Chemical Engineering Journal 325 (2017) 80–90

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

A tri-enzyme magnetic nanobiocatalyst with one pot starch hydrolytic activity

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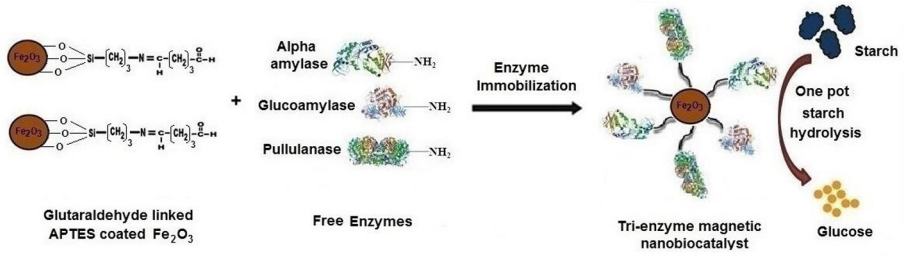
HIGHLIGHTS

- Tri-enzyme magnetic nanobiocatalyst of α-amylase, glucoamylase, pullulanase was prepared.
- Tri-enzyme magnetic nanobiocatalyst was characterized by FT-IR, FE-SEM and XRD.
- Tri-enzyme magnetic nanobiocatalyst improved enzyme stability and catalytic efficiency.
- Tri-enzyme magnetic nanobiocatalyst gained 100% starch conversion in one pot hydrolysis.
- Tri-enzyme magnetic nanobiocatalyst showed excellent reusability in starch hydrolysis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 December 2016
Received in revised form 5 May 2017
Accepted 8 May 2017
Available online 10 May 2017

Keywords:
Co-immobilization
Magnetic nanoparticles
Starch hydrolysis
Alpha amylase
Glucoamylase
Pullulanase

ABSTRACT

An efficient tri-enzyme magnetic nanobiocatalyst for one pot starch hydrolysis was constructed by co-immobilization of commercially available alpha amylase, glucoamylase and pullulanase onto amino functionalized iron oxide magnetic nanoparticles using glutarldehyde as linker. Co-immobilization using 208 mM glutaraldehyde concentration with 4 h linking time and 12 h immobilization time achieved maximum activity recovery of alpha amylase (98%), glucoamylase (92%) and pullulanase (95%) in tri-enzyme magnetic nanobiocatalyst. The prepared nanobiocatalyst was characterized by using X-ray diffraction (XRD), field-emission scanning electron microscopy (FE-SEM) and Fourier transform infrared spectrophotometry (FT-IR). For one pot starch hydrolytic activity, shift in optimum pH from 6 to 7 and temperature from 70 to 80 °C with a high tolerance to alkaline pH and high temperature were observed after co-immobilization of enzymes. The thermal stability in terms of half-life of enzymes in the range of 70–90 °C was three times more for tri-enzyme magnetic nanobiocatalyst than free enzymes. Moreover, the catalytic efficiency of enzymes was found to be higher in tri-enzyme nanobiocatalyst than in free form. The tri-enzyme nanobiocatalyst displayed 100% starch conversion whereas the free enzyme mixture achieved 70% starch conversion after one pot starch hydrolysis in batch mode. At the end, the tri-enzyme nanobiocatalyst remained fully active even after 8 times recycling for one pot starch hydrolysis with cumulative reaction time of 720 min.

1. Introduction

Starch, a water-insoluble polysaccharide of glucose units linked by α (1–4) and α (1–6) glycosidic bonds, is an inexpensive source for production of sugars which are widely used in food industries.

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http://dx.doi.org/10.1016/j.cej.2017.05.054
1385-8947/

In addition to that, the sugars produced can be fermented to produce bioethanol [1]. The major approaches which have been widely used in starch conversion are physical, chemical and biocatalytic procedures. Although the physical and chemical processes have shown their usefulness to a certain extent, these processes are energy consuming and produce many by-products [2]. On the contrary, enzyme-based biocatalytic processes are performed under milder and greener reaction conditions with higher product selectivity [3]. Therefore, enzyme-assisted starch conversion is considered an alternative green approach that reduces process costs, prevents unwanted by-products, and elevates reaction specificity [4]. Starch hydrolytic enzymes alpha amylase (EC 3.2.1.1), glucoamylase (EC 3.2.1.3) and pullulanase (EC 3.2.1.41) are used for two step synergistic hydrolysis (*i.e.* liquefaction and saccharification) of starch to glucose [5].

The major challenges in enzymatic reactions are high cost and time-consuming process of enzyme production, maintenance of enzyme activity upon reaction and the reusability of the enzyme after the reaction [6,7]. To overcome these challenges while keeping the advantages of enzymes, immobilization of enzymes on a suitable prefabricated support has been considered as a graceful approach [8–10]. There are several reports on the immobilization of alpha amylase, glucoamylase and pullulanase individually using solid carriers such as functionalized glass beads [11], polyaniline [12], ion exchange beads [13], gelatin [14], alginate beads [15], chitosan beads [16], mesoporous silica [17], etc., and few works report co-immobilization of any two [18–24]. In these reports, co-immobilized enzymes were found to be more beneficial as they produced larger amounts of glucose by hydrolysing starch compared with both free enzymes and immobilized enzymes alone. Co-immobilization of enzymes also allows consolidation of multi-step enzymatic process into an one pot process without the need of separation of intermediates and offers benefits such as fewer unit operations, less solvent, small reactor volume, shorter cycle times, higher volumetric and space time yields and less waste generation-which translates to substantial economic and environmental efficiency [25]. Recently, we demonstrated consolidation of liquefaction and saccharification by adding co-immobilized alpha amylase, glucoamylase and pullulanase as combined cross-linked enzyme aggregates (combi-CLEAs) to the same vessel containing starch [26]. However, squeezing of combi-CLEA particles occurred while separation from reaction mixture by centrifugation and filtration during repetitive use in batch reaction cycles. This squeezing of combi-CLEA particles elevated mass transfer limitations for macromolecular substrate like starch which resulted in decreased conversion of starch during subsequent batch reaction cycles.

Over the last few years iron oxide magnetic nanoparticles have undergone explosive growth as support for immobilization/co-im mobilization of enzymes, due to their advantages such as high surface area resulting in high enzyme loading capacity, easy surface modification, high dispersion, improved mass transfer, outstanding stability, efficient separation of the enzymes from the reaction mixture using an external magnetic field without centrifugation or filtration [27–31]. So far, several literature reports have studied the supports prepared by iron oxide magnetic nanoparticles for immobilization of alpha amylase, glucoamylase and pullulanase separately [16,32–34] to obtain highly active, stable and easily recyclable biocatalyst. However, there is no report on co-immobilization of these three enzymes onto iron oxide magnetic nanoparticles to carry out multi-step enzymatic starch hydrolysis in the same reaction vessel. Therefore, we envisage the deliberate co-immobilization of alpha amylase, glucoamylase and pullulanase onto iron oxide magnetic nanoparticles to overcome the drawbacks of our previously developed combi-CLEA design as mentioned above. Due to magnetic support, co-immobilized biocatalyst can be easily separated from reaction mixture without squeezing by gentle magnetic field which will avoid mass transfer resistance during repetitive use in batch reaction cycles.

Therefore, in present study, to redesign tri-enzyme biocatalyst for one pot starch hydrolysis, we co-immobilized alpha amylase, glucoamylase and pullulanase onto iron oxide magnetic nanoparticles to develop a tri-enzyme magnetic nanobiocatalyst with one pot starch hydrolytic activity. To the best of our knowledge, this is the first report on co-immobilization of these enzymes onto iron oxide magnetic nanoparticles with one pot starch hydrolytic activity. Covalent attachment of enzyme molecules on iron oxide magnetic nanoparticles gives higher stability and lower enzyme leakage. The resultant multi-enzyme system was characterized and it acted as a green and robust magnetic nanobiocatalyst for one pot starch hydrolysis. Further, the kinetic parameters and thermal stability of free and immobilized enzymes were determined and expressed in terms of thermal deactivation constant (k_d) and half-life ($t_{1/2}$). In addition, prepared nanobiocatalyst was employed for one pot starch hydrolysis. Lastly, reusability was studied up to eight cycles to check its durability and industrial feasibility.

2. Experimental section

2.1. Materials

Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$), glutaraldehyde (25%, v/v), maltose and soluble starch were obtained from Himedia, Laboratories Pvt. Ltd. (Mumbai, India). Rice starch, Pullulan and 3-aminopropyl triethoxysilane (APTES) were purchased from Sigma (St. Louis, USA). Commercial enzyme preparations Thermo-Nzyme L (alpha amylase) and Gluco-Nzyme L (glucoamylase) were purchased from Enzymes India Pvt. Ltd., Chennai and commercial pullulanase preparation was gifted by Riddhi Siddhi Gluco Biols Ltd. (Gokak, India). DNS (3,5-Dinitrosalycyclic acid) was purchased from Merck Specialities Private Limited (Mumbai, India). Glucose estimation (GOD-PAP) kit was purchased from Biolab Diagnostics (I) Pvt. Ltd. Other reagents used were of analytical grade and obtained either from Himedia or Merck.

2.2. Synthesis and amino functionalization of magnetic nanoparticles

Magnetic nanoparticles (MNPs) were synthesized by the co-precipitation of $FeCl_3$ and $FeSO_4$ under basic conditions as described previously with some modifications [35]. Hundred millilitres of $FeSO_4{\cdot}7H_2O$ and $FeCl_3{\cdot}6H_2O$ solution were added into a conical flask to make the molar ratio 1:2 of Fe^{2+} and Fe^{3+}. The solution was heated to 90 °C. Then, sodium hydroxide (5 M, 25 mL) was added slowly under vigorous stirring. The colour of the mixture changed from brown at the initial addition stages to black at the end of the sodium hydroxide addition. The mixture was allowed to complete the oxide precipitation during 30 min. The obtained dark solution was decanted and supernatant was discarded. The black precipitates were washed five times with deionized water until the solution became neutral followed by magnetic decantation and dried under vacuum.

The obtained MNPs were amino functionalized by APTES silanization as reported by Liu et al. [36]. Magnetic nanoparticles (0.5 g) were dispersed in the solvent containing deionized water and ethanol (1:1 v/v, total solution 100 mL) and then APTES (2%) was added into the mixture under mechanical stirring. The reaction mixture was heated up to 70 °C for 3 h then cooled down to room temperature followed by magnetic decantation and thorough washing with ethanol water. Then, the samples were dried in an