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Anticancer Property Of L-Glutaminase Producing Actinomycete *Streptomyces Albogriseolus* Isolated From Estuary Of Uttara Kannada District Against Hela And HepG2 Cell Lines.

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Abstract

L-glutaminase (L-glutamine aminohydrolase EC 3.5.1.2) is an extracellular hydrolytic enzyme having anticarcinogenic potential and is widely used in enzyme therapy especially for acute lymphocytic leukemia. L-glutaminase deaminates L-glutamine to glutamic acid and ammonia. In most tumors glutamine is the primary mitochondrial substrate and is present in circulating blood. It is a source of essential amino acid necessary for development of leukemic cells. The lack or depletion of L-glutamine leads to death of tumour cells. The marine environment is a potential source of bioactive secondary metabolites that provides pharmaceutically important compounds. The present study was focussed on to study the anticancer property of L-glutaminase producing actinomycetes isolated from estuaries of Uttara Kannada district of Karnataka. The isolates were screened for L-glutaminase production by qualitative and quantitative assay and the potent isolate was further subjected for MTT assay to determine its anticancer potential. The study showed 60% of isolates were positive for L-glutaminase production in Rapid plate assay and 85% of isolates were positive in semiquantitative assay. In quantitative assay the isolate *Streptomyces albogriseolus* exhibited high enzyme activity of 24.32+0.02 IU/ml. In MTT assay the isolate *Streptomyces albogriseolus* showed an IC50 value 102.0µg/ml in cervical cancer HeLa cells and IC50 value of 101.2µg/ml in HePG2 cells respectively, and could be used as good source of L-glutaminase.

Keywords: Estuaries, L-Glutaminase, Actinomycetes, Anticancer, Hela, HepG2

INTRODUCTION

The marine microorganisms have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds (Valli *et al*., 2012). Marine organisms have the potential to be a massive resource for the discovery of bioactive natural products (Haggag *et al*., 2014). The sea water of marine environment is saline and is chemically closer to human blood plasma, provides microbial enzymes that are safe with less or no side effects when administered for human therapeutic application (Teja *et al*., 2014). The marine microbial enzymes show a high level of salt tolerance.

The actinomycetes are filamentous prokaryotes and include a diverse group of heterotrophic prokaryotes that form hyphae at some stage of their growth (Rajan *et al*., 2015). Actinomycetes from the unexplored habitats have gained attention for the production of bioactive metabolites such as enzymes, enzyme inhibitors, immunological regulators, anti-oxidation reagents in recent years. The actinomycetes diversity from marine sediments is an inexhaustible resource that has not been adequately exploited. Nearly half of the discovered bioactive secondary metabolites are produced by actinomycetes (Lam 2006). Around 23,000 bioactive metabolites are reported to be produced by microorganisms and are produced by the actinomycetes and represent 45% of all the bioactive microbial metabolites discovered (Abd-Elnaby *et al*., 2016).

Gulve and Deshmukh (2011) have described the Indian coastal area as a significant source of actinomycetes. Okazaki and Okami (1972) reported that actinomycetes are widely distributed in the marine environment. Recent findings have demonstrated that indigenous marine actinomycetes that exist in the oceans are widely distributed in different marine ecosystems (Jensen *et al*., 1991). Marine actinomycetes show a marked chemical and morphological diversity but form a distinct evolutionary line of organisms (Goodfellow and Donnell 1989). Marine actinomycetes have a broad range of enzyme activities and are capable of catalyzing various biochemical reactions. Enzymes *viz* α-galactosidase, L-glutaminase, amylase, protease, L-asparaginase, cellulase have been reported from the marine actinobacteria (Lakshmanaperumalsamy 1978). During the past 30 to 40 years, numerous novel compounds have been isolated from marine organisms, and many of these have been reported to have biological activities, some of which are of interest from the point of view of potential drug development. Microbial enzymes such as asparaginases, glutaminases, and carboxypeptidase deplete the nutritionally essential amino acids and have been suggested for the treatment of human leukemia's and solid tumors (Chandrasekaran *et al*., 1998; Athira *et al*., 2014).

L-glutaminase (L-glutamine aminohydrolase E.C.3.5.1.2) enzyme hydrolysis glutamine to glutamate and ammonia (Nandakumar *et al*., 2003; Sarkar *et al*., 2014; Teja *et al*., 2014). Leukemic cells depend directly on the exogenous supply of L-glutamine from blood and do not rely upon L-glutamine synthetase, unlike normal cells for their survival and growth. Therefore, L-glutamine present in blood serves as a metabolic precursor for protein and nucleotide synthesis of tumor cells. As a result, the L-glutaminase blocks the energy route necessary for the proliferation of tumor cells (Nathiya *et al*., 2012) and causes selective death of L-glutamine dependent tumor cells (Sarada, 2013). L-glutaminase also has its application in biosensors for monitoring the glutamine levels in mammalian and hybridoma cells (Sabu *et al*., 2002; Rajan *et al*., 2015), and in food flavoring, especially in soy sauce and related industries (Karim and Thalij 2016).

The Uttara Kannada District is unique in the Western Ghats, with regards to the distribution of biodiversity. The district has diverse marine habitats that include seashores, hypersaline lakes, estuaries, salt pans, mangroves, etc. The production of L-glutaminase from estuarine actinomycetes of Uttara Kannada district is still scanty. Hence with this view and looking at the wide application of actinomycetes in various fields, the present study is focussed on to investigate the anticancer potential of L-glutaminase producing marine actinomycete *Streptomyces albogriseolus* isolated from estuaries of Uttara Kannada district against HeLa and HePG2 cell lines.

MATERIAL AND METHODS

Isolation of marine actinomycetes

In the present study we have used 43 marine actinomycetes strains (SMRO 1 to SMRO 43) recovered previously from estuaries of Uttara Kannada District, Karnataka, that have shown antibacterial and antiyeast properties (Mesta and Onkarappa 2017).

Screening of marine actinomycetes for L-glutaminase production
Rapid plate method (Qualitative assay)
The isolates were subjected for screening of L- glutaminase production by rapid plate assay (Gulati *et al*., 1997). Minimal glutamine agar (MGA) medium (supplemented with 10% sea water) was used for detecting L-glutaminase activity in which L-glutamine acts as sole carbon and nitrogen source and phenol red acts as pH indicator. Point inoculation was carried out in a Petri dish containing 15-20ml of medium and was incubated at 30ºC for 14 days. One uninoculated plate was kept as control. All the test plates were observed from the second day of incubation. The potent strains able to cleave L-glutamine to L-glutamic acid and ammonia leads to an increase in pH (alkaline), resulting in a change in color of media. The media color change from yellow to pink indicated L-glutaminase activity. No color change in media by the marine actinomycetes indicated negative for L-glutaminase production (Balagurunathan *et al*., 2010).

L-glutaminase production from marine actinomycetes by Submerged Fermentation:
The isolates that showed positive result for rapid plate assay were inoculated into 250ml Erlen Meyer flask containing 100ml of minimal glutamine broth and incubated for 7 days at 30ºC in a rotary shaker at 120rpm. After the incubation period, the broth was filtered through Whatman No 1 filter paper and centrifuged by using cold centrifuge at 10,000 rpm for 30 minutes at 4ºC. The clear supernatant was collected in screw cap bottles and was stored at 4ºC and used as a crude enzyme (Balagurunathan *et al*., 2010).

Semiquantitative Assay for L- Glutaminase production (Agar well diffusion)
Minimal glutamine agar medium was prepared. About 50µl of the crude enzyme was added into the well of 6mm diameter at the center of agar medium followed by incubation at 30°C for 7 days. One uninoculated plate was kept as control. The plates were observed from the second day of incubation (Gulati *et al.,* 1997). The production of the zone around the well from yellow to pink color indicates L-glutaminase activity (Balagurunathan *et al*., 2010). The actinomycete strains with its crude L-glutaminase enzyme that showed maximum zone of color change (from yellow to pink) on minimal glutamine agar medium was selected as a potential strain for further studies.

Nesslerization method (Quantitative assay)
The L-glutaminase activity of marine actinomycetes was estimated quantitatively by following the method of Imada *et al*. (1973).The actinomycete strain that showed the maximum zone of color change (from yellow to pink) on minimal glutamine agar medium in the semiquantitative assay was subjected for quantitative assay. In this assay, L-glutamine was used as a substrate, and the product ammonia released during the catalysis was measured by using Nessler's reagent.

0.1ml of culture filtrate (crude enzyme) was added to 0.2ml of 0.05M Tris HCl buffer (pH 8.5) and 1.7ml of 0.01M L-glutamine and incubated for 10min at 37ºC. The reaction was stopped by the addition of 0.5ml of 1.5M Trichloroacetic acid (TCA), and the precipitated protein was removed by centrifugation at 10,000 rpm at 4ºC. 0.5ml of the supernatant was diluted to 7ml with Distilled water and treated with 1ml of Nessler's reagent and incubated for 10min at room temperature. The absorbance was recorded at a wavelength of 450nm using UV/Visible Spectrophotometer (ELICO SL150, India). One tube was maintained as blank without adding the crude enzyme filterate. The enzyme activity was expressed in the International unit (IU). One International unit of L-glutaminase activity is the amount of enzyme that catalysis the formation of 1µmole of ammonia per ml per minute (µmole/ml/min) under the condition of the assay. The actinomycete strain that showed higher L-glutaminase activity was selected for further studies.

Screening of marine actinomycete *Streptomyces albogriseolus* for anticancer activity
***In vitro* cell viability assay by MTT on HeLa and HePG2 cell lines**
The MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide) was used to assess the viability of the cells (Yoo *et al*., 2002; Sudha and Selvam 2013; Singh *et al*., 2013). The amount of purple formazan formed was determined by measuring the absorbance at 560 nm using a microplate reader. Briefly, 50,000 cells of HeLa and HePG2 were seeded in a 96 well plate and were incubated for 24 hrs at 37ºC in 5% CO_2 incubator. The samples to be tested were added from 0-320µg/ml concentration in DMEM and were incubated for 24 hrs. After the incubation period, 100µl of MTT was added and incubated for 3 to 4 hours. After incubation with MTT reagent, the MTT reagent was discarded by pipetting without disturbing cells and

100µl of DMSO to rapidly solubilize the formazan. The absorbance was measured at 590nm. Inhibition of growth is a measure of cytotoxicity, and the percentage inhibition is calculated as follows:
% Inhibition = 100–[(Mean OD for test sample/ Mean OD for the Control) x100]
The plot of % cytotoxicity versus sample concentration was used to calculate the concentration lethal to 50% of the cells (IC_{50}).

RESULTS

Rapid plate assay

Out of 43 isolates screened for L-glutaminase activity, 26 isolates showed positive result by exhibiting pink coloration of media. The acid-base indicator dye phenol red converts yellow into pink color at basic pH due to the production of ammonia.

Fig 1: Production of L-glutaminase (media colour change from yellow to pink)

Semiquantitative assay

A total of 26 isolates screened for semiquantitative assay, 22 isolates showed positive results by producing pink colouration of media.

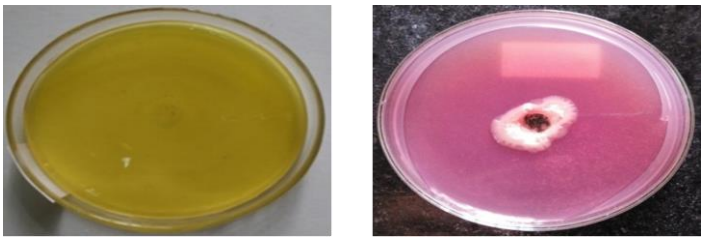


Fig 2: Plates showing production of L-glutaminase

Quantitative Assay (Nesslerization)

The glutaminase activity was determined by estimating the amount of ammonia liberated from the glutamine. The enzyme activity ranged from 0.18+0.03 IU/ml being the lowest to 24.32+0.02 IU/ml being the highest. Out of 22 isolates screened, isolate *Streptomyces albogriseolus* was considered to be high L-glutaminase producing strain about 24.32±0.02 IU/ml compared to others.

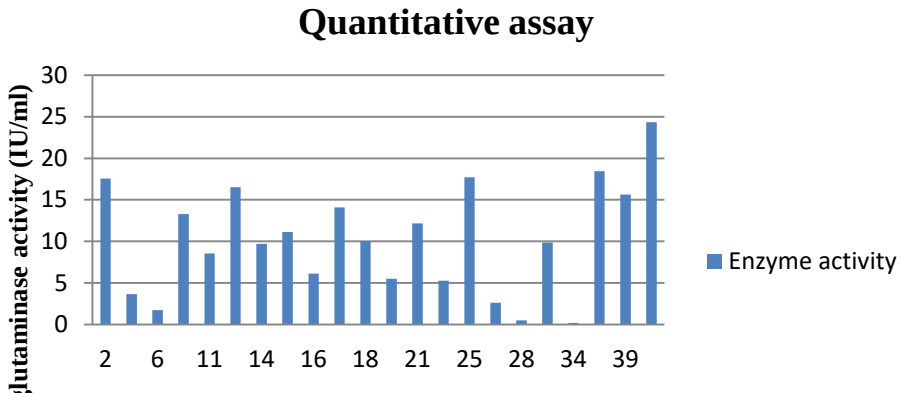
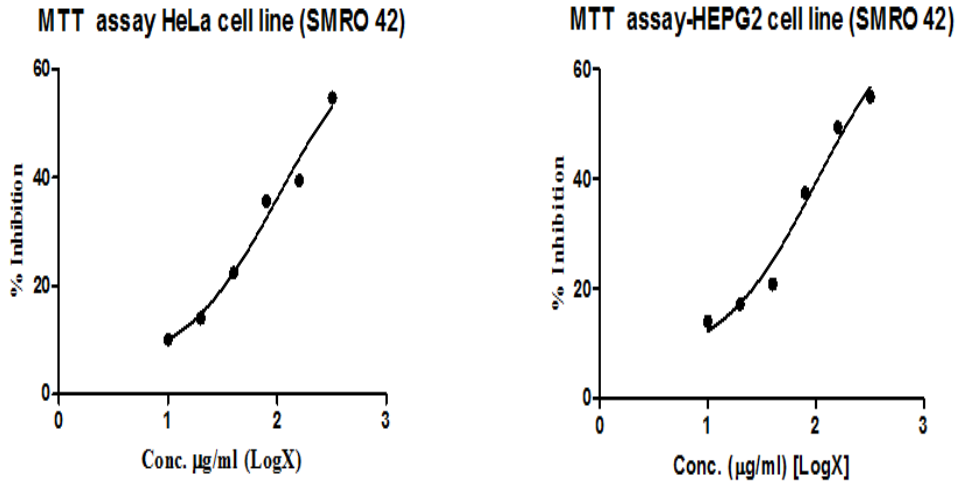


Fig 3: L-glutaminase production by active marine actinomycetes isolates

Screening of marine actinomycete *Streptomyces albogriseolus* for anticancer activity

The L-glutaminase from isolate *Streptomyces albogriseolus* showed an IC_{50} value of 102.0 µg/ml in HeLa cells and 101.2 µg/ml in HePG2 cells.



Bottom	68.64
Top	4.300
LogIC50	2.009
IC50	102.0

Bottom	72.65
Top	6.472
LogIC50	2.005
IC50	101.2

Fig 4: The percent inhibition and viability values of *S. albogriseolus* on HeLa and HePG2 cell lines

DISCUSSION

Microbial enzymes are much preferred because of their eco-friendly nature, economic production, concentration of an enzyme can be increased by environmental and genetic manipulation, easy handling and process modification, consistency, short fermentation time, simple growth requirements and faster growth of the target microorganisms (Dutt *et al*., 2014). Glutaminase from microbial sources has found its application in clinical analysis and also in the manufacture of metabolites and is involved in the synthesis of various nitrogenous metabolic intermediates.

Screening of marine actinomycetes for L-glutaminase production.

Rapid Plate Technique (Qualitative Assay)

Majority of microorganisms, including bacteria, yeasts, molds, fungi, and actinomycetes have been reported to synthesize L-glutaminase enzyme (Teja *et al*., 2014**)** of which actinomycetes are most potent producers (Balagurunathan *et al*., 2010). Earlier studies have documented the capability of microbes producing L-glutaminase by actinomycetes (Abd-Alla *et al*., 2013; Teja *et al*., 2014; Rajan *et al*., 2015), endophytic fungi

(Sajitha *et al*., 2013), *Bacillus subtilis* (Sathish and Prakasham, 2012), marine *Bacillus* (Suneetha *et al*., 2013), *Pseudomonas aeroginosa* (Karim and Thalij, 2016), *Penicillium brevicompactum* (Elshafei *et al*., 2014). L-glutaminase producing actinomycetes were also reported from Marine sediment sample collected from rhizosphere region of the mangrove *Rhizophora apiculata*. Among 20 actinomycetes strains obtained, the potential strain was identified *Streptomyces olivochromogens* (Balagurunathan *et al*., 2010). A similar type of screening was carried out by Yulianti *et al*. (2012) and found that out of 13 marine bacterial isolates, *Pseudomonas aeruginosa* strain CG-T8 showed a positive result for L-glutaminase. In the present study, 60% of the isolates showed positive for L-glutaminase production in a rapid plate assay. Krishnakumar *et al*. (2011) isolated and screened marine actinomycetes for L-glutaminase enzyme from marine sediment samples from Cape Comorin coast. Their findings revealed that out of 25 isolates screened only one isolate showed promising L-glutaminase activity. Similar results were reported by Desai *et al*. (2016). The present study confirms the work and results of Wade *et al.* (1971) and Imada *et al.* (1973) that rapid plate assay is advantageous as this method is quick and results can be visualized directly from the plates.

Semiquantitative assay (Agar well diffusion method)

Submerged fermentation is one of the routinely used procedure for L-glutaminase production from various microorganisms (Sabu, 2003). In the present study, 85% of actinomycete isolates with its crude L-glutaminase enzyme showed positive results by producing media color change from yellow to pink on minimal glutamine agar medium and were selected as a potential strain for further studies. Production of L-glutaminase enzyme from actinomycetes strains by submerged fermentation and confirmation by semiquantitative assay were reported by Dura *et al.*, 2002; Balagurunathan *et al.*, 2010.

Quantitative assay (Nesslerization method)

The enzyme activity ranged from 0.18+0.03 IU/ml being the lowest to 24.32+0.02 IU/ml being the highest. The isolate *Streptomyces albogriseolus* exhibited high productivity of 24.32±0.02 IU/ml. Sivakumar *et al*. (2006) analyzed and reported maximum production of extracellular glutaminase about 17.51 IU/ml by actinomycetes strains isolated from various body parts of fish *Chanos chanos*, and 31.62 IU/ml of L-glutaminase activity was reported in seaweed endophytic fungi – *Penicillium* species by Sajitha *et al.* (2013). The maximum production of L-glutaminase of 18.0 IU/ml at optimum conditions by marine alkalophilic *Streptomyce*s sp. SBU1 was reported by Krishnakumar *et al*. (2011). Similarly L-glutaminase estimation by Nesslerization method from various organisms was performed and reported by (Imada *et al*., 1973; Yulianti *et al*., 2012; Abdallah *et al*., 2013; Suneetha *et al*., 2013; Dhevagi and Poorani, 2016).

Screening of marine actinomycete *Streptomyces albogriseolus* for anticancer activity by MTT assay

L-glutaminase and other amino acid depleting enzymes are known to be potent agents for treating tumors (Holcenberg, 1982). It plays a major role as a therapeutic agent in cancer and HIV (Kumar and Chandrasekaran. 2003). The tumor cells depend on the exogenous supply of L-glutamine as a substrate for growth as they do not demonstrate L-glutamine synthetase. The survival of tumor cells can be blocked by incorporation of L-glutaminase. In the present study, the L-glutaminase from *Streptomyces albogriseolus* exhibited significant anticancer activity against HeLa and HePG2 cell lines. The isolate *Streptomyces albogriseolus* showed an IC50 value 102.0µg/ml in cervical cancer HeLa cells and IC50 value of 101.2µg/ml in HePG2 cells, respectively. Vijayabharathi *et al*. (2011) reported that *Streptomyces* strain isolated from humus soils of Western Ghats possessed anticancer activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) cell lines *in vitro*. Nathiya *et al*. (2012) reported that L-glutaminase from *Aspergillus flavus* KUG009 possessed cytotoxic activity (CTC_{50} 250µg/ml) against MCF-7 (human breast carcinoma cell line) by MTT assay. The extract from marine actinomycete *Streptomyces variabilis* possessed anticancer activity against cancer cell lines. The IC50 value of the extracts was 13.7 µg/ml for a liver cancer cell, 40 µg/ml for skeletal muscle cell, 4.41 µg/ml for cardiac RD and 12.6 µg/ml for cervical carcinoma FL cell respectively [45](Pham *et al*., 2014). Reda, (2015) reported that L-glutaminase produced by *Streptomyces canarius* FR (KC460654) had high efficiency against Hep-G2 cell (IC50, 6.8 µg/ml) and HeLa cells (IC50, 8.3 µg/ml), and moderate cytotoxic effect against HCT-116 cell (IC50, 64.7 µg/ml) and RAW 264.7 cell (IC50, 59.3 µg/ml). The growth of MCF-7 cells was not affected. Naine *et al*., (2015) reported cytotoxic activity of marine *Streptomyces parvulus* VITJS11. Human hepatocellular liver carcinoma cell lines (HePG2) was used to examine the chemopreventive effect of the isolate. The anticancer effect was found with IC50 of 500 µg/ml on HePG2 cell lines.

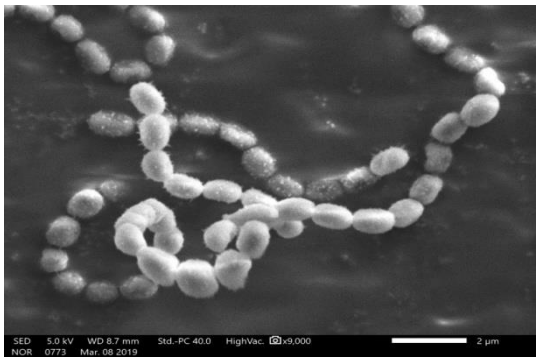


Fig 5: Scanning electron micrograph of *Streptomyces albogriseolus*

CONCLUSION

The marine environments are increasingly appreciated as a reservoir for bioactive natural products. The estuaries are potential ecosystem for novel actinomycetes. The present work revealed the potentiality of marine actinomycetes from estuarine environments to produce L-glutaminase enzyme with anticancer activity. Despite the promise of glutaminase as therapeutic agent, there are only scanty reports on the application of marine glutaminase enzyme as anticancer agent. The anticancer activity of L-glutaminase from *Streptomyces albogriseolus* against HeLa and HePG2 cell lines showed proliferative activity and induced apoptosis. Thus the enzyme L-glutaminase from actinomycete isolate *Streptomyces albogriseolus* proves to be a promising agent for the development of therapeutic anticancer drugs and pharmaceutical preparations.

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