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Original Research Paper

Solid state fermentation with recovery of Amyloglucosidase from extract by direct immobilization in cross linked enzyme aggregate for starch hydrolysis

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ABSTRACT

Main limitations in application of Amyloglucosidase (AMG), an important industrial enzyme for starch hydrolysis, areits high production cost and limited reusability. Immobilization techniques have been tried to address the issue of reusability, however, these techniques either increase the cost of enzyme o rare difficult to scale up owing to special requirements for synthesis of carriers. We tried to address the issue of production cost and reusability for enzyme application by forming carrier free cross-linked enzyme aggregate (CLEA) of AMG from crude SSF extract of *Aspergillus niger* . CLEA recovered 65 percent activity from extract and retained 90% even after 25 cycles of reuse. CLEA showed improved affinity toward substrate; higher storage, thermal and pH stability. Use of cheap SSF and direct immobilization of AMG from extract can reduce the cost of starch hydrolysis.

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1. Introduction

Manufacturing low value; high volume products through enzymatic conversions require addressing issues related to cheap enzyme production, easy and economical downstream processing, operational stability and re-use of the enzymes. Use of enzyme also leads to requirement of extra downstream processing steps for its removal. SSF using biomass residues for low cost production of enzymes had been studied because of several advantages like compactness, greater yield, less investment, low energy demand and waste biomass conversion (Kar et al., 2013). Immobilization of enzymes by binding to carriers, encapsulation in inorganic or organic polymeric gels, cross-linking of the protein molecules also helps to overcome some of these problems (Pascoal et al., 2011). But, the use of carrier or other supportive materials for immobilization have shown to cause problems related to changes in the kinetic parameters of enzymes and introduction of large proportion of non catalytic mass (carrier) resulting in dilution of volumetric activities, decreasing space-time yields and reduced catalyst productivities (Cao et al., 2003; Oh and Kim, 2000).

Carrier-free enzyme immobilization approaches such as cross-linked enzyme crystal (CLEC) (Häring and Schreier, 1999) and cross-linked enzyme aggregate (CLEA) are being used due to their advantages such as: highly concentrated enzyme activity in the catalyst, enhancedstability and low production cost due to the exclusion of an additional carriers (Sheldon, 2007a). CLEC involves crosslinking of crystallized enzyme. Enzymes are crystallized using conventional approach (used for X-ray diffraction) and then these crystals are crosslinked using protein crosslinker such as glutaraldehyde. CLEA on other hand involves precipitation of enzymes using non ionic polymers, salts or water miscible organic solvents as aggregates of protein molecule followed by crosslinking to obtain cross linked aggregates (Cui and Jia, 2015). The major limitation of CLEC methodology is that it essentially requires pure enzymes for enzyme to be crystallized. Whereas, methodology of CLEA does not require purified enzymes, as it involve cross-linking of precipitated enzymes which need not be in it's pure form (Aytar and Bakir, 2008; Pan et al., 2011; Talekar et al., 2012, 2012b). The general methodology of CLEA has been widely used in various earlier studies (Agyei and He, 2015; Cui et al., 2014; García-García et al., 2012; Hormigo et al., 2012; Maria et al., 2011; Pan et al., 2011; Talekar et al., 2012b; Vaidya et al., 2012; Wang et al., 2011) and is also used in this study.

Amyloglucosidase (AMG) (EC 3.2.1.3) is a key enzyme used in the hydrolysis of starch for large scale production of glucose which is being further utilized in many applications. We have earlier shown efficient immobilization of AMG from SSF extract over magnetic nanoparticles (MNP) as carrier (Gupta et al., 2013). But

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the use of MNP as carrier is bound to increase cost of immobilized enzyme as industrial production of MNP is expensive.The average cost of commercial MNP is around 2 USD per mg (http://nanocomposix.com/products/20-nm-magnetite-nanoparticles). Such high cost of MNP makes it difficult to use the technology at industrial scale. Hence in this study, we tried to eliminate carrier by using a carrier free methodology. Advantages of enzyme production using the cheap agricultural residues in solid state fermentation (SSF) and recovery of the enzyme through direct immobilization by CLEA from the crude extract with impurities is expected to serve the need of low cost enzyme in large scale industrial manufacture of low value high volume products.The process would directly reduce the purification/downstream processing cost and small quantity of protein impurities may contribute to the beneficial effect in strengthening the CLEA i.e. kinetic and stability properties of enzyme.No specific study in this direction is available in the literature.The present study focussed on using cheap wheat bran agro residue for SSF. Enzyme in SSF extract was then recovered by direct immobilization in CLEA for production of immobilizedAMG. Screening of precipitating and cross-linking agent for formation of CLEA was done with subsequent optimization of conditions. Kinetic parameters, storage stability and reusability of the immobilized enzyme at different conditions were also analysed.

2. Materials and methods

2.1. Materials

AMG was produced by SSF of *Aspergillus niger* (MTCC 3537). Ammonium sulfate, bovine serum albumin (BSA), dinitrosalicylic acid (DNS), glutaraldehyde, poly ethylene glycol (PEG), polyethyleneimine (PEI) and maize starch were purchased from SD Fine-Chem Ltd, Mumbai, India. Wheat bran was a waste product of wheat flour industry. All other chemicals were of analytical grade and purchased from SD 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 as described in our previous work (Gupta et al., 2013).

2.3. Preparation of spore suspension and enzyme production

The spore suspension was prepared as per the method described in the literature (Gupta et al., 2013; Varalakshmi et al., 2009). Briefly, surface of thallus was scrapped with a sterile loop and 1 ml of supernatant diluted to 0.6 OD (0.95 mg dry weight ml^{-1}) at 600 nm was added to SSF substrate (wheat bran) and fermentation was carried out for 5 days. Extract obtained from SSF after 5 days was used as enzyme source. Detailed procedure can be found in our previous study (Gupta et al., 2013). We have earlier optimized the protocol for amylase production and have shown that enzyme activity in SSF extract is predominantly due to AMG (Gupta et al., 2013).

2.4. Enzyme activity assay

The activity of AMG was assayed on the basis of starch hydrolysis and the amount of hydrolyzed product i.e. glucose was estimated by DNS method (Gupta et al., 2013; Miller, 1959). 12 ml of reaction mixture containing 1 ml enzyme (or precipitated enzyme/CLEA suspended in 1 ml 50 mM phosphate buffer, pH 7.0), 8 ml of 50 mM phosphate buffer (pH 7.0) and 3 ml of 5% w/v gelatinized starch suspension was used in measuring the activity of the enzyme. The reaction was carried out at 37 °C, pH 7.0, 150 rpm for 30 min. For measuring the activity of CLEA, the particulates in the solution were removed by centrifugation at $10{,}000 \times g$ before measurement of absorbance by spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute at pH 7.0 and temperature 37 °C. Specific activity of enzyme was measured as $U\,mg^{-1}$ proteinand $U\,ml^{-1}$. In case of CLEA, specific activity ($U\,ml^{-1}$) was measured based on initial volume of enzyme taken for CLEA formation. Protein content of enzyme was estimated by Lowry method using BSA as standard (Lowry et al., 1951).

2.5. Screening of precipitating agents

Ammonium sulfate (20, 30, 40, 50, 70. 90 and 100% saturation at 4 °C), ethanol (final concentration 5, 10, 20, 40, 50, 60 and 75% v/v) and acetone (final concentration 5, 10, 20, 40, 50 and 60% v/v) were tried for effective precipitation of AMG from SSF extract. 1 ml enzyme extract (2.16 mg protein, 1.3 U AMG activity) was diluted to 8 times by 50 mM phosphate buffer (pH 7.0), precipitantwas added to desired concentration and precipitation of enzyme allowed overnight at 4 °C. Precipitates were centrifuged at $10{,}000 \times g$ for 10 min at 4 °C, redissolved in 50 mM phosphate buffer (pH 7.0) to 1 ml and enzyme activities were measured.

2.6. Formation of CLEA

CLEA was formed by precipitation of enzyme and other proteins (impurities) coupled with cross-linking as described in literature (Kumar et al., 2010). 1 ml enzyme extract (2.16 mg protein, 1.3 U AMG activity) was mixed with appropriate amount of cross-linker to give desired final concentration and volume was made to 8 ml by 50 mM phosphate buffer (pH 7.0). Ammonium sulfate (corresponding to 90% saturation, 5 g) was added to this mixture (8 ml)and incubated at 4 °C overnight. The solution was centrifuged at $10{,}000 \times g$ for 10 min (4 °C) for pelleting CLEA from solution. Pellet was resuspended in 1 ml of 50 mM phosphate buffer (pH 7.0). Suspension obtained was centrifuged again for removal of enzyme which precipitated but was not crosslinked. This procedure was repeated thrice for complete removal of noncrosslinked enzyme.

2.7. Selection of cross-linker

Different cross-linkers such as glutaraldehyde (final concentration 1–15 mM), poly ethylene glycol (PEG) (final concentration 5–40% v/v) and polyethylene imine (PEI) (final concentration 5–40% v/v) were used to form CLEA. Enzyme activity was analyzed both in the CLEA suspended in 1 ml of 50 mM phosphate buffer (pH 7.0) and in supernatant.

2.8. Optimization of CLEA formation

After screening the precipitating agent and cross-linker, physical parameters (pH, temperature and duration of precipitation coupled with cross-linking) were optimized for formation of CLEA. Parameters were optimized by varying one parameter at a time and optimized values were used in the next cycle of optimization. The sequence of optimization was pH, temperature and time duration of precipitation coupled with cross-linking.

2.9. Estimation of kinetic and operational parameters

Michaelis–Menten kinetic parameters (K_M and V_{max}) of free

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