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Full Length Research Paper

A study of acetic and propionic acids from the hydrolyzed sugars obtained from lignocellulosic wastes

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Immobilized cell reactor (ICR) was developed as a novel bioreactor to convert hydrolyzed sugars to organic acids. Sugar fermentation by *Propionibacterium acid-propionici* entraped by calcium alginate was carried out in continuous mode to produce propionic and acetic acids. In continuous fermentation, more than 90 percent of glucose conversion and 60 percent conversion of xylose were obtained at a retention time of 28 h. The present research has demonstrated that the microorganism preferred glucose as carbon source over other carbon sources. The highest sugar concentration (120 g/L) in the ICR column was successfully converted to propionic acid. The achieved results in ICR with high substrate concentration are promising for scale up operation.

Key words: Enzyme, whole cell, immobilized cell reactor, bioreactor, entrapment.

INTRODUCTION

Renewable resources such as biomass can be converted to sugar via hydrolysis, which in turn can be used as feed stocks for the production of chemicals through fermen-tation. Methane and other chemicals such as ethanol, butanol, acetone and organic acids are produced via fermentation processes (Baily and Ollis, 1986; Chum and Overned, 2001; Chibata, 1983; Yamada et al., 2002). Generally in a bioprocess, it is well known that the activities of free enzyme and cell may gradually decline in a bioreactor (Gui et al., 2007; Gikas and Livingston, 1997; Galazzo and Bailey, 1990). In order to stabilize and reuse the biocatalysts for longer duration without losing in downstream, immobilization was the method of choice which was introduced in past two decades (Guy and Thierry, 2004; Yuan et al., 2002). In immobilization of enzymes and microorganisms solidification or solid support was needed to link the protein or entrap the cell. These techniques were gradually introduced into micro-biology and biotechnology (Nagodawithana and Steinkraus, 1976; Xu et al., 2005). As for immobilization of enzymes and free cells, two broad types of methods have been used: attachment to a solid support and entra-pment (Willaert and Baron, 1993; Najafpour et al., 2004).

In an immobilization technique, the cell wall of a microorganism is linked by a chemical agent to the cell wall of the others. Glutaraldehyde was used as the linking chemical agent (Najafpour, 2007; Vega et al., 1988; Yu et al., 2007). This mechanism may explain the stability of the linkages, but the chemical linkage is toxic and growth inhibitor may influence the microbial process (Yamada et al., 2002). These reactions are only partly understood and often lead to decay or death of the microorganisms. The toxicity may be retarded if the binding and linking of agents goes through phase transition, if the chemical be insoluble and solid state then the chance of inhibition is limited (Nagodawithana and Steinkraus, 1976; Najafpour et al., 2004).

The most extensively studied method in cell immobili-zation is the entrapment of microbial cells in a polymer matrix (Najafpour, 1987, 1990). The common matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, photo cross-linkable resins and polyacrylamide. Among all of the above stated ma-trices, polyacrylamide, alginate, and k-carrageenan are widely used (Najafpour et al., 2004; Rafael et al., 2006).

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Table 1. Main application fields of immobilized cell cultures (Najafpour, 2007).

Product	Examples
Enzymes	α-Amylases, cellulase and other cellulolytic enzymes, chitinolytic enzymes, cyclodextrin, glucosyltransferase, l-glutaminase, inulase, lipases, penicillin V acylase, peroxidases, polymethylgalacturonase, alkaline and acid proteases, pullulanases, ribonuclease and xylanase.
Antibiotics	Ampicillin, candicidin, cephalosporin C, clavulanic acid, cyclosporin A, daunorubicin, divercin, kasugamycin, nikkomycin, nisin Z, oxytetracyclin, patulin, penicillin G and rifamycin B
Steroids[a], amino acids,	Androstenedione, hydrocortisone, prednisolone, progesterone, Alanine, arginine, aspartic acid, cysteine, glutamic acid, phenylalanine, serine, tryptophan
Organic acids, alcohols and polysaccharides	Acetic, citric, fumaric, gluconic, lactic, malic, propionic acids Butanol, ethanol, sorbitol, xylitol Alginate, dextran, levan, pullulan, sulfated exopolysaccharides Pigments, vitamins, flavors and aroma.
Environment water treatment	Carbon removal (COD), nitrogen removal (nitrification/denitrification, assimilation), heavy metal removal (Au, Cd, Cu, Ni, Pb, Sr, Th, U, etc), pollutant iodegradation (phenol and phenolic compounds, polycyclic aromatics, heterocycles, cyanide compounds, surfactants, hydrocarbons and oily products.
Biofertilisation	Soil inoculation with plant growth-promoting organisms (*Azospirillum rasilense*, *Bradyrhizobium japonicum*, *Glomus deserticola*, *Pseudomonas fluorescens* and *Yarowia lipolytica*).
Bioremediation	Degradation of pollutants in contaminated soils (e.g. chlorinated phenols), quifers and marine habitats (e.g. petroleum hydrocarbons) by microbial inocula.
Alternative fuels	Dihydrogen and methane productions, ethanol production, biofuel cells
Food processing, alcoholic beverages, milk products	Brewing, vinification, fermentation of cider and kefir, controlled *in situ* generation of bioflavors continuous inoculation of milk (lactic starters), lactose hydrolysis in milk whey.
Biosensors Electrochemical[b]	Acetic acid, acrylinitrile, amino acids, BOD, cyanide, cholesterol, chlorinated aliphatic compounds, ethanol, naphthalene, nitrate, phenolic compounds, phosphate, pyruvate, sugars, sulfuric acid (corrosion monitoring), uric acid, herbicides, pesticides, vitamins, toxicity assays.
Optical	Herbicides, metals, genotoxicant, polyaromatics and toxicity testing.

[a]Obtained by conversion of steroid parent compounds.
[b]

The calcium alginate gel-entrapment is used for the fixation of highly active enzymes (Najafpour, 1987; Riley et al., 1996). Preparation of a uniform calcium alginate gel necessitates maintaining the viscosity of the mixture of calcium alginate and yeast cells between 1000 and 2000 centipoises. Addition of a nonionic surfactant and an unsaturated fatty acid at the time of gel formation is also found to improve the whole cell and enzyme activities (Senthuran et al., 1997).

In the process of immobilization, spherical gel beads were prepared as in the case of calcium alginate gels. The spherical gels are readily obtained by adding sodium alginate solution to calcium chloride solution using a needle. The flask is filled with a calcium chloride solution prior to fermentation, and the dense propagated culture is mixed with sodium alginate solution, added drop wise to form granules. The culture medium is then supplied to the fermentor to initiate the fermentation (Summers, 1924; Takamitsu et al., 1993). The main applications of the immobilized cells and enzyme are summarized in Table 1.

An alternative processes for minimizing propionic acid production costs have been considered in the present research. The purpose of this paper is to produce acetic and propionic acids from the hydrolyzed sugars obtained from lignocellulosic wastes. The carbohydrates were obtained from agro solid wastes by acid hydrolysis. The organic acids were obtained via active biocatalysts which was immobilized *Propionibacterium* spices. The cell walls were cross linked to retain the whole cells inside the immobilized cell reactor (ICR). The hydrolyzed sugars were produced in a continuous process. In this paper, the effects of substrate concentration, retention time on the yield of organic acids production were extensively investigated.

MATERIAL AND METHODS

Experimental reactor system

The ICR was a plug flow tubular column, constructed with a nominal diameter of 5 cm, ID of 4.6 cm, Plexiglas of 3 mm wall thickness and 110 cm length. The medium was fed to the ICR column from a feed tank located above the column. A variable-speed Master flex

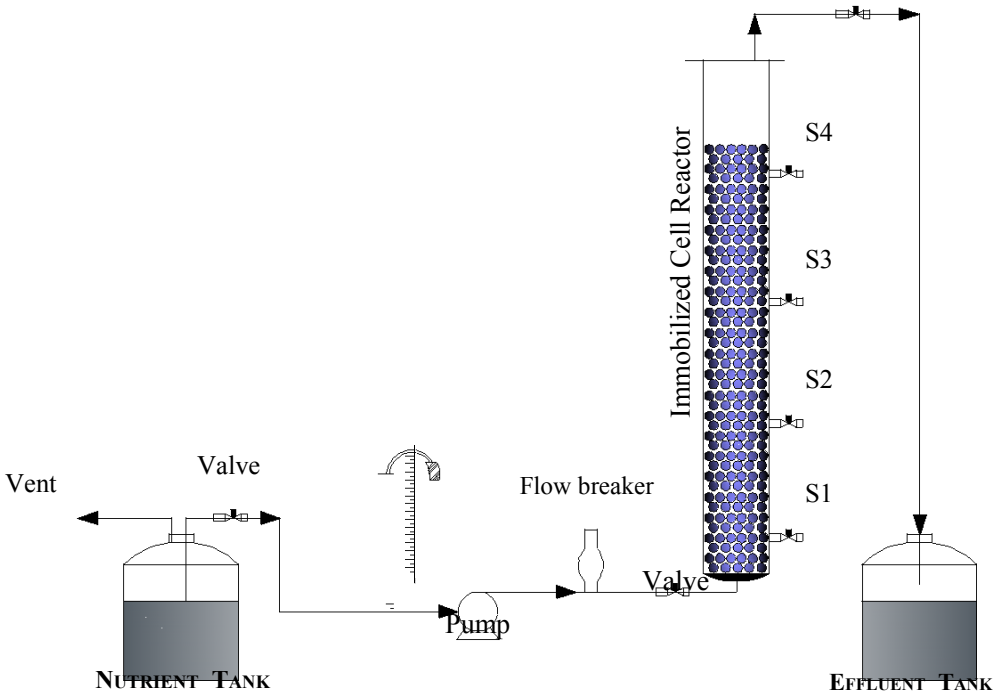

Figure 1. Schematic diagram of ICR experimental setup.

pump, model L/S easy load (Cole-Parmer, Vernon Hills, IL, USA) was used to transfer feed medium from a 20-liter polypropylene autoclaveable Nalgene carboy (Cole- Parmer, Vernon Hills, IL, USA), with the carboy serving as reservoir. The effluent from the column was collected in a 20-liter polypropylene autoclaveable carboy serving as product reservoir. A flow breaker was installed between the column and feed pump, which prevented the growth of microorganism and contamination of feed line and feed tank. The samples from the ICR column were taken from the inlet and outlet compartments of the column.

A 16 h seed culture was harvested at exponential growth phase and the slurry of the microbial culture was converted to droplet form while it was dripped into a 6% bath of calcium chloride using a 50 ml syringe. The bed consisted of uniformly packed 5 mm beads. The solidified beads were transferred to the column. About 75% of the column was packed. The extra space was counted for bed expansion by the fresh media. The void volume was measured by volume of distilled water pumped through the bed. The packed ICR column was used in continuous mode for the duration of fermentation. The fresh feed was pumped in an upflow manner, while sugar and propionic acid concentration was monitored during the course of continuous fermentation. The working volume of ICR after random packing was 960 ml. The bed volume was about 860 ml. The experimental set-up of ICR is shown in Figure 1.

There was no evidence of cell leakage from the beads to the surrounding media, the matrix was permeable to substrate and product, the cell growth and glucose conversion were monitored in the ICR. Overgrowth of beads after a few days of operation was controlled. Carbon dioxide was purged to eliminate overgrowth of beads. The major overgrowth occurred at the entrance region, about the first 30 cm length of the column where the sugar concentration was very high. High sugar concentration of 30, 60, and 120 g/L were used. The microbial overgrowth was controlled with carbon dioxide passing through the bed. There was a maximum 30% increase in the beads diameter at the lower part of column, where the sugar concentration was dominating in this region. The void volume was measured by passing sterilized water. In addition to carbon source, the feeding media consisting of 1 g/L yeast extract was pumped from the bottom of the reactor, while the flow rate was constant for a minimum duration of 5 h.

A seed culture of *Propionibacterium acid-propionici* (American Type Culture Collection, Manassas, VA, USA) was grown in a media of 5 g of glucose, and 0.5 g of yeast extract, respectively, and 1.5 g of KH_2PO_4 and 2.25 g of Na_2PO_4 phosphate buffer up to a total volume of distilled water, 500 ml. The media were sterilized at 121ºC for 15 min. The stock culture of the microorganisms was transferred to the broth media for preparation of seed culture. Sodium alginate (Fisher Scientific, Manchester, UK) was prepared by dissolving 10 g of powder form in 500 ml of distilled water. A separate solution of 120 g of calcium chloride was made in 2 L distilled water. Sodium alginate and calcium chloride solution were autoclaved at 121ºC for 15 min. The sterilized sodium alginate solution and the high cell density of the grown seed culture were thoroughly mixed. Beads were prepared by droplet from a pipette about 5 mm diameter in a sterilized calcium chloride solution. The cell density of seed culture for bead preparation was 2.78 g/L. The wet and dry weights of a 16 h incubated beads sample were 2.8 and 0.42 g, respectively. The moisture content of the beads was 85%. Several methods developed for various technique of immobilization with or without support are shown in Table 2. Also Table 3 summarizes application of immobilization of the microbial cells, where the cells were covalently linked to various supports.

Sugar concentration determination

To determine the concentration of inlet and outlet sugar in the ICR, a reducing chemical reagent, 3,5-dinitrosalicylic acid (98%) solution (DNS) was used (Miller, 1959; Summers, 1924). The DNS solution was prepared by dissolving 10 g of 3,5- dinitrosalicylic acid in 2 M

Table 2. Methods of immobilization.

Attachment	Method/support
Without support	Aggregation of floc for formation of cross-linking
With support	Covalent binding
	Adsorption to ion-exchangers or inorganic molecules
	Biofilm formation
Entrapment	Organic polymer
	Inorganic polymer
	Semi-permeable membrane

Table 3. Microbial cells covalently linked to various supports (Najafpour, 2007).

Microbial species	Support	Product
Acetobactor	Metal hydroxides	Acetic acid
Aspergillus niger	Glycidyl methacrylate	Gluconic acid
Micrococcus luteus	CM-cellulose	Urocanic acid
Saccharomyces cerevisiae	Aminioproply silica	Ethanol
Saccharomyces cerevisiae	Hydrocyalkyl methacrylate	Killer toxin
Saccharomyces cerevisiae	Cellulose	Ethanol
Zygosaccharomyces lactic	Hydroxyalkyl methacrylate	β-galactosidase

sodium hydroxide solution. A separate solution of 300 g sodium potassium tartrate solution was prepared in 300 ml of distilled water. The hot alkaline 3, 5-dinitrosalicylate solution was added to sodium potassium tartrate solution. The final volume of DNS solution was made up to one liter with distilled water. The calibration curve was prepared with 2 g/l sugar solution (Najafpour, 2007; Vega et al., 1988).

Detection of organic acids

Propionic and acetic acids production in the fermentation process were detected with gas chromatography, HP 5890 series II, (Hewlett-Packard, Avondale, PA, USA) equipped with flame ionization detector (FID) and GC column Porapak QS (Alltech Associates Inc., Deerfield, IL, USA) 100/120 mesh. The oven and detector temperature were 175 and 185ºC, respectively. Nitrogen gas was used as a carrier gas. Isopropanol was used as an internal standard.

Microbial cell dry weight and optical density

In batch fermentation, approximately 2 ml sample was harvested every 2 h. The absorbance of each sample during batch fermentation was measured at 620 nm using spectrophotometer, Cecil 1000 series (Cecil Instruments, Cambridge, England). The cell dry weight was obtained using a calibration curve. The cell dry weight was proportional to cell turbidity and absorbance at 620 nm. The cell concentration (dry weight) of 2.1 g/L was obtained from the 24 h culture broth, the free cell samples with absorbance of 1.6 at 620 nm.

In an immobilized cell bioreactor, cells are located close to the nutrient supply, which are likely to maintain higher quality and activity than cells located relatively further away, leading to differentiation in the quality or activity of the immobilized cell population. Typical approaches for measuring diffusivities in immobilized cell systems include bead methods, diffusion chambers and holographic laser interferometry. These methods can be applied to various support materials, but they are time consuming, making it tedious to measure effective diffusivity (D_{eff}) over a wide range of cell fractions (Table 4). For a few systems, the diffusivity ratios are very large (∞). The infinite diffusivity ratio corresponds to a diffusing solute that does not enter the cells (i.e. Dc=0).

RESULTS AND DISCUSSION

The fermentation was performed with pentose and hexose sugars (glucose and xylose) at various concentrations. The flow rate was measured by a volumetric flow-meter. The total initial feed sugars concentration in this study were 30, 60 and 120 g/L. Glucose-xylose mixture was fermented for production of acetic and propionic acids. The sugar consumption profiles, at various retention of time of 5, 12 and 28 h with fixed amount of glucose and xylose (15 g/L of each sugar) along the length of the ICR system are presented in Figure 2. The total sugars consumption at various retention times and the feed media temperature set at 36^{o}C were investigated. The pH of the fresh feed media was adjusted to 7, using a phosphate buffer, but it was found that within the reactor the pH value was drastically dropped to a level of about 5.8. The dash lines show the glucose concentration, and the solid lines are related to xylose consumption trend. It is obvious that glucose was consumed rapidly. The conversion rate in the ICR increased with increasing retention time. As shown in Figure 2, at retention time of 28 h, more than 90 and 60 percent of glucose and xylose were consumed, respectively. At a 12 h retention time, the conversion for glucose

Table 4. Experimental studies of diffusion in immobilized cell and values of D_0 / D_C (Najafpour, 2007).

Cell type	Immobilization support	Substrate	D_0 / D_C
Saccharomyces cerevisiae	Ca-alginate	Glucose	0.1
Baker's yeast	Ca-alginate	Glucose	10.6
Ehrlich ascites tumor	Agar, collagen	Glucose	2.4
Zymomonas mobilis	K-carrageenan Ca-alginate	Glucose	∞
Pseudomonas aerogunosa	Ca-alginate	Glucose	2.3
Saccharomyces cerevisiae	Ca-alginate	Glucose	2.8
Plant	Ca-alginate	Glucose	∞
Baker's yeast	Ca-alginate	Glucose	15.8
Zymomonas mobilis	Ca-alginate	Glucose	∞
Baker's yeast	Ca-alginate	Glucose	∞
Ehrlich ascites tumor	Agar, collagen	Lactic acid	6.7
Clostridium butyricum	Polyacrylamide, agar collagen	Hydrogen	3.2
Escherichia coli	Natural aggregate	Nitrous oxide	3.9
Saccharomyces cerevisiae	Fermentation media	Oxygen	2.3
Saccharomyces cerevisiae	Ca-alginate, Ba-alginate	Oxygen	0.1
Escherichia coli	Fermentation media	Oxygen	2.2
Penicillium chrysogenum	Fermentation media	Oxygen	5.6
Bacillus amilaliquefaciens	Ca-alginate, PVA-SbQ gel	Oxygen	1.8
Saccharomyces cerevisiae	Ca-alginate	Ethanol	0.1
Baker's yeast	Ca-alginate	Ethanol	∞

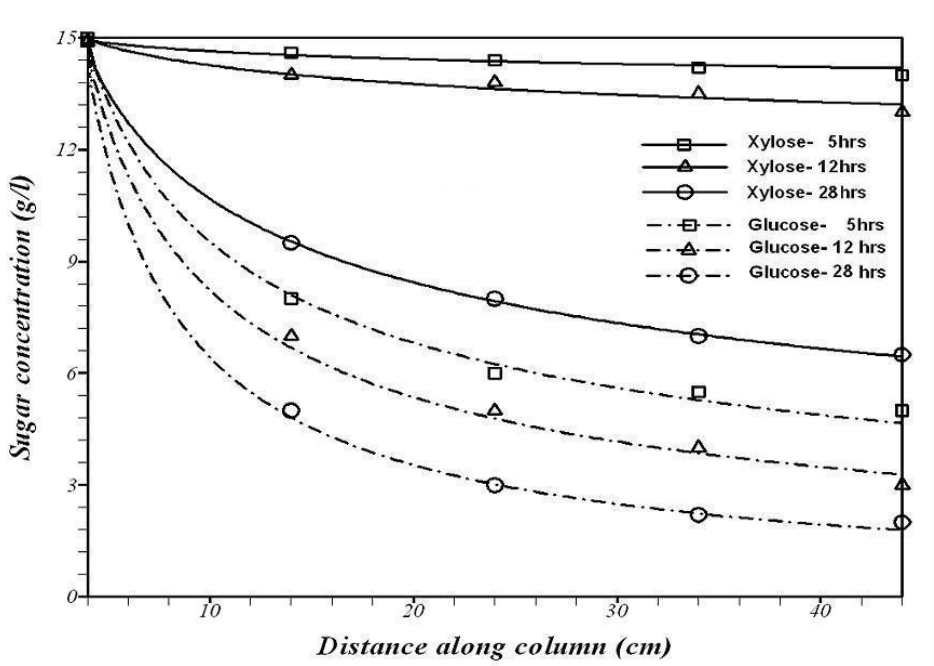

Figure 2. Sugar composition in the ICR fermentation, using *Propionibacterium acid-propionici* entraped by calcium alginate, at 5, 12 and 28 h retention time.

and xylose dropped to 75 and 20 percents, respectively.

The sugar consumption with total concentration of 30, 60 and 120 g/L at pH 7 and 36^{o}C is shown in Figure 3.

The experiments were initially investigated for sugar consumption at a constant retention time of 28 h. This retention time was chosen because of high conversion

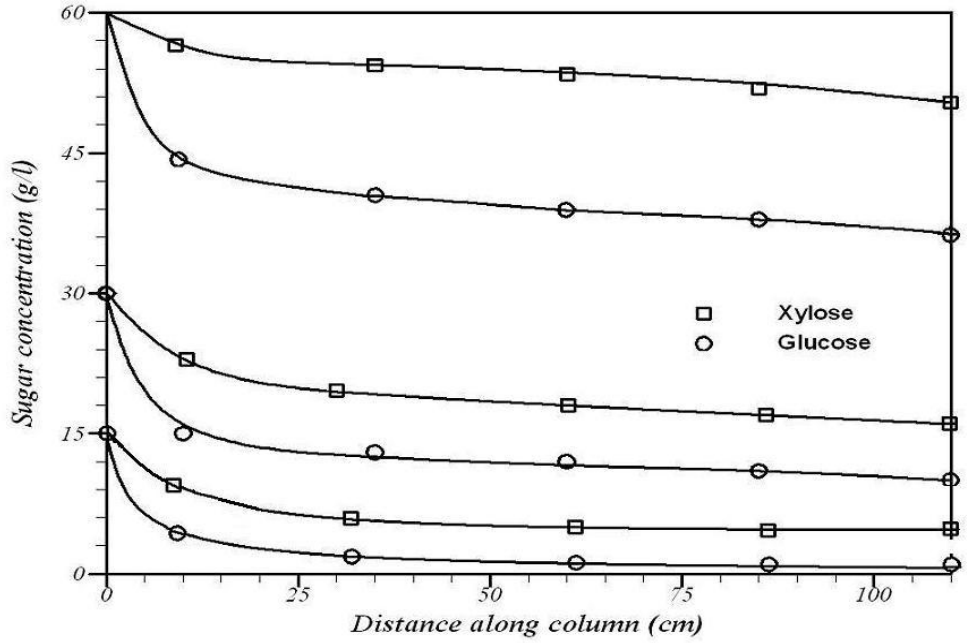

Figure 3. Sugar composition in the ICR fermentation, using *Propionibacterium acid-propionici* entraped by calcium alginate, at 28 h retention time.

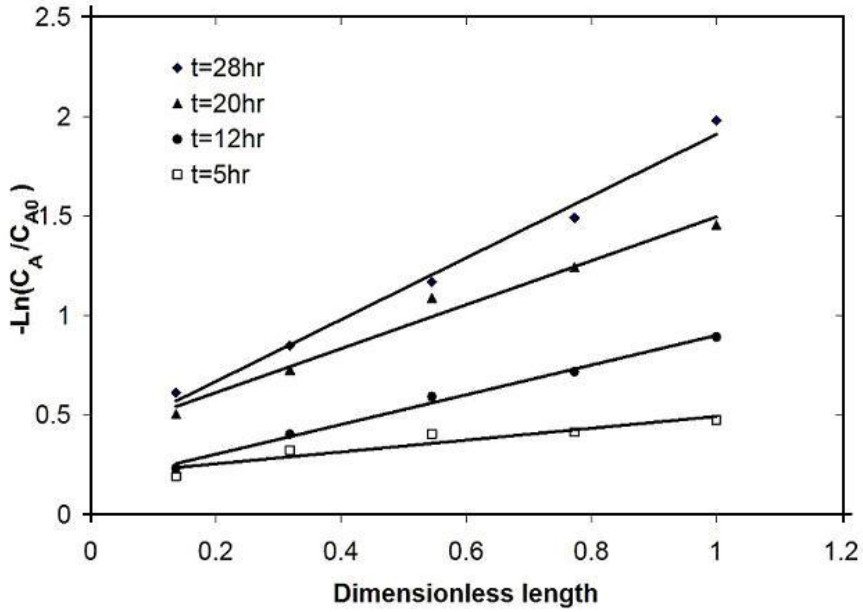

Figure 4. Model test for ICR using *Propionicbacterium acidi-propionici* entraped by calcium alginate at diferent retention times.

level of glucose and xylose. The sugar consumption trends for various concentration of glucose and xylose (30, 60 and 120 g/l) were similar, with a sharp reduction of substrate occurring in the first 25 cm distance along the length of column. A 90 percent conversion of glucose and 60 percent conversion of xylose were obtained with 30 g/L of the total sugars. For the 60 g/L concentration of the total sugar, the glucose conservation was about 63 percent and the conservation of xylose was 46 percent. As the total sugar increased to 120 g/L, the conversion rate of glucose decreased to 41 percent and also the xylose conservation dropped to 17 percent. The results

shown that the longer columns most probably did not help for such cases since the curves as proceeded, flatten out at a nearly constant sugar concentration after a short distance of the column.

Figure 4 shows a plot of linear model, $\ln (C_A / C_{AO})$ as a function of dimensionless column length; the fermentation data obtained at a feed concentration of 15 g/L glucose and 15 g/L xylose at various retention times. As the experimental data were observed, a straight line was obtained at a fixed retention time. The values of the slope were increased with increasing the retention time due to decrease of flow velocity through the column. By increasing the retention time, the effect of organic acid production in ICR system were influenced. As the retention time increased, the acid production was increased. The obtained experimental data were perfectly fitted with the projected linear model.

Conclusions

The use of immobilized cells in industrial processes has attracted attention due to advantages such as an increase in yield and cellular stability and a decrease of process expenses due to the ease for cell recovery and reutilization over traditional processes. This research has shown that both glucose and xylose, which are rich in corn stock hydrolysate can be used equally well to produce propionic acid, but using glucose as carbon source is more efficient than xylose. The immobilized cells could be reused for at least 60 days retaining about 95% of its original activity. Hence there is a great potential for the use of immobilized *P. acid-propionici* cells for the biosynthesis of industrial organic acid. It is feasible to produce propionic acid from mixed sugars and the immobilized method could be a useful model for other biochemical reactions with microbial cells.

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