Immobilization of *Chaetomium erraticum* dextranase (CED) by adsorption on carboxylated multi walled carbon nanotubes (c-MWCNT)

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Research Article

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Abstract

In this study, CED was immobilized onto c-MWCNT by adsorption. Optimization of immobilization conditions (immobilization buffer's pH and molarity, c-MWCNT amount, and immobilization time) was resulted in 100% immobilization yield and 114.13% activity yield. Further, characterization of FCED and ICED was also studied. After immobilization, the optimum pH shifted from 5.0 to 6.0, while the optimum temperature (55 °C) did not change. Furthermore, kinetic constants for FCED and ICED were also determined using the Lineweaver-Burk plot. The K_m value for both FCED and ICED were 54.35 g / L, while V_{max} values for FCED and ICED were 2.77 μmol reducing sugar / L.mg.min and 3.19 μmol reducing sugar / L.mg.min, respectively. Moreover, there was no reduction in the initial activity of ICED after 20 consecutive uses and 30 days of storage at optimal storage conditions. Finally, 17.15% and 17.53% of the dextran in 10% dextran solution (pH 6.0) were converted to reduced sugars (IMOs and Glucose) in 12 hours using FCED and ICED, respectively. Consequently, it can be concluded that ICED obtained in this study can be effectively used for industrial production of IMOs and for hydrolysis of dextran.

1. Introduction

Isomaltooligosaccharides (IMOs) are consist of glucose units linked by α-(1→6) glicosidic bonds with degrees of polymerization (DP) ranging between 2 and 6 [1]. IMOs prevent diarrea, constipation, osteoporosis, and colon cancer by promoting the growth of *Bifidobacteria* in the large intestine of humans and animals [2, 3]. IMOs are produced using such carbohydrates as starch, maltose, sucrose, and dextran [4]. They are produced from starch by using alpha-amylase and neopullulanase [5], from maltose by using cells of *Aureobasidium pullulans* [6], and from sucrose by using dextransucrase and dextranase [7, 8]. These oligosaccharides can also be produced by hydrolysis of dextran with endodextranases [9]. The hydrolysis products of dextran by fungal dextranases contains isomaltose, isomaltotriose and a small amount of glucose [10].

Dextranase (α-1,6-D-glucan, 6-glucanohydrolase; E.C.3.2.1.11) is an enzyme which hydrolyzes the α-1,6 glucosidic linkages in dextran [11, 12]. Dextranases are used in industrial applications, such as chemistry, food, textile industries and pharmacy. Industrial dextranases are isolated from microbial sources such as fungi and bacteria [13–15]. *Chaetomium erraticum* dextranase (CED) is one of the endo-dextranases that hydrolyzes dextran to yield IMOs [16–19].

Enzymes are biocatalysts perform the same reactions under mild conditions compared with chemical catalysts. Hundreds of enzymes are used in various industrial applications, such as chemistry, textile, food, detergent, medicine, and pharmacy [20]. But soluble enzymes have some important disadvantages during industrial applications, such as low operational and storage stabilities, high cost for removal from reaction medium to protect the product contamination with enzymes and lack of continuous operations [21]. Enzyme immobilization technique is one of the strategies to overcome these problems [22]. Enzyme immobilization is restriction of enzyme to a different phase (matrix/support) from the one in which contains substrates and products [23].

There are several advantages of using immobilized enzymes. Besides on the easy operation of the enzyme, it can be easily removal from the reaction medium, thus contamination of the product can be limited or eliminated. Immobilization also allows the efficient recovery and reuse of expensive enzymes, is a must in most applications for economic viability, and make possible their use in continuous, fixed-bed or fluidized bed operation. An another profit is frequently enhanced stability during both storage and operation, e.g., against denaturation by heat or organic solvents or by autolysis [24].

Adsorption, cross-linking, entrapment/encapsulation and covalent attachment are main traditional immobilization methods [21, 22, 24, 25]. Each one of these methods have some advantages and some disadvantages. Adsorption which includes reversible surface interaction between carrier and enzyme [26] can be accomplished by mixing enzyme and support in adequate buffer solution at optimum conditions such as pH and ionic strength [27]. This method is easy, cheap and fast, but weak linkages between enzyme molecules and support can be resulted in the losing the catalytic activity of enzyme due to desorption of enzyme molecules from support under the catalytic reaction conditions. Entrapment of enzyme in a polymeric gel during the formation of gel matrix can be performed by dropping the mixture of enzyme and poly anionic polymers into the solution of polyvalent metal ions under optimum conditions such as ratio of enzyme / polymer, pH and ionic strength [28]. This method is also easy, cheap and fast but mass transfer limitations for the large substrates and product molecules can lead to lower catalytic activity of enzymes [27]. Cross-linking method is linking of enzyme molecules to each other with covalent bonds by using cross-linking reagent such as glutaraldehyde, carbodiimide and diisocyanate to prepare carrierless macro particles. This method is not required using any support material. The clear advantages of this method are highly concentrated catalytic enzyme activity, high stability and low production costs due to the exclusion of an additional (expensive) carrier [24]. Covalent immobilization of enzyme may have some advantages over physical adsorption, such as inhibition of enzyme desorption during processing and increased stability due to multiple points covalent binding [29, 30]. However, it also has a problem: both enzyme and support should be discarded after enzyme inactivation [29]. On the other hand, covalent immobilization is generally resulted in higher reusability, due to low leakage level of enzyme molecules from support [24].

Since first report have been published[31], carbon nanotubes (CNTs) have attracted attentions of scientists working in different fields icluding enzyme immobilization. As seen in Fig. 3, CNTs were classified as single-walled carbon nanotubes (SWCNTs) multi-walled carbon nanotubes (MWCNTs) [32–34]. While SWCTs are composed of tubes with 0.7 nm of outer diameter and single atom tickness of up-rolled graphene layer, MWCNTs are composed of many SWCNT stacked inside of other one, and the outer diameter of MWCTs are less than 15 nm and the lengths are tens of micrometers [35]. Large surface area of SWCNTs is more advantageous for enzyme loading capasity, but MWCNTs are preferable because of their high dispersibility and lower cost [36]. Enzymes have been immobilized on CNTs using adsorption or covalent bonding methods. Optimal enzyme conformations that is necessary for activity can be protected in adsorption, but durability and loos of activity upon leaching of enzymes from matrix is still concern for industrial applications. Covalent enzyme immobilization can improve stability and activity [34].

There are numerous studies in the literature about the use of single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) by using adsorption and covalent binding methods for enzyme immobilization. Immobilization by adsorption is carried out by incubating the enzyme solution with the matrix in a buffer solution at the appropriate time [37]. Since CNTs have a natural affinity for different proteins, adsorption is spontaneous when proteins come into contact with CNTs in a solution [38]. In covalent immobilization, the carboxyl (-COOH) groups on c-CNTs are first activated by N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). The enzyme molecules are then linked to c-MWCNT through these groups [39]. c-MWNTs have been successfully used for immobilization of several enzymes such as *Aspergillus niger* glucose oxidase [40], *Candida antarctica* lipase B [41], Papain [42], *Candida rugosa* lipase [43–45], *Aspergillus niger* inulinase [37], fungal peroxidase [46], α-Amylase and glucoamylase [47], and *Pseudomonas fluorescens* lipase [48].

There are only three studies have been published in literature about the immobilization of CED individually. In the first study, CED has been immobilized on bentonite, hydroxyapatite, and ion exchanger Streamline DEAE by adsorption, on Eupergit® C, Eupergit® C 250L and on aminopropyl silica, by covalent attachment and lastly within alginate beads by entrapment method [49]. In this study, the highest activity yield (41%) achieved with Eupergit® C 250 L. But, adsorption experiments have been resulted in low activity yields. In the second study, CED has been immobilized onto Ca-Alginate Gel Beads with 89.7% activity yield by ultrasound irradiation technique [50]. In this study, immobilization of CED with ultrasound irradiation have been shifted optimum pH from 5 to 6 and optimum temperature from 50 ^{o}C to 60 ^{o}C. Initial activity of immobilized CED with ultrasound irradiation decrease to 20% after sixth usage. In third study [51], CED has been immobilized by adsorption on activated carbon and dextranase located on activated carbon used for the preparing a membrane reactor by using polysulfone. But, in this study, immobilization yield and activity yield were not reported. There are not any report in the related literature about the immobilization of CED by using c-MWCNT. In some study with c-MWCNT, enzyme immobilization resulted in 5 times [45] and 12 times [48] increased activity. Therefore, the main goal of this study was to obtain sustainable higher immobilization yield and activity yield than previous results reported in the literature in the immobilization of CED on c-MWCNT by optimizing the conditions of adsorption.

2. Materials And Methods

2.1. Materials

CED (E.C.3.2.1.11) was provided as a gift by Bio-Cat (Troy, USA). Dextran T70, purchased from Carl Roth GmbH-Co. KG. Polyvinylidene di fluoride (PVDF) membrane (48 mm diameter and 0.1 µm pore size) were purchased from Co. (Cork, Ireland). Carbon nanotubes were purchased from Nanografi Co. (Ankara, Turkey). UV-VIS Spectrometer (UV-6300PC) was purchased from VWR (Radnor, USA). PH meter (Hanna HI 2020 edge), was purchased from Hanna Instruments Ltd. (Bedfordshire, UK). The magnetic stirrer (Heidolph MR Hei-Standard) was purchased from Heidolph UK-Radleys (Shire Hill, UK). Pure water

appliance (Mini Pure 1, MDM-0170) was purchased from MDM Co. Ltd. (Suwon-si, South Korea). Precision scale (Shimadzu-ATX224) was purchased from Shimadzu Corporation (Kyoto, Japan). Orbital shaking heated incubator (Mipro-MCI) was purchased from Protek Lab Group; professional laboratory solutions company (Ankara, Turkey). The vacuum pump (Biobase, GM-0.50A) was purchased from Biobase Biodustry Co. Ltd. (Shandong, China). Bovine Serum Albumin (BSA), sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, sodium sulfite, phenol, and D-glucose were purchased from Sigma-Aldrich (Taufkirchen, Germany). 3.5-dinitrosalicilic acid (DNS) was purchased from Alfa Aesar (Kandel, Germany). Sodium potassium tartrate (Rochelle salt) was purchased from VWR Prolabo Chemicals (Leuven Belgium). Sodium azide was purchased from Merck Millipore (Darmstadt, Germany).

2.2. Determination of protein

The quantities of proteins, amounts of an enzyme in the immobilization solutions before and after immobilization will be determined according to Bradford Protein Assay Method [52] method using UV-VIS Spectrometer. The amount of immobilized enzyme was calculated by subtracting the recovered protein in the supernatant of immobilization buffer from the amount of enzyme used for immobilization [53].

Immobilization yield was calculated by using Equation 1. **See equation 1 in the supplementary files.**

2.3 *Determination of CED activity*

The activity of free CED were determined by reacting the free CED with 1% (w / v) dextran solutions in an incubator orbitally shaken at 150 rpm for 60 minutes under optimum activity conditions (pH for free and immobilized CED was 5 and 6, respectively and temperature was 60 ^{o}C). The amount of reducing sugar released was determined according to DNS (3.5-dinitrosalicylic acid) Method of Miller [54]. One IU CED activity was defined as the amount of enzyme forming 1 µmol reducing sugar equivalent to 1 µmol D-glucose from dextran per minute, under the optimum activity assay conditions. CED activity was calculated by using Equation 2. Activity yield was calculated by using Equation 3.

2.4. Immobilization procedure

CED was incubated with c-MWCNT in sodium phosphate buffer (pH was optimum) at room temperature for proper time in an incubator shaken at 150 rpm. After immobilization, the immobilized CED was filtered using a PVDF membrane by suction under vacuum and washed with 30 mL of 0.5 M sodium phosphate buffer (pH was optimum) and with 30 mL of distilled water as three aliquots, respectively. The supernatant was used for protein assay and the immobilized CED was used for activity assay. Free CED and immobilized CED were labelled as FCED and ICED, respectively hereafter.

2.5. Optimization of immobilization conditions

Some factors, such as pH and molarity of immobilization buffer, enzyme/support ratio and immobilization time affect the immobilization efficiency [55, 56]. Moreover, in some cases the immobilization protocol can able to fix the enzyme form with higher activity, thus the final immobilized preparation may be more active than the native one [57]. Therefore, these factors, should be optimized. The optimum conditions for immobilization were determined by changing individually the factors, (pHs from 3.0 to 7.0; buffer concentration from 0.025 mM to 0.500 mM; amount of c-MWCNT from 25 mg to 100 mg; and duration of immobilization from 1 h to 5 h).

2.6 Optimization of activity conditions

The pH of the reaction medium, temperature, and the concentrations of enzyme and substrate can affect both of the activity and the stability of enzymes [20]. On the other hand, immobilization protocol can also affect the activity conditions. Therefore, each of the mentioned factors were optimized respectively [24, 42, 57].

2.6.1 Effect of pH on the CED activity

Effect of pH on the activity of FCED and ICED were investigated by determining the activity after the reaction between FCED or ICED with 1% (w/v) dextran solutions, at several pHs (3.0-7.0) and 55 ^{o}C for 60 minutes.

2.6.2. Effect of temperature on the CED activity

Effect of pH on the activity of FCED and ICED were determined by conducting the activity assay method with 1% (w/v) of dextran solutions (pH was 5.0 for the FCED and 6.0 for ICED at several temperatures (30 ^{o}C - 70 ^{o}C).

2.6.3 Effect of pH on the stability

In this case, firstly, 200 µL free or 0.785 g wet immobilized CED were incubated with 2.5 mL of 25 mM sodium phosphate buffers with different pHs (3.0-7.0) at 25 ^{o}C for 60 minutes in an incubator that was orbitally shaken at 150 rpm. After then, the retained activities were determined according to the standard activity assay method by adding 2.5 mL of 2% (w/v) buffered dextran solutions (pH was 5.0 for FCED and pH is 6.0 for ICED) into enzyme solutions incubated at different pHs.

2.6.4. Effect of temperature on the CED stability

Firstly, 200 µL FCED solution and 0.785 g wet ICED were incubated with 2.5 mL of 0.25 mM sodium phosphate buffers (for free and immobilized CED, pH was 5 and 6 respectively) at different temperatures

(30 ^{o}C - 70 ^{o}C) for 60 minutes in an incubator that was orbitally shaken at 150 rpm. Then, this solutions containing FCED or ICED were incubated in an ice-bath for 10 minutes to terminate the temperature effect. Lastly, the retained activities were determined according to standard activity assay method by adding 2.5 mL of 2% (w/v) buffered dextran solutions (pH was 5.0 for FCED and pH is 6.0 for ICED) into enzyme solutions incubated at different temperatures.

2.6.5. Kinetic constants

Initial velocities for kinetic parameters were determined by performing the reactions between 200 µL of FCED or 0.785 g of the wet ICED and dextran solutions (pH was 5.0 for FCED and 6.0 for ICED) at several concentrations (0.5 g/L - 20 g/L) for 10 min. K_m and V_{max} were determined from Line-weaver–Burk plots.

2.7 Operational and storage stabilities of ICED

The operational and the storage stabilities of ICED were determined by performing the standard activity assay method after 20 repeated batch experiments and every two days when storing in sodium phosphate buffer (0.5 M, pH 6.0) in the refrigerator at +4 ^{o}C for 30 days, respectively. ICED was used for the determination of storage stability by storing in 5 mL 0.5 M sodium phosphate buffers (pH 6.0) in a refrigerator at +4 ^{o}C until next use. Before each use, the immobilized enzymes were filtered and washed with 10 mL of distilled water using PVD membrane filter on a sintered glass under vacuum.

2.8 Hydrolysis of dextran using FCED and ICED

For the effect of substrate concentration on dextran hydrolysis, 200 µL FCED solutions or 0.785 mg wet ICEDs were reacted with dextran solutions at different concentrations (from 5 g/L to 20 g/L) at optimum pH and temperatures for 10 minutes. For the effect of duration of hydrolysis on the dextran hydrolysis, 400 µL FCED solutions or 1.57 g ICED were reacted with 10 mL of 10% dextran solution (pH 6.0) for 12 hours.

During or after the reactions, 200 µL aliquots withdrawn from reaction mixtures and added to 1800 µL of distilled water. After inactivating the enzymes in boiling water bath for 10 minutes, the released reducing sugars were determined by DNS method of Miller [54].

2.9 Statistical analysis

All experiments were performed in triplicate and the values are given as mean ± the experimental error. Each data represents the mean of three values was calculated using Microsoft Office Excel 2016. The graphics were drafted by using Origin Pro 8.0 software.

3. Results And Discussion

3.1. Determination of protein

The enzyme concentrations in the immobilization buffers before and after immobilization are calculated by using equation obtained BSA standard curve. Accordingly, CED concentration in the commercial liquid enzyme preparation was calculated as 6.686 mg/mL.

3.2. Determination of CED activity

Specific FCED activity was calculated as 0.11 IU/mg by using Equation 2. The activity of commercial liquid CED preparation was also calculated to be 7.36 IU/mL for Dextran T-70 as substrate. The amount of FCED having 1 IU activity was also calculated as 9.1 mg.

3.3. Optimization of immobilization conditions

The factors, such as immobilization buffer's pH and molarity, amount of c-MWCNT versus to fixed amount of CED, and duration of immobilization affect the immobilization efficiency. Therefore these factors were optimized by changing individualy.

3.3.1. Effect of buffer pH on the immobilization efficiency

As seen in Table 2 the highest immobilization yield (51.12 ± 0.04) activity yield (52.54 ± 0.04) was achieved at pH 5. At the lower and higher pH values immobilization yield and activity yield activity are lower. The lowest immobilization yield and activity yield was obtained at pH 3.0. Erhardt and Jördening [49] reported that pI value of CED is 5.1 and maximum activity yield was achieved at pH 5.0. Torras et al. [51] also reported the maximum immobilization yield and activity yield achieved at pH 5.0 for the asorption of CED on activated carbon. Hamzehi and Pflug [58] also reported the highest immobilization yield and activity yield for α-amylase was achieved at pH equal to pI. CED was probably asorbed on c-MWCNT at the active form at pH 5.0. The results suggest that adsorption at pH around pI promotes enzyme binding in the most active conformation. Mansor et al. [59] reported that pK_a of c-MWCNT is 3.68. The net charge of proteins is zero at isoelectric points (pI), positive at pH below and negative at pH above [20]. Both c-MWCNT and CED are positively charged at pH 3.0. c-MWCNT and CED are negatively charged at the higher pH than 5.1. Therefore, the lowest yields achieved at pH 3.0 and decreased yields at the higher pH than 5.0 may were resulted from the repulsion between same charges on both support and the enzyme. Furthermore, pH can encourage the denaturation of enzymes that resulted in the decreased activity [29, 60-62]. On the other hand, the catalytic activity of enzyme depends on conformational structure of the protein, even minor alterations in the tertiary structure of the protein resulted in loss of its catalytic activity [63]. Similar results can be seen in the related literature. For example, Ramani et al. [61]

obtained the maximum activity yield at the optimum pH (5.0) in the immobilization of *Pseudomonas gessardii* acidic lipase with MAC. In another study, Chen et al. [64] also obtained the maximum immobilization and activity yields at the optimum pH of in the immobilization of β-galactosidase on glutaraldehyde activated chitosan beads.

3.3.2. Effect of buffer molarity on the immobilization efficiency

According to the Table 3, the immobilization yield and the activity yield was increasing when molarities increased and reached the maximum values 65.08±0.03 and 66.57±0.05, respectively at 0.5 M. High ionic strength prevents ionic exchange between support and enzyme molecules and immobilzation occured by hydrophobic interaction [65]. On the other hand, dehydration of the protein due to the hydrated effect of salt molecules surrounding the protein at higher ionic strength enhancing the hydrophobic interactions between protein and support [66]. Therefore, the highest activity yield at 0.5 M buffer might due to increased enzyme adsorbed on the c-MWCNT.

3.3.3. Effect of c-MWCNT amount on the immobilization efficiency

As shown in Table 4. The immobilization yield and the activity yield were increased by increasing amount of c-MWCNT. The highest activity yield (75.59 ± 0.05%) was achieved for 100 mg c-MWCNT. Similar results can be seen in other studies in related literature. For example, Garlet et al. [37] reported that amount of adsorbed inulinase from *Aspergillus niger* on c-MWCNT was increased when amount of c-MWCNT increased versus fixed enzyme concentration. The excessive enzyme loading that occured when lower amount of marice ıs used, always causes protein-protein interaction and inhibits the flexible stretching of enzyme conformation, which will result in the steric hindrance and thus the inactivation of an enzyme. That is, the enzyme molecule may be difficult to modulate its most suitable conformation for catching the substrate molecules and releasing product molecules under molecular crowding condition [67]. Recently, several authors have reported the similar effect of support amount on the immobilization [68-71].

3.3.4. Effect of immobilization duration on the immobilization efficiency

As is shown in Table 2, Duration of immobilization did not effect the immobilization yield but the activity yield increased by the duration of immobilization. Activity yield was reached to 114.13% after 5 hours.

As a result of the optimizing of the immobilization conditions, 100% immobilization yield (66.86 mg/g dry weight of c-MWCNT) and 114.13% activity yield (8.40 U/g dry weight of c-MWCNT) was achieved. The immobilization of CED was studied by another researchers. Our activity yield (114.13%) is higher than previous two studies. The maximum activity yields achieved by Erhardt and Jördening [49] and by Bashari et al. [50] were 41% and 93% respectively. This highest yields may be result of natural affinity of

c-MWCNT for proteins [38]. On the based of these results it can be said that c-MWCNT is a better candidate as a immobilization support for the immobilization of other enzymes in the future.

3.4. Characterization of FCED and ICED

3.4.1. Effect of pH on the activity

As well known, enzymes are quite sensitive to excess acidic or alkaline medium, therefore determining optimum pH value is very important to achieve maximum activity [72]. According to Figure 2, optimum pH of CED was shifted from 5.0 to 6.0 after immobilization. This result is agree with the study of Bashari et al. (2014). Similar results can be seen in the related literature. For example, the optimum pH of *Aspergillus oryzae* β-galactosidase shifted from 4.0 to 4.5-5.0 after immobilization with different membranes [73]. In another study, after immobilization with nylon hydrolonmembrane, the optimum pH of *Aspergillus oryzae* β-galactosidase increased from 4.0 to 4.5 [74]. At the end of immobilization, the optimum pH may shift to higher or lower levels depending on the physicochemical properties of the matrix [75]. In addition, change in optimum pH are probably due to changes in the load on the enzyme and matrix [76]. According to an another approach, shifting of optimum pH might be due to the effects of the isoelectric point (pI). As we know, the net charge of the protein is negative at a pH higher than and zero at a pH near the isoelectric point [72]. Since pI of CED is 5.1, the net charge of CED is negative at pH 6.0. On the other hand, ICED is more active than FCED at the pH tested except 5.0. The higher activity of immobilized ANAG was resulted from increased stability bu multi-point attachment during immobilization [77-80].

3.4.2. Effect of temperature on the activity

As shown in Figure 3, optimum tepmperature of CED was not changed after immobilization. But in the study of Bashari et al. [81], optimum tamperature of CED was shifted from 50 oC to 60 oC after immobilization on Ca-Alginate gels. This difference may resulted from the differencies of the structure of two support. On the other hand, it is also seen in the Figure 3 that ICED is more active than FCED at all temperatures tested. It is well known that immobilized enzymes exhibit higher activity than free enzymes at elevated temperatures as immobilization increases the thermal stability of the enzymes. Lower activities exhibited by FCED at lower temperatures than ICED is resulted from the un-establishement of optimal activity conformation of enzyme molecules required to react with the substrate, due to free enzyme molecules have less energy than activation energy at low temperatures [82]. Since the optimal activity conformation of the enzyme molecules is fixed as a result of immobilization, it can be said that the activity of immobilized enzyme is higher than free enzyme because the decrease in temperature does not change the conformation of enzyme [83].

3.4.3. Effect of pH and temperature on the stability

According to Figure 4 ICED was more active than FCED at broader pH range. It also can be seen that ICED is more stable than FCED at the all pH values tested. The reason for the low stability at pH outside the optimum pH range may be due to the leaving of enzyme molecules from the matrix as a result of changes in pH as the binding forces between support and enzyme are weak [84]. On the other hand, as shown in Figure 5, ICED is more stable than FCED at higher temperatures. ICED didn't lose its activity, while FCED lost ~ 4 of its activity at 65 ^{o}C. At the upper temperatures than 65 ^{o}C FCED lost rapidly its activity. When FCED was completely inactivated at 80 ^{o}C, ICED retained 50% of its activity. Furtermore, ICED retained 20% of its activity at 90 ^{o}C. The increased stability of the immobilized enzyme has been linked to a decrease in the mobility of the protein structure caused by the attachment of the enzyme to the support [85]. Increased stability may be the result of immobilization limiting the thermal movement of the enzyme at elevated temperatures. As a result, thermal denaturation may not occur at higher temperatures with an immobilized enzyme. Thermostable enzymes provide higher reaction rates, lower diffusion constraints, higher stability and higher yields [86].

3.5. Kinetic constants

The kinetic constants for the free and for the immobilized CED were also determined by using the Lineweaver-Burk plot (Figure 6). The Km value for both FCED and ICED were 54.35 g / L, while Vmax values for FCED and ICED were 2.77 µmol reducing sugar / L.mg.min and 3.19 µmol reducing sugar / L.mg.min, respectively. The K_m represents the affinity of an anzyme to its substrate. There are negative correlation between the affinity and K_m when K_m decreases the affinity increases [45].

3.6. Operational and storage stabilities of ICED

Since enzymes are expensive components of the applications of food industry, longer operational and storage stabilities of immobilized biocatalysts are very important properties, for lowering the product costs. As shown in Figure 7 and Figure 8, ICED has perfect operational and storage stabilities. This result better than achieved operational and storage stabilities in previous two studies[49, 81]. CED immobilized on all supports lost more than 80% of their activities after six recycle[81]. But, Erhardt and Jördening [49], did not determine the operational and storage stabilities of CED immobilized on Ca-Alginate gels. As a consequent of the results it can be said that ICED obtained in this study is better than ICED obtained in previous studies fort industrial applications.

3.7. Hydrolysis of dextran using FCED and ICED

The optimum condition of dextran hydrolysis was determined by changing the factors such as substrate (dextran) concentration and duration of hydrolysis that affect the hydrolysis yield, individually. According to Figure 9, the released reducing sugars (IMOs and glucose) concentration were increased and reached the maximum level at 100 g/L. As shown in Figure 10, the released reducing sugar concentration was

increased by the incresing hydrolysis time until 10 hours. According to Table 6, 17.15% and 17.52% of dextran in the 10 mL of dextran solutions (pH is 5.0 and 6.0 for FCED and ICED respectively) was converted to reducing sugars at the end of 10 hours, using FCED and ICED respectively. Dextran hydrolysis ratio in our study lower than achieved dextran hydrolysis ratio (~ 60%) in the study of Erhardt and Jördening [49]. This difference may resulted from the difference between the moleculer weights of dextrans. Because, we used dextran T70, but they have used dextan T40.

4. Conclusions

As a result of the optimizing of the immobilization conditions, 100% immobilization yield (66.86 mg/g dry weight of c-MWCNT) and 114.13% activity yield (8.40 U/g dry weight of c-MWCNT) was achieved. Our activity yield (114.13%) is higher than previous studies. Immobilization improved the pH and thermal stabilities of CED. Operational and storage stabilities of CED is higher than previous results reported in the literature. Conseguently, it can be said that ICED obtained in this study is better than ICED obtained in the previous studies.

Declarations

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Tables

Table 1. Immobilization of enzymes using c-MWCNT

Enzyme	Immobilization yield (%)	Activity yield (%)	Operational Stability (%)	Storage stability (%)	References
Aspergillus niger glucose oxidase	unstudied	unstudied	unstudied	unstudied	Soo-Keun et al., 2005 [40]
Candida antarctica lipase B	unstudied	unstudied	Unstudied	55 after 6 months	Pavlidis et al., 2010 [41]
Papain from Genview, USA	18.8	78.9	61 after 7 use	unstudied	Wang et al., 2011 [42]
Candida rugosa lipase	52	48	Unstudied	unstudied	Rastian et al., 2013 [43]
Aspergillus niger inulinase	90	unstudied	Unstudied	100 after 5 weeks	Garlet et al., 2014 [37]
Candida rugosa lipase	86.7	492.5	123.7 after 7 use	unstudied	Rastian et al., 2014 [44]
Fungal peroxidase	100	unstudied	0 after 9 use	34 after 40	Azevedo, 2014 [46]
α-Amylase ve glucoamylase	unstudied	95.1	95.1 after 8 use	unstudied	Feng et al., 2015 [34]
Candida rugosa lipase	85.6	500	unstudied	unstudied	Jamie et al., 2016 [45]
Pseudomonas fluorescens lipase	59	1200	60 after 8 use	unstudied	Zniszczoł et al., 2016 [48]

Table 2. Effect of immobilization buffer pH on immobilization efficiency

Immobilization Buffer pH	Immobilization Yield* (%)	Activity Yield ** (%)
3.0	36.23 ± 0.05	37.48 ± 0.03
4.0	39.41 ± 0.03	41.77 ± 0.02
5.0	51.12 ± 0.04	52.54 ± 0.04
6.0	48.72 ± 0.03	50.04 ± 0.03
7.0	45.37 ± 0.02	46.98 ± 0.05

*200 µL of CED solutions were incubated with 100 mg of CNTs in 5 mL of sodium phosphate buffers (0.5 M) at different pHs and room temperature for 1 hour in an incubator orbitally shaking at 150 rpm.

**200 µL of free or 0.785 g of immobilized CED were reacted with 5 mL of 1% (w/v) buffered dextran solutions (pH 5.0 for the FCED and 6.0 for the ICED) at 55 ºC for 60 minutes in an incubator orbitally shaking at 150 rpm.

Table 3. Effect of immobilization buffer molarity on ımmobilization efficiency

Buffer Molarity (M)	Immobilization Yield* (%)	Activity Yield** (%)
0.025	51.12 ± 0.04	52.54 ± 0.04
0.050	53.36 ± 0.02	54.42 ± 0.05
0.100	57.15 ± 0.04	58.07 ± 0.02
0.250	61.23 ± 0.05	62.41 ± 0.03
0.500	65.08 ± 0.03	66.57 ± 0.05

*200 µL of CED solutions were incubated with 100 mg of CNT in 5 mL of sodium phosphate buffers (pH 5.0) at different concentraions at room temperature for 1 hour in an incubator orbitally shaking at 150 rpm.

**200 µL of free or 0.785 g of immobilized CED were reacted with 5 mL of 1% (w/v) buffered dextran solutions (pH 5.0 for the FCED and 6.0 for the ICED) at 55 ºC for 60 minutes in an incubator orbitally shaking at 150 rpm.

Table 4. Effect of c-MWCNT amount on immobilization efficiency

c-MWCNT (mg)	Immobilization Yield* (%)	Activity Yield** (%)
25	65.08 ± 0.03	66.57 ± 0.05
50	68.31 ± 0.02	69.41 ± 0.05
75	71.17 ± 0.04	72.37 ± 0.02
100	74.29 ± 0.03	75.59 ± 0.05

*200 µL CED solutions were incubated with different amounts of c-MWCNT in 5 mL of sodium phosphate buffers (0.5 M, pH 5.0) and room temperature for 1 hour in an incubator orbitally shaking at 150 rpm.

**200 µL free or 0.785 g immobilized CED were reacted with 5 mL of 1 % (w/v) buffered dextran solutions (pH 5.0 for the FCED and 6.0 for the ICED) at 55 ºC for 60 minutes in an incubator orbitally shaking at 150 rpm.

Table 5. Effect of immobilization duration on immobilization efficiency

Duration of Immobilization (hours)	Immobilization Yield* (%)	Activity Yield** (%)
1	74.29 ± 0.03	75.59 ± 0.05
2	84.12 ± 0.03	85.55 ± 0.03
3	86.63 ± 0.02	89.53 ± 0.04
4	94.16 ± 0.03	101.48 ± 0.02
5	100.00 ± 0.05	114.13 ± 0.03

*200 µL of CED solutions were incubated with 100 mg of c-MWCNT in 5 mL of sodium phosphate buffers (0.5 M, pH 5.0) at room temperature for different duration in an incubator orbitally shaking at 150 rpm.

**200 µL of free or 0.785 g of immobilized CED having were reacted with 5 mL of 1% (w/v) buffered dextran solutions (pH 5.0 for the FCED and 6.0 for the ICED) at 55 ^{o}C for 60 minutes in an incubator orbitally shaking at 150 rpm.

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Table 6. Production of IMOs from dextran by FCED and by ICED*

Enzyme	Hiydrolysis Time (hours)	Dextran (g/L)	Reducing sugars (IMO+Glucose) (g/L)
FCED	0	100.00 ± 02	0.00 ± 03
	2	94.01 ± 04	5.99 ± 02
	4	87.63 ± 05	12.37 ± 05
	6	86.51 ± 03	13.49 ± 04
	8	84.28 ± 04	15.72 ± 03
	10	82.86 ± 02	17.14 ± 02
	12	82.85 ± 03	17.15 ± 05
ICED	0	100.00 ± 04	0.00 ± 02
	2	93.88 ± 03	6.12 ± 05
	4	87.26 ± 05	12.74 ± 03
	6	86.31 ± 03	13.69 ± 04
	8	84.23 ± 04	15.78 ± 02
	10	82.48 ± 02	17.52 ± 03
	12	82.47 ± 05	17.53 ± 05

*1.57 g ICED was reacted with 10 mL of 10% dextran solution (pH 6.0 for 12 hours. During reaction, 200 µL aliquats were taken with two hours intervals and added to 1800 µL of distilled water. After inactivating the enzymes in the diluted samples in boiling water bath for 10 minutes, the reducing sugars released were determined by DNS method.

Figures

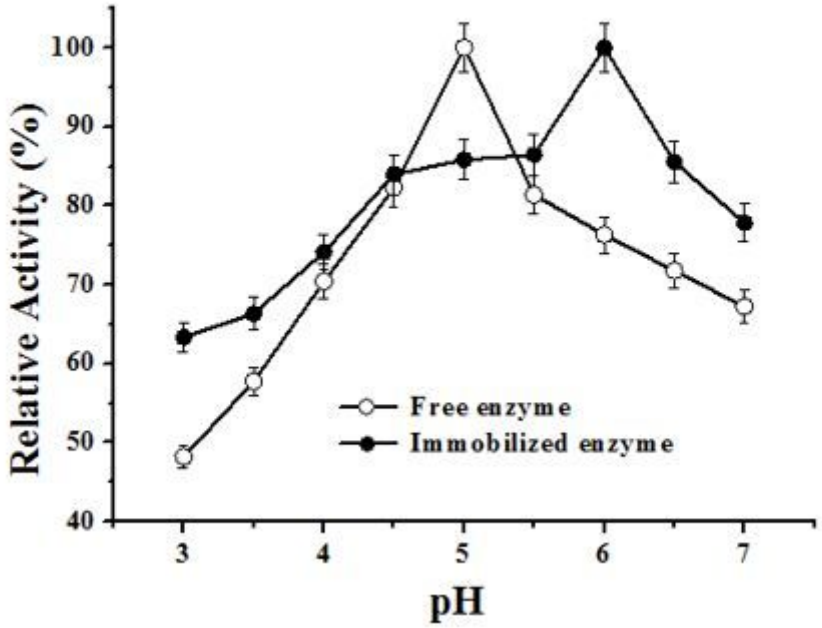

Figure 1

Optimum pH of the free and the immobilized CED

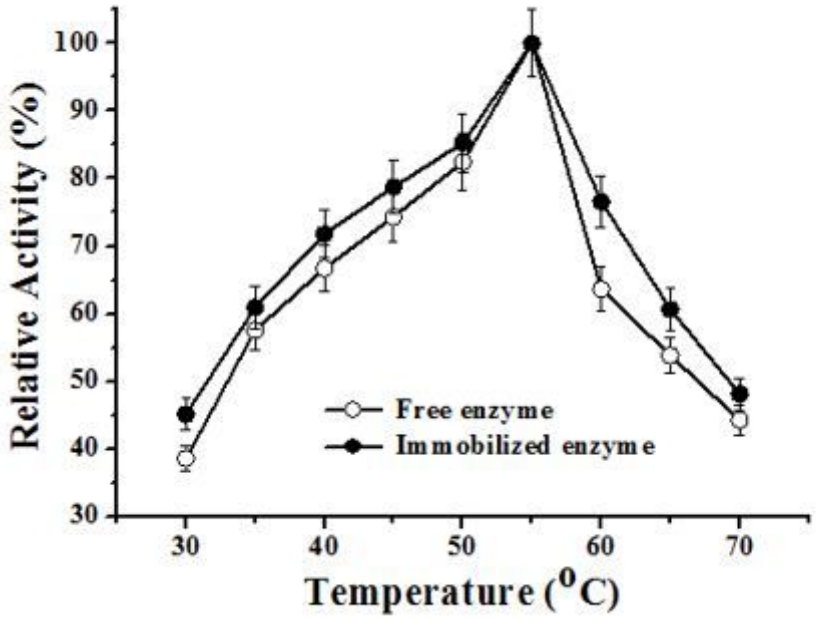

Figure 2

Optimum temperature of free and immobilized CED

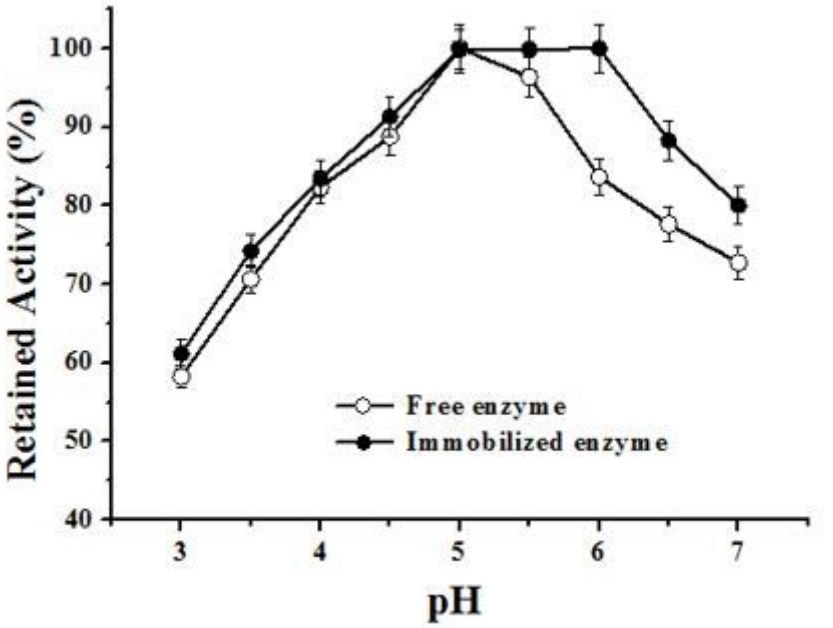

Figure 3

pH stability of free and immobilized CED

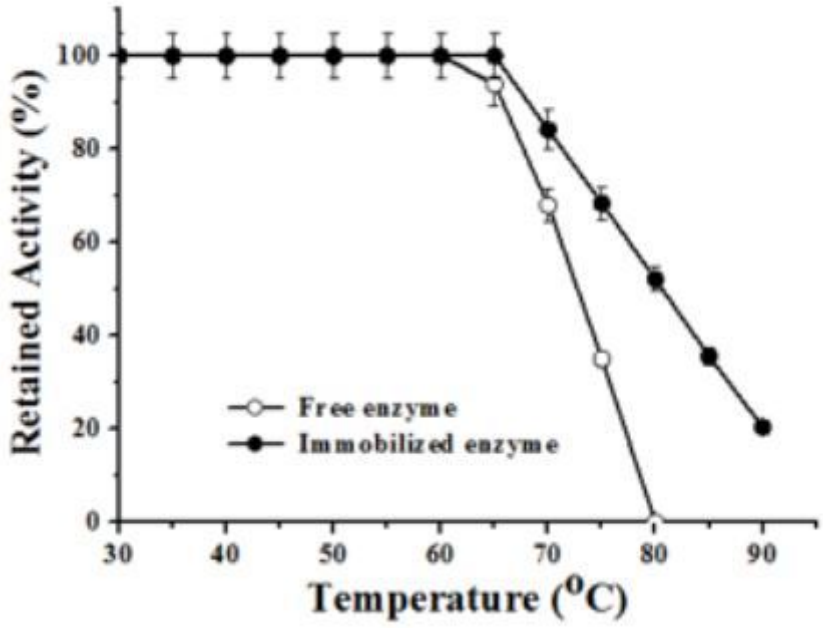

Figure 4

Thermal stability of free and immobilized CED

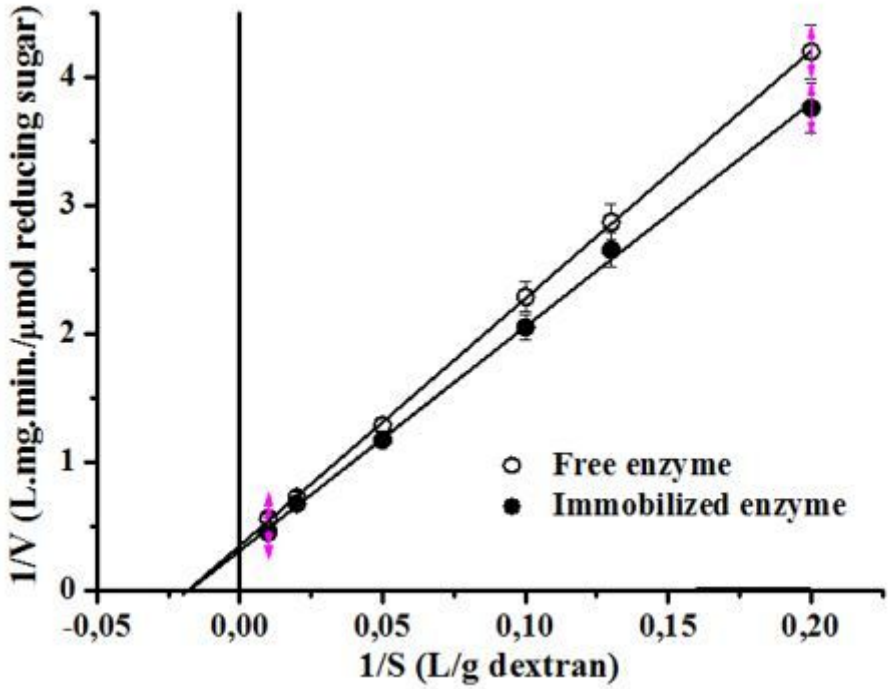

Figure 5

Kinetic constant of free and immobilized CED

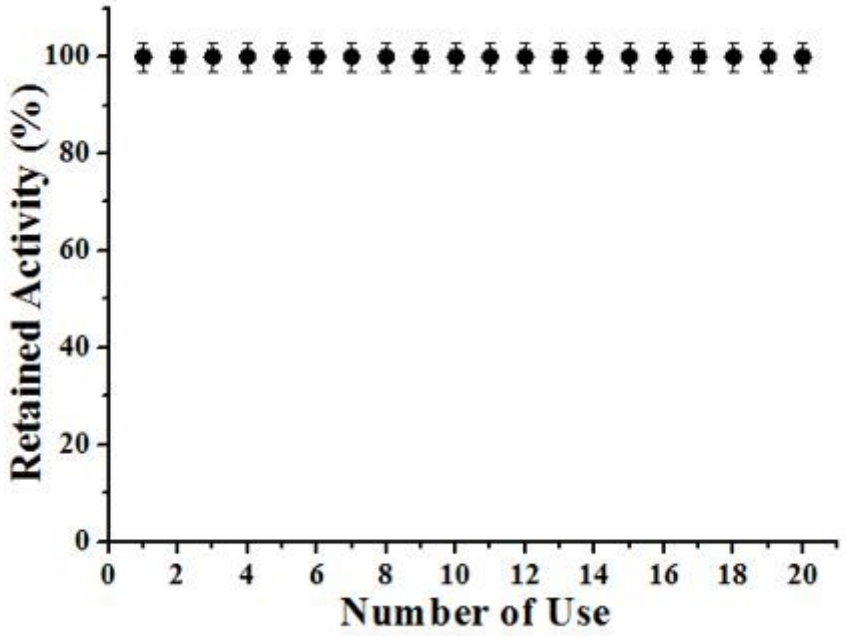

Figure 6

Operational stability of ICED

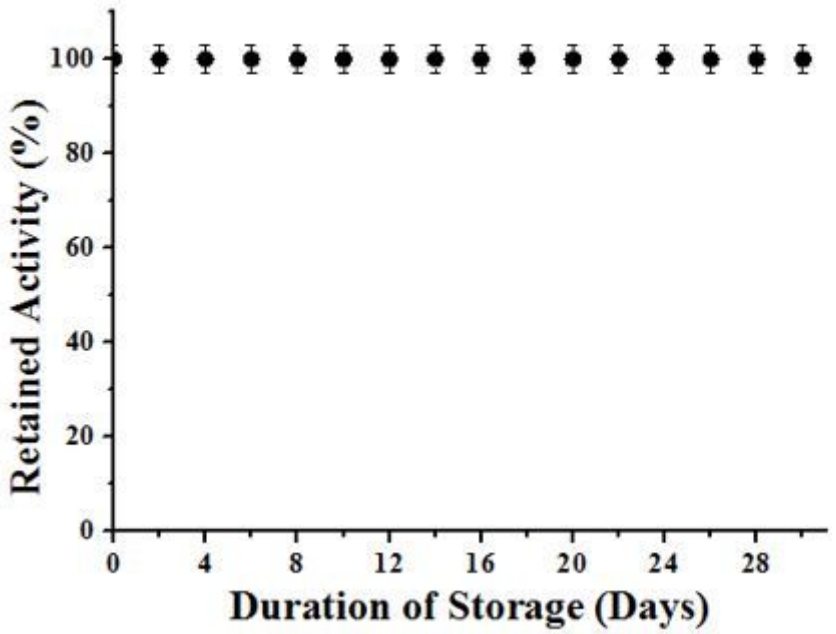

Figure 7

Storage stability of ICED

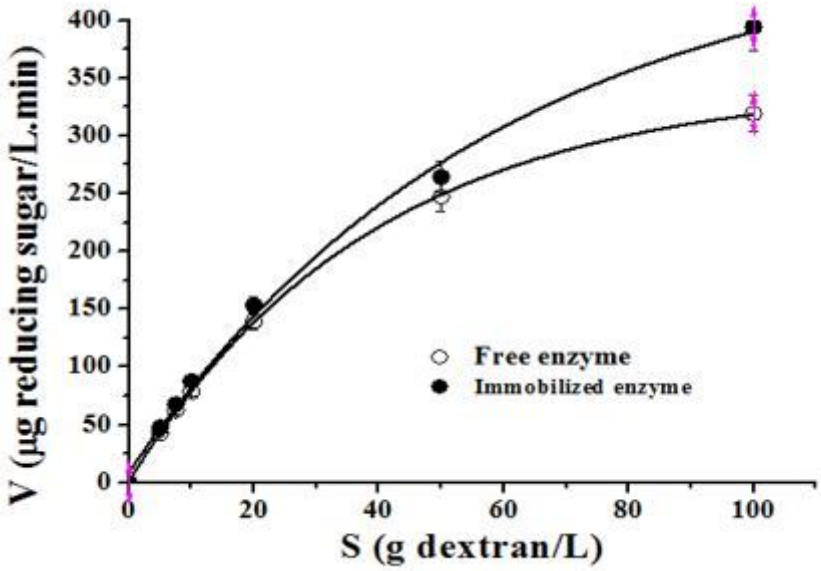

Figure 8

The effect of substrate concentration on the released reducing sugars

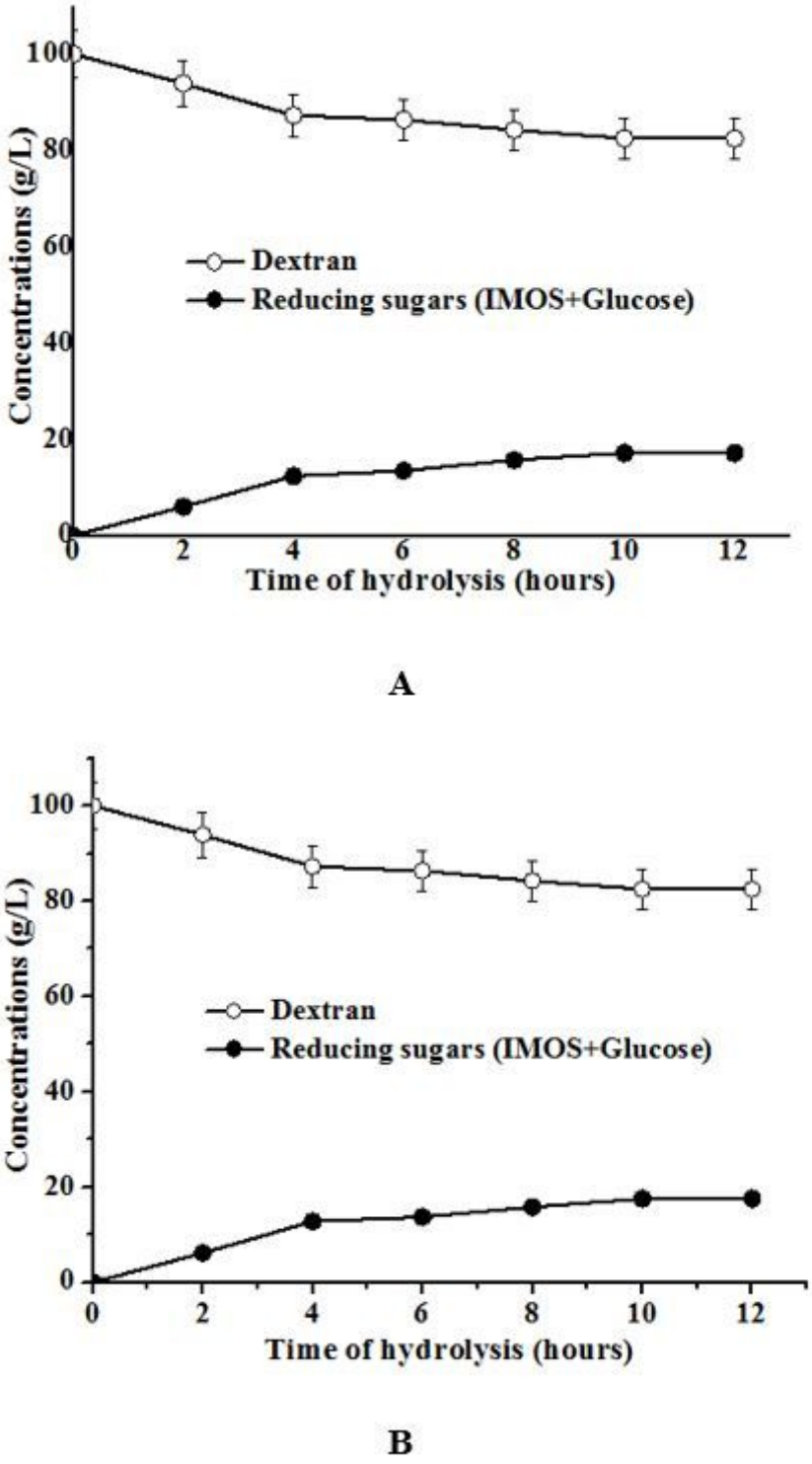

Figure 9

Time course of the production of IMOs from dextran by FCED (A) and ICED (B)

Supplementary Files

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