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Screening and Isolation of L-Asparaginase Enzyme Producing Bacteria from Diverse Niches

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Abstract
L-asparaginase is used as a chemotherapeutic drug against Acute Lymphoblastic Leukemia. Several micro-organisms produce this enzyme but, there has been a problem of hypersenstivity during treatment which restricts its use. Therefore, there is a need to obtain such an enzyme which would be allosterically, pharmacologically, serologically different (from those in use) and showing diminished physiological untoward side effects. This enzyme has attracted its attention in the food industry as well, where its ability to alleviate formation of acrylamide during baking of fried foods has made it a remarkable mitigating agent. Thus, the current study focuses on exploring the diverse niches in order to obtain, enzyme which would be completely inert and render its substantial applications in food industry as well as drug industry. In this study, 27 isolates exhibited maximum L-asparaginase production. These isolates were then streaked over Davis-Mingolis minimal medium containing L-asparagine amino acid as a sole nitrogen as well as carbon source. So, the isolates capable of growing in such a minimal medium were confirmed to be potent strains. These strains were then subjected to quantitative analysis (Nesselerization treatment) for determining maximum enzyme producer. In this process, 4 best potent strains were obtained which exhibited maximum enzyme production within a period of 48 hours with a profound ability to utilize and grow only on L-asparagine. These 4 isolates were then identified by cultural, biochemical and molecular (genetic) techniques.

Keywords
Acute Lymphoblastic Leukemia, Chemotherapeutic Drug, L-asparaginase, Acrylamide, Mitigating agent, Davis-Mingolis Minimal Medium, Nesselerization treatment.

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INTRODUCTION:

Bacterial L-asparaginase enzyme (EC 3.5.1.1) has been widely used as an antineoplastic drug against acute-lymphoblastic leukemia, Hodgkins lymphoma, lymphosarcoma, and also, acute myelocytic leukemia[1]. The anti-cancerous property was demonstrated by Broome [2] and later on proved by Marshburn & Wriston[3]. Kidd demonstrated, in early fifties, that the guinea pig serum, serve as a good source for remission of murine Lymhoma cells. The guinea pig serum was injected intraperitonally to mice transplanted with lymphoma cells. This treatment recovered mice suffering from Lymphoma while those maintained as control mice (possessing lymphoma and not injected by guinea pig serum) died within 20-30 days [4]. Clementi et.al, observed and identified this source to be rich in L-asparaginase [5].

The Commercially available L-asparaginases include, Elspar, Oncospar, Erwinase, and Kidrolase . This enzyme is present in most prokaryotic micro-organisms. But L-asparaginases from a few microbes such as *E. coli,* and *Erwinia carotovora* have shown curable results with potential therapeutic effects [6]. The enzyme is able to reduce the substantial levels of tumors (quantity wise) by making the tumor cells deficient in essential amino acid L-asparagine required for their normal growth and proliferation. The normal and healthy cells have inherent intracellular asparagine synthase enzyme (which the, cancerous cells lack) responsible for production of non-essential amino acid L-asparagine. Therefore, the normal cells can grow in absence of L-asparagine. This amino acid thus, becomes an exogenous source for cancerous cells, for their survival and growth increasing their ability to rapidly enter into metastasis [7]. The enzyme L-asparaginase being amino-hydrolytic, converts L-asparagine to L-aspartic acid and ammonia which depletes this major pool of L-asparagine. The cancerous cells hence become intolerable and cannot upregulate this loss, thereby ultimately getting destroyed.

L-asaparginase has drawn attention in the drug industry as this enzyme can be isolated from a wide variety of micro-organisms such as, *Enterobacter aerogenes, Bacillus cereus, Thermus thermophillus, Pseudomonas sp.,*and also *Erwinia chrysanthemi* in a very affordable rate[8]. The commercially available sources exhibit untoward side-effects such as hypersensitivity in the cancer suffering patients. There is a need of exploring the diverse niches, in order to gain such an essential drug from environment so that this major problem would be completely abolished [9]. This enzyme is utilized in the food industry also, where it has shown its property to alleviate the acrylamide (a carcinogenic agent) formed during baking and heating of starchy foods. The acrylamide formed is been cleaved into soluble aspartic acid thus, minimizing the risk of its direct intake [10].

In the present investigation varied sources such as vermi-compost soil, effluent-treatment plant wastewater, slaughterhouse soil was analyzed for obtaining potent L-asparaginase enzyme producers that would yield pharmacologically active and biologically stable L-asparaginase enzyme.

MATERIALS AND METHODS:

Sample Collection:

Samples such as vermi compost (From, Tuljaram Chaturchand College: **VTC**), milk waste water (From Dynamix Dairy, collection unit, Baramati:: **MWB**) , effluent treatment plant waste water sample (From Tuljaram Chaturchand College :: **ETP**) , Fish gut waste Sample (Fish Sample Baramati: **FSB**), Rhizosphere (roots) soil sample (Rhizosphere soil from botanical garden of Tuljaram Chaturchand College, Baramati: **RZ**) were collected.

Isolation & Screening of Micro-organisms:

These samples were streaked over modified M9 medium[Na_2HPO_4 : 6g/l, KH2PO4 : 3g/l, NaCl : 0.5g/l, L-asparagine : 10g/l , 1M $MgSO_4$: 20 ml/l , 0.1M $CaCl_2.2H_2O$: 10 ml/l , Glucose stock(20g%) : 10 ml /l , phenol red dye : 5 g% , pH : 6.2.][11].

The selected isolates were then streaked over Davis-Mingolis Minimal Medium[Dextrose :1g/l, NH4SO4: 1g/l, K2HPO4: 7g/l, KH2PO4: 2g/l, Sodium Citrate : 0.5g/l, MgSO4 : 0.1g/l, L-asparagine : 10g/l, ph: 7.0] to obtain the potential enzyme producers.

Qualitative methodology:

The above-mentioned samples were inoculated into M9 broth as 1% in concentration and incubated at 37 ^oC for 48 hours. This inoculum (a loopful) were then streaked over M9 agar medium keeping it at 37 ^oC for 72 hours. The colour of the medium is yellow, as phenol red dye is yellow at acidic pH. The isolated colonies over this medium exhibited pink colouration, making the medium alkaline. This changed the colour of the medium to pink [1, 11].

Quantitative methodology.

L-asparaginase assay:

L-asparaginase assay procedure is based on detection of ammonia release in the medium. The assay was carried out according to Marshburn and Wriston *et al*. (1964) with few modifications. Here, cultures were centrifuged at 7000rpm for 15 minutes, while the supernatant was taken as the crude enzyme source for estimation. The cell free extract (1ml) was mixed with 0.2ml of Triss-Hcl buffer (pH: 8.6) and L-asparagine (1.7ml). The reaction mixture was incubated at 37^0 for 10 minutes. The reaction was terminated by addition of 0.05M Trichloro-acetic acid(0.5ml). This solution was then centrifuged once again at 7000 rpm for 10 minutes. The supernatant (0.5 ml) was taken as clear

enzyme source. In this solution Nesseler's reagent (1 ml) was added to detect amount of ammonia released. A standard curve of ammonium sulphate was used for comparison and estimation of ammonia. One unit (IU) of ammonia is defined as the amount of an enzyme that releases 1µM of ammonia per ml per minute[1µM/ml/min.] [3, 12].

Protein estimation:

The protein concentration was determined as per the Lowry et al., method. A standard curve was plotted by using Bovine Serum Albumin as a standard [13].

Cultural and Biochemical characteristics:

Morphological, Cultural and biochemical characteristics were carried out in reference with 9th edition of Bergey's Manual [11, 14].

Molecular Identification of selected isolates:

Cultures were identified by 16S rRNA gene sequencing and compared through BLAST program with other sequences in the Gene Bank. All the sequences were submitted to NCBI database, so as to assign accession numbers. Phylogenetic tree of these isolates were constructed using Neighbor joining method [5 , 15].

RESULTS AND DISCUSSIONS:

L-asparaginase enzyme has been a cornerstone in the drug industry. The hallmark activity to combat cancer has made it a drug of choice during chemotherapeutic treatments against acute lymphoblastic leukemia. It has drawn attention in the food industry also, where it diminishes the acrylamide formed during frying and baking of starchy foods. In the present study, an attempt was made to explore most natural habitats so as to obtain an isolate which would be allosterically different and exhibit minimum side-effects after administration [14, 16].

Research in multiple directions yield unique and characteristic results. The approach as mentioned here of exploring diverse niches so as to obtain pharmacologically active enzyme was followed by most researchers.

Qeshmi *et al*. reported intracellular production of L-asparaginase by using a novel strain, of Bacillus *sp.PG0* [17].

Most of the researchers have tried using fermentation strategies so as to enhance production yield. So this approach was employed by Elizabeth T. *et al, for* the production of L-asparaginase by *Pseudomonas stuzeri* PIMS6 using agro residues [18] .

Research in exploring varied microbes would gain better results. Saxena *et al*., concluded that *Streptomyces phaechromogenes* under submerged fermentation produced glutiminase free extracellular L-asparaginase with maximum L-asparaginase activity [19].

The diversity in medicinal plants have been an unconditional source of antibiot ic in medical field. El-Said *et al*., tested leaves of *Datura innoxia* and *H muticus* medicinal plants for antibacterial & anticancerous activity. In this study, it was concluded that endophytic fungi serve as a reservoir of anticancerous and antibacterial compounds. L-asparaginase production was maximum with *Aspergillus niger* strain [20].

Similarly, Pagalla *et al*., used medicinal plant (*Mentha Spicata*) to produce this enzyme from endophytic bacteria [21].

Marine niches have served as a reservoir of enzymes with high pharmacological activity and efficacy, since ages. Dhevagi and Poorani *et al*., isolated marine actinomycetes *Streptomyces sp.* from Parangipettai & Cochin Coastal area that produced L-asparaginase showing cytotoxic effect on JURKAT cells (Acute T-cell leukemia) and K562 cells (Chronic myelogenous leukemia)[22].

Isolation strategy employed for obtaining potent L-asparaginase producers in current study was as per Gulati R., *et al* [23]. Here, total 14 isolates exhibited viability upto 96 hours over M9 medium. A different approach was employed hereafter. These isolates were checked for their ability to utilize only L-asparagine in Davis-Mingolis Minimal medium. This was compared with simultaneous inoculation in M9 broth. Here, total 4 isolates showed positive reaction in both the media. Therefore, these isolates were identified by through 16SrRNA gene sequencing and phylogenetic tree of these isolates were constructed.

I. In this way, 60 isolates showed positive results. Total 27 isolates from the above-mentioned list showed survival rate i.e. viability for more than 96 hours. These isolates were once again screened over Davis-Mingolis Minimal Medium containing Dextrose and L-asparagine as a sole carbon and nitrogen sources. Here **14** isolates were obtained. The selected isolates from this media were again streaked over the same medium but, with only L-asparagine as a sole nitrogen and a carbon source. In this process **4**, isolates survived in such a minimal medium also, exhibiting appropriate morphological characters with better results.

Table :1. Characteristics of isolates over M9 medium & Davis-Mingolis Minimal Medium (Qualitative methodology).

Sr. No.	Isolates	M9 Medium	Davis-Mingolis Minimal Medium
1.	*VTC(Cottony white)	+ + +	+ + +
2.	VTC(G)	- - -	- - -
3.	MWB(1)	+	- - -
4.	RZ(1)	+	- - -
5.	RZ(2)	+	- - -
6.	RZ(3)	+	- - -
7.	RZ(4)	- - -	- - -
8.	*SG(Off)1	+ + +	+ + + +
9.	SG(W)2	- - -	- - -
10.	*SG(P)3	+ + +	+ +
11.	FSB(1)	+ +	- -
12.	FSB(2)	+ +	- - -
13	ETP(1)	+ +	- - -
14.	*ETP(2)	+ + +	+ +

(+ + + +): High enzyme activity, (+ + +): Moderate enzyme activity, (+ +): Less activity
(- - -): No enzyme activity; *4 isolates were selected in this process.

Nesslerization assay of (14) potential isolates: (Quantitative methodology)

These 14, isolates were then subjected to Nesselerization treatment for quantitave estimation. In this assay, samples showing positive results in qualitative methodology were selected.

(The selected isolates were inoculated into M9 broth, keeping it at 37°C for 48 hours. The colour of broth changed to pink after incubation)

Table 2. Nesselerization assay:

Sr.No.	Isolates	Asparaginase activity in units (µmol/ml)
1.	*VTC (Cottony white)	54
2.	VTC(G)	12
3.	MWB (1)	2.6
4.	RZ (1)	18.66
5.	RZ (2)	21.2
6.	RZ (3)	10.6
7.	RZ (4)	2.6
8.	*SG(Off)1	64
9.	SG(W)2	30.66
10.	*SG(P)3	49.33
11.	FSB (1)	43
12	FSB (2)	39
13.	ETP (1)	20
14.	*ETP (2)	45

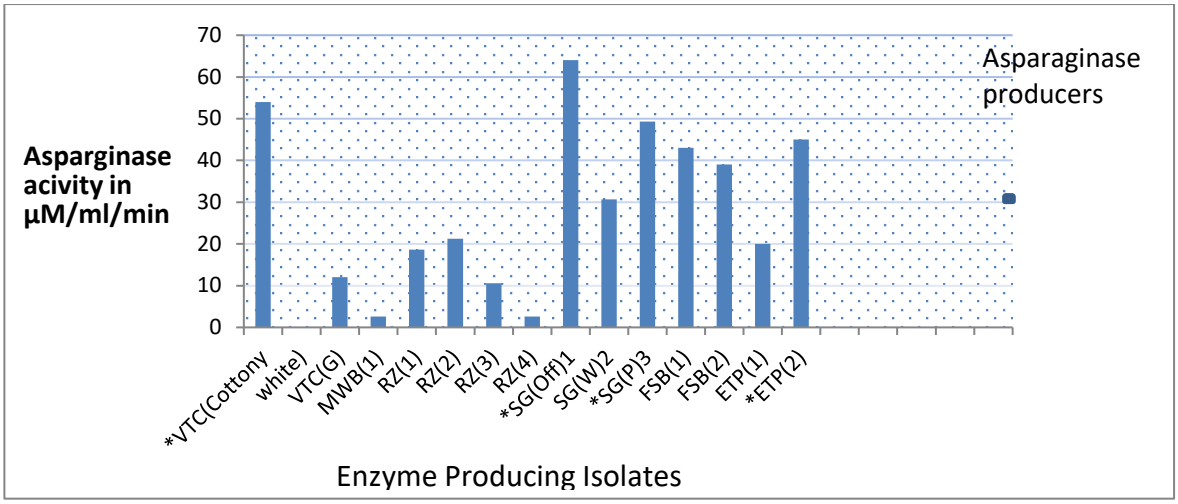


Fig:1 : Graphical presentation of (14) enzyme producers : Nesselerization assay.

II. Morphological and Biochemical Characteristics of potent selected isolates

Table 3: Cultural characteristics of (4) potent isolates:

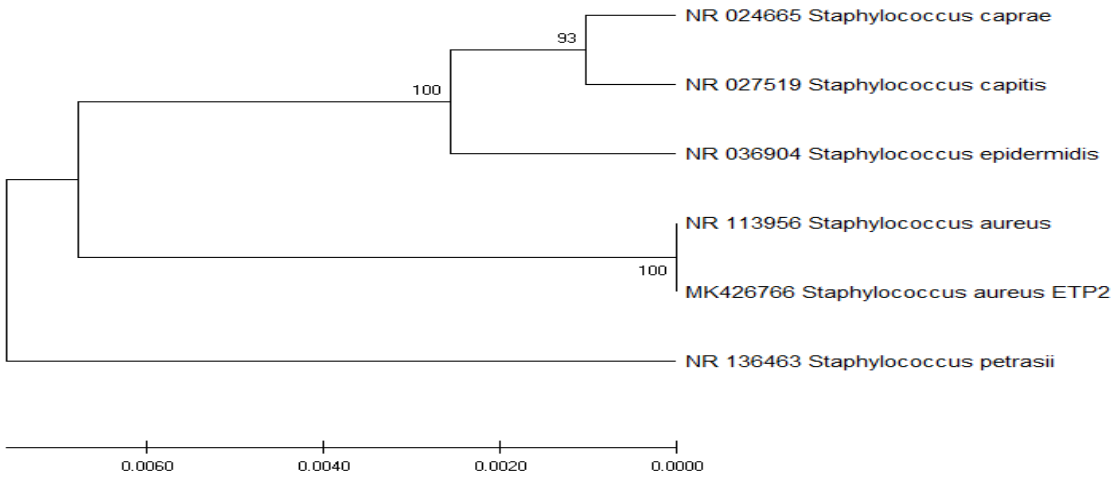
Sr. No.	Isolate	Mophological characters		Biochemical character	
1.	*Staphylococcus_aureus*_ETP2	Gram positive	Cocci	Catalase	+
				Nitrate	+
2.	*Lysinibacillus _sp*_VTCO	Gram Positive	Rods	Oxidase	+
				Urease	+
3.	*Enterobacter_xiangfangensis*_	Gram Negative	Rods	Indole	-
				Oxidase	-
4.	*Micrococcus_aloevera*_SGP3	Gram positice	Spherical Cells	Citrate	-
				Catalase	

(+): positive reaction, (-): negative reaction.

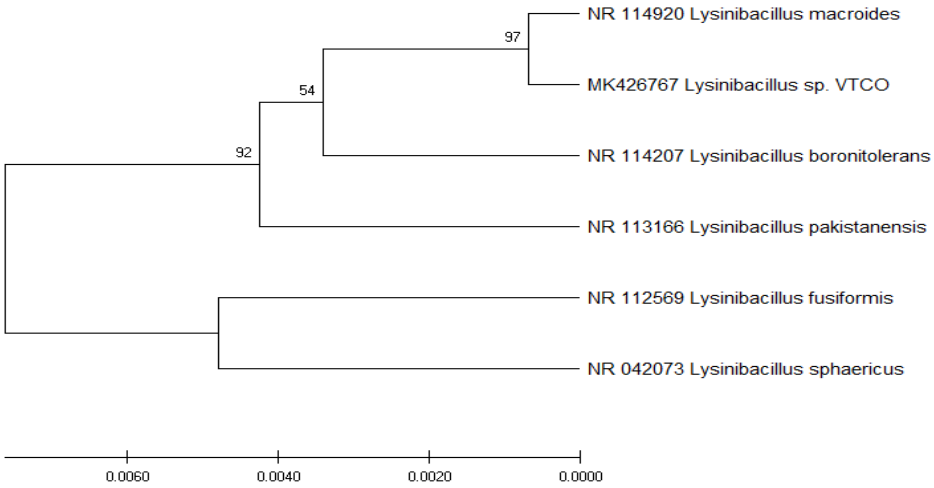
Molecular and Phylogenetic analysis of selected potent strains:

The sequences of following Potent and selected Isolates, were submitted to the genebank. These Sequences were assigned with their respective accession numbers and their phylogenetic tree were constructed.

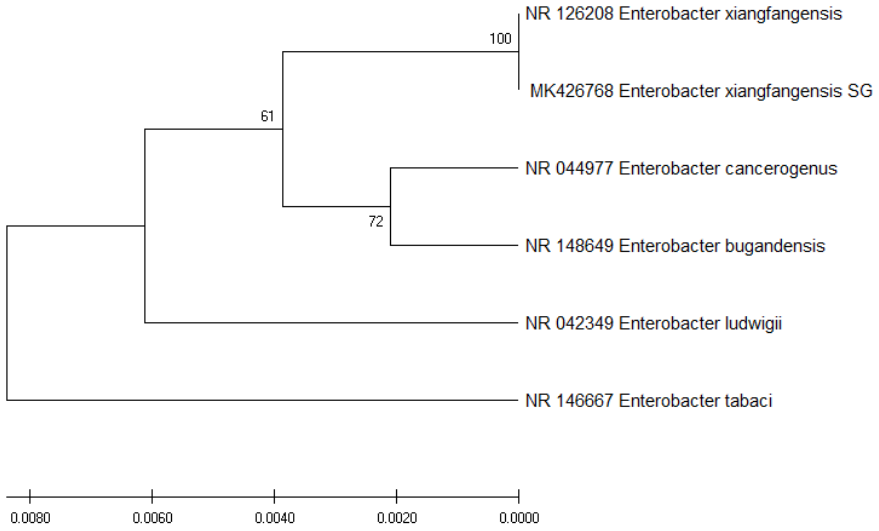
a) *Staphylococcus_aureus*_ETP2 MK426766



b) ***Lysinibacillus _sp_*VTCO MK42676**



c) ***Enterobacter_xiangfangensis_* MK426768**



d) ***Micrococcus_aloevera*_SGP3 MK426769**

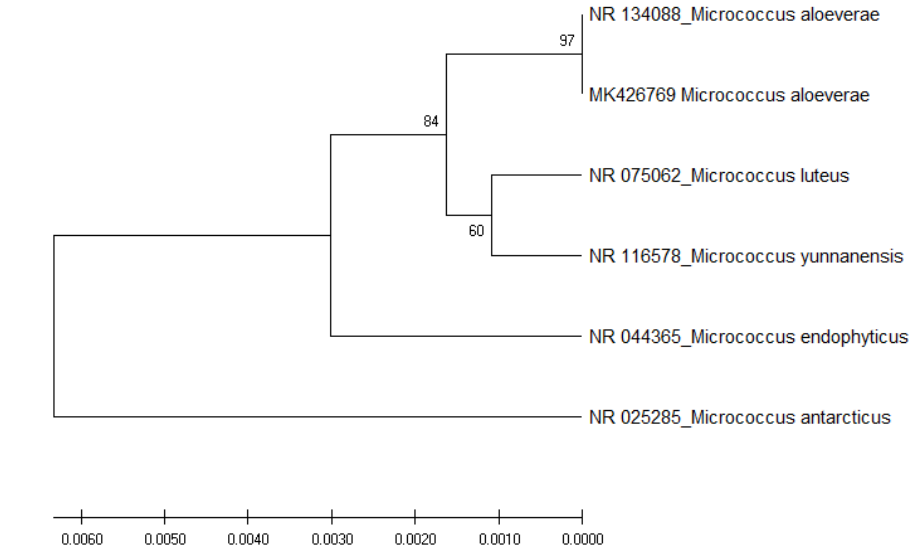


Fig: 2 . Phylogenetic tree of selected isolates(4) with their respective accession numbers.

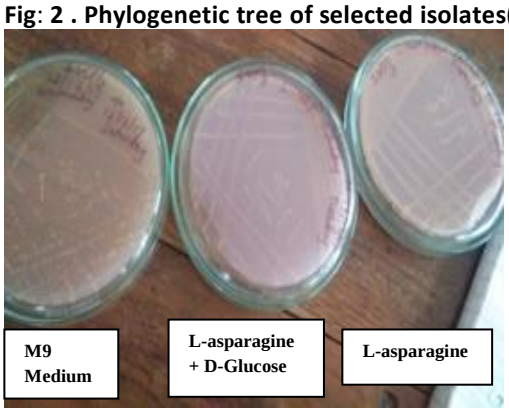


Fig.3 Positive isolates over Davis-Mingolis Minimal Medium

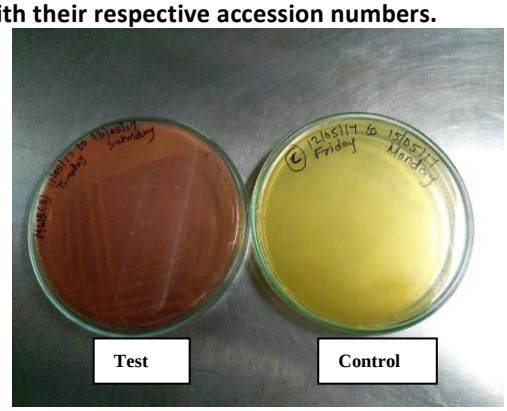


Fig 4: Positive isolates over M9 Medium

Fig. 5: Nesselerization Assay

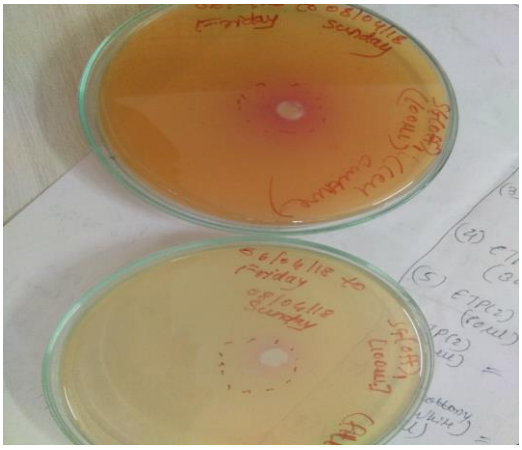


Fig.6: Extracellular enzyme activity (of two selected potent strains) observed in supernatant over M9 Agar plate

CONCLUSION:

In the current study, aim, was to isolate microbes which would be efficient enough to survive on L-asparagine as a growth factor. This can also lower the risks of glutaminase which might serve as a source of hypersensitivity of the drug after administration [24]. Although many reports have shown varied as well as potential L-asparaginase producers, but the characteristic of growing these microbes only over substrate L-asparagine can contribute enormously to higher yield as well as obtaining, genetically stable micro-organisms.

All the (4) cultures were able to grow over both Conventional medium (M9 medium) as well as in minimal medium (Davis-Mingolis Minimal Medium). This is the 1st report so far observed where 4 isolates are showing results in a minimal medium also. Hence, the maximum enzyme producer amongst these isolates would be taken for further large-scale studies.

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