Research Article

Bioethanol Production by *Pichia stipitis* Immobilized on Water Hyacinth and Thin-shell Silk Cocoon

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Abstract
Cell immobilization technique was applied in this study in order to examine effect of immobilized *Pichia stipitis* TISTR5806 on bioethanol production. Water hyacinth (WH) and thin-shell silk cocoon (CC) were used as cell carriers. Characteristics of the cell carriers were examined to explain the mechanism of bioethanol production. Carrier sizes and weights were optimized to improve bioethanol production. Moreover, stabilities of immobilized cells and carriers were evaluated. Because of high porosity, high surface area and good swelling ability of WH, cell immobilized on 1 g WH with 1 cm length produced the highest ethanol concentration at 13.3 g/L. Five cycles of a repeated batch of immobilized cell (IC) system on WH showed stable performance in ethanol production (8.2–10.4 g/L) with large numbers of the immobilized cells. The interaction between the immobilized cells and the WH surface were discovered.

Keywords: Bioethanol production, Cell immobilization, Cell carrier, Natural materials, *Pichia stipitis*

1 Introduction

The second generation of feedstocks for bioethanol production is commonly lignocellulosic material containing cellulose, hemicellulose and lignin. The lignocellulosic material must be pretreated and hydrolyzed to obtain various types of monosaccharides which is a mixture of C5 sugars (xylose and arabinose) and C6 sugars (glucose and mannose). Subsequently, the monosaccharides are generally consumed by microorganism to produce bioethanol during fermentation [1], [2]. *Saccharomyces cerevisiae* is typically claimed as a high bioethanol producing yeast by consuming only C6 sugars and it has been particularly used in industrial bioethanol production [3]. On the other hand, *Pichia stipitis* is one of the high-performance yeasts for simultaneously converting both C5 and C6 sugars to high bioethanol production yield [4]. However, *P. stipitis* has low tolerance to high concentrations of ethanol [5] and inhibitors (hydroxymethylfurfural and furfural) [6]. These drawbacks restrict *P. stipitis* application of bioethanol production from lignocellulosic material in pilot and industrial scales.

In order to enhance cell density, cell stability and cell tolerance, a cell immobilization technique has been extensively recommended [7]. Several literatures reported that the cell immobilization succeeded in bioethanol production enhancement [8], [9]. Moreover,

the immobilized cells are easily separated from broth and recovered to reuse in batch and repeated batch processes leading to reduction of operating time and inoculum preparation cost [10]. However, mass transfer limitation could occur in the immobilized cell (IC) system [11], [12] resulting in low cell growth and low ethanol production.

A cell carrier plays a crucial role in the IC system. High mechanical strength, proper interior structure, good compatibility and non-toxicity are required characteristics of the cell carrier [13]. Natural materials with high porosity and interconnected porous structure, which promote mass transfer of nutrients and cell attachment, are available such as water hyacinth (WH), banana stalk. The structure of the carrier is not only an important factor, but also other properties including surface charge, material composition, wettability, etc. also play critical effects on cell immobilization behaviour [14]. Thin-shell silk cocoon (CC), a protein-based biopolymer, has been applied in various applications such as cosmetics, electrocatalyst [15] enzyme immobilization [16] and cell immobilization [17], [18]. It was reported that *S. cerevisiae* immobilized on CC produced 11.5% higher ethanol production and showed higher cell stability on the fifth repeated batch when compared with suspended cells (SC) [17].

Limited literatures have been studied about effect of *P. stipitis* immobilized by natural materials on ethanol production. The aim of this research is to examine effect of immobilized *P. stipitis* on bioethanol production using WH and CC as cell carriers. Carrier size and weight were optimized in order to improve bioethanol production. Moreover, stabilities of the immobilized cells and the carriers were evaluated under five cycles of a repeated batch operation

2 Materials and Methods

2.1 *Material preparation*

WH was collected from Chao Phraya River, Nonthaburi, Thailand. Only WH stalk with diameter of 1.6 ± 0.2 cm was used in this study. Firstly, WH was cleaned with tap water and was cut into 1 and 2 cm in length. Then, WH was dehydrated in an ethanol series treatment in order to keep its original structure. Dried WH was stored in a desiccator until used. CC was supplied by the Queen Sirikit Department of Sericulture, Saraburi, Thailand. CC was also kept in a desiccator until used without additional treatment.

2.2 *Microorganism and cultivation*

P. stipitis TISTR5806 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). *P. stipitis* stock stored in a refrigerator at 4°C was transferred to Yeast Malt (YM) Agar slant and cultivated under ambient condition (28–32 °C) for 24 h. After that, the cells were transferred to a 50 mL pre-culture medium containing 10 g/L xylose, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.1 g/L NaCl and 0.1 g/L $CaCl_2$, and were incubated at 30 °C, 150 rpm for 24 h.

2.3 *Cell immobilization and ethanol production*

P. stipitis cultivated with the pre-culture medium was concentrated to 7 g/L (dry weight) cell density. A 5 mL of the concentrated cell was transferred to the 50 mL pre-culture medium. Then, the cell carriers were added in order to immobilize cell by the adsorption technique. The cultivation was carried out under the same condition as the pre-cultivation step for 24 h.

2.4 *Batch and repeated batch operations*

For batch ethanol production by the IC system, after 24 h of the immobilization step, the carriers adsorbing cells were transferred into a 100 mL fermenting media consisting of 50 g/L xylose and the same minerals formula as the pre-culture medium, and were incubated at 30 °C, 100 rpm for 144 h. For the SC system, the inoculum of 7 g/L was added into the fermenting medium instead of the carriers. Samples were taken every 24 h for xylose and ethanol analysis. For a repeated batch, after 72 h of each batch, the fermenting medium was withdrawn, and a fresh medium was substituted to start the next batch.

2.5 *Analytical technique*

2.5.1 Characterization of carrier

Swelling ability of the carrier was evaluated by immersing it in a liquid sample until equilibrium state was reached [8]. A weighted carrier (W_i) was immersed

in DI water and fermenting media. After 24 h, the carrier was reweighted (W_s). Swelling ability of the carrier was determined using Equation (1).

$$\text{Swelling ability (times)} = (W_s - W_i)/W_i \quad (1)$$

Liquid displacement method was carried out to determine carrier porosity. A weighted carrier (W_i) was immersed in hexane under vacuum condition. After the carrier was saturated by hexane, it was weighted again (W_h). Volume of the carrier (V) was evaluated using geometry calculation. Porosity of the carrier was calculated by Equation (2)

$$\text{Porosity (\%)} = 100\ ((W_h - W_i)/\rho_h)/V \quad (2)$$

where ρ_h is density of hexane.

Carrier morphology was examined and photographed using CUsmartlens and Micro Stage (Thailand). For observation of interaction between cells and carrier, a fermented carrier was sputtered with gold. The coated carrier was photographed under scanning electron microscope (SEM) (JOEL JSM-5410LV, Japan).

2.5.2 Analysis of xylose and ethanol concentrations, and cell dry weight concentration

3,5-dinitrosalicylic acid (DNS) assay [19] was applied to determine xylose concentration in a fermentation broth sample. After DNS reacted with reducing xylose, optical density of the sample was analyzed using UV-Visible Spectroscopy (T80+, PG Instruments, United Kingdom) at 540 nm. The concentration of reducing xyloses were calculated based on standard curve of xylose.

The fermentation broth was evaluated ethanol concentration by using a gas chromatography (6890, Agilent, United State) equipped with HP-Innowax column and a flame ionization detector. Ethanol productivity was determined by Equation (3).

$$\text{Ethanol productivity (g/L• h)} = \text{Ethanol conc./time} \quad (3)$$

To determine cell dry weight concentration, 5 mL of fermentation broth was centrifuges at 5000 rpm for 10 min and then the supernatant was removed. The cell pellet was mixed with 0.1 M HCl and rinsed with distilled water twice. After that, the cell was dried at 100 °C for 24 h. Finally, the dried cell was weighted and calculated in term of dry weight cell concentration.

2.5.3 Statistical analysis

The experimental data was reported as an average with the standard deviation. The pair comparison of the data was carried out with two-tailed hypothesis by t-test for two-sample equal variance. The pair was considered significant difference by p-value < 0.01 and < 0.05.

3 Results and Discussion

3.1 *Physical characterization of cell carrier*

Natural materials selected to use as cell carriers in this study were WH and CC. During experimental handling, both WH and CC exhibited stable structure and good mechanical strength, which might protect immobilized cells from shear stress caused by agitation and CO_2 evolution [20], [21]. Also, it had been reported that the WH morphology investigated by SEM showed well-shaped fibrils with impact structure [22]. Macrostructures of WH and CC are presented in Figure 1. For Figure 1(c), cross-sectional morphology of WH showed macroporous structure (0.47±0.16 mm) with high porosity of 68.5±5.1% while that of CC illustrated thin layer of fibrous silk covering porous thin shell with 35.4±1.9% porosity, as seen in Figure 1(d). The characteristics of CC such as high biocompatibility and suitable porous structure, promoted yield and activity of the immobilized *S. cerevisiae* [18].

Swelling abilities of WH and CC in water and fermenting medium are shown in Figure 2. Many factors such as structure, composition, crystallinity, hydrophilicity/hydrophobicity affect swelling ability of biomaterials [23]. According to carrier photographs (Figure 1) and their porosities, WH showed macroporous morphology throughout the structure with high void volume and porosity; therefore, both its swelling abilities in water and medium were much higher than those of CC. In addition, swelling ability could be attributed to the crystallinity of a material. With a higher crystallinity, the material structure is much more compact and less flexibility. WH is a floating aquatic plant containing 80–85% by weight of water and low cellulose content

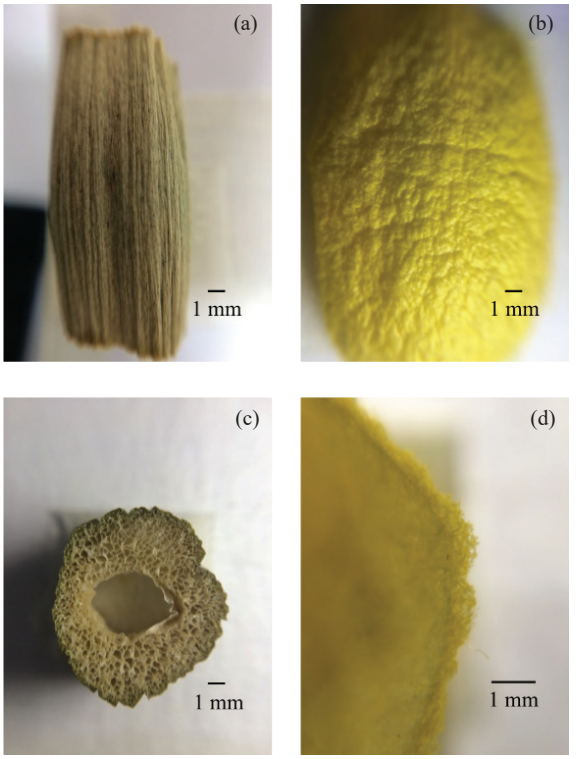

Figure 1: Photographs of WH (a) and CC (b) surfaces, and cross-sectional area of WH (c) and CC (d).

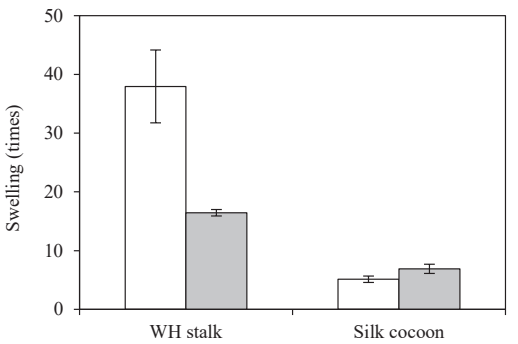

Figure 2: Swelling ability of carriers in water (white) and fermenting medium (grey).

with ~16% crystallinity index [24]. WH greatly adsorbed and swollen in water. However, xylose and minerals in medium mainly affected WH swelling leading to huge reduction of swelling ability. Due to its high crystallinity of 58–62% [25] and its morphology, less than 10 times of the swelling abilities of CC were obtained.

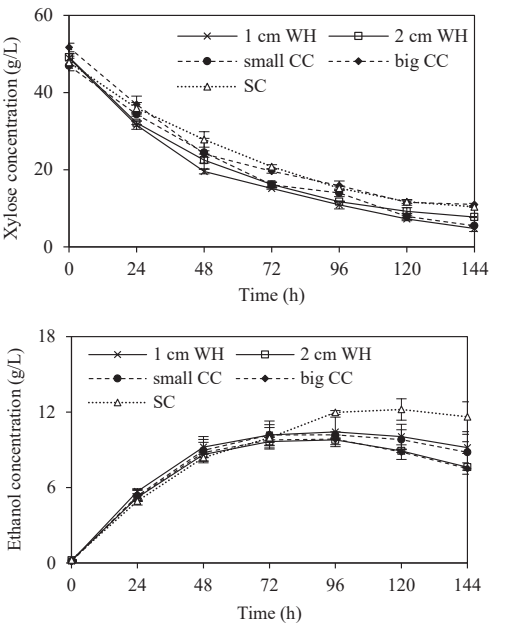

Figure 3: Xylose and ethanol concentrations by the IC systems with varying sizes of WH length: 1 and 2 cm and CC: small and big size, and those by the SC system.

3.2 *Effect of carrier size on bioethanol production*

Size of carrier is considered as an important key of cell immobilization that affects the adsorption of nutrients and the desorption of products and by-products [9]. Although a larger size of carrier could provide a higher surface area for cell attachment, mass transfers of nutrients, products and by-products could be limited. Two different lengths of WH: 1 and 2 cm, and two different sizes of CC: small (1.26 + 0.07 cm width and 2.30 + 0.13 cm length) and big (1.42 + 0.04 cm width and 2.71 + 0.10 cm length) were examined. Xylose consumptions and ethanol productions of the IC system and the SC system are presented in Figure 3. It was found that the highest ethanol production of 12.2 + 0.9 g/L was obtained by the SC system. Due to no restriction of mass transfer, the yeast cells could utilize nutrients directly for growth and ethanol production. Although the immobilized cells on 1 cm WH and small CC consumed the highest amount of xylose (residual xylose concentrations at 144 h of 4.8 + 0.8 and 5.5 + 0.2 g/L, respectively), ethanol concentrations were lower than that of the SC system. It might because

higher total cell concentrations (immobilized cells and free cells) in the IC systems metabolized xylose for cell mass production more than ethanol production. Considering carrier size, cells immobilized on 1 cm WH and small CC produced higher ethanol concentrations than those on 2 cm WH and big CC. As mentioned before, the limitation of diffusion of nutrients might occur in the bigger size of the cell carriers.

3.3 *Effect of carrier weight on bioethanol production*

To compare each material, the WH with 1 cm length and the small size of CC presented higher xylose consumptions and ethanol productions; therefore, they were selected to use as cell carriers in this study. Carrier weights were varied as 0.5, 1 and 1.5 g. Xylose consumption of the IC systems on WH and CC with varying weight are shown in Figure 4(a) and (b), respectively. The increase of the carrier weight provided larger area for cell attachment and growth. However, the crowded numbers of the carriers might cause the restriction of mass transfer in the system resulting in the low xylose consumption and low ethanol production. The cell immobilized on 1.5WH and 1.5CC systems produced ethanol concentrations of 12.0 and 9.6 g/L, respectively. In contrast, for the lowest loading of the cell carriers, the IC systems on 0.5WH and 0.5CC gave 12.5 and 9.8 g/L ethanol productions, respectively.

According to the result of residual xylose concentrations in Figure 4(a) and (b), the IC systems on 1 g of WH (1WH) and 1g of CC (1CC) showed the lowest values in their systems. Therefore, xylose and ethanol concentration profiles of the 1WH and 1CC systems were compared with the SC system, as shown in Figure 4(c) and (d), respectively. As mentioned before, a higher total cell concentration system consumed large quantities of substrates. The residual xylose concentrations at 144 h ordered from low to high values as 1WH, 1CC and SC systems. Although the SC system consumed the lowest amount of xylose, the ethanol production was higher than the IC systems on 1CC. The highest ethanol production of 13.3 + 1.0 g/L was obtained from the IC system on 1WH. To compare with the SC system, it might claim that since 1WH provided the proper porous structure with suitable size and weight, the cells immobilized on 1WH consumed substrates and released products

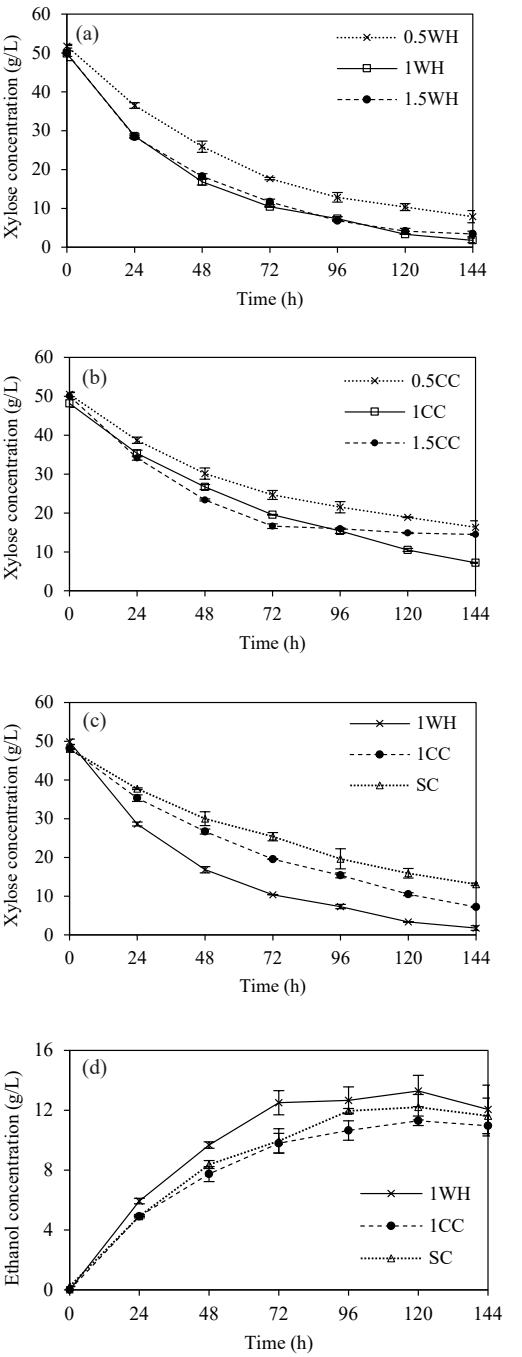

Figure 4: Xylose concentration of the IC system with varying carrier weights (0.5, 1, and 1.5 g) of WH (a) and CC (b), and xylose (c) and ethanol (d) concentrations of the best conditions of the IC systems compared with those of the SC system.

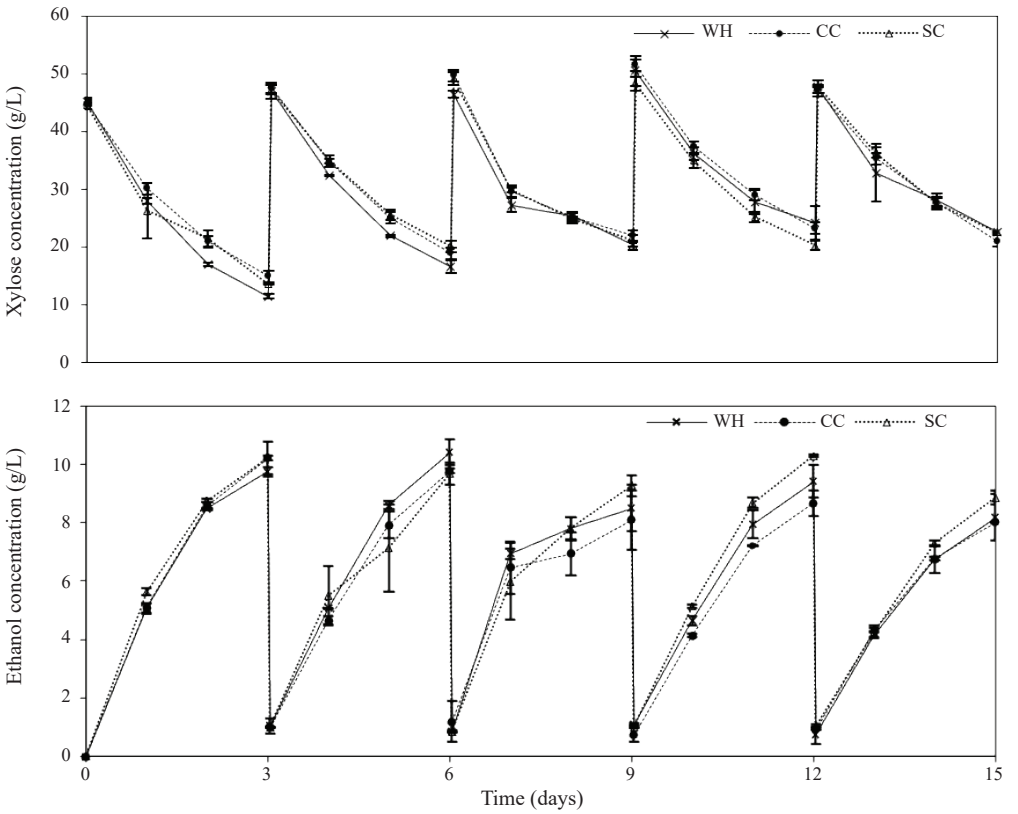

Figure 5: Residual xylose and ethanol concentrations by the IC systems on WH and CC, and those by the SC system under the five cycles of a repeated batch operation.

without mass transfer limitation.

3.4 *Stabilities of immobilized cells and carriers*

Typically, the immobilized cells are protected by the carrier from hash conditions, such as excessive shear force, high concentrations of substrates, products and inhibitors to prolong cell activity and stability [26]. The stabilities of both immobilized cells and cell carriers were evaluated using the five cycles of a repeated batch. From Figure 5, xylose consumption profiles obtained from the IC and the SC systems gradually decreased from the first to the fifth batch, as confirmed by residual xylose concentrations in Table 1. On the other hand, ethanol production from the first and the second batches of the IC system on WH slightly increased. The second batch of the IC system on WH exhibited the highest ethanol productivity and yield. Ranges of ethanol productions of the SC system, the IC systems on WH and that on CC were 8.8–10.3 g/L, 8.2–10.4 g/L and 8.0–10.2 g/L, respectively. Considering the overall repeated batch operation, the results revealed that no significant differences of ethanol production, ethanol productivity and ethanol yields obtained from the IC systems and those obtained from the SC system. Similar result had been reported by Bari *et al.* [27] for ethanol production using *P. stipitis* immobilized in silica-hydrogel film. On the other hand, the positive impact of *P. stipitis* immobilized by calcium alginate bead on ethanol production had been published [21], [28]. Low mechanical strength and restriction of mass transfer are still main drawbacks of the calcium alginate beads [17]. The immobilized cells could be reused five times without any damage of the WH and CC carriers. For long-term operation, the ethanol production efficiency of the IC systems was stable because the immobilized cells stability and activity were kept by the cell carriers. Nevertheless, the IC

Table 1: Parameters of the five cycles of a repeated batch

System	Batch Cycle	Residual Xylose Concentration (g/L)	Ethanol Concentration (g/L)	Ethanol Productivity (g/L• h)	Ethanol Yield (g ethanol/g xylose)
WH	1	11.5 + 0.4[a,b]	9.7 + 0.2[a]	0.135 + 0.002[a]	0.314 + 0.007[a]
	2	16.6 + 1.0[a]	10.4 + 0.4	0.145 + 0.006	0.383 + 0.013
	3	20.3 + 0.7	8.5 + 0.8	0.109 + 0.013	0.280 + 0.012
	4	24.3 + 2.9	9.4 + 0.6	0.131 + 0.008	0.337 + 0.005
	5	22.7 + 0.1[b]	8.2 + 0.8	0.114 + 0.011	0.323 + 0.020
CC	1	14.9 + 0.8[c]	10.2 + 0.6	0.142 + 0.008	0.329 + 0.020
	2	18.9 + 1.2	9.8 + 0.1	0.136 + 0.001	0.359 + 0.006
	3	21.9 + 0.3	8.1 + 1.0	0.112 + 0.014	0.289 + 0.014
	4	23.3 + 0.4[a]	8.7 + 0.4[a]	0.121 + 0.006[a]	0.311 + 0.039
	5	21.4 + 0.5[a,c]	8.0 + 0.1[a]	0.111 + 0.001[a]	0.318 + 0.012[a]
SC	1	13.6 + 0.1[c]	10.2 + 0.1[c]	0.142 + 0.001[c]	0.329 + 0.001[c]
	2	20.2 + 1.0[c]	9.7 + 0.4	0.134 + 0.005	0.356 + 0.017
	3	21.2 + 1.0	9.3 + 0.4	0.129 + 0.005	0.333 + 0.038
	4	20.3 + 0.9[b]	10.3 + 0.1[b]	0.143 + 0.001[b]	0.369 + 0.026
	5	22.5 + 0.2[b]	8.8 + 0.2[b]	0.123 + 0.003[b]	0.350 + 0.003[b]

[a] represents significant difference ($p < 0.05$) between that value compared to that of the SC system in the same batch.
[b] represents significant difference ($p < 0.05$) between that value compared to that of the IC system on CC in the same batch.
[c] represents significant difference ($p < 0.05$) between that value compared to that of the IC system on WH in the same batch.
Bracket symbol represents significant difference ($p < 0.01$) between those two values.

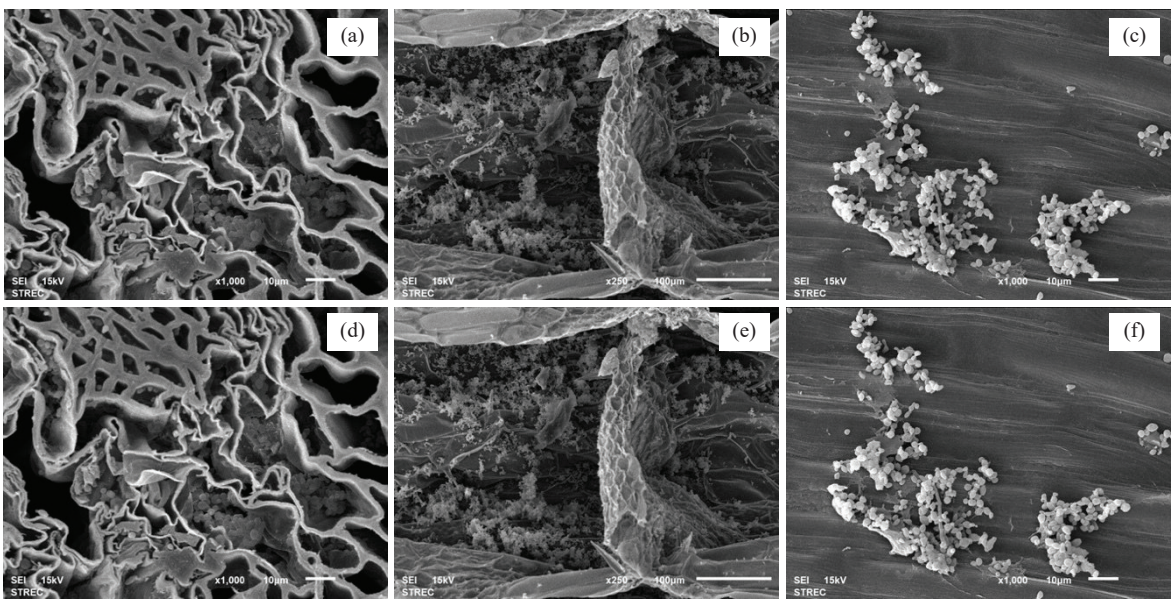

Figure 6: SEM images of WH: horizontal cross-section (a), vertical cross-section (b) and close-up vertical cross-section (c), and CC: inner surface (d), outer surface (e) and close-up outer surface (f) after used as cell carriers in the fifth repeated batch.

systems were much easier to handle than the SC system because the immobilized cells could directly transfer to the fresh media for the next batch operation.

After the fifth batch, the structure of WH and CC remained their original morphologies. The immobilized cells and the carriers were examined by SEM (Figure 6). Large numbers of cells were observed in the interior area of WH without any damage structure, as presented in Figure 6(a) and (b). Lower numbers of cells attached on inner and outer surface of CC, as seen in Figure 6(d) and (e). Comparing between Figure 6(c) and (f), the interaction between the immobilized cells and the WH surface was observed, while self-agglomeration of the immobilized cells was found on the CC surface. Although CC was effectively immobilized *S. cerevisiae* due to its protein composition [17], small numbers of

P. stipitis cells could attach on. This finding could be explained that different strains of yeast cells preferred different characteristics of the cell carriers. Due to the smooth fibers of CC, cells might have difficultly to attach on.

4 Conclusions

The IC systems on WH and CC were applied to produce ethanol by *P. stipitis*. High porosity, adequate void volume, good swelling ability of WH promoted mass transfer of nutrients and cell growth resulting in high ethanol production and high xylose consumption. Cell immobilized on 1 g WH with 1 cm length produced the highest ethanol concentration. For five-cycles repeated batch operation, the stabilities of immobilized cells and system of WH were adequate to produce ethanol in stable concentration range without structural damage. Moreover, large numbers of immobilized cells and the interaction between cells and WH surface were found suggesting the potential of using the IC systems for promoting bioethanol production.

Acknowledgments

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