American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

American Journal of Biochemistry and Molecular Biology 1 (3): 255-264, 2011
ISSN 2150-4210 / DOI: 10.3923/ajbmb.2011.255.264
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Immobilization of Microbial (Wild and Mutant Strains) Amylase on Coconut Fiber and Alginate Matrix for Enhanced Activity

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ABSTRACT

Reports are not available for immobilization of on α-amylase produced by *Bacillus subtilis, Agrobacterium tumefaciens, Escherichia coli, Xanthomonas campestris, Staphylococcus aureus, Aspergillus niger, Aspergillus flavus, Metarhizium anisopliae, Azotobacter chroococcum* and *Rhizopus oryzae* on coconut coir. Hence, an attempt was made in present study to immobilize the free amylase on coconut coir. Cell bound amylase of *Bacillus subtilis, Escherichia coli, Xanthomonas campestris, Aspergillus niger, Aspergillus flavus* and *Rhizopus oryzae* were immobilized by entrapment in calcium alginate. The immobilized enzyme on cells showed higher α-amylase production compared to free cells. The enzyme was characterized by Thin Layer Chromatography (TLC) and confirmed as alpha amylase. A simple and inexpensive method for immobilizing α-amylase on coconut fiber was also adopted. Highest amylase activity was observed in *X. campestris* on immobilized state on coconut fibers (1.708±0.103 U/min/g) and also in alginate matrix (0.948±0.082 U/min/100 beads). Hence, the cells of *X. campestris* were mutated with UV (for different time intervals: 1, 2, 4 and 8 min) and acridine orange (in different concentrations: 1, 2, 4 and 8 μg mL^{-1}). The immobilized catalyst of mutant strain of *X. campestris* AO_4 on alginate matrix exhibited 66.31% (4.125±0.056 U/min/100 beads) higher than that of the parent strain. It exhibited highest activity, when immobilized on coconut coir. Thus the mutant strain of *X. campestris* AO_4 could be used in industries for the efficient conversion of starch to oligosaccharides.

Key words: Microbial α-amylase, immobilization on coconut fiber, mutant strains, *Xanthomonas campestris, Metarhizium anisopliae*

INTRODUCTION

Amylases constitute a class of industrial enzymes which alone form approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals. Although amylases can be derived from plants and animals, those obtained from bacterial and fungal sources generally meet industrial demands (Guzman-Maldonato and Paredes-Lopes, 1995; Rao *et al*., 2007; Ahmed *et al*., 2008; Djabali *et al.,* 2009; Pascoal *et al*., 2010). Immobilization increases the stability and catalytic activity of amylases and the resistance of microorganisms against destroying factors. Industrial development of enzymatic reactors requires the use of immobilized enzymes in order to reduce the cost of the biocatalyst. To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations. In previous works, α-amylase has been immobilized into a large variety of supports such as controlled pore glass, nylon, collagen, sephadex, sepharose and polyaminostyrene (Hornby and Goldstein, 1976; Dey *et al*., 2002; Guisan, 2006; Rao *et al*., 2007; Yugandhar *et al*., 2007; Delcheva *et al.,* 2007; El-Banna *et al.,* 2007; Pramod and Lingappa,

2008; Egwim and Oloyede, 2008; Alkhatib *et al.,* 2010). Also the amylase was immobilized on nitrocellulose membrane and zirconium dynamic membrane (Guisan, 2006; Rao *et al.*, 2007). However, reports on utilization of natural fiber such as coconut coir and drumstick fiber for immobilization of amylase are scarce (Dey *et al.*, 2002). Coconut coir is a product of the coconut industry that has numerous industrial applications including automobile upholstery, bedding and mattresses, drainage filters, insulation, packaging, brush and broom manufacturing, reinforcement of thermoplastics, marine cordage and fishnets and in horticulture as a growing medium/soil substrate. Coir has water holding capacity and a cation-exchange capacity of 39-60 meq/100 g (Dey *et al.*, 2002).

These properties and the fact that it is widely available make coconut fiber a potential hydrophilic support that may be used for immobilization of enzymes by physical adsorption. However, its application has been restricted to the mentioned applications and it has tested as an immobilization matrix for adsorption of enzyme by Dey and their colloquies (Dey *et al.*, 2002). However, coconut fiber has been tested as an immobilization matrix only for adsorption of enzyme from *Bacillus circulans* GRS 313 (Dey *et al.*, 2002). Thus, an attempt was made to test coconut fiber and drumstick fiber as adsorption matrices for immobilization of amylase from *Bacillus subtilis, Escherichia coli, Xanthomonas Campestris, Aspergillus niger, Aspergillus flavus* and *Rhizopus oryzae*. The catalytic behaviour of immobilized amylase from different sources will be discussed and compared with the free enzyme.

MATERIALS AND METHODS

Microorganisms used: Pure culture of *Bacillus subtilis, Agrobacterium tumefaciens, Escherichia coli, Xanthomonas campestris, Staphylococcus aureus, Aspergillus niger, Aspergillus flavus, Metarhizium anisopliae, Azotobacter chroococcum* and *Rhizopus oryzae* were purified from the mother culture of our laboratory. They were isolated from different sources using selective medium and identified by biochemical and microscopic analysis and stored in collection centre. The cultures were used as reference and sub-cultures were used as wild strains for the present study. The fungal strains were maintained on Potato Dextrose Agar (PDA) agar plates. *E. coli, X. campestris, A. tumefaciens* and *A. chroococcum* were maintained on Eosin Methylene Blue (EMB) agar, Luria agar, *Agrobacterium* agar and nitrogen-free agar, respectively. *B. subtilis* and *S. aureus* were grown in nutrient agar (Fig. 1a-g). Study was conducted from September 2008 to August 2010.

Primary screening of amylase producing microorganisms: Primary screening was done by starch agar plate method (Guisan, 2006). Strains of *B. subtilis, E. coli, X. campestris, A. niger, A. flavus* and *R. oryzae* showed maximum starch hydrolysis on starch agar medium. These six strains were selected for further investigation.

Preparation of immobilized cell bound enzyme using calcium alginate: Young fungal mycelia or bacterial cells from liquid cultures were centrifuged at 3000 rpm at 10°C for 8 min and the supernatant liquid was discarded. The pellet was resuspended with a previously autoclaved solution of sodium alginate (HiMedia, Mumbai, India). The solution had been adjusted to pH 7.0 before the autoclaving. Unless otherwise stated, a final concentration of 2% (w/v) sodium alginate and 10% (vol/vol) fungal biomass was used. The alginate-fungus mixture was added drop wise from a height of 20 to 50 mm with sterilized 10 mL glass pipette (nominal diameter, 2.0 mm) into an autoclaved solution of calcium chloride (normally 1%, adjusted to pH 7.0), where beads

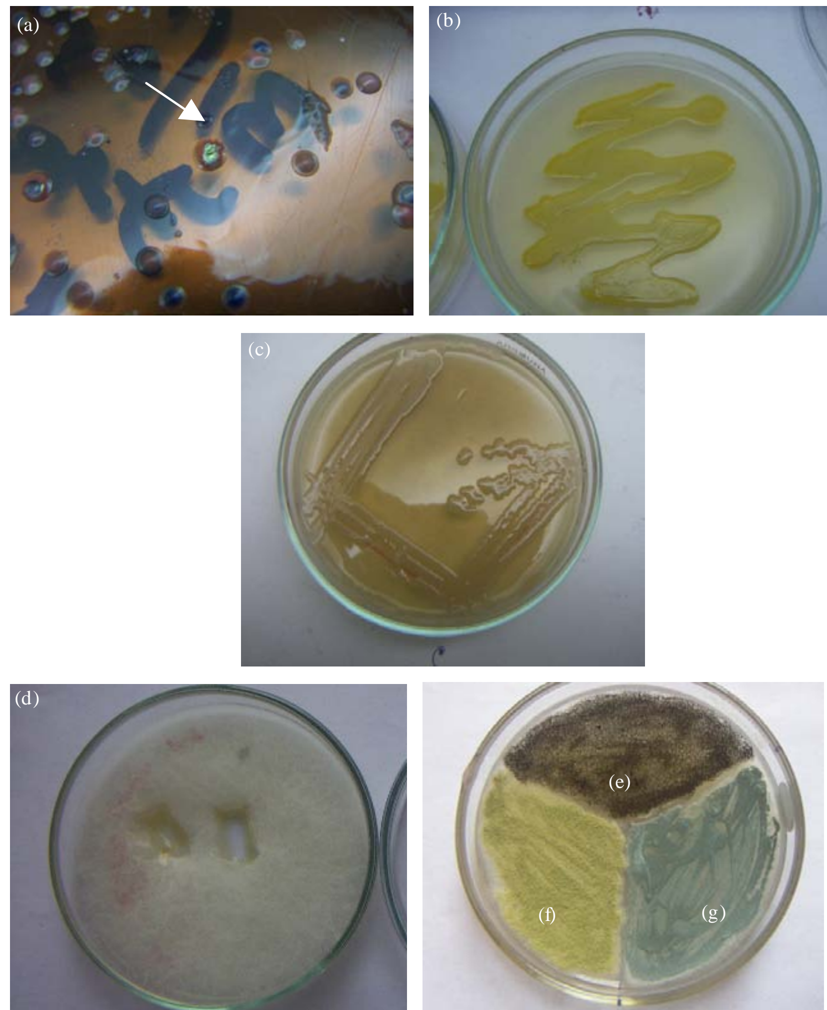

Fig. 1: Microorganisms used in the present study; (a) *E. coli* on EMB agar; (b) *X. campestris* on Luria agar; (c) *A. chroococcum* on nitrogen-free agar; (d) *R. oryzae* on PDA; (e) *A. niger* on PDA; (f) *A. flavus* on PDA and (g) *M. anisopliae* on PDA

formed instantaneously. The beads were left in this gelling solution for 5 min before being harvested by filtration through a 0.18 mm sieve, rinsed with sterilized physiological saline solution and chilled overnight at 4°C and comparative data of their activity was obtained. The preparation of control for immobilized cells was also carried out.

The enzyme production medium contained ammonium dihydrogen phosphate (0.02), manganese chloride (0.05), soluble starch (0.2), beef extract (0.02), distilled water (100 mL). The pH of the medium is adjusted to 6.5 and the flasks were kept on rotary shaker at 27±2°C (for fungi) or 37±2°C (for bacteria) for 72 h. Two sets of production medium were prepared. To one of it, entrapped gel beads of *B. subtilis, E. coli, X. campestris, A. niger, A. flavus* and *R. oryzae* were added to the medium. To the second, unimmobilized cells were added. Control was set up by the medium containing calcium alginate without cells. After incubation, the enzyme was assayed.

Amylase assay: Amylase was assayed by adding 0.2 mL of enzyme (fermented broth supernatant) to 0.5 mL of 1% soluble starch and incubated for 10 min at 37±1°C. The reaction was stopped by

adding 0.75 mL of dinitrosalicylic acid reagent. The final volume was made to 5 mL with distilled water, tubes were kept in boiling water for 6 min, cooled and the absorbency measured at 575 nm with a UV-Visible spectrophotometer.

One unit (U) of amylase was defined as the amount of enzyme that liberates one Mole of reducing sugars, measured as reducing sugar per min under the conditions of assay.

Thin-Layer Chromatography (TLC) analysis: The hydrolyzed products of starch were submitted to TLC on silica gel plates for confirmation as alpha amylase. Enzymes (10 μL) were incubated with 1% of soluble starch (100 μL) in potassium buffer (pH 8.0) at 50°C for 1 h. Aliquots of the reaction mixtures were chromatographed on a silica gel [30 gm of silica gel in 61.5 mL of chloroform: methanol: conc. sulphuric acid (42: 18: 1.5)] with n-butanol, ethanol and water (5: 3: 2) as mobile phase. The products from the incubated content were detected by baking at 120±22°C for 30 min.

Preparation of immobilized enzyme on coconut fibers: The coconut fibers (1 cm) were separated and boiled in water containing 0.01% (w/v) sodium dodecyl sulfate for 1 h. The fibers were dried completely at room temperature. One milliliter of enzyme solution (fermented broth supernatant), along with 0.1 g of the fiber was placed in a shaking water bath at 30°C for a contact time of 2 h. After adsorption the enzyme solution was decanted and saved for subsequent assay. The unbound amylase was washed off with distilled water until no activity was detected in the washing.

Enzyme assay: The reaction mixture, containing 0.5 mL of 1% (w/v) soluble starch solution and 0.05 g of coconut fiber was incubated at 37±1°C in a water bath shaker. Adding 0.75 mL of dinitrosalicylic acid reagent stopped the reaction. The final volume was made to 5 mL with distilled water, tubes were kept in boiling water for 6 min, cooled and the absorbency measured at 575 nm. One unit is defined as the amount of amylase that produced 1 μmol of reducing sugar under assay conditions/g of fiber.

Preparation of mutant strains of *X. campestris*: *X. campestris* which showed maximum starch hydrolysis on starch agar medium and amylase activity on alginate matrix and coconut fibers was selected for further investigation. *X. campestris* cell suspensions (approximately 1.03×10^{2} CFU mL^{-1} in saline solution) were treated with UV (for different time intervals: 1, 2, 4 and 8 min) or with acridine orange (in different concentrations: 1, 2, 4 and 8 μg mL^{-1}). The treated suspensions were appropriately diluted and plated on to starch agar plates. Colonies, producing large clear zones around them were selected (Strain name-mutagen, *X. campestris* AO1-1 μg mL^{-1}, *X. campestris* AO2-2 μg mL^{-1}, *X. campestris* AO3-4 μg mL^{-1}, *X. campestris* AO4-8 μg mL^{-1}, *X. campestris* UV1-1 min, *X. campestris* UV2-2 min, *X. campestris* UV3-4 min and *X. campestris* UV4-8 min) and tested for amylase activity by culturing in a liquid medium and also by immobilization on alginate matrix, coconut coir and drumstick fiber.

Statistical analysis: All experiments were performed in triplicate and the data presented in this work are the mean value of three different experiments. Standard error was calculated by standard statistics (STATISTICA/w 5.0. software).

RESULTS AND DISCUSSION

Primary screening of amylase producing microorganisms: In the search for microorganisms possessing high amylolytic activity, *Bacillus subtilis, Escherichia coli, Xanthomonas campestris, Aspergillus niger, Aspergillus flavus* and *Rhizopus oryzae* exhibited higher amylolytic activity in starch agar medium (Fig. 2, 3a-h) and were selected for further studies. The type of amylase enzyme was identified as α-amylase by analysing the hydrolysed products (maltose and maltotriose) of starch in TLC (Palanivelu, 2001). Dhanasekaran and co-workers adapted the same method to identify the enzyme separated from *Bacillus* species using in thin layer chromatography. They observed that the *Bacillus* sp. what they have isolated produced maltose and maltotriose (Dhanasekaran *et al*., 2006).

The highest (22.00±1.50 mm) activity was observed in *X. campestris* on starch agar plates with clear zone was seen after flooding the plate with iodine as showed in Fig. 2. This result agreed with that of Konsoula *et al.* (2007) on the production of alpha amylase from *Xanthomonas campestris* ATCC 13951.

Amylase immobilization: Number of workers have reported that the addition of various compounds to the enzyme to increase its catalytic activity and stability by preventing spontaneous or heat induced denaturation of the enzymes (Mozhaev *et al*., 1989; Blandino *et al*., 2001; Gupta *et al*., 2003; El-Batal *et al*., 2005; El-Banna *et al*., 2007). Effective enzyme immobilization can be achieved using several techniques including adsorption to insoluble materials, entrapment in polymeric matrix encapsulation, cross linking with a bifunctional reagent or covalent linking to an insoluble carrier (Kara *et al*., 2005). The most important advantages of immobilization methods are the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method. Enzyme immobilization is a very powerful tool that may be considered to solve this cost problem and saving time (Hamilton *et al*., 1999; Akgol and Denizli, 2004). On the other hand, industrial development of the enzyme reactors requires the use of immobilized enzyme in order to reduce the cost of the biocatalyst (Blandino *et al*., 2000).

In the present study, α-amylase produced by *B. subtilis, E. coli, X. campestris, A. niger, A. flavus* and *R. oryzae* was immobilized on different carriers (alginate matrix, coconut coir and drumstick fiber) as an attempt to assess the activity retained upon immobilization in comparison

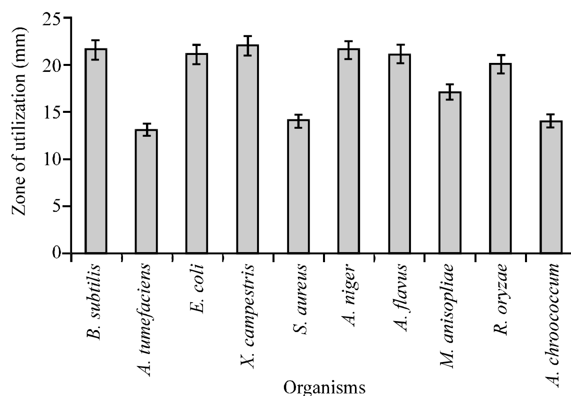

Fig. 2: Zone of starch hydrolysis by different microbes on the starch agar plate C

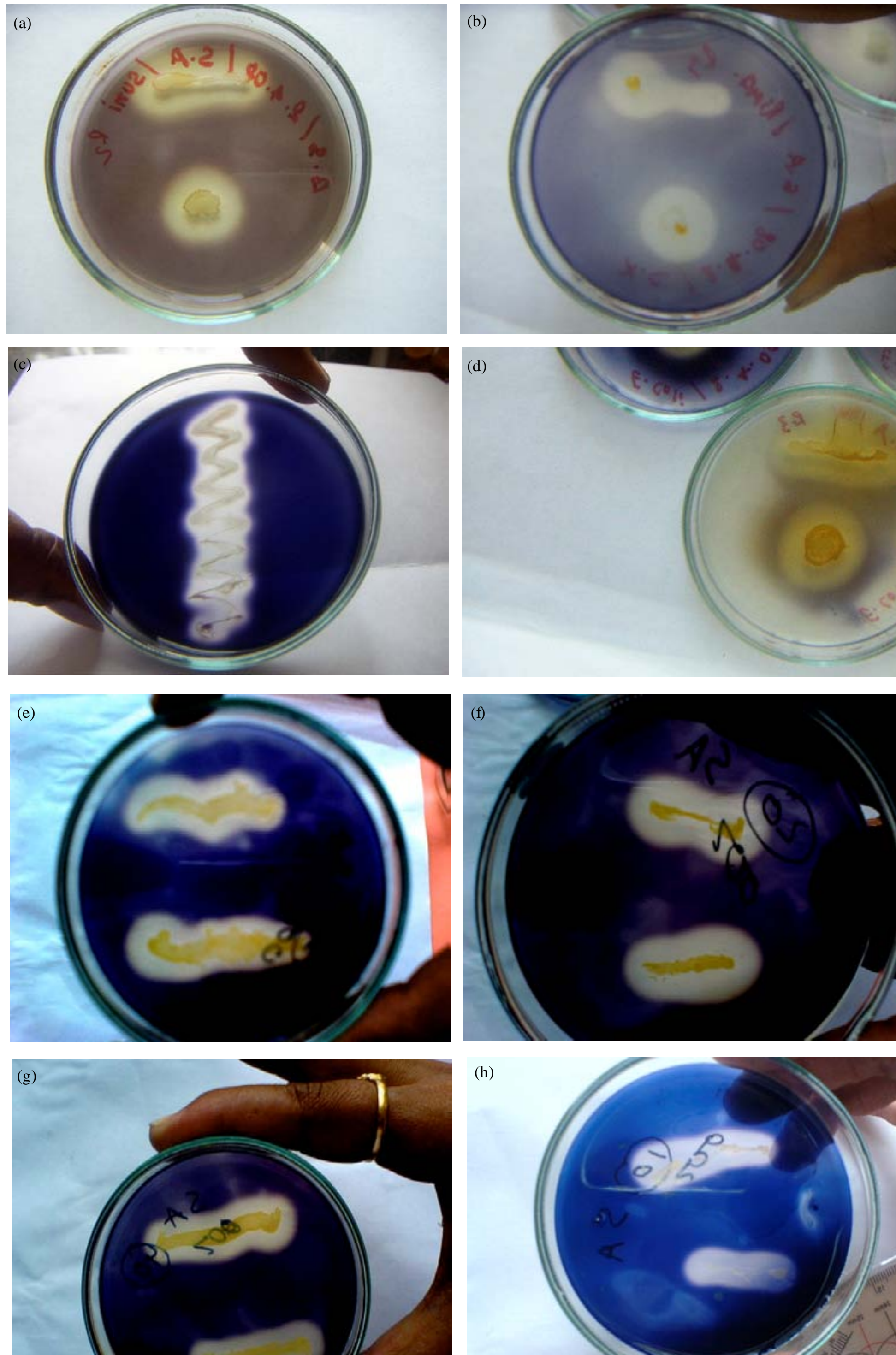

Fig. 3: Screening of the isolates for amylase production in starch agar plate; (a) *B. subtilis*, (b) *X. campestris*, (c) *A. chroococcum*, (d) *E. coli*, (e) *X. campestris* AO3, (f) *X. campestris* AO4, (g) *X. campestris* AO3 and (h) *X. campestris* AO2

to the free amylase and to investigate the operational stability of the immobilized enzyme when compare with the free enzyme. The efficiency of enzyme immobilization was evaluated according to the enzyme activity. Immobilization of amylase on alginate matrix showed that *X. campestris* α-amylase exhibited the highest enzyme activity (0.948±0.082 U/min/100 beads) with 2% calcium alginate. Highest amylase activity was also observed in *X. campestris* α-amylase on immobilized state on coconut fibers (1.708±0.103 U/min/g) (Fig. 4). On the other hand, the lowest enzyme activity (0.647±0.042 U/min/g) was exhibited by *A. flavus* α-amylase immobilized on coconut coir. The current study on the α-amylase from six strains immobilized on coconut coir agrees well with the results reported earlier for *B. circulans* GRS 313 amylase immobilized on coconut coir (Dey *et al.*, 2002). However, all the α-amylase from six isolates showed lower enzyme activity when immobilized on drumstick fibers than that obtained with the other two substrates, alginate matrix and coconut coir (Fig. 4).

Mutagenesis of *X. campestris*: Among the organisms tested, *X. campestris* was the most potent strain for amylase production as it showed maximum starch hydrolysis on starch agar medium and amylase activity on alginate matrix, coconut fibers and drumstick fibers (Fig. 4). Thus *X. campestris* was mutated using ultraviolet irradiation for various time intervals and acridine orange at different concentrations to check its efficacy after mutation.

All the eight mutant strains of *X. campestris* were tested for amylase activity, α-amylase from eight mutant strains of *X. campestris* were analysed in free and in immobilized state on alginate matrix, coconut coir and drumstick fiber. Hyper productive mutants were obtained by these treatments. Under the optimized cultivation conditions, amylase activity of the mutant strain, *X. campestris* AO4 (1.7 μg mL^{-1}) was higher than that of the parent strain (0.52 μg mL^{-1}), *X. campestris* in liquid medium. Immobilized α-amylase from mutant strain, *X. campestris* AO4 on alginate matrix showed higher amylase activity (4.3 μg mL^{-1}) than that of the parent strain, *X. campestris* (0.9 μg mL^{-1}). However, the amylase activity for the immobilized α-amylase from mutant strain, *X. campestris* AO4 on coconut coir and drumstick fibers exhibited lower activity than that of the parent strain (Fig. 5).

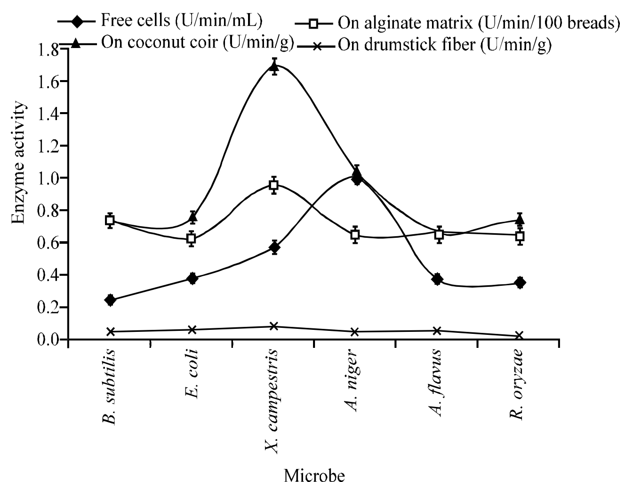

Fig. 4: Activity of free and immobilized α-amylase from bacteria and fungi. Data are given as Means±SD

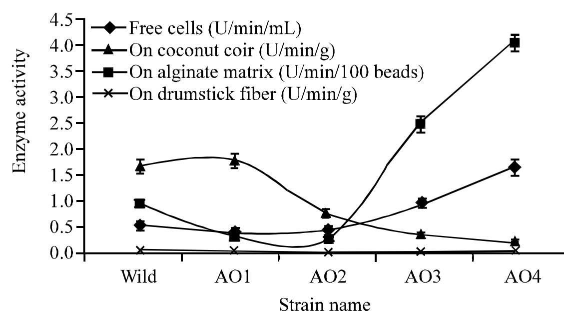

Fig. 5: Activity of free and immobilized α-amylase from mutated *X. campestris* using acridine orange. Data are given as Means±SD. (AO1-*X. campestris* 1 μg mL^{-1}; AO2-*X. campestris* 2 μg mL^{-1}; AO3-*X. campestris* 4 μg mL^{-1}; AO4-*X. campestris* 8 μg mL^{-1})

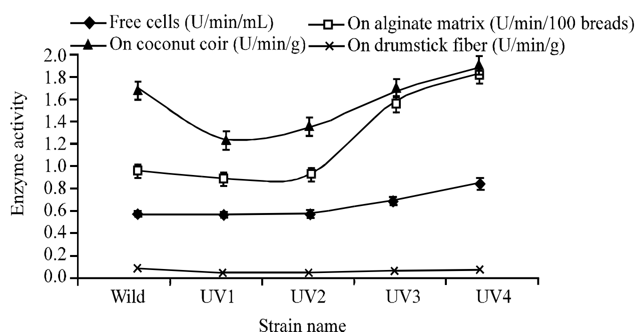

Fig. 6: Activity of free and immobilized α-amylase from mutated *X. campestris* using ultra violet light (UV1-*X. campestris* 1 min; UV2-*X. campestris* 2 min; UV3-*X. campestris* 4 min and UV4-*X. campestris* 4 min)

The amylase activity of immobilized α-amylase from mutant strains on alginate matrix declined slightly after 1 min of exposure to ultraviolet irradiation. But the enzyme activity increased slightly after 2 min of exposure to UV irradiation. However, the enzyme activity of immobilized α-amylase from mutant strains on alginate matrix continued to increase reaching the activity of 1.82 μg mL^{-1} while α-amylase from parent strain (Fig. 6) reached the activity of 0.96 μg mL^{-1} after 4 min of exposure to UV irradiation.

Thus using mutation, amylase activity was improved by as much as about 50-75% in several of the mutants when compared to the parental strain. This clearly suggests that mutation has enhanced amylase synthesis and secretion (Maruo and Tojo, 1985). A preliminary study with one of these hyper producers showed that thermostability of the α-amylase was maintained which suggests that mutation could have occurred in the regulatory gene (Hamilton *et al*., 1999; Akgol and Denizli, 2004) rather than in the structural gene but this must await further investigation. The purpose of the mutation, to make a mutant by altering the regulatory part of the amylase gene was achieved, that can be said from increased production by mutated strains, but gene level studies are needed to confirm the mutation in the regulatory region of the amylase gene.

CONCLUSION

The *X. campestris* AO4 strain mutated in this study could be used in industries for the efficient conversion of locally produced starch to simple sugars. Furthermore, this mutation technique

developed to obtain mutants with enhanced overall amylase production is interesting in that the process is simple, effective and economic. This merits consideration in future mutagenic studies, especially if such studies are being conducted in the underdeveloped countries where cost is a major factor.

ACKNOWLEDGMENTS

Catalyzed and Supported by Vision Group on Science and Technology, Department of Science and Technology, Govt. of Karnataka (VGST/Seed Money 2009-10/LS-N1/10-11/172). The author is thankful to Rev. Dr. Ambrose Pinto, S.J., (Principal), St. Joseph's College (Autonomous), Bangalore for the encouragement. Mrs. B. Bency Borgio is thanked for her continuous encouragement and scientific discussion.

REFERENCES

Ahmed, S.A., E.M.E. Mahdy, O. Hassan, N.A. Saleh and H.A. Goda, 2008. Studies on the activity and stability of immobilized *Bacillus acidocaldarius* α-amylase. Aust. J. Basic Applied Sci., 2: 466-474.

Akgol, S. and A. Denizli, 2004. Novel metal-chelate affinity sorbents for reversible use in catalase adsorption. J. Mol. Catalysis B. Enzymatic, 28: 7-14.

Alkhatib, M.F., M.E.S. Mirghani, I.Y. Qudsieh and I.A.F. Husain, 2010. Immobilization of chitosan onto carbon nanotubes for lead removal from water. J. Applied Sci., 10: 2705-2708.

Blandino, A., M. Macias and D. Cantero, 2000. Glucose oxidase release from calcium alginate capsules. Enzyme Microbial. Technol., 27: 319-324.

Blandino, A., M. Macias and D. Cantero, 2001. Immobilization of glucose oxidase within calcium alginate gel capsules. Process Biochem., 36: 601-606.

Delcheva, G., I. Pishtiyski, G. Dobrev and S. Krusteva, 2007. Immobilization of *Aspergillus niger* pectinase on polyacrylonitrile copolymer membrane. Trends Applied Sci. Res., 2: 419-425.

Dey, G., V. Nagpal and R. Banerjee, 2002. Immobilization of α-amylase from *Bacillus circulans* GRS 313 on coconut fiber. Applied Biochem. Biotechnol., 102-103: 303-313.

Dhanasekaran, D., P. Sivamani, G. Rajakumar, A. Panneerselvam and N. Thajuddin, 2006. Studies on free and immobilised cells of *Bacillus* species on the production of α-amylase. Internet J. Microbiol., 2: 4-4.

Djabali, D., N. Belhaneche, B. Nadjemi, V. Dulong and L. Picton, 2009. Relationship between potato starch isolation methods and kinetic parameters of hydrolysis by free and immobilised α-amylase on alginate (from *Laminaria digitata algae*). J. Food Compos. Anal., 22: 563-570.

Egwim, E.C. and O.B. Oloyede, 2008. Immobilization of α-amylase from Acha (*Digiteria exilis*) on different cellulose fibre materials. Asian J. Biochem., 3: 169-175.

El-Banna, T.E., A.A. Abd-Aziz, M.I. Abou-Dobara and R.I. Ibrahim, 2007. Production and immobilization of α-amylase from *Bacillus subtilis*. Pak. J. Biol. Sci., 10: 2039-2047.

El-Batal, A.I., K.S. Atia and M.A. Eid, 2005. Stabilization of α-amylase by using anionic surfactant during the immobilization process. Radiation Phys. Chem., 74: 96-101.

Guisan, J.M., 2006. Immobilization of Enzymes and Cells. Humana Press Inc., Totowa.

Gupta, R., P. Gigars, H. Mohapatra, V.K. Goswami and B. Chauhan, 2003. Microbial α-amylase: A biotechnological perspective. Process. Biochem., 38: 1599-1616.

Guzman-Maldonato, H. and O. Paredes-Lopes, 1995. Amylolytic enzymes and products derived from starch: A review. Crit. Rev. Food Sci. Nutr., 35: 373-403.

Hamilton, L.M., C.T. Kelly and W.M. Fogarty, 1999. Production and properties of the raw starch-digesting α-amylase of *Bacillus* sp. IMD 435. Proc. Biochem., 35: 27-31.

Hornby, W.E. and L. Goldstein, 1976. Immobilization of Enzymes on Nylon. In: Methods in Enzymology, Jacoby, W.B. and M. Wilchek (Eds.). Academic Press, New York, pp: 118-134.

Kara, A., B. Osman, H. Yavuz, N. Besirilana and A. Denizli, 2005. Immobilization of α-amylase on Cu+2 chelated poly (ethylene glycol dimethacrylate-n-vinylimidazole) matrix via adsorption. Reactive Func. Poly, 62: 61-68.

Konsoula, Z., M. Liakopoulou-Kyriakides, A. Perysinakis, P. Chira, A. Afendra, C. Drainas and D.A. Kyriakidis, 2007. Heterologous expression of a hyperthermophilic α-amylase in xanthan gum producing *Xanthomonas campestris* cells. Applied Biochem. Biotechnol., 118: 1-10.

Maruo, B. and T. Tojo, 1985. Thermostable amylase in *Bacillus licheniformis* by a series of mutations. J. Gen. Applied Microbiol., 31: 323-328.

Mozhaev, V.V., Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov and K. Martinek, 1989. Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures: α-chymotrypsin and laccase in mixed water/alcohol, water/glycol and water/formamides solvents. Eur. J. Biochem., 184: 597-602.

Palanivelu, P., 2001. Analytical Biochemistry and Separation Techniques, A Laboratory Manual. Kalaimani Printers, Madurai, India.

Pascoal, A.M., S. Mitidieri and K.F. Fernandes, 2010. Immobilisation of α-amylase from *Aspergillus niger* onto polyaniline. Food Bioprod. Process., 10.1016/j.fbp.2010.07.002.

Pramod, T. and K. Lingappa, 2008. Immobilization of *Aspergillus niger* in polyurethane foam for citric acid production from carob pod extract. Am. J. Food Technol., 3: 252-256.

Rao, D.M., A.V.N. Swamy and G.S.R. Krishna, 2007. Bioprocess technology strategies, production and purification of amylases: An overview. Internet J. Genomics Proteomics, 2: 1540-2630.

Yugandhar, N.M., C.A.I. Raju, P.J. Rao, K. Jaya Raju and D.S. Rami Reddy, 2007. Production of glutamic acid using *Brevibacterium roseum* with free and immobilized cells. Res. J. Microbiol., 2: 584-589.