Characterization of Cellulose Membranes Produced by *Acetobacter xylinum*

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Abstract:

Cellulose membranes formed by *Acetobacter xylinum* under known cell density in a culture medium were characterized. A dead end testing unit was used for water flux and filtration of *Chlorella* sp. and bovine serum albumin (BSA). This study found that the cells formed membranes faster in sucrose supplemented coconut juice than in the standard Schramn & Hestrin's medium. For two-day formed membranes in the former medium, an increase in cell density from $1x10^8$ to $2x10^8$ cfu.ml^{-1} reduced water flux and, hence, reduced the hydraulic permeability coefficient (L_p) from $3.6x10^{-10}$ to $0.5x10^{-10}$ $m^3N^{-1}s^{-1}$. These membranes were asymmetric-hydrophilic type with thickness less than 6.0 μm. Membrane porosity was found to vary from 1.4% to 2.4%, with the averaged pore size 0.08 μm. Under 100 kPa filtration, two-day formed membranes in sucrose supplemented coconut juice with higher cell density rejected *Chlorella* cells and BSA by 99.8% and 98.4%, respectively.

Key Words: *Acetobacter xylinum*, cellulose membrane, filtration, hydraulic permeability, *Chlorella* sp., BSA

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Introduction

Filter membranes are categorized as symmetric, asymmetric, porous or non-porous, depending on their filtration behavior, materials used and manufacturing processes. Normally, they are manufactured from polymers, composite materials between two polymers, or between polymer and ceramic. Related theories, membrane types, and applications can be studied in the handbook provided by Ho and Sirkar (1992). Porous membranes are used as micro-filtration (MF) and ultra-filtration (UF), whereas dense membranes are used for nano-filtration (NF) and reverse osmosis (RO). These membranes function under pressure driven process. Due to the largest pore size among these membrane types, MF needs lowest pressures up to about 350 kPa when macromolecules and particles ranging from 0.02 to 10μm are filtered. For smaller particles in UF process, the pressures required are higher, up to about 1 MPa. To filter particles smaller than 10 angstrom, NF and RO membranes are used and the operating pressures are between 1-10 MPa. Nowadays, membrane filtration is widely used in many applications, such as production of drinking water, yeast filtration and fruit juice concentrating in food industries, and bacteria filtration in waste treatment (Vera *et al.*, 1997, Kuiper *et at.*, 1998, Jonas and Farah, 1998, and Sadr Ghayeni *et al.*, 1999).

As an agricultural country like Thailand, membrane filtration for food industries and waste recovery is a challenging application. Although the cost of commercial membrane is now much lower than in the past ten years, it is still relatively high compared to agriculture products. This reason forms an obstacle to demonstrate membrane technology for industrial uses. Even in education and research, membrane manufacturing is still costly due to imported substances, such as chemicals and polymers. Therefore, there is a need to seek simple methods and to provide cheap membranes. At least, their performance should be sufficiently reliable for lab-scale usage for the first place. An alternative method to produce membranes of microfiltration range from microorganisms is a trial in this study.

It has been well known that bacteria, such as *Acetobacter, Rhizobium, Agrobacterium* and *Sarcina*, synthesize bio-polymers. Among these, gram-negative *Acetobacter xylinum* is claimed to be an effective cellulose-producing bacterium and is widely used (Jonas and Farah, 1998, and Yang *et al.*, 1998). It can be simply grown in a shallow tray with a culture medium; such as coconut juice, sugarcane juice, vinegar, and fermented beverage (Benziman and Rachamimov, 1962, Weinhouse and Beriman, 1974, and 1976, and Yoshinaga *et al.*, 1997), which are plentiful locally. Cellulose network formed as a sheet floating on the medium surface has been proved to have high tensile strength, elasticity, resilience, durability, shape-retention, and high water-binding capacity, and is nontoxic and non-allergen (Schmitt *et al.*, 1991). It resists heat up to 100° C for at least 3 hours (Chung and Shyu, 1999). Moreover, the high water-binding capacity makes it suitable for filtering colloids or particles of micro to ultra range from solutions. This paper investigated whether cellulose sheets produced by *A. xylinum* could be used directly as a filter membrane. Membrane characterization such as hydraulic permeability, membrane morphology, and tests on cell and bovine serum albumin (BSA) rejection are reported.

Materials and Method

Preparation of bacterial cellulose membranes

Four types of cellulose membrane namely A, C, C12 and C25 were produced by *A. xylinum* TISTR 975 which was obtained from the Thailand Institute of Scientific and Technological Research. The membrane A was prepared with the method described by Hestrin *et.al.* (Hestrin and Schramn, 1954). In brief, it was grown in buffered Schramn & Hestrin's medium (BSH medium) which was composed of 2.0% (w/v) glucose, 0.5% (w/v) yeast extract (Difco Laboratories, USA), 0.5% (w/v) peptone (Difco), 0.27% (w/v) Na_2HPO_4, and 0.11% (w/v) citric acid.H_2O, pH 5.0. *A. xylinum* TISTR 975 was grown as a pre-culture

in the medium of 150 ml at 30°C for 24 h before inoculating it into 500 ml cultured medium. A stainless steel round shallow tray of 39 cm diameter was used. The culture with $2x10^8$ cfu.ml^{-1} was incubated statically at 30°C for 3 days and cellulose pellicle was obtained. The cellulose was harvested and washed with distilled water to remove residual medium components. Then, it was treated with 0.25 N NaOH at 80°C for 2 h to get rid of the microorganisms and proteins (Dubey *et al.*, 2002). The pure cellulose sheet was dried at 37°C over night and kept in a dust free atmosphere until used.

The membranes C was produced from bacteria grown in coconut juice, which was supplemented with 4% sucrose (w/v) at pH 5.0, to reduce the production cost. The inoculum and the culture conditions were the same as for membrane A. The membranes C12 and C25 were prepared in the same method as for membrane C, but the cell density of inoculum was controlled to be $1x10^8$ and $2x10^8$ cfu.ml^{-1}, respectively. The incubation time for these membranes was only two days and the thickness of the films obtained was less than 10 μm. Membrane porosity was estimated from surface pictures of membrane skin layer obtained by scanning electron microscope technique (SEM) with the aid of computer Carnoy program (Lab of Plant Systematics, Belgium).

Hydraulic permeability coefficient (L_P)

Experiments were carried out using distilled water as a feed and permeate volume was collected under various applied pressures. Apparatus and equipment set up for dead-end filtration has been described before (Wanichapichart *et al.*, 2000), as shown in Figure 1, and the tested circular membrane of $1.4x10^{-3}$ m^2 was placed on a porous polypropylene support. In a preliminary study, water absorption of the membrane was tested and it was found to take about 20 minutes before water content in the membrane was maximized. Each membrane was, therefore, immersed in distilled water for at least 1 hour to assure that any change in flux measurement was not due to this water swelling property. According to the Hagen-Poissuille equation (Davis, 1992), hydraulic permeability coefficient (L_p) can be obtained from the slope of graph between the flux (J) and the applied pressure (ΔP). The L_P is related to membrane pore number (N), mean pore size (r), viscosity of feed solution (η) and membrane thickness (Δx) as

$$J = \frac{N\pi r^4}{8\eta\Delta x}\Delta P = L_p\Delta P \qquad (1)$$

Filtration of *Chlorella* sp. and bovine serum albumin (BSA)

A feed solution containing a mixture of fresh water phytoplankton, *Chlorella* sp., of 2.5±0.5 μm average cell size was used. The experiments started with a fixed feed volume (V_f) of 100 ml and with a known cell density (D_f) of $1x10^6$ cfu.ml^{-1}. The feed was stirred continuously to avoid cell precipitation on the membrane surface. Data taking was made whenever the permeated volume (V_p) reached 5 ml under 100 kPa. The cell density of the permeate volume (D_p) was estimated using a compound microscope of 400x magnification. The cell size was measured using an eye piece scale of ±0.1 μm accuracy. As suggested by Devitt *et al.* (1998), cell rejection was estimated from

$$\%rejection = \left(1 - \frac{D_pV_p}{D_fV_f}\right)x100 \qquad (2)$$

For BSA testing, a 1% BSA feed solution was prepared. Analysis for BSA in permeate volume was made using a spectrophotometer (Spectronic Instruments, 20 series) at 750 nm wavelength. Percent rejection of BSA was calculated from a ratio between permeate and feed concentration as $[1-(C_p/C_f)]x100$.

Results and Discussion

Hydraulic permeability coefficient (L_p)

In a preliminary study, membrane C could stand a compressive pressure in the dead end testing unit up to 250 kPa without breaking. This is equivalent to a compressive force approximately 380 N. On a permeated volume of distilled water, Figure 2(a) shows a tendency of linear relation between the flux and the applied pressure. The reverse flux was obtained by reducing the applied pressure back to the lower value. The slightly greater reverse flux than the forward one could be due to some residual pressure in the previous one. The thickness of membrane A and C after three-day culturing was 10.3 μm and 27.5 μm, respectively. This was due to the fact that thicker membrane reduced water flux under the same pressure and, hence, resulted in smaller hydraulic permeability coefficient (L_P). The result implies that cellulose threads were produced more actively in sucrose supplemented coconut juice. Since cell mobility in static culturing could not be manipulated, slight variation in the thickness of the produced membranes from position to position was common and found to be about ±0.6 μm. Accordingly, cell culture period for membrane forming was reduced to two days. It was noted that one-day culturing was not adequate for the cells to form a firm membrane.

Concerning membrane C of lower production cost, Figure 2 (b) shows that membrane C25 possesses much smaller water flux, resulting in a significantly smaller L_p. It is interesting to point out that the cell density of *A. xylinum* between $1x10^8$ and $2x10^8$ $cfu.ml^{-1}$ does not seem to affect the membrane thickness significantly. It was possible that larger cell density increased the cellulose threads, which spread over the entire medium surface in the tray, and hence it might only reduce the number of large pores. To verify this, membrane impedance was studied using the same method as reported by Coster *et al.* (1992). Figure 3 shows that the impedance of C12 membrane is about 1/3 of that of C25 at frequency between 0.1 and 1 kHz. The larger impedance was due to more cellulose threads, which obstructed water passage. This difference was reasonable compared to the permeate fluxes (*see Table 2*).

L_p value was deduced from the slope of the graph between the water flux and the pressure. Unlike nuclear pore membranes produced previously (Wanichapichart *et al.*, 2000), the closer linearity of the flux with the applied pressure of these membranes implies that pore opening faces up directly to the compressive force. Comparing among the produced C12 and C25 membranes, Table 1 shows L_P varying from $0.46x10^{-10}$ for C25 membrane to $3.59x10^{-10}$ $m^3N^{-1}s^{-1}$ for C12 one. As expected, the values are much smaller than that of 0.2 μm MF millipore membrane estimated, as $166x10^{-10}$ $m^3N^{-1}s^{-1}$, in our previous work. Examples of pore distribution and the mean pore size of membranes formed in BSH and in sucrose supplemented coconut juice are shown in Figure 4. It should be noted that the computer program reflected the size of surface pores. However, data in the table implies that the mean pore size of C12 and C25 membrane is 0.08-0.09 μm, independent of the cell density. The produced membranes were of microfiltration range (0.02-10 μm), of which the largest pore was found to be 0.25-0.35 μm.

Evidence of asymmetry membranes

The so-called skin layer of the membrane was the air-facing surface, while it was formed in culturing medium, and the sub-layer was the medium-facing one. This section deals with water flux measurement from skin and sub layer of A and C type membranes. Figure 5(a) shows that permeate fluxes obtained from the skin layer are slightly smaller. The

asymmetric fluxes between the two surfaces still remained when two-day formed membranes were tested, as shown in Figure 5(b). The A25 membrane was formed under the same cell density as C25, except BSH medium was used instead. Compared with C25 membrane, the larger fluxes from skin and sub layer of A25 membrane confirm the slower forming in the BSH medium than in the sucrose supplemented coconut juice. Surface morphology of a C type membrane is shown in Figure 6. Cellulose intertwines and forms a membrane of several pore sizes with a denser skin layer than sub layer. Since the bacteria reside on the medium surface for oxygen requirement, the newly released threads would attach on top of the previously formed layer, leading to some "straight holes" and possibly some tortuous channels from the top surface to the underneath. Such membrane forming was likely to provide narrower pores on the skin layer. This explains why water fluxes obtained from the skin layer are slightly smaller. However, it is plausible to assume cylindrical pores for these thin membranes, since the ratio between passage length and the membrane thickness (definition of tortuosity) should be close to unity.

Filtration of *Chlorella* sp. and BSA

Feed solution of 100 ml fixed volume with *Chlorella* sp. of cell density $1x10^6$ cfu.ml^{-1} was filtered through the dead-end unit. Each 1 ml permeate volume was collected and brought to count for cell density (D_p), and the corresponding cell density (D_f) in the feed was estimated. Table 2(a) shows that permeate fluxes of C12 and C25 membrane are fairly constant at 11.4 and 3.5 $Lm^{-2}h^{-1}$, respectively. The difference in the flux, in spite of having the same surface pore size, could be due to the fewer cellulose threads and also the slightly greater membrane porosity within the C12 than C25 membrane. Moreover, the constant flux implies that the continuous stirring in the feed chamber during filtration helps to protect membrane fouling. Considering the size of *Chlorella* sp., the cells were a lot larger than membrane pores and hence total rejection was expected. In this study percent rejection for *Chlorella* cells is closed to 100% for both membranes. Although Schmitt *et al.* (1991) reported high elasticity property of cellulose membranes, some cells should not be able to penetrate the largest pore under such pressure.

For BSA filtration, Table 2(b) shows a gradual decrease in permeate flux within 40 minute filtration in C12 membrane. This is consistent with the slight increase in % BSA rejection from the membrane. Comparing between two membranes, the smaller BSA rejection for C12 than C25 membrane is expected since permeate flux of the former is greater. However, the fairly constant permeate flux of C25 membrane indicates that there was no BSA fouling during the filtration. The near total BSA rejection could be explained for the thread-like protein in solution not easily aligning itself to be perpendicular to the surface pores during filtration, although its globular size was only 64 angstrom (Baker, 2000) - much smaller than cell size. The averaged BSA rejection of C12 membrane was 67%, while that of C25 membrane was 98.6%.

Conclusion

This study found that cellulose membranes were formed faster by *A. xylinum* in the supplemented coconut juice than in the buffered Schramn & Hestrin's medium. The hydraulic permeability coefficient of the membranes depended on the time and the cell density used during membrane forming. Surface pore size of membranes formed in sucrose supplemented coconut juice was 0.08 μm on average, and they are considered to be microfiltration membranes. Their porosity was found to vary from 1.4% to 2.4%. *Chlorella* and BSA rejection of the membranes formed in $2x10^8$ cfu.ml^{-1} cell density was 99.8% and 98.4%, respectively.

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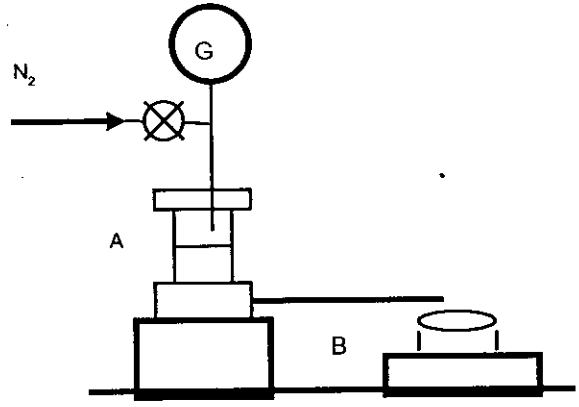
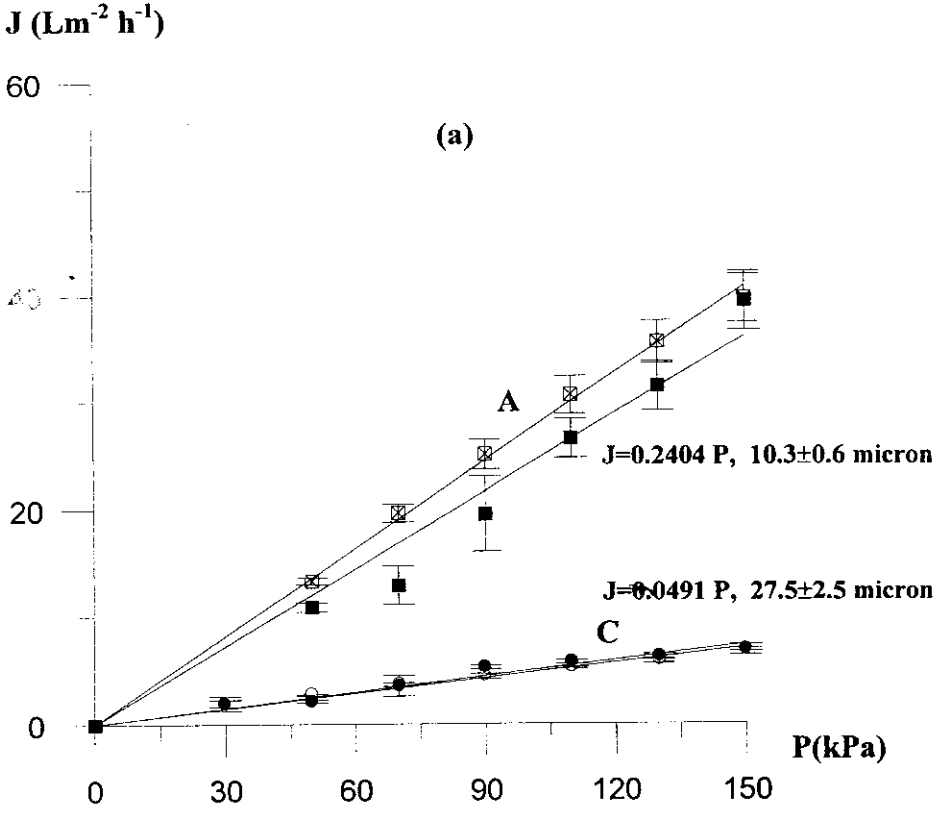


Figure 1

Schematic representation of experimental set up.
A is the feed unit, and B permeate container, G pressure gauge,
⟶ N_2 gas line, and — water line.


J (Lm-2 h-1)
60
(a)
40
20
0
A
J=0.2404 P, 10.3±0.6 micron
J=0.0491 P, 27.5±2.5 micron
C
P(kPa)
0
30
60
90
120
150

(b)

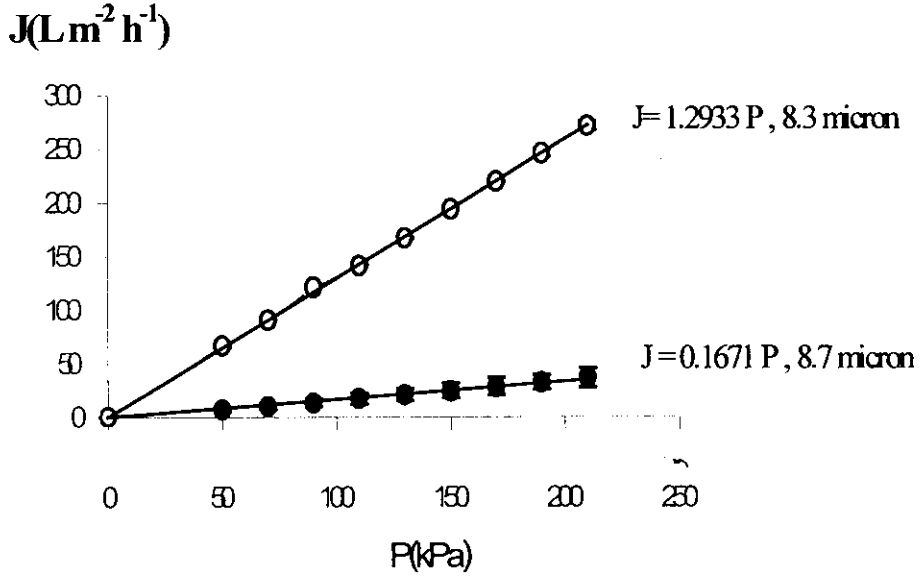


Figure 2 Water flux of membrane A and C types.
(a) Forwards (dark) and reverse (white) fluxes obtained from membrane A (square) and C (circle).
(b) Comparing between fluxes of C12 (white) and C25 (dark) membrane against the applied pressures.
Membrane thickness in micron is indicated.

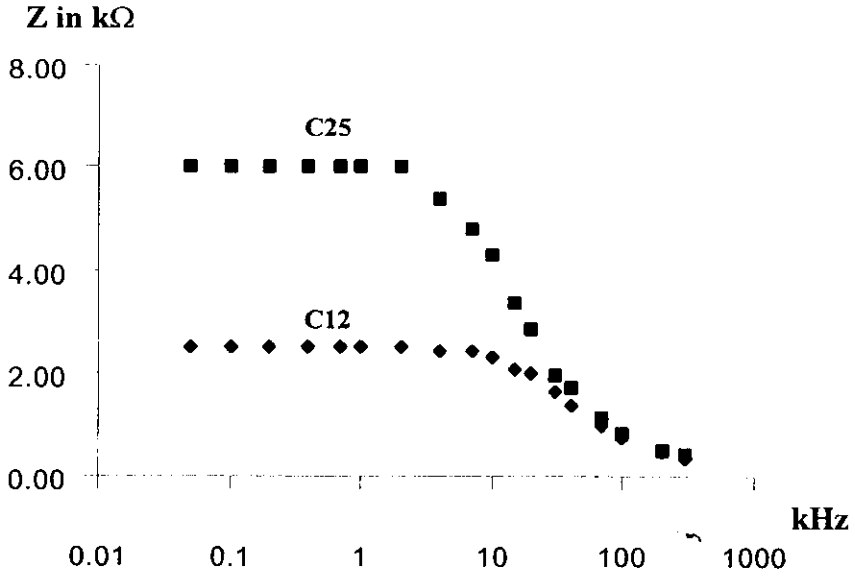
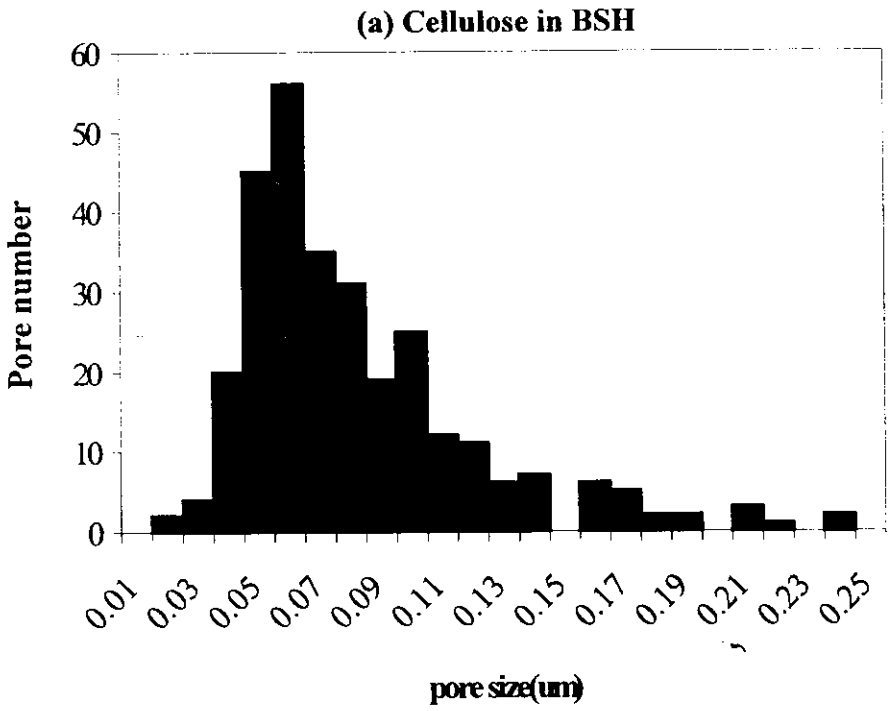


Figure 3 Impedance (Z) of C12 and C25 membrane against the electric field frequency.
Each membrane was immersed in a 1.0 mM KCl solution.



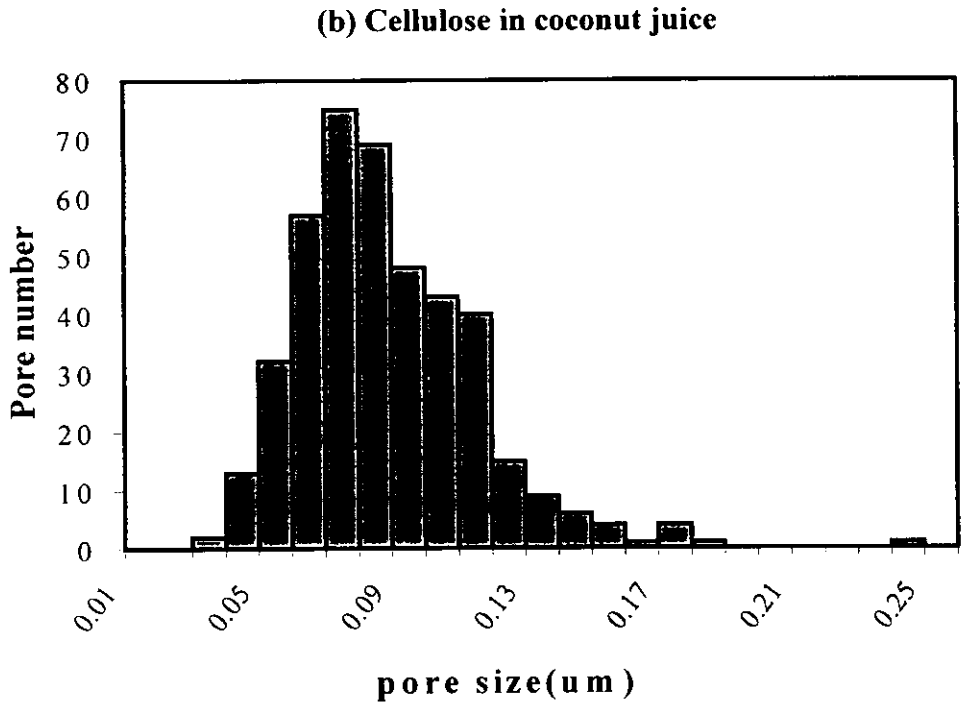


Figure 4 Pore distribution of membranes formed for two days under the same cell density.
(a) membrane A formed in BSH medium
(b) membrane C formed in sucrose supplemented culture

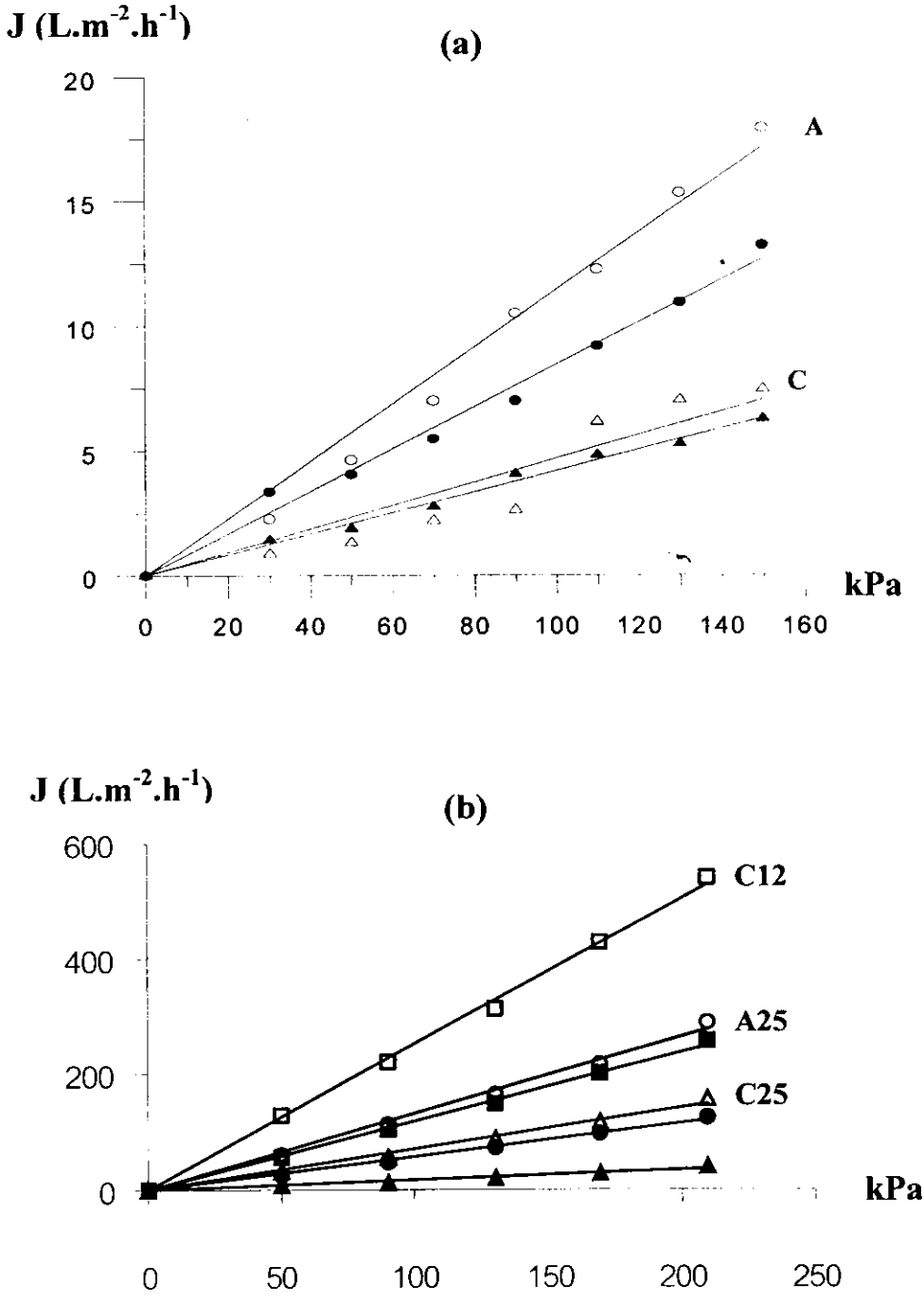


Figure 5 Water fluxes from skin (dark) and sub layer (white) of membranes.
(a) three-day formed membrane A and C.
(b) two-day formed membrane C12, C25, and A25.

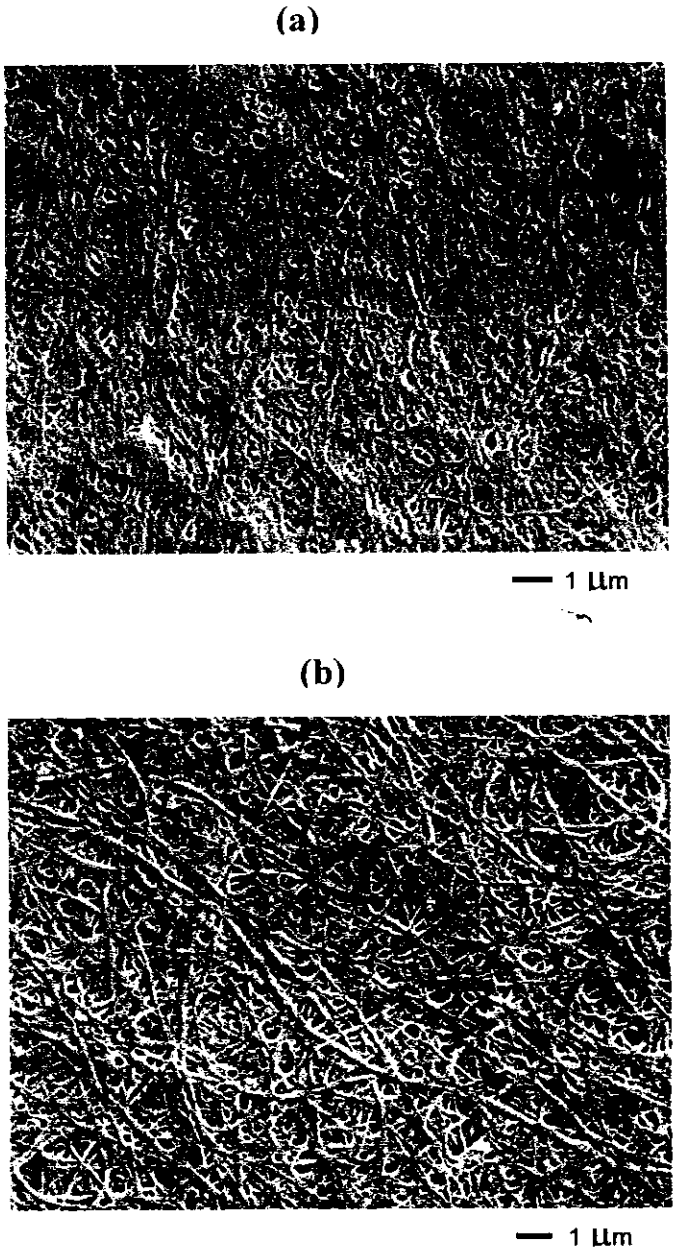


Figure 6
SEM micrographs of skin layer (a) and sub layer (b) of C12 membrane.

Table 1 Comparing estimated values for hydraulic permeability coefficient (L_P), mean pore size (2r), and porosity (ε) between C12 and C25 membranes.

Membrane type	L_P ($m^3N^{-1}s^{-1}$)	2r (μm)	ε (%)
C12	3.59×10^{-10}	0.077	1.45
		0.074	1.78
		0.069	1.85
		0.084	2.36
		0.076±0.006	1.86±0.38
C25	0.46×10^{-10}	0.083	1.56
		0.095	1.40
		0.088	1.37
		0.089±0.006	1.44±0.10

Table 2 Permeate fluxes and % rejection of a cellulose membrane under 100 kPa. Each data was averaged from 2 experiments.

(a) *Chlorella* sp. cells

Membrane	Time (min.)	Permeate flux ($L.m^{-2}.h^{-1}$)	% Rejection
C12	13.3	10.8	99.7
	26.7	11.2	99.8
	39.3	11.6	99.7
	53.5	11.5	99.6
	66.3	11.7	99.7
C25	4.2	3.6	99.7
	8.5	3.5	99.8
	12.7	3.8	99.8
	17.0	3.2	99.8
	21.0	3.6	99.8

(b) BSA

Membrane	Time (min.)	Permeate flux ($L.m^{-2}.h^{-1}$)	% Rejection
C12	5	81	61.6
	20	73	70.1
	40	66	70.6
C25	5	11	99.0
	20	13	98.6
	40	13	98.2