SUCROSE HYDROLYSIS AND PHENOLICS PRODUCTION BY *DAUCUS CAROTA* ENTRAPPED IN HOLLOW FIBER REACTORS

Foreword

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The paper was written by Dr. Wilfredo I. Jose, with Henrik Pedersen and Chee Kok Chin as co-authors. Dr. Wilfredo Jose is a professor of Chemical Engineering at the UP College of Engineering. He was given The Outstanding Chemical Engineer Award for 2007 by the Professional Regulation Commission for his "exemplary achievements in the field of chemical engineering, having demonstrated exceptional professional competence through his various studies and researches in the field of biotechnology and biochemical engineering, with his works having been applied by the industry in various waste management programs." He was also recipient of the Outstanding Young Scientist Award given by NAST-DOST in 1987.

Dr. Jose graduated cum laude with a Bachelor of Science degree in Mapua Institute of Technology, obtained his Master of Science in Chemical Engineering at the University of the Philippines and his M.S. and Ph.D. In Chemical and Biochemical Engineering at Rutgers University in New Jersey, USA.

At the time the paper was written, he was an assistant professor at the UP Chemical Engineering Department. Among his studies related to the paper were: Charcoal-fired Drier with a Novel Furnace Design, Coconut Shell Charcoal as Packing Material for Distillation Columns, Absorbers and Demisters, and Development of a Model for the Waste Utilization Value. He continues to actively engage in research in many areas of chemical engineering, including applications on health and wellness, process intensification and engineering education.

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Carrot cells immobilized in a hollow fiber membrane reactor were investigated for sucrose hydrolysis and formation of phenolics. The reactor system was run in batch, once-thru, and partial recycle configurations. The results showed that hollow fibers are suitable for plant cells, but since they are expensive and fragile, a modified system is proposed.

1. Introduction

Plant cells in culture can produce or transform biochemicals in the same way as microorganisms such as bacteria and yeasts. In order to exploit this potential, existing fermentation techniques and biochemical reactors have been adapted and modified for a variety of plant cell uses such as steroid transformation, alkaloid synthesis, and production of tobacco cell mass, among others (11). Since plant cells grow slowly and exist almost entirely in aggregates, however, immobilization or entrapment should be considered as an attractive policy for plant cell utilization. Brodelius et al, (2) first immobilized plant cells by entrapping the cells in alginate beads and demonstrated the synthetic capabilities of various immobilized plant cells. Other immobilization techniques have been employed successfully since then (1, 2, 4, 5, 6, 9, 12). The most widely used method to date has been immobilization by entrapment in alginate gels, probably because of the simplicity and mildness of the technique. The alginate beads, however, are subject to mechanical and chemical degradation and, in addition, allow for the propagation of free plant cells in the medium. These are potentially aggravating problems in an industrial system that makes use of plant cell reactions. An alternative immobilization technique that is still simple and mild, yet avoids the problems just mentioned, is the use of hollow fiber membrane reactors.

2. Experimental

Daucus carota cells were grown in suspension with Murashige and Skoog (7) medium in rotary shake flasks at 280 rpm. Seven-day-old cultures were used in the experiments.

2.1 Growth and reaction in free cell suspension cultures.

Ten identical flasks were filled with 100 ml of medium inoculated with equal amounts of cells and placed on a rotary shaker at 280 rpm and 25°C. Every two days a flask was analyzed for total phenolics, glucose, sucrose, fructose, and cell mass.

2.2 Initial rate experiments.

Total asepsis was not required for these experiments a run lasted only for a short period of time. Cells were filtered off, washed free of glucose, and resuspended in distilled water. For each trial, one ml of the cell slurry was transferred to a 125-ml erlenmeyer flask and the total volume brought to 50 ml with sucrose solution (concentration between 0.1 and 10% sucrose). The flask was immediately placed on a shaker and one ml of the solution was withdrawn every 5 minutes and analyzed for glucose. At the end of each trial, the cells were filtered off, dried, and weighed. The initial rate is the slope at time zero on a plot of the glucose concentration versus time. The rate was expressed as activity units/mg dry cell. An activity unit is the amount of cells that catalyzes the hydrolysis of one μmol of sucrose per minute.

2.3 Entrapment procedure.

Commercially available hollow fiber membrane reactors were used. (See Fig. 1.) The cells were entrapped outside the fibers and the medium was passed through the fiber lumen to provide the necessary nutrients and substrate and to continuously remove the products. The shell of the reactor was modified so that more plant cells can be loaded. Using a sterile syringe, approximately four ml of a seven-day-old suspension

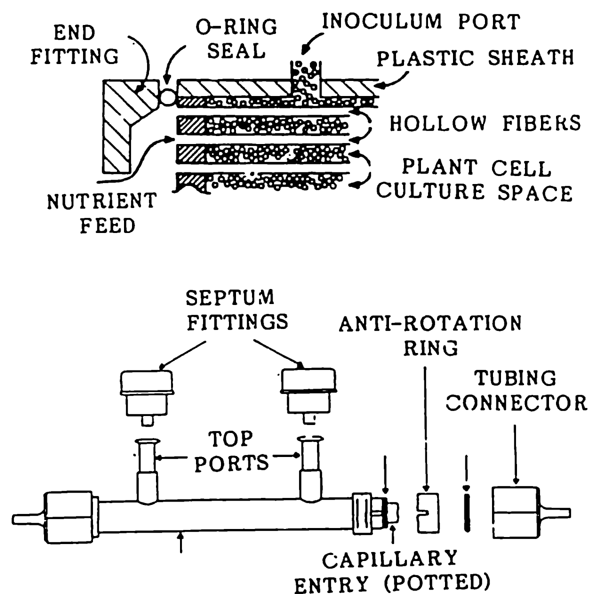


Fig. 1 The hollow fiber reactor (Characteristics: 150 fibers, 200 μm internal dia., 50-75 μm thick, anisotropic, 10,000 Daltons MW cutoff, each fiber 5.7 cm long, extracapillary volume 2.5 ml modified to 4 ml)

was loaded in the shell side of the hollow fiber cartridge (sterile). The reactor ports were then sealed and the immobilized cells were incorporated in the setup.

2.4 Batch experiment (total recycle)

The system, as shown in Fig. 2, (autoclaved and cooled before cell loading) consisted of a reservoir (250-ml Erlenmeyer flask), silicon tubings, the reactor, a sampling device, and an aerating system. The flow rate was maintained at 10 ml/min. The phenolics, glucose, fructose, and sucrose concentrations were measured every two days. The initial and final cell masses (dry weight) were measured.

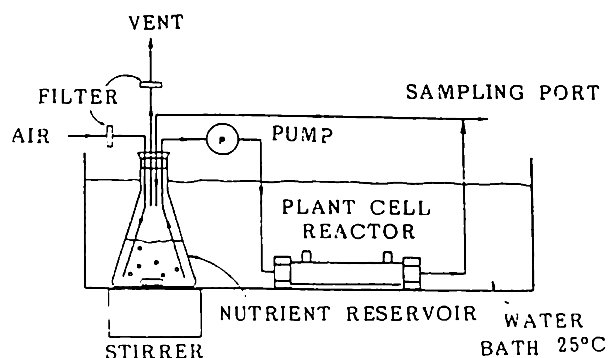


Fig. 2 Setup for the batch operation

2.5 Once-thru experiments

The cells were prepared in the same manner as in the initial rate experiments. Four ml of the resuspended cells was loaded in the reactor. Medium containing 3% sucrose (pH = 4.8 and T = 25°C) was allowed to flow thru the reactor. The effluent was analyzed for glucose at different flow rates (0.1 to 10 ml/min) until a steady state value was obtained. The dry cell mass was determined at the end of a set of runs.

2.6 Continuous mode of operation

The total recycle system was modified by adding a fresh feed input to the reservoir and at the same time withdrawing an equivalent amount of effluent. The setup was autoclaved and cooled before cell loading and filling with the medium which was cold-sterilized with a 0.2 μm filter. During operation, sterile medium was pumped to the reservoir at 5 ml/hr using a peristaltic pump, at the same time that 5 ml/hr was being withdrawn from the system, with 100 ml of fluid kept in the reservoir. The recirculation rate through the reactor was 10 ml/min. The effluent was continuously collected in an automatic fraction collector. The run was repeated for a large reactor (with 20 ml extracapillary space) with feed flow rate at 20 ml/hr and a recycle rate of 50 ml/min.

2.7 Analytical procedures

The phenolics determination was adapted from that given by Hillis and Swain (3) using gallic acid as the standard. Glucose was analyzed using an instrument that employs immobilized glucose oxidase. Sucrose was analyzed by hydrolyzing with invertase and determining the glucose. The fructose was determined by difference ((mols total reducing sugars - mols free glucose)/2). Reducing sugars was determined by the method of Somyogi (10) and Nelson (8). For cell mass determination, the cells were filtered through a weighed glass fiber filter, washed with distilled water, and dried in an oven at 65°C until a constant weight was obtained.

3. Results and Discussion

Plant cells produce phenolics and have acid invertase enzyme at the cell wall as well as in the interior of the cell and we used this reactions to follow the activity of the cells. We compared the performance of the cell suspension with that entrapped in hollow fiber reactors. The mass of the cells in the reactor increased only 3 fold after 20 days while the free cells increased to over 100 fold. The free cells showed a long lag phase, a logarithmic phase, and a stationary phase. The hollow fibers in effect extended the lag phase of the cells. Simple entrapment is thus seen to reduce the growth while maintaining viability.

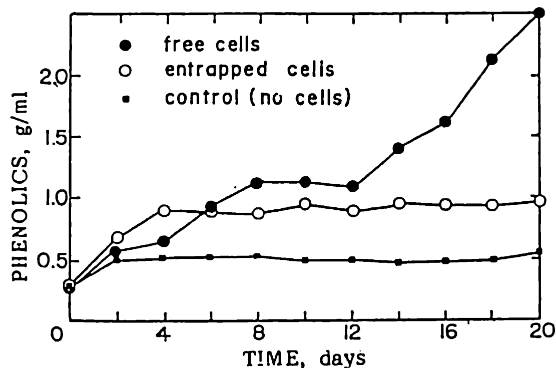


Fig. 3 Phenolics production

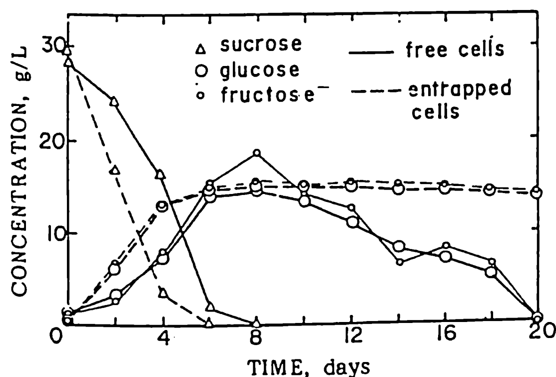


Fig. 4 Sucrose hydrolysis by free and entrapped cells.

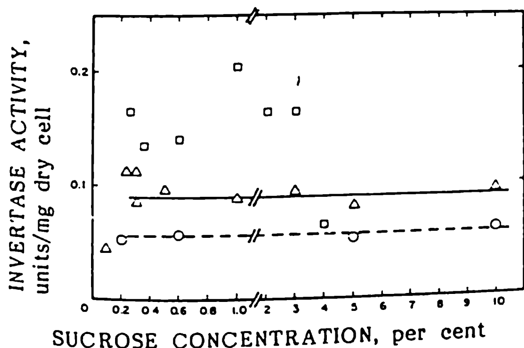


Fig. 5 Initial rate data.

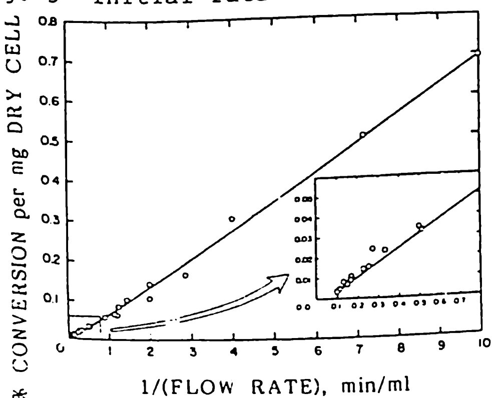


Fig. 6 Data for once-thru experiments.

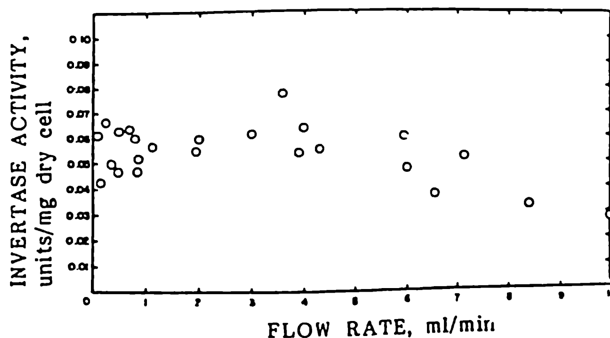


Fig. 7 Invertase activity versus flow rate. Data obtained from once-thru experiments.

We originally intended to focus the investigation on the production of phenolics by Daucus carota but as Fig. 3 shows, the cells do not secrete significant amounts of phenolics. The immobilized cells maintained a constant concentration of 0.8 µg/ml starting at day four (which in effect is only 0.3 µg/ml considering that the control showed 0.5 µg/ml). The medium of the free cells however showed increasing concentration of phenolics. This is mainly due to the stress on the cells caused by nutrient depletion.

Sucrose hydrolysis to glucose and fructose for both free and entrapped cells are shown in Fig. 4. Both systems converted the sucrose completely with six to eight days. The free cells depleted the sugars after 20 days while the entrapped cells utilized only a small fraction of the total sugars. The initial mass of the entrapped cells was twice that of the free cells, and in effect, the free cells was converting sucrose at a slightly faster rate. The difference can be attributed to some inefficiency of cell distribution in the reactor, where the cells had a tendency to settle on the "bottom" side of the horizontal reactor resulting on a decreased effective contact of membrane area with the plant cells. In some runs, we alternated the regular medium alternated with plain 3% sucrose solution every seven days. Sucrose was hydrolyzed every 6 days and the cells were viable up to 28 days.

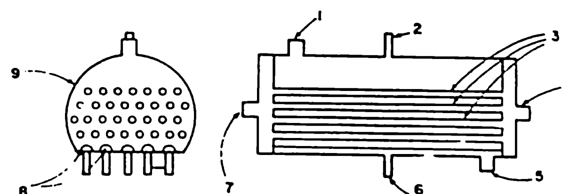
Initial rate experiments with free cells yielded inconsistent results for cells from different batches. For the sucrose concentration range from 0.2 to 10%, a zero-order reaction is indicated (two different batches with points represented by Δ and O respectively in Fig. 5). A wide scatter of points each of a different batch is indicated by □. The variability in activity was mainly due to aggregate size as well as the aggregate size distribution. Maintaining a uniform size distribution for all the runs was difficult.

By using once-thru experiments, the actual reaction rates through the reactor as a function of flow rate can be obtained. To avoid the variation of activity encountered in the initial rate experiments, a set of run was done on a single batch of cells (same batch represented by △ in Fig. 5). Fig. 6 is a typical result which shows % conversion versus the reciprocal of flow rate. The plot is a straight line, indicating that the reaction is still zero order and the diffusional limitation of the hollow fibers is minimal.

The data of Fig. 6 can be converted to invertase activity units per mg dry cell at different flow rates. The normalization is shown in Fig. 7. The batch of cells is the same for as the batch for the data represented by in Fig. 5 (0.09 activity unit per mg dry cell). The values in Fig. 7 are well below the value of 0.09 activity unit per mg dry cell and represent 50 to 70% of the values from initial rate experiments. The discrepancy can be attributed to the inefficient cell distribution within the extracapillary space of the reactor. As was mentioned above, the plant cells always settled after they were loaded and only the half bottom of the fiber bundle (horizontal) were in intimate contact with the plant cells. This in effect gave only a fraction of the bundle to be working. Hence, the inefficiency is observed. Continuous mode of operation was done with small and large reactors which showed consistent performances.

4. Conclusion and Recommendations

We have demonstrated that the hollow fiber cartridge can used as a plant cell bioreactor. The entrapment is gentle, the membrane barrier prevents any cell washout, and large-scale hollow fiber units are commercially available. However, hollow fibers are too fragile for long-term use. Moreover, they are expensive as they are intended for ultrafiltration and other uses. Since plant cells are larger than 25 μm, membranes with larger pores can be used. Therefore, a modified design such as the porous-tube bioreactor (See Fig. 8 and Fig. 9.) is proposed. The hollow fibers are replaced by porous tubes (ceramic, stainless steel, or plastic) with pore size of 15 to 50 μm. High density cell suspension is loaded at the top port and fills the shell to the level of the top row of tubes. Air is bubbled through the shell to provide oxygen and agitation. The production medium passes through the porous tubes. Part of the outlet stream is recycled back. The reactor can be used to recover products from the outlet stream (extracellular products) or plant cells can be harvested from the reactor for extraction of intracellular products. We hope to demonstrate a prototype soon.



1 PORT FOR LOADING PLANT CELLS
2 AIR OUTLET
3 POROUS TUBES
4 MEDIUM OUTLET
5 OUTLET FOR PLANT CELLS
6 AIR INLET
7 MEDIUM INLET
8 AIR SPARGER
9 SHELL

Fig. 9 Porous Tube Bioreactor

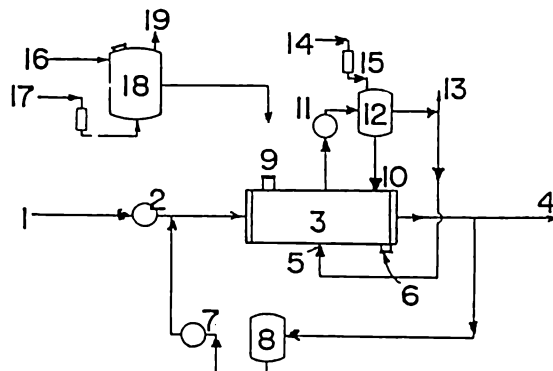


Fig. 10 Proposed New Bioreactor System. 1-Sterile production medium 2- 7- 11-Pump 3-Porous tube bioreactor 4-Stream to product recovery 5-Air sparger 6-Outlet for plant cells 8-Holding tank 9-Inlet for loading plant cells 10-Condensate return 12-Air tank 13-Gas purge 14-Make-up air 15-Filter 16-Sterile growth medium 17-Air 18-Free cells batch tank 19-Vent

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