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Isolation and Screening of L-asparaginase and L-glutaminase Producing Bacteria and their Antimicrobial Potential from Environmental Sources

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Abstract: *Microbes are greatest sources for the bulk production of L-asparaginase and L-glutaminase for its bio-medical applications. Bacteria from terrestrial and marine habitats have long been recognised as an organism of antibiotic producers. L-asparaginase and L- glutaminase is an enzyme produced by various microorganisms which are currently used for the treatment of leukemia, in the food industry as a flavour enhancer and also as enzyme biosensors. The aim of this work was to screen L-asparaginase and L-glutaminase producing bacteria from the aquatic and terrestrial environment and their antimicrobial activity. Out of 537 bacterial strains were isolated from diverse terrestrial soil samples and aquatic area, only 118 strains exhibited enzyme activity. Among these, 15 strains were chosen for further study based on high enzyme activity. All the 15 bacterial isolates were characterised and identified up to genus level. The results revealed that 9 strains belong to Bacillus spp. 3 strains belong to Pseudomonas spp. and the remaining 3 strains found to be E.coli. All the 15 bacterial isolates exhibited potential antibacterial and antifungal activity. The highest antibacterial activity was recorded against Staphylococcus aureus (26 mm) and Pseudomonas aeroginosa (26 mm) by Bacillus flexus RM1 strain. The next highest antibacterial activity was recorded against Shigella flexneri (22 mm) and Salmonella Typhi (20 mm) by Bacillus subtilis RM4 strain. Out of 15 strains, 10 strains were found to active against Vibrio cholerae and Klebsiella pneumoniae. The lowest activity was observed against Bacillus subtilis (10 mm) and E.coli (12 mm). Inhibitory activity was mostly against gram-negative bacteria, such as Vibrio cholerae and Klebsiella pneumoniae. In fungi, the highest activity was in Bacillus flexus RM1 against Aspergillus niger (18 mm) and Cryptococcus neoformans (18 mm) and the lowest activity was noticed against Mucor racemosus (10 mm) and Rhizopus arrhizus (10 mm). From the results it was found that bacteria have great potential for the production of enzymes and antimicrobial compounds.*

Keywords: *Antimicrobial activity, L-asparaginase, and L-glutaminase bacteria.*

I. Introduction

L-asparaginase is an enzyme which converts L-asparagine to L-aspartic acid and ammonia has been used as the chemotherapeutic agent. It has received increased attention in recent years for its anti-carcinogenic potential. L-glutaminase is an enzyme which catalyses the conversion of L-glutamine to L-glutamic acid and ammonia (Bessoumy *et al.* 2004). Tumour cells, more specifically lymphatic tumour cells are unable to synthesize the L-glutamic unlike the normal cells; hence it requires huge amounts of glutamine to keep their rapid growth of normal cells. Thus, the clinical action of L-glutamine lies in the death of L-glutamine dependent tumour cells by depriving L-glutamine (Savithri *et al*. 2003). Among different sources of L-asparaginase and L-glutaminase producers, the principle source of this enzymes is from microbes *Escherichia coli* and *Erwinia* spp, which are currently in medical usage as drugs (commercial brand: *Erwinase* and colpase) in the management of lymphoblastic leukemia because of high substrate affinity (Siddhalingeswara *et al.* 2003). L-asparaginase is present in many animal tissues, bacteria, plant and in the serum of rodents but not in mankind. A variety of microorganisms have been reported for their L-asparaginase producing potencies such as *Enterobacter aerogens, Pseudomonas stutzeri, Staphylococcus aureus* and *Serratia marscescens* (Agarwal *et al.* 2011). Bacteria has a different range of enzymatic activity and capable of catalysing various biochemical reactions. Thus, there is enormous scope for the investigation to explore the possibilities of deriving new products of economic importance. Realising the significance of microbial enzyme, the present work has therefore been dealt with isolation and screening of L-asparaginase and L-glutaminase producing bacteria, and their antimicrobial potential from the terrestrial and aquatic environment.

II. Materials And Methods

Sample Collection and Isolation of Bacteria

A total of 10 marine sediments and 9 terrestrial soil samples were collected in new polythene bag and 3 sea water and 1 freshwater sample were collected in sterile screw capped Mc cartney bottle from different locations in Tamil Nadu, India. 1g of sediment and soil samples were transferred to a conical flask containing 99 ml of phosphate buffered saline (PBS) and tenfold dilution were made by using 9 ml PBS. From the desired dilution, 0.1 ml of diluted sample was spread plated either on ZoBell marine agar or Nutrient agar plates and incubated them at 30°C for 24 to 48 h. A total of 537 single distinct colonies from all the samples were picked up, purified and maintained in nutrient agar slants for further studies.

Screening for L-asparaginase / L-glutaminase production

All the bacterial strains were screened for the presence of L-asparaginase / L-glutaminase enzyme by following the method of Prakasham *et al.* (2010). The isolated strains were inoculated into modified M9 medium [composition (g/L): Na_2HPO_4. $2H_20$, 6; KH_2PO_4, 3; NaCl 0.5; 1 M $MgSO_4.7H_20$, 2; 0.1M $CaCl_2$ $2H_20$, 1 ml; L-asparagine, 10; L-glutamine, 10; 20% glucose solution, 10 ml; phenol red dye as a pH indicator 0.3 ml]. M9 media with the above said compositions is used for L-glutaminase activity, where L-asparagine is replaced with L-glutamine. The media were then autoclaved and poured into sterile petri plates. After solidification and surface drying, the plates were inoculated with 24 h old culture of all 537 bacterial strains and the plates were incubated at 30°C for overnight. Positive control plate with *Serratia marcescens* was also maintained. The pinkish red colonies were considered as L-asparaginase / L-glutaminase producers and picked up from plates, streaked onto nutrient agar slants, incubated at 30°C and stored at 4°C for further studies.

Characterization of potential bacterial strains

All the L-asparaginase / L-glutaminase positive bacterial strains were identified for its morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology, (1994).

Test microorganisms

All the bacterial strains (clinical isolates) were kindly provided by the Microbiology Laboratory, Sri Ramakrishna Medical College and Hospital, Coimbatore. The bacterial strains used in this study were *Bacillus subtilis*; *E.coli*; *Klebsiella pneumoniae*; *Pseudomonas aeroginosa*; *Salmonella* Typhi; *Shigella flexneri*; *Staphylococcus aureus* and *Vibrio cholerae*. The fungal strains used in this study were *Aspergillus niger*; *Cryptococcus neoformans*; *Mucor racemosus*; *Rhizopus arrhizus* and *Candida albicans*. These strains were obtained from National Culture for Pathogenic Fungi (NCPF).

Screening of secondary metabolites for their antimicrobial activity using agar well diffusion method

The metabolites of all 118 isolates were screened for their antibacterial activity using the agar well diffusion method. The inocula were prepared by growing the various test organisms on separate Nutrient agar plates and colonies from the plates were transferred with the help of inoculation loop onto 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Muller-Hinton Agar (MHA) plate was evenly inoculated with the test organisms using a sterile swab. The wells were made with the help of sterile cork borer (6 mm in diameter) and the wells were filled with 200 µl of metabolites from the isolated microorganism. Plates in triplicates were incubated at 37°C for 24 h the inhibition zone was recorded. Kirby-Bauer (1996).

Antifungal activity

Antifungal activity of the metabolites was assessed using the well diffusion method. The spore suspension were prepared (10^6 CFU / ml; 0.5ml McFarland) for each test fungal isolate was evenly swabbed onto the surface of Sabouraud Dextrose Agar (SDA) plates. The plates were kept to dry and a sterile borer (6 mm in diameter) was then used to punch wells in the agar medium. Subsequently, wells were filled with 200 µl of the metabolites obtained from the isolated microorganisms and the plates were incubated at 30°C for 72 h. After incubation period the zone of inhibition was measured in millimeters (mm). All experiments were done in triplicates. NCCLS (2002).

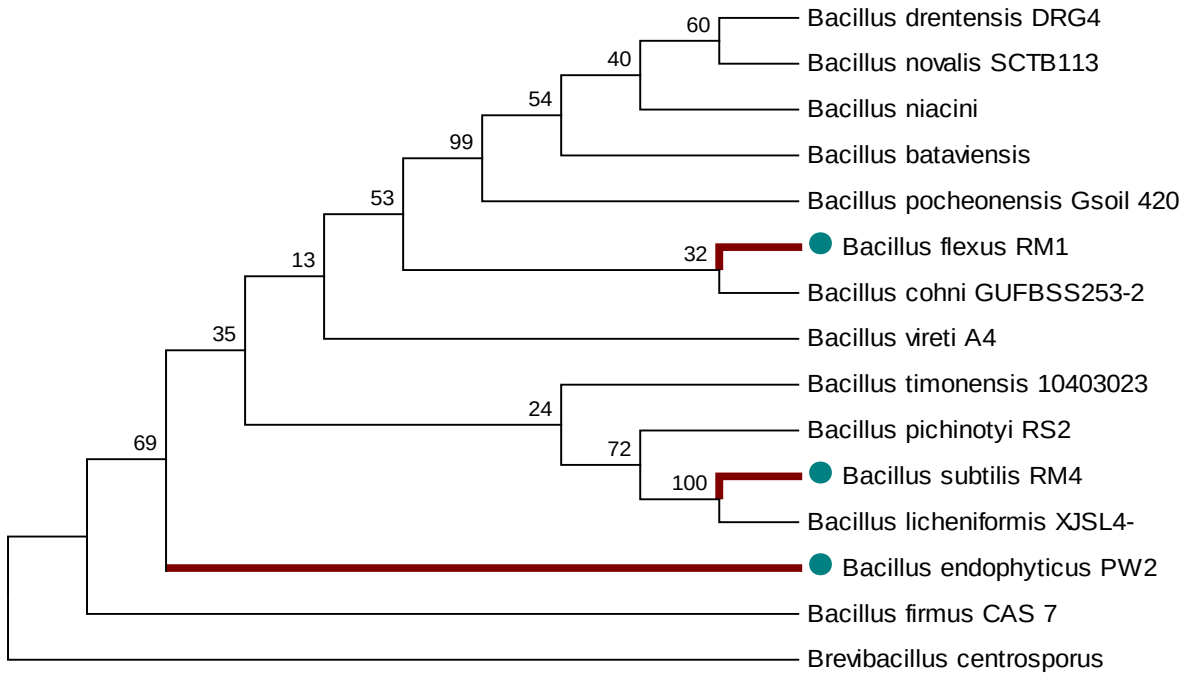
III. Results

Screening of L-asparaginase producing bacteria

A total of 356 bacteria were isolated from terrestrial and aquatic environments and the results are presented in (Table-1). Overall 85 (23.5%) bacterial strains were found positive for L-asparaginase activity in the studied habitat. Among the terrestrial habitat, the maximum number of L-asparaginase producing bacterial isolates were obtained forest soil (33.3%) followed by corn field agricultural soil (28.5%); cotton field

agricultural soil (28.5%); garden soil (22.7%); Jowar field agricultural soil (17.6%); cashew forest soil (11.1%); sesame field soil (10.7%) and paddy field soil (5.2%). In marine habitat, the maximum number of L-asparaginase positive bacterial strains were recorded from the sample of Parangipettai marine sediment and water (47.6%); followed by Tuticorin harbour sediment and water (25.7%); sea water from Velanganni (25.0%); sediment from Kovalam beach (22.7%) and sediment sample from Mandapam (20.0%). In fresh water habitat, the number of L-asparaginase producers was (30.6%). Out of 85 bacterial isolates that exhibited L-asparaginase activity, 9 strains (RM1, RM2, RM3, RM4, ME2, PS1, PS2, PW1 and PW2) showed highest activity and all the strains were identified as *Bacillus* spp. through conventional techniques (Table-3). Among the 9 strains, 3 strains are confirmed by 16S rRNA sequences (Figure-1). RM4 - *Bacillus subtilis* (KU166865); PW2 - *Bacillus endophyticus* (KU166866)*;* RM1 - *Bacillus flexus* (KU166867*).*

Figure 1 – Phylogenetic analysis of 16S rRNA gene for *Bacillus* spp. RM1, RM4 & PW2



Screening of L-glutaminase producing bacteria

A total of 181 bacteria were isolated from marine and terrestrial habitats and the results are presented in (Table-2). Overall 33 (17.6%) of bacterial strains were found positive for L-glutaminase activity in all the samples. Among the marine habitat, the maximum numbers of bacterial isolates were obtained from the sample of Aurovile beach sediment, Pondicherry (27.7%); followed by Muthupet mangrove sediment (23.8%); Kayalpattinam sediment (18.4%); Manapad beach sediment (18.1%); Kannur mangrove sediment (15.0%) and Palaverkadu lake sediment (8.0%). Out of 33 bacteria that showed L-glutaminase activity, 6 bacterial isolates (MS4, KS6, PLS1, PK, MBS and MS2) showed highest activity. Among them 3 strains (MS4, KS6, and PLS1) were identified as *E.coli* and remaining 3 strains (PK, MBS and MS2) belongs to *Pseudomonas* spp. identified through conventional techniques (Table-3).

Table 1- Isolation of L-asparaginase producing bacteria in different samples from various environments

S. No	Habitats			No. of bacterial isolates	No. of L-asparaginase producing bacteria	% of positive L-asparaginase producers
	Terrestrial (Soil)	Aquatic				
		Marine / Brackish (Water or Sediment)	Fresh water			
1	Agricultural soil - Paddy field, Trichy, Tamilnadu	-	-	38	2	5.2
2	-	Sediment -Mandapam coastal area - Rameswaram, Tamilnadu	-	43	9	20.0
3	Forest soil – Kodiyakarai, Nagapattinam. Tamilnadu	-	-	30	10	33.3
4	Forest soil (Cashew) – Cuddalore, Tamilnadu	-	-	18	2	11.1
5	Garden soil –Pudukkottai, Tamilnadu	-	-	22	5	22.7
6	-	Sediment and water Vellar estuary Parangipettai, Cuddalore, Tamilnadu	-	42	20	47.6
7	-	Sediment and water Harbour area Tuticorin, Tamilnadu	-	35	9	25.7
8	-	Sediment - Kovalam beach, Chennai, Tamilnadu	-	22	5	22.7
9	-	Sea water – Velanganni, Nagapattinam, Tamilnadu	-	20	5	25.0
10	Agricultural soil- Cotton fields, Coimbatore, Tamilnadu	-	-	7	2	28.5
11	Agricultural soil- Jowar fields, Tanjore, Tamilnadu	-	-	17	3	17.6
12	Agricultural soil- Corn fields, Coimbatore, Tamilnadu	-	-	14	4	28.5
13	Agricultural soil- Sesame fields, Tanjore, Tamilnadu	-	-	28	3	10.7
14	-	-	Seasonal Niche Trichy, Tamilnadu	20	6	30.6
	Total	-	-	356	85	23.5

Table 2- Isolation of L-glutaminase producing bacteria in different samples from various environments

S. No	Habitats		No. of bacterial isolates	No. of L-glutaminase producing bacteria	% of positive L-glutaminase producers
	Aquatic Marine / Brackish (Water or Sediment)	Terrestrial (Soil)			
1	Sediment - Muthupet Mangrove, Thiruvarur, Tamilnadu	-	42	10	23.8
2	Sediment - Kayalpattinam, Tamilnadu	-	38	7	18.4
3	Sediment - Manapad Beach, Thiruchendur, Tamilnadu	-	22	4	18.1
4	Sediment - Auroville Beach, Pondicherry	-	18	5	27.7
5	Sediment - Kannur Mangrove, Kerala	-	20	3	15.0
6	Sediment– Palaverkadu lake,(Pulicat),Chennai, Tamilnadu	-	25	2	8.0
7	-	Forest soil- Puliancholai, Kolli hills, Tamilnadu	16	2	12.5
	Total	-	181	33	17.6

Table 3 - Biochemical characterization of L-asparaginase / L-glutaminase producing bacteria

S.NO		Strain numbers		
	Biochemical reactions	RM1,RM2,RM3,RM4,ME2,PS1,PS2,PW1 and PW2	MW4,KS6 and PLS1	PK, MBW and MBS2
1	Gram staining	+	-	-
2	Shape	Rods	Rods	Rods
3	Motility	+	+	+
4	Endospore	+	-	-
5	Catalase	+	+	+
6	Oxidase	+	-	-
7	Indole	-	+	+
8	MR	-	-	+
9	VP	+	+	-
10	Citrate	-	+	-
11	Casein	-	-	+
12	Nitrate	+	-	+
13	Mannitol	+	+	-
14	Glucose	+	+	-
15	Sucrose	+	+	+
16	Maltose	+	-	-
17	Lactose	+	-	-
18	Fructose	+	-	-
		***Bacillus* spp.**	***Pseudomonas* spp.**	***E.coli***

Antibacterial and antifungal activity of L-asparaginase and L-glutaminase producing bacteria

Screening of antibacterial activity of 15 isolates was carried out against 8 test strains. The majority of isolates completely inhibited the growth of *Vibrio cholerae* and *Klebsiella pneumoniae*. Among the 15 isolates *Bacillus flexus* RM1 and *Bacillus subtilis* RM4 were found with potential activity against most of the test strains (Table 4) depicts the zone of inhibition exhibited by different bacterial isolates. However, the highest zone of inhibition for antibacterial activity was recorded against *Staphylococcus aureus* (26 mm) and *Pseudomonas aeroginosa* (26 mm) by *Bacillus flexus* RM1. *Shigella flexneri* (22 mm) and *Salmonella* Typhi (20 mm) by *Bacillus subtilis* RM4. The lowest activity was observed against *Bacillus subtilis* (10 mm) and *E.coli* (12 mm).

Among the 15 isolates, 2 isolates *Pseudomonas* spp. KS6 and *Pseudomonas* spp. PLS1 produce no zone of inhibition against test strains. Screening of antifungal activity of 15 isolates was carried out against 5 test strains. The bacterial metabolite exerted strong antagonistic action against the growth of *Aspergillus niger* (18 mm) and *Cryptococcus neoformans* (18 mm) exhibited by *Bacillus flexus* RM1. However, in *Candida albicans* the highest activity was exhibited by *Bacillus* Spp. RM3 (16 mm) and *Bacillus endophyticus* PW2 (16 mm).The lowest antifungal effect was noticed against *Mucor racemosus* and *Rhizopus arrhizus* (Table 5).

Table 4 - Antibacterial activity of L-asparaginase / L-glutaminase producing bacterial isolates

Samples	Zone of Inhibition (mm)							
	Bacillus subtilis	***Escherichia coli***	***Klebsiella pneumoniae***	***Pseudomonas aeroginosa***	***Salmonella* Typhi**	***Shigella flexineri***	***Staphylococcus aureus***	***Vibrio cholerae***
RM1	12	10	14	10	14	18	26	16
RM2	-	-	10	26	-	-	19	10
RM3	-	-	15	23	-	-	18	13
RM4	20	22	18	15	20	22	10	16
ME2	-	-	11	17	-	-	16	-
PW1	-	-	11	-	14	-	-	12
PW2	13	-	12	10	8	-	-	13
PS1	-	-	-	-	15	-	-	11
PS2	-	-	12	-	10	-	9	10
MW4	-	-	10	12	10	12	-	11
KS6	-	-	-	-	-	-	-	-
PLS1	-	-	-	-	-	-	-	-
PK	-	-	-	10	-	10	-	-
MBW	-	-	10	-	-	-	10	10
MBS2	-	-	-	-	-	10	-	-
RF1	14	12	16	26	20	14	18	19

Table 5 - Antifungal activity of L-asparaginase / L-glutaminase producing bacterial isolates

Samples	Zone of Inhibition (mm)				
	Aspergillus niger	***Cryptococcus neoformans***	***Candida albicans***	***Mucor racemosus***	***Rhizopus arrhizus***
RM1	18	18	12	14	12
RM2	-	14	-	-	-
RM3	-	10	16	-	-
RM4	14	16	13	10	10
ME2	-	-	-	-	-
PW1	-	10	14	-	-
PW2	-	12	16	-	-
PS1	-	-	-	-	-
PS2	-	10	12	-	-
MW4	-	-	-	10	-
KS6	-	-	-	-	-
PLS1	-	-	-	-	-
PK	-	-	-	-	-
MBW	-	-	-	-	-
MBS2		-	10	-	-
RF1	14	12	12	14	12

IV. Discussion

Selection of a suitable niche that yields potential producers of any metabolite is one of the most successful ways for assaying at suitable candidates for any target metabolites. There are many reports on asparaginase and glutaminase in soil. The present study was aimed at finding the bacterial isolates which can yield L-asparaginase and L-glutaminase enzyme, that can be employed for an economic and industrial scale production. In this study, totally 85 L-asparaginase producing bacteria were isolated from 14 different places which include marine sediment and sea water, freshwater as well as soil samples from TamilNadu. Among them, 48 L-asparaginase producing bacterial isolates was from marine environment. And the remaining 37 isolate were from terrestrial environment. This is due to the fact that marine sediment provides conclusive environment for microbes with high nutrient content. The biochemical and molecular screening test confirmed all the strains were *Bacillus* spp. similar findings were reported by Gulati *et al*. (1997) and Kamble *et al*. (2010) who isolated *Bacillus* spp. with L-asparaginase activity. Also, in this study investigates 33 L-glutaminase producing bacteria were isolated from 7 different samples (marine sediment, sea water and soil sample). Among them, 31 L-glutaminase producing bacteria belong to marine habitat and only 2 isolates were obtained from

terrestrial environment. This is in agreement with the findings of Pradhan *et al*. (2013) and Upadhyay *et al*. (2013) who reported L-glutaminase producing bacteria from soil samples. This study also revealed that L-glutaminase producing bacterial isolates was identified as *E.coli* and *Pseudomonas* spp. This is in contrast with Suresh Kumar *et al*. (2013) reported that most of the L-glutaminase production is from gram positive bacteria. In the current study all the 15 bacterial potent isolates, 9 L- asparaginase and 6 L-glutaminase producers are not only potent enzyme producers but also they were antibiotic producers. This study, the highest zone of inhibition was recorded against *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Shigella flexneri*, and *Salmonella* Typhi by two potential isolates *Bacillus flexus* (RM1) and *Bacillus subtilis* (RM4). Similarly Zhang *et al*. (2005) reported that 28% of the isolates that showed enzyme activity produced antimicrobial activity also. Bugni *et al*. (2003) also reported that several metabolites of marine isolates showed antibacterial and antifungal potential. These results are in conformity with others who confirmed the effective antimicrobial activity of genus (*Bacillus* spp.) especially against the pathogenic microbes. In our study, the highest zone of inhibition for antifungal activity was recorded against *Aspergillus niger* and *Cryptococcus neoformans*. Whereas, the lowest activity was recorded against *Mucor racemosus* and *Rhizopus arrhizus*. This was in contrast to Tullimilli *et al*. (2014) who reported that maximum activity was against *Mucor racemosus* by L-asparaginase producing bacterial isolates. Though investigations on bacterial metabolites with antifungal potency are scarce, in our study, all the isolates tested showed antifungal activity.

V. Conclusion

Isolation and screening of enzyme producing bacteria from the aquatic and terrestrial environment may provide ample scope to access their therapeutic potential. Results of this study indicate that the potential of the bacteria to produce antimicrobial compounds which can be useful for many therapeutical applications.

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