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Screening and Identification of Asparginase and Glutaminase Producing Halophilic Bacteria from Natural Saline Habitats

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Abstract

Halophiles are excellent source of enzymes that are not only salt stable but also can withstand and carryout reaction efficiently under extreme conditions. Screening of halophiles from 5 different saline habitats led to isolation of 34 bacterial isolates which showed glutaminase and asparaginase activity. Based on primary and secondary screening out of the 34 isolates, the 2 most potent strains were selected for further studies. Those were identified based on 16S rDNA sequencing. These isolates were also screened for various industrially important enzymes such as protease, amylase, phosphatase and cellulase. The present study is also focused on growth study, substrate optimization and enzyme activity of 2 antineoplastic enzymes such as asparaginase and glutaminase at different time intervals.

Keywords: Halophiles, Asparaginase, Glutaminase, Antineoplastic Enzymes.

Introduction

The Marine environment harbors millions of species of microorganisms. They have a diverse range of enzymatic activities and capable of catalyzing various biochemical reactions with novel enzymes (Chandarasekaran, 1997). They are the unique organisms which are a promising source for our Biotechnological future. In course of time, they developed their own mechanism which resulted in the synthesis of new biologically active substances. With these potential microbes are used in Industrial processing, medicines and foods. Due to their biochemical diversity, the enzyme concentration can be increased by environmental and genetic manipulation. In today's Research World, many attempts are being made to replace enzymes. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controlled and reliable.

Halophiles are a group of microorganisms that can grow and often thrive in areas of high salt (NaCl) concentration including various oceans, seas, estuaries, backwaters and hypersaline

lagoons. Most commonly observed halophiles are Archaea and Bacteria such as *Halobacterium*, *Halomonas* and *Salinibacter* (Oren, 2015). Diverse response mechanisms of halophiles under high-salinity conditions cause the production of various valuable biomolecules (Rungaroon *et al.*, 2016). Halophilic microorganisms are a potential source of extremozymes called halozymes like proteases, amylases, nucleases, lipases, cellulases, xylanases, catalases, and esterases, which are capable of functioning under high concentrations of salt, wide range of pH values, and temperatures at which other proteins will usually precipitate or denature (Sonika *et al., 2016).* These halozymes can be exploited in the presence of organic solvents and extremes in temperature and salt content wherever enzymatic transformations are required to function. Over the past few decades several efforts have been put on discovering the potential abilities of these extremophiles and using them efficiently in therapeutic procedures. Therapeutic enzymes like L-asparaginase, L-glutaminase, α- and β-glucosidase and β-galactosidase have been used as antineoplastics (Bar, 1970). The important features of these enzymes that distinguish them from all other types of drugs are high affinity, specificity and catalytic efficiency. Thus, halophiles can be used in extraction of drugs for good use in lymphoblastic leukemia and tumor cells (Pejman *et al.*, 2016).

Biomedical sciences also accentuate the involvement of the enzyme L- Glutaminase and other amino acid depleting enzymes as a therapeutic agent for the treatment of tumor (Holcenberg, 1982). Either the use of enzyme prodrug therapy or the use of antineoplastic enzyme therapy can be used in cancer treatment. Using these treatments tumor proliferation is thus often reduced. Although both normal cells and tumor cells they need them in large amount for cell growth, they cannot synthesize L-asparagine and L-glutamine. Asparagine and glutamine are non-essential amino acids used by immature lymphocytes for their proliferation and run as substrate for respiration, nitrogen for the production of hexosamines, proteins, and macromolecules (Unissa *et al*., 2014). As a result they are considered as one of the key molecules in cancer metabolism through cell proliferation. It has an antiviral effect (Kumar and Chandrasekaran, 2003). The first observation for L-glutaminase as antileukemia agent was by Greenberg *et al,* 1964. It is used in the treatment of cancer (Roberts *et al*., 1970, 2001) and HIV (Schmid and Roberts, 1974). L-glutaminase has been used as biosensors to monitoring L-glutamine level in mammalian and hybridoma cell lines (Huang *et al*., 1995).

The most potent enzymes used for the treatment of leukemia for more than 40 years are bacterial sources which are mostly produced from *Erwinia carotovora* and *E. coli* (Huang *et al*., 1995). Most of Enterobacteriaceae family members produce only L-asparaginase, while *Pseudomonas* species produce both L-asparaginase and L-glutaminase (Imada *et al*., 1973; Dutta *et al*., 2015). *Serratiamarcescens SB08, E. coli, P. pseudoalcaligenes JHS-71,* and *E. carotovora* produce L-asparaginase intracellularly (Venil *et al*., 2009; Sajitha *et al*., 2015; Faret *et al*., 2018), while *P. aeruginosa 50071* and *B. subtilis WB600* produce L-asparaginase extracellularly (Bessoumy *et al*., 2004; Yue *et al*., 2017). On other hand, L-glutaminase produced extracellularly by *Vibrio azureus JK-79* and *P. otitidis* (Kiruthika and Nachimuthu,

2013; Husain *et al*., 2016). Several plants such as tamarind, chilies and tomato contain appreciable quantities of L-asparaginase and L-glutaminase; onions, potatoes and lemons have trace quantities, whereas both enzymes could not be identified in ginger and drumsticks (Bano and Sivaramakrishnan, 1980). The present study dealt with the isolation and identification of halophilic bacteria from different saline habitats in Tamil Nadu, India namely; Kottivakkam, Tiruvottriyur, Thiruvanmiyur, Kovelong and Marina beach.

Methodology

Isolation of Bacteria

Sea water sample was collected from various saline habitats in Chennai, Tamil Nadu viz; Kottivakkam, Tiruvottriyur, Thiruvanmiyur, Kovelong and Marina beach and were brought to the laboratory in well-sealed sterile bottles. The bacteria were isolated by serial dilution and pour plate method on petri plates containing saline nutrient agar medium. The plates were incubated at room temperature. After 72 h of incubation, different bacterial strains were selected based on various morphological characters, and were taken into consideration for further studies.

Salinity

Salinity of the collected water samples was determined in terms of chlorinity. To 10 mL of sea water sample, 7.5 mL of potassium dichromate indicator solution was added. This was titrated against a standard silver nitrate (0.2184 M) solution taken in a burette. End point was indicated by the appearance of pale red color.

The salinity is a constant 1.80655 times higher than the chloride level. Thus, the salinity (S) can be calculated (using the equation Sppt=1.80655 Clppt)

Primary Screening of Asperginase and Glutaminase

The isolated bacterial strains were cultured in the screening medium. Modified M-9 medium was used for rapid plate assay containing $Na_2HPO_4.2H_2O$ 6.0 g/L, KH_2PO_4 3.0 g/L, NaCl 20.5 g/L, $MgSO_4.7H_2O$ 0.5 g /L, $CaCl_2.2H_2O$ 0.15 g/L, Glucose 2.0 g/L, Agar 15.0 g/L and L-asparagine or L-glutamine 5.0 g supplemented with 0.009% Phenol red (Anup *et al*., 2019). The isolated colonies were inoculated into this medium and incubated at room temperature with regular observation of color change around the bacterial colonies. The indicators colour change appeared around bacterial colonies were used as a sign for asparaginase and glutaminase production.

Secondary Screening of Asperginase and Glutaminase

For further verification, the modified M-9 medium was supplemented with 0.007% of Bromothymol Blue dye (BTB). The isolated colonies were inoculated into this medium and

incubated at room temperature with regular observation of color change around the bacterial colonies (Anup *et al*., 2019).

Purification and Maintenance of Culture

The isolated colonies were purified by further repeated quadrant streaking on agar plates containing saline nutrient agar medium. Only the best two out of 34 bacterial strains were chosen for further studies. The culture is maintained on 1.5% sterile agar (w/v) slants at 4˚C and subcultured fortnightly.

Screening for Various Industrially Important Enzymes

Various screening tests for industrially important enzymes were carried out for the selected two bacteria using different plate assays.

Amylase Plate Assay

The saline nutrient agar medium is supplemented with 0.25% starch and bacteria were incubated for 24-48 h. On addition of Logul's solution, clear zone may be seen around the colonies if there is any amylase production.

Cellulase Plate Assay

The bacteria were inoculated on plates containing saline nutrient agar medium supplemented with 0.25% CMC and incubated for 24-48 h. After incubation, the plates were flooded with an aqueous solution of congo red dye (1% w/v) for 15 min. Then, the plates were flooded with 1 M NaCl for 15 min. After that a zone of hydrolysis could be seen as clear area around the colonies, which indicates the cellulolytic activity of the organism.

Phosphatase Plate Assay

The saline nutrient agar medium was supplemented with 1% of tricalcium phosphate and the bacteria were incubated for 48-72 h. If phosphatase is present, a clear zone is observed around the colonies after 72h.

Protease Plate Assay

To the saline nutrient agar medium supplemented with 1% of gelatin, the bacteria were inoculated and incubated for 24-48 h. If protease is produced, a clear zone was observed around the colonies on addition of mercuric chloride solution.

Growth Study

L-asparaginase and L-glutaminase producing strains were inoculated in flasks containing 250 mL saline nutrient broth at room temperature for 72 h. The culture was harvested at every 6 h

interval for a period of 72 h. Growth kinetics was obtained by measuring the cell density at 660 nm at different intervals using UV- Vis Spectrophotometer.

Enzyme Assay

Enzyme activity was determined by nesslerization for both enzymes L-asparaginase and L-glutaminase (Gulati *et al*., 1997; Roberts *et al*., 2001). The isolated strains were inoculated in M-9 broth medium at room temperature. At every 6 h intervals, cell free crude enzyme was prepared by centrifugation at 6000 rpm for 20 min. To 0.5 ml of supernatant, 1.0 mL of 0.1 M sodium acetate buffer (pH 8.5) and 0.5 ml of various concentrations of 0.02 M to 1.0 M asparagine or glutamine solution were added and incubated at 37 °C for 10 min. The reaction was stopped by addition of trichloroacetic acid. The precipitated proteins were removed by centrifugation (1000 xg for 20 min). The amount of ammonia in supernatant was determined by nesslerization and expressed as U/mL. Nessler's reagent was prepared by adding 45.5 g HgI_2 and 35.0 g KI to 1 liter distilled water containing 112 g KOH. 0.5 mL of Nessler's reagent was added to the supernatant and the absorbance was determined at 505 nm using a UV/VIS spectrophotometer. One unit (U) of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 µmole of ammonia in 1 min at the conditions of assay. Different concentrations of ammonium sulphate $(NH_4)_2SO_4$ were used as standards.

Estimation of Protein (Bradford, 1976)

The protein content of the culture filtrate was estimated by the dye binding method of Bradford. To one hundred microlitre of the above protein sample 5 mL of protein reagent was added and mixed well. The optical density of the sample was read at 595 nm against a reagent blank. The amount of protein was calculated by using a standard graph prepared with different concentration of Bovine Serum Albumin (10-100 µg/mL).

Identification of the Bacteria

The isolated bacterial strains were identified by 16S rDNA sequencing. The 16S rDNA sequence was compared with sequences available in public databases, using the BLAST search program on the NCBI web site (http://www.ncbi.nlm.nih.gov) to find closely related bacterial 16S rDNA gene sequences. A phylogenetic tree was constructed by the neighbor joining method and maximum composite likelihood model.

Results and Discussion

Isolation of Bacteria

In the present study seawater sample from five saline habitats namely Kottivakkam, Tiruvottriyur, Thiruvanmiyur, Kovelong and Marina beach was subjected for isolation of bacteria by serial dilution and pour plate method. Based on different morphological

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characteristics, 34 bacterial isolates were selected (Plate 1). These isolates were subjected for primary and secondary screening.

Plate 1.Shows the isolation of bacteria

Measurement of Salinity

Salinity of the five different sea water sample was measured using Mohr's titration method. The initial color of the sample with the indicator is yellow which after reaction with titrant turns pale red (Plate 2).

Plate 2.Shows the color change during titration

When salinity was calculated using the equation Sppt=1.80655 Clppt, the maximum salinity was observed in Kovelong beach sample with a salinity of 8.5 ppt or 89% followed by Thiruvanmiyur beach sample with a salinity of 7.8 ppt or 78% as depicted by Figure 1.

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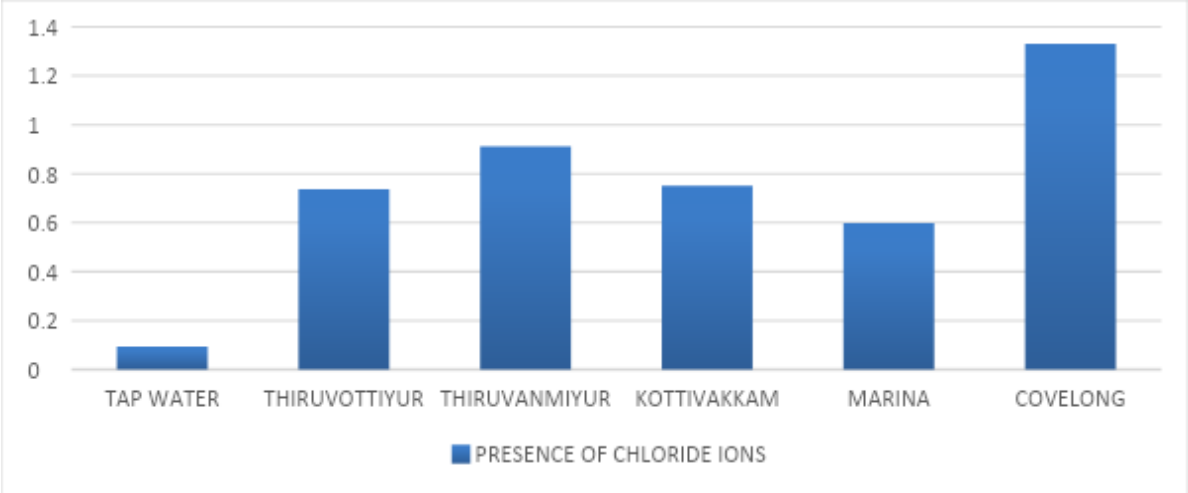

Figure 1.Shows the measurement of salinity of various seawater samples

Primary Screening of Asparaginase and Glutaminase

Total of 34 bacterial strains were isolated from sea water samples. All strains were screened for asparaginase and glutaminase production on M-9 medium supplemented with 0.009% Phenol red. It was observed that there was a gradual change in the colour zone transition around the colonies from buff colour to pink colour indicated the presence of asparaginase and glutaminase production. It was observed that among the isolated strains, , 3 strains (8.823%) have asparaginase activity and 13 strains (38.23%) have glutaminase activity. 14 strains(41.174%) show both asparaginase and glutaminase activity; while 4 strains (11.76%) strains show no asparaginase or glutaminase activity (Plate 3). The results indicated that strains with most asparaginase activity, namely T4, Th2, M6, M3, C8, C4 and C3 have no glutaminase activity (Plate 3).

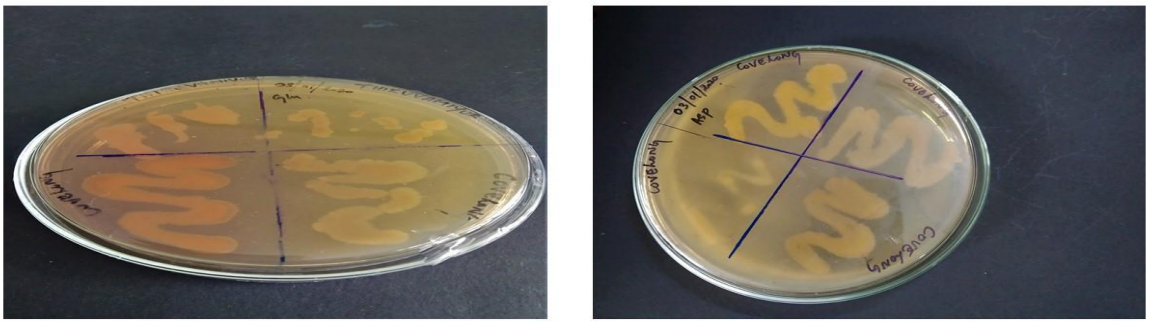

Plate 3.Shows the primary screening of isolated strains

Secondary Screening of Bacterial Colonies

All the 34 isolated strains were screened for asparaginase and glutaminase production on M-9 medium supplemented with 0.007% of Bromothymol Blue dye (BTB) showed a gradual change in the colour zone transition around the colonies from pale green to yellowish green indicated positive result for L-asparaginase and L-glutaminase production. It was observed

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that among the isolated strains, 11 strains (32.34%) have glutaminase activity and only 4 strains (11.76%) have L-asparaginase activity. 7 strains (20.58%) show both asparaginase and glutaminase activity; while 12 strains (35.28%) show no asparaginase or glutaminase activity. The results indicated that strains with the most L-asparaginase activity Th5, M6, M4 and M3 have no glutaminase activity (Plate 4).

Plate 4.Shows the secondary screening of the isolated strains

BTB showed a better zone of hydrolysis whereas plates containing phenol red dye showed less contrast. The results of BTB dye were similar to the results obtained using phenol red. Out of the 34 isolates screened, **strain C5 and strain Th1** showed positive growth of asparaginase activity and glutaminase activities in both primary and secondary screening were selected for further studies.

Purification

Based on primary and secondary screening two strains having maximum potential of asparaginase and glutaminase production were selected and purified by quadrant streaking method. Individual colonies were obtained and selected for further studies (Plate 5).

Plate 5.Shows the purification of bacteria

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Plate Assay for Industrially Important Enzymes

The purified strains were subjected to plate assays for the production of four industrially important enzymes i.e., amylase, cellulase, phosphatase and protease.

Plate Assay for Amylase

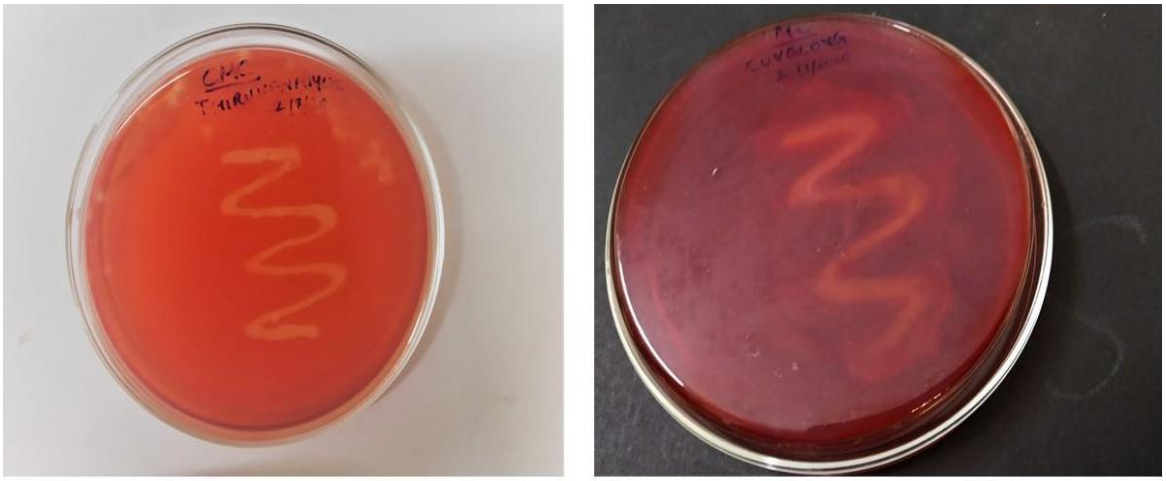
Both the bacteria C5 and Th1 did not show any clear zone around the colonies. Thus there was no amylase production (Plate 6).

STRAIN C5 **STRAIN Th1**

Plate 6.Shows plate assay for amylase

Plate Assay for Cellulase

The isolated bacteria C5 and Th1 were subjected for plate assay to check the production of cellulase. There was a clear zone observed around the colonies, which indicated the presence of the enzyme cellulase (Plate 7).



STRAIN C5 **STRAIN Th1**

Plate 7.Shows plate assay for cellulase

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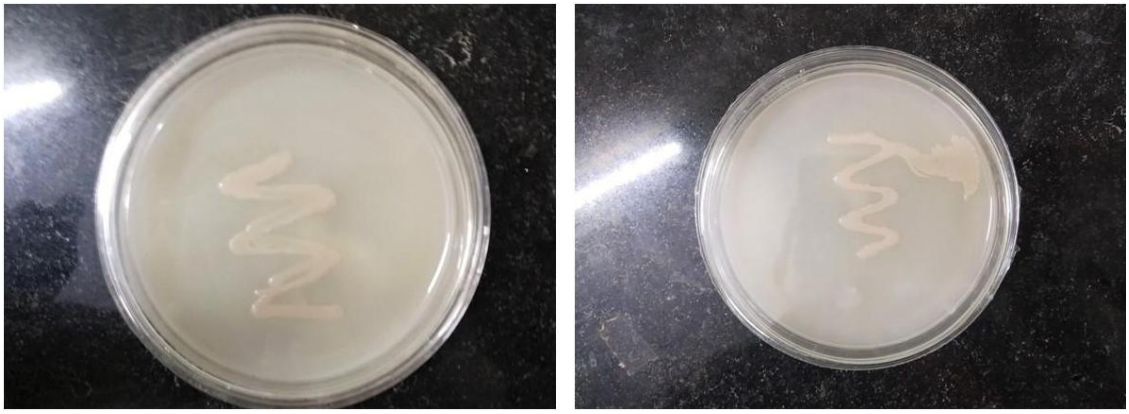
Plate Assay for Phosphatase

The asparaginase and glutaminase producing bacteria C5 and Th1 does not have the ability to produce phosphatase enzyme (Plate 8).



STRAIN C5 **STRAIN Th1**

Plate 8.Shows plate assay for phosphatase

Plate Assay for Protease

Both the bacteria C5 and Th1 showed clear zones after 24-48 h of incubation. Thus, they have the ability to produce protease enzyme (Plate 9).

STRAIN C5 **STRAIN Th1**

Plate 9.Shows plate assay for protease

Growth Study

Both the purified strains were studied for their growth kinetics as depicted by Figure 2. The growth of both the strains were studied for every 6 h over a period of 72 h of incubation at an absorbance of 660 nm.

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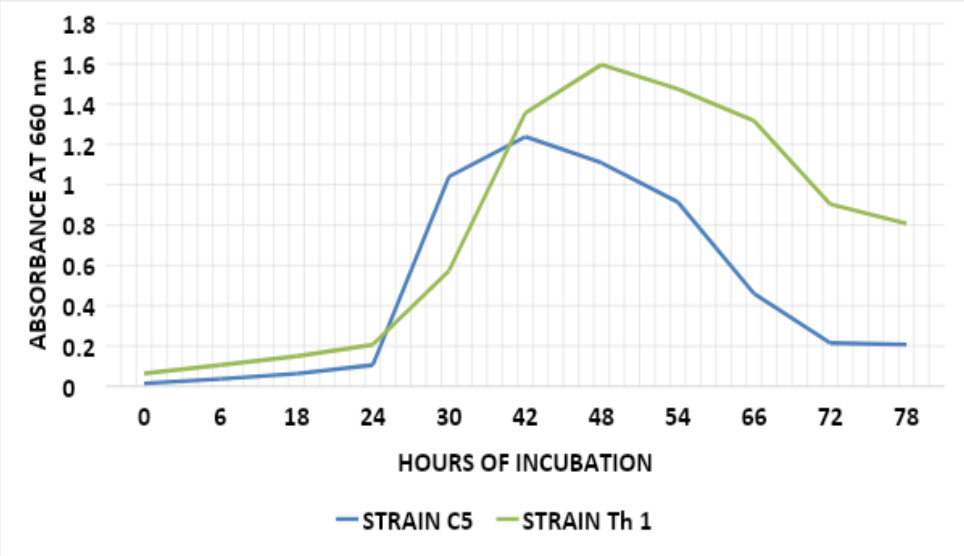

Figure 2.Shows the comparative growth study of C5 and Th1

The growth of the bacteria C5 and Th1 were studied for every 6 h over a period of 72 h of incubation at the absorbance of 660 nm. It was found that Th1 showed the maximum growth at 48 h (Fig. 2) of incubation after which the growth declined and for C5 the maximum growth was observed at 42 h (Fig. 2). Comparison of the growth curve and enzyme production for the selected isolates, C5 and Th1 showed that both enzymes were produced along with the bacterial growth (Fig. 3 and 4). Examining the cell extracts and culture supernatants for asparaginase or glutaminase activity, showed that both enzymes were secreted to the culture media.

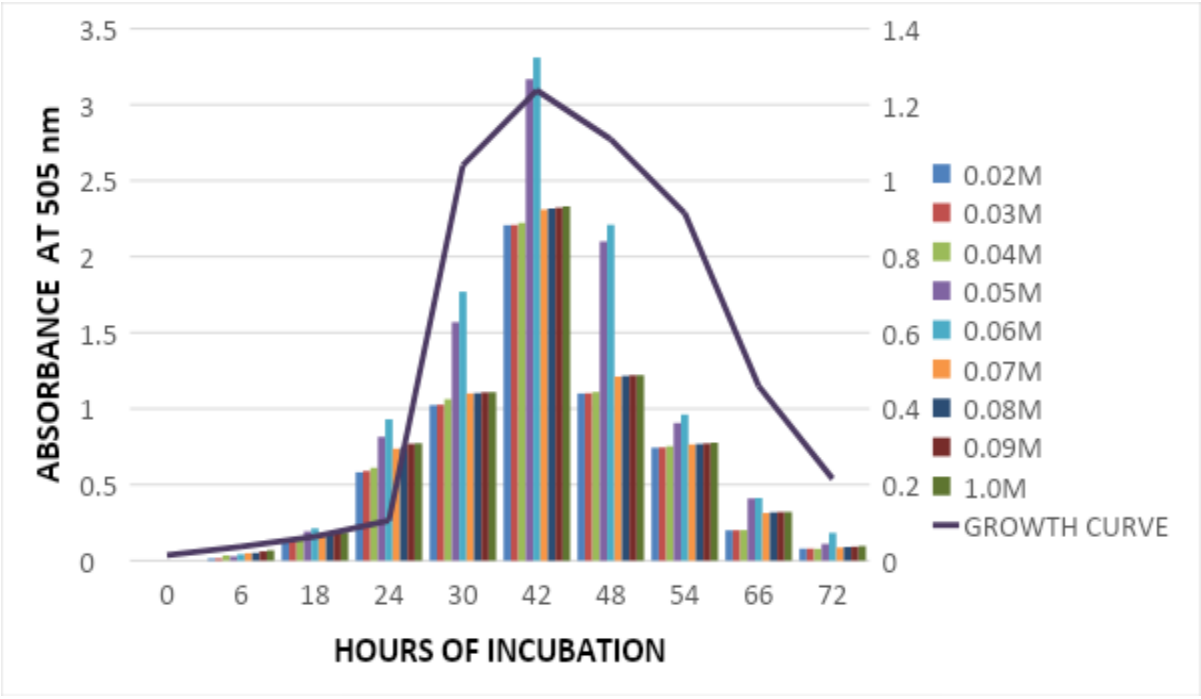

Figure 3.Shows the maximum enzyme production of C5

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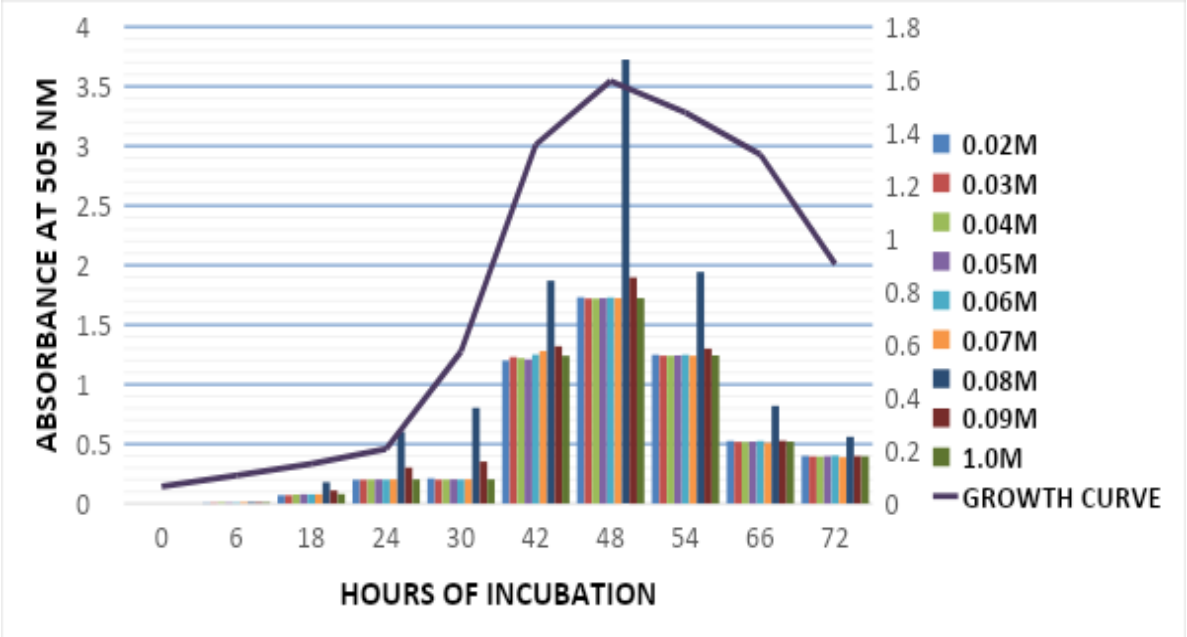

Figure 4.Shows the maximum enzyme production of Th1

Effect of Different Concentrations of Asparagine on Extracellular Protein Content and Asparaginase Production

Among the different concentrations of asparagine ranging from 0.02 M to 1.0 M, C5 produced maximum extracellular protein content of 1064 µg/ml at 48 h and maximum asparaginase production of 7.445 U/ml at 42 h in 0.06 M concentration (Fig.5 and 6). Similarly, Th1 produced maximum extracellular protein content of 1080 µg/ml at 54 h and maximum asparaginase production of 6.038 U/ml at 48 h in 0.08 M concentration (Fig.7 and 8).

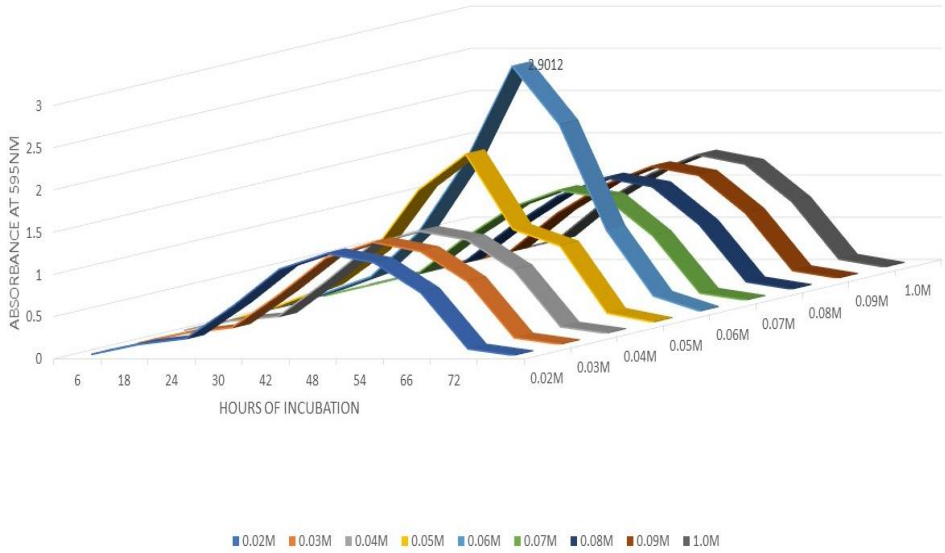

Figure 5.Shows protein estimation in strain C5 with various concentrations of asparagine

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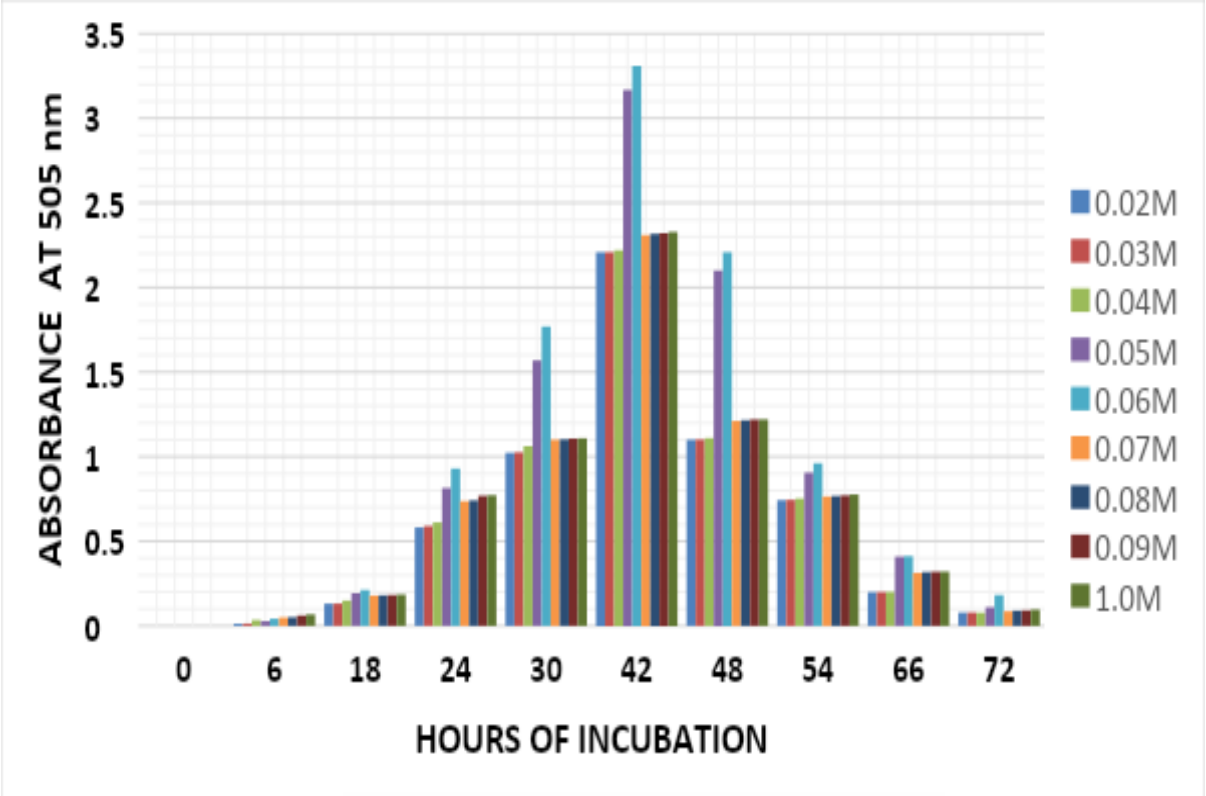

Figure 6.Shows production of asparaginase by C5 at various concentrations of asparagine

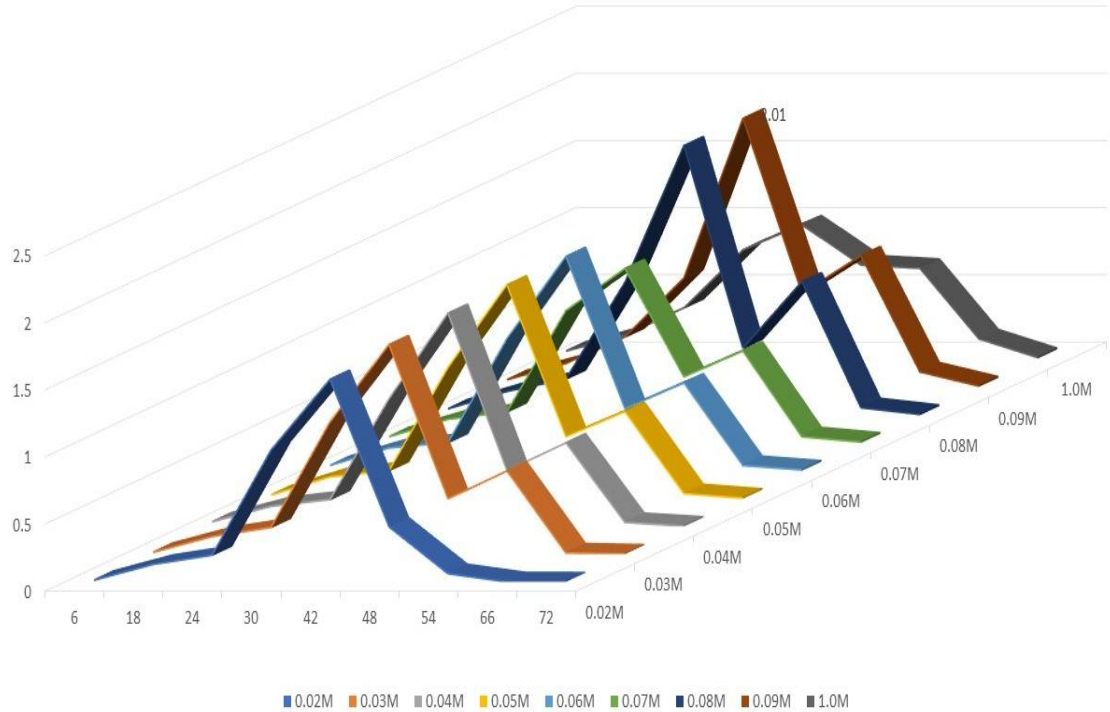

Figure 7.Shows protein estimation of strain Th1 at various concentrations of asparagine

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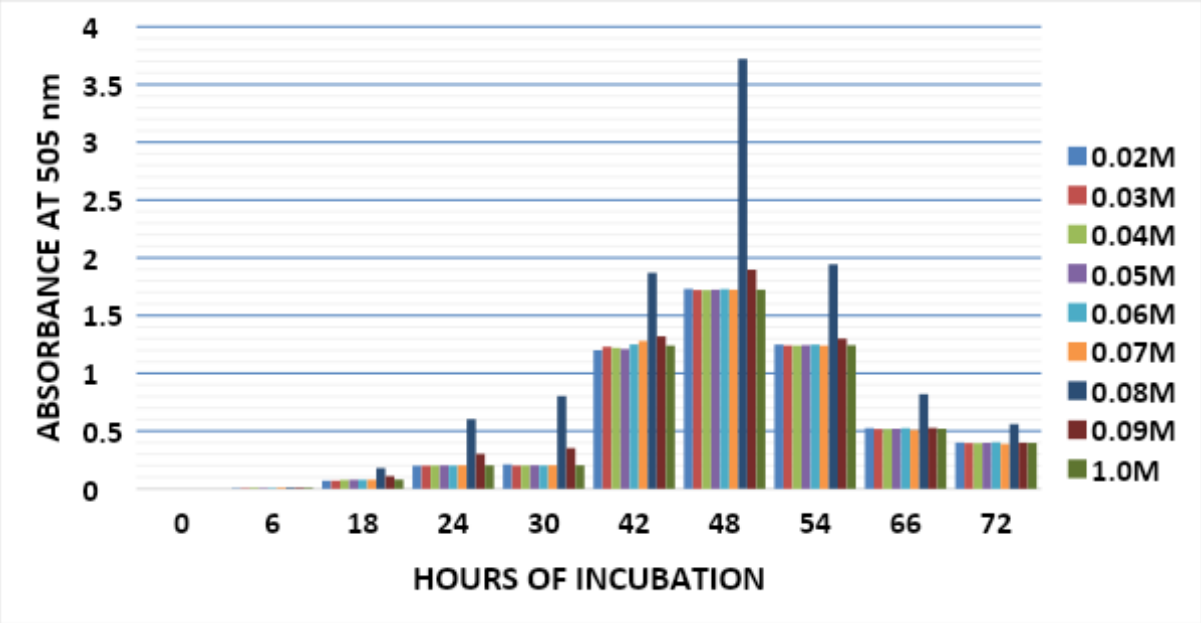

Figure 8.Shows production of asparaginase by Th1 at various concentrations of asparagine

Effect of Different Concentrations of Glutamine on Extracellular Protein Content and Glutaminase Production

The results showed that the different concentrations of glutamine significantly influenced the extracellular protein content and glutaminase production. C5 produced a maximum protein content of 1119 µg/ml at 48 h and maximum glutaminase production of 6.7017 U/ml at 42 h in 0.05 M concentration (Fig. 9 and 10). Similarly, Th1 produced maximum extracellular protein content of 965.75 µg/ml at 54 h and maximum glutaminase production of 6.699 U/ml at 48 hr in 0.1 M concentration (Fig. 11 and 12).

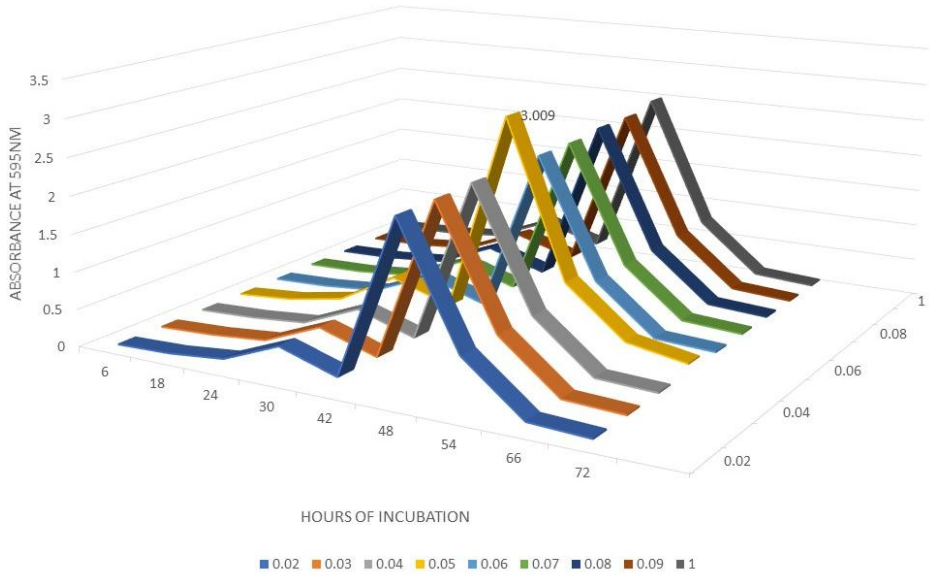

Figure 9.Shows protein estimation of strain C5 with various concentrations of glutamine

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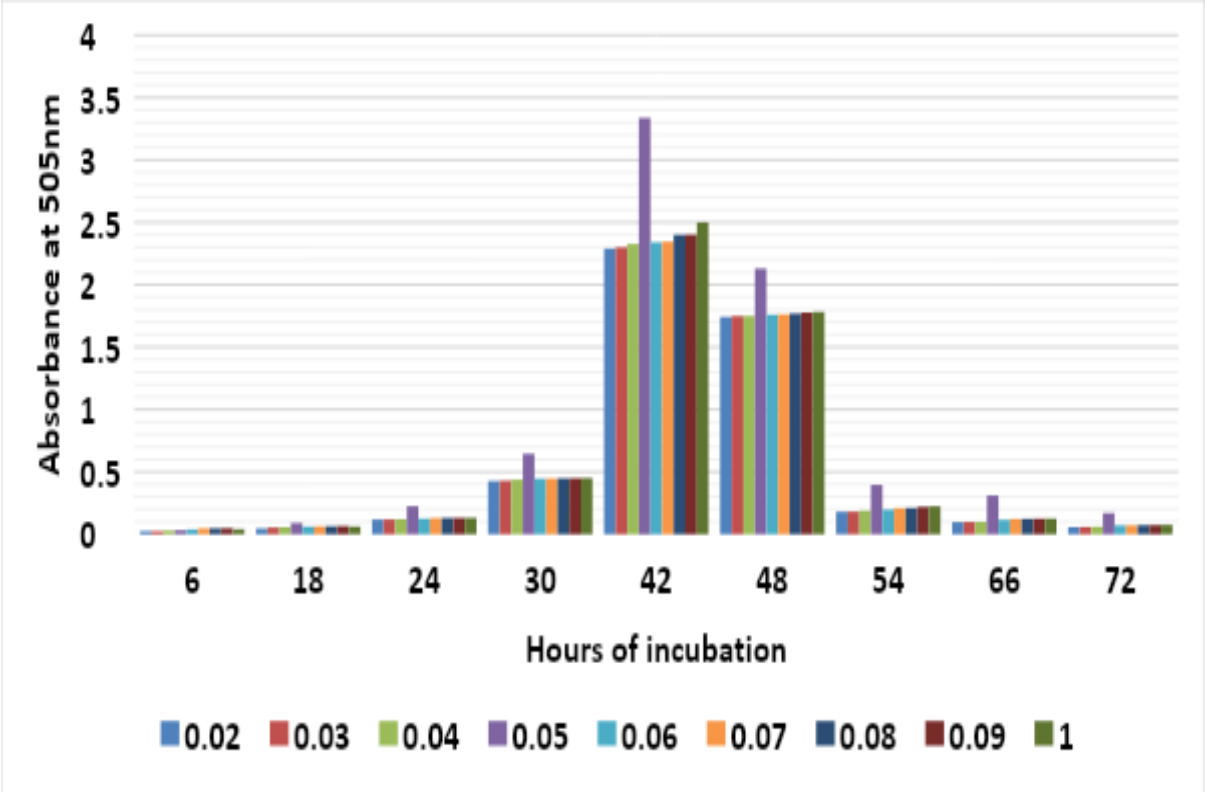

Figure 10.Shows production of glutaminase by C5 at various concentrations of glutamine

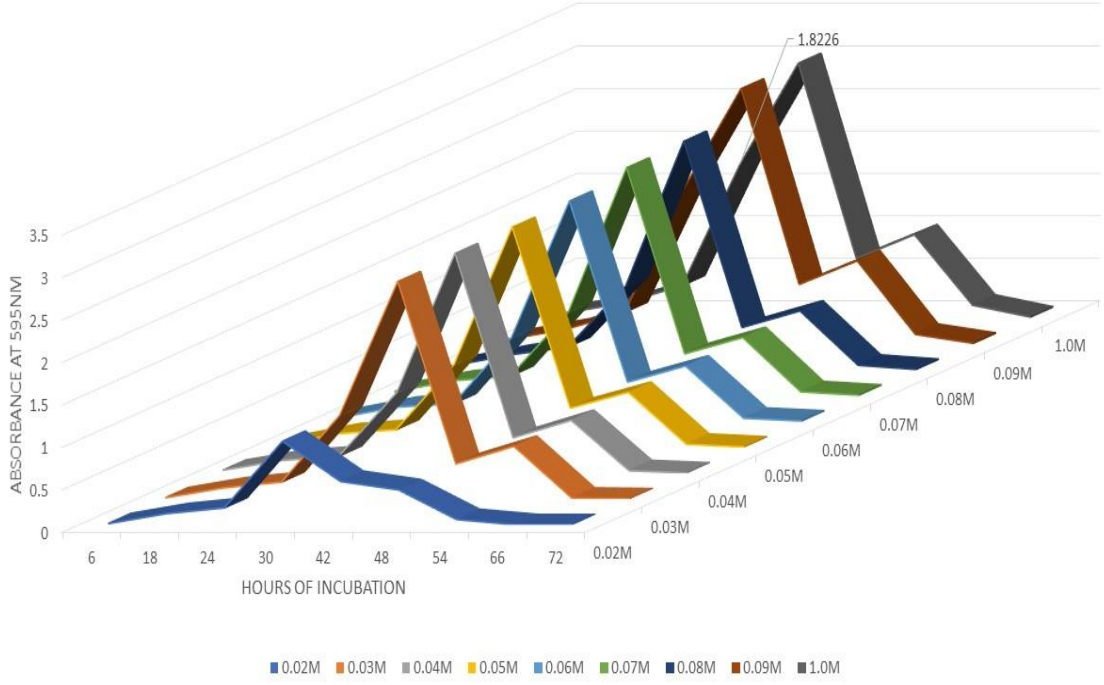

Figure 11.Shows protein estimation of strain Th1 at various concentrations of glutamine

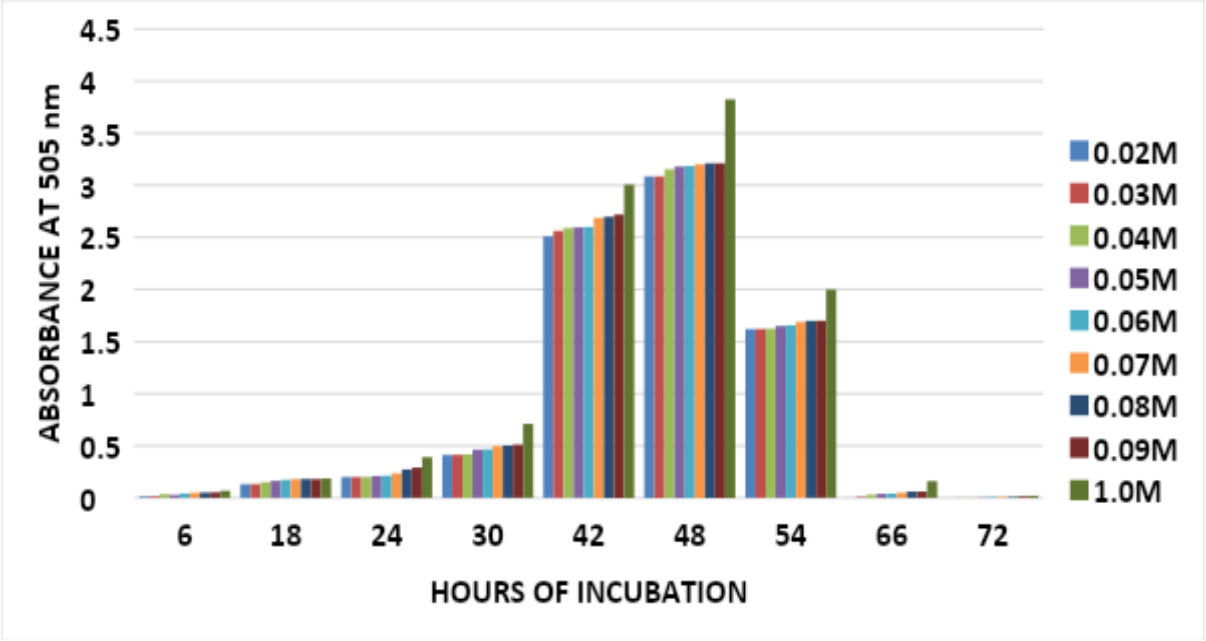

Figure 12.Shows production of glutaminase by Th1 at various concentrations of glutamine

The enzyme production was optimized for both producer strains by response to different concentration of substrates. There are some reports on the L-asparaginase and L-glutaminase production by different bacterial strains, in which 32.3, 51.54 and 135 U/mL of L-asparaginase production were described for *Streptomyces ginsengisoli* (Deshpande *et al*., 2014), *Bacillus cereus* (Thenmozhi *et al*., 2011) and *Streptomyces parvulus* (Rajamanickam *et al*., 2011), respectively. In the study of Ebrahiminezhad *et al*. (2011), considering L-asparaginase production by halophilic and halotolerant bacteria isolated from Maharloo salt lake, *Bacillus* sp. BCCS 034 was found to produce L-asparaginase extracellularly (1.64 IU/mL supernatant).

Iyer and Singhal (2009) reported 119 U/mL of L-glutaminase production by a *Providencia* sp. strain. In another report by Dilara and Emine (2014), 13.75 U/mL of the extracellular enzyme production was achieved by the isolated *Hypocrea Jecorina* strain.

In the present study ***strain C5*** produced a maximum of 7.445 U/mL asparaginase at 0.06 M concentration and it produced 6.707 U/mL of glutaminase at 0.05 M concentration. Based on screening results, it was shown that halophilic and halotolerant bacteria isolated from saline habitats, have the potential of L-asparaginase and L-glutaminase production. To conclude, more studies are needed to explore their potential as antineoplastic enzymes.

Identification

16S rDNA Sequencing

Among the two strains C5 and Th1 it was observed that strain **C5** showed more asparaginase and glutaminase activity. So, it was then identified through 16S rDNA sequencing. It Showed

99% homology with ***Pseudoalteromonas arabiensis*** .Thus the strain C5 was identified as ***Pseudoalteromonas arabiensis***. All the 16S rDNA sequences of related strains have been retrieved from the NCBI database. Genome accession numbers are shown in parenthesis: 0.005 denotes the genetic distance. The phylogenetic relationship of the strain ***Pseudoalteromonas arabiensis*** is shown in Fig. 13.

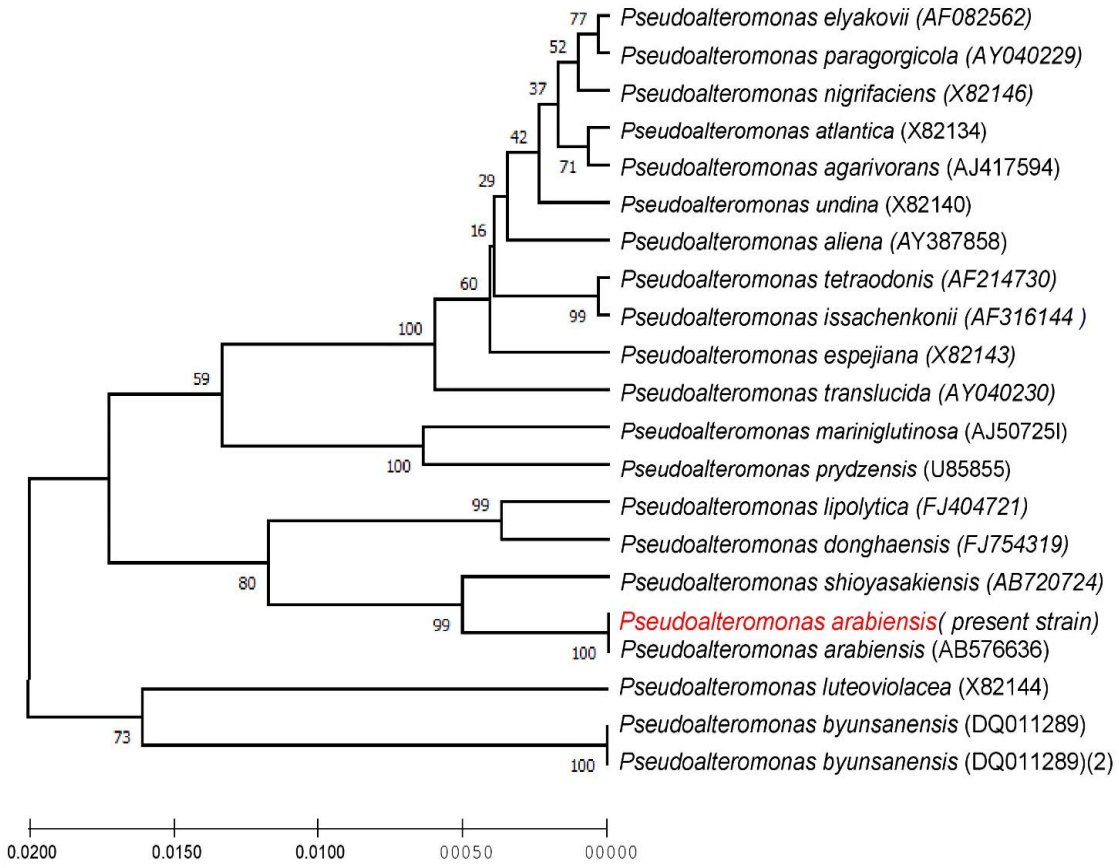

Figure 13.Shows the phylogenetic tree of *Pseudoalteromonas arabiensis*

Conclusion

Halophiles are excellent source of enzymes that are not only salt stable but also can withstand and carryout reaction efficiently under extreme conditions. In this study, screening of halophiles from 5 different saline habitats led to isolation of 34 bacteria. The most potent bacterial isolates in response to glutamine and asparagine were selected for further studies based on primary and secondary screening. These isolates were also screened for various industrially important enzymes such as protease, amylase, phosphatase and cellulase. The present study is also focused on growth study, substrate optimization and enzyme activity of 2 antineoplastic enzymes asparaginase and glutaminase at different time intervals. Strain C5 was identified as ***Pseudoalteromonas arabiensis.*** If more studies are done on halophilic

bacteria, their potential as antineoplastic enzymes can be explored and used in various therapeutical procedures.

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