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Isolation, Screening and Characterization of L-Glutaminase Producing Bacteria from Brackish Water

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Abstract – L-glutaminase is an important enzyme owing to their applications in pharmaceutical and food industries. In the present study, 142 L-glutaminase-producing bacterial strains were isolated from brackish water samples collected from the Lake Mariout at the North of Egypt. Results revealed that the majority of these strains produced the enzyme intracellularly and only 18 strains produced extracellular enzyme. Based on 16S rRNA gene analysis, the strains were identified as *Citrobacter amalonaticus* strain GPBW1, *Legionella feeleii* strain GPBW2, *Enterobacter cloacae* strain GPBW3, *Empedobacter brevis* strain GPBW4, *Burkholderia plantarii* strain GPBW5, *Lysobacter antibioticus* strain GPBW6, *Vibrio parahaemolyticus* strain GPBW7, *Serratia fonticola* strain GPBW8, *Yokenella regensburgei* strain GPBW9, and *Klebsiella oxytoca* strain GPBW10. The study highlighted the promising activity of *Klebsiella oxytoca* strain GPBW10 for L-glutaminase production.

Keywords – Isolation, Screening, Characterization, L-glutaminase, Bacteria.

I. Introduction

L-glutaminase (EC 3.5.1.2) is an amido hydrolase enzyme which catalyzes L-glutamine to L-glutamate and ammonia [1]. It is useful in the treatment of cancer [2]. L-glutaminase is widely distributed in bacteria, actinomycetes, yeast and fungi [1], [3]–[6]. L-glutaminase exhibits anticancer effect by depleting the L-glutamine from the cancerous cells, since these cells more avidly consume L-glutamine for their energy needs and proliferation than normal cells [7]. It is also understood that the cancerous cells cannot synthesis their own L-glutamine and this is the Achilles heel that is exploited by these amino acid depleting anticancer agents [8], [9].

Other than in pharmaceutical applications, L-glutaminase finds applications in food industries as a flavor enhancement agent especially for fermented foods and has also got applications as a biosensor and analytical agent and used for the production of fine chemicals like theanine [10]–[13]. The present study aimed at isolation and characterization of L-glutaminase-producing bacteria from brackish water.

II. Materials and Methods

A. Sample collection

Brackish water samples were collected from the Lake Mariout, North Egypt (31°09'22.2"N 29°53'32.0"E) at 1m depth. Lake-water samples were collected in sterile bottles (100 ml) and transferred to the laboratory in an ice box.

B. Isolation and primary screening of L-glutaminase-producing bacteria

Glutamine-utilizing bacteria were isolated on minimal medium supplemented with L-glutamine (0.1%) as the sole nitrogen source and phenol red as a pH indicator [6]. After incubation at 28°C for 4 days, colonies that showed pink zones indicating L-glutaminase production were picked and re-streaked on fresh plates containing the same medium and

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incubated at 28°C for 48 h. Single discrete colonies were selected and maintained on LB slants for further studies.

C. Secondary screening

The Secondary screening of L-glutaminase activity was conducted the above mention medium (without phenol red and agar). Each bacterial isolate was inoculated into minimal broth and incubated at 28°C for 48 h then the culture was centrifugated at 15000 rpm for 15 min. The cell-free supernatant of each bacterial isolate was assessed for L-glutaminase activity.

D. Enzyme assay

The reaction was conducted by adding 100 µl of crude enzyme to a mixture of 100 µl of 189 mM L-glutaminase solution (prepared in 50 mM Tris–HCl buffer pH 8.6); 1.0 ml of 50 mM Tris–HCl buffer (pH 8.6) and 0.9 ml of deionized water, pre-warmed to 37ºC. The reaction mixture was incubated at 37ºC for 30 min after which the reaction was stopped by adding 100 µl of 1.5 M trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 6000 rpm for 15 min. The ammonia released in the supernatant was determined by adding 0.5 ml of Nessler's reagent to tubes containing 0.20 ml supernatant in 4.30 ml of distilled water. The absorbance was measured at 436 nm against the blanks which received TCA before the addition of 100 µl the crude enzyme; using spectrophotometer (Model 6300, Jenway, United Kingdom). The ammonia produced in the reaction was determined based on a standard curve obtained with ammonium sulfate as standard. One unit of L-asparaginase is defined as the amount of enzyme that liberates 1.0 µmole of ammonia from L-asparagine per minute at pH 8.6 at 37°C [14].

E. Molecular identification of the most active isolates

Genomic DNA from the most active isolates was extracted by GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). The 16S rRNA gene was amplified using universal primers 27F and 1492R. The PCR product was then was sequenced and the sequence was compared to sequences in National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database using Basic Local Alignment Search Tool (BLAST) program and the 16S rRNA gene sequences were deposited in GenBank. The phylogenetic tree of the most active strain was constructed after pairwise sequence alignment of 16S rRNA gene sequence with the closest related species using ClustalW program. Neighbor joining method was used for construction of phylogenetic tree based on bootstrap values (1000 replications with MEGAX software).

III. Results

A. Isolation and primary screening of L-glutaminase-producing bacteria

One hundred and forty-two bacterial strains were isolated from brackish water samples using modified minimal medium, supplemented with phenol red as a pH indicator. The isolated strains showed intense pink zone around the colonies indicating the increase in pH due to ammonia accumulation in the medium as a result of L-glutaminase production. The isolated strains were re-streaked on the same medium twice to ensure their purity.

B. Secondary screening

Extracellular L-glutaminase activity was determined in cell-free supernatant by enzyme assay. Of 142 L-glutaminase-producing isolates, only 18 exhibited extracellular activity. L-glutaminase activity ranged from 0.1 to 5.3 U/ml. The most active isolates (n=10) were selected and identified.

C. Molecular identification and phylogenetic analysis

The most active isolates were identified according to 16S rRNA analysis and the 16S rRNA gene sequences were submitted to the GenBank (Table. 1). *Citrobacter amalonaticus* strain GPBW1, *Legionella feeleii* strain GPBW2, *Enterobacter cloacae* strain GPBW3, *Empedobacter brevis* strain GPBW4, *Burkholderia plantarii* strain GPBW5, *Lysobacter antibioticus* strain GPBW6, *Vibrio parahaemolyticus* strain GPBW7, *Serratia fonticola* strain GPBW8, *Yokenella regensburgei* strain GPBW9, and *Klebsiella oxytoca* strain GPBW10. The phylogenetic tree of *Klebsiella oxytoca* strain GPBW10 showing the superior L-glutaminase activity was constructed (Fig. 1).

Table 1: Identification of L-glutaminase-producing bacteria isolated from brackish water

Strain	Accession number
***Citrobacter amalonaticus* strain GPBW1**	**MN865865**
***Legionella feeleii* strain GPBW2**	**MN865866**
***Enterobacter cloacae* strain GPBW3**	**MN865867**

***Empedobacter brevis* strain GPBW4**	**MN865868**
***Burkholderia plantarii* strain GPBW5**	**MN865869**
***Lysobacter antibioticus* strain GPBW6**	**MN865870**
***Vibrio parahaemolyticus* strain GPBW7**	**MN865871**
***Serratia fonticola* strain GPBW8**	**MN865872**
***Yokenella regensburgei* strain GPBW9**	**MN865873**
***Klebsiella oxytoca* strain GPBW10**	**MN865874**

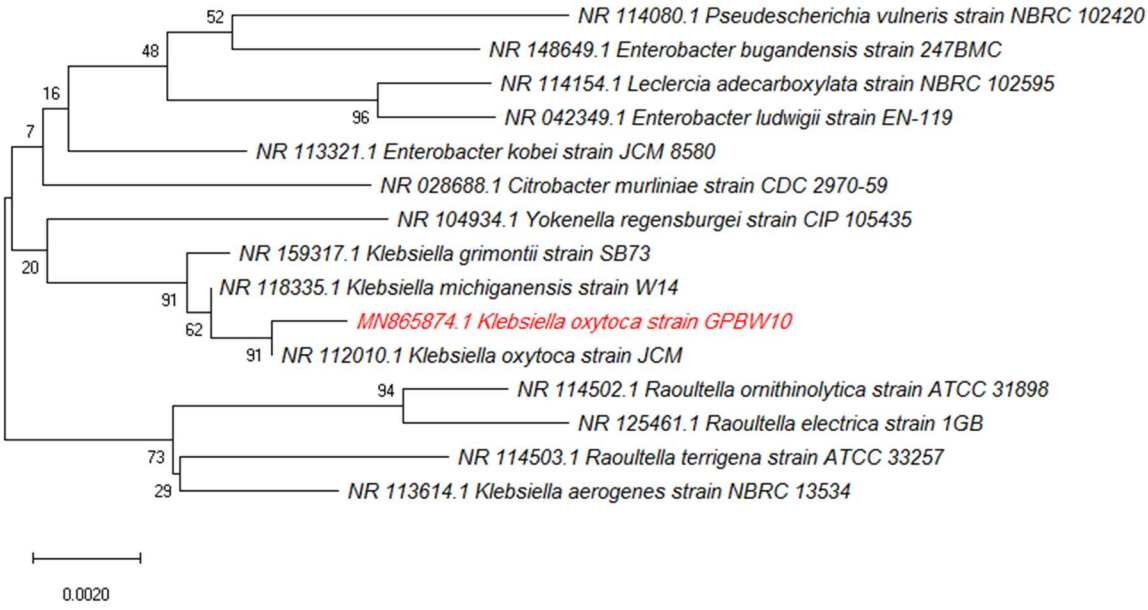

Figure 1: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *Klebsiella oxytoca* strain GPBW10 and the most closely related species.

IV. Discussion

The present work was carried out to identify L-glutaminase-producing bacteria isolated from brackish water samples. Initially, plate assay was used for isolation and screening of L-glutaminase producing microorganisms. L-glutaminase production was visualized by formation of pink zones around the producing colonies by incorporating the pH indicator phenol red in the medium containing glutamine (sole nitrogen sources). At acidic pH, phenol red is yellow and turns pink at alkaline pH, thus a pink zone is formed around L-glutaminase-producing colonies. Similarly, several previous reports documented the isolation of L-glutaminase-producing microorganisms using culture media supplemented with phenol red [15]–[19]. All the 142 isolates were subjected to secondary screening for extracellular L-glutaminase production by quantitative enzyme assay. Results revealed that the majority of the assessed isolates dis not exhibit extracellular L-glutaminase activity indicating the intracellular localization of the enzyme. Only 18 strains were found to produce extracellular L-glutaminase and the secondary screening revealed that *Klebsiella oxytoca* strain GPBW10 exhibits excellent enzyme production (5.3 U/ml). In a recent study, *Bacillus subtilis* OHEM11 isolated from marine habitat produced extracellular L-glutaminase [20]. Also, extracellular L-glutaminase production by halophilic and halotolerant bacteria belong to the genus *Bacillus* and *Salicola* has been reported [21]. Furthermore, L-glutaminase production by various bacteria such as *Kurthia gibsonii* [22], *Weisella cibaria*, *Leuconostoc citreum* [23], *Alcaligenes faecalis* KLU102 [5], *Bacillus cereus* Strain LC13 [4], and

Stenotrophomonas maltophilia NYW [24] has been documented.

V. Conclusion

The present investigation addressed the extracellular production of L-glutaminase by various bacterial strains isolated from brackish water and highlighted the promising activity of *Klebsiella oxytoca* strain GPBW10 regarding L-glutaminase production. However further future studies are aimed to characterize the enzyme at molecular level.

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