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Research Article

Investigation of Efficient Local Bacterial Isolates Producing L-methioninase

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A B S T R A C T

Introduction: L-methioninase is one of the few enzymes that have a high therapeutic value since it was reported as an effective anti-cancer agent against different types of malignant cell lines like lung, colon, and breast. The distribution of methionine-gamma-lyase (MGL) as an intracellular enzyme in all microbial pathogens, but not in humans, makes it a promising drug target for antibacterial, antifungal, and antiprotozoal therapies. MGL is intracellularly present in bacteria. Both gram-negative and gram-positive bacteria have been reported to produce L-methioninase. Screening involves the use of selection procedures for the isolation of high-yielding species from natural sources such as soil, plants, water, and animals containing a heterogeneous large microbial population.

Method: One hundred and thirty-seven samples collected from different sources were screened for the isolation of methioninase-producing bacteria based on morphological and biochemical characteristics and then only the two best isolates for MGL production (17 and 111) were selected. These were subjected to molecular characterisation by 16S rRNA sequencing.

Results: The Blast sequence analysis in the NCBI database showed a sequence similarity of isolate 17 to Serratia sp. with 99% similarity and that of 111 to Serratia marcescens with 99% similarity. Conclusion: The sequences were deposited in the NCBI database as Serratia sp. NDM25 with accession number op564989 and Serratia marcescens NDM73 with accession number op564990.

Keywords: L-Methioninase, Serratia Marcescens, 16S rRNA, Phylogeny

Introduction

L-methioninase is one of the few enzymes that have a high therapeutic value since it was reported as an effective anti-cancer agent against different types of malignant cell lines like lung, colon, and breast. The distribution of methionine-gamma-lyase (MGL) as an intracellular enzyme in all microbial pathogens, but not in humans, makes it a promising drug target for antibacterial, antifungal, and antiprotozoal therapies. MGL is intracellularly present in bacteria. Both gram-negative and gram-positive bacteria have been reported to produce L-methioninase. Screening involves the use of selection procedures for the isolation of high-yielding species from natural sources such as soil, plants, water, and animals containing a heterogeneous large microbial population. Method: One hundred and thirty-seven samples collected from different sources

L-methioninase or methionine gamma-lyase (MGL, EC 4.4.1.11), belongs to the family of pyridoxal L-phosphate (PLP) dependent enzymes which are known to catalyse the alpha, gamma elimination of L-methionine to generate alpha-ketobutyrate, methanethiol and ammonia.[1] L-methioninase is a cytosolic enzyme created by the addition of L-methionine to the culture medium.[2] It catalyses the direct conversion of the amino acid methionine ($C_5H_{11}NO_5$) into α-ketobutyrate, ammonia, and methanethiol (Figure 1).[3]

L-methioninase is one of the few enzymes that have a high therapeutic value since it was reported as an effective anti-cancer agent against different types of malignant cell lines: lung, colon, and breast. The limited distribution of MGL as an intracellular enzyme within all microbial pathogens, but not in humans, makes MGL enzyme a promising drug target for antibacterial, antifungal, and antiprotozoal therapies.[4] It is intracellularly present in both gram-negative and gram-positive bacteria and is also found in fungal species as an intracellular and extracellular enzyme. The enzyme is absent in the mammalian system.[5]

This enzyme was first obtained from the rumen bacteria around the 1950s. It is well-distributed in most organisms, such as bacteria, fungi, protozoa, and plants. However, it is absent in mammals. It has been found in a number of bacterial species, especially in Pseudomonas putida, which is reported to be the best source of MGL.[5] It has been found in various bacteria, some of which are anaerobic like Porphyromonas gingivalis[6] and Treponema denticola[7]. It is also found in eukaryotic pathogens, such as Entamoeba histolytica[8] bacteria like Citrobacter freundii, Aeromona spp., Lactococcus lactis,[9] Clostridium sporogenes,[10] Salmonella spp., Mycobacterium spp., Bacillus spp., and Listeria spp.[11]

MGL for different bacterial species was obtained from several microorganisms such as Citobacter freundii, Bacillus subtilis, and Lactococcus lactis and was purified.[12–14] MGL is found in Eubacteria such as Aeromona ssp., Pseudomonas putida, and Brevibacterium lineus, as well as in Archaebacteria, fungi, protists, and in plants.

For a long period of time, it was only isolated from Clostridium sporogenes, but now, recombinant methionase, from Pseudomonas putida has been found to possess convenient kinetic properties and is more stable. It is remarkable that reports describe MGL in the culture filtrates of a few yeast including Debaryomyces hansenii, Geotrichum candidum, and Saccharomyces cerevisiae.[15]

Materials and Methods

This study was conducted on 137 samples collected from different sources during the period from April to June 2021. Samples taken from the moth and rotten fruits were activated on tryptone soya broth, and incubated at 37 °C for 24 hours. They were then cultured on Tryptone soya agar and again incubated for 24 hours. The growing colonies were transferred to the modified M9 medium. The isolates producing pink-coloured colonies were identified as MGL-producing isolates and were selected for further assays. Stool samples were cultured on MacConkey agar under the same conditions as above. The growing colonies were transferred to the M9 medium and the previous steps were repeated. The soil samples were serially diluted. Each sample (1 gm of soil) was transferred to a sterile container containing 9 ml peptone water. Samples were serially diluted (10^{-3}, 10^{-4}, 10^{-5}) and were plated on the nutrient agar and incubated at 37 °C for 48 hours. The growing colonies were transferred to the M9 medium and the steps were repeated.

For all samples, colonies that grew on the modified M9 medium and gave a pink colour were transferred to Tryptone soya broth or nutrient broth and were incubated at 37 °C for 24–48 hours. They were then preserved in the refrigerator at 4 °C for the purpose of conducting chemical and microscopic examinations. Later, they were tested for their ability to produce the enzyme.

The qualitative detection method was used to test the ability of the isolated bacteria in this study to produce an enzyme. According to a study, the M9 broth medium contains 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as a screening dye inoculated with isolates and incubated at 37 °C for 48 hours.[16] The colour of the medium changed from red to yellow, indicating the ability of the isolate to produce the enzyme. The results were given by looking at a control medium that was not inoculated with bacteria.

The isolates with highly efficient L-methioninase enzyme production, based on the results of the primary screening, were subjected to an additional screening process in which the efficiency of the isolates on enzyme production was estimated quantitatively. In this step, the M9 broth medium was prepared and distributed to plan tubes. Each tube was inoculated with the enzyme-producing isolate according to the initial screening, after which it was incubated under suitable conditions, and separated by centrifugation.

The quantitative assay mixture in a final volume of 1 ml consisted of 20 mM of the amino acid L-methionine in 0.05 M potassium phosphate buffer of pH 7.0, 0.01 mM pyridoxal phosphate (PLP), 0.25 mM DTNB, and 0.3 ml of culture supernatant of the microbial isolate (the crude enzyme sample). The control tube contained heat-denatured (95 °C for 30 min) culture supernatant. This mixture was incubated at 37 °C for one hour and then the developing yellow colour of the assay mixture was read at 412 nm.[17]

The best isolate which showed a high production of MGL activity was subjected to morphological, biochemical and molecular characterisation for identification. The

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preliminary identification was conducted based on microscopic and cultural observations by gram staining.

This was followed by diagnosis by the VITEK 2 system, which was used to confirm the results of the biochemical test and was used according to the manufacturer's instructions (Biomerieux, France). The

molecular identification of the chosen isolates was performed by sequencing the conserved 16S rRNA.

Table 1.Primers Used

Primers' Names	Sequence
27F	5`-AGAGTTTGATCCTGGCTCAG-3`
1493 R	5`-TACGGTTACCTTGTTACGACTT-3`

Table 1 shows the details of the primers used in the study. These primers were supplied by Macrogen Company in a lyophilised form. Lyophilised primers were dissolved in nuclease-free water to give a final concentration of 100 pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10 µl of primer stock solution (stored in a freezer at -20 °C) to 90 µl of nuclease-free water to obtain a working primer solution of 100 pmol/µl. Table 2 shows the PCR component calculation employed in this study.

After PCR amplification, agarose gel electrophoresis was performed to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria. PCR products were sent for Sanger sequencing using ABI13730XL, an automated DNA sequencer, manufactured by Macrogen Corporation, Korea. The results were received by e-mail and analysed using the Geneious software.

Results and Discussion

In this study, 70 isolates of bacteria belonging to different species were selected from various sources. These 70 isolates were selected out of 137 isolates on the basis of their ability to produce the enzyme L-methioninase, which was the main objective of this experiment. This was evaluated based on the formation of pink colonies on the M9 medium containing the amino acid L-methionine (Figure 2). The isolates forming pink-coloured colonies resulting from the production of ammonia by the action of MGL on L-methionine were identified as MGL-producing isolates and were selected for future assays.

These 70 bacterial isolates were subjected to a set of tests in stages to identify the competent isolates for the production of the MGL enzyme. In the first stage, the qualitative detection method was used to test the ability of the isolated bacteria to produce an enzyme by using the M9 broth medium containing DTNB, which is used as a screening

Table 2.PCR Component Calculation

No. of Reactions	2	rxn	Annealing Temperature of Primers (°C)		60
Reaction Volume/Run	25	µl	No. of PCR Cycles		30
Master Mix Components	**Stock**	**Unit**	**Final**	**Unit**	**Volume (µl)**
1 Sample					
Master mix	2	X	1	X	12.5
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease-free water	-	-	-	-	8.5
DNA	10	ng/µl	10	ng/µl	2
Total volume	-				25
Aliquot per single rxn	23 µl of Master mix per tube and add 2 µl of template				
PCR Programme					
Steps	**Temperature (°C)**		**m : s**	**Cycle**	
Initial denaturation	95		05: 00	1	
Denaturation	95		00: 30	30	
Annealing	60		00: 30		
Extension	72		01: 00		
Final extension	72		07: 00		
Hold	10		10: 00	1	

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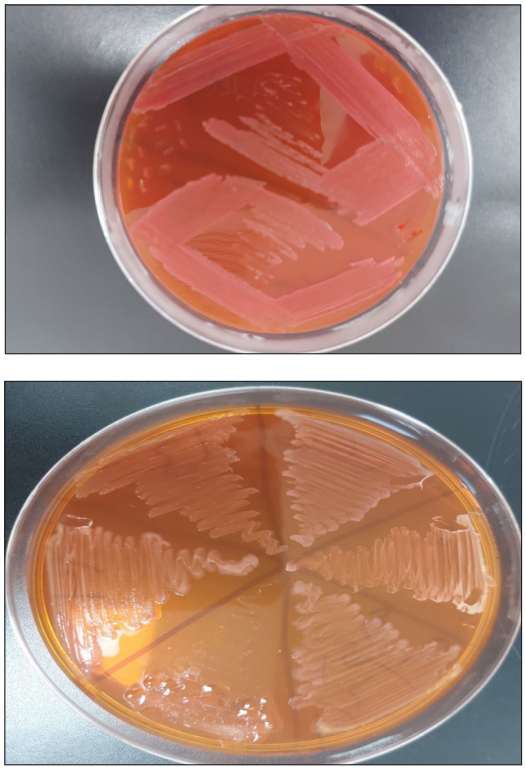

Figure 2.L-methioninase Producing Isolates

dye in media to detect methanethiol which reduces DTNB to form a yellow coloured substance, therefore a bacterial isolate that can change the colour of the medium from red to yellow is considered to be capable of producing this enzyme (Figure 3). It was found that out of 70 bacteria isolated in this study, only 45 isolates had the ability to produce the MGL enzyme.

In the second stage, 15 isolates were selected according to the intensity of the yellow colour in the first stage. These isolates were subjected to an additional screening process in which the efficiency of the isolates on enzyme production was estimated quantitatively.

In this stage, the efficiency of two bacterial isolates was considered the highest among the rest of the 45 isolates.

Identification of the Chosen Isolates

The two isolates with the highest efficiency in MGL production were subjected to identification and characterisation as follows:

Morphological Characteristics

The direct microscopic examination of stained smears of pure bacterial isolates showed that the two bacterial isolates were gram-negative short rods (Figure 4).

The exact diagnosis of samples was performed using the VITEK 2 system. Its results showed that the first bacterial isolate was Serratia plymuthica while the second was Serratia marcescens.

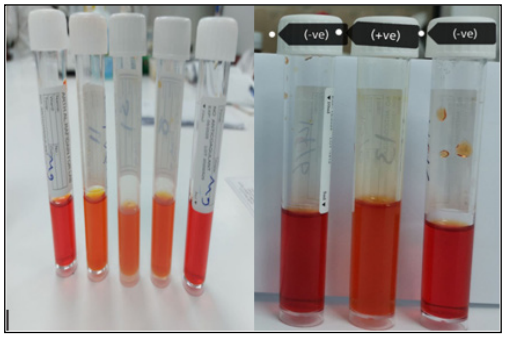

Figure 3.Qualitative Screening

The diagnosis by VITEK 2 was used to confirm the results of the biochemical test. The assay was performed according to the manufacturer's instructions (Biomeriaux, France).

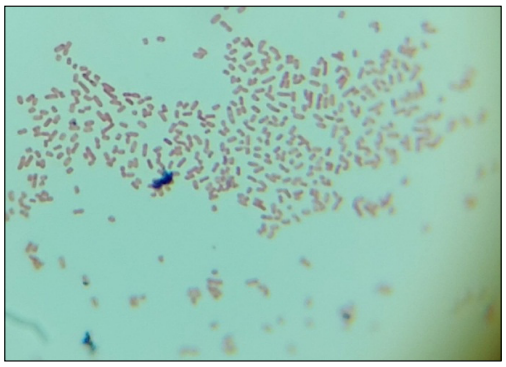

Figure 4.Microscopic Gram-negative Short Rods

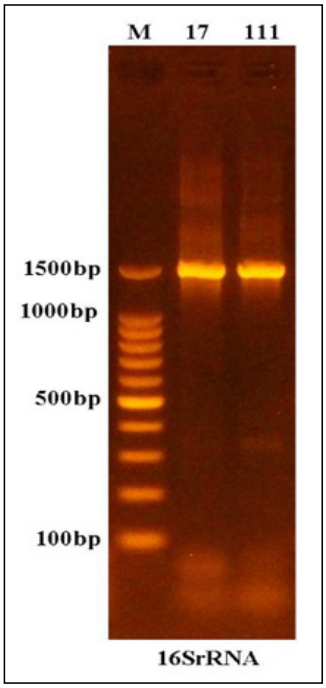

Figure 5.Results of the Amplification of 16S rRNA gene of Unknown Bacterial Species Fractionated on 1.5% Agarose Gel Electrophoresis Stained with Eth. Br. M: 100 bp Ladder Marker. Lanes 17–111 Resemble 1500 bp PCR Products.

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16S rRNA gene sequencing is a routinely used method for the identification of bacterial species.[18] PCR amplification of 16S rRNA fragment from the first bacterial isolate which bore the number (17) generated PCR amplified product of size 1.5 bp and the second isolate which bore the number (111) generated a PCR-amplified product of size 1500 bp (Figure 5).

The BLAST sequence analysis in the NCBI database showed the sequence similarity of isolate number 17 to Serratia sp. with 99% similarity. This result was identical to the conclusion of the morphological and biochemical characterisation. Thus this result revealed that this isolate was a new strain. Neighbour-joining phylogenetic tree analysis indicated that this species could be considered a new strain of Serratia sp. Hence we named this isolate Serratia sp. strain NDM25 with accession number op564989 (Figure 6).

The BLAST sequence analysis in the NCBI database showed the sequence similarity of the isolate number 111 to Serratia marcescens with 99% similarity. This result was identical to the conclusion of the morphological and biochemical characterisation. Thus this result revealed that this isolate too was a new strain. Neighbour-joining phylogenetic tree analysis indicated that this species could be considered a new strain of Serratia sp. Hence we named this isolate Serratia marcescens strain NDM73 with accession number op564990 (Figure 7).

Figure 6.Phylogenetic Tree of Serratia sp. NDM25 & its Relationship with the Closest Strain Sequences by 16S rRNA Gene Sequencing Using the Neighbor-joining Method

Figure 7.Phylogenetic Tree of Serratia marcescens NDM73 & its Relationship with the Closest Strain Sequences by 16S rRNA Gene Sequencing Using the Neighbor-joining Method .

Small ribosomal RNA gene sequencing, especially 16S rRNA sequencing in bacteria, has led to advances on diverse fronts in microbiology. Firstly, the texture of a universal phylogenetic tree classifies organisms into three domains of life, namely Archaea, Bacteria and Eucarya.[19] Secondly, it revolutionises the classification of microorganisms, and the classification of non-cultivable microorganisms is made

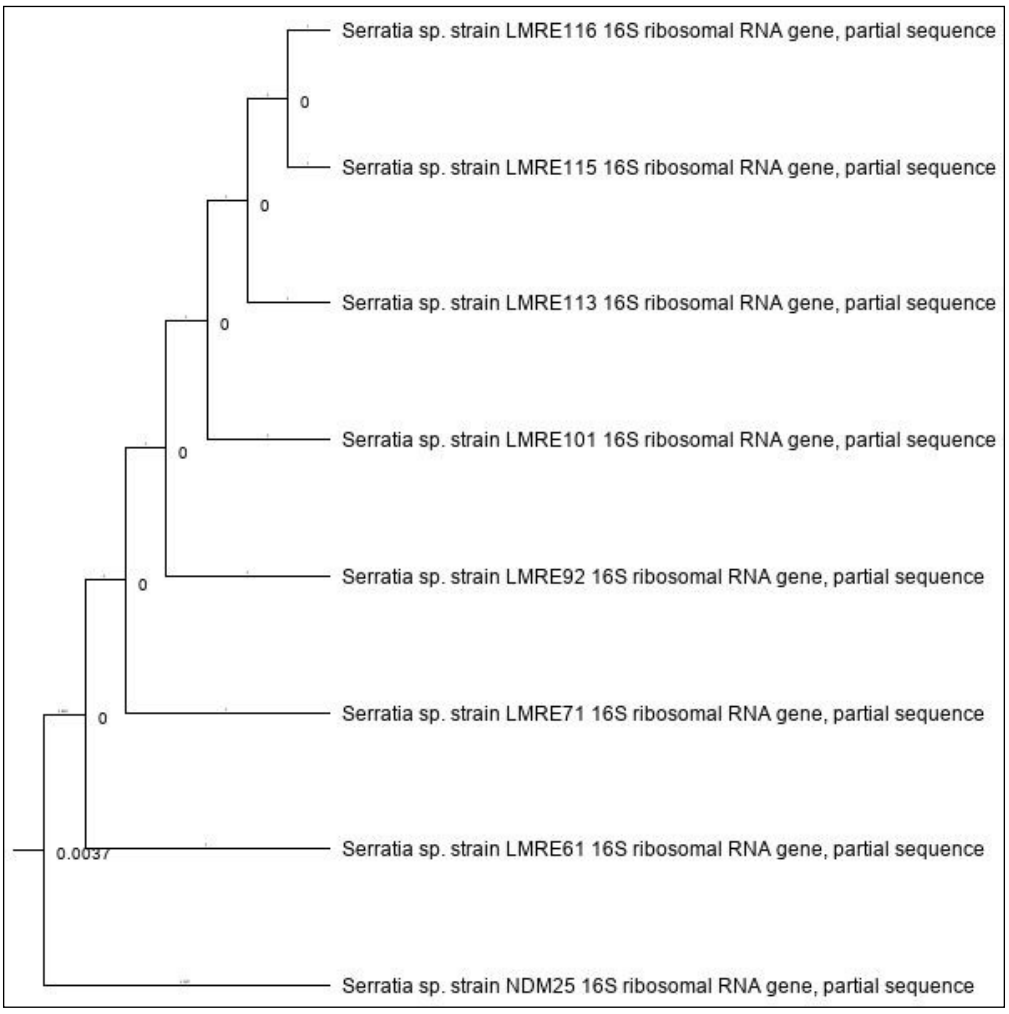

Figure 6

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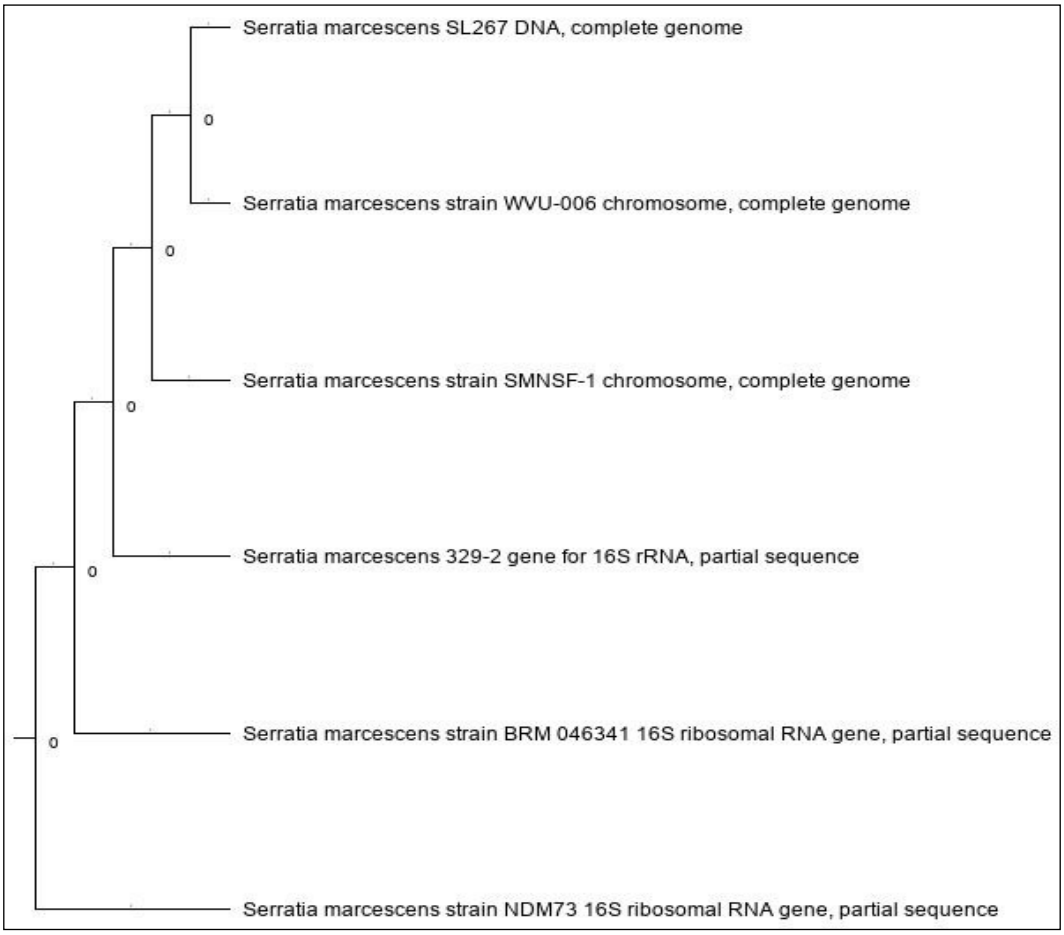

Figure 7.Phylogenetic Tree of Serratia marcescens NDM73 & its Relationship with the Closest Strain Sequences by 16S rRNA Gene Sequencing Using the Neighbor-joining Method

possible by using 16S rRNA gene sequencing.[20] Thirdly, it helps to clarify the relationship of unknown bacterial species with known species. 16S rRNA gene sequencing will continue to be the gold standard for the identification of bacteria in the future.

In order to obtain the highest L-methioninase enzyme producers among the chosen isolates, we conducted the last screening, from which it was found that the highest productive isolate is Serratia marcescens.

Serratia marcescens was identified as an L-methioninase producer due to the presence of pink-coloured colonies as evidence for the production of intracellular MGL by these isolates. This phenomenon is in agreement with that reported by Sundar and Nellaiah,[21] while it does not corroborate with the results of a study reported by Selim et al. on Streptomyces isolates in which it was mentioned that L-methioninase producers were characterised by the presence of pink colour around their colonial growth as evidence for the production of extracellular L-methioninase by these isolates.[17]

Conclusion

Only two isolates (17 and 111) out of 137 samples collected from different sources were selected as per the MGL production. The analysis showed a sequence similarity of isolate 17 to Serratia sp. with 99% similarity and that of 111 to Serratia marcescens with 99% similarity. The sequences were deposited in the NCBI database as Serratia sp. NDM25 with accession number op564989 and Serratia marcescens NDM73 with accession number op564990.

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Conflict of Interest: None

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