Abstract: Bacterial strain C1 isolated from Kolkata area, showed maximum level of L-glutaminase activity in semi-quantitative assay. The crude enzyme from potential stain C1 was produced and partially purified by acetone fractionation and also by ammonium sulphate precipitation and membrane dialysis, following ion exchange chromatography method. In quantitative assay, the partially purified preparation showed 333.3U/ml of enzyme activity. In SDS PAGE the enzyme showed two bands with 45 and 225 kDa respectively. While in Zymography, the enzyme showed two bands with 43 and 225 kDa respectively of the various Physiochemical parameters tested, pH and temperature 37°C was found to influence the production of L-glutaminase enzyme, by the strainC1. The activator manganese sulphate (MnSO4) was found to activate the activity of L-glutaminase enzyme, whereas, the inhibitor EDTA was found to suppress the activity of the enzyme, L-glutaminase.

Keywords: L-glutaminase, manganese, Enzyme, Bacillus Subtilis
INTRODUCTION

Glutaminases belong to a larger family that includes serine-dependent beta-lactamases and penicillin-binding proteins. Many bacteria have two isoforms. This model is based on selected known glutaminases and their homologs within prokaryotes, with the exclusion of highly-derived (long-branch) and architecturally varied homologs, so as to achieve conservative assignments. A sharp drop in scores occurs below 250, and cut offs are set accordingly. This enzyme belongs to the family of hydrolases, those acting on carbon-nitrogen bonds other than peptide bonds, specifically in linear amides. Other names in common use include peptidoglutaminase II, glutaminyl-peptide glutaminase, destabilase, and peptidylglutaminase II. Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products like enzymes. Actinomycetes are considered to be preferred enzymes sources due to their production of extracellular enzymes. They act as decomposers of complex animal and plant materials resulting in release of simple substances, especially carbon and nitrogen which is easily utilized by other organisms, thus performing a vital role in life cycle.

The terrestrial bacterial source of L-asparaginase is currently used for the treatment of leukaemia but this is known to cause a lot of side effects and hence there is a need for an alternative enzyme drug which is more compatible to human blood and induces less or no side effects in patients. The marine environment particularly sea water, which is saline in nature and chemically closer to human blood plasma, can provide microbial enzymes that are safe with no or less side effects when administered for human therapeutic application. Yet another important fact about marine microbial enzyme is that they show high level of salt tolerance. Hence there is an increasing interest in the marine micro-organism for therapeutic purposes. The action of glutaminase plays a major role as therapeutic agent in cancer and HIV. L-glutaminase has inculcated significant buzz in food industry as potential flavour modulating agent, imparting a savoury flavour as it increases foods glutamic acid content. Attempts to increase the glutamate content of Soya sauce using salt and thermo tolerant glutaminase have drawn much attention. It also plays an important role in biosensor as a monitoring agent for glutamine level measurement. Another important application of L-glutaminase is in food industry as flavour enhancing agent. It increases the glutamic acid content of the fermented food there by imparting a unique flavour.

MATERIALS & METHODS

1. Enzyme Purification: Proteins are often produced using large scale fermentation processing in order to acquire purified enzymes. This often presents a challenge as the enzymes must be separated in such way as to maintain its catalytic activity.
1.1. Acetone Fractionation: Water miscible organic solvents like ethanol, methanol and acetone are also good protein precipitations. Precipitation using organic solvent is done at 0°C or subzero temperatures in order to minimize the denaturation of proteins. In this method solvent is cooled overnight and added slowly with constant stirring to ice-cold solution of proteins in cold condition. The precipitates are collected by centrifugation at 10,000 rpm for 20 minutes at 4°C. Advantage of using solvent precipitation is that it can be easily removed by evaporation. The enzyme obtained after centrifugation was mixed with equal volumes of crude acetone. The solution was then kept for 24 hours at 4°C. The solution was the centrifuged at 4°C for 15 minutes at 1000 rpm. Then the supernatant was discarded and the pellets were mixed with phosphate buffer and preserved for later use. The enzyme obtained was then characterized and assayed to check the activity and specificity.

1.2. Ammonium-Sulphate Precipitation: Ammonium sulphate is used for the fractional precipitation of the proteins. First ammonium sulphate of different percentage (20% to 100%) was weighed because the precipitate varies from a strain to strain. Then the sample was kept on magnetic stirrer to mix well and solid ammonium sulphate was slowly added to the sample. The process was done until the sample gets maximum saturation. Keep it overnight at 4°C for complete precipitation. After the centrifugation was done at 10000 rpm for 15 minutes and the supernatant was separated from the pellet was re-suspended in 1 ml of sample buffer. The L-glutaminase enzyme was then further purified by membrane dialysis and stored for later use.

1.3. Dialysis: Dissolve 1 g of DEAE cellulose in distilled water and pour in chromatographic column. Filter it out. Soak the filtrate in 25 ml of 1N HCL and keep it for 30 minutes to activate the charged site of DEAE cellulose resin. Wash twice with distilled water for 30 minutes to remove excess HCL. Again soak the filtrate in 25 ml of 1 N NaOH and keep it for 30 minutes to activate charged site of DEAE cellulose resin. Wash twice with distilled water for 30 minutes to remove excess NaOH. Pour the overnight dialysed enzyme in column incubate for 15 minutes. Elute it in fresh tube and marked it as first elution. Pour 25% fraction of buffer in the column and incubate for 15 minutes. Elute it in fresh tube and mark it as 25% fraction. Pour 50% fraction of buffer in the column and incubate for 15 minutes. Elute it in fresh tube and mark it as 50% fraction. The same process is repeated for 75% and 100% fraction of buffer. All tubes are stored at 4°C for further process.

2. Protein Estimation by Lowry’s Method: Protein can be estimated by different methods described by Lowry and also by estimating the total nitrogen content with (Reagent A: 2% Sodium Carbonate in 0.1N Sodium hydroxide, Reagent B: 0.5% Copper sulphate in 1% Potassium tartrate, Reagent C: Alkaline copper solution -50 ml Reagent A + 1 ml Reagent B (Freshly prepared), Reagent D: FC reagent : distilled water (1:2), Stock protein standard – 50mg BSA in 50ml distilled water, Working protein standard – 10ml stock protein standard in 400ml
distilled water (solution contains 200µg/ml protein), Desired enzyme sample. It can be determined by calorimetrically using phenol reagent by the colour it develops using a specific filter of specific wavelength. The blue colour developed by the reduction of the phosphomolybdic and phosphotungstic components in the Folin-ciocalteau reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by the burette reaction of the protein with the alkaline cupric tartrate are measured in the Lowry’s method.

3. Assay Method of L-Glutaminase

4.3.1. Spectrophotometric Assay: L-Glutaminase activity was determined using L-Glutamine as substrate and the product ammonia, released during the catalysis was measured by using Nessler’s reagent. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of tris HCl buffer (0.05 M, pH 8.0). Then the mixture was incubated at 37°C for 15 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M Trichloro Acetic Acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 436 nm using a visible spectrophotometer. Then a standard graph was plotted (Imada et al., 1973). One international unit of L-glutaminase was defined as the amount of enzyme that liberates one µMol of ammonia under optimum conditions. The enzyme yield was expressed as Units/ml (U/ml). Protein concentration was determined (Lowry et al., 1951) using bovine serum albumin as the standard. The reaction mixture containing 0.5ml of substrate (glutamine - 0.04M). 0.5ml of buffer (Tris HCl - 0.05M and pH 8.0). 0.5 ml of distilled water and 0.5 ml of crude enzyme solution was added and incubated at 37°C for 15 minutes. The reaction was stopped by addition of 0.5ml of 1.5M Trichloroacetic acid (or heated at 60°C for 10 minutes). Then to 3.7 ml of distilled water and 0.2 ml of Nessler’s reagent were added. The OD value was recorded at 436 nm in a UV spectrophotometer. Enzyme and substrate blanks were used as controls.

Calculation

\[
\text{Units/ml/min} = \frac{\mu \text{moles of NH}_3 \text{liberated} \times 2.50}{0.1 \times 30 \times 0.5}
\]

Where, 2.5 = total volume of test tube, 0.2 = volume of Nessler's reagent, 0.5 = volume of enzyme used, 30 = time of assay in volume, 1mole of L-glutamine = 2moles of ammonia (NH3)

The activity of glutaminase is determined by estimating the amount of ammonia liberated from glutamine. The amount of ammonia was measured by adding 100 µl of the crude enzyme to 200 µl Nessler’s reagent and completed to 4 ml by distilled water. Absorbance was measured at
540 nm using a visible spectrophotometer. L-glutaminase activity is expressed in μg ammonia produced per one ml medium. The level of ammonia produced was estimated against the ammonia standard curve using ammonium sulphate.

3.2. Well Based Assay: Minimal glutamine agar medium was prepared and a well with 5 mm diameter was made at the four corners of the agar medium by using well puncher. About 50 μl of different fraction of enzyme was loaded in to the well and incubated at 28°C for 24 hours. After measuring the dimension of the coloured zone, the number of units was determined, thus, proving that the enzyme is extracellular in nature and it formed a clear zone of colouration showing the activeness of L-glutaminase enzyme. One unit of the enzyme activity was defined as the amount of enzyme in 25μl of enzyme solution that produced a clear zone of 1mm² at pH 8 and 30°C for 18 hours. Minimal glutamine agar medium was prepared and control medium was also prepared containing NaNO₃. Using well puncher, 5mm diameter wells were made at four corners of the agar plate. Enzymes were loaded into the wells and incubated for 24 hours at 37°C. The coloured zones thus formed (if any) was measured. One unit of the enzyme activity was defined as the amount of enzyme in 25μl of enzyme solution that produced a clear zone of 1mm² at pH 8 and 30°C for 18 hours.

3.3. Cell Based Assay: Bacterial strain with its crude L glutaminase enzyme showed maximum zone of colour change (from yellow to pink) on minimal glutamine agar medium. After measuring the dimension of the clear zone, the number of units was determined. One unit of the enzyme activity was defined as the amount of enzyme in 25μl of enzyme solution that produced a clear zone of 1mm² at pH 8 and 30°C for 18 hours. Minimal glutamine agar medium was prepared and control medium was also prepared containing NaNO₃. Using inoculating loop, different bacterial strains were inoculated in different plates, in form of small round shape at the centre of the agar medium. The plates were then incubated for 24 hours at 37°C. The dimension of the clear zone, the number of units was determined.

3.4. Biogenic Assay:

3.4.1. Harvesting of spleen cells and preparation of cells: The spleen was transferred to a sterile 100mm diameter petridish filled with 10 ml sterile complete serum free RPMI media. The spleen was teased into single cell suspension by squeezing it with angled forceps or by chopping with fine tip dissecting scissors or by crushing it in mortar after mixing it in 20 ml of phosphate buffer solution. The debris was removed by centrifuging it in 10000rpm for 15minutes at 27°C. The cells were dispersed further by passaging it through a fine mesh metal screen. The spleen cell suspension was transferred to a sterile 50ml conical centrifuge tube filled with sterile complete serum free RPMI media (10:10). The solution was then centrifuged for 5minutes in TH-4 rotor at 1500rpm (500xg) at room temperature & the supernatant was discarded. RBCs
were lysed by resuspending pellets in 5ml of ammonium chloride solution. Let stand for 5 minutes at room temperature. 45ml complete sterile free RPMI media was added and centrifuged for 5 minutes in TH-4 rotor at 1500 rpm (500xg) at room temperature. Pellets were resuspended in 50ml sterile complete serum free RPMI media and centrifuged again as above. Repeat RPMI addition and centrifuge once again (each repeat is a wash). While spleen cells were being washed, separately P-3 myeloma cells was being harvested by transferring the cells to a 50ml conical centrifuge tubes and then centrifuged again (for 5 minutes in TH-4 rotor at 1500 rpm (500xg) at room temperature). Myeloma cells were resuspended in RPMI media and all cells were pooled in one 50ml conical centrifuge tubes. Wash myeloma cells three times following the above step. Separately, the spleen and myeloma cells were resuspended in 10ml complete serum free RPMI media. Cells were counted and viability accessed in each cell suspension using a hemacytometer and tryptan blue exclusion, there should be nearly 100 viability of both suspensions. On the basis of cell counts, the amount of complete serum free RPMI/HEPES/pyruvate needed to plate cells were calculated at - 2.5 x 10^6 total cells/ml. This amount of complete serum free RPMI/HEPES/pyruvate was prewarmed at 37°C water bath.18 well flat bottom plates were prepared by labelling them sequentially.

3.4.2. Monitoring of cells: 0.5ml of glycerol was mixed with 50 ml of water. On the other hand 10 ml of RPMI media was mixed with 5ml glycerol (above solution). Streptomycin sulphate and Gentamycin was added to check any kind of microbial contamination in the media as well as in spleen cell suspension. The wells were then loaded with RPMI media (containing glycerol) and 0.5 ml of serum (obtained from blood) was added. 1 ml of mononuclear cells were also added into the wells. After one day of incubation, wells were checked under an inverted microscope. If seeded with appropriate number of cells, there should be a nearly confluent monolayer of highly viable cells at the bottom of the wells. A drop of ethidium bromide was added in each of the wells and incubated at 37°C for 3 hours. Subsequent growth in cells was then checked under an inverted microscope. 0.5 ml of L-glutaminase enzyme was then added in to the wells and incubated for 48 hours at 37°C. After the addition of ethidium bromide, the cells become cancerous and start to grow indefinitely. The enzyme L-glutaminase when added into the wells reacts with ethidium bromide and detoxifies it. As a result, the cells get time to recover and gets back to its original phase of growth cycle, thus using up the nutrients from the side of the wall of the wells, as a result of which zone formation occurs.

4. Optimization of the L-Glutaminase: The optimization of fermentation medium and the process condition in order to maximize the profits from fermentation processes. The relative activity and the quantitative estimation such as temperature, effect of pH, effect of activator/inhibitor, of L-glutaminase enzyme were estimated by Lowry’s method spectrophotometrically at 436nm.
4.1. Effect of Temperature

Temperature is also a factor in the stability of the enzymes. As with activity, for each enzyme there is also a region of temperature, optimal stability. 0.5ml of Tris HCl buffer with a pH maintained at 7.2 was taken. 0.5ml of substrate (glutaminase) was added in each of the test tube. Then 0.5ml of the enzyme was given in each of the test tube except blank. 0.5ml of water was added. Each test tube was then incubated for 30 minutes at different temperatures (4°C, 27°C, 37°C, 60°C, 80°C, 100°C). 3.7ml of water was added to dilute the solution. TCA solution was added and/or the solution was heated to 60°C for 10 minutes. 0.2ml of Nessler’s reagent was added to the samples and was incubated at 37°C for 30 minutes. The mixture was centrifuged at 15000rpm for 5 minutes. The supernatant was collected and the absorbance was measured at 436 nm.

4.2. Effect of pH

Enzymes are affected by the change in pH. Buffer solution was taken (1ml) in different test tube and the pH was maintained accordingly (3, 5, 7, 9, 11 and 13). 0.5ml of substrate was added in each of the test tube. 0.5ml of the enzyme was then taken in each of the test tube except blank. 0.5ml of distilled water was added. The solution was incubated 30 minutes at 37°C. 3.7ml of distilled water was added to dilute the solution. TCA solution was added and/or the solution was heated to 60°C for 10 minutes. 0.2ml of Nessler’s reagent was added and the solution was incubated for 30 minutes at room temperature. The mixture was centrifuged at 15000rpm for 5 minutes. The supernatant was collected and the absorbance was measured at 436 nm.

4.3. Effect of Activators: To determine whether the activity of L-glutaminase could be affected, each activator was added to the crude enzyme. 1% in 1ml solution of different possible activators was made. 0.5ml of substrate was taken in test tube. 0.5ml of enzyme was added in each of the test tube except blank. 0.5ml of different activators was added. 0.5ml distilled water was added. The solution as incubated for 30 minutes at 37°C. 3.7ml of distilled water was added to dilute the solution. TCA solution was added and/or the solution was heated to 60°C for 10 minutes. 0.2ml of Nessler’s reagent was added and incubated further for 30 minutes at room temperature. The mixture was centrifuged at 15000rpm for 5 minutes. The supernatant was collected and the absorbance was measured at 436 nm.

4.4. Effect of Inhibitors: Inhibitors known to effect L-glutaminase enzyme that were used are: Mercuric chloride (HgCl), Ethylene diamine tetra acetic acid (EDTA), Sodium dodecyl sulphate (SDS), Mercuric chloride (HgCl₂), Hydrogen peroxide (H₂O₂), Sodium azide (NaN₃). To determine whether the activity of L-glutaminase could be affected, each inhibitor was added to the crude enzyme. 1% in 1ml solution of different possible inhibitors was made. 0.5ml of substrate was
taken in test tube. 0.5ml of enzyme was added in each of the test tube except blank. 0.5ml of different inhibitors was added. 0.5ml distilled water was added. The solution was incubated for 30 minutes at 37°C. 3.7ml of distilled water was added to dilute the solution. TCA solution was added and/or the solution was heated to 60°C for 10 minutes. 0.2ml Nessler’s reagent was added and incubated further for 30 minutes at room temperature. The mixture was centrifuged at 15000rpm for 5minutes. The supernatant was collected and the absorbance was measured at 436nm.

5. Gel Electrophoresis (SDS-PAGE): SDS-PAGE analysis of samples: A 10% separating gel was prepared and ingredients were de-aerated for 10 min before addition of 10% ammonium persulfate solution that polymerizes the gel. The separating gel was pipetted into a gel-casting unit and was allowed to polymerize for 30 min. A stacking gel was prepared and was also de-aerated 10 min before adding 10% ammonium persulfate. This gel was pipetted on top of the solidified separating gel and a comb was placed. This gel was allowed to polymerize for 30 min and then the gel was placed into an electrophoresis unit. The upper and lower chambers were filled with tank buffer. Whole cell pellets and membrane pellets were prepared by adding an equal volume of 2× gel loading buffer (~20 µl) in an Eppendorf tube. Additionally, a protein molecular weight standard was prepared by adding 2 µl BioRad SDS-PAGE broad-range molecular size marker with 8 µl of 2× gel loading buffer. All samples were then placed in a boiling water bath for five minutes. Samples were loaded onto the gel (5 µl of whole cell pellets and standard and 15 µl of membrane pellets), and the gel was run at 30 milliamps per gel for approximately one hour or until the dye front reached the bottom of the gel. The gel was placed in Coomasi Blue Stain for 30 min, destained, and stored in 5% acetic acid. After staining and de staining procedure, the gel was observed in a light. Also, it can be observed in natural white light background.

6. High Performance Liquid Chromatography (HPLC): High performance liquid chromatography (HPLC) is now firmly established as the premier technique for the analysis and purification of a wide range of molecules. In particular, HPLC in its various modes has become the central technique in the characterization of peptides and proteins. HPLC arises from the fact that all interactive modes of chromatography are based on recognition forces that can be subtly manipulated through changes in the elution conditions that are specific for a particular mode of chromatography. Solvent (Solvent A: 90% sodium acetate (0.1M; pH 7.2), 9.5% methanol (HPLC grade) and/or aceto-nitrate, 0.5% trifluoroacetic acid (TFA)-Solution B: 100% methanol (HPLC grade) and/or aceto-nitrate-) preparation filters all solvents through a 0.22µm filter before use. This removes particulates that can block the solvent lines or the column and also serves to degas the solvent and absorbance of detector: 260 to 268nm, alternative absorbance: 400 to 650nm.
7. Zymography of L-Glutaminase: Samples are prepared in the standard SDS PAGE treatment buffer but without boiling and without a reducing agent (Distilled water-1.3ml 3mM cholesterol + 1% Triton X-100-1.0ml, 30% acryl amide +1.4% bis- acrylamide-2.65ml, Lower buffer-1.3ml, 10% APS (freshly prepared)-0.05ml, TEMED-10µl). Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100, followed by incubation in an appropriate digestion buffer, for an optimized length of time at 37°C. Glutamic acid is the most commonly used substrate in zymography.

RESULTS

1. Purification of Enzyme:

1.1. Acetone Fractationation: Acetone and enzyme (both with glucose and without glucose) was kept for a day and later centrifuged.

1.2. Ammonium Sulphate Precipitation: The enzymes containing glucose got saturated at 80%, while the enzyme (without glucose) got saturated at 95%.

1.3. Membrane Dialysis: The enzymes obtained after ammonium sulphate precipitation was further purified by membrane dialysis and then subjected to purification by ion exchange chromatography.

2. Assay of L- Glutaminase Enzyme

2.1. Spectrophotometric Assay

2.1.1. Assay of different fraction of enzymes (V_{MAX}):
2.1.2. Assay of crude enzyme

<table>
<thead>
<tr>
<th>Enzyme with glucose</th>
<th>Enzyme without glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 - 40µM – 66.6 U/ml/min</td>
<td>0.2 – 45 µM – 75 U/ml/min</td>
</tr>
<tr>
<td>0.4 – 55 µM – 91.6 U/ml/min</td>
<td>0.4 – 80 µM – 133.3 U/ml/min</td>
</tr>
<tr>
<td>0.6 – 120 µM – 200 U/ml/min</td>
<td>0.6 – 120 µM – 200 U/ml/min</td>
</tr>
<tr>
<td>0.8 - 160µM – 266.6 U/ml/min</td>
<td>0.8 - 160µM – 266.6 U/ml/min</td>
</tr>
<tr>
<td>1.0 - 180µm – 300 U/ml/min</td>
<td>1.0 - 200µm – 333.3 U/ml/min</td>
</tr>
</tbody>
</table>

Crude enzyme showed maximum activity at 1ml concentration (both with and without glucose). Among different fraction of enzyme (with and without glucose), 100% fraction of enzyme, showed maximum activity.
3.1. Cell Based Assay:

C1 and C3 showed zone formation with a diameter of 1cm and 1.2cm respectively.

3.2. Well Based Assay:

Enzyme showed coloured zone formation proving that enzyme (L-glutaminase) is extracellular in nature. The zone formed by Crude enzyme was 1.5cm diameter.

3.3. Biogenic Assay:

Fig: After addition of Ethidium bromide, spleen cells and enzyme (left-control)
Fig: zone formation occurred in plates containing glucose whereas no zones were observed in control media (containing NaNO₃).

Zones were measured to be cm in diameter in case of crude enzyme (with glucose), cm in diameter of crude enzyme (without glucose) and cm in diameter of 50% pure enzyme (without glucose).

4. Protein estimation by Lowry’s method

4.1. Protein estimation by Lowry’s method of different fraction of L-glutaminase enzyme

Fig: protein estimation of L-glutaminase enzyme (with & without glucose)
Thus, among all fraction of enzymes (with glucose), acetone fraction contains the highest concentration (0.28 mg/ml) of protein. Thus, among all fraction of enzymes (without glucose), acetone fraction contains the highest concentration (0.27 mg/ml) of protein.

5. Optimization of the Enzyme

5.1. Effect of Temperature

Thus, the enzyme L-Glutaminase (without glucose) shows maximum activity at 27°C temperature. Then, subsequently at 37°C > 60°C > 80°C > 4°C > 100°C. Thus, the enzyme L-Glutaminase (with glucose) shows maximum activity at 80°C temperature. Then, subsequently at 100°C > 60°C > 27°C > 4°C > 37°C.

5.2. Effect of pH

Thus, the enzyme L-glutaminase (with and without glucose) shows maximum activity at pH 9.
5.3. Effect of Activators:

Thus, Manganese sulphate is the best activator for enzyme L-glutaminase (without glucose). Then, subsequently calcium chloride > ferric chloride > ferrous sulphate > magnesium sulphate. Thus, Manganese sulphate is the best activator for enzyme L-glutaminase (with glucose). Then subsequently ferric chloride > ferrous sulphate > calcium chloride > magnesium sulphate.

5.4. Effect of Inhibitors:

Thus, SDS is the best inhibitor for enzyme L-glutaminase (without glucose). Then, subsequently sodium azide > mercury chloride > hydrogen peroxide > EDTA. Thus, Mercury chloride is the best inhibitor for enzyme L-glutaminase (with glucose). Then, subsequently sodium azide > SDS > hydrogen peroxide > EDTA.
6. SDS PAGE

Two bands of L-glutaminase enzyme were observed on SDS-PAGE as compared to the marker; with 43 and 225 kDa molecular weight respectively.

7. Zymography:

Two bands of L-Glutaminase enzyme were observed on zymography as compared to the marker; with 40 and 225 kDa molecular weight respectively. No casein substrate was digested by the protease enzyme; therefore, we conclude that, only glutaminase enzyme was present in the crude enzyme solution.

DISCUSSION

The partially purified enzyme from *Bacillus subtilis* exhibited maximum L-glutaminase activity at pH 7 to 9 (for 100% fraction with and without glucose 166.60 and 60 U/ml/min, for crude with and without glucose 300 and 333.3 U/ml/min, for ammonium sulphate with and without glucose 333.3 and 58.3 U/ml/min). The enzyme retained 53.3 and 88.6% of the maximum
activity when assayed at pH 6 and 9 respectively. The pH 7 was also the optimum for maximum L-glutaminase activity from marine *Micrococcus Luteus* as reported by Moriguchi *et al.* According to our present investigation the optimum pH of glutaminase enzyme was 9. The enzyme was optimally active (22.3U/ml) at a temperature range of 37°C. Incubation above 40°C promoted remarkable inactivation of L-glutaminase from *Streptomyces avermitilis*, while about 93.5% of the optimum activity remained at 40 ºC. Duraet *et al.* found that maximum activity of L-glutaminase from Debaryomyces spp was at 40°C. The results revealed that in *Streptomyces avermitilis*, as NaCl concentration increase the activity of L-glutaminase enzyme increase until it reach its maximum activity (22.4 U/ml) at 4%. The glutaminase from *Lactobacillus rhamnosus* which showed increased activity in the presence of 5% (w/v) salt was reported by Alexandra *et al.* The best activator for L-glutaminase enzyme (with and without glucose) was found to be Manganese sulphate. The best inhibitor for L-glutaminase enzyme (with and without glucose) was found to be Mercuric chloride and SDS respectively. In contrast, the L-glutaminase activity from *Streptomyces avermitilis* was enhanced by MgSO4 and NaCl with 19.3 and 19.8 (U/ml) activities and 104.9 and 107.6% residual activity respectively. On the other hand, CuSO4, ZnSO4 and EDTA decreased the activity. These results are in accordance to those reported by Lu *et al.* who indicated that the activity of glutaminase from *Actinomucor taiwanensis* was enhanced by addition of MgSO4 and NaCl. L-glutaminase from *Streptomyces avermitilis* showed high stability with the used oxidizing agents. It was highly stable in the presence of sodium hypochlorite and hydrogen peroxide with activity (21.7 and 21.4 U/ml) and (116.3 and 117.9%) residual activity respectively. The molecular weight of L-glutaminase was observed on SDS PAGE showed two bands of 225kDa and 43kDa respectively. While two bands of L- glutaminase enzyme were observed on zymography as compared to the marker; was 40 and 225 kDa molecular weight respectively. No casein substrate was digested by the protease enzyme; therefore, we conclude that, only glutaminase enzyme was present in the crude enzyme solution.

**CONCLUSION**

The L- glutaminase enzyme isolated from Bacillus species is a type of anti-cancer enzyme. It is stable at pH 7 to 9 and temperature 27°C to 37°C. The molecular weight of the enzyme was found to be around 40 to 43 kDa by SDS-PAGE, where as it was found to be around 40 to 45 kDa by zymography. The best activator for L-glutaminase enzyme (with and without glucose) was found to be Manganese sulphate. The best inhibitor for L-glutaminase enzyme (with and without glucose) was found to be Mercuric chloride and SDS respectively. These properties indicates that the possibilities for use of the L- glutaminase enzyme in the medical/ clinical industry. Therefore, this enzyme can be exploited commercially with some modifications.
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