Immobilization of *Saccharomyces cerevisiae* using polyethyleneimine grafted collagen fibre as support and investigations of its fermentation performance

Deyi Zhu, Xia Li, Xuepin Liao, and Bi Shi

**ABSTRACT**

In the present investigation, an collagen fibre (CF), abundant natural biomass, was successfully grafted by polyethyleneimine (PEI). The resultant PEI grafted collagen fibre (CF-PEI) was employed as biocompatible and high cell loading support matrix for the immobilization of *Saccharomyces cerevisiae* cells. The as-prepared CF-PEI immobilized cells (CF-PEI-cell) exhibited high activity and stability for both batch and continuous fermentation. In batch fermentation, CF-PEI-cells showed enhanced stability as compared with other matrices supported cells, and produced an average ethanol concentration of 45.04 g/L with ethanol yield (Y_{P/E}) of 0.46 g/g and glucose conversion efficiency (η) of 90.4%. Continuous fermentation was operated stably in a down-flow trickling bed reactor charged with CF-PEI-cell for a total of 2 months. When the dilution rate was 0.16 1/h, the average ethanol productivity reached 7.18 g/(L h) with η of 88.94%. Further scanning electron microscopy observations confirmed that yeast cells can proliferate on the surface of CF-PEI during ethanol fermentation, which demonstrates that CF-PEI is indeed an ideal matrix for the immobilization of yeast cells.

**Introduction**

Immobilized cell technology has been extensively applied to ethanol fermentation due to its high cell density, tolerance to higher concentrations of substrate and products, easier separation, etc. [1–3]. Cell immobilization by adsorption is often preferred for ethanol fermentation because of its simplicity, low cost and high efficiency [4]. Moreover, the adsorption technique permits sufficient diffusion of nutrients and metabolites, and especially leads to easy evolution of CO_2 during fermentation [5–7].

Selection of the most appropriate support for immobilization is an important factor that determines the activity of the immobilized cells. For successful immobilization, the support must be conducive to cell viability [1–3] as well as easy to use, cheaper, renewable, biodegradable and available naturally in abundance. Recently, various natural biomass materials have been developed as supports for yeast cell immobilization, including grape skins [5], silk cocoons [6], sugarcane bagasse [7], loofa sponges [8] and bacterial cellulose [9]. The most important advantage accruable from such biomaterials is that their use is free from toxicity problems comparing with inorganic or synthetic support materials.

Collagen fibre (CF), an abundant natural biomass, is the major component of the extracellular matrix (ECM). In fact, *in vivo* CF form the natural scaffold for housing the cells, acting as a mechanical support and creating a microenvironment for the cells. Thus, CF and its derivatives have incomparable biocompatibility, which has allowed their application in prosthetic, artificial tissue, drug carrier, food and cosmetics [10–12]. Furthermore, CF has the features of special microstructure, good mechanical strength, resistance to enzymatic cleavage and unavailability for most microorganisms [10,11,13,14]. Therefore, CF has a great potential application as a biocompatible support material for microbial immobilization.

In our previous work [15], a new support matrix, polyethyleneimine grafted collagen fibre (CF-PEI), was developed, and its suitability as a support material was validated by the immobilization of *Microbacterium arborescens* cell. However, the immobilized *M. arborescens* cells were employed in a non-viable form, which catalyses a single-enzyme reaction of isomerization of D-glucose to D-fructose. It is reasonable to expect that CF-PEI will perform much better for immobilizing viable cells.

In this work, the obtained CF-PEI was employed as biocompatible, non-toxic and high cell loading support.
matrix for the immobilization of yeast cells. We systematically investigated the cell viability of immobilized cells and their ethanol fermentation behaviours in batch and continuous fermentation experiments.

Materials and methods

Materials

CF-PEI was prepared as described in our previous work [15]. The as-prepared CF-PEI was characterized using X-ray photoelectron spectroscopy (XPS, Kratos XSAM 800pci, UK) and emission scanning electron microscopy (SEM, JSM-7500F, Japan), and the results are given as supplementary material. Three conventionally used supports, granular activated carbon, cellular glass and porous ceramic, were all purchased from a local supplier. Before use, they were rinsed with distilled water to remove ash and were sterilized at 121 °C for 15 min. The specific surface area of CF-PEI and other supports was determined by Brunauer–Emmett–Teller method using the nitrogen adsorption approach (Micromeritics ASAP 2010, nitrogen adsorption apparatus). Other chemicals were analytical grade or the highest purity commercially available.

Strains

The *Saccharomyces cerevisiae* strain SCY008 utilized in this study was obtained from the Shandong Centre of Industrial Culture Collection (Jinan, China).

Yeast cell immobilization

Prior to immobilization, the *S. cerevisiae* cells were cultured in a propagation medium (50 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone) at 30 °C for 24 h in a rotary shaker (150 rpm) under aerobic conditions. The cells were collected by centrifugation at 9,000 × g for 10 min at 4 °C. The pellet was recovered, washed and resuspended with sterilized 0.9% NaCl solution to obtain a cell suspension of approximately 6.8 × 10⁶ cells/mL. Pretreated supports (CF-PEI, activated carbon, cellular glass and porous ceramic) were soaked into this cell suspension in a ratio of 1:50 (w/v) and were incubated at 30 °C for 6 h. After removing the residual cell suspensions, the immobilized cells were obtained. They were stored in a 4 °C refrigerator for cell viability assay or aseptically transferred into flasks or trickling bed reactor for ethanol fermentation.

The cell loading, Lc (cells/g dry support), on different supports was estimated by counting the cell number of the initial and residual cell suspensions with a Neubauer-counting chamber after suitable dilution.

Cell viability assay

For certain time intervals (three days, one week, two weeks and four weeks), the immobilized cells on different supports were taken out from the refrigerator and washed off with sterilized pH 6.0 phosphate buffer by vortexing gently in a 10 mL centrifugal tube. Subsequently, the viability of yeast cells in the washed buffer was analysed using the methylene blue staining method [16]. The cell viability was calculated by dividing the number of viable cells by the number of total cells. The analysis was duplicated and average values are reported.

Batch fermentation

Synthetic fermentation medium containing 100.0 g of glucose, 1.5 g of yeast extract, 2.5 g of NH₄Cl, 5.5 g of K₂HPO₄, 0.25 g of MgSO₄·7H₂O, 0.01 g of CaCl₂, and 1.0 g of NaCl per litre was sterilized in an autoclave at 116 °C for 20 min and its pH was adjusted to 5.0 using lactic acid prior to fermentation in all cases. Batch fermentation was carried out in 250 mL Erlenmeyer flasks with 120 mL synthetic fermentation medium and immobilized or free cells at 30 °C under anaerobic conditions for 40 h. The initial cells concentration of free and immobilized cells inoculated into media were approximately equal to 8.2 × 10⁶ cells/mL fermentation medium. At the end of each fermentation run, the supports were removed from their broths by filtration with a sterile strainer, and washed once with cold sterilized 0.9% NaCl solution and twice with fresh sterilized fermentation medium for 20 min and its pH was adjusted to 5.0 using lactic acid prior to fermentation in all cases. Batch fermentation was carried out in 250 mL Erlenmeyer flasks with 120 mL synthetic fermentation medium and immobilized or free cells at 30 °C under anaerobic conditions for 40 h. The initial cells concentration of free and immobilized cells inoculated into media were approximately equal to 8.2 × 10⁶ cells/mL fermentation medium. At the end of each fermentation run, the supports were removed from their broths by filtration with a sterile strainer, and washed once with cold sterilized 0.9% NaCl solution and twice with fresh sterilized fermentation medium.

The fermentation broths were collected and centrifuged (9000 × g, 10 min) at 4 °C, and the supernatants were removed to fresh tubes for following analysis. Ethanol concentration (Pe, g/L) was determined using gas chromatography (GC8900A, Chengdu, China) according to Yan et al. [17]. Residual glucose concentration (g/L) was measured using the dinitrosalicylic acid (DNS) method [18]. The ethanol yield (YP/S, g/g) was calculated as a ratio of the ethanol produced to the total glucose utilized. The efficiency of glucose conversion to ethanol (η, %) has been estimated by the ratio of ethanol yield to the theoretical value of ethanol yield (0.51 g/g).
**Continuous fermentation**

A down-flow trickling bed reactor was selected for continuous ethanol fermentation. The major component of the reactor was a jacketed synthetic glass column (4 cm, height of 50.0 cm) packed with approximately 70% volume of CF-PEI-cell. Column temperature was maintained at 30 ± 1 °C by circulating water inside the reactor jacket. The fermentation medium was continuously fed from the top of the reactor by a peristaltic pump with various dilution rates of 0.05, 0.11, 0.16 and 0.21 1/h, which were changed step-wisely from low to high every week. In order to examine the stability of the CF-PEI immobilized cells, the continuous ethanol fermentation system was operated sequentially for 30 days with a dilution rate of 0.16 1/h. Fifty-millilitre samples were collected every day, and the ethanol and residual glucose concentration were analysed. The volumetric ethanol productivity ($Q_e$, g/(Lh)) was defined as the rate of ethanol production divided by the reactor volume.

**Electron microscopic scanning**

The morphological changes of CF-PEI immobilized cells before and after the continuous fermentation were observed using SEM (JSM-7500F, Japan). The samples were washed three times carefully with 0.9% NaCl solution. Subsequently, the samples were fixed with 2% glutaraldehyde 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. The dehydrated samples were dried by a CO$_2$ supercritical drying process, gold-coated by sputtering and photographed.

**Data analysis**

One-way analysis of variance (ANOVA) was performed to determine the statistical significance among multiple means of residual glucose concentration or ethanol concentration in continuous ethanol fermentation. Origin 9.0 software (Northampton, MA, USA) was applied for statistical analysis and significance was determined by a $P$ value of less than 0.05.

**Results and discussion**

**Cell immobilization**

The specific surface areas and *S. cerevisiae* cell loading of four different supports are summarized in Table 1. The cell loading is influenced by several factors, such as surface charges, surface morphology and surface area of the support [16,19]. Since yeast cells have negatively charged surface at physiological conditions [20], a direct cell/support interaction, due to electrostatic forces, can be the essential factor in the adsorption of cells. The surface of CF-PEI was positively charged at pH < 9.7 [15]. On the contrary, the main component of glass and ceramic both is silicon dioxide, which leads to a negatively charged surface [21]. As a result, CF-PEI took the lead in cell loading, although its specific surface area was not the highest.

Additionally, the surface morphology of support also has an important influence on the adsorption of cells: a rough and porous surface with appropriate pore size is necessary [8,16]. Although activated carbon has a rough surface and the highest specific surface area, its unsuitable pore size causes a small accessible surface area for cell absorption. As for CF-PEI, its fibrous morphology with rough surface (see the SEM images in the online supplementary material) is suitable for *S. cerevisiae* cell immobilization. In general, a positively charged matrix with a larger specific surface area, like CF-PEI, is an ideal support for cell loading.

**Cell viability**

The viability of *S. cerevisiae* cells on various supports at different storage times in a 4 °C refrigerator is given in Table 2. A decreasing trend was observed along with the increase of storage time due to the exhaustion of nutrients. Obviously, cells immobilized on CF-PEI exhibited the highest viability over the rest of the supported counterparts, which was not less than 97% within two weeks of storage. Even stored for four weeks, the viability was still higher than 87%, which was three times of the value obtained using cellular glass or porous ceramic as supporting matrices. These results demonstrate that CF-PEI is non-toxic for *S. cerevisiae* cells.

**Table 1.** The specific surface areas and *S. cerevisiae* cell loading of various supports.

| Support       | Specific surface area (m$^2$/g) | $L_e$ ($\times 10^5$ cells/g dry support) |
|---------------|---------------------------------|------------------------------------------|
| Cellular glass | 1.619                           | 6.12 ± 2.23                              |
| Porous ceramic | 1.547                           | 5.36 ± 1.89                              |
| Activated carbon | 12.010                         | 3.78 ± 0.65                              |
| CF-PEI        | 3.790                           | 19.78 ± 3.13                             |

**Table 2.** The viabilities of *S. cerevisiae* cells on various supports at different storage times.

| Support       | Three days (%) | One week (%) | Two weeks (%) | Four weeks (%) |
|---------------|----------------|--------------|---------------|----------------|
| Cellular glass | 97.96          | 91.43        | 54.76         | 27.50          |
| Porous ceramic | 96.84          | 91.03        | 53.30         | 25.72          |
| Activated carbon | 75.95          | 63.79        | 19.64         | /              |
| CF-PEI        | 99.63          | 97.42        | 97.08         | 87.35          |
Batch fermentation

Table 3 summarizes the fermentation kinetics of immobilized S. cerevisiae cells and free cells during ethanol production in a repeated batch process. Among the immobilized and free cells, a remarkably higher activity of ethanol production was observed for CF-PEI-cells. The average ethanol concentration of CF-PEI-cells was 45.04 g/L with \( Y_{P/S} \) of 0.46 g/g and \( \eta \) of 90.4%. For cellular glass immobilized cells, the average ethanol concentration was decreased to 42.57 g/L with \( Y_{P/S} \) of 0.44 g/g and \( \eta \) of 86.1%, and for the porous ceramic group, the \( P_e \), \( Y_{P/S} \) and \( \eta \) were 42.84 g/L, 0.44 g/g and 86.6%, respectively.

For CF-PEI-cells, the ethanol concentration in each cycle was quite stable without obvious decrease. On the contrary, a visible reduction in ethanol concentration was observed in the case of the cellular glass and porous ceramic immobilized cells after the 7th cycle of fermentation, in which \( P_e \), \( Y_{P/S} \) and \( \eta \) were dropped to approximately 40 g/L, 0.42 g/g and 81%, respectively. A similar result has been reported by Rattanapan et al. [7] using sugarcane bagasse as support material for ethanol production. They attributed the reduction of ethanol production to marginal leakage of cells from the support during batch fermentation. Unlike cellular glass and porous ceramic, CF-PEI is positively charged in the fermentation. In this way, CF-PEI-cells exhibited much higher activity and stability of CF-PEI-cells was still appreciable.

Continuous fermentation

Figure 1 illustrates variations of ethanol and residual glucose concentration with the change of dilution. The residual glucose concentration decreased sharply in the first 2 days and stabilized thereafter with the dilution rate of 0.05 1/h, and the maximum glucose utilization of 97.8% (residual glucose concentration, 2.24 g/L) was obtained at this dilution rate. However, when the dilution rate was increased to 0.21 1/h, the residual glucose concentration was clearly increased by the 21st day of the continuous fermentation. As shown in Figure 1, the maximum ethanol concentration of 45.78 g/L was also found at the dilution rate of 0.05 1/h, and this was also clearly decreased when the dilution rate jumped to 0.21 1/h. This indicates that stable continuous operation with high ethanol concentration can be achieved by limiting the dilution rate to less than 0.16 1/h.

In essence, for continuous ethanol fermentation, high productivity is in reverse correlation with the glucose conversion efficiency [17,23]. At a higher dilution rate, more products are converted per hour, but the conversion efficiency is lower. The choice whether to operate at maximal

Table 3. Ethanol concentration (\( P_e \), g/L), ethanol yield (\( Y_{P/S} \), g/g) and efficiency of glucose conversion to ethanol (\( \eta \), %) during the ten-cycle repeated batch fermentation using free cells and immobilized cells.

| Batch | Free cells | Cellular glass immobilized cells | Porous ceramic immobilized cells | CF-PEI immobilized cells |
|-------|------------|---------------------------------|---------------------------------|-------------------------|
|       | \( P_e \) (g/L) | \( Y_{P/S} \) (g/g) | \( \eta \) (%) | \( P_e \) (g/L) | \( Y_{P/S} \) (g/g) | \( \eta \) (%) | \( P_e \) (g/L) | \( Y_{P/S} \) (g/g) | \( \eta \) (%) |
| 1st   | 38.08 ± 0.42 | 0.39 ± 0.02 | 77.2 | 42.58 ± 0.32 | 0.44 ± 0.01 | 86.1 | 42.34 ± 0.26 | 0.44 ± 0.01 | 85.9 | 44.62 ± 0.19 | 0.46 ± 0.01 | 89.9 |
| 2nd   | 38.79 ± 0.64 | 0.42 ± 0.02 | 78.2 | 43.02 ± 0.44 | 0.44 ± 0.02 | 86.6 | 43.66 ± 0.34 | 0.45 ± 0.01 | 87.9 | 45.09 ± 0.47 | 0.46 ± 0.02 | 90.1 |
| 3rd   | 38.62 ± 0.48 | 0.42 ± 0.02 | 78.6 | 43.29 ± 0.40 | 0.44 ± 0.02 | 87.2 | 43.03 ± 0.55 | 0.44 ± 0.02 | 86.7 | 45.28 ± 0.56 | 0.46 ± 0.02 | 90.5 |
| 4th   | 38.18 ± 0.52 | 0.39 ± 0.02 | 77.6 | 42.16 ± 0.28 | 0.44 ± 0.02 | 85.5 | 43.37 ± 0.46 | 0.45 ± 0.02 | 87.4 | 45.3 ± 0.42 | 0.47 ± 0.01 | 91.4 |
| 5th   | 36.29 ± 0.67 | 0.39 ± 0.03 | 76.1 | 42.65 ± 0.35 | 0.44 ± 0.02 | 86.1 | 42.68 ± 0.64 | 0.44 ± 0.02 | 86.3 | 44.78 ± 0.50 | 0.46 ± 0.02 | 90.6 |
| 6th   | 29.47 ± 1.20 | 0.34 ± 0.03 | 67.1 | 41.70 ± 0.55 | 0.43 ± 0.02 | 85.2 | 41.94 ± 0.59 | 0.43 ± 0.02 | 85.1 | 45.25 ± 0.26 | 0.46 ± 0.01 | 90.9 |
| 7th   | 28.1 ± 1.36 | 0.33 ± 0.04 | 66.4 | 40.19 ± 0.89 | 0.42 ± 0.02 | 81.3 | 40.23 ± 0.76 | 0.42 ± 0.02 | 81.1 | 45.32 ± 0.62 | 0.46 ± 0.02 | 90.5 |
| 8th   | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / |
| 9th   | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / |
| 10th  | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / | / / / / / |
| Average | 38.00b | 0.39a | 77.5a | 42.57b | 0.44b | 86.1b | 42.84b | 0.44b | 86.6b | 45.04 | 0.46 | 90.4 |

*Average of the first 5 cycles

*Average of the first 6 cycles.
volumetric ethanol productivity or substrate conversion efficiency has to be made judiciously [17]. In this study, we selected high glucose conversion efficiency as the purpose to evaluate the operation stability of continuous ethanol fermentation (30 days) at the dilution rate of 0.16 1/h and the result is given in Figure 2. ANOVA analysis indicated no significant differences in residual glucose concentration and ethanol concentration within 30 days’ operation ($P > 0.05$). In further detail, the fermentation parameters are summarized in Table 4. During the continuous fermentation, the ethanol concentration was maintained at 44.90 g/L with $Y_{PS}$ of 0.45 g/g and $\eta$ of 88.94%, and the volumetric ethanol productivity ($Q_e$) was about 7.18 g/(L h). It should be noted that the $Y_{PS}$ and $\eta$ were decreased compared to those in the repeated batch fermentation (0.46 g/g and 90.4%, respectively). Similar results have been reported by Yan et al. [17], where the authors concluded that this is caused by the continuous exposure of immobilized cells to high concentrations of ethanol during the continuous operation.

Göksungur et al. [24] conducted continuous ethanol fermentation using Ca-alginate as support with initial sugar concentration of 109 g/L at a dilution rate of 0.22 1/h, and maximum $P_e$ of 46.2 g/L, and $Q_e$ of 10.16 g/(L h) was obtained, but $\eta$ was only 82.9%, which was attributed to the limited nutrient diffusion into Ca-alginate beads. Shindo et al. [25] used an inorganic material, zeolite, as support for immobilization of $S.\ cerevisiae$ and studied ethanol production under continuous

Table 4. Results of continuous fermentation (30 days) using CF-PEI-cell at the dilution rate of 0.16 1/h.

| Parameters                        | CF-PEI-cell |
|-----------------------------------|-------------|
| Initial glucose concentration (g/L) | 102.11 ± 0.48 |
| Residual glucose concentration (g/L) | 3.19 ± 0.05  |
| Ethanol concentration ($P_e$, g/L) | 44.90 ± 0.23 |
| Ethanol yield ($Y_{PS}$, g/g)      | 0.45 ± 0.01  |
| Efficiency of glucose conversion to ethanol ($\eta$, \%) | 88.94 ± 0.13  |
| Volumetric ethanol productivity ($Q_e$, g/(L h)) | 7.18 ± 0.04 |

Figure 2. Continuous ethanol fermentation using CF-PEI-cell in a down-flow trickling bed reactor at a dilution rate of 0.16 1/h for 30 days.

Figure 3. SEM images of CF-PEI-cell before (a,b) and after (c,d) continuous fermentation.
fermentation with initial glucose concentration of 50 g/L at a dilution rate of 0.20 1/h. It was found that continuous fermentation was stable for over 21 d with \( P_e \) of 20 g/L, and \( \eta \) was no more than 82%. Rattanapan et al. [6] reported a stable operation of continuous fermentation (20 days) using silk cocoon as support with a feed sugar concentration of 220 g/L at the dilution rate of 0.04 1/h. The corresponding \( P_e \), \( Q_e \), and \( \eta \) were of 80.6 g/L, 3.2 g/L h) and 87%, respectively. Compared with those results, the ideal glucose conversion efficiency and ethanol productivity obtained in this work are probably due to better mass transfer and higher cell activity of CF-PEI-cells.

Figure 3 shows SEM images of the CF-PEI surface before and after the continuous fermentation operation. As shown in Figure 3(a,c), a dramatic increase in the number of yeast cells was observed after the continuous fermentation operation. High cell densities were clearly confirmed in the entire fibril network of the CF-PEI. Apparently, the speed of cell proliferation was much higher than that of cell leakage, and resulted in higher cell densities on CF-PEI. The high cell densities on CF-PEI should be responsible for its stable operation in continuous ethanol fermentation with high glucose consumption and ethanol productivity.

The findings outlined above indicate that the CF-PEI immobilized cells are stable and efficient in continuous ethanol fermentation. In consideration of the abundance of CF and the ease of the immobilization technique (fast, ethanol fermentation. In consideration of the abundance of CF and the ease of the immobilization technique (fast, diesel-fuel production and winemaking.

Conclusions

Our experimental results demonstrated the potential application of CF-PEI as a biocompatible support for \( S. \) cerevisiae immobilization. Many advantages, such as high cell loading, cell viability, reusability and cell regeneration were achieved and resulted in stable operation both in batch and continuous fermentation process. Since the \( S. \) cerevisiae cells immobilized on CF-PEI showed multiple benefits for ethanol production, further investigation of the physiology changes of immobilized \( S. \) cerevisiae cells is underway.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National High Technology Research and Development Program of China (863 Program) [grant number 2011AA06A108].

References

[1] Kourkoutas Y, Bekatorou A, Banat IM, et al. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol. 2004;21(4):377–397.
[2] Junter G, Jouenne T. Immobilized viable microbial cells: from the process to the proteome... or the cart before the horse. Biotechnol Adv. 2004;22(8):633–658.
[3] Mohd Azhar SH, Abdulla R, Jambo SA, et al. Yeasts in sustainable bioethanol production: a review. Biochem Biophys Rep. 2017;10:52–61.
[4] Borovikova D, Scherbaka R, Patmalnieks A, et al. Effects of yeast immobilization on bioethanol production. Biotechnol Appl Biochem. 2014;61(1):33–39.
[5] Mallouchos A, Reppa P, Aggelis G, et al. Grape skins as a natural support for yeast immobilization. Biotechnol Lett. 2002;24(16):1331–1335.
[6] Rattanapan A, Limtong S, Phisalaphong M. Ethanol production by repeated batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons. Appl Energy. 2011;88(12):4400–4404.
[7] Singh A, Sharma P, Saran AK, et al. Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. Renew Energy. 2013;50:488–493.
[8] Saeed A, Iqbal M. Loofa \( (Luffa cylindrica) \) sponge: review of development of the biomatrix as a tool for biotechnological applications. Biotechnol Progr. 2013;29(3):573–600.
[9] Baldikova E, Pospiskova K, Ladakis D, et al. Magnetically modified bacterial cellulose: a promising carrier for immobilization of affinity ligands, enzymes, and cells. Mater Sci Eng C Mater Biol Appl. 2017;71:214–221.
[10] Blum KM, Novak T, Watkins L, et al. Acellular and cellular high-density, collagen-fibril constructs with suprafibrillar organization. Biomater Sci. 2016;4(4):711–723.
[11] Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharmaceut. 2001;212(1):1–22.
[12] Sionkowska A, Skrzyński S, Śmiechowski, K, et al. The review of versatile application of collagen. Polym Adv Technol. 2017;28(1):4–9.
[13] Orgel JP, Miller A, Irving TC, et al. The in situ supermolecular structure of type I collagen. Structure. 2001;9(11):1061–1069.
[14] Lai ZB, Yan C. Mechanical behaviour of staggered array of mineralised collagen fibrils in protein matrix: effects of fibril dimensions and failure energy in protein matrix. J Mech Behav Biomed. 2017;65:236–247.
[15] Zhu D, Li X, Liao X, et al. Polyethyleneimine-grafted collagen fiber as a carrier for cell immobilization. J Ind Microbiol Biot. 2015;42(2):189–196.
[16] Klonzko P, Margaritis A, Bergougnou M. Effects of surface treatment and process parameters on immobilization of recombinant yeast cells by adsorption to fibrous matrices. Bioresour Technol. 2011;102(4):3662–3672.
[17] Yan S, Chen X, Wu J, et al. Ethanol production from concentrated food waste hydrolysates with yeast cells immobilized on corn stalk. Appl Microbiol Biotechnol. 2012;94(3):829–838.
[18] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426–428.
[19] Mustranta A, Pere J, Poutanen K. Comparison of different carriers for adsorption of *Saccharomyces cerevisiae* and *Zymomonas mobilis*. *Enzyme Microb Tech.* 1987;9(5):272–276.

[20] Van Haecht JL, Bolipombo M, Rouxhet PG. Immobilization of *Saccharomyces cerevisiae* by adhesion: treatment of the cells by Al ions. *Biotechnol Bioeng.* 1985;27(3):217–224.

[21] De Aza PN, Luklinska ZB, Anseau MR, et al. Reactivity of a wollastonite-tricalcium phosphate Bioeutectic® ceramic in human parotid saliva. *Biomaterials*. 2000;21(17):1735–1741.

[22] Yao W, Wu X, Zhu J, et al. Bacterial cellulose membrane – a new support carrier for yeast immobilization for ethanol fermentation. *Process Biochem.* 2011;46(10):2054–2058.

[23] Baptista CMSG, Côias JMA, Oliveira ACM, et al. Natural immobilisation of microorganisms for continuous ethanol production. *Enzyme Microb Tech.* 2006;40(1):127–131.

[24] Göksungur Y, Zorlu N. Production of ethanol from beet molasses by Ca-alginate immobilized yeast cells in a packed-bed bioreactor. *Turk J Biol.* 2001;25(3):265–275.

[25] Shindo S, Takata S, Taguchi H, et al. Development of novel carrier using natural zeolite and continuous ethanol fermentation with immobilized *Saccharomyces cerevisiae* in a bioreactor. *Biotechnol Lett.* 2001;23(24):2001–2004.