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IMMOBILIZATION OF MICROBIAL CELLS FOR THE PRODUCTION OF ORGANIC ACIDS

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Abstract

Techniques of immobilization of microbial cells have been established by multifunctional reagents such as glutaraldehyed on a solid support. Immobilized cell reactor was designed to demonstrate stability of cross linking of whole cells for the production of organic acids. An eighty-six percent conversion of glucose was obtained for an eight hour retention time. The immobilized cell reactor is about 5-8 times faster than the continuous stirred tank reactor with free microbial cells. An appropriate kinetic model was obtained using immobilized *propionibacterium acidi-propionici* on solid support.

Immobilization of Microbial Cells

Since 1978, a number of papers have been published on the potential of using immobilized cells in fuel production. Microbial cells are being used advantageously for industrial purposes such as *Escherichia Coli* for the continuous production of L-aspartic acid from ammonium furmarate [1,2]. The enzymes from microorganisms are classified as extracellular and intracellular enzymes. If whole microbial cells can be immobilized directly, the procedures for extraction and purification can be omitted and the loss of intracellular enzyme activity can be kept to a minimum. The whole cells are used as a solid catalyst when they are immobilized onto a solid support.

There are three general methods available for the immobilization of whole microbial cells: carrier-binding, entrapment, and cross-linking.

Carrier-Binding Method: As shown in Figure la, the carrier-binding method is based on binding microbial cells directly to water insoluble carriers. The binding takes place due to ionic forces between the microbial cells and the water insoluble carriers. This technique has rarely been used however, because of lysis during the enzyme reaction. Microbial cells may leak from the carrier, thereby disrupting the immobilization. Therefore, this method is not applied successfully [3].

Key words: Immobilized Cell Reactor, Organic Acids, Techniques of Immobilization.

Entrapping Method: In applying entrapment, microbial cells are directly entrapped into polymer matrices (see Figure 1b). This method was applied to a number of microorganisms using gelatin, agar, polyacrylamide gel, calcium alginate, etc., as the entrapping agents. The following list of matrices are used extensively for the immobilization of microbial cells:

1. Collagen
2. gelatin
3. agar
4. alginate
5. carrageenan
6. cellulose triacetate
7. polyacrylamide
8. polystyrene

In this method, microbial cells are apparently lysed within the entrapping agent, but they retain the desired enzymatic activity. In order to prepare an efficient immobilization, the type and concentration of a bifunctional reagent for entrappment should be optimized. The advantage of this method is that cell leakage may be minimized, but the bifunctional reagent layer may act as a diffusion barrier, thus causing difficulty in transferring substrate and product through the matrix [5].

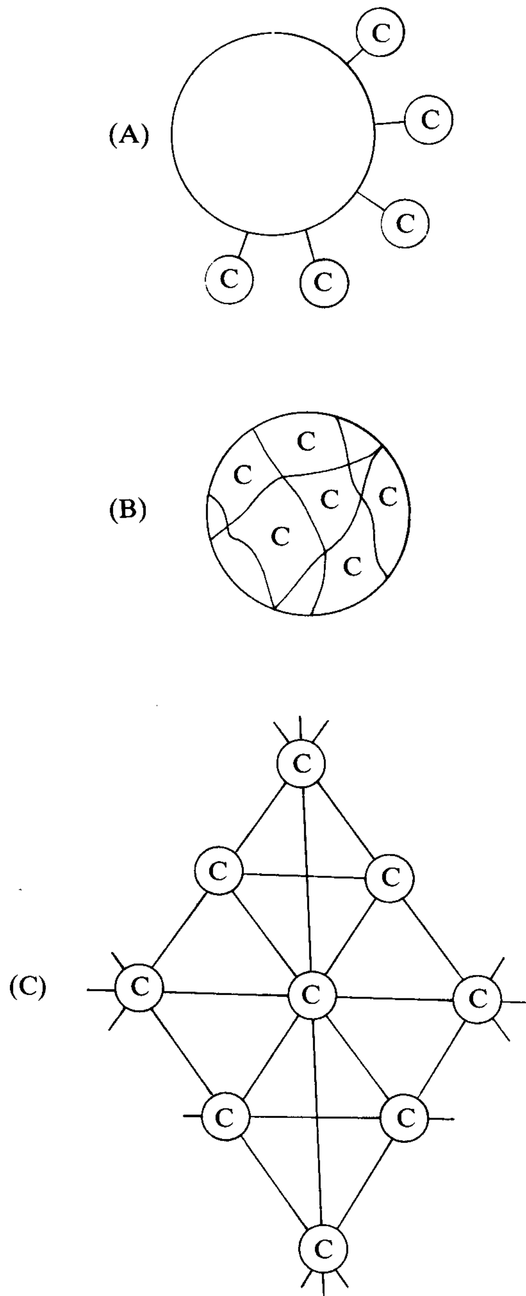

FIGURE 1. Alternative Methods Used To Immobilize Cells (A) Carrier Binding; (B) entrapment; (C) Cross-Linking, [4].

Cross-Linking Method: Microbial cells immobilized by cross-linking with bi - or multi-functional reagents such as glutaraldehyde has been reported to be successful, but active immobilizations have not been obtained with the other reagents such as toluenediisocyanate [3].

The microbial cell wall is composed of lipoproteins, with lipopolysaccharide extending from the cell membrane. Glutaraldehyde reacts with lysine within the protein in the lipid bilayer of the cell membrane. Furthermore, gelatin is physically absorbed to a solid support which provides an active base for glutaraldehyde. Then, glutaraldehyde can provide a covalent link between microbial cells and the gelatin layer which is tightly bound by absorption to a solid support (see Figure 1c).

Glutaraldehyde has been used as cross-linking reagent for ethanol production by Sitton and Gaddy [6] achieving rates as high as 7.4 g/liter hr. ethanol production from glucose. The major advantage of the cross-linking method is that the immobilization reagent does not act as diffusion barrier. Actually a thin film of cells is provided in this system, making it ideal for many applications [7,8].

Advantages and Disadvantages of Immobilized Cells

There are a number of advantages of an immobilized cell system over a batch or CSTR* system. The first and most obvious benefit is the capability of recycling or reusing the micro-organisms since they are retained in the reactor instead of flowing out with the product. Secondly, immobilization can be easily used for a continuous process maintaining high cell density, thus providing a high productivity.

Thirdly, nutrient depletion and any inhibitory compounds do not, in general, have a large effect on the immobilized cells since the cells are fixed by immobilization. In the batch and CSTR fermentations, nutrient depletion and accumulation of toxic by-products are major problems, but immobilized cells are usually unaffected by the accumulation of toxic by-products.

There are, however, disadvantages to using immobilized cells. The cell may contain numerous catalytically active enzymes, which may catalyze unwanted side reactions. Also, the cell membrane itself may serve as a diffusion barrier, and may reduce productivity. Absorption of reaction products to the supporting matrix may sharply reduce productivity if the micro-organism is sensitive to product inhibition. One of the disadvantages of immobilized cell reactors is that the physiological state of the micro-organisms can not be controlled.

Immobilized Cell Reactor Experiments

The ICR experiments were undertaken to determine the performance of immobilized *P.acidi-propionici* in a plug flow tubular reactor. The productivity of the ICR was evaluated by measuring glucose and xylose consumption and propionic and acetic acids production along the length of the reactor.

* Continuous stirred tank reactor.

The Rasching rings were sterilized and dip-coated in sterile 20 percent gelatin and 1.5 percent agar solution. The coated rings were randomly packed in the column. After the coated rings had dried, the packing was sprayed with a 2.5 percent glutaraldehyde solution. An alternative procedure to prepare the packing was also tested and proved to be satisfactory. In using the latter method, the reactor column was filled with clean Rasching rings. A hot gelatin-agar solution was passed through the column, and allowed to wet the entire packing surface. When the coating was dried, the glutaraldehyde solution was passed through the column in a similar fashion. The column was then allowed to stand for 24 hours and then washed with sterile distilled water. The reactor was sterilized by passing ethylene oxide through the column. The ethylene oxide was allowed to stand in the column for eight hours before the system was purged with sterile nitrogen. The feed and product tubing were autoclaved by employing 15 psig steam.

After sterilization, a 24 hour old seed culture was pumped through the column. An adaptation time of about 48 hours was allowed for the establishment of a film of micro-organisms cross-linked to the Raschig rings. Feed media was then pumped into the reactor. An accurate calculation of the retention times in the reactor was difficult; due to the fact that growth and carbon dioxide evolution may take a significant fraction of void volume of the ICR, resulting in a false retention time. The microbial film thickness may be controlled by periodically passing sterile nitrogen or carbon dioxide through the column, to stop cell overgrowth.

A feed concentration of 15g glucose and 15g xylose per liter was used over a feed rate range of 20 ml/hr to 200 ml/hr. Samples were taken at successive points along the reactor length, and the usual analysis for glucose and xylose consumption, organic acids production, and cell density were done. A kinetic model for the growth and fermentation of *P. acidi-propionici* was obtained from this data.

Analytical procedures were set up for measurement of cell density, sugar concentration and organic acid concentrations. The turbidity of the culture (optical density) by reading the absorbances with spectophotometer and cells count. Sugar concentration as single substrate was measured by industrial digital analyzer. Total sugar was evaluated by a reducing agent such as dinitrosalicylic acid in alkaline solution. An orange color is produced which is read on colorimeter at 540 nm. Organic acids were determined by a gas chromatorgraph, with the flame ionization detector.

Results

Table 1 presents the results of the ICR retention time studies, sugar concentrations (dual substrate) studies and cell density. The kinetic model for ICR was derived on the basis of a first order reaction, plug flow and steady state behavior.

$$r_A = k \quad c_A \qquad (1)$$

$$u \frac{dc_A}{dz} = r_A \qquad (2)$$

where

r_A=reaction rate, g/L-hr
k=rate constant, 1/hr
c_A=sugar concentration, g/L
z=axial reactor length, cm
u=bulk fluid velocity, cm/hr

substituting equation (1) into equation (2) yields.

$$u = \frac{dc_A}{dz} \qquad (3)$$

Equation (3) is a linear first order differential equation in concentration and reactor length. Using the separation of variables technique, the equation can be integrated yields.

$$\ln({}^{c}A/c_{A_0}) = \frac{kz}{u} \qquad (4)$$

Figure 2 shows a plot of $\ln({}^{c}A/c_{A_0})$ as a function of dimensionless reactor length for the fermentation data obtained at a feed concentration of 15 g/1 glucose and 15 g/1 xylose at different retention times. As soon, a straight line is obtained at each retention time. The value of the slope increased with increasing retention time due to decreasing velocity through the column. Table 2, also indicates the rate constant in the first order reaction is fixed with different retention time.

The ICR flow rate was 5-8 times faster than the CSTR. The overall conversion of sugars in the ICR at a 12 hours retention time was 60 percent. At this retention time, the ICR was 8 times faster than CSTR, but in the CSTR an overall conversion rate of 89 percent was obtained. At the washout rate for the chemostat, the ICR resulted in a 38 percent conversion of total sugars. Also, the organic acids production rate in the ICR was about 4 times more than the CSTR. At a higher retention time of 28 hours the conversion of glucose in the ICR and CSTR are about the same, but the conversion of xylose reached 75 percent in the ICR and 86 percent in the CSTR.

Table 1. Continuous Fermentation of Dual Substrate (Glucose, Xylose) in ICR at 36°C.

Retention Time (Hours)	Length of Reactor (in)	Substrate Concentration (g/l)		Organic Acid Concentration (g/l)		Reaction Rate of Glucose (g/l - hour)	Cell Density (# cells/ml)
		Glucose	Xylos	Propionic Acid	Acetic Acid		
28	0	15	15	--	--		
	6	6.28	10	1.3	7.78	0.31	9 X 10^{11}
	14	4.52	8.6	1.98	8.45	0.37	9 X 10^{11}
	24	2.13	7.2	2.7	12.4	0.46	9.5 X 10^{11}
	34	1.36	5.4	3.25	17.2	0.49	9.5 X 10^{11}
	44	1.14	3	3.95	19.1	0.495	9.5 X 10^{11}
20	0	15	15	--	--		
	6	7.1	11	1.2	6.45	0.395	9 X 10^{11}
	14	4.72	9.8	1.46	8.1	0.514	9.5 X 10^{1}
	24	2.21	7.9	1.87	12	0.64	9.5 X 10^{1}
	34	1.5	7.15	2.12	16.4	0.675	9.5 X 10^{1}
	44	1.2	5.8	2.4	18	0.69	9.5 X 10^{1}
12	0	15	15	--	--		
	6	9.30	14.5	2.59	1.33	0.475	1.1 X 10^{1}
	14	6.02	14	3.26	2.37	0.75	2 X 10^{10}
	24	3.62	13	4.5	2.45	0.95	3 X 10^{11}
	34	2.65	12	5.07	2.50	1.03	3 X 10^{11}
	44	2.30	10	5.36	2.54	1.06	5 X 10^{11}
5	0	15	15	--	--		
	6	9.8	15	0.50	0.58	1.04	6 X 10^{10}
	14	7.27	14.5	0.60	0.76	1.55	1.8 X 10^{1}
	24	6.05	14	0.65	1.20	1.79	5 X 10^{10}
	34	5.84	14	0.70	1.36	1.83	5 X 10^{10}
	44	5.20	13.5	0.80	1.65	1.96	6 X 10^{10}

Table 2. ICR Kinetic Model for Immobilized *Propionibacterium acidi-propionici.*

Retention Time τ, Hours	**Flow Rate ml/hr**	**Rate Constant hr^{-1}**
5	214	0.7
8	135	0.69
12	90	0.68
20	55	0.68
28	38	0.51

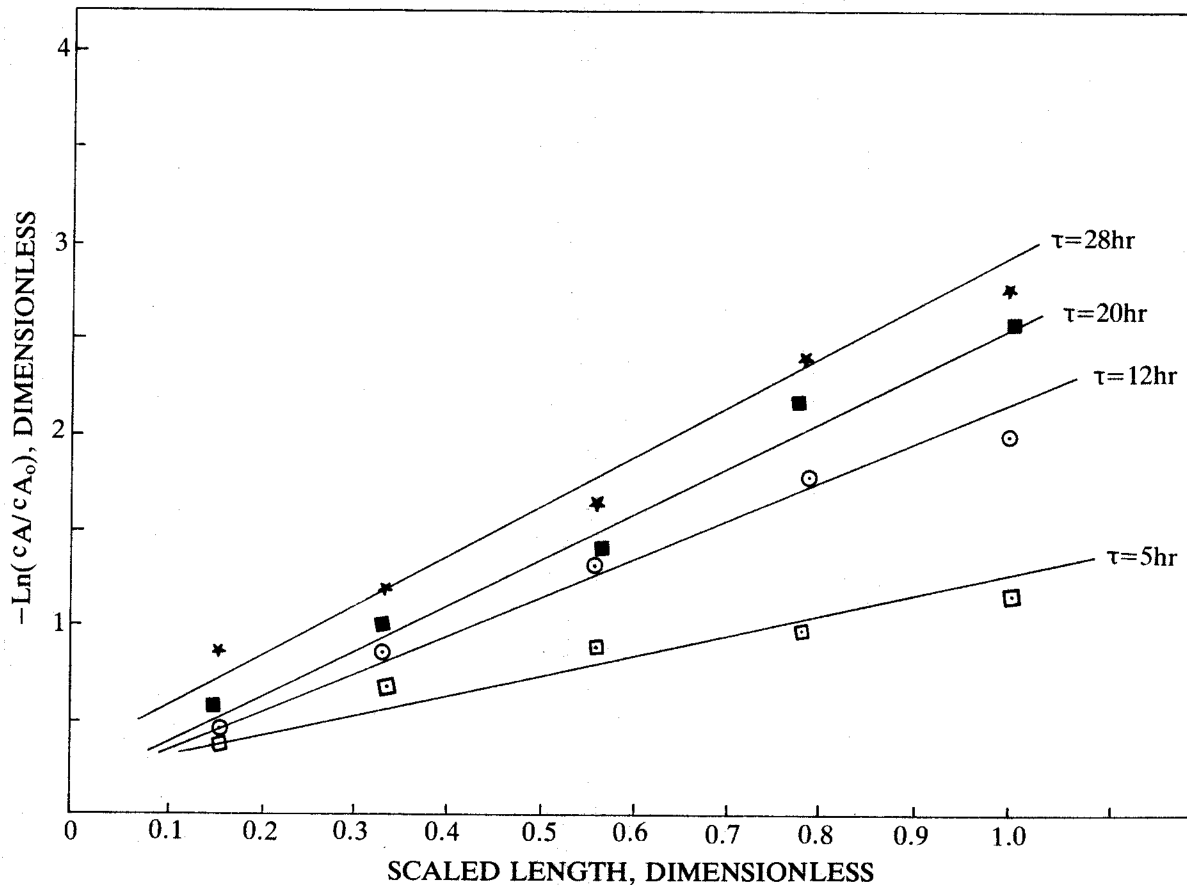

FIGURE 2. MODEL TEST FOR ICR USING *PROPIONIBACTERIUM ACIDI-PROPIONICI.*

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