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Investigations on Glycolipid Production by Pseudomonas Putida grown on Toluene in Batch and Continuous Culture Conditions

Keith Foorest Dockery
Portland State University

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THESIS APPROVAL

The abstract and thesis of Keith Forrest Dockery for the Master of Science in Biology were presented November 18, 1994, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Keith Forrest Dockery for the Master of Science in Biology presented November 18, 1994.

Title: Investigations on Glycolipid Production by *Pseudomonas putida* Grown on Toluene in Batch and Continuous Culture Conditions

Utilization of toluene by *Pseudomonas putida* as its sole carbon and energy source affects morphology, outer membrane protein composition, and glycolipid production. Two strains of *P. putida* were found to utilize toluene and to coexist in continuous and batch culture. The two strains were designated translucent and opaque, based upon their readily identifiable coloration when grown on Luria agar. The translucent strain was the dominant strain in continuous culture conditions. The outer membrane proteins of *P. putida* were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. When toluene is the carbon and energy source, the trend in protein composition was towards a general increase in concentration of lower molecular weight proteins ($<70X10^4$ mol wt). A similar decrease occurred in the concentration of higher molecular weight proteins in the range of $70X10^4$-$9X10^4$ mol wt. *P. putida* produces glycolipids when grown on toluene as a sole carbon and energy source. Three glycolipids have been isolated from chemostat and batch culture spent media, using thin layer chromatography on silica gel G_{F254}. The glycolipids are believed to be previously reported mono- and di-rhamnolipids that function as

biosurfactants. The release of glycolipid into the media is believed to function to emulsify toluene, aiding in toluene uptake.

INVESTIGATIONS ON GLYCOLIPID PRODUCTION BY *Pseudomonas putida* GROWN ON TOLUENE IN BATCH AND CONTINUOUS CULTURE CONDITIONS

by

KEITH FORREST DOCKERY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1995

ACKNOWLEDGMENTS

I wish to thank Dr. Mary L. Taylor for her guidance and assistance. I thank the members of my committee, Dr. David T. Clark, Dr. Richard B. Forbes, and Dr. David A. Dunnette, for reviewing this manuscript. I extend my thanks also to Dr. David H. Peyton for use of his vacuum distillation apparatus and Lisa Kilejian for help in preparation of this manuscript.

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INTRODUCTION

Our dependence upon petroleum has created dangerous reservoirs of environmental contamination: product storage, transfer and disposal. Failure of product storage or transfer systems releases contaminants into the surrounding environment. Disposal practices are often designed only to contain waste rather than to detoxify it into harmless waste products.

Various approaches have been considered and applied to combat the present and growing number of contaminated sites. Bioremediation of contaminated soils, slurries, or water is one method that has been implemented successfully with diverse contaminants. Bioremediation utilizes endemic bacteria to metabolize contaminants, either in situ or after removal, into non-toxic by-products. Success depends upon thorough understanding of physical conditions, nutrient availability and bacterial metabolic capability. Usually conditions and nutrients can be manipulated to increase degradation rates.

Certain bacteria enhance their ability to degrade contaminants by increasing nutrient availability. Increasing nutrient availability is accomplished through release of biosurfactants, such as glycolipids, into the bacteria's environment. Biosurfactants decrease surface tension of the aqueous environment, thereby emulsifying and/or solubilizing the contaminant. As a result, the bacteria increase production of biosurfactants, thereby producing faster biodegradation of contaminants.

Research concerning the production of biosurfactants has applications beyond practical implications of improving biodegradation rates of

environmental contaminants. Other potential benefits of biosurfactant research include their use as fine industrial lubricants (Duvnjak et al, 1982).

Toluene is a common environmental contaminant due to its wide use in industrial processes such as the making of fuels, paints, and adhesives. Toluene is a naturally occuring, clear, colorless liquid (U. S. Department of Health and Human Services, 1994). Toluene enters the environment through leaking underground storage tanks, spills, and landfills. Toluene toxicity is well documented (U. S. Department of Health, Education, and Welfare, 1978), so permanent remediation of toluene contamination is paramount. Toluene can be readily degraded by soil microorganisms, such as *Pseudomonas putida* .

P. putida strains may catabolize toluene using one of the two metabolic pathways elucidated (Appendix D, Figure 13). First, toluene can be catabolized to a cis-dihydrodiol (3-methylcatechol), via a multicomponent dioxygenase system (Yeh, 1977). The 3-methylcatechol aromatic ring structure is then cleaved via the meta pathway into a diacid (Cerniglia, 1984; Atlas and Bartha, 1992). Second, a catabolic pathway coded on plasmid pWW0 (TOL plasmid) converts toluene into catechol, which is available for meta ring cleavage (Assinder and Williams, 1990). The TOL plasmid encodes for two essential functions in the metabolism of toluene. First is the *xyl* gene cluster coding a series of non-specific enzymes (e.g. xylene oxygenase, benzyl-alcohol dehydrogenase, and benzaldehyde dehydrogenases) necessary for successive conversions leading to catechol as the final intermediate before ring fission via the meta pathway. Second is the meta-pathway which opens the ring structures of 3-methylcatechol and catechol (Worsey and Williams, 1975)

The purpose of this study is to improve our understanding of the affects of toluene degradation by *P. putida* by examining: the population dynamics of

P. putida strain variation and competition in continuous culture; changes to the outer membrane structure as function of toluene utiliztion by *P. putida*; and the ability of *P. putida* to produce glycolipids as biosurfactants. Population variation was studied to examine the connection between substrate utilization and strain success as measured by strain dominance in continuous culture. Outer membrane proteins were examined to investigate their relationship with extracellular glycolipid encapsulation of toluene and with subsequent uptake of toluene filled vessicles (Appendix D, Figure 14) (Finnerty and Singer, 1985). Continuous culture has been used to optimize the utilization of hydrocarbon substrates and production of biosurfactants (Atlas and Bartha, 1992; Gruber et al, 1993; Koch et al, 1991; Reiling et al, 1986). The biosurfactants sought in this experiment are mono- and di-rhamnolipids (Rh) . Rh are acylated sugar derivatives, that combined with glycosyl diglycerides, form two classes of glycolipids (Appendix D, Figure 15) (Zajic and Mahomedy, 1984).

MATERIALS AND METHODS

Overview

This section describes the procedures used to establish bacterial strain competition in continuous culture, the effect of toluene on outer membrane protein composition, and the effect of toluene and culture conditions on glycolipid production. Culturing procedures concern plate, batch, and continuous culture techniques, as well as descriptive and quantitative cell measurements. Outer membrane protein procedures involve extraction and separation of individual outer membrane proteins from *P. putida*. Glycolipid production procedures involve extraction and isolation of glycolipids from various culture conditions.

Reagents.

All reagents used were analytical grade.

Culture Methods

P. putida was grown in batch and continuous culture, using a modified mineral salts media and either toluene or succinate as the carbon and energy source.

Media Preparation. The media used in batch and continuous culture is 2xBushnell-Haas Modified Mineral Salts Media (2xMMSM) (Anderson, 1992; Bird, 1994). Analytical reagents used to prepare 2xMMSM were dissolved in 1 l distilled deionized H_2O (ddH_2O), sequentially, as follows: 3.2 g K_2HPO_4; 0.8 g KH_2PO_4; 0.82 g $MgSO_4 \cdot 7H_2O$; 0.08 g $CaCl_2 \cdot 2H_2O$; 2.0 g NH_4NO_3; 0.1 g $FeSO_4 \cdot 7H_2O$. Each reagent was thoroughly dissolved before adding the next.

The solution was then titrated with moderate stirring with 1M NaOH to pH 7.4 at 25 °C. The solution was autoclaved for 20 min at 121 °C. Final pH was 7.00-7.24.

Growth in Batch Culture. Bacteria were grown in succinate stock culture medium prepared as follows: 20 ml 2xMMSM + 0.1% succinate in 125 ml Erlenmeyer flasks. Bacteria were also grown in toluene stock culture medium, prepared as follows: 20 ml 2xMMSM in 125 ml nephelometer flasks (Kontes Glass Co., #4984) with toluene in the sidearms to provide vapor. Both stock cultures were placed at 25 °C at 150 rpm on a rotary shaker and transferred every 2 to 4 days. Stock cultures were checked for purity on Luria agar at 28 °C. Except where noted all plate culturing was performed on Luria agar. Batch cultures were grown at 20% of flask volume at 28-30 °C at 150 rpm for 48 hours.

Preparation of 2xMMSM Agar Plates with Toluene Insert. For recovery and purification of stock cultures, 2xMMSM agar plates were prepared. 200 ml 4xBushnell-Haas Modified Mineral Salts Medium (4xMMSM) was prepared as previously reported (Anderson, 1992) in a 1 l flask. 200 ml 5% purified agar (Difco laboratories, Detroit, MI, No. 0560-01) in a 500 ml flask in ddH_20 was prepared by heating to boiling in microwave to dissolve the agar. The agar and 4xMMSM flasks were autoclaved for 20 min at 121 °C. After autoclaving the 4xMMSM was stirred to resuspend precipitate. The agar and 4xMMSM were mixed, cooled to 50 °C, and poured into sterile pyrex petri dishes with glass inserts for toluene. The glass inserts used were glass, flat-bottomed, cylindrical vessels, approximately 1.5 cm in diameter and 1.5 cm in height.

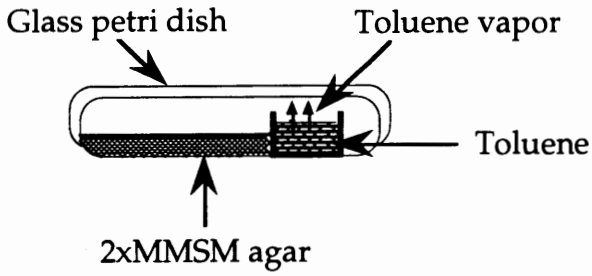


Figure 1. 2xMMSM agar plate with toluene insert

Continuous Culture. Continuous cultures were run using a continuous culture apparatus (New Brunswick Scientific, Model Bioflo C30). The culture volume was 350 ml. Addition of 2xMMSM was added at a dilution rate, D = 0.026 h^{-1}, except where noted. Dilution was controlled by a peristaltic pump. The temperature was controlled by an external heating/refrigerating water recirculation system connected to a heat exchanger in the fermenter vessel. Except where noted, temperature was set at 30 °C. Air flow was set at 250 ml/min. Toluene was used as the sole carbon and energy source. Toluene was added in vapor form by bubbling the air flow through a layer of toluene in a 500 ml Erlenmeyer flask, filled approximately to 250 ml, for the initial run. For the second run air was bubbled through a cylindrical flask filled with toluene to a depth of 8 cm and fitted with a size 10 stopper. The culture was stirred by an internal magnetic impeller at 200 rpm.

The feed rate for medium was adjusted to the peristaltic pump's lowest flow setting, F = 9 ± 2 ml/hr, D= 0.026 hr^{-1}, except where otherwise noted. Flow rate F was determined by calculating an optimum flow rate using the equation, $F = V*0.5*\mu_{max}$, (New Brunswick Scientific Co.), where V is the culture volume, V = 350 ml; μ_{max} is the maximum instanstaneous growth rate, $\mu_{max} = \ln 2/t_{batch}$; and t_{batch} is the generation time of the bacteria in batch culture, t_{batch} = 4.5 hr (Bird,

(Bird, 1994). The dilution rate, D, approximates the instantaneous growth rate, $D \approx \mu$, and is therefore a measure of growth rate.

Collection of Samples. The effluent from the fermenter vessel was collected in 0.5-1 l batches in 1 l Erlenmeyer flasks stored in a cooler filled with ice. Ice was replenished as needed to maintain sample temperature below 1 °C.

Samples of 20 ml or less were also taken aseptically directly from the fermenter vessel. Samples were initially pipetted from the vessel through the rubber seal; later, they were taken via a permanently installed sampler.

Outer Membrane Isolation

Lithium Chloride Extraction. Lithium chloride (LiCl) extraction, as modified from (Johnston et al, 1976), was used to isolate outer membrane proteins. Cultures were grown in 2xMMSM, with either toluene vapor or 0.1% succinate as the sole carbon and energy source. Toluene cultures were incubated at 30 °C with shaking at 150 rpm (Incubator Shaker Model G25, New Brunswick Scientific, Co.) until yellow and turbid, 49 hrs. Succinate cultures were incubated identically until they turned greenish-yellow and turbid.

The cultures were centrifuged at 8,000X*g*, in a GSA, r=5.75 in, rotor for 15 min. For centrifugation, 150 ml Corex No. 1265 tubes were used for toluene cultures and 250 ml Sorvall No. 03069 tubes were used for succinate cultures. The pellets were resuspended in LiCl extraction buffer (200 mM LiCl, 100 mM lithium acetate, adjusted to pH 6.0 with acetic acid) to give 1 gm wet weight cells per 20 ml buffer. The 1 l suspensions were placed in a 500 ml Erlenmyer flasks, containing one layer of 3 mm glass beads, and shaken at 250 rpm for 2 hr at 45 °C. The suspensions were then centrifuged at 12,000X*g* for 15 min at 4 °C. The supernatants were decanted and centrifuged at 25,000X*g* for 15 min at 4 °C. The

resulting supernatants were centrifuged in polycarbonate tubes at 100,000X*g* for 1 hr at 4 °C in a Type 60 Ti fixed angle rotor using an ultracentrifuge (Beckman L20-65B). The pellets were resuspended in 10 mM Tris, 100 mM NaCl, pH 8.0). The preparations were stored at -20 °C.

SDS-PAGE Gel Electrophoresis

The membrane protein preparations extracted with LiCl were separated by Laemmli sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), prepared with 7% and 10% polyacrylamide. For all gels, well size was 30 mm, degassing time was 15 min, and polymerization time was 45 min. Three sets of molecular weight markers (MWM) were used: Molecular Weight Marker Set #1 (Sigma), Molecular Weight Marker Set #2 (Kit No. MW-GF-70, Sigma),and Molecular Weight Marker Set #3 (Gel Filtration Calibration Kit, Pharmacia Fine Chemicals) (Appendix A, Tables V-VII).

MWM were prepared individually in 1 µg/ml concentrations and in sets to final concentrations of 1 µg/ml in sample buffer (7 ml Tris-HCl, 2 ml glycerol, 1 ml 2-mercaptoethanol, 400 mg SDS, and 0.01% bromphenol blue tracking dye). MWM were loaded into sample wells in volumes of 25-70 µl. Membrane protein preparations were to added sample buffer at 1 µl/ml. The protein preparations in sample buffer were loaded into sample wells in volumes of 35-70 µl.

All gels were stained at least overnight in Coomassie Brilliant Blue stain (0.125% R-250 Coomassie, 50% methanol, 10% glacial acetic acid) for protein band visualization. Gels were then treated with destain (100 ml glacial acetic acid, 500 ml methanol, and 400 ml ddH_20) to remove background coloration.

The gels were preserved for analysis by drying in shrink plastic. After drying, R_f values were determined and compared.

Photography. All TLC plates and SDS-PAGE gels were photographed using 100 ASA Ektar print film under overcast daylight conditions.

Glycolipid Extraction

Centrifugation: Recovery of Supernatant: Centrifuge tubes (Corex No. 1265, 150 ml) were filled with culture and centrifuged (Sorvall Superspeed RC2-B) at 16,300X*g*, in a GSA, r=5.75 in, rotor for 30 min. Pellets were discarded. The supernatant was collected.

Extraction of Glycolipid from Supernatant: The supernatant was acidified to pH 3 with concentrated H_2SO_4, and extracted in separatory funnels with four volumes of ethyl acetate. The aqueous layer was drained off and the organic layer was saved. The organic layer was dried with ~3% by weight anhydrous magnesium. The liquid extract was decanted and stored 4 °C in 4 l flask.

Vacuum Distillation in Rotary Evaporator. The ethyl acetate extract was added to a 1 l round bottomed flask in water bath and vacuum distilled at 30 °C in a Büchi Rotavapor-EL apparatus. The final yellow-orange extract was concentrated in 100 ml conical flask. The ethyl acetate distillate was collected in a 2 l round bottomed flask in ice bath for reuse.

Thin Layer Chromatography

Solvent Systems. The solvent systems used were prepared in the following ratios (vol:vol:vol): chloroform:methanol:acetic acid, 65:15:2 (Syldatk et al, 1985); chloroform:methanol:water, 65:25:4 (Ross et al,1991); acetone:n-butanol:water, 8:1:1 (Ross et al,1991).

Materials for Thin Layer Preparation:

Materials used in the preparation of the thin layer chromatograph are listed in Appendix B.

Preparation of Thin Layer. Glass plates were soaked in saturated sodium carbonate solution (after cleaning the glass plate with soap and water). The plates were rinsed well in ddH_2O and dried. Handling by edge of plate only, plates were wiped well with ether or petroleum ether, using a lintless cloth.

The thin layer was prepared according to Operating Manual 103-D, Desaga/Brinkmann Apparatus for TLC and (Touchstone, 1992). The sorbent was prepared as a slurry and applied as follows. 1) 30 g Silica Gel G F_{254} powder was mixed with 65 ml ddH_2O. 2) The mixture was homogenized in a blender for 30 sec. 3) The applicator was set to 250 mm thickness and plates were spread within 3 min. The mixture was not spread if it was too watery. Mixture was permitted to begin thickening, before application to the plate. 4) Plates were dried in a dust free cabinet. 5) Using a razor blade, approximately 3-5 mm was scraped away from the plate edges to minimize the effect of irregularities near the layer's edge upon solvent migration. 6) The plates were activated for at least 30 min at 110 °C.

Glycolipid Detection Methods

All spray reagents were prepared as described by Touchstone (1992), except where otherwise noted.

Nondestructive Reagents. UV absorption was used as a screening method for the presence of distinct spot separation. Bromcresol green was used to detect fatty acids (Appendix C, Tables VIII).

Destructive Spray Reagents

Spray reagents were used to detect lipids, sugars, and glycolipids. Destructive reagents chemically alter the sample. The reagents used for lipid detection were bromthymol blue, molybdophosphoric acid (Stahl, 1969), and rhodamine B (Appendix C, Tables IX-XI). The reagents used for glycolipid detection were Bial's reagent and dephenylamine (Appendix C, Tables XII-XIII). The reagent used for sugar detection was p-anisaldehyde (Appendix C, Table XIV).

Standard Reagents. Standard reagents were chosen according to (Shryock et al, 1984). The sugar standard was α-L-rhamnose. The lipid standard was palmitic acid (Appendix C, Table XV).

Cell Density Determination

Turbidity. Culture turbidity was measured using a Model #800-3 Klett Summerson Photoelectric Colorimeter (Klett Mfg. Co. Inc., NY) using Klett Filter numbers: 52, 54, 56, 62 and 66. Fermenter sample and sample supernatant were measured against a dd$H_2$0 sample to determine the effect of the supernatant coloration upon turbidity measurements. Filter #62 was determined to be the most effective filter for background coloration of the culture. Therefore, filter #62 was used for all turbidity measurements reported.

Viable Cell Count. Viable cell count dilutions were aliquoted into 20 ml test tubes with decanted 2xMMSM as dilutant. Dilutions were spread on Luria agar plates, grown at 28 °C and counted after colony development. Colony forming units (CFU) were determined by plate counts and used as the measure for cell density.

RESULTS

Overview

This section presents the results of experimental methods used to study population dynamics of continuous culture, the effect of toluene on outer membrane protein composition, and the effect of toluene and culture conditions on glycolipid production. The population results show evidence of *P. putida* strain variation and competition in continuous culture. Outer membrane protein results show differences in outer membrane proteins from *P. putida* grown on toluene versus succinate as the carbon source. Glycolipid production results show the extracted glycolipids from various culture conditions.

Population

Colony Type. Two strains of *P. putida* were isolated in batch and continuous culture. The two strains were designated opaque and translucent based on their appearance on Luria agar. Identification was done according to Bergey et al (1974) and Gerhardt et al (1981). The results of the strain determination are shown in Table I. Colonies were readily discerned from one another on Luria, 2xMMSM, and King's B agar.

Both translucent and opaque colony types were shown to be capable of utilizing toluene as the sole source of carbon and energy by isolating each colony type on 2xMMSM agar with glass inserts for toluene. In separate batch culture and Luria plate experiments, pure translucent cultures consistently produced opaque colonies and pure opaque cultures consistently produced translucent colonies.

TABLE I

IDENTIFICATION OF TRANSLUCENT AND OPAQUE STRAINS OF P. PUTIDA

Test	Translucent Strain	Opaque Strain
Gram	(-), rods	(-), rods
Motility	(+)	(+)
Oxidase	(+)	(+)
Oxidation/Fermentation	O(+)/F(-)	O(+)/F(-)
Gelatinase	(-)	(-)
Polyhydroxybutyrate	(-)	(-)
Fluorescence	(+)	(+)
Growth at 41 °C	(-)	(-)
Growth at 4 °C	(-)	(-)
Arginine dihydrolase	(+)	(+)
Toluene utilization	(+)	(+)
Colony description:	translucent	opaque
	light tan	creamy tan
	circular	circular
	entire	entire
	umbonate ("fried egg")	convex

Cell Density Correlation. The Klett unit absorbance data was compared to the viable cell count data (Figure 2) to determine a coefficient of correlation, $R^2 = 0.772$ (CFU/ml). The correlation was low but sufficient to utilize the turbidity measurements in Klett units to calculate cell density values.

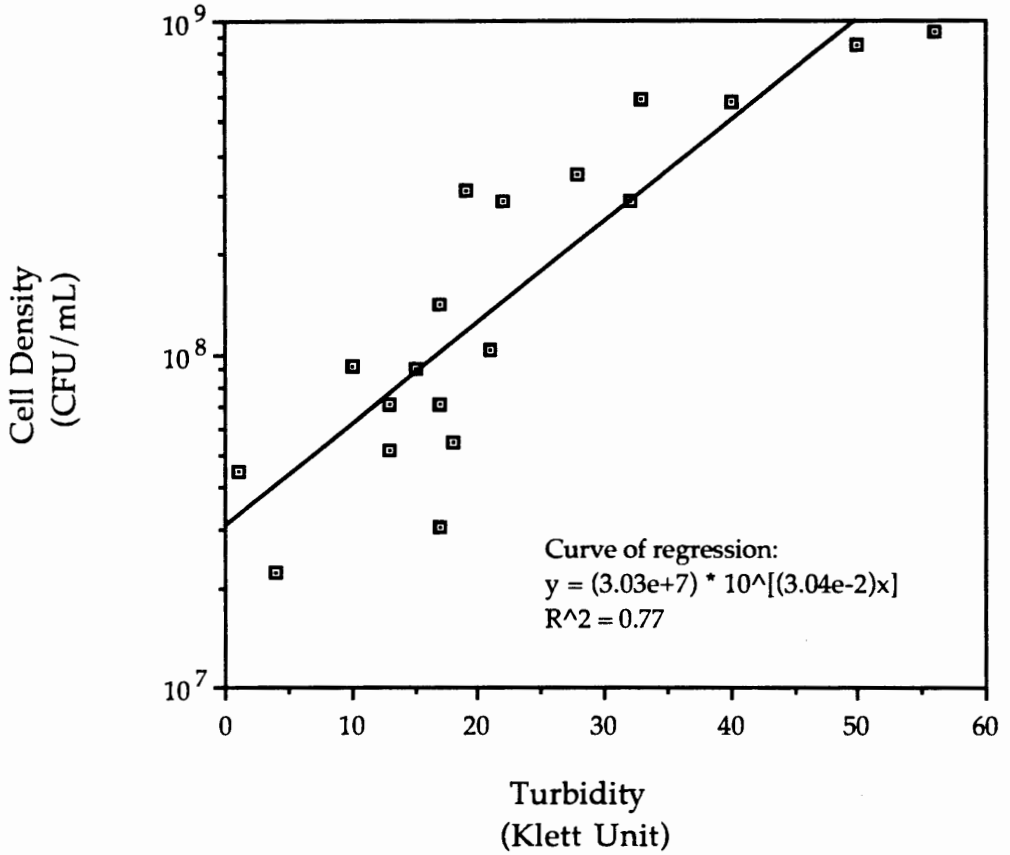


Figure 2. Turbidity-cell density correlation

Chemostat Competition. Cell density values over time were plotted against culture colony type as a percent of total population counts (Figures 3 and 4). Figure 3 shows the competition curve between two variants of *P. putida* in the chemostat at 30 °C and flow rate, $F = 9 \pm 2$ ml/hr.

The initial continuous culture (Figure 3) was inoculated with a translucent culture. Figure 3 shows the presence of 4 colony types as determined by viable cell culture dilutions. The translucent and opaque colonies were shown to be different colony types of *P. putida*. The yellow and white colony types were contaminants present at no more than 0.04% and 5.2%, respectively, of the total population at all times. The yellow and white colony types were not present in the initial inoculum and were found to be incapable of utilizing toluene as a carbon source. As a result of their low percentage of total cells and their inability to utilize toluene, white and yellow contaminant colony types were considered to have a negligible effect on experiments in which they were present.

Figure 3 clearly shows that the dominant organism in the culture changed over time from translucent to opaque, and then back to translucent. This change coincides with problems of restricted air/toluene vapor flow to the fermenter vessel recorded at 218 hr. The total population rebounded steadily until 342 hr, when it had more than quadrupled. By 460 hr the population had dropped to approximately $2X10^8$ (CFU/ml), coincident with the drop in opaque population.

Figure 4 shows greater stabilization in the continuous culture. The second run was inoculated with an opaque culture, but before the first sampling the culture became predominantly (>99%) translucent. The translucent strain remained at greater than 90% of the population for most of the experimental run.

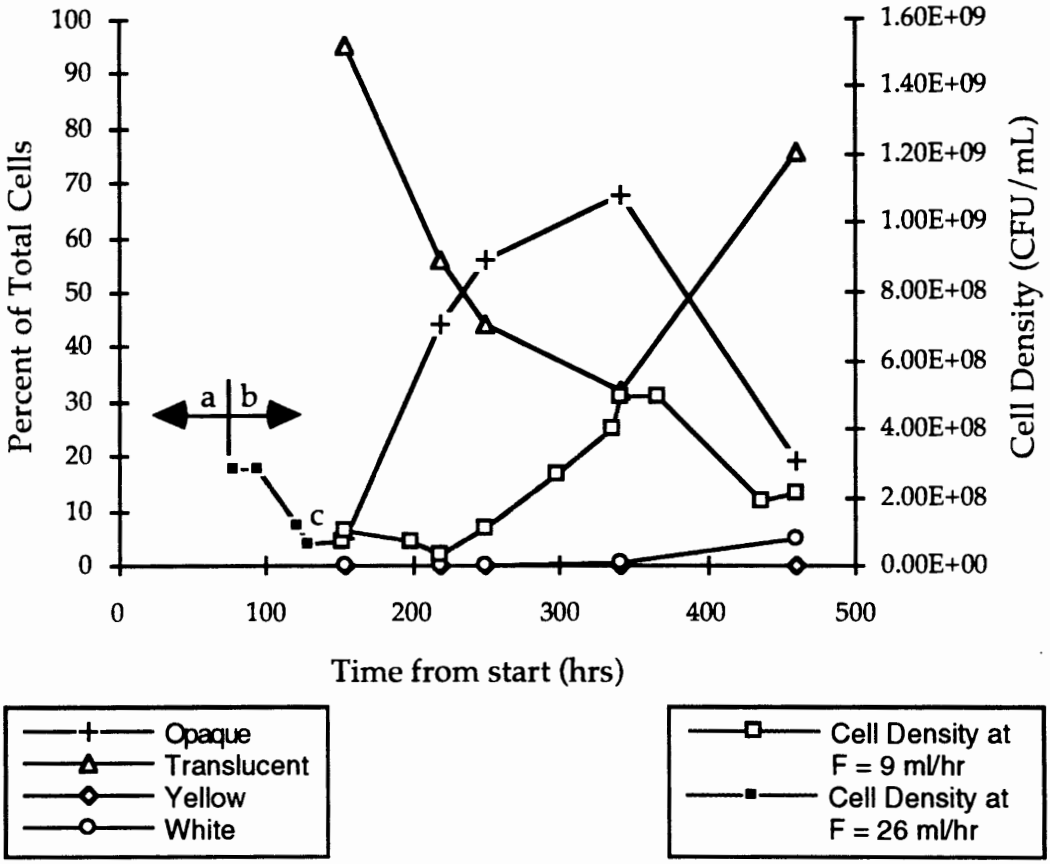


Figure 3. Continuous culture population at 30 °C: initial run

(a) Temperature set at 25 °C before 77 hrs. No population data available.

(b) Temperature set at 30 °C after 77 hrs.

(c) The chemostat flow rate from 0-129 hr was set at an initial flow rate, $F_{initial} = 26 \pm 2$ ml/hr, approximating the calculated value from batch experiments, $F_{calc} = 27.11$ ml/hr. The population washed out, so the flow rate was reduced to $F = 9 \pm 2$ ml/hr, $D = 0.026\ hr^{-1}$, for the remainder of the experiment.

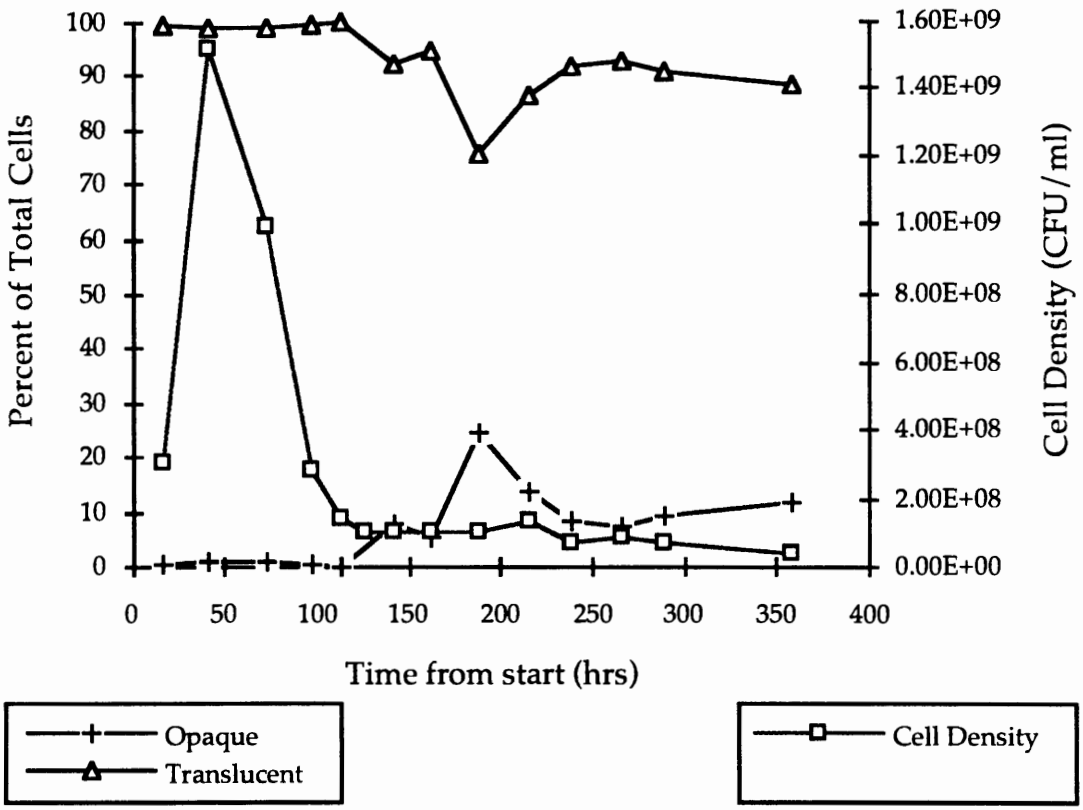


Figure 4. Continuous culture population at 30 °C: second run

Outer Membrane Proteins

Changes in Proteins. The outer membrane protein electrophoregrams showed significant differences in the protein content of the outer membrane of *P. putida* grown on toluene versus succinate control cultures (Figures 5, 6, and 7). As shown in Table II, the most striking differences were in band intensity between corresponding toluene grown and succinate grown outer membrane

protein bands at $83X10^3$, $70X10^3$ $64X10^3$, $59X10^3$, $54X10^3$, $50X10^3$, and $46X10^3$ mol wt. The toluene batch culture bands at $116X10^3$, $41X10^3$, $36X10^3$, $30X10^3$, $26X10^3$, and $11X10^3$ mol wt had no corresponding bands evident in the succinate batch control. Further, the succinate batch control protein band at $90X10^3$ and $32X10^3$ mol wt had no corresponding bands evident in the toluene batch culture. Molecular weights were calculated to the nearest $1X10^3$ mol wt.

Proteins less than $7X10^4$ mol wt increased concentrations, generally by one relative measure of intensity, from moderate to dark, faint to moderate, very faint to faint, or absent to very faint. A similar decrease occurred in the concentration of proteins in the $7X10^4$-$9X10^4$ mol wt. Band 1 at $1.16X10^5$ mol wt appears to be the only band in the toluene culture that is heavier than in the succinate culture. Band 1 does not appear in the 35 µl sample, so its concentration is below that of the very faint bands.

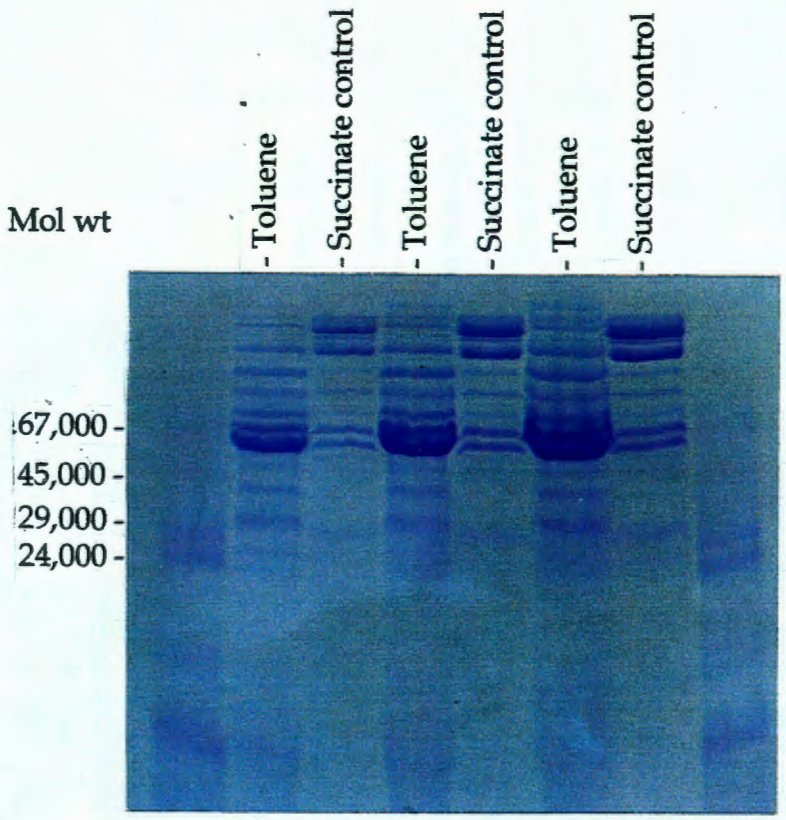


Figure 5. Gel A: 10% SDS-PAGE: Toluene vs. succinate culture.

Gel A was electophoresed at 100 mA for 3 hr. The initial amperage set for the gel was 150 mA, but the setting was progressively lowered to 100 mA within the first 30 min to prevent smiling. MWM set #1 was used for a molecular weight standard.

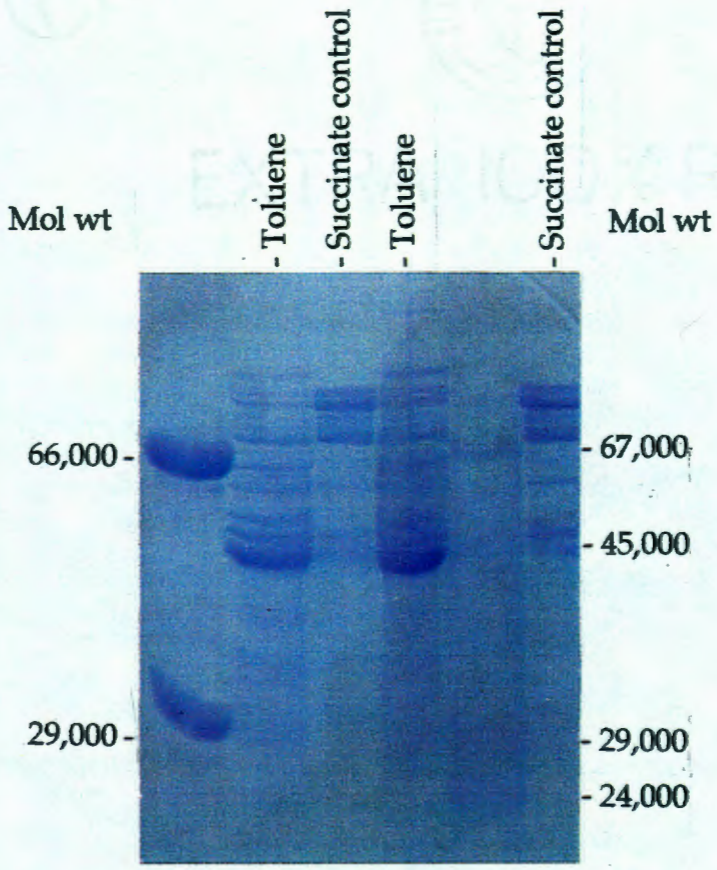


Figure 6. Gel B: 7% SDS-PAGE: Toluene vs. succinate culture
Gel B was electophoresed at 180 volts for 2 hr. MWM sets #1 and #2 were used as molecular weight standards.

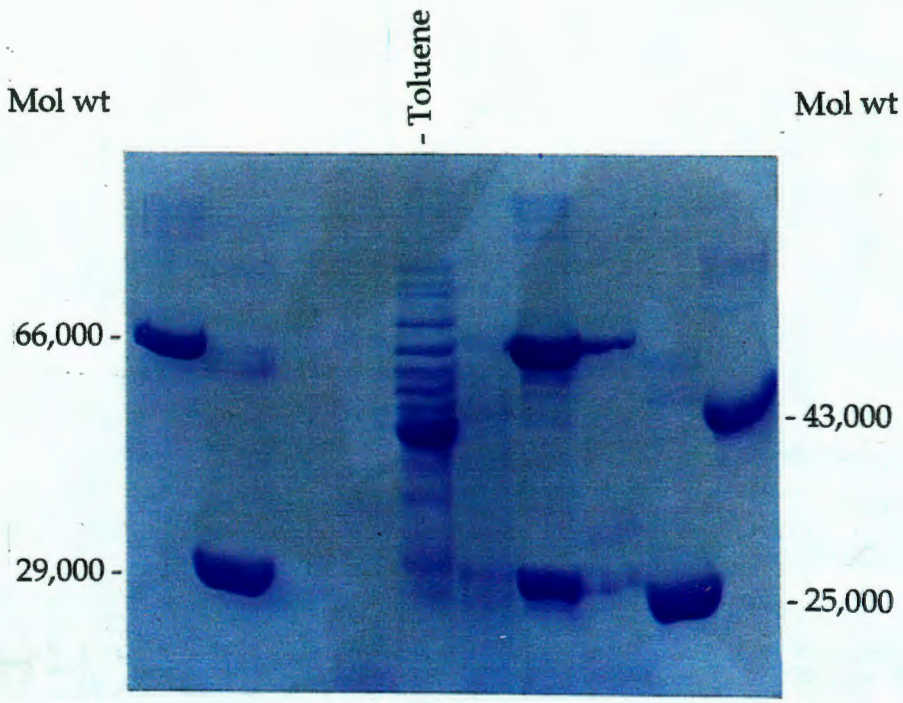


Figure 7. Gel C: 7% SDS-PAGE: Toluene culture and molecular weight markers Gel C was electophoresed at 140 volts for 2.25 hr. MWM sets #1, #2, and #3 were used as molecular weight standards.

Reliability of Data. The high correlations, $R^2 = 0.935$ and $R^2 = 0.980$, determined for Gel A (Figure 8) and Gel B (Figure 9), respectively, show the reliability of the method for assigning molecular weights for the toluene- and succinate- grown cultures' outer membrane proteins. All molecular weight markers were run again on a the second 7% SDS-PAGE, Gel C shown in Figure 7. The result confirmed the band identifications ($R^2 = 0.906$).

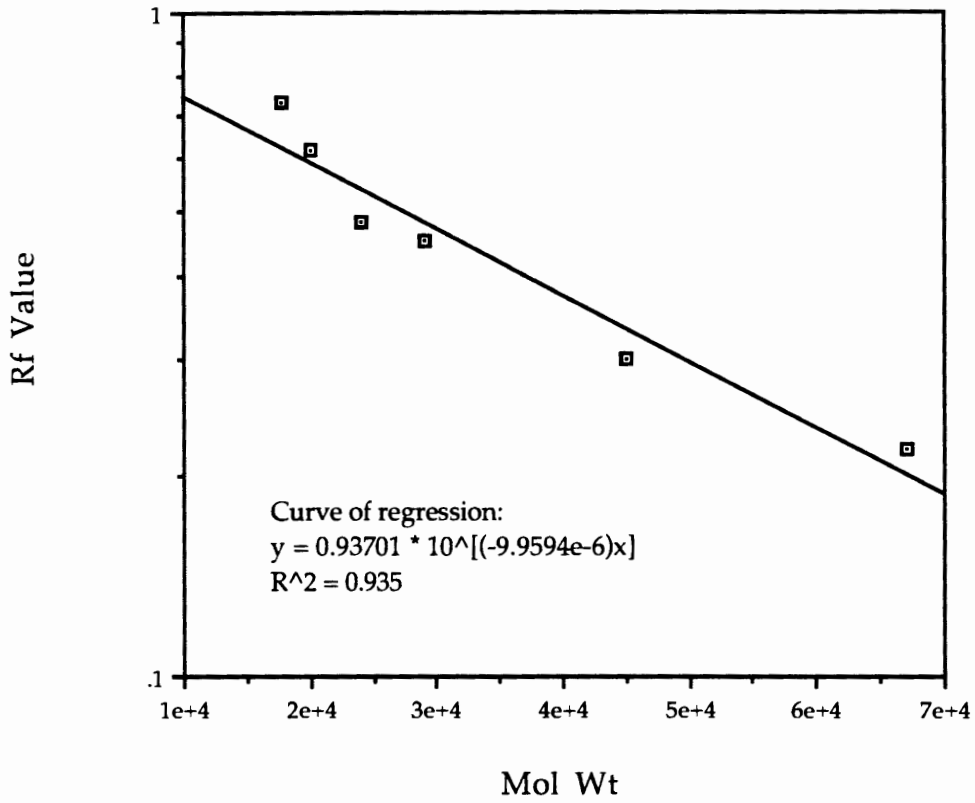


Figure 8. Gel A: 10% SDS-PAGE regression analysis

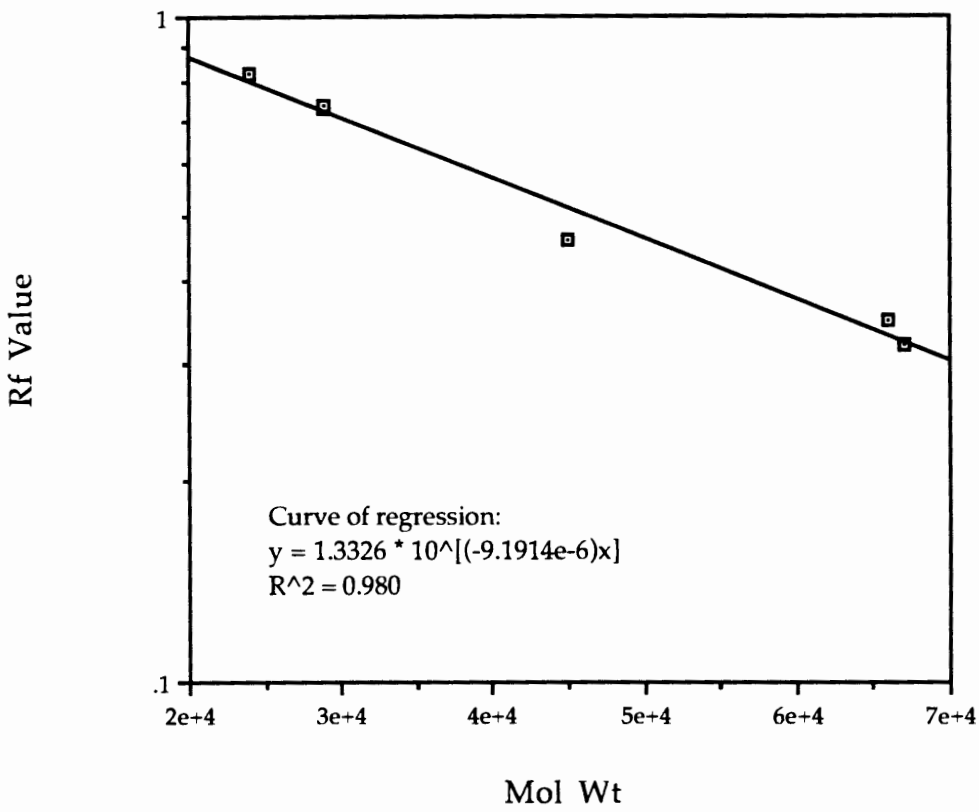


Figure 9. Gel B: 7% SDS-PAGE regression analysis

TABLE II

DIFFERENTIAL BANDING BETWEEN TOLUENE AND SUCCINATE GROWN *P. PUTIDA*

Membranes	Label	Relative Band Intensity	Protein (mol wt X10^3)
Succinate Control	a	very faint	90
	b	moderate	83
	c	dark	79
	e	dark	70
	f	faint	64
	g	faint	59
	h	very faint	54
	i	moderate	50
	j	moderate	46
	n	faint	32
	o	very faint	29
Toluene Grown	1	very faint	116
	3	faint	83
	4	moderate	79
	5	moderate	70
	6	moderate	64
	7	moderate	59
	8	moderate	54
	9	dark	50
	10	dark	46
	11	faint	41
	12	faint	36
	13	faint	30
	14	very faint	26
	15	very faint	29
	16	very faint	11

Thin Layer Chromatography

The solvent used for thin layer chromatography data shown was chloroform:methanol:acetic acid, 65:15:2 (Syldatk et al, 1985).

Toluene Batch Glycolipids. Analysis of repeated TLC of the toluene batch extract yielded two possible glycolipids, designated G1 ($R_f = 0.37 \pm 0.04$) and G2 ($R_f = 0.82 \pm 0.04$) (Table III and Figures 10-12). Differing growth conditions were then compared using the toluene batch culture as a baseline for production of the glycolipids. Results from glycolipid production by *P. putida* under the following conditions are shown in Table IV: 1) growth on succinate in batch culture at 28 °C; 2) growth on toluene in continuous culture at 25 °C; 3) growth on toluene in continuous culture at 30 °C.

Positive identification of G1 and G2 proved to be problematic for the toluene batch on culture comparison plates (Table IV). The G2 spot may have migrated slightly farther as no spot correlating to $R_f = 0.90$ was evident with the diphenylamine (glycolipid detection) spray. Furthermore, the rhodamine (lipid detection) spray yielded a very weak reaction. In previous experimental runs, both diphenylamine (glycolipid detection) and rhodamine (lipid detection) sprays yielded positive results for two toluene batch spots, $R_f = 0.83$ and 0.89.

Rhodamine (lipid detection) stained spots at $R_f = 0.90$, 0.77, 0.66, and 0.35, which were only visible under short ultraviolet light (Table IV). This grouping includes G1 at $R_f = 0.35$ and G2 at $R_f = 0.90$. The toluene batch extract presumably degraded during storage, leaving sample which is below the detection limit in visible light, yet still visible under the more sensitive ultraviolet. No corresponding band was evident for G2 in the diphenylamine (glycolipid detection) test (Table IV). Diphenylamine (glycolipid detection) has no reaction under ultraviolet, thus the G2 spot could not be confirmed with

ultraviolet light to be present at a concentration below the detection limit under normal visible light conditions.

Nonetheless, the G2 spot was presumed to be below the detection limit for the diphenylamine (glycolipid detection) test. Finally, the presence of the G2 spot using p-anisaldehyde (sugar detection) indicates that the sensitivity of this reaction was probably greater than that of the diphenylamine reaction for glycolipids.

Comparison of Culture Conditions. Three continuous culture sample extracts were prepared from effluent continuously collected over specific time periods. The first extract at 25 °C was prepared from sample collected during the initial continuous culture run from 51-76 hr. The second extract at 30 °C was prepared from sample collected during the initial continuous culture run from 194.6-293.7 hr. The third extract at 30 °C was prepared from sample collected during the second continuous culture run from 185.2-241.3 hr.

G1 was not produced under any other conditions except toluene batch culture (Table IV). Continuous culture conditions and succinate batch control produced spots designated G3, $R_f = 0.94$, (Table IV and Figures 10-12), near the expected toluene batch culture spot G2. However, all G3 designated spots were negative or uncertain for the p-anisaldehyde (sugar detection), indicating that the G3 spots were in fact different than the expected G2, which was positive. The diphenylamine (glycolipid detection) test gave positive results for the initial continuous culture grown at 25 °C, $R_f = 0.09$ and 0.85, and the initial continuous culture grown at 30 °C, $R_f = 0.13$ and 0.81.

Yield. The purported glycolipid was extracted as outlined in the materials and methods. The concentrated yellow-orange extracts were applied directly to the thin layer in 1 µl sample sizes. The extract yields of G2 and G3

were determined using an estimate of the bromthymol blue (lipid dectection) test detection limit. According to Touchstone (1992), the detection limit for the bromthymol blue (lipid detection) reaction is 0.1 - 1 mg. In the bromthymol blue test, palmitic acid was applied to the thin layer at, approximately, 0.1 mg and was barely visible. Since the positive spots on the bromthymol blue test for the toluene batch culture were also barely visible, positive reactions were assumed to be approximately 0.1 mg per spot.

The toluene batch which obtained the highest yield of glycolipid G2 was calculated to be approximately 2.55 mg/l. The approximate G3 yields were calculated as follows: initial continuous 25 °C was 0.03 mg/l; initial continuous 30 °C was 0.02 mg/l; second continuous 30°C was 0.87 mg/l; and the succinate batch control was 0.12 mg/l.

TABLE III

BASELINE R_f VALUES AND REAGENT REACTIONS FOR GLYCOLIPIDS PRODUCED BY TOLUENE BATCH GROWN *P. PUTIDA*

Test	Method	Spot G1 Rf	Spot G2 Rf	
Lipid	Bromthymol blue	0.30(+)	0.84(+)	
	Rhodamine	0.28(+)	0.84(+)	
	Molybdophosphoric acid	0.40(?)	0.83(?)	a
Organic Acid	Bromcresol	0.27(+)	0.80(+)	b
Sugar	p-Anisaldehyde	0.40(+)	0.81(+)	
Glycolipid	Bial's Reagent	0.37(+)		
	Diphenylamine	0.37(+)	0.81(+)	

a Controls both negative, but spots stained reddish pink.
b Controls both negative, but samples were positive.

TABLE IV

COMPARISON R_f VALUES AND REAGENT REACTIONS FOR GLYCOLIPIDS PRODUCED BY TOLUENE AND SUCCINATE GROWN *P. PUTIDA*

Sample Culture Extract	p-Anisaldehyde	Rhodamine	Diphenylamine
	Sugars	Lipids	Glycolipids
Succinate Batch Control 28°C		0.08(+)	
			0.78(+)[c]
			0.87(+)
	0.92(+/-)[ag]	0.96(+) g	0.94(+)[dg]
Toluene Batch 28 °C	0.24(-)	0.24(+)	0.25(+)
			0.29(+)
	0.38(+)[e]	0.35(+) [be]	0.37(+)[e]
			0.55(+)
		0.66(+) [b]	
	0.72(-)	0.77(+) [b]	
	0.90(+)[f]	0.90(+) [bf]	
Initial Continuous 25 °C			0.13(+)
			0.85(+)
	0.96(-)g	0.92(+) g	0.94(+)
Initial Continuous 30 °C	0.10(+)		0.09(+)
			0.81(+)
	0.96(-)g	0.92(+) g	0.95(+)g
Second Run Continuous 30 °C	0.16(+)		
	0.92(+/-)[ag]	0.96(+) g	0.95(+)g

a Spots were elongated and faint, so definitive identification was uncertain.
b Weak positive, spots were visible only with ultraviolet light.
c Very strong positive. d Very faint. e G1. f G2. g G3

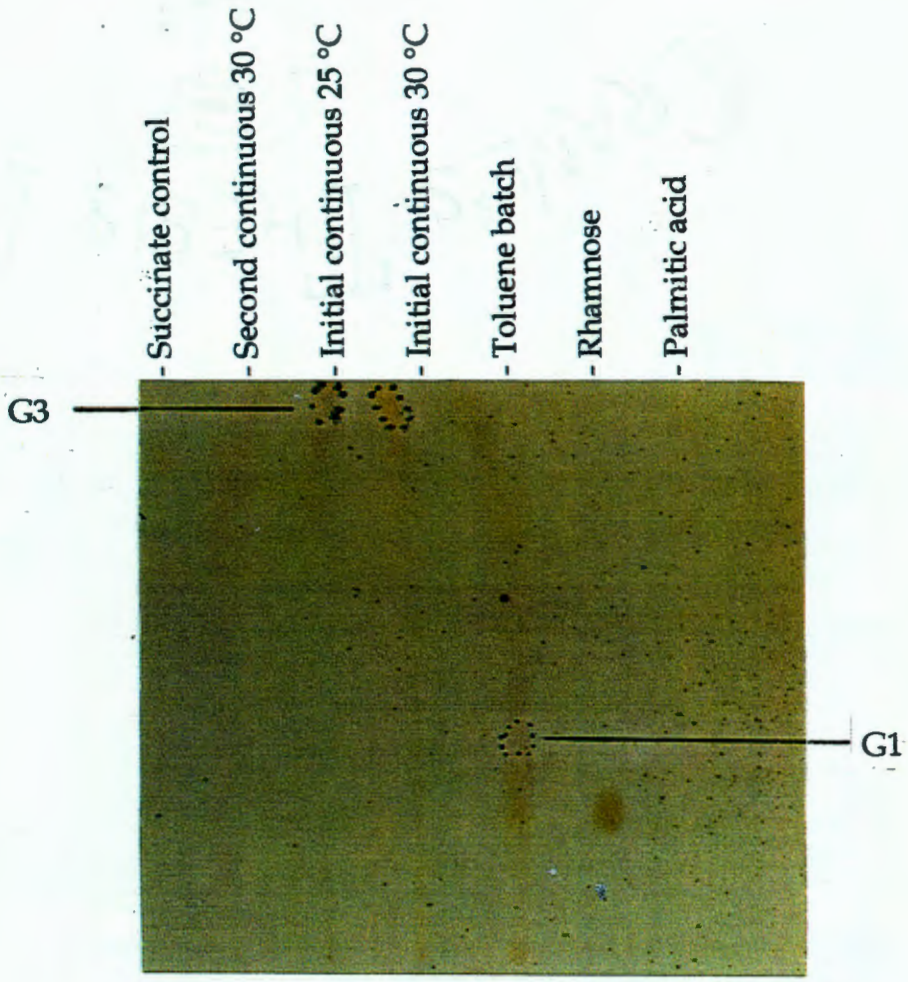


Figure 10. TLC of batch and continuous culture extracts: p-anisaldehyde reagent for visualization of sugars G1, glycolipid 1. G3, glycolipid 3.

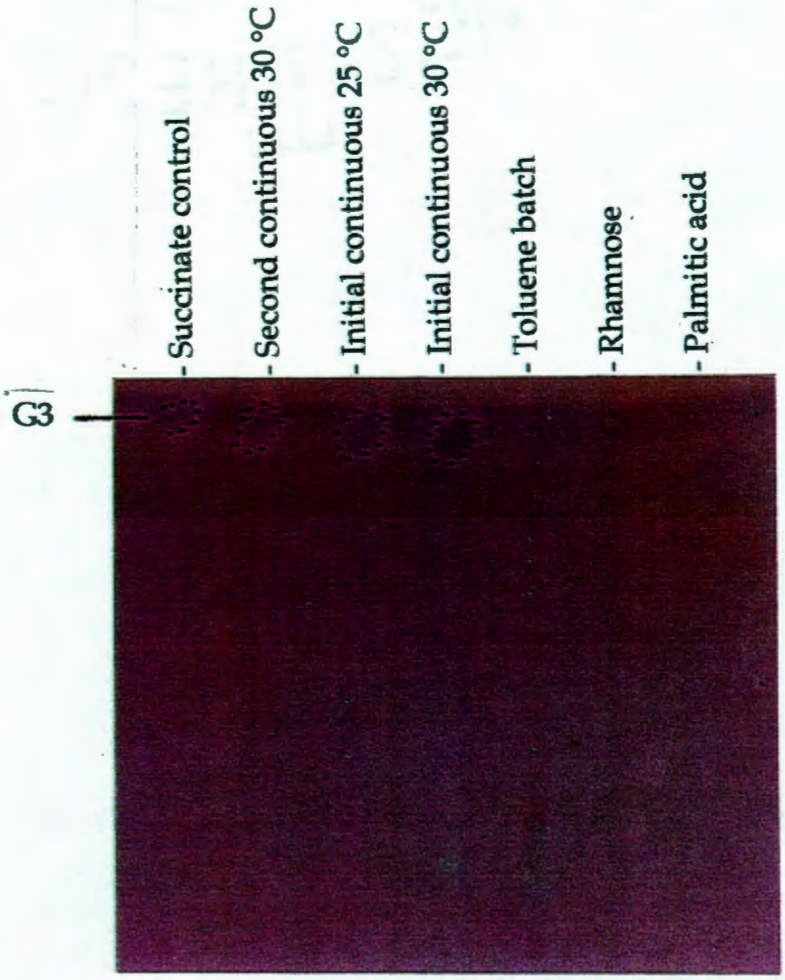


Figure 11. TLC of batch and continuous culture extracts rhodamine B reagent for visualization of lipids G3, glycolipid 3.

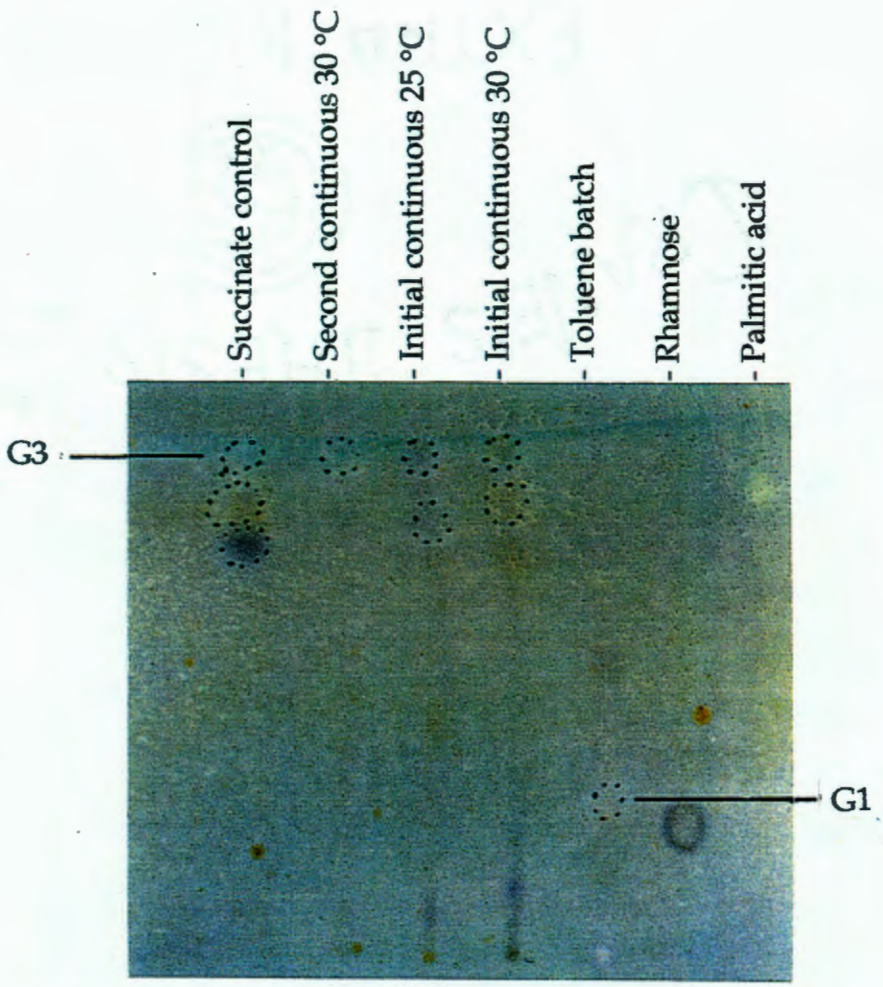


Figure 12. TLC of batch and continuous culture extracts: diphenylamine reagent for visualization of glycolipids G1, glycolipid 1. G3, glycolipid 3.

DISCUSSION

Overview

The discussion section provides analysis of the results from the three phases of study: population dynamics of continuous culture; the effect of toluene on outer membrane protein composition; and the effect of toluene and culture conditions on glycolipid production. The population study shows *P. putida* strain variation and competition in continuous culture. Outer membrane protein composition changes for *P. putida* when it is grown on toluene versus succinate as the carbon source. Glycolipid production is a function of culture method, nutrients, conditions, and possibly species strain.

Population Study

An opaque toluene batch culture was used to inoculate the second continuous flow run. The batch culture was streaked prior to inoculation of the continuous culture system to inspect for the presence of translucent colonies. Though none were evident, it appeared that in the chemostat the translucent colony type rapidly outcompeted the opaque within the first 16 hr, and remained dominant throughout the run. The initial rapid rise in cell density was probably due to an effort to "jump-start" the culture by letting the fermenter run from time 0 hr to 14.25 hr at a flow rate of 0. However, the cell density leveled out when flow was initiated after three volume changes . Based on the consistency of the second continuous run, as compared to the initial continuous run, the translucent colony type was the better competitor at 30 °C, D = 0.026 hr^{-1}, and unrestricted flow of toluene vapor.

The opaque colony competed successfully when conditions limit availability of toluene. Toluene recondensation in the delivery line appeared to have inhibited the free exchange of the toluene saturated air into the continuous culture vessel during the initial continuous run. Toluene vapor was clearly available at some concentration during this period, since it continued to collect in the effluent collection vessel. However, the concentration may have been reduced enough to create conditions that enabled the opaque strain to outcompete the translucent strain for the available toluene.

Reports of *P. putida* strains using differing pathways to compete for toluene in continuous culture have been reported by (Duetz et al, 1994). Two different pathways for the catabolism of toluene by *P. putida* have been characterized (Assinder and Williams, 1990; Yeh et al, 1977). According to Duetz et al (1994), under the low dilution rate conditions D = 0.05 hr^{-1}, the direct-ring attack of toluene (Yeh, 1977) is favored over the TOL pathway. In this study, the opaque strain may be expressing the direct-ring attack of toluene over the energetically more favorable TOL pathway. The translucent strain may be expressing the TOL pathway.

Translucent expression of the TOL pathway and opaque expression of the direct-ring attack pathway could account for the rise in population of the opaque strain under nutrient limiting conditions caused by the recondensation in the air/toluene line in the initial continuous culture. This appears to contradict the effect of dilution rate upon competition, which indicates that the direct ring attack would be favored. However, the maximum dilution rate of 0.026 h^{-1} is well below recorded values of 0.45 h^{-1} (Duetzet al, 1994). The reason for the low dilution rate is probably reflected in the toxic effect of the toluene (Duetz et al, 1994). Therefore, when the toluene influx was reduced in the initial

continuous culture, the conditions more closely approximated those of a low dilution rate system with limited nutrient availability, rather than a low a dilution sytem with high nutrient availability. The low dilution, limited nutrient availability theory would need to be investigated by adjusting toluene substrated concentrations.

Further, it is interesting to note that the opaque strain did not wash out even after approximately 12 volume changes. The relatively constant low level of the opaque strain suggests the close relationship between the opaque strain and the translucent strain. (Persson et al, 1990) determined that organisms cultured at lower dilution rate continuous cultures (0.05 h^{-1}) showed greater morphological change over time than those grown at higher dilution rates (0.15-1). More study needs to be done to determine whether the changes in colony morphology reflect the changes in catabolic pathway of toluene by *P. putida*.

Reproducibility. Quantitative chemostat reproducibility with *P. putida* was found to be problematic under the conditions used. The competition curve resulting form the initial continuous run could not be duplicated in the second continuous culture run. The difficulties in reproducibility resulted from changes in medium flow rate, temperature, and toluene availability, during the course of the initial continuous run. However, qualitative interpretation of the relative competition successes of the translucent and opaque cultures appears to be valid (Duetz et al, 1994).

Outer Membrane Protein

Batch cutltures were chosen for outer membrane protein studies, since the yield of bacteria per culture volume was higher in the batch culture than the continuous culture methods. Individually, faint protein bands were not

considered good indicators of differences between the membrane proteins of toluene batch and succinate batch cultures. However, when faint bands were viewed together with heavier bands to ascertain the shift in membrane composition, the result clearly showed that changes in outer membrane protein composition occur when cultures where grown on toluene versus succinate. The changes were evident from two comparisons: (1) higher band intensities at molecular weights $64X10^3$, $59X10^3$, $54X10^3$, $50X10^3$, and $46X10^3$ for toluene batch versus succinate batch control; and (2) higher band intensities at molecular weight $83X10^3$ and $70X10^3$, for succinate control versus toluene batch.

Also, the absence of corresponding banding at specific molecular weights, between toluene batch and succinate control cultures, provides further evidence that changes in outer membrane composition occur under the different culture conditions. First, no proteins corresponding to toluene control proteins at molecular weights $116X10^3$, $41X10^3$, $36X10^3$, $30X10^3$, $26X10^3$, and $11X10^3$ were evident in the succinate control culture. Second, no proteins corresponding succinate control proteins at molecular weights $90X10^3$ and $32X10^3$, were evident in the toluene batch culure.

Although bands at molecular weight $29X10^3$ were evident in both the toluene batch and succinate control, they could be different bands, but the SDS-PAGE technique utilized was limited to one dimensional, size exclusion electrophoresis, and thus failed to produce the resolution necessary to distinguish the two bands. The apparent correspondence probably results from calculating protein molecular weight to the nearest $1X10^3$ mol wt.

When toluene is the carbon and energy source, the trend in protein composition is towards a general increase in concentration of lower molecular weight proteins ($<70X10^4$ mol wt). A similar decrease occurred in the

concentration of higher molecular weight proteins in the $70X10^4$-$9X10^4$ mol wt. Band 1 (Table II) at $1.16X10^5$ mol wt appears to be the only heavier band in the toluene culture and that was not evident in the succinate control. The fact that band 1 does not appear in the 35 µl sample indicates that it is present at a negligible concentration in relation to the other proteins, and consequently does not affect the general trend towards lower weight proteins.

The function of the proteins was not determined and was beyond the scope of this project. However, it is hoped that further studies will continue to elucidate the structure and function of the different proteins that are produced. A comparison with changes that occur within different layers of the membrane is proposed as a continuing project.

Succinate. Succinate batch culture was used as a carbon and energy source control for toluene grown cultures for all experiments Succinate is a key four-carbon intermediate of cell metabolism. Succinate, a tricarboxylic acid cycle intermediate, will have no direct effect upon the structure of the the outer membrane proteins. Further, uptake of succinate can occur without damaging the outer membrane. Therefore, succinate is an appropriate control substrate for outer membrane studies, as well. The production of alkaline by-products which raise pH in stationary cultures was not a problem in the buffered exponentially growing cultures, which were harvested before reaching stationary phase.

Glycolipid Production

Glycolipid was prepared from cultures grown on toluene in batch and continuous culture and from cultures grown on succinate in batch cultures for a control. Succinate, a tricarboxylic cycle intermediate, is central to metabolism

and is used by many microorgansisms. Therefore, succinate can serve as a control for glycolipid production.

Toluene Batch Glycolipids. Two glycolipids were determined to be present in the toluene batch culture, designated G1 and G2. This is consistent with data that other glycolipids have been found to be produced by *P. putida* during growth on toluene or other xenobiotic substrates (Choe et al, 1992; Hiorayama et al, 1982; Syldatk et al, 1985).

In the toluene batch extract, the G1 was located, within experimental error, at the same position in all runs, suggesting that the separation was effective and reliable. Early experiments established the toluene batch G2 spot location to be $R_f = 0.82$, within the experimental error of $R_f = 0.04$. However, in later experiments the spot believed to be G2 was located at $R_f = 0.90$, which was not within the determined experimental error. This difference could not be accounted for by factors involving the thin layer used or the conditions used, since the control palmitic acid and rhamnose ran within expected ranges. Some degradation of the sample may have occurred in the sample during the 6 month storage at 4 °C, resulting in lower product concentrations. The lower concentration may have permitted the spot to run farther than expected. Based upon the fact that the extract used in early and late experiments was from the same sample extract, the spot ,$R_f = 0.90$ as determined in later experimental runs, was still considered to be the G2, since no other likely explanation accounted for its presence.

Comparison of Glycolipid Extracts. The initial continuous culture percent yields were approximately 20 % of the succinate batch yields. Even though the second continuous culture percent yield was approximately 7 times greater than the succinate batch yields, it was still approximately 30 % of the

toluene batch. The TLC data for the continuous culture samples more closely resembles that of the succinate batch culture than it does the toluene batch culture (Table IV). This was completely unexpected. We had postulated the the glycolipid might be utilized in toluene encapsulation by glycolipid micelles, providing an uptake mechanism for toluene that would protect the cell membrane from degradation by the direct action of toluene. It had been assumed that the glycolipid production was induced by toluene. If this had been the only factor, then succinate cultures would not have produced the G3 glycolipid in similar concentrations as that of the toluene second continuous culture.

The positive values for the diphenylamine (glycolipid detection), at approximately $R_f = 0.09$ and $R_f = 0.81$, Table IV, for the initial continuous runs at 30 °C, were not evident in the second continuous run at 30 °C, suggesting that the spots may be due to the higher concentration of the opaque strain, than translucent strain at the time of sampling. No population data was collected for the continous culture sampling time at 25 °C, so no conclusions regarding *P. putida* strain effect on glycolipid production can be made for this period. However, the population for the initial continuous culture sample at 30 °C, Figure 3, shows that the opaque strain was the dominant strain at the sampling time, supporting the prospect of the opaque strain contributing different glycolipids to the culture. The effect of the opaque strain upon production of glycolipids is further supported by the absence of spots at $R_f = 0.09$ and $R_f = 0.81$, in the extract from the second continuous run at 30 °C. The absence of spots at $R_f = 0.09$ and $R_f = 0.81$ would be expected if the opaque strain were responsible for their production, as is the case in the second continuous culture at 30 °C sample.

SUMMARY

Two strains of *P. putida,* translucent and opaque, were able to coexist in continuous culture. The opaque and translucent strains were able to utilize toluene as the sole carbon and energy source. The translucent appeared to compete better than the opaque strain under stable conditions of high toluene concentration.

Toluene had a significant impact on the outer membrane protein composition. The toluene grown culture proteins had markedly different protein concentrations compared to the succinate batch control proteins. Proteins less than $7.0X10^4$ mol wt increased in concentration in toluene grown organisms versus succinate control, while larger proteins, greater than $7.0X10^4$ mol wt, decreased in concentration.

The production of glycolipid by *P. putida* in batch culture is more efficient than in continuous culture under the conditions tested. This may be due to greater nutrient availability of required intermediates in the more dense batch culture. Glycolipids production in continuous culture resembled succinate batch controls more than toluene batch controls. Temperature variation between 25 °C and 30 °C did not appear to affect glycolipid production. *P. putida* strains may produce different glycolipids under the same culture conditions.

REFERENCES

Anderson, B. C. (1992). Morphology, physiology and fine structure of a toluene oxidizing strain of *Pseusomonas putida*. Master of Science/Biology, Portland State University.

Assinder, S. J., & P. A Williams. (1990). The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microbial Phys., 31, 1-69.

Atlas, R. M., & R. Bartha. (1992). Hydrocarbon biodegradation and oil spill bioremediation. In K. C. Marshall (Ed.), Advances in microbial ecology (pp. 287-338). New York: Plenum Press.

Bergey, D. H., R. E. Buchanan, & N. E Gibbons. (1974). Bergey's manual of determinative bacteriology (8th ed.). Baltimore: Williams & Wilkins, Co.

Bird, S. E. (1994). Rhamnolipid production in a toluene-oxidizing strain of *Pseudomonas putida*. Master of Science in Biology, Portland State University.

Cerniglia, C. E. (1984). Microbial transformation of aromatic hydrocarbons. In R. M. Atlas (Ed.), Petroleum Microbiology (pp. 99-128). New York: Macmillan Publishing Co.

Choe, B.-Y., N. R. Krishna, & D. G. Pritchard. (1992). Proton NMR study on rhamnolipids produced by *Pseudomonas aeruginosa*. Magnetic Resonance in Chemistry, 30, 1025-1029.

Duetz, W. A., C. de Jong, , P. A.Williams, & J. G. vanAndel. (1994). Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl. Env. Microbiol., 60(8).

Duvnjak, Z., D. G. Cooper, & N. Kosaric. (1982). Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. Biotechnol. Bioeng., 24, 165-175.

Finnerty, W. R., & M. E. Singer. (1985). Membranes of hydrocarbon-utilizing microorgansims. In B. K. Ghosh (Ed.), Organization of prokaryotic cell membranes (pp. 1-44). Boca Raton: CRC Press, Inc.

Gerhardt, P., R., G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, & G. B. Phillips (Eds.). (1981). Manual of methods for general bacteriology. Washington, D. C.: American Society for Microbiology.

Gruber, T., H. Chmiel, O. Käppeli, P. Sticher, & A. Fiechter. (1993). Integrated process for continuous rhamnolipid biosynthesis. Surfactant Sci. Ser., 48((Biosurfactants)), 157-173.

Hiorayama, T., & I. Kato. (1982). Novel methyl rhamnolipids from *Pseudomonas aeruginosa*. FEBS Lett., 139(1), 81-85.

Johnston, K. H., K. K. Holmes, & E. C. Gotschlich. (1976). The serological classification of *Neisseria gonorrhoeae*. I. Isolation of the outer membrane complex responsible for serotype specificity. J. Exp. Med.(143), 741-758.

Koch, A. K., O. Käppeli, A. Fiechter, & J. Reiser. (1991). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol., 173(13), 4212-4219.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(680).

New Brunswick Scientific Co., I. Operating manual for Bioflo, Benchtop Chemostat, Model C30.

Persson, A., G. Molin, & C. Weibull. (1990). Physiological and morphological changes induced by nutrient limitation of *Pseudomonas fluorescens* 378 in continuous culture. Appl. Env. Microbiol., 56(3), 686-692.

Reiling, H. E., U. Thanei-Wyss, L. H. Guerra-Santos, R. Hirt, O. Käppeli, & A. Fiechter. (1986). Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Appl. Env. Microbiol., 51(5), 985-989.

Ross, N. W., R. Levitan, J. Labelle, & H. Schneider (1991). Protein and other compositional differences of the extracellular meterial from slimy and non-slimy colonies of non-mucoid *Pseudomonas aeruginosa*. FEMS Microbiol. Lett., 81, 257-260.

Shryock, T. R., S. A. Silver, M. W. Banschback,, & J. C. Kramer. (1984). Effect of *Pseudomonas aeruginosa* rhamnolipid on human neutrophil migration. Current Microbiol., 10, 323-328.

Stahl (Ed.). (1969). Thin layer chromatography: a laboratory handbook. New York: Springer-Verlag, Inc.

Syldatk, C., S. Lang, F. Wagner, V. Wray, & L. Witte. (1985). Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM 2874 grown on n-Alkanes. Z. Naturforsch., 40, 51-60.

Touchstone, J. C. (1992). Practice of thin layer chromatography (3rd ed.). New York: John Wiley & Sons.

U. S. Department of Health and Human Services, Public Health Service , Agency for Toxic Substances and Disease Registry (1994). Toxicological profile for toluene (update) No. TP-93/14).

U. S. Department of Health, Education, and Welfare (1978). Occupational diseases: a guide to their recognition.

Worsey, M. J., & P. A. Williams. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol., 124(1), 7-13.

Yeh, W. K., D. T. Gibson, & T.-N. Liu. (1977). Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Comm., 78(1), 401-410.

Zajic, J. E., & A. Y. Mahomedy. (1984). Biosurfactants: intermediates in the biosynthesis of amphipathic molecules in microbes. In R. M. Atlas (Ed.), Petroleum Microbiology (pp. 221-297). New York: Macmillan Publishing Co.

APPENDIX A

MOLECULAR WEIGHT MARKERS

TABLE V

MOLECULAR WEIGHT MARKER SET #1

Molecular Weight Marker Set #1 (Sigma)	Mol Wt*10^3 (g/mol)
Bovine Serum Albumin (BSA)	67
Ovalbumin	45
Carbonic Anhydrase	29
Trypsinogen	24
Trypsin Inhibitor	20.1
Myoglobin	17.8
Cytochrome C	12.4

TABLE VI

MOLECULAR WEIGHT MARKER SET #2

Molecular Weight Marker Set #2 (Kit No. MW-GF-70, Sigma)	Mol Wt*10^3 (g/mol)
Bovine Serum Albumin (BSA)	66
Carbonic Anhydrase	29
Cytochrome C	12.4
Aprotinin	6.5

TABLE VII

MOLECULAR WEIGHT MARKER SET #3

Molecular Weight Marker Set #3 (Gel Filtration Calibration Kit, Pharmacia Fine Chemicals)	Mol Wt*10^3 (g/mol)
Ovalbumin	43
Chymotrypsinogen A	25
Ribonuclease A	13.7

APPENDIX B

MATERIALS FOR PREPARATION OF THIN LAYER CHROMATOGRAPH

Thin Layer Chromatography Materials

The following list of materials was used in the preparation, development, and spraying of the thin layer chromatographs:

Silica Gel GF-254 (type 60) (EM Laboratories, Inc. Cat. #7730)

Model S-II Applicator (Desaga, Brinkmann, Cat. #250012)

Standard Mounting Board (Desaga, Brinkmann, Cat. # 25005)

Standard Glass Plates 40mm x 20cm x 20cm (Desaga/ Brinkmann)

Brinkmann Drying Racks (Brinkmann, Cat. #251031-B)

Drying Oven-Precision Model (GCA Corp)

Developing Chamber (Brinkmann Cat. #251022)

Standard Labeling Template (Brinkmann #251030)

Gilson Pipetman p20 (Gilson, France #M80-13023)

Aerosol Spray Gun (Brinkmann # 251141) or atomizer

Sorvall Omni Mixer (Ian Sovall, Inc. Norwalk, Conn)

Petroleum Ether or Acetone.

APPENDIX C

SPRAY REAGENTS

TABLE VIII

BROMCRESOL GREEN REAGENT

Reagent:	0.3% bromcresol (Harleco, Philadelphia, PA, #851) in 1:4 H_2O:CH_3OH + 8 drops 30% NaOH per 100 ml.
Procedure:	Spray.
Compounds detected:	Yellow areas for aliphatic carboxylic acids on green background.

TABLE IX

BROMTHYMOL BLUE

Reagent:	0.1% bromthymol.blue (Baker, #D7403-3) in 10% aqueous ethanol made just alkaline with NH_4OH (Baker, #3-9721)
Procedure:	Spray
Compounds detected:	Lipids and phospholipids produce blue-green areas. Sensitivity = 0.1 - 1 mg.

TABLE X

MOLYBDOPHOSPHORIC ACID[a]

Reagent:	5% ethanolic solution of molybdophosphoric acid (Mallinckrodt, #2772).
Procedure:	After spraying, heat at 110 °C until best spot formation is attained, approximately 10 min.
Compounds detected:	Reducing compounds and lipids. Background can be rendered colorless by placing the plate in a tank containing 25% ammonium hydroxide.

a Method from (Stahl, 1969 #64).

TABLE XII

RHODAMINE B

Reagent:	Solution a): 0.2% rhodamine in ddH_2O (Aldrich Chemicals Co., #R95-3). Solution b): 3% H_2O_2. Solution c): 10N KOH.
Procedure:	Spray with a) and observe: also observe under UV. Spraying with b) may enhance color.
Compounds detected:	Lipids produce purple spots on pink-red to blood-red background. Many lipids produce a bright red fluorescence.

TABLE XII

BIAL'S REAGENT (ORCINOL)

Reagent:	Dissolve 0.1 g orcinol (The Matheson Co., Inc., East Rutherford, NJ, #6073) in 40.7 ml concentrated HCl. Add 1 ml 1% $FeCl_3$ (Mallinckrodt Chemical Works, St. Louis, MO, #5029). Dilute to 50 ml.
Procedure:	Spray and heat at 110 °C
Compounds detected:	Glycolipids produce violet spots

TABLE XIII

DIPHENYLAMINE

Reagent:	20 ml 10% diphenylamine (Sigma Chem. Co., St. Louis, MO, #D-2385) in ethanol. 100 ml HCl. 80 ml acetic acid.
Procedure:	Spray and heat at 110°C for 60 min, until positive areas appear.
Compounds detected:	Glycolipids produce blue spots

TABLE XIV

P-ANISALDEHYDE

Reagent:	Dissolve 1 ml p-anisaldehyde (Matheson Coleman & Bell, Norwood, OH, #AX1525 2239) and 1 ml H_2SO_4 in 18 ml ethanol.
Procedure:	Spray and heat at 110 °C for 10 min.
Compounds detected:	Sugars produce blue, green or violet spots in 10 min.

TABLE XV

STANDARD REAGENTS FOR LIPIDS AND SUGARS

Palmitic acid	10% palmitic acid (Matheson Coleman & Bell, Norwood, OH, #p3207) in ethyl acetate or n-propanol.
α-L-Rhamnose	10% α-L-rhamnose in ddH_2O (Sigma Chem. Co., St. Louis, MO, #R-3875)

APPENDIX D

TOLUENE METABOLISM PATHWAY, TOLUENE UPTAKE MODEL, AND RHAMNOLIPID STRUCTURE

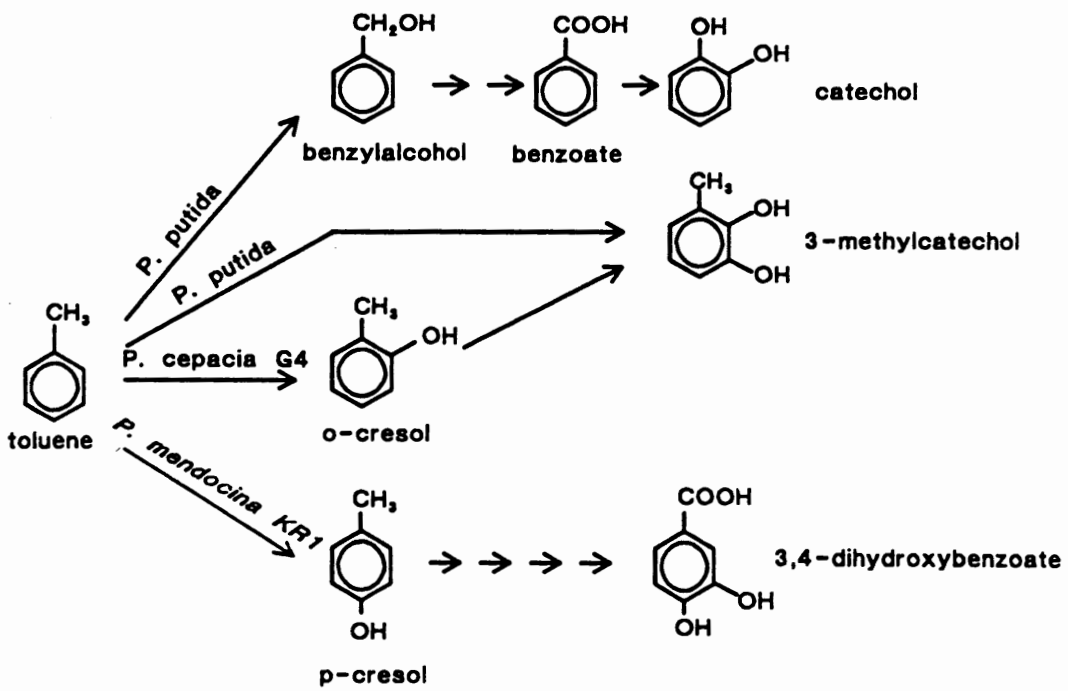


Figure 13. Initial steps in toluene degradation in *Pseudomonas* species (adapted from (Duetz et al, 1994))

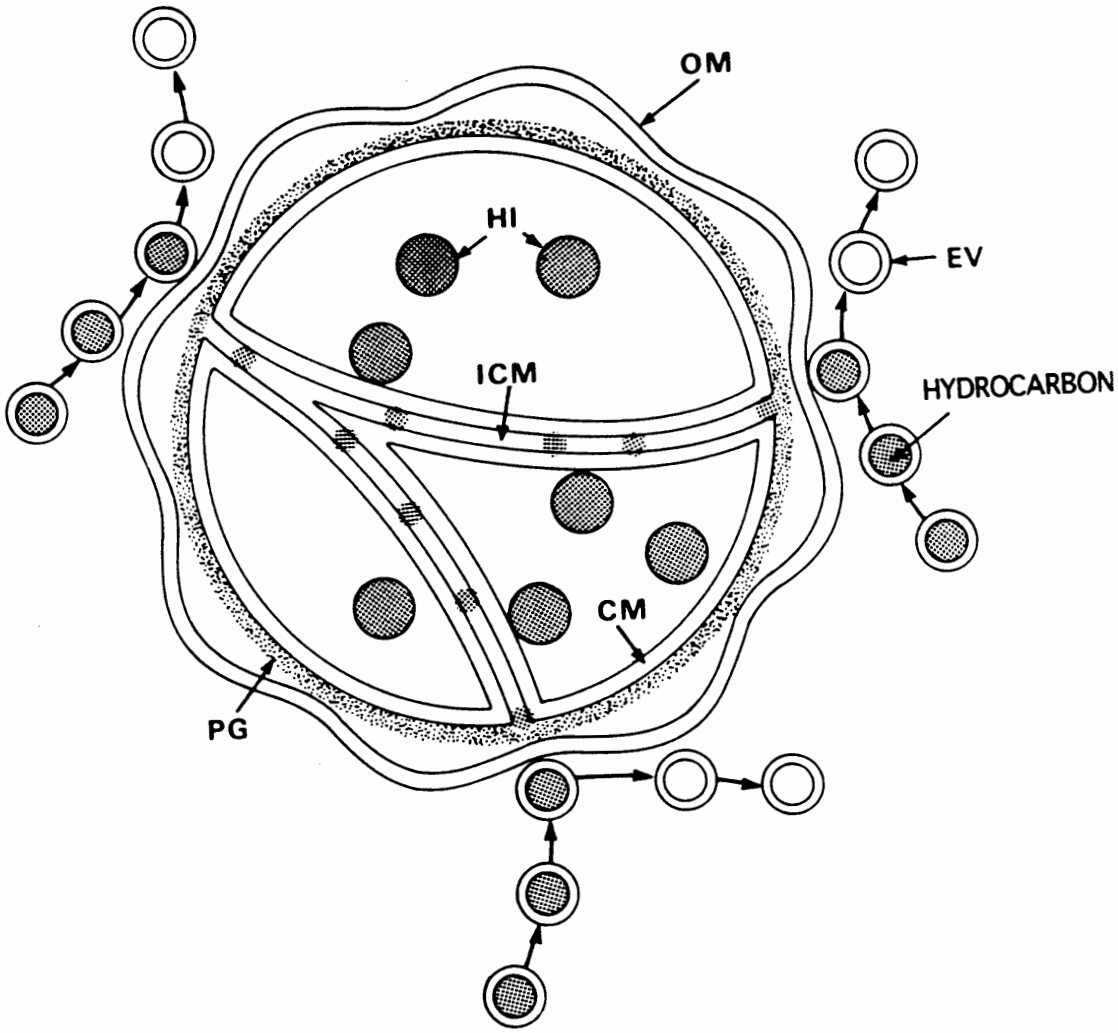
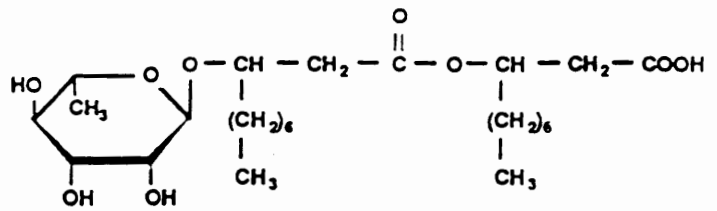
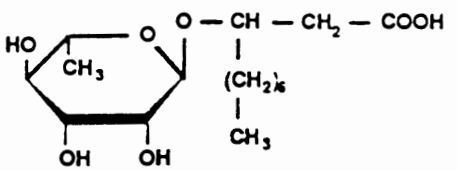


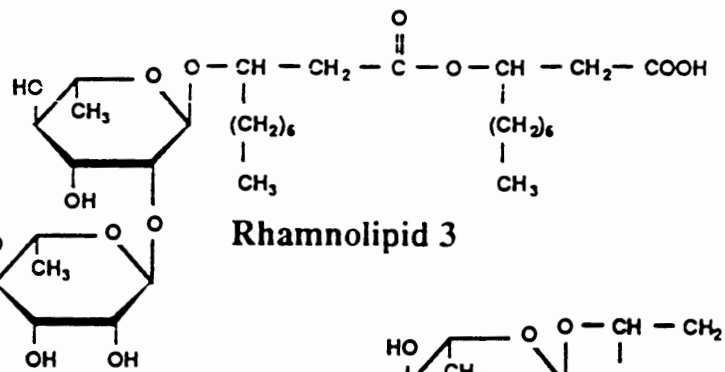
Figure 14. Model of hydrocarbon uptake in bacteria (adapted from (Finnerty and Singer, 1985))



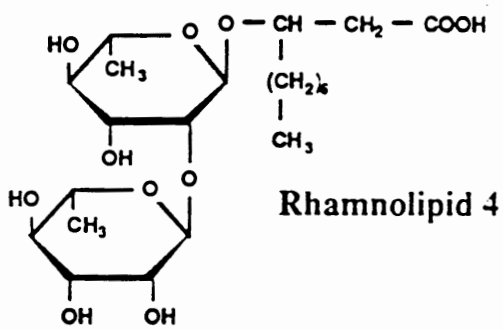
Rhamnolipid 1



Rhamnolipid 2



Rhamnolipid 3



Rhamnolipid 4

Figure 15. Rhamnolipid structures produced by *Pseudomonas* species. HI, hydrocarbon inclusion; ICM, intracytoplasmic membrane; CM, cytoplasmic membrane; OM, outer membrane; PG, peptidoglycan; EV, extracellular vessicle (adapted from (Gruber et al, 1993))