L ASPARAGINASE CHARACTERIZATION AND NANO ENCAPSULATION

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ABSTRACT:

Halophilic bacteria are organisms which inhabit the salt-rich environment were isolated and characterized by conventional and molecular methods. Sediment samples were collected from four thoothukudi salt pan, Tamil Nadu. Totally, 4 halophilic bacteria were isolated and their colony characteristics were recorded. The results of biochemical tests are in accordance with*Bacillus sp. Halobacterium salinarum* , *E.coli* and *Staphylococcus citreus* were identified based on the morphological, physiological and biochemical characterization..In this present study, we have screened moderate Halophilic bacteria for L-asparaginase production ability and showed that intra- and extracellular L-asparaginas was produced by Halophilic bacteria. The isolate T2 identified as*Halobacterium salinarum* only strain found to L asparaginase producer and enzyme assay was increased 34U to 82 U followed by immobilization with alginate and chitosan mixture. The total protein was also recorded higher in immobilized cell culture. It concludes that chitosan was best natural polymer effectively entrap the cells either alone or with alginate and improve the efficiency of cells.

Key words: *Halobacterium salinarum,*L Asparaginase, Immobilization,

INTRODUCTION

L- asparaginase characterization

Halophilic bacteria are belong to Halomonadaceae, a family of Proteobacteria. They are rich in many phylogenetic subgroups. These are aerobic, anaerobic, photoheterotrophic, chemoheterotrophic, and/or photoautotrophicindustrial activities. Itimparts to toxic organic compound contamination of nearby ecosystems, particularly those along coastal areas, which are the gateways forthe transport of manufactured goods worldwide(Wei*et al.* 2015). Microorganisms are living optimally at salty concentrations higer than 100 g/L fall under thecategory of halophiles. Hypersaline coastal habitats harbor unique andancient microorganisms, denoted halophiles,that surviveor even thrive in saline environments (DasSarma 2012).

Even molecular science with recent advancements in technology, the fraction of discovered microorganisms is minor and exploration is still needed. Halophiles are present in most noxious environments on the planet. This changes in the environment from the normal condition has allowed these extremophiles to develop rare skills for survival in nature.Because of halophiles acclimatization ability, their diversification are having large size in all hypersaline biotopes(Sarwar M.K. 2015).

Escherichia coli isproducing Asparaginase enzyme .It is a homotetramer. The molecular weight of the enzyme is 142 kilo Dalton.The enzyme L-asparaginase (ASNase) is used for acute lymphoblastic leukemia treatment. It is a key chemotherapeutic agent and belonging to the N-

terminal nucleophile family. ASNase is an amidohydrolase between Gly167 and Thr168 to become catalytically competent. It requires autocleavage other hematopoietic malignancies.It catalyses to asparagine (Asn) deamidation,resulting in the formation of aspartate(Asp) and ammonia as a by products（Medeiros*et al.*, 2018) .Since leukemic cells are auxotrophic for Asn , a reduction in the blood concentration of this amino acid resulting from ASNase action is an effective therapy for all, because under these conditions the cell cycle arrests in the G1 phase leading to apoptosis.However, the clearance of Asparaginase from blood plasma several methods are used such as immunogenic reactions and pharmacokinetic limitations that is for short half life. The recent new biotechnological techniques are used to reduce these problesms. The alternatives fro asparaginase production by using recombinant forms or different microbial sources have been studied. The employment of pegylation or immobilization methods by using nano encapsulation is as well as studied（Linge Wang*et al.,* 2012) . .

Until now, the first pharmaceutical technological innovation in the nanomedicine field concerning ASNase was the pegylation with the approval of Oncaspar in 2006, but this therapeutic enzyme has been used since 1994 when *Escherichia coli* ASNase was approved. ASNase nanoencapsulation into liposomes and polymersomes method studies are reported over 25 years. However, it has some limitations. Liposomes have the chemical instability that is the main drawback (Shikha *et al.*, 2014).

NANO ENCAPSULATION

Chitoson encapsulation

The one of revolutionary field of research is the targeted drug nanocarriers development. Cancer theranostics is regarded to leads to significant benefits. The nanocarriers enlightened design depends on the targeted cellls of particular characteristics. It is also the tumor microenvironment. Cancer cells targeting has dependent on the particular targeting ligands design and the over expressed receptors identification. The mononuclear phagocyte evade system is the irrespective of the targeting approach for the targeted nanocarriers capacity. However, the targeted nanocarriers still endure in the clinical development phase(Rodriguez G.R. *et al.,* 2013). To the modification of their surface with hydrophilicpolymers is the general strategy to avoid the rapid plasmatic elimination of nanosystems. The hydrophilicpolymers are polysaccharides, poly (ethylene glycol) (PEG) and poly (amino acids). The shield with these hydrophilic polymers enable nanostructures to circulate in the bloodstream for long periods of time and, eventually, to extravasate into the tumor tissues(Romberg *et al.*, 2007).

Collection of Samples

Soil samples were collected in sterile containers after carefully removing the surface layer (up to 10 cm) from salt pan of Thoothukudi District, Tamil Nadu, India. Latitude and longitude coordinates are: 8.764166, 78.134834.

Serial Dilution Method

Bacterial strains are isolated by enrichment method with 10% sodium chloride medium. Isolation and enumeration of bacteria were performed by the serial dilution and plate technique.1ml of the sea sample was taken in test tube containing 9 ml of sterile water. The particulate matter was allowed to settle down and the suspension was serially diluted up to 10-7 times. 1ml of 10^{-6} dilutions is transferred to sterile petri dish and overlaid with sterile molten nutrient agar medium prepared and is mixed added to 20 ml of s thoroughly mixed and poured into Petri plates and incubated at 28°C for 24h days (Anwar huq *et al.*, 2012).

Identification of isolated bacterial colonies

Colony Morphology Morphology of colony like Color, margin, opacity, production of pigments, reverse side color were visually determined by observing the agar plates during the incubation period (Upasana Bhumbla *et al.*, 2020).

Gram's staining : Thin smear of isolated bacterias were prepared on a clean glass slide and air dried. The smear was covered by crystal violet (CV) for 1 min. and then washed. The slide was treated with grams iodine (I) for 1 min. The CVI complex destained by 70 percent ethanol was added over the slide. After 30 sec the slide were washed and counter stained by Safranin for 1 min. and then washed. Stained slide was air dried and observed under 600X magnification on Nikon photomicroscope and their morphology of spore and mycelium was captured and followed by Ann C. Smith and Marise A. Hussey(2005) protocol.

Screening of L-Asparginase: The isolates were screened for L-asparaginase production by plate assay. The medium used for screening was minimal medium. The principle on which this screening was based is as follows: The phenol red indicator is present in the medium. The under alkaline condition turns pink in colour . The enzyme L-asparaginase will act on the substrate L-asparagine enzyme leading to production of ammonia which shifts the pH towards alkaline(Neelima Deshpande *et al,* 2014). If the organism has the ability to produce L-asparaginase enzyme then the pink color zone is will form on the colonies . Thus this qualitative method was used as a screening test for L-asparaginase producing bacteria(Gulati *et al.* 1997).

Effect of Carbon source on L-Asparaginase Production: Minimal medium with 0.1% phenol red was prepared and enriched with 1% of different carbon such as glucose, lactose, Starch, and glycerol. The medium was autoclaved and cooled. Freshly grown 24h culture was inoculated and incubated at 37° C under static condition. Similar set up was prepared and kept under shaking condition at 150 rpm for 24 h at room temperature. The medium contained phenol red indicator which turns pink in colour under alkaline conditions recorded as positive(Zia M.A *et a*l.,2013) .

Immobiliztion: Freshly grown Asparaginase producing bacteria was centrifuged and the cell pellet was diluted in PBS buffer to obtain an of 1 at 600 nm. About 1 ml of cell

suspension was mixed with polymers for entrapment(Alrumman. S. A. *et al.*, 2019).The entrapment of cells were done as follows

Group	Source
Group 1	Native cell
Group 2	0.3% Alginates in $CaCl_2$
Group 3	0.5 % Chitosan in 0.5 m NaOH
Group 4	0.1% of Alginate+ Chitosan (1:1)

Production of L asparaginaase: The selected isolates were allowed to grow on 200 ml M-9 broth was prepared with 1% lactose and 0.01% Asparagine. The enzyme production flasks were incubated on rotary shaker for 24 hours. At the end of incubation period, fermentation medium was taken and centrifuged.protein was precipitated with saturated ammonium sulphate and kept under refrigeration for overnight. Precipitated protein was collected by centrifugation at 10,000 rpm and dialyzed against phosphate buffer to purify the protein.

Estimation of protein: The amount of protein was determined by the method of Lowry *et. al.,* using BSA as the standard protein. The working standard BSA was prepared from stock 1mg/ml as given in table 1. The standard and samples were mixed with alkaline CuSo4 reagent and incubated 30 min. 0.2 ml of phenol follin was added to all tubes and incubated 15 min and reading was taken at 550 nm. Dh2O and reagent used as blank. Standard OD values are plotted on graph against concentration and protein concentration was estimated from the graph.

Enzyme assay: Quantitative enzyme detection was carried out by a Nesslerization method. A 0.5 ml protein, 1.0 ml of 0.1M sodium borate buffer (pH 8.5) and 0.5 ml of 0.04M L-asparagine solution were taken and incubated at 37ºC for 10 minutes. 0.5 ml of 0.1N trichloroacetic acid was added and the reaction was terminated.After it kept for centrifugation.The precipitated protein was separated and direct nesslerization is used to determined the liberated ammonia. 1ml of 1N NaOH and 0.2 ml of 0.1M EDTA was added individually to each sample. After 2 minutes 0.5 ml of nessler's reagent was taken and mixed. Suitable blanks of substrate and enzyme containing sample were included in all assays. After 5 minutes Nessler‟s reagent was taken into the sample, the yellow colour was determined on 117 UV-Visible spectrophotometer (systronics) and the OD of the sample was determined at 450nm(Lalitha Devi.A.S. *et al.,* 2016).

Standard graph: Into a series of test tubes, 2 ml of (0.05M) borate buffer was taken. To this 1 ml of each 1 mM, 0.75 mM, 0.5 mM and 0.25 mM of working solutions ammonium sulphate were added. From this 1 ml sample was withdrawn and delivered into 2.5 ml of 0.1N trichloroacetic acid.1 ml of 1N NaOH was taken to each sample. 0.2 ml of EDTA (0.1M) was taken to each sample to overcome the encountered turbidity. After 2 minutes., 0.5 ml of Nessler reagent was taken . The OD was measured after 5 min. at 425 nm. Blank was prepared by adding 1 ml of water instead of ammonium sulphate solutions(Akhilesh*et al.*, 2012).

The amount of enzyme that released 1 µmole of ammonia from L-asparagine per minute at pH 8.5 at 37ºC is known as one unit (IU).

Biochemical characterization: The isolated bacterial were subjected to morphological, physiological and biochemical confirmations(Sawale et al., 2013)

Indole test: Sterile Indole medium were prepared and sterilized by autoclaving at 15 lbs (121° C) pressure for 15 min. The isolated *Actinomycete*s sp. was inoculated on indole broth and incubated at 35°C for 24 h. After that, few drops of Kovacs reagent were added to the test tubes and the results were recorded(Charles*et al.*, 2015).

Methyl red test: MR-VP was prepared and sterilized by autoclaving at 15 lbs (121° C) pressure for 15 min. Isolated *Actinomycete*s was inoculated and labeled respectively in the MR-VP broth and incubated at 35° C for 24 hours. After incubation 5 drops of 0.4 per cent methyl red reagent were added to each tubes and the color change were observed.

Voges-Proskauer test: Isolated *Actinomycete*s was inoculated and labeled respectively in the MR-VP broth and incubated at 35° C for 24 hours. After incubation 1-2 drops of Barritt's Reagent A (α-napthol) and 2-3 drops of Barritt's B (40 % KOH) were added and color change were recorded.

Citrate utilization test: The isolated *Actinomycetes*colonies werecollected from a straight wire and inoculated into Simmon's citrate slants and incubated overnight at 37° C. If the organism has the capable to utilize citrate, the medium colorwillchange green to blue.

Catalase test: A loop full of culture was placed on the clean glass slide. To the slide 3 percent H_2O_2 were added and allowed to react for 30 sec. The presence of the effervescence was recorded as catalase positive and absence were catalase negative.

Oxidase test: Plates were prepared with isolated *Actinomycetes* sp and sterile oxidase disc were placed over the surface of colonies. The change of color on disc was noted. If the area of inoculation turns dark blue to maroon to almost black, then the result is positive. If a color change does not occur within three min the result are negative.

RESULTS AND DISCUSSION

Colony Morphology And Gram Staining

Totally 18X 10^7 bacterial colonies were isolated by crowded plate method (plate 1). From the present investigation, only 4 morphologically diverse isolates were subjected to further works. Morphologically three distinct colonies were designated as T1-4. Colonies were irregular rhizoidal, pigmented (T3), and others are circular and punctiform either opaque or translucent in nature. All the four isolates were subjected to Gram staining; their results showed that most of the isolates were Gram positive rod shaped bacilli (plate 2). Interestingly, T1, are positive and irregular in shape (pleomorphic). This present study by Sawale*et al.*, (2013) had revealedyhat the presence of cocci , several gram positive rod and gram negative varying size rod shaped bacteria. Among all the isolates, only TSP2 and TSP 3 were found to be Gram negative rod.

Biochemical Tests

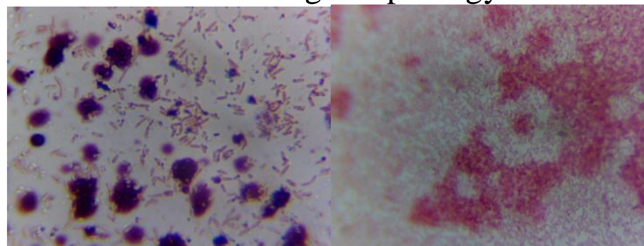
The biochemical tests showed that most of the isolates showed negative for Indole, Methyl red, Voges Proskauer and catalase, oxidase tests (Table 1). Based on biochemical features isolates were identified as, *Bacillus subtilis, , Halobacterium salinarum. E.coli,* and *Staphylococcus citreus* . Antón *et al.,* 2002 had demonstrated the novel halophilic bacteria, *Salinibacter* sp. isolated from solar salterns of Spain. Further biochemical and molecular characterization are used for the confirmation of isolates. Similarly, Saju *et al.*, 2011 isolated and characterized the halophilic bacteria such as *Vibrio fischeri, Halobacillus salinus, Halobacterium salinarum, Bacillus subtilis* and *Staphylococcus citreus* from salt pans of Kovalam, Kanyakumari district.

Table 1: Biochemical Results for Halophilic Bacterial Isolates

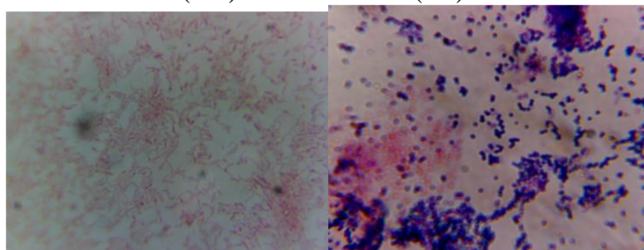
Culture code	Indole	MR	VP	Citrate	Catalase	Oxidase
T1	Negative	Positive	Negative	Positive	Positive	Positive
T2	Negative	positive	Negative	Positive	Negative	Positive
T3	Negative	Negative	Negative	Positive	Positive	Positive
T4	Positive	Positive	Negative	Positiv	Positiv	Positiv

Plate 1: Isolation of Bacterial Colonies by Conventional Method

Plate 2: Gram staining morphology of isolates



T1(G^+) rod T2(G^-) rod



T3(G^-) rod T4(G^+) cocci

Growth and Enzyme screening

Among the four isolated bacterial stains, Isolate designated as T2 and found to be positive on L asparginase production (plate 2).The pink colored zones around the colonies showing L - Asparaginase . Halophiles are salt loving organisms that inhabit hypersaline environments and rare isolates only found to be positive for L asparaginase production during screening(Krishnan*et al.,* 2017) . Microorganisms inhabiting these environments are expected to have proteins with different features than proteins of non-saline environment organisms. They have halophilic enzymes. With modified structure that creates tolerance of high salt concentration and low water activity. Therefore, Halophilic bacteria may contain L-asparaginase with novel immunological properties that can be used in hypersensitive patients (Rohban*et al.*, 2008). The growth rate of OD at 600 nm was 0.18, and 0.2, respectively under staic and shaking condition (plate3). Influences of growth and enzyme production with different carbon source (plate 4) was given in figure 1. It was found that Poor growth rate of *Halobacterium salinarum* was recorded at starch. A moderately growth was given by glucose and significantly at lactose with strong enzyme production . Among the three tested carbons glucose, lactose, and starch lactose was found to be best.Asparagine or glutamine consumption releases ammonia which increases pH and will cause indicator's color change from yellow to red (in phenol red) or blue (in bromothymol blue) (Mahajan *et al.,* 2013).

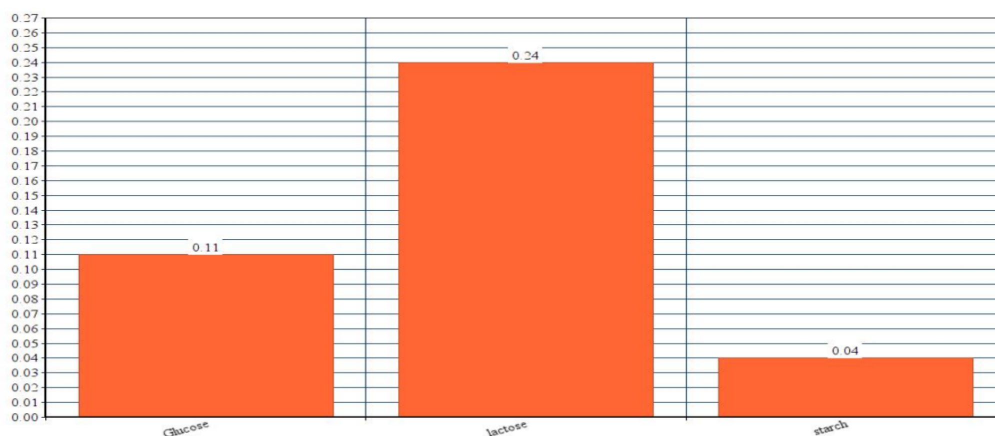


Figure 1: Effect of carbon on growth of *Halobacterium salinarum*

Enzyme assay

The optimal condition for L-glutaminase production was predicted at 37 °C, with 1% lactose. The estimated total protein was given in estimated as 0.15, 0.26, 0.29 and 0.32 mg/ml respective for non-immobilized and immobilized cells. The extra cellular enzyme was isolated by ammonium sulpahte precipitation (plate 6). A extracellular production of the enzymes is the advange of the isolated strain. The downstream processing is easier because of the enzyme secretion to the culture medium and cheaper by omitting the cell disruption. The crude protein enzyme assay given in plate 7. The asparaginase activity

immobilized and non immobilized cell given in figure 2. The maximum enzyme activity was 82 U/g by cells immobilized with mixture of Chitosan and alginate followed by chitosan alone an enzyme activity of 67 U/mL. The predicted enzyme production by alginate immobilized cell checked and the value of 42 U/mL and the control cells are 34U/ml. The immobilized enzyme was more stable at pH 9.0 for 50 min by halophile was reported by El-Refai *et al.*(2014). The immobilized aspargiase enzyme producing cells remained 100% active at temperatures up to 60° C, while the free asparaginase was less tolerant to high temperatures.

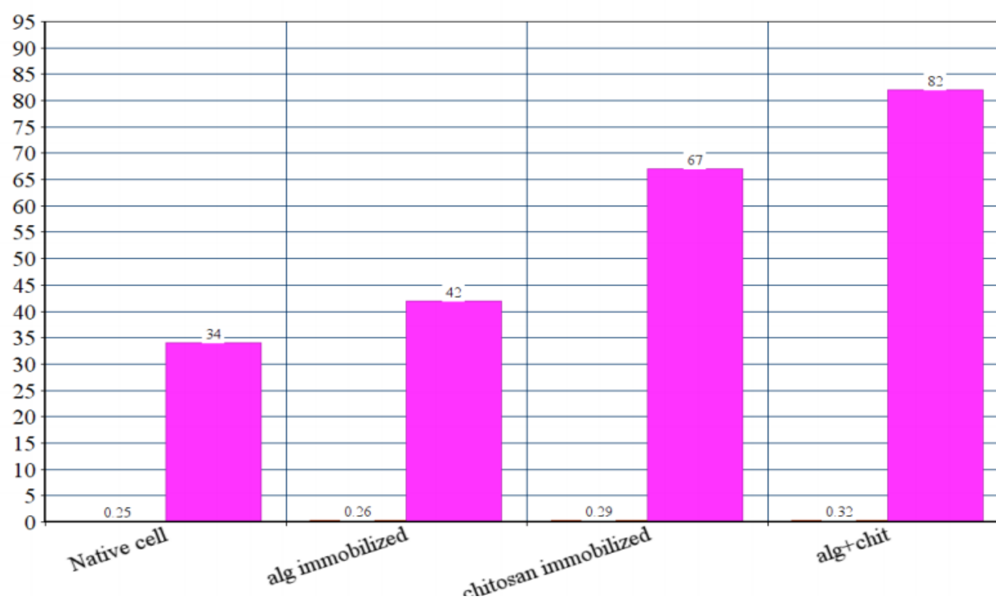


Figure 2: Total protein (mg/ml)and enzyme assay (U/g)of different immobilized group

Table 2: Total protein estimation:

SAMPLE	CONCENTRATON
G1 un immobilized	0.25
G2 chitosan immobilized	0.26
G3 alginate immob	0.29
G4 Chit+alg	0.32

Plate 2: Screening of L-asparaginase plate assay

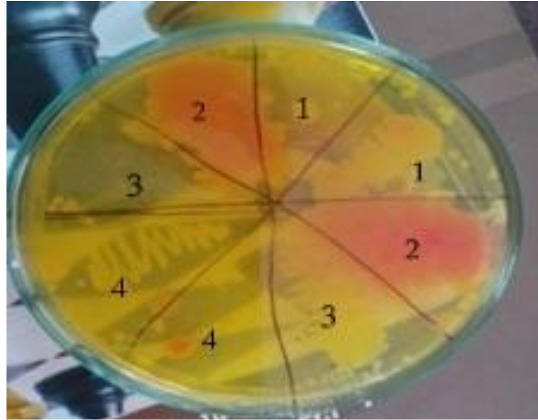


Plate 3: growth under shaking and static

Plate 4: Effect of carbon on Enzyme production (lactose, sucrose, Glucose, xylose, starch)

Plate 5: immobilization of enzyme producer

Plate 6:Total protein

Plate 7:Enzyme assay

SUMMARY AND CONCLUSION

Halophilic bacteria are organisms which inhabit the salt-rich environment were isolated and characterized by conventional and molecular methods. Sediment samples were collected from four thoothukudi salt pan, Tamil Nadu. Totally, 4 halophilic bacteria were isolated and their colony characteristics were recorded. The results of biochemical tests are in accordance with*Bacillus sp.* Halobacterium salinarum , *E.coli* and *Staphylococcus citreus* were identified based on the morphological, physiological and biochemical characterization..In this present study, we have screened moderate Halophilic bacteria for L-asparaginase production ability and showed that intra- and extracellular L-asparaginas was produced by Halophilic bacteria (Alireza *et al*., 2011). The isolate T2 identified as*Halobacterium salinarum* only strain found to L asparaginase producer and enzyme assay was increased 34U to 82 U followed by immobilization with alginate and chitosan mixture. The total protein was also recorded higher in immobilized cell culture. It concludes that chitosan was best natural polymer effectively entrap the cells either alone or with alginate and improve the efficiency of cells.

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