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# Immobilization of amyloglucosidase from SSF of *Aspergillus niger* by crosslinked enzyme aggregate onto magnetic nanoparticles using minimum amount of carrier and characterizations

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ABSTRACT

Immobilizations of enzymes are done for operational stability, recovery and re-use of the enzymes and easy separation of products. Amyloglucosidase (AMG) obtained from solid state fermentation (SSF) of *Aspergillus niger* was directly immobilized by novel technique of crosslinked enzyme aggregate onto magnetic nanoparticles. AMG was covalently linked to the magnetic nanoparticle (MNP) to form a monolayer of AMG (MNP–AMG), followed by crosslinked aggregates with free AMG (which was not immobilized) to yield MNP with high enzyme loading (MNP–AMG$_n$). Under optimized conditions, very high recovery (92.8%) of enzyme activity was obtained in MNP–AMG$_n$ using 14 times less carrier compared to the quantity of carrier required by conventional method. MNP–AMG$_n$ showed enhanced affinity for substrate, thermal stability, storage stability and reusability.



## 1. Introduction

Amyloglucosidase (AMG) (EC 3.2.1.3) is a key enzyme being used for production of glucose from starch which is further utilized as sweetener, substrate for microorganisms producing alcohol, as an instant source of energy in health drinks, production of High Fructose Corn Syrup (HFCS) and in many other applications [1]. The exploitation of enzymes in commercial production of large volume, low value products depend on addressing the issues such as cheaper enzyme productions, easy and economical downstream processing, long-term operational stability and recovery and re-use of the enzymes. It has also been encountered that the use of enzyme in these processes, leads to requirement of extra downstream processing steps for its removal. Various immobilization methods such as binding to a carrier, encapsulation in an inorganic or organic polymeric gel, cross-linking of the protein molecules [2] had been employed to overcome some of these challenges. Studies have been done on immobilization of amyloglucosidase (AMG) and other amylases on carriers (poly[(glycidyl methacrylate)co(ethylene dimethacrylate)] [3], nonporous polystyrene/poly(sodium styrene sulfonate) microspheres [4], polyglutaraldehyde-activated gelatin, chitosan and amberlite beads [5], magnetic nanoparticles [6], glyoxyl-agarose [7], in reverse micelle [8] and in alginate fibres [9].

All these methods have their own advantages and limitations. The use of large amount of carrier or other supportive materials have shown to cause problem such as introduction of large proportion of non catalytic mass (carrier) which results in dilution of volumetric activities (activity per unit volume of enzyme preparations), decrease in space-time yields and lowering of catalyst productivity [4,10]. Carrier-free enzyme immobilization approaches such as cross linked enzyme crystal (CLEC) [11] and cross linked enzyme aggregate (CLEA) have been used to circumvent some of these problems [12]. CLEC require pure enzyme and hence is of limited application. The advantage of CLEA is that it does not require purified enzyme as it involves cross linking of precipitated enzyme, which can combine purification and immobilization into a single unit operation [13–16]. The methodology of CLEA involving cross-linking of precipitated enzyme has been widely discussed in various recent studies [13,15,17–21]. But, complete exclusion of carriers and sometimes, immobilized enzyme of low mechanical strength due to poor cross-linking may not be desirable in many industrial applications.

Association of an enzyme with magnetic particles has an inherent advantage of being easily separable. Attachments of AMG to magnetic nanoparticles (MNP) to form MNP linked AMG (MNP–AMG) has been reported earlier in the literature [6,22–24]. Although magnetic nanoparticles have large surface to volume ratio, attachment of enzyme just as a monolayer over MNP leads to

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amount of enzyme immobilized being limited by amount of particles and thus increase the non catalytic mass considerably. Recently magnetic CLEA of alpha amylase was prepared for its easy separation without clumping [25]. In the present study we have coupled the covalent linking of AMG onto MNP and CLEA in order to overcome the problem of limitation of monolayer immobilization. By optimizing the technique, the amount of particles required for maximum immobilization of the enzyme from the solution has been minimized. Crude enzyme obtained from SSF was directly immobilized to avoid the problem of low cross-linking that causes of poor mechanical strength of CLEA. Presence of non catalytic proteins provides extra amino groups for cross-linking and hence problem of low mechanical strength of CLEA could be circumvented. The detailed procedure could provide benefits of low cost SSF, combining the high recovery and immobilization in single unit operation, easy (using minimum energy) and efficient recovery of the immobilized enzyme by magnetic separations along with excellent kinetic and stability properties.

## 2. Materials and methods

### 2.1. Chemicals

AMG used in this study was produced by *Aspergillus niger* (MTCC 3537). Maize starch, glutaraldehyde, bovine serum albumin, ammonium sulfate and dinitrosalicylic acid were purchased from S. D. Fine-Chem Ltd., Mumbai, India. $FeCl_3$, $FeSO_4{\cdot}7H_2O$ and 3-aminopropyltriethoxysilane (APTES) were obtained from Himedia Laboratories, Mumbai, India. Wheat bran was obtained from local market (a waste product from wheat flour industry). All other chemicals were of analytical grade and purchased from S. D. Fine-Chem Ltd., Mumbai, India.

### 2.2. Microorganism and cultivation media

*A. niger* (MTCC 3537) was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained by sub-culturing every month on Czapek yeast extract agar media containing Czapek concentrate ($NaNO_3$ 30.0 g, KCl 5.0 g, $MgSO_4{\cdot}7H_2O$ 5.0 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, distilled water upto 100 mL) 10.0 mL, $K_2HPO_4$ 1.0 g, yeast extract 5.0 g, sucrose 30.0 g, agar 15.0 g, distilled water upto 1.0 L.

### 2.3. Preparation of spore suspension

The spore suspension was prepared by the method as described in the literature [26]. *A. niger* was cultured on Czapek agar media for 5 days and then surface of thallus was scraped with a sterile loop and suspended in phosphate buffer (50 mM, pH 7.0). The supernatant was diluted to 0.6 OD (0.95 mg dry weight $mL^{-1}$) at 600 nm. Spore suspension was used as inoculum for enzyme production.

### 2.4. Production and identification of enzyme

*A. niger* was inoculated in 250 mL Erlenmeyer flask containing 5 g wheat bran and 8 mL salt solution ($ZnSO_4{\cdot}7H_2O$: 6.2 mg/L, $FeSO_4{\cdot}7H_2O$: 6.8 mg/L, $CuSO_4{\cdot}7H_2O$: 0.8 mg/L) [26]. 1 mL of spore suspension was added as inoculum and fermentation carried out at 25 °C for 5 days. 30 mL phosphate buffer (50 mM, pH 7) was added to the flask after fermentation and shaken at 150 rpm for 30 min. Suspension was centrifuged at 10,000 × *g* for 10 min and supernatant filtered through 0.22 μm filter. Filtrate was used as source of enzyme. 3 mL of 5%, w/v gelatinized starch suspension was added to 1 mL crude enzyme obtained from SSF and then volume made upto 12 mL by 8 mL of phosphate buffer (50 mM). The reaction was carried out at 37 °C, 150 rpm for 30 min. To characterize the enzyme, tests for reducing sugars such as maltooligosaccharides (maltose, maltotriose, etc.) including glucose produced from starch by amylase activities viz. Fehling's test, Furfural test and Osazone test were done. Based on the analysis of the sugars, the amylase activities due to endohydrolases like alpha-amylases and/or exohydrolases including beta-amylases and AMG were identified.

The objective of the study was recovery and immobilization of enzyme from fermentation broth or a semi-purified enzyme. There was lack of a standard, commercially available sample for such purpose and it was also difficult to evaluate the purity the enzyme in detail. AMG from SSF contained impurities and was used as a test sample. The technique is also expected to be valid for other commercial samples.

### 2.5. Immobilization of AMG on magnetic nanoparticles (MNP)

#### 2.5.1. Formation and functionalization of magnetic nanoparticles

$Fe_3O_4$ magnetic nanoparticles (MNP) were prepared by chemical coprecipitation of $Fe^{3+}$ and $Fe^{2+}$ ions with a molar ratio of 2:1 [27]. Iron solution containing 1 M $FeCl_3$ and 0.5 M $FeSO_4{\cdot}7H_2O$ in 0.4 M HCl (9 mL) was added drop wise to solution of 0.5 M NaOH (90 mL) under nitrogenous environment at 80 °C with vigorous stirring. Magnetically attracted particles were separated by magnetic separator and washed many times with deionized water (Milli Q water) (DI) until suspension (MNP suspended in DI) of pH 7.0 was obtained. MNP were again separated using magnetic separator and resuspended in 50 mL of DI. MNP formed were analyzed using transmission electron microscopy (TEM). MNP were treated with APTES and glutaraldehyde to attach functional group as shown in Scheme 1. 90 mL methanol and 1 mL APTES were added to solution of MNP. The mixture was shaken at 200 rpm, 50 °C for 12 h to form APTES linked MNP (AMNP). It was followed by 2 washings with DI. AMNP were separated using magnetic separation and the supernatant was thrown. The volume was made upto 50 mL using 5 mM phosphate buffer pH 7.0. 2 mL glutaraldehyde (25%, v/v) was then added to the AMNP solution. It was incubated at 37 °C for 4 h to form glutaraldehyde linked AMNP (GMNP) and followed by 8 washings with DI, magnetic separation done after each cycle of washing. The GMNP thus obtained were separated using magnetic separation and resuspended in 5 mM phosphate buffer. The GMNP thus formed had iron concentration of 30 mg $mL^{-1}$ determined by method discussed earlier in the literature [28].

#### 2.5.2. Attachment of AMG to MNP

AMG immobilized as monolayer over MNP (MNP–AMG) was formed by attachment of AMG onto functionalized MNP (GMNP). For optimization of GMNP concentration required for efficient activity retention; MNP–AMG was formed by adding GMNP (concentration of iron 0.14, 0.41, 0.7, 0.97, 1.39, 2.78, 5.56, 6.94 and 10.42 mg (mg protein)$^{-1}$) to 100 μL enzyme solution (2.16 mg $mL^{-1}$ protein) and volume was made upto 0.8 mL. It was stirred at 200 rpm, 4 °C for 15 h. MNP–AMG was separated using magnet and its activity was determined based on starch hydrolysis.

#### 2.5.3. Formation of crosslinked aggregates of MNP–AMG and free AMG

In case of MNP–AMG, AMG was linked over the MNP as a monolayer only requiring large amount of MNP carrier. In order to increase the enzyme loading over MNP, free AMG was linked to MNP–AMG to form multilayer of AMG over MNP using CLEA methodology. This preparation of crosslinked aggregates of MNP–AMG and free AMG is referred as MNP–$AMG_n$. MNP–$AMG_n$ was formed by first attaching the AMG to GMNP to form MNP–AMG, followed by precipitation and crosslinking of MNP–AMG and