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Development of a hollow-fiber membrane bioreactor system for the continuous production of 2,5-diketo-D-gluconic acid

Matthew Brock Dalton

To the Graduate Council:

I am submitting herewith a thesis written by Matthew Brock Dalton entitled "Development of a hollow-fiber membrane bioreactor system for the continuous production of 2,5-diketo-D-gluconic acid." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

Paul D. Frymer, Major Professor

We have read this thesis and recommend its acceptance:

Gary Sayler, Charles Moore

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Development of a Hollow-fiber Membrane Bioreactor System for the Continuous Production of 2,5-diketo-D-gluconic Acid

A Thesis

Presented for the Master of Science

Degree

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Matthew Brock Dalton

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Abstract

A hollow-fiber membrane bioreactor system for the continuous production of 2,5-diketo-gluconic acid (2,5-DKG) was constructed and demonstrated. Additionally, key control parameters affecting the production of 2,5-DKG were identified. *Pantoea citrea*, which possesses three membrane bound enzymes that convert D-glucose into 2,5-DKG, was contained within the extracapillary space of the reactor. The system was designed to provide direct oxygenation to the biomass in the reactor, while liquid nutrients were provided through the intracapillary space. Experiments in which biomass concentration within the reactor and substrate throughput rate were varied identified key operating parameters that control product titre and the volumetric production rate of the system. Under the operating conditions used in the experiments, 2,5-DKG concentrations as high as 13 g/L were observed and maintained for 37 hours. Yields as high as 0.54 g 2,5-DKG/g glucose consumed were seen and a maximum volumetric productivity (based upon intracapillary volume) of 3.9 g/L/hr was achieved.

Table of Contents

List of Figures

Page

1. Introduction

L-ascorbic acid, also known as vitamin C, is a necessary component of a healthy diet. A prolonged lack of this chemical leads to scurvy, a common problem on sailing ships of centuries past which necessitated the stocking of large quantities of citrus fruits, a natural source of ascorbic acid, before a voyage. In recent years, increasing attention has been paid to ascorbic acid's role in the human immune system, where it is required for the production of white blood cells, T-cells, and macrophages, as well as its antioxidizing characteristics, which aids in the prevention of heart disease (Hoge, 1999). As a result, the demand for this chemical has drastically increased in the last 20 years, a time during which the annual production approximately doubled every 6 years and reached 35,000 tons in 1984 (Boudrant, 1990). To keep pace with the escalating demand and to maximize the profits obtained from the production of this chemical, the chemical process that has been used to produce ascorbic acid has undergone continual optimization and research has been conducted to further modify the process with biochemical pathways in order to further reduce production costs.

1.1. Reichstein Process

Vitamin C was first isolated in 1928 from orange juice and cabbage juice by Szent-Györgyi (1928), who established its molecular formula and its oxidative characteristics (Crawford and Crawford, 1980). Five years later, the first production process was proposed by Reichstein and Grussner (1934) and it is essentially this process

that is still used to produce ascorbic acid. Predominately a chemical process, the Reichstein process is composed of five steps—four chemical and one biochemical (Kulhanek, 1970). The first step is a chemical hydrogenation of D-glucose to D-sorbitol using a nickel catalyst, yielding a solution composed of at least 20% D-sorbitol (see Figure 1). This solution is then introduced into a fermenter containing either *Acetobacter xylinum* or *Acetobacter suboxydans* and dehydrogenated to L-sorbose. L-sorbose is then condensed with acetone to form 2,3,4,6-diisopropylidene-L-sorbose. This compound is then chemically oxidized using a platinum catalyst to 2-keto-L-gulonic acid, which then undergoes hydrolysis, enolization, and lactonization to form L-ascorbic acid.

Because of its relatively inexpensive starting substrate (D-glucose), the chemical stability of the intermediates, and the improvements made to the process, the Reichstein process has remained the method of choice for the production of ascorbic acid (Boudrant, 1990). Theoretically, by this process one molecule of D-glucose can be converted to one molecule of L-ascorbic acid; however, because of molecular rearrangements three hydrogen atoms and from one to four oxygen atoms cannot come from the D-glucose molecule. Accounting for these considerations, the maximum theoretical yield under these constraints can therefore not exceed 87%. Even with the improvements made through the years to the catalytic materials for the chemical steps and the microorganism selection for the biochemical step, the practical yield of the Reichstein process is currently 50%. This relatively low yield has led to a great deal of research in the past two decades into bacterial fermentation processes capable of producing vitamin C.

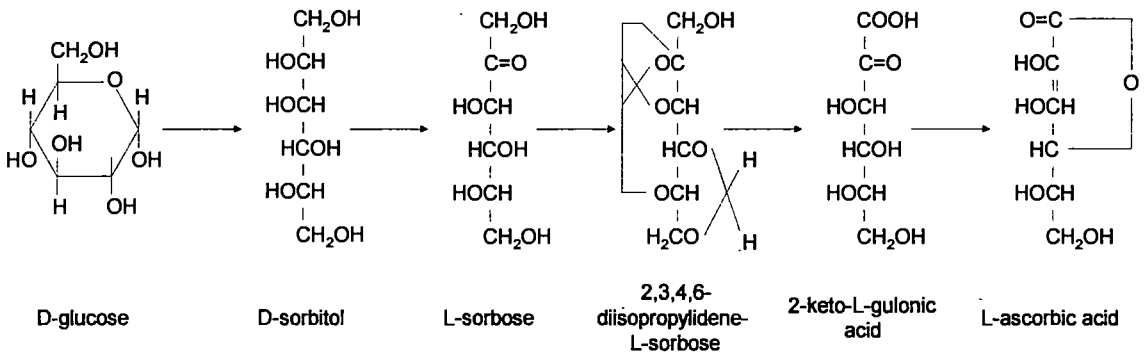

Figure 1. Reichstein Process

1.2. Biological Processes

Bioprocessing is becoming an increasingly popular method for chemical production, and of the types of biological systems available, systems using whole microbial cells are among the most common. Compared to classical chemical operations, biological systems can offer the advantages of multi-step chemical reactions taking place in a single reactor, thus reducing capital costs by eliminating the need for multiple reactors, and reduction or elimination of environmentally hazardous compounds from the process. Both of these advantages are applicable to the process for producing L-ascorbic acid. Replacing the separate chemical steps of the Reichstein process with a biochemical process composed of multiple conversion steps in a single vessel would reduce the capital invest required while simultaneously eliminating an environmentally hazardous chemical (acetone) from the process. Currently, there are six biochemical processes for the production of L-ascorbic acid (Boudrant, 1990). All of these produce 2-keto-L-gulonic acid (2-KLG) as the immediate precursor of ascorbic acid and all but one share at least one other intermediate (D-gluconate). All propose to replace the first four steps of the Reichstein process while leaving in place the final chemical step that converts 2-KLG into L-ascorbic acid.

1.2.1. Sorbitol Pathway

The first of these biochemical processes is the sorbitol pathway, first described by Motizuki and associates in 1966 (Crawford and Crawford, 1980), which utilizes one of several strains of *Pseudomonas* and *Acetobacter* for the single stage fermentation of

sorbitol to 2-KLG. Although the biochemical steps of the fermentation are not known, a possible metabolic pathway has been proposed that contains L-sorbose, L-idose, and L-idonic acid as intermediates (see Figure 2). Yields from this pathway have not consistently exceeded 10%, making it unfit for commercialization at its current level of development.

1.2.2. L-idonic Acid Pathway

The second of these biochemical processes is the L-idonic acid pathway. This multi-step pathway first oxidatively converts D-glucose into D-gluconate, which is then oxidized biochemically in the second step to 5-keto-D-gluconate (5-KDG). The conversion of D-gluconate to 5-KDG was first performed by Boutroux before the turn of the century using *Bacterium oblungus* (Crawford and Crawford, 1980) and many strains of *Acetobacter*, *Bacterium*, and *Pseudomonas* have been used to carry out this fermentation, with the highest yield (90%) coming from *Acetobacter suboxydans* (Boudrant, 1990). The next step utilizes *Acetobacter suboxydans* or *Bacterium gluconicum* (Kulhanek, 1970) to reduce the 5-KDG formed in the previous step to L-idonic acid. Finally, L-idonic acid is biochemically oxidized by *Pseudomonas mildenbergii* or *Pseudomonas fluorescenes* to produce 2-KLG, with yields of 80% and 90%, respectively. Overall, this pathway results in a yield of 2-KLG from D-glucose of up to 65%.

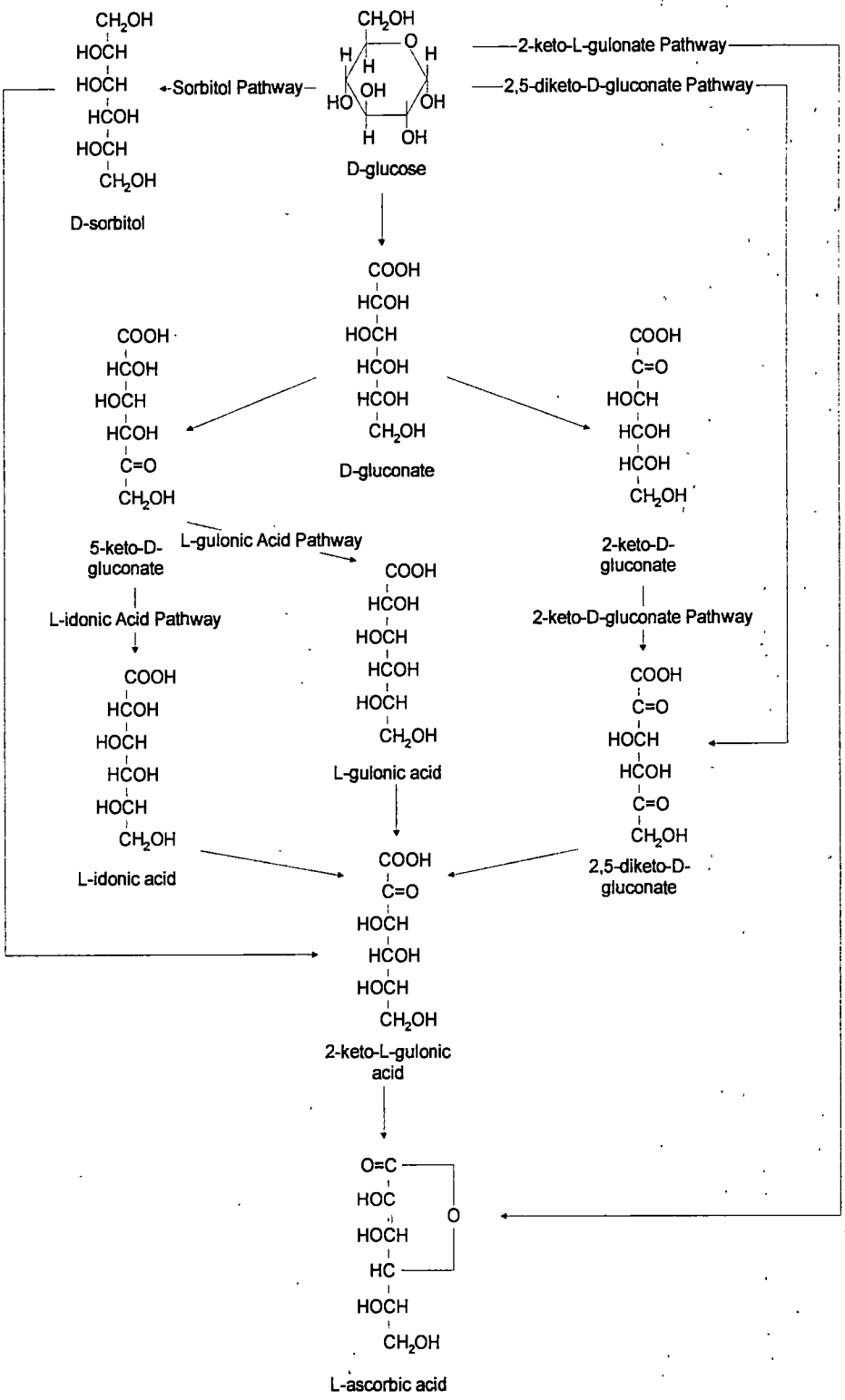

Figure 2. Biochemical Pathways

1.2.3. L-gulonic Acid Pathway

The third of the bacterial fermentation processes for the production of 2-KLG is the L-gulonic acid pathway. The starting material for this process is also D-glucose, which is oxidized to D-gluconate before undergoing a fermentation step that transforms it to 5-KDG. This compound is then reduced to form L-gulonic acid. The final step consists of a bacterial fermentation to 2-KLG using either *Xanthomonas transluscens* or *trifolii*, which gives a 90% yield of L-gulonic acid to 2-KLG (Kita, 1977).

1.2.4. 2-keto-D-gluconate Pathway

The 2-keto-D-gluconate (2-KDG) pathway, another of the processes to make 2-KLG, proceeds in three main fermentation steps that can result in an overall yield of up to 65% (Boudrant, 1990). The first oxidizes D-gluconate using either *Acetobacter suboxydans* or *Erwinia* spp. into 2-KDG, which is then oxidized by one of several *Acetobacter* and *Bacterium* strains into 2,5-diketo-D-gluconate (2,5-DKG). This compound is then reduced in what is the most studied of the three steps into 2-KLG. Initially, this step was carried out by *Brevibacterium ketosporum*, but because of the low yield associated with this organism (no more than 15%) *Corynebacterium* is used with a resulting yield of 80% (Boudrant, 1990).

1.2.5. 2,5-diketo-D-gluconate Pathway

Another of the pathways to produce 2-KLG is the 2,5-DKG pathway. This pathway differs from the above in that 2,5-DKG is made from D-glucose in a single bacterial fermentation. Another fermentation is then carried out to convert the 2,5-DKG into 2-KLG. Sonoyama et al. (1982) developed a process following this pathway using mutant strains of *Erwinia* sp. and *Corynebacterium* sp. to carry out the fermentations. In the first step, D-glucose is converted to Ca-2,5-DKG, with Ca-D-gluconate and Ca-2-KDG as intermediates, in a 26 hour fed-batch fermentation. These reactions are catalyzed by three membrane bound enzymes, D-glucose dehydrogenase, D-gluconate dehydrogenase, and 2-keto-D-gluconate dehydrogenase (Lazarus et al., 1989), which allows the reactions to proceed without the time consuming necessity of transporting substrate or products through the cellular membrane. Accumulation of Ca-D-gluconate and Ca-2-KDG, seen initially in the reactor, ceases after the completion of the D-glucose feed. By carefully controlling the glucose feed and maintaining the dissolved oxygen in the fermentation broth above zero, high productivity can be achieved and yields as high as 95% can be observed (Sonoyama et al., 1982). At the completion of this step, the fermentation broth is treated with sodium dodecyl sulfate to kill the *Erwinia* before feeding the broth to the *Corynebacterium* fermentation. In the second step, the fermentation broth is mixed with D-glucose (a hydrogen donor) and fed to the fermenter over a period of 48 hours at a rate that keeps the Ca-2,5-DKG concentration in the fermenter below 5 g/L. Production of Ca-2-KLG is seen immediately after the start of the broth feed and the yield for this step at the end of the fermentation is 93%, while the

overall yield of 2-KLG from D-glucose in this process is 85%. A variation of this process has been studied by Kita et al. (1981) in which the first step is carried out by *Acetobacter cerenus* with a yield of 90% (Boudrant, 1990). Furthermore, promising results have been reported for the first step by maintaining contact of D-glucose with cellular debris, which has the benefit of reducing the oxygen requirements of the system. Thus, with high product yields for each of the steps in the process and high product concentration in the final fermentation broth, there exists the potential for the commercialization of the 2,5-DKG pathway.

1.2.6. 2-keto-L-gulonate Pathway

The last of the biochemical routes to 2-KLG is the 2-KLG pathway. The unique aspect of this pathway is that it is carried out in a single fermentative step. This one stage conversion of D-glucose to 2-KLG is only possible through the use of mixed cultures (Boudrant, 1990) or genetically engineered organisms (Lazarus et al., 1989). In a mixed culture system using *Erwinia* to produce 2,5-DKG from D-glucose, *Corynebacterium* or *Brevibacterium* mutants to convert the 2,5-DKG to 2-KLG, and a surfactant to kill the first strain at the end of the first phase of the process, the overall yield of 2-KLG from D-glucose can be as high as 80%, which is near that theoretically obtainable in the Reichstein process. Processes using genetically engineered strains typically utilize *Erwinia herbicola* that has been modified to express the NADPH dependent 2,5-diketo-D-gluconate reductase (2,5-DKGR) from *Corynebacterium* (Lazarus et al., 1989). Overall yields of 2-KLG from D-glucose in these systems currently reach values of only

30%. In order to make production of 2-KLG using genetically engineered strains cost effective, methods for efficient transport of 2,5-DKG and 2-KLG across the cellular membrane and an active NADPH regeneration system must be developed.

1.3. Summary

Thus, there are several biochemical alternatives to the Reichstein process for the production of L-ascorbic acid. All propose to replace the first four steps of the Reichstein process while leaving in place the final chemical step that converts 2-KLG into L-ascorbic acid. Of the pathways available, one—the sorbitol pathway—is clearly inferior to the Reichstein system in its current form. Three of the pathways—L-idonic acid, L-gulonic acid, and 2-KDG—offer a marginal improvement over the present overall yield of 50% seen in the Reichstein process, although the potential of the bacterial fermentations must be weighed against the known potential yield of 87% in the Reichstein process. It is the 2,5-DKG and 2-KLG pathways that offer the greatest possibility to replace the current production system for ascorbic acid. Of these, the 2,5-DKG pathway offers the most immediate promise for a cost effective biological route to 2-KLG, for while the 2-KLG pathway would allow a reduction in capital costs by allowing the production of 2-KLG in a single vessel, at present no genetically engineered strains have been produced with a satisfactory yield of 2-KLG from D-glucose. In contrast, yields higher than 90% for each of the two steps in the 2,5-DKG pathway have been achieved, demonstrating its viability as a means of cost effective production of 2-KLG (Sonoyama et al., 1982; Kita et al., 1981).

The production of L-ascorbic acid by one of the biochemical pathways is ultimately dependent upon the bioreactor used for the production of its precursors. Because the final step in the biochemical process, in which 2-KLG is converted to vitamin C, is the same as that used in the Reichstein process, the potential benefits of using a biological alternative to the traditional chemical steps can only be achieved by proper design and operation of a bioreactor for the particular biological pathway chosen. The purpose of this research was to design and operate a continuous bioreactor to be used in the first of the two steps of the 2,5-DKG pathway (the conversion of D-glucose to 2,5-DKG) and to determine the parameters in the system that can be varied to control 2,5-DKG production.

2. Background

Another method of increasing the volumetric production rate of L-ascorbic acid or its intermediates is to develop a continuous process for its production. Past investigations into methods of improving or replacing the Reichstein process have focused upon batch production systems. The fermentation step in the existing Reichstein system is conducted in a batch reactor and the various fermentative routes to 2-KLG that have been proposed have all been studied in batch systems. Development of a continuous production system would eliminate the lost production time while batch fermenters are being sterilized, filled, and emptied. Coupling such a system with a biochemical pathway which reduces the number of steps required to form L-ascorbic acid provides the possibility of improving the production rate of vitamin C, while simultaneously reducing capital costs by reducing the number of reactors needed in the system. In order to achieve this goal, however, it is necessary to develop a reactor that not only allows for continuous operation, but also retains the biomass within the system.

2.1. Continuous Bioreactors

Free cell systems can be extremely effective in efficiently converting substrates into the desired end products when operated under the proper conditions. They are severely limited, however, in their rate of production. Most free cell systems are batch processes, although they can be operated in a continuous mode in the form of cascaded chemostats (Shuler and Kargi, 1992). But even in this continuous style of operation, the production rate is severely limited because operating at dilution rates higher than the

growth rate of the cells results in the biomass being washed out of the system. For this reason, to achieve higher rates of production it is necessary to immobilize the cells within the reactor system.

2.2. Immobilized Cell Reactors

Immobilized cell reactors provide several advantages when compared to a free cell system (Willaert et al., 1996). Systems using immobilized cells can operate at high dilution rates without culture washout, which is one factor in these systems having higher cell densities than free cell processes. Also contributing to the higher cell densities is the lower shear environment for the cells when immobilized, which generally promotes growth. Product streams containing little or no biomass, thus simplifying downstream separations, is another characteristic of immobilized systems. Finally, continuous removal of inhibitory products maintains high levels of product formation. The result of these attributes of fixed cell systems is the capability of operating in a continuous mode with a reactor that has a higher product yield per reactor volume than a corresponding free cell system.

There are a variety of immobilized cell reactors using several different immobilization techniques that can be chosen from to meet the goal of continuous production (Willaert et al., 1996). Two of the most common are packed and fluidized bed reactors, which are very similar to their classical chemical reactor counterparts. In both cases, cells can be immobilized on solid catalyst particles or encapsulated within gels or polymeric beads. Although these reactor types offer the possibility of higher

production rates than the free cell systems, they suffer from some disadvantages. In both cases, flow rates must be kept low enough to prevent catalyst particles from being washed out of the reactor, a problem that is especially evident when using lighter packing such as gel beads. With cells immobilized on catalyst particles, there is still some wash-out of the cell culture with the product stream, thus necessitating a separation step to remove the biomass from the reactor effluent. Encapsulated cells exhibit two primary problems—mass transfer limitations in providing nutrients through the encapsulating material and excessive cell growth that can cause the beads containing the cells to rupture. Additionally, the regeneration of biocatalyst and repacking of the reactor are time consuming processes common to both fluidized and packed beds.

2.3. Hollow-fiber Membrane Reactors

A relatively new type of immobilized cell reactor that overcomes the problems associated with bed-type reactors is the hollow-fiber membrane reactor (Willaert et al., 1996; Bunch, 1988). A reactor of this type (see Figure 3) consists of micro- or ultrafiltration asymmetric hollow-fibers within an outer shell in a configuration analogous to a shell-and-tube heat exchanger. Cells are immobilized on the shell side of the membrane and within its porous support, while nutrient medium is introduced into the reactor by one of two methods. In the first method, often called the direct mode of operation, nutrient medium flows through the tube side of the hollow-fibers. Substrates diffuse through the semi-permeable membrane to the cells which then utilize them to form products that diffuse back through the membrane. The second method, referred to

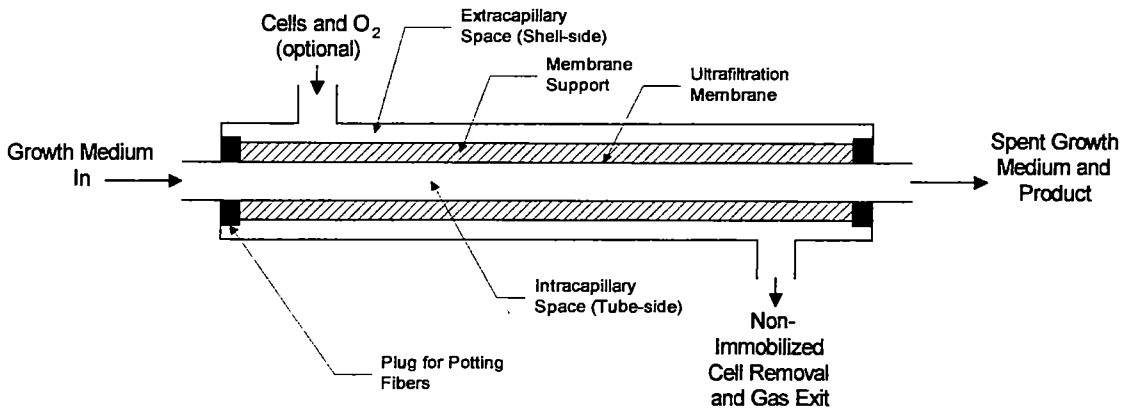

Figure 3. Hollow-fiber Membrane Reactor (Direct Mode of Operation)

as the transverse mode of operation, has the nutrient inlet on the shell side of the reactor and the product outlet on the tube side (see Figure 4). Substrates flow directly to the cells, thus ensuring the cells have the opportunity to utilize all of the substrates, and product and unused substrates flow convectively through the membrane into the tube side where they exit the reactor. A reactor of this type, therefore, benefits from a totally cell free product stream. Additional advantages include less risk of contamination of the cell culture (Inloes et al., 1983), a cell environment with much less shear than other systems, high surface area to volume ratio for the immobilized cells, higher cell densities than other systems, and "lower capital investment and less space required...than with typical production systems" (Willaert et al., 1996).

Of course, as with any reactor system there are some disadvantages associated with hollow-fiber membrane reactors that must be overcome (Willaert et al., 1996, Bunch, 1988). It is often difficult to provide microbial cells with enough oxygen in a hollow-fiber reactor by oxygenating the substrate stream in the tube-side because of mass transfer limitations associated with oxygen diffusion through the membrane and the low solubility of oxygen in water. Also, much like encapsulated cells, excessive cell growth can be a problem in hollow-fiber reactors, particularly those operating in the transverse mode. This phenomenon leads to a thick coating of cells around the fibers, plugging the pores and resulting in an increase in pressure until the membrane ruptures. Thus, to obtain the benefits offered by the hollow-fiber membrane reactor, careful consideration must be paid to the problems associated with this reactor type and methods of overcoming them developed and applied to the reactor design.

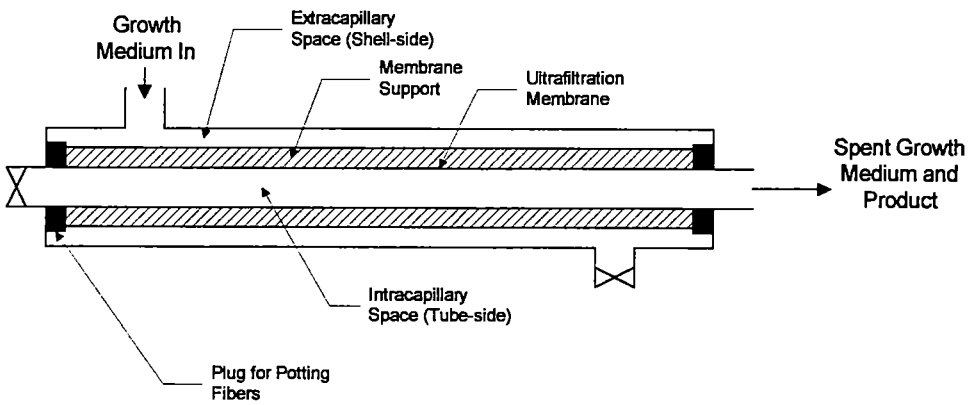

Figure 4. Hollow-fiber Membrane Reactor (Transverse Mode of Operation)

Although there is no evidence of previous attempts at developing a hollow-fiber membrane reactor for the production of L-ascorbic acid intermediates, reactors of this type have been studied in the production of urocanic acid, lactate, β-lactamase, tetracycline, and rifamycin B. In the course of these studies, both modes of operation were explored and a variety of different configurations were utilized, primarily with the aim of improving oxygen supply to the cells. The success of the hollow-fiber membrane reactor in the production of these compounds varied, but these studies do demonstrate both the limitations and potential of this design.

2.3.1. Hollow-fiber Reactor for Production of Urocanic Acid

The first of these systems was used for the one-step enzymatic conversion of L-histidine to urocanic acid (Kan and Shuler, 1978) using Cordis-Dow Artificial Kidneys as hollow-fiber reactors. These devices had an identical shell and tube side volume—100 mL—and contained 13,500 regenerated cellulose hollow-fibers with an inner diameter of 0.2 mm. Liquid nutrients were fed through the tube side of the reactor and inoculated into the shell side was a suspension of *Pseudomonas fluorescens* . This organism contains both L-histidine ammonia-lyase for conversion of L-histidine to urocanic acid as well as urocanase, which is responsible for the degradation of urocanic acid. Consequently, in order to prevent loss of urocanic acid during reactor operation, it was necessary to heat treat the cells to destroy urocanase activity before immobilization. The system was operated with fresh feed entering and a product stream leaving a recycle tank from which medium entered the reactor. A variety of system volumes, feed

substrate concentrations, and flowrates were used in production runs that lasted approximately 12 days. The results from these runs showed significant production of urocanic acid, with conversions of between 20 and 50 %, depending upon operating conditions and perturbations performed on the system. For example, in one run in which flow was stopped for 1.2 days beginning on the second day of operation then resumed at its previous rate, conversion of L-histidine to urocanic acid rose to approximately 50 % by the fourth day and was maintained at this level throughout the rest of the production run. It should be noted, however, that their biological system was extremely simple (a single enzymatic step) and, because of the heat treatment of the cells, did not require oxygen addition to the system. Thus, Kan and Shuler demonstrated the possibility of using a hollow-fiber reactor system for continuous production with microbial cells, but did not explore potentially two of the major limitations of a hollow-fiber reactor system, namely oxygen limitation and excessive cell growth.

2.3.2. Hollow-fiber Reactor for Production of β-lactamase

A system that investigated the first of these potential limitations (oxygen) was that used by Inloes and his associates for the production of β-lactamase (Inloes et al., 1983) and consisted of a single hollow-fiber potted within a 25 cm glass tube. *Escherichia coli* cells were immobilized on the shell side of the membrane and within the porous support of the membrane, liquid nutrients were introduced through the tube side, and sterile, humidified air or oxygen passed through the shell side. Reactor runs of 5 and 23 days were conducted, with β-lactamase production and cell density in the continuous systems

being compared to that in a batch process. In both the long- and short-term continuous runs, bacteria in the continuous reactors were only about 10% as productive as those in the batch system, but because of a cell density approximately four orders of magnitude greater than the batch system, the hollow-fiber reactor was approximately 100 times more productive than the batch process. Additionally, β-lactamase productivity was independent of liquid nutrient flow rate and did not change significantly when oxygen, rather than air, was used to aerate the shell side. These characteristics indicated that the performance of the hollow-fiber reactor was kinetically controlled. Problems associated with excessive cell growth (i.e. fiber rupture), however, were seen in all runs, as was indicated by the appearance of cells in the reactor effluent within 6 hours of inoculation.

Thus, the hollow-fiber reactor designed for β-lactamase production was successful in continuously producing this compound, but a significant problem remained. In order for the reactor design to be useful, some method to prevent the rupture of the hollow-fiber membranes had to be developed. It should be remembered that the cells were contained only within the porous support of the membrane and on the shell side of the membrane itself. This allowed for the passage of air or oxygen through the hollow-fiber membrane of the reactor to provide adequate amounts of oxygen to the cells, however it also created the situation where slight cell growth ruptured the membrane. Therefore, a different method of cell containment and oxygen supply or a means of monitoring and controlling cell growth was necessary to provide a reactor robust enough for long-term continuous production.

2.3.3. Hollow-fiber Reactor and Control Method for Production of Lactate

To address the problem of excessive cell growth, Linton et al. (1987,1989) developed a method for monitoring and operational strategy to control cell growth in a hollow-fiber reactor. Their system was composed of a stainless steel cylinder 15.0 cm long with an inner diameter of 1.1 cm into which nine Romicon PM100 hollow-fibers with a 100,000 molecular weight cutoff were placed. *Streptococcus faecalis* var. *zymogenes* was chosen as the organism to use in the system because it produces two different metabolic end products from glucose depending upon the amount of oxygen present—acetate under aerobic conditions, lactate under anaerobic conditions. The cells were inoculated into the shell side of the reactor and immobilized, as in the previous system, only upon the outer surface of the membrane and within its porous support. Liquid nutrients were pumped into the shell side of the reactor and forced through the hollow-fiber membrane to exit out the tube side of the reactor (transverse mode of operation), while oxygen was supplied through the tube side port opposite the liquid outlet. The system was operated for a variety of times up to 120 hours and the shell side hydrostatic pressure was monitored during all runs.

Over the course of the initial reactor runs, rapid glucose degradation and lactate formation was observed during the first 10 hours of operation. At this point, lactate concentration in the reactor effluent stabilized at 17 mM, which corresponds to an 85 % yield of lactate from glucose, and remained at this level for the rest of the 120 hour production run. At approximately the 30th hour of reactor operation, however, fiber rupture was observed in the system, as evidenced by cells in the reactor effluent. This

corresponded to a reproducible shell side hydrostatic pressure change from the beginning of operation of approximately 1.0 psi. Subsequent runs were then made using the shell side hydrostatic pressure change as a means of predicting membrane rupture. When a hydrostatic pressure change of 0.7 to 0.8 psi was obtained, reactor feed was changed to a nitrogen limited medium. Operating with this strategy, no further increase in shell side hydrostatic pressure was observed after beginning feed of the nitrogen limited medium and there was no evidence of hollow-fiber membrane rupture. An effect was also seen in the formation of lactate. Upon switching to nitrogen-free medium, lactate production continued for the next 40 hours, with a peak yield of 75 %, after which the concentration of lactate in the reactor effluent decreased rapidly (see Figure 5). Shell side hydrostatic pressure was also observed to rise at this point, a phenomenon attributed to debris from cell lysis obstructing the membrane.

Thus, Linton et al. (1987,1989) developed both a means for monitoring cell growth in a hollow-fiber reactor and a method for controlling that growth. They successfully demonstrated that a nitrogen-limited medium could be used to maintain production of lactate while simultaneously preventing further cell growth. While lactate formation during the supply of nitrogen-free medium did not remain at the level initially seen and did not continue for as long a period as it did during continuous feed of growth medium, it is possible that this limitation could be overcome by periodically reintroducing the growth medium to the system to regenerate cells. This would not, however, overcome the obvious oxygen limitation seen in the system. The formation of

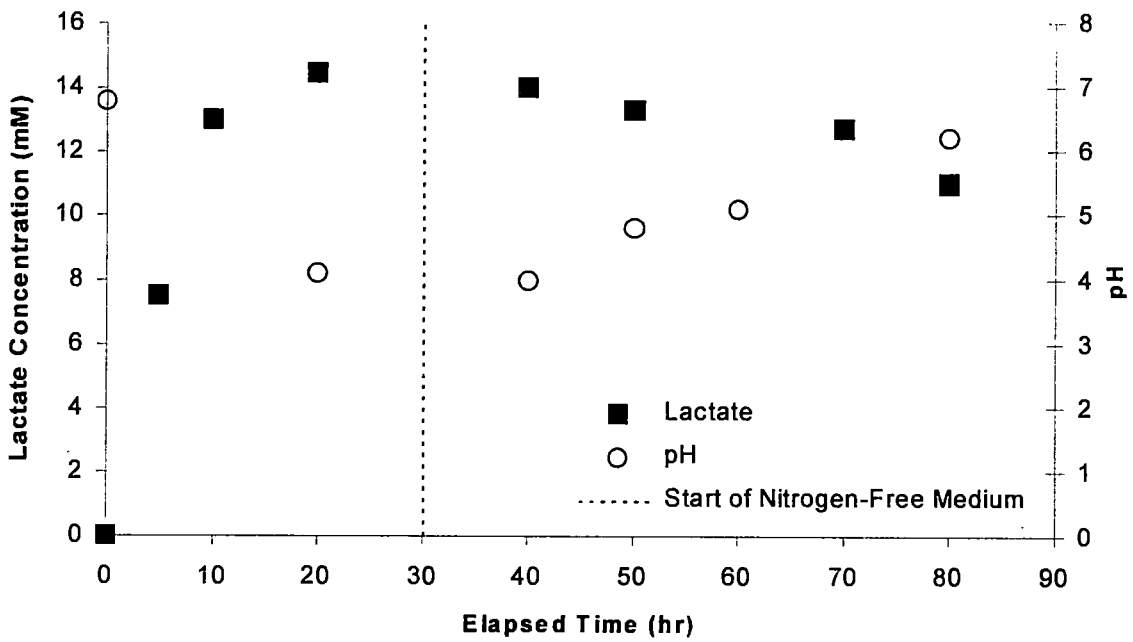

Figure 5. Lactate Production in Hollow-fiber Reactor (adapted from Linton et al., 1989)

lactate rather than acetate clearly indicated that the system was operating under conditions of oxygen limitation. Therefore, in order for the hollow-fiber reactor system to be applicable to processes utilizing aerobic microorganisms, further adjustments to the system design had to be made.

2.3.4. Dual Hollow-fiber Reactor for Production of Tetracycline

The hollow-fiber reactor developed by Robertson and Kim (see Figure 6) for the production of tetracycline from the fermentation of *Streptomyces aureofaciens* addressed the issue of cell containment and oxygen supply (Robertson and Kim, 1985). Rather than immobilizing the cells only on the porous support and outer wall of the hollow-fiber membrane, they decided to use the area exterior to the fibers to contain the cells. This would allow the cells more room to grow, thus at least prolonging the life of the membrane. Oxygen supply, however, then became a problem. This was overcome by introducing a second, oxygen-permeable membrane (in this case silicone) into the reactor. The new configuration for the reactor consisted of three silicone tubules contained within a single polypropylene hollow-fiber. Three of these hollow-fibers were then enclosed within a glass shell, thus forming a dual hollow-fiber reactor. The cells were inoculated into the space between the hollow-fiber and the silicone tubules. Liquid nutrient was passed between the glass shell and the hollow-fibers, while air or oxygen flowed through the silicone tubules contained within each hollow-fiber. As a final means of ensuring excessive cell growth would not cause membrane rupture, cells were grown

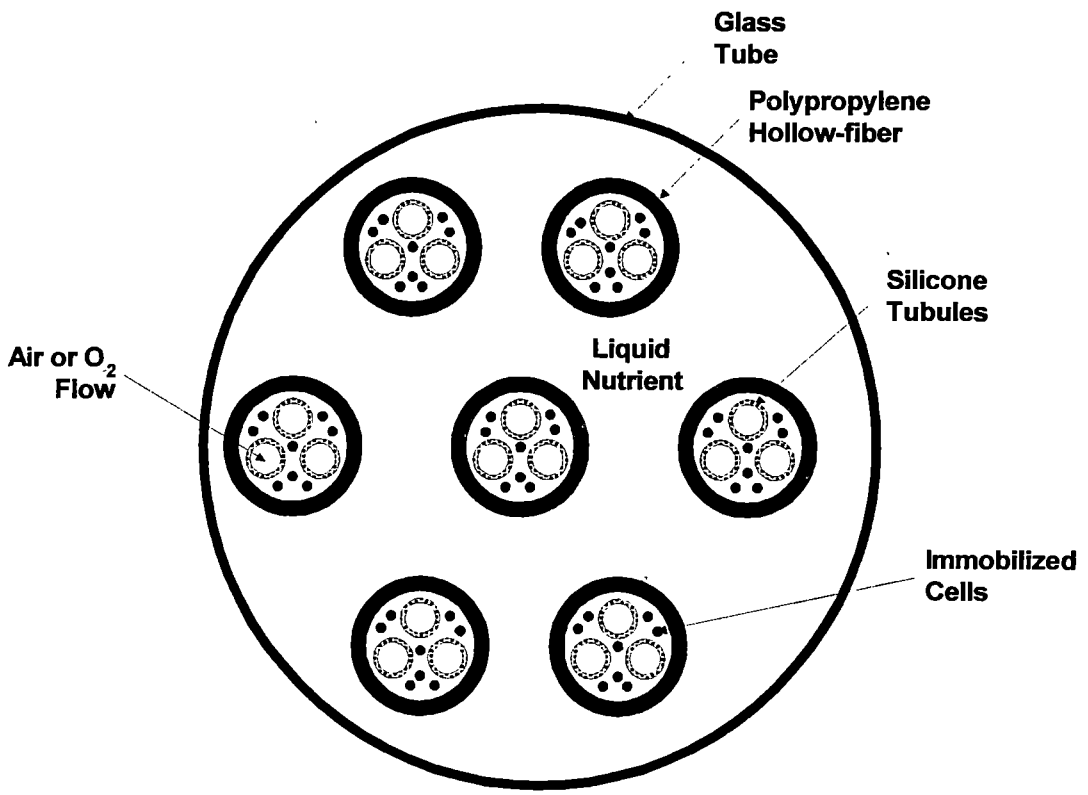

Figure 6. Dual Hollow-fiber Reactor for Production of Tetracycline (adapted from Robertson and Kim, 1985)

separately to stationary phase, introduced into the dual hollow-fiber reactor, and non-growth medium supplied to the system. The completed dual hollow-fiber reactor had an overall length of 19 cm and an outer diameter of 0.7 cm. Ghia-Membrana polypropylene hollow-fibers with an inner diameter of 0.16 cm were used, and of course each of these enclosed three silicone tubules. Continuous reactor runs were made as described above, namely gas and liquid nutrients were fed counter-currently through the silicone tubules and the space between the hollow-fibers and the glass shell, respectively (direct mode of operation). Additionally, runs were made in which liquid nutrient was introduced into the space between the silicone tubules and the hollow-fibers through the inoculum port and forced through the membrane, exiting out the usual liquid nutrient exit port (transverse mode). Direct mode reactor runs were 130 hours long and utilized both air and pure oxygen as the gas nutrient, while transverse mode runs were only 40 hours long. Also, direct mode fermentations were conducted using both a growth medium/non-growth medium feed cycle and a growth medium only feed.

Through the first 40 hours of the initial fermentations, the direct and transverse mode reactors exhibited nearly identical trends in pH and tetracycline production. At this point in the transverse mode fermentation, cell growth had greatly reduced the flow of medium through the hollow-fiber membrane, necessitating an end to this fermentation. During the subsequent 40 hours of the direct mode fermentation, tetracycline production remained essentially constant at 90 μg/mL/h based on interstitial volume (5.5 μg/mL/h based on total reactor volume). At this point in the initial fermentation (80 hours), non-growth medium was fed to the system. The system then exhibited a steady decline in

both productivity and pH until the fermentation was terminated at the 130th hour (see Figure 7). Direct mode fermentations using growth medium throughout the course of the experiment exhibited the same decline in productivity after the 80th hour. Also, using pure oxygen rather than air as the gas nutrient had no effect on the performance of the reactor.

Thus, the results of Robertson and Kim's studies indicated some of the potential for continuous production in hollow-fiber bioreactors. *S. aureofaciens* was successfully immobilized within the system and rupture of the membrane prevented by containing the cells outside of the porous membrane support and providing oxygen through silicone tubules. The insensitivity of the system to changes in gas nutrient indicated that the reactor design was providing adequate amounts of oxygen to the cells. Decline in tetracycline production, however, suggested some changes to the system were necessary before stable continuous production could be achieved.

2.3.5. Dual Hollow-fiber Reactor for Production of Rifamycin B

Building on the work of Robertson and Kim, Chung et al. developed a successful dual hollow-fiber bioreactor (see Figure 8) for the production of rifamycin B (Chung et al., 1987). The reactor, although similar to the previous one (Robertson and Kim, 1985), differed in certain key aspects of its design. Whereas before a single polypropylene hollow-fiber contained three silicone tubules, in this reactor three polypropylene hollow-fibers were confined within a single silicone tube. Ten of these assemblies were then

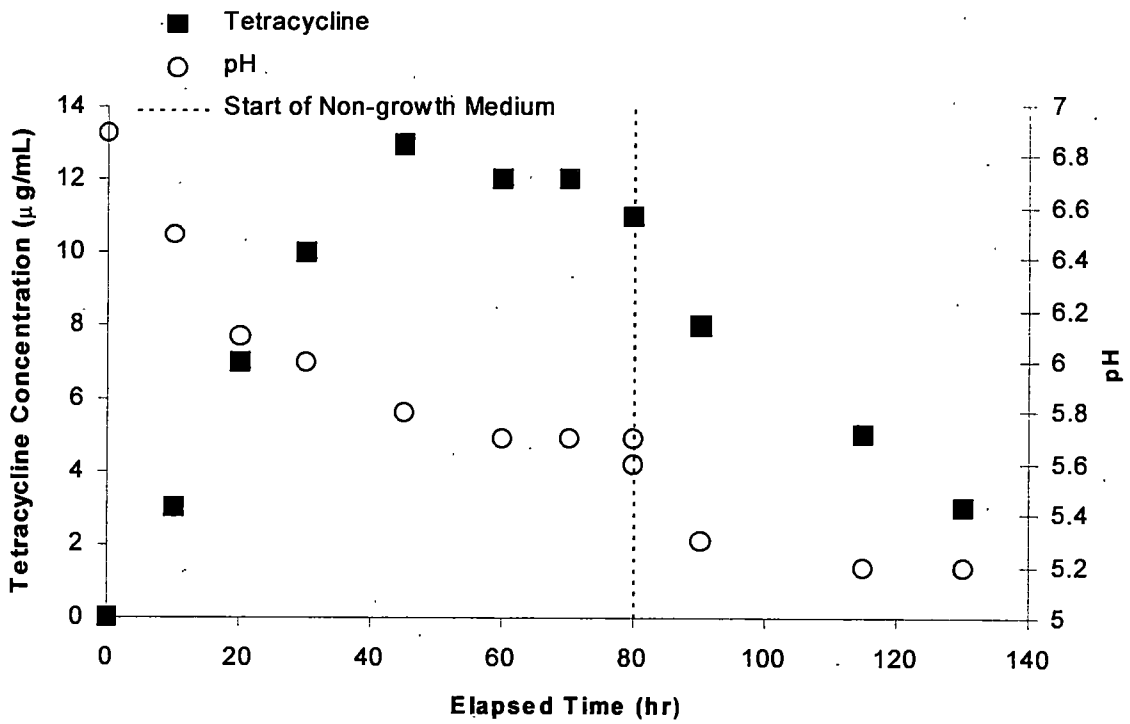

Figure 7. Tetracycline Production in Dual Hollow-fiber Reactor (adapted from Robertson and Kim, 1985)

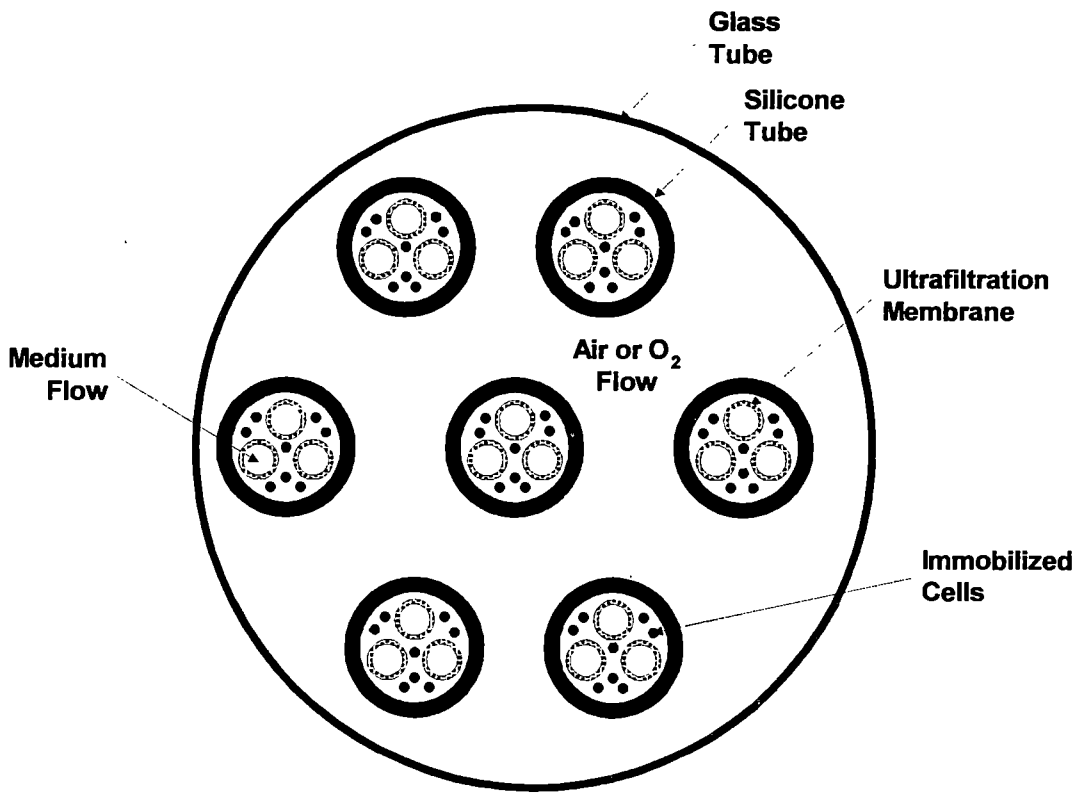

Figure 8. Dual Hollow-fiber Reactor for Production of Rifamycin B (adapted from Chung et al., 1987)

placed within a glass shell. *Nocardia mediterranei* cells were inoculated into the interstitial space between the hollow-fibers and the silicone tube, while liquid and gas nutrients flowed through the hollow-fibers and the space between the silicone tube and the glass shell, respectively.

The resulting dual hollow-fiber reactor had an overall length of 30 cm and an outer diameter of 1 cm. Silicone tubing with an inner diameter of 0.147 cm was used and contained three Enka polypropylene hollow-fibers with an inner diameter of 0.033 cm. Continuous reactor runs of 34 days were made with the reactor operating in the direct mode and liquid and gas nutrients flowing countercurrently. In order to fully test the performance of the system, two liquid nutrient media (one a ten-fold dilution of the other), two gas nutrients (air and pure oxygen), and three liquid nutrient flow rates were used. A batch fermentation of seven days duration was used as a control to evaluate the performance of the dual hollow-fiber system.

The initial studies with the dual hollow-fiber system investigated the effect of liquid nutrient concentration on the productivity of the system. Fermentations used gas and liquid nutrient flows of 100 and 1.7 mL/h, respectively, with the concentrated liquid medium being fed for the first 24 days and the dilute medium for the 10 days thereafter. During the concentrated nutrient period, after day 18 rifamycin B was produced at a steady concentration of 72 mg/L and carbon source (glucose) was nearly consumed. With the introduction of the dilute medium, the production level dropped by half and the glucose concentration began to rise. Although clearly the dilute medium was less productive than the concentrated medium in the continuous system, using dilute medium

in the hollow-fiber reactor resulted in a much higher rate of production than that seen in the batch system using the concentrated medium (115 mg/L/hr on an interstitial volume basis compared to 4.88 mg/L/hr). This trend demonstrated that proper design of the nutrient medium could result in a high production rate with a minimum of substrate since the production level only dropped by half when the substrate concentration was reduced by an order of magnitude.

Substituting pure oxygen for air as the gas nutrient exposed an oxygen limitation inherent in the reactor design. Fermentations using pure oxygen reached a steady production level faster and achieved a higher production rate than those using air as the gas nutrient. After day seven of the fermentation, rifamycin B production stabilized at 95 mg/L until the dilute medium was introduced on day 19, at which point production fell to 40 mg/L. In order to test the regenerative capabilities of the cells within the system, concentrated medium was resupplied to the reactor on day 31, resulting in an increase in production, but to only 78 mg/L. Thus, the cells were somehow damaged during the period of dilute medium feed, possibly by the system becoming more alkaline during this feed cycle.

A final set of experiments were performed with the system in an attempt to increase the concentration of the product in the reactor effluent. By increasing the level of rifamycin B coming from the reactor, downstream purification costs would be lessened, thus increasing the economic viability of the continuous system when compared to the batch process. To achieve this concentration, lower liquid nutrient flow rates (0.25 and 1 mL/h) were used. When operating at 1 mL/h, rifamycin B production was steady at

150 mg/L, however, when medium flow was slowed to 0.25 mL/h production spiked at 560 mg/L and steadily fell over the next six days to 405 mg/L (see Figure 9). The corresponding product yields for these two flow rates were 75 and 37 mg rifamycin B/g glucose. The most likely explanation for the decrease in yield at the lower flow rate was the increase in pH in the system to a level beyond the optimum level reported by Margalith and Pagani (1961).

Overall, the performance of the dual hollow-fiber system was much better than the batch process. Although the specific productivity of the cells in the hollow-fiber reactor was only about 30-40% of those in the batch system, this was more than offset by a cell density 70 times that in the batch process (550 g/L compared to 8 g/L). Consequently, the volumetric productivity of the continuous system was either 22 or 30 times that of the batch system, depending upon which gas nutrient was used (air or oxygen, respectively). This difference in production rates with changing gas feed clearly indicated that the design of the continuous reactor did not fully meet the oxygen demand of the cells, however, the demand was sufficiently met to continuously produce rifamycin B for 40 days at levels much higher than was possible in the batch system. Also, it should be noted that all continuous reactor runs were stopped after an arbitrary number of days, not as a result of a decline in production. All production decreases during a run were the result of changes in the liquid nutrient and, even after these decreases, production stabilized at levels much higher than that seen in the batch fermentation. Thus, although the design of the dual hollow-fiber reactor could still have been optimized to provide greater levels of oxygen to the cells, in this configuration it was still superior

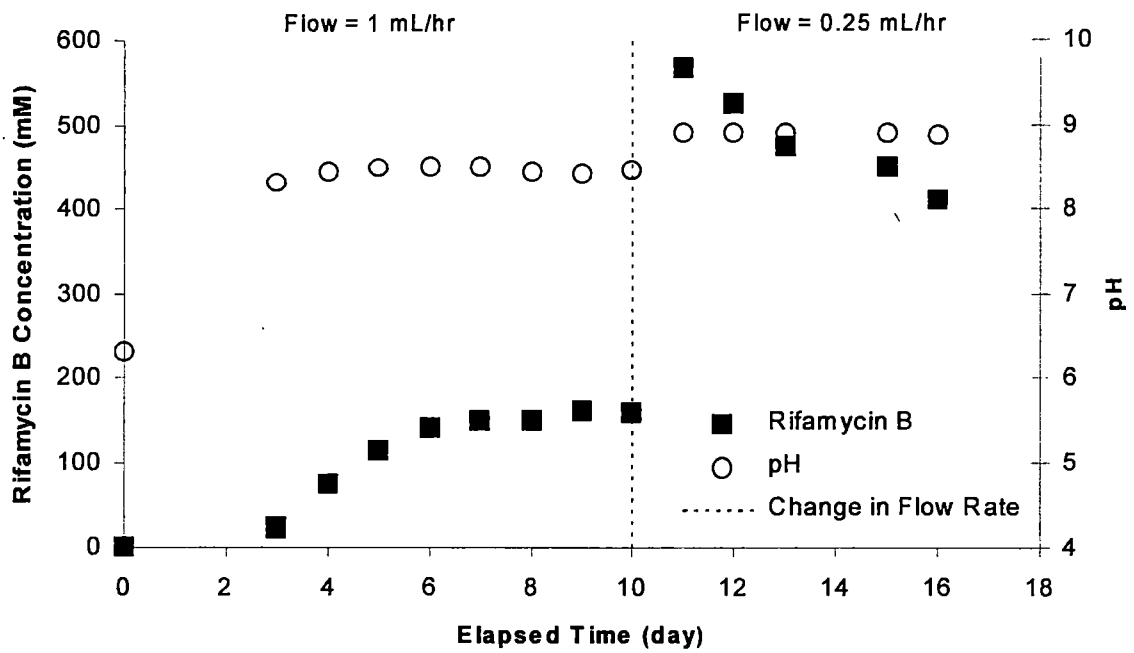

Figure 9. Rifamycin B Production in Hollow-fiber Reactor (adapted from Chung et al., 1987)

to the batch fermentation system and capable of continuously producing rifamycin B for extended periods of time.

3. Materials and Methods

3.1. Hollow-fiber Bioreactor System for the Production of 2,5-DKG

In order to develop a hollow-fiber reactor system for the continuous production of 2,5-DKG, the type of hollow-fiber reactor (single fiber type or dual hollow-fiber), membrane type (ultra- or microfilitration), mode of operation (direct or transverse), and method of oxygen supply had to be addressed. The reactor chosen for this system was a conventional single-fiber-type hollow-fiber reactor. This design was used because of the availability of large commercial hollow-fiber cartridges, which eliminated the task of constructing the reactor itself and also provided the possibility of ease of scale-up to a pilot or industrial scale process. A Spectrum Labs CellFlo hollow-fiber filtration cartridge was used as the bioreactor in the system. The cartridge, constructed from polysulfone, was 50.7 cm long with an outside diameter of 3.12 cm and contained 274 microporous mixed cellulose ester hollow-fibers (0.2 μm pore size) each with an inner diameter of 1 mm. The total surface area of the membranes within the cartridge was 3915 cm^2 and the shell-side and tube-side volumes were 168 and 99 mL, respectively. Tube-side connections were by 0.5 in hose barbs, while shell-side inlet and outlet ports consisted of 0.125 in luer fittings. A hollow-fiber reactor with microporous membranes was chosen in order to reduce the mass transfer limitations associated with systems using ultrafiltration membranes and also to lessen the possibility of fiber rupture. To further address the problem of fiber rupture as well as to allow a higher level of oxygen supply, the reactor was run in the direct mode of operation.

Because of the relatively low flow rates used in the tube-side of the reactor, the reactor was oriented vertically with flow entering the bottom of the cartridge and flowing out the top (see Figure 10). This flow path ensured that the tube side of the reactor would be completely filled with medium, however it also provided the possibility for the biomass to accumulate in the bottom of the shell-side and thus effectively shorten the length of the reactor. To combat this problem, the shell-side cell suspension was recirculated to maintain a uniform biomass concentration along the length of the reactor. Recirculation on the shell-side also allowed for direct aeration of the cell suspension. The shell-side effluent entered a small aeration tank (50 mL Pyrex vial) where sterile filtered air was bubbled through the fluid and was then pumped (Masterflex peristaltic pump using size 15 silicone tubing) out of the tank and into the bottom shell-side port. Additionally, an in-line oxygen probe (YSI Model 53 oxygen probe in a Swagelock housing) was added to the line between the pump and the shell-side inlet to monitor the shell-side inlet oxygen concentration in the reactor.

A Bioengineering AG Fermentor 2000 KLF was used as a feed tank for the reactor system. Sterile filtered air was bubbled through the medium and the tank was agitated at 500 rpm to saturate the medium with oxygen before feeding it to the reactor. Medium was supplied from the feed tank to the tube-side inlet of the reactor using an FMI QV-0 pump and a combination of silicone and Tygon tubing. Effluent flow out of the reactor was controlled by a Masterflex Digital Console Drive peristaltic pump using size 13 silicone tubing. This configuration and pump choice ensured that a consistent

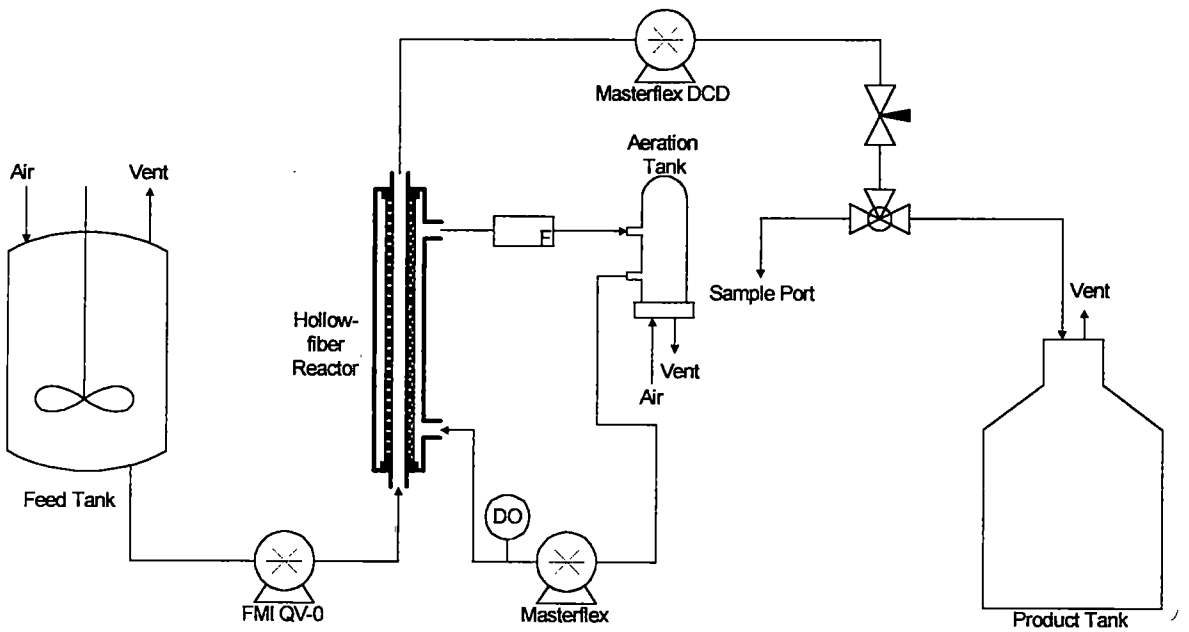

Figure 10. Hollow-fiber Membrane Reactor System

flow in and out of the reactor would be maintained and, thus, that there would be minimal accumulation or depletion of cell suspension in the aeration tank.

Sample acquisition occurred at two locations, at the effluent sample port slightly upstream from the product tank and from the aeration tank on the shell-side. Effluent samples were analyzed for glucose and 2,5-DKG and their pH was measured, while shell-side samples were used for enzyme assays and 2,5-DKG analysis.

Nitrogen limited medium was used in all reactor runs to control cell growth and reduce the possibility of contamination of the reactor system. The nutrient medium was composed of KH_2PO_4 (6 g/L), K_2HPO_4 (4 g/L), glutamic acid (1 g/L), and glucose (50 g/L). Medium was made in 3 L batches in two parts, a phosphate/MSG solution and a glucose solution, with the phosphate/MSG solution being autoclaved while the concentrated glucose solution was filter sterilized. These two solutions were then combined before being added to the feed tank. The Bioengineering feed tank, the product tank, and the aeration tank were all autoclaved prior to system startup, while all tubing and the hollow-fiber reactor were sterilized with a 70 % ethanol solution and then rinsed with sterile distilled water. All air inlet lines passed through a 0.2 μm filter before entering their respective vessels.

3.2. Generation of Hollow-fiber Reactor Inoculum

Pantoea citrea (ATCC 39140) was used to inoculate the reactor for all runs. This organism, originally identified as *Acetobacter cerinus*, contains the three membrane bound enzymes-- D-glucose dehydrogenase (E1), D-gluconate dehydrogenase (E2), and

2-keto-D-gluconate dehydrogenase (E3) – of the 2,5-DKG pathway. *P. citrea*, therefore, posses the capability of producing 2,5-DKG, with D-gluconate and 2-KDG as intermediates, when in the presence of D-glucose (see Figure 11). The inoculum was generated by growing the cells on a fructose and glucose supplemented minimal medium (Genencor, 1998). A 1 mL frozen stock of *P. citrea* was added to 100 mL of this medium in a 250 mL shake flask and allowed to grow overnight at 29°C and 300 rpm. The resulting cell suspension was then used as an inoculum for a New Brunswick Scientific BioFlo 3000 Bench-top Fermentor containing 3.6 L of the above medium. This reactor was operated until the cells reached stationary phase, indicated by an increase in dissolved oxygen concentration and pH (see Figure 12). During the course of this fermentation, the reactor broth was agitated at 1200 rpm, temperature and pH were controlled at 28°C and 6.0, respectively, and sterile filtered air was provided to the system at 2.5 L/min. Once stationary phase had been achieved, the cells were harvested and centrifuged at 10,000 rpm. The supernatant was then removed, the cells resuspended in phosphate buffer, and again centrifuged at 10,000 rpm. After removing the resulting supernatant, the medium to be fed to the hollow-fiber reactor was then added to the cells until the desired optical density was reached. Finally, the shell-side recirculation pump was used to fill the shell-side of the reactor and the aeration tank with the cell suspension.

After inoculation was completed, the shell-side recirculation pump inlet line was reconnected to the aeration tank and all pumps were started. During all runs, the shell-side recirculation flow rate was 167 mL/min, while the tube-side flow rate varied from run to run depending upon the desired residence time. Because of a slight difference in

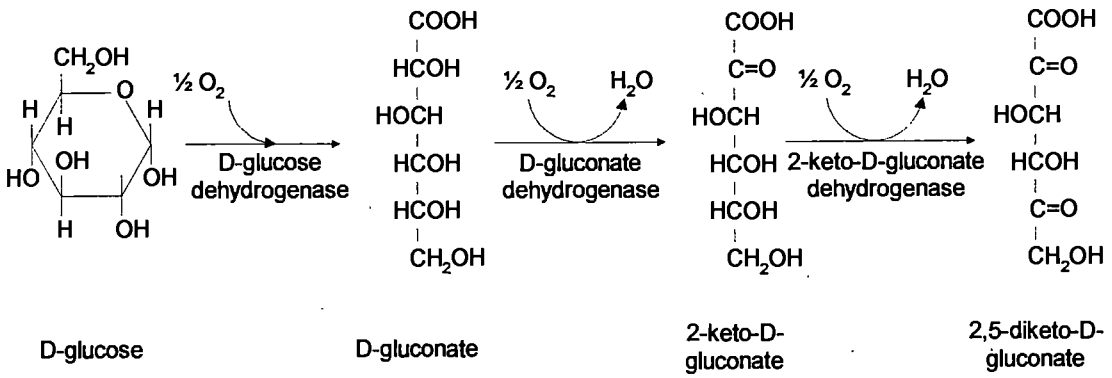

Figure 11. Reaction Scheme for 2,5-DKG Production in *Pantoea citrea*

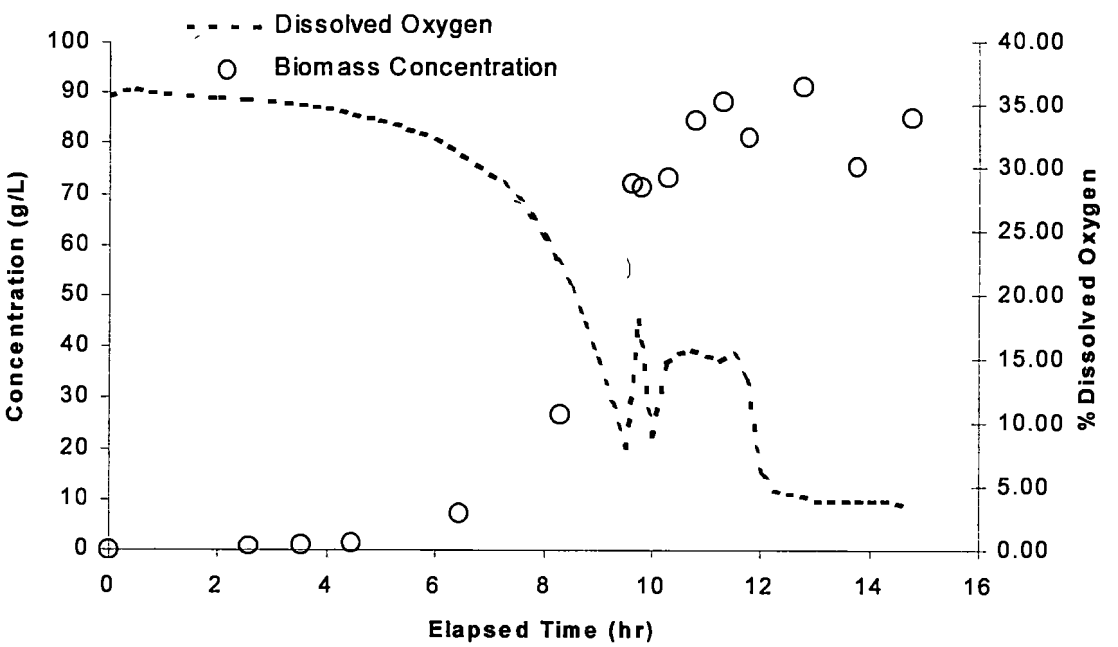

Figure 12. Oxygen Profile of Batch Fermentation of *Pantoea Citrea*

the flow rates of the tube-side inlet and outlet pumps, the volume of cell suspension in the aeration tank would slowly decrease over a number of hours. To prevent emptying of the aeration tank, the tube-side outlet pump was stopped periodically for 4 minute intervals. This forced medium into the shell-side of the reactor, thus increasing the amount of reactor broth in the aeration tank.

3.3. Analytical

Three general types of analyses were performed on samples taken from the hollow-fiber reactor system: analysis of the biomass in the shell-side samples, determination of 2,5-DKG concentration in both shell- and tube-side samples, and determination of glucose concentration in tube-side samples. The optical density of *P. citrea* cell suspensions was measured by spectrophotometry at 600 nm and used in an optical density/dry weight concentration correlation to determine the concentration of biomass present. Furthermore, enzyme assays were performed on the reactor broth to determine the level of activity of the 2-KDG dehydrogenase enzyme (Sun, 1999).

2,5-DKG concentrations in the shell- and tube-side samples were determined by high pressure liquid chromatography. Samples were analyzed with a Perkin-Elmer LC-235 Diode Array Detector at 210 nm, using a SupelcoGel H ion exchange column to perform the separation of 2,5-DKG from the intermediates formed in the fermentation. A phosphoric acid solution (0.5 %) flowing at 0.3 mL/min was used as eluent for the isocratic separation, with a sample run time of 20 minutes. A 2,5-DKG analytical standard provided by Genencor International was used to generate a calibration curve,

allowing the quantification of 2,5-DKG in the reactor samples (see Figure 13). Unfortunately, it was not possible to separate the intermediates D-gluconate and 2-KDG from each other using this analytical system. This lack of a complete separation of all fermentation products meant not only that the concentration of 2,5-DKG could only be compared to the concentration of the combined intermediates, but also that no calibration curve could be produced for the intermediates in the reactor effluent. Because the detector response for D-gluconate, 2-KDG, and 2,5-DKG was approximately the same for a given concentration, it was possible, however, to compare the chromatogram peak sizes of 2,5-DKG and the combined intermediates to determine the relative concentrations of these compounds in the reactor outlet stream.

High pressure liquid chromatography was also used for the detection of glucose in the tube-side effluent, albeit with a different system. A Dionex HPLC system composed of an LC 20 chromatography enclosure, an ED 40 electrochemical detector, an AD 40 autosampler, and a GP 40 gradient pump was used in conjunction with a CarboPac PA10 ion exchange column to separate and quantify the amount of glucose present in the reactor effluent samples. The ED 40 electrochemical detector was operated in pulsed amperometry detection mode, in which the amount of carbohydrate present in a sample is determined by oxidizing the carbohydrate on the surface of a gold electrode and measuring the resulting current change. One hundred mM NaOH flowing at 1 mL/min was used as eluent in the isocratic separation, with a sample run time of 15 minutes. Analytical standards of Fisher Scientific brand D-glucose were used to generate a

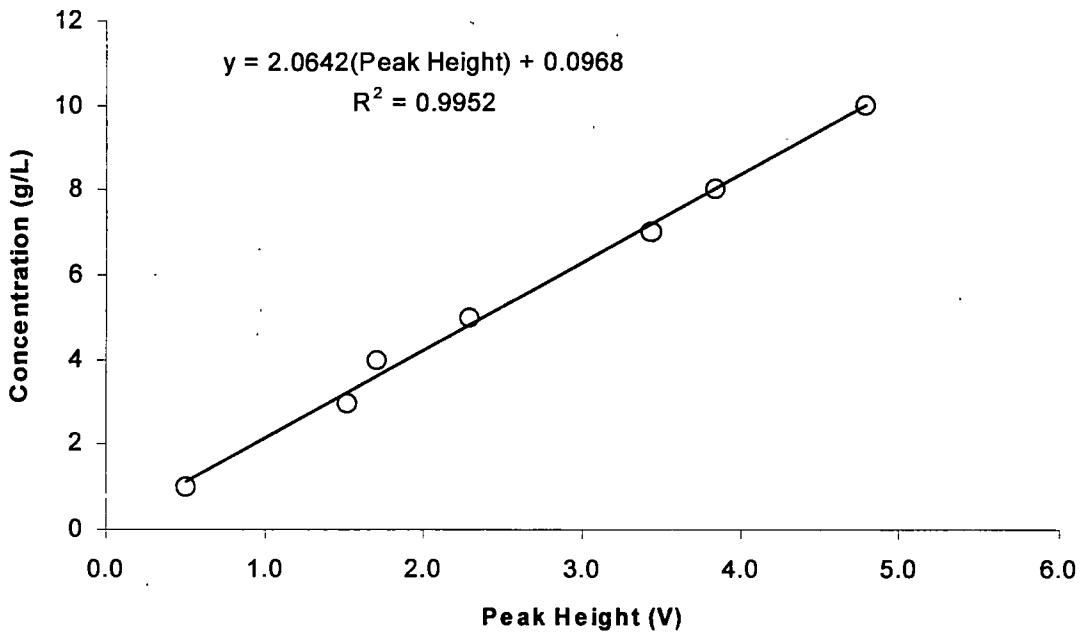

Figure 13. 2,5-DKG Calibration Curve

calibration curve, allowing the quantification of glucose in the reactor samples (see Figure 14)

3.4. Experiments

In order to evaluate the performance of the hollow-fiber reactor with respect to 2,5-DKG production, a series of experiments was conducted in which biomass concentration in the shell-side of the reactor and fresh feed flow rate were varied and the resulting effect on 2,5-DKG production measured (see Table 1). Reactor runs were carried out for 48 to 160 hours for the purpose of not only establishing the steady state concentration of 2,5-DKG in the reactor effluent for a given set of operating conditions, but also to determine if 2,5-DKG production could be maintained over extended periods of time using stationary phase cells with non-growth medium. In all runs, medium in the fresh feed tank was saturated with air before being fed to the reactor and the shell-side

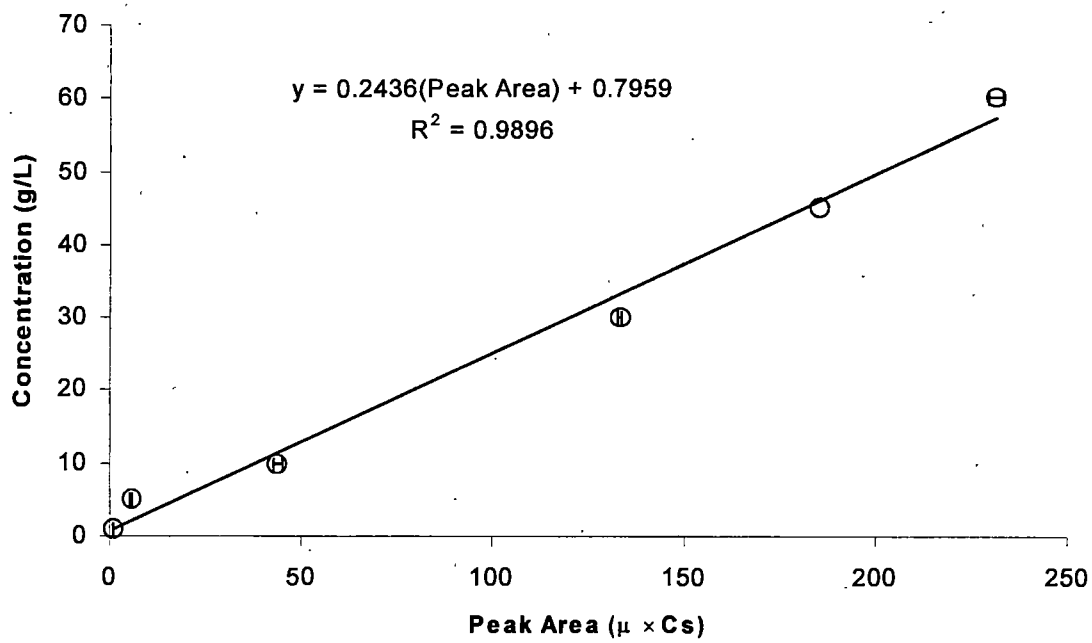

Figure 14. Glucose Calibration Curve

Table 1. Fresh Feed Rates and Biomass Concentrations Used in Hollow-fiber Membrane Reactor

Experiment	Fresh Feed Rate (mL/min)	Biomass Concentration (g dry wt./L)
Case 1	1.65	40
Case 2	1.65	138
Case 3	0.50	147
Case 4	0.50	206

recirculation rate set to 167 mL/min. Furthermore, the glucose concentration in the reactor feed was always held constant at 50 g/L. This concentration was chosen to both ensure that glucose would not be the limiting substrate and to provide a medium comparable in composition to one being used in research conducted in a batch reactor system (Sun, 1999). Tube-side samples were taken every hour for the first several hours of operation, with subsequent samples being taken progressively less frequently until the reactor run was stopped. Samples intended for glucose analysis were placed in a refrigerator until analyzed, while 2,5-DKG samples, if not analyzed immediately upon collection, were frozen at –80°C until such time as they could be analyzed using the HPLC system. Shell-side samples were taken at the beginning and end of each experiment and periodically throughout the course of the run, typically in the morning and the evening of each day of reactor operation. These samples were then frozen at –80°C until they could be analyzed for 2-KDG dehydrogenase activity and 2,5-DKG content.

4. Results and Discussion

4.1. Case 1 (40 g dry wt./L, 1.65 mL/min)

Immediately after startup, the formation of intermediates (gluconate and 2-KDG) was observed in the reactor effluent (see Figure 15). The concentration of the intermediates increased rapidly during the first 9 hours of operation, after which it remained relatively constant for 12 hours at a peak height of approximately 2.7 V. At that point, 21 hours into the run, the concentration of the intermediates increased gradually over the next 9 hours before achieving its final steady state concentration at a peak height of 3.6 V. The concentration of 2,5-DKG in the reactor effluent followed a slightly different trend. After initially rising to a concentration of 0.1 g/L, 2,5-DKG concentration in the reactor effluent remained at this level between the 5^{th} and 10^{th} hour of operation. It then steadily increased over the next 24 hours before reaching a final steady-state level of approximately 0.45 g/L.

The trend in glucose concentration did not directly follow either of the trends observed for 2,5-DKG or the intermediates (see Figure 16). Glucose concentration fell precipitously over the first three hours of operation from an initial value of 56 g/L to 10 g/L. This level was maintained for the ensuing 5 hours, at which point it increased rapidly over the next two hours to approximately 30 g/L. This concentration was roughly maintained over the next five hours before it again rose rapidly between the 17^{th} and 26^{th} hours of operation to its final steady state concentration of approximately 52 g/L.

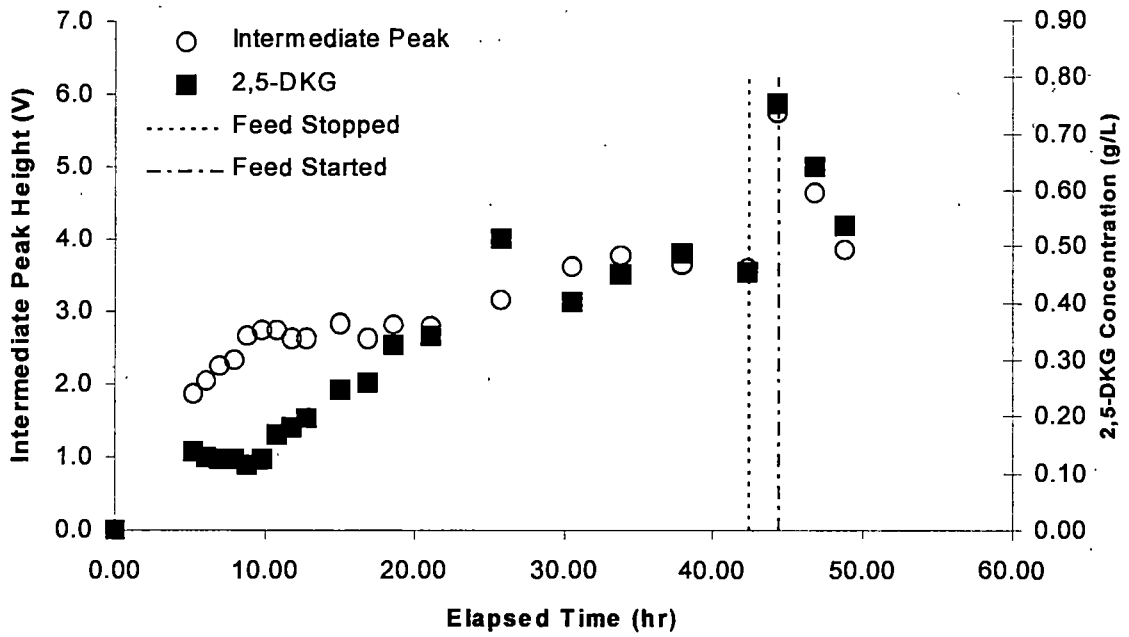

Figure 15. Intermediate Peak Height and 2,5-DKG Concentration in HFR Effluent (40 g dry wt./L, 1.65 mL/min)

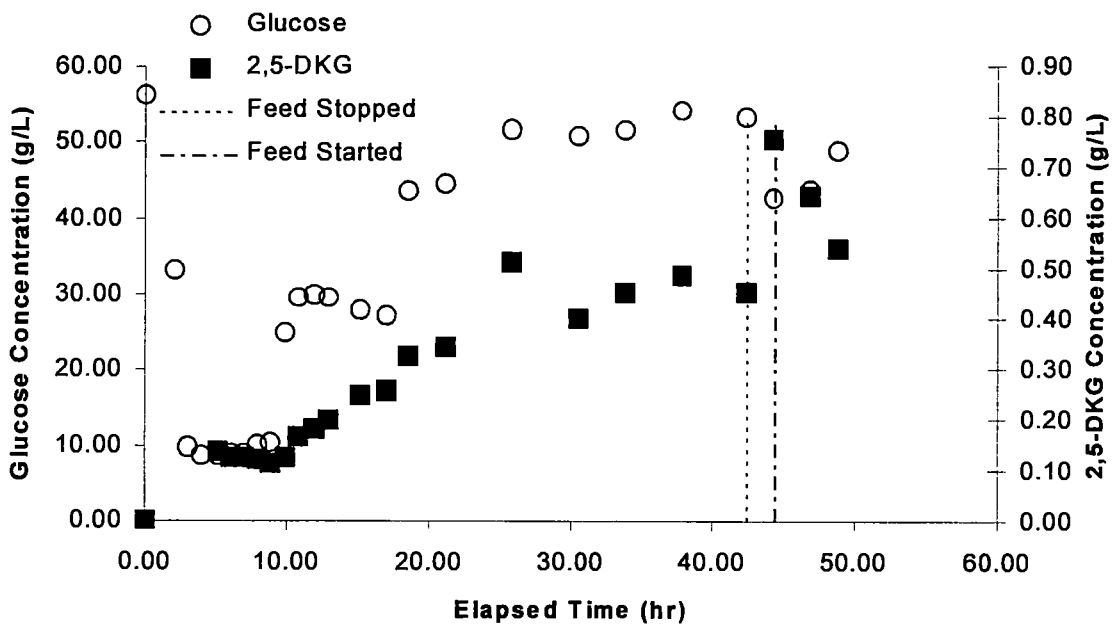

Figure 16. Glucose and 2,5-DKG Concentration in HFR Effluent (40 g dry wt./L, 1.65 mL/min)

At this point in the reactor run (at the 42nd hour of operation), fresh feed to the reactor was stopped for two hours to determine if increased residence time would result in a more complete conversion of substrate to 2,5-DKG. After this two hour interruption of fresh feed, the 2,5-DKG concentration had increased sharply from 0.46 g/L to 0.8 g/L and the intermediate concentration had also increased significantly. Concurrently, the glucose concentration fell to 42 g/L. When the fresh feed was restarted, the concentration of both 2,5-DKG and the intermediates immediately began to decline and the glucose concentration began to increase.

Over the course of this experiment, the pH of the reactor effluent stream declined from an initial value of 6.0 to a final value of 3.6 (see Figure 17). Eighty percent of this decline in pH occurred during the first 7 hours of the reactor run, with the final value after this period of steady decline being 4.1. The ensuing 30 hours was a time of a nearly constant pH reading of 3.9 ± 0.1. It was at this point in the reactor run that the fresh feed to the reactor was interrupted. After this interruption in the fresh feed, the pH had dropped to 3.6, a point at which it remained until the reactor run was stopped.

The specific activity of the 2-KDG dehydrogenase enzyme varied widely over the course of the reactor run (see Figure 18). Initially at a value of 37 units/mg protein (one unit of activity is the amount of 2-KDG dehydrogenase required to convert 1 μmol of 2-KDG to 2,5-DKG per minute), the specific activity fell below 10 units/mg protein within the first 17 hours of the experiment and remained below 10 units/mg protein through the 45th hour. The specific enzyme activity then peaked at the 49th hour at a value of 35 units/mg protein before again declining over the final 17 hours of the reactor

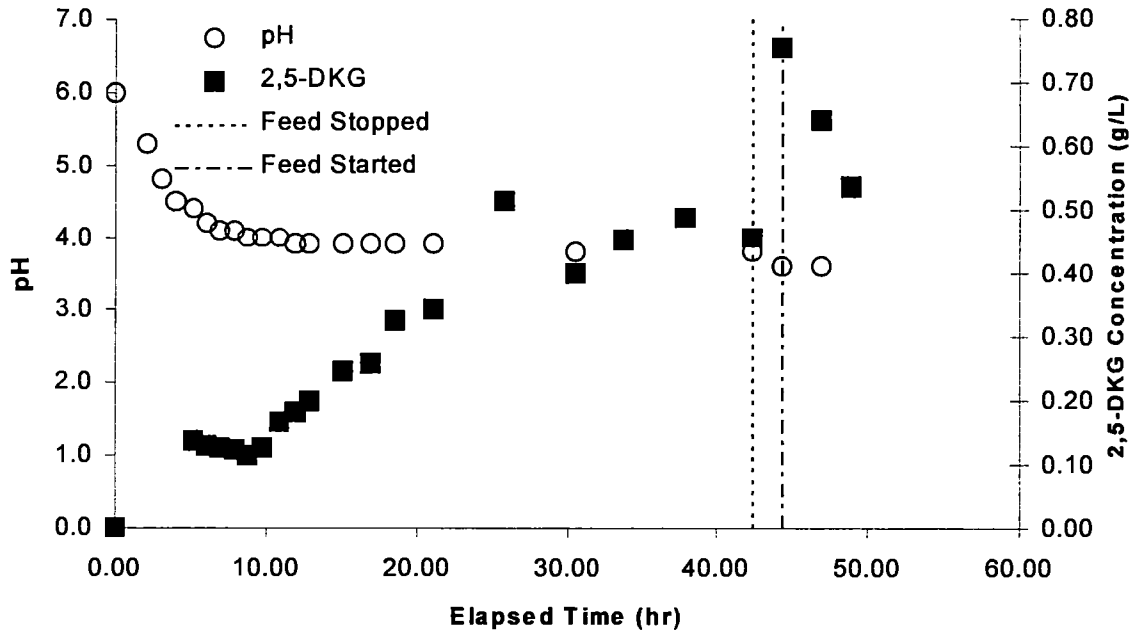

Figure 17. pH and 2,5-DKG Concentration in HFR Effluent (40 g dry wt./L, 1.65 mL/min)

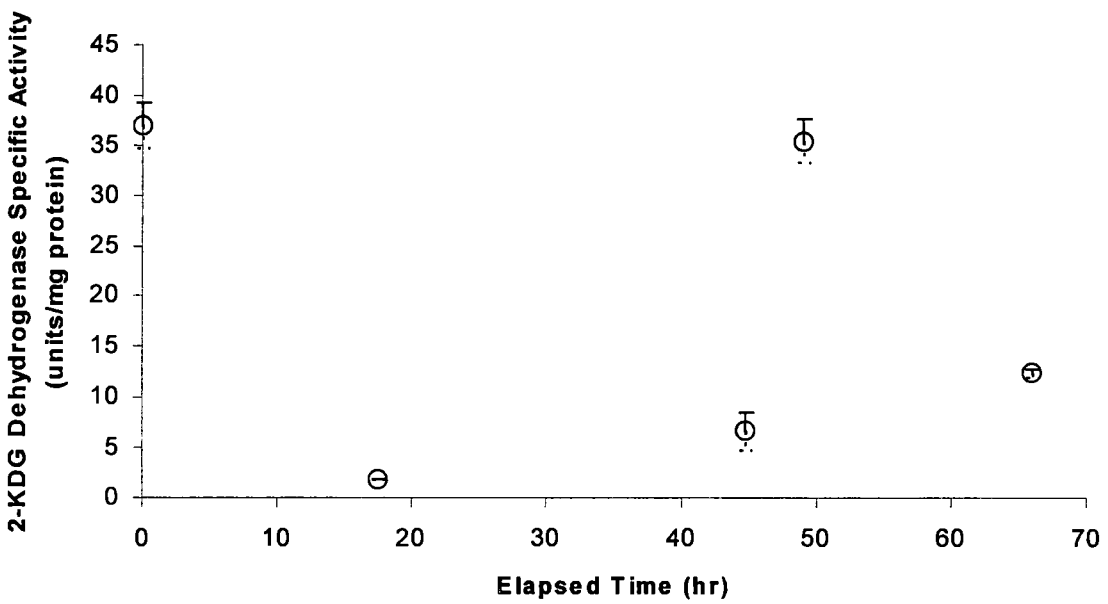

Figure 18. Specific 2-KDG Dehydrogenase Activity for the HFR Shell-Side (40 g dry wt./L, 1.65 mL/min)

run. It should be noted that the peak in specific enzyme activity did not occur until after the interruption in the fresh feed to the reactor.

The results from this reactor run demonstrate that it is indeed possible to produce 2,5-DKG in a hollow-fiber reactor. The steady state productivity of 2,5-DKG based upon tube-side, shell-side, and total volume was 0.45, 0.26, and 0.17 g/L/hr, respectively, and the 2,5-DKG yield during this period was 0.11 g 2,5-DKG formed/g glucose consumed. The initial rapid formation of gluconate and 2-KDG and their subsequent high concentrations throughout the rest of the experiment indicated an obstacle to the formation of higher levels of 2,5-DKG. That is, something seemed to inhibit the formation of 2,5-DKG as indicated by the accumulation of intermediates. Because the gluconate and 2-KDG coeluted in the reactor samples, it was not possible to measure the levels of these compounds individually and compare them to the concentration of 2,5-DKG observed. An indication of the relative amounts of 2,5-DKG and its intermediates can be obtained, however, by comparing the size of their respective chromatogram peaks. Throughout the reactor run, the intermediate peak size was much larger than that for 2,5-DKG, roughly differing by an order of magnitude. In batch reactor runs, the intermediate concentration also initially outpaced the 2,5-DKG concentration, but eventually the intermediates formed were converted into 2,5-DKG. This trend suggested that an increase in residence time would increase the concentration of 2,5-DKG in the effluent and reduce the level of intermediates in that stream. This, however, was not the case. Allowing the medium to reside in the reactor for two hours without fresh feed resulted in an increase in both 2,5-DKG concentration and the

intermediate concentration. Clearly then, with this level of biomass in the reactor, residence times would have to be extremely large for adequate concentrations of 2,5-DKG to be produced.

4.2. Case 2 (138 g dry wt./L, 1.65 mL/min)

As in the experiment conducted at low biomass concentration, the formation of intermediates was observed immediately after the start of the reactor run. Intermediate concentration increased sharply during the first hour of operation, after which it gradually increased over the next 16 hours before leveling off for 6 hours at a peak height of approximately 1.3 V (see Figure 19). The following 51 hours of reactor operation witnessed wide variations in the intermediate concentration, after which the concentration stabilized and declined steadily over the next 38 hours. During the final 33 hours of the reactor run, only a slight decline in intermediate concentration was observed, with an average peak height over this period of 1.8 ± 0.05 V.

Initially, the 2,5-DKG concentration followed a different trend than the intermediates, although eventually it exhibited the same changes as the intermediates. Over the first hour of operation, the concentration of 2,5-DKG rose to 0.65 g/L, after which it declined over the next 6 hours to a value of approximately 0.14 g/L, a level that was maintained for 21 hours. At the 32nd hour of operation, the 2,5-DKG concentration began an increase that continued steadily for the next 57 hours, with the exception of an interval between the 63rd and 79th hour, to a concentration of 3.6 g/L. The final 59 hours of the reactor run saw a gradual decline in 2,5-DKG concentration to 2.8 g/L.

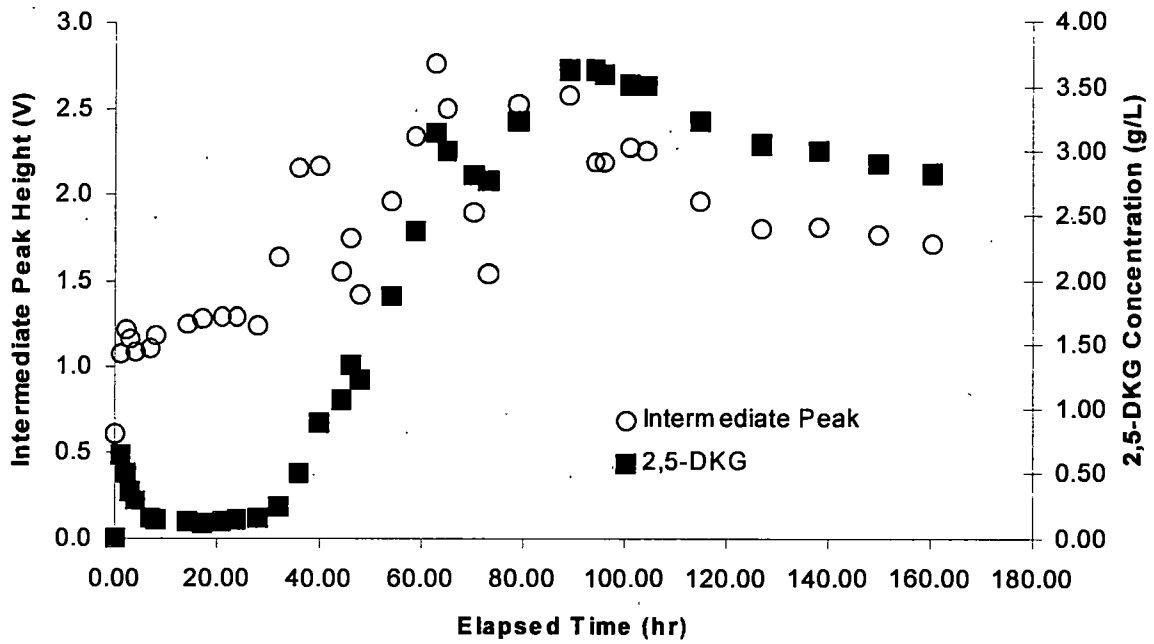

Figure 19. Intermediate Peak Height and 2,5-DKG Concentration in HFR Effluent (138 g dry wt./L, 1.65 mL/min)

Glucose concentration in the reactor effluent fell rapidly over the first four hours of operation from an initial value of 53 g/L to 12 g/L and remained at that level for the next four hours (see Figure 20). During the following 27 hours, the glucose concentration varied widely from a low of 34 g/L to a high of 47 g/L, after which it stabilized at a steady state value of about 49 g/L. From the 65th to the 104th hour of operation, which corresponds approximately with the period of fluctuation in intermediate and 2,5-DKG concentration, the glucose concentration fluctuated before regaining its previous steady state value of 49 g/L for the final 45 hours of the reactor run.

The trend in pH over the course of this reactor run was similar to that seen in the experiment conducted at the lower biomass concentration, in which the pH attained a relatively stable value after initially dropping rapidly (see Figure 21). Within the first 2 hours of starting the reactor, the pH had fallen from 6.0 to 4.7. For the following 26 hours, the pH remained essentially constant at a value of 4.6 ± 0.09, after which it slowly fell during the ensuing 66 hours to 3.4, a value which was maintained (± 0.05) for 21 hours. The pH then gradually rose during the final 33 hours of the reactor run to a final value of 3.7. Thus, during the final 114 hours of this experiment the average pH of the reactor effluent was 3.7, with a standard deviation of only 0.2.

Specific 2-KDG dehydrogenase activity, as in the previous experiment, varied significantly over the course of the reactor run (see Figure 22). From an initial specific activity of 15 units/mg protein, the specific enzyme activity fell over the first 24 hours of the reactor run to a value of 8 units/mg protein. Specific activity then rose sharply during

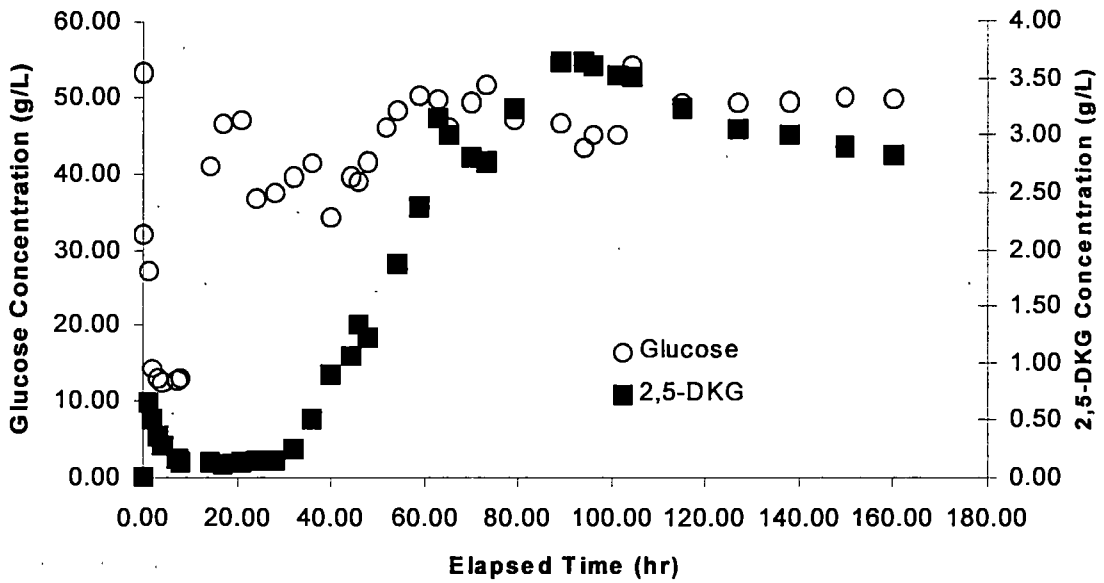

Figure 20. Glucose and 2,5-DKG Concentration in HFR Effluent (138 g dry wt./L, 1.65 mL/min)

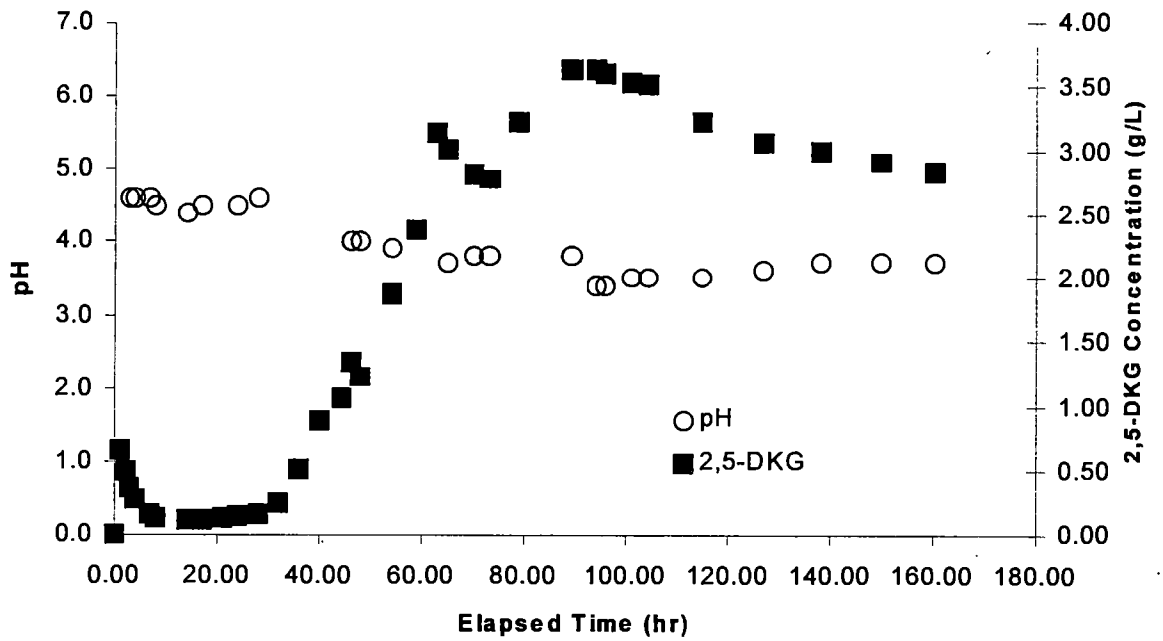

Figure 21. pH and 2,5-DKG Concentration in HFR Effluent (138 g dry wt./L, 1.65 mL/min)

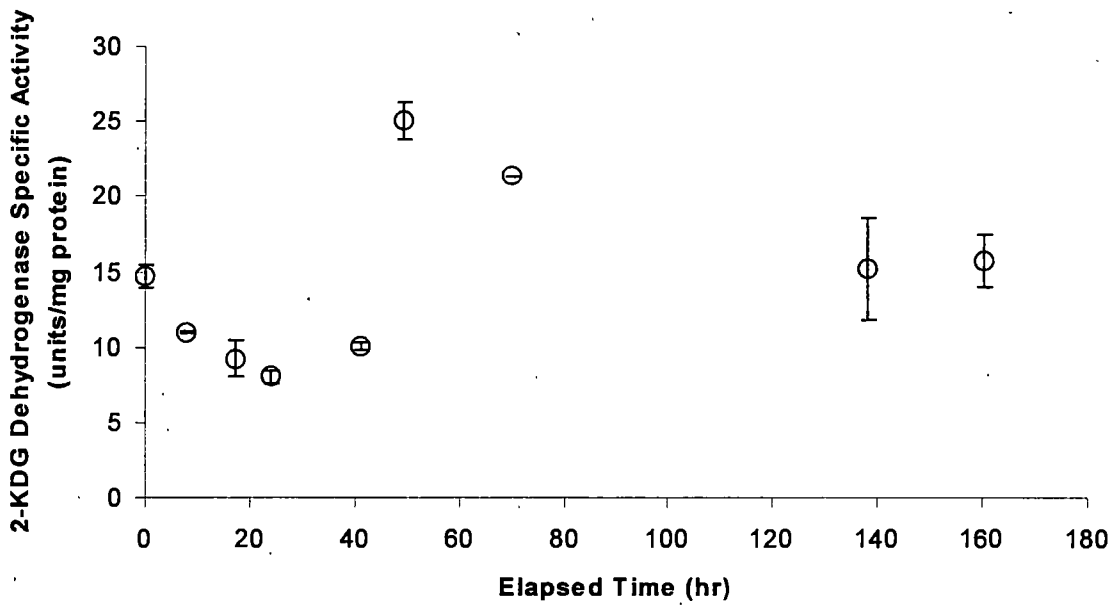

Figure 22. Specific 2-KDG Dehydrogenase Activity for the HFR Shell-Side (138 g dry wt./L, 1.65 mL/min)

the following 25 hours, coinciding with the increase in 2,5-DKG concentration in the reactor effluent, before reaching a peak value of 25 units/mg protein at the 49th hour of the reactor run. The final 111 hours of the experiment witnessed a gradual decline in specific enzyme activity to a final value of 16 units/mg protein.

Results for the reactor run at a high biomass concentration showed a significant improvement in 2,5-DKG productivity over that seen in the low biomass case. Raising the biomass concentration to 3.5 times that used in the previous run resulted in a peak 2,5-DKG concentration 8.1 times that in the earlier experiment. During the period of gradually declining 2,5-DKG concentration at the end of this run, the product concentration was an average of 6.7 times higher than the steady state value observed in the other reactor. In addition to the higher biomass concentration in this reactor, the increase in specific 2-KDG dehydrogenase activity between this experiment and the one at a lower biomass concentration may also account for the increased concentration of 2,5-DKG in the effluent. Whereas in the previous reactor run the specific activity of the 2-KDG dehydrogenase enzyme fell below 5 units/mg protein, in the experiment conducted at the higher biomass concentration the specific activity never fell below 8 units/mg protein. The peak productivity of 2,5-DKG based upon tube-side, shell-side, and total volume was 3.43, 2.04, and 1.28 g/L/hr, respectively, while the approximate corresponding values during the final period of gradually lessening product concentration were 2.82, 1.67, and 1.05 g/L/hr. Therefore, the peak productivity of 2,5-DKG in this experiment was 7.6 times that in the previous case, while productivity during the final period was 6.3 times the earlier steady state productivity.

Unlike the previous case, in this experiment the chromatogram peak size of 2,5-DKG was larger (after the 63rd hour) than that for the intermediates, differing by a factor of approximately 1.3. The decline in product concentration between the 63rd and 79th hour was most likely attributable to a leak in the shell-side recirculation line which was discovered during this period. This leak resulted in a loss of biomass, which would explain the decrease in 2,5-DKG production. After this leak was detected, the line was repaired and more biomass was added to the system to raise the concentration to its previous level, restoring the productivity of the system.

4.3. Case 3 (147 g dry wt./L, 0.50 mL/min)

Following the established trend, intermediates were formed immediately after reactor startup and their concentration increased steadily over the first 20 hours of the reactor run to a peak height of 3.7 V (see Figure 23). At that point, the intermediate concentration dropped sharply, disappearing entirely at the 45th hour of operation. This marked the first time during a reactor run that the concentration of intermediates in the reactor effluent dropped to undetectable levels. Throughout the rest of this experiment, the amount of intermediates in the reactor effluent remained below the detection threshold of the analytical system.

The concentration of 2,5-DKG in the reactor effluent also increased rapidly at the beginning of the reactor run. During the first 45 hours of reactor operation, the concentration of 2,5-DKG in the product stream increased steadily, reaching a final value

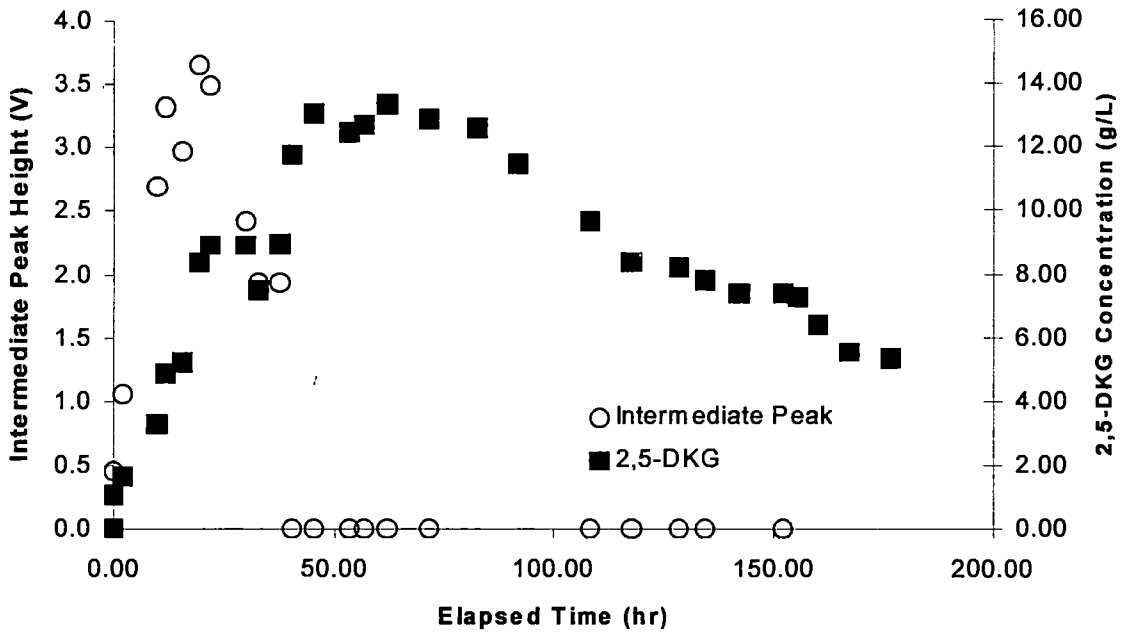

Figure 23. Intermediate Peak Height and 2,5-DKG Concentration in HFR Effluent (147 g dry wt./L, 0.50 mL/min)

of 13 g/L. This approximate concentration was maintained for the next 37 hours, after which time 2,5-DKG concentration declined gradually over the final 85 hours of the reactor run to a value of 5.4 g/L.

The trend in glucose concentration observed in this reactor run did not follow that seen with either the intermediates or 2,5-DKG (see Figure 24). Immediately following reactor startup, the concentration of glucose in the reactor effluent fell precipitously to a value of 2.3 g/L. Over the next 57 hours, the glucose concentration leaving the reactor slowly increased to 5.2 g/L, after which it rose steadily for 60 hours, peaking at a value of 50 g/L at the 118th hour of operation. Glucose concentration then fell sharply between the 118th and 134th hours to 36 g/L before leveling off and maintaining a concentration of approximately 34 g/L for the final 34.5 hours of operation.

As in the case of intermediate concentration in the effluent, the pH of the reactor effluent followed a similar trend as that observed previously (see Figure 25). From an initial value of 6.0, the pH fell steadily over the course of the first 19 hours of the reactor run to a value of 3.9, after which it gradually declined during following 38 hours, reaching a value of 3.0 by the 57th hour of reactor operation. This pH level was approximately maintained for the next 85 hours, before beginning a gradual rise at the 152nd hour to attain a final value of 3.7.

The specific activity of the 2-KDG dehydrogenase enzyme in this experiment behaved in a similar manner as in the previous experiment (see Figure 26). After initially dropping from 40 units/mg protein to 20 units/mg protein in the first 20 hours of reactor operation, the specific activity gradually increased during the next 145 hours, reaching a

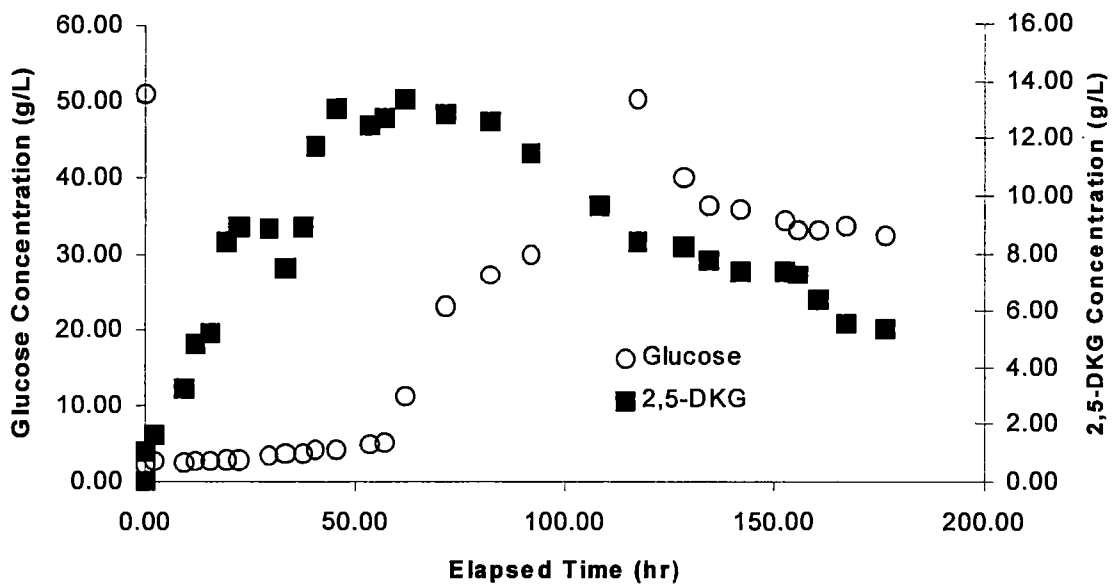

Figure 24. Glucose and 2,5-DKG Concentration in HFR Effluent (147 g dry wt./L, 0.50 mL/min)

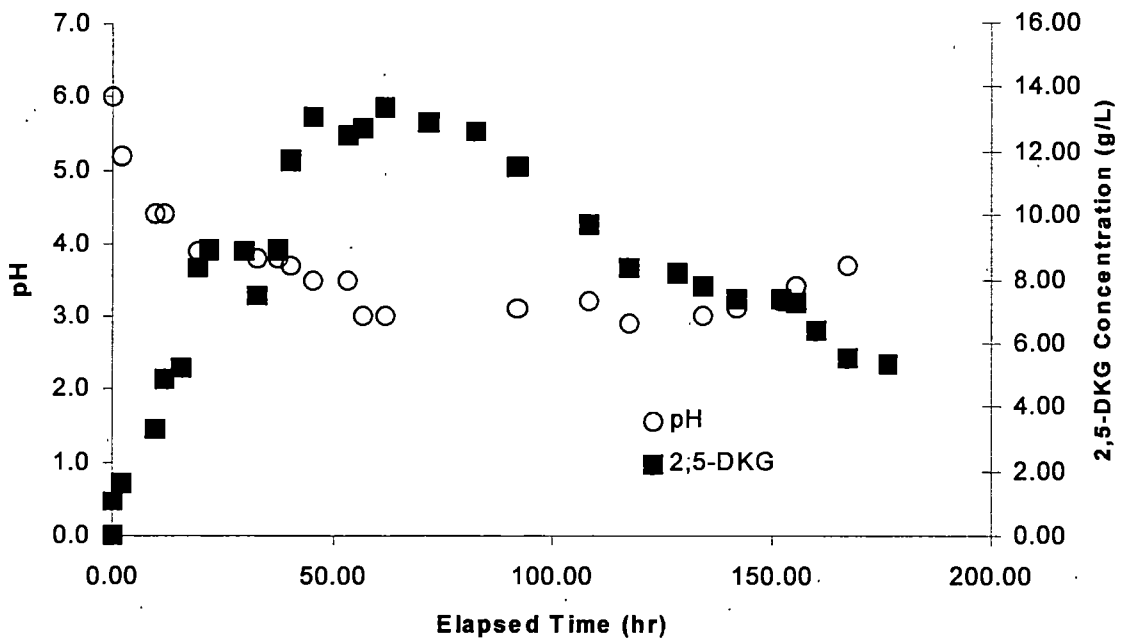

Figure 25. pH and 2,5-DKG Concentration in HFR Effluent (147 g dry wt./L, 0.50 mL/min)

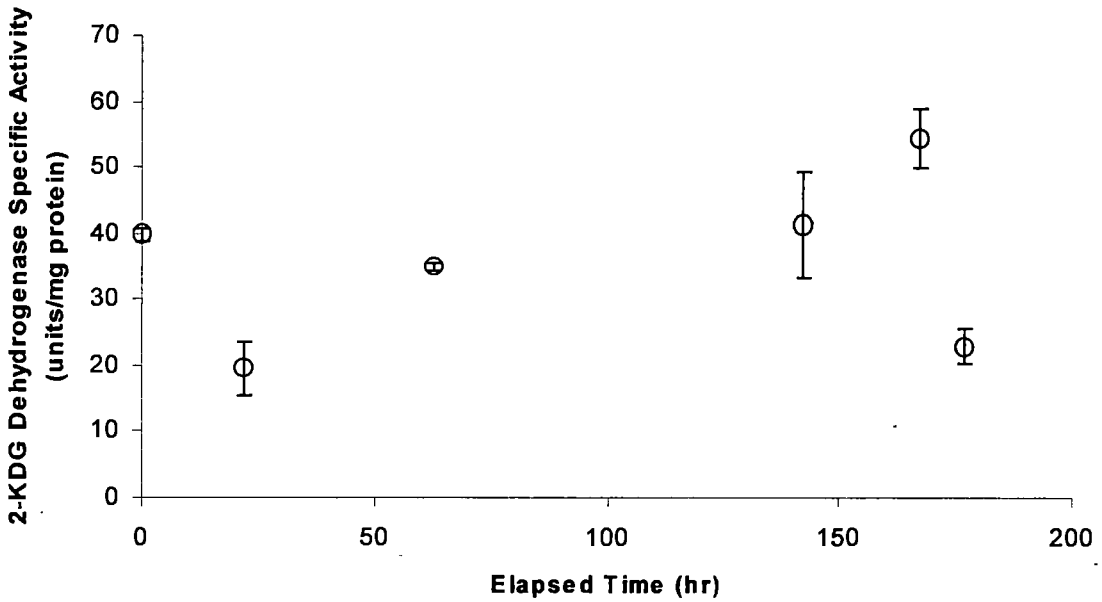

Figure 26. Specific 2-KDG Dehydrogenase Activity in HFR Shell-Side (147 g dry wt./L, 0.50 mL/min)

peak value of 54 units/mg protein by the 167th hour of operation. Specific activity then fell sharply to a final value of 23 units/mg protein 177 hours after the reactor was started.

As is clearly indicated by the 2,5-DKG profile for this experiment, this reactor run was far more productive than either of the two previous cases. Reducing the throughput rate of the reactor by a factor of 3.2 resulted in a peak 2,5-DKG concentration 3.6 times that seen in case 2. Considering that the biomass concentration in this experiment was only slightly higher than in the previous experiment (1.07 times higher) and that all other parameters were held constant, the increase in residence time is the most likely reason for this increase in product concentration. This is further suggested by the fact that the concentration of 2,5-DKG in the reactor effluent increased by a factor nearly identical to that by which the throughput rate was reduced. A higher specific 2-KDG dehydrogenase activity over the course of this experiment compared to that in the previous reactor run is also a likely contributor to the higher effluent concentration. In this run, the specific enzyme activity never fell below 20 units/mg protein (2.5 times the lowest level observed in the previous run), while in the case 2 experiment the specific activity was 20 units/mg protein or higher for only a fraction of the time the reactor was being operated. As another indication of the higher specific enzyme activity in this reactor run, the average specific activity over the course of the entire run was 35 ± 12 units/mg protein compared to 15 ± 9 units/mg protein. Unfortunately, since the product stream concentration did not increase by a factor larger than that by which the flow rate was reduced, the productivity of the system operating at high biomass concentration/low flow rate (case 3) was not significantly higher than the system operating at high biomass concentration/high flow

rate (case 2). During this reactor run the peak steady state productivity based upon tube-side, shell-side, and total volume was 3.90, 2.31, and 1.45 g/L/hr, respectively. Furthermore, because the glucose concentration varied greatly during the period of peak steady state production of 2,5-DKG, calculation of the yield of 2,5-DKG from glucose over this region can provide only an approximate value. During the period of maximum steady state production of 2,5-DKG (between the 45th and 82nd hours) the average concentration of glucose was 12.7 g/L and resulting in a yield of 0.34 g 2,5-DKG formed/g glucose consumed.

4.4. Case 4 (206 g dry wt./L, 0.50 mL/min)

As in all previous experiments, intermediate concentration increased rapidly throughout the early stages of this reactor run, although for the first 16 hours of this reactor run the intermediate concentration was below the detection threshold (see Figure 27). After this initial lag in intermediate production, the concentration of intermediates in the reactor effluent increased steadily over the course of the next 52 hours, reaching a peak 68 hours after starting the reactor at a peak height of 2.5 V. The concentration then declined steadily for 25 hours until, at the 98th hour of operation, the intermediate concentration again dropped below the detection threshold.

The trend in 2,5-DKG concentration in the reactor effluent generally followed that for the intermediates during the first half of the reactor run. After rising to 3.6 g/L within the first 8 hours of reactor operation, 2,5-DKG concentration then fell over the next

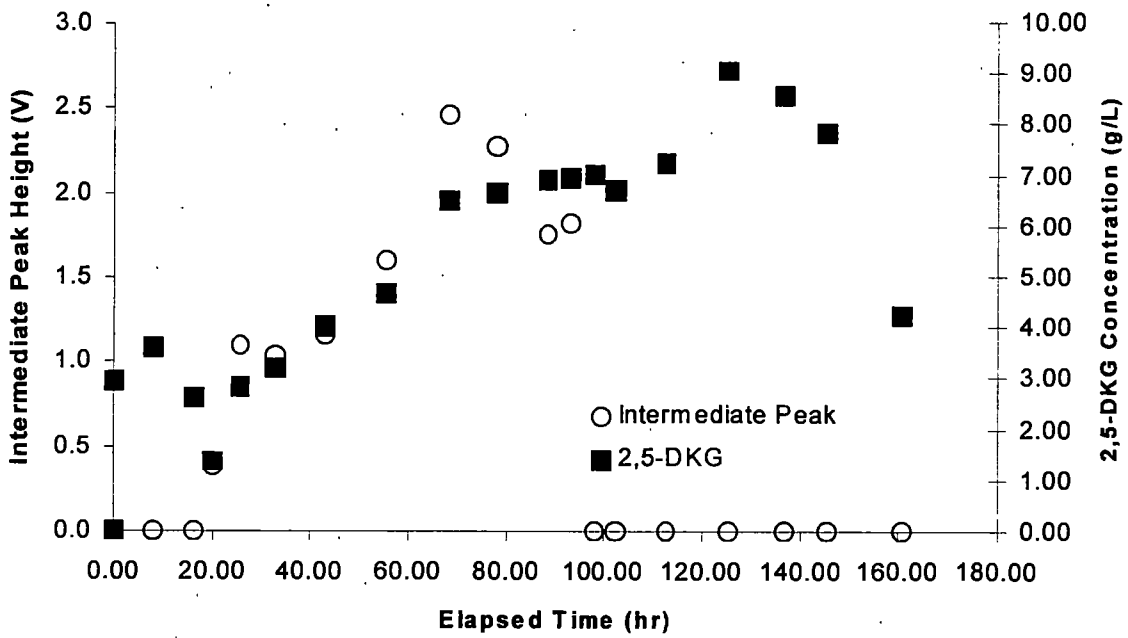

Figure 27. Intermediate Peak Height and 2,5-DKG Concentration in HFR Effluent (206 g dry wt./L, 0.50 mL/min)

12 hours to 1.4 g/L. From this low point in the 2,5-DKG profile, the concentration increased steadily for 48 hours, after which 2,5-DKG concentration increased only slightly over the ensuing 45 hours. During this period of slight increase, the average concentration of 2,5-DKG in the reactor effluent was 6.9 ± 0.2 g/L. After this period of relative stability, the concentration increased rapidly between the 125th and the 112th hour of operation, before again falling over the final 36 hours of the experiment to a final value of 4.2 g/L.

The concentration of glucose in the reactor effluent was more stable during this experiment than any of the others (see Figure 28). After dropping immediately after reactor startup to 2.2 g/L, glucose concentration gradually rose over the first 32 hours of operation to reach 6.3 g/L. The following 80 hours were a period of gradually falling and rising glucose concentrations in which the average value was 4.5 ± 0.4 g/L. Glucose concentration again rose during the final 48 hours to achieve a final value of 26.7 g/L.

As in previous experiments, pH dropped in the early stages of this reactor run, stabilizing at a value significantly lower than that at the beginning of the reactor run (see Figure 29). Unlike the previous cases, the decline in pH occurred over a much longer period of time. The pH remained essentially constant during the first 33 hours of operation at 5.5 ± 0.1 before beginning a steady decline over the following 25 hours. At this point (the 68th hour) in the reactor run the decline in pH slowed significantly, but continued for the next 69 hours. It then rose gradually over the final 24 hours to a final value of 3.8. The pH change during the final 48 hours of the reactor run was very slight, with the average value over this period being 3.4 ± 0.3.

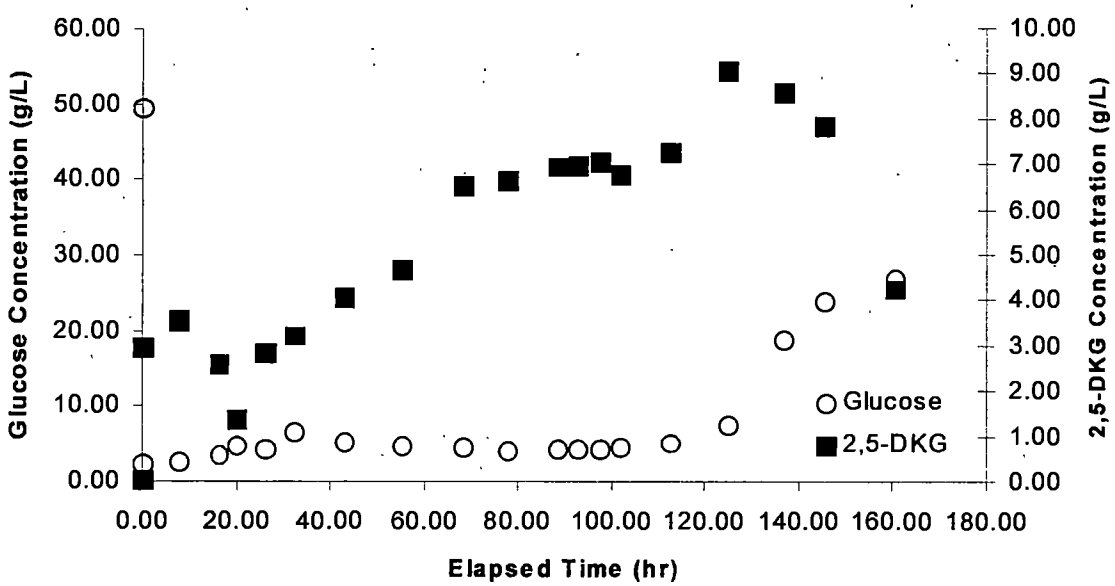

Figure 28. Glucose and 2,5-DKG Concentration in HFR Effluent (206 g dry wt./L, 0.50 mL/min)

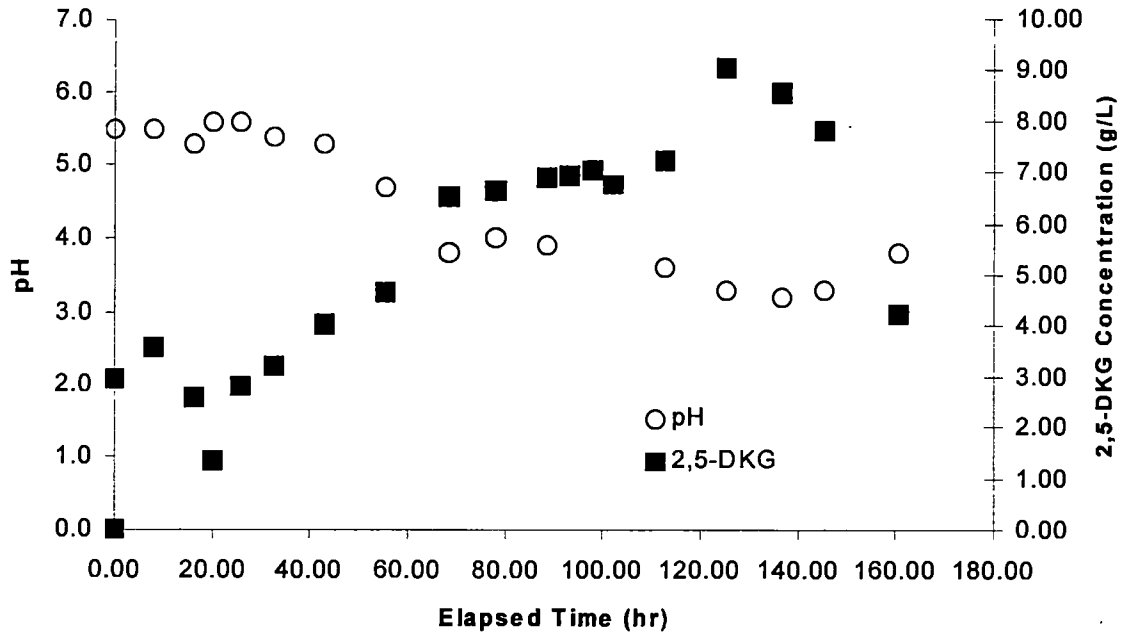

Figure 29. pH and 2,5-DKG Concentration in HFR Effluent (206 g dry wt./L, 0.50 mL/min)

Fewer biomass samples were taken in this experiment than in any of the other reactor runs, thus preventing the determination of the precise trend of specific 2-KDG dehydrogenase activity for this run (see Figure 30). Because of similar trends in each of the previous cases and the data obtained from this run, however, it is reasonable to assume a profile in which a decline in specific enzyme activity was followed by an increase to levels approximating those in the beginning. It is also possible to compare the level of specific activity throughout this run with those of the previous experiments. The specific enzyme activity over the course of this experiment averaged 21.5 ± 2 units/mg protein, a level comparable to that seen in the high biomass concentration/high flow rate case (case 2), but lower than that observed in the run using a high biomass concentration and low flow rate (case 3).

Thus, the results from this reactor run indicate that a limit had been reached in the production of 2,5-DKG in the system as it is presently configured and operated. Biomass concentration in the reactor was increased by a factor of 1.4 above that in the previous reactor run, but the concentration of 2,5-DKG during the period of peak production declined by a factor of 0.7. Considering the period of nearly constant 2,5-DKG production, in this experiment the concentration in the effluent fell by a factor of 0.5 from that in the high biomass concentration/low flow rate case. Productivity in the system was also lower than that seen in the previous two runs, with a steady state productivity based upon tube-side, shell-side, and total volume of 1.65, 0.98, and 0.61 g/L/hr, respectively, approximately 42% of that in the previous cases. Furthermore, the yield observed in this

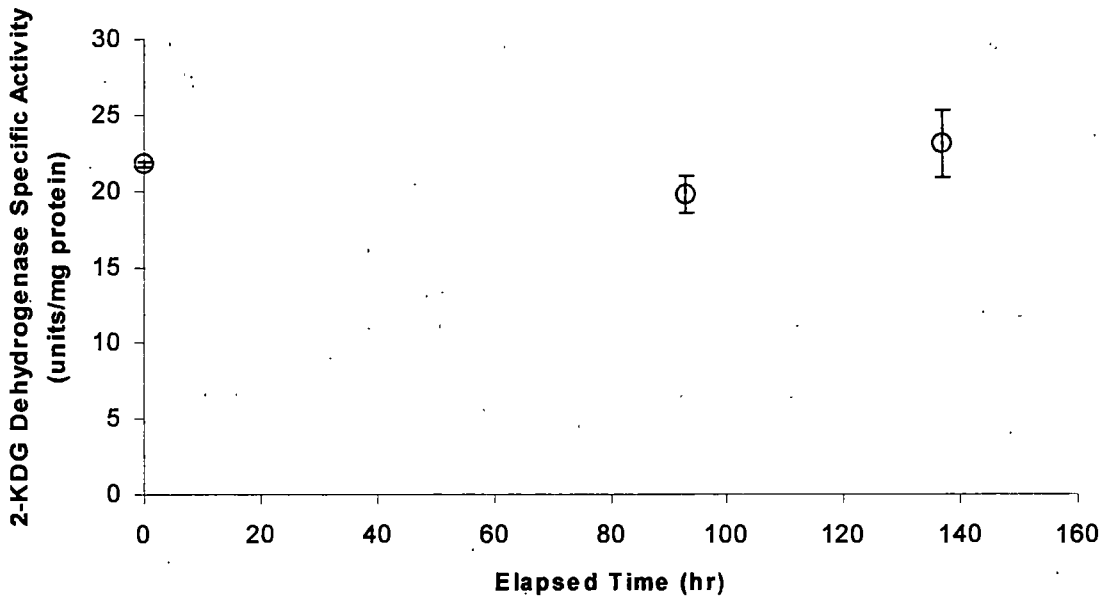

Figure 30. Specific 2-KDG Dehydrogenase Activity in HFR Shell-Side (206 g dry wt./L, 0.50 mL/min)

reactor run, 0.15 g 2,5-DKG formed/g glucose consumed, was substantially lower than that seen in the two previous experiments.

The most likely cause of the decline in production with an increase in biomass concentration was an oxygen limitation in the system that developed when the concentration of cells in the reactor was increased. It was anticipated that eventually an increase in biomass concentration would not prompt an increase in effluent product concentration because the amount of membrane bound enzymes in the higher level of biomass would require a greater amount of oxygen than was available in the system. This situation would result in an under utilization of the available biomass. The decline in product concentration with an increase in biomass concentration suggests that when biomass concentration is increased beyond a certain level, the increased demand on oxygen by cellular respiration reduces the amount of oxygen available for other reactions, such as the three membrane bound reactions in this system. This reduction in available oxygen would result in less 2,5-DKG being formed.

Another possible cause of the decline in 2,5-DKG production with an increase in biomass concentration is the onset of mass transfer limitations at the higher biomass level. The cell suspension used in this reactor run was extremely viscous, possibly causing a reduction in the flux of substrate and product through the hollow-fiber membrane. Additionally, the increased viscosity could reduce the efficiency of the aeration tank, thus lessening the amount of oxygen being supplied to the system. In either case, the mass transfer limitation would cause the amount of 2,5-DKG in the outlet stream to be reduced.

5. Conclusions

The results from the hollow-fiber reactor experiments show that it is, indeed, possible to produce 2,5-DKG continuously in a hollow-fiber rector and indicate some of the parameters that control the productivity of the system and the concentration of the product stream (see Table 2). Reactor runs were carried out for up to 180 hours, with some level of 2,5-DKG production during that entire period. Variations in effluent product concentration were observed during the course of each experiment and the maximum titre of the product stream varied from run to run, but in all cases 2,5-DKG production was observed immediately after reactor startup and began to increase steadily after several hours of operation. Effluent product concentrations as high as 13 g/L were observed for several hours and under two different sets of operating conditions, productivities as high as 3.4 g/L/hr (based on tube-side volume) were achieved. These results were obtained using a nitrogen limited non-growth medium, demonstrating the capability of *Pantoea citrea* to continually produce 2,5-DKG for periods as long as a week on a minimal medium.

From variations made to the fresh feed rate to the reactor system, it can be seen that the residence time of glucose in the hollow-fiber reactor can be an effective means of controlling the product titre in the reactor system. The increase in 2,5-DKG concentration in the reactor effluent following the two hour interruption of fresh feed during the low biomass concentration/high flow rate experiment (case 1) was the first indication that the throughput rate could be used to control product concentration. Further evidence of this capability was obtained from the two reactor runs made at high

Table 2. Summary of Experimental Results

Flow Rate (mL/min)	Biomass Concentration (g dry wt./L)	Peak 2,5-DKG Concentration (g/L)	Average Enzyme Activity (units/mg Protein)	pH	Productivity ($g/L_{tube}/hr$)	Productivity (g 2,5-DKG/g biomass/hr)	Yield (g 2,5-DKG/g glucose consumed)
1.65	40	0.45	18 ± 16	3.9 ± 0.1	0.45	0.0067	0.11
	138	3.6	15 ± 9	3.7 ± 0.2	3.43	0.016	0.54
0.50	147	13	35 ± 12	3.1 ± 0.1	3.90	0.013	0.34
	206	6.9	22 ± 2	3.4 ± 0.3	1.65	0.0047	0.15

biomass concentration (cases 2 and 3) in which the flow rate through the system was varied. When the fresh feed rate was reduced by a factor of 3.3 (a 30% reduction in flow rate), the concentration of 2,5-DKG in the product stream increased by an almost identical factor—3.6. Considering that all other parameters were held nearly constant (none differing by more than 7% between the two runs), clearly the change in throughput rate brought about the change in product titre. Unfortunately, this parameter does not appear to be effective in controlling the productivity of the reactor system. Because the product concentration and fresh feed rate varied in a nearly one-to-one ratio, the increase in product titre was balanced by the reduction in flow rate, resulting in nearly identical volumetric productivites in the two experiments.

In contrast, the concentration of biomass in the hollow-fiber reactor appears to be a viable means of controlling both product titre and volumetric productivity. This capability is indicated by the results obtained from operating the reactor system at the same high flow rate (1.65 mL/min) while varying biomass concentration in the shell-side of the reactor. When the concentration of cells in the reactor was increased by a factor of 3.5, the concentration of 2,5-DKG in the reactor effluent increased by a factor of 8.1. All other parameters were again held constant, indicating that the change in biomass concentration was the governing factor in the increase in product titre. Additionally, since the flow rate through the reactor was held constant, the increase in effluent 2,5-DKG concentration was not offset by a reduction in flow, therefore the volumetric productivity of the system also increased. With the increase in cell concentration, the peak steady state productivity in the reactor system increased by a factor of 7.5. There is

apparently a limitation, however, on the amount which the product titre and volumetric productivity may be increased by changes in the biomass concentration. This limitation is suggested by the results from the experiments conducted at a low flow rate (0.5 mL/min) while biomass concentration was varied. The cellular concentration in the shell-side of the reactor was increased by a factor of 1.4 over that used in the initial high biomass concentration/low flow rate experiment. This change resulted in a decline in product concentration by a factor of 1.9, while volumetric productivity decreased by a factor of 2.4. The most likely explanation for the reduction in product titre and system productivity is that an oxygen limitation was created by the increased amount of biomass in the system. Increasing the biomass in the system also increased the demand on the available oxygen by cellular respiration, thus reducing the amount of oxygen available for the membrane bound enzymes to convert glucose into 2,5-DKG. Therefore, in order to use biomass concentration as a control parameter for 2,5-DKG concentration and system productivity it must be recognized that, when raised above a certain level, the amount of oxygen in the reactor must be increased along with cellular concentration.

Finally, data from this research and that conducted by Sun (1999) in a batch reactor, coupled with that found in literature (Linton et al., 1987, 1989; Robertson and Kim, 1985; Chung et al., 1987) indicate that pH control is necessary in the hollow-fiber reactor in order to significantly increase the product concentration, system productivity, and long term stability of production above the levels observed in the current reactor system. In three of the hollow-fiber reactor systems described previously, a decline in product concentration was accompanied by a change in pH to a level outside the optimum

range (Linton et al., 1987, 1989; Robertson and Kim, 1985; Chung et al., 1987) for the cells being used. Chung et al. (1987) goes so far as to say that "pH control remain(s) as a means for further improvement of productivities" in his hollow-fiber reactor system. Clearly then, the possibility has been established that pH may need to be controlled in a hollow-fiber reactor. That this parameter need be controlled in this system is indicated by the level outside the optimum pH range (5.0-7.5) to which the pH fell in all experiments conducted in the present system configuration. In all cases, during the period of product titre decline, the pH of the reactor effluent fell to and was maintained at 4.0 or lower. Further evidence that pH needs to be a control variable is found in research conducted by Sun (1999). In a batch fermentation of *Pantoea citrea* to produce 2,5-DKG, he discovered that the productivity without pH control was an order of magnitude lower than it was when pH control was utilized. Therefore, the control of pH in the hollow-fiber reactor studied would need to be initiated in order to significantly increase the productivity of the system.

Thus, a hollow-fiber membrane reactor for the continuous production of 2,5-DKG has been successfully developed. Additionally, key operating and control parameters—namely substrate residence time, biomass concentration, and reactor pH—have been identified. Modification of the current reactor system to control one or more of these parameters—most particularly pH—and development of a process control strategy should result in a dramatic increase in the productivity of the hollow-fiber reactor system. With increased productivities and product titres, this system could then provide the basis for an

industrial scale hollow-fiber membrane reactor, making feasible a biochemical route to ascorbic acid production.

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