CHAPTER-III MATERIALS AND METHODS

3.1. ISOLATION AND SCREENING OF BACTERIAL SPECIES/STRAINS

3.1.1. Collection of Soil Samples

Soil samples were collected from different agricultural fields where farm produces like jowar, bajra, rice, sugar cane, ground nut, wheat, potato, maize and various cereals are cultivated as well as from the fruit farms and plantain plots of Kalaburagi District, Karnataka, India. Soil samples from 3-4 cm depth were collected employing sterile spatula and transferred to sterile plastic containers. The collected samples were transferred and maintained in the laboratory in aseptic condition. They were further processed for isolation of the soil bacteria.

3.1.2. Screening of Potent $\alpha$-Amylase Producing Soil Bacteria

Initially, the soil samples were screened for the bacterial flora through serial dilution and spread-plate technique as per standard procedures. One gram of each soil sample was serially diluted with sterilized distilled water in the range of $10^{-1}$ to $10^{-6}$. Thereafter, from dilutions of $10^{-5}$ and $10^{-6}$, 0.1 ml suspension was transferred and equally spread on nutrient agar plates in aseptic conditions. These plates were incubated at $37^0$ C for 24 h. Individual bacterial isolates with similar morphological appearances were picked up and sub-cultured further to obtain pure cultures. Pure cultures were maintained on starch agar slants $4^0$ C.

3.1.3. Primary Screening for $\alpha$-Amylase Producers

Rapid plate assay method was employed for primary screened to evaluate whether the isolated bacterial strains could produce $\alpha$-amylase. Employing substrate enrichment technique, the pure cultures maintained in the laboratory were sub-cultured in nutrient broth and a loopful 18-24 h culture of each isolate was streaked centrally in petri plates containing starch agar medium (10 g starch, 3 g beef extract,
15 g agar per 1000 ml and pH adjusted to 7.00). The inoculated plates were incubated at 37°C for 24-48 h. The amylase producers exhibited a clear hallow surrounding the streaks indicating hydrolysis of starch, which was confirmed by flooding the plates with 1% iodine solution for 30 sec and draining off the excess iodine.

3.1.4. Secondary Screening for α-Amylase Producers

The α-amylose producer isolates were once again centrally inoculated on starch agar medium in petri plates and incubated at 37°C. After 24 h, each plate was flooded with 1% iodine solution for 30 sec and excess iodine solution was drained off. The potent α-amylose producing bacterial strains exhibited clear zones of hydrolysis, which were recorded. The morphological and physiological tests were carried out as per Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994) for primary identification of the top-ranking four isolates exhibiting higher diameters of hydrolytic zone.

3.2. BACTERIAL SPECIES/STRAIN IDENTIFICATION

3.2.1. Presumptive Identification (upto generic level) of the Bacterial Species/Strain

The four top ranking amylase producer species were observed for their colony characteristics (such as size, shape, colour, surface, margin, elevation, consistency and opacity), staining characteristics (Gram’s), microscopic observations, biochemical characteristics (indole, methyl red, Voges-Proskaeur, hydrolysis of starch, casein and gelatin), catalase and urease activities, utilization of citrate and sugars (lactose, glucose, maltose, sucrose and mannitol) that assisted in their identification upto generic level. All the four isolates belonged to the genus Bacillus.

The α-amylose producing capability of these four bacterial strains was confirmed over three generations. The strain, KLMI4, that exhibited maximum hydrolytic zone among these four was subjected to further identification based the
16S rRNA gene sequencing and PCR amplification for confirmatory identification upto species level.

3.2.2. Production of α-Amylase in Batch Cultures (Submerged Fermentation)

3.2.2.1. Cultivation Medium

Basal medium (0.4% NH₄HPO₄, 0.05% MgSO₄.7H₂O, 0.1% KCl and 1% soluble starch in distilled water with pH adjusted to 7.0 ± 0.2) served as the medium for submerged fermentation by the selected bacterial strains. This cultivation medium (100 ml/flask) was dispensed into each of the 250 ml Ehrlenmeyer flasks and autoclave sterilized at 121°C for 15 minutes. This medium was employed in studies relating α-amylase production by the top ranking four bacterial isolates and in process parameter optimization studies.

3.2.2.2. Preparation of the Inoculum

A loopful of each of these four strains was inoculated into 100 ml autoclave sterilized nutrient broth (pH 7.0) in an Ehrlenmeyer flask and incubated at 37°C. An 18-24 culture with OD of 0.5 to 1.0 (approximately 3x10⁶ CFU/ml) was maintained as the stock inoculum and as per requirement the stock inoculum was pipetted out and seeded into the fermentation broth as the inoculum in all the further studies. All the experimental studies were conducted in triplicates and mean of the readings was taken as the result.

3.2.2.3. α-Amylase Production through Submerged Fermentation (SmF)

The amylase production by the identified bacterial strain, *Bacillus megaterium* KLM14 was carried out through SmF in 250 ml Ehrlenmeyer flasks. The autoclave sterilized basal mineral medium in the flask was aseptically inoculated with one ml of the bacterial inoculum (approximately 3x10⁶ CFU/ml) and incubated at 37°C in a rotary shaker incubator at 200 rpm. Samples were withdrawn from the flasks at
intervals of 24 h to estimate the enzyme activity. The studies were conducted in triplicates.

3.2.2.4. α-Amylase Assay

Estimation of α-amylase activity was determined as per Miller (1959) using 3,5-dinitrosalicylic acid (DNS) to estimate the reduced sugar formed due to enzymatic hydrolysis of the soluble starch. Suitable volume of sample withdrawn from the fermented broth was centrifuged in a refrigerated centrifuge (Gyrogen 1580R) at 10,000 rpm at 4°C. To 1 ml of the supernatant containing the crude enzyme, 1 ml of 1% (w/v) soluble starch in citrate buffer (pH 7.0) was added and incubated at 37°C for 30 min. The reaction was stopped by adding DNS (2 ml). The mixture was then heated in a boiling water bath for 5 min and cooled down. Absorbance was read at 540 nm in a double beam spectrophotometer (Systronics M2011). One unit of enzyme activity (IU) is defined as the amount of amylase required to catalyze the liberation of one mole of reducing sugar per min under assay conditions.

3.2.2.5. Identification of the Bacterium KLMI4 by 16S rRNA gene sequencing and PCR amplification

After morphological and biochemical characterization, the most potent α-amylase producing bacterial isolate, KLMI4 was subjected to 16S rRNA gene sequencing for identification. A single bacterial colony was inoculated into 50 ml nutrient broth and grown to absorbance of 0.5 to 1.0 at 600 nm.

The cells were collected by centrifugation at 5000 rpm at 4°C for 10 min. Genomic DNA was isolated from the pellets by using Biopure™ kits for bacterial genomic DNA isolation. The universal forward primer: 5’-AGAGTTTGATCCTGGCTCAG-3’ and reverse primer: 5’-ACGGCTACCTTGTTACGACTT-3’ were employed to amplify the 16S rRNA of the bacterial strain.
PCR amplification was performed maintain the following conditions: 1 cycle at 94°C for 60 sec (initial denaturing), 35 cycles at 94°C for 60 sec (denaturing), 53°C for 45 sec (annealing), 68°C (extension) and 1 cycle at 68°C for 10 min (final extension). Amplified PCR product was subjected to electrophoresis using agarose gel 1.2 % in TAE buffer and stained with ethidium bromide for visualization. The PCR product was purified by washing with sodium acetate and 70% ethanol and further eluted from the gel. Forward and reverse sequencing reactions of PCR amplicon were carried out on ABI3730XL sequencer for obtaining the sequence.

The sequence of 16S rRNA (1367 bp) was aligned by using the BLAST (Basic Local Alignment Search Tool) program to identify the most similar sequence in the database (Altschul et al., 1990) and has been deposited in the GenBank (National Centre for Biotechnology Information, NCBI, Bethesda, Maryland, USA) under accession number KY029077. The 16S rRNA sequences of different strains of *Bacillus megaterium* and its related species and genera were downloaded from GenBank database (http://www.ncbi.nlm.nih.gov/entrez). An attempt was made to align for constructing a neighbour-joining phylogenetic tree using ClustalW algorithm with the help of MEGA software version 4.1 (Tamura et al., 2007).

### 3.3. OPTIMIZATION OF PROCESS VARIABLES FOR α-AMYLASE PRODUCTION THROUGH SUBMERGED FERMENTATION OF THE BASAL MEDIUM

Being a sensitive process, bio-fermentation depends not only on the organism and methodology adopted but also on various environmental and cultivation conditions. Selection of a proper source of the enzyme as well as providing optimum fermentation conditions play a significant role in production of the desired end-product at industrial scale. Hence, it becomes necessary to evaluate the impact of various physical parameters and optimal nutritional requirements on the process in
order to attain maximum possible production of the enzyme. Hence, a few selected fermentation variable parameters like pH, temperature, inoculum size and different nutrients were optimized sequentially to improve the yield of the desired enzyme through submerged fermentation employing the process of one variable at a time (OVAT). Once a variable is optimized, it was retained in the subsequent studies wherein other variables are to be optimized.

### 3.3.1. Optimization of pH

The pH of the fermentation broth before inoculation was adjusted to different levels. The studies were carried out to determine the influence of pH on α-amylase activity (in the range of 5.0 to 10.0 with intervals of pH 0.5). After adjusting initial pH employing 0.1N HCl and 0.1N NaOH solutions, the individual flasks were each inoculated with 1 ml bacterial suspension and incubated at 37°C. Samples were withdrawn from each flask for estimating α-amylase activity every 24 h over the period of fermentation.

### 3.3.2. Optimization of Temperature

In order to evaluate the influence of temperature, the flasks with fermentation medium inoculated with the bacterial suspension and optimum initial pH adjustment were incubated at different temperature levels in rotary shaker incubators. Herein also exploratory studies were initially carried out in temperature levels of 21°C to 49°C (with intervals of 2°C). Samples were withdrawn from each flask for estimating α-amylase activity at regular intervals of 24 h.

### 3.3.3. Optimization of Initial Inoculum Size

The seed inoculum was prepared as earlier to yield approximately 3x10^6 CFU/ml of bacterial suspension. Different volumes of inocula yielding 1.0x10^6 cfu/ml, 2x10^6 cfu/ml, 3x10^6 cfu/ml, 4.5x10^6 cfu/ml and 6.0 x10^6 cfu/ml) were dispensed
aseptically into the experimental flasks to determine the optimum levels of inoculum size on α-amylase production. The flasks were incubated a rotary shaker incubator under optimum pH and temperature conditions established earlier. Samples were withdrawn from each flask to estimate α-amylase activity at regular intervals of 24 h.

### 3.3.4. Optimization of Substrate Concentration

Temperature, pH and inoculum size being optimized, it was considered worthwhile to optimize the substrate concentration too for maximal production of α-amylase by the bacterial strain under consideration. Starch concentration (w/v) was varied in the range of 0.5 g to 6.0 g per 100 ml (0.5, 1.0, 1.5, 2, 3, 4, 5 and 6 g/l) of the substrate. After inoculating the suspension, the flasks were incubated and after every 24 h interval, samples were withdrawn from each flask to estimate the enzyme activity.

### 3.4. PROCESS ECONOMIZATION FOR α-AMYLASE PRODUCTION IN SUBMERGED FERMENTATION BASAL MEDIUM

#### 3.4.1. Influence Carbon sources on Enzyme Production

Carbon sources play an important role in the process of α-amylase production by an organism. Hence, it was felt worthwhile to evaluate the influence of various sugars as C sources and various organic and inorganic N sources on α-amylase production by the organism under submerged fermentation studies.

Sugars play an important role in α-amylase production in submerged fermentation. Hence, different sugars like fructose, meso-inositol, glucose, galactose, sucrose, arabinose, raffinose, lactose, maltose, starch, dextrin, xylose, glycerol and trehalose were individually and separately incorporated into the basal medium at concentrations of 1 g/100 ml. After conducting the SmF studies under optimized conditions, samples were withdrawn at intervals of 24 h and α-amylase activity was estimated as detailed earlier.
3.4.2. Influence Nitrogen sources on Enzyme Production

Nitrogen sources (both organic and inorganic) play an important role in the maintenance and activities of the bacteria and living organisms in general. Peptone, various other complex organic compounds like tryptone, yeast extract, beef extract, corn steep liquor, casein (both hydrolyzed and soluble) and urea are known to influence bacterial activity. Hence, studies were conducted to evaluate the influence of these organic compounds on α-amylase activity of the bacterial strain under consideration through submerged fermentation using basal medium as the substrate.

3.4.2.1. Influence of Peptone on α-Amylase Production

Initially, the peptone requirement of the organism was determined by adding different concentrations of peptone (0.5 to 4.0%) to the substrate and autoclave sterilized before inoculating the bacterial suspension. The optimized fermentation parameters were applied and SmF was conducted over a period of 96 h. Samples were collected at intervals of 24 h to estimate the α-amylase activity.

3.4.2.2. Influence of Other Organic Nutrients on α-Amylase Production

Peptone at 1.5% effected optimum α-amylase production. Hence, the influence of other organic nutrients (tryptone, yeast extract, beef extract, corn steep liquor, both hydrolyzed and soluble casein as well as urea) on α-amylase production by the bacterium was evaluated by adding them at 1.5% concentration (w/v) individually and separately to the fermentation medium and autoclave sterilizing before inoculating the organism. Samples from the individual flasks were withdrawn at intervals of 24 h and α-amylase production was estimated. The study was conducted in triplicate.

3.4.3. Influence of Inorganic Nutrients on α-Amylase Production

Various inorganic salts are known to stimulate growth and activity of microorganisms. They also enhance soil fertility. Hence, studies were carried out to
evaluate the influence of especially some nitrogen based and other inorganic salts (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate and ammonium bisulphate) on the $\alpha$-amylase production by the bacterium under consideration. Each of these salts was dispensed at concentrations of 1.0% (w/v) into the fermentation medium separately and autoclave sterilized. After inoculating the medium with the fermenting bacterial suspension, the experimental flasks were incubated in the conditions detailed earlier. Samples were withdrawn aseptically at 24 h intervals and $\alpha$-amylase production was estimated. The study was conducted in triplicate.

3.4.4. Influence of Metal Salts on $\alpha$-Amylase Production

Some metal ions act as co-factors for microbial enzymes. It is also known that some microorganisms are dependent on calcium for their activities. Hence, studies were carried out to evaluate the influence of some metal ions and calcium salts on the potency of the bacterium to produce $\alpha$-amylase through SmF. The metal ions selected for the study were magnesium sulphate (MgSO$_4$.7H$_2$O), manganese sulphate (MnSO$_4$.7H$_2$O), copper sulphate (CuSO$_4$), zinc sulphate (ZnSO$_4$.7H$_2$O), ferrous sulphate (FeSO$_4$.7H$_2$O) and chloride as well as sulphate forms of calcium (CaCl$_2$ and CaSO$_4$). Each of these was dispensed into 100 ml fermenting medium separately at the rate of 1% (w/v) and autoclave sterilized before inoculating the fermenting organism. Then the flasks were incubated at 37$^\circ$ C in a rotary shaker at 200 rpm. At intervals of 24 h, samples were withdrawn from the flasks for estimation of the enzyme. The study was conducted in triplicate.

3.4.5. Influence of C: N Ratio on $\alpha$-Amylase Production

The ratio of carbon to nitrogen in the fermenting medium is known to influence the process of $\alpha$-amylase production. To evaluate the influence of C:N ratio
on the enzyme production by *B. megaterium* KLMI4 through submerged fermentation, two sets of studies (one with complex organic N$_2$ source, the peptone and the other with inorganic N$_2$ source, ammonium bisulphate) were carried out. Starch was the sole C source in both sets.

3.4.5.1. Influence of C: N Ratio using Peptone as N$_2$ Source

In this set, starch served as the sole carbon source and peptone served as the source of nitrogen. Starch was incorporated at 1.5% (w/v) and peptone was varied in the range of 1.0 to 2.5%. After adding the required C and N$_2$ sources to the fermentation medium, it was autoclave sterilized at 121$^\circ$ C for 15 min. After cooling to room temperature, bacterial suspension was inoculated into each of the experimental flasks under aseptic conditions and incubated in a rotary shaker incubator at 200 rpm and 37$^\circ$ C. At every 24 h interval, required quantity of sample was withdrawn to estimate $\alpha$-amylase activity.

3.4.5.2. Influence of C: N Ratio using Ammonium Bisulphate as N$_2$ Source

In this set, starch served as the sole carbon source and ammonium bisulphate served as the nitrogen source. Starch was incorporated at 1.5% (w/v) and ammonium bisulphate was varied in the range of 1.0 to 2.5%. After adding the required quantities of C and N$_2$ sources to the fermentation medium, it was autoclave sterilized at 121$^\circ$ C for 15 min. After cooling to room temperature, bacterial suspension was inoculated into each of the experimental flasks under aseptic conditions and incubated in a rotary shaker incubator at 200 rpm and 37$^\circ$ C. At every 24 h interval, required quantity of sample was withdrawn to estimate $\alpha$-amylase activity.

3.4.6. Overall Influence of Nutrients on $\alpha$-Amylase Production

After establishing the optimum physico-chemical parameters for SmF of the fermenting medium as well as the different organic and inorganic nutrients, it was
considered worthwhile to obtain an overall picture when all these in combination
influence the enzyme production by the microbe under consideration. For this
purpose, the basal fermentation medium was supplemented with 1% fructose, the
most effective of the sugars, 1.5% peptone, and 1% each of ammonium bisulphate and
calcium sulphate and autoclave sterilized at 121°C for 15 min. After allowing it to
cool to room temperature, bacterial suspension (having approximately 3x10^6 CFU/ml)
was dispensed into each flask aseptically and incubated in a rotary shaker incubator
with 200 rpm at 37°C. The study was run in triplicate. Samples were withdrawn from
each flask every 24 h and analyzed for α-amylase activity.

3.4.7. Bacterial Cell Immobilization

3.4.7.1. Cultivation of B. megaterium KLMI4

The organism, B. megaterium KLMI4, was inoculated (3x10^6 cfu/l) into
100 ml nutrient broth in Ehrlenmeyer flask and incubated at 37°C in a rotary
incubator for 48 h. The bacterial culture obtained thus was then aseptically introduced
into the centrifuge tubes and centrifuged at 4°C at 6000 rpm for 15 min. The pellets
formed were collected and washed thrice in phosphate saline buffer (pH 7.0). This
biomass was thoroughly mixed in 10 ml sterilized distilled water and used for
immobilization.

3.4.7.2. Immobilization in Sodium Alginate

Sodium alginate (3.0 g) was added to 90 ml distilled water and heated to
complete dissolution. The slurry thus formed was autoclave sterilized and then
allowed to cool down to room temperature. The rich cell suspension of B. megaterium
KLMI4 was poured into this slurry and thoroughly mixed with the help of a sterile
glass rod to obtain a uniform distribution of the bacterial cells in the slurry.
This mixture was dispensed into the pre-chilled calcium chloride solution (0.2M) with
the help of a sterile syringe from a height of 20 cm. Thus formed beads were kept in
the same solution at 4°C for an hour for curing. Then the beads were washed thrice
with sterile distilled water and stored at 4°C in aseptic conditions until further use in
the experiments. The experimental flasks containing basal medium amended with
1.5% starch were inoculated with alginate beads (30% v/v) and fermentation was
carried out at optimized conditions. In repeated batch fermentation studies, the
previously used beads were washed with sterile distilled water and reused
for experimentation. Appropriate controls inoculated with free bacterial cells
(3x10^6 cfu/ml) were maintained.

In the studies involving repeated batch fermentation, the gradual leakage of
the cells from the immobilized beads was determined by bacterial load in the
supernatant medium in each cycle at the end of 48 h.

3.4.8. Use of Agro-wastes for Economization of α-Amylase Production through
Submerged Fermentation.

3.4.8.1. Preparation of the Agro-wastes for fermentation

Different locally available agro-wastes like groundnut oil cake, green gram
husk and rice husk were obtained and employed to evaluate whether these could
support α-amylase production by the bacterium in concern through submerged
fermentation. Each was thoroughly cleaned to remove any foreign impurities, finely
ground and sieved through 85 mesh standard sieves and fine powder was once again
ground in a mixer to obtain finer powder. The green gram was purchased in the local
market; foreign materials were removed, and the grains were dipped in water for
30 min to loosen the husk. Then the moistened grains were heat-dried overnight at a
temperature of 60°C in a hot air oven. It was de-husked next day in a grinder.
Approximately 150 to 250 g husk was obtained from one kg of whole grains.
Required quantities of each agro-waste was finely ground and sieved through 85 mesh
standard sieve to remove any coarse particulates. The sieved husks were individually ground once again in a mixer to obtain fine powders.

3.4.8.2. Composition of Agrowastes

The finely ground agro-wastes were individually assessed for their gross organic contents (carbohydrate, protein and fats).

3.4.8.2.1. Estimation of total carbohydrates

The total carbohydrate content of the different agro-wastes was estimated as per the method of Dubois et al. (1956) wherein 100 mg of finely ground substrate is hydrolyzed with 5 ml of 2.5 N HCl, keeping it in a boiling water bath for 3 h and after cooling neutralized with sodium bicarbonate until effervescence of the liquid ceases, making the volume to 100 ml with distilled water and centrifuging to collect supernatant for analysis of the carbohydrates by addition of phenol and reading the colour at 490 nm in a double beam spectrophotometer (Systronics M2011).

3.4.8.2.2. Estimation of total protein

Total protein content of the different agro-wastes was determined by colorimetric method of Lowry et al. (1951). Each of the finely ground substrate (500 mg) was mixed with 10 ml Tris-HCl buffer, centrifuged in a refrigerated centrifuge (Gyrogen 1580R) and the supernatant obtained was used for protein estimation. Bovine serum albumin was used as standard protein and the Folin-Ciocalteau reagent was employed. The colour developed was read at 660 nm in the Systronics M2011 spectrophotometer.

3.4.8.2.3. Estimation of total fats

Total fat content of the agro-wastes was estimated through the Soxhlet method by extracting with petroleum ether. Known quantity of each agro-waste was extracted with petroleum ether in a Soxhlet apparatus for 6 h and the extract thus obtained was
decanted and evaporated in a pre-weighed dry beaker at 80°C and kept in a desiccator. The beaker was re-weighed. The difference sample weight gives the quantity of the fat present in the agro-waste.

3.4.8.3. Preparation of the Fermentation Medium

Required quantities of each agro-waste were separately dispensed into beakers containing 100 ml of distilled water and boiled for 10 min. The suspension was cooled and filtered through Whatman filter paper no. 1. The filtered extract volume was made up to 100 ml with distilled water and 0.5% NaCl was added. This served as the fermentation medium to evaluate α-amylase production by the bacterial strain.

3.4.8.4. Optimization of the Process Parameters using Agrowastes as substrate

Extracts of different quantities (1, 3, 5, 7 and 10 g) of each of the agro-wastes were prepared as detailed above, amended with 0.5% NaCl and made up to 100 ml were dispensed separately in 250 ml Erlenmeyer flasks and autoclave sterilized at 121°C for 15 min and cooled to room temperature. Thereafter the extract was aseptically inoculated with bacterial suspension (3x10⁶ CFU/ml) and incubated in a rotary shaker incubator at 200 rpm and 37°C over a length of time. Required quantities of samples were withdrawn every 24 h and analyzed for α-amylase activity as detailed earlier. Studies were conducted in triplicate.

3.4.8.4.1. Optimization of pH

The pH of the green gram husk extract before autoclave sterilizing was adjusted to different levels using 0.1N NaOH and 0.1 HCl. Studies were conducted in pH range of 5.0 to 10.0 (with intervals of 0.5). After initial pH levels adjustments, the individual flasks were each inoculated with bacterial suspension (3x10⁶ CFU/ml) and incubated at 37°C in a rotary shaker incubator. Samples were withdrawn from each
flask for estimating $\alpha$-amylase activity at regular intervals of 24 h. Study was conducted in triplicate.

3.4.8.4.2. Optimization of Temperature

In order to evaluate the influence of temperature, the flasks with fermentation medium inoculated with the bacterial suspension and optimum initial $p\text{H}$ adjustment were incubated at different temperature levels in rotary shaker incubators. Studies were carried out at temperature levels of 21 to 49$^\circ$C with intervals of 2$^\circ$C. Samples were withdrawn from each flask for estimating $\alpha$-amylase activity at regular intervals of 24 h. Study was conducted in triplicates.

3.4.8.4.3. Optimization of Initial Inoculum Size

The seed inoculum was prepared as earlier, and it was diluted, if necessary, to yield 3x$10^6$ CFU/ml of bacterial suspension. Different volumes of inocula (1x$10^6$ cfu/ml, 2x$10^6$ cfu/ml, 3x$10^6$ cfu/ml, 4.5x$10^6$ cfu/ml and 6.0x$10^6$ cfu/ml) were dispensed aseptically into the experimental flasks containing green gram husk to determine the optimum level of inoculum size to effect maximum $\alpha$-amylase production. The flasks were incubated under optimum $p\text{H}$ and temperature conditions established earlier. Samples were withdrawn from each flask to estimate $\alpha$-amylase activity at 24 h intervals. The study was conducted in triplicates.

3.4.8.5. Influence of Carbon and Nitrogen Sources on $\alpha$-Amylase Production

Carbon and nitrogen sources play an important role in the process of $\alpha$-amylase production by a microorganism. Hence, it was felt worthwhile to evaluate the influence of different sugars as C sources and various organic and inorganic N sources on $\alpha$-amylase production by the organism under submerged fermentation of green gram husk.
3.4.8.5.1. Influence of Carbon Sources

Carbon sources play an important role in enzyme production in submerged fermentation. Hence, different sugars like fructose, meso-inositol, glucose, galactose, sucrose, arabinose, raffinose, lactose, maltose, dextrin, xylose, glycerol and trehalose as well as starch were individually and separately added to the green gram husk extract at concentration of 1 g/100 ml. After submerged fermentation under optimized conditions, samples were withdrawn at intervals of 24 h and $\alpha$-amylase activity was estimated as detailed earlier. The study was conducted in triplicate for each C source.

3.4.8.5.2. Influence Nitrogen Sources

Nitrogen sources also play an important role in the maintenance and activities of the bacteria and living organisms in general. Peptone, various other complex organic substrates like tryptone, yeast extract, beef extract, corn steep liquor, casein (both hydrolyzed and soluble) and urea have were used to observe their influence on bacterial activity.

3.4.8.5.2.1. Peptone Supplementation and $\alpha$-Amylase Production

Initially, the peptone requirement of the organism was determined by adding different concentrations of peptone (0.5 to 4.0%, with intervals of 0.5%) to the extract and autoclave sterilized before inoculating the bacterial suspension. Maintaining the already optimized fermentation parameters as detailed earlier, SmF was conducted over a period of 72 h. Samples were collected every 24 h to estimate the $\alpha$-amylase activity.

3.4.8.5.2.2. Supplementation of other Organic Nutrients

Since 1.5% peptone was required for optimum $\alpha$-amylase production, the influence of other organic nutrients (tryptone, yeast extract, beef extract, corn steep liquor, both hydrolyzed and soluble casein as well as urea) on $\alpha$-amylase production by the bacterium was evaluated. These were added at concentrations of 1.5% individually
and separately to the fermentation medium and autoclave sterilized before inoculating the organism. Samples from the individual flasks were withdrawn every 24 h and \( \alpha \)-amylase production was estimated. The study was conducted in triplicate.

3.4.8.5.3. Supplementation of Inorganic Nutrients

Various inorganic salts are known to influence growth and activity of microorganisms. Hence, studies were carried out to evaluate the influence of some nitrogen based and other inorganic salts (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate and ammonium bisulphate) on the \( \alpha \)-amylase production by the bacterium. Each of these salts (1% w/v) was dispensed into the fermentation medium separately and autoclave sterilized. The inoculated flasks were incubated in the same conditions as detailed earlier. Required samples were withdrawn aseptically at intervals of 24 h and \( \alpha \)-amylase production was estimated.

3.4.8.5.4. Supplementation of Metal Salts

Some metal ions act as co-factors for microbial enzymes. It is also known that some microorganisms depend on calcium for their activities. Hence, studies were carried out to evaluate the influence of some metal ions and calcium salt on the potency of the microbe under consideration to produce \( \alpha \)-amylase through SmF. The metal ions selected for the study were magnesium sulphate (MgSO\(_4\).7H\(_2\)O), manganese sulphate (MnSO\(_4\).7H\(_2\)O), copper sulphate (CuSO\(_4\)), zinc sulphate (ZnSO\(_4\).7H\(_2\)O), ferrous sulphate (FeSO\(_4\).7H\(_2\)O) and calcium chloride (CaCl\(_2\)) and calcium sulphate (CaSO\(_4\)). Each of these (1 g) was dispensed into 100 ml of the fermenting medium separately and autoclave sterilized before inoculating the fermenting organism. Then the flasks were incubated at 37\(^0\) C and samples were withdrawn every 24 h from the flasks for enzyme estimation.
3.4.8.5.5. Overall Influence of Nutrient Supplementation on α-Amylase Production

After establishing the optimum physico-chemical parameters for SmF of the fermenting medium as well as the different organic and inorganic nutrients, it was considered to obtain an overall picture when all these in combination influence the enzyme production by the microbe under consideration. For this purpose, the most influencing sugar (fructose, 1% w/v) as well as peptone (1.5%), ammonium bisulphate and calcium sulphate (each 1% w/v) were added to the green gram husk extract and autoclave sterilized at 121°C for 15 min. After the fermenting medium cooled to room temperature, bacterial suspension (approximately 3x10⁶ CFU/ml) was inoculated into each flask aseptically and incubated in a rotary shaker incubator with 200 rpm at 37°C. The study was run in triplicate. Samples were withdrawn from each flask every 24 h and analyzed for α-amylase activity.

3.5. PROTEIN ESTIMATION

*B. megaterium* KLMI4 was subjected submerged fermentation to produce α-amylase using starch supplemented basal medium under optimized conditions. To 100 ml of medium in a 250 ml Ehrlenmeyer flask, an inoculum (3x10⁶ cfu/ml) of 24 h overnight culture was added and incubated at 37°C to obtain a rich bacterial culture. After fermentation for 48 h, the culture was centrifuged at 8000 rpm for 10 min to obtain the enzyme protein as supernatant. Thereafter, the crude enzyme protein was estimated employing the Folin-Ciocalteau reagent as per Lowry’s method (1951), the colour developed was read as optical density (OD) in a UV spectrophotometer at 660 nm and by referring to the bovine serum albumen (BSA) standard graph.
3.6. ENZYME PURIFICATION

The crude enzyme extract was subjected to purification through the following steps:

3.6.1. Salt precipitation

Crude supernatant collected from the fermentation flask was placed in a beaker on top of a cool pack. Ammonium sulphate was added to it with continuous stirring to reach 80% saturation. Thereafter it was incubated at 4°C overnight. The precipitated protein was centrifuged in a refrigerated centrifuge at 4°C at 10,000 rpm for 10 minutes. The pellet was collected and dissolved in 50 mM Tris-HCl and this was further subjected to dialysis.

3.6.2. Dialysis

In order to remove the traces of ammonium sulphate, the sample was placed in a dialysis bag (pre-boiled in 100 ml distilled water for 10 min to open the pores, then in 2% sodium bi carbonate solution for 10 min to remove glycerine coating and finally in 100 ml distilled water for 10 min to remove residual sodium bicarbonate) was suspended in a beaker containing distilled water and kept in a refrigerator overnight. Then the protein present in the bag was dissolved in 50 mM Tris-HCl.

3.6.3. Ion-Exchange Chromatography

A properly washed column was loaded with 10 cm of DEAE-cellulose slurry and left undisturbed for 1 h for establishment of the column matrix. To this column, 10 ml of 50mM Tris-HCl buffer was loaded and thereafter it was eluted from the column. This process was repeated twice. The sample (10 ml) was poured in the column and it was eluted from the column. The effluent was poured back in the column and re-eluted. This was repeated thrice, in order to ensure the binding of all the protein of the sample with the DEAE cellulose. Thereafter, 40 ml of 25 mM
NaCl in 50 mM tris-HCl buffer was applied to the column and the effluent was collected in fractions.

3.7. ENZYME CHARACTERIZATION

3.7.1. Molecular Weight Determination

SDS-PAGE

The molecular weight of the protein in the enzyme was determined by SDS-PAGE by using 12% separating gel and 4% stacking gel with 10% SDS. The gel was further stained with bromophenol blue and later destained. The standard protein markers used in the process are 66KDa, 45 KDa, 35 KDa, 25 KDa, 18 KDa and 14 KDa.

3.7.2. Crude Enzyme Characterization

3.7.2.1. Enzyme stability at different pH levels

Fractions of the crude enzyme were maintained separately at different pH levels ranging from 5.5 to 10.0 employing different buffers. Citrate phosphate buffer for pH 5.5, phosphate buffer for pH 6.0 to 7.5, tris-HCl buffer for pH 8.0 and glycine-NaOH buffer for pH levels from 8.5 to 10.0 were employed. The enzyme fractions were incubated at 37° C for a period of 1.0 h and thereafter the enzyme activity was assayed as per the DNS method (1959) and the results are presented in terms of relative activity.

3.7.2.2. Thermostability of the Crude Enzymes

Fractions of the crude enzyme (at pH 8.0 using tris-HCl buffer) were incubated for 1 h at different temperature conditions ranging from 21° to 51° C and thereafter assayed for its activity as per the DNS method (1959). The results are presented in terms of relative activity.
3.7.3. Characterization of Purified Free and immobilized Enzyme

Immobilization of Purified Enzyme

The purified enzyme obtained after ion exchange chromatography with 150 IU/ml enzyme activity was entrapped on sodium alginate as per Naganagouda and Mulimani (2006). Solutions of 2% calcium chloride in 25 ml distilled water and 3% sodium alginate in 10 ml distilled water were prepared separately. Calcium chloride solution was chilled. The enzyme (5 ml) was dispensed into the sodium alginate solution and mixed well. This mixture was dispensed into pre-chilled calcium chloride at 4°C through a hypodermic syringe from a height of 20 cm allowing the formation of beads. The beads were then cured for 2 h in calcium chloride solution and thereafter the beads were washed with sterile distilled water and stored at 4°C in Tris-HCl buffer (pH 8.0) until further use. The activity of the immobilized enzyme was assayed by incubating in buffer with 1.5% starch in 50 mM Tris-HCl buffer (pH 8.0) with 1 g beads at 37°C for 30 minutes and estimating the reducing sugars released from the starch. The studies were carried out in triplicate.

Estimation of Enzyme Immobilized in the Alginate Beads:

Entrapment of the enzyme into the beads was determined by taking into consideration the difference between the activity of the free enzyme and that remaining in the washed water as well as the filtered calcium chloride solution in the following manner:

\[
\text{Immobilization yield}(\%) = \frac{(\text{Activity of added enzyme}) - (\text{Activity of incubated solution} + \text{Activity of washed buffer})}{\text{Activity of added enzyme}} \times 100
\]

Physico-chemical Properties of the Free and Immobilized Enzyme:

Various physico-chemical properties of the free and immobilized enzyme like temperature and pH tolerance, reusability, storage stability and kinetic measurement
were studied. The relative activity of the enzyme entrapped in the alginate beads was considered to be hundred per cent in all these studies.

### 3.7.3.1. Thermostability of the Enzyme

The influence of the temperature on the free and immobilized enzyme was studied in the following manner. The free enzyme (1.0 ml) was added to tris-HCl buffer (pH 8.0) with 1.5% starch and incubated for 30 min at different temperature levels (21° to 61° C) in constant temperature water baths and then assayed for enzyme activity employing the DNS method (Bernsfelden, 1959). In the same way, 1.0 g beads with immobilized enzyme were introduced into tris-HCl buffer with 1.5% starch and incubated at different temperatures as detailed above. After 30 min, the supernatant solution was assayed for enzyme activity by the DNS method. The results are presented in terms of relative activity.

### 3.7.3.2. Enzyme Stability at different pH

The free (1 ml) as well as immobilized enzyme (1.0 g beads) were separately added to buffers with 1.5% starch for studying the tolerance of both forms of the enzyme to different pH levels (pH 5.0 to 10.0). Different buffers were used for different pH levels: sodium citrate buffer for pH 5.0, sodium phosphate buffer for pH 6.0 to 8.0, glycine-sodium hydroxide buffer for pH 9.0 and pH 10.0. The two preparations (free enzyme and enzyme embedded in the alginate beads) with different buffers were incubated for 30 min at 37° C and thereafter the enzyme activity was assayed as detailed above. The results are presented in terms of relative activity.

### 3.7.3.3. Reusability of Immobilized Enzyme

The Erlenmeyer flasks (50 ml) in triplicate with 10 ml tris-HCl buffer with 1.5% starch were added with immobilized beads and incubated at 37° C for 30 min. Thereafter, the supernatant solution was assayed for amylase activity as per the DNS
method briefed earlier. This constituted the first cycle of use of the immobilized beads. Then, the supernatant was drained off and the beads were washed thrice with the buffer. The beads were subjected to fermentation of the fresh reaction mixture as in the first cycle and enzyme activity was assayed by considering the enzyme activity in the first cycle as 100%. In this way, the immobilized beads were used repeatedly and at the end of each cycle, enzyme was assayed.

3.7.3.4. Storage Stability of the Free and Immobilized Enzyme

It was considered worthwhile to estimate the length of time the enzyme, either in free or immobilized condition, can be stored. The two forms of the enzyme were stored in tris-HCl buffer (pH 8.0) at 4°C separately over a span of 30 days. Every 5 days the residual activity of the enzyme was assayed as mentioned above taking into consideration its initial activity in each form, the results being expressed as percentage of respective initial activity.

3.7.3.5. Kinetic Analysis of Free and Immobilized forms of α-amylase

Kinetic parameters of free and immobilized forms of α-amylase were estimated by measuring the initial reaction rates using starch concentrations in the range of 0.1-1.5% at pre-determined optimum pH and temperature of each form. For both forms of enzyme (free and immobilized), the Michaelis-Menton kinetic behaviour was observed. $K_m$ and $V_{max}$ values of free and immobilized α-amylase were calculated from Lineweaver-Burke plot.

3.8. APPLICATIONS OF α-AMYLASE

The enzyme, α-amylase, finds applications in various industries. Desizing capacity and its use along with detergent formulations for washing performance are some of the applications and these applications were studied due to their feasibility in the present laboratory conditions.
3.8.1. α-Amylase as Desizing Agent in Textile Industries

One meter length of coarse white cotton cloth was freshly purchased from the local market. It was stiff due to presence of starch and it was cut into several equal sized pieces of 5x5 cm dimensions. Each was weighed on an electronic balance. Each strip was immersed in crude enzyme solution (94 IU/ml and pH 8.0) and placed in an incubator at 37° C for 30 min. Then it was rinsed thoroughly in tap water, oven dried and was again weighed. The difference between the two weights indicated the amount of starch removed. Total starch present in the cloth piece was determined by hydrolyzing it with 0.1N sulphuric acid. The percentage of starch removal or desizing was calculated applying the following formula:

\[
\% \text{ Desizing by the enzyme} = \frac{\text{weight of starch removed by enzyme}}{\text{total starch present in the cloth strip}}
\]

In order to evaluate the use of α-amylase produced by B. megaterium KLMI4 as a desizer in the textile industries, the following parameters were studied: 1) effect of enzyme concentration on desizing; 2) effect of temperature on desizing; 3) pH on desizing and 4) time profile for desizing.

3.8.1.1. Effect of Enzyme Concentration on the process of Desizing

The influence of crude enzyme concentration on the process of desizing was studied by keeping the cotton cloth pieces immersed in separately in distilled water with varying the enzyme concentration in the range of 50 to 400 IU/ml (with 50 IU/ml intervals) at room temperature of 37° C for a period of 30 min. Thereafter, the amount of starch removed was determined using the method described above. The study was conducted in triplicates.

3.8.1.2. Effect of Temperature on the process of Desizing

The influence of temperature on the process of desizing was studied at various levels: 21, 25, 29, 33, 37, 41 and 43° C, by incubating the immersed cloth pieces
(in distilled water with optimum enzyme concentration) at specific temperature level for 30 min and estimating thereafter the percentage of desizing. The study was conducted in triplicates.

3.8.1.3. Effect of pH on the process of Desizing

The influence of pH on the process of desizing was studied in the range of 5.5 to 10.0, with intervals of pH 0.5 by immersing the stiff cloth pieces in distilled water (maintaining optimum enzyme concentration and temperature as noticed in the earlier steps) with different pH levels for 30 min. and thereafter estimating the percentage of desizing as detailed earlier. The study was conducted in triplicates.

3.8.1.4. Time Profile for the Desizing of the Cloth

Maintaining the optimum enzyme concentration of pH and temperature, the cloth pieces were immersed in crude enzyme (94 IU/ml) for 15, 30, 45 and 60 min separately. The percentage of desizing was estimated as earlier. The study was conducted in triplicates.

3.8.2. α-Amylase as Additive In Detergent Formulations

Studies regarding the utility of α-amylase produced by B. megaterium KLMI4 in detergent industry were carried out in two parts. The first one comprised of evaluation of the residual enzyme activity in the presence of detergent powders/granules, while the second one was regarding the washing performance of the enzyme in the presence of the commercial detergents.

3.8.2.1. Residual Enzyme Activity in the presence of Detergents

Five commonly available powder/granular detergents (Surf Excel, Wheel, Tide, Ariel and Ghadi) were obtained from the local market and were diluted in tap water separately to get 7 mg/ml concentrations in order to simulate normal washing practices. Each was heated at 80° C for 30 min. in order to inactivate their intrinsic enzymes, if any. To each detergent solution was added the crude α-amylase with
93 IU/ml and the mixtures were incubated at 37\(^\circ\) C for different periods (15, 30, 45, 60 and 75 min). The residual activity of the enzyme was assayed withdrawing 0.5 ml aliquots after these periods. Then they were assayed for residual enzyme activities. The activity of the crude enzyme, without any detergent supplementation, was 100%.

3.8.2.2. Evaluation of Washing Performance

This study was carried on small cotton cloth pieces of 5x5 cm dimensions. Each piece was spread-stained with blood (100 µl), chocolate (heat liquified) and barbecue sauce and allowed to dry. The dried cloth pieces were soaked and incubated at 40\(^\circ\) C for 60 min under agitation in 100 ml each of normal tap water, Tide detergent solution (as it exhibited maximum compatibility with the enzyme and in 7 mg/ml concentration) and water amended with the crude enzyme. Afterwards, the pieces were taken out, rinsed with normal tap water, dried and visually examined to evaluate the removal of the stains.