GENETIC STABILITY OF PLASMIDS pRO101 AND pRO103 IN ALCALIGENES EUTROPHUS AEO106 GROWN IN EXPANDED BED BIOFILM REACTORS

By

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CHAPTER I

INTRODUCTION

Research Overview

The focus of this thesis involves the development of bench scale expanded bed biofilm reactor systems designed to biologically remove 2,4-dichlorophenoxyacetic acid (TFD) from a feed stream. The development of the reactor system has been a collaborative venture between the Department of Civil Engineering and the Department of Microbiology at Oklahoma State University. The reactor system has been used to meet both applied research goals concerning waste treatment reactor design as well as more fundamental objectives concerning the genetic stability of a pure culture used as the inoculant. The investigation of the degradative capacity and system design has been the research objectives of the project collaborators, and their findings have been reported elsewhere (Yang 1990). This thesis addresses the genetic stability of the reactor inoculant.

Biofilm reactors utilize a support material on which bacteria attach and form a film of bacterial growth called biofilm. The biofilm covered support material provides a method for maintaining a high concentration of bacteria within the reactor, resulting in a very efficient and useful

waste treatment system. The reactors in this study utilize monoculture biofilms developed by inoculating with well characterized pure cultures.

The pure culture inoculum used in this study carries genes which encode a catabolic pathway for the degradation of the herbicide 2,4-dichlorophenoxyacetic acid (TFD). The genes encoding the degradation pathway are plasmid-borne and are induced in the presence of TFD. Bacterial strains displaying inducible and constitutive expression of the TFD degradative pathway were compared in the biofilm reactors with regards to degradation efficiency and plasmid stability.

Methodology was developed for maintaining a monoculture system and for monitoring the stability of the cultures within the system. The feasibility of a monoculture reactor system and the culture and genetic stability of the bacterial inoculant were studied during long-term operation under various growth conditions, in order to answer fundamental questions regarding what factors influence the culture and genetic stability.

Parallel experiments were carried out on pure cultures of bacteria grown in chemostats, comparing the genetic stability of both the inducible and constitutive strains under various growth conditions.

Environmental Pollutants

Toxicity and Persistence

During the last few decades, there has been a dramatic increase in the production and use of a vast number of synthetic chemicals (Chakrabarty 1980). The numerous uses and the many benefits that these synthetic compounds provide have made them an essential part of our modern society. Yet the benefits provided to society are not without cost. Large quantities of halogenated compounds used as refrigerants, solvents, flame retardants, insecticides, and herbicides have been released into the environment, polluting the soil and water and causing grave health problems. Many of these compounds are chlorinated hydrocarbons, which are often very toxic and resistant to natural biological degradation and nutrient cycling processes (Atlas and Bartha 1987). Their recalcitrance has resulted in the accumulation of pollutants to levels that pose serious health concerns to a growing society (Alexander 1981). Regulating usage, developing methods for treating industrial effluents, and developing remediation techniques for contaminated sites are all essential steps that must be addressed by government regulators, engineers, and scientists, in order to curb increasing pollution problems.

The persistence of synthetic compounds in soil and aquatic ecosystems is dependent upon the rate at which they

can be degraded by biotic or abiotic activities. Abiotic processes contribute to degradation, but biotic processes play the major role in complete mineralization (USEPA 1988). Understanding the biotic degradation processes is therefore essential so that methods can be developed for the effective removal of toxic compounds from polluted soil and water, and so that industrial waste effluents can be treated to eliminate sources of environmental pollutants.

Pesticide Pollution

Modern agriculture has relied on a variety of synthetically produced chemicals, very often chlorinated hydrocarbons, as insecticides, fungicides, and herbicides. Benefits from these pesticides have included a decrease in the percentage of household income spent on food and an increase in food quality. However, pesticides are a major source of pollution and have contributed to the toxic compounds found in the soil and water. With a growing world population and the increased agricultural productivity that pesticide use provides, it is likely that pesticide pollution will be a continuing problem. It is therefore crucial that research efforts focus on methods for minimizing further pesticide pollution.

Pollution Remediation

Microbial Biodegradation of Chlorinated Hydrocarbons

Microbial degradation of xenobiotics has gained increasing attention as a means of controlling or eliminating xenobiotic compounds in the environment (Ghosal et al. 1985). Since naturally occurring compounds containing carbon-halogen bonds are rare, halogenated hydrocarbons have accumulated in the environment and in turn have exerted strong selective pressures on microorganisms to evolve methods for detoxification and degradation (Chakrabarty 1980). Bacteria have the ability to evolve quickly, adapting to and utilizing new substrates. Numerous bacterial strains that are capable of degrading a variety of xenobiotic compounds have been isolated and characterized. Studies involving these strains have revealed that in many cases the degradative genes are found clustered on plasmids (Chakrabarty 1980). The transmissible nature of these plasmids has no doubt played a role in the evolution of pathways for degrading these new classes of environmental pollutants (Pemberton 1980).

Waste treatment reactors have been designed and applied to the treatment of industrial effluents, and to remediate contaminated surface and ground waters via "pump and treat" processes. In-situ treatment of soil and water is another area of application that is making use of the catabolic

abilities that microorganisms have evolved for degrading synthetic compounds.

Genetically Engineered Bacteria

Studying the transmissible nature, physiology, and genetic basis of the plasmid mediated biodegradation process may allow the construction of genetically improved strains that may be used for enhanced removal of some of the more recalcitrant compounds. Recombinant DNA technology has facilitated the development of microbial strains with improved biodegradation capabilities. A vast potential exists for the application of these improved strains toward environmental pollution problems.

Possibilities exist for utilizing engineered bacteria both for waste treatment processes as well as for in-situ environmental remediation techniques. (Kilbane 1986). Other potential uses of recombinant organisms designed for release into the environment exist. Besides waste treatment and environmental remediation, other uses include enhanced food and agriculture production, metal and mineral leaching, and biocontrol of insects (Gillet 1986). Improved prospects involving the deliberate release of engineered bacteria into the environment are becoming increasingly feasible. Yet, public and scientific apprehension concerning potential adverse environmental effects has hindered the use of engineered bacteria. Plasmid mobility, stability, survival and fate are crucial factors that must be modeled in

controlled laboratory conditions before any biotechnology product such as an engineered plasmid is applied to a task outside the laboratory.

Purpose of this Research

It is the focus of this research to better understand factors that influence plasmid stability and maintenance within a bacterial host by studying the stability of an inducible and constitutive TFD degrading plasmid during long-term growth under varying conditions within the biofilm reactors and chemostats. The maintenance of a plasmid and expression of associated enzymes in the absence of any plasmid encoded benefits, might result in plasmid loss. Such a situation could occur during bacterial growth under non-selective conditions. The constitutive strain may carry an additional metabolic load due to non-induced gene expression. The energy required for expression may place selective pressure on the population favoring plasmid free cells or cells with reduced plasmid size. Energy required for plasmid maintenance and gene expression are two important aspects that may have a role in the stability of a plasmid during conditions that might favor plasmid-free cells (Caulcott et al. 1987, Keshavarz et al. 1985).

Understanding factors which influence the maintenance and stability of an engineered plasmid is of both fundamental and practical importance. Not only is there immense potential for using engineered bacteria in managing

wastes and toxic substances, but many industrial processes rely on plasmid encoded pathways in which plasmid stability is crucial.

Risk assessment parameters concerning the fate and stability of engineered plasmids released into the environment are other important areas for study. The research carried out in this study has possible implications regarding environmental risk.

Objectives and Goals of this Research

This study focuses on monitoring the stability of an engineered plasmid in a biofilm reactor system with the hope of understanding better the factors involved in plasmid maintenance and stability within the bacteria. It is the intent of this study to investigate specific microbial strains containing natural and genetically engineered degradative plasmids, examining the genetic stability of the engineered plasmids under various laboratory controlled conditions. This research has employed monoculture biofilm reactor systems that were developed for this study, so that long-term growth of the cultures could be monitored with regards to plasmid stability. Because the physiology and genetics of the pure culture are partially known, a more detailed and fundamental study of the reactor inoculum can be achieved than could be with a less characterized mixed consortia. Additionally, the use of a monoculture permits the strain of interest to be studied without the complexity

of a mixed culture, while allowing the feasibility of a pure culture waste treatment system to be assessed. Chemostats were also used in this study to maintain cultures over a long-term growth period so that comparisons could be made between the plasmid stability in immobilized bacteria, as in the biofilms, versus free bacteria grown in the chemostats. Both constitutive and inducible strains were available for comparing any differential stability between the two strains when grown as free cells in chemostats.

The major objectives of this study were:

* Develop reactor systems and determine the conditions necessary to establish and to maintain monocultures (physical design, aseptic sampling and operating techniques, etc.)

* Develop protocols for monitoring the growth and stability of the engineered cultures (enumeration of bacteria in biofilm, selective plating techniques, etc.)

* Compare the fate, survival, and genetic stability of the inducible and constitutive strains as free and immobilized cells, under induced and starvation conditions.

* Compare the biofilm growth between the inducible and constitutive strains.

* Monitor the biofilm culture for contaminating species capable of incorporating the plasmids.

* Monitor the transmissibility and stability of the plasmids in the contaminating species.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Biofilm Reactor Systems

Biodegradation of chemical compounds in nature is enhanced by the formation of immobilized biofilms upon soil particles (Atlas and Bartha 1987). Engineering processes have been developed that take advantage of this property, and several types of biofilm reactor systems have been employed in research and industry. Trickling filter and fluidized bed reactors are two examples of biofilm systems that have been widely used in municipal waste treatment. Biofilm reactors have displayed high removal efficiencies both in research and industry and have been used both for aerobic and anaerobic degradation of wastes. Biofilm systems have demonstrated organic removal capacities twice that of suspended microbial reactor systems under aerobic conditions (Jewell and MacKenzie 1972).

The expanded bed biofilm reactors, under development by Jewell and coworkers since 1970, utilize a support material for bacterial immobilization and retention, and a low upflow velocity for bed expansion. The expanded bed biofilm system has been utilized by several researchers in the last few years for anaerobic treatment of waste (Schraa and Jewell

1984, Switzenbaum and Jewell 1980, Rittmann and Brunner 1983). The degradation of chlorinated organic compounds using expanded bed biofilm reactor systems was described by Bouwer and McCarty (1982).

Biofilm systems offer several advantages over other waste treatment methods and have gained increasing popularity in recent years. The increased surface area that the support material provides allows increased biomass concentrations within the reactor, resulting in a very efficient and smaller waste treatment system. Another advantage that biofilm systems have over other waste treatment methods such as fluidized suspended growth systems is the ability to withstand a great deal of oscillation in the components of the feed stream, including periods of starvation (Rittmann and Brunner 1983).

In the biofilm reactor, a support material such as sand, coal, activated carbon, or diatomaceous earth is coated by microbial growth. The support material and biofilm are expanded and mixed within the reactor by the hydraulic flow. This expansion and mixing of the bed minimizes stagnation and mass transfer limitations, thus increasing the efficiency of the system. The expanded bed design in this study uses high liquid velocities controlled by a recirculation pump to expand the support material and maintain a completely mixed hydraulic regime. This avoids the possibility of differential growth rates and biofilm development in the support bed, while allowing a

representative sample to be taken at any time and from any portion of the bed. Numerous support materials have been used in biofilm reactors, each having certain advantages based on the application. Diatomaceous earth has been used as an effective support material in biofilm systems (Schraa and Jewell 1984, Clarkson et al. 1987), and was chosen as the support material for this study. Diatomaceous earth provides a very light-weight support with a porous surface that facilitates biofilm attachment and growth.

Most biofilm reactor systems reported in the literature have utilized mixed uncharacterized reactor inocula. Mixed cultures are usually obtained at a pollution site where the microbial population has adapted capabilities for degrading the chemicals of interest. Little information exists concerning the application of monoculture biofilms to waste treatment applications. Well characterized monocultures have the advantage over mixed cultures of being able to respond to sporadic contamination by chemicals at lower concentrations for which a mixed culture might not be adapted or induced.

Most of the biofilm reactors that have been used in research and industry employ mixed cultures. Aseptic operation has not been considered in the design of these types of systems. Maintenance of a monoculture requires aseptic operation and sampling, therefore the physical design of the reactors requires that these conditions can be easily maintained during long-term operation. These

considerations dictate the possible designs of a monoculture system and have influenced the design, operation, and experimental use of the reactor system used in this study.

2,4-Dichlorophenoxyacetic Acid

During the last few decades, chlorinated derivatives of phenoxyacetic acid have been applied as herbicides. Their extensive use has resulted in the rapid evolution and dissemination of degradative pathways in soil and aquatic bacteria. Consequently, the herbicide 2,4-dichlorophenoxyacetic acid (TFD) is degraded by several organisms belonging to a variety of genera and is not considered to be a recalcitrant compound. It has a half-life from one to six weeks in the soil and from a few days to several months in surface waters (Ware 1988). Many other halogenated compounds are more recalcitrant and pose a greater pollution problem than TFD. The physical properties of TFD are given in Table I.

TFD is a systemic herbicide used to control broadleaf weeds. It is applied directly or as salts or esters, and is sold under various names including Amidox, Amoxone, and Aqua Kleen. It is most often applied by aerial spraying. Surface waters are more frequently contaminated than ground water due to runoff from agriculture applications. Surveys have detected levels up to 0.5 ppb in drinking water supplies but most are below 0.1 ppb (Ware 1988). The United

States Environmental Protection Agency (USEPA) has set a drinking water standard for TFD at 0.1 ppm (USEPA 1980). Although TFD has been detected in some foods, significant and consistent levels have not been detected in recent years and therefore drinking water supplies are the main concern to human health.

TABLE I

PHYSICAL PROPERTIES OF TFD (Ware 1988)

Property	Value
Chemical Abstract Services number	94-75-7
Chemical formula	$C_8H_6O_3Cl_2$
Molecular weight	221
Physical State	White crystalline powder
Boiling point	160°C at 0.4 mm Hg
Melting point	138°C
Water solubility	540 mg/L

Cultures, Strains, and Plasmids

The bacterial degradation of TFD has been investigated by numerous research groups, and several bacterial strains that are capable of TFD degradation have been identified. (Fisher et al. 1978, Don et al. 1985, Amy et al. 1985). The cultures used in this study are derivatives of Alcaligenes eutrophus JMP134 carrying the plasmid pJP4. This strain was

first reported by Don and Pemberton (1981), isolated by its ability to grow on TFD as its sole carbon and energy source. Plasmid pJP4 is 80 kilobases (kb) in size and carries the genes essential for TFD degradation, 3-chlorobenzoate (3CB) degradation, and mercury resistance. Plasmid pJP4 has a broad host range, is self transmissible, and belongs to the P1 incompatibility group (Don and Pemberton 1985, Don et al. 1985).

The TFD pathway is induced in the presence of TFD or 3CB. Several restriction maps for this plasmid and similar plasmids have been published (Don and Pemberton 1985, Don et al. 1985, Ghosal et al. 1985). The metabolic pathways for the degradation of TFD and 3CB utilize enzymes common to the degradation of chlorocatechol to chloromaleylacetic acid. Don et al. (1985) have proposed a degradative pathway and the enzymes involved (Figure 1). Most of the catabolic functions of plasmid pJP4 have been mapped to the BamHI C fragment shown in Figure 2. The TFD monoxygenase, tfdA, has been mapped to the HindIII B fragment (Streber et al. 1987). Harker et al. (1989) have identified a regulatory gene tfdR which controls expression of the TFD monoxygenase. Deletion of the tfdR gene from pJP4 allows constitutive expression of the entire pathway in the absence of any induction (Harker et al. 1989).

Alcaligenes eutrophus AEO106 is the plasmid cured derivative of JMP134. In studies by Harker et al. (1989) this strain was used as the host for carrying engineered

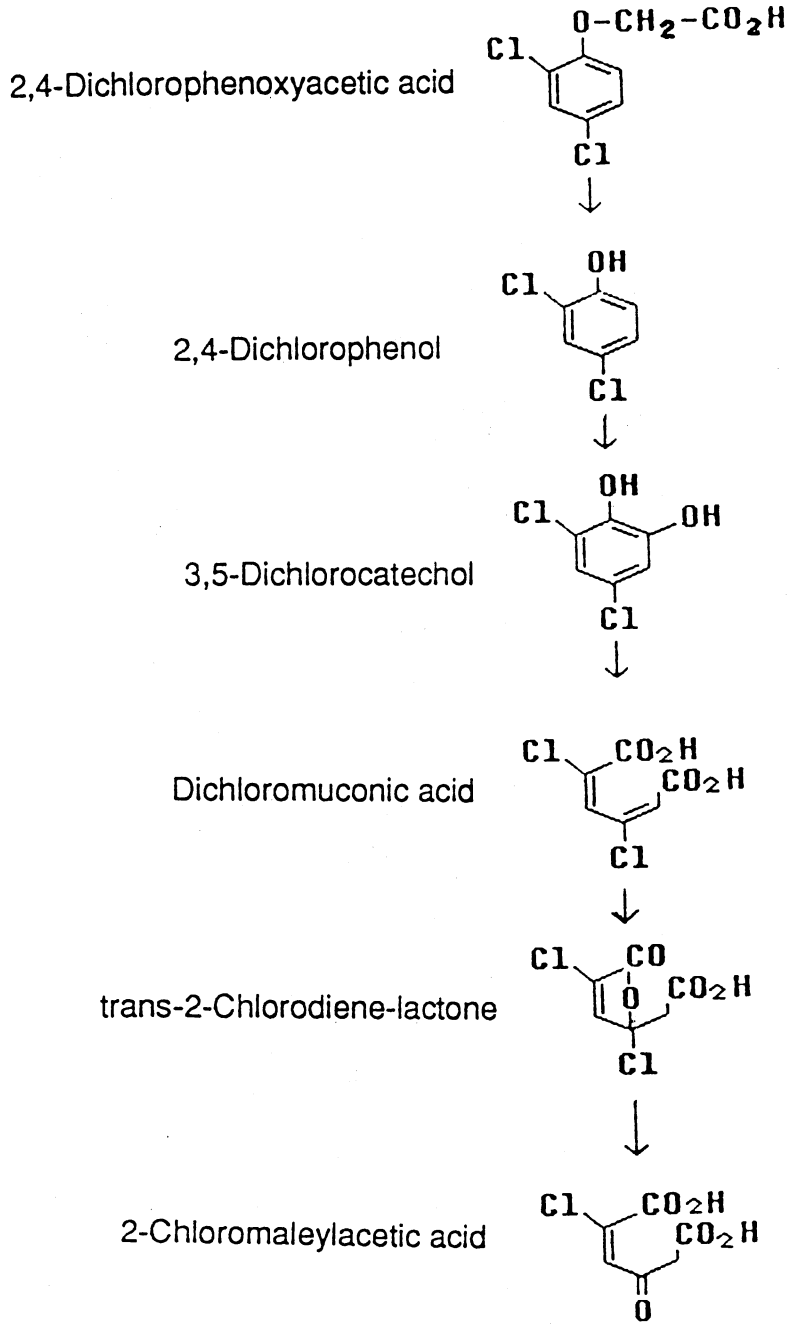


Figure 1. TFD Pathway Encoded by pJP4 (Don et al. 1985)

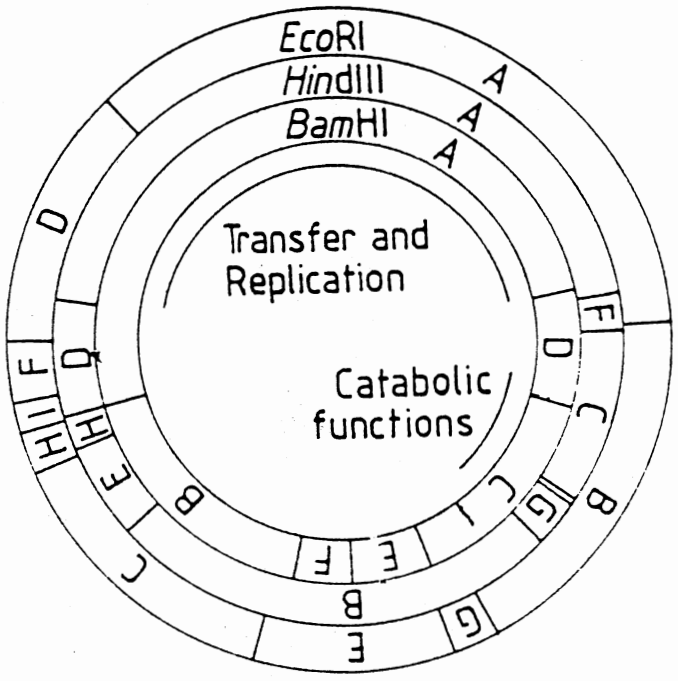


Figure 2. Restriction Map of pJP4 (Streber et al. 1987)

derivatives of pJP4. Plasmid pRO101 is a derivative of pJP4 obtained by the insertion of a Tn1721 transposon into a nonessential region of the plasmid. Plasmid pRO103 was produced by the deletion of a 3.9-kilobase fragment within the EcoRI E fragment of pRO101 and constitutively expresses the TFD pathway. Both engineered plasmids have been transformed into the aforementioned strain, creating two new strains, AEO106 pRO101 and AEO106 pRO103, used in this study.

Many other bacterial strains have been isolated that are proficient in degrading more recalcitrant pollutants and more toxic compounds. The strains chosen for this study have been well characterized and engineered variants exist, providing a choice system for study.

Plasmid Stability

Plasmid stability here is defined as the ability of host bacteria to maintain a plasmid unchanged during growth, expressing the plasmid's phenotypic characteristics. Interest in the factors that affect the stability of a plasmid has increased due to observations by many that both genetic and physiological parameters can alter the ability of the host microorganism to retain a plasmid (Caulcott et al. 1985, Sayadi et al. 1988). Understanding what factors influence plasmid stability and how these factors function to influence plasmid stability is an important area of study. Commercial processes and waste treatment methods

involving plasmid encoded processes are examples of areas in which plasmid stability is crucial. The genetic mobility and stability of an engineered plasmid is also an important factor in the evaluation of engineered bacteria for potential environmental risks.

Many bacteria contain plasmids which give the host a certain growth advantage during particular growth conditions (Dykhuizen and Hartl 1983). Under non-selective conditions, possessing a plasmid can be a disadvantage to the bacterial host due to the expense of energy required for plasmid replication and maintenance without any subsequent energy benefits. Additionally, constitutive expression of plasmid pathways during non-inducing growth conditions might also result in plasmid instability. One possible result from these types of energy drains is that the plasmid-free bacteria would outgrow the plasmid-containing bacteria and soon the plasmid is lost from most of the population. Yet, to a population as a whole it is an important evolutionary property that at least a small percentage of the population retain such plasmids in the event the environmental conditions change.

Commercial use of plasmid-coded gene products or application of plasmid mediated waste treatment methods is possible only if the plasmids can be stably maintained in the host. Studies have shown that recombinant plasmids are often not maintained within the host under non-selective conditions. The stability of plasmids in host cells may be

affected by several factors, such as the physiological consequences of gene expression on plasmids. It has been observed that modifications such as the insertion of highly expressed DNA into a plasmid may result in instability (Imanaka et al. 1980, Caulcott et al. 1985). Genes that are constitutively expressed during growth conditions in which the expressed gene product provides no energy benefit may result in a metabolic load on the bacteria. This metabolic load might then result in loss of a portion of the plasmid or the entire plasmid.

Growth conditions of the host cell may also affect the stability of plasmids. Godwin and Slater (1979) have shown that nutrient limitations result in plasmid loss. It has been shown in previous papers that immobilization of whole cells can lead to increased plasmid stability (Nasri et al. 1987, Sayadi et al. 1987, Sayadi et al. 1988).

Risk Assessment

Predicting the potential for adverse effects of engineered organisms released into the environment depends on an understanding of the fate and movement of engineered genetic material. Controlled laboratory studies of engineered microbes is an essential and required step before the organism can be released into the environment.

CHAPTER III

MATERIALS AND METHODS

Reactor Materials

The major components of the reactor system and a brief description of each are listed in Table II.

Reactor Design

The physical design of the reactor system was one of the first objectives of this research project. The development and modification of the reactor system was carried out in conjunction with the project collaborators. The pure culture requirements of the reactor system dictated the physical design. Several basic parameters relative to the reactor design such as the support medium, aeration system, the reactor hardware, and the pumping systems were modified and adjusted until a practical operating system was developed. Consideration was taken in choosing reactor materials so that all the reactor materials were autoclavable. The reactor configuration is shown in Figure 3.

The reactor consists of a glass chromatography column 3 cm in diameter by 80 cm in length and 160 ml total volume. The column was fitted with fluoropolymer endplates, teflon

TABLE II

DESCRIPTION OF REACTOR COMPONENTS

PART	DESCRIPTION
Reactor column	Fisher & Porter Co., 80 x 5 cm, 160 ml volume
Peristaltic pumps	Cole Parmer Instrument Co., Masterflex Model # 7553-30
Pump heads	Cole Parmer Instrument Co., Masterflex
recirculation	Model # 7015-20
2,4-D feed	Model # 7014-20
dilution feed	Model # 7016-20
Tubing	Cole Parmer Instrument Co., Masterflex Tygon, Masterflex Silicone
recirculation	Model # 6419-15, inside diameter = 4.8 mm
2,4-D feed	Model # 6419-14, inside diameter = 1.6 mm
dilution feed	Model # 6419-16, inside diameter = 3.1 mm
Oxygen electrodes	Yellow Springs Instrument Co., Clark oxygen probe, Model # 4004
Oxygen monitor	Yellow Springs Instrument Co., Biological oxygen monitor, Model # 53
Aeration tube	Modified test tube, 15 cm x 2cm, containing air stone
Biofilm support	Diatomaceous earth, sieved to size of 600 μm or less
Sampling apparatus	New Brunswick Instruments, chemostat sampler

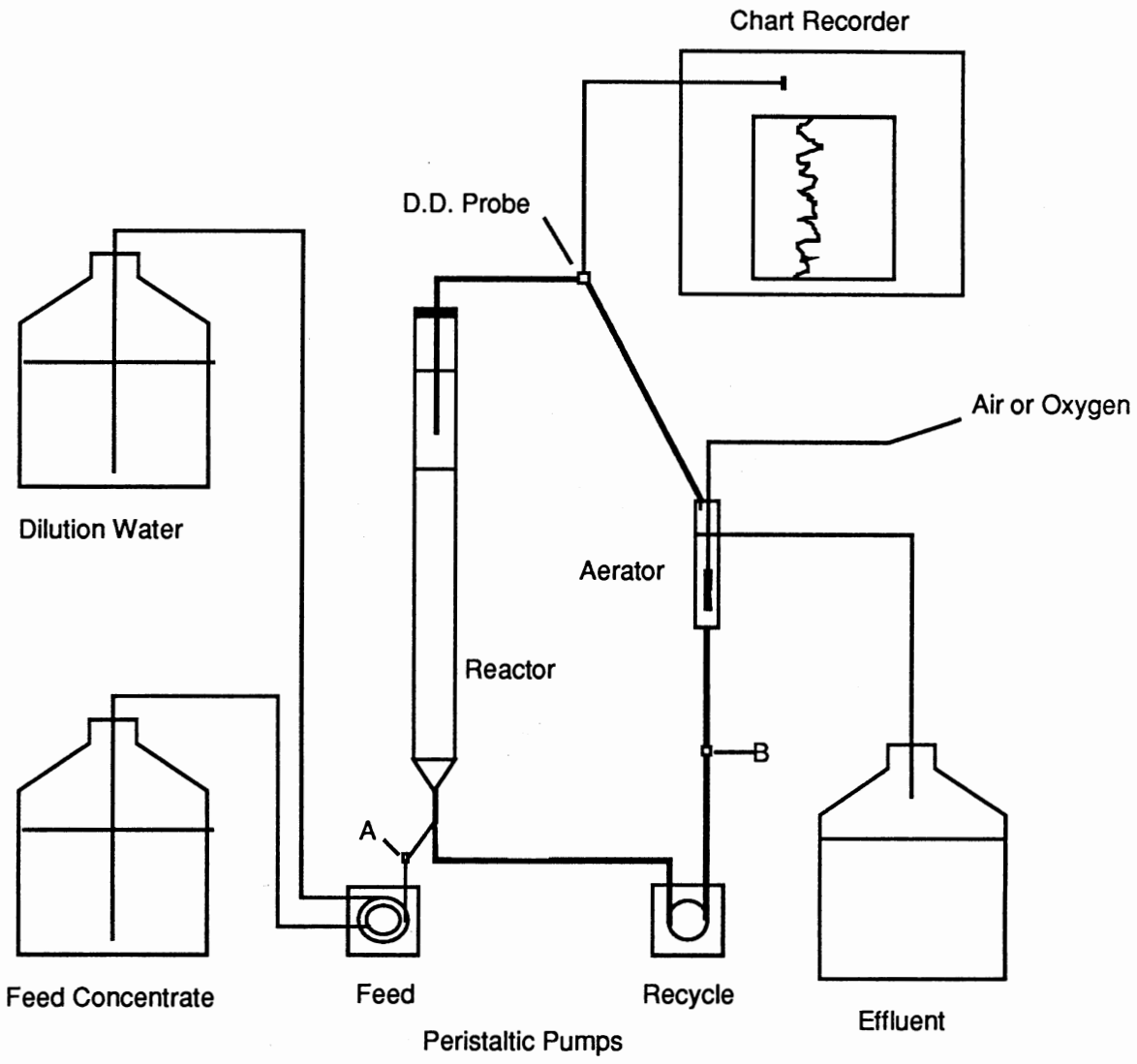


Figure 3. Reactor Diagram

seals, and a stopcock. The column contained 80 ml of diatomaceous earth. Silicone tubing was used throughout, except for 12 inch segments of tygon tubing within the pump head. The silicone tubing was attached to the funnel shaped glass stem at the bottom of the reactor where the feed line and recirculation line were brought together via a plastic Y-tubing connector. The top of the column was plugged with a two-hole rubber stopper. One of the holes contained a short segment of glass tubing to which the recirculation line was attached. The biofilm sampling apparatus filled the second hole. The recirculation line was attached to an aeration tube and then connected from the bottom of the aeration tube to a recycle pump. The recirculation pump was connected to the plastic Y-connector at the bottom of the column, completing a circular flow path.

Two twenty-liter glass carboys were used for the TFD concentrated feed and the dilution water which were introduced into the reactor system by way of a peristalic feed pump with two pump heads: one for each of the liquid streams. The two streams were brought together at a T-connector and this combined stream was then connected to the Y-connector at the bottom of the reactor column where the feed and recirculation streams mix. The combined hydraulic flow of the feed and recirculation lines was directed into the bottom of the column which results in an expansion of the diatomaceous earth bed and a completely mixed hydraulic regime.

Diatomaceous earth was sifted through a No. 20 mesh sieve (W.S. Tyler Co.) so that the particle size of the earth would be less than 600 μm. The reactors were filled with 80 ml of earth.

An in-line aeration tube was used to maintain aerobic conditions within the reactor by injecting compressed air or oxygen as needed to maintain dissolved oxygen levels. The aeration tube consisted of a 2 cm diameter by 15 cm glass tube. The top of the tube was closed with a two hole rubber stopper. The recirculation line entered through one whole and emptied into the tube. Air or oxygen was introduced into the system through the other hole in the stopper and was attached to an aeration stone placed in the middle of the tube. A glass sidearm on the aeration tube permitted effluent liquid overflow to a carboy. The aerated liquid was pumped through a glass arm in the bottom of the tube where the recirculation line was attached.

Sampling was carried out by way of a sampling assembly that ran through the rubber stopper in the top of the reactor column. The sampling line extended down into the expanded bed and attached to a collection vessel tube at the top. By applying suction to this sampling device a sample of the reactor support material could be taken from any level within the bed.

Dissolved oxygen probes (YSI 4004, Yellow Springs Instruments) were placed in the recirculation line before and after the aeration tube. A biological oxygen monitor

(YSI Model 53, Yellow Springs Instruments) and a chart recorder connected to the oxygen probes allowed the dissolved oxygen level to be monitored. The oxygen level measured in the recirculation line just before the aeration tube was maintained above 2 ppm by injection of air or pure oxygen as required.

All reactor components were thoroughly autoclaved and carefully handled during the set-up process. The feed carboys were autoclaved and the media components carefully added according to the method described in Appendix A. In-line 0.45 μm air filters (Gelman Sciences Inc.) were used for filtering the air or oxygen and placed on the carboys as pressure vents.

Chemostats

Two New Brunswick brand model C-30 chemostats were used for the experimental steps involving free cells, and operated according to the methods given in Chapter IV.

Media

Phosphate buffered minimal medium (MMO) supplemented with TFD or casamino acids (CAA, Difco) was used as the culture medium for the biofilm reactor system and the chemostats. The MMO medium has been described by Stanier et al. (1966). Appendix A describes the components and preparation of MMO and all other media. The TFD (Aldrich Chemical Co.) was made at a 5% stock concentration in water

and added to the MMO to get the final desired concentration.

The 5% TFD stock was prepared by heating the water and TFD mixture on a hot plate until the solution was very warm to the touch. The solution was then titrated with 10N NaOH until all TFD went into solution.

Bottles of TFD feed for the reactors were made at concentrations of 800, 1600, and 3200 mg/L. Dilution bottles containing autoclaved distilled water and buffer solution (solution A) were used to dilute the reactor feed to concentrations of 200, 400, and 800 mg TFD/L.

Screening for mercury resistance was done by using brain heart infusion (Difco Laboratories, Detroit, Mich.) agar plates with 25 ug of HgCl per ml (Hg^{25}). Tetracycline resistance was screened using TNA agar plates with 25 ug of tetracycline per ml (Tc^{25}). Both selective media were prepared as agar plates. See Appendix A for a complete description and preparation methods for all of the media.

Cultures

Alcaligenes eutrophus AEO106 pRO101 and AEO106 pRO103 were used as reactor and chemostat inoculants. The cultures were maintained on a complex medium (TNA) containing tetracycline at 25 ug per ml. All cultures of AEO106 were grown at 30°C. A single isolated colony was passed twice on a TNA selective plate. Growth from the selective plate was then used to inoculate a 100 ml liquid culture of MMO containing 0.05% TFD and 0.3% CAA. From this culture a one

ml sample of cells was spun down and washed with buffer. The washed cells were used to inoculate a MMO 0.05% TFD liquid culture, and after sufficient growth this culture was used to inoculate the reactors and chemostats. Generally a 10% volume of culture was used as inoculant for the reactors and chemostat.

Viable Counts Enumeration

Viable counts of the biofilm were measured by serial dilutions and spread plating. First, a sample of the diatomaceous earth support was taken using the sampling assembly. The liquid concurrently removed during sampling was decanted and a 0.5 ml sample of the diatomaceous earth was measured in a marked test tube. This sample was washed gently with 1 ml of MMO, the liquid decanted and the procedure repeated. This step was necessary to insure that the viable count represents only those bacteria attached as biofilm, not bacteria in the surrounding reactor liquid. Next 2 ml of MMO was added and the test tube was capped and vortexed for 30 seconds to remove the attached biofilm. To this an additional 3 ml of MMO was added to give a total liquid volume of 5 ml, and this tube was designated as a 10^{-1} dilution. Serial dilutions of this tube were made and spread plated on TNA agar plates and incubated at 30°C. The colonies on the plates were counted after they grew to a clearly visible size.

Viable counts of the cultures grown in the chemostat

were measured using the same procedure, except that a liquid sample was taken.

Plasmid Stability Assay

Plasmid stability was monitored by replica plating the colonies from the non-selective TNA plates to mercury plates and tetracycline plates. The number of colonies growing on the selective plates was expressed as a proportion of the number growing on the non-selective plates. This value was taken to represent the proportion of the population carrying the plasmid. Plating on both of the selective plates allows plasmid stability as well as transposon stability to be monitored. Stock cultures of AE0106 and AE0106 pRO101 were also plated as controls on each selective plate. Plating directly from the reactors or chemostats to the selective media gave false results indicating plasmid and transposon loss. This observation is further discussed in the results section and is the basis for the methodology described here.

Plasmid DNA Isolation

Plasmid DNA was isolated in small quantities using the modified method of Birnboim and Doly (Birnboim and Doly, 1979; Ish-Horowitz and Burke, 1981). The complete procedure is given in Appendix B. Restriction enzyme digests were done as recommended by the manufacturer. DNA samples were visualized by gel electrophoresis and photography, using a

polaroid camera and a UV transilluminator as described by Maniatis (1982). A horizontal electrophoresis unit and an 0.8% agarose gel were used to screen plasmid preparations and to separate restriction fragments. Ethidium bromide was incorporated into the agarose (10 ug/ml), permitting quick visualization of the DNA bands.

TFD Assay

Samples (1 ml) of the reactor and chemostat effluent were centrifuged in an Eppendorf microcentrifuge for 3 min at maximum speed to remove cells. The supernatant was placed in a microcuvette (path length 10 mm) and the UV-absorbing properties measured by scanning spectra between 320 nm and 240 nm. All spectrophotometric measurements were performed using a model UV-160A spectrophotometer (Shimadzu). Under these conditions TFD exhibits a maximal absorbance at 283 nm. TFD removal was measured by the change in absorbance at 283 nm between the feed spectrum and the effluent spectrum.

A standard curve was constructed by measuring the absorbance at 283 nm of a series of TFD concentrations (50, 100, 150, 200, 250 mg/L). Influent and effluent TFD concentrations were monitored by sampling at septums B and A, respectively (Figure 3), comparing the absorbance spectrum to the standard curve.

Strain Identification

The identification of all bacterial strains was done using Rapid NFT diagnostic strips (API Analytab Products, Plainview, New York). The test provides a standardized method combining 20 biochemical and assimilation tests for the identification of gram-negative, nonfermentative bacteria. Each test strip contains dehydrated substrates that are reconstituted when a bacterial suspension is added. For the enzymatic test, color changes are observed according to the metabolic endproducts. The assimilation tests are observed for growth, based on the ability of a bacterial strain to assimilate a single carbon source. The results are grouped according to a coding principle, and a codebook is used for the identification of the unknown. Strain identification tests were performed on the pure cultures before inoculating the reactor and chemostat systems and during and after the long-term operation of these systems.

CHAPTER IV

RESULTS

Development of Methodology

Reactor Setup and Operation

The development of experimental methods for operating and monitoring the reactor system was the first objective of this project. The reactor modifications were made during the initial stages of this study during which CAA was used as the carbon source in the reactor feed. Maintenance of a monoculture system proved to be a difficult task. The complexity of the reactor configuration allowed contaminating bacteria to become established in the reactors, requiring that the system be taken apart, sterilized, and set up again. In the early stages of this project, the reactor system required constant maintenance to ensure that the system remained operational. The tygon tubing used in the recirculation and feed pumps became worn after extended operation and would often break and become a suspected source of contamination to the monoculture system. This problem was lessened by putting tubing connections before and after the pump head so that this portion of tubing could be easily replaced on a regular basis.

Air lines were another suspected contamination point. Tubing was originally connected directly to the compressed air lines on the lab benches. The reactors were quickly contaminated by other bacteria. In-line 0.45 μm bacterial air filters (Gelman Sciences Inc.) were installed to prevent this problem.

Additional contamination problems resulted from the sampling methods. Originally, the rubber stopper in the top of the reactor was removed for sampling so that a sterile scoop or pipet could be lowered into the reactor support material. This method proved to be inadequate with regard to the aseptic conditions required to maintain a monoculture. To prevent this type of sampling contamination, a sampling assembly was placed on the reactor so a sample of the biofilm could be taken without opening the system and compromising the monoculture conditions. Syringe septa were also placed in-line for easy sampling of the reactor liquid using a sterile syringe. These septa were placed in the recirculation line after the aeration vessel, and in the feed line past the mixing point of the dilution water and feed stock. A photograph of the final operating reactor is shown in Figure 4.

Plasmid Stability Assay

A practical and reproducible protocol was developed to determine the composition of the biofilm. The sampling, sample preparation, and plating are described in Chapter III.

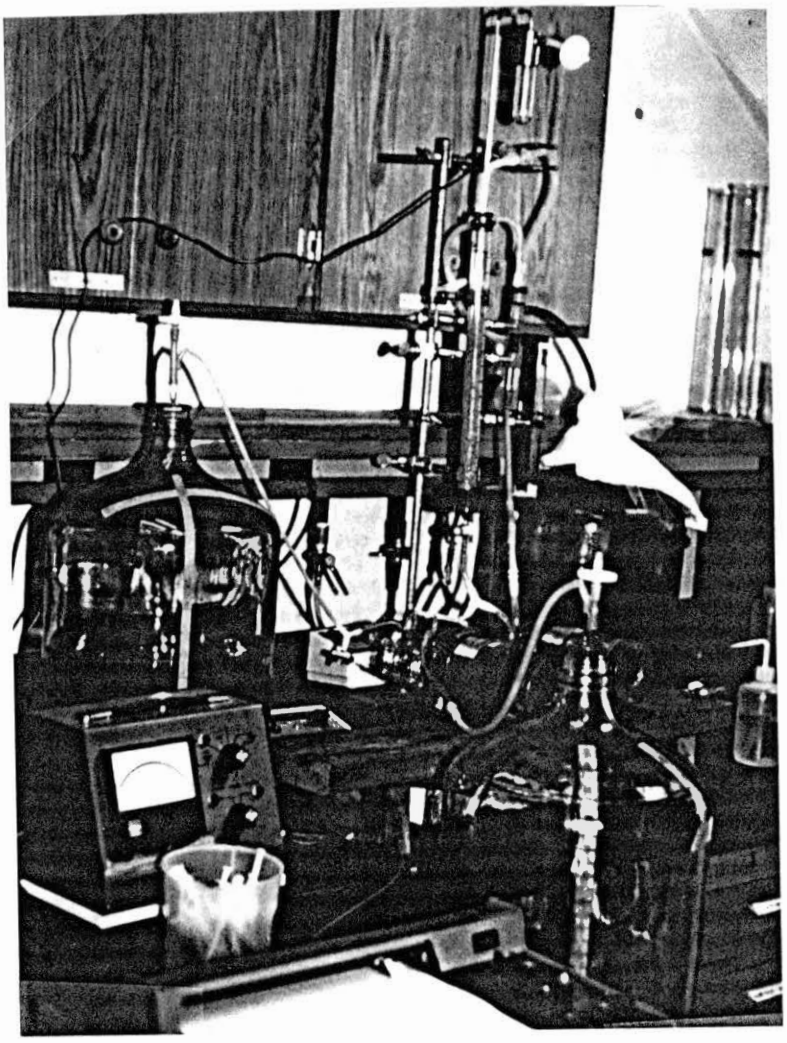


Figure 4. Photograph of Reactor System

Variations of this procedure were tested, and it was determined that the results from this method were representative of the biofilm population. These variations included additional vortexing and washing of the biofilm to determine if the procedure efficiently removed the bacteria from the support material.

Initially, samples were withdrawn from the reactors and plated both directly on the selective media (TNA Tc^{25}) and on non-selective media (TNA), then replica plated after growth onto the selective media. Viable counts of the biofilm plated on the non-selective media were much higher than the counts plated directly on the selective media for both AEO106 pRO101 and AEO106 pRO103. The ratio of the bacteria expressing resistance markers when directly plated on the selective media to the total viable count is given as a percentage in Figure 5. The percentage of the total bacteria population that grows on the selective agar drops with time, indicating an apparent loss of the plasmid. Yet, nearly all these colonies when plated first on TNA and then replica plated on to the Tc^{25} and Hg^{25} grew normally. The ratio of the number of colonies replica plated on the selective plates to the viable counts on the non-selective plates (total viable count) is shown as a percentage in Figure 6. These results indicate that the plasmid carrying the resistance markers was still present in the colonies that did not grow when directly plated on the selective

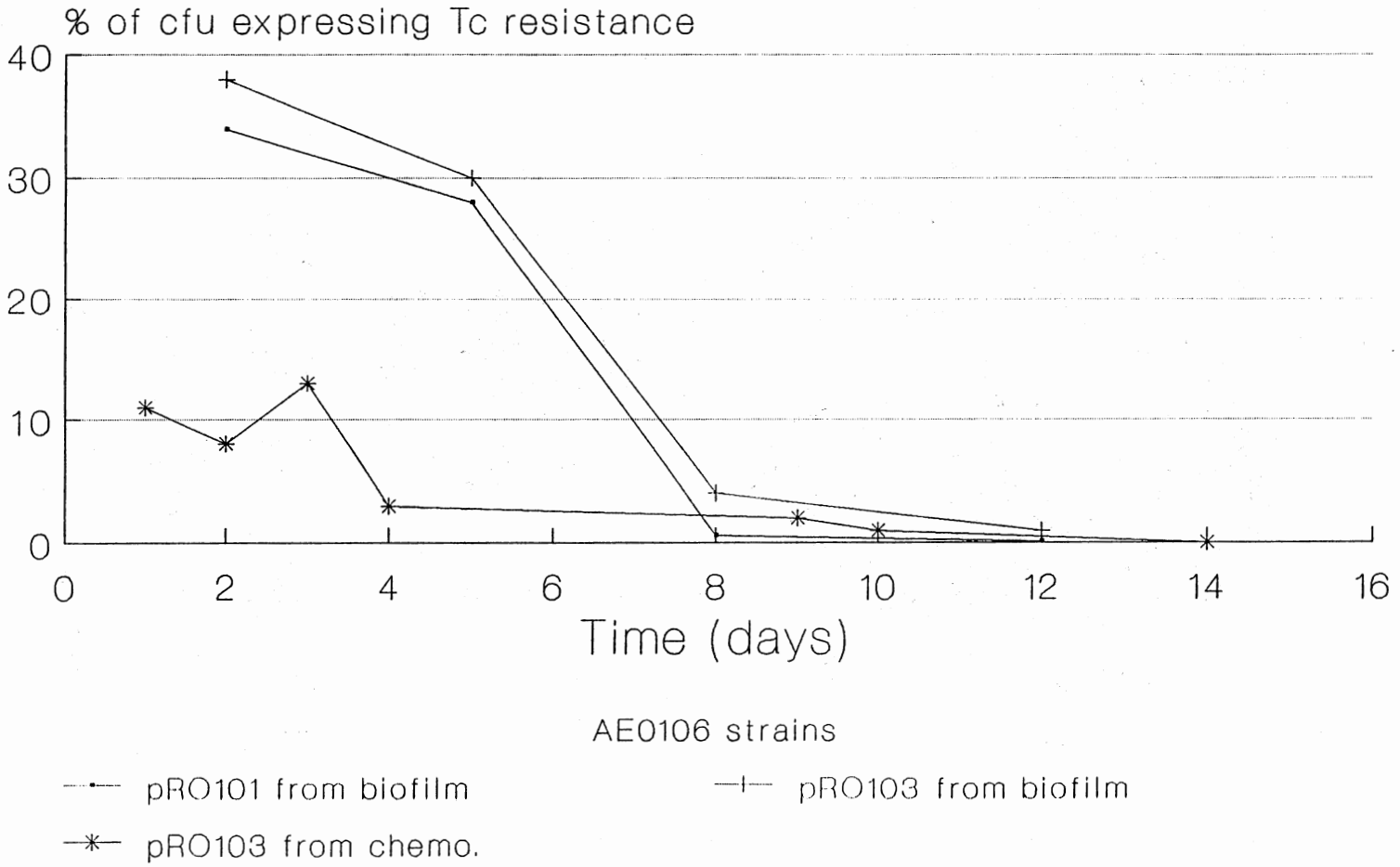
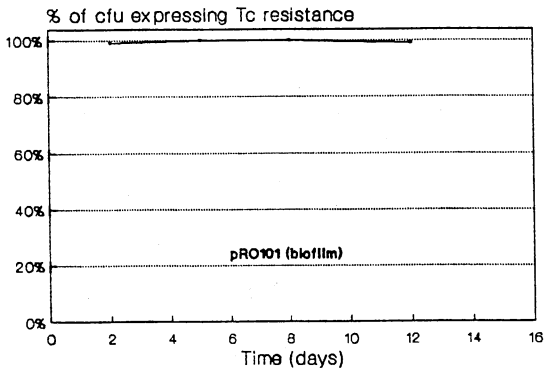
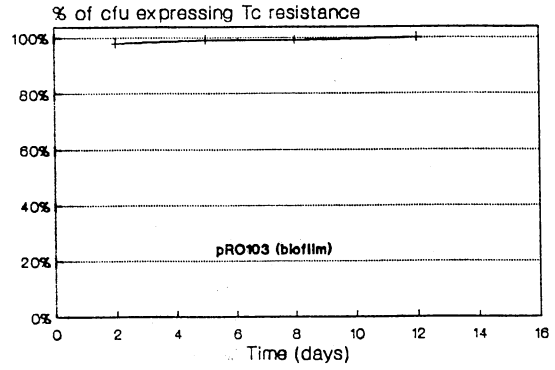


Figure 5. Results from Direct Plating on Selective Media





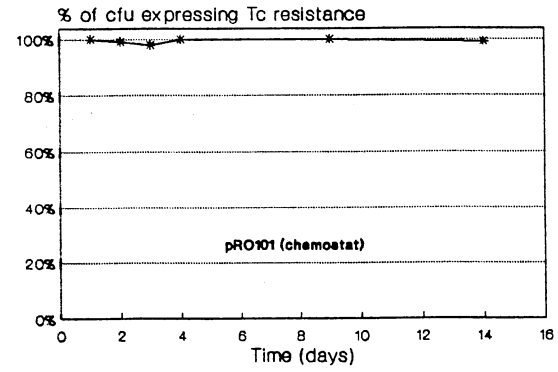


Figure 6. Results from Replica Plating to Selective Media

media. Plasmid isolation from these colonies in all cases revealed a plasmid of the appropriate size (Figure 7).

It is likely that the expression of tetracycline resistance is not immediate, resulting in fewer colonies when directly plated on the selective media. After these observations, all further plasmid stability assays were performed by first plating on the non-selective media and then replica plating from the non-selective media to the selective one.

Experimental Phase

Growth on CAA

After the physical design of the reactor system had been established and methods had been developed for lessening contamination problems, a pair of identical reactors were set up and fed with MMO, plus CAA as the non-selective carbon source. CAA allows rapid cell growth without inducing the TFD pathway. Under these non-inducing conditions, any plasmid instability in the constitutive or inducible strains should be observed.

Cultures of AEO106 pRO101 and AEO106 pRO103 were grown to saturation in 100 ml MMO plus 3% CAA. A 10% volume inoculum was used to inoculate the reactors, which were maintained for several days. Viable counts from the reactor biofilms were monitored during this time period.

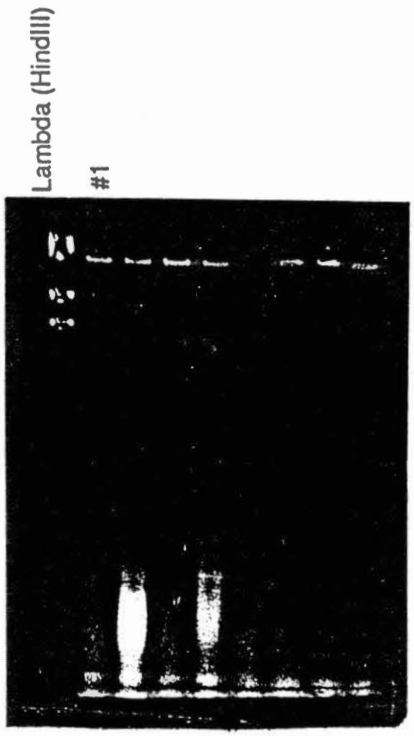


Figure 7. Plasmid DNA Isolation (Agarose gel showing plasmids isolated from colonies that did not express tetracycline resistance upon direct plating; Lane 1 is pRO101 stock, remaining lanes contain plasmid DNA from random colonies.

The use of CAA as a sole carbon source again allowed quick establishment of contaminating bacteria and prevented the long-term monitoring of the strain of interest. Several contaminating bacteria were detected on the non-selective plates as early as one week after the reactors were inoculated. Several attempts were made to set up sterile reactors and maintain a monoculture, but the complexity of the system proved to be a major barrier in maintaining a pure culture when CAA was the carbon source. The CAA provided an easily utilized carbon source for any contaminating bacteria which then quickly grew and hindered the study of the desired culture.

Growth on TFD

Due to the difficulty in maintaining pure cultures when CAA was used as the sole carbon source in the reactors, the reactors were taken apart, sterilized and assembled again. The two reactors were filled with MMO media containing 200 mg/L TFD and inoculated with cultures of AE0106 pRO101 and AE0106 pRO103. Continuous TFD feeding was not started until significant TFD removal was observed. On the fifth day after inoculation both reactors were fed at a loading rate of 4 g/L*d. Initial experiments involved monitoring microbial population, dissolved oxygen, residual TFD concentration, and the stability of the plasmid within the systems during increased loading rates of TFD. The increased loading rate was achieved by increasing either the

TFD concentration in the influent or by increasing the feeding rate so that the loading rate was doubled at each of five stages as shown in Table III.

TABLE III

REACTOR OPERATING CONDITIONS

Stage	*Target TFD Conc. (mg/L)	Flow Rate (L/d)	Loading Rate (g/L*d)
1	200	2.16	4
2	200	4.32	8
3	200	8.64	16
4	400	8.64	32
5	800	8.64	64

* Actual TFD conc. in influent varied slightly.

Viable counts were measured during the five stages. Triplicate samples were taken, and Figure 8 shows the results.

Both the inducible and constitutive strains showed an increase in biofilm concentration during the first three stages. Viable counts for AE0106 pRO101 were on the average higher throughout the test period. The difference could be attributed to reactor conditions or to growth differences in the strains. Both reactors reached a stable biofilm concentration by the third stage, and after this point little fluctuation in counts was noted. Dissolved oxygen levels were maintained above 2 ppm with compressed air. At a loading rate of 16 g/L*d pure oxygen injection was required. This corresponds to the point where the biofilm

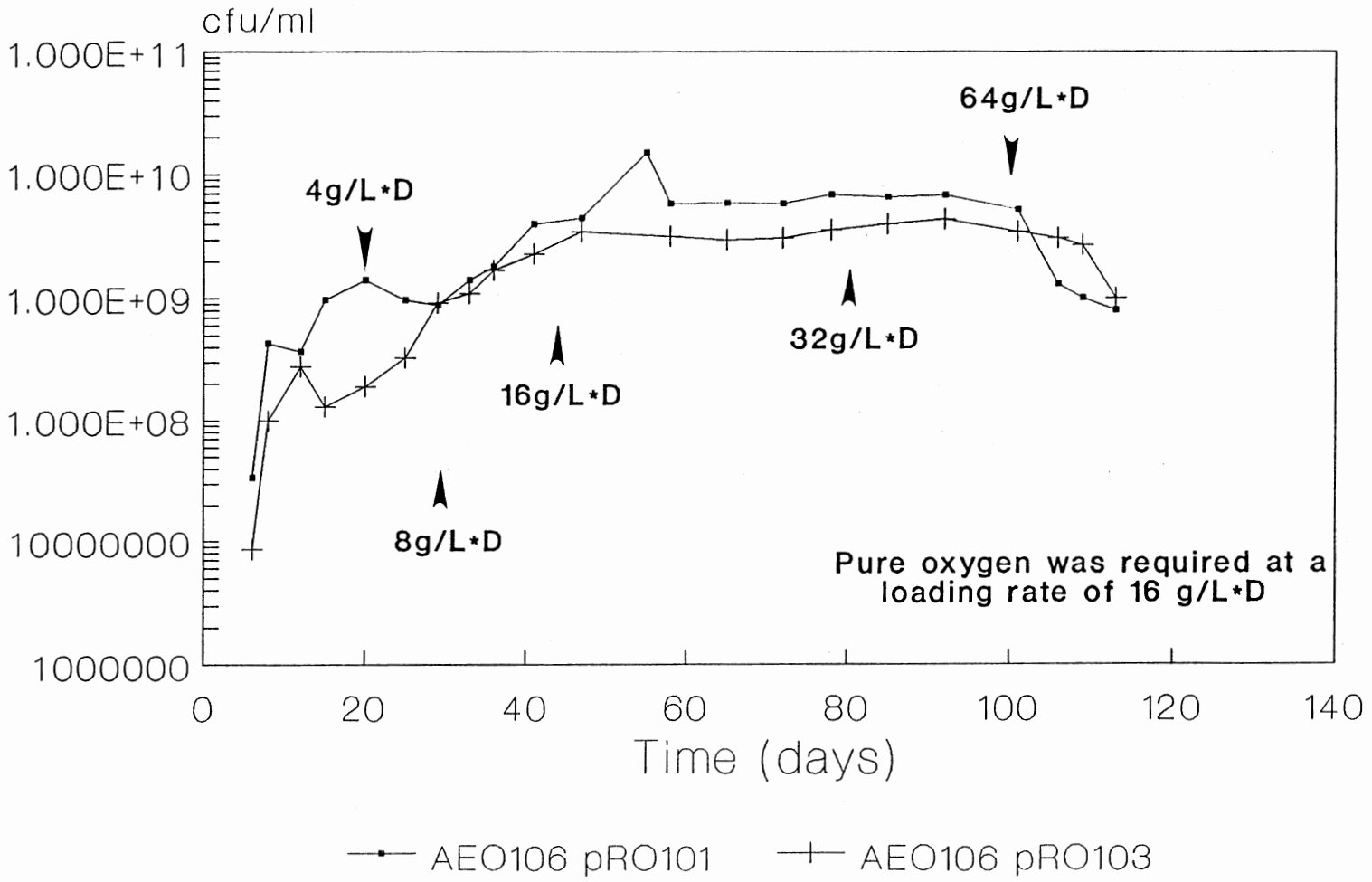
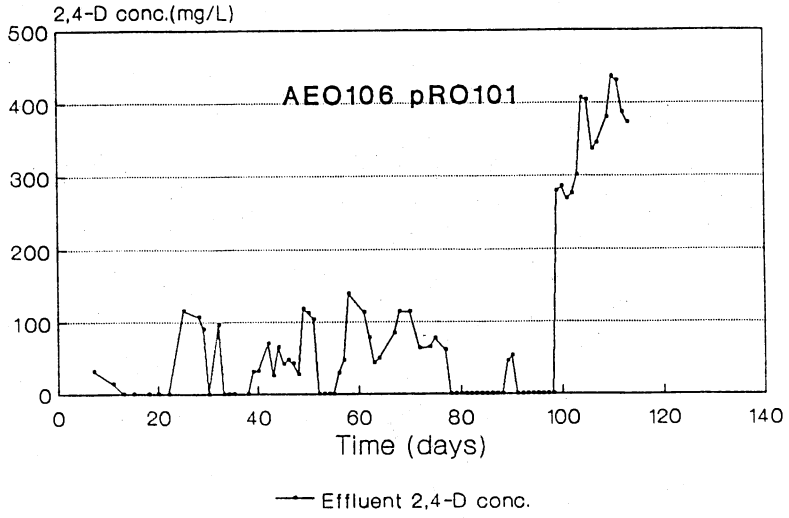


Figure 8. Viable Counts During Five Stage Operation

concentration stabilizes. The pure oxygen probably contributed to the stable biofilm concentrations. Before the addition of pure oxygen, dissolved oxygen levels were possibly not adequate to maintain the biofilm at a high biomass concentration resulting in fluctuating biofilm concentrations. At the highest loading rate, 64 g/L*d, a drop in viable counts was noted for both cultures. Analysis at this point showed an increase in the TFD concentration in the effluent. Figure 9 shows the effluent TFD concentrations as calculated by the standard curve during the five stages of operation. Both reactors showed removal of the TFD to levels below detectable limits up to the last loading rate. A slight increase in effluent TFD is seen after each doubling but drops quickly below detectable levels.

The drop in viable counts in Figure 8 coincides with the increase in effluent TFD. This reflects possible toxic effects of the increased TFD concentrations or the metabolic intermediates of TFD degradation.

During the operation of both reactors, plasmid stability was measured. Figures 10 and 11 show the percentage of the colonies for both AEO106 pRO101 and AEO106 pRO103 respectively, that grew when replica plated on both of the selective media. During the five stages of operation, nearly one hundred percent of the colonies grew on the tetracycline and mercury plates. This indicates that both pRO101 and pRO103 were genetically stable when grown on



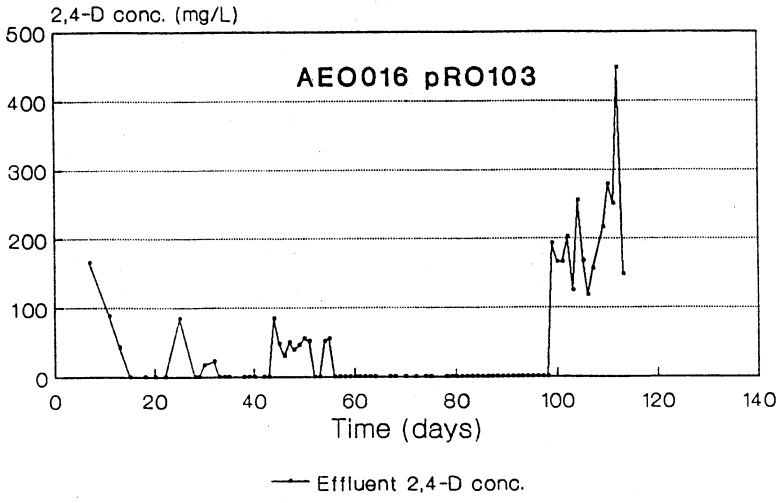


Figure 9. Effluent TFD Concentration During Five Stage Operation

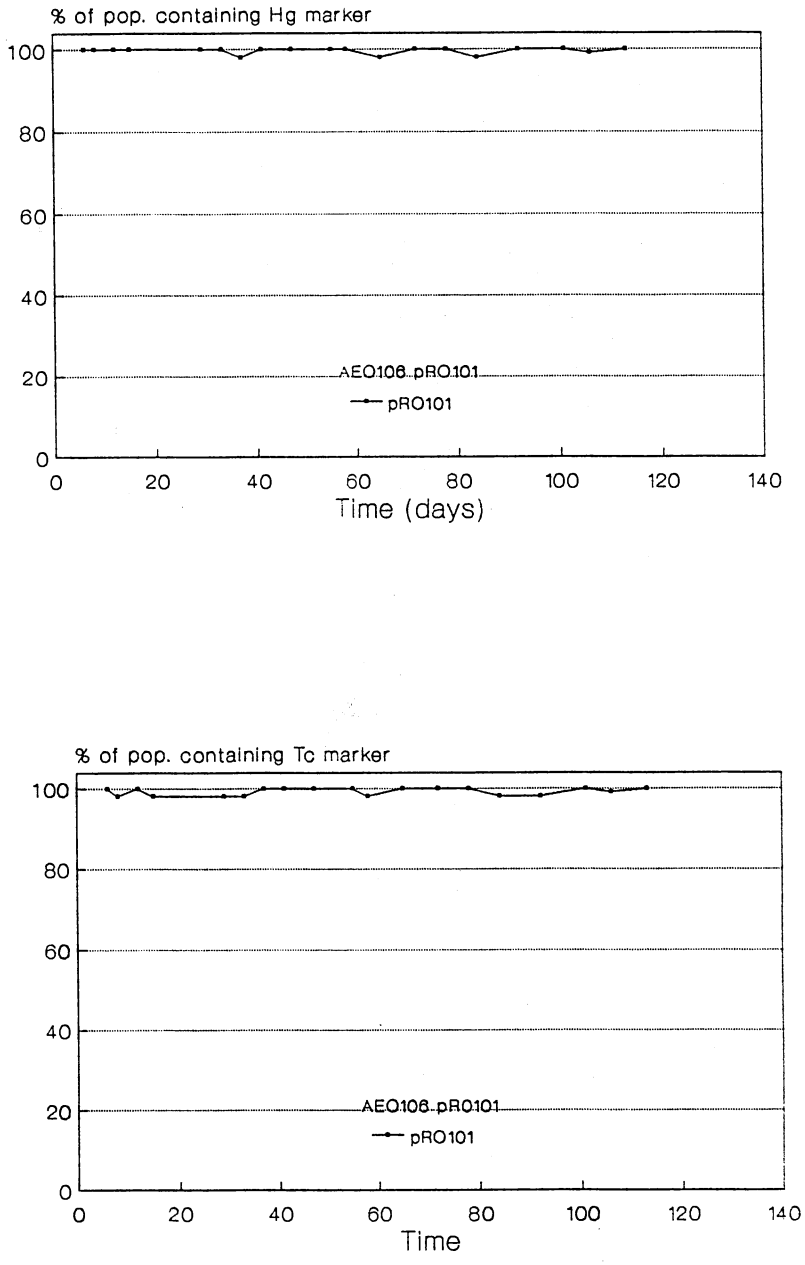


Figure 10. pRO101 Stability During Five Stage Operation

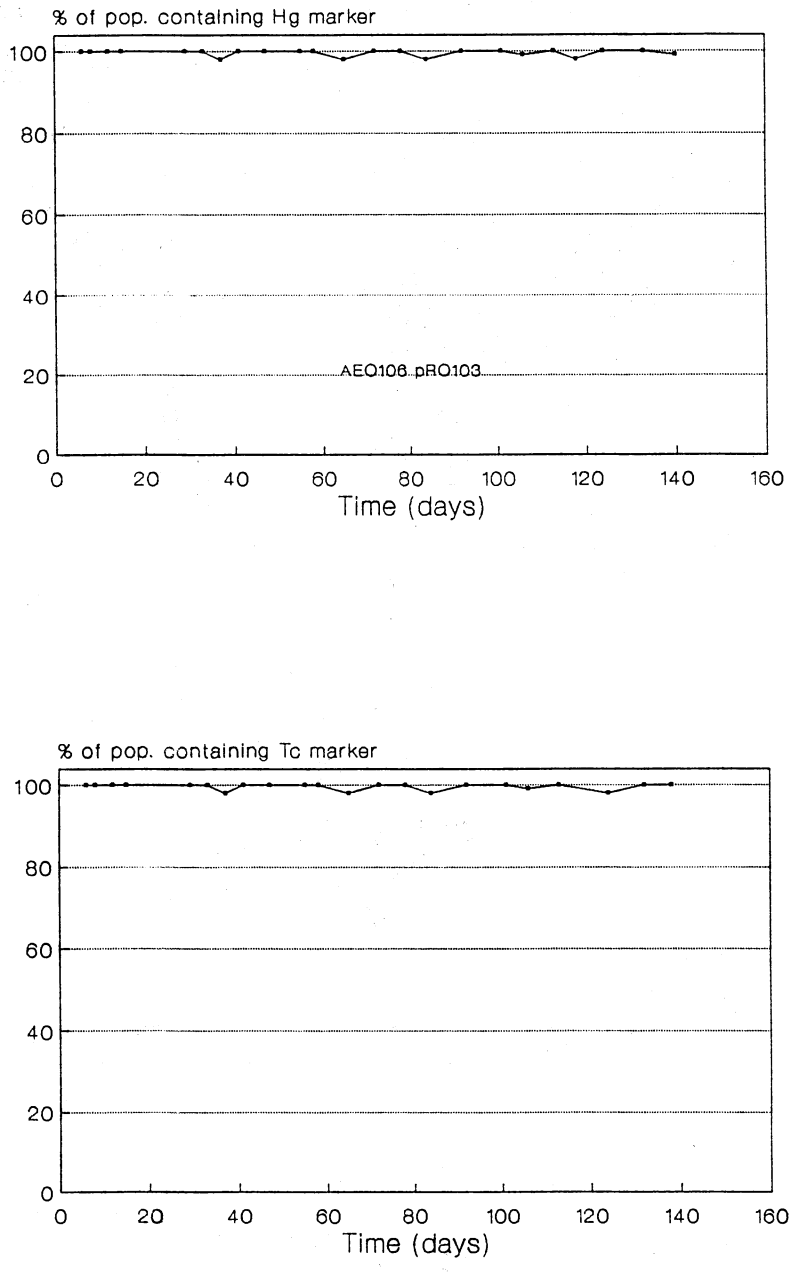


Figure 11. pRO103 Stability During Five Stage Operation

TFD as sole carbon source, even when both culture populations decreased in the fifth stage. The small percentage of the population that did not grow on the selective plates could be the result of mutational changes in the plasmids or actually reflect the loss of the plasmid or transposon from the culture. Plasmid isolation from the random colonies revealed plasmids, confirming the results of the plasmid stability assay. No isolation ever indicated complete plasmid loss even in the few colonies that did not grow when replica plated on the selective plates.

During the five stages of operation, one dominant contaminating bacterial species was noted. One pale yellow colony consistently appeared on the non-selective plates in the AEO106 pRO101 reactor. It also grew when replica plated on the mercury and tetracycline agar plates. API diagnostic strips identified the bacterium as Pseudomonas maltophilia and a literature review on this strain also corresponded with this identification (Bergey's Manual of Determinative Bacteriology, 8th edition). Attempts to grow this strain on TFD as a sole carbon source failed. A plasmid of similar size to pRO101 was isolated from this contaminant (Figure 12). The ratio of Pseudomonas maltophilia to the total population over the five stage period is given in Figure 13. The contaminating bacteria remained at a relatively low number throughout the first four stages and significantly increased during the fifth. The contaminating bacteria complicated the monitoring of the strain of

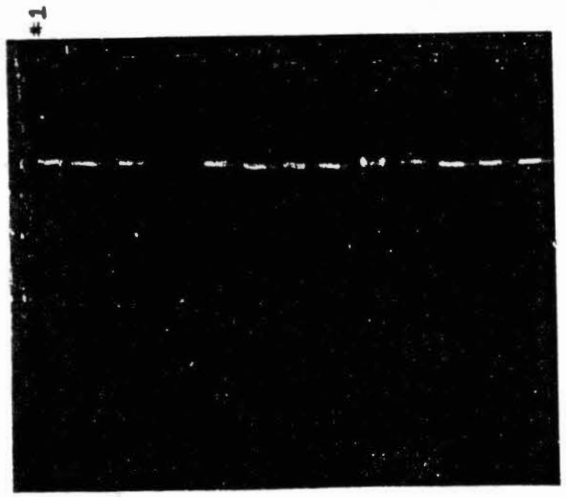


Figure 12. Plasmid DNA Isolation From Ps. maltophilia Agarose gel showing plasmids from colonies that did not express tetracycline resistance upon direct plating; Lane 1 is pRO101 stock, remaining lanes contain plasmid DNA from random colonies.

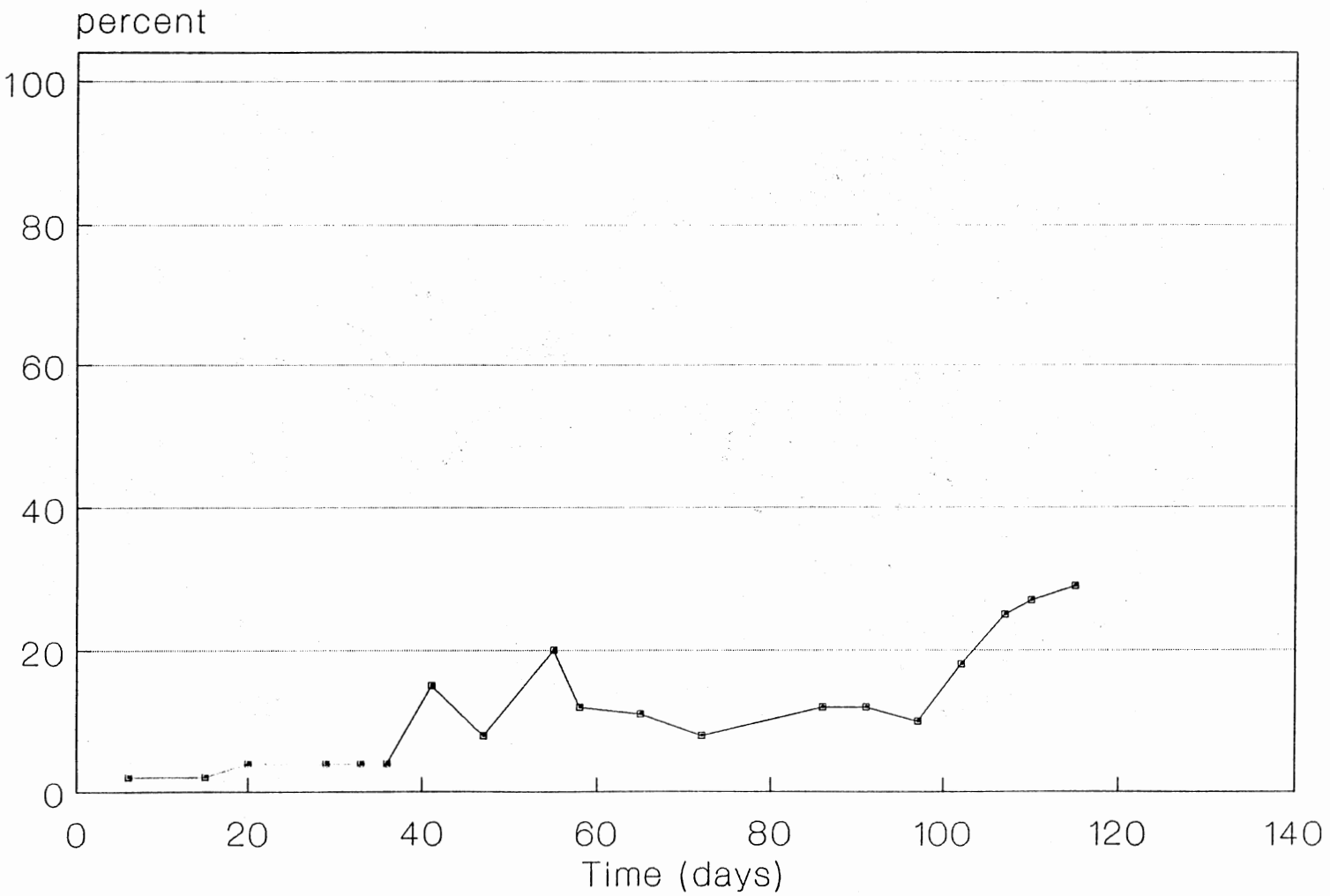


Figure 13. Growth of Ps. maltophilia (% of total pop.)

interest and therefore the reactor system was taken down and sterilized before the next experimental stage. Pseudomonas maltophilia was also isolated on two other occasions: once again in the pRO101 reactor and also in the pRO103 chemostat.

Starvation Conditions

The next series of experiments was carried out to determine what effect non-selective conditions would have on the stability of the inducible and constitutive plasmids. Due to the fact that the reactor could not be successfully maintained on CAA as sole carbon source without contamination, the reactors were subjected to non-selective conditions by depriving the biofilms of any carbon source. The biofilm composition and plasmid stability were monitored during conditions in which the reactors were alternately fed TFD followed by starvation of carbon source.

After the reactors were sterilized and set up, they were again inoculated and biofilms were established. Both reactors were filled with 200 mg/L TFD and inoculated with AEO106 pRO101 and AEO106 pRO103 respectively. The reactors were not fed continuously until the TFD level within the system decreased. The reactors were then maintained as continuous cultures. A loading rate of 8 g/L*d was maintained throughout so that pure oxygen would not be required to maintain oxygen levels. The biofilm composition was again monitored by taking samples and plating. Once a

stable biofilm was established on a 2% TFD feed, the feed was switched to MMO with no carbon source, and the culture and genetic stability were measured. After the biofilm density decreased to a low level the TFD was added back to the feed and the biofilms were reestablished to the previous levels; the starve/feed cycle was then repeated. Figures 14 and 15 show the results of the viable count measurements for the reactors containing AEO106 pRO101 and pRO103 respectively. The results from the plasmid stability assay are given in Figures 16 and 17.

During the operation of the reactor systems, yellow colonies were again noted on the plates from the pRO101 reactor during the second starvation cycle. API diagnostic tests identified the contaminant as Pseudomonas maltophilia. Figure 18 plots the ratio of Ps. maltophilia to total bacteria within the reactor. The contaminating bacteria remained at a low density during the TFD feeding stage but increases greatly during carbon limitation. Plasmid isolation from the contaminant revealed a plasmid of similar size to pRO101. All attempts to grow the isolate on TFD as sole carbon source failed. The AEO106 pRO103 reactor remained as a pure culture.

Plasmid stability was also monitored in the Ps. maltophilia strain. Figure 19 shows the percentage of the Ps. maltophilia colonies containing the selective markers during the last starvation cycle in AEO106 pRO101 reactor.

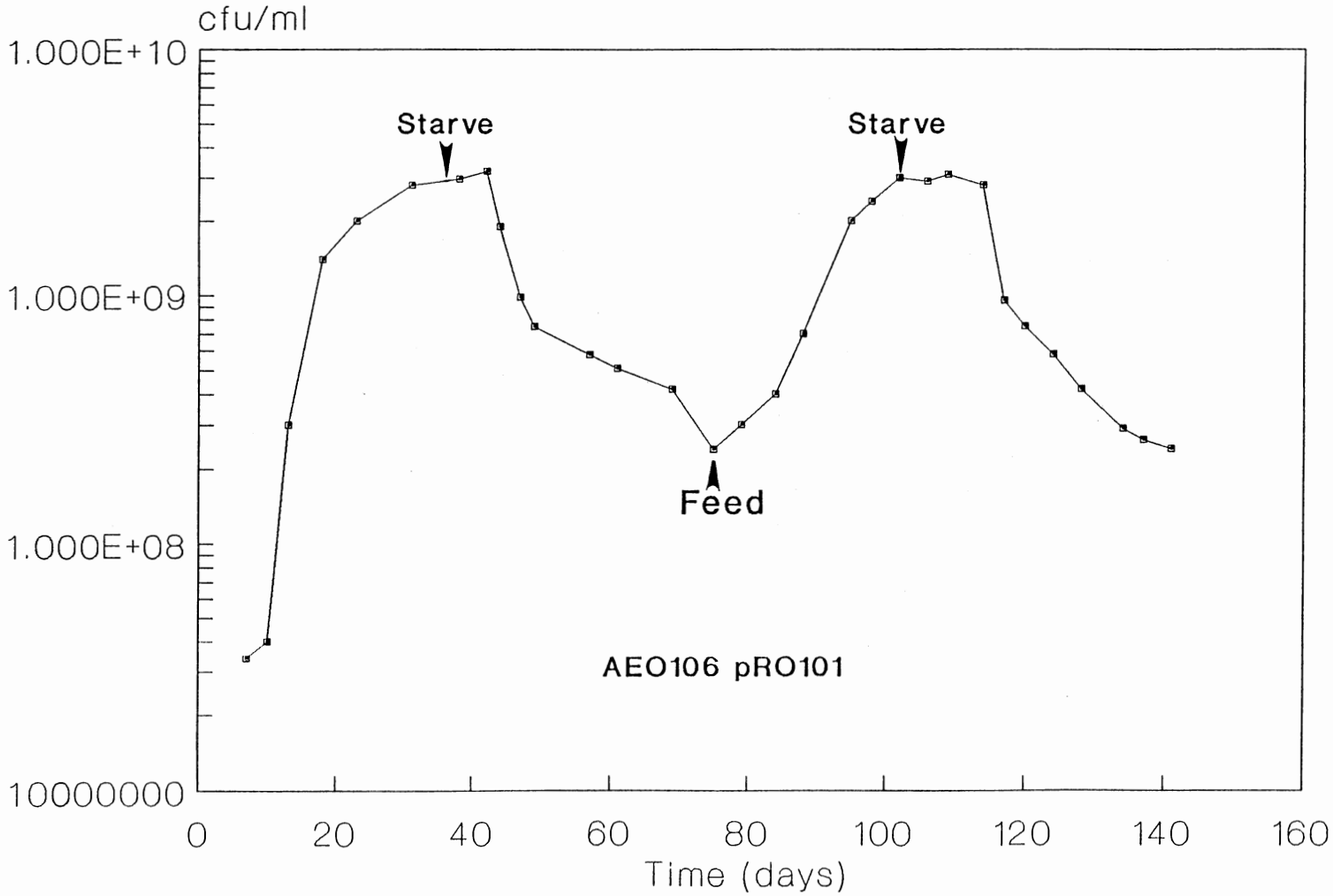


Figure 14. Viable Counts During Feed/Starve Cycles

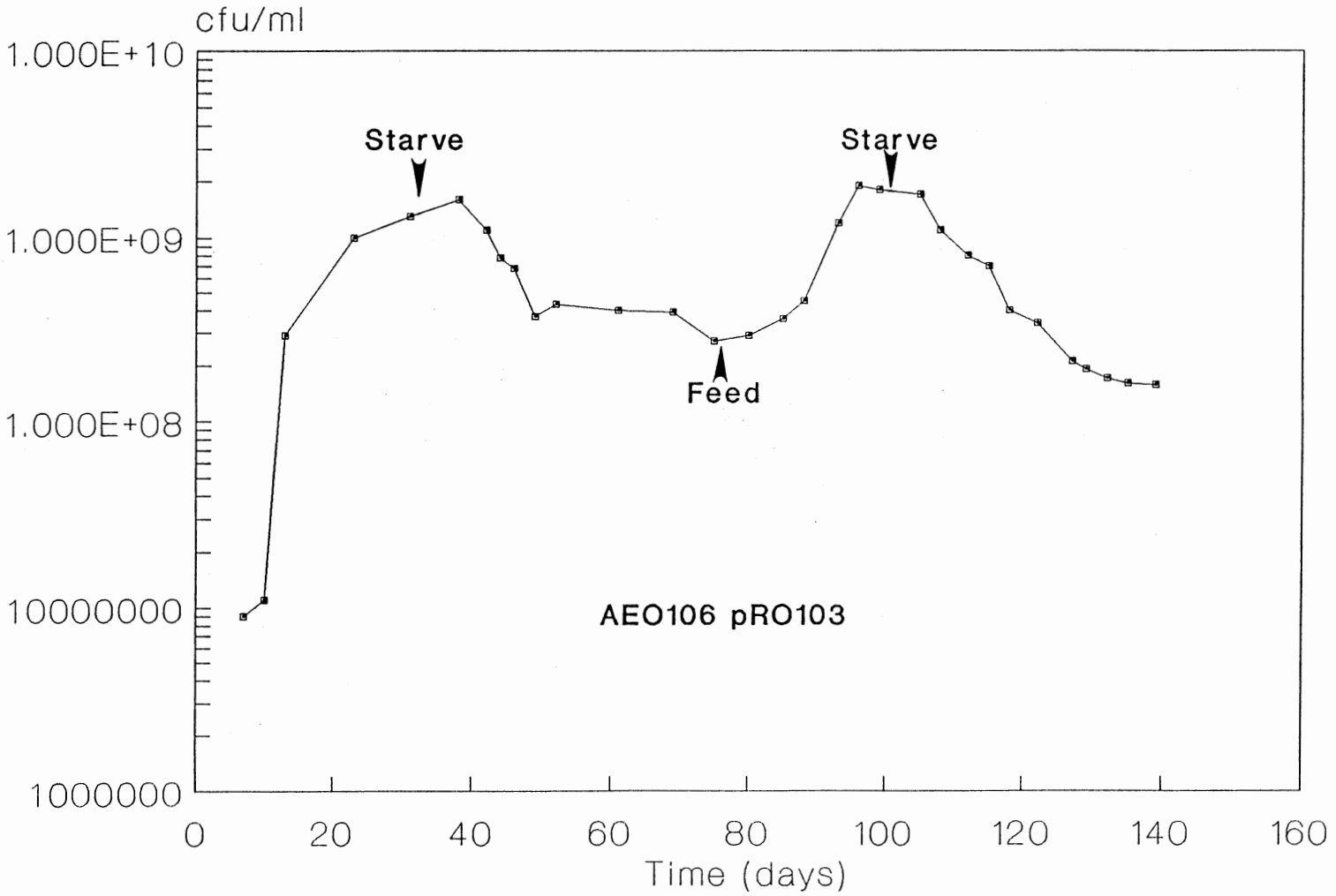


Figure 15. Viable Counts During Feed/Starvation Cycles

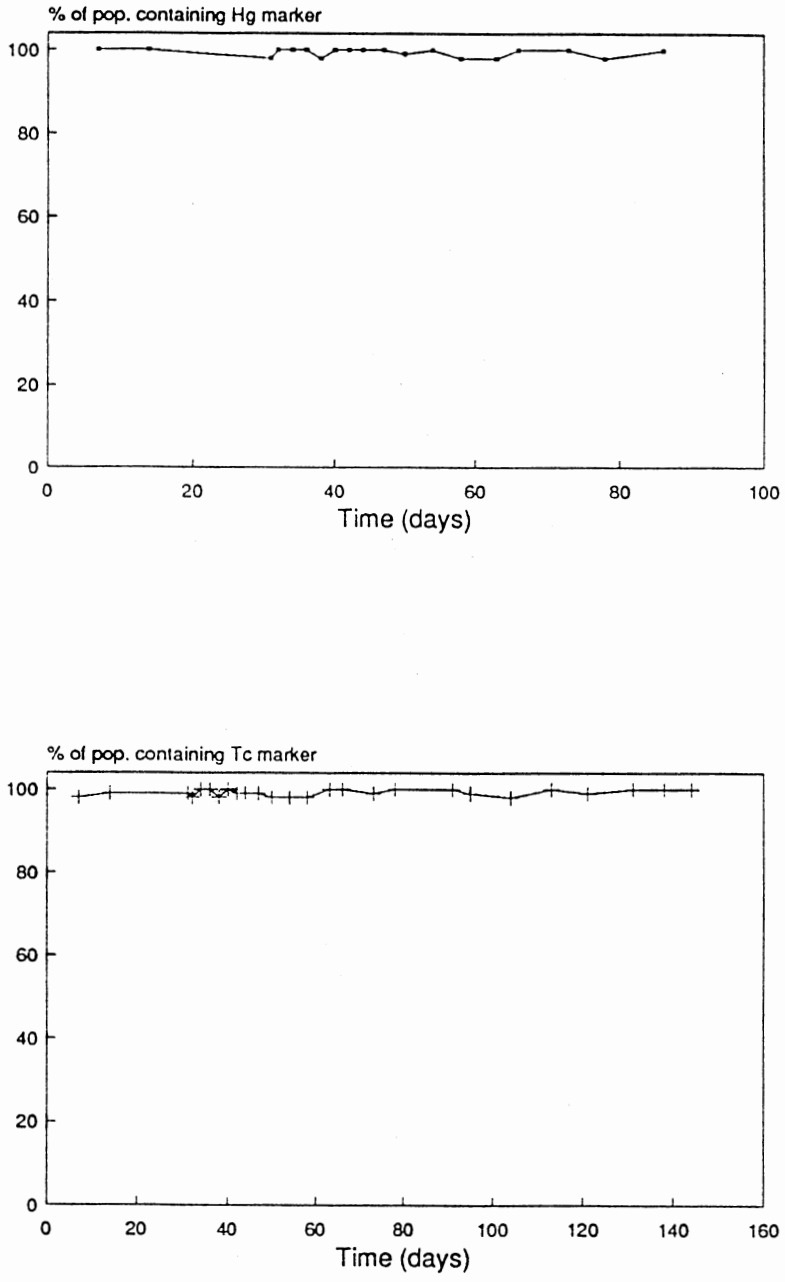


Figure 16. pRO101 Stability During Feed/Starve Cycles

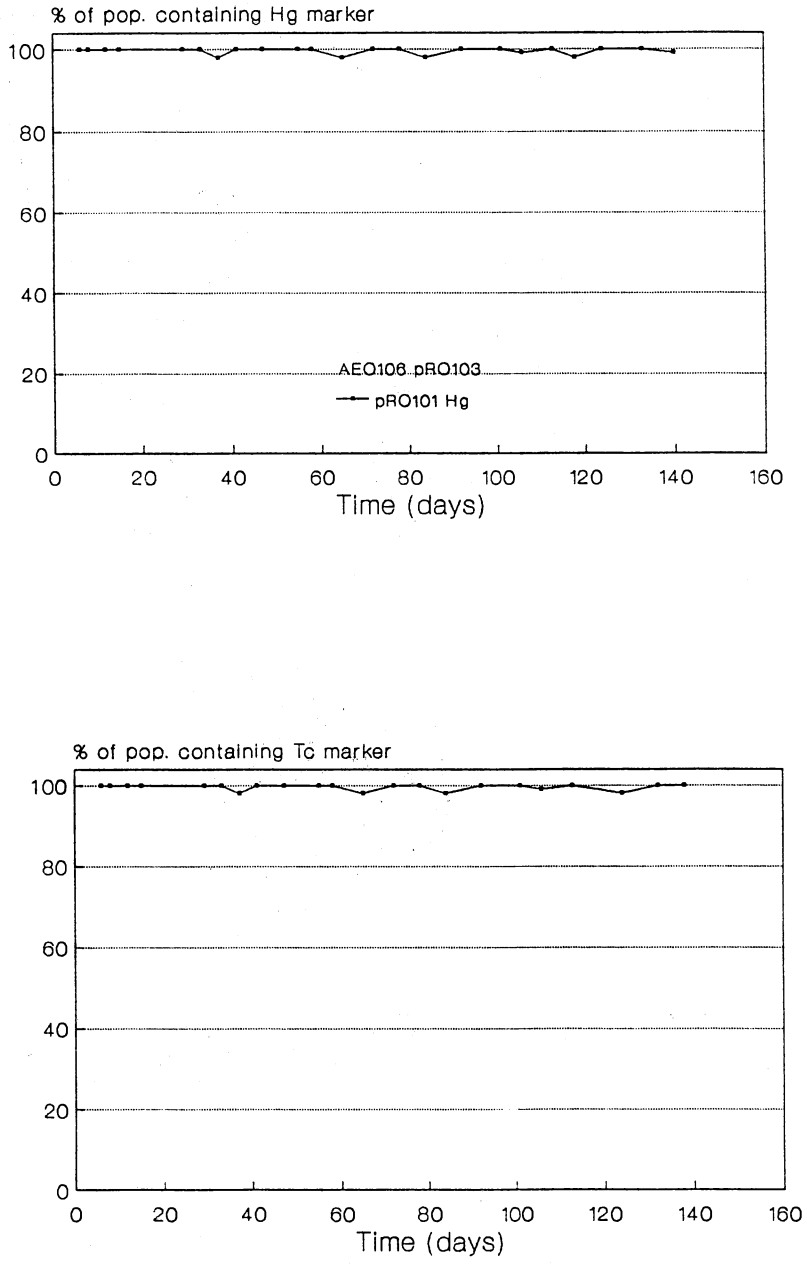


Figure 17. pRO103 Stability During Feed/Starve Cycles

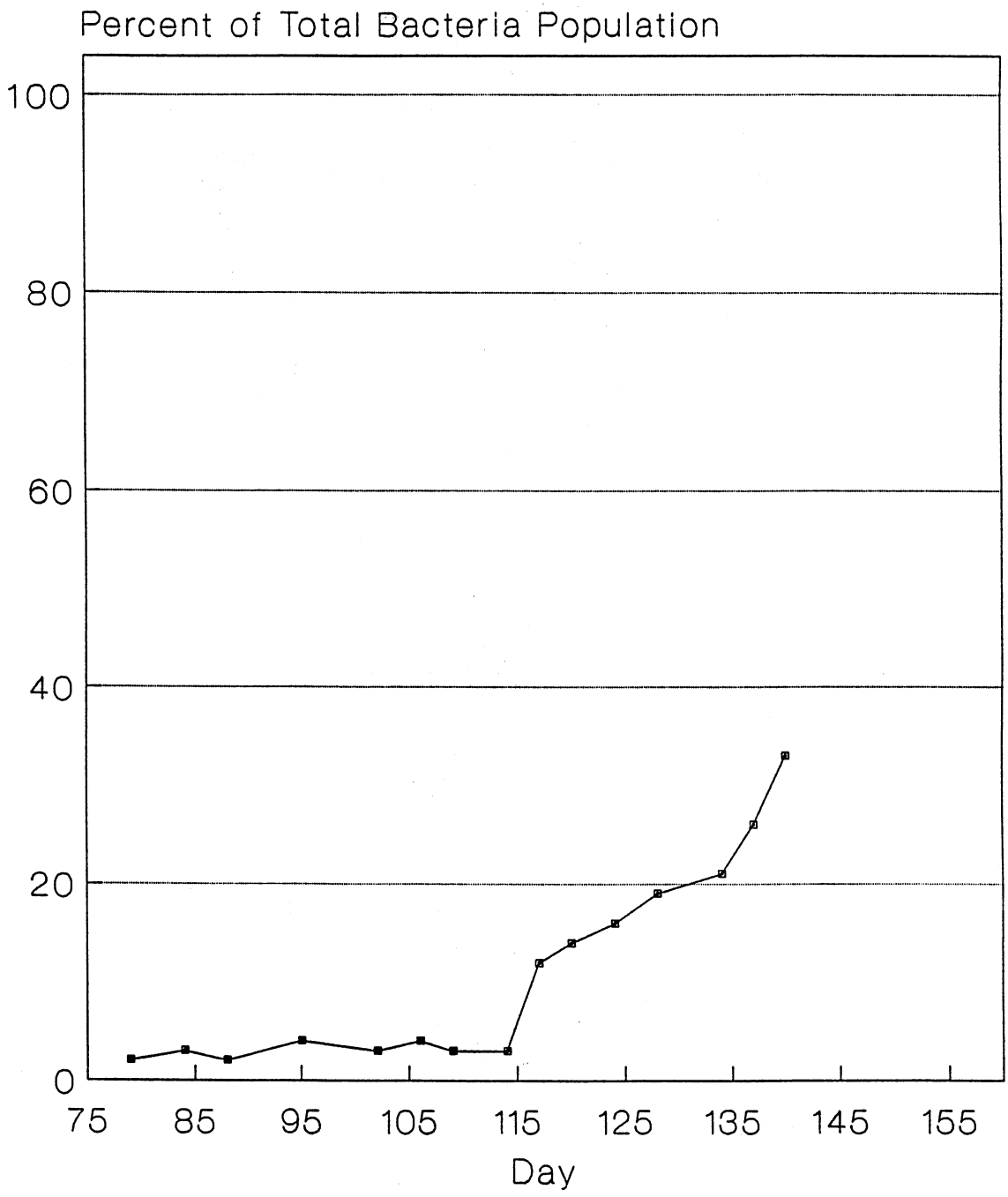
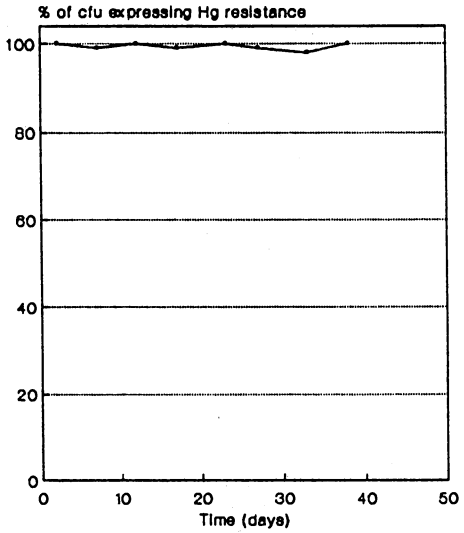


Figure 18. Ratio of Ps. maltophilia to Total Population in the AEO106 pRO101 Reactor During the Second Feed/Starve Cycle



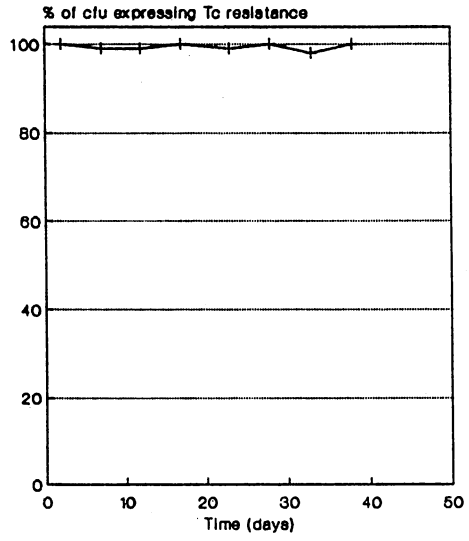


Figure 19. Stability of Plasmid in Ps. maltophilia During the Second Starvation Period in Reactor AEO106 pRO101

Chemostat Studies

One liter New Brunswick chemostats were set up to parallel the experiments carried out in the reactor systems. The chemostat vessel was filled with sterile MMO. A 10% inoculum of an overnight liquid culture grown in MMO plus 0.05% TFD was used to inoculate the chemostat. The chemostat systems were fed with 2% TFD at a feed rate of 2.4 ml/min. The OD was monitored during initial feeding to insure that the starting culture would not be washed out upon continuous feeding. The operating conditions for the chemostats are given in Table IV. The chemostats were maintained at room temperature and aerated with sterile filtered air from the built-in air pumps. Sterile samples were taken through the sampling assembly and dilutions were made and plated on the non-selective agar plates. The replica plating procedure was used to monitor the selective markers. The selective markers were monitored during growth in the presence of TFD in the feed solution, with interval periods of carbon starvation.

TABLE IV
CHEMOSTAT OPERATING CONDITIONS

Feed rate	2.4 ml/min
TFD feed conc.	2% in MMO
Operating temp.	room temp.
Chemostat volume	1.2 L
Stir rod RPM	100

Figures 20 and 21 show the viable counts measured during the feeding/starvation cycles for pRO101 and pRO103 respectively. The stability of the resistance markers during these experiments is shown in Figures 22 and 23.

Discussion

The development of a monoculture reactor system proved to be difficult. Maintenance of the reactor system with CAA as sole carbon source for an extended time permitted contamination of the system and complicated the monitoring of the AEO106 strains. Contaminants quickly grew in the reactor system, becoming a major portion of the total population and thereby obscuring the study of the desired strain. The use of TFD as sole carbon source prevented the rapid growth of most contaminants. Long-term maintenance of a monoculture was possible during selective growth in one of the biofilm reactors during one of the experimental trials.

One recurring contaminant was identified as Pseudomonas maltophilia. The contaminant was mercury and tetracycline resistant and carried a plasmid of similar size to the pJP4 derivatives, indicating plasmid transfer to the contaminant. It remained only a small portion of the total population in the reactors maintained on TFD, but grew to higher levels when the AEO106 culture began to decline during high loading rates or starvation. All attempts to grow Ps. maltophilia on TFD as sole carbon source failed.

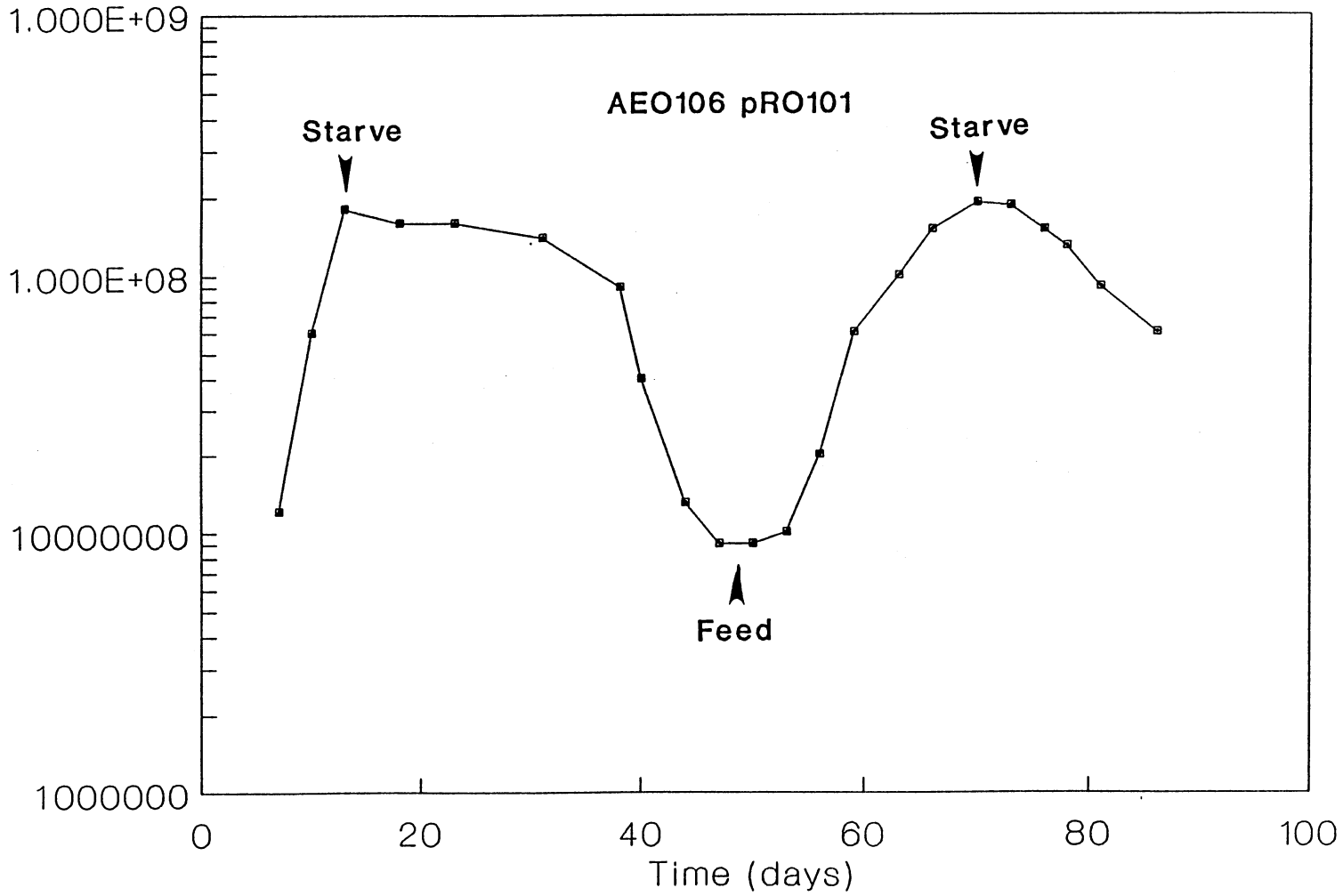


Figure 20. Viable Counts During Feed/Starve Cycles in Chemostats

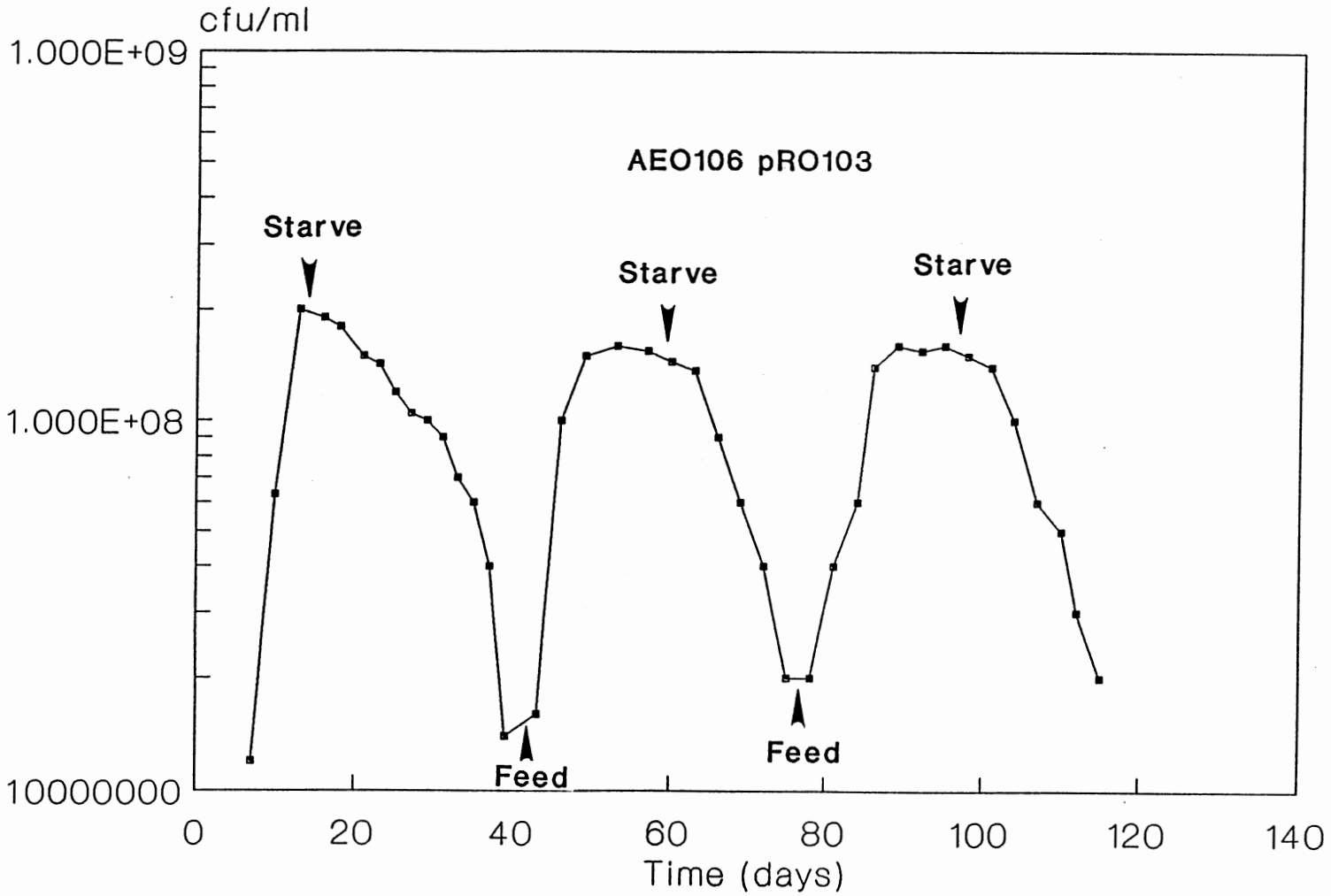


Figure 21. Viable Counts During Feed/Starve Cycle in Chemostats

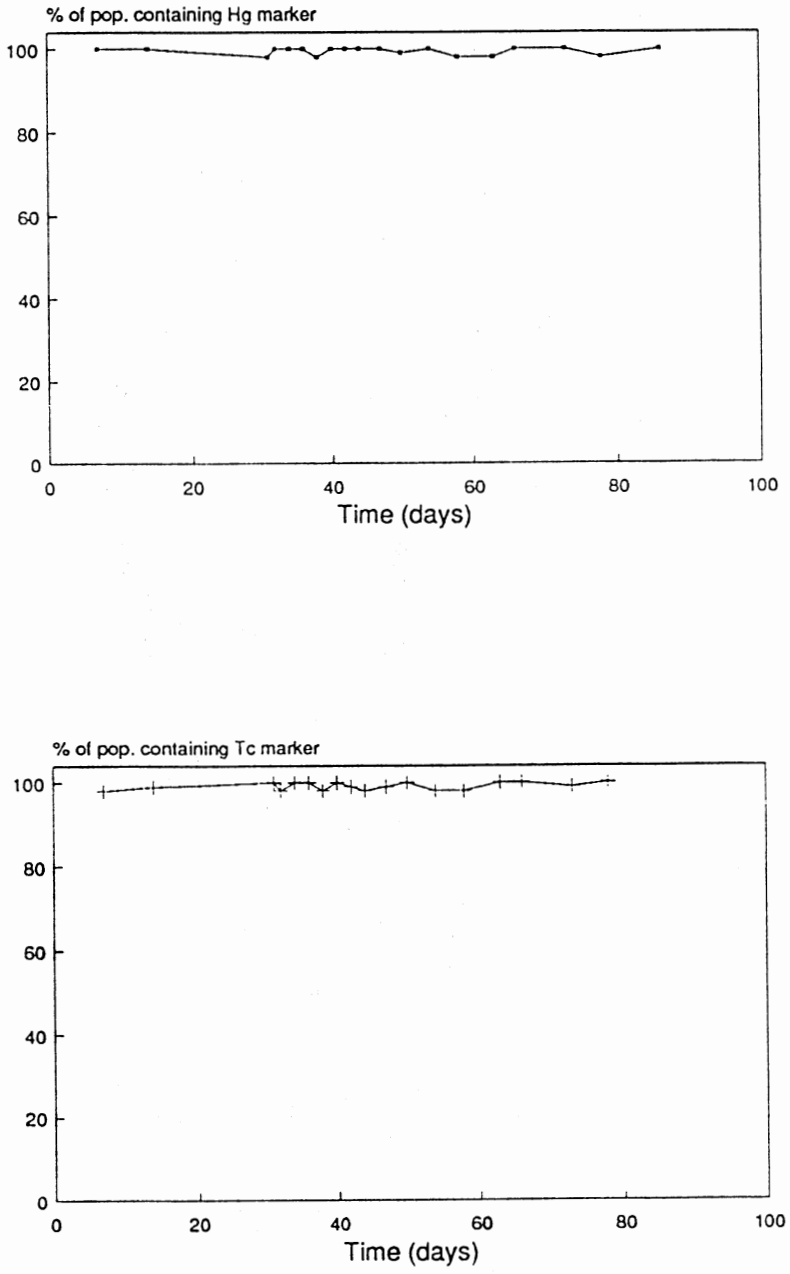


Figure 22. pRO101 Stability During Feed/Starve Cycles

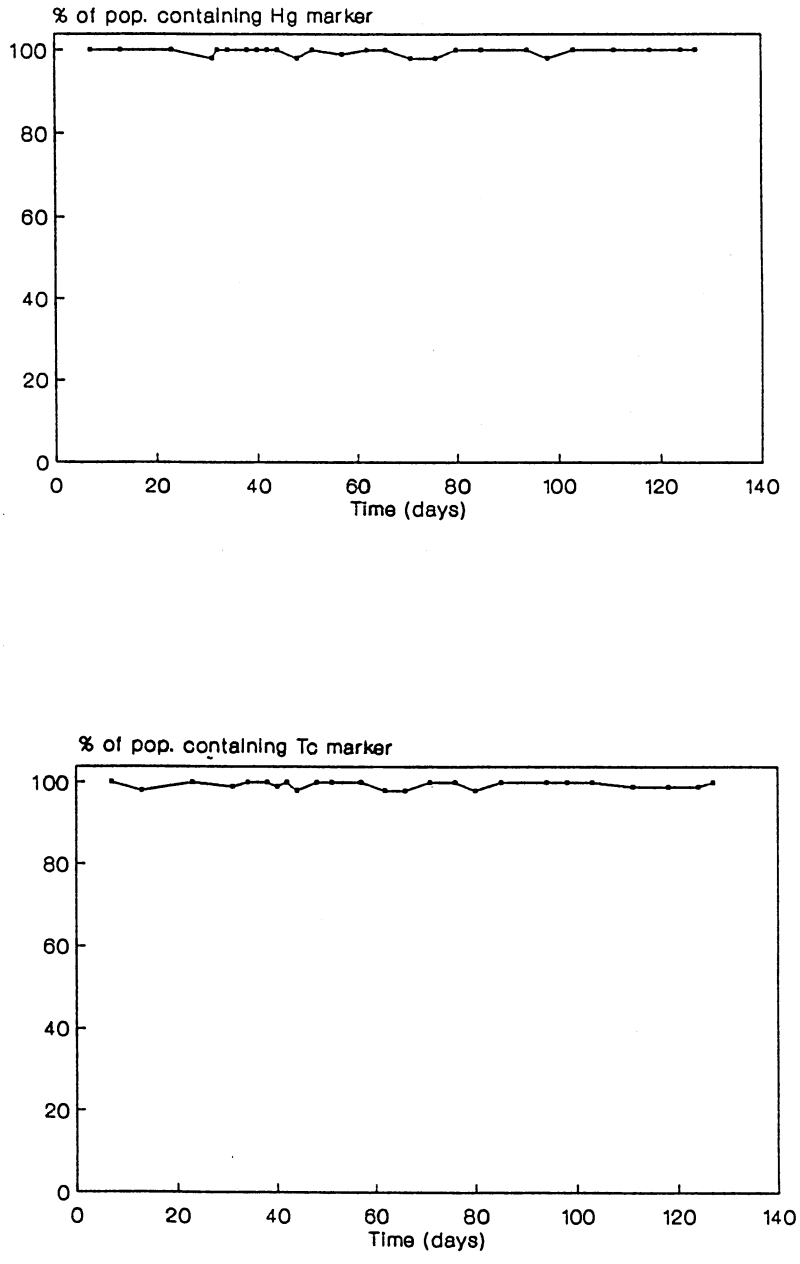


Figure 23. pRO103 Stability During Feed/Starve Cycles

Plasmid stability assays indicated that both the tetracycline and mercury markers were retained during growth on TFD and during carbon starvation for both strains of AEO106, both in the biofilm reactors and chemostats. Plasmid isolation confirmed that the plasmids were maintained during all of the growth conditions. In no case was the complete loss of either plasmid detected. The selective markers were maintained in the Ps. maltophilia during the time plasmid stability was monitored.

Due to the time involved in the development of the procedures and reactor system, and the time involved in the conducting each experiment in the reactor system, additional variations in operating conditions and further repetition of the experiments could not be carried out. Further studies involving the non-engineered strain JMP134 pJP4 were not conducted but is one possible area for follow up studies. Additionally, further studies involving the growth rates of the plasmid free versus the plasmid containing cells might be of interest.

CHAPTER V

SUMMARY AND CONCLUSIONS

Maintenance of a plasmid during conditions in which the plasmid provides no benefit to the bacteria is unfavorable and in most cases bacteria have evolved a system in which the majority of the population are plasmid free (Chakrabarty 1980). The small portion of the population that retain the plasmid are still present in the event that conditions change and the plasmid is needed. The evolution of such a system is energy efficient, since energy would be wasted on the maintenance and replication of a plasmid during non-selective conditions. An extra metabolic burden would be carried by a constitutively expressed plasmid during non-selective conditions and exert further selective pressure toward plasmid free cells. The experiments in this study were designed and conducted based on these observations.

Maintenance of monoculture biofilms during non-selective conditions was not possible. Bacterial contaminants grew easily in the reactors under these conditions. Maintenance of monocultures was difficult even when the cultures were maintained under selective conditions. Both plasmids pRO101 and pRO103 were maintained stably within AEO106 during growth on TFD and when starved

of TFD in both the biofilm reactor system and chemostats. The maintenance of these engineered plasmids under non-selective conditions (starved of TFD) did not result in any detectable plasmid loss or transposon loss from the bacteria. In addition, the constitutive expression of the TFD pathway by pRO103 had no significant effect on the stability of the plasmid during growth under non-selective conditions. No plasmid loss was detected in either the free cells grown in the chemostats or the immobilized cells grown as a biofilm. In addition, no loss of transposon was noted.

From these observations it appears that under the conditions chosen in this study, the maintenance and expression of the plasmids pRO101 and pRO103 produced no observable selective pressures toward plasmid free cells. These observations oppose the notion that degradative plasmids exist as mobile DNA reservoirs in order to minimize the total DNA content within a population. Other degradative plasmids, such as the TOL plasmid, which encodes for toluene degradation, have been reported to be maintained within the bacteria even when it reduces the growth rate of the host (Shapiro et al. 1980).

Several factors have been reported that influence the maintenance of low copy plasmids under non-selective conditions (Caulcott et al. 1987). DNA sequences have been identified that are involved in plasmid maintenance (Meacock and Cohen 1980). Austin and Abeles (1983) report plasmid maintenance regions in P1 plasmids that promote

equipartition, and despite the fact that there may be only two copies of the plasmid in a cell, daughter cells lacking plasmids are rare.

It has also been found that growth conditions as well as the immobilization of cells can alter the stability of plasmids (Godwin and Slater 1979, Nasri et al. 1987). The findings presented here indicate no detectable differences in plasmid stability between the immobilized or free cells; in both the plasmids were stable.

Other explanations exist for the stability of plasmids pRO101 and pRO103. The deletion of the 3.9 kb fragment from pRO101 may carry positive regulatory elements for genes in the TFD pathway. Using oxygen uptake studies, Harker (1989) has observed that the constitutive expression of the enzymes by pRO103 in the TFD pathway is at lower levels than in the induced state. Perkins et al. (1989) and Kaphammer et al. (1990) have reported a positive regulatory gene, tfdS, which regulates the expression of the second enzyme in the TFD pathway and maps within part of the 3.9 kb deletion fragment absent in the constitutive strain. Therefore, the constitutive expression of the TFD pathway by plasmid pRO103 may not place an excessive metabolic burden on the cell.

The stability of the plasmids could also be due to other unknown functions of the plasmid. A large portion of the plasmid has not been fully characterized and could carry genes that are beneficial or necessary to the cell, thereby preventing the selection of plasmid-free cells.

Both strains showed no observable differences in growth characteristics. Both also reacted with a drop in viable counts at high loading rates of TFD, possibly due to toxic effects of high concentrations of TFD or intermediates in the TFD pathway. The viable counts in the biofilm reactors were slightly higher for pRO101 throughout, but could be a result of differences in reactors. Yang (1990) did observe measurable differences in biomass between the two strains that were not reflected in the viable count measurements.

Ps. maltophilia was identified as a reactor and chemostat contaminant. The density of the contaminant remained low and only increased when the AE0106 cultures decreased. All attempts to grow the contaminant on TFD as sole carbon source failed. However, Ps. maltophilia expressed tetracycline and mercury resistance. Kukor et al. (1989) have shown that some strains of Pseudomonas carry pJP4 but cannot grow on TFD as sole carbon source due to the absence of a chromosomally encoded maleylacetate reductase gene. Pseudomonas maltophilia is therefore probably degrading TFD but is unable to utilize this pathway for energy requirements. This is indicated by the low levels of the contaminant in the reactors and by the fact that only when AE0106 cultures decline does the population increase. It is probable that the contaminant is using any available carbon from the biofilm. As the biofilm density decreases then a carbon source from dead cells is made available.

The transfer of pRO101 to Ps. maltophilia was observed within the biofilm reactor. Plasmid pRO101 was maintained within the bacterium even during non-selective conditions. Plasmid pJP4 derivatives have been observed to freely transfer to numerous strains (Don and Pemberton 1981) which has likely played a major role in the widespread occurrence of populations capable of degrading TFD. Such ease of transfer may pose environmental risk considerations concerning the use of engineered derivatives of pJP4. However, the broad host range of plasmids encoding for the degradation of toxic chemicals might lead to useful applications in contaminated areas suited to a particular host.

During this study effective protocols were developed for the monitoring and maintenance of the biofilm reactor system. It was observed that the direct plating of bacteria on tetracycline plates resulted in little growth due to factors other than plasmid loss.

The application of this type of reactor as a monoculture system for waste treatment seems unlikely. Monocultures could not be maintained during long-term operation even during stringent laboratory handling. The parameters for the reactor design and operation conditions could be applied to mixed culture systems and may be of benefit there.

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APPENDIX A

MEDIA PREPARATION

Mineral Medium -- MMO Salt Base (per 1 liter)

Distilled water	970 ml
Solution A	40 ml
Solution B	20 ml
Solution C	10 ml

each component is autoclaved separately, then combined

Solution A: 1 M sodium/potassium phosphate buffer

Na_2HPO_4 (1 M)	220 ml
KH_2PO_4 (1 M)	190 ml

final pH should be 6.8

Solution B:

Distilled water	to 1000 ml
nitrilotriacetate	10.00 g
$MgSO_4$	14.45 g
$CaCl_2$ $2H_2O$	3.34 g
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	9.25 mg
$FeSO_4$ $7H_2O$	0.1 g
Metal "44"	50 ml

Solution C: 10% $(NH_4)_2SO_4$

Distilled water	100 ml
$(NH_4)_2SO_4$	10 g

Metal "44"

Distilled water	100 ml
EDTA	0.25 g
$FeSO_4$ $7H_2O$	0.5 g
$MnSO_4$ H_2O	0.154 g
$CuSO_4$ $5H_2O$	0.039 g
$Co(NO_3)_2$ $6H_2O$	0.025 g
$ZnSO_4$ $7H_2O$	0.11 mg
$Na_2B_4O_7$ $10H_2O$	0.018 mg
H_2SO_4	a few drops to retard precipitation

TNA

Distilled water	500 ml
Tryptone	2.5 g
Yeast extract	1.25 g
Dextrose	0.5 g
NaCl	4.25 g
Agar	10.0 g
$CaCl_2$ (1%)	10 ml

do not add $CaCl_2$ with antibiotic

BHI

Distilled water	500 ml
Brain heart infusion	18.5 g
Agar	10 g

Tetracycline

Added to TNA to 25 ug/ml

Stock preparation of 12.5 mg/ml in 1:1 EtOH/water

Mercury

Added to BHI to 25 ug/ml

Stock preparation of 50 mg/ml of Hg^+, using $HgCl_2$

APPENDIX B

PLASMID ISOLATION PROCECURE

Mini-Lysis: Modified Birnboim Doly procedure.

1. make 2 ml turbid suspension of culture in dist. water.
2. put 0.75 ml of this suspension into a 1.5 ml tube.
3. centrifuge 1 min., discard supernatant.
4. add 100 µl lysozyme to pellet, sit static 5 min. at room temperature.
5. add 200 µl of Alkaline SDS, mix.
6. incubate on ice, 10 min.
7. add 50 µl high salt solution; mix.
8. incubate on ice, 5 min.
9. microfuge 2 min.
10. transfer 400 µl of supernatant to fresh 1.5 ml tube.
11. add 1 ml of cold 100% ethanol.
12. incubate on ice 20 min.
13. microfuge 2 min.; discard supernatant; vacuum dry 5 min.
14. add 100 µl buffer.
15. add 50 µl of 7.5 M NH_4O acetate (cold); mix.
16. incubate on ice 20 min.
17. microfuge 2 min.; transfer supernatant to fresh 1.5 ml tube.
18. add 300 µl cold 100% EtOH.
19. incubate on ice 20 min.
20. microfuge 2 min.; discard supernatant.
21. vacuum dry pellet for 10 min.
22. add 40 µl cold .04M sodium phosphate buffer; mix.

Lysozyme solution
2 mg/ml lysozyme, 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), prepare fresh.

Alkaline SDS
0.2 N NaOH, 1% (w/v) SDS, prepare fresh.

High salt solution
29.4 g potassium acetate, 11.5 ml acetic acid, distilled water to 100 ml.

VITA

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Canidate for the Degree of

Master of Science

Thesis: GENETIC STABILITY OF PLASMIDS pRO101 AND pRO103 IN ALCALIGENES EUTROPHUS AEO106 GROWN IN EXPANDED BED BIOFILM REACTORS

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