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Article

Screening, Identification and Production of L-asparagine from *Lactobacillus reuteri* of Healthy Mother Milk

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ABSTRACT
Human breast milk (HBM) may benefit Lactobacillus reuteri, a probiotic strain isolated from a mother's breast milk. It has been shown to inhibit pathogen growth, modulate the immune system, and have strong anti-inflammatory effects. This study aimed to isolate and screen Lactobacillus reuteri for L-asparagine production. Lactobacillus reuteri were grown in submerged fermentation and liquid media and used for secondary screening by the Nesslerization method. The isolates were identified based on morphological, cultural, and biochemical tests and API 50 to identify Lactobacillus reuteri. According to the findings, L-asparaginase isolated from bacteria showed positive L-asparaginase activity on a modified medium using phenol red as an indicator dye. However, bacterial isolates showed the highest enzyme activity, exhibiting a pink zone of hydrolysis of around 7.0 cm after 48h. and 4.92 IU/mg of specific activity. These results illustrated the importance of these isolates to produce and control L-asparagine for biomedical production in the future.

Keywords: *Lactobacillus reuteri*, L-asparagine, L-asparaginase

INTRODUCTION

L-asparaginase is a critical enzyme that has been isolated and described for decades to investigate and evaluate its biological activities1. The enzyme's ability to convert L-asparagine into aspartic acid and ammonia is responsible for its anti-cancerous activity2. L-asparagine can hydrolyze L-glutamine. Bacterial L asparagines are divided into two groups based on their affinity for the substrate L-asparagine, cell location, and glutaminolysis activity3. The first type of enzyme is found in the cytoplasm and is constitutively produced. They have a lower affinity for L-asparagine and hydrolyze L-glutamine. Second-type enzymes with reduced af-

finity for L-glutamine and higher affinity for L-asparagine are found in the periplasm4. The L-asparagine is found in the cytoplasm, and L-asparagine II with periplasmic origin5. However, only the second one has anti-cancer activity. Some studies described I as a constitutive enzyme and the second as secreted only as a response to exposure to low concentrations of nitrogen6.
Most of the microbial L-asparagine is intracellular except a few, which are secreted outside the cell7. Extracellular L-asparagine is more advantageous than intracellular type because of the higher accumulation of enzymes in culture broth under normal conditions, easy extraction, and downstream processing8. Lactic acid bacteria (LAB) are among the most thoroughly studied microorganisms and are widely employed in the production of secondary metabolites around the world.9, 10, 11. The potential to be employed as probiotics, which can aid in maintaining good health and preventing disease12,13. Purification of L-asparaginase is critical for its characterization and therapeutic application with fewer side effects. Thus, the current study aimed to isolate and screen the lactic acid bacteria, Lactobacillus reuteri, for the production of Asparagine.

MATERIALS AND METHODS

Sample collection and identification

Three ten isolates of Lactobacillus reuteri isolated from mother milk were plated in de Mann–Rogosa–Sharpe (MRS) agar and incubated at 37 oC for 48 h anaerobically. The distinct colonies based on their colony morphology from each plate were further purified by subsequent subculturing in the same media 3–4 times to ensure their purity. To screen LAB, the isolates were subjected to Gram's staining reaction, endospore staining, oxidase, and catalase activities and identified by API 50 13.

Isolation and primary screening of L-asparaginase-producing bacteria (semi-qualitative assay)

All isolates were screened for L-asparaginase activity using the modified M9 medium (1 l: 3.0 g KH_2PO_4; 6.0 g $Na_2HPO_4 \cdot 2H_2O$; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 0.014 g $CaCl_2.2H_2O$; 2.0 % (w/v) glucose, and 15.0 g agar) combined with a pH indicator (phenol red) supplemented with 0.005% phenol red dye plat were inoculated with test organisms by using one loopful culture of bacteria and incubated at 37°C for 48d h. A set of plat medium contains L-asparagine as the sole source of nitrogen. L-asparaginases hydrolyses L-asparagine into L-aspartic acid and ammonia. This can be easily detected by the change in the medium's pH due to ammonia production. The color change of the medium (from yellow to pink) indicates positive L-asparaginase production. L-asparaginase activity of each bacterium was recorded by measuring a pink zone in the plat and depending on the L-asparaginase activity 14. The enzyme index was determined using the following equation:
Hydrolysis zone = diameter of zone/diameter of bacterial colony

Secondary screening of L-asparagine-producing bacteria (quantitative assay)
Isolated microorganisms from the previous screening were cultured in liquid media with Asparagine Modified broth medium at pH 6.5. All experiments for quantitative screening were done in triplicate after incubation at 37°C for 48 hrs. Culture broth was at 10,000 rpm for 10 min, supernatants were collected, and enzyme assay was done by Nesslerization 15.
Asparagine Assay
The activity of L-asparaginase was measured by modified method 16. The L-asparaginase catalyzes L-asparagine to L-aspartic acid and ammonia, and the latter reacts with Nessler's reagent to produce an orange-colored product. The reaction mixture containing 1.5 mL of 0.04 M L-asparagine was prepared in 0.05 M Tris-HCl buffer, pH 8.6, and 0.5 mL of the enzyme to make up the total volume to 2 mL. The tubes were incubated at 37 °C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). The blank was run by adding TCA, followed by enzyme preparation. The reaction mixture was centrifuged at 10,000 ×g for 5 min at 4°C to remove the precipitates. The ammonia released in the supernatant was determined using a colorimetric technique by adding 100 µL Nessler's reagent into the sample containing 100 µL supernatant and 800 µL distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min, and OD was measured at 425 nm. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One unit of L-asparaginase activity is defined as the amount of the enzyme that liberates 1 µmol of ammonia per minute at 37°C.

RESULTS

Sample collection and Lactobacillus reuteri identification
To perform limited biochemical tests, 30 colonies were selected from MRS plates, and out of these, Gram-positive and catalase-negative isolates were selected to screen LAB.
Semi-quantitative L-asparagine production screening
The semi-quantitative screening for L-asparagine production was done by the agar plate assay method, which was achieved by detecting the ability to form a pink zone around each colony when grown on L-asparagine. L-asparagine-media containing phenol red. The pink color was directly related to the quantity of enzyme produced. L reuteri. No. 25 could be considered the highest L-asparagine producer, which gave a clear pink zone of hydrolysis around (7.0 mm) after 48 h of cultured on agar medium containing L-asparagine and monitored during 48 h of incubation. The isolates varied in growth on this medium; 20 of L. reuteri isolates appeared to give a clear pink zone of 6.3mm during 48 hr., but the other 3 isolates could not grow. Results indicate that the isolates L. reuteri are varied in their ability to utilize L-asparagine, the sole nitrogen and carbon source in this medium, and the faster may show active L-asparagine utilizing enzymes that facilitate bacterial growth.
Quantitative screening of L-asparagine production
To select the highest productive isolate of L-Asparaginase enzyme, produced with variable degrees of production. L reuteri. isolates were cultured in L-Asparagine broth. These active L-Asparagine bacterial isolates revealed different specific activities (SA) ranging between 0.25 and 4.92 U/mg proteins. The more active L-Asparagine productive isolates had different specific activities ranging between

4.5 and 4.92. Produced pink weakly color in L-Asparagine Produced pink - hydrolysis broth medium during 48hr and then converted to pink colored after 48 hr. Of incubation at 37℃, which indicated ammonia production as a final product of L-Asparagine L. reuteri no.25 gave the highest specific activity (4.92 U/mg proteins). Moreover, specimens L. reuteri no. 22 showed specific activity (4.5 U/mg proteins).

***L. reuteri* Isolate**	**Pink zone (mm)**	**Specific activity (U/mg)**	**Intensity of color**	***L. reuteri* isolates**	**Pink zone (mm)**	**Specific activity (U/mg)**	**Intensity of color**
1	6	4.30	+++	21	5.0	3.10	++
2	5.6	3.80	++	22	6.3	4.51	+++
3	6.8	3.0	++	23	6	4.0	+++
4	5	3.60	++	24	2.9	3.16	++
5	4	2.92	++	25	7	4.92	+++
6	2	1.80	+	26	-	-	-
7	3	2.30	+	27	5.5	3.22	++
8	4.4	3.10	++	28	4.2	2.59	+
9	2	1.20	+	29	2	0.25	+
10	5.1	3.70	++	30	3.30	2.80	+
11	4.2	3.06	++	31	6.5	3.90	++
12	5.3	3.75	++	32	-	-	-
13	6	4.20	+++	33	4	3.0	++
14	3.9	2.90	+	34	4.20	3.50	++
15	6.3	4.0	++	35	2	1.00	+
16	3.8	2.50	+	36	4	2.91	+
17	-	-	-	37	5	2.90	+
18	4.2	2.90	+	38	6.1	3.60	++
19	2	0.50	+	39	3	1.30	+
20	1	1.98	++	40	5	3.98	++

Table1. The specific activity of L- Asparagine from *L. reuteri*. Isolates

DISCUSSION

The isolates were identified based on morphological, cultural, and biochemical tests, and API 50 to identify the [13] *Lactobacillus reuteri* isolates. In recent years, breast milk has been hypothesized to be a continuous source of lactic acid bacteria [17, 18].

The findings are in agreement with Savitha et al. The media color changes of the medium from yellow to pink were considered an indication of L-asparagine production. Since the active L-asparagine-producing isolates increased, it appeared as a pink zone around colonies related to L-asparaginase enzyme action that disrupted L-asparagine and released ammonia, which raised the pH number similar to this study 20. Similarly, 21 screened Bacillus sp as L-asparaginase-producing bacteria from this semi-quantitative assay.

According to the results, L. reuteri no. 25 was selected to prove its ability in L-Asparagine production by optimization. Similarly, 22, they found the Production of L-Asparagine from Lactobacillus plantarum. the impact of carbon and nitrogen sources on enzyme activity

CONCLUSIONS

The present study reports the screening of L-asparaginase-producing *Lactobacillus reuteri* from mother milk, emphasizing the potential LAB working for variables influencing the production enzyme. Further studies were recommended on the complete purification and characterization of L-asparaginase to evaluate its in vivo toxicity.

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Conflicts of Interest: The authors declare no conflict of interest.

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