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Polyethyleneimine-grafted collagen fiber as a carrier for cell immobilization

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Abstract Collagen fiber (CF), an abundant natural biopolymer, features many favorable properties that make it a potential carrier for cell immobilization. In the present investigation, CF was grafted with polyethyleneimine (PEI) using glutaraldehyde (GA) as the cross-linking agent, resulting in the formation of a novel CF based carrier (CF-PEI). The properties of CF-PEI as a carrier were evaluated by the immobilization of *Microbacterium arborescens* (CICC 20196), which has glucose isomerase (EC 5.3.1.5) activity. It was found that *M. arborescens* cells immobilized on CF-PEI exhibited higher glucose isomerization than those using activated carbon or anion exchange resin as the carriers. The Michaelis constant (K_m) of the isomerization reaction for the CF-PEI-immobilized *M. arborescens* cells was 0.528 mol/L, which was slightly higher than that of free cells (0.473 mol/L). In addition, the apparent activation energies (E_a) of free and immobilized cells on CF-PEI were almost the same at 60 kJ/mol. In an isomerization reaction of glucose to fructose in a fixed-bed reactor, CF-PEI-immobilized *M. arborescens* cells showed appreciable activity and operational stability. The corresponding isomerization ratio was as high as 41 % for 20 days, and the half-life was about 40 days.

Keywords Collagen fiber · Polyethyleneimine · Cell immobilization · *Microbacterium arborescens* · Glucose isomerization

Introduction

Immobilized cells have many advantages over free cells, including enhanced fermentation productivity, improved downstream processing, strengthened cell stability and easy recovery, [11, 15, 23] etc. Conventional methods used for the immobilization of cells include adsorption, covalent bonding, entrapment and encapsulation. Among these methods, adsorption is preferred because this method has minimal effect on cell physiological activity and it is easy to operate [4, 15].

An important issue for cell immobilization by adsorption is the choice of carriers. Usually, materials used as carriers should have good chemical, physical and biological stability during the immobilization processes and subsequent application conditions. An ideal carrier should have sufficient mechanical strength, adequate functional groups to bind cells and high loading capacity of cells. More importantly, the carriers should also be non-toxic for the immobilized cell [16, 23, 33]. If possible, material availability and cost-effectiveness should also be considered.

Collagen fiber (CF), an abundant natural biopolymer, features many favorable properties for using as a carrier. CF is biocompatible and non-toxic [18]. CF has the chemical and biological stability, which are related to its unique

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structure. Moreover, the stability of CF can be further enhanced by the use of covalent cross-linking agents [36]. The alignment of the collagen molecules in the CF and the stabilization in this position by the intermolecular cross-links endow CF with a high degree of mechanical strength [25]. It has been reported that CF has a tensile strength equal to that of wood or steel wire [24]. As a fibrous matrix, CF can provide adequate supporting surfaces for cell adsorption, and its fibrous morphology can lead to both reduced pressure drops and low mass-transfer resistance [4, 14, 37].

Based on these favorable properties, it is reasonable to expect that CF can serve as a good carrier for cell immobilization. However, the thermal stability and surface charge of CF should be further improved before its application in cell immobilization. A common method to enhance thermal stability of CF is introducing chemical cross-links among collagen molecules. Glutaraldehyde (GA) is a dialdehyde cross-linker that has been extensively used in polypeptide and protein cross-linking because of the high activity of its aldehyde groups towards the amino groups of proteins. This treatment allows collagen-based materials to improve its mechanical strength, resistance to biodegradation and thermal stability [9, 31, 35]. The positively charged CF is favorable for cell immobilization due to the fact that bacterial cells are often negatively charged when cultivated at physiological pH values [34]. Polyethyleneimine (PEI) is a synthetic polymer with a large number of primary, secondary and ternary amine groups. PEI in its protonated form has been the most commonly used cationic polymer as a stabilizer of colloid and a gene delivery agent. Due to the high affinity of positively charged PEI with negatively charged species, many investigators have focused their attention on PEI as a surface modifying agent in immobilized cell technology [8, 14, 27]. PEI is also non-toxic and has been approved by the FDA for the use in food as a secondary direct food additive [3].

The objective of this research is to develop PEI and GA-modified collagen fiber (CF-PEI) as a novel supporting matrix, and further to evaluate its potential for cell immobilization. *Microbacterium arborescens* cells (CICC 20196) were chosen to be immobilized on CF-PEI, and then glucose isomerase activity was investigated. For comparison, *M. arborescens* cells were also immobilized on anion exchange resins and activated carbon, and their activities in glucose isomerization were evaluated under the same experimental conditions. As demonstrated in the following experiments, *M. arborescens* cells immobilized on CF-PEI exhibited much higher activity and stability than those immobilized on activated carbon or resin.

Materials and methods

Materials

Collagen fiber was prepared by the procedures reported in our previous work [20]. Polyethyleneimine with a molecular weight of about 10,000 was purchased from Aladdin (Shanghai, China). Standard anionic polyelectrolyte potassium polyvinyl sulphate (PVSK) solution and standard cationic polyelectrolyte poly-diallyldimethylammonium chloride (poly-DADMAC) for colloidal titrations were obtained from Wako Pum Chemicals Industry in Japan and Sigma Chemical Company, respectively. Carbazole was chemically pure grade, and L-cysteine hydrochloride and xylose were biochemical grade.

A granular activated carbon with a diameter of 1.0 mm and a strong anion exchange resin (Amberlite IRA-400) with quaternary ammonium groups as the anionic binding sites, were purchased from a local supplier. Activated carbon was rinsed with distilled water to remove ash, and sterilized at 121 °C for 15 min. Amberlite IRA-400 was pretreated with acid and/or alkali and converted into its OH^- form prior to use.

Bacterial strain and culture medium

Microbacterium arborescens (CICC 20196), producing intracellular glucose isomerase, was obtained from the China Center of Industrial Culture Collection (CICC). Luria–Bertani (LB) medium contained 10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl. The cells were routinely maintained at 32 °C on LB agar plates with 1.5 % agar and LB medium with 2 g xylose per liter added as an inducer for the isomerase production.

Preparation of PEI-grafted CF

PEI was grafted to the CF by dissolving 1.8 g of PEI in 300 mL of distilled water and mixing with 30 g of CF. The mixture was stirred at 30 °C for 8 h. Then, the PEI-grafted CF was collected by filtration, and transferred into 200 mL of 1.5 % GA solution (w/v) at 35 °C for 1 h. When the reaction was finished, the product was washed three times with distilled water and dehydrated using absolute ethanol. Finally, the dehydrated product was dried in an oven at 45 °C for 12 h, after which the PEI and GA-modified CF (CF-PEI) was obtained. It should also be noted that the amounts of PEI and GA used in our experiments have been optimized. Increasing amounts of PEI can provide CF more positive charge, which is beneficial for the immobilization of *M. arborescens* cells on CF. However, when the amount of PEI exceeded 6 % of the CF weight, further

increases of PEI showed limited influences on strengthening the positive charge of CF surface. Hence, the amount of PEI was fixed at 6 % of the CF weight. Additionally, the use of GA is to cross-link the CF so as to improve the thermal stability of CF. Based on our results, when the concentration of GA solution reached 1.5 % (w/v), the resultant materials were very stable in the process of glucose isomerization.

Surface charge density determination of CF and CF-PEI

The surface charge density of CF and CF-PEI was determined by back titration with a Mütek Particle Charge Detector PCD-03 (Herrsching, Germany) at a series of pH values [28]. In this study, the CF and CF-PEI samples were ground in a mortar and pestle and sieved through a 100-mesh screen. For positive surface charge measurements, an anionic polyvinyl sulphate (PVSK) solution was employed as a neutralizing agent and a cationic polyelectrolyte (poly-DADMAC) was used as titrant. For negative surface charge measurements, the sequence of these two standard polyelectrolytes was reversed.

Thermal stability determination of CF and CF-PEI

Denaturation temperature of CF and CF-PEI was measured using a differential scanning calorimeter (DSC, 200PC, Netzsch, Germany), to indicate their thermal stability. Samples in aluminum pans were scanned in nitrogen atmospheres in the range of 30–100 °C. The temperature corresponding to top of the endothermic peak was determined as the denaturation temperature.

Cell immobilization procedure

M. arborescens cells in stationary phase were harvested by centrifugation, washed and re-suspended in 0.02 M PBS solution (pH 7.4) to obtain a cell suspension with an OD600nm of 1.0 (about 5×10^8 cfu/mL). 1.0 g of activated carbon, CF, CF-PEI, and Amberlite IRA-400 were added to individual in 50 mL of cell suspensions in 100 mL Erlenmeyer flasks. The flasks were gently shaken at 25 °C for 1 h. The concentration of free cells in the supernatant was determined by standard plate count method. The number of immobilized cells, Q_e (CFU/g), can be calculated as:

$$Q_e = \frac{(C_0 - C_e)\, V}{M}, \tag{1}$$

where C_0 is the initial cell concentration (CFU/mL), C_e is the final cell concentration (CFU/mL), M is the mass of carrier (g) and V is the volume of cell suspensions (mL). All experiments were repeated five times.

Morphology observation of samples

All samples were washed three times with 0.02 M PBS solution (pH 7.4). Subsequently, the samples were fixed with 2 % GA for 12 h at 4 °C and sequentially dehydrated for 10 min in 25, 50, 75, 90 and 95 % ethanol solutions and then twice for 20 min in absolute ethanol, and then they were air-dried and gold-coated by sputtering. The morphology of samples was observed with a field emission scanning electron microscope (JSM-7500F, JEOL Ltd., Japan).

Glucose isomerase activity determination

M. arborescens cells used in the study produce glucose isomerase (EC 5.3.1.5), also known as xylose isomerase, which can catalyze the isomerizations of D-glucose to D-fructose and D-xylose to D-xylulose, respectively. The food industry employs the isomerization of D-glucose to D-fructose to produce high-fructose corn syrup (HFCS). Thus, we selected the isomerization of D-glucose to D-fructose to evaluate the activity of immobilized *M. arborescens*. The activity of glucose isomerase was determined by measuring the production of fructose according to the method described by Yu [38]. Briefly, 2 M glucose in 0.05 M phosphate buffer (pH 7.5) containing 0.02 M $MgSO_4$ served as a substrate. It was incubated with free or immobilized cells at 60 °C. One unit of the glucose isomerase (GIU) is defined as the amount of enzyme required to convert 1 μmol of glucose per min under the assay conditions. The glucose isomerase activity of immobilized cells was expressed in GIU per gram carrier.

Kinetic parameters of glucose isomerase in free and immobilized cell

The kinetic studies of the isomerization reaction catalyzed both by free and immobilized cells were carried out at various concentrations of glucose solutions (0.2, 0.4 0.6, 0.8, 1.0, 1.5 and 2.0 mol/L) by measuring the initial reaction rate. The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) of the glucose isomerization reaction were obtained from Lineweaver–Burk plots.

Apparent activation energy (E_a) was determined from the Arrhenius plot based on the data of maximum reaction rate (V_{max}) at various temperatures. $\mathrm{Ln}V_{max}$ was plotted against $1/T$ (in Kelvin) and the slope was used to determine E_a from Eq. (2):

$$E_a = -\text{slope} \times R \tag{2}$$

where R (gas constant) = 8.314 J/K.

lsomerization of glucose to fructose in fixed-bed reactor

Three glass columns (30 cm long and 1.0 cm in diameter) were filled with three kinds of immobilized cells (CF-PEI, activated carbon and Amberlite IRA-400 immobilized cells, respectively) and thermostatically controlled at 60 °C. A peristaltic pump was used to deliver the substrate solution continuously at a flow rate of 1.5 mL/h. The substrate solution was 0.02 M phosphate buffer solution (pH 7.5) containing 2 M glucose and 0.02 M $MgSO_4$. The products were sampled at regular intervals and the concentration of fructose was determined using the cysteine–carbazole method [38].

Results and discussion

Surface charge of CF and CF-PEI

Supplementary material 1 illustrates the immobilization of bacterial cells on CF-PEI. Firstly, PEI was coated onto the surface of CF to increase its positive charge. Then, GA was used as a cross-linking agent to graft PEI onto CF and improved its thermal stability. As shown in Fig. 1, the surface charge density of CF-PEI was higher than 1.2 meq/g in the pH range of 3.5–7.0, while it was only 0.2 meq/g for that of CF. Note that the point of zero charge (PZC) of CF-PEI was 9.7, while it was only 5.6 for that of CF. This result suggested that the grafted PEI can significantly increase the surface positive charge of CF, which was very beneficial for the immobilization of cells.

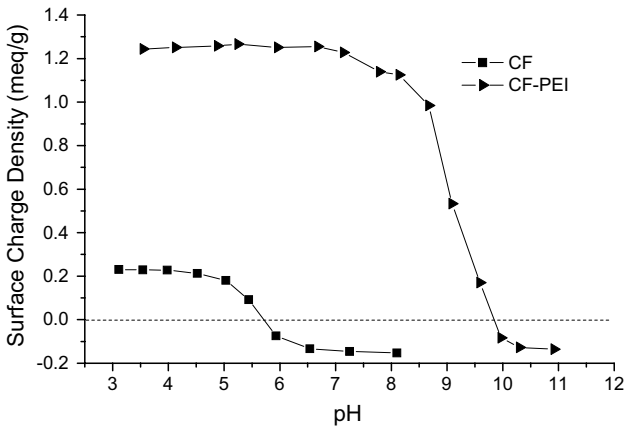

Fig. 1 Surface charge density of CF and CF-PEI

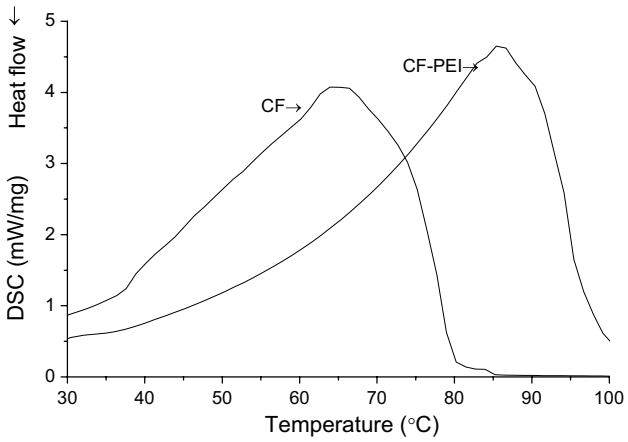

Fig. 2 DSC *curves* of CF and CF-PEI

Thermal stability of CF and CF-PEI

Denaturation temperature is often used to evaluate the degree of thermal stability of collagen-based materials [5]. Basically, as CF is progressively heated, a threshold temperature is reached at which disruption of the hydrogen bonding in collagen molecule triple helix occurs, causing the triple helices to unwind.

Compared with CF, the denaturation temperature of CF-PEI was significantly increased to about 85 °C, as shown in Fig. 2. This suggested that the GA effectively improved the thermal stability of CF [9], which made it more applicable to use as a carrier in the reaction of glucose isomerization.

Cell immobilization

Activated carbon and ion exchange resin are conventional materials used as supporting matrices in industry because these matrices are easily available and have very stable properties for repeated experiments [26, 32]. Hence, activated carbon and Amberlite IRA-400 were also employed as matrices for the immobilization of *M. arborescens* cells. The adsorption capacities of CF, CF-PEI, activated carbon and Amberlite IRA-400 to *M. arborescens* cells are summarized in Table 1. The adsorption capacity sequence of these carriers to *M. arborescens* cells was found to be CF-PEI > Amberlite IRA-400 > CF > activated carbon. Compared with CF, the adsorption capacity of CF-PEI to *M. arborescens* cells was substantially increased. As shown in Fig. 1, the PZC of CF-PEI increased to 9.7 after PEI was grafted, suggesting that CF-PEI has a positive charge at pH < 9.7. Amberlite IRA-400, an anionic exchange resin, is also positively charged. Therefore, the adsorption capacities of CF-PEI and Amberlite IRA-400 to *M. arborescens* cells are higher than those of CF and activated carbon. This suggests that electrostatic interaction is the main immobilization mechanism of *M. arborescens* cells on CF-PEI.

In addition, the surface morphology of carriers has an important influence on the adsorption of cells [10, 12, 14]. As shown in Table 1, the adsorption capacity of CF-PEI is

Table 1 Influences of supporting matrices on the amount of immobilized *M. arborescens* cells and the activity of glucose isomerization

Supports	Q_e ($\times 10^{10}$cfu/g dry carrier)	Glucose isomerase activity (GIU/g dry carrier)
CF	0.23 ± 0.04	–
CF-PEI	1.21 ± 0.31	260.15 ± 13.13
Amberlite IRA-400	0.71 ± 0.22	128.75 ± 6.92
Activated carbon	0.14 ± 0.06	55.90 ± 7.44

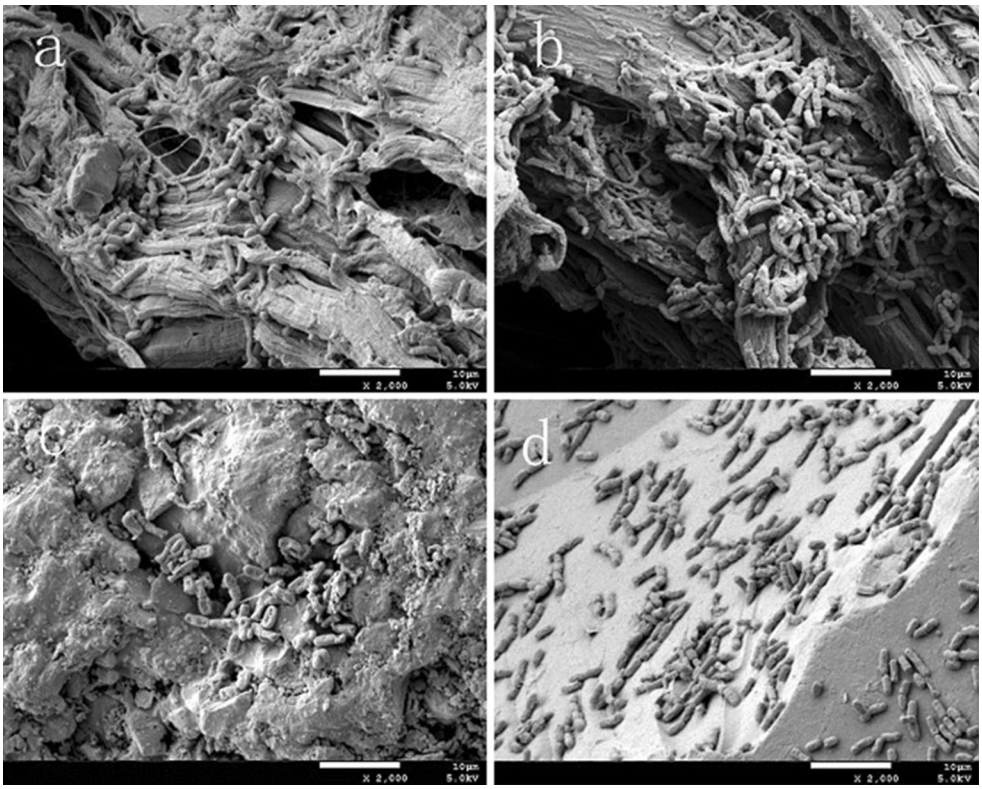

Fig. 3 SEM images of *M. arborescens* cells immobilized on CF (**a**), CF-PEI (**b**), activated carbon (**c**) and Amberlite IRA-400 (**d**); ×2,000

higher than that of Amberlite IRA-400 even though CF-PEI and Amberlite IRA-400 are both positive charged. As illustrated in SEM images, collagen fibers have rough, uneven surfaces and a complicated morphology with many interstitial spaces (Fig. 3a, b). IRA-400 has a smooth surface (Fig. 3d) that would provide less accessible surface area for cell adsorption.

Although activated carbon has a high specific surface area, its accessible surface area for cell absorption is small, and the number of adsorbed cells may not be proportional to the specific area of the carrier [21, 29]. It has been reported that the pore size of carriers must be 2–5 times greater than the cells for the adsorption of microbial cells [29]. As shown in SEM images of Fig. 3, *M. arborescens* cell is rod-shaped, about 2.5 μm long and 1 μm in diameter. However, the pores in activated carbon are mesopores and micropores, with diameters not larger than 50 nm [22]. In this case, cells can only adhere on the outer surface of activated carbon (Fig. 3c), resulting in a low adsorption capacity.

Glucose isomerase activity of immobilized cell

The glucose isomerase activities of three kinds of immobilized cells are listed in Table 1. The isomerase activity per gram CF-PEI was found to be two times and five times higher than that of Amberlite IRA-400 and activated carbon, respectively. This was largely due to the fact that the cell immobilization capacity of CF-PEI was much higher than activated carbon and Amberlite IRA-400.

Kinetic parameters

As summarized in Table 2, the apparent K_m and maximum velocity (V_{max}) of the glucose isomerization reaction of free and immobilized *M. arborescens* cells were obtained

Table 2 Kinetic parameters of glucose isomerization using free and immobilized cells

	K_m (mol/L)	V_{max} (mmol/L min^{-1})	E_a (kJ/mol)
Free cell	0.473	0.219	61.28
CF-PEI-immobilized cell	0.528	0.155	60.80
Amberlite IRA-400-immobilized cell	0.547	0.086	60.24
Activated carbon-immobilized cell	0.504	0.040	59.41

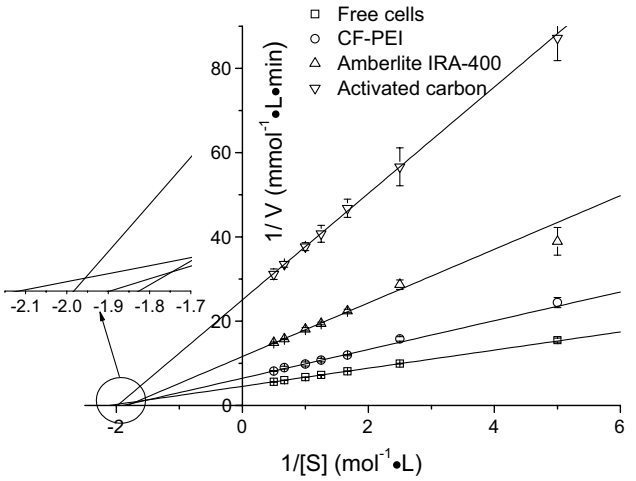

Fig. 4 Lineweaver–Burk *plots* of glucose isomerization reaction rates obtained using free cells and immobilized cells at 60 °C and pH 7.5

from Lineweaver–Burk plots (Fig. 4). Commonly, the increase in K_m indicates that the immobilized cells have a relatively lower affinity for its substrate than that of free cells, and this results in a decrease in cell activity. Conversely, little change in K_m suggests that the maximum activity of cell after immobilized was preserved. Accordingly, the carriers should have appropriate affinity with cells to ensure adequate immobilization capacity and higher activity. It was found that the K_m value for the CF-PEI-immobilized cells was 0.528 mol/L, only an 11.6 % of increase over the free cells (0.473 mol/L). In the case of activated carbon, its K_m value for the immobilized cells was smaller than other carriers, but its immobilization capacity is much lower. It has been reported that the increase of K_m for the immobilized cells is strongly related to the property of carriers and the immobilization approaches. Kasumi and co-workers [13] entrapped *Streptomyces* sp. cells in chitosan/gelatin and the K_m of glucose isomerase increased from 0.22 to 0.51 mol/L. Kumakura

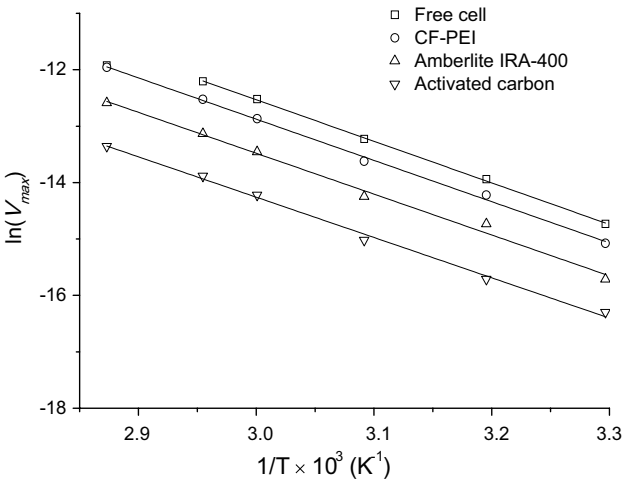

Fig. 5 Arrhenius *plots* for the determination of activation energy (E_a)

and Kaetsu [17] reported that the K_m value of glucose isomerase in *Streptomyces phaeochromogenes* immobilized in radiation-induced cast-polymerization, increased from 0.33 to 0.85 mol/L. Therefore, it can be concluded that CF-PEI is an improved carrier for the adsorptive immobilization of *M. arborescens* cells.

In the case of maximum velocity (V_{max}), the activity sequence is CF-PEI-immobilized cells > Amberlite IRA-400-immobilized cells > activated carbon-immobilized cells, which is accordance with their activity sequence, as shown in Table 1.

Activation energy is another important parameter for immobilized enzymes or cells because it may indicate the behaviors of mass transfer in the reaction process [2]. Figure 5 shows Arrhenius plots of the isomerization reaction from glucose to fructose using free and immobilized cells. The apparent activation energy (E_a) was calculated from the slope of the plot and is listed in Table 2. There is no significant difference between free cells and immobilized cells. Other researchers have found that the activation energy will decrease if the catalytic reaction of immobilized cell systems is subjected to internal diffusion limitation [2, 13, 19, 30]. This depends on the carriers and immobilization approaches [6]. Since CF-PEI is in fibrous state and the cells are adsorbed on its outer surface, the internal diffusion limitation is negligible [1, 7]. Therefore, the higher reaction rate of CF-PEI-immobilized *M. arborescens* cells in the isomerization reaction of glucose to fructose can be expected. In summary, the values of K_m, V_{max} and E_a can be used to estimate the quality of carriers.

Fixed-bed operation

Continuous isomerization reactions of glucose to fructose were performed using fixed-bed reactors packed with three

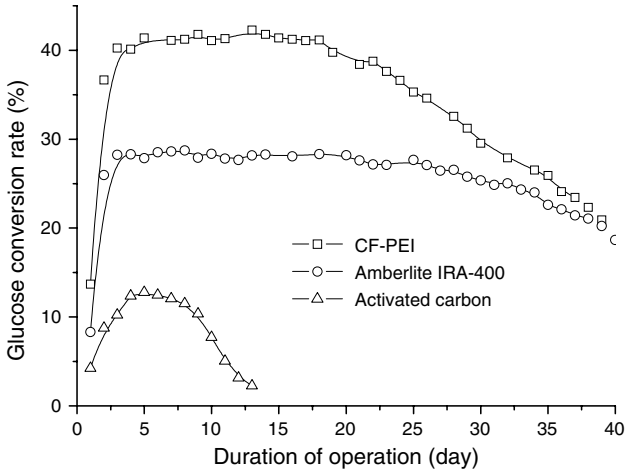

Fig. 6 Influences of supporting matrices on the operation stability of immobilized *M. arborescens* cells

kinds of immobilized *M. arborescens* cells. As shown in Fig. 6, the isomerization ratio of CF-PEI-immobilized cells is about 40 % from 4 to 20 days, much higher than that of Amberlite IRA-400 and activated carbon-immobilized cells. In addition, the half-life of CF-PEI-immobilized cells is as long as 40 days, implying its practical application. It was reported that the half-life of *Streptomyces olivochromogenes* cells immobilized on ion exchange resin were in the range of 8–40 days [32]. In contrast, the isomerization ratio of *M. arborescens* cells immobilized on activated carbon was low, and activity was quickly lost due to loss of cells. This confirms that the suitable affinity of carrier to the cells is important to obtain immobilized cells with high activity and stability.

Conclusions

Chemically modified collagen fiber (CF-PEI) can be used as an effective carrier for the adsorptive immobilization of *M. arborescens* cells. Due to its affinity with cells, the kinetic parameters (K_m and E_a) of CF-PEI-immobilized cells were similar to those of free cells. In fixed-bed bioreactors, CF-PEI-immobilized cells showed pretty good activity and excellent durability. Considering that CF is biocompatible, non-toxic and inexpensive, the CF-PEI matrix prepared by our method may also be extended for the immobilization of other biological cells.

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