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Cytotoxicity of L-Glutaminase from Estuarine Actinomycete SMRO 38 against HeLa and HePG2 Cell Lines

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ABSTRACT: Marine actinomycetes are the biotechnologically important prokaryotic organisms with high G+C content. These actinomycetes are physiologically and metabolically diverse from terrestrial organisms and have a diverse range of metabolic activities and metabolites. They are responsible for production of bioactive secondary metabolites, antibiotics, anticancer agents, immunosuppressive agents and enzymes. 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 present study was focused on to isolate and determine the cytotoxic activity of L-glutaminase of potent marine actinomycetes from sediment and water samples of different estuaries Sharavathi, Aghanashini and Kali of Uttara Kannada district. A total of 42 isolates were obtained from the sediment and water samples. Preliminary screening for L- glutaminase was done by Rapid plate assay using Modified M9 medium containing L-glutamine and phenol red as indicator. Out of 42 isolates, 26 isolates were found to be positive by showing change in media colour from yellow to pink and 5 isolates were chosen for cytotoxic activity which was evaluated by toxicity on cell lines. Metabolites in the extract of culture SMRO 38 proved to have cytotoxic effect against HeLa and HePG2 cell lines.

KEYWORDS: Estuaries, L-glutaminase, actinomycetes, cytotoxic activity, HeLa, HePG2

I. INTRODUCTION

Actinomycetes are the economical and biotechnologically valuable class of prokaryotes. They are main source of clinically important antibiotics [1], Sharmin et al. [2]. The discoveries of novel bioactive metabolites from terrestrial actinomycetes is decreasing and therapeutic compounds from new sources are to be explored. So much interest is given for screening of the actinomycetes from diverse environments [3]. The limited availability to control cancer drug resistance and emergence of new pathogens has led to search of new and novel drugs. The microorganisms especially actinomycetes and their bioactive products are found in unexplored habitats Naine et al. [4]. Actinomycetes from extreme habitat will provide a valuable resource for novel products of enzymes and antimicrobial agents.

Indian coastal region is an ideal location for harvesting many valuable species producing bioactive compounds with novel chemical entities. Recently much attention has been directed towards biologically active compounds from natural products. The discovery of new active metabolites must be followed by adequate biological testing Jemimah et al. [5]. The Uttara Kannada district is unique in the Western Ghats, with regards to the distribution of biodiversity. This unique

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feature is probably due to its central location where takes place biogeographical transition between the northern and southern Western Ghats. Five major rivers of Uttara Kannada viz., Kalinadi, Gangavali (Bedti), Aghanashini, Sharavati and Venkatapura have their sources in the Sahyadris and flow west through the district into the Arabian Sea. Some of the magnificent spectacular waterfalls in the district such as the Jog falls, the Lushington falls (Unchalli) and Magod falls are associated with the rivers Sharavati, Aghanashini and Gangavali respectively. Also, where these rivers meet the sea, there are some of the finest estuaries of the west coast.

Microbial enzymes play a major role in the diagnosis, curing, biochemical investigation and monitoring of many diseases. Microorganisms represent an excellent source of many therapeutic enzymes owing to their broad biochemical diversity [6]. L-glutaminase is an amidohydrolase gaining importance on its potential anticancer activity. It is used in enzyme therapy for cancer especially for Acute lymphocytic leukemia Sivakumar et al. [7],[8],[9] and HIV virus therapy [10], Suneetha et al. [11] Rajan et al. [12].

Glutaminase enzyme deaminates L-glutamine into L-glutamic acid and ammonia. It is a non essential amino acid. The primary substrate for L-glutaminase is present in circulating blood and serves as fuel for cell growth and nucleic acid synthesis [13][14]. The action of L-glutaminase plays an important role in the nitrogen metabolism of both prokaryotes and eukaryotes Sajitha et al. [9]. L-glutaminase produced by *Alkaligenes faecalis* KLU102 isolated from marine realm (Bay of Bengal) exhibits potential anticancer activity [15]. In most tumors glutamine is the primary mitochondrial substrate Wise et al. [16]. The cancer cells consume glutamine faster than that of other amino acids and its uptake is directly proportional to its supply [17]. The cancer cells are incapable of producing their own glutamine but normal cells can do so Wise et al. [16].

L-glutaminase production from estuarine actinomycetes is still scanty. Hence with this view the present study is focused on to investigate the cytotoxic potential L-glutaminase of the marine actinomycetes SMRO 38 isolated from estuaries of Uttara Kannada district against HeLa and HePG2 cell lines.

II. MATERIALS AND METHODOLOGY

Isolation of Actinomycetes

Starch casein nitrate agar (SCN), Kuster agar media (KUA), Actinomycetes isolation agar (AIA) (Himedia), Oat meal agar (OMA) (All media were supplemented with 10% sea water) were used for isolation of actinomycetes. The sediment and water samples collected from different spots of estuaries were subjected for serial dilution and plating followed by incubation at 30°C for 7 -14 days. The colonies developed on the plates were enumerated and were separated and subcultured on agar slants. The media were supplemented with Gresiofulvin and Flucanazole to prevent the fungal contamination. The colonies obtained were identified in accordance with guidelines given by Shirling and Gottlieb in International Streptomyces Project (ISP) [18],[19].

Characterization of Actinomycetes

The microscopic observation was done by cover slip method to identify the isolates upto genus level. The structures observed under oil immersion objective (100x) were compared with Bergeys manual of Determinative Bacteriology. The biochemical characterization was done by assays including starch hydrolysis test, casein hydrolysis test, urea hydrolysis test, Methyl red test, Voges proscuer test, Hydrogen sulphide production test, Citrate utilization test, Nitrate reduction test, Carbohydrate fermentation test (Lactose, Maltose, Starch, Dextrose, Sucrose) [19],[20].

Screening for L-glutaminase enzyme by Rapid plate assay

The isolates obtained were subjected for rapid screening of L- glutaminase production by rapid plate assay [21] Minimal glutamine agar (MGA) medium was used for L-glutaminase in which L-glutamine acts as carbon and nitrogen source and phenol red acts as pH indicator. Point inoculation was carried out in a petridish containing 20-25ml of medium and was incubated at 30°C for 14 days. One uninoculated plate was kept as control. All the plates were observed from second day of incubation. The media colour change from yellow to pink indicated L-glutaminase activity Balagurunathan et al., [22],[23],[24].

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Quantitative Assay

Quantitative assay of L-glutaminase was carried out. L-glutamine was used as a substrate and the product ammonia released during catalysis was measured by using Nessler's reagent. The enzyme activity was expressed in International unit (IU) Imada et al. [25].

Cytotoxic activity

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. [26],[27],Singh et al. [28]. The amount of purple formazan formed was determined by measuring the absorbance at 560 nm using a micro plate reader. Briefly 50,000 cells of HeLa and HePG2 were seeded in a 96 well plate and were incubated for 24 hrs at 37°C in 5% CO₂ 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. Absorbance was read at 590nm.

Inhibition of growth is a measure of cytotoxicity and the percentage inhibition is calculated as follows:

$$\% \text{ Inhibition (\% cytotoxicity)} = 100 - \left[\frac{\text{Mean OD for test sample}}{\text{Mean OD for the Control}} \times 100 \right]$$

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration lethal to 50% of the cells (IC₅₀).

III. RESULTS

Isolation and Identification of Actinomycetes

The dilution and plating method yielded 43 isolates. Majority of the isolates belonged to the genus *Streptomyces*.

Figure 1: Representative Actinomycetes isolates from Estuaries of Uttara Kannada District showing different mycelial colours (a) grey (b) brown (c) light pink (d) yellow (e) white

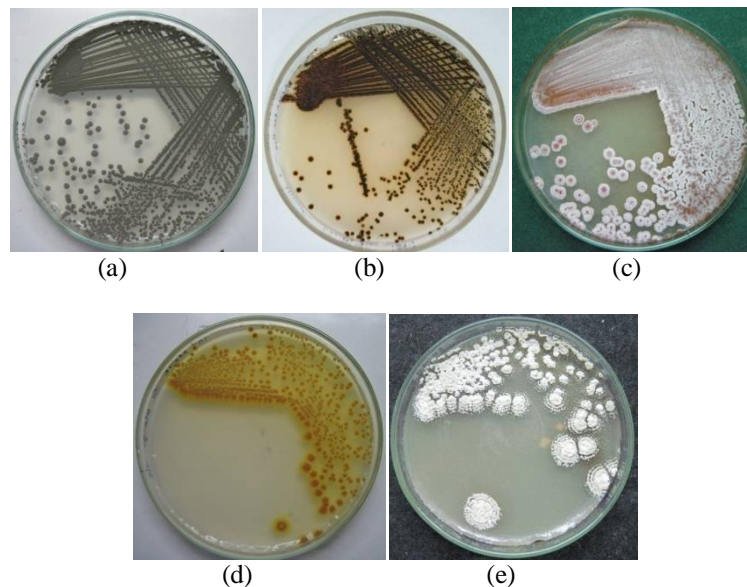


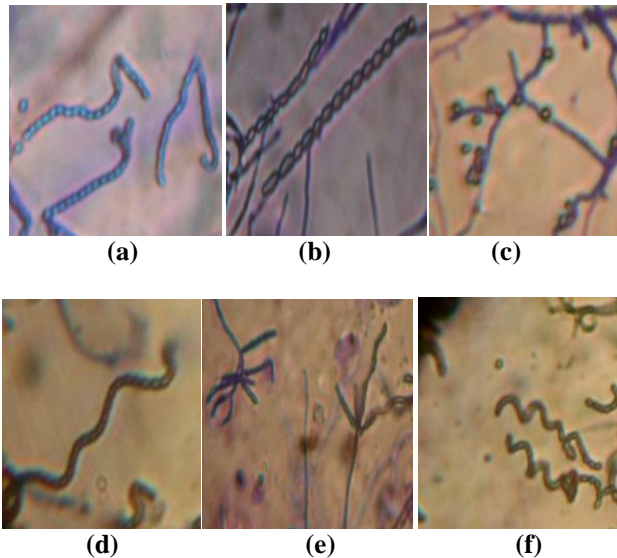
Figure 2: Spore chain arrangement (a-Hooks, b- Rectus/ Straight, c-Micromonospora, d- Flexibilis, e- Verticillium, f- Spiral)

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Biochemical characterization

The biochemical characterization showed varied activities of the isolates. All the 43 isolates were positive for starch hydrolysis. 36 isolates were positive for catalase test. 28 isolates were positive for gelatin hydrolysis, 41 isolates were positive for urease test. 32 isolates were positive for H₂S production. 37 isolates were positive for nitrate reduction. 38 isolates were positive for citrate utilization. 38 isolates were positive for casein hydrolysis. All the 43 isolates were negative for methyl red and voges proscuer test (Table 2). The carbohydrate utilization tests showed variable results and showed acid and alkaline production. The acid production was observed by colour change from red to yellow and there was no accumulation of the gas in the Durham's tube. The alkali production was observed by colour change from red to deep pink. 8 isolates showed positive for acid production in Dextrose, 7 isolates in Lactose and Maltose, 6 isolates in Sucrose and only 2 isolates in Starch. Majority of the isolates showed positive results for alkali production in all the tested sugars.

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Table No 2: Biochemical characteristics of Actinomycetes isolates (Starch hydrolysis, Casein Hydrolysis, Nitrate Reduction, Catalase, Citrate Utilization, Urease test, H₂S test)

Isolate No	Starch hydrolysis	Casein Hydrolysis	Nitrate reduction	Catalase test	Citrate utilization	Urease test	H ₂ S test
SMRO 1	+++	++	+++	+	+		
SMRO 2	++	+++	++	+	++		
SMRO 3	+++	++	+	+	++	+	++
SMRO 4	+	-	+	+	+++	+	-
SMRO 5	+++	+	+	+	++	+	+
SMRO 6	++	++	-	+	+	-	++
SMRO 7	+++	++	+++	+	-	+	+++
SMRO 8	+++	++	+	+	-	+	-
SMRO 9	++	++	++	+	++	+	+
SMRO 10	+	+++	++	+	+++	+	++
SMRO 11	+	++	++	-	-	-	+++
SMRO 12	++	++	+++	+	++	+	-
SMRO 13	+	++	++	+	-	+	++
SMRO 14	+++	+	++	+	++	+	+
SMRO 15	++	++	++	+	+	+	-
SMRO 16	+++	+	-	+	+	+	+++
SMRO 17	+	++	+++	+	+	+	++
SMRO 18	+++	+++	++	+	+++	+	+++
SMRO 19	+	++	++	+	++	+	++
SMRO 20	++	-	+	+	++	+	-
SMRO 21	+++	+	+	+	+	+	++
SMRO 22	++	++	+	-	+	+	+
SMRO 23	+++	++	++	+	++	+	-
SMRO 24	+++	++	+++	+	++	+	+++
SMRO 25	+++	+	++	-	++	+	++
SMRO 26	+++	+	+++	-	+	+	+++
SMRO 27	++	++	++	+	++	+	+
SMRO 28	++	+	+	-	-	+	-
SMRO 29	++	+	-	+	+	+	++
SMRO 30	+++	+++	+	+	+++	+	++
SMRO 31	+++	-	++	+	++	+	+
SMRO 32	++	++	+++	+	++	+	-
SMRO 33	+++	-	++	+	++	+	+
SMRO 34	++	+	-	+	+	+	++

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SMRO 35	++	+++	+++	+	++	+	+++
SMRO 36	+++	+++	++	+	++	+	++
SMRO 37	+++	-	++	+	++	+	-
SMRO 38	++	++	+++	+	++	+	++
SMRO 39	++	++	+++	+	+	+	+
SMRO 40	++	++	+++	+	+++	+	-
SMRO 41	+++	++	++	-	+	+	++
SMRO 42	++	+++	-	+	+++	+	++
SMRO 43	+++	+++	-	+	++	+	-

(‘-’ Negative, ‘+’ Positive, ‘++’ Good, ‘+++’ Very good)

Rapid Plate Assay: Out of 43 isolated actinomycetes screened for L-glutaminase activity, 26 isolates showed positive results by exhibiting pink zones. The results are tabulated in Table no 3.

Table no 3: Primary screening of L-glutaminase (Rapid Plate Assay)

Isolate No	Glutaminase	Isolate No	Glutaminase	Isolate No	Glutaminase
1	+	16	+++	31	-
2	++	17	++	32	+
3	-	18	+++	33	-
4	-	19	+++	34	++
5	+++	20	-	35	-
6	++	21	+++	36	-
7	-	22	-	37	-
8	+	23	+++	38	+++
9	+++	24	-	39	++
10	-	25	++	40	-
11	++	26	-	41	-
12	++	27	+++	42	+++
13	-	28	+++	43	-
14	+++	29	+		
15	++	30	++		

Where (-) negative, (+) positive, (++) good, (+++) very good

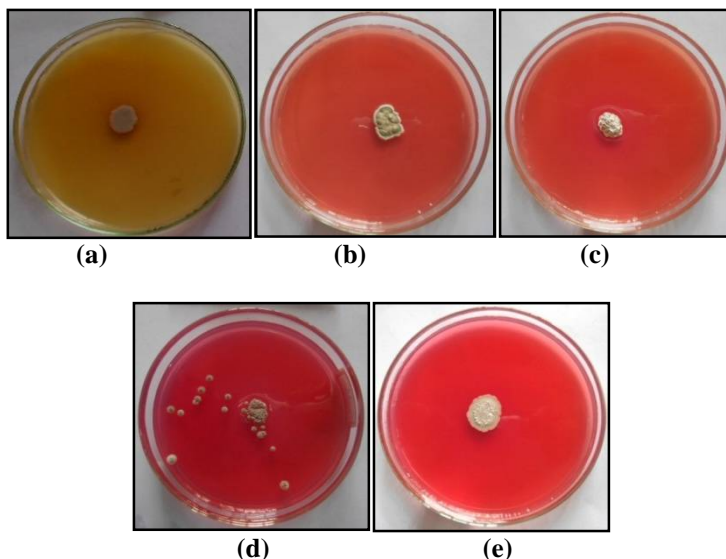
Figure 3: Production of L-glutaminase (media colour change from yellow to pink). Isolates (a) SMRO 10 showing negative result, Isolates (b) SMRO 09 (c) SMRO 16 (d) SMRO 21 (e) SMRO 27 showing positive result.

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Quantitative Assay

Out of 26 isolates, Isolate SMRO 38 was considered to be the best and high L-glutaminase producing strain. The maximum enzyme activity was showed at 18.45 IU/ml.

Cytotoxic activity

For cytotoxic studies HeLa and HePG2 cancer cells were choosen. The cancer cells were exposed to various concentrations of the extracts. Cell viability was evaluated by counting live cells after 24 and 48h of exposure. The isolates SMRO 38 showed an IC_{50} value 116.1 μ g/ml in cervical cancer HeLa cells and IC_{50} value of 108.1 μ g/ml in HePG2 cells respectively. The percent inhibition and viability values are shown in the Table no 4.

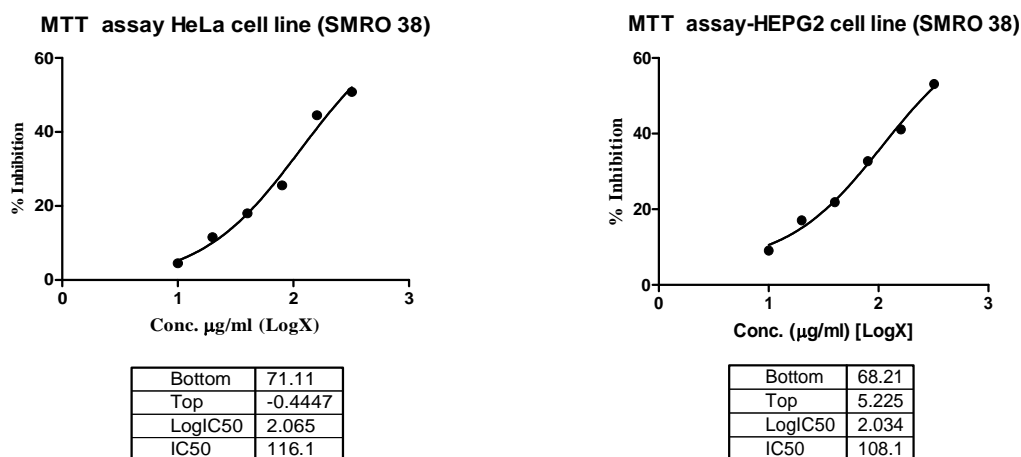


Table No 4: The percent inhibition and viability values of SMRO 38 on HeLa and HePG2 cell lines

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IV. DISCUSSION

Marine actinomycetes have attracted the special attention due to their diversity and capacity to produce biologically active secondary metabolites. These organisms are found to be potent sources of anticancer agents that target proteasome function. Actinomycetes are Gram positive eubacteria with high G+C content, found in terrestrial, fresh water and marine water and are responsible for the production of geosmin responsible for characteristic earthy odour Devi et al. [29], Sirisha et al. [30]. L-glutaminase an alternative to asparaginase has been focused upon as component of Chemotherapy regimens in the treatment of Malignancies. The role of L-glutaminase contributes to its anticancer activity that reduces the viability of the cell and further progression of the disease is avoided Goranjana et al.[31]. HeLa cells display a greater addiction to glutamine Reitzer et al.[32]. Glutaminases from microbial origin are supposed to be more stable than from animal and plant sources [6],[8], Nathiya et al. [33]. Few reports are available regarding antimicrobial activities of actinomycetes from Karwar coast [1] of Uttara Kannada district but studies on L-glutaminase have not yet been focused. Hence the present study was focused on to evaluate the cytotoxic potential of L-glutaminase from estuarine actinomycete SMRO 38 against cancer cell lines.

In the present study, water and sediment samples were collected from different sites of Sharavathi, Aghanashini and Kali estuaries of Uttara Kannada district, Karnataka, and isolation was carried out. Similar studies have shown that diverse group of actinomycetes are present in the sediment sample of marine environment Gao et al.[34],[29], soil samples Vanmathi et al.[35], Priya et al. [36], farming soils Oskay et al. [37], soil samples from sea shores and estuary Valli et al. [38], saline waters Sharma et al. [39], Sagar et al. [40], mangrove forests (Haque et al. [41] medicinal plant rhizosphere soil Khamna et al. [42].

A total of 43 actinomycetes isolates were obtained from the water and sediment samples collected from three Estuaries. The information on characteristic spore arrangement by cover slip method and biochemical characterization studies assists classification of actinomycetes as members belonging to the genus *Streptomyces* Gautham et al.[43], Kekuda et al. [44]. Similar studies carried out by researches revealed that *Streptomyces* members are abundant in marine environment. These results are in agreement with findings of [30] who have isolated actinomycetes from sediment samples and have observed that Starch casein media yielded good growth of actinomycetes isolates.

Rapid plate assay is a simple method to detect the production of L-glutaminase by actinomycetes isolates. The active strains producing glutaminase changed the colour of the medium from yellow to pink. The change in the pH indicates breakdown of amide bond in L-glutamine and the ammonia liberated. Earlier studies have been documented about the capability of microbes of producing L-glutaminase by actinomycetes Abd-Alla et al. [45], [12], endophytic fungi [9], *Bacillus subtilis* [10], marine bacillus [11]. 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* [22].

The amount of enzyme produced by the isolates were determined by Nesslerization method. The quantitative assay revealed that Isolate number SMRO 38 exhibited high productivity of 18.45IU/ml. It was found to have produced higher amounts of L-glutaminase compared to other isolates studied. Sivakumar et al. [7] 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 by seaweed endophytic fungi – *Penicillium* species (Sajitha et al.[9].

Present investigation revealed cytotoxic potential of L-glutaminase from SMRO 38. A dose dependent effect on HeLa and HePG2 cells was observed. The enzyme had an IC50 value of 116.1µg/ml for HeLa cells and 102 µg/ml for HePG2 cells respectively that induced a partial reduction in the cell viability. These results collaborate the findings of [15] that confirmed the anticancer activity of bacterial L-glutaminase with IC50 value 12.5 µg/ml. Dutt et al. [46] studied Antitumour property of L-glutaminase from *Aspergillus oryzae*. MTT assay showed that L-glutaminase was toxic to MCF7 cells with IC₅₀ value of 283.288 µg/ml. In [4], the authors reported cytotoxic activity of marine *Streptomyces parvulus* VITJS11. Human hepatocellular liver carcinoma cell lines (HePG2) was used to examine chemo preventive effect of the isolate. The cytotoxic effect was found with IC50 of 500 µg/ml on HepG2 cell lines.

V. CONCLUSION

Actinomycetes are potent producers of secondary metabolites. The present work reveals that Isolates from estuarine actinomycetes of Uttara Kannada district are found to be potent producers of L-glutaminase enzyme and L-glutamine

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from estuarine actinomycetes colony SMRO 38 have inhibitory action against the growth of human cancer cell lines. Anticancerous property shown by the metabolite need to be further exploited. Sequencing studies of the potent isolate is under progress.

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