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Identification of highest L-Methioninase enzyme producers among soil microbial isolates, with potential antioxidant and anticancer properties

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ABSTRACT

Search for new pharmacological agents is a priority area of research in cancer biology due to the inefficiency of the existing therapeutic approaches toward this disease. L-methioninase enzymatic approach has shown considerable beneficial effects in reducing cancer burden in experimental models. In this regard, the present study is aimed at isolating soil microorganisms that produce L-methioninase with highest enzyme activity and evaluating their anticancer and antioxidant potentials. Fifty different soil samples were collected and screened for L-methioninase producers, from which two isolates with highest L-methioninase activity, KA-S2 (with 32.43 ± 2.16 U/mL activity) and TN-S33 (with 122.76 ± 2.12 U/mL activity) were selected. These promising isolates were subjected to molecular characterization by 16s rRNA sequencing. The BLAST sequence analysis in the NCBI database showed the sequence similarity of KA-S2 isolate to *Streptomyces diastaticus* with 99.55% similarity and that of TN-S33 to *Methylobacterium aminovorans* with 99.86% similarity. The sequences were deposited in the NCBI database as *Streptomyces* sp. JUBTK2 with the Accession number MN372078 and *Methylobacterium* sp. strain JUBTK33 with the Accession number MN372079.1. When the enzymes from these isolates were checked for antioxidant and anticancer activities, they demonstrated high antioxidant and anticancer potentials. It can be concluded from the present study results that the enzyme extracted from the new isolates has high L-methioninase activity coupled with promising anticancer potential.

1. INTRODUCTION

Cancer is a complex disease involving multistep processes comprising metabolic and behavioral changes of the cell, causing them to proliferate excessively in an uncontrolled manner. Genetic, epigenetic, and other factors are also contributing to the disease process of cancer [1]. Understanding the responsiveness of cancerous cells to different treatment modalities is a major challenge to the scientific community. The conventional treatment strategies of surgery, chemo, ionization/radiation, and ultrasound therapies lack complete effectiveness in reducing cancer and usually cause serious side effects to the healthy cells of the body [2].

A new approach for the treatment of cancer is the use of enzymes that block the availability of chief nutrients required for the rapid growth of cancer cells. The deficit of such nutrients is key for halting cancer cell growth. Few microbial enzymes such as L-Asparaginase, L-arginase, and L-methioninase are being explored toward cancer therapy [3]. Hence, the search for new biological sources that produce L-methioninase would be of great significance toward cancer drug discoveries.

Cancer cells have a higher requirement for the amino acid L-methionine as they lack methionine synthase, unlike normal healthy cells [4]. The use of L-methioninase depletes the plasma L-methionine, alters methionine dependent metabolic activity in cancer cells and halts their growth. Targeting essential amino acid L-methionine depletion by enzymatic degradation appears to be a promising approach in treating tumor cells [5,6].

L-methioninase has been reported from bacteria such as *Pseudomonas putida, Clostridium sporogenes, Aeromonas* sp., *Citrobacter intermedius, Brevibacterium linens, Trichomonas vaginalis, Porphyromonas gingivalis, Treponema denticola,* and *Streptomyces* sp [7]. Bacteria are also well known for their diverse secondary metabolites having antioxidant capabilities that can specifically target cancer cells and cause tumor regression [8,9]. Although L-methioninase production from fungal source has been reported as efficient and easier, the anaphylactic reactions triggered by them is of great concern [10]. It appears that exploring bacterial sources for L-methioninase would be a safer alternative as compared to fungal sources. Hence, in the present study, we aimed to isolate microbes

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from various soil sources, screen them for identifying the highest L-methioninase producer and to analyze the antioxidant, anticancer activity of the promising isolates.

2. MATERIALS AND METHODS

2.2. Soil Samples

Soil samples from various unexplored ecological niches such as dense forest, isolated ponds, abandoned mining area (Kolar gold mines in Karnataka), hill top, surrounding of rocks, soil near water fall, landslide, and agricultural lands across different states in India were collected and stored in dry sterile containers. The samples were further used for the isolation of bacteria, actinomycetes, and fungi.

2.3. Chemicals

The chemicals used in the current study include L-methionine (Merck, Germany). Pyridoxal-5-phosphate and L-ascorbic acid (HIMEDIA), Coomassie Brilliant Blue, Bovine Serum Albumin (BSA), MEM media, fetal bovine serum, trypsin, di-Sodium monohydrogen phosphate ($Na_2HPO_{4)}$, monopotassium dihydrogen phosphate ($KH_2PO_{4),}$ di-potassium monohydrogen phosphate (K_2HPO4), sodium chloride (NaCl), magnesium sulfate ($MgSO_{4,}$ calcium chloride ($CaCl_2$), D-Glucose, phenol red, agar, trichloroacetic Acid, and Nessler's reagent (Potassium tetraiodomercurate (II)), all of analytical grade.

2.4. Rapid Screening of L-Methioninase Producers

The soil samples were serially diluted (10^{-3}, 10^{-4}, and 10^{-5}) and were spread plated onto modified M9 media used for rapid screening of L-methioninase producing microorganisms. Modified M9 media contained Na_2HPO_4, 6.0 g/l; L-methionine, 5.0 g/l; KH_2PO_4, 3.0 g/l; NaCl, 0.5 g/l; $MgSO_4$, 0.24 g/l; $CaCl_2$, 0.011 g/l; D – Glucose, 2.0 g/l; and phenol red as an indicator and 2% Agar with a pH of 7.0. The plates were incubated at 37°C for 3 days for isolating bacteria and actinomycetes and at room temperature (26±°C) for 5–6 days for isolating fungi. The positive colonies, which were pink in color, were selected and maintained as pure cultures [11].

2.5. Extraction of L-methioninase from the Production Media

The selected colonies were transferred to L-methioninase production media containing sodium citrate, 0.5 g/l; $MgSO_4$, 0.1 g/l; ammonium sulfate, 1.0 g/l; K_2HPO_4, 7.0 g/l; KH_2PO_4, 2.0 g/l; D-glucose, 4.0 g/l; DL-leucine, 0.2 g/l; and thiamine hydrochloride, 0.005 g/l [11]. The microbial cultures were transferred aseptically to 100 ml of this defined medium, each in separate 250 ml Erlenmeyer flasks. The flasks were incubated in a shaker incubator at 37°C for 2–3 days. After this period, the media were centrifuged at 10,000 rpm for 15 min at 4°C; the supernatant having the enzyme was collected and stored at −20°C for further use.

2.6. Protein Estimation

The protein content of the enzyme samples (microbial culture supernatants) was determined by Lowry's method [12], using bovine serum albumin as the standard at 200 µg/mL concentration.

2.7. L-methioninase Assay

The culture supernatants containing the crude enzymes were used for L-methioninase activity assay by direct Nesslerization method according to the standard protocol with some modifications [13]. The assay was performed by incubating 1 mL of 1% L-methionine as the substrate prepared in phosphate buffer (50 mmol and pH 7.0), followed by 0.1 mL of pyridoxal phosphate (100 µmol) with 1 mL of crude enzyme extract. The contents were mixed and incubated for 60 min at 37°C. The enzyme catalyzed reaction was arrested by adding 0.1 mL of Trichloro acetic acid (1.5 mol). The ammonia released was determined by adding 0.5 ml of Nessler's reagent ($HgCl_2$, KI, and NaOH) and the developed color was measured spectrophotometrically at 430 nm against the enzyme blank [14].

The unit of enzyme activity in the crude extract was expressed as µmol/ml. The specific activity was calculated using total protein content and expressed as unit activity per milligram protein (U/mg).

2.8. Partial Purification of L-methioninase

The crude enzyme was subjected to partial purification by acetone precipitation method [15]. The crude enzyme and chilled acetone (1:4) were mixed well and incubated over night at −20°C. The mixture was centrifuged at −4°C at 10,000 rpm for 20 min. The precipitate collected was dissolved in 50 mM potassium phosphate buffer of pH 7.0.

2.9. Lyophilization of the Sample

The above partially purified enzyme was lyophilized to obtain the enzyme in powder form. The powder was suitably dissolved in phosphate buffer for estimating enzyme and specific activities.

2.10. Antioxidant Assay by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) Method

The radical scavenging activity of the crude enzyme was determined following DPPH assay with slight modifications in the protocol [16]. Different concentrations of the crude sample were prepared using phosphate-buffer of pH 8. 200 µl of the sample with 100 µl of the DPPH was incubated at room temperature (26±2°C) for 30 min in the dark. The absorbance was recorded at 517 nm in an ELISA reader. L-ascorbic acid was used as the positive control. The percentage of DPPH radical scavenging activity was calculated as per the following formula.

$$\% \text{ DPPH scavenging activity} = \frac{Control\ OD - Test\ OD}{Control\ OD} x100$$

2.11. Biochemical Characterization of the Selected Isolates

The selected isolates were subjected to biochemical characterization by performing catalase, oxidase, Simmon citrate, indole [17], triple sugar, urea, methyl red, Voges–Proskauer, and tryptophan tests as per standard protocols given in Bergey's manual [18-21].

2.12. Molecular Characterization by 16s rRNA Sequencing

Molecular identification of the promising bacterial isolates was carried out by 16s rRNA sequencing (outsourced to Applied Biosciences, Bengaluru) using sequence scanner software 2v 2.0. The phylogenic tree was constructed using the Mega X (version 10.1.7) software.

2.13. Cell Cytotoxicity Assay

The anti-cancer activity was determined following the standard protocol of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay [22]. The assay was carried out using different concentrations of the crude and partially purified enzyme samples on HepG2 liver cancer cell line.

2.14. Statistical Analysis

All experiments for enzyme activity and total protein content of various bacterial and fungal samples were performed in triplicates. Statistical

significance was calculated using one-way ANOVA with GraphPad Prism 6.0 software for all independent triplicates experiments. Values are expressed as mean ± SEM, and reported as significant when $P < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Rapid Screening

A total of fifty soil samples were collected. All the fifty samples from different places of India were screened for L-methioninase producing microorganisms. These samples were categorized based on the states of samples collected as Karnataka, Kerala, Rajasthan, Tamil Nadu, Shillong, and Assam [Table 1]. Out of these 41 samples showed L-methioninase activity, while three samples from Karnataka, three from Rajasthan, and two from Shillong had no L-methioninase producers. The bacterial samples designated as KA-S2 and TN-S33 showed the highest L-methioninase activity with 32 U/ml and 122 U/ml, respectively, with the corresponding specific activities being 124 and 164 U/mg, as shown in Figures 1 and 2. The bacteria, KA-S2 was isolated from the soil surrounding the bottom of a rock at Revana Siddeshwara hills, also known as SRS Hills in Karnataka (Location- 12°38'25.4"N 77°19'35.4"E). The actinomycete, TN-S33, was isolated from the soil of fenugreek farm land near Sethuvalai, Tamil Nadu (Location- 12°53'35.8"N 79°00'52.7"E). These isolates were chosen for further studies. The bacterial isolates from Kerala, Assam, and fungal isolates of different places showed lesser L-methioninase activities [Figures 3 and 4].

3.2. Antioxidant Activity

The role of bacteria as sources of natural antioxidants has been reported earlier [23] and antioxidants were reported as to play a significant role in cancer with the ability for tumor regression [8,9]. In the present study, the isolates KA-S-2 and TN-S-33 were checked for their antioxidant properties as both these isolates demonstrated highly promising L-methioninase activities. As per the results of the DPPH assay, at higher concentrations of the samples (100 and 200 µg/mL), the percentage scavenging activities were 65–67% which is very close to that of the positive control, ascorbic acid [Figure 5]. These results showed that the L-methioninase samples possess good capability for scavenging free radicals, thus indicating the potential of these microbial isolates toward anticancer application studies. The antioxidant property of microbial extracts varies depending on the concentrations of the metabolites present in them [24,25].

3.3. Characterization of Selected Isolates

The results of the various biochemical tests carried out to characterize the promising isolates KA-S-2 and TN-S-33 are represented in Figures 6 and 7 and Table 2.

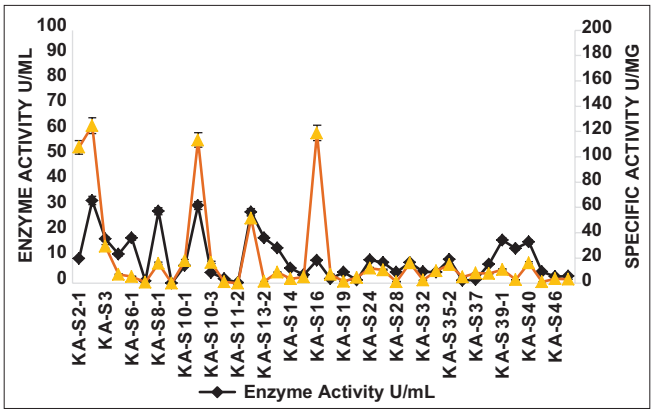

Figure 1: Enzyme activity and specific activity of the bacterial isolates from Karnataka (KA: Karnataka, S: Sample, Numbers represents the samples). The values are expressed as mean±SE

Table 1: Soil samples from different locations used for isolation of microbes that produce L-methioninase

State	Place	Source of soil	Geographical Coordinates	Number of Positive Bacterial Isolates	Number of Positive Fungal Isolates
Karnataka	SRS hills	Hill	12°38'25.4"N 77°19'35.4"E	2	
	Kolar Gold Field - Cyanide Treated sample	Mining area	12°57'42.3"N 78°16'14.6"E	2	2
	Mysore - Chamundi Hills	Hills	12°16'30.92"N 76°40'12.53"E	2	
	Manchinbele dam	Hills	12°52'01.3"N 77°20'04.0"E	2	
	Sunkapura region	Pond area	14°33'37.1"N 75°24'50.8"E	6	2
	Tavarekere market	Waste Dump Area	12°58'03.0"N 77°24'01.4"E	1	
	Chikmagalur	Hill	13° 14' 8.5° N, 75° 29' 0.2° E	5	3
	Bettampady	Dam Area	12°39'56.5"N 75°11'52.4"E	1	2
	Poultry Farm	Farm Land	11°59'58.2"N 76°43'14.5"E	1	
	Ragi Farm	Farm Land	13°07'59.9"N 78°10'19.9"E	4	1
	Kolar meat dump Area	Waste Dump Area	13°08'10.3"N 78°07'44.8"E	2	1
	Mullayanagiri surroundings	Hills	13°23′26″N 75°43′18″E	1	
	Mullayanagiri	Hills	13°18'58.0"N 75°46'19.2"E	4	1
	Tumkur 1- coconut farm A1	Farm Land	13°25'35.0"N 76°34'08.8"E	2	1
	Chikmagalur Land slide area 2	Hill	13°07′46.24″N 75°16′06.79″E	2	2
Kerala	Kerala - Reserve area of Malampuzha dam forest	Dam Area	10°49′49.8″N 76°41′1.5″E	3	1
Tamil Nadu	Tamil nadu Farm Land	Farm Land	12°53'35.8"N 79°00'52.7"E	6	5
	Kodaikanal hills	Hill	10°14'28.7"N 77°30'32.4"E	2	
Assam	Kaziranga forest	Hill	26°34'39.0"N 93°10'16.0"E	3	1

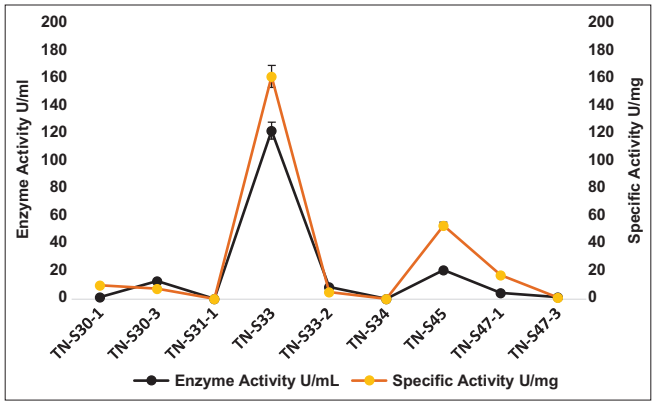

Figure 2: Enzyme activity and specific activity of the isolates of Tamil Nadu. (TN: Tamil Nadu, S: Sample, Numbers represents the sample). The values are expressed as mean±SE.

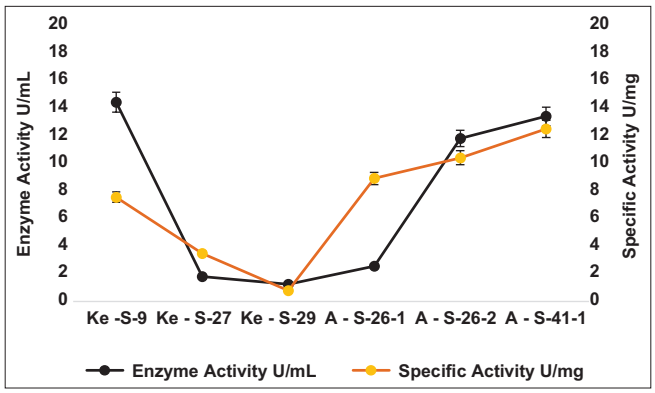

Figure 3: Enzyme and specific activity of the isolates collected from Kerala and Assam soil (KE: Kerala, A: Assam). The values are expressed as mean±SE

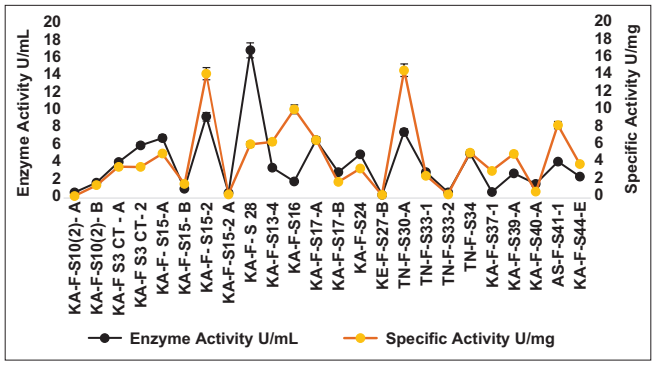

Figure 4: Enzyme and specific activity of the Fungal isolates (KA: Karnataka, TN: Tamil Nadu, KE: Kerala, A: Assam). The values are expressed as mean±SE

3.3.1. Biochemical and molecular characterization of KA-S2

The morphological observation of KA-S2 indicated that it belonged to the Actinomycetes family. The physiological and biochemical characteristics of KA-S2 presented in Table 2 indicate that it is Gram-positive, catalase positive, oxidase negative, methyl red, and Voges-Proskauer test results were positive. The triple sugar and Simmon Citrate Agar tests were found negative. These results suggest the isolate as a strain of *Streptomyces* sp. [Figure 7]. The molecular identification of KA-S2 was carried out by 16s ribosomal RNA partial

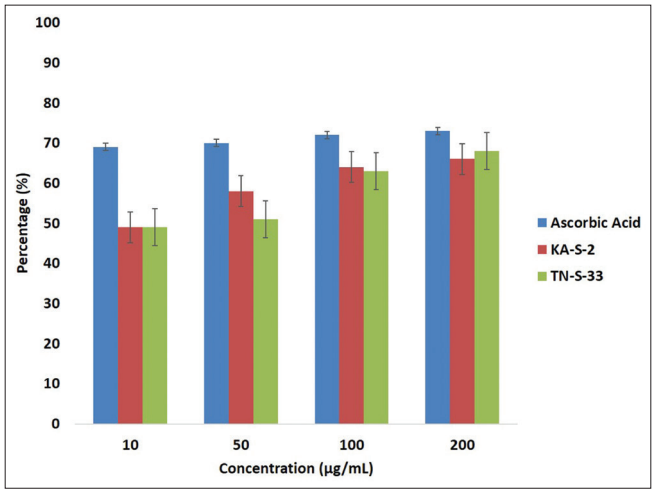

Figure 5: DPPH scavenging activity of microbial isolates. The values are expressed as mean±SE.

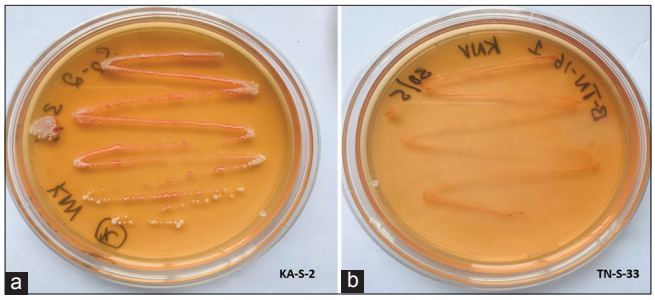

Figure 6: The L-methioninase producing isolates: a- KA-S-2; b- TN-S-33

Table 2: Biochemical tests of the selected KA-S-2 and TN-S-33 isolates

Biochemical tests	Results	
	KA-S-2	TN-S-33
Catalase test	+	+
Simon citrate agar test	–	–
Oxidase test	–	–
Methyl red test	+	+
Triple sugar iodine test	–	–
Voges–Proskauer test	+	–
Indole test	–	–
Urease test	+	–

sequencing. The nucleotide sequence thus obtained was analyzed in the NCBI database to determine the sequence similarity using the BLAST program. The results indicated the highest similarity of 99.55% to *Streptomyces diastaticus*. On considering all the related sequences of the same species from Genbank database, a phylogenetic tree was constructed with the help of Mega X software (version 10.1.7) using neighbor-joining method which showed a bootstrap value of 32% with *Streptomyces diastaticus* [Figure 8]. A low bootstrap value of 32% (<50) indicates that it might be a novel strain of *Streptomyces* sp. as it excludes the possibility of specific relatedness between the two species. Thus, on the basis of this, KA-S2 can be assigned as a new species of *Streptomyces,* designated as *Streptomyces* sp. JUBTK2. The 16S rRNA gene sequence was deposited in the NCBI database with the Accession number MN372078.

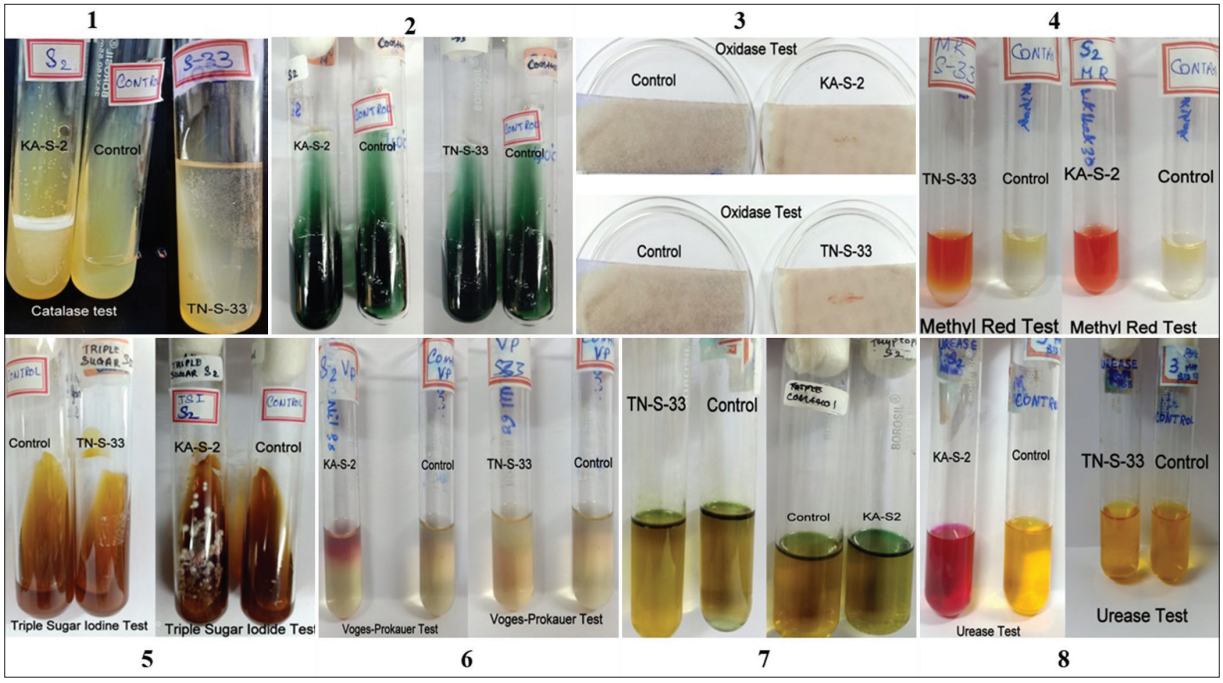

Figure 7: (1) Catalase, (2) Simon Citrate Agar, (3) Oxidase, (4) Methyl red, (5) Triple sugar iodine, (6) Voges–Proskauer, (7) Indole, (8) Urease tests

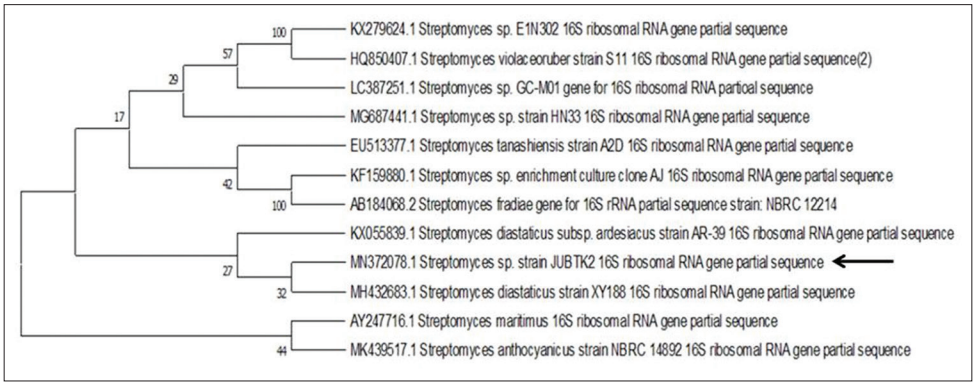

Figure 8: The phylogenetic tree of *Streptomyces* sp JUBTK2 and the relationships with the closest species based on 16S rRNA gene sequencing using the neighbor-joining method. Bootstrap values are shown at the nodes

3.3.2. Biochemical and molecular characterization of TN-S-33

The isolate, TN-S-33 was found to be Gram-negative, catalase positive, oxidase negative, hydrolyzing capacity of citrate and triple sugars negative, methyl red test positive, and Voges–Proskauer test negative as per the results of biochemical tests [Table 2 and Figure 7]. The isolate was further subjected to molecular characterization through 16s rRNA sequencing.

The 16s rRNA partial nucleotide sequence obtained was analyzed in the NCBI database for sequence similarity using BLAST analysis program which revealed the highest similarity of 99.89% to *Methylobacterium aminovorans*. After analyzing related sequences of the same species from the Genbank database, a phylogenetic tree was constructed with Mega X software (version 10.1.7) using neighbor-joining method. The results showed a bootstrap value of 42% with *Methylobacterium aminovorans*. A lower bootstrap value excludes the possibility of specific relatedness between the two species suggesting it as a novel strain of *Methylobacterium* sp. The 16S rRNA gene sequence was deposited in the NCBI database and was saved as *Methylobacterium* sp. strain JUBTK33 with the Accession number MN372079.1 [Figure 9]. To the best of our knowledge, based on the available data [26], this is the first report on this particular species possessing L-methioninase activity.

3.4. Partial Purified Sample

The partially purified sample after acetone precipitation was reconstituted using phosphate-buffered (pH 7) to prepare a solution of 1 mg/mL concentration and used for determining the enzyme and specific activities. Enzyme activity and total protein content were represented as mean ± Standard errors ($n = 3$). The enzyme activity of *Streptomyces* sp. JUBTK2 was 32.4 U/mL which increased to 47.9 U/mL after partial purification while the specific activity was 162 U/mg protein for the crude sample and 282 U/mg protein for the acetone purified sample indicating 1.7 folds purification [Table 3]. In a recent study of L-methioninase from different isolates of *Streptomyces* sp., the highest specific activity reported was 40.9 U/mg protein [26]. In another very recent report, the highest enzyme activity of L-methioninase from *Streptomyces* DMMMH60 after optimization of culture conditions was 60.7 U/mg [27], while the *Streptomyces* sp. JUBTK2 isolate in

Table 3: L-Methioninase activity and specific activity of the selected bacterial isolates. The values are expressed as mean±SE

S. No.	Bacterial isolate code	Sample	Enzyme activity U/mL	Total protein content µg/mL	Specific activity U/mg
1.	*Streptomyces* sp. JUBTK2	Crude sample	32.43± 2.16	210±0.012	162
		Partially purified	47.94±1.93	175±0.026	282
2.	*Methylobacterium* sp. Strain JUBTK33	Crude enzyme	122.76±2.12	700±0.088	175
		Partially purified	103.05±3.91	175±0.020	606

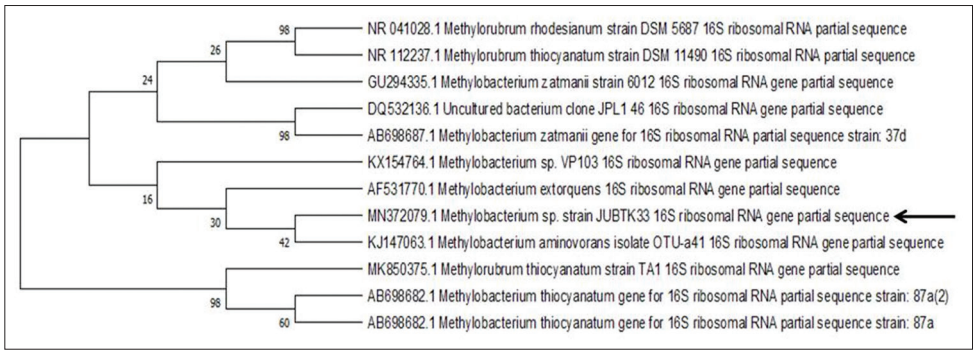

Figure 9: The phylogenetic tree of *Methylobacterium* sp. Strain JUBTK33 and the relationships with the closest species based on 16S rRNA gene sequencing using the neighbor-joining method. Bootstrap values are shown at the nodes

the current study demonstrated higher specific activity of 162 U/mg protein thus proving it to be a highest producer of L-methioninase enzyme among the soil bacteria collected from various locations in India. In the case of *Methylobacterium* sp., the enzyme activity was 122.76 U/mL for the crude sample, 103.0 U/mL for partially purified sample. As compared to a recent report of L-methioninase activity from *Hafnia alvei*, a novel bacterial isolate, the L-methioninase activity of our isolate, *Methylobacterium* sp. is much higher [7]. The specific activities of L-methioninase from *Methylobacterium* sp. was 175 U/mg protein for the crude sample which increased to 606 U/mg protein for the acetone purified sample indicating 3.5 folds purification by acetone precipitation method. In another study [27], it was reported that they could purify the L-methioninase from a *Streptomyces* sp. through Sephadex-G column resulting in a specific activity of 260.5 U/mg and a purification of 3.15 folds. When compared to this study, our isolated bacterial L-methioninase, we hereby report a higher enzyme activity, specific activity of 606 U/mg proteins, which is again a higher degree of purification with 3.5 folds higher than the crude enzyme. All these indicate that both the isolates in the current study are having high enzyme activities and show promise toward future characterization and anticancer application studies.

3.5. Cytotoxicity Assay

The cell cytotoxicity (MTT) assay was performed on the liver cancer cell line HepG2 with the crude extracts of the two isolates at different treatment concentrations. The results suggested that *Methylobacterium* sp. exhibited cytotoxicity at 10 µg/mL concentration with 77% viability and *Streptomyces* sp. exhibited cytotoxicity at 50 µg/mL concentration with 71% viability of the treated HepG2 cells [Figure 10]. Further increase in the treatment concentrations to 100 and 200 µg/mL did not cause any significant cytotoxic effects to the cancer cells. Among the two isolates, *Streptomyces* sp. had a higher effect on the cancer cells. Degradation of methionine using methioninase enzyme in methionine dependent (MET-dp) cancer cells in culture results in inhibition of mitosis, cell cycle arrest predominantly in the late S/G2 phase of the cell cycle, followed by apoptosis of the cells [26]. The current study results are also supporting the use of L-methioninase for anticancer

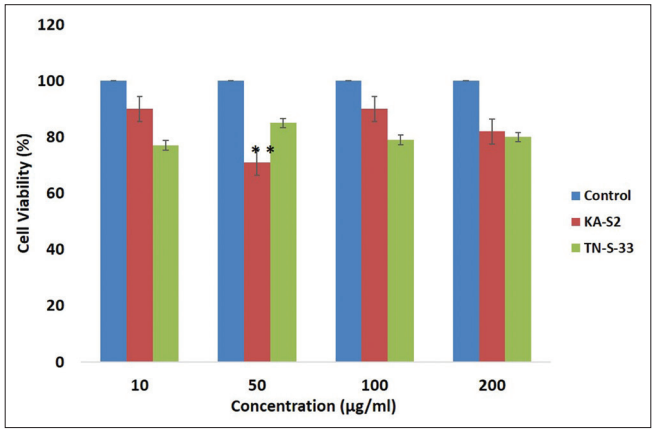

Figure 10: Percentage viability of HepG2 cells treated with L-methioninase from KA-S2 and TN-S-33. The values are expressed as mean±SE. The level of significance is indicated as **P*<0.05, ***P*<0.01 in comparison to the untreated control

applications since we observed inhibitory effects of crude enzymes at low concentrations. There are some earlier studies that reported the cytotoxicity of the purified L-methioninase extracted from *Streptomyces* sp. [28,29]. However, this is the first study, to the best of our knowledge, to report the cytotoxicity of L-methioninase from *Methylobacterium* sp.

4. CONCLUSION

Through the current study of screening microbial isolates from various soil samples for L-methioninase production, two novel isolates, identified as *Streptomyces* sp. JUBTK2 and *Methylobacterium* sp. JUBTK33, were found to be the highest producers of L-methioninase enzyme that could be partially purified. The enzyme was found to possess promising anti-oxidant and anticancer activities and hence can be taken up for future purification and application studies. This is the first report of L-methioninase from *Methylobacterium* sp. These two

new isolates offer further opportunities to produce L-methioninase for cancer therapy.

5. CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

6. FINANCIAL SUPPORT AND SPONSORSHIP

None.

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