5. RESULTS

5. 1. Sample collection

In the present study, 254 sediment samples were collected from six separate expeditions of Bay of Bengal, Tamilnadu, India. Air dried sediment samples were sieved to exclude large mineral and organic matter particles and ground in a pestle and mortar and kept at 55°C for 10 minutes. The samples thus obtained were taken for further screening for production of enzyme. The sites of sample collection are presented in table 1.

5. 1. 1. Enrichment and isolation of enzyme producing marine actinomycetes

One gram each of the sieved sediment samples were treated with 100 ml of tapioca effluent and incubated at an ambient temperature for about a week at 200 rpm in an orbital shaker. From the effluent, the diluted samples were plated on to Starch casein nitrate agar medium supplemented with phenol red indicator. The colonies with powdery consistency which appeared in different colors were observed from sediment enriched on starch casein nitrate agar medium.

The organisms would form a pink - colour zone in the medium, which was indicative of the ability of an organism to produce the L-asparaginase enzyme. This characteristic of the organism has been used for primary screening of enzyme producing isolates. The pink color zone was observed around the colonies as positive L-asparaginase producer and the colonies were further purified by streaking on Starch casein nitrate agar medium. The pink colour colonies of L-asparaginase producing actinomycetes on SCA agar medium has been shown in plate 1. Based on their colony morphology and pink zone forming ability, 29 actinomycetes strains were selected (Table 2) for further screening for enzyme producing attribute.

5. 1. 2. Screening for L-asparaginase producing marine actinomycetes.

The actinomycete isolates (3, 6, 10, 14, 23 and 28) obtained were screened for L-asparaginase producing activity. The six isolates were assayed for L-asparaginase producing ability by a modified method of Mashburn and Wriston method.

The results of screening of actinomycete isolates for L-asparaginase activity is presented in table 3. The isolates that showed characteristic L-asparaginase producing
ability were then subjected to further identification, characterization and optimization procedures.

5. 1. 2. 1. Identification of actinomycetes

Strains were preliminarily identified according to colony characteristics in terms of colony morphology, aerial hyphae, spores, pigment production on AIA and SCA medium. Morphological (Gram staining), cultural/physiological and biochemical characterization were carried out to identify the actinomycetes. Based on the cultural and microscopic appearance about 89 percent of the isolates were presumed to be in genus *Streptomycetes* and 11 percent non *Streptomycetes*. The *Streptomycetes* strains (Isolate - 3, 6, 10, 14, 23 and 28) that had L-asparaginase activity on phenol red incorporated SCA medium were screened and concluded that strain number 6 was found to be producing significant range of L-asparaginase activity. Farther than six strains, strain number 6 had expressed higher activity, which was shown by the color intensity. The isolated strain number 6 was characterized by morphological, biochemical tests and 16S rRNA sequence.

The isolated strain number 6 was found to be Gram positive. The colonies on media showed substrate mycelium with olive gray to dark reddish brown and aerial mycelium white to pinkish, especially on Czapek agars. Spores were moderate gray on most media and reddish gray on Czapek agar. Spore chains were observed to be in long, open spirals. The spore surface was spiny and melanin pigments were not produced on Czapek agar. The results are presented in table 4.

5. 1. 2. 2. Physiological and Biochemical properties of isolated strain

The Physiological and biochemical properties of isolated strain number 6 also were very similar to those of *Streptomyces radiopugnans*. The results of physiological characterization, biochemical and carbohydrate utilization tests have been presented in table 5 and 6.

The results of physiological characterization reveals that the actinomycetes isolated strain number 6 showed tolerance to NaCl levels from 4 to 10 per cent, whereas higher concentration of 13 per cent shows intolerance, which shows that salt tolerance in the isolate was upto 10 per cent NaCl concentration. The optimum temperature for
growth of the organism has been identified as 40°C and optimum pH for growth of the organism as 7.4.

Biochemical test results have been shown in table 6. Liquefaction of gelatin, citrate, urea, arginine, lysine, ornithine decarboxylase, galactose orthonitrophenol and voges - proskauer tests were all positive but indole and H₂S were negative.

Strain showed carbohydrate fermentation pattern as in D-Glucose, Galactose, D-Mannitol, D-Fructose, L-Rhamnose, Erythritol, Melibiose, Sucrose, Cellulose, L-Arabinose, but could not utilize Lactose, D-Xylose, Inositol, D-Mannitol, and Raffinose.

The actinomycete isolates were then subjected to molecular identification to characterize the genus/species level identity.

5. 1. 2. 3. 16S rRNA gene sequencing and phylogenetic analysis

The chromosomal DNA was isolated from actinomycetes strain and their 16S rRNA sequence was obtained. The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer. The complete 16S rRNA sequence of strain number was generated (Genebank accession No. HQ623051). Phylogentic analysis of 16S rRNA sequences of the strain and related taxas have shown that almost complete 16S rRNA sequence of the strain had 99 per cent similarity to that of Streptomyces radiopugnans (Genbank accession no. DQ912930). The results of DNA coding for 16S rRNA has been shown in plate 2. The results of 16S rRNA sequence and Clustal W phylogenetic trees were constructed using Neighbor - Joining methods from MEGA version 5. The results have been presented in fig. 3. As a result, the strain MS1 was identified as Streptomyces radiopugnans MS1.

The taxonomic ranking of the isolate Streptomyces radiopugnans MS1 has been referred and presented as follows; Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycinae; Streptomycetaceae; Streptomyces; Streptomyces radiopugnans.
5. 2. Mass cultivation of the organism for enzyme production

The *S. radiopugnans* MS1 was inoculated in Minimal broth media (1 per cent inoculum) and incubated at 28°C for 48 hrs. The log phase culture (5 per cent v/v) from the Minimal broth medium was transferred to 250 ml Erlenmeyer flask containing 50 ml of the culture medium and grown at 28°C for 7 days with 250 rpm to provide aerobic conditions. From this, 1 percent v/v of the culture medium was transferred to production medium for further optimization studies.

5. 2. 1. Optimization of culture conditions for L-asparaginase production

5. 2. 1. 1. L-asparaginase production from *S. radiopugnans* MS1

The physiological conditions for characteristic L-asparaginase production like incubation period, pH, temperature, and different carbon and nitrogen sources were optimized to obtain maximum enzyme production using standard enzyme production media. The culture free samples from the production media were collected every 24 hrs by centrifugation at 10,000 rpm for 30 minutes as enzyme source for assay of L-asparaginase activity. The results for optimization of *S. radiopugnans* MS1 for L-asparaginase production was presented.

The results for the effect of incubation period on growth and L-asparaginase production by *S. radiopugnans* MS1 was presented in Table 7.

The study on effect of incubation period of L-asparaginase production shows that growth of the actinomycete has increased from 12 hrs to 168 hrs. There was an increase in biomass as understood by the dry weight (mg/ml) values upto 168 hrs. Whereas, the L-asparaginase enzyme activity kept increasing up to 96 hrs of incubation showing the logarithmic growth phase of the *S. radiopugnans* MS1 and thereafter decreased gradually up to 168 hr of the study period showing the stationary growth phase of the organism.

Optimization of the L-asparaginase activity for pH had been done for a range of 2.0 to 11.0 at the required culture conditions. The results for optimization of pH for growth of the *S. radiopugnans* MS1 is presented in table 8.

Optimum pH was observed at 8.0 with a maximum enzyme activity recorded as 4.92 (IU/ml). Temperature optimization for maximum L-asparaginase activity was measured for temperature range from 10 to 80°C. The results of temperature optimization for enzyme production are presented in table 9.
Lower temperatures upto 30°C and higher temperatures above 30°C have shown decrease in growth and enzyme activity representing the lower activity in these temperatures. The optimum enzyme activity was observed at 30°C, with an activity of 4.88 IU/ml.

Optimization of inoculum size for maximum L-asparaginase activity was measured for a range of inoculum sizes from 0.5 per cent to 5.00 per cent. The results of optimum inoculum size for enzyme production are presented in Table 10.

The optimum enzyme activity was observed at with the inoculum size of 2.5 percent with an activity of 4.86 IU/ml. Lower inoculum sizes and higher inoculum sizes than 2.5 per cent has shown lesser growth of the organism and lesser L-asparaginase production.

Different carbon and nitrogen sources required for the growth of the organism were optimized by shake flask method. The result of effect of carbon sources for growth and enzyme production is presented in Table 11.

Carbon sources such as glucose, fructose, and mannose, galactose, lactose and wheat flour and tapioca effluent had been used for optimization of carbon requirements for enzyme production. Highest L-asparaginase activity (3.56 IU/ml) was observed at 2 per cent concentration of substrate using tapioca in medium as compared to other carbon sources.

Various nitrogen sources were evaluated for optimization of suitable nitrogen source for L-asparaginase enzyme activity. The results for nitrogen source supplementation have been presented in Table 12.

Nitrogen sources such as tryptone, yeast extract, corn steep liquor, peptone and casein hydrolysate had been used. Among the various nitrogen sources used for the study, Corn steep liquor showed maximum production of L-asparaginase with nitrogen activity of (3.77 IU/ml) at 2.00w/v concentration as compared to other nitrogen sources.

Effect of various amino acid supplements for L-asparaginase production has been optimized. The results have been presented in Table 13.

Out of twenty different amino acids tested for the study, only nine amino acids L-asparagine, L-histidine, L-proline, L-glycine, L-cysteine, L-glutamic acid, L-gutamine, L-theonine and L-serine were preferable nitrogen sources for the activity of
L-asparaginase when added to the medium as compared to other amino acids. The L-asparaginase activity reached up to maximum (3.77 IU/ml) when L-asparagine was used as nitrogen source.

The summary of optimum physiological/cultural parameters for growth of *S. radiopugnans* MS1 and L-asparaginase production is presented in table 14.

5.3. **Enzyme purification and quantification**

L-asparaginase was purified from crude extract by the ammonium sulfate, DEAE cellulose, Sephadex G - 200 and Prep cell Model 491. A sharp distinctive single peak of L-asparaginase was obtained from 12th fraction of the purified enzyme in chromatographic purification indicating the purity of the enzyme (Fig. 4, 5 and 6).

The partial purification of the L-asparaginase crude extract was observed in the ammonium sulfate (80 per cent) precipitation. 80 per cent extract from the above procedure showed that most of the enzyme activity was preserved in the precipitate. The total protein decreased from 4339.82 to 316.99 mg by ammonium sulfate precipitation step. The specific activity increased to 938.89, 2118.53 and 5035.28 IU/mg after the DEAE cellulose column, Sephadex G - 200 and Prep cell Model 491 steps, respectively (Table 15).

L-asparaginase was purified to homogeneity after Prep cell Model 491 separation with 80.72 per cent of yield. The enzyme was purified approximately 249-fold with a specific activity of 5035.28 IU/mg.

5.3.1. **Molecular mass determination of L-asparaginase**

DISC - PAGE of the enzyme preparation from different purification steps has showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of the Prep cell Model 491. It revealed only one distinctive band which was indicative of the pure preparation of L-asparaginase through the above procedures.

The molecular weight of the subunits of L-asparaginase was found to be approximately 33.3 kDa by SDS - PAGE analysis. The purified L-asparaginase has an approximate molecular weight of 133 ± 2.5 kDa as assessed by Native PAGE. The results of molecular mass determination are presented in plate 7 and 8.
5. 3. 2. Estimation of Isoelectric focusing (pI) and 2D analysis of L-asparaginase

The isoelectric focusing (IEF - PAGE) performed in a PROTEAN IEF (Bio-Rad, Hercules, CA) System using ReadyStrip™ IPG strips (Bio-Rad) has showed that the pI of purified L - asparaginase from *S. radiopugnans* MS1 was close to 5.6 (Plate 9).

5. 3. 3. *De novo* sequencing analysis for L - asparaginase

Ten targeted protein spots (Plate 10) were identified by MALDI - TOF - TOF followed by peptide sequencing using PEAKS Studio 5.3 *de novo* sequencing software (fig. 11). The generalized schematic of the methodology used in the current study to compile a database for *Streptomyces radiopugnans*. The detailed information of the confirmed protein characterization are elaborated with respect to the precursor mass, *m/z* error (ppm), PEAKS score for confidence interval (per cent) for the PEAKS *de novo* generated peptide sequences and their corresponding homology searches. The MS/MS spectrum was analyzed by PEAKS *de novo* sequencing software to generate “GSLVLLAADGSVDLALGDPAAPVFPR” (Fig. 12). The peak homology search of this peptide was almost 95 per cent identical to that of amino - terminal amino acid sequence of *Streptomyces* sp.

5. 3. 4. Amino acid composition

Aminoacid composition of the L-asparaginase enzyme was carried out from the purified enzyme fraction using a Beckman Amino Acid Analyzer (Model 119 GL). Amino acid contents of the purified *S. radiopugnans* MS1 L-asparaginase has been shown in fig. 13. The purified enzyme was rich in alanine and leucine. For comparison, the amino acid composition of the enzyme with the compositions of other bacterial L-asparaginase has been shown in table 16. The *S. radiopugnans* MS1 L-asparaginase has an amino acid composition generally similar to that of other *Streptomyces* L - asparaginase.

The amino acid profile of *S. radiopugnans* MS1 was compared with the other species of *Streptomyces* to analyse the homogeneity in aminoacid profile of the isolate of the present study. The table 16 presents the various aminoacids possessed by the organism. This profile could be used for structure building using bioinformatics tools.
5.4. Analysis of Enzyme properties

5.4.1. Kinetic parameters of the purified L-asparaginase

The $K_m$ and $V_{max}$ of purified L-asparaginase from *S. radiopugnans* MS1 were 0.0598 mM and 3.5478 IU/μg, respectively (Fig. 14). This indicates that the high affinity of the enzyme to the substrate. L-asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity.

5.4.2. Effect of pH, temperature on activity and stability of purified enzyme

The purified L-asparaginase was subjected to different pH from 2 to 12, to check for the stability of the enzyme and to measure its activity. The enzyme showed stability at pH 5.0 - 11.0. The results of effect of pH on enzyme activity are presented in fig. 15.

The purified L-asparaginase was active at a pH range of 5 - 12. The maximum L-asparaginase activity was obtained at pH 6.0. The activity of L-asparaginase from *S. radiopugnans* MS1 showed alkaline optimum pH 6 - 7.

While the temperature optimum for enzyme activity was measured, the purified enzyme exhibited maximum activity at 40°C. The results have been presented in fig. 16.

The enzyme activity measured at 40°C was found to be maximum and thus was taken as the optimum temperature for activity and its activity decreased sharply above 90°C with a loss of 53 per cent of its original activity at 80°C.

*In vitro* half-life of L-asparaginase has been studied to understand the stability of the enzyme. The result showed that in vitro half-life of the L-asparaginase was 48 hrs (Fig. 17).

No significant activity was lost when the purified enzyme was pre-incubated at 40°C for 60 minutes and beyond this temperature the enzyme became increasingly stable till 80°C.

5.4.3. Influence of metal ions and reagents on L-asparaginase activity

L-asparaginase activity was assayed in the presence of various ions and reagents (Table 17). Among the ions tested, significant loss of activity was observed only with Cu$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Fe$^{3+}$ and Mg$^{2+}$ but Na$^+$, K$^+$ and EDTA acting as an inducer. The data designate that asparaginase might not be the metalloenzyme. Based on amino acids tested, only L - cysteine and histidine kindle the comparative activity, while others
had not shown any apparent result. Inhibition of enzyme activity in presence of Cu$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$ might be indicative of essential vicinal sulfhydryl groups of the enzyme for productive catalysis. In addition, inspiration of the establishment with reducing agents like reduced glutathione (GSH), 2 - mercaptoethanol (2 - ME) and dithiothreitol (DTT) and inhibition in the presence of thiol group blocking reagents, specifically, P - chloro mercury benzoate and iodiacetate provided supplementary confirmation for the role of sulfhydryl groups in the catalytic activity of the enzyme. The enzyme entirely lost its activity at 5.0M urea and only 5.0 M sodium dodecyl sulfate (SDS).

5. 5. Genetic Engineering of the L-asparaginase and Cloning into E. coli host cells

5. 5. 1. Gene cloning and sequence analysis

Approximately 50 clones of L-asparaginase genes were screened by colony PCR (Plate -18). The Multiple cloning Sites (MCS) (152 bp) of pET32a (Fig. 19) along with the inserts were amplified from the clones using T$_7$ forward and reverse primer and were sequenced. The results of agarose gel electrophoresis of S. radiopugnans MS1 L-asparaginase amplification and agarose gel electrophoresis showing the pET32a cloned L-asparaginase sequence. Lane marker: DNA marker (1Kb) used as standard has been presented in lane B. The results are presented in plate 20. The sequence was then confirmed by BLAST analysis against the sequence present in NCBI data base. The primers T$_7$ promotor and terminator were used to amplify the complete asp gene from E.coli BL21 (DE3) containing the recombinant plasmid. The resulting 1700 bp PCR product was sequenced using the same primers which were used for amplification (Plate 20).

5. 6. Purification of recombinant L-asparaginase

The recombinant L-asparaginase secreted into the culture supernatant was purified in a single step by Ni - NTA affinity chromatography. One hundred milliliter of the supernatant was collected and treated and then loaded on to the affinity column. After washing, the bound recombinant L-asparaginase was eluted with elution buffer. SDS - PAGE analysis of the purified recombinant L-asparaginase revealed a single protein band migrating at approximately 55.3 kDa (plate 21). The specific activity of the recombinant L-asparaginase increased from 106.34 IU/mg to 742.86 IU/mg, which was a 6.9 - fold increase during this purification step. Overall yield of 7800 IU/ml that was
approximately 10.5 mg/ml of the purified recombinant L-asparaginase was achieved at the shake flask level. This corresponded to a recovery of 136.8 per cent from the initial recombinant L-asparaginase which was present in the culture medium. The results of purification status of recombinant L-asparaginase from *E.coli* BL 21 has been shown in table 18.

5.7 Application of L-asparaginase

5.7.1 Exposure of Hepatocellular carcinoma to L-asparaginase

5.7.1.1 *In vitro* L-asparaginase cytotoxicity assay in HepG2 cell line

Cytotoxicity results of Recombinant L-asparaginase has been summarized in table 19. L-asparaginase showed positive activity and high selectivity against HepG2 cell line (SI = 10.87), when compared with commercially available L-asparaginase (6.99) melphalan (SI = 2.43 and 5-FU (SI = 1.62). This study, not only estimates IC50 value but also determines that the Selective Index (SI) of the enzyme which was comparatively very high than the other standard commercial product. Thus the data demonstrates the effectiveness of the indigenously produced recombinant L-asparaginase in the therapeutic applications of HepG2 cell line (Plate 22).

5.7.2 Effects of recombinant L-asparaginase on DNA synthesis in tumor cells

Results to estimate the effect of the recombinant L-asparaginase on DNA synthesis in tumour cells were processed with Cell fit software package. Compared with the control group, treatment with recombinant and commercially L-asparaginase had significantly increased the number of HepG2 cells at the G0/G1 stages (65.3 and 45.3 per cent respectively). On the other hand, the number of cells at the S stage was dramatically decreased, indicative of reduction in DNA synthesis due to interception at the G0/G1 stages by L-asparaginase treatments. Meanwhile, the appearance of the sub-peak at the G1 stage before the G0/G1 stages was due to apoptosis. It suggested that the accelerated tumor cell death was achieved through cell apoptosis after treatment with L-asparaginases (Table 20 and Plate 23).

5.8 Cytokines mRNA expression of HepG2 cells treated with L-asparaginase by RT-PCR

The cytokines secreted by tumor cells can promote immune suppression and angiogenesis, which in turn eventually facilitate tumor survival and metastasis. To
investigate the physiological and pathological roles of L-asparaginase in HepG2 cells, HepG2 cells were treated with L-asparaginase for 72 hrs, and then the supernatants were collected to facilitate determination of the levels of positive control group which was treated with imported commercially available L-asparaginase.

The negative control group received 0.9 per cent normal saline PCR. The unstimulated HepG2 cells (controls) were significantly expressed or secrete VEGF, IL-4, IL-8, and IL-10 and detected down-regulated mRNA expression for VEGF and IL-8 where as up-regulated mRNA for IL-4 and IL-10 in HepG2 cells after L-asparaginase stimulation.

5. 8. 1. Isolation of mRNA

The RNA was extracted from the HepG2 cell using modified protocol chomczynki and Sacchi. The Quality assessment of RNA extracted from HepG2 was done by agarose gel electrophoresis. Plate 24; shows the electrophoretic separation of RNA. (Lane A) shows the presence of the two intact ribosomal RNA bands (28S and 18S) with the intensity of the 28S ribosomal band approximately twice that of the 18s band. This was the indirect indication that the mRNA was intact without any degradation in this preparation. To purify the mRNA from this total RNA, 250.0 µg of total RNA was used. Plate 24 (Lane B) shows the mRNA purified using mRNA mini purification kit (Qiagen). Total mRNAs appeared as continuous smear from 500bp to 5 Kb. Appearance of intact residual 28S and 18S ribosomal RNA indicated the intact presence and enrichment of mRNA. Distribution of mRNA centered in the 1 - 3 Kb regions indicated that the majority of the mRNAs are in this size range. The continuous smear of mRNA was not degraded. The purity of the RNA preparation was assessed by spectrophotometric analysis prior to Reverse transcription. Results shown in Table 21 indicate that the extracted RNA and mRNA are intact and are of high quality suitable for RT - PCR analysis.

5. 8. 2. cDNA synthesis

From cDNA synthesis, 5.0 µg of intact mRNA was used as starting material. First strand cDNA was synthesized by reverse transcription using mRNA as a template primed by Oligo Not I adapter primer. After reverse transcription, the product was electrophoresed through 1.0 per cent agarose gel. First strand cDNA (mRNA: cDNA
hybrid) appeared as a smear from 500bp to 7Kb plate 25 (lane A). Second strand cDNA was synthesized using enzyme cocktail provided by manufacturers. Plate 25 (lane B) shows the distribution of second strand cDNAs similar to the distribution of the first strand cDNA. First and second cDNAs appeared in a size, ranging from 500 bp to 7 Kb.

5.8.3. RT PCR analysis

5.8.3.1. Expression of VEGF, IL-4, IL-8, and IL-10 were analyzed by qPCR

5.8.3.1.1. Analysis Parameters

The real-time PCR reactions were performed in duplicates for both target and normalizer genes and the analysis parameters have been presented below.

Analysis Mode: Normalized expression (ΔΔCq)

Chart Data: Relative to control

Scaling options:

Chart Error: ± 1.0 SEMs

5.8.3.1.2. Target Names

<table>
<thead>
<tr>
<th>Name</th>
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5.8.3.1.3. Target Stability

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<tr>
<td>GAPDH</td>
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<td>2.9626</td>
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</table>

Average Coefficient Variance: 0.9405  Averages M - Value: 2.9626

5.8.3.1.4. Inter-run calibration

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<td>-1.6720</td>
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Average ΔCq: -1.6717

Average ΔΔCq: 0.0000
To determine effects of VEGF, IL-4, IL-8 and IL-10 on HepG2 cells were incubated for 72 hrs. Treatment with L-asparaginase had down-regulated constitutive VEGF and IL-8 and up-regulated IL-4 and IL-10 mRNA levels in HCC cells (HepG2), as measured by real-time PCR. VEGF, IL-4, IL-8, and IL-10 mRNA expression was normalized to GAPDH. The results have been presented in table 22a and table 22b (fig. 26).

5. 9. Cytokine analysis using multiplexed flow cytometric bead-based assays

The cytokines production was evaluated in HepG2 cellular supernatants after incubation with L-asparaginase at 48 and 72 hrs by Bio-Plex assay. The results obtained were compared with untreated cells used as control. These experiments showed that at the increasing ratio of two molecules concentrations, two pro-inflammatory cytokines such as VEGF and IL-8 decreased whereas the anti-inflammatory cytokines such as IL-4 and IL-10 increased in statistically significant way at concentrations increasing of L-asparaginase. However, in HepG2 treated at 72 hrs a significant reduction was observed only using 0.5 U/ml in agreement with the results of cytotoxicity test. The results have been presented in table 23a, table 23b, table 24a and table 24b (Fig. 27 and Fig 28).

5. 10. Bioinformatic Characterization of L-asparaginase

5. 10. 1. Sequence and structure alignment of L-asparaginase

5. 10. 1. 1. Characterization of protein sequence

The multiple alignment result showed significant homology was found with *S. radiopugnans* MS1 and homology with *Streptomyces* sps. The multiple alignment score was showed that they are very close to each other ranging from 50 to 79 per cent as shown in table 25. fig. 29 shows the result of the multiple sequence alignment of the mentioned organisms using CLUSTALW program. The multiple sequence alignment also revealed that there are stretches of amino acids that are exclusive to *Streptomyces* sps L-asparaginase.

BLOCK analysis had indicated that there were six blocks found which was of L-asparaginase II family at positions like 13 - 52, 53 - 82, 122 - 166, 206 - 239, 246 - 296 and 302 - 322 its block sequence were
PVLAEVVRSGTEGHHRGSLVLLAADGSDLALGDPAAP,  
FPRSSNKPMQAAAILRAGLELSGERLALAA,  
AEAYLAAGRVSPEPLTMNCGBKHALAVCVRNGWDTATYLDPAHP,  
AFRAFVTAEPRGSAERRVADAMRAHPEYVAGTRRP,  
EVPGLSKMGAEEAVQAVALADGRALAFKIDDGSTRALGPVLARALELGLGVD  
RIGRAPLLGGAEEVGRIRAFA respectively.

5. 10. 2. Characterization of Sequenced L-asparaginase

Further analysis with “SMART” tool indicated that there was one domain present in the sequence between 15 and 321 with an E-value $4.70 \times 10^{-2}$. The identification of conserved domains from the sequence was done through “Motif Scan”. The results indicate that amino acids from 15 - 321 were highly conserved for L-asparaginase II family.

“PRED TMBB” tool was used to analyze the amino acid positions showed that 65 - 75 and 90 - 102 amino acids were in transmembrane, where 1 - 64 and 103 - 322 amino acid were in inner membrane and 76 - 89 amino acids were present in outer membrane (Fig. 30). Sequence scored a value of 2.835, which was lower than the threshold value of 2.965. The difference between the value and the threshold indicates the possibility of the protein being an outer membrane protein.

5. 10. 3. Prediction of protein structure

The result obtained from “SOPMA” was presented in the form of graphics (Fig. 31). The tool described that about 39.75 per cent of amino acids presented in Alpha helix, 10.25 per cent of amino acids in beta turn, 36.02 per cent of amino acids in random coil, 13.98 per cent of amino acids in extended strand and rest of all amino acids in bridge and turn.

The homology model was builted from sequence of L-asparaginase from *S. radiopugnans* MS1 and as per multiple alignment result *Strptomyces* L-asparaginase used as a template structure. The homology modelling was done through the swiss model server and phyre2 were founded as the best server among of all others software or server. The predicted structure quality was good through different software as compared with others. Fig. 32 and Fig. 33 showed the structure obtained through “swiss model workspace server” and “Phyre 2” respectively.
5.10.4. Characterization of Structure

The amino acid composition of the L-asparaginase has been showed in table 26a and table 26b. The sequence of L-asparaginase was analyzed by the software ‘Protparam’ in order to find the physical and chemical properties (Table 27).

5.10.5. Evaluation and validation of refined model

The model generated by the above method was subjected to validation using the following softwares: Ramachandran Plot using RAMPAGE server: The Ramachandran plot for the modeled structure showed 87.9 per cent of the residues in the most favored region, 6.4 per cent in the additional allowed region, and 5.8 per cent in the generally allowed region and no residues in the unfavorable region (Fig. 34) and Ramachandran Plot Statistics from Richardson's lab (Fig. 35). L-asparaginase was validated with Phyre2 177 residues (55 per cent of your sequence) have been modelled with 96.6 per cent confidence by the single highest scoring template (Fig. 36).

5.10.6. Comparison of the structure of L-asparaginase

From the time when the alignment results of S. radiopugnans MS1 L-asparaginase showed homology to Streptomyces pristinaespiralis ATCC 25486 L-asparaginase II. The structure of L-asparaginase was superposed with the Streptomyces pristinaespiralis ATCC 25486 (Sippl 2008). L-asparaginase II, which showed that in 10 residues pairs that were structurally equivalent the identities of the overall structure were around 84 per cent (Fig. 37).

5.10.7. Binding site analysis of L-asparaginase

Once final model was build and validated, the possible binding sites of L-asparaginase were searched using Phyre2 server. Eight different Ligand cluster were identified and predicted binding aminoacid were Ala (158), Thr (159), Met (254), Gly (255), Ala (256). (Fig. 38) and Hetrogens present in predicated Binding site (SUC and CFX) (Fig. 39).