Research Article

Characterization and Bioprocessing of Oncolytic Enzyme - L-asparaginase isolated from Marine *bacillus* AVP 14†

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ABSTRACT

To characterize and bioprocessing of oncolytic enzyme-L-asparaginase from novel marine bacteria by statistical optimization for maximum asparaginase production. Out of 47, 9 bacterial isolates obtained from mangrove sediments showed positive L-asparaginase activity. One of the novel marine Bacillus AVP 14 was identified as *Bacillus subtilis* by 16S rRNA partial sequence and characterized for growth optimization and maximum production of anticancer enzyme. Solid state fermentation of L-Asparaginase production by using sesame oil cake, Groundnut oil cake, Red gram husk, Bengal gram husk, coconut oil cake, Green tea, Coffee was also done. *Bacillus subtilis* AVP 14 identified as potent extracellular producer of L-Asparaginase (347.42 IU) and showed 15 folds enhancement of L-asparaginase production (5,205 U/gds) under submerged fermentation condition in 5 days in presence of inducers and activators. The results emphasizes that submerged fermentation with Red gram yields more production than others and also revealed that marine bacillus is potential producer of L-asparaginase, highly significant pharmaceutical product.

Keywords: Oncolytic enzyme, L-asparaginase, Mangrove sediment, *Bacillus subtilis,* 16S rRNA, Solid state fermentation, Bioprocessing.

INTRODUCTION

L-Asparaginase has proved to be particularly promising in the treatment of acute lymphocytic leukemia. 60% incidence of complete remission has been reported in a study of almost 6,000 cases of acute lymphocytic leukemia. This enzyme is administered intravenously. The manufacture of this enzyme for use as a drug is an important facet of today's pharmaceutical industry. Tumor cells are deficient in aspartate ammonia ligase activity, which restricts their ability to synthesize the normally non-essential amino acid, L-asparagine[1]. L-asparaginase catalysis the conversion of l-asparagine to l-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions. Supplementation of L-asparaginase results in continuous depletion of l-asparagine and deprives the survival of cancerous cells. This phenomenal behavior of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase.

The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma[2,3]. The role of L-asparaginase in lymphocytic leukemia cells treatment is based on the fact that these cells are not capable of synthesizing L-asparagine and they rely on the exogenous sources to get hold of L-asparagine[4]. On the contrary, normal cells are protected from L-asparagine starvation due to their ability to generate this essential amino acid[5]. The anti-neoplastic activity is attributed to the depletion of L-asparagine by the action of L-asparaginase[4].

Marine microbes represent a potential source for commercially important bioactive compounds[6,7]. Among marine microorganisms, Bacteria have gained special importance as the most potent source of antibiotics and other bioactive secondary metabolites[8,9]. These secondary metabolites serve as model systems in discovery of new drugs[10]. Most of the studies on Bacteria have focused on antibiotic production, only few reports have dwelt on their enzymatic potential[11]. Microorganisms, particularly Marine bacteria have tremendously influenced the development of medical science. Marine bacteria have shown that they are a rich source of structurally unique, bioactive compounds[12]. Marine Environment is the Potential Source for L-Asparaginase Producing Microorganisms[13]. The enzyme L-asparaginase (E.C.3.5.1.1) from bacterial source has been used as a therapeutic agent in the treatment of acute lymphoblastic leukemia[14,3].Though many L-asparaginase producing species are available, only *E. coli* and *Erwinia carotovora* asparaginases are currently in medical use as drugs in the treatment of lymphocytic leukemia, because of high substrate affinity[3,15] and rapid clearance of the enzyme from the media of the reaction[2,16].

Recent literature revealed the marine actinobacteria such as *Streptomyces* isolated from marine sponge *C.diffusa* found to be potential L-Asparaginase producer but much attention has not been paid on marine Proteobacteria for a major tax of marine environment for L-Asparaginase production.

Present research describes characterization and bioprocessing of L-asparaginase from novel marine *Bacillus subtilis* AVP14 by optimization and solid state fermentation.

MATERIALS AND METHODS

Sample collection

Marine soil samples were aseptically collected from marine sediments of Nizampatnam, Guntur, A.P, India. In a sterile container for the isolation of L-Asparaginase producing organisms under laboratory conditions.

Isolation and Screening of Bacteria

Marine soil sample was serially diluted for the isolation of L-Asparaginase producing bacteria according to the method[17]. The culture was maintained in M-9 medium slant (The composition of 1 L of M-9 medium is (g/L): $Na_2HPO_4.2H_20$, 6 g; KH_2PO_4, 3g; NaCl 0.5g; L-asparagine, 5g; 1M $MgSO_4.7H_20$, 2 ml; 0.1M $CaCl_2$ $2H_20$, 1 ml; 20% Glucose stock solution 10 ml, Agar, 20 and pH 7.0 in distilled water with phenol red (2.5%): 0.04-0.36 ml indicator). The slant was incubated at 30 °C for 24 h and stored at 4°C ± 1°C. Stock culture was transferred to fresh M-9 medium every 3-4 weeks.

Effect of Physico-Chemical Parameters

Effect of different temperature ranges (25°C, 37°C, 50°C, 100°C), different incubation periods(24 hrs, 48 hrs, 72 hrs), pH values(7, 9, 10, 12, 14), salinity concentrations (0.5%, 5%, 10%, 15%, 20%), different carbon sources (21), different Nitrogen sources (11), different amino acids (16) and metal ions/mineral salts (51) on enzyme production was studied.

Inoculum Preparation

Inoculum was prepared by the addition of 2 ml of sterile distilled water to the culture grown in Modified M-9 medium at room temperature for 3 days, collected the inoculum after vigorous shake for 1 min.

Determination of L-asparaginase Activity

The rate of hydrolysis of L-Asparagine was determined by measuring the ammonia released using Nessler's reaction[18]. The color reaction was allowed to develop for 10 min and the absorbance read at 480 nm with a spectrophotometer. The ammonia liberated was extrapolated from a curve derived with ammonium sulphate. One unit (U) of L-Asparaginase was defined as that amount of enzyme which liberates 1 µ mole of ammonia per minute under the assay conditions[19].

Partial Purification of Enzyme

The culture filtrate was filtered through Whatmann No. 1 filter paper and centrifuged at 8000 rpm for 10 min at 4°C. The culture filtrate (crude enzyme) was brought to 45 per cent saturation with ammonium sulphate at pH 8.4 and kept overnight in a cold room at 4°C. It was thereafter subjected to centrifugation at 8000 rpm for 10 min at 4°C. The precipitate was discarded, while the supernatant was brought to 70 per cent saturation with ammonium sulphate and centrifuged at 8000 rpm at 4°C for 10 min. The precipitate from this step was collected and stored at 4°C.

Kinetics of the L-asparaginase Enzyme

To measure the kinetics of L-asparaginase, Michaelis constant (Km) and Maximal velocity (Vmax) of the partial purified enzyme was determined. They are one of the important parameters for the evaluation of the potential usefulness of the enzyme for anti-leukemic therapy. They were determined using L-asparaginase as substrate in the range of 0.01M-1M concentration. Each reported velocity I the mean of at least three measurements. The apparent Km was determined[20].

Enzyme Production under Solid State Fermentation (SSF)

The culturable microorganisms can be manipulated and processed due to their small size and huge reproduction capabilities[21]. The scaling up and mass production are relatively easy in microorganisms where they can be grown in large-volume. Many microorganisms can be stored for an indefinite time, ensuring availability of the targeted source organism. The microorganisms can be manipulated both physico-chemically and genetically to increase yields of desired natural products[5,22]. Coconut oil cake, Coffee, Green tea, Groundnut oil cake, Red gram and Sesame oil cake, procured from a local oil extracting unit of Guntur, Bapatla and Narasaraopet, Andhra Pradesh, India were used as the substrate. The substrate was dried at 60°C for 72h to reduce the moisture content to approx. 5% and ground to a desired size. Ten grams of each substrate was measured into 250 ml Erlenmeyer flasks into which a supplemental salt solution was added properly to get the desired moisture level. The salt solution composed of 6.0 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, and 0.5 g/L NaCl. L-asparagine was supplemented as inducer for synthesis of enzyme L-asparaginase[17]. Content was mixed properly and autoclaved at temperature 121°C (pressure 15 psi) for 15 min. The sterilized fermentation media was inoculated with 2.0 ml of inoculum, mixed thoroughly and incubated at 37°C for 5 days in a stationary condition. Each experiment was done in triplicate.

Extraction of Crude Enzyme

The recovery of crude L-asparaginase from the fermented material was done by simple extraction method. For this, the fermented substrate was mixed thoroughly with 50 ml of 50 mM phosphate buffer (pH 7.0) and the contents were agitated for 1 h at room temperature in a rotary shaker at 150 rpm. The liquid was filtered off through Whatmann No.1 filter paper and the resulting clear filtrate. The filtrate was then centrifuged at 8000rpm for 15 min to get enzyme as clear supernatant[23].

Assay of L-asparaginase Activity

Enzyme activity of the bacterial isolate AVP 14 was determined by quantifying ammonia production with Nessler's reagent. Add 0.1ml sample of culture filtrate (enzyme solution), to 0.9 ml of 0.1 M Tris-hydroxy methyl amino methane buffer (pH 8.5), and 1 ml of 0.04 M L-asparagine and incubated for 10 min at 37°C. The

reaction is stopped by the addition of 0.5 ml of 15% (w/v) Trichloroacetic acid. After centrifugation, a 1 ml portion of the supernatant fluid is diluted to 3 ml with distilled water and treated with 1.0 ml of Nessler's reagent and 1.0 ml of 2.0 M NaOH. The absorbance of the resultant color was read after 20 min at 480nm in UV/Visible Spectrophotometer. The OD was then compared to a standard curve prepared from solutions of ammonium sulfate as the source. Blank was prepared by without asparaginase enzyme sample (Produced in production medium). One unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 µmole of ammonia in 1 min at 37°C.

Molecular Identification

Pure culture of AVP 14 bacterial isolate was grown until log phase achieved and genomic DNA was isolated essentially[24]. The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) and 27F (5´ AGAGTTTGATCMTGGCTC AG- 3´) as per the conditions[25]. The PCR product was sequenced at Macrogen South Korea. The sequences obtained were compared with those from the GenBank using the BLAST program[26] and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree[26,27].

RESULTS AND DISCUSSION

Isolation & Screening of Bacterial Species

Marine Actino bacterial isolated PDK 7 & PDK 8 isolated from coastal areas of Parangipettai and Cochin showed potential L-asparaginase activity[28]. Out of 10 marine action bacterial strains, 3 isolates S3, S4, K8 and marine Streptomyces sp.[20] strain EPD 27 were reported to be potential producers of extracellular anti leukemic enzyme L-Asparaginase[29].

Dharmaraj reported S. noursei MTCC 10469 isolated from marine sponge *Callyspongia diffusa* as potential l-asparaginase producer[30].

In present study, bacteria habituated to mangrove sediment were screened for L-asparaginase production. Out of 47 bacterial isolates 9 isolates were identified as potential producers for L-Asparaginase (AVP 11to AVP 19) as shown in (**Figure 1**).

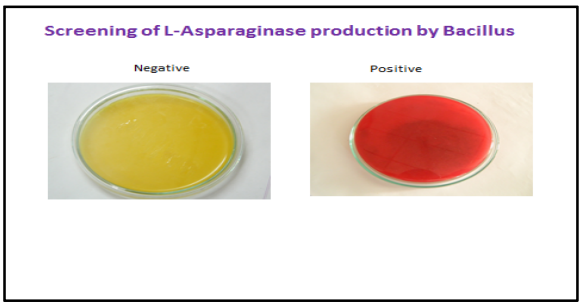

Figure-1: Screening of L-Asparaginase producer

One of the selected potential isolate were identified as genus Bacillus by Cultural, Morphological, and Biochemical characterization(**Table 1**) according to the data of Bergy's manual of systemic bacteriology[31].

Table 1: Morphological, Physiological and Biological Analysis of Bacteria Isolated From Marine Environment

Isolated strain	AVP 14
Morphological analysis	
Gram staining	Positive
Shape	Rod
Physiological analysis	
Starch hydrolysis	Positive
Lipase	Positive
Urease	Positive
H2S Production	Negative
Protease	Positive
Gelatin hydrolysis	Negative
HCN Production	Positive
Litmus	Positive
Biochemical analysis	
Indole	Negative
Methyl red	Negative
Vogues Proskeur	Negative
Citrate	Positive
Catalase	Negative
Oxidase	Negative

Molecular Identification of AVP14

A 1466 bp PCR product of gene was amplified from the genomic DNA of AVP 14. A sequence similarity showed that the 16srDNA gene sequence of AVP 14 had 99% similarity to the 16srDNA of *Bacillus subtilis* strain (AC:JNO3355) and *Bacillus* species PPB2 (AC:HM771657). The sequence was blast in NCBI and for analysis. Based on Phylogenetic tree of *Bacillus subtilis* AVP 14 16S rRNA gene analysis revealed that AVP 14 was closely related to *Bacillus subtilis* (Figure 2) and sequence was deposited in NCBI as *Bacillus subtilis* AVP 14 with accession number KF527828.

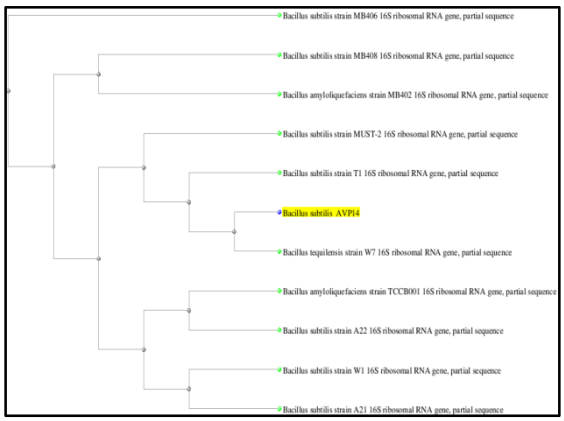

Figure 2: Phylogenetic tree of Bacillus subtilis AVP 14 based on 16S rRNA gene analysis

Optimization of L-asparaginase Production

Optimization of physico chemical parameters like pH, temperature and salinity plays an important role in production of biocatalysts. Temperature is one of the most critical parameter to be optimized in any bioprocess[33]. pH of the medium strongly affects many enzyme processes and transport the various components across the cell membrane.

Effect of Temperature

In present study optimization revealed that AVP 14 showed maximum production (1.69 IU/ml) at 37°C after 72 hrs of incubation (Figure 3). Production of L-asparaginase varies with incubation period at 37°C indicating optimization of incubating period also exhibit a significant role (Figure 3).

3a 3b 3c

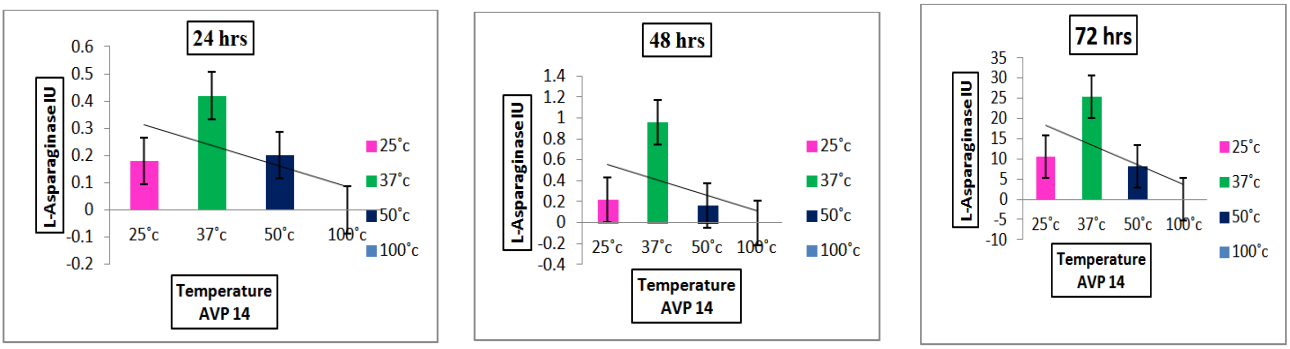

Figure 3: Effect of Temperature

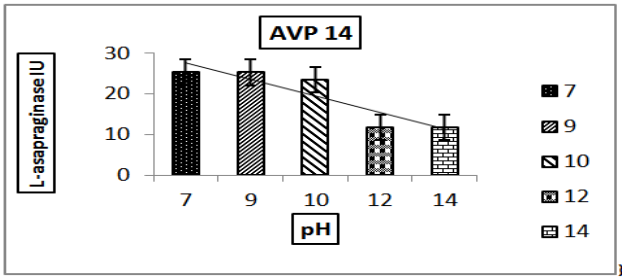

Figure 4(a): Effect of pH

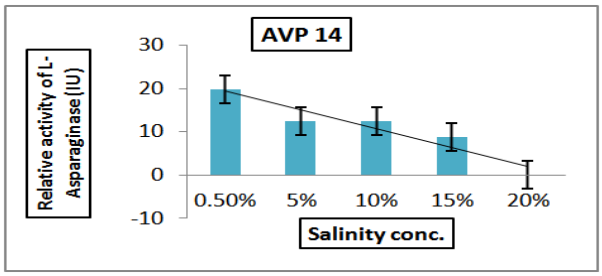

Figure 4(b): Effect of Salinity

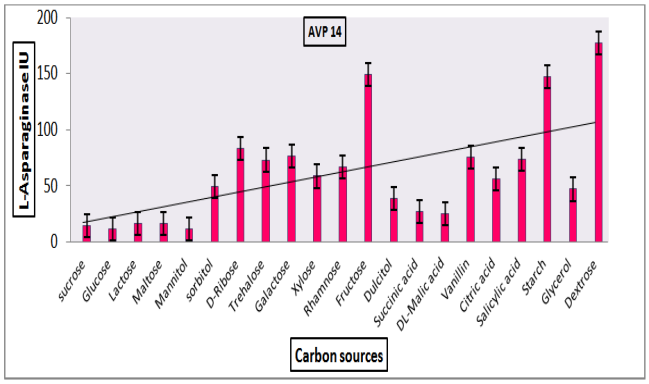

Figure 5(a): Effect of Carbon Sources

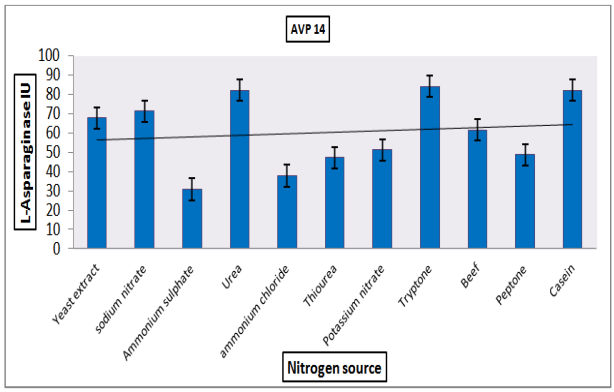

Figure 5(b): Effect of Nitrogen Sources

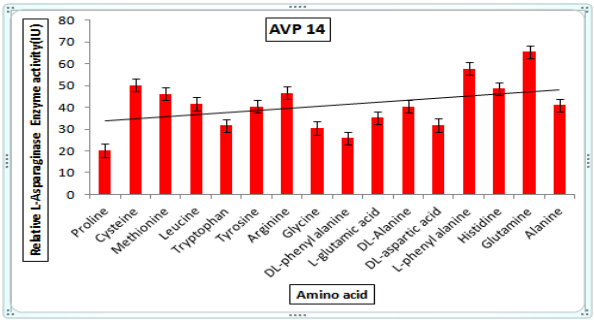

Figure 6(a): Effect of Amino acids

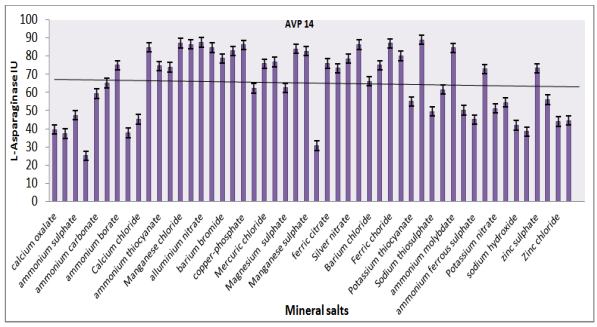

Figure 6(b): Effect of Metals/Mineral salts

Effect of pH and NaCl

It is observed that enzyme production of AVP 14 showed variation at different pH. L-asparaginase production was found to be maximum (25.36 IU/ml) at pH 7 and pH 9 and observed to be gradually decreased beyond pH 9 (Figure 4 (a)). Percentage of NaCl concentration also effect the L-asparaginase production of AVP 14. The production was inversely related with increasing concentration of NaCl and found to be maximum (19.85 IU/ml) at 0.5% concentration (Figure 4(b)). Earlier literature revealed that L-asparaginase production in *Streptomonas albidoflavus* was observed to be high (11.9 IU/ml) at 40°C at pH 7.5[34]. In contrast present result showed that the marine bacteria are relatively more potential than actinobacteria.

Effect of Carbon Sources and Nitrogen Sources

Maltose and Yeast extract were proved to be best carbon and nitrogen sources for the production of L-Asparaginase enzyme from *S.albidoflavus* whereas *S.gulbergensis* requires maltose and L-asparagine[34,35]. Isolate AVP 14 showed maximum l-asparaginase production with Dextrose (177.45 IU/ml) and Tryptone (84.1 IU/ml), the carbon and nitrogen source respectively. (Figure 5(a) and 5(b))

Effect of Amino acids and Effect of Metals/Mineral salts

Amino acid Glutamine and Calcium carbonate were observed to be potential inducers for l-asparaginase production of 65.54 IU/ml and 89.01 IU/ml respectively (Figure 6(a) and 6(b)).

After optimization Dextrose, Tryptone, Glutamine and Calcium carbonate were selected as potential inducers and enhancers.

Enzyme Kinetics of Partial purified L-Asparaginase of AVP 14

An attempt was made to evaluate the extent of improvement in production of L-asparaginase in the modified formulated production media with necessary inducers and enhancers. 5 folds of enhancement in L-asparaginase production observed with modified production medium indicating highly significant improvement so far observed. Enzyme kinetics, Vmax and Km values were studied at different substrate concentrations (0.01-1 M) and at different incubation periods (24 hrs, 48 hrs and 72 hrs).

At 24 hrs of incubation, Vmax value of L-asparaginase is 86 and Km value is 0.18. At 48 hrs, Vmax is 173.16 and Km value is 0.2 and at 72 hrs AVP 14 showed 202.94 Vmax value and 0.3 Km value. Enzyme kinetics study revealed that L-Asparaginase of AVP 14 showed Vmax at 0.7 M substrate. (Figure 7). Enzymatic activity of the strain AVP14 measured at physiological temperature showed lower Km values. The Km values obtained were closer those of some mesophilic L-asparaginase of earlier studies.

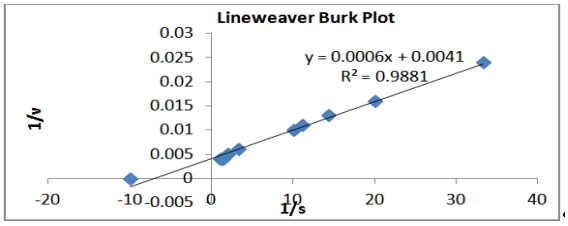

Figure 7: Kinetics of Partial purified L-Asparaginase of AVP 14

Solid State Fermentation (SSF)

Solid state fermentation (SSF) has gained fresh and plentiful attention of researchers to overcome the drawbacks of submerged fermentation. SSF has several advantages over submerged fermentation such as lesser energy requirements, very low risk of bacterial contamination, lower need of water and less environmental concerns regarding the disposal of solid waste[36]. Additionally, the utilization of agro-waste solid as a substrate for carbon and energy requirement under SSF makes this approach environmental friendly. Because of optimum moisture level and low volume of medium per unit weight agro industrial byproducts are generally used as solid substrates for bioprocessing of enzymes. SSF of L-asparaginase of fungal origin was extensively studied than bacterial L-asparaginase. Earlier studies revealed that incubation period for production of L-asparaginase production varies from species to species *Aspergillus terrus* showed maximum L-asparaginase production at 96 hrs of incubation under SSF and showed variation in production of L-asparaginase with substrate and incubation period[38-43].

In present study an attempt was made for maximum production of L-asparaginase with 6 different solid substrates of different composition of carbon and energy sources. Out of the six substrates tested for their suitability to support SSF production of L-asparaginase, Red gram husk appeared to be the best substrate supporting maximum enzyme activity of 246.32 IU after 5 days of incubation. Groundnut oil cake supported 166.54 IU, Coconut oil cake supported 152.53 IU, Green tea supported 136.76 IU, Sesame oil cake supported 124.26 IU activity while coffee supported 102.20 IU for L-asparaginase activity (Figure 8). As maximum activity was seen using Red gram husk therefore, Red gram husk might be the potential solid substrate for bioprocessing.

COC-coconut oil cake, SOC-sesame oil cake, GOC-Groundnut oil cake

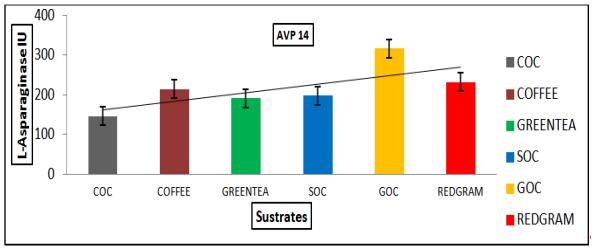

Figure 8: Solid State Fermentation

CONCLUSION

A novel bacterial strain namely: *Bacillus subtilis* AVP 14 was isolated from Nizampatnam marine soil sediment and can be considered as a candidate for L-asparaginase production in large scale industry to be used in the treatment of lymphoblastic leukemia. Optimization of culturing parameters could be the decisive factor that favoured better productivity.

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