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Identification ,Screening and Characteristics of of L-asparaginase produce from *Lactobacillus rhamnosus*

Rasha Mohammed Sajet Al-Oqaili [1,2] **Stry@uomustansiriyah.edu.iq**

Seyyed Meysam Abtahi Froushani [1] **meysamabtahi@hotmail.com , sm.abtahi@urmia.ac.ir**

Likaa Hamied Mahdi [2] **likaahamied@yahoo.com, Likaahamied@uomustansiriyah.edu.iq**

[1,] **Department Microbiology, Faculty Veterinary medicine, Urmia University,Iran**

[2,] **Department of Biology, College of Science, Mustansiriyah University,Iraq**

Corresponding author E-mail: **Stry@uomustansiriyah.edu.iq**

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Abstract

The study aimed to purify and characterize the enzyme L-asparaginase from *Lactobacillus rhamnosus* to produce L-asparagine, *Lactobacillus rhamnosus* was cultured using liquid media, fermentation, and then the Nesslerization method was used. *Lactobacillus rhamnosus* is a probiotic bacteria strain isolated of the mother's humans breast milk. It has been shown to inhibit the growth of pathogens, This bacteria modulates the immune system and has severe anti-inflammatory effects.The isolates bacteria were recognize build on various tests, including cultural, morphological, chemical, and API 50 to recognize isolates *Lactobacillus rhamnosus*. Through the apparent results, it was shown that the bacterial isolates produced the L-asparginase enzyme on the modified medium and the color changed from yellow to red as a positive result in the presence of phenol red dye as an indication of the result.However, the isolates bacteria showed the highest of enzyme production with pink inhibition zones of approximately 14.81 mm after two days and 99.137 IU/mg of specific activity.In this study, the pH and temperature were evaluated for the production of L-asparaginase enzyme and the maximum production was at pH 6.5 and at a temperature of 35°C. Investigations have indicated that L-asparaginase in the future may have an important and essential role in uses clinical and its applications in clinical

Keywords: *Lactobacillus rhamnosus*, L-asparaginase, Characteristics, probiotic, breast milk healthy mother

Introduction

L-asparaginamide hydrolase "EC 3.5.1.1". It catalyzes the hydrolysis of the asparagine substrate into two substances, ammonia and raspartic acid [1]. Furthermore, the enzyme L-asparaginase can hydrolyze L-glutamine. Asparaginases produced by bacteria are split into two (2) groups with respect to their accord for substrates, glutaminolytic action and intracellular location . Type I enzymes are localized in the cytoplasm and both L-asparagine and L-glutamine hydrolases are expressed with low affinity. The location of type II enzymes is in the peripheral region and their affinity is high for L-asparagine and low for L-glutamine [2]. There are many strains of bacteria that have the capacity to product the enzyme L-asparaginase, including *Streptomyces brollosae* [3]*Halomonas elongate* [4], *pseudoradiation* [5] *Helicobacter pylori* [6], *Vibrio cholerae*[7],*Bacillus licheniformis*[8],*Rhizomucor miehei*[9]and *Enterobacter* cloacae [10]. L-asparaginase has many industrial uses and biotechnological applications, which include medicine,chemotherapy, food industry, treatment of non-Hodgkin lymphoma and treatment of acute lymphoblastic leukemia one of the leading childhood cancers[11]. When high temperatures are used in cooked foods, acrylamide is formed. Therefore, this enzyme can also be used to reduce acrylamide[12]. On the other hand, there are harmful side effects caused by treatment, including, for example, toxicity to normal cells, pancreatitis, seizures, allergic reactions, infections, fever, and diabetes [13,14].Therefore, there is a need for new rasparaginase-producing sources from probiotic microorganisms that have anticancer activity and could be a better alternative to overcome the disadvantages. There are different types of lactic acid bacteria (LAB): *Lactococci* spp, *Streptococci* spp, and *Lactobacilli* spp that are generally considered safe and because they possess enzymes have been widely studied [2]. The term "probiotic" has been in use for several decades, with the Food Organization in United Nations and the (WHO)Organization in2001 defining the term as "providing health benefits to the host when consumed in sufficient quantities." microorganisms that cause The immunomodulatory effects of probiotic bacteria were hypothesized by Metchnikoff over 100 years ago [15]. Probiotics have been shown to be beneficial for a wide range of conditions ranging from antibiotic-associated diarrhea, traveler's diarrhea, irritable bowel syndrome, neonatal necrotizing colitis, colon cancer prevention, dental caries, Helicobacter pylori ulcers, enteritis and hepatic encephalopathy [16,17]. The scope of interest and research in the field of probiotics has expanded significantly in recent years, especially in cases of intestinal infections and colon carcinogenic [12]. To avoid bacterial resistance to antibiotics, we can use probiotics represented by lactobacilli with antibacterial ability to improve human and animal health [4].The purification and characterization of the L-asparaginase enzyme is very important in terms of its application and therapeutic uses with the least percentage of side effects. Therefore, the present study aimed to isolate,detection and characterize the probiotic isolate *Lactobacillus rhamnosus* to produce the asparaginase enzyme, purify it and characterize it.

Materials and methods

Clinical isolates and media

43 isolates of *Lactobacillus rhamnosus* bacteria. Isolated from breast milk healthy mother . All isolates were cultured in de Mann–Rogosa–Sharpe MRS [18]. It was initially diagnosed microscopically and biochemical tests, and the API 50 kit was used to confirm the diagnosis of *Lactobacillus rhamnosus* bacterial isolates.

Screening test of *Lactobacillus rhamnosus* isolates for L-asparaginase

Specific agar for the detection of L-asparaginase was prepared as previously described[19],and used this agar for semi-quantitative preliminary screening of L-asparaginase production and was also selected for enzyme production. The isolates that gave the highest enzyme production were treated with higher concentrations than the other isolates[20].

Quantitative screening of L-asparaginase

Quantitative tests were performed to determine the enzymatic activity of each isolate according to the method described in [21].

Preparation and purification of L-asparaginase

Lactobacillus rhamnosus isolate number 8 was used to isolate and purify the crude L-asparginase enzyme [20,22]. After the modified procedure, the cultures were incubated at 37°C for 48 hour in anaerobic conditions . Centrifugation was used at 10,000 rpm for 15 minutes to pellet the bacterial cells at 4°C, then pH 7. Supernatant were transferred and used temperature 4°C for storage until assayed . L-asparaginase get of *L. rhamnosus* isolate No 8 was subjected to ion-exchange chromatography used DEAE cellulose and gel filtration used Sephadex-G100 using 0.2 M phosphate buffer 7.7 with a linear gradient of NaCl (0.0 to 0.5 M). It was purified by measuring the protein concentration at each step [23]. Purified L-asparaginase was separated on a gel (10% SDS) and stained [20]. Additionally, L-asparaginase activity was measured according [24].

Characterization of the purified L-asparaginase

Molecular weight (MW) of the purified. L-asparaginase

The molecular weight (MW) of L-asparaginase purified from L. rhamnosus isolate 8 was predestined by sodium dodecylsulfate-SDS-polyacrylamide gel electrophoresis-PAGE in Presence of misfolded labeled proteins . To represent the relationship between the log molecular weight of standard proteins versus (Ve/Vo), a standard curve was used [25].

pH effects on L-asparaginase stability:

To investigate the effect of different pH on L-asparaginase, preparations of purified L-asparaginase were regulated to different pH values (from 2 to 10)[26].

The **effects of temperatures on L-asparaginase stability**

The effect of temperature was evaluated by incubating the L-asparaginase for 15 min at 4, 20,25, 30, 37, 4 0, 45, 50, 60, 70,80, 90, 100, and 121 °C. The residual activity was then assayed against the indicator isolates [27].

The effects of reducing and chelating agents on L-asparaginase stability

Differen reducing and chelating agents as 2-mercaptoethanol, EDTA, and sodium azide were added to the purified L-asparaginase enzyme at concentrations five mM [20,28], andeffects observed.

The effects of surfactants and detergents on L-asparaginase stability

The effect of various non-ionic surfactants/detergents and ionic surfactants/detergents: EDTA , Mercaptoethanol ,Tween 80, Urea,Sodum azide and SDS "1.0%, each w/v" were used [20,28].

The effects of metal ions and inhibitors on L-asparaginase

In DW "distilled water" were prepared Solution of Mg,CO,CU,ZN,P,Na,Ca,Ba,Mn and Fe at 5 mMconcentration. Take one volume of the purified enzyme and mix it with a similar volume of 5 mM Mg, CO, Cu, ZN, P, Na, Ca, Ba, Mn and Fe, each mixture separately and then incubate the mixtures for 60 minutes at a temperature of 37 C. Theremaining e nzymatic activity was measured under standardassay conditions. The control element was the use of the enzyme solution with the addition of any of the mineral solutions used[20,28].

Statistical analysis

Oneway analysis of variance (ANOVA) test was performed to assess the intergroup variation and the p-value of $P \leq 0.05$ was considered signi ficant .

Results

The production and the detection of L-asparaginase

Enzyme L-asparaginase producing testing appeared Table 1. that all the *L. rhamnosus* were capable to production L-asparaginase .The results also showed that isolate *L. rhamnosus* No. 8 is the best isolate for producing and purifying the L-asparaginase enzyme .It was used as an L-asparaginase producer and the specific activity 99.137±2.12a (U/mg).

Table 1:Semi Quantitative Screening of L- asparaginase

Isolates	Pink zone(mean ±sd) (mm)	*Isolates*	Pink zone(mean ±sd) (mm)
no.1	12.5±2.3	**no. 18**	**9.11 ±0.5610**
no. 2	11.7±1.7	**no. 19**	**0±0**
no. 3	0±0	**no. 20**	**0±0**
no.4	7.55±2.9	**no. 21**	**13.07 ±0.92**
no. 5	9.8±1.7	**no. 22**	**6.12 ±0.51**
no. 6	0±0	**no. 23**	**11.51 ±1.06**
no.7	13.01±2.1	**no.24**	**0±0**
no. 8	14.81 ±5.1	**no. 25**	**7.98 ±0.71**
no. 9	0±0	**no. 26**	**10.05 ±1.02**
no.10	7.03±1.01	**no.27**	**6.18 ±0.86**
no. 11	9.44±2.04	**no. 28**	**0±0**
no. 12	12.51 ±1.76	**no. 29**	**13.88 ±3.92**
no.13	10.49 ±2.05	**no.30**	**0±0**
no. 14	8.94.22 ±1.52	**no. 31**	**0±0**
no. 15		**no. 32**	**6.94 ±1.03**
no.16	**11**.85 ±3.02	**no.33**	**10.11 ±2.08**
no. 17	7.65±2.03		

Table 2-Quantitative Screening of L- asparaginase

Isolates	Enzyme activity	Protein mg/ml	Specific activity (U/mg)
no.1	**10.61**±o.84b	**0.148**±0.01	**81.23±4.06c**
no. 2	**8.03**±1.2bc	**0.143**±0.03	**55.91±3.21d**
no. 7	**14.02**±o.65a	**0.151**±0.02	**92.63±2.07a**
no.8	**14.572**±o.93a	**0.159**±0.02	**99.137±2.12a**
no. 12	**12.91**±o.22ab	**0.136**±0.02	**89.75±3.91b**
13	**5.999**±o.26bc	**0.142**±0.01	**46.305±4.25d**
no.16	10.52±1.1b	**0.139**±0.06	**87.15±3.15b**

no. 21	**14.082**±o.45a	**0.153**±0.02	**91.04±1.02ab**
no. 23	**3. 81**±o.47c	**0.12**±0.04	**37.34±2.14e**
no.26	**7.192**±1.31bc	**0.104**±0.01	**77.52±3.15c**
no. 29	**14.07**±o.63a	**0.151**±0.02	**92.63±2.07ab**
no. 33	11.95±o.33ab	**0.147**±0.01	**86.03±1.09b**
LSD	5.031**	**NS**	**7.98****

2Similar letters in the same coloumn refer to insignificant differences.

Purification of L-asparaginase

To purify the enzyme L-asparaginase there are a number of sequential steps.The enzyme was successfully purified using the first step of 70% ammonium sulfate precipitation, followed by the second step, which is saturation, then the dialysis step, and then the chromatographic purification step. The first peak represents L-asparaginase extracted at a concentration of 5-10% o 1 mueller (M) sodium chloride (NaCl), and the second (2nd) peak represents L-asparaginase purified of *L. rhamnosus* isolate No. 8 obtained between 40-50% o 1 mueller (M) sodium chloride (NaCl).

Characterization of L-asparaginase from *L. rhamnosus* isolate# 8

The molecular weight (MW)

Determination ofmolecularweight by gel filtration chromatography showed that the molecular weight t (MW) of L-asparaginase purified from *L. rhamnosus* –isolate no. 8, as stated by the standard curve, as 32kDa Figure 1.

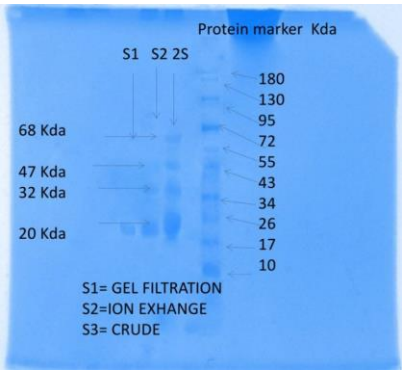

Figure 1: SDS-PAGE chromatograph of L-asparginase

Effect of temperatures and pH on Asparginase activity

Different temperatures and pH were used to study their effect on the purified L-asparginase enzyme. The enzyme gave a clear activity in a wide range of temperatures extending from 4 to 121°C

The enzyme was active over a broad range of temperatures extending from 4 to 121 °C , where enzyme activity was observed at an optimal temperature of about 35-40 °C (Figure 2A). There was an increase in the activity of the L-asparaginase enzyme at 25°C, while a rapid decrease in enzyme activity was observed at 60°C. The results of L-asparaginase activity were observed at a wide range of pH 2 to 10. (Figure 2B) indicates that the purified L-asparaginase enzyme shows optimal activity at pH 6.5 and the activity with L-asparaginase II shows a wider optimal range (pH 6.5-7.5).

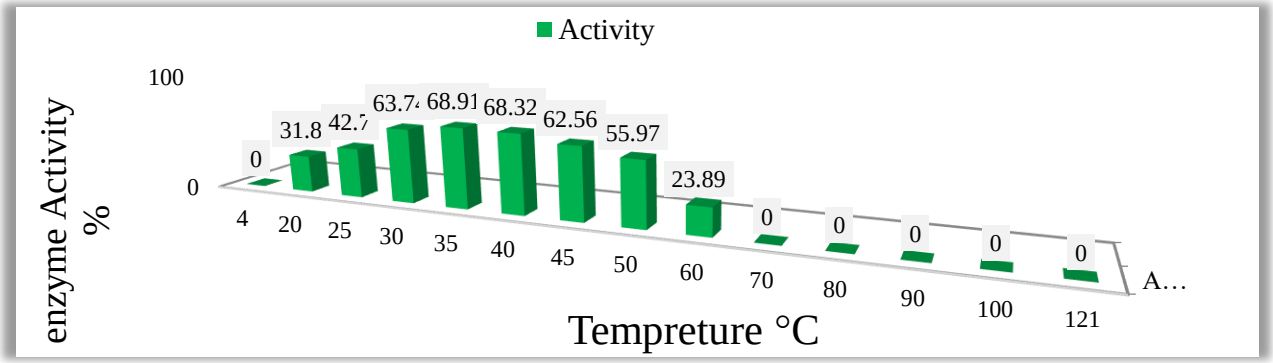

Figure 2A : The effect of temperatures on L-asparginase

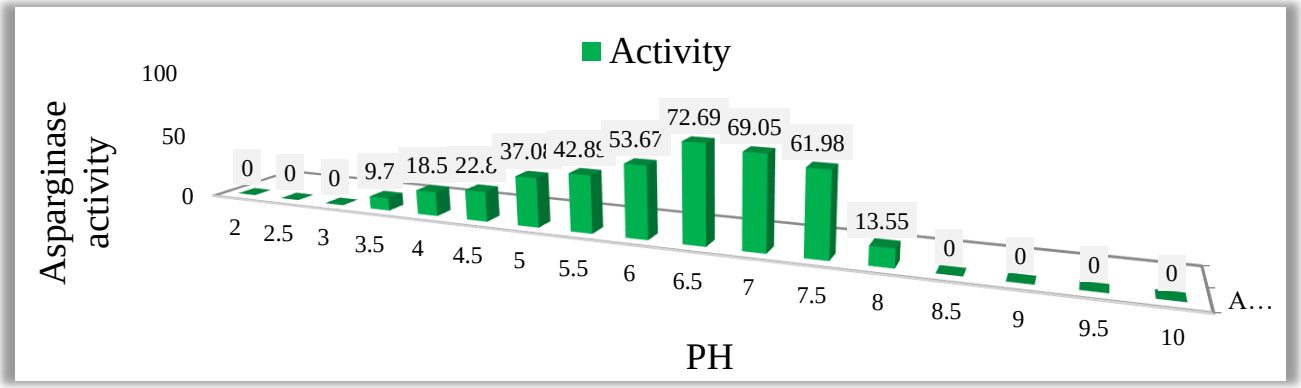

Figure 2B : pH values effect on L-aspasparginase

Effects of pH on L-asparaginase stability

The purified enzyme L-asparaginase from *L. rhamnosus* isolate No. 8 was stable at pH ranging from 6 to 6.5. At these values, L-asparaginase remains active, and it was observed at pH values of 5,5.5,7 and 7.5 that L-asparaginase was forfeit 80%, 93%, 98% and 81% respectively of its activity. L-asparaginase forfeit 62% of its activity at pH value 4.5. While at pH 3.5, 4, and 8, L-asparaginase forfeit 17%, 33%, and 22%, respectively, Figure 3.

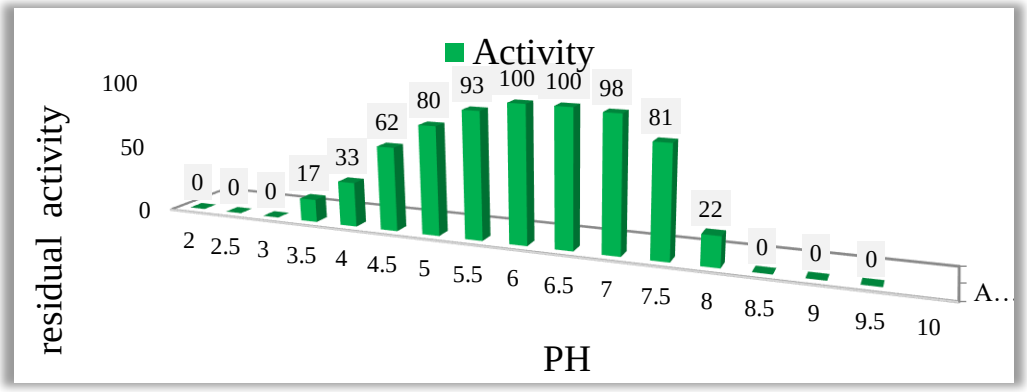

Figure 3 : pH stability of Asparginase at different pH values.

Thermal stability of L-asparaginase

L-asparaginase enzyme purified from L. rhamnosus isolate No. 8 presented high thermal fastness at temperatures amidst 30°C and 40°C for 15 minutes.Furthermore, the L-asparaginase forfeit 45.12%, 66.08 %, 74.89 %,44.16% and 25.8% of its activity at 20,25,45, 50, and 60 °C temperatures, respectively Figure 4.

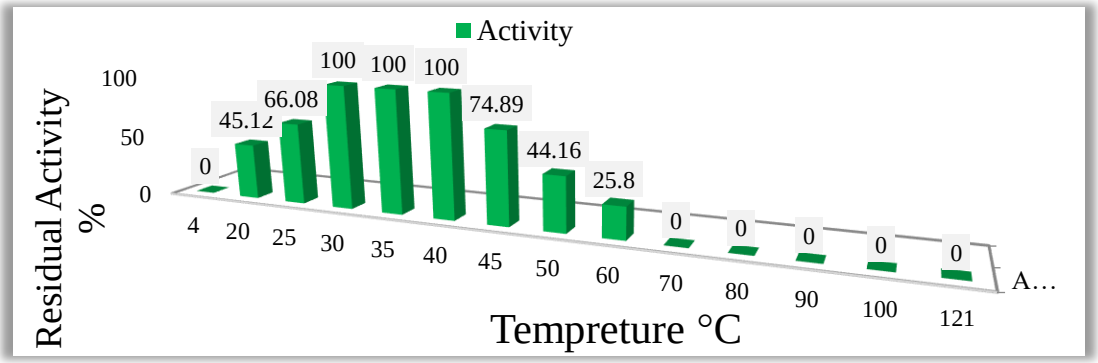

Figure 4 : thermal stability of purified Asparginase at different temperatures

The effects of detergents & surfactants on L-asparaginase stability

The purified enzyme L-asparaginase activity was found to be at 111%, 108%, 103%, 81% and 71% at 0.1% of Tween 80,EDTA ,Mercaptoethanol,SDS and Sodium azide respectively. At 1% urea, the minimum enzyme activity was measured at 65%. Figure 5.

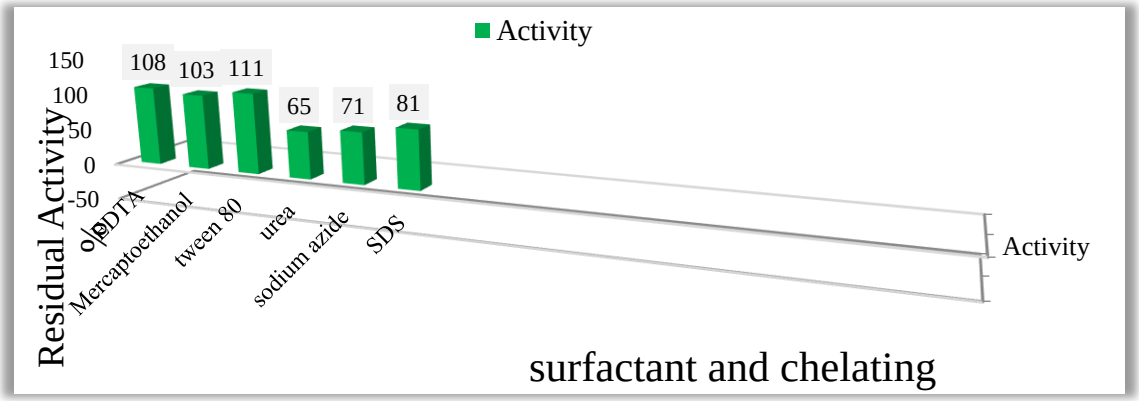

Figure 5: Effect of surfactant and chelating on stabilityof purified Asparginase

Effects of metal ions on purified L-asparaginase stability

Effect of some inorganic ions on enzyme L-asparaginase activity was studied in figure 6 that the enzyme activity enhanced considerably in the presence of Mn,Fe, Mg,Co ,Ni,Ca and Na (120,117,115,109,105,83 and79)% and maximal in the presence of P123% On the other hand, slight decrease in the presence of Ba,Cu and Zn remaining activity around 71,63and 55 %, in L-asparaginase activity**.**

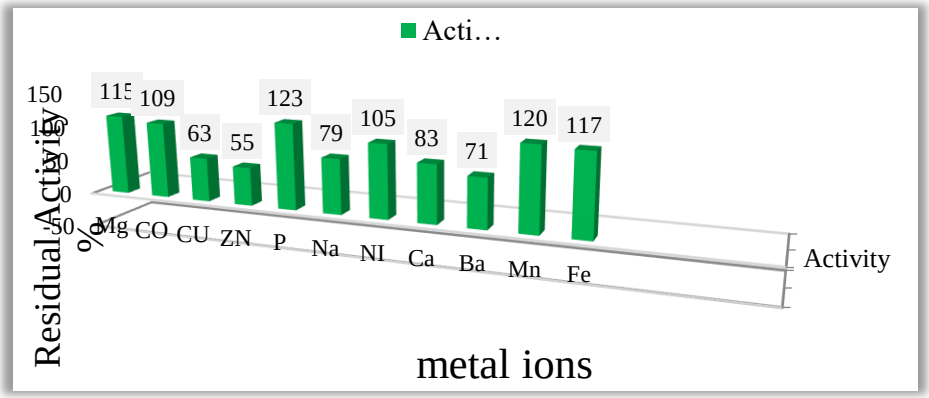

Figure 6: effect of metal ions on asparginase activity

DISCUSSION

The isolates *L. rhamnosus* were diagnosed build on microscopic and chemical tests, and the diagnosis was confirmed by API 50 [29]. In recent years, studies and research have hypothesized that breast milk is a continuous source of lactic acid bacteria [30].All isolates isolated from burn wounds in this study be back to *L. rhamnosus* and were all have the ability to producing the enzyme L-asparginase, although the production capacity varied among the isolates [31].The results are consistent with [19,20]. As stated by to the results, *L. rhamnosus* isolate No. 8 was chosen to demonstrate its capacity to produce enzyme L-asparaginease and the results were similar to [32], where they found that the production of L-Asparagine from *Lactobacillus plantarum* showed that the enzyme activity is affected by carbon and nitrogen sources. The transformation of the yellow color into a pink color in the medium used when growing bacteria is an indication of the isolates' production of asparaginine. Increased numbers of active isolates producing L-asparagine appear as pink areas around the colony, associated with the action of the enzyme L-asparaginase, which inactivates L-asparagine and releases ammonia, resulting in The pH increased as in this study [33]. Similarly, [34]. *Bacillus* sp was investigated as an L-asparaginase able producing bacterium of that semi-quantitative analysis The pH of the medium has a significant influence on many enzymatic processes and the movement of various components across the cell membrane of microorganisms [35]. The optimal pH for enzyme L-asparaginase produce is dependent on the specific bacterial strain, and its dissimilaritys can be ascribe to leavening conditions and specified genetic characteristics of the microbial types [36]. In this study, maximum L-asparaginase production happened at pH 6.5. This was a similar result

to that observed by [37], who reported that L-asparaginase production by *B. subtilis* happen optimally at pH 6.5. Furthermore, [38] reported that L-asparaginase production by *B. licheniformis* happen in the range of pH 5.5 to 7.5, with maximum enzyme production happening at pH 6.0.Maximum enzyme productivity of the L-asparaginase evaluated in this study was obtained at 35 °C, similar to the optimal temperature for the producing of enzyme L-asparaginase by bacteria *Streptomyces olivaceus* was 35 °C[39]. In contrast, the optimal producing of L-asparaginase from *Bacillus* happened at pH 6–7.5. This feature is a key requirement for the antitumor activity of L-asparaginase with an optimum temperature of 37°C.

Conclusions

Probiotic LAB is a very important part of the larger healthcare. Due to their biodiversity, they can be used as an important scientific basis for the development of probiotics, and rich LAB resources are available.On the other hand, lactic acid bacteria isolated from fermentation of traditional foods and healthy people are relatively safe due to their biological properties.In this study, the enzyme L-asparaginase from breast milk was examined, and the enzyme was isolated and purified to produce L-asparaginase. However, extensive research and studies are needed applying the enzyme to human clinical trials to evaluate this possibility.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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