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Isolation and Characterization of L-asparaginase isolated from soil isolate *Bacillus sp.*

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ABSTRACT

L-asparaginases are the therapeutic proteins and are accounted for around 40 % of anti-leukemic and antilymphoma agents it is also used in the design of biosensors a diagnostic biosensor. The soil samples were collected from different locations around Hubli city. The soil isolates showing potential L-asparaginase activity were identified and characterized. The partially purified L-asparaginase form soil isolate *Bacillus Sp.* showed a specific activity of 6.0IU/mg. The L-asparaginase isolated showed maximum activity at 37^0c with glucose and beef extract as carbon and nitrogen source respectively. The L-asparaginase for soil isolate *Bacillus sp.* Can be utilized as commercially viable L-asparaginase enzyme to be used in pharmaceutical and food industry.

Keywords: L-asparaginases, soil Isolates, *Bacillus sp.*

Introduction

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1), the enzyme which converts L-asparagine to Laspartic acid and ammonia, has been used as a chemotherapeutic agent (Fisher and Wray, 2002). It has received increased attention in recent years for its anticarcinogenic potential (Manna et al., 1995). The scientists observed that that lymphomas in rat and mice regressed after treatment with guinea pig serum. Later it was found out that it is not the serum itself which provoke the tumor regression, but rather the

enzyme asparaginase (Krishnapura *et.al.*,2015). L-asparaginases are the therapeutic proteins as they are accounted for around 40 % of anti-leukemic and antilymphoma agents. L-asparaginase enzyme is also isolated from the various fungal sources else than the bacterial sources (Dalfard *et.al.*,2015).

L-asparaginase causes certain side effects, and despite its potential antileukemic activity, utilization of L-asparaginase by leukemic patients causes lethality to normal cells. L-asparaginase produces a broad range of symptoms such as edema, skin rashes, fever, hepatic dysfunction, diabetes, leucopenia, pancreatitis, neurological seizures, and hemorrhage. some hypersensitivity reactions, mild allergic reactions, and anaphylactic shock are also caused by the usage of asparaginase based drugs. Adolescents appear to beat higher risk of neurotoxicity caused by L-asparaginase, which results in depression, fatigue, lethargy, dizziness, and agitation. The toxicity of L-asparaginases is believed to be brought about by its glutaminase action (Batool *et.al.*,2015).

L-asparaginase is a type of intracellular enzyme produced by microorganisms that can be used as an effective anti-leukemia agent. The enzyme restricts the availability of L-asparaginase to the leukemia cells inducing efficacious and selective inhibition of the protein synthesis. This activity has encouraged the search for new sources of L-asparaginase that present potential for therapeutic use.

There is a need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. This may require the screening of soil samples from various sources for isolation of potential microbes, which have the ability to produce the desired enzyme. The enzyme is produced throughout the world by both submerged and solid-state fermentations. Hence need to isolate a bacterial strain with potent L-asparaginase activity, and to study the enzyme production by purifying and characterising it.

Materials and Methods

Isolation of L-asparaginase producing bacteria from soil samples

Samples were collected from University of Agriculture Science college Dharwad, and serially diluted with sterile distilled water and grown on agar based modified M9 medium. The composition of the medium (g/L): $Na_2HPO_4.2H_2O$, 6; KH_2PO_4, 3; NaCl 0.5; L-asparagine, 5;and 1 mol/L $MgSO_4.7H_2O$, 2ML;0.1mol/L $CaCl_2$ $2H_2O$, 1 mL; 20% glucose 10 mL, agar, 20 and pH 7.0 in distilled water to1L with

phenol red (2.5%):1 to 2 drops. The plates were then incubated at 37^0c for 24 hours, to obtain colonies with pink zones around the them. The colonies obtained were further purified by streaking techniques on M9 medium plates. The purified culture was further maintained at 4^0c for further use.

L-asparaginase assay

The isolated colonies which exhibited highest zone diameter were transferred to 250 mL Erlen-meyer flasks 50 mL modified basal broth medium containing (g/L): L-asparagine, 3.0; glucose, 2.0; Na2HPO4.2H2O, 6.0; KH2PO4,3.0; NaCl, 0.5; MgSO4.7H2O, 0.5; CaCl2.2H2O, 0.015; yeast extract,1.0 and peptone,1.0 and initial pH was maintained at 6.5 and incubated in a shaker incubator (150 r/min, 37 °C) for 36 h. After incubation, the cells were removed by centrifugation 6000×g for 5 min. The supernatant was used to assay extracelluar LAse activity.

L-asparaginase activity was measured by direct Nesslerization of ammonia. The modified method of was Wriston . The L-asparaginase catalyzes L- asparagines to Laspartic acid and ammonia and the latter react with the Nessler's reagent to produce an orange colored product. The enzyme assay mixture consisted of consisted of 100 µL of freshly prepared L-asparagine (189 mmol/L) in Tris-HCl buffer (pH 8.6) and 100 µL of crude extract of the enzyme. The reaction was incubated 37 °C for 30 min and the reaction was stopped by adding 100 µL of 15% trichloroacetic acid (TCA).

The reaction mixture was centrifuged at 6000×g for 5 min at 4 °C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 500 µL Nessler's reagent into the sample containing 200 µL supernatant and 4.3 mL distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min, scanned for e_{max}. OD was measured at e_{396} nm against the blanks that received TCA before the addition of crude enzyme. The ammonia produced in the reaction was determined based the standrad curve obtained with ammonia sulfate. One international unit (IU) of LAse activity was defined as the amount that liberates 1 µmol/L of ammonia /min at 37^0c.

Results and discussion

Isolation

The samples from two different sites were collected and cultured on M9 media and A total 10 bacterial colonies isolated. Out of 10 isolates 2 were selected on basis of different morphological appearance were sub cultured in order to obtain pure culture on slants and from slants were streaked onto media with quadrant streaking.

In the study bacteria were isolated from soil sample obtained from Agricultre university dharwad, Nirani Sugar factory mudhol. Total 10 isolates were showing the 2 isolates good activity .

Isolates were subjected to quantitative L–asparaginase activity the isolates 2 having highest activity 6.7 IU/mL was selected and subjected for molecular characterization by 16sRNA sequencing the isolate 2 which was identified tentatively as B*acillus sp* .and phylogenetic tree was constructed. Using distance matrices by neighbour-joining model of the MEGA6.1 program, with substitution method maximum composite likelihood[tamura,*et al.* 2013].

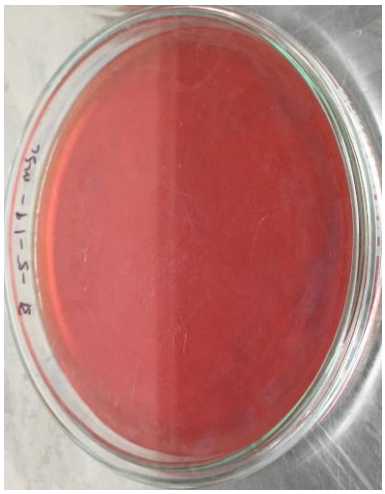
L-asparaginase isolated from soil shown activity of 0.7, 0.5, 0.6, 6.7, 5.0 (IU/mL) at pH 4,5,6,7,8 respectively. highest activity was obeserved at pH 7 .

L-asparaginase isolated from soil shown activity of at 0.2, 0.4, 0.7, 6.7, 0.2 (IU/mL) temperature 10^0C, RT, 22^0C, 37^0C, and 45^0c respectively. highest activity was obeserved at 37^0C.

CONCLUSION

In the present study an effort was made to produce L-asparaginase from microbial source at lower cost by using various low cost substrates. The soil samples were collected from different locations around Hubli city. The soil isolates showing potential L-asparaginase activity were identified and characterized. The partially purified L-asparaginase form soil isolate *Bacillus sp.* has showed a specific activity of 6.0IU/mg. The L-asparaginase isolated showed maximum activity at 37^0c with glucose and beef extract as carbon and nitrogen source respectively . The L-asparaginase for soil isolate *Bacillus sp.* Can be utilized as commercially viable L-asparaginase enzyme to be used in pharmaceutical and food industry.

Before Incubation After Incubation developed pink colour surrounding the growing in modifies M9 medium microorganisms



Morphology Characteristic

S. L. No.	Character	Observation
1	**Gram's reaction**	**Gram positive**
2	Cell shape	Rods
3	Arrangement	Short chains
4	**Configuration**	**Circular**
5	Margin	Entire
6	Elevation	Flat

7	Surface	Dry
8	Opacity	Opaque

S L. No.	Tests	Result
1	Indole test	-
2	Voges Proskuer test	-
3	Methyl red test	+
4	Citrate utilization	-
5	Nitrate reduction	+
6	Oxidase test	-
7	Catalase test	-

Molecular characterization by 16s rRNA sequencing.

395	**AGAGTTTGATCMTGGCTCAG**
396	**TACGGYTACCTTGTTACGACTT**

Photographs:

1

Fig1: Genomic DNA Loaded on 1% Agarose Gel

Lane Description:

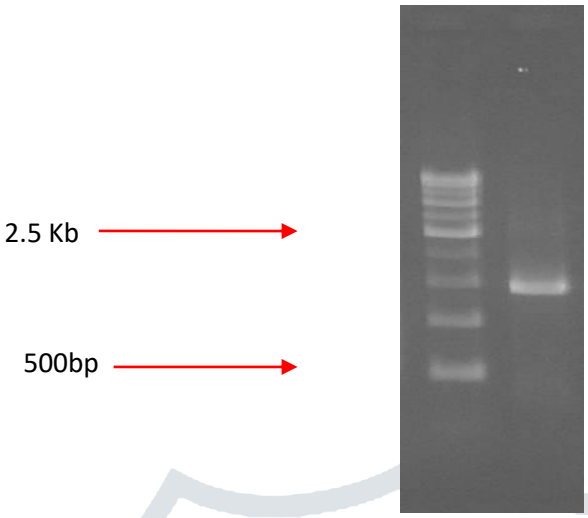


Fig2: PCR Amplicons (~1.5kb) Loaded on 1% Agarose Gel

ALIGNED SEQUENCE

CTGTTCACAATCGTGTCCTCATCATCTCCCCAGTGGGTAGGAGAGGACCCAAGGGAAAAA

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CTCTCTGGAGATGATGCAAAGATGGTTCTGCTGACTGCACCAACATGGGCCCACCGGGCA

CTTCCCTCGCTGGTGATGCTCCTGCTCACTGGCGACCCGGTGCCTACCCCCCCCCCCAGG

GGGATCGGCCACACTGGGACTGAGATACCGCCCTTATTCCTACGGAAAGCATACATGGGG

AATCTTCAGAAAAGCACCCCAGACTGACTCTGTAACGCCGTGTGAGTGATTCACGCTCCC

ATGACAAGAAAAGTCTAGGACGAGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTG

ACGGTACCTAACCAAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG

TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTG

ATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG

AAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA

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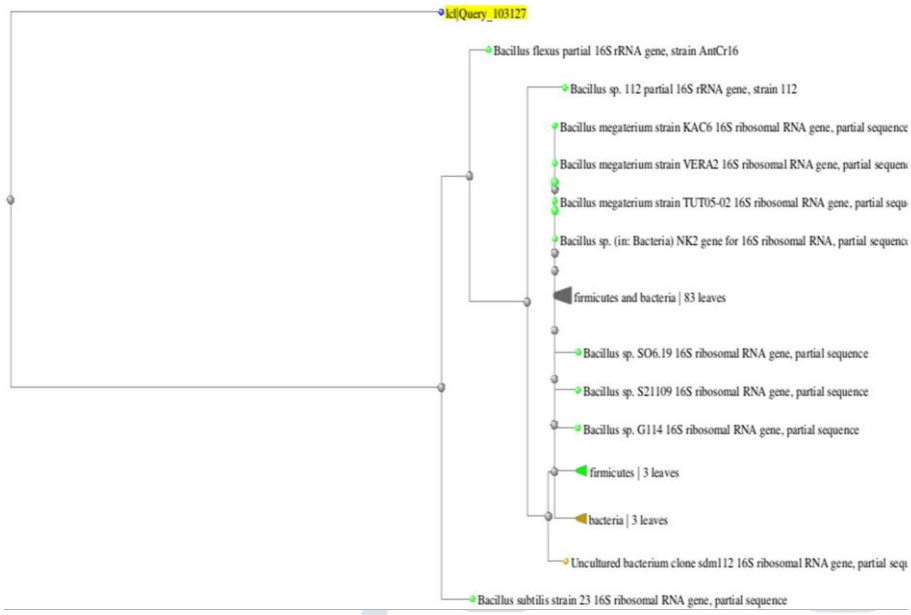
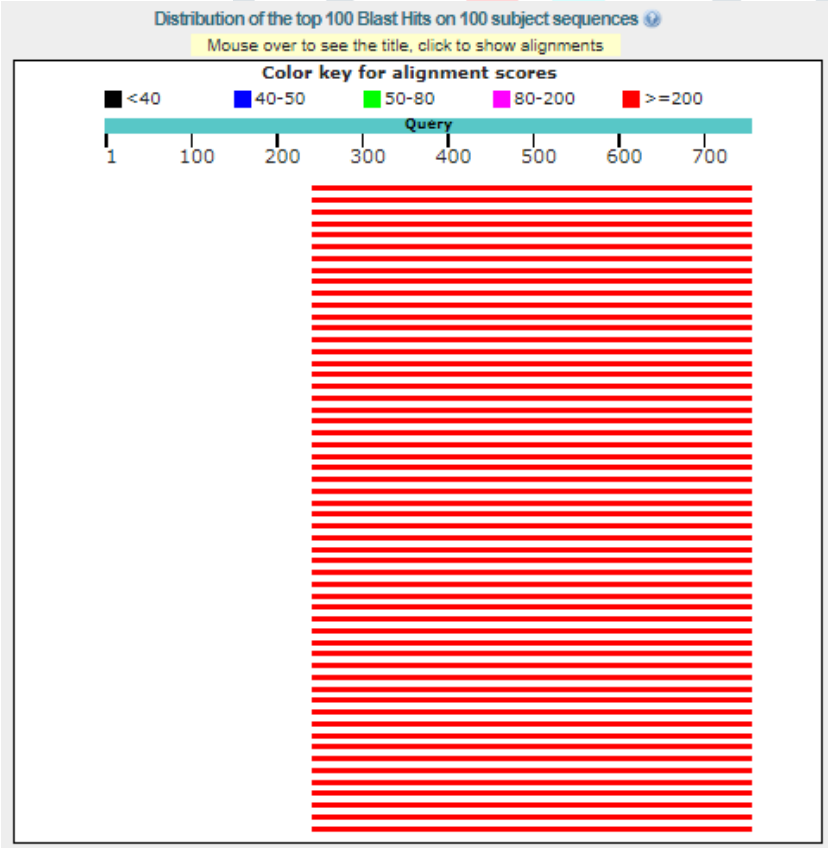
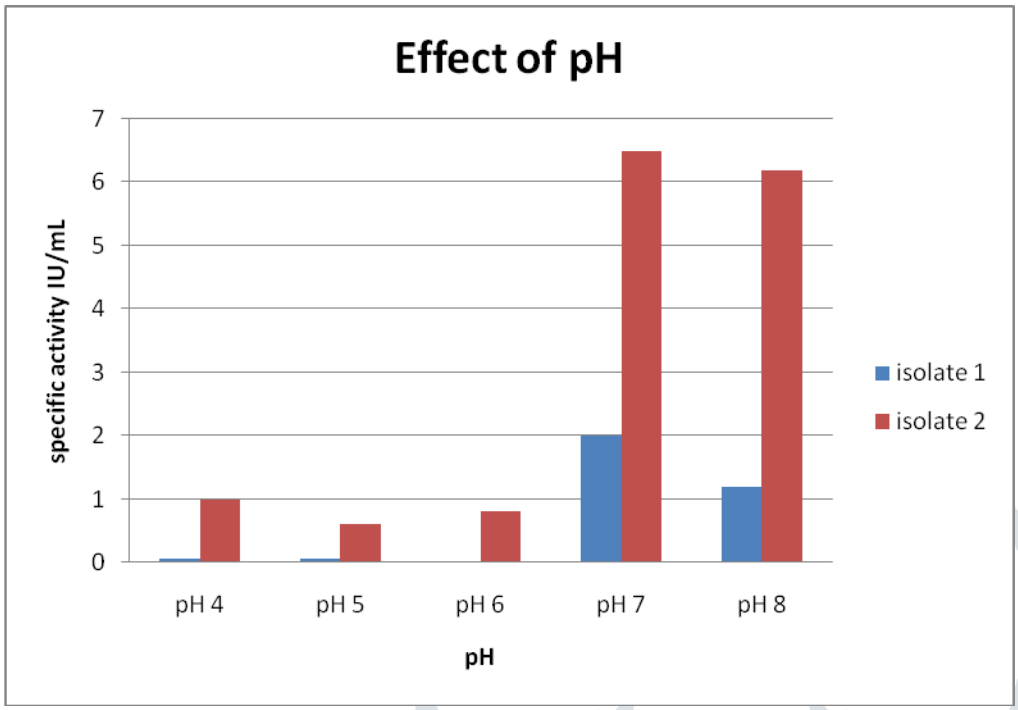
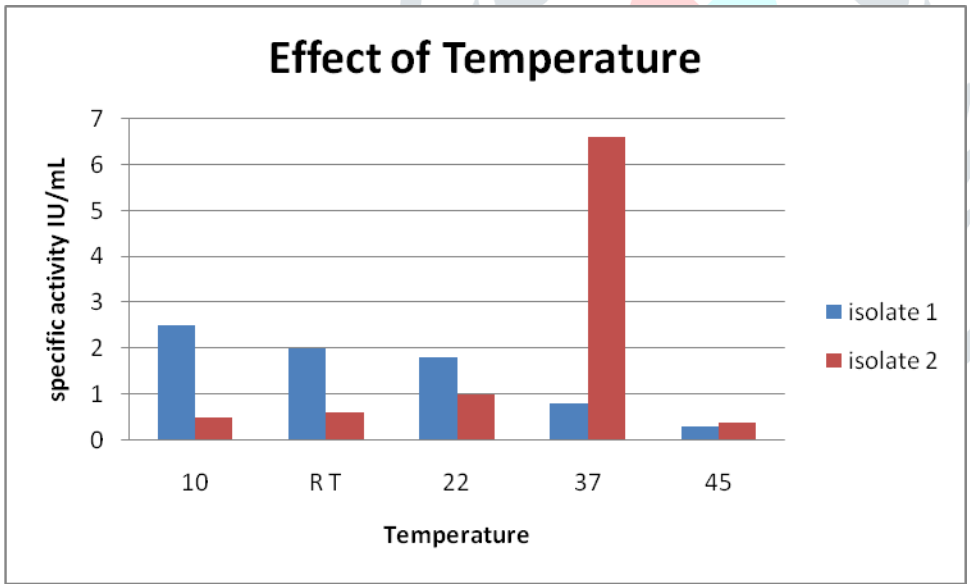
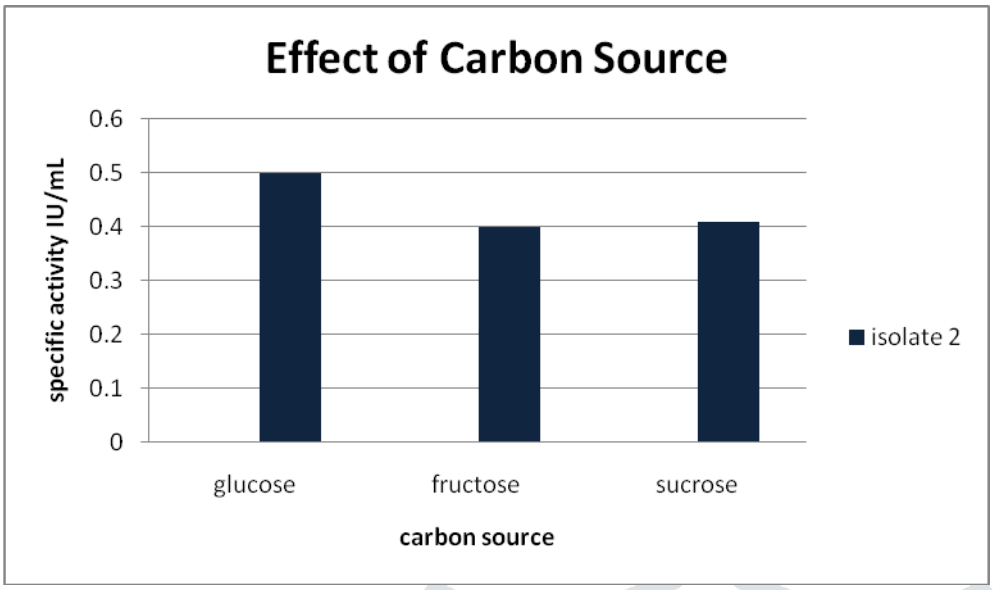


PHYLOGENETIC OF BLAST SEQUENCE




Effect of pH
specific activity IU/mL
0
1
2
3
4
5
6
7
pH 4
pH 5
pH 6
pH 7
pH 8
pH
isolate 1
isolate 2


Effect of Temperature
specific activity IU/mL
0
1
2
3
4
5
6
7
10
R T
22
37
45
Temperature
isolate 1
isolate 2


Effect of Carbon Source
specific activity IU/mL
0.6
0.5
0.4
0.3
0.2
0.1
0
glucose
fructose
sucrose
carbon source
isolate 2