Without knowing the existence of the macro-biomolecules, i.e., the enzymes which are part and parcel of all the living beings, man continued to enjoy their fruits ever since he evolved on the earth. It is estimated that the numerous enzymes so far described (the list is broadening due to formation of newer enzymes through genetic engineering techniques) catalyze more than 5000 biochemical reaction types, most of the enzymes being highly specific regarding their substrate and end-products. And, now the period has come to observe the rule of enzymes in the entire world.

It is interesting to note that one of the components of the very first enzyme complex discovered in the first half of the 19th Century and laid the foundation of enzymology, the amylase has been ruling the life activities since several millennia and global market in terms of requirement and production. Especially a single enzyme, α-amylase, playing an important role in day-to-day life, has nearly a share of 25-30% of the total enzyme market globally and the demand continues to progressively increase concurrently every year with the population growth. Though α-amylases from a few bacterial species are being used industrially, there is always a scope for venturing into isolation of local strains which are capable of producing the enzyme and produce the enzyme in an economical way. With this objective in mind the present investigations have been carried and presented in this thesis.

The summary of the results and the conclusions arrived at based on the results obtained and presented in the thesis, are briefly presented herein:

1. Chapter I: In this chapter are presented a brief introduction to the historical development of the branch of enzymology is given and the significance of the enzyme, the α-amylase, that laid the foundation for the development of this
important aspect of science and technology is highlighted. This justifies the need for undertaking the present work. Along with this, a broad outline of the aims and objectives of present study is given.

2. Chapter II: This chapter covers an exhaustive literature on the relevant aspects of the enzyme, the principle aspects of its production, the importance of basic variable parameters of the fermentation process, economization of the process of fermentation aiming at reducing the cost of production of the enzyme (through supplementation of different organic and inorganic nutrients, substituting the substrate by cheap, easily and locally available agro-wastes aiming at economization as well as environmental pollution abatement, purification and characterisation of the enzyme as well as immobilization of whole cells and enzyme, along with application of the enzyme for industrial purposes, especially in textile and laundry as well as dish washing detergent formulations.

3. Chapter III: In this chapter are detailed the materials used as well as the methodology adopted for conducting the present investigation. Various aspects covered are: screening of the local $\alpha$-amylase-producer bacteria from different agricultural fields, production of $\alpha$-amylase by the isolated bacterial strains employing starch containing basal medium in batch cultures, selection of the best $\alpha$-amylase-producing bacterial strain based on starch hydrolysis in starch agar plates and in basal medium, identification of different isolates up to generic level as well as confirmation of the best $\alpha$-amylase-producer strain by 16S rRNA gene sequencing, DNA extraction and PCR amplification in the first part. Thereafter, methodology adopted for maximum production of $\alpha$-amylase by the test strain by evaluating the optimum process variable
parameters ($p$H, temperature, inoculum size and substrate concentration) through OVAT system has been described. The methodology adopted for process economization for maximum enzyme production through nutrient supplementation, and different aspects of bacterial immobilization, process economization by enzyme production using different agro-wastes and nutrient supplementation to the agro-wastes have been described in details. The methods adopted for characterization and purification of the enzyme produced by the test organism, immobilizing the enzyme to achieve further economization of the process, etc. have been described. Finally the methods adopted to evaluate the enzyme for use in textile industry and for inclusion in the detergent formulations are detailed.

4. Chapter IV: In this chapter, the results obtained during the study have been presented. The results are summarized in brief as below:

i. Out of the 1062 local isolates, 100 isolates exhibited $\alpha$-amylase producing capability through rapid plate technique of starch hydrolysis. Of these, 4 strains that produced maximum zone of hydrolysis were identified upto generic level through microbiological observations and biochemical tests. They were observed to belong to the genus *Bacillus*. They were then screened for $\alpha$-amylase production in basal medium containing 1% as substrate consistently over three generations. One isolate, KLMI4, exhibited highest $\alpha$-amylase activity and this was subjected to species confirmation through DNA extract and PCR amplification and 16S rRNA sequencing. Using the BLAST tool, the organism was identified as a strain of *Bacillus megaterium* and named as *Bacillus megaterium* KLMI4. This strain was used in further investigations conducted.
ii. The studies conducted to optimize the basic variable submerged fermentation parameters revealed that the optimum pH, temperature, inoculum size and substrate concentration for maximum α-amylase activity were 8.0, 37\(^0\)C, 3\(\times\)10\(^6\) cfu/ml and 1.5\% (w/v) starch, respectively. Enzyme activity increased step-wise as each parameter was optimized. The ultimate yield after optimization of variable parameters was noticed to be 93 IU/ml.

iii. The process economization studies revealed that fructose (252 IU/ml) closely followed by maltose (240 IU/ml) were the best sources of C, peptone at 1.5\% concentration (175 IU/ml) was the most beneficial amongst the organic N\(_2\) sources, ammonium bisulphate (1\% concentration) was the most beneficial (125 IU/ml) amongst the inorganic N\(_2\) sources and calcium sulphate (1\% concentration) was the most beneficial (181 IU/ml) amongst the various metal salts. The C:N ratio was observed to 1:1 with peptone (250 IU/ml) and 3:4 with ammonium bisulphate (190 IU/ml) for maximum α-amylase activity.

iv. With all the parameters and nutrients at optimized levels, α-amylase activity of 395 IU/ml was achieved after 48 h fermentation employing basal medium; thus a 4.16 fold increase in enzyme yield when compared to that recorded after optimizing the basic variable parameters.

v. Cells of \(B.\) \textit{megaterium} KLMI4 upon alginate immobilization retained enzyme activity for prolonged period than the free cells and showed that the beads could be repeatedly reused for 5 cycles of 48 h, retaining 60\% activity at the end of the 5\textsuperscript{th} cycle.
vi. The use of three agro-wastes (rice husk, groundnut oil cake and green gram husk) as substrates replacing starch showed positive responses. Of the three wastes, the green gram husk led to $\alpha$-amylase yield of 42 IU/ml by *B. megaterium* KLMI4 to the maximum extent. In these studies, the fermentation medium contained only the green gram husk extract and 0.5% common salt in 100 ml distilled water when compared to the costlier components of the basal medium. Thus the cost of enzyme production is lowered sufficiently. A broad evaluation of total carbohydrate, protein and fats showed that green gram husk contained maximum carbohydrate content and least contents of proteins and fats amongst the three agro-wastes. The optimization of the basic variable parameters of submerged fermentation revealed the optimum level of $pH$, temperature and inoculum size to be the same as that in the studies with basal medium, i.e., 8.0, 37$^0$ C and 3x10$^6$ cfu/ml, respectively. The yield of enzyme after parametric optimization was 128 IU/ml compared the 93 IU/ml with basal medium, an increase of nearly 37%.

vii. The attempts to further economize the process of green gram husk extract with nutrients like sugars, organic and inorganic N sources yielded similar results as observed in studies with basal medium, the only difference being enhanced yields of the enzyme with green gram husk extract.

viii. With all the parameters and nutrients at optimized levels, $\alpha$-amylase activity of 513 IU/ml was achieved after 48 h fermentation of green gram husk extract; thus, almost 1.30 fold increase in enzyme yield is observed when compared to that recorded with the basal medium (with all optimum parameters and nutrients). Thus, the green gram husk proves to be an
efficient and much cheaper alternative than starch for α-amylase production by *B. megaterium* KLMI4.

ix. The crude enzyme obtained through submerged fermentation was purified through the steps of ammonium sulphate fractionation, dialysis and DEAE-cellulose chromatography, ultimately yielding 41.31% enzyme yield achieving a 50.77 fold purification. The specific activity of the purified enzyme reached 2142.85. The protein content of the purified enzyme is 0.07 mg/ml, while that of the crude enzyme is 8.60 mg/ml. The molecular weight of the enzyme is estimated to be 45 kDa through SDS-PAGE.

x. The crude enzyme is stable (>60% activity) in the range of pH 7.0 to 9.5 and it is stable with > 40% activity in the temperature range of 21^0^ to 45^0^ C.

xi. The immobilization yield of the purified enzyme in alginate beads is 83.33%. The purified enzyme activity was maximum at 41^0^ C, indicating a shift in temperature tolerance due to immobilization. This is further confirmed by the observation that while the activity free cells was nil at 61^0^ C, that of the immobilized enzyme was nearly 40% at this temperature. Thus, immobilization of the enzyme imparted it the ability to be active for a longer period as well as to withstand temperature levels higher than the free enzyme can.

xii. Immobilization also enabled the entrapped enzyme to be used and reused repeatedly several times, i.e., for 8 cycles, with fairly good relative activity above 50%. Because of immobilization the enzyme can be stored for
25 days with residual activity of above 60% when compared to only 35% residual activity of the free enzyme after 25 days storage.

xiii. Immobilization also imparted more substrate hydrolysis capacity as observed from the requirement of 12 mg/ml substrate to express 100% relative activity when compared to the 10 mg/ml substrate required by the free enzyme.

xiv. The kinetic studies revealed that the $K_m$ and $V_{\text{max}}$ values of the free enzyme to be 0.66 and 166.66, while those of the immobilized enzyme to be 2.70 and 142.85, respectively.

xv. Attempts have been made to study two of the applications of $\alpha$-amylase produced by *B. megaterium* KLMI4, i.e., enzyme application in textile industries and its incorporation in the detergent formulations.

a. $\alpha$-Amylase is used in textile industries for desizing the fabric, i.e., to remove starch from the woven yarn. The optimum concentration of the enzyme for desizing has been observed to be 300 IU/ml and an efficiency of 71% desizing is achieved. The optimum temperature has been observed to be 37\(^{\circ}\) C, implying that the cost of raising the temperature does not arise. The optimum $pH$ is 8.0 and the studies on the time profile revealed 71% desizing in contact time of 30 minutes.

b. In the studies pertaining to the feasibility of incorporation of the $\alpha$-amylase produced by *B. megaterium* KLMI4 into detergent formulations, compatibility of the crude enzyme with five locally available detergents (Surf Excel, Wheel, Tide, Ariel and Ghadi) has been evaluated. It is observed that on the whole, the enzyme is compatible to very good extent with all the detergent formulations.
However, its stability in the presence of the detergents over 15 min contact period has been observed to be in the following order: Tide=Ariel>Surf Excel>Ghadi>Wheel. With incubation time extension to 75 minutes, the loss of activity is observed to be 7% and 8% in case of Tide and Ariel, respectively, and loss of relative activity of 17% (Surf Excel), 40% (Ghadi) and 46% (Wheel) has been recorded. Further, the stains of blood, chocolate and barbecue sauce were removed to very good extent by incorporating the enzyme with the detergents.

5. Chapter V: In this chapter, a detailed discussion of observations made during the present investigation are presented with reference to the studies reported by the earlier researchers in the field. The present study reveals that the locally isolated soil bacterium, *B. megaterium* KLMI4 is a potent producer of α-amylase. Optimization of the basic process variables, supplementation of carbon and nitrogen sources as well as metal salts help in obtaining much higher enzyme yield. Higher production of the enzyme employing green gram husk as an alternate carbon source assists in reducing the process cost. Immobilization of the bacterial cells as well as the enzyme assisted in further economization of the process due feasibility of reuses. The kinetic analysis of both the free and immobilized enzyme have been carried out probably for the first time for a strain of *B. megaterium*. The enzyme characteristics like pH and thermostability associated with the results obtained regarding its possible applications indicate the enzyme can be successfully used as desizer and as a supplement in the detergent formulation at either room or mild warm temperatures. Since there is a scope for further economization of the
production of enzyme, further studies on inducing mutations, either through physical or chemical means and also recombinant gene technology for this bacterium may be carried out. Since, the enzyme of this bacterium is proposed to be used as an ingredient of detergent formulations, recombinant gene technology for inducing the same organism to co-produce other important enzymes like lipase and protease, may also be attempted.

The bibliography referred in the present thesis is presented in the References section at the end of the thesis.