Materials and Methods
3. MATERIALS AND METHODS

3.1 ISOLATION OF *ASPERGILLUS TERREUS* STRAINS FROM SOIL SAMPLES

Soil samples collected from various places of Gulbarga were used for the isolation of *Aspergillus terreus* strains as per the method of Seifert (1990). The isolated (Plate - 1) strains were tentatively identified in the laboratory as described by Rapper and Fennell (1965) and were maintained on potato dextrose agar (PDA) slants. Further, the tentatively identified strains were labeled serially as KLS1 to KLS35.

3.2 RAPID PLATE ASSAY FOR SCREENING OF L-ASPARAGINASE PRODUCERS

The strains obtained from the above steps were subjected for rapid screening of L-asparaginase production by plate assay (Plate - 2) as per Gulati et al. (1997).

The modified Czapek Dox’s medium was supplemented with phenol red (2.5% prepared in ethanol and the pH was adjusted to 7.0) dye. The media was autoclaved and plates were prepared. Control plate was maintained without asparagine (instead containing NaNO₃ as nitrogen source). The plates were inoculated with *Aspergillus terreus* strains isolated from the soil. The zone and colony diameter was measured after 48 hrs. The isolate, which has given maximum zone of clearance, has been selected for further study.
Plate – 1: *Aspergillus terreus*
PLATE – 1
Plate – 2: Rapid plate assay for screening of L-asparaginase producers

a. Control

b. Assay plate
3.3 IDENTIFICATION OF STRAINS

The strain which produced maximum zone of diameter \((A. terreus KLS2)\) on rapid plate assay was sent to Agarkar Research Institute, Pune for further identification and confirmation. The strain was identified and confirmed as \(Aspergillus terreus\) Thom Var. Therefore, further studies were conducted by employing \(A. terreus\) KLS2 strain for the production of L-asparaginase under solid state fermentation.

3.4 COLLECTION OF SUBSTRATES

In order to produce good amount of L-asparaginase through solid state fermentation various substrates having sufficient amount of carbohydrates and proteins have been chosen. The substrates like rice chaff (husk), pigeon pea waste, groundnut cake, banana wastes and carob pods were collected (Plate – 3). All the above mentioned agro based materials except carob pods were collected from local places in Gulbarga. The carob pods were collected from the carob tree \((Ceratonia siliqua)\).

3.5 SCREENING OF AGROBASED WASTE SUBSTRATES FOR L-ASPARAGINASE PRODUCTION THROUGH SOLID STATE FERMENTATION

20 g of each of the above substrates rice chaff (husk), pigeon pea waste, banana waste, groundnut cake and carob pods were taken in 250 ml conical flask and rehydrated to 65% of moisture content by adding sterile distilled water for all flasks. The cotton plugged flasks were autoclaved at 121°C for 15 min and allowed to cool at
Plate – 3: Collection of substrates for solid state fermentation

1. Rice chaff
2. Pigeon pea waste
3. Ground nut cake
4. Banana waste
5. Carob pods
room temperature. The contents of the flasks were inoculated with 1 ml of inoculum
(1 x 10^7 spores/ml). The flasks were mixed thoroughly by gently beating on the palm
of the hand and incubated in a slanting position at room temperature for 7 days. After
incubation (Plate – 4) the moldy substrates were analyzed for L-asparaginase
production.

Further, the results indicate that the maximum L-asparaginase production in
deseeded carob pods and hence the studies were continued with carob pods as
suitable substrate for the production of L-asparaginase and the detailed study of carob
pod is explained in detailed in next steps.

3.6 BIOCHEMICAL ANALYSIS OF CAROB POD

Carob pods (Plate – 5) were subjected to deseeding process the kibbles (pods)
were chopped into fine powder using waring blender at high speed. Thus obtained
powder of carob substrate was analyzed for the following biochemical constituents.

3.6.1 Estimation of moisture content

About 20 g of pulverized particles of carob substrate was taken in a clean
petridish and weighted. The initial weight of deseeded carob substrate along with
petridish was noted and the same was dried in an oven at 60°C for 4-6 hrs. Care
should be taken to avoid the charring of substrate. After drying, the petridishes
were cooled in a desiccator and final weight was taken. The difference in weight
obtained after drying process was taken as the moisture content of the substrate
(Ranganna, 1977).

3.6.2 Estimation of total carbohydrates

100 g of powdered carob substrate was taken in a tube and hydrolyzed with
Plate – 4: Moldy growth on the substrate
PLATE – 4
Plate – 5: Carob pod’s (*Ceratonia siliqua*)
PLATE – 5
5 ml of 2.5 N HCl by keeping it in a boiling water bath for 3 hrs. Later it was cooled to room temperature and neutralized with sodium carbonate until the effervescence of liquid ceases. Then the volume was made up to 100 ml by adding distilled water and centrifuged to collect the supernatant for analysis of total carbohydrate content as per the method of Dubios et al. (1956).

3.6.3 Estimation of protein content

The deseeded 5000 mg of carob powder was ground with 5-10 ml Tris-HCl buffer and centrifuged. The supernatant obtained was used for protein estimation by using Folin-Ciocalteaue reagent as described by Lowry et al. (1951). Bovine serum albumin was used as the standard protein. The colour developed was read at 660 nm in a Systronic 505 spectrophotometer.

3.6.4 Estimation of soluble fats

The deseeded 100 g of carob powder was extracted with 75 ml petroleum ether for 16 hrs in a Soxhlet apparatus. The extract obtained was decanted into a small, dry and pre-weighed beaker and the residual ether content was evaporated on a heating pan kept at 30°C. The difference in the weights of the empty beaker alone and after evaporation of the ether gives the amount of soluble fat material in the sample.

3.6.5 Estimation of trace elements

The carob pod husk (10 g) was treated with 50 ml of 1:1 HCl and decomposed in an Erlenmeyer flask by heating and if necessary HNO₃ was added for further decomposition. The decomposed samples were filtered through Whatman No. 1 filter paper and the filtrate was analyzed for determination of trace metals by an atomic absorption spectrophotometer (Model Thermo Jarvell Ash, USA Smith-Hieftje-1000).
as described in AOAC (1975).

3.7 PRODUCTION OF L-ASPARAGINASE THROUGH SOLID STATE FERMENTATION

Solid state fermentation (SSF) was carried out using deseeded carob pod as substrate in the present study by employing *Aspergillus terreus* KLS2 strain. Hence a detailed methodology of SSF process for L-asparaginase production was mentioned herein.

3.7.1 Preparation of Inoculum

Spore suspensions were prepared from 168 hrs., old cultures grown on PDA slants by adding 10ml of sterile distilled water containing 0.01% Tween–80 and suspending the spores with a sterile loop. One ml of the spore suspension containing about 1 x 10^7 spores/ml was used to inoculate experimental media in the flasks (Lingappa and Vivek Babu, 2004).

3.7.2 Preparation of substrates for solid state fermentation

The deseeded carob pods were dried at 52^oC for 6 hrs in an oven. Then the kibble was chopped into small particle size of 2 mm and pulverized in a waring blender at high speed. Thus obtained carob substrate was analyzed for total fermentable sugars and pH was adjusted to 4.5 units as described by Roukas (1994). Then the required amount of substrate was taken in a 250 ml Erlenmeyer flask and rehydrated using distilled water to get desired moisture level (Lingappa and Vivek Babu, 2004). The cotton-plugged flasks were autoclaved at 121^oC for 15 min and allowed to cool.
3.7.3 Solid state fermentation methodology

SSF was carried out in 250 ml Erlenmeyer flasks. The flasks containing 20 g (prepared and pretreated deseeded carob pods) substrate were autoclaved at 121°C for 20 min and cooled to room temperature. Then the flasks were inoculated with one ml of spore suspension and the contents were thoroughly mixed by gentle tapping. Thus prepared flasks were kept in slanting position and incubated for a period of 7 days at 35°C in a humidity chamber (65-70% relative humidity) (Lingappa and Vivek Babu, 2004).

3.7.4 Extraction of fermented substrate

The samples were with drawn periodically at 24 hrs in aseptic condition 1 gm of moldy substrate was taken into a beaker and distilled water (1:10) was added to it. The contents of flasks were allowed to have contact with water for 1 hr with occasional stirring with a glass rod. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged. The supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay.

3.7.5 Assay of L-asparaginase from crude extract

Assay of enzyme was carried out as per Imad et al. (1973). 0.5 ml of 0.04 M asparagine was taken in a test tube, to which 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added
to 3.7 ml distilled water and to that 0.2 ml Nessler’s reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit.

3.7.6 **International Unit (IU)**

One IU of L-asparaginase is the amount of enzyme which liberates 1 μmol of ammonia per minute per ml [μ mole/ml/min].

3.8 **OPTIMIZATION OF FERMENTATION PARAMETERS FOR L-ASPARAGINASE PRODUCTION**

The production of L-asparaginase under SSF mainly depends on various factors like initial moisture content, pH, temperature, inoculum size, particle size and bed depth. Hence, these parameters must be optimized in order to achieve higher yields of L-asparaginase. During this optimization process, once a particular parameter was optimized, the same optimum condition of that specific parameter was employed in the subsequent studies wherein another parameter is to be optimized.

3.8.1 **Effect of initial moisture content on L-asparaginase production**

A set of conical flasks containing 20 g of substrate (2 mm size) were moistened with an appropriate amount of distilled water in order to obtain different moisture levels like 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75%. The contents were autoclaved at 121°C for 20 min., and inoculate each flask with one ml of spore inoculum of *A. terreus* KLS2. Thus prepared flasks were mixed thoroughly and incubated in a humidity chamber (60-75%) at 35°C temperature for a period of 7 days (Lingappa and Vivek Babu, 2004).
3.8.2 Effect of initial pH on L-asparaginase production

The flasks containing 20 gm of substrate were mixed with acid/alkali moistening solution to obtain required pH. The pH was adjusted in the range of 3-7 with increments of 0.5. Thus prepared flasks were cotton plugged and autoclaved at 121°C for 15 min. The flasks were inoculated and incubated as described by Lingappa and Vivek Babu (2004).

3.8.3 Effect of initial temperature on L-asparaginase production

The ground substrates about 20 g were taken separately in 250 ml Erlenmeyer flasks and prepared for solid state fermentation as described by Lingappa and Vivek Babu (2004). Thus prepared flasks were incubated at different temperatures like 25, 30, 35 and 40°C.

3.8.4 Effect of initial bed depth on L-asparaginase production

The substrate was taken in 250 ml beaker and adjusted to bed height of 10, 20, 30, 40 and 50 mm. Thus prepared media were inoculated and incubated as described earlier.

3.8.5 Effect of initial particle size on L-asparaginase production

The ground substrates were sieved through sieves having different sizes, like 2, 4 and 6 mm and each particle size was separately used in studies.

3.8.6 Effect of inoculum size on L-asparaginase production

The inoculum was prepared separately at different levels i.e., $1 \times 10^5$ to $1 \times 10^{10}$ spores/ml as described by Vergano et al. (1996) and then fermentation studies were carried out.
3.9 PROCESS ECONOMIZATION

3.9.1 Influence of nutrients on L-asparaginase production

Under the present study nutrient sources like carbon, nitrogen, amino acid, phosphate, alcohol and metal ions were supplemented to the carob substrate in order to achieve higher yields of L-asparaginase under solid state fermentation.

3.9.1.1 Influence of carbon source on L-asparaginase production

A set of conical flasks with 20 g of carob substrate were amended with different carbon source with concentration of 0.05, 0.10, 0.15 and 0.20 M. The different carbon sources like, monosaccharides (glucose, fructose) and disaccharides (sucrose) were used under the present study.

3.9.1.2 Influence of inorganic nitrogen source on L-asparaginase production

A set of conical flasks with 20 g of carob substrate were prepared for SSF and supplemented with different inorganic nitrogen sources like ammonium sulphate, ammonium chloride and urea at concentrations ranging from 0.065-0.26% with increments of 0.065%.

3.9.1.3 Influence of organic nitrogen source on L-asparaginase production

A set of conical flasks with 20 g of carob substrate were prepared for SSF and supplemented with different organic nitrogen sources such as peptone, yeast extract and tryptone at concentrations ranging from 0.25% to 1.25% with increments of 0.25%.

3.9.1.4 Influence of phosphate source on L-asparaginase production

A set of conical flasks with 20 g of carob substrate were prepared for SSF and supplemented with different phosphate sources like, diammonium hydrogen
phosphate, dipotassium hydrogen phosphate and potassium dihydrogen phosphate at concentration ranging from 0.05 to 0.20 M with increments of 0.05 M.

3.9.1.5 Influence of alcohol source on L-asparaginase production

A set of conical flasks containing 20 g of carob substrate was prepared for solid state fermentation as described earlier and supplemented with glycerol at concentration of 0.1, 0.25, 0.50 and 1.0%.

3.9.1.6 Influence of trace metal ions on L-asparaginase

The different trace metals such as Cu$^{+2}$, Zn$^{+2}$, Mn$^{+2}$ and Fe$^{+2}$ were supplemented with the percentage of 0.1%, -0.3% with increment of 0.1%. All these metal ions were prepared by using double distilled water and added individually and kept for fermentation as described earlier.

3.9.1.7 Influence of combination of nutrients on the production of L-asparaginase

A set of conical flasks with 20 g of carob substrate were prepared for solid state fermentation and the medium was supplemented with all the nutrients at their optimum level in mixed concentrations in order to evaluate the beneficial influence on the L-asparaginase production.

3.10 MUTATION

The strain *Aspergillus terreus* KLS2 was subjected to UV irradiation to induce mutation for better yield of L-asparaginase. Spore suspensions of the *A. terreus* KLS2 (Plate – 6a) were irradiated using a 15W Phillips UV lamp at varying distances (5, 10, 15 and 20 cm) for 10 min. The irradiation was performed in a dark room and the irradiated suspensions were protected from light until plating was done on Czapek Dox agar in order to minimize any photo-reactivation effects as described by
Plate – 6:

a. *Aspergillus terreus* KLS2

b. *Aspergillus terreus* KLS2 mu
Gardener et al. (1956) and Banik et al. (1975). Under the present study mutant strain was isolated and labeled as *A. terreus* KLS2 mu (Plate – 6b).

### 3.11 Purification of L-Asparaginase

The fermented substrate is dissolved in known volume of buffer and placed on a shaker for 1 hr to release the enzyme. The content was filtered through Whatman No.1 filter paper and centrifuged. The supernatant was treated as crude extract and used for purification as explained below.

The partial purification of enzyme has been carried out as per the method described by Sadashivam and Manikam (1998). The cell free supernatant was precipitated with different concentrations of ammonium sulphate i.e., from 10 - 90%. The precipitate was dissolved in small amount of 50 mM glycine-NaOH buffer (pH 8) and dialyzed over night against the same buffer. The dialyzed enzyme was used for ion exchange chromatography studies like DEAE-Cellulose Chromatography and Gel-filtration methods as described by Naukamura (1971) Mesas et al. (1990) respectively. Thus purified enzyme was subjected to SDS-PAGE (Plate – 7) and used for the following studies.

### 3.12 General Properties of L-Asparaginase

For the study of general properties of L-asparaginase purified enzyme was used for the following studies.
Plate – 7: SDS-PAGE of L-asparaginase obtained from *A. terreus* KLS2
3.12.1 Effect of pH on enzyme activity

The optimum pH of the purified enzyme was studied over a range of pH 4.0-11.0 with asparagine as a substrate dissolved in different buffers. The buffers used were citrate-phosphate pH 4-8 and glycine–NaOH, pH 9-11. The experiments were conducted as per the method described by Gaffar (1976).

3.12.2 Effect of temperature on enzyme activity

The optimum temperature of L-asparaginase activity was determined with assay reaction mixture incubated at different temperature from 30 to 75°C. The residual activity was measured as described by Gaffar (1976).

3.12.3 Effect of pH on stability of enzyme activity

The pH stability of L-asparaginase was determined by pre-incubating the enzyme for 30 and 60 min for 45°C in buffers of various pH values. The residual activity was measured as described by Gaffar (1976).

3.12.4 Effect of temperature on stability of enzyme activity

The stability of enzyme to temperature was determined as per Gaffar (1976). The reaction mixture (without substrate) containing enzyme and buffer was pre-incubated for 30 and 60 min with different temperatures ranging 30-75°C and cooled. The residual activity was measured as per Gaffar (1976).

3.12.5 Substrate specificity

The reaction mixtures contained enzyme, buffer and different substrates, were used under the study as described by Imada et al. (1973) and Mannan et al. (1995). L-asparagine, D-asparagine and glutamine were used as substrates in the present study.