L-Asparaginase Production by *Aspergillus unguis*

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Abstract

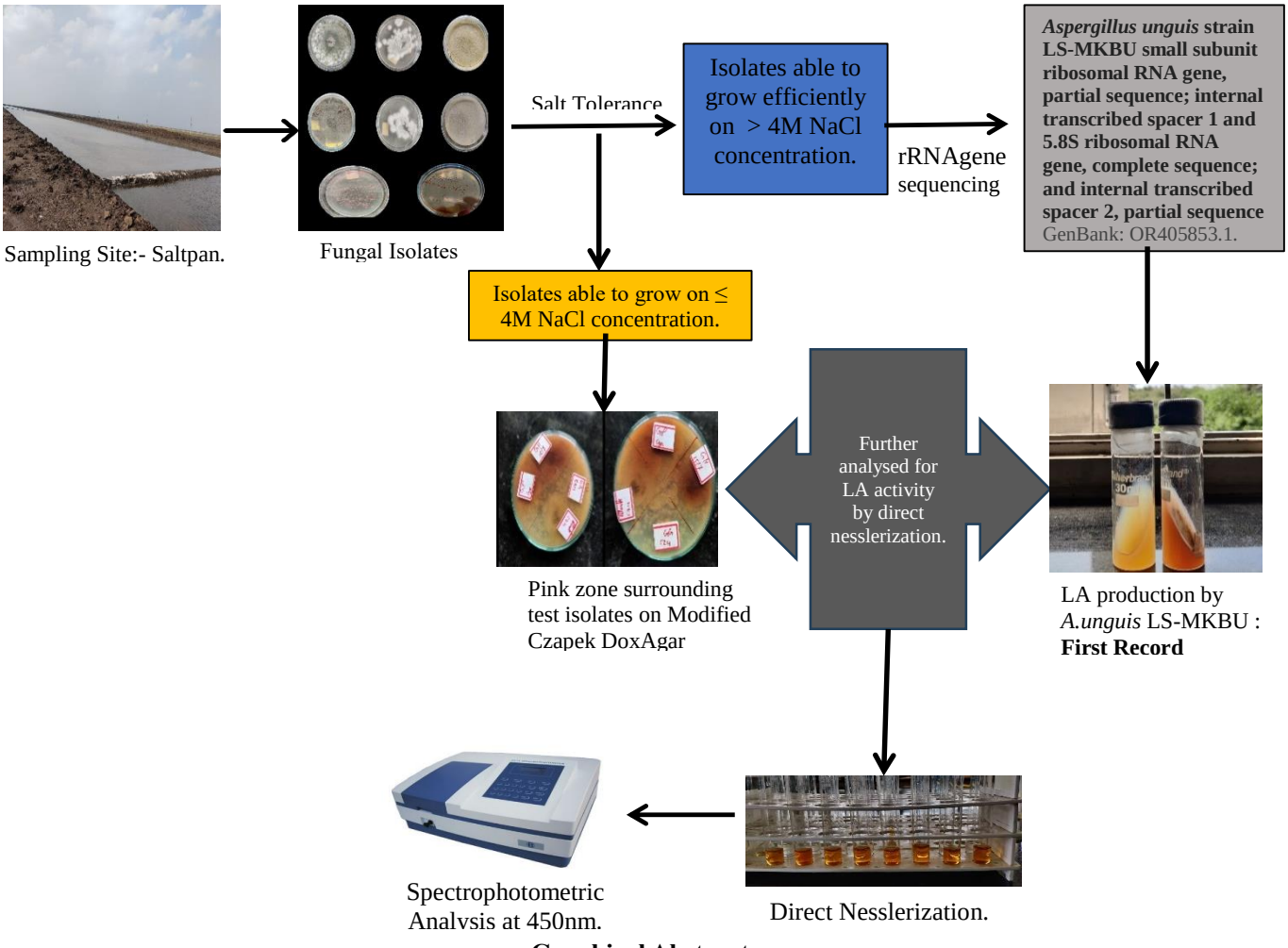
Demand for L-asparaginase (LA) has increased worldwide due to its diverse applications, particularly in cancer therapy and in food industry. While various microbial sources have been extensively studied for LA production, exploration on fungal LA, especially from hypersaline environments hitherto remains untapped. The objective of the work was to explore LA production potential of fungi isolated from saltern. The isolation yielded seven fungi (RJ1 to RJ7) and all the isolates exhibited LA production potential. Upon examining salt tolerance (2M-5M), isolate RJ1 efficiently grew at all test concentration with optimum growth at 3M salt, hence it was subjected to molecular identification and was identified as Aspergillus unguis strain LS-MKBU.

Effect of sugar concentration on LA production by A. unguis LS-MKBU was also examined. The present work is a report on LA production by saltern-derived fungi. This work unveils A. unguis as a novel LA producer. The findings contribute to enzyme biotechnology especially from hypersaline environment, opening avenues for novel applications and future research.

Keywords: L-asparaginase, saltern, halophilic fungi, *Aspergillus unguis*.

Introduction

Enzymes are fundamental to life, serving as molecular catalysts that accelerate biochemical reactions within cells. The diversity of enzymes reflects the complexity and versatility of biological systems, playing indispensable roles in DNA replication, protein synthesis, energy production and nutrient metabolism[13,21,24,39,40]. This catalytic efficiency is central to cellular dynamics, enabling organisms to respond to environmental cues, to regulate biochemical pathways and to adapt to changing conditions[5,13,21,24,31,39,40].



Graphical Abstract

Enzymes exhibit remarkable diversity in structure, function and regulation, reflecting their evolutionary adaptation to specific biological niches and contributing to organismal homeostasis and adaptation[28,32,38,54]. Among these enzymes, L-asparaginase (LA) (E.C. 3.5.1.1), an enzyme with anticancer properties, is extensively utilized in the chemotherapy of acute lymphoblastic leukaemia (ALL) in children and other cancers. LA functions by irreversibly catalysing hydrolysis of L-asparagine into L-aspartic acid and ammonia, which leads to disruption of tumour cell metabolism and DNA structure, making it a vital component of modern cancer treatment strategies[3,6,7,10,11,35,49,51].

The deficiency of asparagine synthetase in tumour cells makes their dependence on circulating plasma pools for L-asparagine, making them susceptible to LA, thereby inducing nutritional stress that inhibits vital biosynthetic pathways, including DNA, RNA and protein synthesis, leading to programmed cell death and cell cycle arrest, predominantly in G0/G1 phase, while normal cells remain unharmed; this enzymatic process is crucial for depleting asparagine in malignant cells[2,19,20,36,44,47]. LA is also known for its capacity to inhibit acrylamide formation in baked and starchy goods through the reduction of asparagine levels because during preparation of baked foods or starchy foods asparagine naturally present in the starchy foods forms some heterocyclic amines and acrylamide, a carcinogen due to Maillard reaction (responsible for giving baked or fried foods their brown colour, crust and toasted flavour). Upon adding LA before baking the food. Asparagine irreversibly gets converted into aspartic acid and ammonium. Thus, asparagine does not take part in the Maillard reaction and this significantly leads to lower acrylamide formation without change in the taste and appearance of the product[4,22,30,33,41,42,46,48,55,56].

LA also exhibits applications other than therapeutics and food industries, with emerging potentials in several novel areas[35]. LA has been used in biosensors for rapid detection of L-asparagine levels in blood and food samples, aiding in cancer therapy dosage adjustments and food quality assessments[35,50,53]. Furthermore, LA contributes to the biosynthesis of amino acids like aspartic acid, serving as a precursor for other amino acids synthesis[3,35]. LA-based biocalcification in bio-grout shows better result in ground improvement techniques, giving advantages over traditional urease-based methods[25,29,35]. LA has also demonstrated anti-biofilm activity against drug-resistant bacteria and potential antimicrobial properties, suggesting applications in combating microbial infections[35,37,52].

Furthermore, LA's role in plant growth promotion and as a potential anti-insecticidal agent against pests like whiteflies highlights its applicability and unrecognized potentials in agricultural and environmental sectors[8,9,14,35]. Although these applications are recently underexplored, ongoing research and advancements are expected to unveil new dimensions of LA's utility across various fields. With increasing applications of LA and limitations of available anticancer LA, there is an ongoing quest to find effective and safe LA variants, however, hypersaline environments have not been explored so far for this purpose. Recently, fungal diversity from hypersaline habitats has been paid attention[12,17,23,34,43]. The present study focuses on LA production potential of fungi isolated from saltern.

Material and Methods

Sampling site, sample collection and isolation of fungi from saltpans: Bhavnagar (21°46′N 72°09′E/21.77°N 72.15°E), a coastal city situated on the eastern coast of Saurashtra region, covers approximately 53.30 km² and is known for being Gujarat State's foremost salt producer, with an annual output of about 35,000 tons.

Sampling was conducted in late February 2023, coinciding with the salt production month. Water samples were aseptically collected in 300ml sterile screw cap bottles by submerging the bottle mouth downwards to a depth of approximately 20cm and then inverting it when filled. The collected samples were promptly transported to the laboratory in an ice-box and processed within 6 hours of collection. In the laboratory, the samples were inoculated in Czapek Dox agar plates amended with 3M NaCl using the serial dilution method. Dilutions ranging from 10^{-1} to 10^{-6} were prepared and from each dilution, a fixed volume was plated onto Czapek Dox agar plates containing 3M NaCl and incubated at room temperature for 5-20 days. After incubation period, morphologically distinct isolates were subcultured onto respective media and stored at 4°C for further analysis.

Salt tolerance of the fungal isolates: Salt tolerance of the fungal isolates was examined by growing the fungal isolates in medium amended with various NaCl concentration ranging from 2M to 5M at an interval of 1M.

Screening of fungi for LA production

(i) Primary screening: Fungal isolates were screened primarily for their LA production ability by using modified Czapek Dox agar medium containing 0.009% phenol red. Young fungal growth on Czapek Dox agar plates was cut from the edges and 2-5mm agar square block was placed on modified Czapek Dox agar plates and incubated for 20 days. The plates were observed for development of pink zone surrounding the colony at 24h interval. Test fungal colonies surrounded by a pink zone were considered as LA positive subjected to secondary screening. The experiment was performed in triplicate.

(ii) Secondary screening: Following the agar plate screening, the cultures showing potential for LA production were further assessed in modified Czapek Dox broth amended with 0.009% phenol red[48]. Flasks containing 50 ml of the Czapeck dox medium were inoculated with respective test isolate and then incubated at room temperature for 15 days. Development of pink color in the broth was monitored

at 24h interval. Simultaneously, pH of the broth was also monitored. Cultures that exhibited a pink colour change in the broth within the 15-day incubation period were considered positive for LA production and quantification of LA activity was carried out.

The fungi exhibiting pink colour change in the broth were tested for activity staining as well to further confirm LA production. For this, a gel was prepared using 1% L-asparagine in 1% agarose dissolved in 0.05M phosphate buffer. Wells were created in the gel and 20 µL of sample was added from our test culture filtrate into these wells. The gel was left to incubate overnight. After incubation, Nessler's reagent was added to the well and observed for the development of a brown zone around the wells[26].

For estimation of LA activity, once pink colour developed in the modified Czapek Dox broth, the biomass was separated by filtration and the culture filtrates were used for assessing LA activity by direct nesslerization process[21] where quantitative estimation of LA production was done spectrophotometrically by quantifying ammonia released after catalysis of L-asparagine and was detected by estimating the optical density at 450nm. The LA activity was expressed in U/ml. In order to carry out specific activity determination, the culture filtrates were also subjected to total extracellular protein estimation (by Lowry's method) and specific activity of LA (U/mg) was determined.

(iii) Molecular identification of fungal isolate: All the isolates were identified tentatively based on morphological microscopic examinations. The isolate capable of growing luxuriantly in 2-5M salt concentration was subjected to molecular identification based on amplification of ITS-rDNA fragment. The isolate was encoded as HF1 for this purpose. Based on the sequence similarity search (using BLAST) of the amplified gene product, the test fungus was identified.

Effect of carbon source concentration on LA activity by isolate HF1: In order to see the effect of carbon source concentration on LA activity of the isolate HF1, the isolate was inoculated in modified Czapek Dox medium containing varying concentrations of glucose (0.3%, 0.5% and 0.8%) and 3M NaCl. After initiation of pink hue in the broth, filtrate was withdrawn for next 6 days and LA activity was measured, pH of the filtrate was also monitored. Total protein content of the filtrate was also estimated and specific activity was calculated.

Results and Discussion

Sampling site, sample collection and isolation of fungi from saltpans: The isolation yielded seven fungal isolates which were named as RJ1, RJ2, RJ3... RJ7. Based on colony morphology and microscopic observations, all the isolates have been primarily identified as aspergilli. Hitherto mycobiota from salterns along Bhavnagar, India have not been reported. The present study is a report on fungi from saltern of this region.

Salt tolerance of the fungal isolates: RJ1 was the only isolate which was able to grow profusely even at 5M NaCl concentration within 4 days of incubation at RT. Fungal isolates RJ2-RJ7 were able to grow efficiently at 3M NaCl concentration within 4 days and at 4M NaCl concentration within 6-8 days of incubation period at RT. Hence, among all halophilic fungal isolates, RJ1 has a remarkable tendency of growing even at 5M NaCl.

Screening of fungi for LA production:

(i) Primary screening: All the seven isolates exhibited pink zone surrounding their growth on modified Czapek Dox agar plates amended with phenol red, suggesting LA production (Figure 1). RJ1, RJ4, RJ5, RJ6 and RJ7 displayed pink zone after 4 days of incubation while in case of isolates RJ2 and RJ3 pink zone was observed after day 8. Hence, halophilic fungi from salterns are a potential source of LA. Interestingly, 100% positive results were obtained in the present study. In case of marine-derived fungi from Bhavnagar coast, 70% of the isolates were found to be LA producers[48].

(ii) Secondary screening: As in plate assay, in broth also all the test isolates exhibited pink coloration indicating LA production. In activity staining on agarose gel plate, development of yellow-brown ring surrounding the filtrate further confirmed LA activity (Figure 2). Salterns were previously considered to be inhabited only by halophilic bacteria, archaea and algae but not by fungi at all.

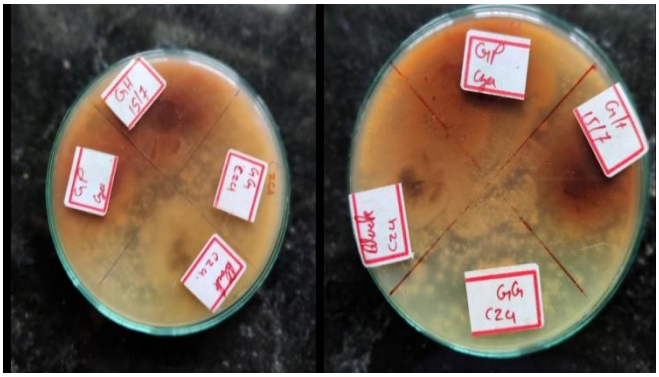


Figure 1: Development of pink zone surrounding test isolates on Modified Czapek Dox Agar indicating LA production

However, such hypersaline environments have been identified later to possess rich fungal diversity with biotechnological potentials[15,16,27,49]. LA activity shown by all isolates in the present study suggests that this field is very promising and worthy of attention. Quantitative analysis of LA activity revealed RJ7 to be the highest producer with specific activity of 25.06 U/mg among all the fungal isolates (Figure 3). RJ1 with LA activity 2.275 U/ml and specific activity 9 U/mg was quite behind RJ7, however, due to its adaptability at all test NaCl concentrations (2M-5M), it was selected for further studies. Enzyme production can be enhanced by employing optimization strategies[45,46]. An increase in pH upto 8.5 was also observed in all isolates.

Figure 2: Activity staining of LA produced by*Aspergillus unguis strain LS-MKBU*

Molecular identification of fungal isolate: Molecular identification of isolate RJ1 (encoded as HF1) was carried out. The selection was based on the ability to grow profusely in the range of 2M to 5M NaCl concentration. The isolate HF1 was identified as *Aspergillus unguis* strain LS-MKBU and the sequence has been deposited to NCBI GenBank with Accession No. GenBank: OR405853.1.

Effect of carbon source concentration on LA activity by isolate HF1: The isolate HF1, identified as *Aspergillus unguis* strain LS-MKBU was examined for effect of carbon source concentration on LA activity at three different concentrations of glucose and it was observed that 0.3% sugar supported an increase in specific activity almost nearly four times (Figure 4) and it was more than the most efficient isolate RJ1.

Optimization of various parameters always influences enzyme production. While just by varying one parameter such an increase is observed, by employing systematic optimization strategies, several fold increase in activity can be envisaged. Vala et al[45] had observed 108.62% rise in LA production by a marine-derived *Aspergillus niger*. They further reported bench scale production of LA and antiproliferative activity against cancer cell lines using this strain[47]. Haseena et al[18] have reported LA from halophilic bacteria from salt pans of Tamil Nadu, India. However, LA of fungal origin from saltern has been reported here. Optimization for enhanced production and purification of LA is in progress in our laboratory.

Conclusion

Fungi from saltern revealed occurrence of LA. Interestingly 100% test isolates exhibited LA activity. The present finding reports LA activity from *Aspergillus unguis*. A noteworthy rise in specific activity was observed by varying carbon source concentration. The present finding paves the way to explore hypersaline environment for newer sources of LA, a commercially relevant enzyme.

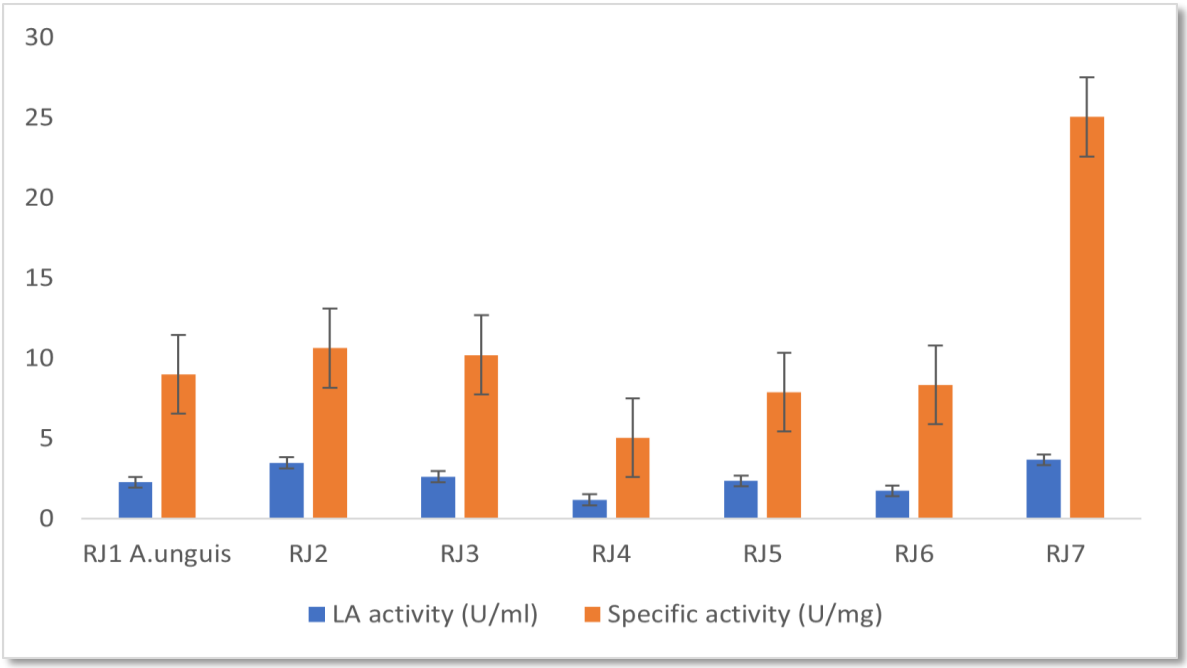


Figure 3: LA activity of fungal isolates RJ1-RJ7 after 15 days of incubation in Modified Czapek Dox Broth

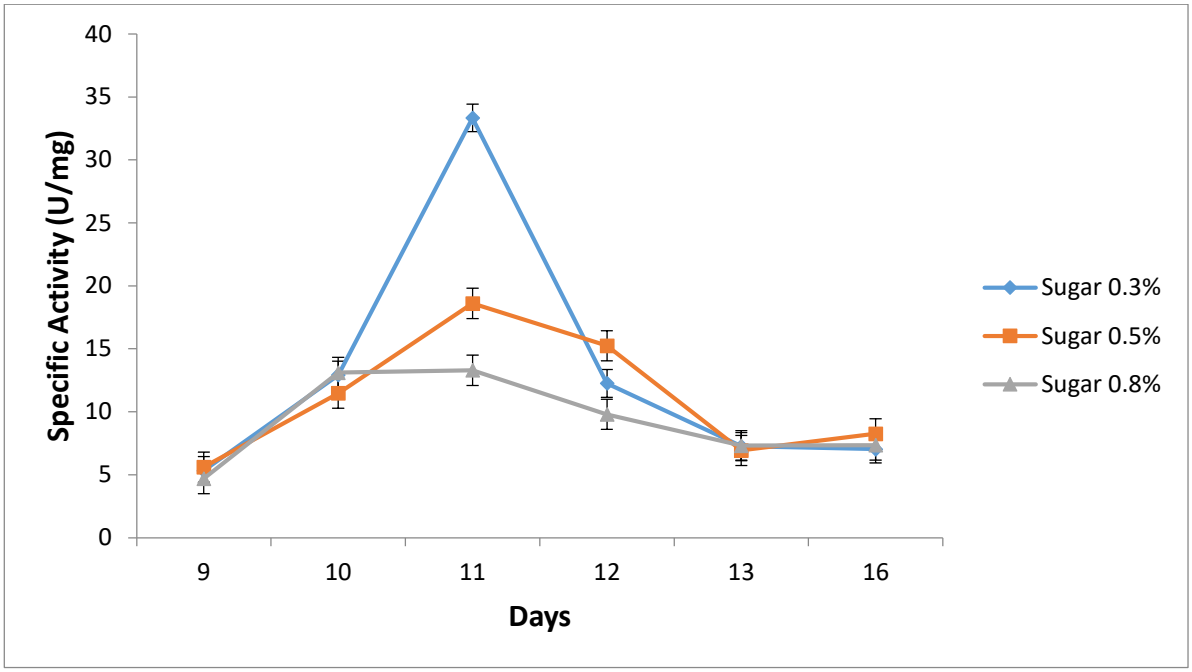


Figure 4: *Aspergillus unguis* LS-MKBU Specific activity for LA at various sugar concentrations

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(Received 14th October 2024, accepted 24th December 2024)