Summary

Amylase, a class of industrial enzymes, occupied major portion of the enzyme market. Among amylases, application of alpha amylase is most prevalent in industrial sector. The α- amylases randomly hydrolyzes α- 1,4 glycosidic linkages in starch, glycogen and related polysaccharides yielding oligosaccharides, maltose and glucose. This enzyme is ubiquitous and obtained from various sources like animal, plant and microbes (bacteria and fungi). But among the sources fungi *Aspergillus* have been widely used for the commercial production of acidophilic amylase. Fungal amylase is now extensively used in many sectors like production of ethanol, fructose syrup, detergent, textile desizing and also in pollution control. The present investigation was undertaken with an aimed to find the acidophilic amylase which can digest different raw starchy food stuffs and food in heterogenous condition, to use this enzyme in digestive syrup producing industry.

The result of present Ph.D work entitled "Studies on acidophilus amylase from newly isolated soil fungus and its potential application in starch saccharification" have been summerized below

At first fifty different fungal isolates were isolated from soil sample of Municipal Garbage, Midnapore bus stand; Vidyasagar University canteen; paddy field of Gopegarh; potato cultivation field of Dharma with predominant in starchy waste. In primary screening among fifty (50) fungal strains twenty five (25) α- amylase producing fungal strains were isolated for secondary screening. Among the twenty five (25) strains only four (4) strains were selected for secondary screening on the basis amylase production on starch containing liquid medium. Among them, one was chosen and designed as RBP7 for highest amylolytic activity. Microscopic observation of the isolated fungi showed morphology similar with *Aspergillus* sp. Phylogenetic analysis based on 18S r DNA sequences showed that the isolate has maximum similarities with *Aspergillus niger* species. The sequence was submitted to GenBank under the accession number KX 100578.1.

Potato peel the solid substrate before and after fermentation was studied with SEM which revealed the structural changes. The control potato peel showed a regular alignment of cells without any damage under scanning electron microscopy. After fermentation the surface of potato peels become rough, slacken and dispersed. This is due to the hydrolytic effect of α-amylase.

In SSF the predicted value of α- amylase (1112.25 U/gds) was obtained from RSM at the following fermentation condition: potato peel concentration (1.24981 g/10 ml), incubation temperature (44.457 °C), initial pH (2.73955) and inoculums concentration (0.987851 ml/g). In OVAT maximum amylase production (1090.40 U/gds) was observed at pH 3.0, temperature 40 ^{o}C, amount of substrate material 1.0 (g/10ml), inoculums concentration 0.75 ml/g substrate which was in good assessment with the predicted results (1112.25 U/gds) of RSM. The enzyme production was increased in RSM than OVAT due to the effect of interactions between four different variables. In case of SmF the maximum amylase production (115.8 U/ml) in OVAT was observed at potato peel 4g/100ml, inoculums concentration 1.25 ml (%V/V), pH 4.0, temperature 35 ^{o}C, which was in good assessment with the predicted results (131.96 U/ml) of RSM at potato peel 4.276 g (%, w/v), inoculums concentration 1.242 ml (%, v/v), pH 4.314, temperature 34.03 ^{o}C. The α-amylase production ability of *Aspergillus niger* RBP7 was compared with the reference strain *Aspergillus niger* MTCC 281 where the isolated strain *Aspergillus niger* RBP7 produce more amylase than standard strain.

The α- amylase from *Aspergillus niger* RBP7 was subsequently purified with size exclusion chromatography using a Sephadex G100 column. The molecular weight of enzyme was found to be approximately 37.5 kDa. Finally the enzyme purified about 9.31 fold with specific activity of 335.61. The activity recovery was 7.19%. The protein band of purified α- amylase was confirmed by the zymogram analysis. The purified enzyme (single band) hydrolyzed the starch present in the zymogram showing a clear zone around the blue starch iodine complex background.

It was found that the hydrolytic efficiency of the experimental α- amylase was differing substrate to substrate and the descending order of efficiency as follows: soluble starch >

amylose > amylopectin. The enzyme was unable to cleave pullulan. The kinetic parameter (V_{max}, $K_{m,}$ K_{cat} and catalytic efficiency) values of enzyme revealed that K_m of 1.4 mg/ml, V_{max} of 0.992 µmol/ min, K_{cat} of 2.979 min^{-1} and catalytic efficiency of 5.069 mg/ml/min for soluble starch.

The maximum activity of α- amylase of the experimental organism was showed at pH 3.0. The enzyme showed the stability in a broad range of pH (2.0 - 6.0) at 60 to 300 min of incubation period. The purified amylase showed maximum activity at 45 ℃ and the stability of the enzyme was observed between 40 - 70 ℃ at 20 to 120 min of incubation.

Temperature quotient (Q_{10}) is mainly used to study the dependence of enzyme catalytic reaction rate on temperature. Result showed that temperature quotient (Q_{10}) of the acidophilic amylase is 1.065 at 45 ^{o}C.

The relative activity of enzyme showed more than 80% in presence of 1% EDTA. Therefore, metal chelation has no major inhibitory effect on enzyme activity. The inhibitory agents like phenylmethylsulfonyl fluoride (PMSF) is a serine protease inhibitor. Phenylmethylsulfonyl fluoride (PMSF) at 3%, had no immense effect on the relative activity of amylase which was 87.82%. Beta mercaptoethanol is mainly used to reduce disulfide bonds. In presence 3% β- mercaptoethanol the relative activities were 92.84% therefore, it had no major effect on amylase activity. No major inhibition of enzyme activity in presence of β- mercapto ethanol indicating that there was no involvement of SH- group at the active site of enzyme. The amylase activity more than 30% was retained in presence of different surfactant (SDS, tween 60, tween 80, triton X).

The purified amylase could retain about 90 % of its activity after 15 days of incubation at room temperature and retain 97% activity in freezing condition. Enzyme retains its 95.4 % activity after 15 days of incubation.

The crude amylase of *Aspergillus niger* RBP7 was used to digest the raw starches (taro, yam, malanga and sweet potato). The relative conversion efficiency at 1 h showed that 10% and 9.3 % obtained for yam starch and taro starch are significantly higher than 4.9 % and 4.85 % obtained for the malanga starch and sweet potato starch respectively. The paper

chromatography (PC) analysis of the starch digest, showed as the maltose predominant product of hydrolysis with small amount of glucose for all the starches tested. The appearance of maltose and low level of glucose on the paper chromatography of starch digestion by amylase of *Aspergillus niger* RBP7 indicated that the crude enzyme consists principally of α-amylase.

The catalytic effect of different commercially available enzymes Carmozyme™, Vitazyme™, Aristrozyme™, Aristrozyme™ are compared with amylase of RBP7 on different raw starch corn starch, wheat starch, cassava starch, potato starch, soluble starch etc. From the experiment it was observed that with respect to amylase from RBP7, Vitazyme has highest relative activity (28.23%) when it works on wheat starch where as Unienzyme has lowest relative activity (0.12%) when it works on cassava starch with respect to amylase from RBP7. In case of production of reducing sugar Unienzyme produced 87.5 U/ml and Vitazyme produced 49.4 U/ml reducing sugar by hydrolyzing soluble starch and potato starch respectively. Degradation of starch in pure form at acidic pH is not the attestation of its application as digestive enzyme. To validate the same, lunch food ingredients was digested with the purified amylase of *Aspergillus niger* RBP7 and the performance was compared with some commercially available digestive tonic. The result revealed that liberation of reducing sugar from the meal was significantly higher after digestion with RBP7 enzyme and among the tested enzyme only carmozyme imparted similar digestive capacity. The result revealed that the amylase of *Aspergillus niger* RBP7 have immense potential to degrade starchy materials present in lunch food ingredients in heterogeneous condition.

The cytotoxicity of the *Aspergillus niger* RBP7 amylase was tested but it shows no significant effect on normal Human intestinal epithelial (InEpC), indicating its non-toxicity to the cells.

The biotransformation of waste materials (Papper pulp industrial waste and Sugar cane bagasse waste) by purified amylase of *Aspergillus niger* RBP7 showed large amount of total carbohydrate, reducing sugar and total solid present in both samples. The amount of total solids in pulp and paper industrial waste is higher than sugar cane bagasse waste. The

production of reducing sugar and total carbohydrate both are higher in pulp and papper industrial waste than sugar cane bagasse waste

The sequential diversity of functionally similar proteins from different genus, reflects the genetic adaptation. Whereas sequential diversity of functionally similar proteins from same genus and species reflect protein's interaction with their diversified external environment. Therefore, in present study it was clearly observed that acidic environment governed the required changes among protein sequences of acid α-amylases from different strains of *Aspergills niger*. Beyond strain specificity environmental adaptation played an important role in α-amylase protein sequence diversity. The specific change of His to Glu at the Ca^{2+} binding amino acid residue within all acid α-amylase is an important clue to make genetically engineered potent α-amylase, which may functionally active in low pH condition.

At the end of the work it can be concluded that fungal strain *Aspergillus niger* RBP7 secrets significant amount of acidophilus amylase by both solid state and submerged fermentation. In Both the solid state and submerged fermentation by RBP7 was carried out by following OVAT and RSM methods sequentially. The highest acidophilus amylase was produced in solid state fermentation where potato peel used as substrate. The α- amylase production ability was compared with the reference strain *Aspergillus niger* MTCC 281. The newly isolated strain was better to produce α- amylase than reference strain. The purified acidophilus amylase obtained from *Aspergillus niger* RBP7 shows some unique features that is the enzyme is highly acidophilic, remain stable during storage and also active in presence of light. Paper chromatography analysis showed that hydrolysis of different food stuffs (yam, taro, malanga, sweet potato) by acidophilus amylase produced maltose and glucose. This acidophilic amylase can also hydrolyze heterogeneous food and different waste materials, moreover, this enzyme has no cytotoxic effect therefore this enzyme should be used as digestive syrup producing industry. In this study it was clearly observed that acidic environment governed the required changes among protein sequences of acid α-amylases from different strains of *Aspergillus niger*. Beyond strain specificity environmental adaptation played an important role in α-amylase protein sequence diversity. The specific change of His to Glu at the Ca^{2+}binding amino acid residue within

all acid α-amylase is an important clue to make genetically engineered potent α-amylase, which may functionally active in low pH condition.