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Screening & isolation of L-asparaginase producing bacteria from diverse niches

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Abstract

L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extracellular enzyme that has been used as a chemotherapeutic agent against, acute lymphoblastic leukemia & lymphosarcomas. The natural varied sources viz., vermi compost, milk waste water, roots of Sadaphuli plant soil sample, slaughter house soil sample, effluent treatment plant(waste water), & fish gut samples were used in order to obtain maximum L-asparaginase producing novel micro-organisms. The primary screening & isolation carried out by determining zones of colouration by diffusion assay(qualitative study over M9 medium), yielded 16 L-asparaginase producing isolates, from the above mentioned natural sources. The quantitative analysis (carried out by Nesselerization treatment) showed, SG(Off)1 to be the potent & maximum L-asparaginase producer (55.231 U). The secondary screening done with Davis-Mingolis minimal medium containing L-asparagine as the only carbon & nitrogen source, showed 4 isolates(amongst the 16) to be best L-asparaginase producers. But, the overall qualitative & quantitative study indicated SG(Off)1 to be efficient l-asparaginase producer. Therefore, this efficient isolate was characterised & identified morphologically, biochemically, & genetically. This potent isolate (obtained from slaughter house soil) was identified as , *Enterobacter xiangfangenesis* .

Keywords: L-asparaginase, *Enterobacter xiangfangenesis*, Nesselerization, M9 medium, Davis-Mingolis minimal medium, natural.

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1. Introduction

L-asparaginase has varied applications in pharmaceuticals, medical & food industry. According to numerous studies, L-asparaginase inhibits cancer by converting

L-asparagine (an essential ingredient for malignant cell growth) into L-aspartic acid and ammonia.[1] As a result, L-asparaginase is an important remedy of acute lymphoblastic leukaemia and melanosarcoma .[2]

L-asparaginase is frequently employed in the food industry because it destroys the carcinogenic component acrylamide, which is generated when starchy, baked, and fried foods are heated above 1000 O C. Acrylamide was recognized as a carcinogen by the International Agency For Research On Cancer (IARC) in 1994.[3]

In 2000, the WHO (World Health Organisation) has declared acrylamide as a carcinogenic molecule supporting IARC's decision. Thus, the ability of enzyme L-asparaginase to counter acrylamide production , has placed it in the food industry as a potential & irreplaceable enzyme used before heating of carbohydrate rich foods .[4]

The global demand for this enzyme, which was 380 million dollars in 2017 and is expected to climb to 420 million dollars by 2025, is essential evidence of its global importance. The efficiency & outstanding properties of this enzyme has placed it as an essential drug , in medical industry because , now it is been tested against various other cancers as well .[5]

Bacteria, fungi, algae, plants, and some animals produce L-asparaginase. It is also present in serum of rodents , but, not in that of humans. The discovery of novel L-asparaginase is an essential task, in order to obtain L-asparaginase other than those derived from E.coli & Erwinia sp. The modification of microbes such as genetic mutation , gamma irradiation , can render pharmaceutically potent L-asparginases with better stability & efficacy , also use of L-asparginase enzyme directly can reduce the carcinogenic acrylamide content from food. By exploring newer diverse niches , serologically altered enzyme having unusual properties can be obtained. This could lead to the isolation of a novel isolate with a wider range of structural and functional features, making it more suitable for use in medicines and food processing.[5]

Asparaginase formulations derived from Erwinia chrysanthemi (Erwinia asparaginase) & E. coli (E.coli asparaginase) bacteria, as well as a PEGylated version of E. coli asparaginase, have been extensively engaged in the medication of acute lymphoblastic leukemia. However, in humans, L-asparaginase therapy has some side Impacts, such as hypersensitivity reactions, including anaphylaxis. [6] These unfavourable side impacts are caused by development of anti-asparaginase antibodies or by the L-glutaminase activity of the L-asparaginase enzyme. As a result, new microbial strains from various environments must be discovered that can produce therapeutically high yields of L-asparaginase.[7]

L-asparaginases produced from a variety of microorganisms i.e. from highly diverse niches, comprise potential & novel properties with respect to Km values, molecular weight , structural orientations & their responses with effector molecules.. The

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ideology of researchers now-a-days has been that the characteristics of altogether novel L-asparaginase could surely combat & eliminate the risk of allergic reactions. [8-9]

The current research therefore, focuses on isolating microorganisms that produce the L-asparginase enzyme by exploring natural habitats such as, Vermicompost, Milk waste water, Roots of Sadaphuli plant soil sample, Slaughter house soil sample, and Effluent treatment plant (waste water) sample , fish gut sample .[10] The screened isolate was then identified & characterized morphologically, biochemically & genetically.

2. Materials and Methods

2.1 Sample collection :

Samples collected were as follows

Table 1. The details of the different samples collected for isolation of the L-asparaginase producing bacteria.

Type of Sample	Location	Collection method	Sample Code
Vermi compost	Tuljaram Chaturchand College Baramati .	It was collected with sterile spatula & brought in sterile plastic bags. [11]	**VTC**
Milk waste water	Dynamix Dairy, unit, Baramati .	Milk waste water was collected , from Dynamix Dairy, unit, Baramati , in sterile bottles (50 ml).[12]	**MWB**
Effluent treatment plant waste water sample	Tuljaram Chaturchand College , Baramati campus.	The Effluent treatment plant waste water was brought in sterile bottles (50 ml).	**ETP**
Fish gut waste Sample	Fish market Baramati .	Fish gut & blood was collected from fish market Baramati. The samples were brought in sterile containers in cooling conditions to the laboratory. The samples were then analysed the next day.[13]	**FSB**
Rhizosphere (roots)soil sample	Rhizosphere soil from botanical garden of Tuljaram Chaturchand College , Baramati .	The soil was taken from a depth of 10-15 cms, using a scrapper & collected in sterile polythene bags. These bags were brought to the laboratory immediately & analysed.[14-15]	**RZ**
Slaughter house sample	Gunawdi , Baramati	The slaughter house soil where animals are cut for meat consisted of blood & gut pieces . This soil was taken from a depth of 10-20 cms in polythene bags , brought to the laboratories & then analysed.[16]	**SG**

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2.2 Chemicals

The analytical grade M9 medium, Nessler's

Reagent, ingredients & other compounds used were acquired from Hi-Media(India). [17]

2.3 Qualitative methodology

2.3.1 *Enrichment, isolation & primary screening of L-asparaginase producing bacteria.*

Samples from diverse niches (mentioned above) were inoculated as 1 gm% into 100ml M9 broth (which is originally yellow) as containing 0.05% Phenol Red dye (The dye at alkaline pH changes from yellow to pink which indicates , the production of extracellular L-asparginase enzyme produced by the micro-organisms). This broth was then placed for 24 hours in an incubator at 37 ° C . For the isolation of L-asparaginase synthesizing bacteria, a loopful of this enriched broth was streaked over M9 agar media (Originally yellow coloured plates, before streaking). These M9 Agar plates after streaking were kept at 37 ° C for 48 hour.

[**M9 Agar media (g/l)** : Na_2HPO_4 : 6g/l, KH_2PO_4 : 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, Agar : 2%.]

2.3.1.1 *Primary Screening*

The plates were examined & checked for well isolated colonies. L-asparaginase producing organisms change the pH of the medium to alkaline conditions, thus making the medium pink coloured. Uninoculated samples served as control where $NaNO_3$ in M9 medium was taken as a sole nitrogen source. The selected isolates were then subjected to diffusion assay, to determine the efficieny of L-asparaginase production. The isolates exhibiting maximum zone of colouration were selected as positive isolates.

These potent L-asparaginase producers were checked for L-asparaginase production by Nesseleriztion assay quantitatively. In this study , the selected isolates, were inoculated as a loopfull into M9 broth (10 ml) & then incubated at 37 ° C for next 24 hours. The cell mass was separated by centrifugation at 7000 revolutions per minute for about 20 minutes, and the cell free supernatant was utilised to calculate extracellular L-asparaginase activity . The amount of ammonia (U ml^{-1}) liberated was estimated by comparing with the Standard graph of ammonium sulphate. The isolates were characterised morphologically, biochemically to classify to genus level. The isolates selected in this course, were considered for secondary screening.[18]

2.3.1.2 *Standard graph of ammonium sulphate.*

1.0 mM ammonium sulphate working solution was prepared. To 3.7 ml of double distilled water in sugar tubes 100µl of different concentrations of ammonium sulphate solution with the use of Nessler's reagent, the final volume was increased to 4.0 ml. A spectrophotometer set to 450 nm was used to measure the intensity of the coloured reaction product. The graph was plotted and used as the ammonium sulphate standard graph.

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2.3.1.3 *Protein estimation.*

The protein estimation was followed by Lowry OH. et al.,(1951) by considering Bovine serum albumin as reference (standard).[19]

2.3.1.4 *Secondary screening of microorganisms that produce L-asparaginase*

The selected isolates from primary screening were streaked over Davis Mingolis minimal medium (containing L-asparagine as nitrogen while dextrose as carbon source). The production of L-asaparaginase takes place in presence of only L-asparagine or not, was still not clear. Hence, isolates grown on the above-mentioned minimum medium were streaked over a minimal medium that included only L-asparagine as a carbon and nitrogen source. These selected isolates were then characterised to classify to species level, microscopically, morphologically, biochemically & genetically.

[Davis - Mingolis Minimal Medium (g/l) : Dextrose : 1g/l, NH_4SO_4 : 1g/l, K_2HPO_4 : 7g/l, KH_2PO_4 : 2g/l, Sodium Citrate : 0.5g/l, $MgSO_4$: 0.1g/l, L-asparagine : 10g/l, pH : 7.0]

3. Quantitative methodology

3.1 *Assay of L-asparaginase*

3.1a *Nesselerization assay*

The L-asparaginase assay is based on the detection of ammonia production in the medium. With a few adjustments, the assay was carried out according to Marshburn and Wriston *et al.* (1964).[20] Cultures were centrifuged for 15 minutes at 7000 rpm, and the supernatant was used to estimate the crude enzyme source. 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 °C 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 1µmol of ammonia per ml per minute [1µmol/ml/min.] (Marshburn & Wriston *et al.*, 1964).[20]

4. Identification of the selected isolate

4.1 *Morphological Characteristics.*

The 9th ed. of Bergey's Manual of Determinative Bacteriology was used to compare the morphological properties of potent strains.. The colony characteristics like size, shape, opacity etc. were recorded and accordingly, isolates were classified & placed into different groups. The colony characteristics, Gram character, motility etc. was checked by using standard protocols. [15]

4.2 *Biochemical Characteristics.*

Finally, biochemical characterization was done , which helped to identify organism up to genus level. The biochemical activities of oxidase & indole synthesis, catalase , nitrate , methyl red , Voges proskaur , citrate were carried out by the potent isolates. The results were compared to Bergey's Manual of Determinative Bacteriology, 9th edition.[15]

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4.3 *Molecular identification of selected isolates.*

16 S rRNA gene sequencing was carried out to identify the culture. The sequences were compared to other gene sequences using the BLAST tool. It was also submitted to NCBI database, so as to assign accession number. Then Neighbor joining method was employed in order to generate a phylogenetic tree of selected isolate. [21]

5. Results & Discussion

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 altogether, drawn interest in the food sector, where it decreases the development of acrylamide during the frying and baking of starchy foods. The goal of this study was to look into as many natural habitats as possible in order to find an isolate that would be allosterically distinct and produce minimal adverse impacts after delivery as drug. 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.[22]

Qeshmi et al. (2016) used a novel *Bacillus sp.*PG02 strain to produce L-asparaginase intracellularly.[23]

Most of the researchers have tried using fermentation strategies so as to enhance production yield. T. E. et al.(2015) used this strategy to synthesize L-asparaginase from agricultural wastes using *Pseudomonas stuzeri* PIMS6. Research in exploring varied natural resources & microbes would gain novel results.[24]

The diversity in medicinal plants have been an unconditional source of antibiotic in medical field. Ahmed El-Said et al., (2016) 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.[25] Similarly, Pagalla U. et al., (2016) used medicinal plant (*Mentha Spicata*) to produce this enzyme from endophytic bacteria. [26]

Marine niches have served as a reservoir of enzymes with high pharmacological activity and efficacy, since ages. Marine actinomycetes *Streptomyces sp.* from the Parangipettai and Cochin Coastal areas produced L-asparaginase and were lethal to K562 cells and JURKAT, according to Dhevagi & Poorani et al., (2005).[27] Edward J. et al., (2016)[28] screened marine sediments, fresh waters, soil samples near seashore, which gave 85 L-asparaginase producing bacteria.[28]

Sindhwad P. & Desai K. (2015) found that supplementing the medium with galactose (2%) and asparagine (0.1) as carbon and nitrogen sources boosted L-asparaginase production (157.03 IU/ml) in the marine isolate *Bacillus pumilis*.[29] Rudrapati P. , Audipudi A V. (2015) reported novel marine *Bacillus subtilis* AVP14 out of 47 bacterial isolates that exhibited highest L-asparaginase activity.[30] In this study, *Bacillus subtilis* AVP14, produced extracellular L-asparaginase (347.42 IU), that raised the L-asparaginase

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production by 15 times under submerged fermentation (5.205 U/gds).[30] Razik – Abdel Noha E. et al., (2019)[31] showed out of 53 isolates , screened from soil samples after optimization increased L-asparaginase production to 4.835 U/ml & 5.221 U/ml respectively.[31] In a study carried out by Dharmaraj S. (2011) , a marine sponge *Callyspongia diffusa* was used to isolate an actinomycete , viz., *Streptomyces noursei* MTCC 10469 using ISP specific medium. [32]

Isolation strategy employed for obtaining potent L-asparaginase producers in current study was as per Gulati R., et al .(1997).[18]

In our study a total 109 isolates were obtained after enrichment & screening over M9 medium. The isolates were subjected to diffusion assay, where zones of colouration produced by L-asparaginase enzyme were measured. In this study, 16 isolates exhibited maximum L-asparaginase zones. The amount of enzyme (in U ml^{-1}) produced by these 16 isolates were then estimated by Nesselerization treatment.[29]

Primary classification of screened isolates (16) up to genus level was done. The morphological, biochemical characterization was done according to 9th edition of Determinative Bacteriology.

The ability of every isolate to produce L-asparaginase , whether involves L-asparagine & dextrose in combination or, occurs due to L-asparagine only, was not understood. Therefore, in the 1st experiment Davis-Mingolis minimal medium containing L-asparagine & dextrose were taken into consideration. However, all the isolates (16) were observed to be growing , in this minimal medium. The L-asparaginase production whether involves both L-asparagine & dextrose or is it elucidated by L-asparagine alone , was still not clear. As a result, the following experiment considered Davis - Mingolis minimal medium using only L-asparagine as a nitrogen and carbon source. Here, only **4 isolates, amongst the 16** , were growing. Therefore, these isolates were **considered to be potent L-asparaginase** producers **from total 109** isolates screened for isolation.[31]

The identification of these isolates, was carried out morphologically, biochemically & genetically. **The 4, isolates in the primary screening also exhibited maximum zones of colouration of L-asparaginase enzyme. But the efficiency of L-asparaginase production elucidated by SG(Off)1 (amongst the 4 isolate) was found to be highest.** Therefore identification of this particular isolate by molecular technique was carried out & finally the sequence was submitted to NCBI so as to assign respective accession number. The phylogenetic tree of this isolate were then constructed. [31]

5.1 Screening &Isolation of L-asparaginase Producing bacteria

From primary screening , 16 isolates exhibited maximum L-asparaginase activity (observed by Diffusion assay). When these isolates were streaked over Davis-Mingolis minimum medium containing L-asparagine

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Sources	A M9 Medium	B Diffusion Assay	C L-aspara+Dextrose	D L-asparagine
VTC	32	2	2	1
RZ	13	4	4	0
SG	49	4	4	2
MWB	5	2	2	0
FSB	5	2	2	0
ETP	5	2	2	1
Total	**109**	**16**	**16**	**4**

as the sole source of carbon & nitrogen only four potent isolates grew. The isolate, SG(Off)1 (out of 4 isolates) was then identified up to species level by molecular techniques.

Table 2 Screening of L-asparaginase producing isolates

A : Isolates growing over M9 medium.

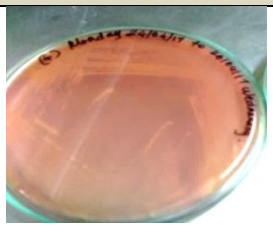
B : Isolates checked for L-asparaginase activity by Diffusion assay.

C : Davis Mingolis minimal medium containing L-asparagine & dextrose

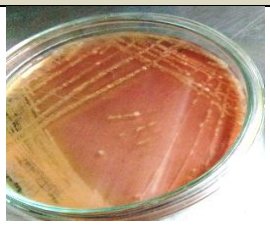
D : Davis Mingolis minimal medium containing only L-asparagine.

5.2 Qualitative methodology :

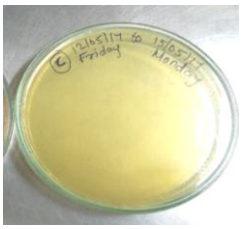
Potent isolates (4) growing over M9 Agar medium



VTC (Cottony White)

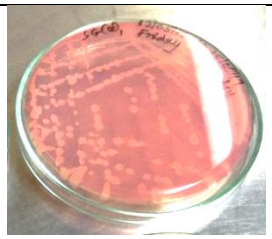


SG(Off)1



Control

SG(P)3



ETP2

Potent isolates (4) grown on Davis Mingolis - Minimal Medium, which solely contains L-asparagine as a carbon and nitrogen source.

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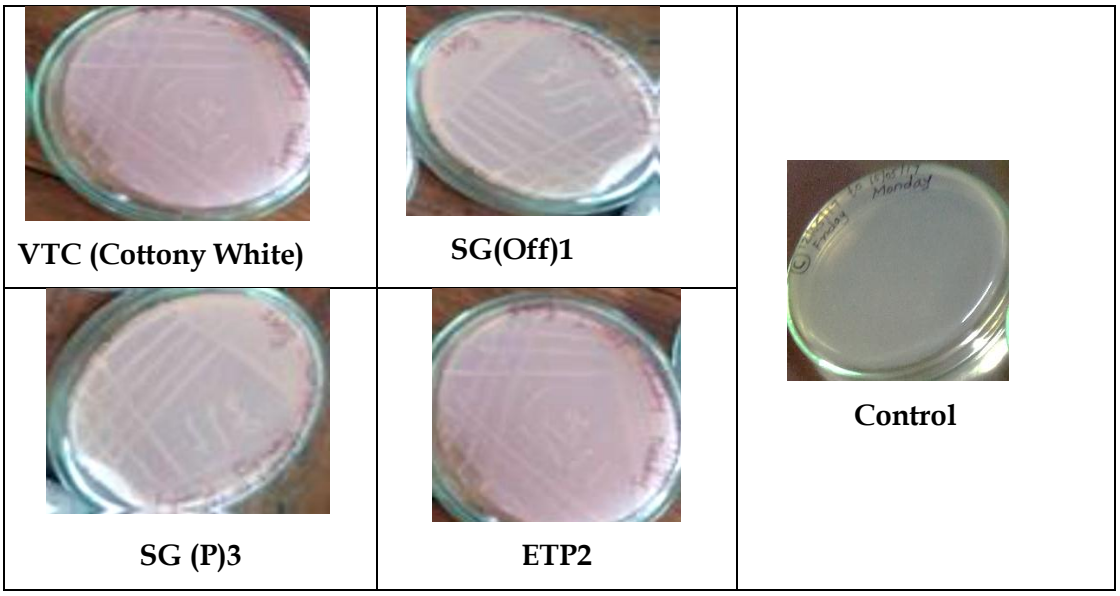

Figure. 1. Selected potent L-asparaginase producing isolates (4)

Table 3. L-asparaginase producers (16) showing maximum pink coloured zone diameters

Sr. No	Isolate Code	Zone Diameter (in cms)
1.	VTC(G)	1.1
*2.	VTC(Cottony white)	1
3.	RZ 1	0.2
4.	RZ 2	0.2
5.	RZ 3	0.2
6.	RZ 4	0.3
7.	MWB(3)	0.3
8.	MWB(4)	0.3
*9.	SG(Off)1	1.3
*10.	SG(P)3	0.7
11.	SG(W)2	0.7
12.	SG(Y)2	0.4
13.	FSB(1)	0.3
14.	FSB(2)	0.4
15.	ETP(1)	0.3
*16.	ETP(2)	0.2

In this (qualitative) study, **SG(Off)1** showed maximum zone of colouration of L-asparaginase production. Upadhyay R et al., (2012)[11] revealed similar pink coloured zone diameters.[42] Jain et al., (2012)[33] & also, Patil & Jadhav (2017)[34] reported smaller diameters of about 1.9 cms, 1.0 cms, & 0.8cms

5.2a ***Standard graph of ammonium sulphate.***

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In the range of 0-1.5 mM, a standard graph was plotted. The selected isolates were compared for L-asparaginase activity with the standard graph. Vachani J., Desai B. (2018) used standard curve range (0.05-0.5) mM/ml. [3]

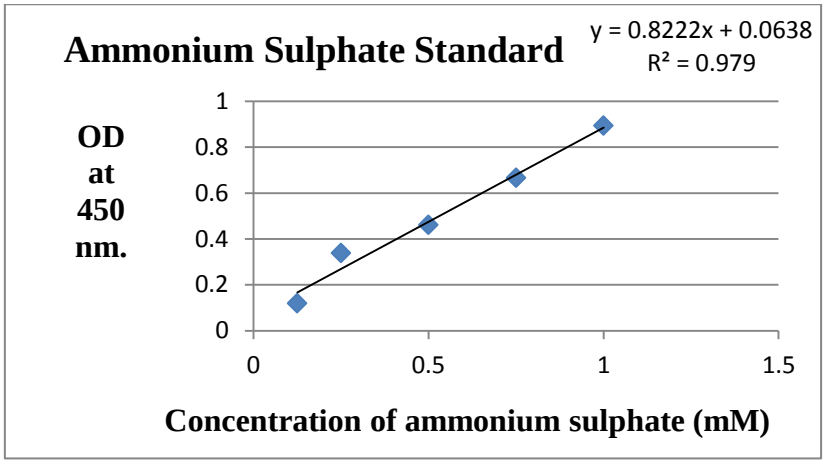

Figure 2. Standard graph for estimation of L-asparaginase activity (NH_3 production) of unknown isolates

5.2b ***Standard graph of Protein.***

In the range of 0-600µg/ml, a protein standard graph was plotted. Test (unknown) samples protein content was compared to the standard.

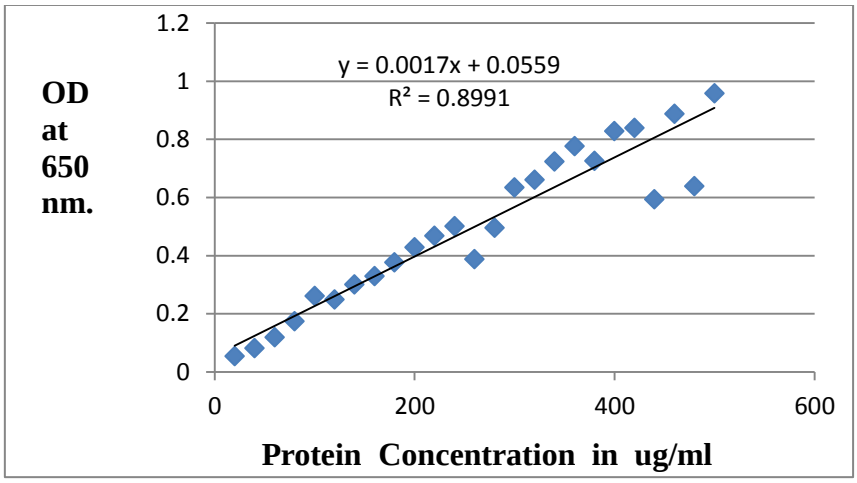

Figure 3. Protein estimation by Lowry. Method.

5.3 Process of screening & isolation of selected isolate SG (Off)$_1$

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The isolate SG(Off)1 (out of 4 isolate) showed ability to grow over M9 medium as well as Davis - Mingoli's minimal medium comprising of L-asparagine as the sole source of carbon & nitrogen . This isolate also showed maximum zone of colouration indicating as the highest L-asparaginase producer out of all the (109) isolate.[31]

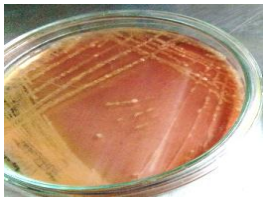
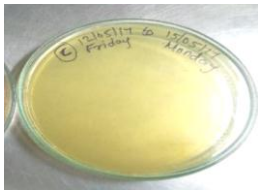
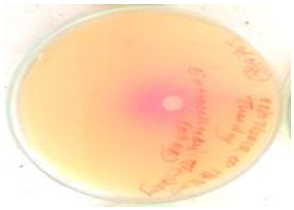
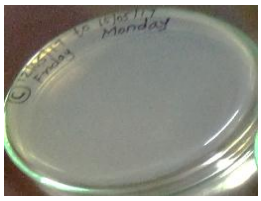
Screening techniques		$SG (Off)_1$	Uninoculated Control
1.	M9 Medium		
2.	Diffusion assay. (Primary Screening)		
3.	Davis-Mingolis Minimal Medium. (Secondary Screening)		

Figure 4. Screening techniques used for selected isolate.

5.4 Quantitative methodology :

5.4.1 Nesselerization assay :

For quantitave estimation, the isolates (16) were subjected to Nesselerization procedure. Samples that yielded positive findings in qualitative methodologies were chosen for this assay. (The isolates were inoculated into M9 broth and placed in incubator for 24 hours at 37 ○ C) After incubation, the colour of the broth changed to pink). The amount of ammonia released was calculated and compared to a standard graph of ammonia.[31] Bhargavi M., & Jayamadhuri

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R. (2016)[36] disclosed highest L-asparaginase production (11.202 µmol/ml/min)

estimated by Nesselerization assay at 37 ○ C after five days of incubation by *Lactobacillus salivarius*.[36] Vacchani J. , Desai B., (2018)[35] investigated L-asparaginase activity by Nesselerization treatment & found out GI3 isolate (with highest zone of L-asparaginase i.e. 52mm) produced 18.66 IU/ml.[35] Fatima N. et al.,(2019)[37] assessed L-asparaginase production (143.55 U/ml) by Nesselerization treatment elucidated by *Pseudomonas aeruginosa*.[37]

Table 4. Nesselerization assay of selected isolates (16)

Sr. No	Isolate Code	L-asparaginase activity in U
1.	VTC(G)	14.676
*2.	VTC(Cottony white)	26.838
3.	RZ 1	10.624
4.	RZ 2	6.569
5.	RZ 3	11.030
6.	RZ 4	8.596
7.	MWB(3)	4.541
8.	MWB(4)	6.569
***9.**	**SG(Off)1**	**55.231**
*10.	SG(P)3	18.73
11.	SG(W)2	10.624
12.	SG(Y)2	10.218
13.	FSB(1)	2.514
14.	FSB(2)	2.108
15.	ETP(1)	10.62
*16.	ETP(2)	15.08

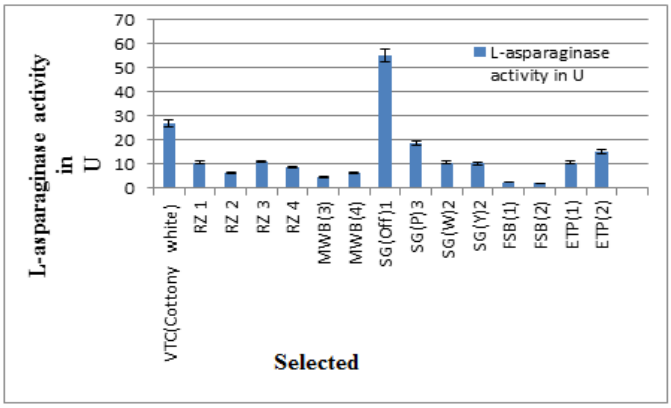

Figure 5. Quantitative estimation of selected isolates by Nesselerization assay.

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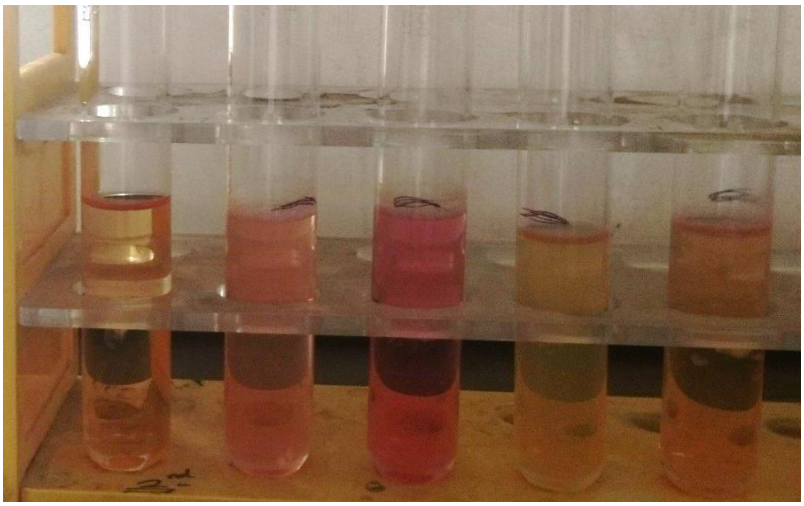

Figure 6. L-asparaginase activity by selected isolates (4) estimated by Nesselerization assay

Table 5 Selected isolates (16) with highest L-asparaginase activities

Sr. No	Isolate Code	Zone Diameter (in cms)	L-asparaginase activity estimated by Nesselerization assay [U]
1.	VTC(G)	1.1	14.676
*2.	VTC(Cottony white)	1	26.838
3.	RZ 1	0.2	10.624
4.	RZ 2	0.2	6.569
5.	RZ 3	0.2	11.030
6.	RZ 4	0.3	8.596
7.	MWB(3)	0.3	4.541
8.	MWB(4)	0.3	6.569
***9.**	**SG(Off)1**	**1.3**	**55.231**
*10.	SG(P)3	0.7	18.73
11.	SG(W)2	0.7	10.624
12.	SG(Y)2	0.4	10.218
13.	FSB(1)	0.3	2.514
14.	FSB(2)	0.4	2.108
15.	ETP(1)	0.3	10.62
*16.	ETP(2)	0.2	15.08

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The overall L-asparaginase production was seen to be higher in all respect with **SG(Off)1** isolate. The identification of this isolate by cultural , morphological, biochemical & genetic analysis was carried out.

This helped us to consider **SG(Off)1** altogether as a potent & efficient micro-organism to be used for further statistical studies (data not shown).

6. Cultural, microscopic , biochemical & genetic characteristics :

Vacchani J & Desai B (2018) reported (out of 49 isolates) , 30 isolates as the best L-asparaginase producers.

6.1 Microscopic Observations of selected (4) isolates :

The positive isolates in our study were characterized & identified in terms of morphology & biochemical analysis. [35, 38]

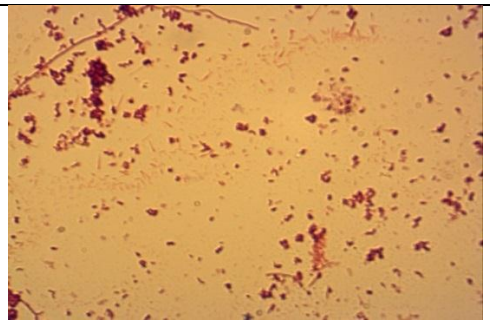

Figure 7. *VTC(Cottony white) : Gram Positive isolate.

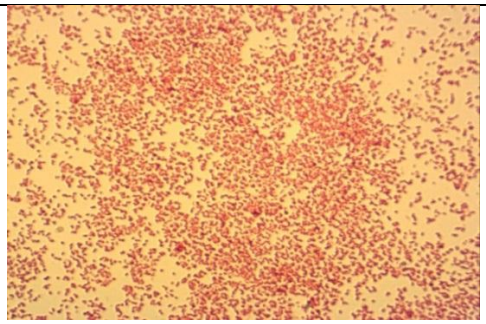

Figure 8. *SG(Off)$_1$: Gram negative isolate.

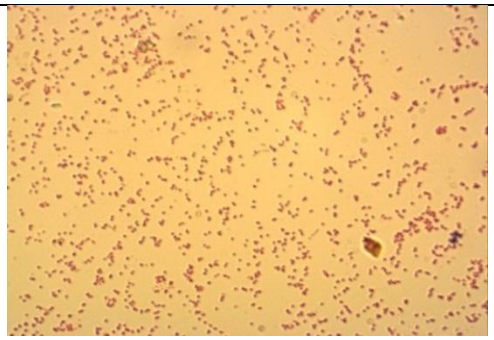

Figure 9. *SG(P)$_3$: Gram Positive isolate.

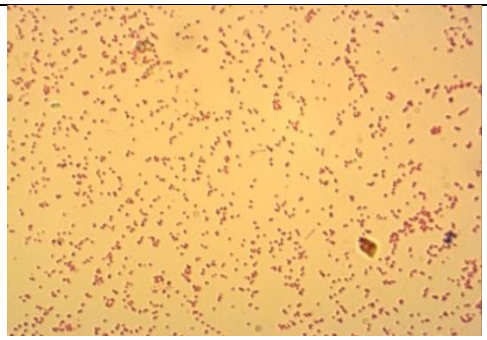

Figure 10. * ETP(2) : Gram Positive isolate.

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Table 6 Colony Characteristics of selected (16) Isolates in reference with Bergey's Manual of Determinative Bacteriology, 9th edition

1.	VTC(G)	1	Circular	Light Green	Undulate	Concave	Opaque	Consistancy	Gram Positive
2.	*VTC(Cottony white)	0.5	Circular	White	Circular	Concave	Translucent	Sticky	Gram Positive
3.	RZ 1	1	Irregular	White	Round	Convex	Translucent	Hard	Gram Negative
4.	RZ 2	1.2	Irregular	Light Brown	Round	Convex	Opaque	Consistant	Gram Positive
5.	RZ 3	1.3	Circular	Light Yellow	Round	Convex	Opaque	Consistant	Gram Negative
6.	RZ 4	1.0	Circular	Light Yellow	Round	Convex	Opaque	Hard	Gram Positive
7.	MWB(3)	1.1	Circular	White	Circular	Convex	Opaque	Sticky	Gram Positive
8.	MWB(4)	0.6	Circular	White	Circular	Convex	Opaque	Sticky	Gram Positive
9.	*SG(Off)$_1$	0.67	Circular	Off White	Circular	Concave	Opaque	Hard	Gram Negative
10.	SG(W)$_2$	0.7	Circular	White	Concave	Concave	Opaque	Hard	Gram Positive
11.	*SG(P)$_3$	0.8	Circular	Pink	Circular	Concave	Translucent	Consistant	Gram Positive
12.	SG(Y)$_2$	1	Circular	Yellow	Circular	Concave	Translucent	Hard	Gram Positive
13.	FSB(1)	0.5	Circular	White	Circular	Concave	Translucent	Consistant	Gram Negative
14.	FSB(2)	0.4	Circular	White	Round	Concave	Translucent	Hard	Gram Negative
15.	ETP(1)	0.36	Circular	White	Round	Concave	Opaque	Sticky	Gram Positive
16.	*ETP(2)	0.49	Circular	White	Round	Concave	Opaque	Sticky	Gram Positive

Table 7. Cultural characteristics of selected isolate [SG(off)1]

[(+) = positive reaction **, (-) =** negative reaction **]**

Cultural characteristics	SG(Off)1	Reactions
Biochemical characteristics	Indole	-
	Oxidase	-
	Catalase	+
	Nitrate	+
	Methyl red	+
	Voges proskaur	+
	Citrate	+
	Motility	+
	Growth on MacConkey agar	+
	Maltose	+

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7. Molecular identification of selected isolate & accession numbers :

Culture was identified genetically by 16 S rRNA gene sequencing & compared through BLAST program with other gene sequences. The sequence was submitted to NCBI database, so as to assign accession numbers.

The selected isolate SG(Off)1 was identified as *Enterobacter xiangfangensis* with accession number as MK426768. A phylogenetic tree was generated using the Neighbor joining method.[21]

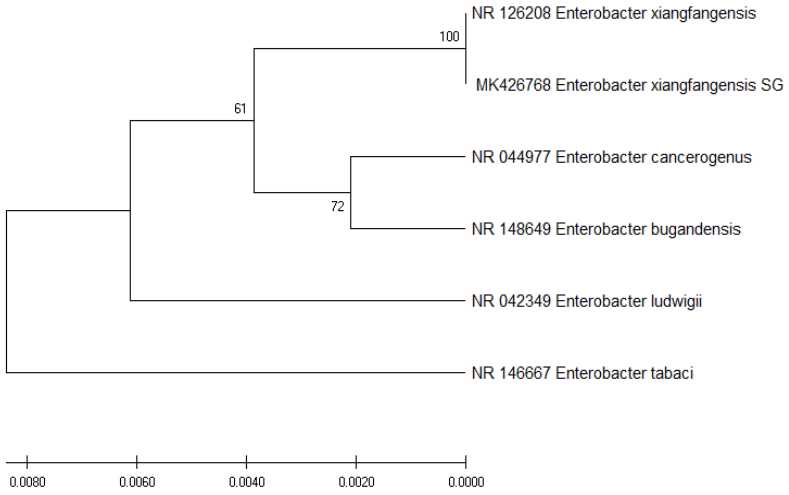

Figure 11. Phylogenetic tree & accession no. of selected isolate *Enterobacter xiangfangensis* MK426768.

Conclusion

Isolation & screening (over M9 medium) gave 109 positive L-asparaginase producing isolates from varied natural sources. The determination of zone of colourations of these isolates screened 16 isolates showing highest zone diameters. In the secondary screening, four isolates were identified using Davis-Mingolis minimal medium with L-asparagine as the only carbon & nitrogen source (out of 16 isolates). In this study, SG(Off)1 was the best L-asparaginase producer (obtained from slaughter house soil), which was identified morphologically, biochemically & genetically as *Enterobacter xiangfangensis* .

Therefore, the current research gave the indegenious knowledge of highly enriched natural habitats consisting of potent & maximum L-asparaginase producing novel micro-organism.

Funding

No

Ethical issue

No

Conflict of interest

no

Acknowledgments: NIL

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