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# Xylitol fermentation by *Candida subtropicalis* WF79 immobilized in polyacrylic hydrogel films

**Wen-Chang Liaw*, Wen-Shion Chang**, Kuan-Pin Chen*, Yee-Fan Chen***, and Chee-Shan Chen***,†**

*Department of Chemical Engineering, National Yunlin University of Science and Technology,
123, Section 3, University Road, Touliu, Yunlin 640, Taiwan, R.O.C.
**Jin Wen Institute of Technology, 99, An-Chung Road, Shin-Tien, Taipei County, 231, Taiwan, R.O.C.
***Department of Applied Chemistry, Chaoyang University of Technology,
168, Gifeng E. Rd., Wufeng, Taichung County, 413, Taiwan, R.O.C.
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**Abstract**−Polyacrylate hydrogel films were used as immobilization matrices for cell entrapment of the yeast strain, *Candida subtropicalis* WF79, isolated from sugarcane bagasse. The hydrogel films with immobilized viable yeast cells were employed for the bioconversion from xylose to xylitol. The hydrogel was prepared from the monomers of 2-hydroxyethyl methacrylate (HEMA), methacrylic acid (MAA), and N,N-dimethyl acrylamide (DMA) with polyethylene glycol diacrylate (PEG-DA, weight-average molecular weight Mw=400 and 1,000 g/mol) as the crosslinking agent. The mechanical properties and fermentation yields of the immobilized polyacrylate hydrogel film with different monomeric formulation were investigated. Fermentation was carried out in a medium of 100 mL aqueous solution containing various amount of xylose. The conversion rate of xylose to xylitol conversion reached a maximum value of 80% after 120 h. The bioconversion activity gradually declined, after 720 h, to 65% conversion at 1,080 h.

Key words: Xylitol, Polyacrylate, Hydrogel, Immobilization, *Candida*

## INTRODUCTION

Xylitol is a five-carbon sugar alcohol which has a sweetening power comparable to that of sucrose, and is one of the valuable rare sugars with properties beneficial to health Although xylitol can be metabolized by humans and many other animals, its metabolism is not insulin controlled and is therefore used as an alternative sweetener for diabetics. Furthermore, since xylitol cannot be metabolized by bacteria living around teeth that are usually responsible for dental caries, it is suitable in chewing gums, toothpaste and as an additive in a variety of foods [1,2].

Although, xylitol is found naturally in fruits [3] like strawberries and pears, the quantity is too small to be economically extracted. Currently, the most important process adopted in the production of xylitol is the chemical reduction of xylose. However, due to the necessary high pressure and temperature, as well as the requirement for multiple purification steps, such processes are not economically favorable for feasible subsequent production of pure xylitol. A competitive alternative for the production of xylitol would be bioconversion by yeasts, or purified enzymes [4-11].

Immobilized cell systems for bioconcension have been traditionally considered as an alternative for increasing the process' overall productivity while reducing production costs [12]. The continuous process is advantageous when immobilized microorganisms are utilized to allow their catalytic activity to be continuously active, and consequently, increasing the volumetric productivity. Various immobilization strategies for the yeast bioconversion, such as solid supports of cells on non-woven fabrics, ceramics, polyamide and microporous glass bead have been reported [13-17]. Another type of cell immobilization is, in spherical-beads geometry, gel entrapment of cells in calcium alginate or carrageenan [18-21], has also been reported.

A thin film structure is another alternative that provides large contact area and shorter diffusion path as required for a successful cell entrapment immobilization system. From this view point, the search for suitable matrices with high mechanical strength, while being biocompatible, to maintain the integrity of such films would be desirable for a durable fermenting system. In this regard, the hydrogel films that are widely used commercially for contact lenses may be a good candidate for such application [23,24].

In this study, we utilized a crosslinked polyacrylic hydrogel film, similar to the material for making soft contact lenses, to entrap the yeast *Candida subtropicalis* WF79 cells to carry out the xylose to xylitol bioconversion. The formulation of acrylic monomer for making the hydrogel films and mechanical properties of the immobilized films were studied. Processing variables such as initial xylose concentration, cell loading were varied to understand their effects on xylitol production.

## MATERIALS AND METHODS

### 1. Microorganism and Inoculums

The yeast strain used in this research was isolated, by using selective medium, from waste sugarcane bagasse collected in central Taiwan. Isolated yeast strains that are capable of converting xylose to xylitol were then screened, on the basis of high yield and fast conversion, for the strain to be used in xylitol production. The yeast

†To whom correspondence should be addressed.
E-mail: csc@cyut.edu.tw
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**Table 1. Preparation of acrylic hydrogel materials of immobilized films (in grams)**

| Batch | HEMA[a] | PEG-DA[b] Mw: 1000 | PEG-DA[b] Mw: 400 | MAA[c] | DMA[d] | Initiator[e] | Cell wt. |
|---|---|---|---|---|---|---|---|
| A | 90 | 10 | - | 0 | 0 | 1 | 5.75 |
| B | 80 | 10 | - | 10 | 0 | 1 | 5.75 |
| C | 70 | 10 | - | 20 | 0 | 1 | 5.75 |
| D | 50 | 10 | - | 40 | 0 | 1 | 5.75 |
| E | 80 | 10 | - | 0 | 10 | 1 | 5.75 |
| F | 90 | - | 10 | 0 | 0 | 1 | 5.75 |
| G | 80 | - | 10 | 10 | 0 | 1 | 5.75 |
| H | 70 | - | 10 | 20 | 0 | 1 | 5.75 |
| I | 50 | - | 10 | 40 | 0 | 1 | 5.75 |
| J | 80 | - | 10 | 0 | 10 | 1 | 5.75 |

[a]HEMA: 2-hydroxyethyl methacrylate
[b]PEG-DA: polythylene glycol diacrylate (Mw=400, 1,000 g/mol)
[c]MAA: methacrylic acid
[d]DMA: N,N-dimethyl acrylamide
[e]Initiator: Benzoin isopropyl ether

strain obtained was identified by the API 20C AUX yeast identification system (bioMmerieus, France) [25,26]. Cells of the yeast *Candida subtropicalis* WF79 were maintained at 4 ºC on malt extract agar slants. The inoculum was prepared by cultivation of the yeast in 125 mL Erlenmeyer flasks with liquid medium consisting of yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and xylose 20 g/L. Cells were cultivated at 100 rpm and 30 ºC for 24 h and were recovered by centrifugation (2,000×g, 20 min, 10 ºC).

**2. Preparation of Acrylic Hydrogel as Carriers for Cell Immobilization**

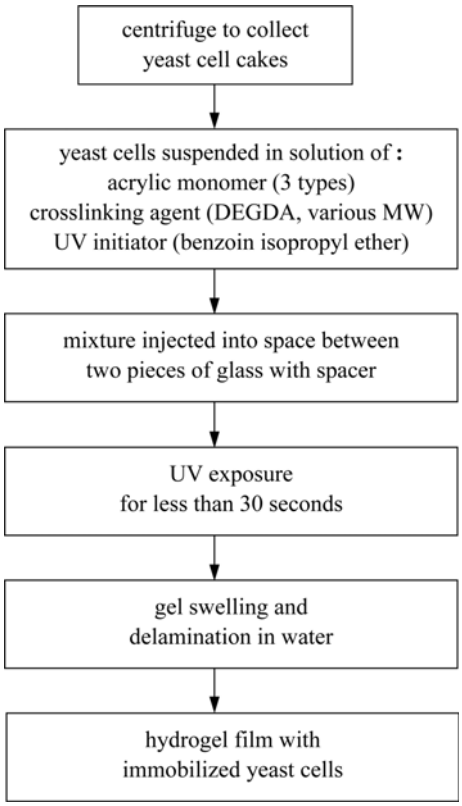
The acrylic hydrogel was chosen to be the immobilization carrier. To make these carriers, the following polymer components were first mixed with viable yeast cells: 2-hydroxyethyl methacrylate (HEMA), methacrylic acid (MAA) and N-N′-dimethy acrylamide (DMA). Then, the different weight-average molecular weights of polyethylene glycol diacrylate (PEG-DA, MW=400, 1,000 g/mol) and 1 wt% of benzoin isopropyl ether were added (Table 1) to the above solution. The acrylic hydrogel film was prepared by injecting the above mixture into the space between two pieces of glasses (100 mm×100 mm) separated by 0.2 mm spacer, followed by free-radical reaction initiated by UV light. Time length of exposure to UV light was kept shorter than 30 sec to minimize UV damage to the microorganism. After the reaction was completed, the glass sheets were put into the distilled water for 30 min to allow for gel-swelling, and then the acrylic hydrogel film was obtained after delamination. The synthesis procedure is shown in Scheme 1.

**3. Fermentation Conditions**

The composition of the fermentation medium was yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and xylose 100 g/L. Experiments were conducted in 250 mL Erlenmeyer flasks with working volume of 100 mL. The medium containing flasks was plugged with foam caps and autoclaved for 15 min at 121 ºC. After cooling, different numbers of pieces of cell immobilized films were put into each flask and incubated in an enclosed rotary type shaker at 30 ºC. Speed was adjusted to 50, 75, and 100 rpm.

**4. Analytical Methods**

Cell dry weight was correlated to absorbance at 570 nm by comparing against a calibration curve. D-xylose, xylitol, glucose and ethanol concentrations were quantified by high performance liquid chromatography (HPLC) equipped with an RI detector. The separation column was Rezex ROA-organic acid type (Phenomenex, Torrance, California, USA). Mobile phase used was 0.005 N $H_2SO_4$ at a flow rate of 0.6 mL/min. The HPLC column was operated at room temperature.




**Scheme 1. Procedure for preparing hydrogel films with immobilized yeast cells.**

The tensile strength of the cell immobilized films was determined

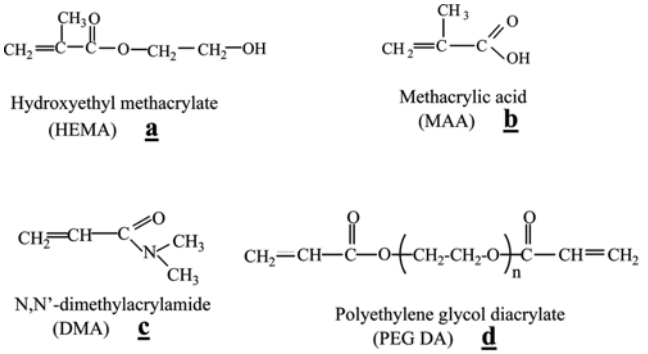



**Fig. 1. Chemical structures of hydrophilic acrylic monomers and cross linking agents.**

by the method specified by the American Society for Testing and Materials (ASTM) D638 employing a dumbbell shaped #2 sample. The rate of stretch was 20 m/min. The tensile strength was expressed in kg/cm$^2$. Water content of the film was calculated according to the following equation:

$$\text{water content}=[(\text{wet weight}-\text{dry weight})/\text{wet weight}]\times 100\%$$

## RESULTS AND DISCUSSIONS

The cell immobilized films produced, according to the aforementioned procedures, have high water content because of the adoption of highly hydrophilic HEMA (Fig. 1a) as the main acrylic monomer. Such an attribute is desirable in this kind of biological application, as is the long history of the uses of carrageenan and alginate.

HEMA is a versatile and inexpensive hydrophilic acrylic monomer that has wide industrial applications. Song and Lee [27] used HEMA to enhance the hydrophilicity of a polysulfone membrane for oily wastewater treatment. After polymerization of hydrophilic acrylic monomers, such as HEMA, MAA (Fig. 1b) or DMA (Fig. 1c), the product is still water soluble. However, prior to polymerization, if hydrophilic cross-linking agent PEGDA (Fig. 1d), is added, the polymer will form an insoluble networked matrix. During the gel-swelling and delamination period, in distilled water, after the polymerization and network-forming process, water molecules tend to diffuse into the network. Therefore, this networked polymer has the special characteristic of swelling in the water to form a hydrogel (Scheme 1).

The hydrogel used in this research has the advantage of being biocompatible and is suitable for immobilizing microorganisms for fermentation. Because of the strong mechanical properties of polyacrylate resin, it is feasible to tailor the material into thin films while retaining their structure for an extended period of time. A good example of application of this category of hydrogel would be the well known traditional soft contact lenses. In this study, we modified the formulation (crosslinking agent of different molecular weight, types of monomer) for making soft contact lenses for the purpose of cell immobilization. The process of cell immobilization was conducted by suspending the yeast cells in the mixture of hydrophilic acrylic monomers, crosslinking agents and photoinitiator, and stirring for even cell distribution. The formulations of the components for synthesizing the polymers of hydrogel and yeast cells for immobilization are listed in Table 1.

After polymerization of the these monomers initiated by UV light, the polymer film with entrapped yeast cells was delaminated and swelled in distilled water to obtain the yeast cell immobilized hydrogel film. When the yeast cells are entrapped in the matrices of polyacrylate hydrogel film, they can still have enough nutrient supply for metabolism, and because of the thin film geometry, maximum contact area provides optimal opportunities for exchanging chemical compounds between the solid and liquid phases. Meanwhile, good mechanical properties of the polyacrylate hydrogels allow the films to maintain their shape while offering repeated usage and easy product separation.

Besides xylitol fermentation, the yeast Candida has been used for other purposes (e.g., lipase) [28,29]. For xylitol fermentation, the percentage bioconversion is usually lower for an immobilized system than for a free cell system. Santos et al. [16,17] studied the xylitol production from sugarcane bagasse hydrolysate using *Candida guilliermondii* immobilized on porous glass and zeolite, and found that the xylose-to-xylitol conversion decreased from 72% (free cells) to 53% (porous glass) and 52% (zeolite) upon immobilization. Carvalho et al. studied the Ca-alginate immobilization system [19-22,32-34], operated in different modes (batch, repeated batch, stirred tank reactor). They optimized the stirred tank reactor operation (using response surface method, adjusted air flow rate, agitation speed, initial cell concentration, initial pH) and improved the xylitol conversion from 47% to 81%. Yahashi et al. [30,31] used non-woven fabric immobilization, and attained a high yield of 69%. In this study, the maximum xylitol conversion was 81%, which is 90% of the performance of the same system using free cell fermentation (91%). This is merely to point out the range of xylitol yield in the literature for relative comparison.

### 1. Mechanical Properties of Immobilized Cells Film

The tensile strength and water content of the polyacrylic hydrogel with or without immobilized cells are shown in Table 2. Water content for the hydrogels from batch A with and without cell immobilization was 39.3% and 39.1%, respectively. Similar trends that the hydrogel films of water content decreased slightly upon cell immobilization were observed for other batches (Table 2). Should further increase of water content be desirable, the addition of mono-

**Table 2. Tensile strength and water content of the acrylic hydrogel films with and without immobilized cells**

| Batch | Tensile strength (kg/cm$^2$) | | Water content (%) | |
|---|---|---|---|---|
| | no cells | with cells[a] | no cells | with cells[a] |
| A | 2.26 | 2.53 | 39.3 | 39.1 |
| B | 1.76 | 1.89 | 42.4 | 41.3 |
| C | 3.52 | 3.59 | 27.1 | 24.1 |
| D | 4.18 | 4.47 | 21.6 | 22.9 |
| E | 1.56 | 1.87 | 41.3 | 41.2 |
| F | 3.15 | 3.26 | 30.3 | 29.8 |
| G | 2.36 | 2.79 | 38.2 | 37.5 |
| H | 2.13 | 2.24 | 30.2 | 29.5 |
| I | 4.56 | 4.89 | 24.6 | 23.7 |
| J | 2.36 | 2.95 | 38.5 | 37.1 |

[a]Cell content of these films was 5.75 mg dry cell per g total dry weight.

mers such as methacrylic acid (MAA) or N,N-dimethylamide (DMA) may serve the purpose.

Formulation of the polymer is a factor that can be manipulated to affect the water-holding capacity of the hydrogel film. The intent of the use of MAA was to increase the number of water affinitive carboxylic acids in the matrix and lead to the increase of water-holding capacity. Table 2 showed that, when 10 wt% of MAA was used, for both sizes of molecular weight of cross-linking agent (MW of PEG-DA=400 and 1,000 g/mol), water content of the films with no cells increased to 42.4% and 38.2% (batches B and G), as compared to 39.3% and 30.3% (batches A and F). For the immobilized cell films (batches B and G) the water content was 41.3% and 37.5%, respectively, while that for batch A and F (no MAA were added) was 39.1% and 29.8%. The contribution of MAA in increasing water holding capacity can thus be justified.

It is desirable to have a mechanically strong immobilization matrix without compromising mass transfer rate. As shown, adding 10 wt% of MAA served the purpose of enhancing the mass transfer rate, but at the price of decreased mechanical strength. The tensile strengths of the films with Mw=1,000 g/mol of PEG-DA, upon addition of 10% MAA, dropped from 2.26 to 1.76 Kg/cm$^2$ (Table 2, batches A & B, no cells), and with Mw=400 g/mol of PEG-DA, dropped form 3.15 to 2.36 Kg/cm$^2$ (Table 2, batches F & G, no cells). Similar results were obtained for films with cell immobilization (Table 2). The tensile strength was comparable to that of contact lenses, 1.23 to 5.87 Kg/cm$^2$ [23,24].

Following the above rationale, we went further to increase the MAA concentration to 20 wt% and 40 wt% (Table 1, batches C, D, H and I). Table 2 shows that, from 10 (batches B, G) to 20 wt% MAA (batches C, H), the water content decreased significantly. Further decrease in water holding capacities was observed when MAA content was increased to 40 wt% (Table 2, batches D, I). Meanwhile, the tensile strength measurements showed that while losing water-holding capacity, the films gained tensile strength upon further increasing the MAA concentration (Table 2, batches C, D, H, I, compared to batches B, G). This observation of losing water-holding capacity while gaining mechanical strength led to the conclusion that we should stick to the formulation of 0 or 10 wt% MAA, on the basis of enhanced water-holding capacity, for good mass transfer, while maintaining satisfactory mechanical strength.

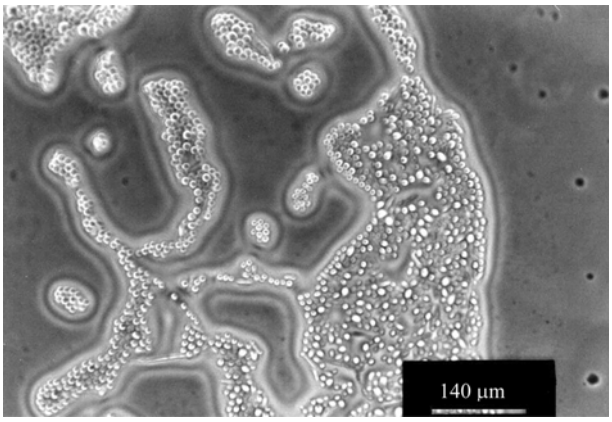



**Fig. 2. Microscopic view (16×10) of the immobilized film of formula A.**

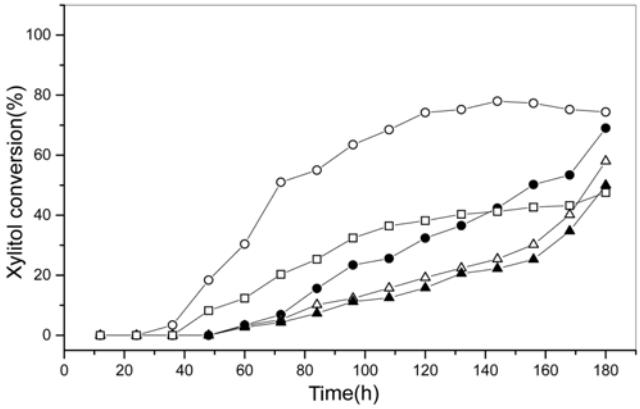



**Fig. 3. Effect of film formulations on fermentation yield (Fermentation medium contained 100 g/L D-xylose, cell loading was 5.75 mg/g film, one piece of film, shaker rate=75 rpm); (○) Formulation A; (●) Formulation B; (△) Formulation C; (▲) Formulation D; (□) Formulation E.**

In this study, DMA was another candidate that could also increase the films' water-holding capacity (batches E and J). However, it was found that the hydrogels failed to form films if the DMA content was higher than 10%, and only this set of data regarding formulations with DMA is shown in Table 2. As can be seen in Table 2, the water-holding capacities and mechanical strengths of the films using DMA were comparable to those of the films with MAA. Fig. 2 shows a typical microscopic view of a cell immobilized film, formula A with cell immobilization. The characteristic pseudomycilial nature of *Candida* could be seen in Fig. 2.

**2. Effect of Film Formulation on Fermentation Yield**

As described above, the addition of MAA or DMA was expected to be a design factor to manipulate the water-holding capacity of the hydrogel. Consequently, changing the water content might affect the permeability of fermentation medium and oxygen gas, and therefore the mass transfer rate, which in turn, will affect the availability of substrates outside the film, to the immobilized yeast cells.

Fig. 3 compares the effects of different formulations of hydrogels on fermentation yields. 100 mL of fermentation medium loaded with one piece of the film of formulation A (5.75 mg yeast cell per g film and totally have 123.4 mg of yeast per 100 mL of fermentation medium) gave a 78% xylitol conversion at 144 hrs. For formulations B, C, and D, although containing higher amount of MAA, their conversions were not as good as that for batch A. Films of formulation B containing 10 wt% MAA, had the highest water holding capacity, but a xylitol conversion of merely 69% was obtained. Films of formulation E containing 10wt% DMA, also had higher water content than formulation A, but the xylitol conversion of 43% was even less, despite a good start at the early stage of fermentation (Fig. 3). These observations showed that, in our case, by increasing the water holding capacity, the fermentation yield did not increase accordingly. The reason awaits further investigation. Such result led to the decision of producing the cell immobilized films without using MAA and DMA, and subsequent experiments were therefore conducted with films of formulation A.

**3. Effect of Xylose Concentration on Fermentation Yield**

As xylose concentration in the medium directly affects the op-

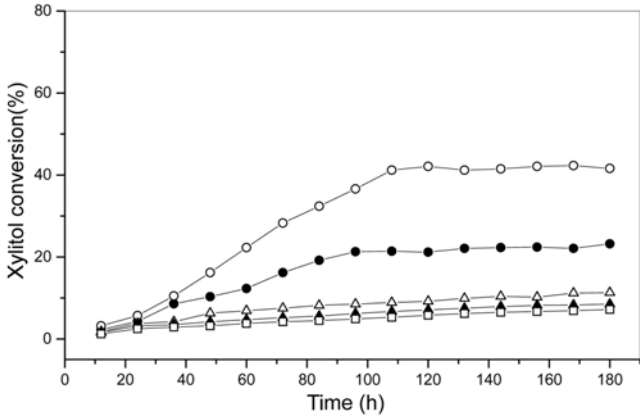



**Fig. 4. Effect of initial xylose concentration on xylitol fermentation yield (film formulation A, one piece of film, xylose=100 g/L, cell loading was 0.57 mg/g film, shaker rate=75 rpm). (●) xylose=80 g/L; (○) xylose=100 g/L; (△) xylose=125 g/L; (▲) xylose=150 g/L; (□) xylose=200 g/L.**

eration costs of a fermentation plant while high sugar concentration imposes osmotic pressure on yeast cells, it is necessary to study its effect on the fermentation. Fig. 4 compares the time courses of the fermentations for broths of different initial xylose concentrations. Initial xylose concentration of 100 g/L gave the highest xylitol conversion, whereas those with 150 g/L or 200 g/L initial xylose concentrations led to decreased xylitol conversion, probably due to substrate inhibition. Adjusting xylose concentration to 125 g/L or 80 g/L did not help in increasing the xylitol conversion.

**4. Effect of Cell Loading on Fermentation Yield**

It was expected that a higher cell loading would result in faster xylitol conversion while maintaining similar yield. Cell loading was varied to study its effect on xylitol conversion. Fig. 5 compares the time courses of fermentations with cell immobilized films of various cell loading. Generally, the xylitol conversion rate was expected to increase with increasing cell loading in the film (Fig. 5). However, it turned out to be a different story when it came to xylitol conversion, the highest conversion was attained at cell loading of 5.75 and 11.5 mg/g film. Further increase of cell concentration (to 23 mg/g film) did not lead to increased conversion rate or yield. The reason could probably be that too densely populated yeast cells hindered mass transfer.

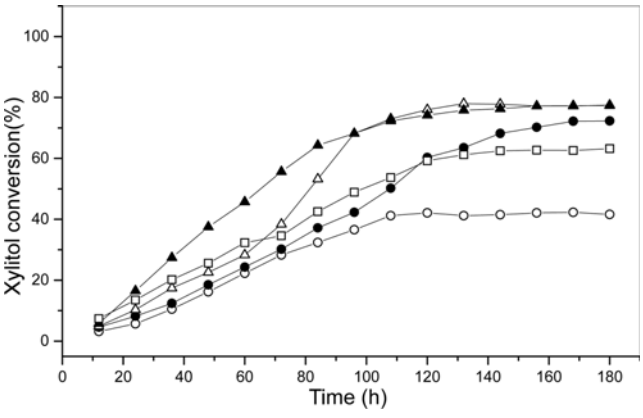



**Fig. 5. Effect of cell loading on xylitol conversion rate (film formulation A, xylose=100 g/L, one piece of film, shaker rate =75 rpm); (○) cell loading 0.57 mg/g film; (●) cell loading 1.15 mg/g film; (△) cell loading 5.75 mg/g film; (▲) cell loading 11.5 mg/g film; (□) cell loading 23 mg/g film.**

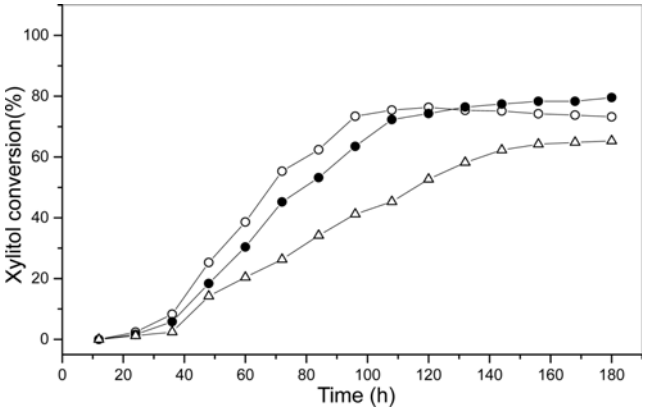



**Fig. 6. Effect of shaker rate on xylitol yield (film formulation A, one piece of film, xylose=100 g/L, cell loading was 5.75 mg/g film); (○) 100 rpm; (●) 75 rpm; (△) 50 rpm.**

**5. Agitation Effect**

Besides the formulation of the supporting matrix, concentration of starting material and cells, agitation is another factor that will influence the mass transfer and dissolution of oxygen gas. In order to study this, the rotary shaker was set at 50 rpm, 75 rpm and 100 rpm while the initial xylose concentration was fixed at 100 g/L and the cell loading was 11.5 mg/g film. Fig. 6 shows that, at 50 rpm, the xylitol conversion was 62%; at 75 rpm, xylitol conversion increased to 74%; and at 100 rpm, the conversion reached 75% in a shorter period of time (120 h), but started to decrease afterwards. A possible explanation would be that increased agitation improved the mass transfer rate, while concomitantly increasing the oxygen concentration in the fermentation broth. For xylitol fermentation by *Candida* sp., a suitable oxygen concentration is desirable, but beyond a certain critical value, oxygen would begin to interfere with

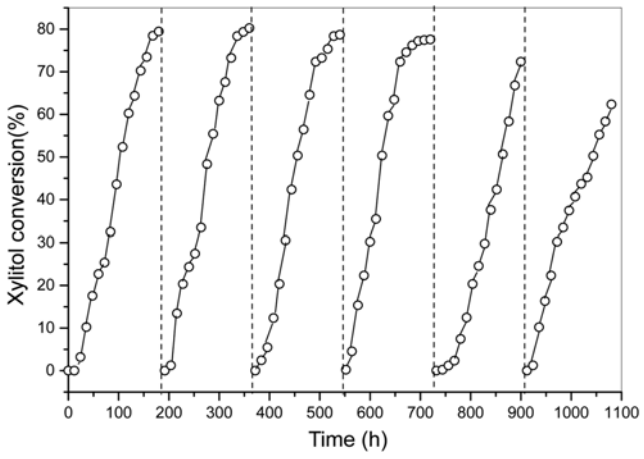



**Fig. 7. Durability test of xylitol fermentation using one piece of immobilized film (film formulation A, xylose=100 g/L, cell loading was 5.75 mg/g, film, shaker rate=75 rpm).**

the xylose reduction reaction.

**6. Durability of the Cell Immobilized Film**

One of the main objectives in developing an immobilized cell system is to use it for continuous production. Hence, whether or not such immobilized film can sustain long-term operation without losing its biological activity is of the utmost importance. In this study, in order to test the durability of these films, the same set of cell immobilized films was used repeatedly. For every 180 h, the fermentation broth was replaced with fresh medium.

Fig. 7 depicts the time courses of fermentation using one piece of the cell immobilized film. In this case, the cell immobilized films lasted longer than two months. No significant decay in terms of biological activity before 720 h was observed; the xylitol conversion maintained at about 80%. After 720 h, xylitol conversion started to decline to about 65% at 1,080 h. It is quite likely that after long-term operation, accumulated cell growth inside the films interfered with mass transfer. To improve this, designing an optimal nutrient composition that directs most of the energy to xylitol conversion and cell maintenance, instead of cell growth, would be important in future researches.

## SUMMARY

This study utilized cross-linking to immobilize the yeast strain *Candida subtropicalis* WF79. Thin films made in this research offered satisfactory mechanical strength while maintaining the viability of the yeast cells. These immobilized cell films successfully converted xylose to xylitol with a maximum xylitol conversion of 81%. Experimental results also showed that these thin films with immobilized yeast cells are suitable for long-term, continuous operation.

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