



# A novel recycling system for nano-magnetic molecular imprinting immobilised cellulases: Synergistic recovery of anthocyanin from fruit and vegetable waste



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## HIGHLIGHTS

- Molecular imprinting magnetic nanoparticles have been developed to immobilise the cellulases.
- It has good reusability and stability in bio-catalytic processes.
- The immobilised amylase was enhanced than the free enzyme.
- Novel material could effectively treat with FVW and recycle the anthocyanin from it.

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## ABSTRACT

Fruit and vegetable waste (FVW) is becoming a serious problem in developing countries. Enzymolysis is a potentially useful method for the treatment of FVW. In the present study, novel recycled magnetic molecular imprinting immobilised cellulases were prepared based on magnetic modified chitosan (MCTS) and Fe<sub>3</sub>O<sub>4</sub>. The properties of obtained were characterised by IR and grain-size measurements. Evaluation of a single factor affecting the loading efficiency of supports and the mixed immobilised enzymes showed better capacity than single immobilised, or free, enzymes. The immobilisation process could improve cellulase stability and repeatability of the method. Meanwhile, the kinetic parameters were also verified. The immobilised enzymes retained most of their capacity after 60 days' storage while free enzymes lost it within 30 days. Tests showed that the immobilised enzymes developed excellent capacity and five anthocyanins were collected.

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## 1. Introduction

Fruit and vegetable waste (FVW) has become key in modern cities around the world. In recent years, concern has increased about FVW from mega-cities in China, such as Beijing or Shanghai, which have large populations and produce a significant amount of waste. According to a recent report, there are about 1.3 million tons (per day) of FVW are greatly generated approximately in Chinese cities (Wu et al., 2015). On the other hand, the modern food industry also produces FVW and most of them are sent for energy recovery (Edo et al., 2016). Of this huge quantity, because it does no good to choose residues for disposal, the technology for FVW treatment and increasing the added value thereof is becoming increasingly important (Garcia-Peña et al., 2011). On the other

hand, they not only pose serious environmental risks, but also an immense loss of nutrients and bio-ingredients therefrom. Those ingredients in FVW are waste and also need to be recycled, such as natural pigments, for instance. Pigments of fruit and vegetable origin, which are currently enjoying high market demand, are β-carotene, lutein, and anthocyanin.

Many FVW have been proven to be sources of food supplementation, flavours, and nutrition-rich animal feed (Christen et al., 2000). Recently, many industrial or economic products were obtained from FVW, such as citric acid and pectinaceous products from apple pomace (Dhillon et al., 2011; Garna et al., 2007), D-psicose (Patel et al., 2016) and lactic acid (Wu et al., 2015) from fruit and vegetable residues. However, most of those products focused on the FVW fermentation, few studies pay close attention to the anthocyanins in FVW. Anthocyanins often couple with polysaccharides, such as starch or cellulose. Meanwhile, low concentrations of anthocyanins in the FVW are noteworthy.

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Anthocyanin is a well-known natural alternative to synthetic pigments, which come from many flowers, fruits, and vegetables. Meanwhile, different types of anthocyanins responsible for colours, such as red, blue, and purple (Gizir et al., 2007; Kamiloglu et al., 2015) are eagerly sought after. The anthocyanins not only have the colourant properties, but also possess a wide range of human health benefits. Some research shows that anthocyanins reduce risk of cardiovascular and cerebrovascular disease such as coronary heart disease. Moreover, many clinical results show that anthocyanins could protect and improve visual acuity and kill cancer cells (Alasalvar et al., 2005). According to the literature, there are about more than 500 anthocyanins including cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin which have been isolated from plants and most of them from fruit and vegetables (Algarra et al., 2014; Fernandes et al., 2014). As can be seen, pigments, especially anthocyanins are important ingredients in fruit and vegetables. Anthocyanins using can be maximised by recycling them from fruit and vegetable waste, however, the complex recycling process costs too much. Therefore, a lower cost technology of anthocyanin recycling should be used in the treatment of FVW before fed into power stations (Wu et al., 2015).

Recently, the biotechnological potential of enzymes in the degradation of biomass has been observed. While, some inevitable disadvantages of free enzymes, such as instability, difficulty in their separation/extraction, and poor reusability, raise their cost of use, and limit their further development. Immobilisation of enzymes on solid supports is crucial and immobilised enzymes have been become one of the most effective ways of enzyme modification (Bayramoglu et al., 2013; Li et al., 2015; Wang et al., 2013). Entrapment, physical adsorption, and covalent attachment are common methods, which could enhance recyclability, ease separation, improve stability, and reduce the reuse-cost (Gülay and Şanlı-Mohamed, 2012; Kannoujia et al., 2009; Mendes et al., 2013). Different immobilisation support materials can provide the improved retention of catalytic activity, stability, and durability (Singh et al., 2015).

Recently, magnetic composites with a core-shell structure have showed great potential for application in enzyme immobilisation, especially cellulase (Chang et al., 2009; Hu et al., 2015; Huang et al., 2015; Li et al., 2015; Li et al., 2014). In this composite, ferro-ferric oxide is widely used as the core surrounded by a silica shell of magnetic composites because of the super-paramagnetism and easy recovery therewith. Starch is a cheap, non-toxic, harmless bio-resource supporting material modification (Szymanowski et al., 2005). The starch is easy to modify due to its rich hydroxyl content: modified starch is also used as a polymeric template for the preparation of magnetic composites. Meanwhile, the rich hydroxyl content can easily link with enzymes.

In this work, a simple preparation process was developed for magnetic composites, consisting of an amination starch coated on Fe<sub>3</sub>O<sub>4</sub>. Then, anthocyanin based on those composites was prepared. Three kinds of cellulases (endoglucanase, cellobiohydrolase, and  $\beta$ -glucosidase) were immobilised on those composites. Then, the effects of pH, temperature, and reusability on the catalytic activity of immobilised enzymes were investigated. Finally, immobilised enzymes were applied to the recycling of anthocyanins in FVW in a fermentation tank.

## 2. Material and methods

### 2.1. Materials

3 kinds of cellulases, endoglucanase (EG), cellobiohydrolase (CBH), and  $\beta$ -glucosidase (BG) were all sourced from *Aspergillus niger*; iron(II) chloride tetrahydrate (FeCl<sub>2</sub>·4H<sub>2</sub>O), iron (III) chloride

hexahydrate (FeCl<sub>3</sub>·6H<sub>2</sub>O), acetic acid, glutaraldehyde (GDA, 25%, v/v), sodium hydroxide (NaOH), and ammonium hydroxide (NH<sub>4</sub>-OH, 28 wt%) were purchased from Sigma. Chitosan (CTS, 80–95% acetylation), liquid paraffin, and Span-80 were obtained from Sino-pharm Chemical Reagent Co., Ltd (Shanghai, China). Ethylene glycol dimethacrylate (EGDMA, 98%), 2, 2'-azobis (2-methylpropionitrile) (AIBN, 99%) and methacrylic acid (MAA, 98%) were obtained from Aladdin Reagent Co., Ltd (Shanghai, China). All reagents were of analytical grade. Double-distilled ultrapure water was purified with a Purelab-ultra (Organo, Tokyo, Japan).

### 2.2. Preparation of Fe<sub>3</sub>O<sub>4</sub> nanoparticles

Fe<sub>3</sub>O<sub>4</sub> nanoparticles are prepared by co-precipitating Fe<sup>2+</sup> and Fe<sup>3+</sup> ions (Massart and Xe, 1981). Ferric and ferrous chlorides (molar ratio 2:1) are dissolved in the water at 40 °C under an N<sub>2</sub> atmosphere, NH<sub>3</sub>·H<sub>2</sub>O solution was added dropwise to prepare iron oxides. The pH of the final mixtures was controlled to within 10–11, the whole reaction process was performed under mechanical agitation. The mixtures were aged at 70 °C for 1.5 h and then washed several times with distilled water until the supernatant pH was neutral. The obtained Fe<sub>3</sub>O<sub>4</sub> nanoparticles were dried in a vacuum oven at 60 °C for 12 h.

### 2.3. Preparation of magnetic modified CTS (MCTS) microspheres

Some 1.5 g of CTS was dissolved in 50 mL 2% (v/v) of acetic acid solution. Then 0.5 g Fe<sub>3</sub>O<sub>4</sub> nanoparticles were dispersed into the CTS solution under mechanical stirring for 2 h at 1500 rpm. 100 mL paraffin and 4 mL emulsifier (Span-80) were poured into the prepared CTS dispersion solution under mechanical stirring at 40 °C. After stirring for 1 h, 2 mL glutaraldehyde (25%, v/v) was slowly dropped into the reaction system and stirred in a water bath at 50 °C for another 1 h, and then NH<sub>3</sub>·H<sub>2</sub>O solution was added dropwise to reaction system. The pH of the final mixtures was controlled within the range of 9–10 and stirred in a water bath at 70 °C for another 1 h. Then, this was added into the adipaldehyde and stirred for another 5 h. The resulting product was then centrifuged and washed with petroleum ether, ethanol, and distilled water, several times, respectively. Finally, the product (MCTS) was dried in a vacuum oven at 60 °C for 12 h.

### 2.4. Preparation of surface molecularly imprinted polymers on magnetic microspheres

The anthocyanin (AOC) CAP-imprinted polymer shells were coated on the surface of MCTS microspheres prepared by situ precipitation polymerisation, where methacrylic acid and EGDMA were used as the functional monomer and cross-linking agent, respectively. Briefly, methacrylic acid (0.5 mmol), EGDMA (1.5 mmol), and AOC (0.125 mmol) were dissolved in acetonitrile (50 mL) to self-assemble in the dark at room temperature. MCTS microspheres (150 mg) were dispersed into the above solution by ultrasonication and then AIBN (10 mg) was added as the initiator. These mixtures were purged with nitrogen for 45 min in an ice bath. To ensure homogeneous dispersion of the MCTS microspheres, the reaction was carried out in a water bath oscillator at 200 rpm. The prepolymerisation was first undertaken at 50 °C for 6 h, then, the cross-linking polymerisation was implemented at 60 °C for 24 h. The resulting MMIPs were separated from the mixed solution with the help of an external magnet, and were then washed with acetonitrile and ethanol several times, with the mole ratio of MAA to EGDMA kept at a constant value of 1:4. The template AOC of the polymers were extracted with a mixture of methanol and acetic acid (9:1, v/v) until no release of AOC was detected by UV-vis spectroscopy. The corresponding magnetic

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