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Extraction of Anticancerous Enzymes from *E.coli* and a New Method to Study its Activity

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Abstract

L-asparaginase is the most promising anti-tumour enzyme that reduces the level of *L-asparagine* (an important nutrient for cancer cells) resulting in cancer cell starvation which leads to the cell death. There are many sources of asparaginase but bacterial source i.e. *E.coli* and *Erwinia spp* is mostly use as a therapeutic agent against leukaemia. In this study screening of different bacteria was done by rapid plate assay for asparaginase producing capability then intracellular and extracellular Asparaginase were extracted from its potent producer i.e. *E.coli* which was isolated from sewage water. The activity of enzyme was determined by using new procedure i.e. rapid activity analysis on agar plate and in tubes. Our results shows that an intracellular asparaginase enzyme was found to be more active against asparagines as compare to the extracellular enzyme. This enzyme isn't only the requirement of therapy for tumour cells but also it is use in food industry so in upcoming years the demand for asparaginase will get increase and our new method for intracellular enzyme extraction and activity analysis method (quantitative and qualitative method) will gain its importance for being easy, less expensive, using less volume of enzymatic extracts and giving better results as compare to the any other activity analysis method.

Keywords: Asparaginase (ASNase); Acute lymphoblastic leukaemia (ALL); Chronic lymphoblastic leukaemia (CLL); Asparagine (Asn); Blood cancer; Therapies

Introduction

Cancer is included in the list of top 10 deadly diseases [1]. According to the recent studies, researchers have reported new cases of cancer in 2008 were 12.7 million and the death ratio was upto 7.6 million. While in 2012 this ratio was increased i.e. about 8.2 million were died and new cases were 14.1 million [2]. The specialized cancer agency of the World Health Organization released the latest data according to which about 28 types of cancer in 184 countries worldwide are becoming a global burden [3].

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. It is not just one disease but many diseases. There are more than 200 different types of cancer. This can occur in any organ [4]. Leukemia is a Cancer that cause large numbers of abnormal blood cells to be produced and enter the bloodstream [5].

Leukemia can be divided into Acute lymphoblastic leukemia (ALL) or Chronic lymphoblastic leukemia (CLL). The Symptoms for leukemia may include paleness, weight loss, repeated infections, fever, and nose bleeds or other hemorrhages. These symptoms appear soon in ALL while appear lately in CLL [6].

Acute leukemia can appear and progress suddenly and require urgent treatment [7]. Now-a-days the treatment and survival rate has been increased about 80-85% as compare to 0-5% in 1960s [8]. Viral infections, genetic factors and exposure to chemical carcinogens are some of the Factors responsible for causing ALL. Treatment strategies followed for curing ALL includes chemotherapy or radiation therapy, wherein, chemotherapy involves the use of the enzymatic drug i.e. *L-Asparaginase* which is the key drug in treating ALL [9].

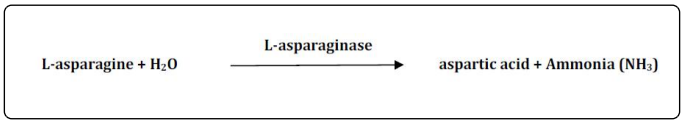
The first discovery of the tumor-inhibitory properties of ASNase was 50 years ago by Kidd who observe that guinea pig (spp of rodent) serum treated lymphoma-bearing mice [10]. In the 1960s, after the continue research in this field, Broome reported that ASNase activity in guinea-pig serum was responsible for the anti-lymphoma. It causes the deletion of asparagine (Asn), due to which alterations occur in metabolism of lymphoma cells so it was found to be responsible for the tumor growth inhibition. The final proof that ASNase was the tumor-inhibitory agent of guinea pig serum was furnished by other investigators such as Yellin and Wriston in 1966. They isolated the enzyme and demonstrated that it was strongly inhibitory to lymphoma tumors [11]. Moreover, Mashburn, Wriston and other found that asparaginase of *E.coli* cause a profound lowering in the level of free asparagine in the blood of treated mice and also in the tissues hence it possess an anti-tumor activity [12]. So the native *E.coli* ASNase was then developed as a drug for use in patients [13]. *L-asparaginase* is use now a day for the treatments of acute lymphoblastic leukemia, chronic lymphoblastic leukemia, myeloblastic leukemia, melanosarcoma, hodgkin's disease and non hodgkin's lymphoma [14].

L-asparaginase is a hydrolase which catalyzes the conversion of *L-asparagine* which is an endogenous amino acid required by the neoplastic or cancerous cells such as lymphoblast [15]. *L-asparagine* is actually require by both normal and cancerous cells but the difference is that cancerous cells are unable to synthesize their own asparagine and they totally rely upon the asparagine present in the blood of patient while normal cell can synthesize their own asparagines by enzyme asparagine synthase, this is basically the principle of asparaginase activity. This approach leads to the therapeutic use of asparaginase as an anticancerous agent [16].

Asparaginase has tetrameric structure i.e. consist of 4 subunits and each sub-unit has weight of 32-38 kDa [17]. Each subunit consists of 326 amino acid residues [18]. This anti-tumor enzyme is considered as a cornerstone of treatment protocols for acute lymphoblastic leukemia [19].

The reason it is preferred for the purpose of therapy is that it is biodegradable, non-toxic and can be administered at the local site quite easily. Other agents are found quite painful when administered to the patient and also these are quite costly [20]. It is universally use to treat acute lymphoblastic leukemia (ALL) in both phases of cancer i.e induction and post induction [21].

The administration of the enzyme i.e. *L-asparaginase*, Destroys the free source of asparagine, Starving and Killing Cancer cells [21]. When asparaginase act upon asparagine it results in the production of aspartate and ammonia [22]. This reaction can be expressed as:



This equation describes that when asparaginase act upon *L-asparagine* amino acid it utilize water and convert *L-asparagine* into aspartic acid and ammonia [23].

Due to the ammonia production the pH gets increase (alkaline) this parameter (ph) can be used in labs when checking the activity of *L-asparaginase* or screening the asparaginase producers.

Three main types of asparaginase have been used to date: native asparaginase derived from *E.coli* (*E. coli*-asparaginase: Kidrolase, EUSA Pharma; Elspar, Ovation Pharmaceuticals; Crasnitin™, Bayer; Leunase®, sanofi-aventis; Asparaginase medac™, Kyowa Hakko); a pegylated form of the native *E. coli*-asparaginase (polyethylene glycol [PEG]-asparaginase: Oncaspar™, Enzon Pharmaceuticals Inc) and an enzyme isolated from *Erwinia chrysanthemi*, referred to as *Erwinia asparaginase* (Erwinase®, EUSA Pharma) [24]. ELSPAR can be used primarily and can be combine with other chemotherapeutic agents. It is a white crystalline powder that is freely soluble in water. ELSPAR can be given intravenously. When administering ELSPAR, the volume at a single injection site should be limited to 2 ml. For more than 2 ml of volume second injection site should be use [25].

The patient to which the dose is given will be kept under observation in 1 h after giving or administration of the drug to observe any sort of reaction or allergies. The frequency of doses depends upon the personal health of the patient such as its liver function and age [26].

There are two types of Asparaginases produced by *E.coli* which are given below:

Asparaginase I

Asparaginase II

Asparaginase-I is an extracellular enzyme while asparaginase II is an intracellular enzyme [27]. Asparaginase II is found to be more active *in vivo* when checked or tested upon mice, Dog and humans [28]. Asparaginase II has more affinity towards the asparagine substrate as compare to the asparaginase I so it is mostly use more as a therapeutic agent [29]. The administration of such an enzyme protein sometime produces the corresponding antibody resulting in allergy or neutralization of drug effect [30]. Development of antibodies and hypersensitivity to asparaginase are common and different studies were carried out to study those reactions and it was found that mostly these reactions can't lead to a highly clinical condition [31].

Antibodies produced in response to Asparaginases do not always lead to clinical hypersensitivity, but may instead cause rapid inactivation of the asparaginase. This is commonly referred to as 'silent hypersensitivity' or 'silent inactivation'. Development of anti-asparaginase antibodies can thus confer resistance to asparaginase therapy and is associated with higher plasma levels of asparagine and reduced therapeutic efficacy in some studies. When patients have allergy to one type of Asparaginase, they are switched to another product [32].

To overcome this problem, native *E.coli* asparaginase was conjugated to polyethylene glycol (PEG) to formulate Pegylated asparaginase, a preparation with decreased immunogenicity and increased circulating half-life [33]. Due to increase in half life the frequency of administration decreases [34]. Half-life can increase upto 14 days and its activity also gets enhanced so the serum of the patient lacks the asparagine which leads to the starvation of the tumor cells. Asparaginase produced by *E.coli* has longer half-life than that of *Erwinia spp* when the patients which are allergic to *E.coli* asparaginase are switched to Erwinia's asparaginase [35]. *E.coli* derived ASNase is known to retain activity for two days at room temperature and seven days when refrigerated [36]. While Erwinia derived asparaginase was known to lose activity soon after freezing [37].

Asparaginase production is affected due to the poor optimization of different factors such as aeration and agitation, pH and incubation time, carbon sources, nitrogen sources and inoculums level [38]. It was found that culture medium supplemented with maltose as carbon source, and Yeast extract (2%) which serve as a good nitrogen source are recommended for the production of *L-asparaginase*. The optimum pH for enzyme production is 7.5 and temperature is 35°C. The release of intracellular asparaginase from the cells was found to be high when the species was treated with cell disrupting agents like lysozyme [39]. Due to the hazards of chemotherapeutic drugs and its painful effects *L-asparaginase* are emerging as safer source use for treatment of cancer.

There are so many different kinds of techniques which can be used for the production, isolation and purification of Asparaginase for example by submerged technique (SF) and solid state fermentation technique (SSF). Solid-state fermentation (SSF) is more effective technique as the yield of the product is several times higher than that of submerged fermentation (SF) [40].

In solid state fermentation method inoculum is prepared first by inoculating organism in asparagine containing broth. Solid substrate can be taken in 250 ml Erlenmeyer flask, and moistened with distilled water then add inoculums in it and incubate it for 3-7 days afterwards extracellular enzyme can be extracted by centrifugation [41].

For Asparaginase production under submerged condition, suitable broth supplemented with Asparagine can be used in which culture or desired organism is inoculated and incubated for 3-7 days (depending upon type of inoculums). The culture medium can be filtered using Whatman filter paper, the filtrate was centrifuged and the clear supernatant was used as the crude extra-cellular enzyme source, Sonicator can be used in both process for intracellular enzyme extraction [42].

Commercially, *L-Asparaginase* is produced throughout the world by submerged fermentation (SmF). This methodology has many

disadvantages such as the low concentration product and difficult to handle due to contamination risk. Therefore, this methodology is a cost intensive and it can be highly problematic. An alternative solution is solid state fermentation (SSF) which is offering a wide range of advantages as compare to SmF. It is a very effective technique, as the yield of the product is many times higher when compared to that in SmF and it also offers many other advantages too such as risk of contamination is decreased, it is simple method [43]. Confirmation of *L-Asparaginase* can be done using thin layer chromatography, Nesslerization method, Rapid plate assay etc. Silica gel and phenol red is use in case of thin layer chromatography where Enzyme activity can be check by spraying ninhydrin reagent and observing red spot [44]. The pH indicator phenol red in medium is a fast method for screening *L-asparaginase* producing bacteria through after 24 h of incubation. The enzyme L–Asparaginase was routinely screened in culture filtrates by using Nessler's reagent but the procedure is lengthy and time consuming, that's why the use of rapid plate assay for screening *L –asparaginase* producing microorganisms can be use which is sensitive and rapid procedure that may directly give potential asparaginase activity. It is generally agreed that *L –asparaginase* production is accompanied by an increase in pH of the culture filtrates. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink. So the pink zone is formed around microbial colonies which are capable of producing *L–Asparaginase* because when asparaginase acts upon asparagine it is converted into aspartic acid and ammonia, which is responsible to increase the pH of the medium [45]. Phenol red exhibits a gradual transition from yellow to red over the pH range 6.8 to 8.2 above pH 8.2, phenol red turns a bright pink color [46]. The enzyme is highly stable at a wide pH range of 3-12 so the changing of pH in the medium will affect its activity i.e. increase or decrease but will not completely suppress it [47]. In this research 4 species of bacteria were screened for the production of asparaginase i.e. *E.coli* (isolated from sewage water), *Bacillus spp*, *Pseudomonas spp* and *Staph spp* (Obtained from Jinnah university for women research laboratory). Among all other Asparaginase producing bacterial species, the reasons for emphasizing upon *E.coli* to be use in rapid plate assay is given below:

Asparaginase obtain from *E.coli* is considered as first-line therapy for many ALL treatment protocols [48].

It can produce extracellular as well as intracellular Asparaginase.

Isolation and screening method for *E.coli* is very easy.

The enzyme isolated from it is found to be active even at higher temperature 55°C. Considering all these characteristics, the production of *L-Asparaginase* from *E.coli* is recommended for industrial production.

It is one of the most potent producers [49] and the richest source of biologically active *L-asparaginase* [50].

It has high generation time i.e. 20 minutes. So it would require less time to grow.

There are many easily available medium which are selective for the growth of *E.coli* such as EMB and Endo-agar.

A systematic and more in-depth investigation on screening, optimization of production parameters and purification of the enzyme from alternative sources is need of the day. Since *L-Asparaginase* is also used in food processing, a demand for *L-Asparaginase* is expected to be increased in up-coming years. Hence development of an economically good process is necessary [51]. Asparaginase enzyme has many roles and uses it isn't only an anti-leukemic enzyme, it is also use in food industry because in different baking products asparagines is found which when heated convert into acryl amide which is a suspected carcinogen present in starchy food. Asparaginase can be used before cooking such food products, so that the Asparagines will convert into Aspartate and Ammonia thus acryl amide willn't form so it reduce the risk factor for cancer. Asparaginase can also be used to detect asparagine containing food. Asparaginase can effectively reduce the level of acryl amide up to 90% in a range of starchy foods without changing the taste and appearance of the end product [52]. The enzyme can be easily cleared from our blood because Our immune system is designed to destroy large, multi-subunit proteins, so *L-asparaginase* is rapidly cleared from the blood in a day or so [53].

In this study methods of isolation, identification, screening, production, extraction and two types of assays for Asparaginase are performed. In the extraction method rather than using a sonicator, the cell disruption of *E.coli* was performed by using lysozyme obtained from egg white. It was known that *L-Asparaginase* could be liberated from Escherichia coli (*E.coli*) cells by either ultrasonic disruption of the cells or by some other mechanical means. It has now been found that such cell lysis can be accomplished by means of lysozyme [54]. Using lysozyme will not only reduce the cost of the method but also it was found to be easy and effective method and can be a good alternative to other methods for cell disruption. To obtain Asparaginase, the use of lysozyme for cell disruption will gain importance in future because:

Using lysozyme in method will result in least contamination.

Lysozyme is easy to obtain from many sources.

Obtaining method of lysozyme are less expensive.

Lysozyme is stable and active at pH 2.0-10.0 [55] so the type of media use in study won't affect its activity.

It can tolerate high temperature range i.e. 30°C to 90°C [56].

Lysozyme requires less time to break cell i.e. 30 minutes [57].

Lysozyme can be easily removed during purification.

Using lysozyme will reduce the cost of the method as compare to using an expensive machine i.e. sonicator.

It won't affect the activity of asparaginase.

Now-a-days Asparaginase is more prefer as compare to the radiations therapy and other chemotherapeutic drugs because Radiation therapy has many disadvantages. In this therapy high-energy radiations are used to kill cancer cells. It can cause sunburn-like skin changes and hair loss in the treated area. Radiation to the belly (abdomen) can sometimes cause nausea, vomiting, or diarrhoea. Radiation to large parts of the body can cause fatigue and an increased risk of infection. It can also effect affect sexual development and the ability to have children [58]. It is known that high radiation dose can damage the cellular DNA and may lead to different types of cancer. So the radiations therapy which you think is saving you from cancer can even become a cause of cancer too if a proper dose at proper time isn't provided so it is a risky treatment. Children whose treatment for leukaemia has included radiation therapy of the brain may have some decrease in their learning ability. There are some steroids which can be used to treat leukaemia or other type of cancers instead of using

Asparaginase but it can lead to the complete Bone damage or thinning of the bones (osteoporosis) [59]. So the chemotherapy is often used instead, as it may work more quickly [60]. Chemotherapy is a common treatment for cancer patients in addition to surgery and radiation therapy. Some chemotherapeutic drugs can be effective in decreasing tumour size or even finish it, and may increase life time [61]. The reason for *L-asparaginase* being most preferred drug is that it is safer, less toxic and well tolerated drug. If given intravenously, *L-Asparaginase* will result in an anaphylactic reaction [62]. Asparaginase isn't known to transfer through breast milk to the baby so it can't affect the baby [63]. It can not only cure but prevent cancer too as it is the main agent of chemotherapy in treating leukaemia and use in food industry to break asparagine before its conversion to a carcinogen on heating. These reasons make Asparaginase a corner stone to be studied in future.

In this study along with the rapid plate assay, tube assay was also done which can confirm the enzyme activity efficiently as that of plate assay except that it is qualitative method while plate assay is a quantitative method (zone can be measure). An advantage for using tube assay is that it is less expensive than that of plate assay and show results in same time as that of plate assay (18-24 h).

In the present study, along with the use of lysozyme for extraction of intracellular enzyme, new procedure i.e. plate and tube assay for enzymatic activity is introduced in which modified nutrient agar plate (contain nutrient agar, asparagine and phenol red with pH 6.8) and modified nutrient broth (contain nutrient broth, asparagine and phenol red with pH 6.8) were used to measure the asparaginase activity. These are quantitative and qualitative analysis respectively. In the future this study will be very helpful because using lysozyme instead of sonicator or many other cells disrupting techniques will save cost of the process and it is also easier to understand and perform. Not only this, the new procedure introduced for the testing of asparaginase activity will be much better than using Nesslerization method because it had some disadvantages which had been overcome by the plate and tube assay for enzymatic activity such as large volume is required in Nesslerization while small amount just a drop of extract will be required to perform its assay. As there is shortage of the most promising anti- tumour enzyme i.e. Asparaginase , it has high demand and its cost is also very high it is very clear that in the future methods will be required by which asparaginase can be available to each and every patient being less expensive, more affective and easily available. Using the method describe in this study the shortage of asparaginase can be overcome making it less expensive, easily available and this study will prove to be more helpful for the production of asparaginase on large scale.

Materials and Method

Materials

Nutrient agar, triple sugar iron (Tsi) agar, nutrient broth, Citrate agar, Eosin methylene blue agar and L-Asparagine was purchased from Hi-media laboratories (India) and almost other all chemicals, dye and equipments were purchased locally (Pakistan), 4 bacterial spp were obtained from departmental cultural stock of Jinnah university for women Karachi, Pakistan while one bacterial strain of *E.coli* was isolated from sewage water near Jinnah university for women Karachi Pakistan. Modified nutrient agar plate and broth (pH: 7) was used which was supplemented with 10 g of asparagine and 3-drops of phenol red dye, the pH of the medium was maintained using NaOH 1 mol^{-1} . Media was autoclaved, tubes and plates were prepared.

Method

Samples Collection

Sterile containers and syringes for the collection of urine, stool and sewage water was obtained from Dr. Essa laboratory and diagnostic centre Karachi.

Stool sample: A stool sample was collected in container from 3 years old boy avoiding any contamination from urine.

Urine sample: A mid-stream urine sample was collected in a sterile and labelled container.

Sewage sample: A sterile disposable syringe was use through which the sewage water was collected from area near Jinnah University for Women Karachi. About 10 ml of sample was collected in a sterile syringe.

All three samples were sent to the research laboratory of Jinnah University for women Karachi in order to isolate *E.coli*.

Isolation of E. coli from Samples

Urine and stool sample were directly inoculated on EMB agar as it is easy to detect *E.coli* on it. Then the plates were incubated for 24 h at 37°C.

In an aseptic condition 5 sterile test tubes were labelled as 10-1, 10-2, 10-3, 10-4 and 10-5. Each tube was containing 9ml of saline. 1 ml of the sewage water sample was added in to the first tube and then serial dilution was performed, from the last tube (10-5) 1 ml was added on Eosin Methylene Blue agar and spread plate method was performed using a sterile cotton swab. This plate was then incubated for 24 h at 37°C.

Purification

From all three samples mixed cultures were obtained then the suspected colonies for *E.coli* was selected and streaked on EMB agar plate once again using four way streak methods, same colonies were inoculated in TSI slant and citrate slant. All the three plates and 6 slants (two slants i.e. TSI and citrate for each sample) were incubated for 24 h at 37°C. Next day *E.coli* was found to be isolated from all the three samples which were further streaked on to EMB agar plate to maintain pure culture of *E.coli* because all of them were used to screen asparaginase production.

Screening for Asparaginase Production

Cultures of *Pseudomonas spp*, *staph spp*, *E.coli* and *bacillus spp* were obtained from the departmental culture stock of Jinnah Univeristy for Women research laboratory and the isolated strains of *E.coli* (from samples) were screened for Asparaginase production. Two modified nutrient agar plates were taken and devided into sectors. Each portion of both plates were labelled with the culture name that will be streaked on that part. The unlabelled area will remain unstreaked and will serve as a negative control. On plate-1 of modified nutrient agar all the lab obtained cultures i.e. *Bacillus spp*, *Pseudomonas spp*, *Staph spp* and *E.coli* were inoculated while on plate-2 isolated strain of *E.coli* from urine stool and sewage water samples were inoculated. Plates were kept in an incubator for 24 h and 37°C.

After the completion of incubation time, plate was taken out and the production of Asparaginase was observe by the change in the agar i.e. pinkish colour surrounding the colonies indicates asparaginase producing organisms [64].

Production of Asparaginase

Isolated strain of *E.coli* from sewage water was found to be the potent asparaginase producer, it was then streaked on Eosin methylene blue agar this same strain of *E.coli* was then streaked on asparagine containing media (modified nutrient agar) and non-asparagine containing media (only containing phenol red) to serve as negative control and Plate was kept in an incubator for 24 h and 37°C.

On the very next day pure culture of this strain of *E.coli* was inoculated into 5 ml of nutrient broth using sterile wire loop and the nutrient broth tube was incubated at 37°C for 24 h in an incubator.

For the production of Asparaginase, production media was prepared in a 250 ml Erlenmeyer flask which contained 0.5% of NaCL (5 g), maltose sugar (10 g/L), KH2PO4 (0.75 g per ltr) and *L-asparagine* (100 g/L) were added pH was maintained as 7.4 then the medium was Autoclaved at 121°C for 15 minute and at 15 lb pressure. Using sterile syringe medium was added into the cuvette and its optical density was note down at 460 nm after that 1 ml of the overnight bacterial broth (O.D>0.5) was inoculated in 100 ml of production media. It was then incubated at 37°C for 72 h at 120 rpm in an orbital shaker incubator (SARTOMAT CT Plus, Sartorious Germany Pvt. Ltd.)

Enzymes Extraction

After 3-days of incubation flask was taken out In an aspetic environment (use 75% alcohol to clean bench tops and work area to maintain sterility), using a sterile syringe medium was taken out from production media and added into the cuvette and its optical density (OD) was noted using spectrophotometer (Milton roy spectronic) at 460 nm. That's the OD of the growth in the medium. Note down the pH of the production medium. Low ph confirmed the enzyme production.

Extracellular enzyme extraction

In an aseptic environment take appropriate quantity from production media (72 h incubated) using sterile syringe and add it in to eppendorfs, centrifuge it at 10,000 rpm for 15 minutes at 4°C using a micro centrifuge machine (Bio maker). The pellet and the supernatant will get separated. Supernatant can be considered as a crude enzyme extract (extracellular enzyme) While the pellet contains cell debris [65].

Take supernatant from eppendrof using a sterile syringe, add it in a sterile cuvette and Note down the O.D of the supernatant at 460 nm. Collect all supernatant in a test tube carefully using sterile syringe marked it as WL (i.e without lysis or extracellular enzyme extract). Put a cotton plug on it and preserve this in freezer until use (in a sterile condition).

Intracellular enzyme extraction

In an aseptic environment, Take a hen egg, clean it with alcoholic cotton wool then poke a hole in it using a sterile forcep, collect all the egg white in a sterile dropper bottle very carefully. This egg white contains lysozyme in it. Transfer production media using sterile syringe into sterile eppendorfs, centrifuge it at 10,000 rpm at 4°C for 15 minutes using micro centrifuge machine.

Supernatant and pellet will get separated. Discard the supernatant using sterile syringe carefully, and in pellet add a small amount of distilled water in order to suspend pellet in it and then Add freshly obtained egg white (lysozyme) from dropper bottle in a sterile environment. Leave it for 4 hours at 37°C. 15-30 minutes are enough for lysozyme to perform lysis of bacterial cell wall) occasionally finger vortexing was performed in this 4 h duration.

Microcentrifuge it on 10,000 rpm at 4°C for 15 minutes using microcentrifuge machine. Pellet and supernatant will get separated, Supernatant are considered to contain the type-2 Asparaginase or intracellular asparaginase [66]. Supernatant are transferred into a cuvette in an aseptic environment using sterile syringe and note down the OD of this supernatant at 460 nm using a spectrophotometer. Supernatant is added into a sterile test tube, mark it as L (lysis or intracellular). Put cotton plug on it and place it in freezer until use.

Rapid Plate Assay

The plate assay to detect Asparaginase activity was performed on Modified nutrient Agar plate which was orange in color (pH of this medium was kept >6.8 at which phenol red turns into orange) the reason for keeping the medium in orange colour is that it would help to read and measure the red zone on contrast of orange color of agar. A control agar (nutrient agar with just phenol red, no asparagine) was also used in this assay to serve as a negative control. Using a sterile borer 2 holes were made on test and control agar plate. Both holes were labelled from back of plate as L (with lysis extraction) and WL (without lysis extraction) i.e for intracellular enzyme and extracellular enzyme extracts respectively.

Using a sterile syringe a drop of an extract of intracellular and extracellular enzymes were placed in the holes of both plates and both of the plates were incubated at 37°C for 24 h. Pinkish red zone around the holes will indicate the enzyme activity because the enzyme will cause the asparagine to get broken down and ammonia will release which will increase the pH (alkaline) and the phenol red present in the medium will turn into pinkish red. Zones were Measured in mm and noted down.

Rapid Broth Assay

Take 2 modified nutrient broth tubes (nutrient broth containing asparagine and phenol red) and 2 control tubes (nutrient broth containing phenol red only). Label one modified nutrient broth tube and one control tube as L and remaining one modified nutrient broth and one control broth tube was labelled as WL for intracellular and extracellular enzyme assay respectively.

Using a sterile syringe take about 0.5 ml of intracellular and extracellular enzyme extract and add it into their respective labeled modified broths tubes and control tubes. Incubate all tubes at 3°C for 24 h. Next day observe the change in colour from orange to red in asparagine containing broth tubes indicating enzymatic activity.

Results

We have performed this research to screen different Asparaginase producing organisms, grew them in production media then Extract 2 types of an anti-leukemic enzyme from *E.coli* i.e. intracellular and extracellular enzyme and checked its activity *in vitro* by a new procedure using modified nutrient agar plate and modifies nutrient broth tube assay which is easy and less expensive as compare to Nesslerization.

Results of Isolation and Identification

From all the three sample (sewage water, stool and urine) *E.coli* was isolated only from sewage water which was identified using Eosin Methylene Blue agar (EMB), TSI, citrate slants and Gram staining technique. The results we obtained are shown in Table 1.

Samples	Gram Stainings			TSI	Citrate	Emb Agar
	color	arrangement	shape	A/A		
Sewage	pink	Scattered	Rods	Gas +	Negative	Green metallic sheen bearing colonies
water Urine sample	pink	Scattered	Rods	A/A	Negative	Green metallic sheen bearing colonies
Stool sample	pink	Scattered	Rods	A/A Gas +	Negative	Green metallic sheen bearing colonies

Table 1: Analysis of *E.coli* from different samples.

We observed that the suspected colonies from all the three samples were detected as *E.coli* i.e. the Tripple sugar iron slant gave acidic butt with acidic slant and gas production was also positive, the citratase enzyme production was negative which indicated the *E.coli*. The colonies on eosin methylene blue agar (EMB) were having grean metallic sheen confirming the *E.coli* organism.

Results of Screening Assay for Asparaginase Producers

The pure culture of *E.coli*, *Pseudomonas spp*, *Bacillus spp*, *Staph spp* obtained from Jinnah university cultural stock and the isolated strains of *E.coli* were screened on modified Nutrient Agar for the detection of enzyme production in which asparagine acted as a substrate for Asparaginase enzyme. The Asparaginase positive colonies were identified by the occurrence of pink color around colonies of organisms which is due to the deamination or the release of ammonia from asparagine. This ammonia production increases the pH of the medium making it pinkish or pinkish purple.

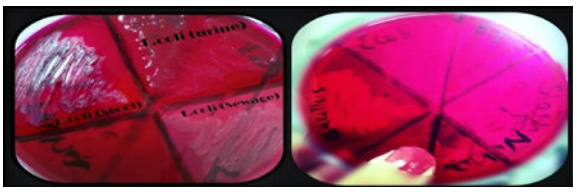


Figure 1: Screening of isolated strains of *E.coli* (left) and obtained pure cultures (right).

Among the pure cultures obtained from lab the pure cultures of *E.coli*, *Pseudomonas spp* and *Staph spp* were found to be the Asparaginase producer while *Bacillus spp* wasn't considered as potent Asparaginase producer because All the asparaginase positive organisms turn agar color into Pinkish purple colour which could be easily compare to the area where *Bacillus spp* grew i.e. there was no pink colour surrounding its growth as shown in Figure 1, so it was considered as asparaginase negative strain i.e. unable to produce asparaginase.

E.coli is commonly known as colon bacteria and easily available in sewage water with faecal contamination it was isolated in this study from different samples (urine, stool and sewage water sample) All the isolated strains were screened for the production of *L-asparaginase* from which the strain of *E.coli* which was isolated from the sewage water was found to be the potent asparaginase producer. Before selecting this strain for asparaginase production this same strain was once again streaked on asparagine containing media and non-asparagine containing media (to serve as a negative control).

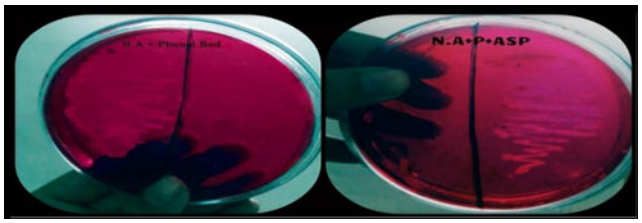


Figure 2: Asparaginase producing strain of *E.coli* on control (left) and modified agar plate (right).

It was clearly observed that the when strain of *E.coli* grew on asparagine containing media it turns the medium into bright pinkish colour as compare to the non-asparagine containing media which indicates its potency to produce asparaginase and utilize asparagine in media and releasing ammonia by the degradation of asparagine as it can be seen in Figure 2. This Asparaginase producing strain of *E.coli* was then streaked on EMB agar plate using 4-way streak method to preserve strain and to use the isolated colonies in production and extraction steps.

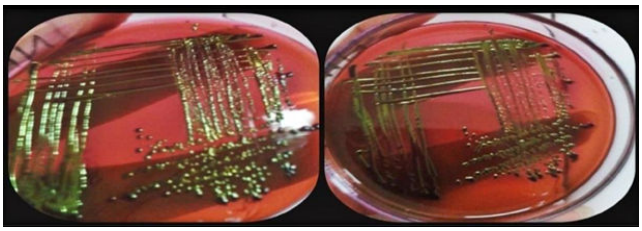


Figure 3: Asparaginase producing strain of *E.coli* on EMB agar.

Figure 3 is showing the asparaginase producing strain of *E.coli* giving its characteristic green metallic sheen on EMB agar plate after the day of incubation. A colony was picked up from this and

inoculated into the 5 ml nutrient agar broth and incubated for 24 h and 37°C , then next day 1 ml from 24 h incubates inoculums was added into the production media. After 3 days of incubation of production media got fully turbid due to the growth of organism which can be seen in Figure 4. At this time the pH of the production media was checked, low ph of the production media after its incubation time gets completed indicates the production of asparaginase [32]. We have found that the pH of our production media was low down i.e. from 7.4 to 5.0 which indicate the production of asparaginase.

Figure 4: Turbidity in production media.

The Optical densities of medium after growth of inoculum, extracellular enzyme extract and intracellular enzyme extract using spectrophotometer at 460 nm were noted down which are mentioned in the Table 2.

S:No	Sample	O.D at 460nm
1	Blank	0
2	Medium	0.432
3	Extracellular enzyme extract (WL)	0.22
4	Intracellular enzyme extract (L)	0.187

Table 2: Optical densities of medium and extracts.

The activity of both enzyme extracts were measured in which Reddish color zone was obtained surrounding both extracts of intracellular and extracellular enzymes on modified nutrient agar plate it was due to the broke down of asparagine into ammonia and aspartate , the release of ammonia increase the pH of agar and the orange color of agar turned into red. No zones were observed on control plate (modified nutrient agar plate which don't contain asparagine) as shown in Figure 5.

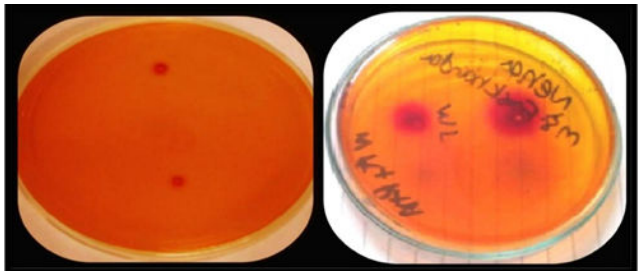


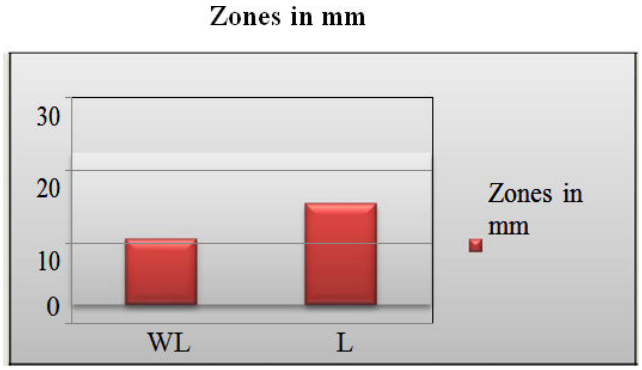
Figure 5: No zone on control plate (left) and Zones obtain on test plate (right).

Both of the zones were measured in mm, their measurements are mentioned in Table 3.

Extracts	Zones in mm
Extracellular enzyme extract (WL)	13 mm
Intracellular enzyme extract (L)	20 mm

Table 3: Zones measurements in mm.

The measurements of zone indicates that the activity of intracellular Asparaginase is more on Asparagine as compare to extracellular Asparaginase which is an authentic results i.e. The type-2 Asparaginase is more effective on asparagine as compare to the type-1 asparaginase as said by Gokbudget [31]. The zones are also shown graphically in graph-1.



Graph-1: Activity of type 1 and 2 Asparaginase according to the zone's measurements.

Tube assay was also done but it has a limitation i.e. it's a qualitative analysis, you can't measure the activity of enzyme. The tube assay was performed in modified Nutrient Broth and negative control broth (modified nutrient broth with no asparagine) for intracellular and extracellular enzyme extracts. The pH of the medium was kept 6.8 again to see the color change clearly. Due to the breakdown of Asparagine into ammonia and aspartate the ph is increased and the color of broth change from orange to reddish as shown in Figure 6 while no color change was observe in control tubes.

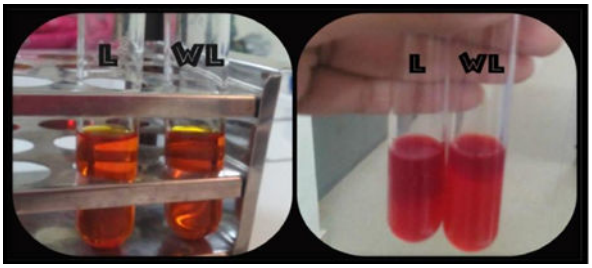


Figure 6: No change in color in control tubes (left) change of color in assay tubes (right).

Discussion

We have performed this research in which we have screened different organisms for Asparaginase production. Among pure cultures obtained from Jinnah University for Women Karachi *Pseudomonas spp*, *Staph spp* and *E.coli* were found to be the potent Asparaginase producer while among isolated strains of *E.coli* (from stool sample, sewage water sample and urine sample), the strain obtained from sewage water was found to be the potent asparaginase producer. These organisms have capability to produce asparaginase, utilize asparagine and produce aspartate and ammonia which increases the pH of the agar medium in the presence of phenol red acting as an indicator this turns the medium into pink while no pink color was seen on asparaginase negative strains. The principle for pink zone formation due to the production of ammonia in an asparagine and phenol red containing agar was also used by Mohana Priya [67,68], Amrutha V. Audipudi [69] and Prasad Talluri [70], according to them asparaginase producing organism turn the modified agar plate into pink color. After our screening step we have selected *E.coli* for our production and extraction process among all asparaginase producing bacteria because Asparaginase extracted from *E.coli* is mostly use in the treatment of leukaemia [71].

E.coli has high generation time so using *E.coli* will reduce the waste of time and produce more quantity of asparaginase in less time. Debajit Borahi [32] also used strain of *E.coli* isolated from the sewage water which was also able to produce asparaginase. The use of asparaginase specially asparaginase II has importance in the treatment of human neoplastic disease its use is increasing day by day so the enzyme need to be produce in large quantity [72]. In asparaginase producing strains, the existence of both periplasm and cytoplasmic enzyme have been reported [73] we have successfully extracted both intracellular and extracellular asparaginase from asparaginase producing strain of *E.coli*, Then we have checked their activity by a new technique in which we have used an asparagine and phenol containing nutrient agar. According to our results Asparaginase I had low affinity towards asparagine hence we observe smaller red zone on agar plate while we have observed larger zone around the extract of asparaginase II which indicates that it has more affinity towards asparagine this result was found to be authentic because according to the Yogitha N. Srikhanta [74] and Gokbudget [31] It was clearly stated that Asparaginase I is extracellular enzyme having low affinity towards asparagine while Asparaginase II is intracellular enzyme which have high affinity towards asparagine.

Enzyme which we had isolated from *E.coli* found to have an anti-leukemic activity *in vitro* i.e. it can cause depletion of asparagine. Death of leukemia cells induced by *L-asparaginase* from *E.coli* was observed by Ueno et al. [75]. To decrease the cost of the process we've used nutrient agar throughout in the study because Nutrient agar is highly available agar we have made certain modifications in the agar composition just for the sake of enzyme production and activity detection, we have also used egg white-lysozyme to break cells of *E.coli* rather than using expensive chemicals or machines, we have performed the activity testing of extracted asparaginase on agar plate rather than Nesslerization because Nesslerization to detect asparaginase activity not only require more volume of extract than we have used on agar but also some chemicals are required to perform it which could increase the cost of process. Using this method the treatment of leukemia could become very easy and affordable as well, as the method is very cost effective and reliable.

Conclusion

Our aim was the screening, production, extraction of *L-Asparaginase* from *E.coli* and to develop a new and rapid procedure to analyze activity of the Asparaginase rather than using Nesslerization technique. In our study we have found that *Pseudomonas spp*, *E.coli* and *Staph spp* were the asparaginase producers we have selected *E.coli* to be use in production and extraction steps. As the isolation and screening method for *E.coli* is very easy, and the generation time of *E .coli* is very low so high amount of asparaginase can be produce by the strains of *E.coli* in less time, considering all these characteristics, the production of *L-asparaginase* from *E.coli* is recommended for industrial production of Asparaginase. As there is shortage of the most promising anti- tumor enzyme i.e. Asparaginase , it has high demand and its cost is also very high it is very clear that in the future methods will be required by which asparaginase can be available to each and every patient being less expensive, more affective and easily available. Using the method describe in this study the shortage of asparaginase can be overcome making it less expensive, easily available and this study will prove to be more helpful for the production of asparaginase on large scale. The new procedure for testing the activity of asparaginase can be very helpful in future because there are certain disadvantages of Nesslerization which can overcome by using this method such as in Nesslerization large volume of sample is required [76]. While our procedure requires just a drop of extract to determine the enzymatic activity. Our activity analysis not only uses less volume of the extract but it is also a quantitative analysis which make it more precious. Use of lysozyme in this study for the purpose of extraction also makes the whole procedure more inexpensive. Further future studies can be done using this procedure in which the purification of the enzyme extract can be done before testing the product *in vivo*. Large scale production of asparaginase is recommended by this method being easy, less expensive and effective process.

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