PRODUCTION OF ACETIC ACID BY FERMENTATION WITH PROPIONIBACTERIA

FINAL REPORT
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Final Report

to the

Iowa Highway Research Advisory Board

on

Production of Acetic Acid by Fermentation with Propionibacteria
(HR-321)

by

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Abstract of HR-321
Production of Acetic Acid by Fermentation with Propionibacteria

This project was undertaken jointly with a project supported by the Iowa Corn Promotion Board. Together the projects aimed at producing the organic acids, propionic acid and acetic acid, by fermentation. The impacts were to provide agriculturally-based alternatives to production of these acids, currently produced mainly as petrochemicals. The potentially high-demand use for acetic acid is as the "acetate" in Calcium Magnesium Acetate (CMA), the non-corrosive road deicer.

Fermentation was, however, far from being an economically acceptable alternative. Gains were made in this work toward making this a feasible route. These advances included (1) development of a variant strain of propionibacteria capable of producing higher concentrations of acids; (2) comparison of conditions for several ways of cultivating free cells and establishment of the relative benefits of each; (3) achievement of the highest productivity in fermentations using immobilized cells; (4) identification of corn steep liquor as a lower cost substrate for the fermentation; (5) application of a membrane extraction system for acid recovery and reduction of product inhibition; and (6) initial use of more detailed economic analysis of process alternatives to guide in the identification of where the greatest payback potential is for future research.

At this point, the fermentation route to these acids using the propionibacteria is technically feasible, but economically unfeasible. Future work with integration of the above process improvements can be expected to lead to further gains in economics. However, such work can not be expected to make CMA a less expensive deicer than common road salt.

Project Title: Production of Acetic Acid by Fermentation with Propionibacteria (HR-321)

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Introduction

Acetic acid, which is the most costly ingredient of the road deicer calcium magnesium acetate, (CMA) is produced by several microorganisms providing an alternative to production from petroleum feedstocks. On such group of organisms is the propionibacteria, which produce acetic acid along with their primary product, propionic acid. Propionic acid is an important mold inhibitor used in the preservation of foods and feeds. It is currently produced commercially by chemical synthesis from petroleum, but is also known to be produced by the propionibacteria and a few other microorganisms in fermentation processes. In addition to producing propionic and acetic acids, the propionibacteria synthesize a number of other compounds that have potential commercial use: vitamin B12, which is currently produced by fermentation with other microorganisms; and bacteriocins, which are proteins with antimicrobial properties that may be used as natural food preservatives.

An advantage of using the propionibacteria in fermentation processes stems from the fact that they are anaerobic organisms and do not require aeration in the fermenter. The need to force air through a fermenter can greatly boost fermentation costs. Barriers to the development of an economically viable fermentation process for the production of propionic acid and other valuable end products are the slow growth rate of the propionibacteria and the relatively low levels of the acids that are accumulated in the fermentation medium. This project addressed means to overcome these barriers.

Traditional batch fermentation methods for organic acid production typically yield relatively low cell densities and very low concentrations of acids after incubation periods that are often very long. The microorganisms are inhibited at propionic acid concentrations above about 2% in the medium. Strain improvement to produce a more acid-tolerant strain, as well as fermentation processes such as fed-batch, cell-recycle, and immobilized-cell that allowed greater cell densities to be maintained in the fermenter, were employed to obtain higher concentrations of organic acids in the fermentation broth.

At the concentrations in which organic acids are produced in fermentation processes, distillation to recover the acids is not feasible. Drying is very energy-intensive. Our approach has been to increase the concentration of acids by membrane processing before subsequent concentration steps, such as drying, are applied. Such recovery methods have also been integrated with the fermentation to reduce product inhibition of the metabolizing cells. We previously studied electrodialysis as a means of concentrating the salt form of these acids. Our results showed that this method was effective at removing the acids, but could only concentrate the product to about 10% acid (Weier *et al.*, 1992).

Extraction is a traditional separation method that has not always worked well for acid recovery from fermentation broths. Some of the problems have been the formation of emulsions on contacting the two phases, little control over the interfacial areas between the broth and extractant, and lack of effective extractants for organic acids. Our research has built on progress in two areas to overcome these obstacles. The first area is the use of membranes to carry out emulsion-free extraction with independent control of interfacial area at levels higher than can be achieved with traditional extraction methods. By confining the interface of the broth and extractant to the membrane, emulsification is eliminated. The product must now pass the added barrier of the membrane, but the high interfacial area combined with a good choice of membrane results in

overall performance the match of the best of traditional methods. The second advance has been in the understanding of extraction chemistry allowing development of much better extractants. Much more effective extraction of propionic and acetic acids has been achieved by combining the organic solvent with a co-extractant (e.g. trioctylphosphine oxide - TOPO).

Previously, Dr. Christopher Nelson of Kemin Industries in Des Moines calculated that propionic acid might be produced by fermentation at a material cost of $0.14 per pound, assuming that a yield of 40 g propionic acid could be obtained from 100 g corn (dry basis), and that corn cost $3.00 per bushel. After experiments in batch fermentation systems gave yields of approximately 20 g propionic acid per 100 g corn, he estimated that ingredient costs alone would set the price of the acid at $0.30-0.35 per pound. It is difficult for fermentation-derived propionic acid to be economically competitive with acid produced by chemical synthesis under current market conditions. Oil prices would need to reach $50 per barrel to increase propionic acid cost to about $0.70 per pound. However, propionic acid sold as a natural preservative can command a higher price. For example, mold inhibitors sold for agricultural use may cost between $0.50 and $2.00 per pound, and typically contain 50% propionic acid. Fermentation-derived propionic acid can compete more successfully in this market. Sale of the propionic acid would be critical to the economic production of the acetic acid portion of the product. Coproducts of the fermentation process (vitamin B12, bacteriocin, cell mass) could also be sold to improve the economics of the process.

There is considerable interest in using acetic acid in the form of CMA as a road deicer. Acetic acid, the primary ingredient of CMA, is produced as a coproduct in the propionic acid fermentation. Benefits of using CMA as a road deicer include a reduction in corrosion of motor vehicles and in breakdown of road surfaces.

Current U. S. production of propionic acid by chemical synthesis from petroleum feedstocks is about 50,000 tons per year. Major uses for propionic acid are as mold inhibitors in bread and other foods (28%), grain preservation (27%), cellulose plastics (20%), herbicides (20%), and miscellaneous uses (5%) (Chem. Marketing Reporter, 1982).

If the cost of production of propionic acid is reduced sufficiently, its use as a preservative for high-moisture grain could be increased, thus increasing the market for the fermentation even further. Benefits of high-moisture grain preservation over dry preservation include avoidance of costs and problems associated with drying, reduction of field and harvest losses, and better feed efficiency.

An additional advantage to producing these products by fermentation is that corn can be used as the substrate. If our fermentation process, which uses products from hydrolyzed corn or by-products from corn wet-milling as fermentation substrate for the production of propionic acid, competes well enough with chemical synthesis to account for 10 to 20% of the yearly production of propionic acid, this could create a demand for 50 million pounds (about 1 million bushels) of corn annually, assuming 40% conversion of hydrolyzed corn into propionic acid. To produce enough CMA to replace all the salt used on Iowa roads yearly, approximately 10 million bushels of corn would be required as fermentation substrate.

Objectives

The overall objective of this project was to improve the economics of propionic and acetic acid production by fermentation by overcoming the two economic/technical obstacles that are primary causes of high production costs: 1) low productivity in the fermenter; 2) difficulty of recovery of the acids from the dilute fermentation broth. Approaches to overcoming these obstacles included: 1) development of an improved strain of *Propionibacterium*; 2) use of various fermentation systems to increase cell density and rate of production of propionic acid; 3) use of membrane-based acid recovery systems.

Methods and Procedures

1. Development of an improved strain of *Propionibacterium*. To be able to introduce genes into the propionibacteria to improve their performance in fermentations, it is necessary to have reliable means of transferring DNA into these organisms. No such reliable means have yet been developed. Techniques of DNA transfer that were attempted include: transformation (uptake of purified DNA by whole cells); protoplast transformation (the cell walls of the organism are first removed through a series of enzymatic steps, and the resulting protoplasts are then incubated with DNA and placed in a special medium to allow them to regenerate cell walls); electroporation (whole cells or protoplasts are mixed with isolated DNA and exposed to very high electrical fields for short periods of time, to induce "holes" in the cell wall and membrane and allow uptake of DNA); and conjugation (incubation of propionibacteria with a donor culture that carries DNA that can move into a recipient cell). Various strains of propionibacteria were used as recipients in these studies. Donor cultures included strains of *Staphylococcus aureus, Enterococcus faecalis, Lactococcus lactis,* and *E. coli*. The DNA species used in transfer studies were plasmids and transposons that contained genes for antibiotic resistance that could easily be detected in the recipient strains.

As an alternative to gene transfer methods, simple selection of a strain that could grow in the presence of high concentrations of propionic acid was also done. Strain P9 was serially transferred into fresh growth medium that contained ever increasing concentrations of propionic acid. At each acid concentration the growth of the culture was monitored, and the culture was transferred to a higher acid concentration only after growth was sufficiently rapid. At each new round of incubation the pH of the medium was adjusted to 7.0.

2. Use of various fermentation systems to increase cell density and rate of production of propionic acid. Growth and acid production were compared in the following fermentation systems: batch fermentation, in which cells were inoculated into the medium and harvested after the desired incubation time; continuous culture, in which the growth medium containing a limiting nutrient was continuously added to the fermenter as an equal amount of culture was removed; fed-batch fermentation, in which the concentration of substrate was increased in a stepwise fashion to match the increase in cell density as growth progressed; cell recycle operations, in which a portion of the culture was harvested, cells were separated from the growth medium, and were pumped back into the fermenter; continuous product removal (extractive fermentation), in which a portion of the culture was removed from the fermenter, cycled through the membrane apparatus to remove some of the accumulated acid, and returned to the fermenter; and immobilized-cell fermentation, in which cells were trapped in calcium alginate gel beads and used in batch, fed-batch, and continuous fermentation operations. Most fermentations were conducted in bench-top fermenters with accessory controllers to keep all environmental conditions constant (B. Braun Biotech, Allentown, PA). Some fermentations were conducted in flasks or in stirred bottles with manual control of pH. Organic acid concentrations were measured by high-performance liquid chromatography (HPLC).

3. Use of membrane-based acid recovery systems. Membranes and extractants were combined in various configurations. First, two different geometries of the membrane system were used: a flat sheet membrane that separates feed from recovery compartments, and membranes in the form of hollow fibers with the feed stream outside the fibers and the recovery stream inside. Second, membranes of different composition were tried, that could take up solvent into their pores and allow selective transfer of organic acids from the aqueous broth into the solvent phase. Third, several different organic solvents and extractants were tested for their compatibility with the membranes and with the propionibacteria, and for their ability to partition organic acids between the aqueous and the solvent phases. Fourth, the nature of the stripping or recovery solution was

varied: an alkaline sodium hydroxide solution used with the flat sheet membrane or an organic solvent used with the hollow fiber membrane.

Testing of transport of organic acids across flat sheet membranes was conducted in a small diffusion cell constructed by C. E. Glatz. The flat sheet membrane recovery apparatus was constructed from a stainless steel ultrafiltration apparatus (Pharmacia Biotechnology, Piscataway, NJ) with peristaltic pumps to control flow of feed and stripping solutions. The hollow fiber recovery apparatus was constructed in the Chemical Engineering Department with the assistance of the Chemistry Glassblowing Shop. Concentrations of organic acids were measured by HPLC.

Results

1. Strain improvement. Results summarized here can be found in the dissertation of S. A. Woskow and the publication of Woskow and Glatz.

Attempts to develop a reliable gene transfer system in the propionibacteria were not successful. An improved method for production of protoplasts, in which the cells were first grown in the presence of 1% threonine to weaken their cell walls, and were treated with a combination of lysozyme and chymotrypsin to quickly produce osmotically fragile protoplasts, was developed. Transformation of these protoplasts with *S. aureus* DNA that carried genes for antibiotic resistance yielded some antibiotic-resistant colonies, but the donor DNA could not be found in these organisms by standard methods of agarose gel electrophoresis.

In another series of experiments, a streptomycin-resistant strain of *Propionibacterium thoenii* was developed and used as a recipient in conjugation experiments with four different donor strains: two *Enterococcus faecalis* strains that carried different conjugative plasmids, and one *Lactococcus lactis* strain and one *Enterococcus faecalis* strain that carried different conjugative transposons. In these trials usually no gene transfer was seen. A few colonies appeared that had the antibiotic resistances of both the donor and recipient parents. However, the presence of donor DNA could not be demonstrated in these isolates.

In attempts to develop an electroporation method for transformation of *Propionibacterium* cells, four different recipient strains, 13 different buffers, and three different plasmid vectors were tested. As was the case with the other methods tried, antibiotic-resistant colonies appeared on some plates. However, the presence of the donor DNA could not be demonstrated in these isolates.

In all of these attempts to transfer foreign DNA into the propionibacteria, it is possible that the donor DNA was rapidly destroyed in the recipient cells by intracellular nucleases. The propionibacteria may have very active restriction systems that identify new DNA as foreign and destroy it. It may be necessary to develop restrictionless strains of propionibacteria to be used as recipients, or to use the plasmids native to the propionibacteria as vectors to introduce genes into these organisms. It is extremely important to develop a reliable method of gene transfer in the propionibacteria if genetic engineering is to be used for strain improvement.

In contrast to the problems encountered in gene transfer studies, a propionate-tolerant strain was developed by transferring a strain (*P. acidipropionici* P9) into broths which had a steadily increasing concentration of propionic acid. The variant strain, designated P200910, grew at higher propionate concentrations (through 8% propionic acid) than could its parent. In fermentations under controlled conditions, strain P200910 produced a higher ratio of propionic acid to acetic acid, utilized sugar more efficiently, and produced more propionic acid per gram of biomass than did P9. Strain P200910 continued to produce propionic acid after growth had stopped, which made it well suited for fed-batch fermentation. In a semicontinuous fermentation (concentrated fresh medium added at regular intervals as an equal volume of spent medium was removed) of laboratory medium by strain P200910, a high concentration (47 g/liter) of propionic acid was obtained. Strain P200910 was considered to be a significantly improved strain for propionic acid fermentation.

Hyun-Dong Paik (see M. S. thesis) was able to confirm the increased propionate tolerance of strain P200910, but he also demonstrated that strains P9 and P200910 behaved similarly in the presence of increasing concentrations of acetate (Figures 1 and 2). We have also observed in other studies that strain P200910 does not tolerate low pH better than does its parent. We have concluded that strain P200910 is more tolerant of the propionate ion at neutral pH than is its parent, and is the best organism to use for fermentations performed under conditions of controlled pH.

2. Use of various fermentation systems. Results summarized here can be found in the theses of S.-K. Park, H.-D. Paik, and F. Ozadali, and in their manuscripts in preparation.

A systematic study was conducted by S.-K. Park under standardized conditions to compare various fermentation systems for propionic acid production. Strain P9 was grown in synthetic medium with various carbon sources in batch, fed-batch, continuous culture, and continuous culture with cell recycle processes. The following conclusions can be made from the results obtained.

In batch fermentation, final propionic acid concentrations were greater when glucose or lactate rather than maltose were used as carbon sources. The optimum initial substrate concentration was 2 to 3%. Complete substrate utilization and maximum organic acid production occurred after about 50 hours of incubation, so productivity (acid produced per hour of incubation) was low.

Fed-batch experiments were carried out through 250 hours of incubation using different feeding schedules. Cultures grown on glucose or maltose produced more biomass and propionic acid than did those grown on lactate. Acid productivity was approximately the same as obtained in batch fermentation. Because accumulated substrate concentrations were not high (3 to 4%), the final propionic acid concentrations also were not high (2 to 2.5%) and did not inhibit the culture. The culture seemed to be able to continue acid production at least through this 250-hour incubation period.

In continuous culture fermentations, low dilution rates (0.03 to 0.27 hr^{-1}) were required because of the slow growth rate of the propionibacteria. Maximum propionic acid concentration in the culture was measured at dilution rates of about 0.10 hr^{-1}. When 50 or 80% of the cells were recycled back to the fermenter to increase the cell density in the vessel, the measured concentrations of biomass and organic acids in the broth both increased, but the biomass concentration increased more than did propionic and acetic acids. We hypothesized that the recycle process may have damaged or even killed cells and thus decreased their ability to produce acids.

In all types of continuous culture operations the concentrations of acids in the broth at any time were low, but the overall acid productivities were high, compared to batch or fed-batch processes. Thus, the question of what type of system to use depends on the efficiency of acid recovery processes. If a system is available that can effectively remove very low concentrations of organic acids from fermentation broth, then continuous culture operations with higher productivity values should be favored. However, if recovery methods must have a relatively high concentration of organic acids to be effective, then fed-batch fermentation processes would be preferred.

Fed-batch fermentation studies were continued by Ozadali with strain P9 in both laboratory medium and in corn steep liquor medium (25% w/v powdered corn steep liquor in distilled water). The total fermentation time was 150 hours, and the total amount of substrate added (7.2% glucose in laboratory medium, 4.7% lactic acid in corn steep liquor) was greater than in previous studies by Park. Corn steep liquor was an excellent medium for growth and propionic acid production; final propionic acid concentrations were about 3.5% in both laboratory medium and corn steep liquor. Because of the shorter incubation time used, productivities were higher than in previous studies. Data from typical fed-batch fermentations are illustrated in Figures 3 and 4.

Data from batch fermentation experiments were used in a mathematical modelling study. A modified Gompertz microbial growth equation showed excellent fit to the experimental data for biomass production at six different initial glucose concentration levels. A reasonable fit was also

obtained by using a modified Logistic equation. To model product formation, the Luedeking and Piret equation was fitted to the experimental data by using a Fortran algorithm. The ability to fit these modelling equations to experimental data is a first step toward predictive modelling of fermentation behavior in new, untried conditions.

These studies were all conducted with strain P9. Its propionate-resistant variant, strain P200910, was used by H.-D. Paik to optimize a cell immobilization procedure. These immobilized cells were tested for stability and were used in batch, fed-batch, and continuous fermentations of laboratory medium and corn steep liquor medium for propionic acid production.

The final immobilization procedure was as follows. A 2-liter culture incubated 2 days at 32C was centrifuged and the cells were recovered as a concentrated suspension (about 2.3×10^{10} cells per ml). This suspension was mixed with a sterile saline solution and a sterile 2.5% sodium alginate solution at a volumetric ratio of 3:1:6 (cells:saline:alginate). The mixture was slowly extruded through the tip of a 5-ml pipet into a sterile 0.1 M calcium chloride solution to form beads of approximately 3.5 mm diameter. The beads were cured by incubation at 37C for 3 hr and were stored in sterile 0.05 M calcium chloride solution at 4C until use within 3 days. The viable cell count per gram (wet weight) of beads was about 9.8×10^9.

Fermentations were conducted by using 0.1 g (wet weight) beads per ml of medium. A typical batch fermentation of laboratory medium is illustrated in Figure 5. Substrate utilization and acid production occurred much more rapidly than in fermentations using free cells (36 hr vs.60 to 72 hr.) This can be attributed to the fact that a large population of active cells is already present in the beads; the time needed for the free cell population to build up is eliminated. The amounts of propionic and acetic acid produced and the yields of acids from substrate were higher than the values obtained for free cells under similar conditions. This difference may be attributed to the fact that with immobilized cells more of the available carbon can be used to produce organic acids rather than biomass.

Beads were stable to repeated use. After a series of 10 consecutive 72-hr fermentations, the viable cell concentration was about 8.0×10^8 per gram of beads, numbers of viable cells released into the medium were very low, and organic acid production was relatively constant over the course of the 10 fermentations. If acids accumulated the amount of new acid produced was significantly reduced; the immobilized cells were affected by the acid concentration in the medium.

Fed-batch fermentations were conducted both in laboratory medium and in corn steep liquor medium (30% (wt/vol) liquid corn steep liquor in distilled water). Figures 6 and 7 show typical fermentations. The amounts of propionic acid obtained in these fermentations (between 5 and 6%) were the highest concentrations achieved to date in our laboratory and are higher than reported literature values. Acid production was still continuing when the fermentations were terminated at 250 hr. It is likely that continued incubation would have resulted in even greater acid concentrations, but the rate of acid accumulation would have slowed. Corn steep liquor without added nutrients was an excellent medium for organic acid production.

Continuous culture fermentations were also conducted with immobilized cells. Concentrations of organic acids in the medium were low, but were stable through dilution rates of 0.2 to 0.3 hr^{-1}. Faster dilution rates and thus greater volumetric productivity values were obtained with immobilized cells compared to free cells.

3. Use of membrane-based acid recovery systems. Results summarized here can be found in the theses of M. Solichien, F. Ozadali, and D. O'Brien, and in their manuscripts in preparation.

The supported liquid membrane in the flat sheet configuration successfully removed propionic and acetic acids from aqueous medium. Celgard 2500, a microporous polypropylene membrane, was the best membrane available. Decane and dodecane were the best solvents with which to load the membrane, not only because they were harmless to the propionibacteria but also because they gave the best transfer rates. Trioctylphosphine oxide (TOPO) facilitated transport and

10% (wt/vol) TOPO gave optimum transfer. The optimum feed and recovery stream flow rates through the apparatus were 16 ml/min and 8.8 ml/min, respectively. The transfer rate was constant over at least 24 hr, but decreased upon longer operation, probably because of membrane instability and loss of solvent from the membrane. When organic solvent rather than sodium hydroxide solution was used as the stripping solution, membrane stability was greater. Sodium propionate and sodium acetate could be recovered from the stripping solution by crystallization.

The flat sheet apparatus was successfully connected to a 600-ml working volume fermenter and used to recover organic acids on-line during a 150-hr fed-batch fermentation. About 40% of the organic acids produced were recovered from the fermentation broth, and the total amounts of organic acids produced in the extractive fermentation were about twice the amounts produced in a nonextractive control fermentation.

The optimum design for the hollow fiber extraction module was as follows. The fibers were hydrophobic isotactic polypropylene (Celgard X20 400 microporous membranes from Hoechst Celanese Corporation) with a porosity of 40%, 0.65 x 0.19 mm pore cross section, 400 micron inner diameter (i.d.). The glass shell had dimensions of 8 mm i.d., 10 mm o.d., and 19 cm length. The module contained 96 fibers (183.9 cm^2 total area), which were sealed into the glass tube at each end with epoxy. The supporting and stripping solution was 20% TOPO in kerosene. Flow rates through the apparatus were 40 ml/min for the aqueous feed stream and 24 ml/min for the solvent recovery stream. This design provided a significant advantage over the flat sheet design in that the supporting and stripping solution was continuously renewed, and thus the apparatus could be operated indefinitely. During protracted operation the solvent reservoir was replenished with fresh solvent every 48 hr.

The hollow fiber extraction module was successfully connected to a 650-ml working volume fermenter and used to recover organic acids in an extractive fed-batch fermentation. The culture (strain P9) was grown in batch mode for 20 hr, at which time the pH was adjusted from 7.0 to 5.5 (the lower pH is needed for better recovery of the organic acids), continuous pumping of the culture through the extraction module was begun, and the first feeding of fresh medium to the culture was given. Two extractive fermentations and two nonextractive control fermentations were conducted; the total concentrations of acetic acid and propionic acid produced in these fermentations are shown in Figures 8 and 9. The extractive fermentations produced slightly more acetic acid than did the control fermentations, but the propionic acid concentrations produced were essentially the same in the four fermentations. This lack of increased acid production in the extractive fermentations might be attributed to the excellent performance of the control fermentations, and possible stress on the microbial cells caused by pumping through the extraction module.

Even though higher organic acid production was not seen in the extractive fermentations, promising results were obtained. The extraction module was successfully operated for 150 hours. Averaged over the two extractive fermentations, 22% of the acetic acid and 44.5% of the propionic acid produced were extracted from the fermentation broth into the recovery solvent.

4. Economic analysis. We have initiated efforts to use process economics to guide choice of research priorities. The software package BioPro Designer (Intelligen Systems) has been purchased and is being used to economically evaluate alternative processing strategies. "What if" analysis will then be used with this package to check the impact of improvements in microbial strain, separation performance, and fermentation productivity on process economics. In doing this we have had to work with the software developer to accomodate some of the process innovations we have developed.

Summary and Conclusions

The following are the significant accomplishments of this project.

1. A propionate-tolerant variant strain, *P. acidipropionici* P200910, was developed by adapting a strain to steadily increasing concentrations of propionate. This strain performed better than its parent in fermentations at controlled pH, and is the organism of choice for further fermentation trials. The method used to produce this strain should be useful in producing other strains with tolerance to organic acids.

2. Performance data were obtained for batch, fed-batch, continuous culture, cell recycle, and extractive fermentations under standard conditions. These data can be used as control values for further studies, and can be used to select the best fermentation system for particular situations.

3. Cells of strain P200910 were successfully immobilized and were found to be stable and active for extended periods of time (at least 30 days). Fed-batch fermentation using immobilized cells gave the highest rate of acid production and the highest acid concentration achieved to date, and is the method of choice for further studies.

4. Corn steep liquor without added nutrients was found to be an excellent medium for growth of free cells and for acid production by free or immobilized cells. It is a good choice as a cheap substrate for large-scale fermentation.

5. Both flat sheet and hollow fiber extraction modules were used to recover propionic and acetic acids on-line during extractive fermentations. The hollow fiber module could be used for a longer period of time because its supporting and stripping solvent could be renewed, and is the design of choice for further studies. Almost half of the propionic acid produced during the fermentation was extracted, and the apparatus should be useful in recovering acids during fermentation as well as in keeping acid concentrations in the fermenter at subinhibitory levels.

6. A software package that calculates process economics was obtained, and is being used to evaluate the impact of various process improvements on the economics of propionic and acetic acid fermentation.

Recommendations

1. While progress has been made on all objectives, this process is not yet competitive with petrochemical sources of the acids; hence, commercialization is not yet recommended.

2. We have yet to consolidate our advances in individual strategies for improved productivity into an integrated process. We believe such integration will bring further enhancements in productivity, the critical area for making this economically feasible and such integration should be pursued.

3. Economic analyses will be important to guide which of the strategies are likely to have the biggest impact and should be used to guide future research.

4. It remains our view that the cost of acid production will continue to make CMA a more expensive deicer than NaCl when one considers only the cost of deicing.

5. We plan to continue to pursue multi-sponsor funding of this research and encourage continued involvement of the Iowa Highway Research Advisory Board as long as they continue to have an interest in alternative production processes that offer additional economic benefit to Iowa from value-added production from agricultural materials.

Sources of Additional Information based on work cosponsored by this project and the Iowa Corn Promotion Board.

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11. Ozadali, F., B. A. Glatz, C. E. Glatz, and D. O'Brien. Fed-batch and extractive fermentation for propionic acid production. *Proceedings of Corn Utilization Conference IV, 1992.*

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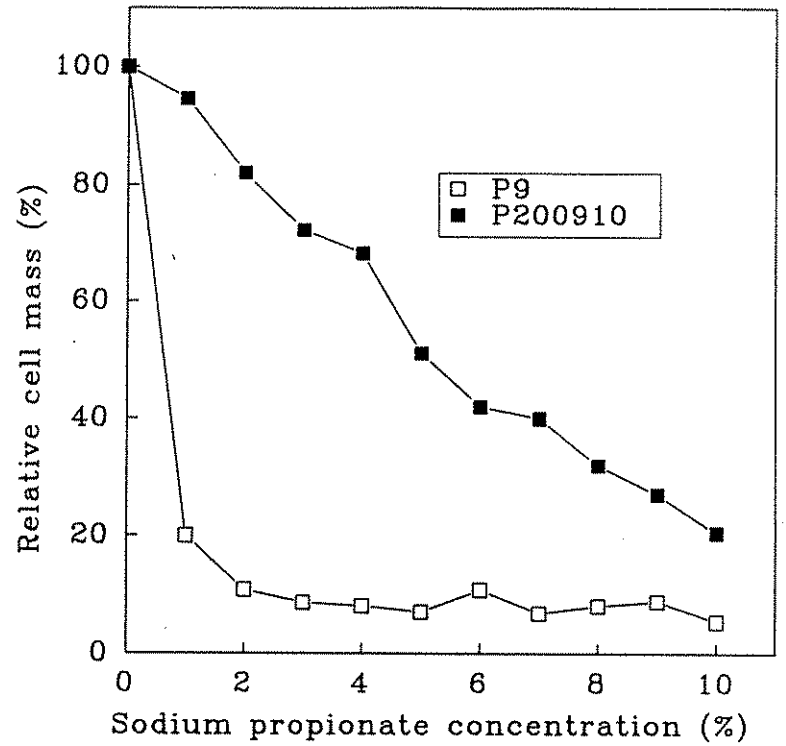
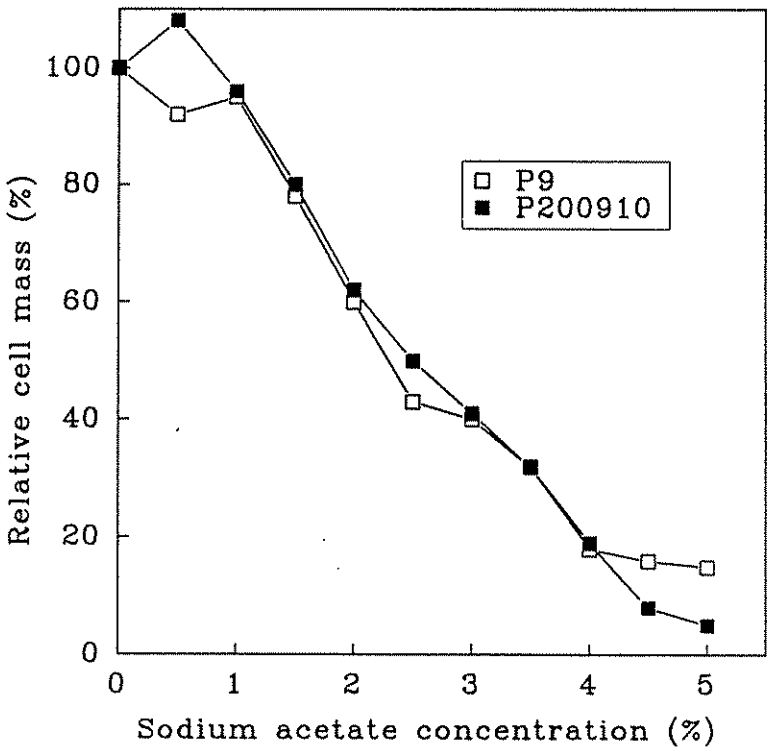
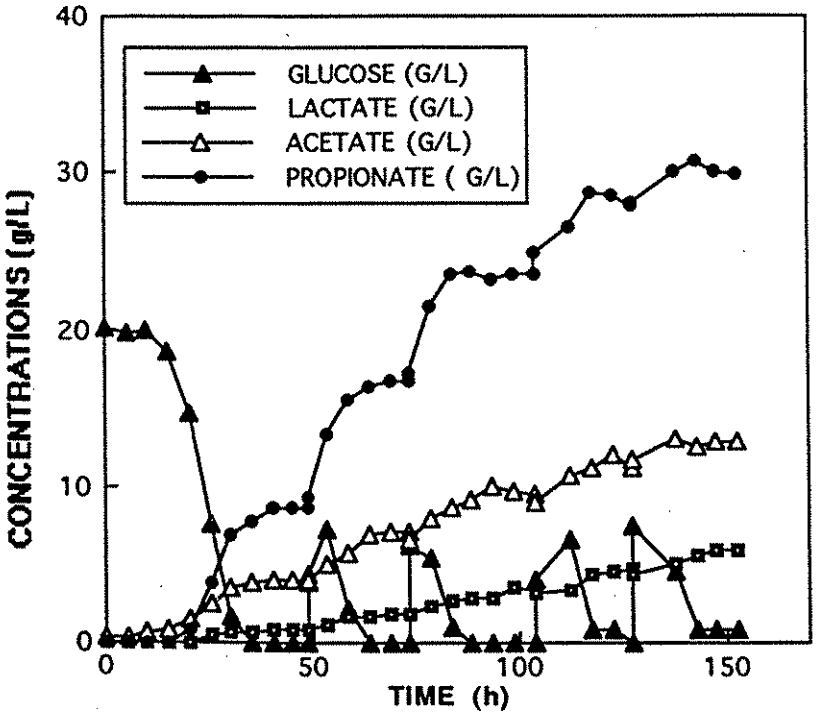
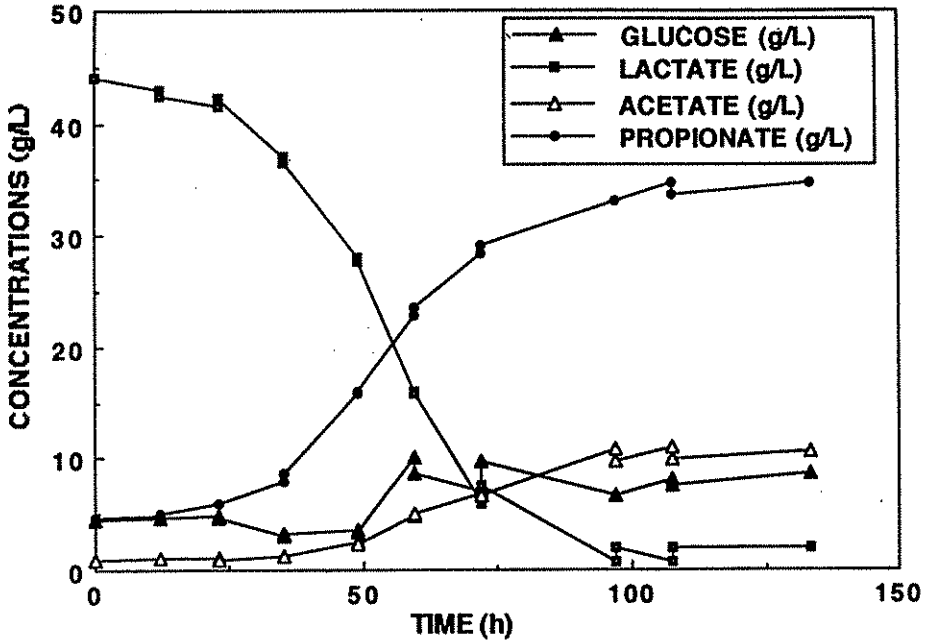
13. Ozadali, F., B. A. Glatz, C. E. Glatz, and D. O'Brien. Optimization and modelling of fed-batch and extractive fermentation for propionic acid production. Abstract 216 in *Book of Abstracts, 1992 IFT Annual Meeting.*
14. Ozadali, F., B. A. Glatz, D. O'Brien, and C. E. Glatz. Mathematical modelling of and extractive fermentation for propionic acid production by *Propionibacterium acidipropionici.* Abstract 8f in *Extended Abstracts of the American Institute of Chemical Engineers 1992 Summer National Meeting.*





Figures 1 and 2. Relative growth in 24 hr in the presence of various concentrations of sodium propionate (top) or sodium acetate (bottom) compared to growth without acids.





Figures 3 and 4. Fed-batch fermentation by free cells of strain P9 in laboratory medium (top) and in corn steep liquor medium (bottom).

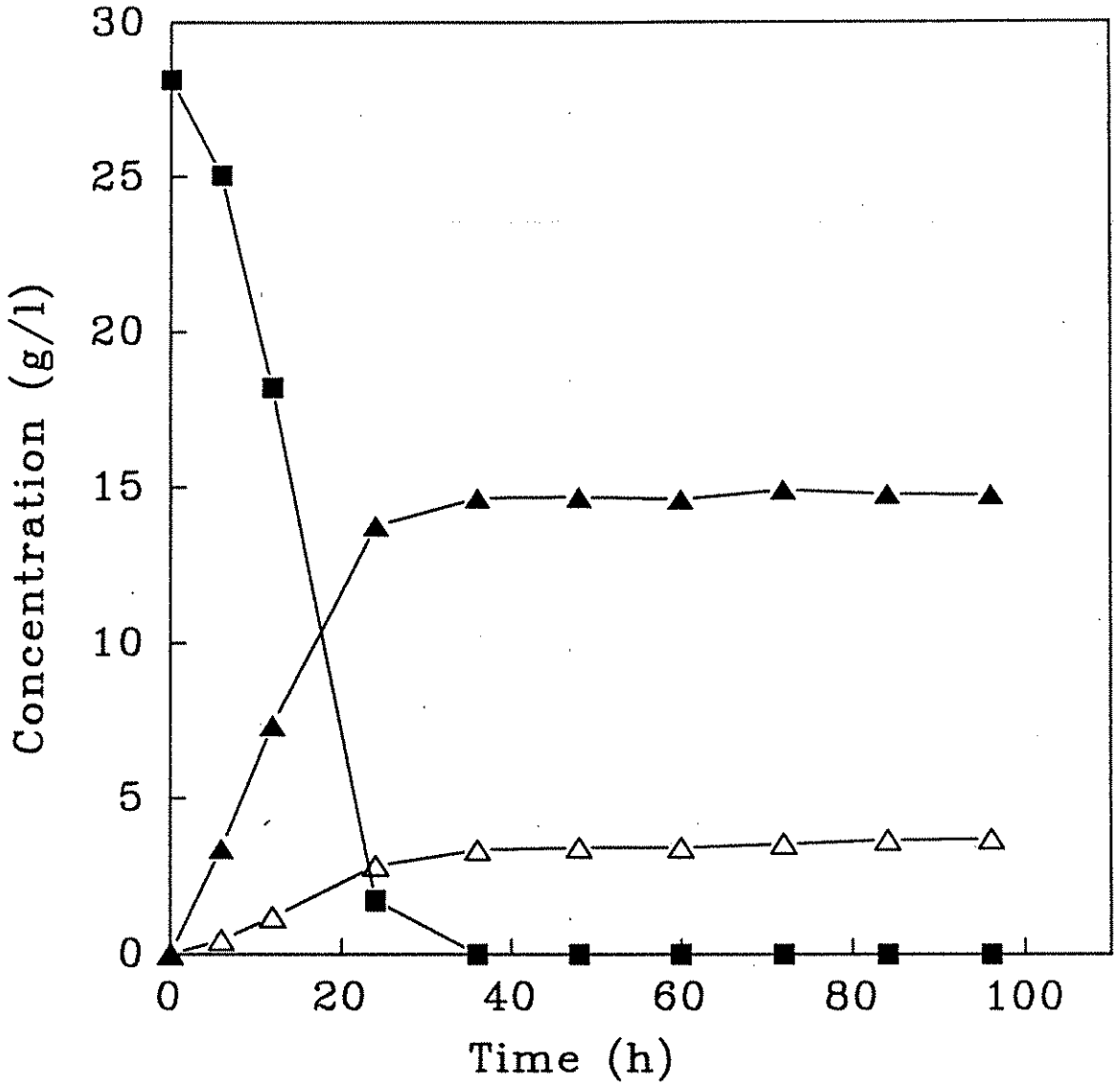
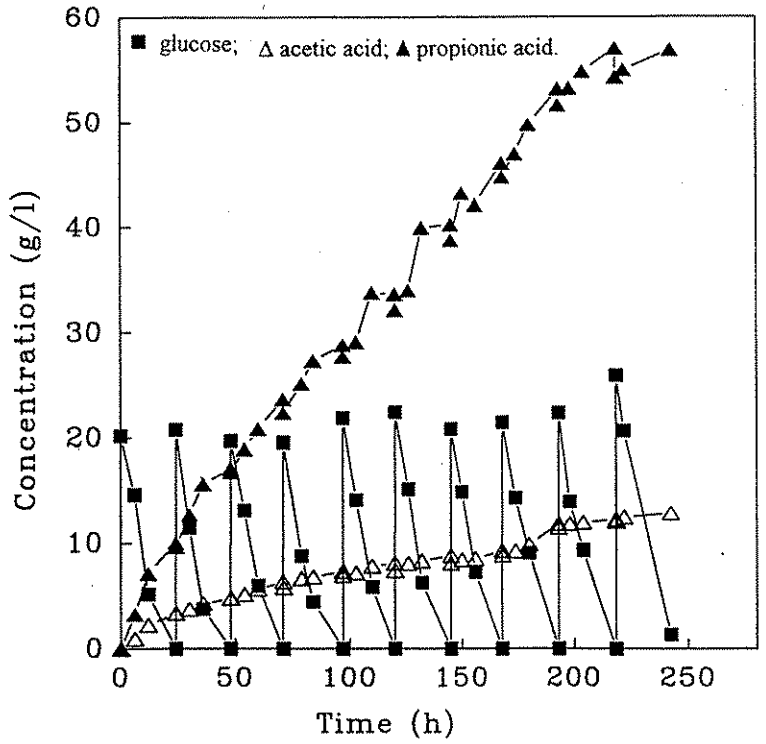
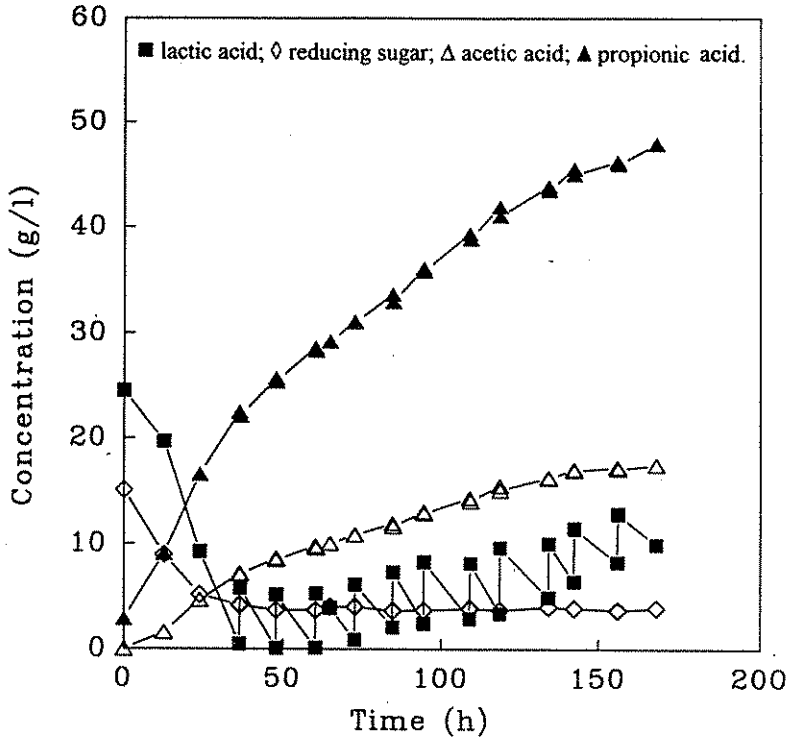
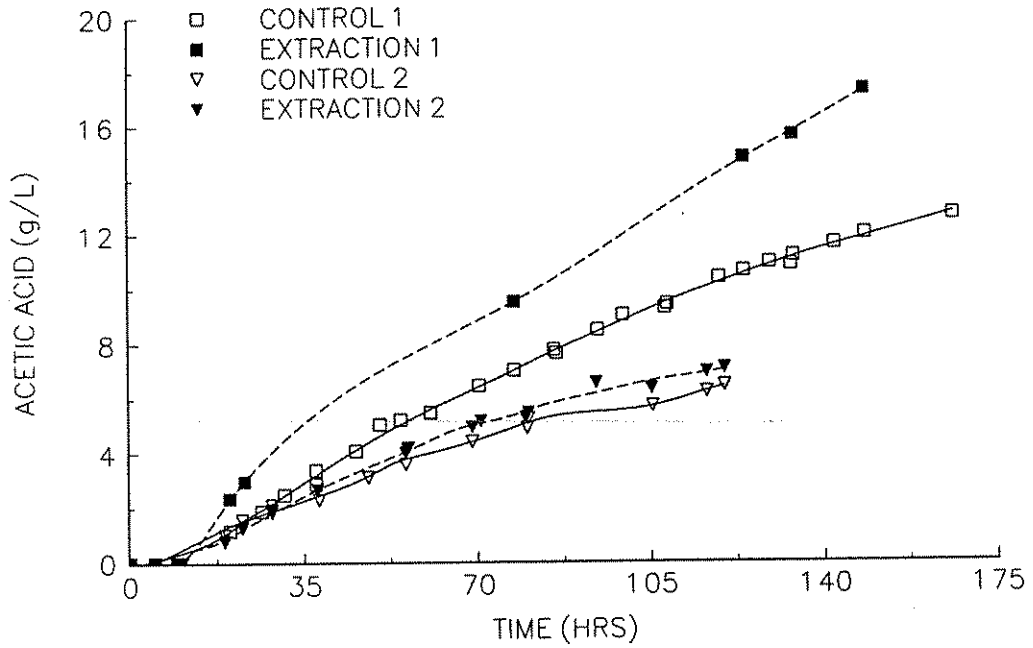


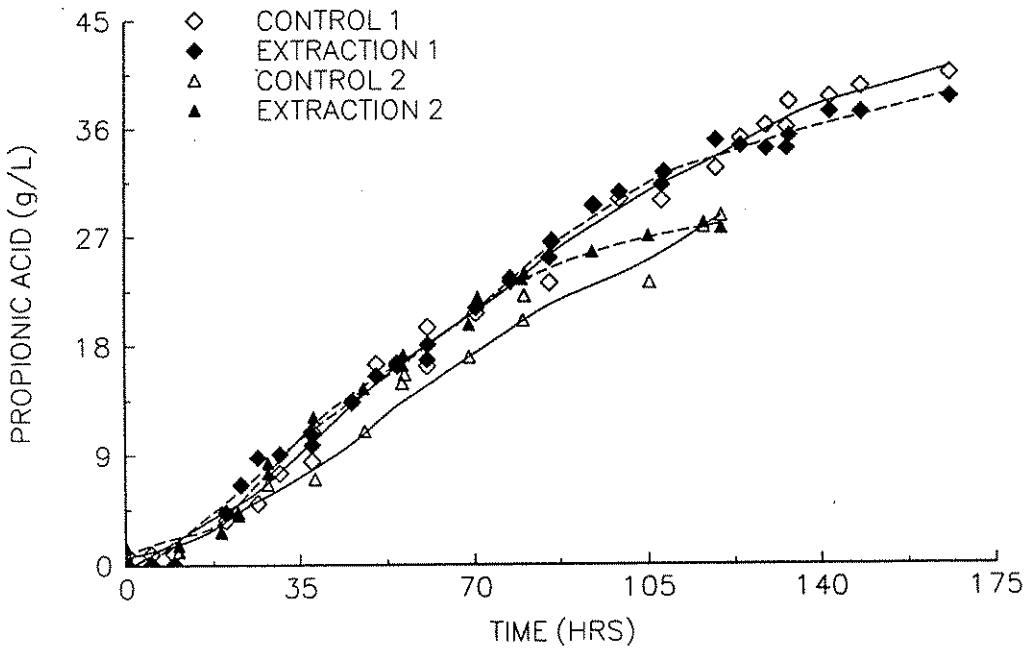
Figure 5. Typical batch fermentation by immobilized cells of strain P200910 in laboratory medium.





Figures 6 and 7. Fed-batch fermentation by immobilized cells of strain P200910 in laboratory medium (top) and corn steep liquor medium (bottom).





Figures 8 and 9. Total concentrations of acetic acid (top) and propionic acid (bottom) produced in extractive and control (nonextractive) fermentations by free cells of strain P9.

Final Report Addendum
(Summary of Economic Analysis)

and

Request for Unfunded Extension to June 30, 1995

to the

Iowa Highway Research Advisory Board

on

Production of Acetic Acid by Fermentation with Propionibacteria
(HR-321)

by

E.G. Hammond, B.A. Glatz, and C.E. Glatz
Iowa State University

April 27, 1993

This research was supported by the Highway Division of the Iowa Department of Transportation and the Iowa Highway Research Advisory Board. The opinions, findings, and conclusions expressed in this publication are those of the authors and not necessarily those of the Highway Division of the Iowa Department of Transportation.

FInal Report Addendum

In our final report presentation to the Iowa Highway Research Advisory Board, we presented a summary of cost studies we had completed after preparation of the final report. We were asked to provide an addendum to include that information and given a no-cost extension until April 30, 1993 to do so. During that period we have refined the analysis so that the figures below differ somewhat from those presented to the Board in February. That information follows.

Procedure

To assess our progress in achieving a more economically viable fermentation route to propionic and acetic acids, we compared flow sheets for acid production based on (1) a fed-batch fermentation based on the yield and productivity data obtained at the beginning of this project; and (2) a continuous, extractive fermentation using immobilized cells based on yield and productivity data obtained from three separate studies during the grant period of continuous, extractive, and immobilized cell fermentations (the combined strategies have not yet been carried out). Extractor design was based on our measured partition coefficients; other separation data were from the literature or default values of the design package used.

Both cases were based on the same fermentation media, production scale, and downstream recovery process (microfiltration and drying of cell mass, extraction of combined acids, distillation to recover extraction solvent and isolated acetic and propionic acids). The complete economic analysis was obtained using the process design package BioPro Designer™. This package provides costs typical to the fermentation industry for equipment, installation, site preparation, labor, utilities, and waste treatment. Production costs were divided among the products (acetic acid, propionic acid, and dried cell mass) in the ratio of the late 1992 selling prices of each.

Results

Case 1 Production Costs:

Acetic Acid	$3.56/kg
Propionic	$4.71/kg

Case 2 Production Costs:

Acetic Acid	$1.57/kg
Propionic	$2.08/kg

Comparison to Bulk Quantity Market Prices:

Acetic Acid	$0.73/kg
Propionic Acid	$0.97/kg

Conclusions

1. Case 1 already represented a significant improvement over traditional batch fermentation.

2. Comparison of the two cases shows a reduction in acid production costs by a factor of 2.3, an indication of the gains that can be made through research on innovative fermentation and product recovery strategies.

3. Fermentation is not yet competitive with petroleum based products and is not likely to become so while world prices for petroleum and natural gas remain low.

Request for No-Cost Extension to June 30, 1995

We ask that the Board extend the project completion date of HR-321 to June 30, 1995. No additional funding is requested. The only "cost" will be that equipment purchased on this contract will be fully depreciated as of December, 1994. Equipment of original purchase value of $23,500 has now been in use for 39 months resulting in a current value of $8225, based on 5-year, straight line depreciation.

Rationale

Continuing funding for this work has been requested from the Midwest Biotechnology Plant Consortium (Energy from Biomass Program) for a period of two years, beginning July 1, 1993. MPBC requires matching funds from industry and other non-Federal sources. We have already had our proposals for matching funds approved by the Iowa Corn Promotion Board, Iowa State University, and New Energy Company of Indiana. MPBC will notify us of their decision June 1, 1993. The total requested of MPBC and matching agencies was $288,337 for the two-year period. The requested extension is to the end of that two-year period.

By granting this extension, the Iowa Department of Transportation would receive:

1. A reproducible-quality copy of the most complete of the annual progress reports (2) to the Iowa Corn Promotion Board or the Midwest Biotechnology Plant Consortium.

2. A separate summary of the economic evaluation of the most favorable process at the completion of the project.

3. Acknowledgement of support in publications resulting from this work.

In this way, at no additional cost, the DOT would be kept informed of the potential for alternative sources of acetic acid and share in the sponsorship of continued research in this area.

Final Report Addendum

to the

Iowa Highway Research Board

on

Production of Acetic Acid by Fermentation with Propionibacteria (HR-321)

by

E.G. Hammond, B.A. Glatz, and C.E. Glatz
Iowa State University

July 1995

(revised August 1995)

This research is being supported by the Project Development Division of the Iowa Department of Transportation and the Iowa Highway Research Board. The opinions, findings, and conclusions expressed in this publication are those of the authors and not necessarily those of the Project Development Division of the Iowa Department of Transportation.

Abstract

This addendum to our Final Report of February 7, 1993 covers the period of the unfunded extension of HR-321. As per the extension agreement, we are providing a single copy of the progress reports that we have submitted to the Iowa Corn Promotion Board, who have continued funding of this project during the time of the extension. The Years 1 and 2 Progress Reports cover that period and these follow. We appreciate the extension offered by the Iowa Highway Research Advisory Board.

During this period we have been able to make further progress on increasing fermentor productivity and carried out much of the preliminary work required to switch to a new extractant system for product recovery. That system, Alamine-304 in 2-octanol showed partition coefficients for extraction of propionic acid from aquuous solutions that were four times higher than with the TOPO-kerosene system used earlier. Experiments are underway to determine whether this holds true for extraction of the combined acetic and propionic acids from the actual fermentation broths. On the fermentation side, continued development of methods to immobilize cells has raised fermentation productivity by a factor of two from that reported in our previous report.

In terms of production cost of the two acids our estimates of lowest attainable costs based on a continuous fermentation are (with values from the previous report given in parentheses):

Acetic Acid:	$0.99/kg	($1.57/kg)
Propionic Acid:	$1.32/kg	($2.08/kg)

A more conservative assessment would be that based on a fed-batch fermentation. While we have not yet carried out that specific fermentation, data we have gathered lead to the prediction that the resulting production costs would be (values in parentheses are for this case using productivity at the beginning of the grant period and reported in the previous report):

Acetic Acid:	$2.75/kg	($3.56/kg)
Propionic Acid:	$3.67/kg	($4.71/kg)

Even the best case gives a production cost 35% higher than the bulk selling price for these acids produced from petroleum. Hence, we continue to believe that the fermentation route can not be competitive without a significant rise in oil prices.

Production of Propionic and Acetic Acids by Extractive Fermentation
Year 1 Progress Report

We have made progress toward two of our project goals during this first half year of support: operation of our fermentation system at high cell densities to increase organic acid production, and development of improved membrane extraction systems for acid removal. Our work in these two areas is summarized below.

Fermentation at high cell densities. As an alternative to immobilizing cells in alginate gel beads, we have tested the ability of our standard acid-producing strain, *P. acidipropionici* P9, to form a biofilm on support materials that are packed into a reactor. Often biofilms contain a higher density of metabolically active cells than do gel beads, and problems of limited diffusion of nutrients into the beads are eliminated.

The support materials were composed of polypropylene, or polypropylene mixed with four different combinations of agricultural materials (soy hulls - zein; corn starch - zein; corn hulls - zein; and oat hulls - zein). The chips were prepared by high-temperature extrusion of the desired materials in a Brabender PL2000 twin-screw extruder. The material was extruded as 3-mm diameter rods, which were air-cooled and then cut into chips 2 to 3 mm long. These chips can be sterilized in the autoclave.

The fermentation system consisted of 50-ml plastic syringes each packed with a different type of chip. One control syringe contained polypropylene chips. Another syringe was used without chips to provide a control culture of free cells. A semidefined laboratory medium provided glucose as a substrate. After sterilization, the reactors were placed in a water bath at 32C, filled with medium, and inoculated with strain P9. The cultures were allowed to grow as a batch culture for 32 hr, and then fresh medium was introduced at a constant rate. A range of flow rates (0.06, 0.12, 0.24, 0.48, and 0.96 ml/min) was tested. These corresponded to dilution rates of 0.18 to 2.88 hr^{-1}, which were much higher than the rates that can be used for free cell continuous culture. Every 5 to 10 hr samples of the effluent medium were taken, and analyzed for optical density (to measure concentration of free cells), pH, % residual glucose, % propionic acid, and % acetic acid. Analysis for glucose and acids was by HPLC. At the end of the experiment, the chips from each syringe were removed and observed for the presence of a biofilm. The chips were treated with a stain that colors microbial cells, and the intensity of the color that developed indicated the extent of film formation. The chips were also dried and weighed to detect any weight changes due to biofilm formation, and were vigorously shaken to evaluate the extent of clumping caused by a biofilm.

Our results indicate that biofilm reactors for efficient propionic acid production show great promise. Strain P9 formed a biofilm on the chips that contained agricultural materials, but not on the polypropylene chips. The concentration of free cells in the four biofilm reactors was much higher than in the controls. The pH of the medium coming from the biofilm reactors was significantly lower than that from the controls, and the measured concentrations of acids from the biofilm reactors were also significantly higher than in controls. However, not all of the glucose in the incoming medium was utilized.

During the next year, we will test additional combinations of agricultural materials in the chips, and select the optimum composition to be used. We will use a

modified fermentation system that allows the medium to be recirculated into the reactors after the free cells have been separated from it, for more efficient substrate utilization. We will compare the behavior of strain P200910 to its parent P9 in the optimum biofilm system, and we will also test corn steep liquor medium as substrate.

Improved acid extraction system. We have used a supported liquid membrane system to recover organic acids from the fermentation broth and to maintain the acid concentrations in the broth at low enough levels so that the cells are not inhibited. The best extractant combination that we have used to date was 20% TOPO in kerosene, but this system was not as efficient as we would like to see. Many new potential extractants are appearing on the market, and we have begun to examine them as potential components of our system.

The first extractant system being tested is Alamine-304 in 2-octanol. Alamine-304 is a water-insoluble tertiary amine produced by Henkel Corporation. Such tertiary amines have used successfully by others in organic acid extraction (Tamada and King. 1990. Ind. Eng. Chem. Res. 29: 1329-1338.) The 2-octanol is a more polar diluent than kerosene, and will increase the extracting power of the nonpolar Alamine. Extraction effectiveness is measured by thoroughly mixing an aqueous solution of the organic acid with the Alamine-octanol extractant, allowing equilibrium to be reached, and measuring the resulting acid concentrations in the aqueous and organic phases. The partition coefficient K_d, which is the ratio of the organic phase acid concentration to the aqueous phase acid concentration, is then calculated. High K_d values indicate effective extraction.

To date, we have mixed aqueous solutions of acetic acid and of propionic acid in the concentration range 0.5 to 16%, with Alamine-octanol mixtures that ranged from 5 to 100 % Alamine, at different pH values, and have calculated the partition coefficients of the acids into the organic phase. Results have shown that the concentration of Alamine significantly affects efficiency of acid extraction, as measured by K_d. For acetic acid extraction, the optimum Alamine concentration was 20%; for propionic acid extraction, the optimum was 40%. The K_d value tended to increase as the starting acid concentration decreased, which suggested that the most efficient acid extraction would be obtained at relatively low acid levels. Extraction was more efficient as pH decreased. This is expected, since the extractant interacts with the undissociated form of the acid, which predominates at low pH. However, at pH 5.0, the highest pH tested to date, the highest K_d value obtained for propionic acid was 19, which compares very favorably to the best K_d values (around 5) that we had obtained previously with TOPO in kerosene as extractant.

The Alamine-octanol extractant shows great promise, and during the next year we will test its effectiveness at higher pH values. We will also obtain partition coefficients for other possible amine extractants, such as Alamine-336, Adogen-283-D, and Aliquat-336. We will test the possible toxicity of these extractants to the propionibacteria by pumping a culture through a hollow fiber extraction module that contains the extractant, and measuring changes in culture viability with time.

The work described in this progress report has been conducted by two graduate students: Ferhan Ozadali, a Ph.D. candidate in Food Science and Human Nutrition, and Zhong Gu, an M.S. student in Chemical Engineering.

Production of Propionic and Acetic Acids by Extractive Fermentation
Year 2 Progress Report

To increase the economic viability of production of propionic and acetic acids via fermentation, we proposed to increase the productivity of the fermentation and improve acid recovery methods. We have followed the strategies of using higher cell densities in the fermenter, continuous extraction of acids as they are produced, and better strains of propionibacteria. We have made progress in all of these strategies, as follows.

Identification of strains of propionibacteria that work best in different fermentation systems. We tested some additional propionibacteria, strains P4(produces polysaccharide), P20(produces polysaccharide), P38(forms clumps), P58(resists solvents), P68(produces high acid), and P127(produces bacteriocin), in addition to strains P9 and P200910, which we have been using as standard strains for organic acid production for characteristics that would give them advantages in different types of fermentations. By following growth, acid production, and pH of the medium in single-stage batch fermentations, we observed that *P. thoenii* P20 resists low pH conditions best and would work well in a single-stage batch fermentation without pH control. By allowing strains to form biofilms on various support materials and measuring acid production after repeatedly removing the spent medium and adding fresh medium, we found that *P. thoenii* P127 forms strong biofilms and may attain the highest cell density in a biofilm reactor. By counting numbers of viable cells upon extended storage, we determined that *P. acidipropionici* P200910 survives well when immobilized in calcium alginate beads and continues to produce acid for extended periods of time in the immobilized state.

Identification of materials that support good biofilm formation. We have continued work described in last year's progress report, in which we tested biofilm formation by strain P9 on various support materials formed form polypropylene plus mixtures of zein and corn starch, soy hulls, oat hulls, or corn hulls. We have added support materials made with soy protein rather than zein, as well as additional materials such as glass beads, ceramic saddles, fire bricks, wood chips, and paper filters. Materials that have a rough surface, are open in texture to allow permeation of growth medium, and withstand the physical forces of pressure, liquid flow, and high temperature work well as supports in biofilm reactors. Best materials identified to date are fire bricks, paper filters, and composite materials of polypropylene, corn starch, and zein. Strains P9 and P127 have produced the most acid in biofilm fermentations. We have been able to increase flow rates of fresh medium in continuous culture significantly, to dilution rates (D) of 10.8 h^{-1} at this point. In contrast, the maximum dilution rate that we have been able to maintain in continuous culture in free-cell reactors has been about 0.75 h^{-1}. These much higher flow rates for biofilm reactors provide an excellent opportunity to increase acid productivity significantly over free-cell fermentation. Results of typical fermentations are shown in Table 1 and Figure 1.

Improvement of immobilization in calcium alginate beads. The highest propionic acid concentrations (between 5 and 6%) that we have yet obtained in our laboratory were produced in fed-batch fermentations using cells immobilized in calcium alginate beads. We have continued our study of alginate immobilization and the use of

alginate beads in fermentations to improve performance. We have counted the viable cells in beads stored in different media for extended periods, and compared acid production by cells immobilized in small (2 mm diameter) vs. large (4 mm diameter) beads. Cells of strain P200910 remained viable for over a month of storage at refrigeration temperature, and continued to produce acid upon extended incubation in sequential fermentations. Bead diameter did not affect the amount of acid produced by immobilized cells in model fermentations. This result was somewhat unexpected, because the bead diameter should affect the diffusion of substrate into the beads and the diffusion of acids out of the beads. We will continue comparisons of different bead sizes under controlled conditions in a larger fermenter, test beads with more cells per gram and add more beads per ml of medium to see if acid production increases.

Toxicity testing of Alamine 304. We have used a supported liquid membrane system to recover organic acids from the fermentation broth and to maintain subinhibitory acid concentrations in the broth. The best extractant combination used was TOPO in kerosene, but this system was not as efficient as we would like to see. The usefulness of new extractants depends not only on their ability to remove acids from an aqueous system but also on their possible toxicity to the propionibacteria. We have been testing Alamine 304, a water-insoluble tertiary amine, in octanol. It is much more effective than the TOPO system in recovering acids from water(partition coefficients for propionic acid are around 5 for the range of propionic acid concentrations we would expect to find in fermentation broth.) We have examined the potential toxicity of Alamine 304/octanol for three strains of propionibacteria: P20, P58, and P200910. The organisms were inoculated into NLB which contained 30% v/v Alamine 304-1 in 2-octanol, provided in two different exposure modes: 1. The NLB was pre-equilibrated with the solvent for several hours before inoculation, so that the cells were in direct contact with solvent. 2. Pieces of the hollow fiber membranes were soaked with solvent, rinsed, and added to NLB at a fiber length to medium volume ratio similar to that used for on-line acid extraction. Cells were in contact with the membrane, not with the free solvent, in a manner most similar to what would happen in on-line extraction. Controls received pieces of membrane to which no solvent had been added, or contained growth medium only. The TOPO-kerosene solvent system was tested again in the same manner. Cells at early-, mid-, and late-log phases were tested separately, to determine if growth phase affected sensitivity to the solvent. Continued growth was monitored by measuring OD at 650 nm. Viable cells in cultures that did not show growth were counted by standard plate counting procedures, to determine if cells in these cultures were killed or only inhibited by exposure to the solvent system.

Typical results are illustrated in Figure 2. The Alamine inhibited culture growth if it was equilibrated with the growth medium, but not when it was confined to the fibers. TOPO equilibrated with the medium might have been slightly inhibitory. Age of the culture upon exposure to the solvents did not matter for strain P20; similar results were seen for all. Viable counts of P20 were not significantly different from controls in cultures exposed to TOPO or to Alamine-saturated fibers, but the presence of Alamine in the medium reduced viable counts by 99% or more. Strain P200910 was inhibited by both TOPO and Alamine equilibrated with the medium, but was not significantly

inhibited by these solvents confined to the hollow fibers. Cells were rapidly killed upon exposure to these solvents in the growth medium; viable counts of cultures exposed to solvent-soaked fibers were essentially the same as in control cultures. Late-log phase cells seemed to be more resistant than early- and mid-log phase cells. These results suggest that the Alamine 304/octanol system might be effectively contained by the hollow fiber membranes and be used for on-line extraction without significant culture inhibition.

Budget Notes

This study has required the efforts of two Principal Investigators plus two graduate students for the past two years. With the awarding of a matching grant from the Consortium for Plant Biotechnology Research (CPBR), a third graduate student has been added. We have provided a budget page that shows the current year's budget to demonstrate the matching support from CPBR, the New Energy Company of Indiana (our industrial partner in CPBR), and Iowa State University. We have also provided a budget page with the request for next year's budget. This request includes funding for one graduate assistantship (plus benefits), supplies for the student, travel funds to present results at national meetings, and user fees for the ISU Fermentation Facility. These user fees were instituted at the beginning of this fiscal year to enable the Facility to become more self-supporting. Fees are charged per fermentation or per hour of usage of other equipment. The amount in this budget request is enough for 20 bench-scale fermentations. The amount requested for travel funds has been increased to $1000 because the previous amount requested($500) was insufficient for even one person to travel to a meeting. We will submit a request to CBPR next spring for the second year's funding for that grant.

Other Accomplishments

Our funding from the Iowa Corn Promotion Board was instrumental in our obtaining a grant from the Consortium for Plant Biotechnology Research (which required matching funding) and from the New Energy Company of Indiana. These additional funds have allowed us to add a third student to this project. In addition to Ferhan Ozadali, a PhD student in Food Science and Human Nutrition, and Zhong Gu, a PhD student in Chemical Engineering, David Rickert, an MS student in FSHN, has begun work with us.

We have made three presentations of results from this project this year:

Ferhan Ozadali presented results of biofilm fermentations at the Annual Meeting of the American Society for Microbiology in May.

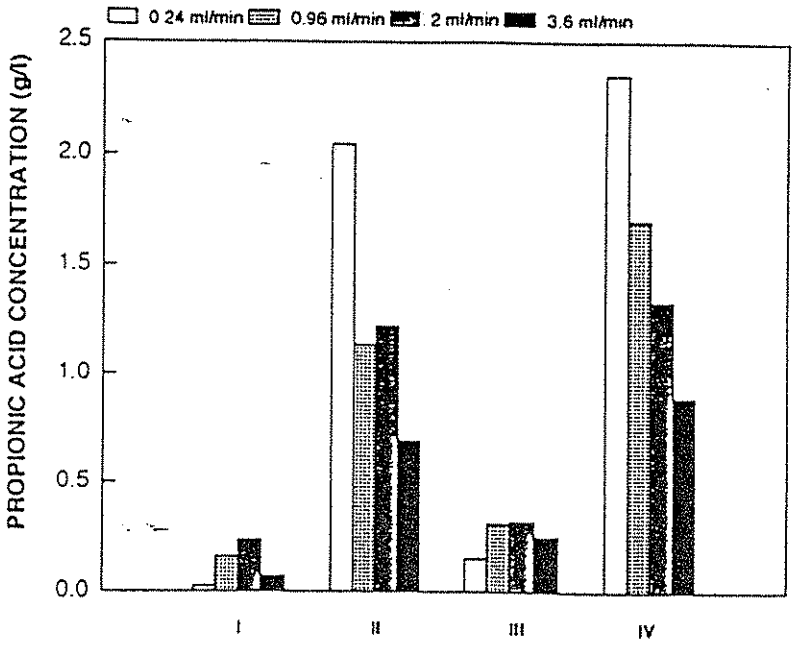
Bonnie Glatz presented fermentation results at the 7th Annual Colorado Biotechnology Symposium in September.

Zhong Gu presented results of toxicity testing at the 24th Annual Biochemical Engineering Symposium in September.

Table 1. Acid production by P9 and P127 biofilms on fire bricks in continuous fermentation at different dilution rates.

Dilution rate D (h^{-1})	Propionic acid conc. P (g/L)		Acetic acid conc. A (g/L)		Productivity P D x P (g/L/h)		Productivity A D x A (g/L/h)	
	P9	P127	P9	P127	P9	P127	P9	P127
0.68	2.26	3.30	0.00	1.12	1.54	2.24	0.00	0.76
1.37	2.28	4.11	0.00	1.43	3.12	5.63	0.00	1.96
2.74	2.68	3.73	0.85	2.49	7.34	10.22	2.33	6.82
5.49	3.68	4.96	1.43	1.87	20.20	27.23	7.85	10.27
8.57	3.16	3.26	1.44	1.23	27.08	27.93	12.34	10.54

Figure 1. Production of Propionic Acid by Strain P127 Immobilized on Various Supports in Continuous Culture at Various Dilution Rates



REACTOR I : CONTROL (FREE CELL)
REACTOR II : FIRE BRICKS
REACTOR III : CORN STARCH + ZEIN + POLYPROPYLENE
REACTOR IV : PAPER FILTER

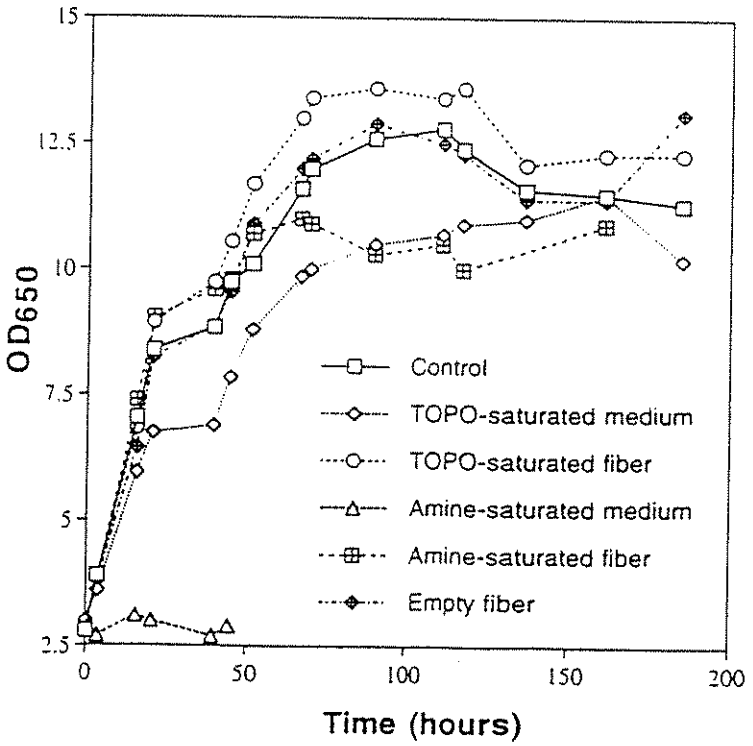



Figure 2. Growth of Mid-Log Phase Cells of Strain P20 Subjected to Various Means of Exposure to Organic Solvents