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Screening and isolation of medicinally important L-asparaginase enzyme from a newly isolated species

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ABSTRACT
The L-Asparaginase activity was determined by detection of ammonia or L-aspartic acid. The assay procedure is based on direct Nesslerization of ammonia. The Nine samples were collected at various places of Andhra Pradesh with a viewto isolate potent L-Asparaginase producing microorganisms.A total of 148 colonies were selected and isolated from all the samples based on Lasparaginase enzyme producing microbial strains were identified by their pink colour zones around the colonies. The selected isolates were transferred onto nutrient agar slants and incubated for 24 h. Out of 148 isolates, 46 were selected based on their macroscopic characters, eliminating those that appeared close to each other. The secondary screening was carried out for detection of L-asparaginase positive cultures.After secondary screening, better enzyme producing isolates (5 numbers) were selected and they are designated as MS-3, MS-6, MS-8, MS-11 and MS-15. Among them, the strain MS-6 showed the maximum enzyme production, hence further studies were focused on this strain. For the identification of the promising isolate MS-6, the preliminary morphological, physiological, biochemical tests, utilization of carbon and nitrogen sources were done in our laboratory. A detailed screening of the literature indicated that our isolate is closely related to B. cereus. Hence, the cultural properties of our isolate were compared with reportedproperties of B. cereus. In view of the general agreement and more similarities and a few differences, our isolate MS-6 can be considered to be as a new strain of cereus.

Keywords: L-asparaginase enzyme, ammonia or L-aspartic acid, cultures and B.cereus

INTRODUCTION
Therapeutic enzymes are widely distributed in plant and animal tissues and microorganisms including bacteria, yeast and fungi. Although microorganisms are potential sources of therapeutic enzymes, utilization of such enzymes for therapeutic purposes is limited because of their incompatibility with the human body. A major potential application of therapeutic enzymes is in the treatment of cancer. Asparaginase has proved to be promising for the treatment of acute lymphocytic leukaemia. L-asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonium.

Asparagine is an amino acid required by cells for the production of protein. Asparagine is not an essential amino acid in normal cells and they synthesize this amino acid by the catalytic activity of asparagines synthetase from aspartic acid and glutamine. This enzyme has been isolated, purified and experimentally studied in detail as an antileukaemia agent in human patients (Clavell et al., 1986; Story et al., 1993) and observed its high potential

against childhood acute lymphoblastic leukaemia during theinduction of remission or the intensification phases of treatment (Hill et al., 1967; Oettgen et al., 1967).

The chemical name for L-asparaginase enzyme is mono methoxy polyethylene glycol succinimidyl L-asparaginase. L-asparaginase is modified by covalently conjugating unit of mono methoxy polyethylene glycol (PEG), forming the active ingredient PEG-L-asparaginase (derived from Escherichia coli). Asparaginase catalyzes the hydrolysis of asparagine to aspartic acid and ammonia. Pegasparginase a pegylated form of the enzyme L-asparaginase derived from E.coli is an oncolytic agent used in combination with chemotherapy for the treatment of patients with acute lymphoblastic leukemia who are hypersensitive to native forms of L-asparaginase.

L-asparaginase production is highly influenced by carbon and nitrogensources in Staphylococci and repressed by L-asparagine and L-aspartic acid (Mikuchi et al., 1997) while the enzyme production was inhibited by the presence of glutamine and urea in Aspergillus tamari and Aspergillus terreus (Sarquis et al., 2004). A typical L-asparaginase production pattern was noticed by Escherichia coli, where conventional aerobic environment yielded in large quantities of cells with minimum enzyme while anaerobic fermentation reversed cell and enzyme production yields (Boeck et al., 1970).

Leukemic cells are unable to synthesize asparagine due to a lack ofasparagine synthetase and are dependent on an exogenous source of asparagine for survival. Rapid depletion of asparagine which results from treatment with the enzyme L-asparagine, kills the leukemic cells. Normal cells are less affected by the rapid depletion due to their ability to synthesize asparagines.

Methotrexate is another common anti-tumor drug. L-asparaginase and methotrexate work against each other & treatment is associated with acute side effects that include unpredictable toxicities such as allergy (20%), thromboembolic events (2 to 11%) and severe pancreatitis (4 to 7%).

MATERIALS AND METHODS

All the chemicals used in this study were of analytical grade.

Determination of L-Asparaginase Activity

The L-Asparaginase activity was determined by detection of ammonia or L-aspartic acid. The assay procedure is based on direct Nesslerization of ammonia. Ammonia is estimated by detecting the optical density at 425nm.

Estimation of Extracellular Protein

To 1 ml properly diluted protein sample (10-60 mg/ml), 5 ml alkaline solution (working solution) was added, mixed well and incubated at 37oC for 10 min. To the above mixture 0.5 ml Folin Ciocalteau reagent was added, mixed well and incubated at 37oC for 30 min. The absorbency of the colour was measured at 280 nm in a spectrophotometer.

Screening and Isolation of L-Asparaginase

The soil samples were collected from various places of Andhra Pradesh with a view to isolate potent L-Asparginase producing microorganisms. All the samples were collected in sterile screw capped tubes and care was taken to the points of collection had as widely varying characteristics as possible with regard to the organic matter, particle size, colour of soil and geographical distribution.

About 1 gm of each of the above samples was taken into separate conical flasks each containing 100 ml of sterile water. The suspension was kept on rotary shaker for 30 min and kept aside to settle the suspending matter. One ml of the supernatant was serially diluted with sterile water. One ml each, of these dilutions were added to 20 ml of sterilemodified M9 medium (as described below) maintained at 45oC, mixed thoroughly and plated in 10 cm dia. sterile petridishes and incubated at 37oC. Antifungal agents (Fluconazole-75 µg/ml, Ketoconazole-75 µg/ml) were incorporated to control the fungal contamination. After 24 h of incubation, the selected bacterial colonies with pink zonesaround them were picked up and transferred onto M-9 medium slants (Gulati et al., 1991).

The composition of M-9 medium (M. Sunitha et al, 2010) is (g/L): Na2HPO4.2H20, 6; KH2PO4, 3; NaCl 0.5;L-asparagine, 5; 1M MgSO4.7H20, 2 ml; 0.1M CaCl2 2H20, 1 ml; 20% Glucose stock 55 solution 10 ml, Agar, 20 and pH 7.0 in distilled water to 1 L with phenol red (2.5%): 0.04-0.36 ml indicator.

Secondary Screening

Detection of L-Asparaginase Positive Cultures

In the present investigation, a novel and semi-quantitative plate assay for screening L-asparaginase producing microorganisms is reported. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing asparagines (as sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase.Submerged fermentation studies were also carried out in order to compare the results obtained with those of plate assay.

Shake flask fermentation

Procedure for production and assay of L-asparaginase

The selective promising isolate MS-6 were subcultured onto M-9 medium (M. Sunitha et al, 2010) and incubated at 37oC for 24 h. Here, the selective wild strain compared with test organisms Serratia marceans and Erwinia carotovora. The growth contents of each slant was suspended in 5 ml of sterile water and transferred into 250 ml EM flask containing 50 ml M-9 medium.

Identification of the Isolate MS-6

For the identification of the promising isolate MS-6, the preliminary morphological, physiological, biochemical tests, utilization of carbon and nitrogen sources were done in our laboratory. The isolate was also sent to MTCC for its identification. The MTCC has done all the relevant tests for its identification and the isolate was identified as a Bacillus cereus strain and it was deposited in the MTCC with accession No 7409. The taxonomical studies indicated in table 1-7

The isolate exhibited good growth on all media. The cultural characteristics, physiological and biochemical properties, carbon source utilization pattern, utilization of nitrogen sources, resistance or sensitivity to various antibiotics etc were conducted. Different microbial identification tests were performed as per Bergeys manual for selected microbial species.The following biochemical reactions were determined employing the prescribed media: gelatin hydrolysis, coagulation and peptization of milk, casein hydrolysis, starch hydrolysis, nitrate reduction, carbon source utilization, sodium chloride tolerance, effect of inhibitory compounds on growth, effect of various nitrogen sources on growth, resistance to various antibiotics, growth temperature range, chemical tolerance and cell wall composition.

RESULTS

A total of 148 colonies were selected and isolated from all the samples based on Lasparaginase enzyme producing microbial strains were identified by their pink colour zones around the colonies (Fig 1). The selected isolates were transferred onto nutrient agar slants and incubated for 24 h. The number of isolates from each sample is given in Table 1. Out of 148 isolates, 46 were selected based on their macroscopic characters, eliminating those that appeared close to each other. Standard graph was prepared by treating 1ml of 0.25, 0.5, 0.75 and 1mM ammonium sulphate with trichloroacetic acid, NaOH and Nessler's reagent. After secondary screening, better enzyme producing isolates (5 numbers) were selected and they are designated as MS-3, MS-6, MS-8, MS-11 and MS-15. Among them, the strain MS-6 showed the maximum enzyme production, hence further studies were focused on this strain. To perform further investigation, the selected MS-6 strain was grown on M-9 medium and incubated at 37oC for 18 h and stored until use at 4oC in refrigerator.

This test detects the presence of the enzyme amylase, which hydrolyses starch, used to identify typical starch hydrolyser by the action of amylolytic enzymes. For this test, starch agar plates were inoculated with a loop-full of organism at the center and after 24 h of incubation, the plates were flooded with 2.0 ml of Lugol's Iodine solution (3 g of potassium iodide and 2 g of iodine dissolved in 300 ml of water). Hydrolysis of starch was noted by the formation of clear zone around the growth. The width of the hydrolyzed zone around the growth versus the width of the growth was measured and the ratio was recorded.

DISCUSSION

The most significant characteristics of our isolate MS-6 are summarized below.

The isolate grew well on most of the media. The isolate was non-chromogenic and it did not produce any other soluble pigment. Gram staining was done to know the morphology of the strain under the microscope. The strain retained violet colour after gram staining indicating the selected isolate is gram-positive. The external morphological features indicated that it may be Bacillus

species. Further, different biochemical tests were performed to confirm the nature of the bacteria. (i.e. Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Indole test, H2S production test, Catalase activity and Methyl red and Voges-Proskauer test)

The taxonomic studies of the isolate MS-6 are as follows: The isolate was H2S production is positive. It exhibited slight tyrosinase activity. It could hydrolyze starch casein and gelatin. It could not reduce nitrite. It exhibited good growth at 25°C. It could tolerate the pH levels between 5.0 and 12. It could not grow above 1% NaCl level. It exhibited moderate growth on valine, methionine, hydroxy proline, threonine & cysteine-HCl. It showed sensitivity to penicillin G, ampicilin, tetracycline, chloramphenicol, gentamicin, rifampicin Amoxicillin and Cloxacillin, all the concentrations which are described in Table 3.

FIGURES AND TABLES

Fig. 1: Agar Plate Showing Microbial colonies and inhibition zone

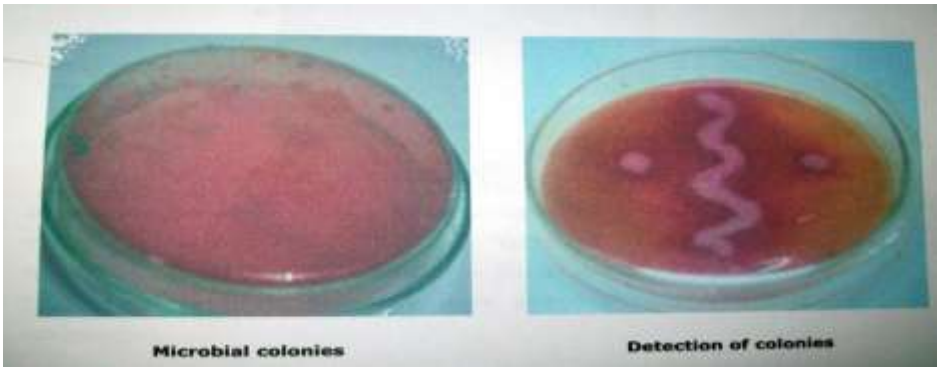

Fig. 2: Detection for isolated microbial strain

Table 1: L-asparaginase producing strains` from various samples

Sample No.	No. of cells per gm. of the sample	No. of selected isolates
I	6.24×10^5	32
II	2.64×10^3	10
III	9.96×10^4	18
IV	2.76×10^5	22
V	4.6×10^4	16
VI	5.21×10^3	06
VII	9.1×10^3	13
VIII	1.5×10^3	14
IX	2.12×10^4	15

Table 2: Comparision of MS-06 with Erwiniacarotovora and Serratiamacerans

Organism	Activity(IU/ml)
MS-06	3.14
Erwiniacarotovora	1.46
Serratiamacerans	1.22

Table 3: Morphological Characters of the isolate MS-6

Tests	
Colony Morphology	**Observation**
Configuration	Circular or irregular
Margin	Entire
Elevation	Flat
Surface	Dry and Granular
Pigment	No pigmentation
Opacity	Opaque
Gram's reaction	Gram positive
Cell shape	Rods
Size (µm)	Length-4µ, Width-1µ
Arrangement	Short chains or pairs
Spore(s)	
Endospore	Endospores present
Position	Central
Shape	Oval
Motility	Positive

Table 4: Resistance of isolate MS-6 to various antibiotics

Antibiotic (μg/ml)	Growth response	Result
Penicillin G (10 IU)	-	Sensitive
Ampicillin (100)	-	Sensitive
Tetracycline (50)	-	Sensitive
Chloramphenicol (100)	-	Sensitive
Gentamicin (100)	-	Sensitive
Rifampicin (50)	-	Sensitive
Amoxicillin (50)	-	Sensitive
Cloxacillin (50)	-	Sensitive

Table 5: Growth of isolate MS-6 in the presence of various nitrogen sources

Nitrogen source (0.1% w/v)	Growth response
L-asparagine (positive control)	+++
Methionine, Hydroxy proline, Valine, Threonine & Cysteine HCl	++
Phenylalanine, Serine, Arginine, Histidine, Potassium nitrate	-

- : No growth; ++ : Moderate growth;

Table 6: Biochemical Tests of the isolate MS-6

Tests	Result
Growth on MacConkey agar	-
Indole test	+
Methyl red test	-
Voges Proskuer test	+
Citrate utilization	-
Gas production from glucose	-
Casein hydrolysis	+
Esculin hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	+
Nitrite reduction	-
H_2S production	-
Catalase test	+
Oxidase test	+
Arginine dihydrolase	+
Ornithine decarboxylase	-
Lysine decarboxylase	-
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
ONPG test	-
Lysozyme resistance test	+

Phosphatase test	+
OF test	-
Haemolysis on Blood agar	Beta-Haemolysis

Table 7: Physiological Tests of the isolate MS-6

Tests Growth at temperatures	Result
4ºC	-
10ºC	-
15ºC	+
25ºC	+
30ºC	+
37ºC	+
42ºC	+
50ºC	-
60ºC	-
Growth at pH	
pH 4.0	-
pH5.0	+
pH 6.0	+
pH 7.0	+
pH 8.0	+
pH 9.0	+
pH 10.0	+
pH 12.0	+
Growth on NaCl (%)	
2.0	+
4.0	+
6.0	+
8.0	+
10.0	-
Growth under anaerobic condition	+

CONCLUSION

A detailed screening of the literature indicated that our isolate is closely related to B. cereus. Hence, the cultural properties of our isolate were compared with reported properties of B. cereus. In view of the general agreement and more similarities and a few differences, our isolate MS-6 can be considered to be as a new strain of cereus.

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