Biodegradation of aromatic compounds in a self-cycling fermenter (SCF).

by

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ABSTRACT

The Self-Cycling Fermentation (SCF) technique was applied to the biodegradation of several aromatic compounds by *Pseudomonas putida* and *Pseudomonas fluorescens*. The SCF technique was shown to be a useful research tool in aromatic biodegradation studies as well as a potential pollution treatment method. Advantages of SCFs include stable and highly repeatable performance and almost complete substrate consumption. Biomass concentration, cycle time and the minimum dissolved oxygen level were monitored from cycle to cycle and the variation of these parameters during steady-state operation was less than 5%. The aromatic compounds used in this study were sodium benzoate, p-anisaldehyde and 4-methoxy-benzylidine-4-n-butylaniline (MBBA). Consumptions of 93-100% were achieved for these compounds using the SCF technique. Consumption rates of aromatic compounds were shown to be considerably higher than in conventional fermenters. Cycle time was found to be a useful parameter for comparing growth of different organisms on aromatic substrates. The cycle time was dependant on substrate concentration for p-anisaldehyde. This dependence was shown to be due to the presence of an unidentified, inhibitory intermediate. A mathematical model is presented to predict steady-state cycle times for different substrate concentrations in inhibitory cases. The use of mixed cultures in SCFs was also examined.

RÉSUMÉ

La technique de fermentation auto-cyclique (FAC) fut appliqueé à la biodégradation de plusieurs composés aromatiques par *Pseudomonas putida* et *Pseudomonas fluorescens*. La technique de FAC a été demontrée comme étant un outil de recherche utile dans les études de biodégradation aromatique et aussi comme méthode potentielle de traitement de la pollution. Les avantages de la technique de FAC comprennant ses performances hautement reproductibles et la consommation presque complète du substrat. La concentration en biomasse, la dureé d'un cycle, et la concentration minimale en oxygène dissous furent enregitrés d'un cycle à l'autre et la variation de ces paramètres au cors de l'opération en état stationnaire était inférieure à 5%. Les composés aromatiques utilisés dans cette étude furent le benzoate de sodium, le p-anisaldehyde et le 4-methoxybenzylidine-4-n-butylaniline (MBBA). Dés consommations de 93 à 100% furent obtenues pour ces composés utilisant la technique de FAC. Les vitesses de consommation se sont avérées être considérablement supérieures à celles observées en feremetation conventionnels. Cette étude a mis en évidence l'interêst de la durée d'un cycle comme paramètre pour comparer la croissance des différents microoganismes sur les substrats aromatiques. La durée d'un cycle s'est montrée dépendance est dûe à la présence d'un composé intermédiare non identifié et inhibiteur. Un modèle mathématique est présenté pour prédire la durée d'un cycle lors de l'état stationnaire pour différentes concentrations de substrat dans des cas d'inhibition. Nous avons également étudié l'utilisation de cultures mixtes en FAC

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Aromatic Compounds as Pollutants

Aromatic compounds are substances which incorporate the benzene ring in their structure. The term "aromatic" was first used to describe these compounds because many of them have a pleasant smell. However, this is certainly not true of all aromatic compounds, particularly the more complex hydrocarbons. Nevertheless, the name has been retained since it is useful to classify these compounds separately.

The aromatic nucleus has a large negative resonance energy; consequently benzene and its derivatives form a very stable group of compounds. The stability of the aromatic ring gives these compounds unique physical, chemical and biological properties and it is these properties that make them so useful in many industries. It is also these properties that make them pollutants of special concern. The chemical and biological stability of these compounds, in particular, makes them difficult to breakdown in the environment and they are thus very persistent pollutants.

Aromatic compounds can be subdivided into two general classes. *Mononuclear* aromatic compounds are those that contain only one aromatic ring in their structure. Mononuclear aromatic compounds are used extensively in fuels and also as industrial solvents. *Polynuclear* aromatic compounds are those containing two or more aromatic rings in their

structure. Polynuclear aromatic compounds are generally less volatile than mononuclear aromatic compounds. Most compounds containing three or more rings in their structure are solids. Polynuclear aromatic hydrocarbons are used in the synthesis of dyes, polymers, explosives, pesticides, drugs, and many other products of everyday use.

Aromatic compounds occur naturally in the environment mainly in fossil fuels, but it is the increased environmental presence of these compounds due to industrial emissions that is the subject of recent concern. Aromatic compounds are released into the environment by industries that utilise aromatic compounds directly (e.g. dyes, pharmaceutical) or those industries that utilize aromatic containing fossil fuels (petroleum refining, coking) (32). Industrial effluents from coal gasification and liquefaction processes, coke and carbon black production, petroleum processing, aluminum production, iron and steel works and fossil fuel combustion seem to add a particularly high input of aromatic compounds into the environment (3,5,20).

Aromatic pollutants are released into the environment in various forms. Combustion operations release aromatics into the atmosphere in gaseous form as well as solid residue form if the operation leaves an ash residue. More commonly aromatic compounds, and aromatic hydrocarbons in particular, are entrapped in effluent wastewaters that are released into nearby waterbodies. Due to their hydrophobic properties and limited water solubilities aromatic hydrocarbons tend to adsorb to particulates and eventually settle in the sediments of rivers, lakes, or oceans. Aromatic hydrocarbons are also released, to a lesser extent, directly into

waterbodies and sediments through spills and chronic leaks.

Several studies have attempted to quantify aromatic hydrocarbon emissions into the environment (5,20,24). Johnson and Larsen (24) compared aromatic hydrocarbon levels in marine and freshwater sediments worldwide. The aromatic hydrocarbon levels ranged from 5 ppb (nanograms of aromatic hydrocarbon per gram of sediment) for an undeveloped strip of land in Alaska and 1.79×10^6 ppb for an oil refinery in England. Sediments from areas surrounding other industries ranged from 198 to 232,000 ppb.

The concern about the emission of aromatic compounds has been mounting in recent years since many compounds in this important class of chemicals are extremely toxic. Furthermore, studies have shown many of these compounds to be carcinogenic in experimental animals (32,14,40) and a potential health risk to man. It is also believed that animal cells are incapable of breaking down many of these compounds and they will thus localise in animal tissue with prolonged exposure.

1.2 Microbial Degradation of Aromatic Hydrocarbons

The ability of microbes to grow on hydrocarbons, both aliphatic and aromatic, was recognised at least as far back as 1946 when Zobell (46) investigated the growth of various microorganisms on different hydrocarbons as a sole source of carbon.

In the years following this study, the interest of the scientific community in the microbial degradation of hydrocarbons continued to grow, motivated primarily by environmental

concerns. The research focused mostly on oil spills and the biological fate of petroleum compounds. Today research in the field is still driven by environmental concerns although the scope of interest has broadened beyond petroleum spills to include industrial effluents as well a wider variety of compounds including CFCs and PCBs.

There are numerous species of bacteria that are capable of degrading hydrocarbons. This is not surprising; hydrocarbons are naturally occurring organic compounds and many microorganisms have consequently evolved to utilize these abundant compounds as a carbon source Several studies (2,10,23,31,43) report as many as 22 genera of bacteria that are capable of degrading hydrocarbons, including aromatics. Some genera of yeasts (28), fungi (25) and algae (2) have also been reported to be capable of hydrocarbon degradation. Atlas (1) provides an excellent review of the various studies of hydrocarbon degradation.

It should be noted that although many microbes have hydrocarbon degrading capabilities, the rate of degradation varies dramatically from microbe to microbe and hydrocarbon to hydrocarbon. Aromatic hydrocarbons, in particular, seem more resistant and difficult to attack. Lee (29) found that organisms in river water were easily able to degrade alkanes, while low-molecular-weight aromatics were degraded more slowly and high-molecular-weight aromatics were resistant to microbial attack. Herbes and Schwall (21), in a study of petroleum-contaminated sediment, found that turnover times for polyaromatic hydrocarbons ranged from 7.1 h for naphthalene to 400 h for anthracene and 30,000 h for benz(a)-pyrene. These observations are consistent with experiences with oil spills where aromatic compounds

are the most persistent components. The range of microbial species with aromatic hydrocarbon degrading capabilities also seem much more limited and this may be another important reason for their persistence in contaminated environments.

The bacterial degradation of aromatic hydrocarbons is generally believed to proceed through an oxidative pathway involving the formation of dihydroxy compounds (diols) followed by cleavage and formation of a diacid such as cis,cis-muconic acid. Gibson (15,16) offers a review of aromatic degradation pathways.

Figure 1.1 shows an example of the dihydroxy degradation pathway. The degradation of benzene by *P. putida* proceeds via the formation of catechol. Catechol and another dihydroxy compound, protocatechuic acid, seem to particularly important intermediates and have been shown to play a role in the ring fission pathways of many different aromatic compounds (15). Among these compounds benzene, benzoic acid, salicylic acid and phenol have been shown to proceed via a catechol intermediate for various species of bacteria, while m-cresol, p-cresol, and p-aminobenzoic acid proceed through a protocatechuic acid pathway (15).

Substitution into the aromatic ring, such as methyl substitution, seems to inhibit initial oxidation (9). Initial enzymatic attack for different aromatic compounds, may be either on the alkyl substituent or alternatively, directly on the ring (16).

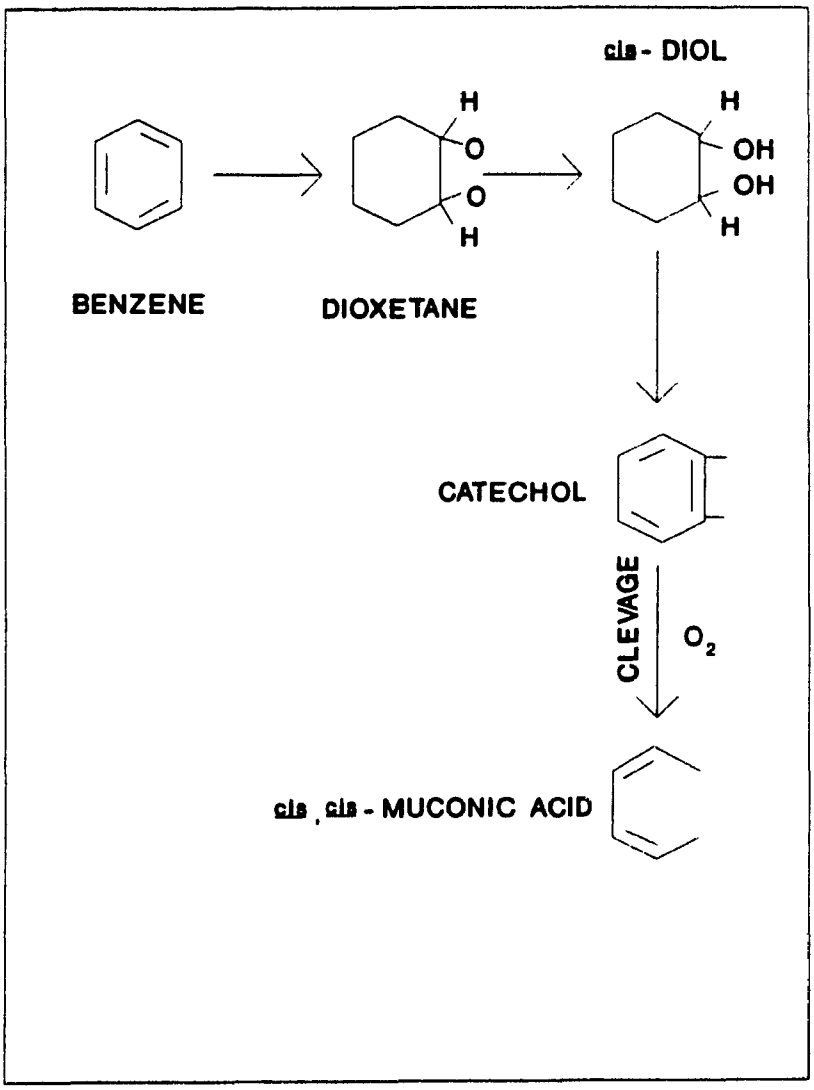

Figure 1.1 Degradation pathway of benzene by *P. putida* via the formation of catechol.

1.3 Problems in Current Aromatic Hydrocarbon Biodegradation Research

Although much progress has been made in the area of aromatic hydrocarbon degradation several problems are quite apparent in the current research effort.

Perhaps the most striking of these is the general lack of repeatability and agreement of data from study to study. This is perhaps not too surprising because of the very nature of biological research. The growth parameters of a living microbe are very dependant upon the composition of the medium and the condition of the environment to which the organism is subjected. Even small variations in the microbe's environment may have significant effects on the behaviour of the microbe. Furthermore, the microbes themselves will evolve from generation to generation to better deal with their environments. Thus, many different strains of the same species of bacteria may exist and they will behave very differently even in identical environments.

The lack of repeatability of data in hydrocarbon degradation studies is further aggravated by the fact that most of these studies have been carried out in batch fermenters. Little control is available over a batch fermentation. The effect of the state and size of batch inocula on the subsequent "lag phase" and exponential growth period associated with batch fermenters remains unclear. Further difficulties arise when studying aromatic hydrocarbon degradation in batch fermenters including:

1. The composition of the broth is continually changing and it is difficult to control the microbe environment both during the fermentation and from run to run.
2. Because of the changing nature of the broth, it is difficult to analyze for intermediate metabolic compounds.
3. Because cell growth in batch fermenters is unsynchronised, the microbes in the broth will be at different "ages" or stages of their life cycles and thus observations made are "averaged" results of the whole life cycle. Consequently, it is difficult to establish the steps by which the microbe breaks down the hydrocarbon substrate.

Current studies have also failed to show that the high or complete aromatic substrate consumption is possible. While the number and range of aromatic compounds that have been shown to be biodegradable is impressive, the extent and rate of biodegradation, when reported, is often poor. Table 1.1 shows the percentage of substrate that was consumed in several recent studies for different aromatic compounds. The consumption of the aromatic compounds falls far short of completion in all of these studies. The extent of biodegradation or consumption is a key factor in the development of biological systems to deal with aromatic hydrocarbon wastes. If such systems are to be used industrially to treat effluents, aromatic hydrocarbon levels must be reduced sufficiently to meet environmental regulation standards. The extent and rate of consumption of aromatics in such systems also play a key role in the financial and practical viability of such systems.

TABLE 1.1 Consumption of Aromatic Compounds Reported in Recent Studies

Type of Aromatic Compound	Reported Consumption (%)	Type of Culture	Ref.
Resorcinol Catechol Phenol	86 77 86	Anoxic mixed culture	Godbole & Chakra-barti 1991 (17)
Toluene and Benzene	50-60	Methano-genic mixed culture	Grbic-Galic & Vogel 1987 (19)
PCBs	<2	Mixed culture in overland flow treat-ment system	Pardue et al. 1988 (33)
p-Toluene sulfonic acid Phthalimido Phthalic acid Benzoic acid 2-Methylaniline Stylene glycol Toluene Bisphenol 2,4-Diaminotoluene N-Methylaniline Naphthol 4-Chloro-2-methyl aniline 2-4-Dinitrotoluene p-Toluidine-m-sulfonic acid 2-Chloro-5-amino-p-toluene p-Methylthio-phenol	90[a] 88 88 86 83 76 60 57 53 42 8 7 1 0 0 0	Activated sludge from wastewater treatment plant	Matsui et al. 1988 (30)

[a]Consumption values in the study by Matsui et al. are based on TOC removal values in a 24hr test period.

Finally, there is still a general lack of understanding of microbial aromatic hydrocarbon biodegradation. Although aromatic degradation pathways for simple compounds and certain organisms are well understood and accepted, these are by no means the only pathways bacteria utilise to break down these compounds. Many proposed pathways remain to be investigated, and their relative importance under different environmental conditions must be determined Biological processes are complex and this is by no mean a trivial task. Greater progress will be made with the development of better methods and newer techniques for studying bacterial aromatic hydrocarbon degradation.

1.4 Mixed Culture Growth

The vast majority of studies aimed at investigating the metabolic pathways of hydrocarbon biodegradation have focused on pure cultures. However, the study of mixed cultures is an area of increasing interest because such systems may offer several advantages over pure culture systems. These systems, for example, may be more adaptable to different hydrocarbon substrates because a mixed culture operation will have a much larger gene pool than a pure culture system. Furthermore, mixed cultures may offer higher overall hydrocarbon consumption rates since mixed cultures can take advantage of synergistic interactions between several hydrocarbon-degrading species. Mixed culture studies are also of great interest since the majority of existing wastewater processing operations utilise mixed cultures. If such

systems are to be used to treat more difficult wastes and hydrocarbons a better understanding of mixed culture interactions is required.

The simplest mixed culture interaction is pure competition and is the subject of many recent theoretical (34,41,45) and experimental (11,18,44) studies. Other interaction have also been recently investigated (12,26).

1.5 The Self-Cycling Fermenter

The Self-Cycling Fermenter (SCF) was first developed by Sheppard and Cooper (36-39) and was later used by Brown and Cooper (6-8). It is essentially a computer controlled aerobic fermenter which uses the dissolved oxygen level in the fermenter broth as the control parameter.

The basic operation of an SCF can best be understood by considering a typical self-cycling fermentation as shown is Figure 1.2. The figure shows the variation of dissolved oxygen, biomass and the limiting nutrient level in an SCF. At the start of the fermentation an inoculum is introduced into a known volume of broth in the fermenter. As the inoculum starts to grow the respiration rate in the fermenter begins to increase and the dissolved oxygen (D.O.) level in the fermenter drops. The D.O. continues to decrease as the biomass in the fermenter builds up and nutrients are consumed. At some point, a limiting nutrient in the fermenter will be exhausted. Growth thus stops and the respiration rate slows down. As less oxygen is being consumed, a minimum is observed in the D.O. level. The fermenter is now "harvested" (at time

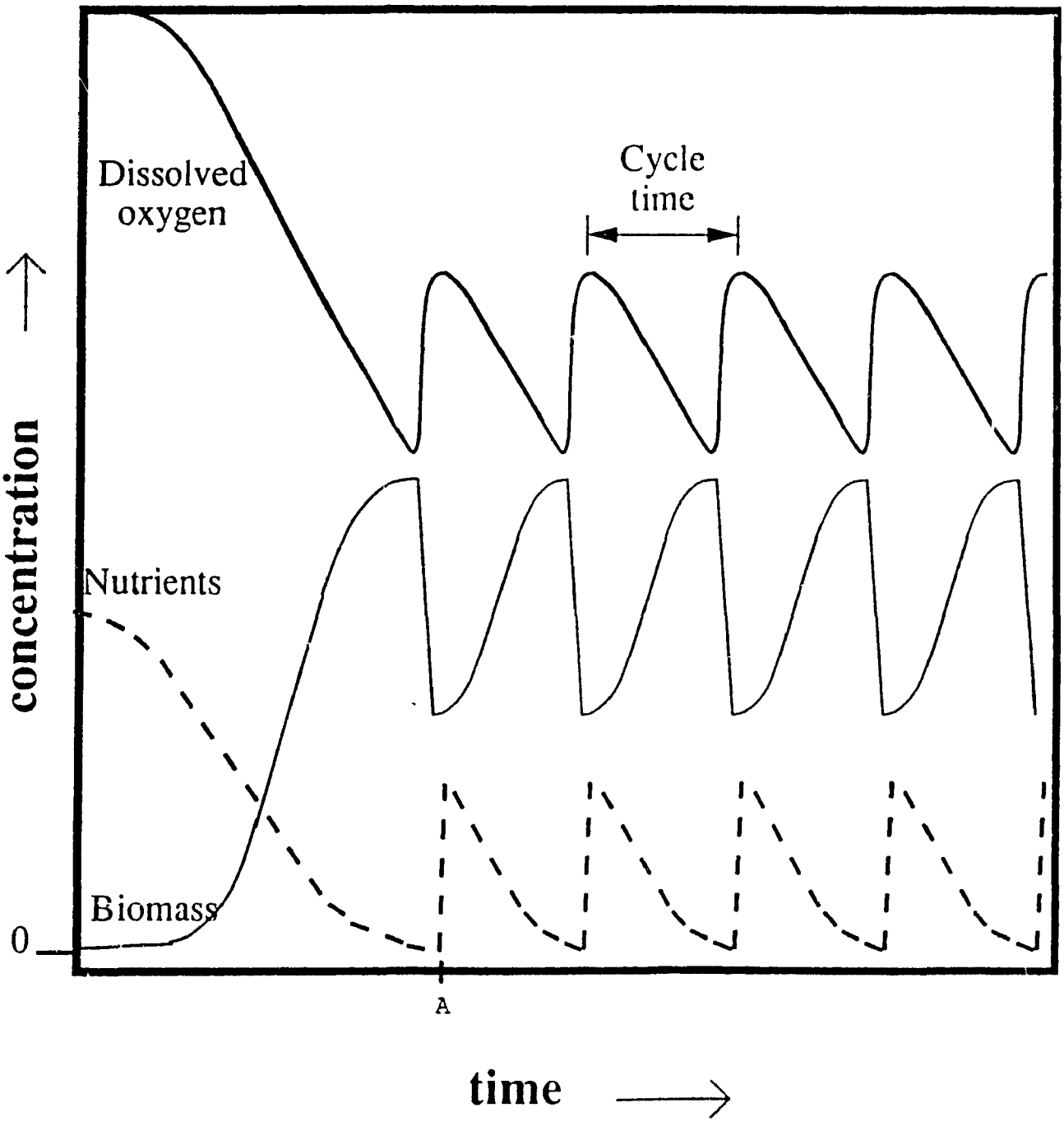

Figure 1.2 Behaviour of dissolved oxygen level, limiting nutrient concentration, and biomass in a Self-Cycling Fermenter.

A in Figure 1.2), the harvesting process removes half of the fermenter broth and replaces it with an equal volume of fresh medium. This is why a drop in biomass level is observed at point A, as well as an increase in restoration of nutrient level. Note that the D.O. level also rises since the fresh medium will also contain a significant amount of dissolved oxygen. Since the limiting nutrient has now been replenished, growth can continue as before. The D.O. concentration starts to drop until the nutrient is once again exhausted and a minimum D.O. level is observed. The fermenter is then harvested as before and the cycle repeats itself.

In a typical SCF a steady-state is quickly established after the first few cycles. The D.O. cycles become repeatable and are characteristic of the organism and substrate used in the fermentation. The cycle time is the distance between any two peaks in figure 1.2 and is also characteristic of the organism and the substrate. The minimum D.O. is the level of D.O. at the harvest point or end of cycle. It is usually a good indication of the biomass in the fermenter (lower minimum D.O. levels are observed for higher biomass) (6,8).

The SCF technique is then essentially a series of batch fermentations carried out in sequence, where half of the broth from one batch acts as the inoculum to the next. However, SCFs differ from batch fermentations in several fundamental ways. An SCF eliminates the "lag phase" associated with batch fermenters. Also, the different "batches" or cycles in an SCF run are identical and very reproducible unlike different batch runs. Furthermore, the pulsing of fresh medium into the fermenter from cycle to cycle induces a synchronisation of the fermenter

cell population and the resulting cell age distribution is much smaller than in a batch fermenter (6,8).

These fundamental differences give the SCF technique several advantages over other fermentation methods. SCFs are very stable and data are generally very repeatable. When running with the carbon source as the limiting nutrient, the consumption of the carbon substrate is complete. In addition, the high degree of cell synchronisation associated with SCFs makes this fermentation technique a powerful tool in cell cycle studies. The synchronisation eliminates the "averaging" effect due to simultaneously observing organisms of many different ages and thus simplifies monitoring for metabolic intermediates at a particular stage of the cell cycle.

Sheppard and Cooper (37,39) have used the SCF technique to investigate the growth of *B. subtilis* on glucose and showed that growth of *B. subtilis* was very stable and repeatable in such a system. Brown and Cooper (7,8) used the SCF technique to degrade a simple aliphatic hydrocarbon (hexadecane) by *A. calcoaceticus*. Hydrocarbon consumption was complete and repeatable from cycle to cycle. To date, the SCF technique has not been applied to more complex hydrocarbons such as aromatics. This is the purpose of this study.

2. OBJECTIVES

This study is part of a multi-component project aimed at developing the self-cycling fermentation technique (SCF). The research effort undertaken in this work was aimed at expanding previous work done with simple hydrocarbon SCFs to include more complex aromatic hydrocarbon substrates. More specifically, the objectives of this study were:

1. To apply the SCF technique to the biodegradation of aromatic compounds.

2. To investigate the stability and reproducibility of SCF data for aromatic compounds.

3. To compare the degradation of aromatic compounds by different microorganisms by use of SCF data.

4. To determine if high or complete aromatic substrate consumption is possible in SCFs.

5. To investigate the growth of a mixed culture in the self-cycling fermenter.

3. MATERIALS AND METHODS

3.1 Selection of Test Organisms and Substrates

The selected microorganisms for the pure culture aromatic compound studies were *Pseudomonas putida* (ATCC 12633) and *Pseudomonas fluorescens* (ATCC 13525). The *Pseudomonas* bacteria are known for their abilities to degrade aromatic compounds and are the test microorganisms in many aromatic degradation studies. Selection of these well studied bacteria made it easy to compare our results to other work. *Rhodoccocus rhodochrous* (ATCC 13808), *Nocardia erythropolis* (ATCC 4277) and *Acinetobacter calcoaceticus* (ATCC 31012) were selected for the mixed culture studies. These bacteria were selected because of their hydrocarbon degrading abilities as well as their distinct appearance on nutrient agar plates, which facilitates cell counts in mixed culture experiments. *Rhodoccocus rhodochrous* appears in red colonies on nutrient agar plates, while *N. erythropolis* and *A. acinetobacter* grow in compact colonies of distinct shape that can be differentiated from other species.

The aromatic compound substrates used were: sodium benzoate, p-anisaldehyde and 4-methoxybenzylidine-4-n-butylaniline (MBBA). Benzoates are pollutants of some importance, moreover, since they are perhaps among the easier aromatic compounds to degrade, they have been used as substrates in several recent studies (20,25). p-Anisaldehyde is a more difficult hydrocarbon substrate while MBBA is a liquid crystal polynuclear aromatic hydrocarbon containing two aromatic rings in its structure.

3.2 Maintenance of Microorganisms

All cultures were maintained on nutrient agar plates (Difco 0001) at 4°C. New plates were prepared monthly, by transferring an inoculum of the microorganism from a single pure colony on any of the stored culture plates to a fresh nutrient agar plate. New plates were then allowed to grow in an incubator at 30°C for 24 hours before refrigeration.

3.3 Medium and Growth Conditions

The mineral medium used for all experiments was a defined mineral salt medium used by Brown and Cooper (6,8) to grow *A. calcoaceticus* on a variety of hydrocarbons in a SCF. The mineral medium consisted of 1.0 g NH_4CL, 2.78 g Na_2HPO_4, 2.77g KH_2PO_4 and 20 ml of trace elements solution, dissolved in 1 litre of distilled water. The trace element solution contains 10.0 g nitriloacetic acid, 7.056 g $MgSO_4$, 2.514 g $CaCl_2$, 9.25 mg $(NH_4)6Mo_7O_{24}.4H_2O$, 99 mg $FeSO_4.7H_2O$ and 50 ml of a trace metal solution, dissolved to 1 litre with distilled water and adjusted to pH 6.8 with KOH. The trace metal solutions consisted of 2.768 g EDTA.$2H_2O$, 5.00 g $FeSO_4.7H_2O$, 1.54 g $MnSO_4.7H_2O$, 0.392 g $CuSO_4.7H_2O$, 0.240 g $Co(NO_3)_2.6H_2O$, and 0.177 g $Na_2B_4O_7$ dissolved to 1 litre with distilled water. A few drops of 6 N H_2SO_4 were added to the trace metal solution to retard precipitation.

The inocula for all experiments were prepared in shake flasks maintained at 30°C in a gyratory shaker (New Brunswick Scientific Co., Incubator Model G25) at 300rpm. The inoculum was first grown in 100 ml of nutrient broth for 24 hours. A 5% (vol/vol) inoculum

was then transferred from the nutrient broth flask into a second flask containing 100 ml mineral salt medium and the test substrate at a similar concentration to the planned experimental concentration. The inoculum was allowed to grow in the mineral salt shake flask for at least 1 week or until significant growth was apparent in the shake flask. A second transfer into mineral salt medium containing the test substrate was then carried out (3% vol/vol inoculum transferred), prior to inoculation in the fermenter. These transfer steps acclimated the inoculum to the test substrate and allowed an easier and quicker start-up in the fermenter. A 1% (vol/vol) inoculum was usually used in the fermenter.

The sodium benzoate substrate was added as a concentrated solution to the fermenter by a peristaltic pump. p-Anisaldehyde and MBBA are liquids and were added directly through a syringe pump (Sage instruments Model 355). The syringe was ethanol sterilized while the tubing of the peristaltic pump was autoclaved at 120°C for 30 minutes. No need was found to sterilize the substrates themselves; sodium benzoate is used as a food preservative and bacterial growth in a concentrated solution is unlikely, and similarly, bacterial growth in the pure p-anisaldehyde and MBBA is unlikely. Various substrate concentrations were used, but all the experiments were run with the aromatic substrate as a limiting nutrient. The limiting concentrations were determined as described below.

The fermenter was aerated at 0.5 l/min flowrate in all experiments. Brown and Cooper (6,8) measured $K_L a$ in an identical set-up and the same flowrate and found it to be equal to 145 hr^{-1}. The fermenter volume was kept constant at 1 litre in all experiments.

3.4 Limiting Aromatic Substrate Concentration Determination

The range of limiting concentrations for any substrate was determined by a procedure similar to the trial-and-error approach used by Brown and Cooper (8) in a SCF. An amount of the substrate was added to the fermenter containing the test organism in fresh medium, and the D.O. profile was observed while growth proceeded. When a minimum in the D.O. profile was observed, an additional amount of substrate was added. The minimum in the D.O. profile indicated that growth had stopped because a certain component of the broth had been exhausted. If the D.O. profile began to drop again on addition of more substrate, it is was an indication that the limiting component that had run out is indeed the substrate. If the D.O. did not fall on addition of more substrate, then some other component of the medium had been exhausted and the substrate concentration had exceeded the limiting concentration range. The D.O. profile was seen to drop in such cases, if fresh medium was added to the reactor and the exhausted component thus replenished (the D.O. initially increased on addition of substrate since more oxygen was also introduced into the system, but began to fall after several minutes).

This procedure can be repeated at increasing substrate concentration to establish the limiting range, or alternatively it can be used to confirm that certain selected substrate concentrations are limiting. Other procedures to determine the limiting range of growth substrates can be used, but this was found to be the quickest method and was generally adopted for all substrates.

3.4 Biomass Determination

All biomass measurements were made on a dry weight basis. Duplicate 25 ml samples were used for each biomass measurement. The samples were centrifuged (Du Pont Instrument RC-5 Superspeed Refrigerated centrifuge) for 20 minutes at 10,000 X g and 4°C. The supernatant was then decanted and the pellet collected was transferred to an oven (Fisher Isotemp Model 126G) and allowed to dry to constant weight at 105°C. Biomass measurement are reported as dry cell weight in grams per litre of broth.

The deviation between duplicate samples was on average less than $\pm$4%.

3.5 Aromatic Compound Analysis

3.5.1 Sodium Benzoate Test

The concentration of sodium benzoate was determined using a procedure based on the UV absorbance method described by Holder and Vaughan (22) to measure the concentration of benzoic acid.

An extraction step was added to the procedure since growth of the test bacteria on non-aromatic compounds was found to produce substances such as proteins that absorb in the aromatic region. Measuring the sodium benzoate content of samples directly using UV absorbance was thus avoided since similar interfering substances could be produced during growth on the sodium benzoate as substrate

Samples for the adjusted procedure were prepared by acidifying a 10 ml sample using 0.5 ml of 0.5 1M HCl in a test tube. The acid added was sufficient to drop the pH level to below pH=2. The acidified sample was vortexed at high speed (Vortex-Genie) and allowed to stand for 5 minutes. 5 ml of iso-octane were then added and the mixture vortexed at high speed for 1 minute. The mixture was then allowed to stand for 30 minutes. A 3.5 ml sample from the iso-octane phase was transferred to a 10mm UV cell and the absorbance measured at 275nm.

Figure 3.1 shows the calibration curve for the above test. Sodium benzoate was fairly soluble in the aqueous phase even at low pH and there was a considerable extraction loss. This extraction loss accounted for the non-zero intercept of the curve. In samples containing less than 0.05 g/l sodium benzoate, the extraction loss exceeded the sample concentration an no sodium benzoate was detected in the iso-octane phase.

It was, however, still possible to measure sodium benzoate concentration levels below 0.05 g/l by "spiking" the test sample with a known volume of concentrated benzoate solution. All experimental samples were "spiked" with 0.5 ml of a 2.5 g/l sodium benzoate solution. This increased the sample sodium benzoate level by an amount equivalent to the content of a 0.125 g/l benzoate sample. The actual sodium benzoate level of the "spiked' sample was then determined by subtracting 0.125 g/l from the observed concentration.

A minimum detection level of 0.003 g/l was possible using this technique. The standard deviation of error for three identical replicates was $\pm$3.5%.

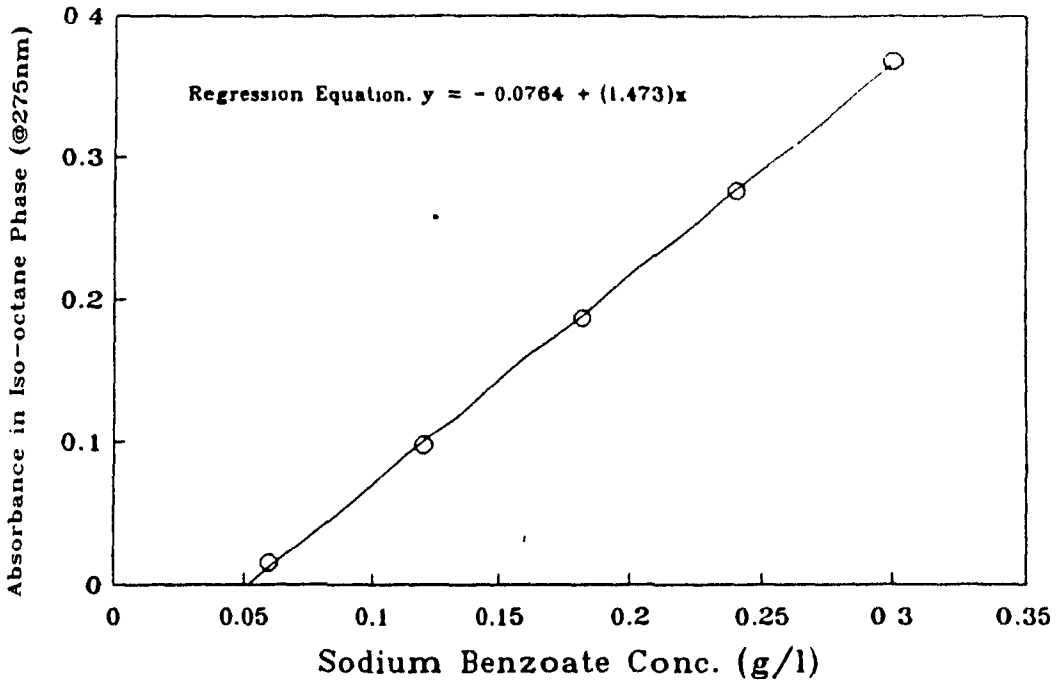

Figure 3.1 Sodium benzoate calibration curve

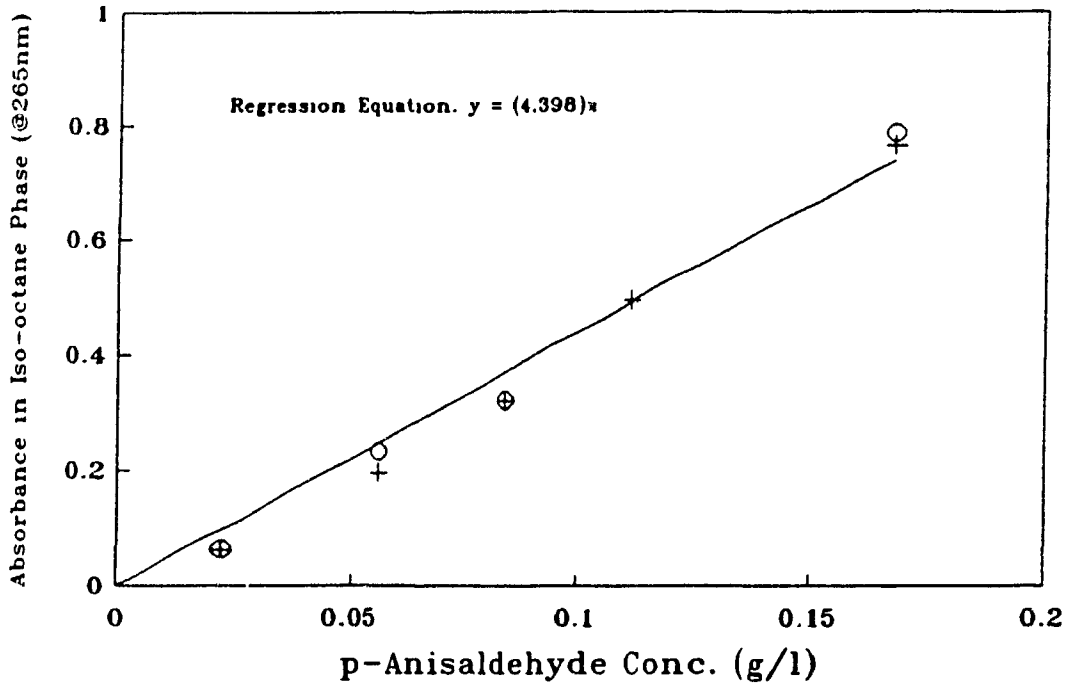

Figure 3.2 p-Anisaldehyde calibration curve. Replicate measurements were performed at some concentrations.

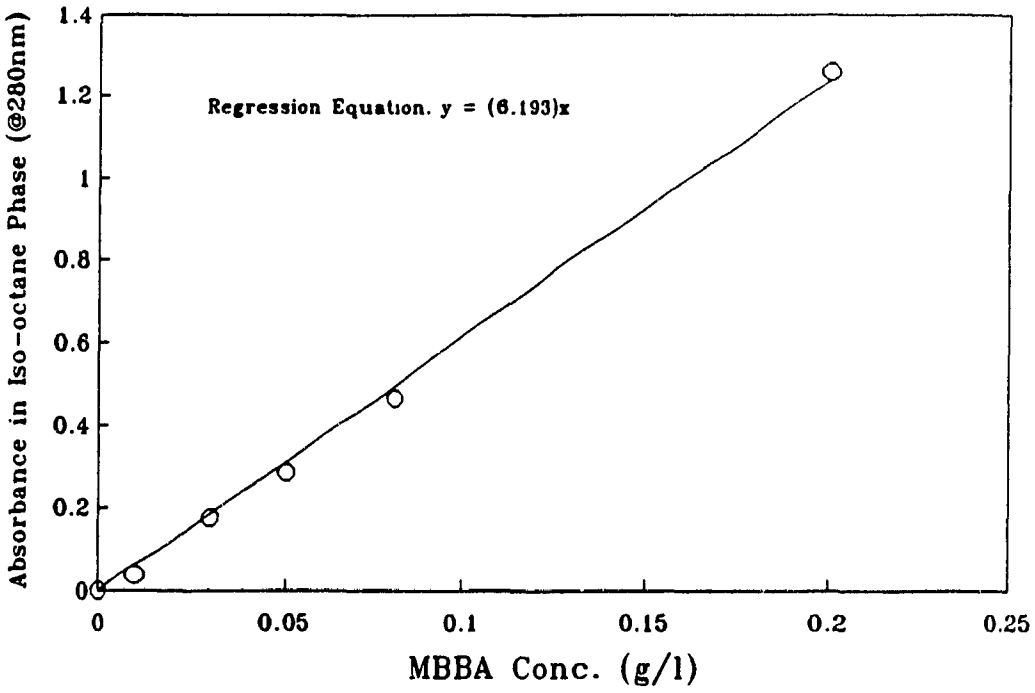

Figure 3.3 MBBA calibration curve

3.5.2 p-Anisaldehyde Test

p-Anisaldehyde concentrations were determined by an UV absorbance procedure identical to that used for the benzoate test. Absorbances were measured at 265nm. No acidification was performed for the p-anisaldehyde test. Figure 3.2 shows the p-anisaldehyde test calibration curve and regression equation.

There were no significant extraction losses and thus no "spiking" of samples was necessary. The calculated coefficient of correlation (R^2) for the calibration curve was 0.981. The minimum detectable level of p-anisaldehyde using this procedure was 0.002 g/l. The standard deviation of error for four identical replicates was $\pm$4%.

3.5.3 MBBA Test

MBBA concentrations were also determined by the same UV absorbance procedure . The iso-octane volume used for the MBBA test was 10 ml and no acidification was required. Absorbances were measured at 280nm. Figure 3.3 shows the MBBA test calibration curve and regression equation.

Once again the calibration curve showed that there were no significant extraction losses. The coefficient of correlation (R^2) for the calibration curve is 0.997. The minimum detectable level of MBBA using this procedure was 0.001 g/l.

3.6 Cell Counts

3.6.1 Cell Count Method

The standard cell count method (35) was used for all cell count determinations. 100 ml Nalgene autoclavable bottles were used for the dilutions. The dilutions were carried out with sterile distilled water which was transferred to the dilution bottle by an autoclavable dispenser (Brinkham). Volumes transferred from dilution bottles and from original samples typically ranged from 0.1 to 1.0 ml in volume (transferred by volumetric pipettes). Dilution and sample bottles were inverted 20 times before volume transfers to ensure that the contents are well mixed and that the withdrawn sample is a representative one. A volume of 0.2 ml was used for spreading on nutrient agar plates which were then allowed to grow for 24 hours. Spreading was done by a glass spreader on a rotary plate stand.

As the expected cell density of the samples varied from experiment to experiment, an initial set of test dilutions were carried out. An experimental sample was selected and cell count plates were prepared from two or three different dilutions. The dilution that resulted in a plate concentration of approximately 30 to 300 colonies/plate was selected as the ideal dilution.

Two or three plates were prepared for each sample and cell counts were reported as the average of these. The typical standard deviation in counts from three replicate plates was $\pm$ 6%

3.6.2 Determination of Mixed Culture Ratios

Mixed culture ratios were determined directly by cell counts. Cell count plates were prepared as described above, and the colonies of each bacteria were counted individually.

Typical standard deviations in ratios determined from three replicate plates ranged from 6-9%, which is considerably higher than the error associated with a pure culture plate count. The higher spread is due to the increased error in differentiating the two types of cell colonies. It was also found that for some mixed cultures the growth rate of plate colonies of one bacteria were considerably different from growth rates for the other. It was thus difficult to determine an ideal growth time for the plates such that the plate colonies are large enough to count but not too large to overlap.

3.6.3 Determination of Cell Synchrony

Cell synchrony was measured using the method of Blumenthal and Zahler (4). Intra-cycle samples of 1 ml were taken from the fermenter at regular time intervals. Cell counts were then performed on these samples according to the method described above. The results were plotted as described by Blumenthal and Zahler (4) and a "synchrony index" (F) was determined A synchrony index of zero indicates unsynchronised growth, while a value of one indicates perfect synchrony. Values of F greater that 0.5 are generally considered synchronous.

3.7 The Self-Cycling Fermenter Setup

A schematic of the SCF setup is given in Figure 3.4. Essentially, the SCF setup consisted of a recirculating cyclone column reactor, the dosing and harvesting units, and the computer and solenoid valves control system. The cyclone column was the heart of the SCF setup and contained the fermenter broth. The broth was continuously recirculated around a closed loop by a pump. The dosing and harvest unit removed broth from the fermenter at the end of cycle and replenished it with fresh medium. Fresh substrate was added to the reactor at the start of a cycle by a syringe or peristaltic pump. Monitoring of dissolved oxygen and control of the SCF was done through a computer which controlled a series of solenoid valves. The system is described in detail by Sheppard and Cooper (36-39) and Brown and Cooper(6-8).

The sampling system of the SCF setup was expanded to include eight sampling bottles and an overflow vessel (although Fig. 3.4 only depicts four of the eight sample bottles in the sampling system). On completion of the fermenter harvest the main solenoid leading to the sampling system opens and the broth from the harvest vessel passes to the overflow vessel in the sampling system. The overflow vessel retains 100 ml of the harvested broth and discards the additional volume to a waste collection jar. After the harvest vessel is filled the solenoid leading to the sampling system closes and one of the solenoids leading to the sampling bottles opens and passes the broth retained in the overflow vessel to the sample bottle. The sampling system rotates between collection bottles from cycle to cycle. All the sample bottles were refrigerated at 4°C.

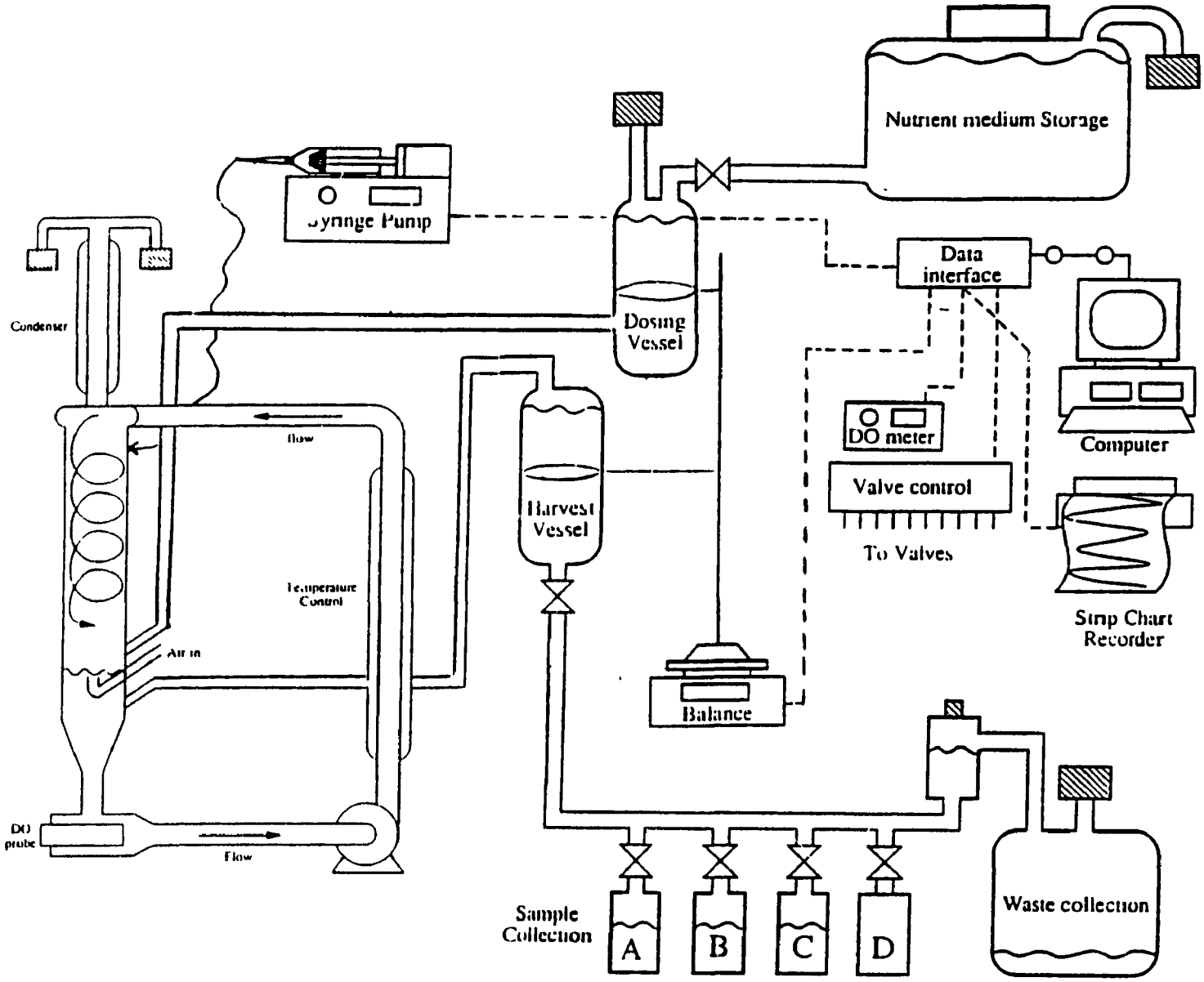

Figure 3.4 Self-Cycling Fermenter Apparatus

The expanded sampling system allowed longer unattended operation times of the SCF since sample bottles had to be changed every 8 rather than 4 cycles. The overflow vessel reduced the amount of unwanted harvest broth. The collected volume of 100 ml was sufficient to carry out all the described tests.

4. RESULTS

4.1 Self-Cycling Fermentations using Aromatic Compounds

4.1.1 SCFs with Sodium Benzoate as Substrate

Several self-cycling fermentations (SCFs) using sodium benzoate as the substrate were carried out with either *P. fluorescens* or *P. putida* as the test organism.

Figure 4.1 shows the dissolved oxygen (D.O.) profile versus time for *P. fluorescens* grown on 0.15 g/l of sodium benzoate. Figure 4.2 shows data measured for each cycle of this fermentaion. The minimum D.O. is the oxygen level detected when a minimum is recognised in the dissolved oxygen profile of any particular cycle. Biomass measurements were made on harvest samples collected at the end of each cycle.

For the first cycle, the D.O. profile started to drop 9-10 hours after inoculation (1% vol/vol). The observed period of the D.O. cycles grew shorter and the pattern became steady after 5 or 6 cycles In fact, steady-state was obtained for all of the parameters by the seventh cycle. This steady-state was stable and was maintained for 40 cycles. The average steady-state cycle time was 72.0 min with a standard deviation of $\pm$1.31 min. The corresponding steady-state biomass and minimum D.O. levels were 0.178 g/l ($\pm$ 0.024 g/l) and 25.81 % of saturation ($\pm$0.35%) respectively.

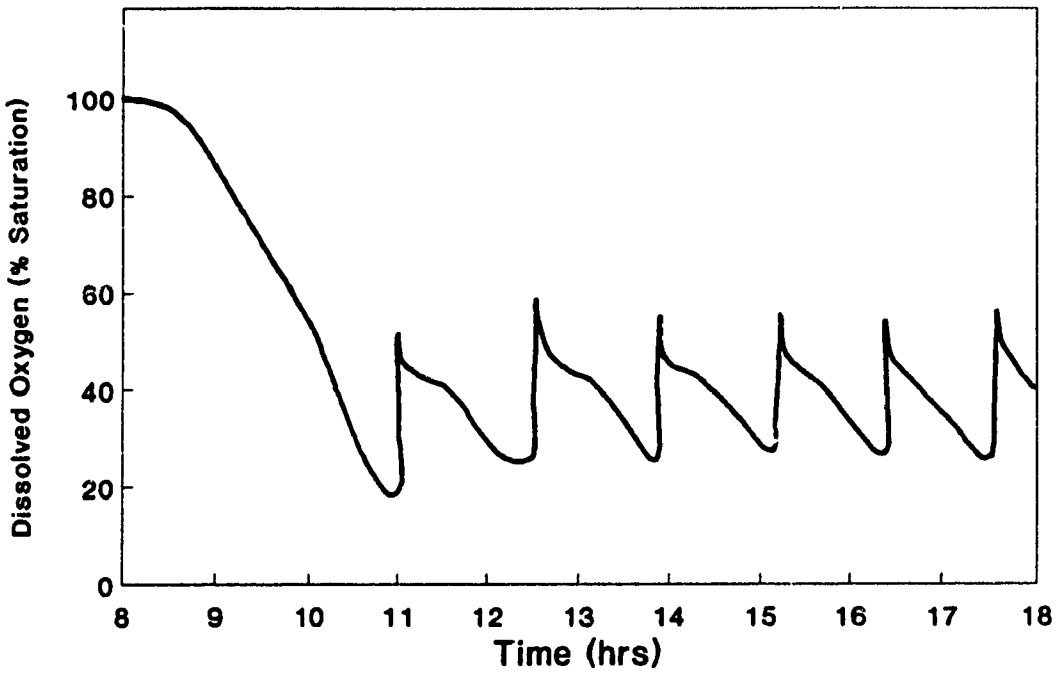

Figure 4.1 Dissolved oxygen profile from a SCF in which *P. fluorescens* was grown on 0.15 g /l of sodium benzoate.

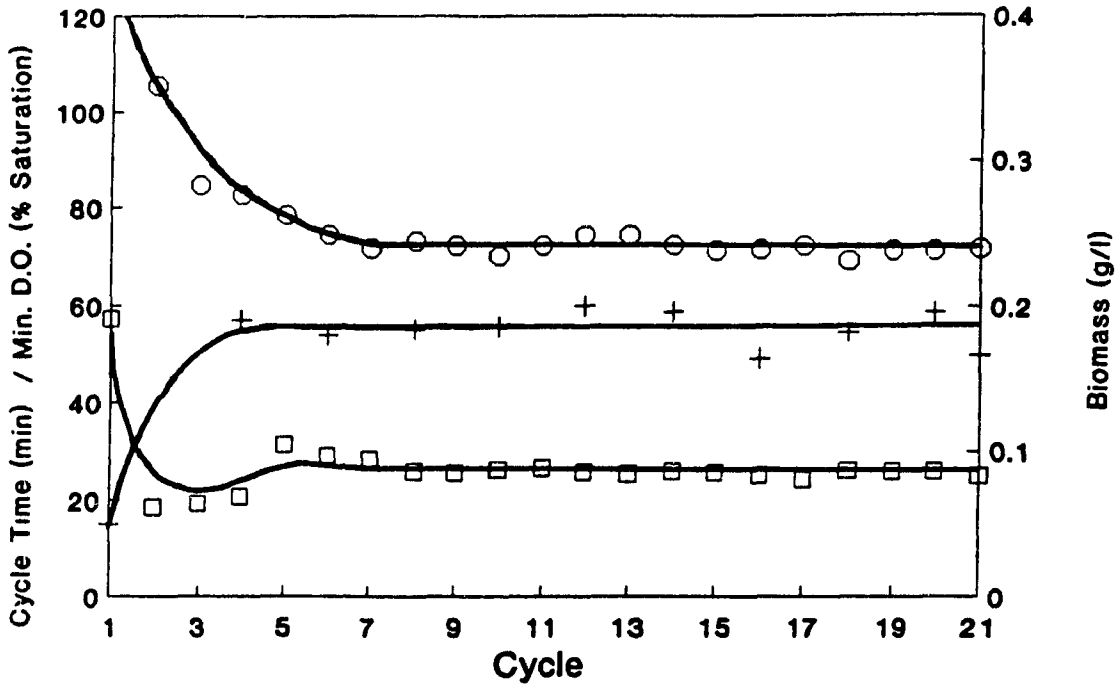

Figure 4.2 Measured cycle time (○), minimum dissolved oxygen level (□) and biomass (+) for *P. fluorescens* grown on 0.15 g/l sodium benzoate.

A steady-state operation was also obtained for *P. putida* grown on a similar sodium benzoate concentration. Figure 4.3 shows parameters for *P. putida*. The steady-state cycle time for *P. putida* grown on sodium benzoate, of 47.62 min (±2.86 min), was considerably shorter than that for *P. fluorescens*. The steady-state biomass and minimum D.O. level were 0.198 g/l (±0.015 g/l) and 80.35% of saturation (±0.78%) respectively.

The state and size of the inoculum had no effect on the steady-state conditions observed for different sodium benzoate SCFs. Inocula prepared in nutrient broth and not acclimated to the sodium benzoate substrate also lead to the same steady-state. Several sodium benzoate SCFs were also initiated by changing or "switching" the substrate to sodium benzoate in SCFs which had attained steady-state on another substrate. Sodium benzoate SCFs initiated in this way also lead to the same steady-state conditions.

The number of cycles required before attaining steady-state was also 6 for *P. putida*. The time required for the first cycle ranged from 13-16 hours for *P. fluorescens* and 10-14 hours for *P. putida* for 1 % vol/vol substrate acclimated inocula. The time required for the first cycle was 2-3 hours longer in SCFs where an inoculum which was not previously acclimated to the sodium benzoate substrate was used. Increasing the inoculum size from 1% vol/vol to 2% vol/vol resulted in a decrease in first cycle times of 1/2 to 1 hour. Sodium benzoate SCFs initiated by "switching" generally reached steady-state more quickly requiring only 3 cycles and a first cycle time of 4-5 hours. This is not surprising since the biomass accumulated before

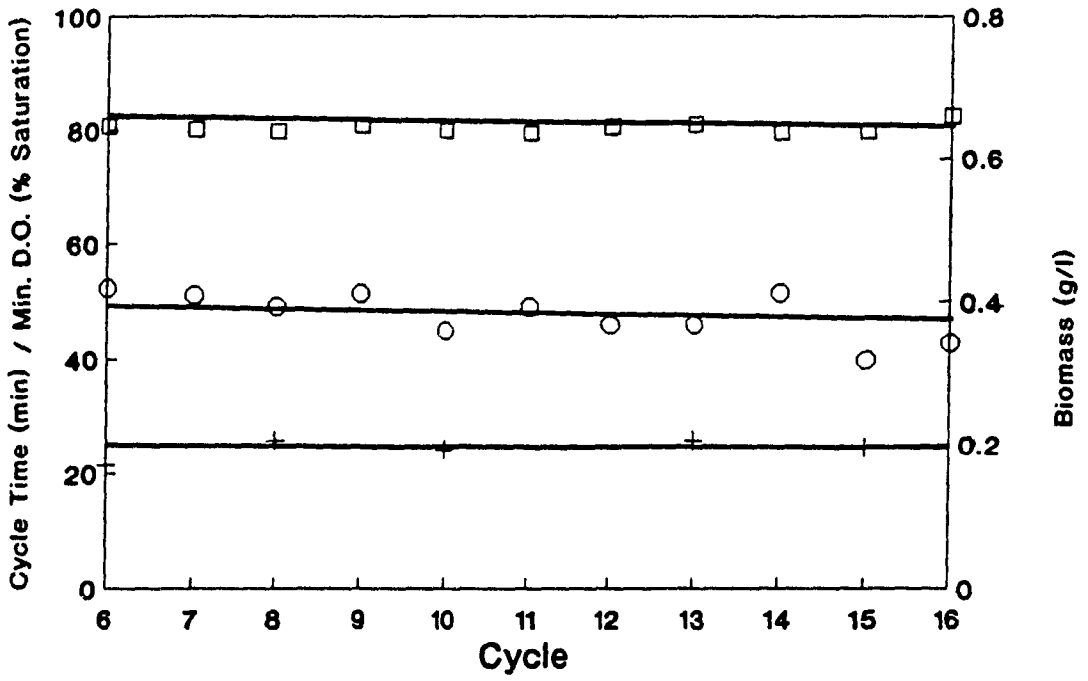

Figure 4.3 Steady-state biomass (+), cycle tine (○) and minimum dissolved oxygen level (□) for *P. putida* grown on 0.14 g/l sodium benzoate.

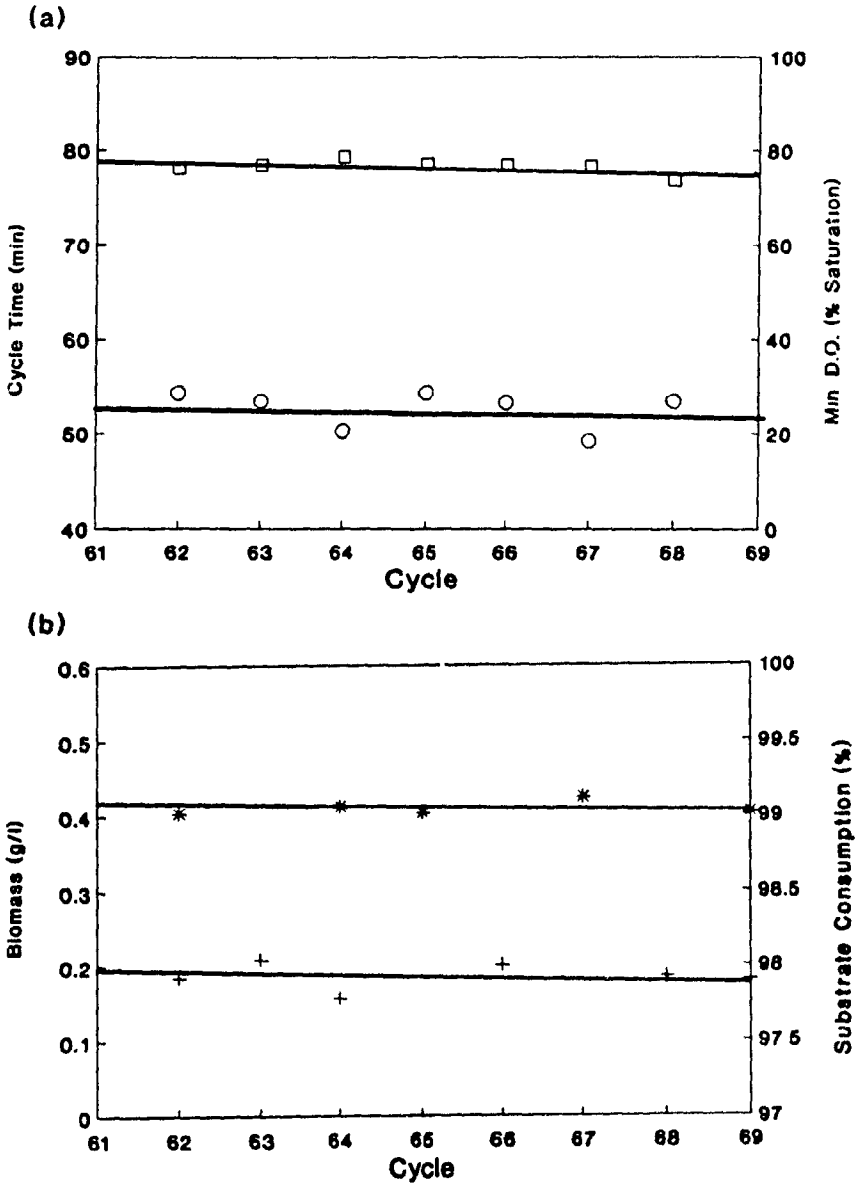

Figure 4.4 Steady-state biomass (+), cycle tine (○), minimum dissolved oxygen level (□) and percentage substrate consumption (*) for *P.putida* grown on 0.14 g/l sodium benzoate.

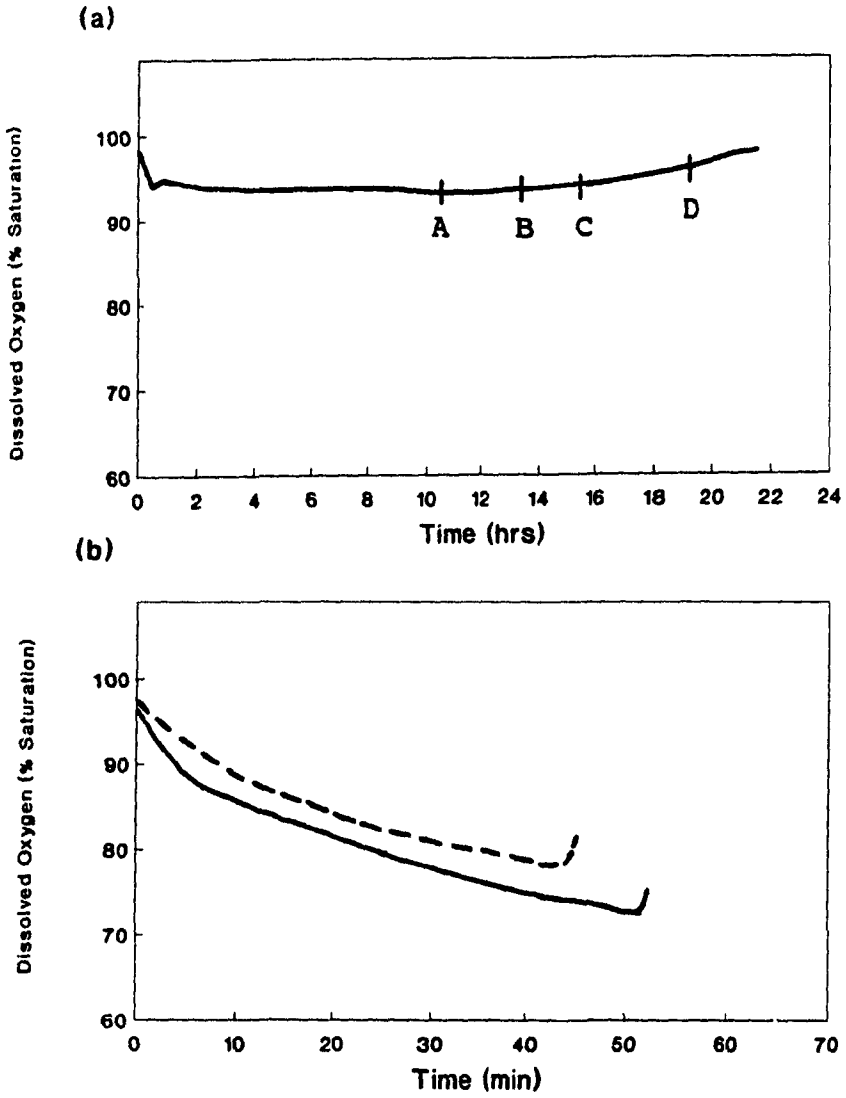

Figure 4.5 (a) Steady-state dissolved oxygen profile for *P. putida* grown on 0.60 g /l of MBBA (b) Steady-state dissolved oxygen profiles for *P. putida* grown on 0.14 g /l of sodium benzoate (broken line) and 0.165 g/l p-anisaldehyde.

changing substrate essentially provides a very large inoculum for the first cycle of growth with sodium benzoate as substrate.

4.1.2 SCFs with p-Anisaldehyde as Substrate

A steady-state was also obtained for *P. putida* when grown on p-anisaldehyde in the SCF. The steady state was maintained for over 60 cycles. Figure 4.4 shows the steady-state parameters for growth on 0.165 g/l of p-anisaldehyde. The average steady-state cycle time was 51.6 min (±0.78 min). The minimum D.O. (Fig 4.4a) and biomass (Fig 4.4b) were also constant from cycle to cycle with average values of 76.4% (±1.33%) and 0.188 g/l (±0.020 g/l) respectively. Figure 4.4b also shows that the percentage consumption of the p-anisaldehyde substrate was constant and close to 100% in each cycle.

Observation made during SCF initiation using p-anisaldehyde as substrate differed considerably to observations from the sodium benzoate SCFs. Steady-state was obtained after 6 or 7 initial cycles of growth. This is similar to the number of initial cycles in the sodium benzoate experiments, however, the initial cycles are much longer in comparison to the steady-state cycle time. The first cycle time for p-anisaldehyde SCFs varied from 21-26 hours. The first cycle to steady-state cycle time ratio thus ranged between 18.1 to 22.4 while the range is 12.6-17.7 for *P. putida* grown on sodium benzoate for SCFs with the same inoculum size. The initiation of p-anisaldehyde SCFs was also generally more sensitive to the state of the inoculum. Furthermore, if the inocula were not acclimated to the p-anisaldehyde in shake flasks, as

described above, the fermentation was not successful. SCFs initiation through substrate "switching" as described in the sodium benzoate experiments were also unsuccessful. Such SCFs showed no sign of growth even after several days following inoculation and also became foamy possibly due to cell lysing.

Steady-state was not obtained with *P. fluorescens* and p-anisaldehyde as substrate. Growth of *P. fluorescens* on p-anisaldehyde in shake flasks was poor even after several transfers, requiring 4-5 weeks before showing any visible signs of growth. No growth was detected in the SCF after 2 weeks when both acclimated inocula and non-acclimated inocula from nutrient broth were used. Inocula size range from 1-5% vol/vol.

4.1.3 SCFs with 4-methoxybenzylidine-4-n-butylaniline (MBBA) as Substrate

Initial SCFs experiments using 4-methoxybenzylidine-4-n-butylaniline (MBBA) as substrate were unsuccessful with both *P. putida* and *P. fluorescens* due to difficulties in monitoring for a minimum level in the D.O. profile during growth.

Figure 4.5 illustrates the D.O. profile during growth of *P. putida* on MBBA as compared to the D O. profiles for a cycle of growth of *P. putida* in the sodium benzoate and p-anisaldehyde SCFs. The MBBA growth profile observed was very "shallow" and did not have a distinct minimum. Both the benzoate and p-anisaldehyde profiles were deeper and showed very distinct minima. The rise in the D.O. level at the end of a cycle in these SCFs was sharp and easily recognised by the computer cycling algorithm. The minimum in D.O. profile

for MBBA growth, by contrast, had an extended period of an almost flat D O. signal before beginning to rise very gradually.

In the initial experiments the sensitivity of the computer cycling algorithm was increased so as to allow the fermenter to cycle when a very small rise in the D O level was detected. However, even with the increased sensitivity the minimum was hard to pinpoint and uneven cycling resulted. The computer algorithm, with the same sensitivity setting, recognised points A,B, or C on Figure 4.5a as the cycle minimum. With such wide fluctuation in the recognition of the cycle minimum, no steady-state was possible. Significant MBBA build-up was also noted from cycle to cycle.

In later experiments another approach was taken. The sensitivity of the algorithm was decreased. The computer thus cycled only when it detected quite a large rise in the D O. level. In effect, the computer cycled the fermenter well past the minimum at point D (Figure 4 5a) This approach led to a steady-state operation at a substrate concentration of 0 715 g/l for *P fluorescens* and 0.610 g/l for *P. putida*. Steady-state was maintained for 10 cycles for both *P putida* and *P. fluorescens*. Steady-state cycle times were 14.5 hr ($\pm$0.5 hr) for *P. fluorescens* and 18.3 hr ($\pm$0.7 hr) for *P. putida*.

Table 4.1 presents other measured parameters for the MBBA experiments and compares these to parameters from the sodium benzoate and p-anisaldehyde experiments.

TABLE 4.1 Steady-state parameters for *P. fluorescens* and *P. putida* grown on various aromatic compounds.

ORGANISM	P. fluorescens		P. putida		
SUBSTRATE	Sodium Benzoate	MBBA	Sodium Benzoate	p-Anis-aldehyde	MBBA
CONC. (g/l)	0.140	0.715	0.150	0.165	0.610
CYCLE TIME[a]	72.0min (1.3)	13.5hr (0.5)	47.6min (2.9)	51.6min (0.56)	18.3hr (0.7)
MIN D.O (%)	26	92	80	76	93
BIOMASS(g/l)	0.178	0.228	0.198	0.188	0.207

[a]Standard deviations are shown in brackets under the average value and are expressed in the designated units.

4.2 Rates of Metabolism of Aromatic Substrates in SCFs

Table 4.2 shows the percentage of the substrate which was consumed in steady-state cycles of growth for the benzoate, p-anisaldehyde and MBBA experiments. The percentage substrate consumption was determined by comparing the substrate concentration in samples harvested at the end of a cycle to the substrate concentration at the beginning of the cycle. The substrate consumption rate is the average amount of substrate consumed in steady-state cycles divided by the average cycle time. Table 4.2 also presents the specific consumption rate, defined as the consumption rate per biomass.

The percentage substrate consumption in the sodium benzoate experiments was 93.5 % for *P. fluorescens* and 93.7 % for *P. putida*. p-Anisaldehyde consumption by *P putida* was almost complete and averaged 99.1 %. MBBA consumption was complete in all steady-state samples analyzed.

4.3 Extended Cycle Studies

The effect of cycle "extension", or delaying the cycling of the SCF for a short time after detection of a minimum in D.O level, on substrate consumption was investigated. Cycle extension was achieved by decreasing the minimum detection sensitivity of the cycling algorithm.

Figure 4.6 illustrates the results of the extended cycle studies for *P. putida* grown on sodium benzoate. Decreasing the minimum sensitivity in the control algorithm resulted in a slight increase in cycle time from an average of 46.4 min to 48.7 min. This 2.3 minute extension in cycle time resulted in a significant increase in substrate consumption from 95.18 % for the four standard cycles shown in Figure 4.6 to a 98.02 % average for the extended cycles. Further cycle extension is possible but was not investigated.

4.4 Substrate Concentration Effects in SCFs

Figure 4.7 shows the steady-state cycle time and minimum D.O. level for *P. putida* grown on 0.15 g/l, 0.51 g/l, and 0.67 g/l of sodium benzoate. The average cycle time for the cycles grown on 0.15 g/l of sodium benzoate was 47.6 min (±2.86 min). When the sodium benzoate concentration was increased to 0.51 g/l the cycle time initially increased sharply for one cycle but quickly re-established a steady-state after another two cycles at a cycle time of 47.0 min (±2 48 min). A further increase in sodium benzoate concentration to 0.67 g/l resulted in a similar initial long cycle, but the system quickly re-equilibrated at a steady-state cycle time of 46.5 min (±0.73). The observed steady-state minimum D.O. levels decreased from an average of 77.54% (±0.50%) , to 45.23% (±1.34%) and to 17.88% (±2.31%) of saturation respectively for the increasing sodium benzoate concentrations. The minimum D.O. is a good indication of biomass and the trend reflects an increase in steady-state biomass for higher sodium benzoate concentrations. Dry weight measurements of selected samples at the three

TABLE 4.2 SCF substrate consumption rates for *P. fluorescens* and *P. putida* grown on various aromatic compounds.

ORGANISM	P. fluorescens		P. putida		
SUBSTRATE	Sodium Benzoate	MBBA	Sodium Benzoate	p-Anis-aldehyde	MBBA
CONC. (g/l)	0.140	0.715	0.150	0.165	0.610
Substrate Consumption (%)[a]	93.5 (5.9)	100 (0)	93.7 (4.0)	99.0 (0.04)	100 (0)
Consumption Rate (kg/m3 day)	2.618	1.269	4.252	4.559	0.800
Specific Consumption Rate (kg/day per kg biomass)	14.71	5.566	21.47	24.25	3.864

[a]Standard deviations are shown in brackets under the average value and are expressed in the designated units

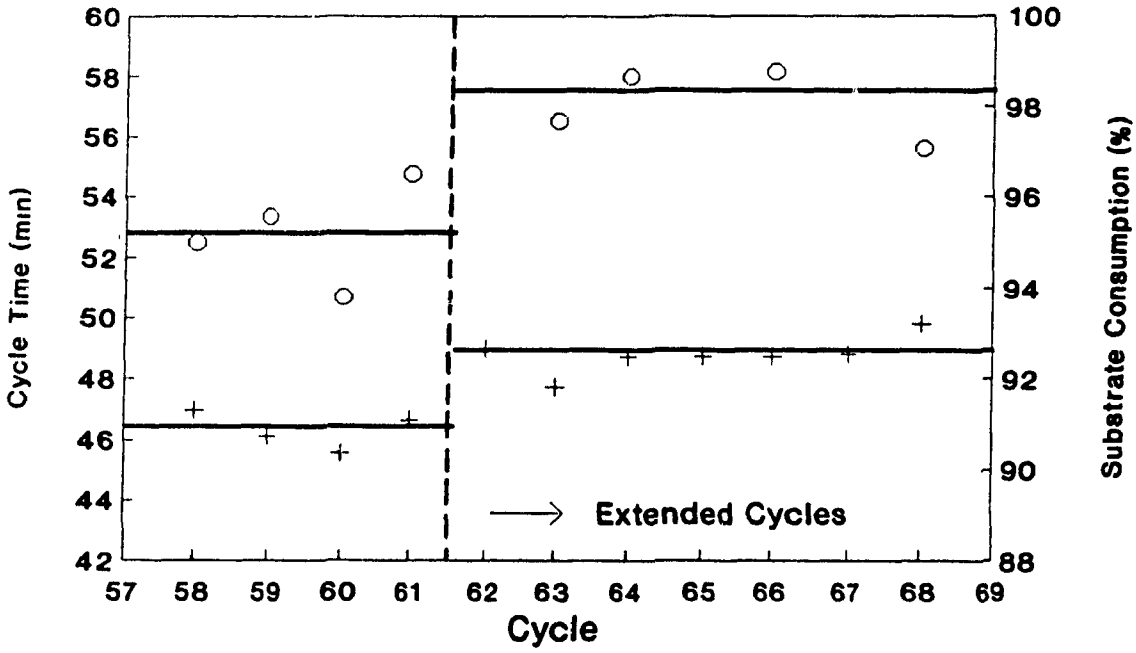

Figure 4.6 Effect of cycle extension on cycle time (+) and substrate consumption (○).

different sodium benzoate concentrations showed average steady-state biomass concentrations of 0.198 g/l (±0.015 g/l), 0.441 g/l (±0.017 g/l), and 0.513 (±0.036 g/l) respectively.

Observations made for *P. putida* grown on different concentrations of p-anisaldehyde were very different. Figure 4.8 shows the steady-state cycle time and minimum D.O. responses when the p-anisaldehyde concentration was increased from 0.165 g/l to 0.377 g/l to 0.473 g/l respectively. As in the sodium benzoate experiment, the observed steady-state minimum D.O. levels decreased for the higher p-anisaldehyde concentrations, indicating an increase in steady-steady biomass. The average steady-state biomass concentrations were 0.188 g/l (±0.020 g/l), 0.339 g/l (±0.024), and 0.445 g/l (±0.025 g/l) respectively for the increasing p-anisaldehyde concentrations. The steady-state cycle times established after a substrate concentration increase, however, were not constant for different substrate concentrations as was the case in the sodium benzoate SCFs. Steady-state cycle times were significantly higher for higher substrate concentrations. The average steady-state cycle time at 0.165 g/l, for example, was approximately 52 min and was increased to 70 min when the concentration was increased to 0.377 g/l. When the p-anisaldehyde concentration was further increased the new steady-state cycle time again increased to 99 min. The steady-state cycle time behaviour for *P. putida* on p-anisaldehyde was thus not independent of the substrate concentration in the concentration range investigated. Figure 4.9 is a plot of the cycle times measured at various concentrations of p-anisaldehyde and shows this very clearly.

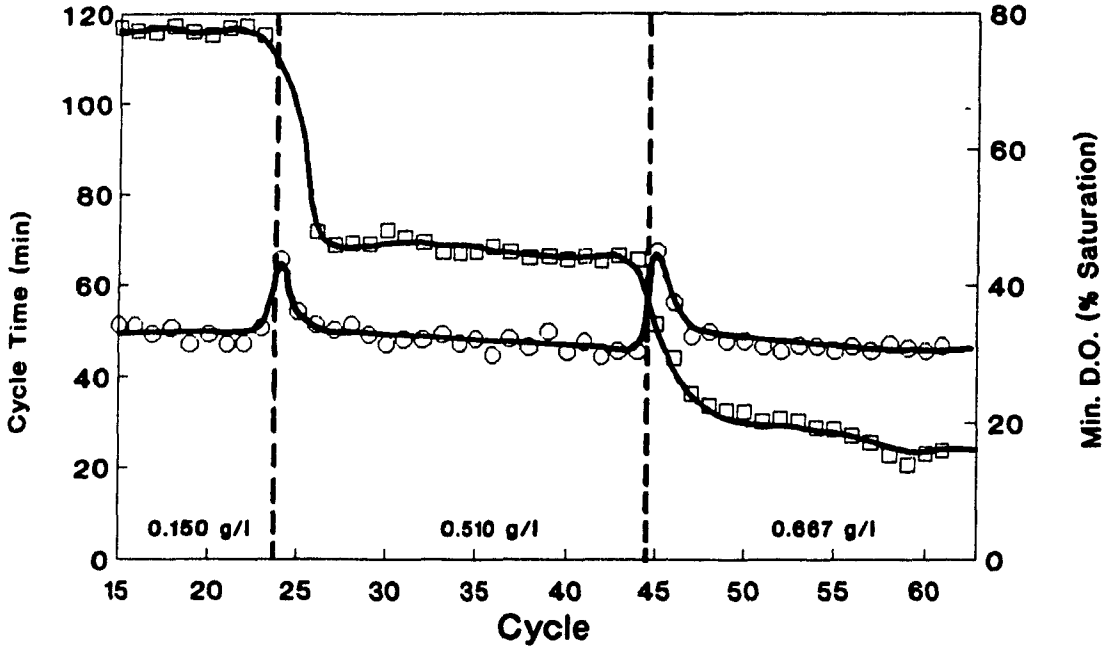

Figure 4.7 Cycle time (○) and minimum dissolved oxygen level (□) response for *P. putida* when sodium benzoate concentration at the beginning of each cycle was increased from 0.150 g/l to 0.510 g/l to 0.667 g/l.

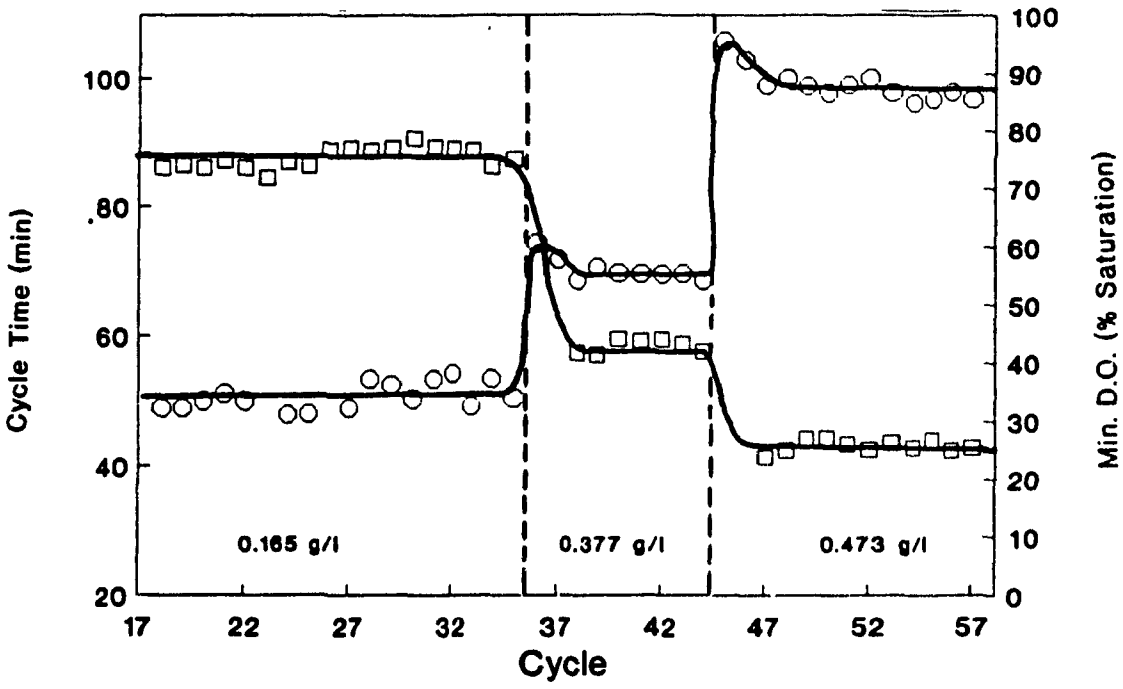

Figure 4.8 Cycle time (○) and minimum dissolved oxygen level (□) response for *P. putida* when p-anisaldehyde concentration at the beginning of each cycle was increased from 0.165 g/l to 0.377 g/l to 0.473 g/l.

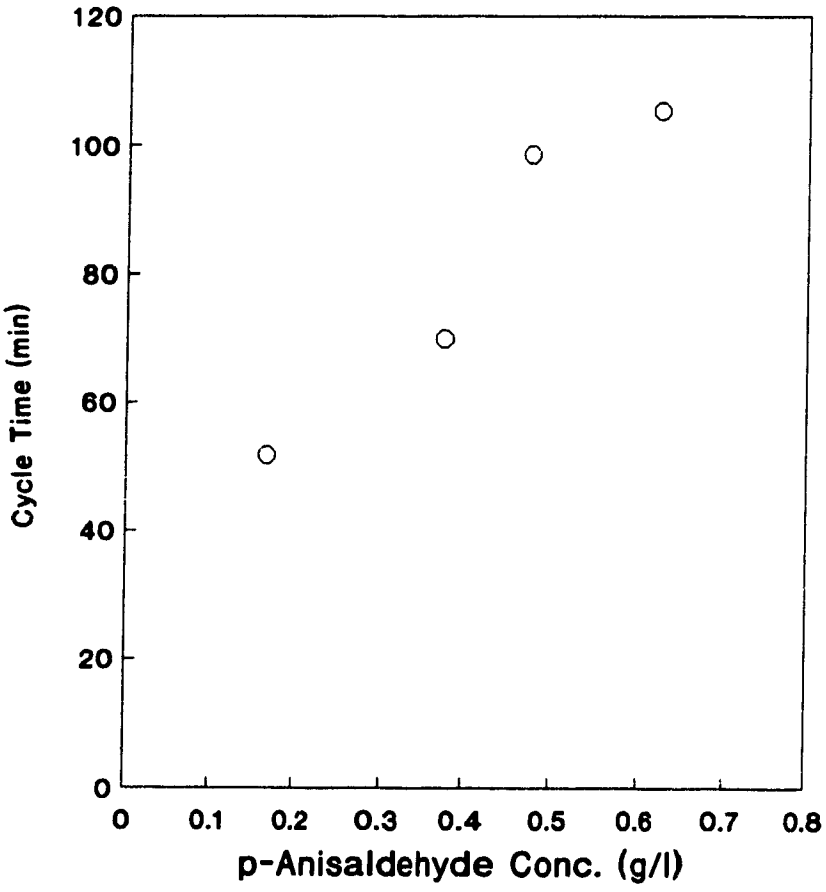

Figure 4.9 Observed steady-state cycle times of *P. putida* when grown on different concentrations of p-anisaldehyde.

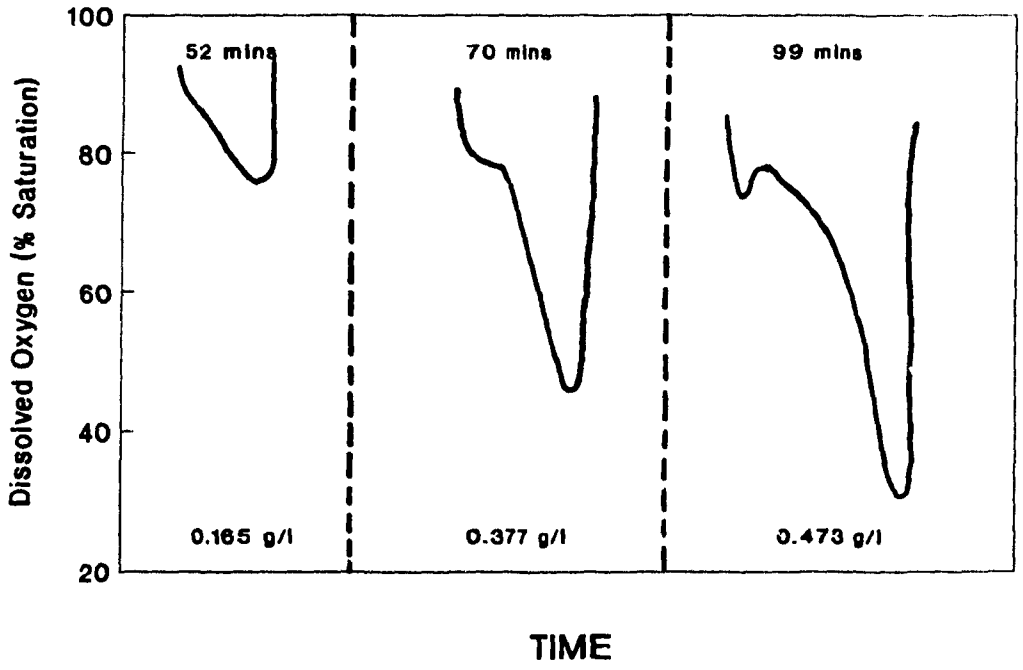

Figure 4.10 Steady-state dissolved oxygen profiles for *P. putida* when grown on 0.165 g/l, 0.377 g/l and 0.473 g/l of p-anisaldehyde.

Figure 4.10 shows the dissolved oxygen cycle profiles for *P. putida* on p-anisaldehyde concentrations of 0.165 g/l, 0.377 g/l and 0.473 g/l. The cycles were "deeper" for higher concentrations and this is expected since the increased biomass at higher concentration results in a higher oxygen consumption. However, the shape of the cycle was also significantly different from concentration to concentration. When the concentration was increased from 0.165 g/l to 0.377 g/l a secondary minimum in the dissolved oxygen level that is not present in the cycle at the lower concentration, appeared at beginning of the cycle. The secondary minimum became pronounced when the concentration was further increased to 0.473 g/l.

SCF experiments using p-anisaldehyde and a second strain of *P. putida* were also carried out. Figure 4.11 shows the cycle times and biomass concentrations observed at p-anisaldehyde concentrations of 0.272 g/l and 0.558 g/l. The steady-state cycle time at 0.272 g/l averaged 59.3 min ($\pm$2.53 min). Upon increasing the p-anisaldehyde concentrations to 0.558 g/l, the familiar increase in cycle time was observed, however, no immediate new steady-state was established. The observed cycle time continued to drop over the next 12 cycles and eventually seemed to level out at a cycle time of 64 minutes. The observed biomass concentration, by contrast, was quick to come to its new steady-state value increasing from 0.333 g/l ($\pm$0.009 g/l) to 0.446 g/l ($\pm$0.013 g/l) within 3 or 4 cycles after the increase in p-anisaldehyde concentration.

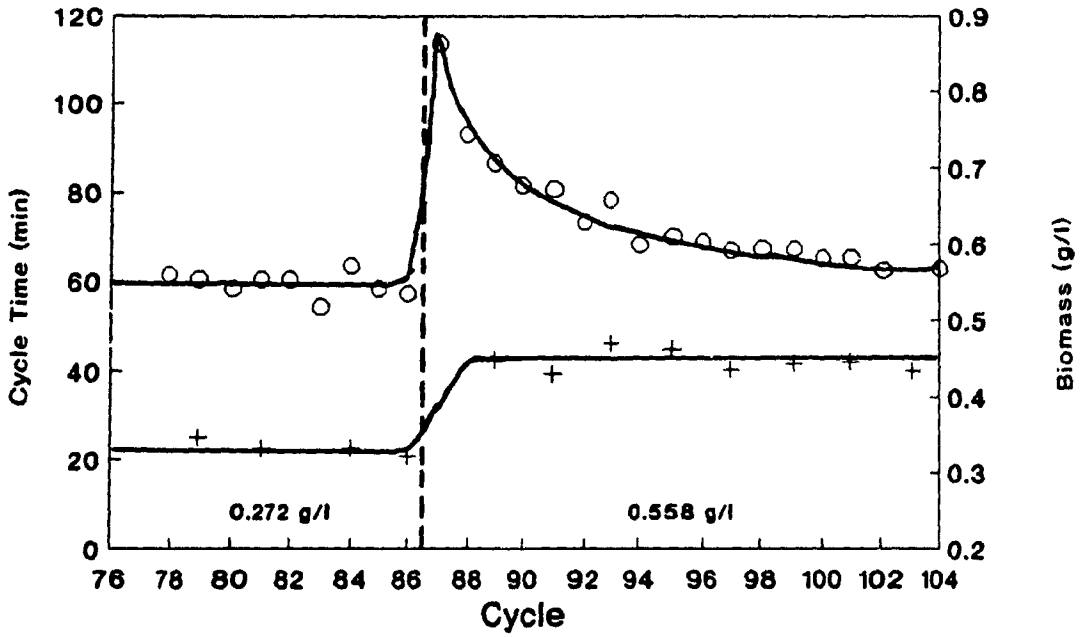

Figure 4.11 Cycle time (○) and biomass (+) response for *P. putida* Strain B when grown when p-anisaldehyde concentration at the beginning of each cycle was increased from 0.272 g/l to 0.558 g/l.

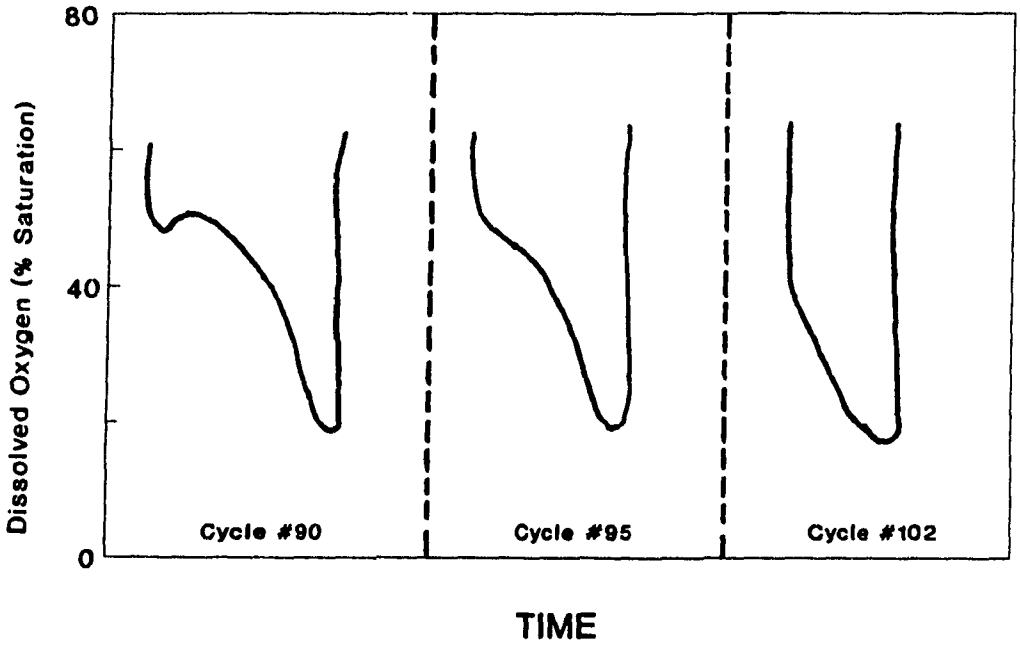

Figure 4.12 Dissolved oxygen profiles for various cycles of growth of *P. putida* Strain B after switching p-anisaldehyde substrate concentration from 0.272 g/l to 0.558 g/l.

Figure 4.12 shows the dissolved oxygen profile for cycle 90, 95, and 102. The figure shows that a significant change in the shape of the D.O. profiles occurred during the period of decreasing cycle time. In particular, the secondary minimum observed in early cycles became less prominent and finally disappeared completely by cycle 102.

4.5 Intra-Cycle Cell Counts

An intra-cycle cell count was performed on samples taken from one cycle of growth of *P. putida* Strain B on 0.272 g/l of p-anisaldehyde. The results are presented in Figure 4.13. The number of cell forming colonies (CFU) remained fairly constant from the beginning of the cycle until about three quarters of the cycle had elapsed. The CFU then rose sharply and doubled in value by the end of the cycle.

4.6 Mixed Culture Growth in SCFs

SCF experiments using several combinations of two bacterial species were carried out, but none of the SCFs were successful failing to produce repeatable results.

The D.O. trace from experiments involving *N. erythropolis* and *R. rhodochrous* became noisy very soon after start-up. The noise level continued to increase as these experiments progressed and a quickly dropping dissolved oxygen level was also observed. The dissolved oxygen level in experiments involving these organism dropped all the way to the zero level

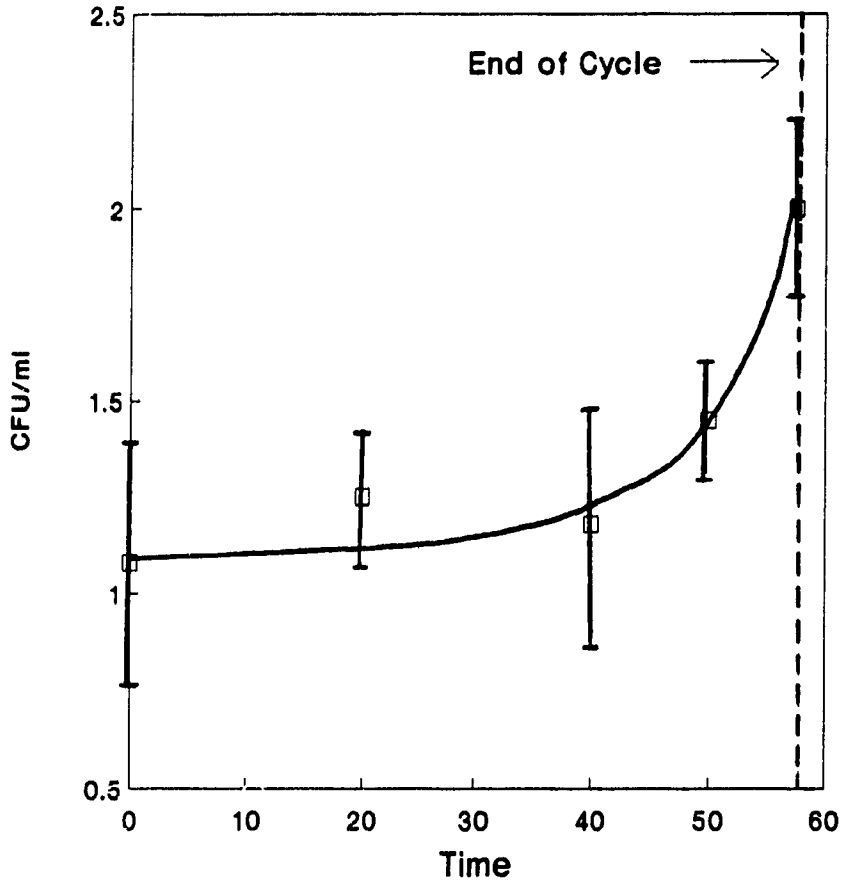

Figure 4.13 Intra-cycle cell count preformed on samples taken from one cycle of growth of *P. putida* Strain B on 0.272 g/l of p-anisaldehyde. Error bars represent three standard deviations.

within several hours after start-up. Cycling the SCF and increasing the air flow rate both failed to change the zero dissolved oxygen level reading. Significant growth was noted on the probe tip and the fermenter walls.

Experiments involving other organisms all failed to produce a steady-state operation. The observed ratio of species as determined by cell counts contained a large degree of error. The standard deviation of error between replicate ratio measurements was often greater than 25%. Observations regarding the makeup of the mixed culture in these experiments were thus inconclusive.

5. DISCUSSION

5.1 Reproducibility and Stability of SCFs using Aromatic Hydrocarbons

SCF operation was possible with all three aromatic substrates used in this study (Figures. 4.2-4.4, Table 4.1). Many successive, repeatable cycles were obtained for all of these systems. The deviation of steady-state cycle time from the average value was less than 5% (Table 4.1). These observations are comparable to results reported by Brown and Cooper (6-8) for SCFs with simple hydrocarbons, where the deviation of average steady-state cycle times for several different simple hydrocarbon SCFs was less than 3%. Other measured parameters were also constant from cycle to cycle with deviations from the steady-state average values of less than 5%.

The stability of the SCFs is reflected in the system response to upsets or to deliberate changes made in substrate concentration or the type of substrate. The results presented for the growth of *P. fluorescens* on different concentrations of sodium benzoate (Figure 4.8) illustrate this point. When the sodium benzoate concentration was increased, the cycle time initially became longer but the system quickly re-equilibrated to the original steady-state cycle time within 3 cycles. The higher substrate concentration supports a higher steady-state biomass concentration which builds up during these initial long cycles. The sodium benzoate SCFs initiated by changing the substrate from p-anisaldehyde or MBBA to sodium benzoate also

adjusted to the new substrate after only 3 cycles. Similar observations were made when system upsets due to mechanical malfunctions (such as improper harvesting due to valve failures) were encountered during the course of this study. The system re-equilibrated to steady-state within two cycles after the malfunction was corrected in all such cases.

The initiation of SCFs was generally more difficult for aromatic compounds than start-ups described in earlier SCF studies using simpler hydrocarbons (6-8). Acclimation of the organism to the substrate prior to inoculation seems to be the most important factor necessary for initiating SCFs with aromatic substrates. Brown and Cooper (7,8) used *A. calcoaceticus* inocula from nutrient broth directly to start-up hexadecane self-cycling fermentations. They found that the organism was able to successfully acclimate to the new substrate in the first cycle. This was also true for the test organisms used in this in this study with sodium benzoate as substrate. However, acclimation to the p-anisaldehyde and MBBA substrates directly in the SCF was unsuccessful and had to be done in shake flasks prior to inoculation as described above. These observations can perhaps be explained by the high shear conditions within the pump in the recirculating column reactor of the SCF. The high shear conditions can cause the cells in the inoculum to lyse, and because the cells are slow to acclimate to these difficult substrates, most will die before steady growth can begin. In the case of easier substrates such as sodium benzoate the organisms acclimate quickly to the substrate and enough cells build up to overcome the losses due to the high shear rate.

5.2 Cycle Time and Substrate Concentration Effects in SCFs

5.2.1 The Significance of Cycle Time

In a self-cycling fermentation, half of the fermenter broth is harvested at the beginning of each cycle and the end-of-cycle biomass remains constant from cycle to cycle. It thus follows from a simple mass balance that the test organism must be doubling once in each cycle and the cycle time in SCFs is equivalent to the doubling time of the test organism. This argument is further strengthened by intra-cycle cell count observations made during a single cycle of growth in an SCF. Figure 4.13 shows the results of such an intra-cycle study performed on samples from a cycle of growth of *P. putida* on p-anisaldehyde. The cell concentration was initially 1.08 x 10^7 CFU/ml and remained constant until about 75% of the cycle had elapsed and then began to rise sharply reaching a final value of 2 x 10^7 CFU/ml, or approximately double the initial cell concentration, by the end of the cycle. Therefore, the cycle time can be equated to the doubling time of the test organism.

It is important to note that the doubling of cells during the cycle of growth studied did not occur gradually during the cycle but was limited to a very short time period at the end of the cycle. This indicated that the culture growth was synchronous. The cell synchrony index, F, for the growth of *P. putida* on p-anisaldehyde can be determined from the intra-cycle cell count data (Figure. 4.13) according to Blumenthal and Zahler's (4) equation:

$$F = N/N_o - 2^{t/g}$$

N and N_o are the final and initial cell concentrations. The ratio t/g is the ratio of observed doubling time to the total cycle time and is 0.25 from the figure. The calculated F value is 0.66. An F value greater than 0.5 generally reflects a high degree of synchrony (4).

Brown and Cooper (6,8) proved that the cycle time was equivalent to doubling time for *A. calcoaceticus* grown on ethanol in an SCF. They also suggested that this equivalence between cycle time and doubling time is a property of the SCF technique and the synchronous nature of SCF cultures and that this equivalence will hold true for other organisms and substrates. The observations presented here for *P. putida* grown on p-anisaldehyde lend further support to this conclusion. The doubling time of an organism is an indication of the growth rate of the organism under the specific fermentation conditions and has been shown to be independent of substrate concentration for synchronous cultures(4). It is thus valid to make comparisons of growth rates of different organisms by comparing their cycle times in SCF runs of identical conditions. The cycle time of an organism in an SCF is an easily determined parameter (recorded on-line by the control computer) and does not involve the tedious biomass measurements at different fermentation times that are necessary for growth rate determination in traditional batch fermenters.

5.2.2 Effect of Substrate Concentration on Cycle Time in SCFs

Since the doubling time of an organism is independent of substrate concentration for synchronous cultures increasing the substrate concentration in a SCF is expected to merely

increase the steady-state biomass level while the steady-state cycle time remains constant.

This effect was clearly seen in the results presented for *P. putida* grown on different sodium benzoate concentrations (Figure 4.7). The observed steady-state cycle time remained constant for the different sodium benzoate concentrations. The biomass concentration also behaved as expected, increasing stepwise with each increase in sodium benzoate concentration.

The results presented for *P. putida* grown on different concentrations of p-anisaldehyde, however, were very different. For these experiments the cycle time increased considerably for each increase in substrate concentration (Figures 4.8 and 4.9). A possible explanation for this behaviour can be inferred from the dissolved oxygen profiles observed at the different concentrations of p-anisaldehyde (Figure 4.10). A secondary minimum appeared in the dissolved oxygen profiles during each cycle, when the concentration was increased. This phenomenon was not observed for the sodium benzoate experiments. Brown and Cooper (6) observed a similar secondary minimum when *A. calcoaceticus* was grown on ethanol in a SCF. They showed that the secondary minimum was the result of an accumulation of an inhibitory breakdown product of ethanol. The accumulation of such an inhibitory intermediate caused a decrease in overall growth rate and thus a decrease in respiration rate which was reflected in the decreased downward slope of the dissolved oxygen curve. As the organism began to assimilate the inhibitory intermediate the growth rate started to increase again and the original downward slope of the dissolved oxygen profile is once again observed.

The presence of an unidentified inhibitory intermediate in the experiments with

p-anisaldehyde as substrate would thus explain the appearance of a secondary minimum in the D.O. profiles from these fermentations. Furthermore, it is likely that an increase in initial substrate concentration in the fermenter also leads to an increased concentration of the inhibitory intermediate and thus the secondary minimum was more pronounced for the higher p-anisaldehyde concentrations. The presence of higher concentrations of the inhibitory intermediate as a result of increased p-anisaldehyde concentrations also explained the longer cycle times observed when the substrate concentration was increased.

The results from SCF experiments involving *P. putida* Strain B (Figure 4.11) grown on two different concentrations of p-anisaldehyde were different yet again. An initial cycle time increase was observed when the p-anisaldehyde concentration was increased from 0.272 g/l to 0.558 g/l in these experiments. However, the cycle time did not become steady and gradually grew shorter from cycle to cycle although the p-anisaldehyde concentration at the beginning of each cycle was kept constant at 0.558 g/l (Figure 4.11). A secondary minimum was observed in D.O. profiles for cycles at the beginning of this period and became less pronounced as the cycle time continued to fall (Figure 4.12). This observation, coupled with the fact that the biomass concentration was steady in this period suggests that the gradually decreasing cycle time may be due not to system instability, but to culture acclimation. The *P. putida* Strain B had developed the ability to overcome the negative effect of the inhibitory intermediate from cycle to cycle. The observed secondary minimum in D.O. profile thus slowly became less pronounced and finally disappeared by the time the cycle time had resumed to 64 minutes. This

period was almost identical to the cycle time observed for the stable system before the concentration of the substrate was increased. The strain of *P. putida* used in the earlier experiment did not have this ability to overcome the inhibitory effect of p-anisaldehyde resulting in the dramatically different behaviour.

5.3 Rates of Metabolism of Aromatic Substrates in SCFs

5.3.1 Aromatic Substrate Consumption

Various differences in the abilities of *P. putida* and *P. fluorescens* to degrade the different aromatic test compounds are apparent from the percentage substrate consumption and consumption rate data.

The percentage consumption of sodium benzoate by *P. putida* and *P. fluorescens* for comparable benzoate concentrations was essentially the same (Table 4.2). However, the cycle time required to achieve this consumption was significantly lower for *P. putida*. The consumption of oxygen by *P. putida* was also lower. The steady-state minimum D.O. level was 80.35 % of saturation ($\pm$0.78%) or a factor of 3.11 higher than the level in the *P. fluorescens* fermentation. The steady-state biomass for the *P. putida* fermentation was 0.198 g/l ($\pm$0.015 g/l). Thus, while less oxygen was consumed in the *P. putida* fermentation, more biomass was generated in comparison to the *P. fluorescens* fermentation.

The consumption of p-anisaldehyde by *P. putida* was almost complete and considerably

higher than the sodium benzoate consumption reported for *P. putida* (Table 4.2). However, the cycle time required to achieve this consumption was also higher and thus the consumption rate is not much greater than that for sodium benzoate (Table 4.2).

MBBA consumption by both *P. putida* and *P. fluorescens* was complete. This does not indicate, however, that these organisms degrade the MBBA substrate readily. This is reflected in the consumption and specific consumption rates; the cycle time required for the consumption of the MBBA substrate was an order of magnitude longer than for SCFs using these organisms with other substrates (Table 4.2). The calculated consumption and specific consumption rates are thus both significantly lower for the MBBA cases.

Figure 4.6 shows that the percentage substrate consumption in SCFs can be increased by cycle extension. The "extension" of a cycle by a short period of time past its minimum D.O. level to achieve higher consumptions can be achieved in one of two ways: imposition of a specified time delay after a minimum is recognised before cycling, or decreasing the minimum detection sensitivity of the cycling algorithm so that cycling is imposed only after the D.O level is well past its minimum and on its way up. The latter method was selected to obtain the data presented in this figure.

The cycling of the SCF was also delayed past the minimun D.O. level in the MBBA experiments (Figure 4.5a). While cycle extension was necessary in this case to achieve repeatable cycling, the resulting complete substrate consumption (Table 4.2) further justifies the use of cycle extension as a method of increasing substrate consumption.

It is important to note, however, that while percentage amount of substrate consumed may be improved by cycle extension, the overall consumption rate may drop. The consumption rate in standard cycles in Figure 4 6 is 19.70 kg/ m^3 day while the consumption rate in the extended cycles is 19 33 kg/ m^3 day. The decision to use or not to use extended cycles may thus depend on the relative importance of high consumption rate versus the need to meet a certain end-of-cycle substrate concentration criterion such as those set by environmental regulations.

5.3.2 Comparison of Aromatic Substrate Consumption Rates in SCFs and other Fermenters

Table 5.1 compares percentage substrate consumption and consumption rate data from the SCF sodium benzoate experiments to two recent studies of benzoate degradation in the literature In their study, Matsui et al. (30) report on the removal rates of several waste substrates by activated sludge taken from an existing industrial wastewater treatment plant. The Kobayashi et al. (27) study reports on the development of an anaerobic digester for the degradation of benzoates. Both studies report above average benzoate consumptions compared to other data in the literature.

The percentage consumption of the sodium benzoate consumed in the SCF is slightly higher than that in aerobic fermenter used by Matsui et al. for similar benzoate concentrations. However, the 24-hour time period required to achieve this consumption in the fermenter used by Matsui et al. is considerably higher than the 47.6 min or 72.0 min period for *P. putida* and

TABLE 5.1 Comparison of Sodium Benzoate Consumption in Various Fermenters

Reference			Matsui 1989	Kobaya-shi 1988
Fermenter Type	SCF	SCF	Sequential Batch Acticated Sludge AEROBIC	Anaerobic Digester ANAEROBIC
Culture	P.putida	P.fluor.	Mixed	Mixed
Fermtr. Vol.(l)	1.0	1.0	2.0	3.0
Conc. (g/l)	0.150	0.140	0.150	3.000
% Consumption	93.7	93.5	86	100
Time required	47.6 min	72.0 min	24 hrs	5-7 days
Consumption Rate (kg/m_3 day)	4.252	2.618	0.129	0.300

P. fluorescens cultures in the SCF. The difference in consumption rates is thus more significant. The consumption rates of the benzoate in the two SCF experiments are 4.252 and 2 618 kg/m^3 day - more than ten times higher than the reported consumption rate of 0.129 kg/m^3 day in the fermenter used by Matsui et al.

The anaerobic digester used by Kobayashi et al. is capable of handling much higher concentrations of benzoate than the self-cycling fermenter. They report operating at a benzoate concentration of 3000 mg/l and achieve complete utilisation at this concentration. However, the time required for the anaerobic degradation of the benzoate is again considerably higher than degradation times in the SCF (degradation times are in the order of days for the anaerobic digester, while SCF degradation times are in the order of minutes). The consumption rate is therefore low at 0.3 kg/m^3 day. The SCF consumption rates represent a tenfold improvement over the anaerobic digester.

5.4 Development of an Inhibition Model in SCFs

5.4.1 Definition of model parameters and equations

A simple model can be developed to describe the behaviour of cycle time as well as substrate consumption rates for different substrate concentrations.

We have already seen that for simple substrates the steady-state cycle time is constant for different substrate concentrations within the limiting range while the steady-state biomass increases for higher substrate concentrations. Thus, if the substrate concentration in SCFs is doubled, the fermenter builds up a biomass level sufficient to consume the increased concentration in the same cycle time. The consumption rate, defined as the mass of substrate consumed per unit time per unit volume, has effectively doubled. The dependence of consumption rate on substrate concentration is, therefore, linear or first order and can be expressed as follows:

$$r = dN_{So}/dt \times 1/V = dC_{So}/dt = KC_{So} \qquad [1]$$

where N_{So} is the mass (or number of moles) of substrate added in each cycle, C_{So} is the substrate concentration, V is the fermenter volume and K is the consumption rate constant. Note that N_{So} and C_{So} are steady-state, start-of-cycle parameters. The actual amount of substrate in the fermenter is transient within any particular cycle and continually decreases as the substrate is consumed. N_{So} and C_{So} refer to the initial amount of substrate added at the beginning of each cycle. Similarly, the actual consumption rate within a cycle changes, **r** merely describes the average for the whole cycle.

It is likely that the biomass increases associated with increasing substrate concentrations are also linearly related to substrate concentration through a substrate yield. Thus, we may write:

$$X = Y_{X/S}C_{So} \qquad [2]$$

where X is the steady state cycle biomass concentration measured at the end of the cycle and $Y_{X/S}$ is the biomass substrate yield. It follows from this equation that the specific consumption rate **r'**, defined as the consumption rate per biomass, is constant for different cycle substrate concentrations. In other words, although the observed overall consumption rate increases for increasing substrate concentrations, the mass of substrate consumed by a single cell remains constant. The specific consumption rate can be expressed as follows:

$$r' = r / XV = K' \qquad [3]$$

where K' is the is the specific consumption rate constant. The relationship between K' and K can be obtained by combining equations [1], [2] and [3]:

$$K' = K / Y_{X/S}V \qquad [4]$$

Figure 5.1 shows the of dependence of consumption rate on the cycle substrate concentration in comparison to that of specific consumption rate.

The effect of an inhibitory substrate or intermediate can also be incorporated in the model. If we assume that an increase in substrate concentration also leads to an increase in the breakdown intermediate concentration the effect can be expressed in terms of the cycle substrate concentration for both the substrate and intermediate inhibition cases. In the presence of an inhibitor we expect a slowdown in metabolic activities and thus the amount of substrate that each cell consumes is reduced. An increase in the concentration of the inhibitory agent causes more of a slowdown (Figure 5.1). If we further assume the slowdown effect is a simple first order one, we can write:

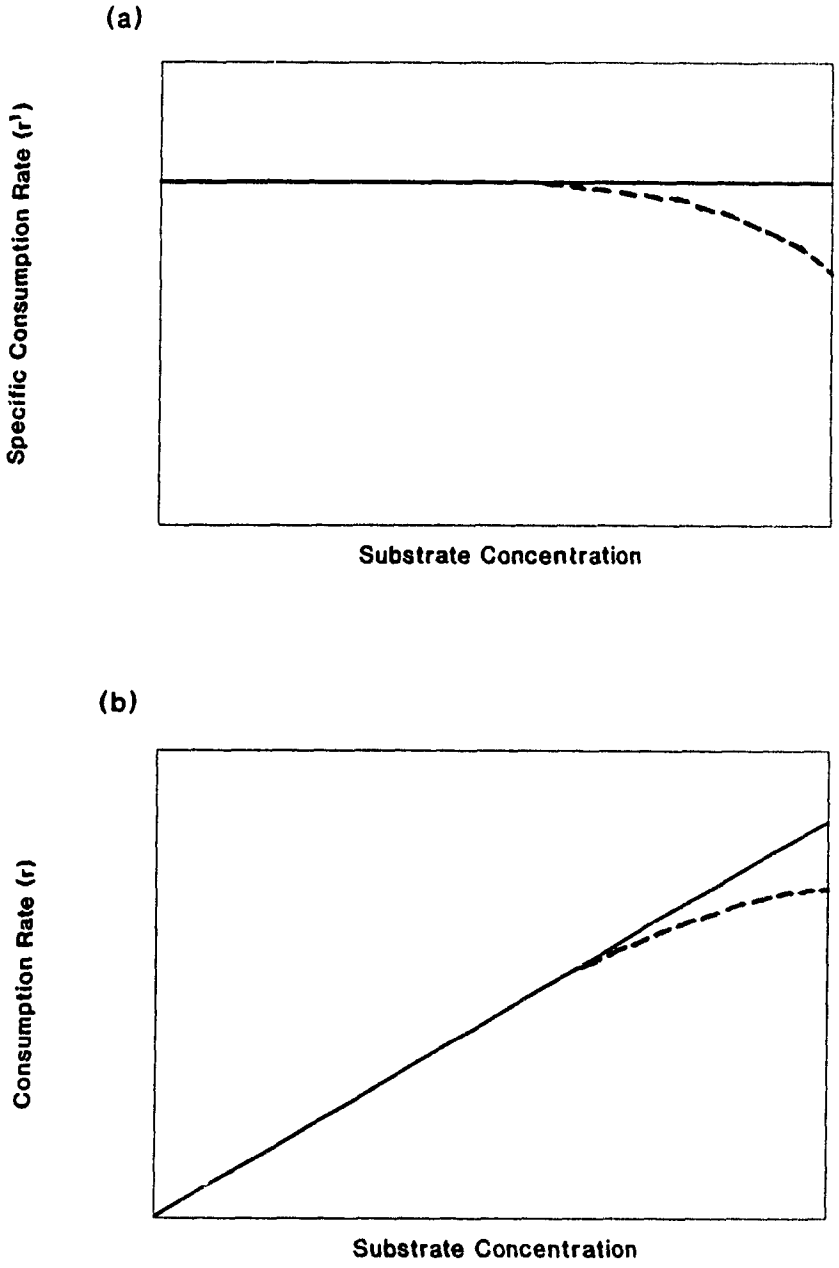

Figure 5.1 Dependence of (a) specific consumption rate and (b) consumption rate the cycle substrate concentration. Broken line shows the expected behaviour for inhibitory substrates.

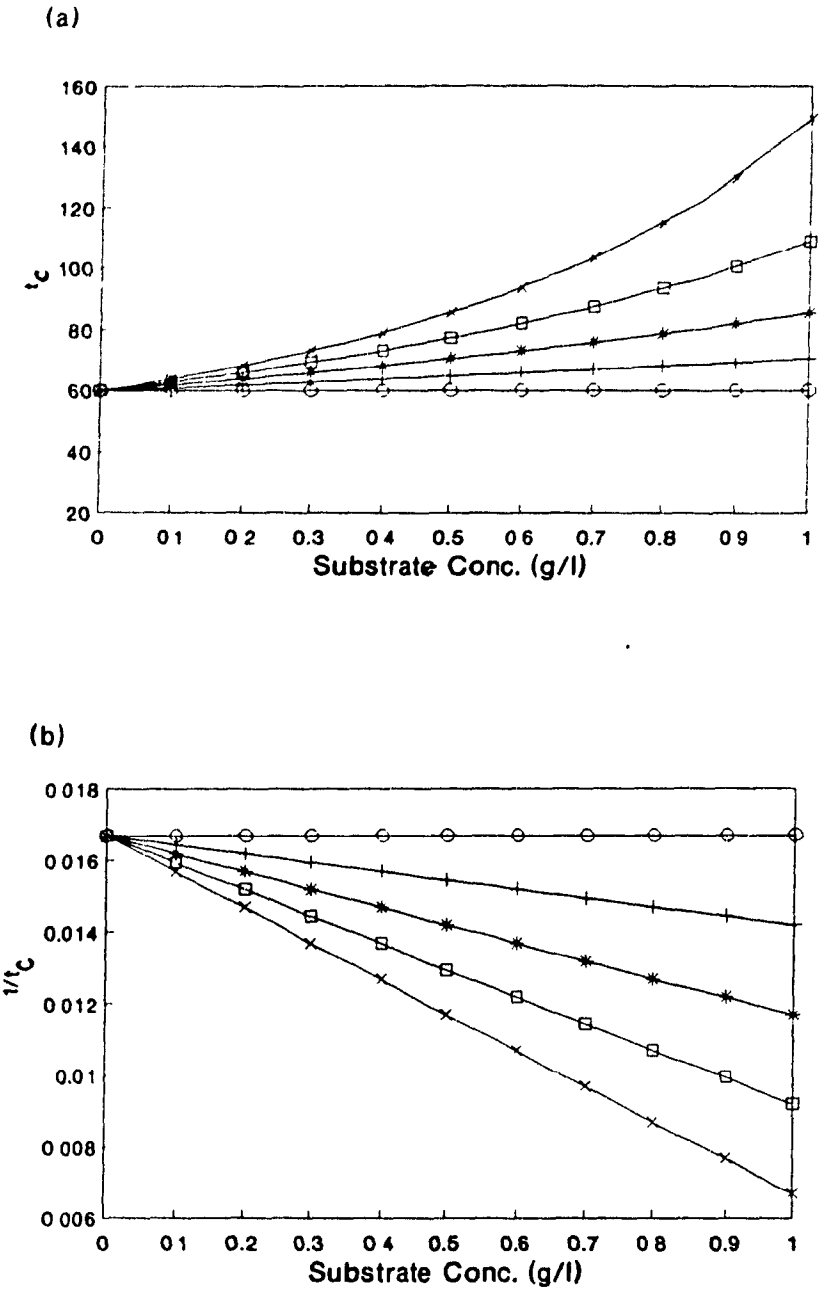

Figure 5.2 Behaviour of (a) cycle time and (b) inverse cycle time for various substrate concentrations according to proposed model. Curves shown for Ki=0 (○), Ki=0.0025 (+), Ki=0.005 (*), Ki=0.0075 (□) and Ki=.01 (×) with K fixed at 0.0167.

$$r' = K' - K_i'C_{So} \qquad [5]$$

where K_i' is the specific inhibition constant From the relationship between r and r' it can be shown that,

$$r = KC_{So} - K_iC_{So}^2 \qquad [6]$$

where Ki is the inhibition constant and is related to Ki' by the following

$$K_i = K_i'V \qquad [7]$$

An expression for cycle time, t_c, can be derived if the assumption of complete substrate consumption in each cycle is made. The cycle time is then merely the cycle substrate concentration divided by the average consumption rate. Thus,

$$t_c = C_{So} / r = 1 / K - K_iC_{So} \qquad [8]$$

Inverting equation [8] we get,

$$1 / t_c = K - K_iC_{So} \qquad [9]$$

Thus, K_i and K can be determined from the slope and intercept of a plot of the inverse of cycle time versus cycle substrate concentration. Figure 5.2a illustrates the behaviour of cycle time versus cycle substrate concentration for different values of K_i with K=0.0167. When K_i = 0 the substrate is not inhibitory and the steady-state cycle time is unaffected by the cycle substrate concentration. For $K_i > 0$ an increase in substrate concentration causes an increase in steady-state cycle time. Higher values of K_i indicate a higher degree of inhibition and thus the increase in cycle time is more pronounced for increased substrate concentrations. Figure 5.2b shows the inverse cycle time versus concentration plots for these curves.

5.4.2 Application of Inhibition Model

Figure 5.3 shows a plot of inverse cycle time for P. putida grown on four different concentrations of p-anisaldehyde. K_1 and K as determined by a regression fit were calculated to be 0 02276 $g^{-1}s^{-1}$ and 0.02267 s^{-1} respectively. The regression has an R^2 value of 0.93.

A plot of cycle time versus substrate concentration is given in Figure 5.4; the curve illustrated was calculated from equation [8] using the constant values above.

The simple inhibition model presented thus gives a reasonable prediction of cycle time behaviour with p-anisaldehyde substrate concentration for *P. putida* (Figure 4.13). Although the model is an obvious oversimplification of complex biochemical interrelationships some physical significance may be attached to both the consumption rate coefficient and the inhibition coefficient The consumption rate constant is mathematically the inverse cycle time at zero substrate concentration. Alternatively this limiting case can be thought of as an indicator of cycle time at a substrate concentration so dilute that it is no longer inhibitory. It is independent of substrate concentration and thus can be used in comparing run data for inhibitory substrates regardless of substrate concentrations used. The inhibition coefficient, K_1, is an indicator of the substrate toxicity. The relative increase in cycle time for a unit increase of substrate concentration is higher for a higher K_1 value. Larger K_1 values thus indicate higher substrate toxicities and vice versa.

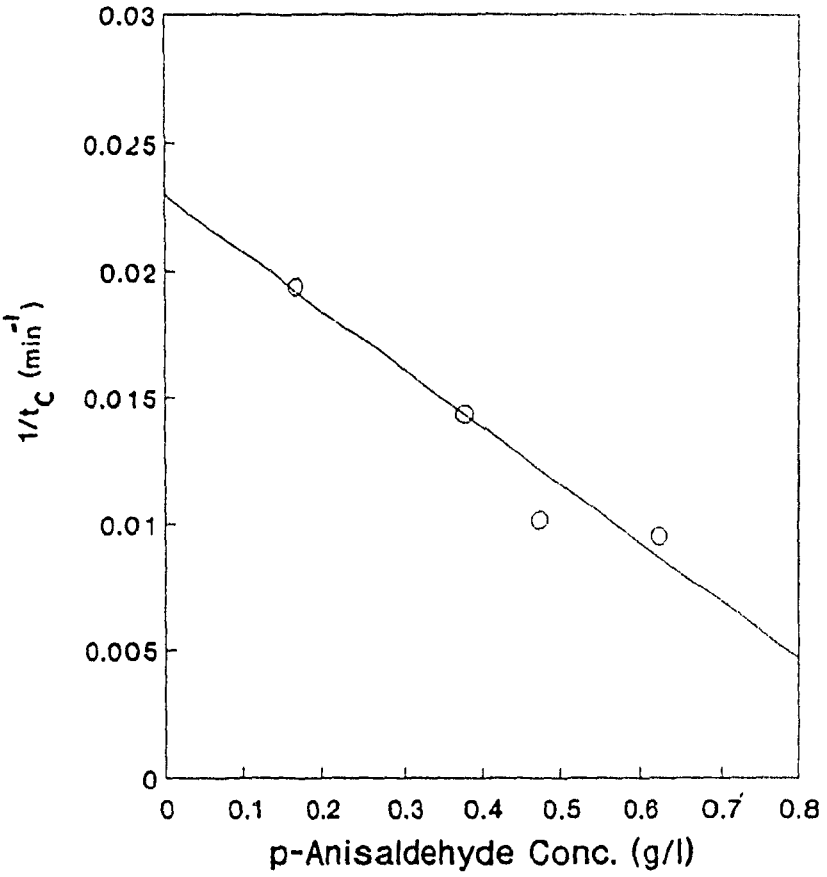

Figure 5.3 Inverse cycle time plot for *P.putida* grown on various p-anisaldehyde concentrations. Regression line is shown.

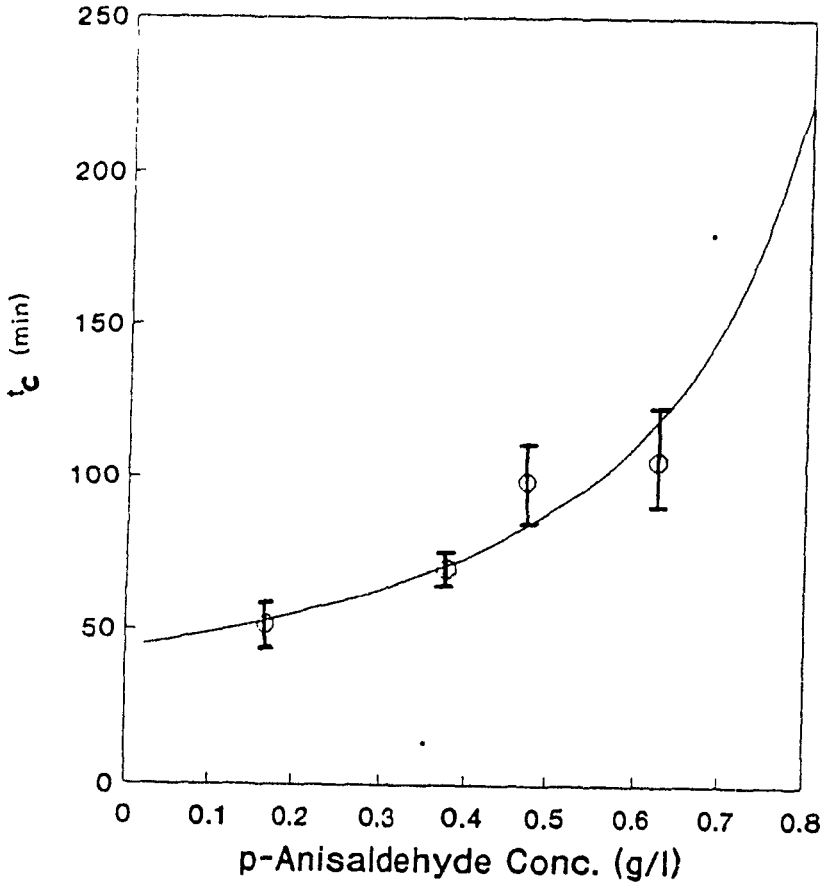

Figure 5.4 Predicted substrate concentration cycle time dependence curve for *P. putida* grown on p-anisaldehyde. Experimental points also shown. Error bars represent three standard deviations.

5.5 Mixed Culture Growth in SCFs

The large error in the measurement of species ratios in the mixed culture experiments were the main reason that data collected from these experiments was not repeatable or conclusive. The large error was due to limitations specific to the cell count procedure. Cell counts are tedious and notoriously inaccurate and this was certainly true in the case of the mixed culture counts preformed in these experiments. Furthermore, the inaccuracies of the cell count procedure were compounded by errors due to difficulties in differentiating the two cultures. Although, the cultures were chosen so that their colonies in agar plates were as different in appearance as possible, there were always some colonies on each plate that were difficult to differentiate. The use of pigmented organisms in mixed cultures has had some success (26,44), but, clearly, the availability of such organisms that are capable of degrading hydrocarbons is limited. If useful information is to be collected from mixed culture studies in SCFs, a better technique for determining the makeup of the mixed population is required. Davison and Stephanopoulos (11,12) used organisms of different cell size in their mixed culture studies and determined the makeup of mixed populations by measuring the cell size distribution of a sample of the population. Measurements of the mixed population's makeup were more accurate than cell count techniques using this method. The procedure also has the advantage of being faster than cell counts. However, the procedure is again limited to only certain organism combinations such that there is a sufficient difference in the cell size distributions of the mixed culture species.

The observations made in experiments involving *N. erythropolis* and *R. rhodochrous* reflect the fact the not all organisms may be suited to growth in the present SCF setup. Both organisms are filamentous in morphology and are likely to attach to surfaces during growth. The attached growth of these organisms on the surface of the dissolved oxygen probe head accounts for the zero dissolved oxygen level in experiments involving these organisms. It was thus impossible to correctly monitor the actual oxygen level in the fermenter broth and successful cycling was infeasible as a result. An alternative means of monitoring oxygen consumption in SCFs using an on-line gas analyzer is currently being studied (42). The analyser monitors the oxygen and carbon dioxide levels in the off-gases from the fermenter rather than the broth itself. This technique may be useful in overcoming the problem of improper D.O. readings due to the organism attaching itself to the tip of the D.O. probe. However, another problem may persist with filamentous organisms even with an improved oxygen monitoring system. The tendency of such organisms to also attach themselves to the fermenter walls means that most of the biomass will remain behind when harvesting the fermenter. The removal of half of the fermenter biomass at the end of each cycle - a basic requirement of stable SCF operation - may thus be difficult to achieve and unstable SCF operation may result.

6. CONCLUSIONS

Several important conclusions can be drawn from the study that has been undertaken in this report:

1. The biodegradation of aromatic compounds is possible using the SCF technique.

2. Stable steady-state SCF operation is possible using aromatic compounds over extended periods of time and many cycles. The SCF data obtained from aromatic substrate fermentations are reproducible.

3. SCFs provides a viable means by which to achieve almost complete consumption of various aromatic substrates.

4. The consumption rate of sodium benzoate in SCFs is considerably higher that reported for other fermenters.

5. Cycle time, which has been shown in this work, as well as in previous studies (6,8,34,36), to be equivalent to cell doubling time, is a useful indication of growth rate. However,

the cycle time is dependent on substrate concentration for inhibitory compounds. Comparisons of growth rates using cycle time must thus be made carefully for these compounds.

6. The SCF technique can be used to adapt some microorganisms to a difficult-to-degrade substrate with inhibitory intermediates.

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