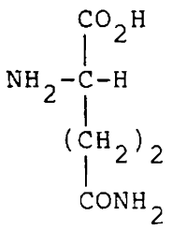
2. MATERIALS AND METHODS

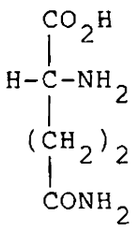
2.1 SUBSTRATE

L-Glutamine (HI-Media) was used as the substrate for growth and enzyme production by L-glutaminase producing bacteria.

Chemical names of glutamine are 2-amino 4-carbomylbutanoic acid and α-amino γ-carbamidobutryic acid. Empirical formula for glutamine is $C_5H_{10}O_3N_2$; C-41.09% H-6.90%; O-32.84% and N-19.17% having a molecular weight of 146.15. Glutamine is found to occur as optical isomers ie., L-Glutamine and D-Glutamine. (Greenstein & Winitz, 1961).



L-Glutamine



D-Glutamine

Wheat gluten hydrolyzate and beet sugar molasses are two natural sources of glutamine.

2.2 ISOLATION OF GLUTAMINASE PRODUCING BACTERIA FROM MARINE ENVIRONMENT

2.2.1 Samples

Both water and sediment samples of marine and estuarine environments of Cochin were screened for isolation of L-glutaminase producing bacteria over a period of six months from July 1988 to December 1988.

2.2.2 Collection of samples

Water samples were collected using sterilized glass containers from the surface region. Sediment samples were collected using Peterson grabs from 1 m depth of water and middle portion of the sediment was aseptically transferred to sterile polythene bags using sterile spatula. The samples were immediately taken to the laboratory (within a period of 2 hours) and processed for bacteriological analysis.

2.2.3 Preparation of media

Since no direct media was reported in literature for the direct isolation of glutaminase producing bacteria

from natural environments, an attempt was made to develop a mineral salts basal medium supplemented with L-glutamine as the sole carbon source.

The composition of the Mineral Salts Glutamine Agar medium (MSGA) after standardisation is as below:

KH_2PO_4	:	0.10 g
$MgSO_4 7H_2O$	:	0.05 g
$NaNO_3$	:	0.01 g
$CaCl_2$	:	0.01 g
$FeCl_3$	:	0.01 g
L-Glutamine	:	1.00 g
NaCl	:	1.00 g
Agar	:	2.00 g
Distilled water	:	100 ml
pH	:	7 ± 0.2

Medium was autoclaved and used.

2.2.4 Plating procedures

Serially diluted water and sediment samples were plated on the Mineral Salts Glutamine Agar (MSGA) medium

under aseptic conditions employing pourplate technique. Plates were incubated at room temperature (28 ± 2°C) for a period of 3-7 days and colony counts were made on the 3rd, 5th and 7th days. All the bacterial colonies developed on MSGA medium were assumed as glutaminase producers since glutamine was the only carbon source in the medium and utilization of glutamine required the presence of glutaminase. L-glutaminase producing bacterial populations are (GPB) expressed in terms of No. ml^{-1} of water and No. g^{-1} of dry weight of sediment.

2.2.5 Isolation and maintenance of cultures

Isolates were subcultured on nutrient agar and after repeated purification, one set of subcultures were stocked under mineral oil (sterilized liquid paraffin was used). Another set of subculture was used as working cultures for further studies. They were maintained at room temperature and subcultured once in two weeks. At regular intervals of one month their purity was checked.

2.2.6 Identification of bacteria

All the isolates were assigned to various genera based on their morphological and biochemical characters

outlined in the Bergey's Manual of Systematic Bacteriology (Bucchanan & Gibbons, 1974). The selected strains of Pseudomonas and Vibrio which were used in the later studies were further identified upto their species level based on the schemes suggested in Bergey's Manual of Systematic Bacteriology (Kreig & Holt, 1984).

2.3 SELECTION OF POTENTIAL L-GLUTAMINASE PRODUCING BACTERIA FOR FURTHER STUDIES

Selection of potential L-glutaminase producing bacteria was carried out in two stages. Initially, all the strains were rechecked for their efficiency to grow in a mineral medium containing L-glutamine as the sole source of carbon. This was performed by inoculating a loopful of 12 hours old agar slope culture into 10 ml of MSG broth (MSGB) (Section 2.2.3) without agar and incubated for 24 hours at room temperature. Turbidity resulted due to the growth of bacteria was measured in terms of OD at 660 nm using a UV-visible spectrophotometer (Hitachi Model 200).

In the second stage of selection, 100 strains, that recorded higher levels of growth in MSG broth were further tested for their enzyme production as detailed below:

2.3.1 Media

Mineral Salts Glutamine Agar (MSGA) medium mentioned earlier (Section 2.2.3) was used as a broth (MSGB) without agar. Fifty ml of MSGB (Mineral Salts Glutamine Broth) taken in 250 ml Erlenmeyer conical flasks were autoclaved and used.

2.3.2 Preparation of inoculum and inoculation procedures

Inoculum for secondary screening was prepared as follows:

1. Initially a loopful of 24 hours old agar slope culture was transferred to 10 ml of NBG (Nutrient Broth added with Glutamine) and grown for 24 hours at room temperature (28 ± 2°C).

2. One ml of the cultured broth was then aseptically transferred into another 50 ml of NBG media and incubated for 24 hours in a rotary shaker (150 rpm) at room temperature (28 ± 2°C).

3. Cells were harvested by centrifugation (MB centrifuge model MB 20) at 5000 rpm for 20 min.

4. The harvested cells were made upto 10 ml volume using physiological saline (0.85% NaCl) after repeated washing with the same.

5. The prepared cell suspension was used as inoculum at 1% level for further inoculation of 50 ml MSGB.

6. All the flasks were uniformly inoculated and incubated on rotary shakers (150 rpm) for a period of 24 hours at room temperature (28 ± 2°C).

2.3.3 Measurement of growth

The growth of bacteria in the MSG broth was followed by estimating the turbidity of the broth by taking the absorbance at 660 nm in a UV-Visible Spectrophotometer (Hitachi Model 200).

2.3.4 Enzyme production

Enzyme production was measured by following the procedure of Imada et al., (1973) with slight modifications. The reaction mixture containing 0.5 ml of enzyme preparation plus 0.5 ml of phosphate buffer (0.2 M) pH 8 (unless

otherwise mentioned) plus 0.5 ml of 0.04 M L-glutamine and distilled water (0.5 ml) to a total volume of 2.0 ml was incubated for 30 min. at 37°C and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloroacetic acid. The precipitated protein was removed by centrifugation at 10,000 rpm for 20 min. To 0.1 ml of this supernatent, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added and the color developed after 10 min. was measured at 450 nm in a UV-Visible Spectrophotometer. Enzyme and substrate blanks were separately included in each assay.

Enzyme production was assessed in terms of enzyme activity which is expressed in terms of international units. One international unit of the enzyme is defined as the amount of enzyme that liberates 1 μ mol of ammonia under optimal assay conditions.

2.3.5 Determination of enzyme protein

Enzyme protein was measured according to the method of Lowry et al., (1951) using Folin Ciocaltaeu's reagent

2.3.6 Selection of strains

All the 100 strains were ranked in terms of their level of enzyme production and the top four ranked strains were used for further studies.

2.4. GROWTH STUDIES

Growth studies were carried out for the four selected strains of bacteria belonging to the species of Pseudomonas and Vibrio (two each) as detailed below:

2.4.1 Optimization of growth conditions for maximal enzyme production by bacteria

Optimal conditions required for maximal growth and L-glutaminase production by the selected strains were determined by subjecting them to various incubation temperatures, different levels of pH, substrate concentrations, NaCl concentrations, additional carbon and nitrogen sources, glucose concentrations, inoculum concentrations in the growth media and different incubation periods.

2.4.1.1 Media

Mineral salts glutamine broth with the composition mentioned earlier (Section 2.2.3) was used for these studies

unless otherwise stated. The prepared media were dispensed in 100 ml aliquots in 250 ml Erlenmeyer conical flasks, autoclaved and used for optimization studies.

2.4.1.2 Preparation of inoculum and inoculation procedures

The procedure described in preveious section (2.3.2) was followed for the preparation of inoculum and inoculation of media. Concentration of inoculum was at 1% level unless otherwise stated.

2.4.1.3 Measurement of growth

Growth was measured according to the procedures mentioned in section 2.3.3.

2.4.1.4 Enzyme production

Enzyme production in the media was estimated in terms of enzyme activity as per procedures mentioned in section 2.3.4 except for the incubation temperature as 40°C and incubation time as 15 min. instead of 30 min. since it was observed that maximal amounts of enzyme units are obtained under these conditions. pH of the buffer varied from 6 to 8 according to the optimum pH of enzyme from each organism.

2.4.1.5 pH

Optimal pH for maximal growth and enzyme production was determined by subjecting the organisms to various pH levels (pH ranging from 4-11) adjusted in the culture broth (MSGB) using 1 N NaOH or 1 N HCl. After inoculation and incubation for 24 hours at room temperature (28 ± 2°C), the culture broths were centrifuged and growth and enzyme production were determined as per the procedures described under sections 2.3.3 and 2.4.1.4.

2.4.1.6 Temperature

Optimal temperature for maximal growth and enzyme production was estimated by incubating the MSG broth inoculated with the test strains at various temperatures (15, 25, 30, 35, 45 and 55°C) for a total period of 24 hours. Growth and enzyme production were determined as per the procedures mentioned earlier (Section 2.3.3 and 2.4.1.4).

2.4.1.7 Substrate concentration

Optimal substrate concentration that favours growth and enzyme production of the strains was checked by growing them in MSG broth supplemented with different

glutamine concentrations (0.25, 0.5, 1, 2, 3%). After 24 hours of incubation at room temperature (28 ± 2°C) the growth and enzyme production were estimated as per the procedures mentioned under sections 2.3.3 and 2.4.1.4).

2.4.1.8 NaCl concentration

Optimal NaCl concentration that promotes maximal growth and enzyme production of the organisms was determined by subjecting them to different NaCl concentrations (0, 1, 3, 5, 7 and 10%) adjusted in the MSG broth. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were analyzed according to the procedures described under sections 2.3.3 and 2.4.1.4).

2.4.1.9 Carbon sources

Requirement of additional carbon sources other than glutamine for enhanced enzyme yield was tested by the addition of glucose, galactose, starch, maltose, lactose, Na_2CO_3 and trisodium citrate in the MSG broth at 1% level. After 24 hours of incubation at room temperature (28 ± 2°C), growth and enzyme production were estimated (Sections 2.3.3 and 2.4.1.4).

2.4.1.10 Nitrogen sources

Requirement of additional nitrogen sources other than glutamine for enhanced growth and enzyme production was estimated by the addition of various nitrogen sources viz., peptone, beef extract, yeast extract, glutamic acid, lysine, $NaNO_3$ and KNO_3 at 1% concentration in the MSGB. After 24 hours of incubation at room temperature growth and enzyme production were estimated as mentioned earlier (Section 2.3.3 and 2.4.1.4).

2.4.1.11 Glucose concentration

Since glucose was found to enhance enzyme production during the studies, optimal requirement of glucose level in the culture medium was estimated by incorporating different concentrations of glucose (0.5, 1, 2, 3%) along with 1% glutamine in the MSGB. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were estimated (Section 2.3.3 and 2.4.1.4).

2.4.1.12 Inoculum concentration

Optimal inoculum size that yields maximal growth and enzyme production was determined in MSG broth at their

optimal growth conditions determined earlier by inoculating the broths with various levels of the prepared medium (1-7%). After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production in the media were estimated according to the procedures mentioned under sections 2.3.3 and 2.4.1.4).

2.4.1.13 Incubation time

Optimal incubation time that leads to maximal growth and enzyme production of the strains was estimated by incubating culture flasks for various incubation periods upto a maximum of 48 hours. Growth and enzyme production in the broths were estimated according to the procedures described under sections 2.3.3 and 2.4.1.4.

2.4.2 Growth curve

Growth curve studies for all the four strains of bacteria were carried out in nutrient broth (HI-media) and MSG broth at the optimal conditions standardized earlier. The prepared media were dispersed in 50 ml aliquots in 250 ml conical flasks, autoclaved and inoculated with 0.5 ml of the prepared inoculum and incubated at room temperature (28 ± 2°C). Samples were drawn at regular intervals and

growth was determined by measuring the turbidity at 660 nm in a UV-Visible Spectrophotometer besides enumeration of TVC on plates employing pourplate technique. From the results obtained growth curve was constructed.

2.5 COMPARISON OF INTRACELLULAR AND EXTRACELLULAR GLUTAMINASE PRODUCTION BY BACTERIA

A comparative study was made, on the extracellular and intracellular glutaminase production by the strains, by cultivating them in nutrient broth (HI-media) with and without the addition of glutamine and in MSG broth (composition mentioned earlier under section 2.3.1). Inoculation and incubation procedures were as detailed earlier under section 2.3.2. After 24 hours of incubation the cells were harvested by centrifugation at 5000 rpm for 20 min. and the supernatent was partially purified by $(NH_4)_2SO_4$ fractionation followed by dialysis and analyzed for extracellular glutaminase activity as per the procedures mentioned earlier under section 2.4.1.4.

Intracellular glutaminase production by cells was tested as stated below. The harvested cells were subjected to osmotic shock using 30% sucrose solution (Cedar & Schwartz, 1967) which resulted in cell lysis and liberation

of enzymes. The lysed cell suspension was centrifuged as mentioned above and the supernatent was fractionated with $(NH_4)_2SO_4$ followed by dialysis and later tested for glutaminase activity as mentioned under section 2.4.1.4.

2.6 ENZYME STUDIES

2.6.1 Isolation of enzymes

2.6.1.1 Media

The enzyme production medium (EPM) was designed based on the data obtained from the studies conducted for optimization of growth conditions for maximal enzyme production in MSG broth. The final composition of EPM after standardisation is as follows:

KH_2PO_4	:	0.10 g
$MgSO_4 7H_2O$	:	0.05 g
$NaNO_3$	:	0.01 g
$CaCl_2$	:	0.01 g
$Na_3C_6H_5O_7 2H_2O$	:	0.01 g
NaCl*	:	1 g
Glucose	:	0.5%
Glutamine	:	1%
pH	:	6 ± 0.2
Distilled water	:	100 ml

* For V.cholerae, no NaCl was used

Prepared medium was autoclaved and used.

2.6.1.2 Preparation of inoculum

a) A loopful of 18 hours old slope culture was transferred aseptically to 10 ml of EP medium and incubated for 24 hours at room temperature (28 ± 2°C).

b) One ml of the 18 hours old culture was then transferred to 50 ml of EP medium and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for a further period of 24 hours.

c) Cells were harvested by centrifugation at 5000 rpm for 20 min. washed repeatedly with physiological saline and suspended in 10 ml of the same saline.

d) The prepared cell suspension was adjusted to a concentration of 1×10^6 cells ml^{-1} and used as inoculum.

2.6.1.3 Enzyme production in the medium

Hundred ml of EP medium taken in 1000 ml conical flask was inoculated with 3 ml of the prepared inoculum aseptically and incubated on a rotary shaker (150 rpm) at 35 ± 2°C for 18 hours. Later the culture broth was centrifuged at 5000 rpm for 20 min. at 4°C and the cell free extract was used as the crude enzyme for further studies.

2.6.2 Enzyme assays

2.6.2.1 Buffers

Different buffers with various pH ranges were used for the assay of glutaminase. They included acetate buffer (0.2 M) with a pH ranging from 4 to 5, phosphate buffer (0.2 M) pH 6 to 8 and glycine-NaOH buffer (0.2 M) of pH 9-11.

2.6.2.2 Determination of enzyme activity

Enzyme activity was measured according to the procedure mentioned earlier (Section 2.4.1.4).

2.6.2.3 Determination of enzyme protein

Enzyme protein was estimated as per the procedure outlined under section 2.3.5.

2.6.3 Purification of enzymes

Enzyme purification was carried out following the methods suggested by Hartman (1968) and Roberts (1976). The cell free extract obtained after centrifugation (Section 2.6.1.3) was purified by $(NH_4)_2SO_4$ fractionation followed by dialysis and ion exchange chromatography.

2.6.3.1 $(NH_4)_2SO_4$ fractionation

The enzyme was purified by $(NH_4)_2SO_4$ precipitation. It was done by adding $(NH_4)_2SO_4$ (Sisco-enzyme grade) slowly and increasing the concentration upto 50-80% saturation along with continuous stirring using a magnetic stirrer, at 4°C, in an ice bath. The precipitate obtained for each saturation was removed by centrifugation at 5000 rpm for 30 min. and dissolved in phosphate buffer (0.2 M) of pH 6 or 8, (varied with organism). Enzyme activity and protein content of each fraction was determined according to the procedures mentioned in sections 2.4.1.4 and 2.3.5.

2.6.3.2 Dialysis

The precipitate obtained after $(NH_4)_2SO_4$ fractionation was dissolved in phosphate buffer (0.2 M) (pH 6 or 8) and dialyzed against the same buffer extensively at 4°C for 24 hours. Enzyme activity and protein content of the dialyzate were determined according to the procedures mentioned under sections 2.4.1.4 and 2.3.5.

2.6.3.3 Chromatography upon seralite SRA-400

The dialyzate was further purified by anion exchange chromatography, upon seralite SRA-400.

1. A strongly anionic exchange resin (seralite SRA-400, SRL) was packed in a column (5 x 30 cm) and kept at 4°C.

2. The packed column was equilibrated with sodium phosphate buffer of pH 8 (0.2 M) containing 1 mM EDTA.

3. The dialyzate was adjusted to the pH 8 with dilute NaOH (0.1 M) and added to the column.

4. The column was washed with sodium phosphate buffer of pH 8 (0.2 M) containing 1 mM EDTA.

5. Elution was carried out using the sodium phosphate buffer (0.2 M) of pH 8 containing different concentrations of NaCl (0.1, 0.2, 0.3, 0.4 M) and 1 mM EDTA in a sequential manner along with increasing concentration of NaCl in buffer. Flow rate was adjusted to 5 ml/10 min. and the fractions of 20 ml were collected using Redifrac fraction collector (Pharmacia). Each fraction was analyzed for enzyme activity and protein as mentioned earlier (Sections 2.4.1.4 and 2.3.5). The fractions with higher specific activities were pooled and concentrated using ammonium sulphate (50-60%) at 4°C (Section 2.6.3.1). The precipitate obtained was dissolved in a minimal volume of phosphate buffer of pH 6 to 8 (0.2 M) and dialyzed as per the procedure mentioned in section 2.6.3.2.

2.6.3.4 Chromatography upon seralite SRA-120

The dialyzate obtained after anion exchange chromatography was further purified by cation exchange chromatography using seralite SRA-120.

1. A cationic exchange resin (Seralite SRA-120, SRL) was packed into a column (5x30 cm) and kept at 4°C.

2. The packed column was equilibrated with sodium phosphate buffer (pH 6), 0.2 M containing 1 mM EDTA.

3. The dialyzate was adjusted to pH 6 with dilute HCl (0.1 N) and added to the column.

4. The column was washed with sodium phosphate buffer of pH 6 (0.2 M) containing 1 mM EDTA.

5. Column was eluted with sodium phosphate buffer (0.2 M) of pH 6 containing 1 mM EDTA and various concentrations of NaCl (viz., 0.1, 0.2, 0.3, 0.4 M) in a sequence of increasing concentration of NaCl. Flow rate was adjusted to 5 ml/10 min. and 20 ml fractions were collected. Each fraction was analyzed for enzyme activity and protein

according to the procedures mentioned under sections 2.4.1.4 and 2.3.5. Active fractions were pooled, and concentrated and stored at 4°C and used for further characterization studies.

2.6.4 Characterization of glutaminase

The purified enzyme was characterized for its activity at various pH, temperature, substrate concentration, incubation time, NaCl concentration, substrate specificity and inhibition by heavy metals and other substances. The stability of the enzyme at different pH and temperatures was also studied.

2.6.4.1 Effect of pH on activity and stability of the enzyme

Effect of pH on the activity of the enzyme was studied as per the following procedure.

A mixture containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of 0.04 M glutamine was incubated with 0.5 ml of buffers of different pH ranging from pH 4-10 (prepared using acetate buffer pH 4-5), phosphate

buffer (pH 6-8) and glycine--NaOH buffer (pH 9-10) for 15 min. at 40°C. Later the enzyme activity in the reaction mixture was determined as mentioned earlier (Section 2.4.1.4).

Stability of the enzyme at various pH (viz., pH 4-10) was determined by incubating 1 ml of enzyme with 1 ml of buffers for 1 hour (prepared with acetate buffer pH 4-5, phosphate buffer pH 6-8, glycine--NaOH buffer pH 9-10).

A mixture containing 0.5 ml of this treated enzyme solution, 0.5 ml of distilled water, 0.5 ml of phosphate buffer of pH 6 or 8 (0.2 M) was incubated with 0.5 ml of 0.04 M glutamine for 15 min. at 40°C. Enzyme activity remained in the reaction mixture was determined according to the procedure mentioned under section 2.4.1.4.

2.6.4.2 Effect of temperature on activity and stability of enzyme

Effect of temperature on the activity of the enzyme was estimated according to the following procedure.

0.5 ml of purified enzyme added with 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate

pH (pH 6 or 8), (0.2 M) was incubated with 0.5 ml of 0.04 M glutamine at different temperatures (30, 40, 50, 60 and 70°C) for 15 min. Later the enzyme activity in the reaction mixture was determined according to the procedures mentioned under section 2.4.1.4.

The stability of the enzyme at various temperatures was determined by incubating 0.5 ml of the purified enzyme with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8), (0.2 M) at different temperatures of 30, 40, 50, 60 and 70°C for 1 hour.

The treated enzyme buffer mixture was later incubated with 0.5 ml of distilled water and 0.5 ml of glutamine for 15 min. at 40°C. Enzyme activity remaining in the reaction mixture was estimated following the procedures mentioned under section 2.4.1.4.

2.6.4.3 Effect of substrate concentration on activity of the enzyme

Effect of substrate concentration on the activity of purified enzyme was determined by incubating 0.5 ml of enzyme with 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) with

different concentrations of glutamine (viz., 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.06 M, 0.08 M and 1 M) for 15 min. at 40°C. Reaction mixture was analyzed for glutaminase activity as per the procedures mentioned under the section 2.4.1.4). Michaelis - Menten constants (Km) of enzymes were evaluated from Line weaver - Burk plots of the data.

2.6.4.4 Effect of incubation time on activity of the enzyme

Effect of incubation time on the rate of hydrolysis of glutamine by purified enzyme was determined according to the following procedure.

Different aliquots of reaction mixture containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) was incubated with 0.5 ml of 0.04 M glutamine at 40°C for different time intervals of 3, 5, 10, 15, 20 and 30 min. Enzyme activity in the reaction mixture was estimated as per the procedures mentioned under section 2.4.1.4.

2.6.4.5 Effect of NaCl concentration on activity of the enzyme

Effect of NaCl concentration on the activity of enzyme was estimated by incubating the reaction mixture

containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of phosphate buffer of pH 6 or 8 (0.2 M) and different concentrations of NaCl (viz., 0, 1, 3, 5, 7, 10, 15, 20 and 25%) for 15 min. at 40°C. Glutaminase activity in the reaction mixture was determined as per the procedures mentioned under section 2.4.1.4.

2.6.4.6 Determination of substrate specificity

0.5 ml of purified enzyme solution was incubated with 0.5 ml of distilled water, 0.5 ml of phosphate buffer (0.2 M) of appropriate pH (pH 6 for all strains except P.fluorescens ACMR 171 for which pH 8 was used. Buffer with pH 7.5 was used for all strains when tested with asparagine) and 0.04 M of asparagine, glutamine, glutamine plus asparagine separately for 15 min. at 40°C. Later enzyme activity was estimated following the procedures mentioned under section 2.4.1.4.

2.6.4.7 Effect of heavy metals on the activity of enzyme

0.5 ml of purified enzyme was incubated with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) and 0.5 ml of glutamine and 0.5 ml of 1 mM of heavy metals such as Pb, Co, Mn, Hg, Cu, Fe, Ag, Ca and Zn separately

at 40°C for 15 min. Glutaminase activity in the reaction mixture was measured according to the procedures mentioned under section 2.4.1.4.

2.6.4.8 Effect of other substances activity of enzyme

Effect of other substances including EDTA, phosphate, borate and tris ions, aspartic acid, glutamic acid, phenolphthalein, bromocresol purple and α-keto glutarate on the activity of the enzyme was determined by the following procedure.

0.5 ml of purified enzyme was incubated with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) plus 0.5 ml of the above mentioned substances at different concentrations (phosphate at 0.4 M; borate and tris ion at 0.2 M; glutamate and aspartate at 30 mM, EDTA at 0.1 mM, α-ketoglutarate at 2 mM, phenolphthalein and bromocresol purple at 1 mM) and 0.5 ml of 0.04 M glutamine at 40°C for 15 min. Enzyme activity was estimated as per the procedures mentioned under section 2.4.1.4.

2.7 FERMENTATIVE PRODUCTION OF GLUTAMINASE BY SOLID STATE FERMENTATION (SSF)

2.7.1 Preparation of solid substrate

Commercially available wheat bran of <0.24 cm particle size was used in the solid state fermentation (SSF)

studies for the production of glutaminase. However the exact size of particles were optimised first and later that sized particles were used in all the later studies. Composition of wheat bran is shown in Table 1. The wheat bran medium for SSF was prepared as detailed below: (Ramesh, 1989).

Hundred gram of wheat bran taken in 1000 ml Erlenmeyer conical flask was thoroughly mixed with MSG solution (Mineral Salts Glutamine Solution - composition as given under section 2.3.1 but for the volume of distilled water. Volume of the water was separately standardised to give 60% moisture content to the final wheat bran medium) and the flask was autoclaved for 60 min. and allowed to cool down to room temperature (28 ± 2°C).

2.7.2 Inoculation and incubation

The prepared inoculum (Section 2.6.1.2) was adjusted to a ratio of 20 mg dry cell equivalent/100 g wheat bran (Ramesh, 1989) and added to the sterilized moist wheat bran in the flasks. The contents were mixed thoroughly and the flasks were incubated in a slanting position at 35°C in an incubator with 65-70% relative humidity (Ramesh & Lonsane, 1987a) for 24-28 hours.

2.7.3 Extraction and recovery of enzyme

2.7.3.1 Optimization of extraction parameters

Extraction of glutaminase from bacterial wheat bran (BWB) after SSF was optimized for maximal enzyme recovery which included drying temperature of BWB, extraction medium, buffer system, pH of extraction, ratio of bran to buffer, extraction temperature and contact time were optimized (Kumar & Lonsane, 1987) as detailed below:

2.7.3.1.1 Drying temperature

Effect of drying temperature on enzyme recovery was studied by drying the BWB at various temperatures of 30°C, 40°C and 50°C for 1 hour.

Later the enzyme was extracted under arbitrarily selected conditions including 0.2 M phosphate buffer of appropriate pH (pH 6 or 8, varied with the organism) in a 1 : 5 ratio (bran to buffer) and a contact time of 90 min. at 30°C. It was carried out in two stages. In the first stage the BWB was contacted with 300 ml of buffer for 60 min. with occasional stirring. Then the slurry was squeezed through a dampened cheese cloth. In the second stage the left over solids were again contacted with another

200 ml of the same buffer for another 30 min. and later the slurry was squeezed as mentioned earlier. The extracts were pooled and centrifuged for 20 min. at 8000 rpm in a refrigerated centrifuge (Ramesh & Lonsane, 1990). The cell free clear extract obtained after centrifugation was used for estimation of enzyme activity (Section 2.4.1.4).

2.7.3.1.2 Extraction medium

Appropriate medium which shall yield maximal enzyme recovery on extraction was determined using ethanol, distilled water, distilled water plus 1% NaCl, tap water and 0.2 M phosphate buffer of appropriate pH (pH 6 or 8). Enzyme extraction and estimation of enzyme activity were carried out as per the procedures mentioned earlier under sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.3 pH of extraction media

Effect of pH of the buffer used for extraction were determined by using buffers of pH ranging from 5-8 (Acetate buffer, 0.2 M of pH 5 phosphate buffer 0.2 M, of pH 6-8). Enzyme recovery and estimation of enzyme activity were done according to the procedures mentioned under section 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.4 Different buffer systems

Influence of different buffer systems on the enzyme recovery was analyzed by using 0.2 M acetate buffer of pH 6, phosphate buffer of pH 6, phosphate buffer of pH 8 (0.2 M), borate buffer of pH 8 (0.2 M) according to the optimum pH. Enzyme extraction and enzyme activity estimation were carried out as per the procedures mentioned in sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.5 Ratio of bran to buffer

Effect of bran to buffer ratio on the enzyme recovery was determined by adding buffer to fermented BWB in different ratios of 1:3, 1:5 and 1:10. Enzyme recovery and estimation of enzyme activity were carried out as per the procedures mentioned under section 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.6 Effect of contact time

Effect of contact time of buffer with fermented BWB was tested by contacting the BWB with buffer for various time intervals of 30, 60, 90, 120 min. Enzyme recovery and estimation of enzyme activity were done according to the procedures mentioned in sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.7 Effect of contact temperature

Effect of contact temperature on enzyme recovery was determined by keeping BWB and buffer in contact at different temperatures of 25, 35, 45°C. Enzyme recovery and estimation of enzyme production were performed by following the procedures mentioned under sections 2.7.3.1.1 and 2.4.1.4.

2.7.4 Effect of operational parameters on enzyme production by SSF

Effect of pH, temperature, moisture content, substrate concentration, NaCl concentration, inoculum size, particle size of wheat bran and various incubation time on enzyme production by SSF was studied as detailed below:

2.7.4.1 Measurement of enzyme production

Enzyme production was determined according to the procedures mentioned earlier under section 2.4.1.4.

The enzyme units were calculated according the methods suggested by Ramesh (1989) for the total volume of the extract after centrifugation divided by the initial weight of wheat bran (in g) gives the units of enzyme per gram of commercial wheat bran. The enzyme units are expressed per gram of commercial wheat bran.

2.7.4.2 Effect of particle size of wheat bran

The effect of particle size of wheat bran on enzyme production during SSF was determined by using wheat bran of different particle size. Commercial wheat bran was graded into various fractions of their particle size using sieves of mesh size ranging from 7-14. The fractions were having particle size of greater than 2.41 mm, 1.41-2.06 mm and less than 1.20 mm. Inoculation and incubation, enzyme recovery and enzyme estimation were carried out as mentioned earlier. (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.3 Effect of moisture content of WB medium

Hundred gram of commercial wheat bran of optimised particle size was weighed and distributed in 1000 ml Erlenmeyer conical flasks. The moisture content of the WB medium was adjusted to various levels ranging from 20-60% (w/w) by varying the water content of the MSG solution. Inoculation and incubation were carried out as mentioned earlier (section 2.7.2). After 48 hours of incubation the contents in the flasks were subjected to analysis for estimation of enzyme production (Sections 2.7.3.1.1 and 2.4.1.4)

2.7.4.4 Effect of pH

Effect of pH on the enzyme production by SSF was determined by subjecting the organisms to various pH levels adjusted in the MSG solution from pH 4-10 (Section 2.3.1). Inoculation, incubation, enzyme extraction and estimation of enzyme activity were carried out following the procedures mentioned earlier under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.5 Effect of temperature

Effect of temperature on enzyme production by SSF was determined by incubating the inoculated wheat bran medium (Section 2.7.1) at different temperatures of 25, 35, 45 and 55°C. Inoculation, incubation, enzyme recovery and estimation of enzyme production were done according to the procedures mentioned in section 2.7.2, 2.7.3.1.1, and 2.4.1.4.

2.7.4.6 Effect of substrate concentration

Effect of substrate concentration on enzyme production by SSF was determined at different substrate concentrations (0.25, 0.5, 1, 2, 3%) in the MSG solution added to the wheat bran. Inoculation, incubation, enzyme recovery and estimation of enzyme production were carried out according to the procedures mentioned in sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.7 Effect of NaCl concentration

Effect of NaCl concentration on enzyme production by SSF was carried out by adjusting the NaCl concentration of MSG solution to various levels of 0, 1, 3, 5, 7% and subjecting the organisms for enzyme production. Inoculation, incubation, enzyme extraction and estimation of enzyme production were done according to the procedures mentioned earlier in sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.8 Effect of carbon sources

Effect of additional carbon sources on enzyme production by SSF was determined by incorporating carbon sources viz., glucose, galactose, maltose, starch, lactose, Na_2CO_3 and trisodium citrate at 1% concentration level to the WB medium (Section 2.7.1). Inoculation, incubation, enzyme extraction and estimation of enzyme production were done according to the procedures mentioned under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.9 Effect of nitrogen sources

Influence of extra nitrogen sources on enzyme production by SSF was determined by the addition of beef extract, yeast extract, peptone, glutamic acid, lysine, KNO_3

and $NaNO_3$ at 1% level separately to the wheat bran medium (Section 2.7.1). Inoculation, incubation, enzyme recovery and estimation of enzyme production were carried out as per the procedures mentioned earlier (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.10 Effect of inoculum concentration

Effect of inoculum concentration on enzyme production by SSF was determined by using different levels of inoculum. Inoculum ratio was adjusted to 10, 20, 30 and 40 mg dry cell equivalents/100 g of wheat bran. Inoculation, incubation, enzyme recovery amd estimation of enzyme production were carried out according to the procedures mentioned earlier (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.11 Effect of incubation time

Effect of incubation time on enzyme production by SSF was determined by incubating the inoculated wheat bran medium (Section 2.7.1) for a total period of 48 hours and estimating the enzyme production at regular intervals of 6 hours. Inoculation, incubation, extraction of enzyme and enzyme production were estimated according to the procedures mentioned under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.