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A Study on Isolation, Purification, and Microencapsulation of L-Asparaginase From Soil Bacteria

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Abstract
L-asparaginase is considered to be a therapeutic enzyme, which is used to treat acute lymphoblastic leukaemia. Soil serves as potential source of bacteria, which can produce L-asparaginase enzyme. In the present study, L-asparaginase enzyme producing bacteria was isolated from soil, using serial dilution technique on modified M-9 media and screened for the production of L-asparaginase enzyme. Out of 6 bacterial strain one pink coloured bacterial strain were subcultured on modified M-9 broth. Morphological and biochemical analysis was done for bacterial identification and confirmed through 16S rRNA analysis as *Enterobacter cloacae.* The isolated bacterial strain was further subjected to enzyme production and purification. The activity of the enzyme after ammonium sulphate precipitation 0.025 unit/mg, dialysis 0.015 unit/mg, and ion-exchange chromatography 0.013 unit/mg was recorded. The molecular weight of the finally purified enzyme was found to be 35 kDa. Further the purified enzyme was encapsulated in sodium alginate microsphere and size ranged in 1 mm. In vitro release study was done and the rate of release of encapsulated enzyme was recorded by estimating of enzyme activity as 0.039 unit/mg.

Keywords
Soil, Bacteria, *Enterobacter cloacae,* L-asparaginase enzyme, Microencapsulation.

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INTRODUCTION:
Cancer is the bunch of disease, where cells grow abnormally in particular area and form tumour. It has ability to spread all over the body which cause abnormal death. Cancer cell failed to achieve the death signal and grow uncontrollably. Every year cancer patients are increasing at all age groups. L-asparaginase is an enzyme, which has antineoplastic properties. Since it has discovered it is used to treat acute lymphoblastic leukaemia in children. Primarily L-asparaginase activity found in Guinea pig serum, used against acute lymphoblastic leukaemia, was reported by Broome's (1961). Several experiments were done by many researchers Mashburn and Wriston (1963); old *et al.,* (1963), to prove Broome's statement. After that, in 1964, Yelllin and Wriston presented that guinea pig serum which was substantially homogeneous had shown anti-lymphomatic activity (Helen *et al.,* 1969). L-asparaginase is present in serum of related species of

guinea pig like Agouti and in that era, it was the best source of L-asparaginase for research purpose. It is present in plants, micro-organism, and some rodent's blood. At first, L-Asparaginase catalyst has been accounted from the two bacterial sources, to be specific: *Escherichia coli* and *Erwinia carotovora*. Most of the bacteria such as *E. coli, Pseudomonas aeruginosa, Pyrococcus furiosus, Erwinia ap*. etc have ability to produce L-Asparaginase enzyme (M.Bhargavi and R.Jayamadhuri, 2016). Industrially *E. coli* and *Erwinia ap.* are used as an L-asparaginase source. Actinomycetes contains different kinds of rare and impressive characteristics (Dharna Shah and Anjali Soni, 2016). Number of researchers have reported on the microbial strains that produce L-asparaginase such as *Pseudomonas fluorescens*, *Serratia marcescens*, *Escherichia coli*, *Erwinia carotovora, Proteus vulgaris*, *Saccharomyces cerevisiae* etc. Comparatively it was identified that *Enterobacteriaceae* family are the best source of L-asparaginase enzyme. For industrial use L-asparaginase produced from *Escherichia coli* (Jorge Javier Muso Cachumba *et al.,* 2016) were used.

L-asparaginase is used as immobilized and PEGylated form. Immobilization of enzyme preserve the activity and half-life of L-asparaginase enzyme. For immobilaization of L-asparaginase enzyme so many kinds of carriers and techniques are available now a days. The present study focused on isolation and purification of L-asparaginase enzyme from soil bacteria and microencapsulation were performed to determine the release time of encapsulated enzyme.

METHOD AND MATERIALS

1. Collection of soil sample

Two soil samples were collected from different places of India. One soil sample was collected from Odisha forest and second soil sample was collected from Madhya Pradesh sewage area.

2. Isolation of bacteria

Isolation of bacteria was done by serial dilution method. Sterilized Modified M9 media was used to isolate the L-asparaginase producing bacteria (modified M9 media composition per liter- KH_2PO_4 – 3 gm, Na_2HPO_4 – 6 gm, NaCl – 0.5 gm, $MgSO_4$ – 0.12 gm, $CaCl_2$ – 1 mg, Glucose – 3 gm, L-asparagine – 10 gm, Phenol red – 2.5%, Agar – 20 gm). The sterilization of the media was carried out in an autoclave at 121°C at 15lbs pressure for 30 mins. Approximately 20 ml of media was poured into pre-sterilized petri dishes and allowed it to solidify. 100µl of serially diluted Soil sample from 1st to 6th tubes were spread on 6 different M-9 agar media plate along with control and incubated for 48h at 30˚C.

2. SCREENING OF L-ASPARAGINASE PRODUCING BACTERIA

After 48 h of incubation enzyme producing bacteria was determined by mild pink colour colonies.

3. IDENTIFICATION OF L-ASPARAGINASE PRODUCING BACTERIA

The selected strain from M-9 media was subjected to identification using morphological, biochemical and phylogenetic study.

3.1. Morphological analysis

Morphological identification of L-asparaginase producing bacterial isolate was done by using Gram's staining. After staining bacterial smear were seen under microscope and identified the bacteria according to their shape and gram negativity or positivity.

3.2. Biochemical analysis

Biochemical tests such as, , Starch hydrolysis, Production of pectiolytic enzyme, Hydrolysis of gelatin, Casein hydrolysis, Urease test, Hydrogen sulfide production, Fermentation of carbohydrate, Reaction in Litmus milk, Methyl red test, Voges proskauer test, Indole production, Catalase, Carbohydrate catabolism, Citrate utilization test were performed on potential bacterial isolate and compared with Bergey's manual.

3.3. Molecular and phylogenetic identification

According to morphological and biochemical identification results potential isolate was selected for molecular and phylogenetic identification. The isolated genomic DNA was used as a template in PCR amplification of 16S rRNA.The Universal primers used for amplification of 16S rRNA are 16S-RS-F (forward) 5'CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'-GGGCGGWGTGTACAAGGC-3'. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA. The amplification reactions was performed in a PCR thermal cycler (GeneAmpPCR System 9700, Applied Biosystems) that was programmed as follows: initial denaturation at 95ºC for 5 mins; 35 cycles of denaturation of 95ºC for 30 sec, annealing at 60ºC for 40 sec, extension at 72ºC for 60 sec and final extension at 72ºC for 7 mins. The PCR products were stored at −4ºC for further use. The amplified products were quantified in 1.2% agarose gel electrophoresis. PCR product (5 L) and 1 L loading dye was loaded along with 5 µL of 1 kb ladder into the separate wells. The gel was run at 100 volts for 1 hour and the amplified products were visualized under UV transilluminator.

4. Production of L-asparaginase

The isolated and screened bacterial strain was cultured for the production of L-asparaginase enzyme into sterilized Modified M9 broth containing chemical composition per litre- KH_2PO_4 – 3 gm, Na_2HPO_4 – 6 gm, NaCl – 0.5 gm, $MgSO_4$ – 0.12 gm, $CaCl_2$ – 1 mg, Glucose – 3 gm 1% L-asparaginase and 2.5% phenol red and incubated for 48 h at 30˚C into shaker incubator

5. Preparation of sample for protein estimation and enzyme assay.

After 48 hours the bacterial broth culture was centrifuged at 4˚C at 10000 rpm for 30 min. The collected supernatant was used for protein estimation and enzyme activity studies.

6. Protein estimation

Protein was estimated by Lowry's method. Protein concentration was measured with bovine serum albumin as the standard.

7. Estimation of enzyme activity

The quantitative enzyme estimation was performed on selected microbial culture by Nesslerization method. 100 µl enzyme mixed with 200 µl of 0.05 M Tris-HCL buffer, 1.7 ml of 0.01 M L-asparagine and then incubated for 30 min at 30˚C temperature. After incubation, reaction was stopped by adding 500 µl of 1.5 M TCA and centrifuged for 10 min at 10000 rpm. Supernatant was taken for enzyme estimation. 500 µl supernatant was mixed with 6 ml of distilled water and then 1 ml of Nessler's reagent and kept for 10 min incubation at room temperature. O.D was taken at 450 nm.

8. Purification of enzyme

The extracted supernatant was subjected to the following purification studies.

8.1. Ammonium sulphate precipitation

The crude enzyme supernatant was precipitated in 80% saturated ammonium sulphate solution and kept it overnight at 4˚C. The pellet was collected after centrifugation at 10000 rpm for 10 min and flushed out the supernatant. Then the pellet was dissolved in 50mM Tris-HCL buffer pH 8.5. Estimation of protein and enzyme was done after ammonium sulphate precipitation and remaining pellet solution was used for further purification through dialysis.

8.2. Dialysis

Partially purified enzyme extract was subjected to further purification using dialysis method. 3 mm Pre-treated dialysis bag were used. The dissolved precipitate was dialyzed against same 50mM Tris-HCL buffer overnight. After dialysis, protein and enzyme estimation was done and remaining purified enzyme was subjected to ion exchange chromatography.

8.3. Ion-exchange chromatography

Ion-exchange chromatography was performed on dialyzed enzyme sample. DEAE-cellulose column was prepared and pre-treated with 0.1M Tris-HCL buffer pH 8.6. Dialyzed sample were loaded on to pre-treated DEAE-cellulose column and allowed the enzyme sample to bind with the gel ions and then elute the unbound sample. The column was washed using 0.1M Tris-HCL buffer pH 8.6 and then eluted the absorbed enzyme sample using a linear gradient of NaCl. Different fractions were collected, and all fraction were subjected to enzyme estimation.

9. Molecular weight determination of L-asparaginase enzyme

The enzyme fraction which showed highest enzyme activity was used for molecular weight determination by SDS-PAGE using Modified Laemmli method

10. Microsphere preparation

Preparation of microsphere was done using Ionotropic gelation method. 50 ml of 1.8% sodium alginate solution was prepared and 0.432 mg gelling agent pectin was mixed thoroughly using magnetic stirrer. Homogenous solution of sodium alginate was mixed into 50 ml of 10% calcium chloride solution using 10 ml syringe and stirred at 100 rpm. The formed microsphere was washed and dried at 50˚C in hot air oven.

11. Microencapsulation of L-asparaginase enzyme into microsphere

Microencapsulation of L-asparaginase was performed using sodium alginate polymer by ionotropic gelation method.

12. SEM analysis of microsphere

SEM analysis also was done to study the shape and size in varying magnifications.

13. In *vitro* release study of encapsulated L-asparaginase enzyme

0.1 gm microsphere was crushed carefully by the help of mortar and pestle and dissolved in 50mM Tris-HCL buffer containing L-asparagine. Reaction mixture kept in rotatory shaker and each ½ hour sample are collected. Collected sample was centrifuged for 10 min at 10000 rpm and the supernatant was subjected to enzyme estimation by nesslerization method.

RESULT

1. Isolation of bacteria

The present study was attempted to isolate L-asparaginase producing bacteria from soil, because previous studies clearly reveals that soil has acted as a rich source for microorganism, which has a very peculiar characteristic.

2. Screening of L-asparaginase producing bacteria

The serially diluted soil sample was inoculated in the M-9 media for 48 hours. It is picturized in Fig. No. 1.

Figure No. 1. Isolated L-asparaginase producing Bacteria on M-9 media.

Four light pink colour producing bacterial colonies was subcultured on modified M-9 media for screening, is shown in figure no 2.

Figure No. 2. Primary screening of L-asparaginase producing colony.

By visualization one of the colonies which appeared pink in color, were picked and streaked out on different M-9 media containing plate for secondary screening shown in fig. no.3.

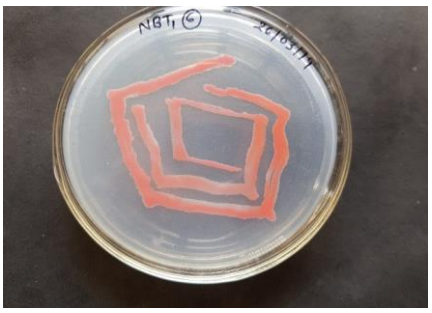

Figure No. 3. Secondary screening of L-asparaginase bacteria.

3. Identification of L-asparaginase producing bacteria

3.1. Biochemical characterization

One strain which appeared dark pink in colour was considered for the present study and subjected to biochemical characterization. By analyzing the biochemical test and comparing with the Bergey's manual, the L-asparaginase producing isolated strain is characterized to be *Enterobacter cloacae*. The findings are tabulated in table no.1. The present findings correlate with M.R. Bhat *et al.,* 2015.

S.no.	Biochemical Test	Result
1	Starch hydrolysis	-
2	Production of pectiolytic enzyme	-
3	Hydrolysis of gelatin	-
4	Casein hydolysis	+
5	Urease test	+
6	Hydrogen sulfide production	-
7	Fermentation of carbohydrate	+
8	Reaction in Litmus milk	-
9	Methyl red test	-
10	Voges proskauer test	+
11	Indole production	-
12	Catalase	+
13	Carbohydrate catabolism	+
14	Citrate utilization test	+

Table No.1. Biochemical analysis of L-asparaginase producing bacteria

3.2. Phylogenetic stud

The preliminary characterized bacterial strain was subjected to 16s rRNA sequencing. The genomic DNA was isolated, subjected to PCR amplification and then to DNA sequencing. The sequence obtained was subjected to BLAST analysis. The online program BLAST (NCBI-2012) was used in identifying the related sequence with known taxonomic information available at the data bank of national center for biotechnology information. The phylogenetic tree constructed is picturized in fig.no. 4.

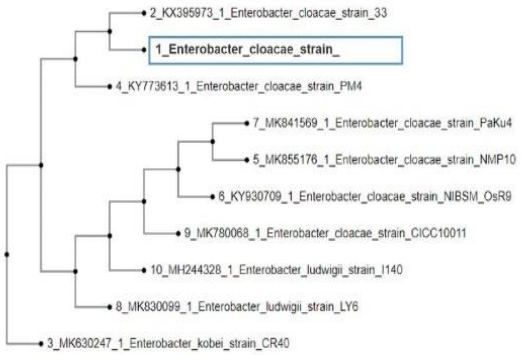

Figure No. 4. Phylogenetic tree of L-asparaginase producing bacteria.

4. Production of L-asparaginase

The pink color colony of *Enterobacter cloacae*, isolated from the plate was further incubated in the M-9 broth for the production of L-asparaginase enzyme. After 48 hours, the turbidity and the colour of the broth increased, which indicated the production of L-aspraginase enzyme. This is evidenced in fig. no. 5.

Figure No.5. Production of L-asparaginase bacteria in broth culture

5. Purification of L-asparaginase enzyme

The results of purification steps of L-asparaginase, isolated from *Entrobacter cloacae* is summarized in table 2.

The enzyme was subjected to purification ranging from Ammonium sulphate precipitation to DEAE-cellulose Ion exchange chromatography.

The protein concentration ranged from 1.09 to 0.32 mg/ml and enzyme activity from 0.028 to 0.013 unit/ml. The present results are correlated with the findings of P Dhevagi and E Poorani, 2006.

S.No.	Purification Steps	Total Recovery		Specific activity mg/ml	Fold purification	% yield
		Protein mg/ml	Enzyme unit/ml			
1.	Crude enzyme	1.09	0.028	0.025	-	-
2.	Ammonium sulphate precipitation	0.85	0.025	0.029	1.16	89.28%
3.	Dialysis	0.38	0.015	0.039	1.56	53.57%
4.	Ion-exchange chromatography	0.32	0.013	0.040	1.6	46.42%

Table No.2. Purification steps and activity of L-asparaginase enzyme from *Enterobacter cloacae*

6. Characterization of the purified enzyme

6.1. Determination of molecular weight

The purified enzyme was subjected to molecular weight determination. The image of SDS PAGE is picturized in fig.no. 6.

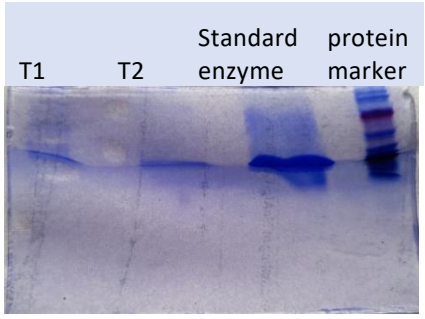

Figure No. 6. Molecular weight determination of L-asparaginase by SDS-PAGE.

From the Figure no. 6, it is clear that the isolated L-asparaginase enzyme and the standard enzyme appeared as single bands. The molecular weight was compared with the standard marker enzyme and ladder protein MBT092. The molecular weight of extracted L-asparaginase enzyme from *Entrobacter clocae* is calculated to 35kDa.

7. Microsphere preparation

A good microsphere was prepared using sodium alginate calcium chloride cross linking. Present study is correlated with studies of K. Nagasree *et al.,* 2016.

8. SEM analysis

This was confirmed using the SEM analysis. The image of SEM is picturized in Figure No. 7.

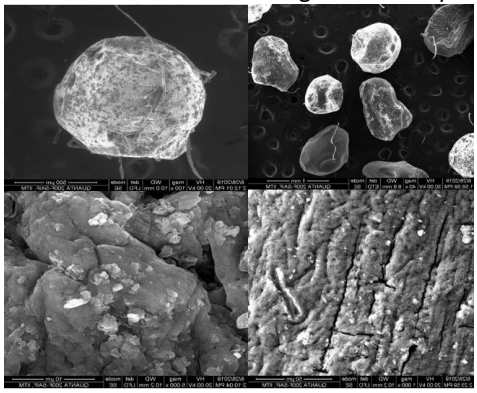

Figure No. 7. Scanning electron microscope of sodium alginate microsphere.

9. Microencapsulation of L-asparaginase enzyme

During the preparation of microspheres, the purified L-asparaginase was attempted for encapsulation. This was supported by release study.

10. *In vitro* release of L-asparaginase enzyme

The encapsulated microsphere was incubated in the Tris-HCL buffer and a steady release of the enzyme was seen. The rate of release is presented in Table No. 7 and figure no.9.

Time Duration(min)	Enzyme activity (unit/mg)
30	0.010
60	0.025
90	0.030
120	0.039

Table No. 3. In vitro release study of enzyme in the time intervals

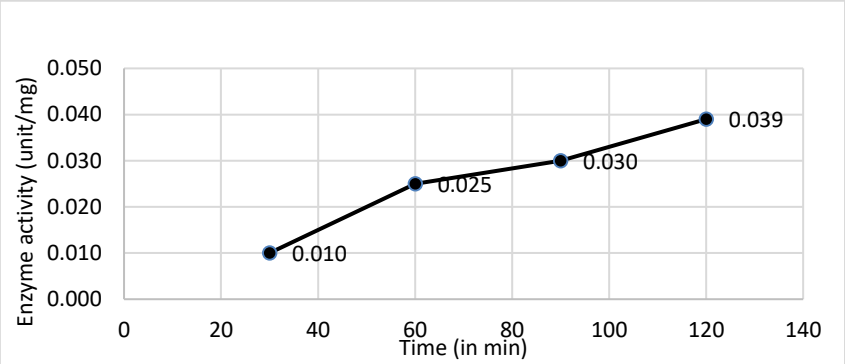

Figure No. 8. *In vitro* release study of enzyme in time intervals.

The results represent that the release of enzyme has started slowly by 30 min and extended till 120 min in a very steady manner.

DISCUSSION

1. Isolation of bacteria

The micro-organism capable of producing L-asparaginase enzyme was isolated from soil through serial dilution technique.

2. Screening of L-asparaginase producing bacteria

Few organisms capable of producing L-asparaginase enzyme were grown in the plate containing M-9 media. During the growth of the bacteria, L-asparaginase has produced. It has utilized L-asparagine to produce aspartic acid in the media resulting in pH change indicated by the change in colour of phenol red, reflecting the colour of the organism to appear pink. Thus growth of the micro-organisms on M-9 media is a very sensitive test to screen the production of the enzyme by the bacteria.

3. Identification of L-asparaginase producing bacteria

3.1. Phylogenetic study

The phylogenetic analysis tree was constructed with the CLUSTAL X program (Thompson et al., 1997), which involved alignment by the neighbor-joining method (Satoa and Nei, 1987). Thus, the isolated strain was confirmed to be *Enterobacter cloacae.* The findings are supported by Biswaprakash Pradhan *et al.,* 2013.

4. Production of L-asparaginase

During the enzyme production the turbidity of the medium indicated the growth of the organism and the asparaginase producing colonies appeared pink in color due to the deamination or release of ammonia from asparagine. The ammonia production had increased the pH of medium and showed pink colour.

5. Purification of L-asparaginase enzyme

The enzyme activity range seems to be decreasing, but the specific activity has increased. This highlights the role of purification.

6. Determination of molecular weight

The present study is supported by the findings of Soni Yadav *et al.,* 2014. The single bond indicates the purity of the enzyme. Also, the isolated enzyme and standard enzyme reflects the same molecular weight, which highlights that enzyme is isolated as a highly purified form.

7. SEM analysis

From the images received it is clear that, the mean diameter of the bead is 1 mm in size. The beads appeared to be more or less spherical in shape. The present findings were supported by German A. Islan & Guillermo Raul Castro, 2014.

8. *In vitro* release of L-asparaginase enzyme

The release of enzyme was confirmed by assaying the enzyme activity in the supernatant. Hence, the sodium alginate has served as potent polymer for microsphere preparation and maintaining the activity of the enzyme stable for a long duration. This study was further confirmed by the findings of Nagpal *et al.,* 2012.

CONCLUSION

Thus, the study attempted would serve as a boon to the modern medicine. Soil is the considered as a precious source of bacteria for the production of a valuable enzyme L-asparaginase. Thus, the enzyme with further studies can be considered as an alternative natural medicine source for the treatment of Leukaemia.

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