

# Isolation and Genetic Identification of L-Asparaginase Antitumor from *Streptomyces sp.*

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## Abstract

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is a supplementary cellular enzyme used as an anticancer substance. 31 *Streptomyces* strains isolated from rhizosphere of some plants in different regions of Saudi Arabia. The highest 11 *Streptomyces* isolates in L-asparaginase activity were selected for further molecular analysis based on differences in 16S rRNA gene sequence. Alignment analysis using the BLAST tools confirmed that all isolates are related to *Streptomyces* with a similarity of 97% to 100%. And partial-length gene sequence was sent into NCBI Gen Bank and received the accession numbers (*Streptomyces griseorubens* MN588164, *Streptomyces alboflavus* MN588165, *Streptomyces werraensis* MN588166, *Streptomyces spororaveus* MN588167, *Streptomyces chryseus* MN588168, *Streptomyces globosus* MN588169, *Streptomyces roseolilacinus* MN588170, *Streptomyces xanthophaeus* MN588171, *Streptomyces lavendulae* MN588172, *Streptomyces tuius* MN588174, and *Streptomyces tendae* MN588175).

**Key words:** Anticancer, L-asparaginase, *Streptomyces*

## Introduction

L-asparaginase is the hydrolyzate linkage of amide in L-asparagine to L-aspartic acid and ammonia (Kumar and Verma, 2012) and has been thoroughly investigated as the first enzyme with anti-leukemic activity (Savitri and Azmi, 2003). In chemotherapy for acute lymphoblastic leukemia (ALL), antineoplastic agent is used (Narta *et al.*, 2007).

The increased attention to L-asparaginase was related to many of the features used in potential industrial fields (Pedreschi *et al.*, 2008) and was modified as anti-leukemia therapy in lymphoblastic leukemia (Patil *et al.*, 2011; and Pieters *et al.*, 2011 and Jain *et al.*, 2012). *Erwinia chrysanthemi* and *E. coli* have been used as effective drugs for lymphoblastic leukemia (Kozak *et al.*, 2002; and Graham, 2003) without toxicity (Duval *et al.*, 2002).

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*Streptomyces* found in marine and terrestrial reigns (Pathom-Aree *et al.*, 2006) and produce 50% of the discovered bioactive secondary metabolites (Manivasagan *et al.*, 2013). L-asparaginase can be produced from *Streptomyces sp.* (Basha *et al.*, 2009), and *Streptomyces gulbargensis* (Amena *et al.*, 2010). *Streptomyces noursei* MTCC 10469 isolated from the marine sponge *Callispongia diffusa* (Dharmaraj, 2011).

*Streptomyces aurantiacus* (Gupta *et al.*, 2007), which were classified in several ways as ribosomal proteins (Ochi, 1995), fatty acid (Zhang *et al.*, 2007) and the 16S rRNA gene (Ramos *et al.* 1997; Prasad *et al.* 2013; Radhakrishnan *et al.*, 2013). Moreover, 16S rRNA is suitable for phylogenetic analysis (Song *et al.*, 2001).

## Materials and Methods:

Considering the importance of soil properties, Sixty-one soil samples from plant rhizosphere were collected in Jeddah city, from coastal soil and from algae in Thual. In addition, some samples of cultivated soils in Al-Baha were also collected and studied. Soil samples collected from plants rhizosphere were 10 to 20 cm in depth and were stored at 4 °C until use. (Alhaithloul H. A. S. 2019; Anisimova, 2019)

### *Streptomyces* spp. Isolation:

All soil samples were placed in the oven to dry at a temperature 55° C to ensure the killing of the highest possible number of vegetative reproductions of bacterial species., 90 ml of sterile dist. H<sub>2</sub>O from each sample was added to 10g, then serial dilution was prepared. Starch nitrate agar medium was inoculated for *Streptomyces* isolation (Waksman, 1962) with 0.1 ml of three different dilutions (10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup>). Three replicates were used for each dilution and the samples were incubated at 28± 2°C for 7 days to give *Streptomyces* colonies.

### Purification of isolated *Streptomyces* strains:

*Streptomyces* colonies were characterized by their sharp edges, cretaceous form, and adhesion to the growth plate. Repeat sub-culturing for colonies to achieve pure culturing strains. Purified *Streptomyces* strains were isolated on 30% (v/v) glycerol at -20° C.

*Detection of L-asparaginase activity from Streptomyces isolates* (Dhanam and Kannan, 2015).

By rapid plate assay on M9 agar medium, an indicator was added to the media, pink colour zones around the colonies were reflected L-asparaginase activity (Rajguru and Deshmukh, 2016).

*Quantitative of L-asparaginase activity Using the agar well Diffusion Technique (Rajguru and Deshmukh, 2016)*

This technique was based on observations of greater enzyme production extracted from an extracellular source to cultures. Prepared plates contain M9 agar medium with red phenol.

The plates were pierced at diameter 8mm, then 100 µl of the crude enzyme concentrations were poured into the agar for each strain. At room temperature, leave the plates for 24 hours, and then observe diffuse into the medium by measuring the zone diameter (mm) of the L-asparaginase activity that appears as a pink-coloured area around the well.

*Measurement of L-asparaginase activity using Nesslerization method:*

Quantitative assay of L-asparaginase according to Mashburn, 1964. Measurement of ammonia release using the Nessler's reaction. this mixture was incubated and after 10 min at 37° C 0.5 ml of 1.5 M Trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation (10,000 rpm) for 5 min. 0.5 ml of diluted supernatant is diluted to 7.0 ml. H<sub>2</sub>O and 1.0 ml of Nessler's reagent were added and incubated at room temperature for 10 min. UV/Visible Spectrophotometer was used to measure the absorbance at 450 nm.

*Molecular characterization of bacterial isolate.*

using GeneJET (Thermo Fisher Scientific) Genomic DNA Purification Kit for Genomic DNA isolation. By using the highly conserved global primers pA 5'AGA GTT TGA TCC TGG CTC AG 3' and pH 5' AAG GAG GTG ATC CAG CCG CA 3', the amplification of the 1500 bp fragments representing the full length of the 16S rRNA gene was amplified (Edwards *et al.*, 1989). 50 ng DNA, 1 µL of each 10 µM primer, 12.5 µL GoTaq® Green Master Mix (Promega, USA) and sterile dH<sub>2</sub>O up to 25 µL.

*PCR amplification conditions:*

Initial: incubation at 94 °C for 5 minutes, Denaturation: 94 °C for 1 min , Annealing: 55 °C for 1 min and Extension: 72 °C for 2 min , Number of cycles: 35. Final extension: 72 °C for 10 min.

Obtained nucleotide sequences of the genes 16S rRNA were aligned using codon code aligner software (Barth *et al.*, 2007). The BLAST algorithm and RDP database were used to analyzed and compared with those in GenBank to check for close evolutionary relatives. In order to characterize the strain, the nucleotide sequence of the gene of 16S rRNA was determined and the phylogenetic tree was constructed using the Neighbour- Joining (N-J) method Clustal-W in MegaAlign tool from DNASTAR version 12.3.1. DNA Star inc.

Final phylogenetic tree obtained using iTOL tree of life tool from Letunic and Bork (2019)

## Results:

*Isolation and purification of Streptomyces isolates*

As shown in Table 1, there are 42 isolates of *Streptomyces* obtained from north Jeddah isolates and from rhizosphere plants of Jeddah gardens, 3 isolates collected from the sea coastal soil in Thual and 2 isolates obtained from green algae. No isolate was obtained from the sea shell sample. 5 *Streptomyces* isolates from rhizosphere plants were obtained from Al Baha.

Table 1. Numbers and locations of Isolated *Streptomyces*.

| Samples reagions                              | Isolates       | Samples sources               | No. of Samples | No. of isolates |
|---|----------------|-------------------------------|----------------|-----------------|
| From Jeddah province                          | Soil           | from Plant Rhizosphere plants | 49             | 42              |
| (Jeddah province) Jeddah city and Thual coast | Marine samples | From Red sea shore soil       | 4              | 3               |
|   |                | Green algae                   | 3              | 2               |
|   |                | seashells                     | 3              | 0               |
| AlBaha  | Soil           | Plants Rhizosphere            | 5              | 5               |

*Detection of L-asparaginase activity (Qualitative assay):*

Purified colonies of isolated *Streptomyces* strains were evaluated for their ability to L-asparaginase activity in plates containing M9 agar medium with phenol red. as shown in Table 2 and figure 1, most of the strains isolated had L-asparaginase activity, ranging from 100% to 59.62%. The percentage of the *Streptomyces* that have L-asparaginase activity from soil were higher than that of sea isolate.

Table 2: L-asparaginase activity of *Streptomyces* isolates (Qualitative assay)

| Samples regions          | Isolates number | Positive L-asparaginase activity | % of Positive of L-asparaginase activity |
|--------------------------|-----------------|----------------------------------|--|
| Soil from Jeddah city    | 42              | 22                               | 52.38                                    |
| Soil from Thual coast    | 3               | 3                                | 100                                      |
| Green Algae              | 2               | 2                                | 100                                      |
| Soil from Al Baha region | 5               | 4                                | 80                                       |

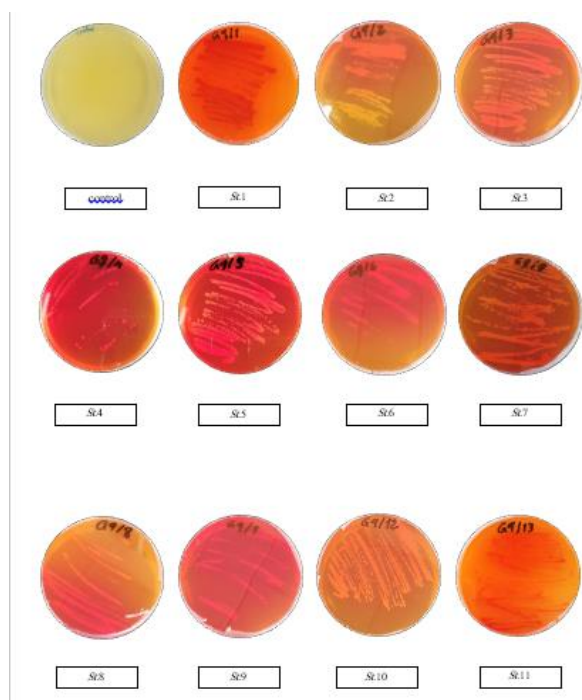


Figure 1: Qualitative Assay to Highest Isolates of Streptomyces Strains

*Quantitative assay of L-asparaginase activity by agar well diffusion technique:*

Quantitative assay of L-asparaginase in cell-free filtrate performed by diffusion method in a pore on M9 solid media plates, with red phenol, after 24 h of incubation, the measurement of pink area in millimetres were recorded. Streptomyces L-asparaginase activity based on the diffused of during 24 h, According to Figure 2, there was no appearance of a pink area on plates number (3, 8, 9), While the pink area was appeared in plates number (1, 2, 4, 5, 6, 7, 10 and 11) and was measured. The number of samples (10, 1 and 11) was recorded as the highest score, and then the number of samples (6, 5, 7, 4 and 2) recorded the lowest score, respectively. According to Fig. 3, which shows the quantitative assay of L-asparaginase by plates, the different pink zones mean the different of L-asparaginase diffused in solid agar supplemented with L-asparagine compared with control.

*Estimation of L-asparaginase activity using nesslerization under Submerged Culture:*

The highest L-asparaginase activity of 31 *Streptomyces* isolates in Cell-Free Filtrate was determined by Nesslerization reaction. The Results in Figure 4 show the different enzyme activity between the isolates showing the highest L-asparaginase activity from the *Streptomyces* isolates. The highest L-asparaginase activity was from 11 *streptomyces* isolates.

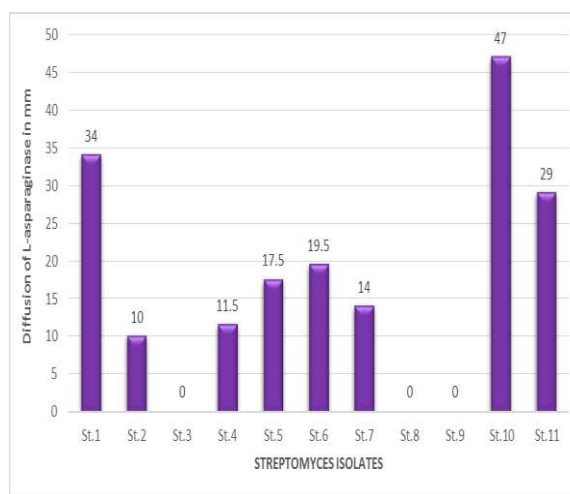


Figure 2 Quantitative assay of L-asparaginase activity by agar well diffusion technique

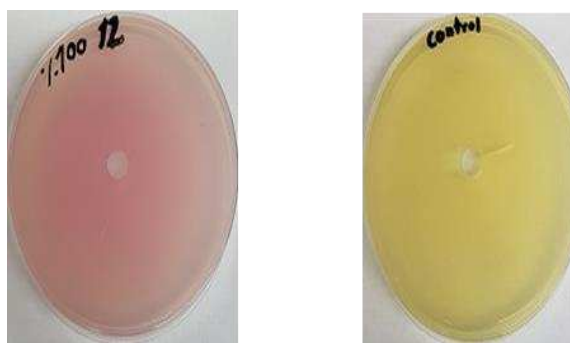


Figure 3: L-asparaginase quantitative in agar, supplemented with L-asparagine.

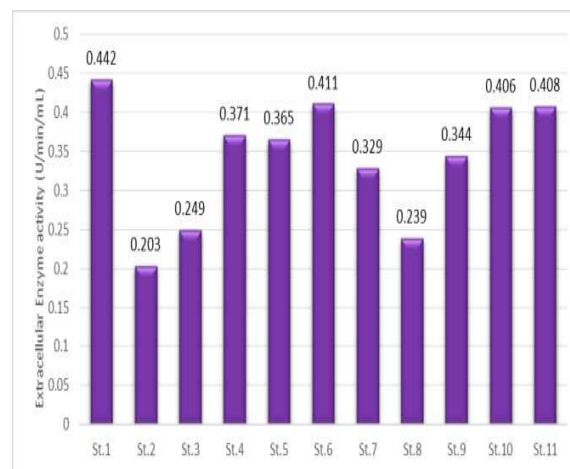


Figure 4: Estimation of L-asparaginase activity of *Streptomyces* isolates in Submerged Culture

*Morphological and Molecular identification*

- *Morphological characteristics*

The Results of Table 3 showed some biological studies on the twelve strains in terms of the outward appearance of the strain from the front and back also the pigments that produced in starch nitrate medium. While the internal form or spore chains of the *Streptomyces* strains

*Molecular identification of bacterial isolates based on 16S rDNA*

After performing successful PCR as shown in figure 4 at the appropriate length, which is 1.5 kbp, DNA sequences were analyzed using gene bank nucleotide blast alignment tools and showed that the isolates were identified as *Streptomyces* with similarity percentages 97 - 100% (Table 4). Based on comparative analysis of 16S rDNA sequences of modified and merged isolates selected with the sequence of the closest species recovered by the NCBI BLAST tool, these strains showed taxonomic dependence with isolated strains. The results of phylogenetic relationships of bacterial strains and closely related species were observed using the interactive tree of life (iTOL), ver. 3.4.3 after all sequences were subjected to NCBI blast search tool (Figure 5).

Table 3: Morphological Properties

| No. of isolates | Gram Stain | Arial Mycelium      | Substrate Mycelium  | Diffusible Pigment | Spore Arrangement |
|-----------------|------------|---------------------|---------------------|--------------------|-------------------|
| St.1            | +          | Gray                | Black               | Light brown        | Linear            |
| St.2            | +          | White to creamy     | White to creamy     | -                  | Linear            |
| St.3            | +          | Gray                | Grayish white       | -                  | Spiral            |
| St.4            | +          | White to light gray | White to light gray | -                  | Linear            |
| St.5            | +          | Creamy              | Orange              | Yellow             | Spiral            |
| St.6            | +          | grayish brown       | Gray to bieage      | -                  | Linear            |
| St.7            | +          | White               | Creamy              | -                  | Hook              |
| St.8            | +          | Red brick           | Red brick           | -                  | Linear            |
| St.9            | +          | White               | Creamy              | -                  | Spiral            |
| St.10           | +          | light grayish red   | Red brick           | -                  | Spiral            |
| St.11           | +          | white               | orange              | Yellow             | Hook              |

Table 4: Molecular identification of isolates based on 16S rDNA gene.

| Strains | Strains                          | Gene bank accession numbers | Identity % | Coverage % |
|---------|----------------------------------|-----------------------------|------------|------------|
| St.1    | <i>Streptomyces griseorubens</i> | MN588164                    | 100%       | 100%       |
| St. 2   | <i>Streptomyces alboflavus</i>   | MN588165                    | 100%       | 100%       |
| St. 3   | <i>Streptomyces werraensis</i>   | MN588166                    | 100%       | 100%       |

|        |                                    |          |        |      |
|--------|------------------------------------|----------|--------|------|
| St. 4  | <i>Streptomyces spororaveus</i>    | MN588167 | 99.63% | 100% |
| St. 5  | <i>Streptomyces chryseus</i>       | MN588168 | 99.17% | 100% |
| St. 6  | <i>Streptomyces globosus</i>       | MN588169 | 99.37% | 99%  |
| St. 7  | <i>Streptomyces roseolilacinus</i> | MN588170 | 100%   | 100% |
| St. 8  | <i>Streptomyces xanthophaeus</i>   | MN588171 | 99.61% | 100% |
| St.9   | <i>Streptomyces lavendulae</i>     | MN588172 | 99.60% | 100% |
| St. 10 | <i>Streptomyces tuirus</i>         | MN588174 | 100%   | 100% |
| St. 11 | <i>Streptomyces tendae</i>         | MN588175 | 97.84% | 99%  |

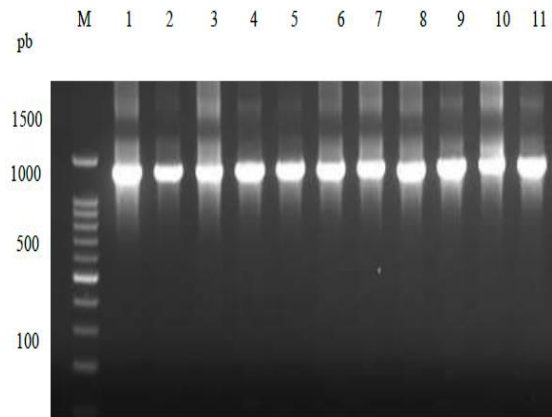


Figure 5: The 1500 bp 16S rDNA amplicon agarose gel electrophoresis after the purification compared with DNA Ladder 1kb on a 1 % agarose gel. Lane 1: 1kb DNA ladder and Lane 2-12 eleven of bacterial isolates.

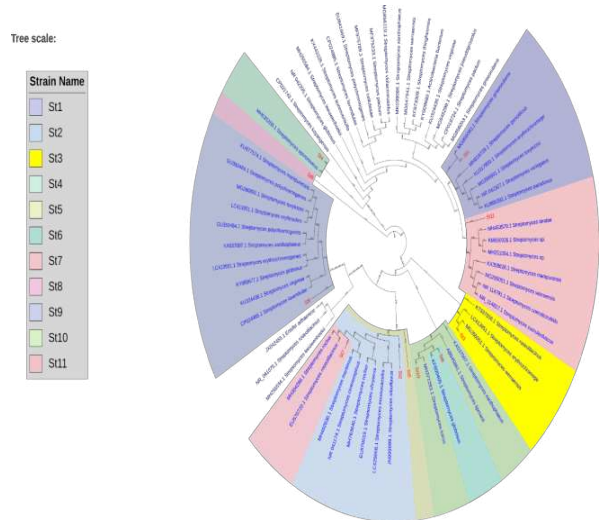


Figure 6: Neighbor- joining phylogenetic tree based on 16S rRNA sequence analysis and the relationship between *Streptomyces* and the most closely related bacterial species.

## Discussion

Fifty-two different actinomycetes isolates for L-asparaginase, showed 31 positive reactions. These strains were taken for molecular identification. Actinomycetes reactions varies related to the amount of organic matter in plant (Germida *et al.*, 1998). (Tewtrakul and Subhadhirasakul, 2007). root exudates increase the growth of actinomycetes while antimicrobial compounds from the root can reduce the number of soil bacteria and fungi. Actinomycetes are widely distributed in the soil (Velayudham and Murugan, 2012).

Pure colonies from *Streptomyces* showed approximately 31 of ASNase activity on plate based on the extent of the zone (pink in colour) around the colonies on the Modified M9 plates (Dhanam and Kannan, 2015). Each isolate was subjected to screen for L-asparaginase activity on M9 agar (Baskar and Renganathan, 2012). The formation of a pink zone around the bacteria was a sign of ASNase production (Asselin *et al.*, 1993). Most of the isolated strains have L-asparaginase activity, varied from 100% (isolates that obtained from Soil from the shore of Red Sea coast and Green Algae) to 59.62% (isolated from Soil Al Baha region and Soil from Jeddah province) (Maldonado *et al.*, 2009) showed that actinomycetes are always present in marine sediments, but their numbers are less than soil and these findings are in agreement with the results of this study.

In general, actinomycetes are known to be capable of producing biological activities such as pesticides, herbicides, antibiotics and enzymes including asparaginase (Prapagdee *et al.*, 2008; Boroujeni *et al.*, 2012). Soil samples in Egypt were actinomycete potent antimicrobial compound producing actinomycete were isolates as yellow, grey and white colour series (Atta, 2015). Actinomycetes, especially *Streptomyces* spp. (Dhevagi and Poorani, 2006; Narayana *et al.*, 2007). *Streptomyces* Genus is a good source of L-asparaginase compared to bacteria and fungi (Sahu *et al.*, 2007). Actinomycetes revealed to excellent resource for L-asparaginase, as *Streptomyces griseus* (Narayana *et al.*, 2007) and in Egypt (EL-sabbagh *et al.*, 2013).

Production and estimation of L-asparaginase anticancer enzyme under SMF by 11 of 31 *Streptomyces* sp. In the diffusion method plates, there were different pink zones ranged from 10-47 mm, as a result of L-asparaginase hydrolysis activity as explained (Gulati *et al.*, 1997). Our results in accordance with that found by Wakil and Adelegan (2015) with zone ranged from 30-50 cm, and Darwesh *et al.* (2018) ranged from 30-40 mm. while from 8-13 mm (Devi and Ramanjaneyulu, 2016) and 90mm by (Gulati *et al.*, 1997) in soil bacterial isolates.

More than 75 % of industrial enzymes produced by SMF, which support the use of genetically modified organisms to a greater extent than SSF, and widely SMF is used lack of paraphernalia regarding the production of many enzymes. Highly critical related to metabolism exhibited by microorganisms is different in SSF and SMF, and in a flux of nutrients and efflux of waste needs to be carried out based on metabolic parameters (Subramaniam and Vimala,

2012). Enzyme activity was determined in culture (Dhevagi and Poorani, 2006). Nesslerization reaction was used to estimate the extracellular L-asparaginase activity.

Eleven isolates were selected for further molecular analysis and identification using DNA sequencing of 16S rDNA. To confirm the accuracy, 1500 bp amplicons produced using 16S rDNA universal primers (Edward *et al.*, 1989) were separated on 1 % agarose gels, and then the fragments with the molecular sizes represented the PCR product of 16S rRNA were purified to be ready for sequenced.

DNA sequences were analyzed using nucleotides blast alignment tools of gene bank and showed that the isolates were identified as *Streptomyces* with similarity percentages 100 % (99% until 97%). Based on comparative analysis of 16S rDNA sequences mapped and selected isolates with sequences of nearest type species retrieved by NCBI BLAST tool, these strains showed taxonomic dependence with isolated strains. Isolates were submitted as *Streptomyces griseorubens* MN588164, *Streptomyces albobiflavus* MN588165, *Streptomyces werraensis* MN588166, *Streptomyces spororaveus* MN588167, *Streptomyces chryseus* MN588168, *Streptomyces globosus* MN588169, *Streptomyces roseolilacinus* MN588170, *Streptomyces xanthophaeus* MN588171, *Streptomyces lavendulae* MN588172, *Streptomyces tuius* MN588174 and *Streptomyces tenda* MN588175.

The 16S rRNA sequence is used to identify *Streptomyces* sp (Khamna *et al.*, 2009; Deshpande *et al.*, 2014). Even though 16S rRNA gene sequencing is very useful to classifying bacteria, it has a little amount of phylogenetic power (Patel *et al.*, 2001; Bosshard *et al.*, 2006; Mignard and Flandrois, 2006).

## Conclusion

Out of 31 L-asparagine strains, only 11 were potential strain for L-asparaginase production. The Organism did not produce any pink zone in control M-9 agar plate incorporated without L-asparagine and the pink zone is only in L-asparaginase production.

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