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Pseudomonas aeruginosa is One of A Novel Organism Involved in the L-asparaginase Production

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L-asparaginase (E.C.3.5.1.1) enzyme was purified to homogeneity from *Pseudomonas aeruginosa*. In this study, investigation, partial purification and characterization of L-asparaginase enzyme from a *Pseudomonas aeruginosa* strain was studied by using different analytical approach. After screening for the presence of L-asparaginase activity in *Pseudomonas aeruginosa* strain which was isolated from soil, it was decided to choose intracellular enzyme sample for characterization and purification studies. Strain improvement of the production strain by chemical and UV mutagenesis. The optimum pH range for activity of L-asparaginase was alkaline pH ranges, about pH 8.0. The optimum temperature was dedicated as 40 ^{O}C. The molecular weight of L-asparaginase was determined Protein profiling by SDS PAGE as revealed the activity responsive protein bands were appeared on gel (160 kDa). The influence of the enzyme based on the certain physical and chemical characteristics.

Key words: L-asparaginase, *Pseudomonas aeruginosa,* enzyme, Mutagenesis.

Enzymes are the chemical keys to every vital life function. They are special proteins found in foods or made by the body. Microbial cells contain or produce a variety of enzymes. Enzymes are the biological catalysts for the biochemical reactions leading to microbial growth and respiration as well as to the formation of fermentation. Asparagine can be produced within a cell through an enzyme called "asparagines Synthetase" or it can absorb in to the cell from the outside. 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 (Castaman *et al*., 1993).

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The enzyme was produced optimally by bacteria grown between p^{H} 7 and 8 at 37^{o}C. Although some enzyme was formed aerobically, between 100 and 1000 times more asparaginase II was produced during anaerobic growth in media enriched with high concentrations of a variety of amino acids. Cultural and nutritional requirements for maximum L-asparaginase synthesis were determined (Keating *et al*., 1993). Enzyme synthesis was not induced by terminating aeration, agitation in the absence of glucose, nor was it induced in the presence of glucose when aeration was continued.

The determination of protease production was performed to explain the decrease of L – asparaginase activity (Manna *et al*., 1995). After 60h, it was demonstrated that the protease activity could be responsible for decrease in L-asparaginase activity since protease levels increase when L-asparaginase levels decrease (Balco *et al*., 2001).

L-asparaginase is present in many animal tissues, bacteria, plants and in the Serum of certain rodents, but not in mankind. L-asparaginase is produced by a large number of microorganisms that include E.coli (Conea *et al*., 2000, Derst *et al*., 1994*), Erwinia Cartovora*, *Corynebacterium glutanicum*, *Candida utilis*, *S.aureus*, *Thermus thermophilus* and *pisum Sativum*. L-asparaginase catalyzes the hydrolysis of L-asparaginase into L-asparate and ammonia. L-asparaginase activity was found in varying quantities in different organisms and appears to be more common among gram negative bacteria. Antitumour activity was shown by the enzymes from *Mycobacterium tuberclosis* (Story *et al*., 1993), *Bacillus subtilis* (Fisher *et al*., 2002).

The carbon and nitrogen sources most suitable for L-asparaginase production by *Pseudomonas aeruginosa* was reported by (Abdel Fattah *et al.,* 2000). The proline 2% medium was observed to be the best condition for L – asparaginase production. Indeed, the highest L-asparaginase activity level was present by *A. terreus* at 48hrs. (Khamna *et al*., 2009). The production of L-asparaginase, an enzyme widely used in cancer chemotherapy, is mainly regulated by carbon catabolite repression and Oxygen. (Asselin *et al*., 2006). The presence of carbon sources, other than starch, in the growth medium or amino acids, other than L – asparagines inhibited the enzyme biosynthesis. (Prakasham *et al*., 2010).

The objective of our study was to understand the relationship between various factors and the response values and to determine the optimum medium components for maximum production of intracellular L-asparaginase form *Pseudomonas aeruginosa.*

MATERIALS AND METHOD

Sample collection

The Garden soil samples were aseptically collected in Tuticorin District. The samples were taken to the laboratory for the further studies. It is stored as 4°C.

Isolation of Bacteria

2 gm of soil samples were taken and serially diluted and plated on Nutrient agar plate. The plates were incubated at 37°C for 24hrs. After incubation, the colonies were selected for further analysis.

Screening of L ˇ asparaginase producing bacteria

Totally 15 colonies were isolated and screened for enzyme production. The colonies were streaked in the L – asparaginase plates and control plates in which L – asparaginase was omitted. The plates were incubated at 37°C for 48 hrs. The positive strains produce pink colour around their growth. This is because the L – asparaginase converts L – asparagines in to aspartic acid and ammonia and the colour of the media was also changed from light yellow to pink by the change in the P^H of the medium using phenol red as indicator.

Microscopic Examination

Gram s Staining

A loopful of culture was taken from the isolated strains and it is smeared over a slide, then a crystal violet was flooded over the smear for 60 sec or 1 min, the crystal violet was washed with distilled water after washing a few drops gram's iodine was flooded over the smear for 60 sec. The iodine was decolorized by using decolorizer (ethyl alcohol). Then a saffranin was flooded over the smear for 30 sec. The saffranin was washed with distilled H_2O. The slide was dried, and it is observed under microscope. The observed results were tabulated.

Motility test

Vaseline was applied on the four corners of a clean coverslip. A loopful of the isolated organism was placed in the cover slip. The concavity slide was placed over the cover slip with the concave surface facing down. The preparation was observed under microscope.

Indole Production Test

The isolate were inoculated in the sterile tryptone broth and incubated at 37°C for 24 – 28 hrs. After 48 hrs of incubation 1ml kovac's reagent was added and results were observed after 15 minutes.

Methyl Red Test

The isolates were inoculated in the MR-VP broth tubes. The tubes were incubated at 37°C for 48 hrs. After 48 hrs of incubation 5 drops of methyl red reagent was added and the results were observed.

Voges Proskauer Test

The isolates were inoculated in the MR – VP broth. Incubated at 37°C temperature for 48 hours. After 48 hours of incubation 1 ml of

potassium hydroxide 40% and 3ml of 5% alphanapthol solution in absolute ethanol was added and the results were recorded.

Citrate utilization test

The isolates were inoculated in Simmon's citrate agar slant. The tubes were the incubated at 37°C for 96 hrs and results were observed.

Starch hydrolysis test

The isolates were inoculated as single streak on starch agar plates and incubated at 37°C for 24 hrs. After incubation the plates were flooded with iodine solution and the results were observed.

Casein hydrolysis

The isolation were inoculated as single streak on skim milk agar plates and incubated at 37°C for 24 hrs. After incubation, the results were observed.

Catalase Test

First one or two drops of 10% hydrogen peroxide were placed on a clean slide on to that a loopful of isolated organisms was placed and the results were noted.

Triple Sugar Iron Agar Test

The organisms were streaked on a slant of TSI agar and incubated at 37°C for 24 hrs and results were observed.

Endospore Staining

Air dry (or) heat fixation of bacterial smear with minimal flaming. Place the slide over a beaker of boiling water with the bacterial film on the tipper slide and flooded the slide with 5% aqueous solution of malachite green and leave it to act for 1 minute while the water continues to boil and then wash in cold water. Treat with 0.5% saffranin (or) 0.05% basic fusion for 30 seconds, the results were recorded.

Stock preparation

The culture was maintained on tryptic soy agar slants that contained tryptic casein, (1.5%), soy peptone (0.5%), Nacl (0.5%), and agar (1.5%). The culture was incubated at 37°C for 18 hrs and stored at 4°C.

Inoculum Preparation

Transfers the single slant cultures in to 50 ml aliquots of the defined inducible medium. These were placed in 250ml Erlenmeyer flasks and used as the standards inoculate.

Effect of P^H on L - asparaginase activity

The culture was inoculated in to the asparaginase production medium and P^H of the medium was varied from P^H 2 to P^H 11. After the incubation was over, the supernatant was used for crude enzyme activity. The growth rate measured at 540nm for 1hr intervals.

Effect on Temperature on L-asparaginase activity

The culture was inoculated in to the asparaginase production medium and it was incubated at different temperatures 25°C, 30°C, 35°C, 37°C, 40°C, up to 60°C. After the incubation was over the supernatant was used for crude enzyme activity. The growth rate measured at 540 nm for 1 hr intervals.

Asparaginase Production

3ml of inoculum culture was inoculated in to the following suspension media.10g of soya bean meal were moistended with 10ml of 0.01M phosphate buffer P^H 7.4, and placed in 250ml Erlenmeyer flask were incubated at 37°C for 4 days. The extracellular crude enzyme was prepared at the end of the fermentation period by the addition of 90 ml of a 0.01M phosphate buffer P^H 7 to the fermented medium, shaking for 15 min followed by centrifugation at 8000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation

Purification of L-asparaginase

Ammonium Sulfate Fractionation

Powdered Ammonium sulfate was added to 80% Saturation. The mixture was left for 12h at 4°C, followed by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in a 0.01M phosphate buffer P^H 8.5 and dialyzed overnight against the same buffer at 4°C.

Disc ˘ PAGE

1gm of agarose was added to the 100ml of IXTBE buffer. Melt the agarose completely and cooled the agarose to 50°C and add comosine brilliant blue to a final concentration of 0.5µg/ml mix well. Pour the agarose in a present template in a well forming comb place it horizontally on a leveling table and put an appropriate well forming comb. It was important that there are no air bubbles in the gel as there will affect the migration of DNA fragments during electrophoresis. To remove the comb and sealing tapes carefully. Mount the template in an appropriate template in an appropriate electrophoresis stand and fill the tank with IXTBE buffer just to remove the gel up to 1mm. Load the 50µl of samples in to wells. Connect the electrophoresis apparatus to a power pack and

run at 50 volts using IXTBE as the running buffer. Check the samples or migrating in the right direction towards anode. Stop electrophoresis by running of the power supply when the dye bromophenol blue has migrated a distance judged sufficient for separation of samples. Visualized the bands on UV transilluminator

UV ˇ Mutagenesis

1ml of bacterial culture was placed in a sterile petridish and exposed to ultraviolet light at a distance of 15cm for one minute. The mutant strain culture was inoculated in sterilized 250ml production media prepared by moisioning 10g soya bean meal with 10ml of 0.01M phosphate buffer. The P^H of the media, and temperature maintained as 8 and 37°C. After incubation of 4 days the growth rate measured at 540nm.

Chemical Mutagenesis

A chemical mutagen (NTG) was transferred to the 10 ml of specified inoculum culture. After treatment with the mutagens the cells were filtered on membrane filters and grown overnight by overlying the membrane on solid growth media. These filters were then transferred to a detection medium satisfied with 2% agar, containing 10m L-asparagines and 20% sucrose. After 6 hrs of incubation at 37°C of the above medium the filter paper was removed and the underlying solid detection media was blotted for 1 min by using whatman no. 1 filter paper. After drying, the filter paper was sprayed with 0.5% ninhydrin in acetone.

The mutated (*Pseudomonas*) culture was transferred to the production media prepared by poisoning. 10g soya bean meal with 10ml of 0.01M phosphate buffer. The P^H of the media, and temperature maintained as, 8 and 37°C. After incubation of 4 days the growth rate measured at 540nm.

Immobilization

Immobilization of L-asparaginase producing micro organism was done by sodium alginate entrapment method. Initially 3 to 6% of sodium alginate was used. This was dissolved in 0.1% sodium chloride solution. This sodium alginate solution was mixed and allowed to stand for 6 to 8 hrs. To this 15ml of fresh bacterial cells were added with constant stirring using a sterile syringe, this cells suspension was added drop wise to the beaker containing 4% calcium chloride. The beads were kept in calcium chloride solution for stabilization.

Asparaginase production using immobilized cells.

The beads were transferred to the production media, and incubated at 37°C for 4 days. The P^H of the media was maintained at 8.

RESULTS AND DISCUSSION

The total heterotropic bacterial population of the soil sample was isolated. Totally 15 colonies were selected and screened for the production of L-asparaginase enzyme. L-asparaginase production was detected by the appearance of pink zone around the colonies (materials and methods). The positive colonies were identified up to generic level (Amena *et al*., 2010).

Among the 15 strains isolated, *Pseudomonas aeruginosa* comprised highest incidence (40%), Bacillus (26.6%) *E.coli* (13.3%), *Vibrio* (13.3%) and *Enterobactor* (6.6%) Table1. In the present study the predominantly producing strain i.e *Pseudomonas aeruginosa* used for further analysis.

Characterisation of Organisms

The organism has been characterized as

Table 1. Occurrence of L-asparaginase producing bacteria in soil sample

S. No	Oraganisms	No of colonies tested	No of colonies producing L-asparagine	Percentage (%)
1.	*Pseudomonas*	15	6	40
2.	*Bacillus*	15	4	26.6
3.	*E. coli*	15	2	13.3
4.	*Vibrio*	15	2	13.3
5.	*Enterobacter*	15	1	6.6

belonging to the genus *Pseudomonas aeruginosa.* It is a gram negative motile rod, 1.2 to 2.3µm. Producing greenish fluorescent colonies on Pseudomonas Selection agar.

Effect of p^H on L-Asparaginase production

The p^H influence on the L-asparaginase activity was studied using a 0.01M phosphate buffer of different P^H values ranging from 2.0 to 11. The growth rate and enzyme activity gradually increased until P^H 8 at which the maximum activity was observed. At higher P^H the growth rate and enzyme activity decreased. The growth rate was observed using 540 nm Table 2.

Effect of temperature on growth rate of L ˘ asparaginase producing bacteria

The effect of temperature on L-asparaginase activity was studies in the ranges of 25°C to 60°C. The highest production occurred at 37 to 40°C. At highest and lowest temperature the growth rate was low table 3.

Table 2. Effect of P^H on growth rate

S.No	P^H range	Growth rate(OD)
1.	2	0.14
2.	4	0.28
3.	6	0.44
4.	8	0.61
5.	10	0.57
6.	12	0.46
7.	14	0.32

Table 3. Effect of Temperature on growth rate

S.No	Temperature in celsius	Growth rate (OD)
1	25	0.28
2	30	0.56
3	35	0.89
4	40	1.05
5	45	0.59
6	50	0.18

Table 4. Different growth rate in optimum P^H and Temperature

S. No.	Production Strain	Growth Rate (OD)
1.	Chemical Mutagen	1.25
2.	Normal	1.08
3.	U.V. Treated	0.85
4.	Immobilized cells	1.18

Effect of chemical mutagenesis and UV mutagenesis on enzyme activity

The optimum growth rate obtained at 35°C to 40°C and at P^H. At the same temperature and P^H, the chemical mutagen exhibit highest growth rate, and the UV mutagen exhibit lowest growth than the normal production table 4.

Detection of enzyme activity using SDS page

SDS-PAGE has showed that the enzyme is one band. By uses a standard protein with known molecular weight. It was found that the apparent molecular weight of Pseudomonas aeruginosa L-asparaginase was 160 kDa (fig1).

Detection of asparaginase in chemical mutagenesis

The appearance of blue sports on whatman no.1 filter paper indicates the presence of Asparaginase. The convertion of asparagine to aspartic acid was indicated by a blue spot given by aspartic acid correspond the position of a colony. Absence of blue spot indicated the position of a colony with impaired conversion.

Immobilization

The production of L-asparaginase was carried out with immobilized cells of the production strain *Pseudomonas aeruginosa.* L-asparaginase is an enzyme commercially produced by Bacteria, and it destroys asparaginase external to the cell.

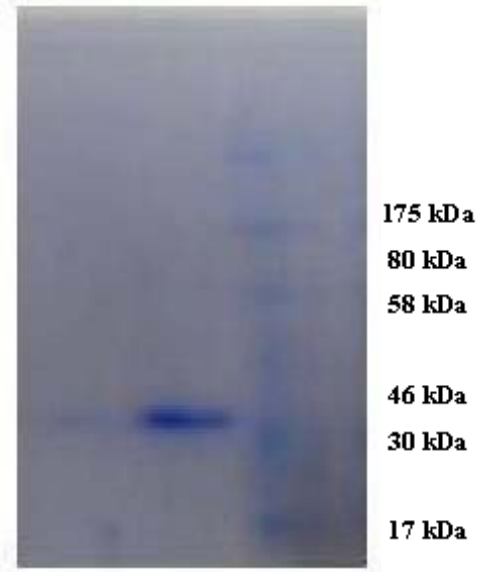

Fig. 1. SDS-PAGE Polyacrylamide gel electrophoresis of purified L-asparaginase from pathogenic *Pseudomonas aeruginosa*.

CONCLUSION

In this study, soil sample was collected from Tuticorin district and screened for the producers of L-asparaginase. Among the screened organisms *Pseudomonas aeruginosa* were selected for the production of L-asparaginase (Prakashan *et al*., 2007). The P^H influence of the L-asparaginase activity was studied using a 0.01M phosphate brffer at different P^H values, ranging frim 2.0 to 11.0. The enzyme activity gradually increased until P^H 9, at which time the maximum activity was observed (Narayana *et al*., 2007).

L-asparaginasse is present in many tissues, bacteria and in the serum of rodents. It is produced by large number of microorganisms include *Escherichia coli, Erwinia caratovora*, *Pseudomonas* sp and *Bacillus* sp., etc. (Abdel *et al*., 2002). Studied that *Pseudomonas aeruginosa* is one of a novel organism involved in the L-asparaginase production (Bessoumy *et al*., 2004).

In our study, the influence of P^H on L-asparaginase activity was seen and at P^H 8, the maximal production of L-asparaginase was observed. In this study immobilized production strain shows high rate of L-asparaginase production. The effect of temperature on growth and enzyme formation in batch fermentation was studied. The optimum temperature for growth and L-asparaginase production were 28°C and 24 °C respectively. At 20 °C the total enzyme activity was close to that synthesized at 24 °C. (Dhevagi *et al*., 2006).

The effect of temperature on L-asparaginase activity and his results shows at temperature of 37 °C maximal L-asparaginase production has obtained (Sumitha *et al*., 2010). In our study maximal rate of production was obtained at temperature of 37 °C. In our study strain improvement technique was performed using mutagenesis. UV-mutagenesis and chemical mutagenesis were performed with the production strain. Highest productivity of L-asparaginase was found with chemical mutagen.

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