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Short Communication

Isolation and molecular characterization of L-asparaginase producing *Streptomyces plantensis* from Virudhunagar region

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ABSTRACT

Cancer is one of the most deadly diseases worldwide and the incidence of cancer is on the rise in both the developing and developed countries. The current treatment does not only affect the cancer cells but also pose harmful to the normal cells. The use of nutritional starvation can be explored to minimize the damage to the normal cells by use of enzymes like L-asparaginase. In our work we have isolated a *Streptomyces* capable of producing L-asparaginase and molecular characterization was done on the isolate. The gene sequence is submitted in the GenBank with the accession number KJ408782. This organism can be further explored for the production of L-asparaginase, a potential anti cancer enzyme.

Keywords: Cancer, L-asparaginase, molecular characterization, GenBank.

INTRODUCTION

Cancer is a disease of worldwide importance. The incidence of cancer in the developed countries is rising, and its mortality occupies the second rank in the order of death causes. Similar tendency can be observed in the developing world, the gradual improvement in the life expectancy is also associated with an elevated cancer incidence and mortality. Accordingly, that malignancy will be soon a global problem with its entire consecutive burden. Therefore, it is easy to understand that cancer therapy is in the focus of common interest. For the time being the treatment of any malignancy is based on surgery, radiotherapy and drug therapy. This complex approach is capable to cure approximately half of the cancer patients, while the other half of the affected individuals may have only prolonged survival or even no benefit at all from the treatment. While therapeutically results obtained by surgery and radiotherapy – which are loco regional interventions – are close to their maximum accomplishment, success of drug therapy - the only systemic approach - is far from being satisfactory. Consequently, great expectation is attached to the further development of antitumour drug therapy [1].

In the first four decades in the history of chemotherapy the concept was generally accepted that malignant tumour cells could be killed through various inhibitory mechanisms of their cellular division by anticancer drugs. This period can also be considered as cellular chemotherapy. The revolutionary discoveries in molecular biology, however has made it obvious, that specific targets can also be identified in tumour cells. We may call this period simply molecular chemotherapy.

In India it is estimated that there are 20 to 25 lakhs cancer patients at any given point of time with about 7 lakh new cases coming every year and nearly half die every year [2]. Two-third of the new cancers are presented in advance and incurable stage at the time of diagnosis. More than 60% of these affected patients are in the age group of 35 and 65 years. The number of cancer cases will be almost three times the current number because of the increasing life expectancy and changing life styles concomitant with development. The age adjusted incidence rate per lakh for all types in India in urban areas range from 106-130 for men and 100-140 for women but still lower than USA, UK and Japan rates. 50% of all male cancers are tobacco related and 25% in female (total 34% of all cancers are tobacco related). There are predictions

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which estimates a 7-fold increase in tobacco related cancer morbidity in between 1995-2025. To control this problem the Government of India has launched a National Cancer Control Programme in 1975 and revised its strategies in 1984-85 stressing on primary prevention and early detection of cancer.

There are more than 100 types of cancer. Cancer begins inside a cell, the basic building block of all living things. Whenever the body needs more cells, older ones die off and younger cells divide to form new cells that take their place. When cancer develops, this process of orderly producing new cells breaks down and the cells continue to divide when new cells are not needed, and a growth or extra mass of cells called a tumor is formed. Over time, changes may take place in tumor cells that cause them to invade and interfere with the function of normal tissues. It takes many years for the development of a tumor and even more years until detection of a tumor and its spread to other parts of the body. People who are exposed to carcinogens from smoking cigarettes may generally do not develop detectable cancer for 20 to 30 years. There are evidences to suggest that permanent changes in our genes may also be responsible for tumor development which can be inherited or acquired throughout one's lifetime. Scientists have identified more than 300 altered genes that can play a role in tumor development. An alteration in the oncogenes can signal the cell to divide out of control whereas on the other hand, an alteration in tumor suppressor genes will allow cells with damaged DNA to continue dividing, rather than repairing the DNA or eliminating the injured cells [3].

Three classes of carcinogenic agents can be identified; chemicals, radiant energy, and oncogenic viruses. Chemical and radiant energy are documented causes of cancer in humans, and oncogenic viruses can cause cancer in animals and a few types of human cancers. Malignant tumor can metastasize—a process during which cancer cells escape from the tumor, enter the lymphatic system or to the bloodstream, and spread to nearby parts of the body and reach to sites far away from the original tumor. The malignant tumors develops under many steps over several years. If the tumor is detected earlier it is less likely that it will spread to other parts of the body.

In the past 25 years, enormous progress has been made in defining the molecular events that take place as a normal cell becomes malignant and the critical genes thought to be involved. Most cancers are named for the organ or type of cell in which they begin to grow, such as lung cancer, stomach cancer, breast cancer, or colon cancer. Melanoma is a cancer of cells in the eyes, skin, and some other tissues, known as melanocytes. Lymphomas are cancers that develop in the lymphatic system and leukemias are cancers of the blood cells. Carcinomas are cancers that develop in the epithelial tissue that lines the surfaces of certain organs, such as the skin, lung, breast or liver. Cancers that develop in the epithelial tissue of specific organs are called carcinoma of the lung, or carcinoma of the breast. Another type of cancers is sarcomas; which arises from cells in bone, fat, cartilage, muscle and connective tissue. In chemotherapy, we can target the weakness of the cancer cell and attack them. Since cancer cells divide at an unusually rapid rate, the cell division process becomes a drug target [4]. The best drugs can attack the features that are unique to this cancer cells. One such example is L-asparaginase.

Asparagine is an aminoacid which is required by our cells to build proteins. Asparagine is synthesized in our cells by the enzyme asparagine synthetase. So, most cells can make their own asparagine and does not look for exogeneous supply for asparagine. L-Asparaginase therapy takes advantage of this fact. L-Asparaginase cleaves asparagine into aspartate and ammonia. L-asparaginase is normally involved in balancing the levels of amino acids for use in protein synthesis. But when a large dose is introduced it will circulate in the blood and continually break down all the asparagine that it finds, ultimately leading to starvation of the cells that rely on the blood-borne supply.

The enzyme cuts off the supply of asparagine in blood there by depriving the cancer cells of the aminoacid and eventually they die. But the problem with the use of large protein like L-asparaginase is that our immune system destroys and rapidly clears this enzyme from the blood in a day or so. Since its a foreign protein, it may cause allergic reaction as well. This can be overcome by coating the enzyme and protecting it from immune system by use of poly ethylene glycol molecules.

The aim of this work is to produce an immunologically and enzymologically compactable new type of L-asparaginase. This can be done by identifying new strain by means of screening from soil and optimization of various production parameters.

MATERIALS AND METHODS

The specimens used in the studies were isolated from the soils of Virudhunagar region. 4 soil samples were collected from the various agricultural fields of region. The soils were

collected at the depth of 10 to 20 cm below the soil surface. The upper region was excluded. Soil samples were brought to the laboratory in aseptic condition. Samples were air dried at room temperature for 7 to 10 days packed it by air tight sealing.

Rapid plate method for the isolation of L-asparaginase producing microorganism form soil was performed [5]. M9 media containing Disodium hydrogen phosphate-6.0 gm; potassium di-hydrogen phosphate-3.0 gm; Sodium chloride-0.5 gm; L-asparagine-5.0 gm; magnesium sulphate-2.0 ml; calcium chloride-1.0 ml; 20% of glucose stock solution-10.0 ml; Agar-20.0 gm; Distilled water-1000.0 ml was prepared and sterilized. The serially diluted soil samples were inoculated on the solidified plates and kept for incubation for 18 hours at 37˚C.

L-Asparaginase Assay: The medium was centrifuged at 5000 rpm for 10 minutes and the supernatant was treated with phosphate buffer pH 7.0 and again centrifuged for 10 minutes at 5000 rpm. The supernatant was taken as the crude enzyme preparation. Enzymatic activity of the isolated L-asparaginase was done by nesselerization method and the amount of enzyme activity was calculated using the standard curve [6]. One international unit of L-Asparaginase activity is defined as that amount of enzyme which catalyzes the formation 1 micro mole of ammonia per minute under the conditions of the assay.

Molecular Identification:

Primer design: *Streptomycis plantensis* WAS1 genus specific primers (forward: 5' CTTAACACATGCAAGTCGAA-3' and reverse: 5' GTTACGACTTCGTCCCAAT 3') were designed based on the homologues regions specific to *Stretomycis plantensis* WAS1 genus.

DNA Isolation And Amplification Of 16S RNA Gene Of *Streptomycis Plantensis* WAS1 genus By Polymerase Chain Reaction (PCR): The template genomic DNA from *Streptomycis plantensis* WAS1 genus was isolated following the protocol [7]. In polymerase chain reaction, the specific primers forward and reverse were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR condition were 94˚C for 2 min, and the 94˚C for 1 min, 60˚C for 1 min, 72˚C for 3 min, a total of 30 cycles, with the extension at 72˚C for 10 min [8].

Agarose Gel Electrophoresis: Required amount of agarose (w/v) was weighed and melted in 1× TBE buffer (0.9 M tris-borate, 0.002 M EDTA, pH 8.2). To this 1-2 µl ethidium bromide was added from the stock (10mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb which was removed after solidification and the gel was placed in an electrophoresis chamber containing 1× TBE buffer. The products were mixed with 6× loading buffer (0.25% bromophenol blue) 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60 V [9].

Eluting DNA From Agarose Gel Fragments: Ethidium bromide stained agarose gel was visualized under a transilluminator and the fragment of interest was excised. The excess liquid was removed and the agarose fragment was placed in the spin column and centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. This eluent was rechecked by running on an agarose gelfor the presence of ethidium bromide stained DNA. This eluted DNA was used directly in manipulation reaction. This DNA fraction was subjected for sequencing.

Sequencing and Chimera Checking: The eluted PCR product was directly sequenced using Streptomycis plantensis WAS1 genus-specific primers without GC-Clamp at Ohmlina centre for molecular research, Chennai. The sequencing reactions were carried out with ABI PRISM dye Terminator cycle sequence ready reaction kit, All sequence exhibiting less than 95% sequence similarity to existing sequences in GenBank were checked using CHIMERA-CHECK program at the Ribosomal Database Project (RDP) using default settings [10]. All representative sequence corresponding to bands were Streptomycis plantensis WAS1 species.

Phylogenetic Placement: The sequences were compared to the sequences in GenBank using the BLAST algorithm [11] and RDP database to search for close evolutionary relatives.

RESULTS AND DISCUSSION

The soil samples were plated on the medium and the plates containing pink colour colonies were taken as L-asparaginase producing micro organisms. The organisms were isolated and were subcultured for further works. The isolates were taken for production and the isolate which produced maximum L-asparaginase activity was taken for molecular characterization.

Evolutionary relationships of 18 taxa: The evolutionary history was inferred using the Neighbor-Joining method [12]. The optimal tree with the sum of branch length = 0.01736315 is shown. The percentage of replicate trees in which

the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [13]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [14] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1322 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 [15].

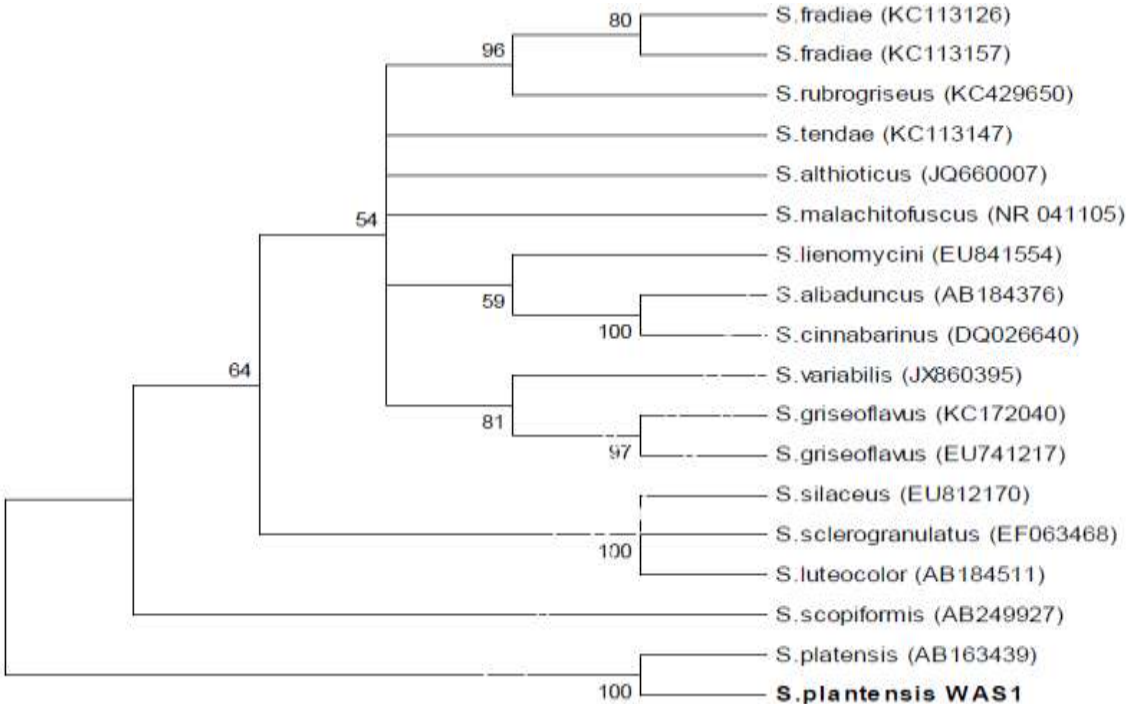
Phylogentic Tree Builder is a software which uses sequences aligned with System Software aligner. In this a distance matrix is generated using the Jukes-Cantor corrected distance model. In this when generating the distance matrix, only the alignment model positions are used whereas alignment inserts are ignored and the minimum comparable position is 200. The phylogenetic tree is created using Weighbor with length size 1000 and alphabet size 4.

Bootstrap: Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making these phylogenetic trees, the approach is to create a pseudo alignment by taking random positions of the original alignment. Here some columns of the alignment could be selected more than once or not selected at all. The pseudo alignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated almost 100 times and a majority consensus tree is displayed showing the number (or percentage) of times a particular group was on each side of a branch without concerning the sub grouping.

Gen Bank Accession Numbers: The representative sequence of the soil *Streptomycis plantensis* WAS1 genus species was deposited in Gen Bank of National Centre for Biotechnology Information (NCBI). The Gen Bank Accession number is KJ408782.

CONCLUSION

The isolated soil microbe has got the potential for production of the anti cancer enzyme L-asparaginase and was also found to be a new strain. Further the medium requirements needed to be optimized and the anti cancer activity of the purified enzyme has to be studied in animal models.



Phylogenetic tree showing the placement of the new soil isolate.

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