isolates were bacilli, and 35 isolates

were cocci. All these isolates were negative toward the catalase test, spore formation, and lactic acid production on a chalk agar plate, as shown in Figure (1).

Figure (1): A clear zone on MRS agar medium with 0.5% $CaCO_3$ illustrates the production of lactic acid.

3.2 Screening for the β-galactosidase enzyme

The rapid plate assay method showed ten isolates only positive from 68 LAB isolates for the production of β-galactosidase enzyme on an X-gal-containment medium, as shown in Figure (2). On the other hand, the quantitative assay method showed the most potent enzyme-producing strain is MH-010, which was isolated from fermented camel milk, as shown in Table (2).

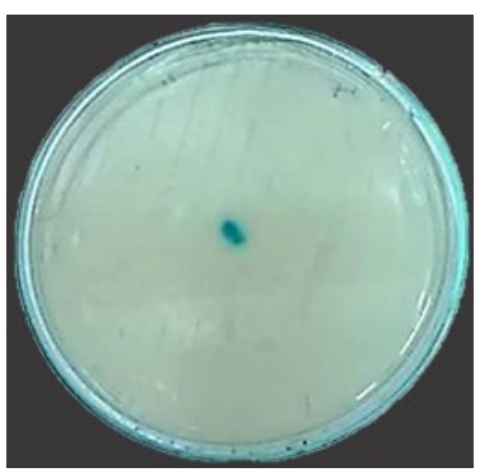

Figure (2): Lactic acid bacterial strain with blue colonies on X-gal-contained medium.

Table (2): Quantitative assay of β-galactosidase enzyme produced by LAB strains isolated from fresh and fermented milk.

Isolation source	Code of isolate	Enzyme Activity U/ml
Cow milk	MH-01	3.43±1.95
Fermented cow milk	MH-02	5.04±1.15
	MH-03	4.57±0.80
	MH-04	2.06±0.15
Camel milk	MH-05	4.02±0.11
	MH-06	3.80±0.13
	MH-07	4.38±0.70
Fermented camel milk	MH-08	2.94±0.80
	MH-09	2.45±0.12
	MH-010	5.49±0.13

3.3 Characterization of isolate MH-010

3.3.1 Acid and bile salt tolerance tests

The data indicated that the growth rate of the bacterial strain MH-010 decreased over time. However, the strain MH-010 was able to grow until 3 hours in the MRS broth medium adjusted to pH 2.0 and supplemented with bile salts of 0.3%, individually, at a percentage of 55.33±4.50 and 19.33±4.04, respectively, as shown in Figure (3).

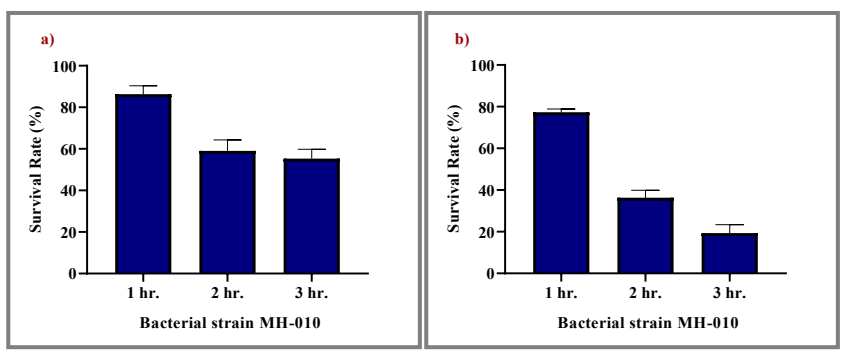

Figure (3): The survival rate of bacterial strain MH-010 at an acidic pH value of 2.0 (a) and bile salt of 0.3% (b).

3.3.2 Phenol resistance test

The CFU/ml of the bacterial isolate MH-010 was determined using MRS agar plates following a 24-hour incubation in an MRS broth medium that included 0.4% and 0.6% phenol. Comparing the phenol concentrations to the MRS control without phenol, an inhibitory impact was seen, as shown in Figure (4). The obtained data showed a decrease in the log CFU/ml of bacterial isolate MH-010 at phenol 0.4% and 0.6% compared with phenol 0.0% with a percentage (3.38±0.16, 3.19±0.0361, and 8.0867±0.07), respectively, as shown in Figure (4).

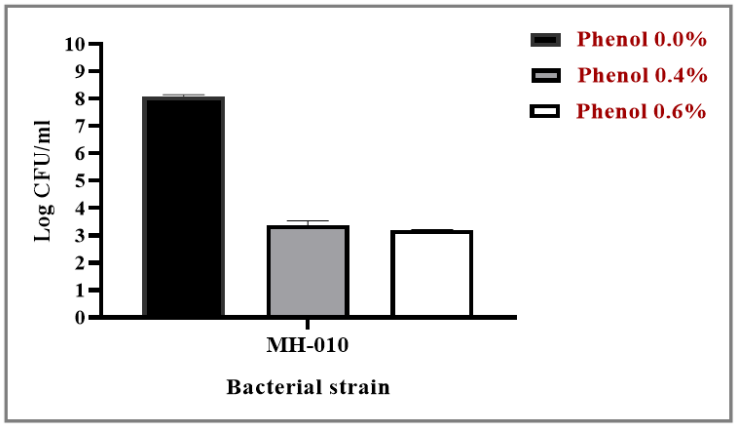

Figure (4): Log CFU/ml of bacterial isolate MH-010 at 0.4% and 0.6% phenol.

3.3.3 Adhesion tests

As documented in Figure (5), the isolate MH-010 exhibited auto-aggregation activity after 2 h of incubation at 37 °C with a percentage of 96%. On the other hand, the co-aggregation ratio between the target isolate and *Klebsiella* sp. was 82.33%. Also, the result showed the bacterial isolate MH-010 had hydrophobicity greater than 40%.

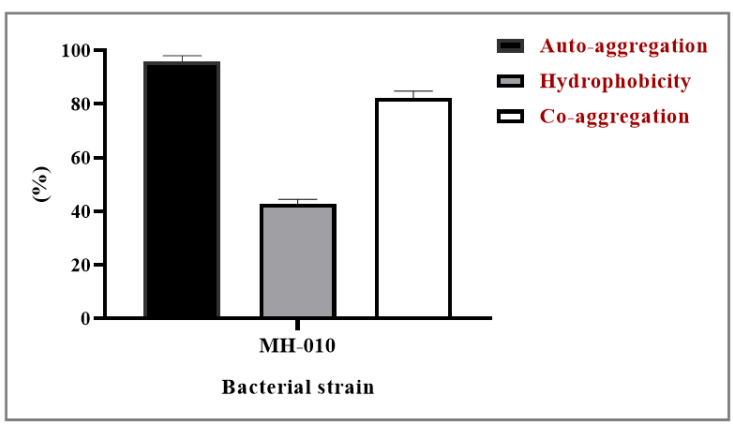

Figure (5): Auto-aggregation, hydrophobicity, and co-aggregation of bacterial isolates MH-010.

3.4 Molecular identification of the bacterial isolate MH-010

Genomic DNA for the isolate MH-010 was subjected to PCR to amplify the 16S-rRNA gene using universal primers (16S 27F and 16S 1492R). The amplified PCR amplicons were then subjected to DNA

sequencing. The resultant 16S-rRNA sequences in Table (3) were analyzed using the basic local alignment search tool (BLAST) at the NCBI database.

Table (3): Partial sequence of 16S-rRNA gene for the bacterial isolates MH-010.

```
   1 aaggggataa cacttggaaa caggtgctaa taccgtataa caatcaaaac cgcatggttt
  61 tgatttgaaa ggcgctttcg ggtgtcgctg atggatggac ccgcggtgca ttagctagtt
 121 ggtgaggtaa cggctcacca nggccacgat gcatagccga cctgagaggg tgatcggcca
 181 cattgggact gagacacggc ccaaactcct acgggaggca gcagtaggga atcttcggca
 241 atggacgaaa gtctgaccga gcaacgccgc gtgagtgaag aaggttttcg gatcgtaaaa
 301 ctctgttgtt agagaagaac aaggatgaga gtaactgttc atcccttgac ggtatctaac
 361 cagaaagcca cggctaacta cgtgccagca gccgcggtaa tacgtaggtg gcaagcgttg
 421 tccggattta ttgggcgtaa agcgagcgca ngcggtttct taagtctgat gtgaaagccc
 481 ccggctcaac cggggagggt cattggaaac tgggagactt gagtgcagaa gaggagagtg
 541 gaattccatg tgtagcggtg aaatgcgtag atatatggag gaacaccagt ggcgaaggcg
 601 gctctctggt ctgtaactga cgctgaggct cgaaagcgtg gggagcaaac aggattagat
 661 accctggtag tccacgccgt aaacgatgag tgntaagtgt tggagggttt ccgccnttca
 721 gtgctgcagn tancgcatta agcantccnc ntggggagta cgnccgcaag gttgaaactc
 781 aaaggaatng ncgggggccc gcncaagcgg tggagcatgt ggtttaattn gaagcaacgn
 841 gaaganccct nccnggtctn gncatccttt gaccactnta gagatagagc ttcccnttcg
 901 ggggcaaagt gacaggtggt gcatggtngt ngtcagctcg ngtcgtgaga tgttgggtta
 961 agtcccgcaa cgagcgcaac ccttattgtt agttgccatc attcagttgg gcactntagc
1021 aagactgccg gtgncaaacc ggaggaaggt ggggatgacg tcaantcatc atgccccttа
1081 tgacntgggt acaccgtgct caatgggaag tacaacgagt ggaagtcgcg aggtaagtaa
1141 ttcttaaagc tttctcagtt cggattgcag gctgcaactc gcctgcatga agccggaatc
1201 gtagtaatcg cggatcagca cgccgcggtg aatacgttcc cgg
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Based on their 16S-rRNA gene sequence and the morphological characters in Figure (6), isolate MH-010 was identified as *Enterococcus lactis*, with a similarity of 99.33%–99.56%. The obtained 16S-rRNA sequence (1193 bp) was submitted to GenBank under accession numbers (PP803411), and phylogenetic analysis of sequences was done, as shown in Figure (7).

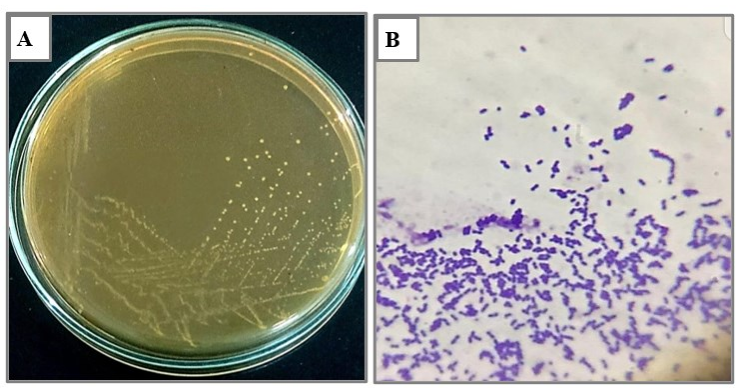

Figure (6): Growth of bacterial isolate MH-010 on MRS agar medium and colony morphology following incubation for 24 h, while (B): Gram staining following incubation for 16 h.

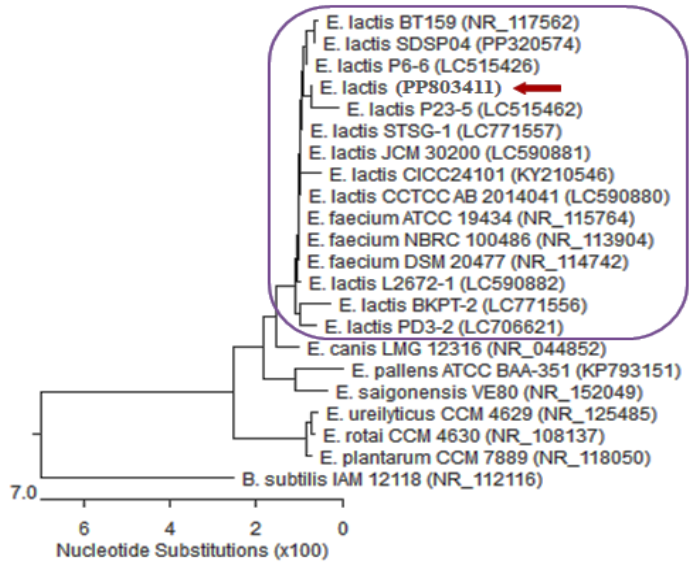

Figure (7): Phylogenetic tree of 16S-rRNA sequences of *Enterococcus lactis*.

4. Discussion

Milk samples from cows and camels were collected from Assuit and Sohag governorates in Egypt for the isolation of LAB. Milk has a high nutritional content and combined with high water activity at a near-neutral pH, it stimulates the growth of numerous microbes (Vargas-Ramella *et al*., 2021). The current study found camel milk is the most abundant source in isolate number, with 36 isolates (52.94%), followed by cow milk with 32 isolates (47.06%), as shown in Table (1). Previously, different LABs were isolated from raw and fermented milk (cow and camel), as documented by (Edalati *et al*., 2019; Nagyzbekkyzy *et al*., 2020; Vantsawa *et al*., 2017; Taye *et al*., 2021). According to Phyu *et al*. (2015) and Mulaw *et al*. (2019), LABs are defined as gram-positive, catalase-negative, non-spore-forming, and lactic acid-producing isolates. The β-galactosidase enzyme is one of the most important enzymes used in food processing and offers nutritional, technological, and environmental applications (Kazemi *et al*., 2016). So, the isolated LABs were primarily screened on an X-gal-contained medium for observing blue-colored colonies, which indicated β-galactosidase enzyme production by bacterial isolates. The current results are in agreement with Kolev *et al*. (2022), who found the same color area in an X-gal-contained medium around the growth of LABs after the incubation period. The quantitative assay of the enzyme showed the bacterial strain MH-010 was the most potent isolate from 10 isolates in enzyme activity, which was 5.49±0.13 U/ml,

according to the o-nitrophenol (ONP) standard curve. Depending on the previous studies, the bacterial isolate MH-010 was selected for the study of the probiotic's properties. Survival of bacterial strains in low pH conditions is a more accurate indication of the ability of strains to survive passage through the stomach (Zielińska *et al*., 2015). Also, bile salt tolerance is a crucial selection factor for probiotic isolates to survive in the small intestine (Mulaw *et al*., 2019). On the other hand, phenol tolerance is required for isolates to survive in gastrointestinal conditions because gut bacteria can deaminate aromatic amino acids received from food proteins, which may generate phenols (Divisekera *et al*., 2019; Singhal *et al*., 2019; Yadav *et al*., 2016). Consequently, the adhesion tests (hydrophobicity, co-aggregation, and auto-aggregation) of the bacterial isolate after tolerance tests (low pH, bile salts, and phenol) are very important for the probiotic determination of the bacterial strain. In the current study, the bacterial strain MH-010 showed good results toward low pH 2.0 and 0.3% (v/v) bile salts in survival rate after 3 hours. At the same time, the phenol and adhesion tests were suitable to prove that the bacterial strain MH-010 is a probiotic strain. The optimal base pairs (bp) of 16S-rRNA was 1500 bp, according to (Patel, 2001), and in the present study, it was 1193 bp, which is sufficient for comparing the sequences with other sequences in GenBank. The probiotic strain MH-010 was identified according to 16-rRNA as *Enterococcus lactis* (PP803411).

5. Conclusion

Enterococcus lactis (PP803411) strain was isolated from fermented camel milk, which can produce the β-galactosidase enzyme when growing in an MRS broth medium. In the future, we will focus on the optimization conditions for the production and purification of the β-galactosidase enzyme from such strain.

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