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Isolation and Purification of Asparaginase from Proteus mirabilis

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ABSTRACT

10 Bacterial strains were isolated from the soil which was collected from the garbage dump areas. The strains were screened for asparaginase production and the isolate AKNP03 2018 showing maximum L-asparagine hydrolysis was selected for further studies. The isolate AKNP03 2018 was identified as Proteus mirabilis based on Bergey's Manual. Different production media were prepared and out of those NH4Cl production media was the best. It was further modified in terms of nitrogen, carbon sources, effect of metal ions and pH. Production of asparaginase was done by submerged fermentation and crude enzyme was purified by salt precipitation and dialysis.

Key words: Asparaginase, L-asparagine, NH4Cl, Fermentation, Dialysis

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INTRODUCTION

Enzymes or we can say biocatalysts which are produced in human body through amino acids that the body gets by digesting food proteins. They control all the biochemical processes and accelerate in the human body within a single second several millions of enzyme mediated chemical reaction occur in the body. Many number of enzymes act as perfectly matched orchestra so as to ensure that the complex life mechanisms and processes occur in the right direction. The enzymes which are present in the body are essential for life and health so it is important that they function optimally and are in sufficient amount. They have a proteinaceous character and designed in such a way that it carries out a specific task. Each enzyme needs a specific substrate which fits together properly and modification can take place. The enzymes in which microbes are involved they play an important role in the diagnosis, curing, biochemical investigation and monitoring of many deadly diseases. Enzymes are manufactured and processed in such a way that they can be used as drugs which in today's era is an important facet in pharmaceutical industry [1].

L-asparaginase is being used as an effective antineoplastic agent. This enzyme falls in the amidase group that hydrolyses the amide bond present in L-asparagine and converts it to L- aspartic acid and ammonia [2]. It's the foremost enzyme with anti- leukemic activity and it's been researched by researchers in a thorough manner worldwide [3]. The necessary information for producing L- asparaginase as a possible antineoplastic agent was created that disclosed that serum of guinea pig is that made supply of L-asparaginase. In 1953 Kidd displayed and described the ability of guinea pig serum that might inhibit the growth of transplantable lymphoid tumours in mice and rat and even spontaneous and radiation-induced leukemia's in mice. In 1957, Tsuji discovered that there can be deaminization of L-asparagine by extracts by *E.coli* to L-aspartic acid and ammonia. The purification of L-asparaginase from cell extracts of *E.coli* and it also displayed and described its tumour repressive activity that was similar to that of guinea pig serum. For many years L-asparaginases of Erwinia chrysanthemi and *E.coli* have been employed as effective drugs in the treatment of acute lymphoblastic leukaemia and leukaemia lymphosarcoma [4] but their therapeutic response rarely occurs without some evidence of toxicity (Duval et al., 2002). In today's world L-asparaginase constitutes one of the most biotechnologically and biomedically important group of therapeutic enzymes which account for about 40% of total worldwide enzyme sales. If we take it on global scale the requirements of anti-leukemic and anti-lymphoma agents they are far greater than those of other therapeutic enzymes of which L-asparaginase contributes one third. L-asparaginase is a very important and it can be used in

combination with other drugs in the treatment of acute lymphoblastic leukaemia, Hodgkin disease, acute myelocytic, acute myelomonocytic leukaemia, chronic lymphocytic leukaemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma [5].

Acute lymphoblastic leukaemia

Leukaemia as we know is a malignant cancer of the bone marrow and blood which is characterized by an uncontrolled accumulation of abnormal blood cells which leads to the inhibition of normal blood cells functions and many times death. It causes more deaths than any other form cancer among children around the age of twenty [6]. Acute lymphoblastic is a cancer of white blood cells which fight against infection characterized by the excessive multiplication of malignant and immature WBC (lymphoblast) in bone marrow. This cancer can be treated by chemotherapy, steroids, radiation therapy and intensive combined treatments including bone marrow or stem cell transplants. The drugs which are commonly used for this cancer are prednisolone, dexamethasone, vincristine, L-asparaginase, daunorubicin, cyclophosphamide, cytarabine, etoposide, thioguanine, mercaptopurine, hydrocortisone, etc. There are variety of drugs available today but their efficacy in treatment of cancer at third and fourth stage is doubtful.But scientist believe that treating the patient suffering with this type of cancer should be treated with this enzyme L-asparaginase and not by chemotherapy because the enzyme is more safer to use .

Types of L-asparaginase

This enzyme has two related families which are designated as type 1 and 2. These types are according to the terminology in *E.coli*. L-asparaginase-1 (AnsA) is a low affinity enzyme which is found in the cytoplasm. The synthesis of cytoplasmic L-asparaginase 1 is constitutive whereas L-asparaginase-2(AnsB) has a high affinity periplasmic enzyme which is activated during anaerobiosis. AnsB is changed by aeration, carbon source and variation of available amino acids. If we go further only the type- 2 enzyme shows the substantial anti-tumour activity (Table 1).

Table 1: This table depicts the microbial sources of L-Asparaginase

S.No	Bacteria's Name
1	*E. coli*
2	*Erwinia cartovora*
3	*Pseudomonas stutzeri*
4	*Pseudomonas acidov oras*
5	*Erwinia aroideae*
6	*Thermus thermophilus*
7	*Thermus aquaticus*
8	*Staphylococcus aureus*
9	*Staphylococcus sp.-6A*
10	*Vibrio succinogenes*
11	*Citrobacter freundi*
12	*Proteus vulgaris*
13	*Zymomonas mobilis*
14	*Bacillus subtilis*
15	*Bacillus licheniformis*
16	*Bacillus circulans MTCC 8574*
17	*Enterobacter aerogenes*
18	*Serratia marcescens*

MECHANISM OF ACTION OF L-ASPARAGINASE

The mechanism of this enzyme explains that lymphocytic leukemic cells are deficient in L-asparagine synthetase so that they can make their own asparagine. When these cells are treated with L-asparaginase which catalyzes the conversion of L-asparagine to L-aspartate and ammonia.as a result of which the growth of cells is critically affected with the rapid depletion of L-asparagine from the blood supply and the surrounding tissue [7]

As we know that cells require a continuous supply of amino acid L-asparagine so that they can build proteins and many cells need the enzyme asparagine synthetase so that they can make their own asparagine. The enzyme takes L-aspartate and addition of an amine takes place which forms the characteristic amide group of

asparagine. L-asparaginase works in an opposite manner, by taking L-asparagine and pulling of its amine and in turn releases l- aspartate and ammonia [8]. In our human body L-aspartate plays a major role by acting as a precursor of ornithine in the urea cycle. It also takes part in transamination reactions by forming oxaloacetate in the gluconeogenic pathway leading to glucose formation. But if we introduce a large dose of this enzyme into the blood then it will circulate and continually break done all the asparagine it finds which ultimately starves the cells that rely on blood- borne supply. This enzyme cuts off the supply of L-asparagine in the blood and the cancer cells die as they are not able to build the proteins. When L-asparaginase is given it also prevents free diffusion of L-asparagine from the surrounding tissues to the cancerous cells. If there is a high pressure in the blood stream, then these enzyme molecules may pass into the intracellular spaces from fine capillaries and they will catalyze the hydrolysis of L- asparagine [9].

MATERIAL AND METHOD

Collection of soil sample

Three different samples were collected from different garbage dump areas of Lucknow for my project work and Table 2 gives the information regarding the location, type and colour of samples that were collected.

Table 2: This table depicts the collection of soil sample.

S. No	Name Of The Sample	Colour	Name Of The Place
1	Soil Sample (Garbage Dump)	Dark Brown	Vibhuti Khand Railway Crossing
2	Soil Sample (Garbage Dump)	Light Brown	Vibhuti Khand Green Café
3	Soil Sample (Garbage Dump)	Blackish Soil	Polytechnic

Isolation of bacteria from soil sample by serial dilution

Serial dilution agar plating method or viable count method is one of the most commonly used methods. It is a series of sequential dilutions used to reduce a dense culture of cells to more usable concentrations.

Colony morphology study

Large number of colonies grows on the agar plates so to distinguish between the different colonies; colony morphology of the bacterial colonies is studied. Colony morphology represents the important information regarding the identification of the organism.

Purification of obtained mixed culture by continuous quadrant streaking

A pure colony is generally isolated from the mixed culture by picking up single colony through inoculum loop and then streaked on the NA plates. The principle of streaking is that the individual colonies are allowed to grow onto the new media by providing them chance to grow into the individual colonies. Hence, a pure colony is obtained.

There are two types of streaking method one is zigzag and the other is quadrant. Again quadrant streaking divided into two parts continuous and discontinuous quadrant streaking. Continuous quadrant streaking was done in order to obtain well isolated colonies. This allows sequential decrease in the number of original microbial material over an entire surface of the plate. By every quadrant streaking the number of colonies decreases as a result we get pure isolated colonies.

Screening of the purified culture

Screening is the procedure to identify whether the selected microbe have the potential to secrete the desired secondary metabolite or not. Asparaginase producing bacteria was detected with the screening media in which carbon source is limited and the additional substrate is provided in the form of L-asparagine in order to check whether the bacteria is able to degrade the substrate which we have provided or not. The presence of zone of hydrolysis is noted. This screening is said to be primary screening. The isolated pure strains are subjected to secondary screening for the extracellular production of asparaginase. This is visualized by the presence of the zone of hydrolysis of the pate after the treatment with the phenol red.

Identification of the purified culture

Gram staining

Gram staining is a differential staining process which was developed by Dr. Gram HC, a Danish physician, for the identification and the classification of the bacteria into two categories: Gram +ve and Gram –ve, as well as whether the bacteria is endospore forming or not simultaneously providing the information regarding the cellular morphology and the arrangements of the cells. The bacteria are subjected to four different chemicals:

- Crystal Violet (Primary stain).
- Iodine Solution (Mordant).
- Ethyl Alcohol (Decolorizing agent).
- Safranine (Counter stain).

Gram positive cells exhibit a thick peptidoglycan cell wall that has an ability to retain the crystal violet iodine complex, while gram negative cells have only thin layer of peptidoglycan and is not able to retain the crystal violet, when treated with ethyl alcohol. Besides, the gram positive cells have the cross linked peptidoglycan layer preventing the cells from decolorizing when treated with ethyl alcohol. As the gram negative cells get dissolved

with ethyl alcohol the negative cells get other stains like Safranine resulting in the purple colour

Mannitol fermentation test

Mannitol test is performed to check whether the Mannitol is oxidized by the bacterial culture or not. The ability of the Mannitol fermentation of the bacterial culture is tested by using the phenol red mannitol broth. Phenol red is a pH indicator which detects the change in the pH. In mannitol fermentation broth, glucose which is a reducing sugar oxidizes the mannitol to the carboxylic acid, the pH of the broth decreases and shifts towards the acidity. The change in the colour occurs. Phenol red is at pH 7 and change to yellow colour at pH less than 7.

Optimization of production media for maximum yield of asparaginase enzyme

The production media is inoculated with the culture and growth which was observed by calculating the OD. For the good production the best conditions for growth were identified and that production media was preferred. Different modifications was done like changes in nitrogen source, carbon source, metal ion concentration and effect of ph.

Production of asparaginase

It is also known as shake flask fermentation. In this process, the microorganism is allowed beneath the surface in the liquid media which is vigorously agitated in the large tanks of the fermenter. This can be either an open tank or closed tank in batch or continuous type and are generally made up of the non-corrosive material. In batch fermentation, the organism is grown in the known amount of culture media for a defined period of time and the cell mass is separated from the media before further processing while in continuous culture, the culture media is withdrawn depending on the rate of production and in flow of media.

Extraction of crude enzyme

For the production and extraction of enzyme, enzyme producing microbes were cultivated in the fresh culture media for the optimized period and the enzyme is recovered. The enzyme extract is centrifuged, to recover cell debris, cell organelles and sometimes other molecular aggregates, leading to the partial purification of the enzyme. It is also known as the characterization of the enzyme, since depending upon its mass and shape. The enzyme will move at a definite speed and occupy the characterization position in the centrifuge tubes.

Salt precipitation

The crude enzyme we extracted is present in the solution forming the homogenous mixture with the help of the hydrogen bonds so making it difficult for us to extract our protein from the mixture. In salt precipitation, we use some other compound that has the greater affinity to form the hydrogen bonds with the water other than our protein, hence precipitating our protein out. At low salt concentrations the presence of the salt stabilizes the various charged groups on the protein molecules, thus attracting the protein in the solution and enhancing the solubility of our protein. This is commonly known as salting in. however; as the salt concentration is increased a point of maximum solubility is reached. Further, increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there is not sufficient amount of water molecules available to interact with the protein molecules. This phenomenon of protein precipitation in the presence of excess of salts is known as salting out. Of these the ammonium sulfate has been the most widely used chemicals because it has high solubility, easily available and relatively cheap.

Dialysis of the salt precipitation protein

Dialysis is a common laboratory practice and operates on the principle of diffusion and osmosis. Typically, a solution of the protein is placed in the semi-permeable bag such as cellulose membrane with the pores and, bag is sealed. The dialysis bag has the cut off 1000 Da. The dialysis bag is placed in the container of different solution or the pure water. Molecules small enough to pass through the pores tend to move in or out of the bag in the direction of decreasing concentration. Large molecules that have the dimensions significantly greater than the pore diameter were retained in the bag.

Protein estimation and enzyme activity of pure enzyme by lowry's method and asparaginase assay

The Lowry's protein assay method for protein concentration determination is one of the most venerable and widely used protein assays. Hydrolysis is probably the most accurate method of determining protein concentration followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein, and absolute concentration cannot be obtained. The Lowry's procedure is sensitive, and is moderately constant from protein to protein. The Lowry's protein estimation has been so widely used that it is completely acceptable alteration to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude are involved.

The method is based on the burette reaction, in which the peptide bonds of proteins react with copper under alkaline condition to produce Cu+, which reacts with the Folin reagent, and phosphorus molybdenum state is reduced to hetero polymolybdenum blue by the copper catalysed oxidation of aromatic amino acids. The action results in a strong blue colour, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 gm of protein/ml, and is best used on solution with concentrations in the range 0.01%-mg/ml of protein.

RESULTS

Results are explained in the form of Tables and Figures (Tables 3 to Table 14), (Figures 1 to Figure 8).

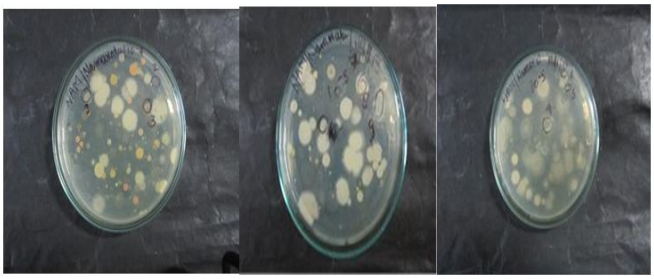

Figure 1: This image shows the mixed culture plate.

Table 3: Colony morphology of different bacterial colony.

Culture	Shape	Margin	Elevation	Pigmentation	Texture	Surface	Opacity
AKNP 01 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 02 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 03 2018	Circular	Entire	Flat	Off-white	Hard	Rough	Opaque
AKNP 04 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 05 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 06 2018	Irregular	Curl	Raised	Off-white	Soft	Smooth	Opaque
AKNP 07 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 08 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque
AKNP 09 2018	Filamentous	Curl	Flat	Off-white	Rough	Hard	Opaque
AKNP 10 2018	Circular	Entire	Flat	Off-white	Soft	Smooth	Opaque

Table 4: Results of screening of purified cultures.

Serial No.	Culture Name	Remarks
1	AKNP 01 2018	-
2	AKNP 02 2018	-
3	AKNP 03 2018	++
4	AKNP 04 2018	-
5	AKNP 05 2018	-
6	AKNP 06 2018	-
7	AKNP 07 2018	-
8	AKNP 08 2018	+
9	AKNP 09 2018	-
10	AKNP 10 2018	-

Table 5: Staining and biochemical tests of AKNP 03 2018.

Serial No.	Test	Result
1	Gram Staining	-vie (Rod Shaped)
2	Glucose Fermentation Test	-vie
3	Lactose Fermentation Test	-vie
4	Mannitol Test	-vie

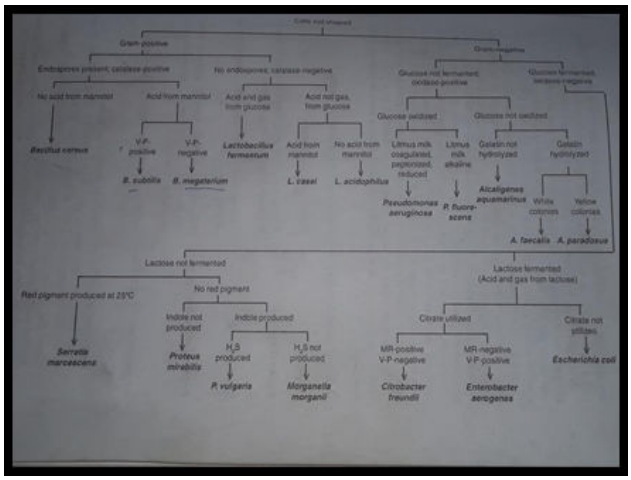

Figure 2: Bergey's manual.

Table 6: Selections of different production media in 24 hrs.

Serial No.	Production media	O.D. at 480 nm in 24hrs
1	Blank	0
2	PM 1	0
3	PM 2	0.1
4	PM 3	0.19
5	NH2	0.06

Table 7: Selections of different production media in 48 hrs.

Serial No.	Production media	O.D. at 480 nm in 48hrs
1	Blank	0
2	PM 1	0.01
3	PM 2	0.07
4	PM 3	0.36
5	NH2	0.13

Table 8: Effect of nitrogen sources varied in the production media.

Serial No.	Nitrogen sources in the production media	O.D. at 480 nm	NH2 Released (mg/ml)	Enzyme activity (U/ml/min)
1	Blank	0	0	0
2	Tryptone (MM 1)	0.29	0.37	0.06166
3	Yeast (MM 2)	0.63	0.8	0.133
4	Peptone (MM 3)	0.44	0.55	0.091
5	Urea (MM 4)	0.35	0.45	0.075

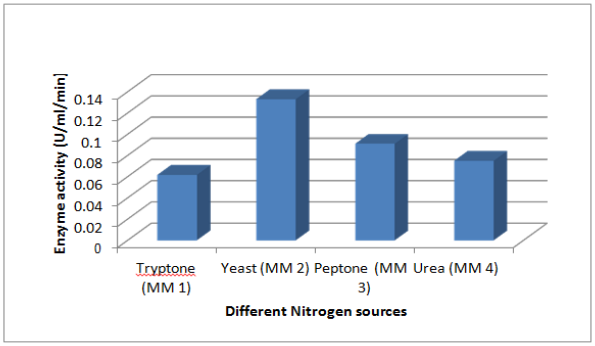

Figure 3: Graph showing effect of different nitrogen sources on production of enzyme.

Table 9: Effect of carbon sources varied in the production media.

Serial No.	Carbon sources in the production media	O.D. at 480 nm	NH2 Released (mg/ml)	Enzyme activity (U/ml/min)
1	Blank	0		
2	Sucrose (MM 5)	0.58	0.75	0.125
3	Lactose (MM 6)	0.34	0.42	0.07
4	Dextrose (MM 7)	0.35	0.45	0.075
5	Mannitol (MM 8)	1.09	1.35	0.225

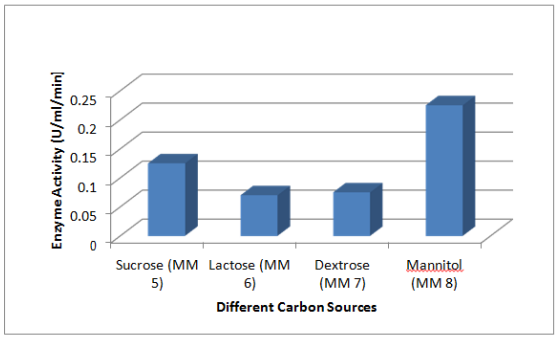

Figure 4: Graph showing effect of different carbon sources on production of enzyme.

Table 10: Effect of metal ion varied in the production media.

Serial No.	Metal Ion in the production media	O.D. at 480 nm	NH2 Released (mg/ml)	Enzyme activity (U/ml/min)
1	Blank	0	0	
2	MgSO4 (MM 9)	0.33	0.4	0.066
3	CaCl2 (MM 10)	0.37	0.46	0.076
4	ZnSO4 (MM 11)	0.23	0.29	0.043
5	CuSO4 (MM 12)	0.35	0.45	0.075

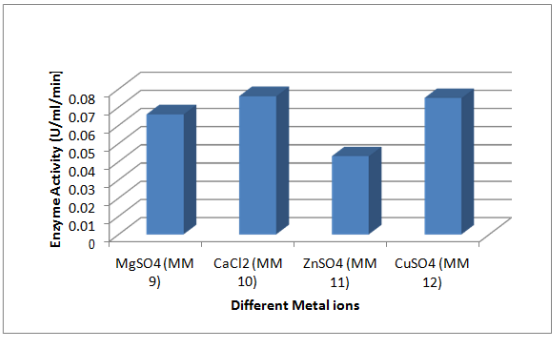

Figure 5: Graph showing effect of different metal ion on production of enzyme.

Table 11: Effect of pH on enzyme activity on the culture AKNP03 2018.

Serial No.	pH	O.D. at 480 nm	NH2 Released (mg/ml)	Enzyme activity (U/ml/min)
1	Blank	0	0	
2	MM 13 (5)	0.22	0.28	0.0466
3	MM 14 (7)	0.13	0.165	0.0275
4	MM 15 (9)	0.38	0.49	0.0816
5	MM 16 (11)	0.25	0.32	0.0533

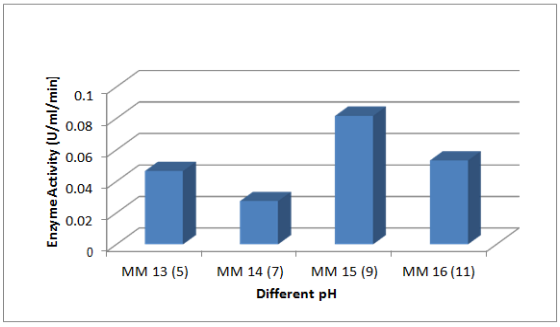

Figure 6: Graph showing effect of different ph on production of enzyme.

Table 12: Asparaginase estimation method.

Serial No.	Time in 48hrs	O.D. at 480 nm	NH2 Released (mg/ml)	Enzyme activity (U/ml/min)
1	Blank	0		
2	PM 1	0.54	0.66	0.11

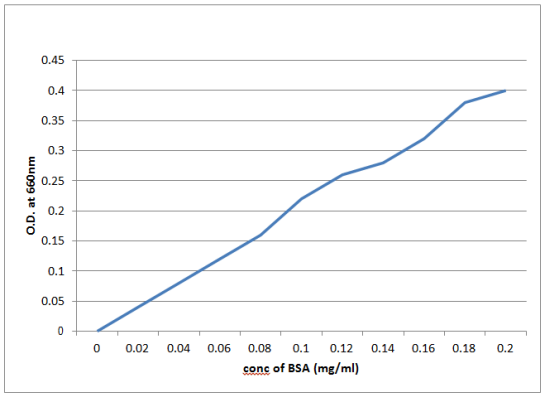

Figure 7: graph showing lowry's assay.

Table 13: Standard graph of lowry's assay.

Test tubes	Vol. of BSA (ml)	Vol. of Distilled water(ml)	Vol. of reagent D(ml)	Incubate at room temperature for 10 min	Volume of FC (ml)	Incubate in dark for ½ hour	OD at 660
1	0	1	0.5				0
2	0.1	0.9	0.5				0.04
3	0.2	0.8	0.5				0.08
4	0.3	0.7	0.5				0.12
5	0.4	0.6	0.5				0.16
6	0.5	0.5	0.5				0.22
7	0.6	0.4	0.5				0.26
8	0.7	0.3	0.5				0.28
9	0.8	0.2	0.5				0.32
10	0.9	0.1	0.5				0.38
11	1	0	0.5				0.4

Table 14: Protein content in pure enzyme.

Serial No.	Enzyme type	O.D. at 680 nm	Conc. of protein(mg/ml)
1	Blank	0	0
2	Pure Enzyme	0.13	0.175

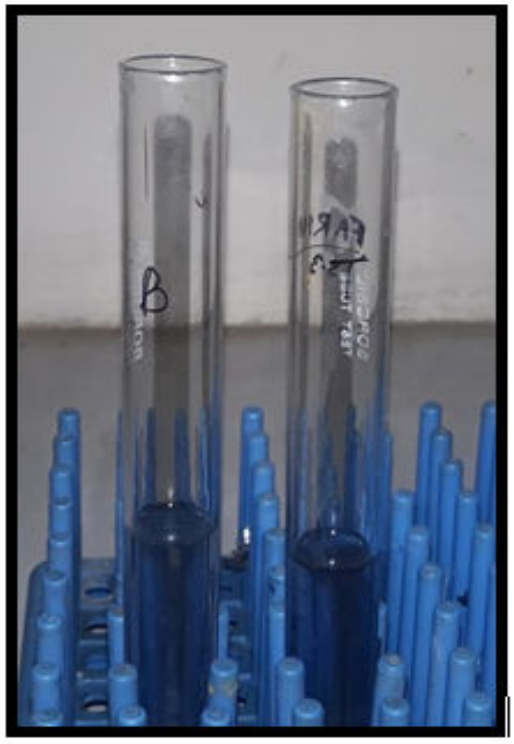

Figure 8: Lowry's assay.

DISCUSSION

Microorganisms were isolated from the soil by serial dilution and agar plate method [8]. The isolate which were purified was named as AKNP03 2018. The culture were grown on minimal agar media (pH 7) supplemented with 3.5g/l L-asparagine and then the screening of plates was done for asparaginase activity by adding the phenol red indicator the maximum zone of hydrolysis was observed in AKNP03 2018 plate [9].

The isolates morphological and taxonomical characteristics were studies according to the bergey's manual and the isolate was identified as *Proteus mirabilis*.

Enhancement of production media was done by asparaginase estimation method. Under different sources the growth of asparaginase enzyme was observed. The different sources which were used were carbon and nitrogen sources, metal ion and pH and it was seen that mannitol, yeast served the best for the growth but pH and metal ion were not reliable [10].

Partial purification was done by ammonium salt precipitation method. In this method the enzyme was centrifuged and then in the supernatant ammonium was added to lower down the saturation in the cold condition after which the dialysis was performed;

After this Lowry's assay was performed. Firstly reagent C was added and incubated at room temperature for 10min. and then reagent D was added which was again incubated for ½ hour in the dark. After this O.D. was taken at 680nm and reading was noted.

CONCLUSION

The present study entitled as "Isolation and Purification of Asparaginase from *Proteus mirabilis*." was done in the lab of MRD life sciences (P) Ltd Lucknow.

Study begins with the isolation of asparaginase producing bacteria from soil by serial dilution and agar plate method.

The isolate was screened for asparaginase activity, selection of different media, and enhancement of production media by asparaginase estimation method and then in that applying various sources and observing the result, submerged fermentation. The results which were obtained we could draw following conclusions which are as follows:

Among all the 10 isolates only AKNP03 2018 was recognized as the best isolate for the asparaginase production. Then different production media were made and from that best production media was taken. In that production media different sources were used. The growth of the isolate was found to best in yeast (nitrogen source) and mannitol (carbon source) whereas pH and metal ion did not serve the best for the growth.

Partial purification of the crude enzyme was done by salt precipitation and dialysis. After which the Lowry's assay was performed.

Later I would like to do further work in my project by fully purifying the enzyme and then applying recombinant DNA technology approaches and try to insert it in the leukemic mice and observe the results.

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