

# BIOTECHNOLOGY PROGRESS

## Biological Production of Acrylic Acid from Cheese Whey by Resting Cells of *Clostridium propionicum*

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Biological production of acrylic acid from cheese whey has been demonstrated. Lactose in sweet whey fortified with yeast extract was first converted to a stoichiometric mixture of propionic and acetic acids in 70 h by a coculture of *Lactobacillus bulgaricus* and *Propionibacterium shermanii*. Further conversion of propionate to acrylate was accomplished by resting cells of *Clostridium propionicum* in systems in which methylene blue acted as an electron acceptor. A maximum acrylate yield of 0.133 mmol/g of wet cell was achieved for periods of 6 h, after which resting cell activity declined substantially. Bioconversion of propionate to acrylate could also be carried out by cells immobilized in calcium alginate beads with no decrease in cell productivity or initial reaction rate.

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### Introduction

Renewable resources could represent an alternative source of many industrial chemicals if the appropriate biological pathways of suitable microorganisms are considered. The present capabilities of genetic engineering for the transfer of specific catalytic functions between organisms can only provide increased opportunities for the future manufacture of chemical commodities from biomass. In the U.S., over 50 billion lb of fluid whey are produced annually (American Dairy Products Institute, 1986) as the main byproduct of cheese manufacture. Although an excellent source of lactose and protein, only 45% of the whey produced in the U.S. is further utilized. Consequently, whey processing and disposal have long constituted a major problem for the dairy industry. Acrylic acid is a billion pound per year industrial feedstock presently derived from petroleum. The objective of this study was to evaluate the biological production of acrylic acid from cheese whey via a two-step process in which the lactose is first fermented to a mixture of propionic and acetic acids. In the second stage, propionate is oxidized to acrylate by resting cells of *Clostridium propionicum*.

The first-stage whey fermentation was accomplished by a coculture of *Lactobacillus bulgaricus* and *Propionibacterium freudenreichii* subsp. *shermanii* (hereafter *P. shermanii*). The growth and metabolism of these organisms have been recently reviewed (Marshall and Law, 1983; Moat, 1985) and extensively studied due to their importance in the production of yogurt and cheese, respectively. *C. propionicum* is an obligate anaerobe capable of growth on amino acids such as alanine, serine, and threonine as well as lactate, pyruvate, and acrylate (Cardon and Barker, 1946). Carbohydrates are not fermented. The metabolism of *C. propionicum* is unusual in that it produces propionic acid through direct reduction of lactate rather than by the propionate-succinate pathway. Recent investigations (Akedo et al., 1983; Schweiger and Buckel, 1985) have strongly suggested that an acrylate moiety is an intermediate in the direct reduction of lactate to propionate in *C. propionicum* (lactate → lactyl-CoA → acrylyl-CoA → propionyl-CoA → propionate) and provides the basis for a possible biological route to acrylic acid from whey. Conditions for the bioconversion of propionate to acrylate by this organism have been developed by Dalal et al. (1980). In this study, sweet whey was used as the initial substrate for acrylate production. The bioconversion of propionate to acrylate by resting cells of *C. propionicum* was studied in two systems: in a free cell suspension and immobilized in calcium alginate beads.

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**Table I. Optimization of Lactic Acid Production by *L. bulgaricus***

experimental conditions			initial	initial	final	yield <sup>b</sup>
lactose, <sup>a</sup> %	yeast, %	temp, °C	[lactose], mM	[lactate], mM	[lactate], mM	
2	0	35	54.2	11.6	209.6	0.91
			64.6	12.3	247.8	0.91
2	0	45	54.5	11.4	203.8	0.88
			64.3	11.2	237.8	0.88
2	1	35	55.9	10.6	216.3	0.92
			64.0	12.1	223.3	0.82
2 <sup>c</sup>	1	45	55.3	11.7	222.7	0.95
5	0	35	137.1	19.2	431.9	0.75
			153.5	22.8	486.7	0.76
5	0	45	138.0	24.2	430.9	0.74
			161.1	20.8	424.4	0.63
5	1	35	136.6	22.2	569.0	1.00
			160.5	19.1	637.8	0.96
5 <sup>c</sup>	1	45	137.4	22.1	572.7	1.00

<sup>a</sup> Lactose from spray-dried whey. <sup>b</sup> Yield = (final [lactate] - initial [lactate])/4 initial [lactose]. Stoichiometry: lactose → 4 lactate.  
<sup>c</sup> Not duplicated.

## Materials and Methods

**Organisms.** Cultures of *L. bulgaricus* (NRRL B-548), *P. shermanii* (NRRL b-4327), and *Propionibacterium acidi-propionici* (NRRL B-3569) were obtained from the Northern Regional Research Center, U.S. Department of Agriculture (Peoria, IL). *C. propionicum* (ATCC 25522) was purchased from the American Type Culture Collection (Rockville, MD). These organisms were maintained under the following conditions: *L. bulgaricus*, 33 °C, medium consisting of 55 g of lactobacilli MRS broth (Difco; reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned) per liter, pH 6.5; *P. shermanii* and *P. acidi-propionici*, 33 °C, medium consisting of 36 g of spray-dried sweet whey (Atlantic Processing, Inc., Allentown, PA), 10 g of yeast extract (Difco), and 15 g of calcium carbonate per liter, pH 7.1 (lactose concentration in medium was approximately 2.5%); *C. propionicum*, 33 °C, medium consisting of 3 g of L-alanine, 0.2 g of cysteine, 3 g of peptone (Difco), 4 g of yeast extract, 0.5 g of MgSO<sub>4</sub>, 0.01 g of FeSO<sub>4</sub>, 5 mL of a 0.1 M potassium phosphate solution, pH 7.1, 2.5 mL of saturated CaSO<sub>4</sub> solution, and 1 mL of 0.2% resazurin solution per liter. All cultures were transferred three times weekly in test tubes and maintained at all times under anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI).

The plating media for these organisms were as follows: *L. bulgaricus*, MRS broth plus 15 g of Difco bacteriological agar and 7.52 g of Difco lactose per liter (lactose was added to promote growth of cells that had been previously grown in whey or in media containing lactose as the primary carbon source. Since the primary carbon source in MRS broth is glucose, the added lactose reduced the lag phase); *P. shermanii* and *P. acidi-propionici*, 37 g of Difco brain heat infusion medium with 15 g of Difco bacteriological agar per liter; *C. propionicum*, growth medium plus 20 g of Difco bacteriological agar per liter. Plates were incubated at 33 °C in the anaerobic chamber.

**Experimental Procedures.** Shake-flask experiments were employed in studies of the first-stage fermentation of sweet whey to propionic and acetic acids. Experiments were conducted in 250-mL trypsinizing flasks (Bellco Glass, Vineland, NJ) with internal baffles and a side arm fitted with a rubber septum for sparging of the contents with an anaerobic gas mixture (4% H<sub>2</sub>, 10% CO<sub>2</sub>, balance N<sub>2</sub>). Experiments were conducted to determine the effects of

inoculum size, yeast extract concentration, whey (lactose) content, and temperature on acid yield and fermentation time. The base medium consisted of spray-dried sweet whey powder and yeast extract with calcium carbonate added for pH control. Unless otherwise specified, experiments were initiated with a 10% inoculum of a 24-h culture. *L. bulgaricus* cultures were switched to a whey medium from the glucose maintenance medium prior to an experiment. Upon inoculation of the flask containing sterile media, it was sparged for 3 min with anaerobic gas through a syringe needle in the side-arm septum, placed on an orbital shaker, and incubated at the desired temperature. Samples were withdrawn through the side-arm septum.

All experiments involving *C. propionicum* reported here utilized resting cells grown at pH 7.1 and 33 °C and harvested in the following manner. Growth medium with 8 g of alanine/L was inoculated with 10% (by volume) of a 24-h culture grown in the same medium. Cells were centrifuged at 10000g for 5 min at 10 °C. The cell paste was resuspended in 0.1 M sodium phosphate buffer, pH 7.1, with 0.3% Na<sub>2</sub>S·9 H<sub>2</sub>O and recentrifuged. After decanting of the supernatant, the resulting pellet was returned to the anaerobic chamber and taken up in a small volume of reaction solution buffer and the experiment was initiated. Experiments verified that these cell-harvesting procedures did not result in any significant loss of cells and that all cells were viable at the initiation of experiments.

Resting cells of *C. propionicum* were immobilized in calcium alginate by mixing the cell paste from centrifugation with the appropriate volume of a 22.2 g/L sodium alginate solution and discharging through a syringe into a stirred 20 g/L calcium chloride solution. The resulting beads, 2–3-mm diameter, were washed with 50 mM Tris [Tris(hydroxymethyl)aminomethane] buffer before use.

Resting cell experiments involving free or immobilized cells of *C. propionicum* were conducted in 35-mL glass vials or 150-mL beakers, respectively, with agitation accomplished by a magnetic stirrer. The reaction solution contained 200 mM propionate, 50 mM lactate, 50 mM Tris buffer, and 2 mM methylene blue. Aeration, necessary to reoxidize the reduced form of methylene blue and thus insure an adequate supply of the electron acceptor, was provided through a small diameter polyethylene tube. Unless otherwise stated, experiments were conducted at 23 °C, pH 8.5, and under aeration. Cell concentration in the experiments ranged from 2 × 10<sup>9</sup> to 8 × 10<sup>9</sup> cells/mL. Propionate to acrylate bioconversion yields were determined in a simulated whey broth (SWB) and a fermented whey broth (FWB), both augmented with methylene blue. FWB was the broth that resulted from the fermentation of sweet whey to propionic and acetic acids by a coculture of *L. bulgaricus* and *P. shermanii*. The compositions of these reaction media are as follows: SWB, 264 mM propionate, 34 mM lactate, 50 mM Tris buffer, and 2 mM methylene blue; FWB, 279 mM propionate, 28 mM lactate, 117 mM acetate, 0.5 mM lactose, and 2 mM methylene blue.

**Analytical.** Analyses for organic acids and lactose were performed by gas chromatography or HPLC; detailed procedures are reported elsewhere (O'Brien and Senske, 1989). Cell wet weights were determined by filtration of an aliquot of cell cultures through a 0.2-μm filter.

## Results and Discussion

**Whey Fermentations.** The objective of this phase of the study was to develop a whey-based medium and

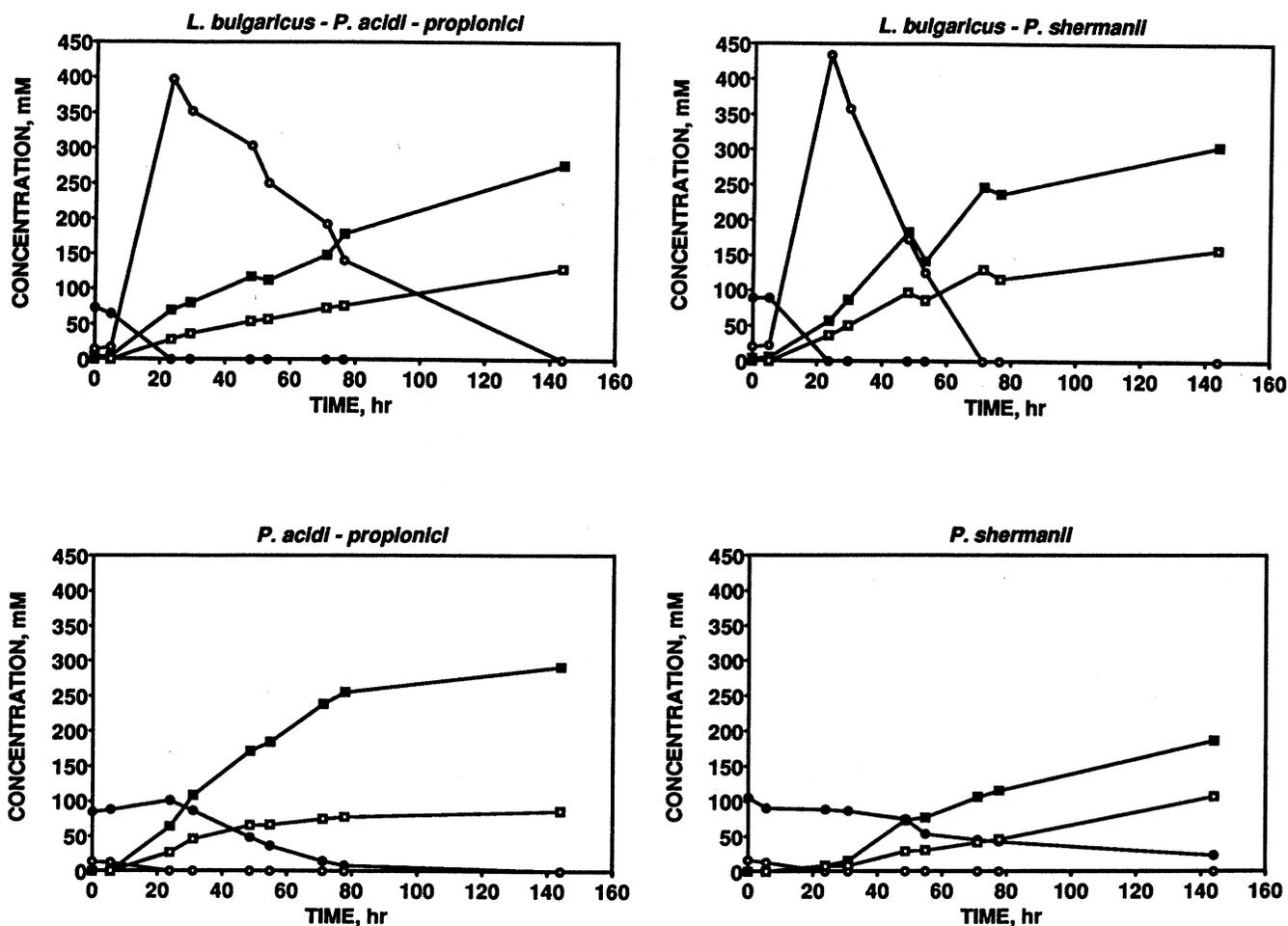


Figure 1. Effect of *L. bulgaricus* coculture on the fermentation of sweet whey by *P. acidipropionici* and *P. shermanii*. Symbols: lactose, ●; lactic acid, ○; propionic acid, ■; acetic acid, □.

fermentation conditions under which lactose is rapidly converted to propionic acid. Cocultures of the homofermentative lactobacillus *L. bulgaricus* with *P. acidipropionici* and *P. shermanii* were chosen for investigation since several studies have shown a reduction in overall fermentation time by this approach (Johns, 1952; Liu and Moon, 1982).

Medium development for *L. bulgaricus* were based upon supplementation of sweet whey with yeast extract. Factorial experiments designed to evaluate the effects of the major variables lactose concentration, yeast extract level, and temperature (35 °C vs 45 °C) on lactate yield and fermentation time demonstrated that practically complete conversion of lactose to lactate can be achieved with yeast extract supplementation (Table I). Under optimum conditions (45 °C, 1% yeast extract), a fermentation yield of 100% was achieved in 12 h.

Typical coculture fermentations of a supplemented whey medium by *L. bulgaricus* and either *P. acidipropionici* or *P. shermanii* are shown in Figure 1. For comparison, pure culture fermentations of the same medium by each propionibacterium are also presented. Coculture did not improve the *P. acidipropionici* fermentation. For *P. shermanii*, the advantages of coculture over pure culture fermentation are a significantly increased acid yield and a substantially reduced fermentation time. In coculture, *P. shermanii* acid yield increased from 70% to 100% (based on lactose) with a propionate/acetate ratio of approximately 2.0, the fermentation time decreased from 145 h to approximately 70 h, and lactose was completely consumed. *P. shermanii* was determined to be the more

suitable organism for coculture with *L. bulgaricus*, on the basis of shorter fermentation time as indicated by complete lactose and/or lactate consumption. Effects such as these are well-known for dairy bacteria (Marshall and Law, 1984; Liu and Moon, 1982) and can be attributed to preference of lactate over glucose by *P. shermanii* (Lee et al., 1974) and cooperative stimulatory effects from products liberated during fermentation. Accordingly, a coculture of *L. bulgaricus* and *P. shermanii* was utilized for propionic acid production from a fortified sweet whey medium.

Further studies were conducted to optimize experimental conditions for the coculture (Table II). These studies utilized a full-strength whey medium containing 5% lactose. Yeast extract concentration, inoculum size, and inoculum ratio did not substantially affect the acid yield in the *L. bulgaricus*-*P. shermanii* fermentation. These experiments established the conditions for the fermentation of a fortified sweet whey medium as a pretreatment for the subsequent bioconversion of propionate to acrylate (Table III).

**Production of Acrylate from Propionate by *C. propionicum* Resting Cells: (A) Free Cell Experiments.** Accumulation of acrylate via oxidation of propionate by *C. propionicum* resting cells can only be accomplished by addition of an electron acceptor to the medium. In the presence of oxygen or other electron acceptors, acrylate is not metabolized by *C. propionicum* (Dalal et al., 1980). Furthermore, consumption of the electron acceptor will allow the formed acrylate to quickly be reduced to propionate. On the basis of prior work by Akedo (1983), methylene blue was chosen as the electron acceptor. Even

**Table II. Acid Production from Sweet Whey by a Coculture of *L. bulgaricus* and *P. shermanii***

yeast ext, %	inoculum		cells, <sup>b</sup> cells/mL		initial [lactose], <sup>c</sup> mM	final [lactate], mM	final [acetate], mM	final [propionate], mM	yield <sup>d</sup>
	size (total), % (v/v)	ratio, <sup>a</sup> v/v	<i>P. shermanii</i>	<i>L. bulgaricus</i>					
1	5	5:3	$9.4 \times 10^7$	$2.2 \times 10^6$	150.6	0	188.3	323.0	0.85
1	5	1:1	$9.6 \times 10^7$	$2.0 \times 10^6$	148.3	0	193.3	325.7	0.88
1	10	5:3	$2.2 \times 10^8$	$9.3 \times 10^5$	146.2	0	173.3	279.7	0.77
1	10	1:1	$1.6 \times 10^8$	$6.2 \times 10^6$	149.1	0	185.0	294.6	0.80
2	5	5:3	$8.3 \times 10^7$	$7.8 \times 10^6$	147.7	0	175.0	260.8	0.74
2	10	5:3	$2.2 \times 10^8$	$7.6 \times 10^6$	145.6	0	183.3	289.2	0.81
2	10	1:1	$1.1 \times 10^7$	$1.7 \times 10^6$	143.6	0	183.3	233.8	0.73
2	5	1:1	$1.0 \times 10^8$	$7.9 \times 10^5$	151.5	0	201.3	246.0	0.74

<sup>a</sup> Ratio of *P. shermanii* to *L. bulgaricus*. <sup>b</sup> Initial concentration of cells in medium. <sup>c</sup> Lactose from spray-dried sweet whey. <sup>d</sup> Based on stoichiometry: lactose  $\rightarrow$   $\frac{2}{3}$  propionate +  $\frac{4}{3}$  acetate +  $\frac{4}{3}$  CO<sub>2</sub>; yield = (final [propionate] + final [acetate]) / ( $\frac{12}{3}$ )(initial [lactose]).

**Table III. Experimental Conditions for Fermentation of Sweet Whey**

medium	6.2% spray-dried sweet whey powder (4.5% lactose in medium)
inoculum	1.0% yeast extract 5% (v/v) <i>L. bulgaricus</i> , $1.1 \times 10^8$ cells/mL (typical) 5% (v/v) <i>P. shermanii</i> , $3.2 \times 10^9$ cells/mL (typical)
temperature	33 °C
pH	7.0
time	50–70 h

**Table IV. Effect of Cell Age on Bioconversion of Propionate to Acrylate by Resting Cells of *C. propionicum*<sup>a</sup>**

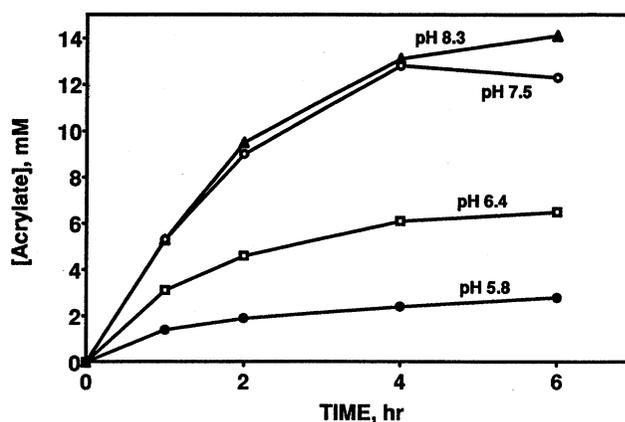
cell age, h <sup>b</sup>	cell wet weight, g	[acrylate], mM <sup>c</sup>	acrylate yield, mmol/g of wet cell
6.5	0.68	3.9	0.034
8.0	0.70	8.5	0.073
12.0	0.80	23.9	0.180
14.0	0.82	30.9	0.226

<sup>a</sup> 167 mM propionate, 37 mM lactate, 42 mM Tris buffer, and 1.7 mM methylene blue, pH 8.70. <sup>b</sup> All cells grown from the same inoculum. <sup>c</sup> Measured after 4 days' incubation (aerobically) on orbital shaker.

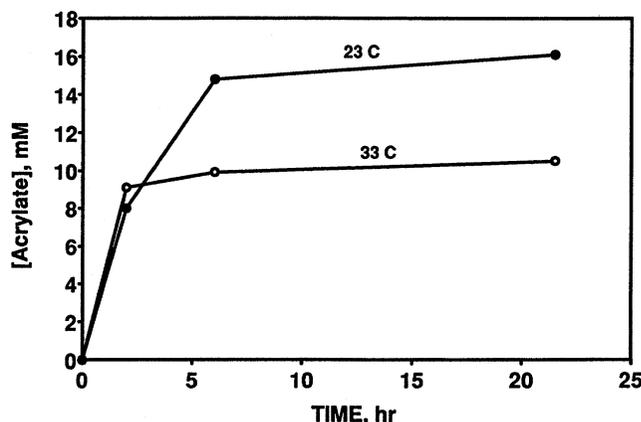
though molecular oxygen can serve as the electron acceptor, only low levels of acrylate are formed, presumably because *C. propionicum* does not have the catalase or superoxide dismutase enzymes to scavenge the free radicals formed. Lactate was also added to synthetic media, since it was found to be stimulatory to acrylate formation (Dalal et al., 1980).

Controlled experiments in synthetic media were conducted to determine the conditions appropriate for maximum productivity of acrylate by *C. propionicum* resting cells. The effect of cell age on acrylate productivity is presented in Table IV. A cell age of 14 h, late exponential growth, was utilized in all further resting cell experiments. The cell concentration at this cell age was approximately  $2 \times 10^8$  cells/mL. The medium pH dramatically affects both the yield of acrylate and the initial rate of formation (Figure 2). *C. propionicum* resting cells grown at 33 °C were incubated with propionate, lactate, and methylene blue at 23 and 33 °C (Figure 3). The initial acrylate production rate is unaffected, but increased temperature leads to a more rapid loss in bioconversion capability. In summary, under the conditions studied, highest yields of acrylate were achieved with a cell age of 14 h, a temperature of 23 °C, and a pH between 7.5 and 8.5.

**(B) Immobilization of *C. propionicum* Resting Cells.** Immobilization of cells or enzymes is often employed to increase the total productivity and lifetime of these



**Figure 2. Effect of pH on acrylate production by resting cells of *C. propionicum*.**



**Figure 3. Effect of temperature on acrylate production by resting cells of *C. propionicum*.**

catalysts. Batch experiments incorporating *C. propionicum* cells immobilized in calcium alginate beads were conducted to determine if cell immobilization could increase the productivity of and extend the useful life of *C. propionicum* cells. Results are presented in Table V and Figure 4. Cell yields, expressed as mmol of acrylate produced/g of wet cells, were measured at 6 h, after which little further acrylate production took place (as is generally true in free cell incubations). At low cell density in the calcium alginate beads, immobilized cell productivity and initial rate of acrylate formation were approximately equal to or slightly higher than those observed for free cells, falling off dramatically as cell density was increased. This phenomenon can be attributed to mass transfer effects inherent in such systems: inadequate rate of diffusion of reactants to the cells or inhibition of the cells by high

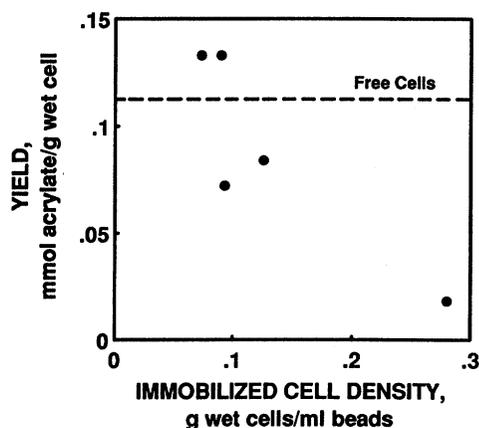
**Table V. Biological Production of Acrylate from Propionate by *C. propionicum* Resting Cells**  
(I) Immobilized Cells

wet cell weight, g	total vol, mL	cell density, g of wet cell/mL		yield, <sup>a</sup> mmol of acrylate/g of wet cell	initial rate, mmol of acrylate/(g of wet cell·h)
		total vol	beads		
1.80	70	0.026	0.090	0.133	0.025
1.68	73	0.023	0.073	0.133	0.026
3.26	76	0.043	0.126	0.084	
4.21	35	0.120	0.280	0.018	
3.09	83	0.037	0.093	0.072	

(II) Free Cells

reference	cell density, g of wet cell/mL	yield, <sup>b</sup> mmol of acrylate/g of wet cell	initial rate, mmol of acrylate/(g of wet cell·h)
this work	0.147	0.111	0.027
this work	0.134	0.115	0.023
Dalal et al., 1980	0.053		0.024 <sup>c</sup>
Dalal et al., 1980	0.100		0.020 <sup>c</sup>

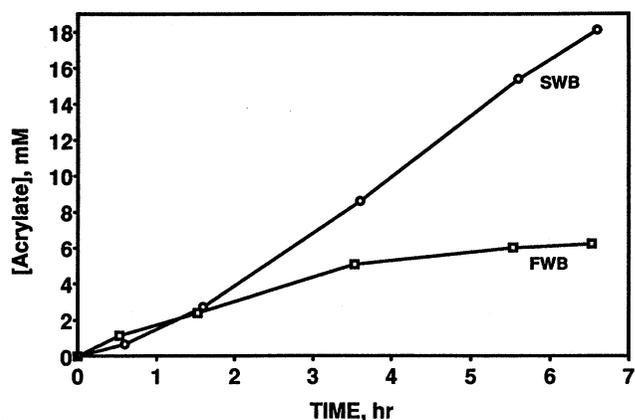
<sup>a</sup> Measured at 6 h. <sup>b</sup> 200 mM propionate, aerated, 37 °C, pH 7.5. <sup>c</sup> 200 mM propionate, 2.2 mM methylene blue, 37 °C, pH 8.5.



**Figure 4.** Acrylate yield by resting cells of *C. propionicum* immobilized in calcium alginate. For experimental conditions, see Table V.

product acrylate concentrations inside the beads. As indicated in Table V, these results are in close agreement with other published data of acrylate production by *C. propionicum* (Dalal et al., 1980). Immobilization did not extend the useful lifetime of *C. propionicum* cells for acrylate production, approximately 6 h. This may be partially explained by the observed physical deterioration of the alginate beads employed in this study. The rather short period of acrylate production activity by *C. propionicum* cells, free or immobilized, can be attributed to the known effects of acrylate inhibition, poisoning by molecular oxygen or methylene blue, or possibly cell death. These results have shown that bioconversion of propionate to acrylate by resting cells of *C. propionicum* immobilized in calcium alginate can be accomplished with no loss of acrylate yield or initial reaction rate.

**Bioconversion of Propionate in Fermented Sweet Whey to Acrylate.** Propionate in fermented sweet whey can be converted to acrylate by resting cells of *C. propionicum*. A comparison of acrylate production in FWB vs the synthetic medium SWB is shown in Figure 5. Further experiments were not performed to elucidate the exact reasons for the more rapid loss of acrylate production rate in FWB, but inhibition of *C. propionicum* growth by acetate (not present in SWB) at the concentration level in FWB has been reported (Akedo, 1983). This result represents the first demonstration of biological production of acrylic acid from a whey product. For economic application of this research, further work is needed to extend the activity of *C. propionicum* resting cells beyond



**Figure 5.** Comparison of acrylate yield by *C. propionicum* resting cells in a synthetic whey broth (SWB) and sweet whey previously fermented by a coculture of *L. bulgaricus* and *P. shermanii* (FWB).

the 5–6 h reported here or to otherwise modify the organism to more effectively utilize this unique biochemical pathway.

### Summary and Conclusions

This study was undertaken to explore the potential for a biological route to acrylic acid from whey in order to increase the utilization of this dairy industry byproduct. For propionic acid production from whey, incorporation of *L. bulgaricus* into a coculture fermentation with *P. shermanii* resulted in complete lactose utilization, increased acid yield, and decreased fermentation time.

Oxidation of propionate to acrylate was accomplished via the “acrylate” pathway of *C. propionicum*. Up to 18.5% conversion of propionate to acrylate was achieved by resting cells (Table IV). Immobilization of *C. propionicum* resting cells in calcium alginate beads resulted in no loss of cell activity. At low immobilized cell densities, acrylate yield and initial acrylate production rate were equivalent for immobilized and free cell systems. Biological production of acrylic acid from sweet whey fortified with yeast extract has been demonstrated.

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