Screening For Factors Affecting Cellulase Adsorption from Solutions by Modified Coffee Residues

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***Abstract*—**In the present study, the modified coffee residues were used as low cost adsorbent for immobilization of cellulase enzyme. In the batch model for adsorption, the experimental parameters: the temperature, the initial cellulase concentration and the agitation rate of adsorption process were investigated. The maximal percentages of immobilization yield (approximately 28%) and immobilization efficiency (approximately 48%) were obtained with the temperature of 40 °C, the initial cellulase concentration of 1 mg/mL and the agitation rate of 120 rpm. The state of dynamic equilibrium was reached after 60 minutes and the pseudo-second-order model was predicted to follow the process kinetic.

***Keywords*—** adsorption, cellulase, coffee residues, enzyme.

I. Introduction

THE use of enzymes has gradually been extended in recent decades to a variety of fields, from industrial applications up to research development [1]. Cellulase (EC 3.2.1.4) is an important member of a key biocatalyst enzyme, which basically hydrolyzes β-1,4-glycosidic bonds of the crystalline complex of cellulose materials [2], thus it bears the responsibility for the bioconversion of cellulose into the soluble sugars. Biotechnology of cellulase began in early 1980s, first annotated in animal feed followed by food applications [1]. Nowadays, it is often used as a model enzyme in biochemistry, food, beverages, clinical diagnosis and environmental engineering [1]-[3].

Some drawbacks that limit the free cellulase practical utility are mainly referred to its stability and reusability [4]. It is estimated that free cellulase was of low specific activity [5]. The multimeric cellulase structure also may tend to dissociate, resulting in their rapid inactivation [4]. In addition, the free enzyme is unable to be recycled [5]. To obviate some of these problems, the immobilization of cellulase on solid supports has been of great concern and suggested as a viable alternative [6]. During immobilization the enzymes were anchored in a solid matrix [1]. The process has advantages since it enables the use of very low amounts of cellulase with high hydrolytic activity and the possibility of reuse [4]. It also may result in an improved stability, simple separation from reaction mixture, possible modulation of the catalytic properties and facilitates prevention of microbial growth, as well [4].

Many immobilization techniques of cellulase have been studied extensively. Cellulase has been immobilized on a number of insoluble and soluble carriers [2], [5], [7]-[14]. At last, nano-scale materials have also been applied in the research of enzyme immobilization [15]-[17]. Although synthetic materials are efficient at recognizing and binding molecular targets, the preparation of those solid carriers may require an additional cost due to the commercialized chemicals for synthesized purpose or could be limited by problems in its recovery. With this respect, the need appeared for the development of cost-effective adsorption materials for use in the preparation of biocatalysts, taking into account that the matrices should provide a biocompatible and an inert environment for the native structure of enzymes [2]. The particular benefit may occur if the used materials originated from recycled waste.

This study investigates the possible use of spent coffee grounds, as an abundant renewable source, for the solid carrier purposes for enzyme immobilization. The coffee residues have a distinct advantage, because of the ascertained high adsorption capacity. Many studies have revealed its great affinity to uptake many different types of adsorbates [18]-[23]. The aim of this project was to study the feasibility of using activated carbon originated from coffee waste in the batch model for adsorption of an enzyme. The carrier material was prepared in a non-toxic manner, considering the further

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product application in animal feed purposes. A commercial cellulase from *Trichoderma reesei* was selected as a model enzyme. The use of coffee for immobilizing cellulose is a new attempt, thus the activity of the immobilized enzyme preparations was investigated under different experimental conditions and compared with those of free enzyme counterpart. The usefulness of pseudo-first- order and second-order models for the adsorption kinetics of enzyme was also discussed.

II. Materials And Methods

A. Preparation of enzyme

Cellulase preparation (derived from *Trichoderma reesei* ATCC 26921) was used as adsorbate for this investigation and it was purchased from Sigma-Aldrich®, Denmark. The working enzyme solutions of different cellulase concentrations (from 1 to 7 mg/L) were prepared fresh before each experiment in pH 4.8 tri-sodium citrate buffer ($Na_3C_6H_5O_7$).

B. Preparation of adsorbent

The espresso coffee was used as raw material from ''Doncafe – Espresso Aromatico – cialde'', Strauss Adriatic d.o.o, Šimanovci, Serbia. After preparing coffee beverage, spent coffee residues were collected and subjected to polyphenol compound extraction [24]. The obtained solid phase of extraction process was separated and further treated with an oxidizing agent. Based on our preliminary experiments, the polyphenol extracted coffee residues were treated with chlorine dioxide solution (1:6 w/v) prepared by 27 % sodium chlorite ($NaClO_2$) and 10 % citric acid ($C_6H_8O_7$) in distilled water (ratio $NaClO_2 : C_6H_8O_7 : dH_2O$ = 1:5:14). After 30 min of oxidation reaction, whole mixture was filtered and well washed with distilled water and then dried in an oven, for overnight, at 105°C. Thus prepared coffee material was employed as adsorbent in the further adsorption experiments.

C. Batch experiments

Batch experiments were carried out in 250 mL Erlenmeyer flasks which were placed on a translatory shaker (IKA – KS 4000i control, Staufen, Germany). According to our preliminary experiments, 1 g of the adopted adsorbent mass was mixed with a pre-determined initial concentration of the enzyme (1:60 w/v). The experimental parameters: the temperature, the initial cellulase concentration and the agitation rate of adsorption process were investigated. Each batch experiment was done in triplicate and performed under the conditions where one parameter changed while the other parameters were held constant. After 30 minutes of the cellulase immobilization, enzyme samples were collected filtrated, carriers were washed and thereafter, cellulase activity was measured using UV/VIS spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, USA) at 540 nm. In order to determine the success of enzyme immobilization, two terms were used: the immobilization yield (%) and the immobilization efficiency (%) [25].

The immobilization yield (%) is used to describe the percentage of total cellulase activity from the free cellulase solution that is immobilized onto the carrier (1):

$$Yield\ (\%) = 100 \cdot \left(\frac{immobilized\ activity}{starting\ activity}\right) \quad (1)$$

The immobilized activity was correctly determined by measuring the total activity from the starting enzyme solution subtracting the total residual enzyme activity that remains in the enzyme solution after immobilization.

The immobilization efficiency describes the percentage of bound enzyme activity that is observed in the immobilizate (2):

$$Efficiency\ (\%) = 100 \cdot \left(\frac{observed\ activity}{immobilized\ activity}\right) \quad (2)$$

All the terms above were calculated by using total activities (units, i.e. μmol/min) [25].

D. Measurement of cellulase activities and protein determination

Cellulase activities (μmol/mL·min) were determined on the basis of the sugar content, that originated from the enzymatic hydrolysis, by the 3,5-dinitrosalicylic acid reagent (DNS) method, using glucose as the standard, with slight modifications [26]-[29]. 1% Avicel (w/v) (purchased from Merck, Germany) dissolved in pH 4.8 tri-sodium citrate buffer was used for substrate medium. 500 μl of substrate was incubated with residual or immobilized enzyme sample in a test tube for 30 or 60 minutes at 50 °C, respectively, for determination of residual and immobilized enzyme activity. The reaction was stopped by adding 1ml of DNS reagent and thereafter, samples were boiled for exactly 5 min. After cooling in a water bath and dilution with distillate water, samples were filtrated and measured spectra-photometrically. For each sample, the sample control was prepared at the same time, but with an alteration that DNS was poured first and then the samples.

One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol of glucose equivalents per minute at 50 °C and pH 4.8. The unit was calculated as follows (3):

$$activity\ of\ cellulase\ (\mu mol/mL \cdot min) = \frac{1000 \cdot W}{M \cdot V \cdot t} \quad (3)$$

where *W* is the amount of released glucose equivalents, *M* is the molecular weight of the glucose, *V* is the volume of the measured sample and *t* is the reaction time, respectively [4].

The protein concentration in solution was measured by the Bradford protein assay using BSA (bovine serum albumin) as standard [26], [30].

E. Adsorption kinetic studies

Adsorption kinetics experiments were conducted by 3 g/L of adsorbent mass in different Erlenmeyer flasks containing 180 mL of the cellulase solution on a translatory shaker (120 rpm), for two hours. The initial cellulase concentration was fixed at 3 g/L, while the reaction temperature was varied (30, 40 and 50 ˚C). The samples were collected at pre-determined time intervals and analyzed spectro-photometrically for the cellulase adsorption capacity.

The equilibrium adsorption capacity of cellulase on the activated carbon, q_e (U/g) was calculated according to the (4):

$$q_e = \frac{(C_0 - C_e)}{m} \cdot V \quad (4)$$

where C_0 (U/mL) and C_e (U/mL) are the initial and the equilibrium concentration of cellulase solution, respectively, V (mL) is the volume of cellulase solution, and m (g) is the mass of activated carbon [1].

III. Results And Discussion

A. Effect of temperature

The effect of temperature on the immobilization yield and the immobilization efficiency of the free and immobilized cellulase onto coffee adsorbent were examined (Fig. 1). The effect was studied in 30, 40, 50 and 60 °C, whereas other parameters: the initial cellulase concentration (1 g/L) and the agitation rate (130 rpm) were held constant. Parallel with adsorption experiments the blank experiments were monitored for thermo-stability investigation of the cellulase enzyme, because of compensating for free enzyme deactivation under the immobilization conditions [25]. It was observed that in 60 °C the blank experiment of free enzyme activity was decreased due to denaturation of the enzyme structure and loss of the active conformation of the cellulase molecule [31]. So, the maximum of the immobilization yield at 60 °C was not real and show no noticeable actual enzyme activity because of the denaturation. At other temperatures (30, 40 and 50 °*C*), the enzyme have shown the thermo-stability in the time duration of the adsorption experiments. The immobilization yield and the immobilization efficiency at 30 °C were slightly lower than at 40 and 50 °C, which were similar one to another. Our results were agreed with the literature data of cellulase adsorption by another researcher [29], [31], [32]. For further continuation of the research, it was adopted the temperature of 40 °C, because of practicality and applicability of the cellulase adsorption onto coffee carrier.

B. Effect of initial cellulase concentration

The effect of initial cellulase concentration was studied in 1, 3, 5 and 7 g/L, whereas other parameters: the temperature (40 °C) and the agitation rate (130 rpm) were held constant. The influence of this effect on the immobilization yield and the immobilization efficiency of the free and immobilized cellulase onto coffee adsorbent were presented on Fig. 2. The percentage of immobilization yield was increased with decreasing of the initial cellulase concentration from 5 to almost 28 %.

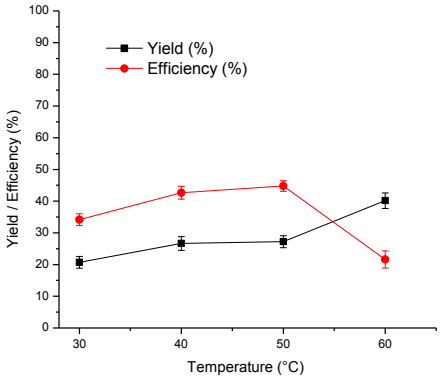

Fig. 1 Temperature influence on cellulase adsorption

The number of available adsorption sites onto coffee adsorbent decreased as the number of cellulase adsorbed increased. Thus, with higher cellulase concentration a lot of cellulase molecules remain non related [1]. On the other hand, the immobilization efficiency was decreased, too, but not drastically with an increasing of the initial cellulase concentration. Molecules of cellulase adsorbed were probably interfered with each other to express their actual activity.

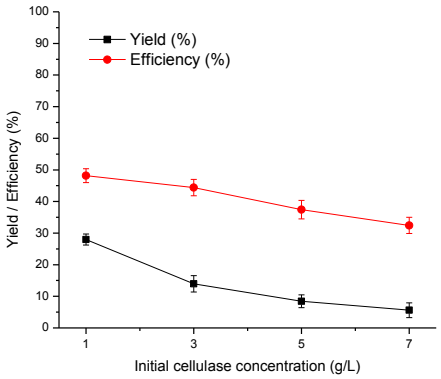

Fig. 2 Initial enzyme concentration influence on cellulase adsorption

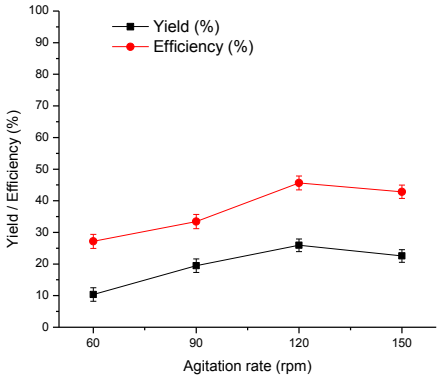

Fig. 3 Agitation rate influences on cellulase adsorption

TABLE I
KINETIC MODEL PARAMETERS FOR CELLULOSE ADSORPTION ON COFFEE RESIDUES AT DIFFERENT TEMPERATURES

Parameters		Pseudo-first order kinetic model			Pseudo-second order kinetic model		
Temperature (˚C)	$q_{e,\,exp}$ (U/g)	k_1 (1/h)	$q_{e,cal}$ (U/g)	R^2	k_2 (g/U·min)	$q_{e,cal}$ (U/g)	R^2
30	1.3536	0.0683	1.1718	0.7872	0.1237	1.4094	0.9884
40	1.2495	0.0704	0.4228	0.7413	0.4234	1.2713	0.9992
50	0.9950	0.0774	1.3826	0.5033	0.0434	1.2154	0.9885

C. Effects of agitation rate

The effect of the agitation rate was studied in 60, 90, 120 and 160 rpm (Fig. 3), whereas other parameters: initial cellulase concentration (1 g/L) and the temperature (40 °C) were held constant. It is evident from Fig. 3 that the maximum of the immobilization yield and the immobilization efficiency were obtained with 120 rpm. *T*he overall amount of coffee adsorbent of 1 g could not contact the cellulase in the solution because of the turbulence was not sufficiently strong when the agitation rate was between 60 to 90 rpm [18]. The strong agitation rate of 160 rpm, probably hinders the binding of cellulase particles to coffee surface active cites or affects desorption of already sorbed enzyme from the adsorbent. Qi, Chen, Su and Wan (2011) [26] have adopted the agitation rate of 150 rpm for their investigation for enzyme adsorption and recycling during hydrolysis of wheat straw lignocellulose, which was correlated with our results.

D. Adsorption kinetics of cellulase

The effect of time on the adsorption capacity, observed on three different temperatures is presented in Fig 4. It is clear that temperature affects the degree of the adsorption capacity, but on the other hand, it does not influence the equilibrium attainment. As the reaction time rises to 60 minutes, the adsorbed enzyme increased. After this point, the amount of enzymes being adsorbed onto the material was in a state of dynamic equilibrium with the amount of enzymes desorbed from the adsorbent [33]. It is considered that no enzyme molecules were further removed from the solution.

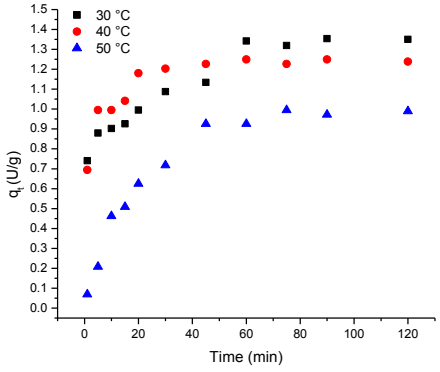

Fig. 4 Effect of contact time for cellulase adsorption onto coffee residues (adsorbent mass 1 g/L, cellulose concentration 3 g/L, 120 rpm)

The amount of enzymes adsorbed at the equilibrium time reflected the maximum enzyme adsorption capacity of the adsorbent under the conditions used in this study [33]. The temperature of 40 ˚C was chosen for further enzyme adsorption experiments. A simple kinetic analysis of adsorption, based on pseudo-first and pseudo-second order models, were employed to fit experimental data. The linearized form of pseudo-first (5) and pseudo-second order kinetic model (6) are presented as follows:

$$\log(q_e - q_t) = log q_e - \frac{k_1}{2.303} \cdot t \tag{5}$$

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_e^2} + \frac{1}{q_e} \cdot t \tag{6}$$

where k_1 (1/min) and k_2 (g/U·min) are the rate constants of the pseudo-first-order and pseudo-second-order adsorption kinetics, respectively, q_e is the ratio of enzyme adsorbed on the surface of the adsorbent at equilibrium (U/g) and q_t, is the ratio of enzyme adsorbed any time (U/g) [1].

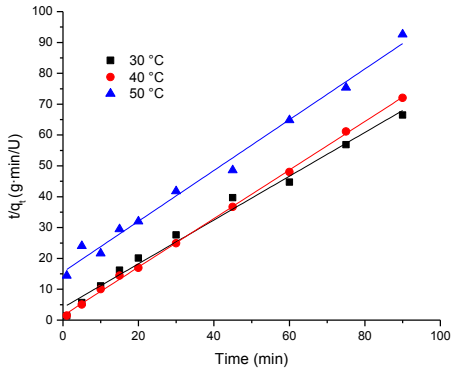

Fig. 5 Pseudo-second-order kinetic plot for the adsorption of cellulase on coffee residues (adsorbent mass 1 g/L, cellulose concentration 3 g/L, 120 rpm)

The corresponding kinetic parameters from the pseudo-first and pseudo-second-order linear plots were obtained at three different temperatures and were reported in Table 1. The validity of the order of adsorption processes was based on two criteria, the first based on the regression coefficients and the second one on predicted q_e values. Bearing that in mind, the immobilization of cellulose onto coffee residues follows the second-order kinetic model (Fig. 5). The correlation coefficients were approximately or greater than 0.99, with the calculated q_e values in good agreement with the experimental q_e values. Based on a principle of pseudo-second order kinetics, the rate limiting step of the immobilization process may be chemical sorption. Daoud, Kaddour and Sadoun

(2010) [1] also found that the adsorption of cellulase from *Aspergillus niger* on a commercial activated carbon follows the kinetic of pseudo-second order model in the same manner.

IV. Conclusions

The aim of this project was to study the feasibility of using low cost coffee residues in the batch model for adsorption of cellulase. To investigate the potential application of this carrier in enzyme immobilization, the properties, such as optimum operating temperature, enzyme concentration and agitation rate are reported, providing an overview of their impact on the process. The equilibrium time of adsorption was found to be 1 h, which was comparable with the processes where the carrier was commercial activated carbons. The process kinetic was founded to be predicted by pseudo-second-order model.

The results have shown that coffee residues may be successfully used as a good supporting material for the immobilization of cellulose, conducted under mild and environmentally friendly conditions. The product obtained in this study is non-toxic, thus could be safely implemented in animal feed applications.

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