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ISOLATION, PRODUCTION & SCREENING OF ANTI-CANCER ENZYME L-GLUTAMINASE FROM *Bacillus subtilis*

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ABSTRACT

In the present study, L-glutaminase producing bacteria were isolated from sedimant samples collected from the area of Kolkata. L-glutamiase from selected five bacterial isolates were produced by submerged fermentation. The potential soil bacterial isolate was identified as Bacillus subtilis based on its morphological, cultural, biochemical and physiological characteristics.

KEY WORDS

L-glutaminase, Serial dilution method, Gram staining, Biochemical characterization of microorganisms, Production of L-glutaminase.

INTRODUCTION

L-Glutaminase (L-Glutamine amid hydrolase EC. 3.5.1.2) the enzyme deaminating L-glutamine plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes. L-glutamine constitutes a large proportion of the available free amino nitrogen of tissues, blood and of the metabolic nitrogen pool and is an important non-toxic, temporary reservoir of ammonia nitrogen in micro organisms which can be drawn upon for synthetic purposes. It also acts as a direct precursor for glutamic acid in the metabolism of certain tumours which consequently furnish the carbon for the partial operation of tri-carboxylic acid cycle from and QC-ketoglutarate to oxaloacetate. Reactions catalysed by glutamine constituted the primary mechanism of ammonia production in the body and it plays an important role in the acid base control of body fluids .

Almost all living cells produce L-glutaminase but only certain microbial strains have the potential for industrial production of this enzyme. It is ubiquitous from the presence point of view in plants, animals and microbes both in prokaryotes and eukaryotes. Among some well studied genera in microbes worth mentioning from study perspective are *E. coli, Pseudomonas sp., Brevibacterium sp., Vibrio costicola, Streptomyces rimosus, Streptomyces avermitilis and Streptomyces labedae, Streptomyces gresius, Hypocrea jecornea, Zygosaccharomyces sp.,*

Bacillus sp. and Micrococcus luteus, Acinetobacter species, Hansenula, Cryptococcus, Candida, Aspergillus oryzae and Beuveria bassiana etc.

This enzyme is involved in glutamine catabolism in micro-organisms. Mammalian cells also synthesis this enzyme which is involved in the generation of the energy using glutamine as the major respiratory fuel. Thus, many types of tumour cells as well as actively dividing normal cells exhibit high rates of glutamine utilization. Cancer cells, especially Lymphatic tumour cells cannot synthesize L-glutamine and hence require large amount of L-glutamine for their rapid growth. Thus these cells depend on the exogenous supply of L-glutamine for their survival and rapid cell division. Hence, the use of amidases deprives the tumour cells from L-glutamine and causes selective death of L-glutamine dependant tumour cells.

In micro organisms the intracellular levels of glutamine are determined by rates of enzymatic synthesis and degradation. Glutamine synthetase catalyses the synthesis while glutaminase catalyses the hydrolytic degradation of glutamine and splits off the R-amide of glutamine as ammonia.

The action of glutaminase directly opposes that of glutamine synthetase, so their coupling would result in a futile cycle of amide synthesis and degradation. Ability of the enzyme to bring about degradation of glutamine poses it as a possible candidate for enzyme therapy which may soon replace or combined with L-asparaginase in the treatment of acute lymphocytic leukaemia. It is found that administration of L-glutaminase will deplete L-glutamine which is required for asparagine synthesis in the body of patient thereby inhibiting asparagine dependent protein synthesis and eventually the synthesis of DNA and RNA. However, the large scale application of glutaminase in cancer chemotherapy it's still under experimental conditions and not much information is available.

MATERIALS AND METHODOLGY

1. Isolation of Microorganisms from soil sample

The micro organisms used in this work was isolated from soil. Minimal essential media was made followed standard conditions and bacteria were isolated by serial dilution method using sterile distilled water. The bacterial colonies were then isolated using the two methods: Serial dilution method, Spread plate technique

2. Serial dilution method

This method was first developed by **Joseph** and later perfected by **Robert Koch**. There are two variation of the dilution plating technique – the spread plate and the pour plate method. In the first method, a measured quantity of a serially diluted sample of the original culture is spread evenly on the surface of the solidify growth medium. On incubation at an optimum temperature, each viable bacterial cell forms a discrete colony on the surface of the medium. About 1gm of soil sample was mixed with 100ml

of distilled water, shaken vigorously, this is the stock solution. From the stock solution 1ml of solution was transfer to 9ml sterile distilled water aseptically, shaken vigorously. This is solution of 10^{-1} dilution and was repeated 9 more times to the dilution till 10^{-10} and 0.1ml of each solution was transferred aseptically to each plate containing nutrient media (for bacteria) and spread with spreader on the surface on the media. The plate incubated for 24hour at 37 ^{0}C in incubator. Pink colony was observed on the agar plate.

3. Spread plate technique

In this technique, micro-organisms are spread over the solidified agar medium with a sterile L-shaped glass rod while the Petridish is spun on a turn table. At first autoclave minimal essential media poured in petridish. 10^{-1}, 10^{-2}……. 10^{-10} concentrations of solution were transfer in solidified minimal essential media. Sterile the bent portion of the glass rod in the Bunsen burner flame. Cool the glass rod for 10-15 seconds. Remove the cover of petridish and spin turn table. Lightly touch the sterile bent rod to the agar surface and move it back and forth while the turntable is spinning for the spreading the culture over the agar surface. Incubate all the 4 plates in an inverted position at 37 ^{0}C for 24 to 48 hours. All the incubated plates were observed and some identical colonies were selected and marked. After selection of colonies pure culture technique was done.

4. Pure culture technique

A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new sterile growth medium in such a manner as to disperse. After the completion of incubation in spread plate technique, appearance of the discrete well separated colonies were examined. The next step was to subculture some of the cells from one of the colonies to separated agar plates or nutrient agar slants with a sterilized inoculating needle or loop for further examination and use. The tip of the loop was touched to the surface of a selected discrete colony or the agar streak plate. The nutrient agar plate was uncovered and the loop was inserted and inoculated by drawing it lightly over the surface in a zigzag manner and the plate was covered. The cultures were kept in a bacteriological incubation in an inverted position for 24-48 hours. After incubation the plate were observed for the growth of pure colonies.

5. Screening of the L-Glutaminase enzyme

Minimal glutamine agar medium was prepared and used for detection of L-glutaminase producing bacteria. Components of MGA (g/L) include 0.5 KCl; 0.5 MgSO4; 1.0 KH2PO4; 0.1 FeSO4; 0.1 ZnSO4; 0.5 NaCl; 10.0 L-glutamine and supplemented with 0.012 g phenol red and pH adjusted at 6.8, in which L-glutamine act as the sole carbon and nitrogen source and phenol red as pH indicator. The colour change of the medium from yellow to pink is an indication of the extra cellular L-glutaminase production by the colony. This colour change is due to change in the pH of

the medium, as L-glutaminase causes the breakdown of amide bond in L-glutamine and liberates ammonia. All the bacterial isolates collected were streaked on minimal glutamine agar medium. After four days of incubation at 30°C, the change of medium colour from yellow to pink around the colony indicating positive response. The secondary screening for the highly producer isolates was done by the same previously method but in liquid medium and the pink colour degree of the supernatant after filtration and centrifugation of the medium at 3000 rpm for 15 min was measured at 540 nm using a visible spectrophotometer.

6. Gram staining

Gram staining (or Gram's Method) is a method of differentiating bacterial species into two large groups (gram positive and gram negative). The name comes from its inventor, Hans Christian Gram. Most common bacteria are either gram positive or gram negative (based on cell wall structures). Gram positive cell walls consist of several layers of peptidoglycan (cross – linked by teichoic acid, lipoteichoic acid). Gram negative cell walls have one layer of peptidoglycan surrounded by a lipid based outer membrane. In the 1880's, Hans Gram developed the differential method of staining that bears his name. While we still don't know exactly gram positive bacteria end up looking different from gram negative bacteria, gram staining is still an important way to characterize bacteria. Prepare heat fix thinly spread smears of fresh bacteria. Stain with crystal violet for 1 minute. Wash in tap water no longer than 2 second. Flood the smear with grams iodine for 1minute. Wash in tap water and drain carefully. Wash in 95%ethyl alcohol for decolourisation. Wash in tap water for 2seconds to remove the ethyl alcohol. Flood smear with safranine for 1minute Wash with tap water and Air dried Place a drop of emulsion oil on the slides in the field of smear. The smear was observed, using the 100x lenses.

7. Biochemical characterization of microorganisms

7.1. Methyl red assay: The methyl red test is employed to detect the ability of micro organisms to oxidise glucose. MR broth was prepared by dissolving peptone and phosphate and the pH was adjusted to 7.6, 5ml amount of media was dispensed in test tubes and sterilized at 121 ^{0}C for 15 minutes. Glucose solution was sterilized by filtration and 0.25ml was added in each test tube which will give a final concentration of 0.5%. Test culture was inoculated in the MR broth and incubated at 37 ^{0}C for 48 hours. After incubation 5-6 drops of methyl red solution was added. A bright cherry red colour indicating a pH of 4.2 or less in a positive test is observed. Yellow or orange colour indicates a negative reaction. A weakly positive test will be red- orange.

7.2. Voges Proskauer test : VP broth was prepared by dissolving peptone and phosphate and pH was adjusted to 7.6, 5ml of media was dispensed in test tubes and sterilized at 121^{o}C for 15 minutes. Glucose solution was sterilized by

filtration and 0.25ml was added to each tube which will give a final concentration of 0.5%. The test culture was inoculated in the VP broth incubated at 37 ^{0}C for 48 hours. After 48 hours of incubation, 1ml of 40% potassium hydroxide (plus creatine) and 3ml of a 5% solution of alpha naphthol were added in absolute ethanol. A positive reaction is indicated by the development of a pink colour in 25 minutes, crimson in 80 minutes.

7.3. Indole production assay: The indole is detected by calorimetric reaction by p- dimethyl amino benzaldehyde (Kovac's reagent).vPeptone broth was prepared and the test culture was inoculated in the test tubes and the tubes were incubated at 37 ^{0}C for 24 hours. After incubation 0.2ml of Kovac's reagent was added for 5 minutes. A cherry red colour in the alcohol indicates a positive reaction.

7.4. Starch Hydrolysis test: 75ml of starch agar plates were prepared. A single line of streak of the organisms was made across the centre of the starch agar plates. The plates were incubated for 37 ^{0}C for 24 hours for sufficient growth. After incubation the plates were flooded with iodine solution. Hydrolysis is indicated by the clear zones around the growth and uncharged starch gives as blue colour. The medium should preferably contain no glucose as this may diminish starch hydrolysis. The iodine normally used for Gram's stain is suitable.

7.5. Catalase test: A nutrient agar plate was streaked with test culture and the plates were incubated at 37 ^{0}C for 24 hours. After incubation 1 ml of 3% hydrogen peroxide was added to the plates after 5mintues the plates were examined immediately for the evolution of bubbles which indicates a positive test.

7.6. Urease test: This is the positive test for the presence of urease. The test culture were inoculated heavily ever the entire slope culture and incubated at 37 ^{0}C for 24 hours and the reaction recorded after 4, 8, 12 and 48 hours of incubation. A positive urease reaction is indicated by a change in the colour of the medium from yellow to purple colour.

7.7. Nitrate reduction test: 50ml of nitrate medium was prepared and sterilized at 121 ^{0}C for 15minutes. After sterilization, inoculate the medium with culture and incubate at 35 ^{0}C for 96 hours. Following incubation add 1ml of test reagent of the test culture. A red colour developing within a few minutes indicates the presence of nitrate and hence the ability of the organism to reduce nitrate.

7.8. Casein hydrolysis: The skim milk plates were prepared and the cultures were inoculated and incubated at 37 ^{0}C for 24 to 48 hours. It is used to observe for clearing around colonies of casein hydrolysing organisms to detect false clearing. 10% solution of $MgCl_2$ was paired in 20% HCl over this medium. The disappearance of cleaned area indicates false hydrolysis.

7.9. Simmon citrate agar test: Simmon citrate medium (a modification of koser's medium with agar and as indicator) dispensed in test tubes and

was sterilized at 121 ^{0}C for 15 minutes and allowed to set as slopes. The organisms from a saline suspension was inoculated to test on agar slopes and incubated at 37^{0}C for 96 hours. A positive test shows a blue colour on a streak of growth. Retention of original green colour and no growth on the line of streak indicates a negative reaction.

7.10. Gelatin hydrolysis test: The gelatine agar medium was prepared and sterilized at 121 ^{0}C for 15 psi for 15 minutes. After sterilization, the test organism was streaked in the agar plate and incubated at 27 ^{0}C for 2-5 days. Deep gelatine inoculated tubes that remain liquefied produce gelatinase and show positive test for gelatine hydrolysis and those tubes remain solid demonstrate negative reaction for gelatine hydrolysis.

7.11. Triple sugar iron test: The triple sugar iron agar medium was prepared and sterilized at 121 ^{0}C for 15 psi for 15 minutes. After sterilization, slant was prepared. The test organism was streaked in the agar plate and incubated at 27 ^{0}C for a day.

7.12. Hydrogen sulphide test: Hydrogen sulphide Agar medium was prepared and sterilized at 121 ^{0}C for 15minutes at 15psi the medium was poured into the tubes and allowed for solidification. Then the organisms were inoculated and incubated at 27 ^{0}C for 2-5days.

8. Production of L-Glutaminase enzyme

L-glutaminase from all the bacterial cultures were produced by shake flask fermentation using Minimal glutamine medium (MGM - g/l). One medium contained MGM along with glucose, while the other medium contained MGM without glucose (composition shown in table). One ml of inoculums was transferred aseptically to 200ml and 250ml of MGM medium. All the flasks were incubated in rotary shaker at 28°C. After incubation, the fermentation medium was removed and centrifuged using cooling centrifuge at 10,000 rpm for 30 minutes at 4°C. The cell free supernatant was collected in screw cap tube and stored at 4°C to use as crude enzyme.

Table 1: Composition of the production media

Minimal Glutamine Medium (With Glucose)	Minimal Glutamine Medium (Without Glucose)	Concentrations (g/l)
Glutamic acid	Glutamic acid	10.0
K2HPO4	K2HPO4	1.0
CaCl2	CaCl2	0.1
NaNO2	NaNO2	0.1
Na3C6H5O7	Na3C6H5O7	0.1
NaCl	NaCl	25.0
MgSO4	MgSO4	0.5
Phenol red	Phenol red	0.012
	glucose	10

RESULTS & DISCUSSION

1. Serial dilution method: Five fractions were taken 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} and following spread plate technique they were spreaded in agar plates containing minimal glutaminase medium. They were then incubated at 37°C for 24 hours and bacterial colonies were observed.

2. Isolation of micro organism from soil:

Fig 1: Bacterial colonies obtained on agar plates

The micro organisms were obtained from serial dilution method, spreaded in minimal glutaminase media. The bacterial colonies were identified after screening of the samples and then further were pure culture and identified by observing their physical characteristics and biochemical mechanisms with the help of different biochemical tests. The bacterial strain thus producing the appropriate type and amount of enzyme was then selected for production of L-glutaminase enzyme on large scale.

3. Screening of the sample:

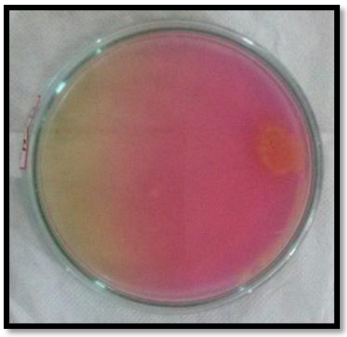

Fig 2: Screening of the sample

Pink colour was observed. It shows that L-glutaminase was being produced by the bacteria, which takes up the glutamic acid present in the media as its substrate and produces ammonia which in turn changes the media alkaline (due to phenol red, as an indicator, that gives pink colour).

4. Colony morphology:

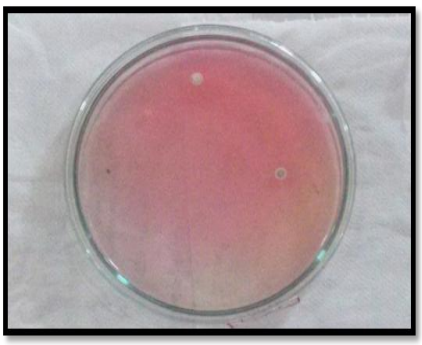

Fig 3: Bacterial plate selected for physical characterization

Table 2: tabulation of the physical characteristics of the bacterial colonies that grew on the agar plate

Sl no	Colony	Form	Elevation	Margin	Colour	Transparency	Size	Surface
1.	C1	Circular	Entire	Raised	White	Translucent	Small	Smooth
2.	C2	Circular	Entire	Raised	White	Translucent	Small	Smooth
3.	C3	Puncti form	Entire	Raised	White	Translucent	Small	Smooth
4.	C4	Circular	Entire	Raised	White	Translucent	Small	Smooth
5.	C5	Circular	Entire	Raised	White	Translucent	Small	Smooth
6.	C6	Circular	Entire	Convex	Yellow	Translucent	Small	Smooth

5. **Pure culture of bacteria:** The strains selected after screening was then pure cultured and the biochemical test was performed on the different cultures obtained.

6. **Gram staining of bacteria:**

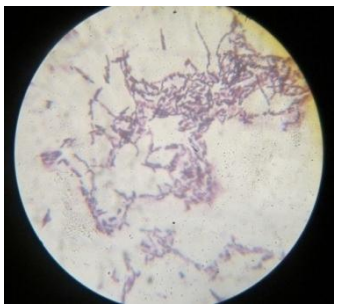

Fig 4: Purple colour; bacillus (rods in chain)

7. Biochemical test:

Table 3: The biochemical test result

Biochemical Test	Strain c1	Strain c2	Strain c3	Strain c4	Strain c5
Methyl Red Test	+	+	+	+	+
Vogas Proskauer Test	-	-	-	-	-
Indole Production Test	-	-	-	-	-
Starch Hydrolysis Test	+	+	+	+	-
Catalase Test	+	+	+	+	+
Urease Agar Test	-	-	-	-	-
Nitrate Reduction Test	+	+	+	+	+
Casein Hydrolysis Test	+	-	+	-	-
Simmon Citrate Agar Test	+	+	+	+	+
Gelatin Hydrolysis Test	-	-	-	-	-
Triple Sugar Iron Test	+	+	+	+	+
Hydrogen Sulphide Test	+	-	-	-	-

8. Production Media:

Fig 5: Production media with glucose (yellow colour) & production media without glucose (pink colour)

CONCLUSION

Glutaminase activity is widely distributed in micro organisms including bacteria, yeast and fungi. The L- glutaminase enzyme isolated from *Bacillus species* is a type of anti-cancer enzyme. L-glutaminase is produced by *Bacillus subtilis* from environments may hold more potential in the treatment of leukemia which is reported to cause allergic reactions. Further their commercial production using *Bacillus subtilis* could make possible its wide application in cancer chemotherapy.

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