DEGRADATION OF ORTHO PHENYL PHENOL BY STREPTOMYCETES IN CONTINUOUS CULTURES

Andreas Escher

INTRODUCTION

Phenolic compounds are quite commonly used as pesticides. Its degradation in soils is in absence of sun light relatively slow, since it is mediated by microbial activity alone. Investigations showed that mainly some fungi and streptomycetes are responsible for the degradation of these xenobiotic compounds. The mechanisms, however, are not well understood and it appears that traditional microbial assays, i.e. batch cultures and enrichment plates, are not very helpful. The intention of this summer work was to develop some different techniques for a better understanding of the mechanisms and the kinetics of biodegradation of phenolic compounds by streptomycetes. Ortho phenyl phenol has been selected since it is the most toxic compound of the different phenols and its possible products would be less toxic (no secondary inhibition by the product).

Xenobiotic compounds, i.e. phenols, can be detoxified by microorganisms either by metabolism or cometabolism. Studies at DuPont (J. Romesser, 1983) showed biodegradation of phenols with some fungi and actinomycetes. These experiments have been made with high amounts of biomass grown in batch cultures, decanted and resuspended in a solutions containing high amounts of substrate, nutrients, and xeonbiotic compounds. The focus of these studies was not to study growth in presence of toxic compounds, but metabolism, i.e. mineralization, and cometabolism of these compounds

The goal of the work was: (a) to measure the toxicity in terms of growth inhibition; (b) to determine whether streptomycetes metabolize or cometabolize phenolic compounds under conditions of continuous culturs; and (c) to measure whether energy is required or gained by degradation of toxic phenolic compounds.

Continuous Culture Dynamics

The use of chemostats with a steady state allows the application of simple mass balances to determine the rates of bioreactions. In the absence of xenobiotic compounds, i.e. carbon limitation, the system can be defined as follows:

1. $\underbrace{\frac{Vdc}{dt}}_{\text{accumulation}} = \underbrace{F(c_0 - c)}_{\text{flow in minus flow out}} - \underbrace{VK}_{\text{reaction rate}}$

By dividing this equation by volume, one obtains:

2. $\frac{dc}{dt} = D(c_0 - c) - K$

where D is the dilution rate. Under steady state conditions (d/dt = 0) it becomes:

3. $D\ (c_0 - c) = K$

Without xenobiotics the system can be defined as follows:

4. $\frac{ds}{dt} = D\ (s_0 - s) - \frac{1}{y}\ ux$ Substrate s

5. $\frac{dx}{dt} = -Dx + ux$ Biomass x

where: s substrate concentration $[ML^{-3}]$
s_0 substrate concentration inflow $[ML^{-3}]$
μ growth rate $[t^{-1}]$
y yield [biomass/substrate]

Under steady state (d/dt = 0) we can obtain:

6. $D(s_0-s) = \frac{1}{y}\ \mu x$

7. $D = \mu$
8. $y = \frac{x}{s_0-s}$

Generally this yield is constant for a large range of different growth rates (no maintenance and no product formation).

In the presence of xenobiotics the system of Equation 4 and 5 expands to three new equations:

9. $\frac{ds}{dt} = D(s_0-s) - \frac{1}{y}\ \mu x$ Substrate s

10. $\frac{dx}{dt} = -Dx + \mu x\ k(A) + xK'\ (s,x,A)$ Biomass x

11. $\frac{dA}{dt} = D(A_0 - A) - K(s,x)$ Xenobiotics A

where A is the concentration of xenobiotics, K(s,x) the degradation rate as a function of substrate of biomass or both together, k(A) the growth inhibition term due to xenobiotics and K' (s,x,A) growth on xenobiotics as a carbon source.

With xenobiotics, Equation 8 can still be calculated but it represents now a different yield, the observed yield. The comparison of the two different yields, measured with and without xenobiotics, allows one to determine whether energy is required or gained by degradation of xenobiotic compounds.

If growth follows Michaelis - Menten equation:

12. $\mu = \frac{\mu_{max} s}{K_s + s}$

k(A) can be estimated by comparing μ versus s in both sets of experiments, with and without xenobiotics.

Therefore studies in continous cultures are useful to determine the key functional process in degradation of xenobiotic compounds.

METHODS

Cultures

Two strain of streptomycetes, isolated by J. Romesser, have been used. Streptomyceae grusolus, # 11 796 and Streptomyceae griseus, # 10 137. In addition, a Gram negative cocci with an intense yellow pigment, which is tolerant to ortho phenyl phenol has been isolated. This organism has been used in coculture studies.

Growth Media

Different growth media have been used: Glycerol-Arginine for plates and a glucose medium for liquid cultures.

Glycerol-Arginine:

glycerol	20.0 g
L-arginine	2.5 g
NaCl	1.0 g
$CaCo_3$	0.1 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
K_2HPO_4	2.0 g
agar	20.0 g
dist. H_2O	1 000.0 ml

adjust to pH 7

P-buffer:

KH_2PO_4	13.6 g
NA_2HPO_4	26.8 g
vitamin solution	10.0 ml

Glucose nutrient stock solution (10 lt)

$FeCl_3 \cdot 6H_2O$	0.75 g
$MnSO_4 \cdot H_2O$	0.009 g
$ZnCl_2$	0.08 g
$CuSo_4$	0.05 g
$CoCl_2 \cdot 6H_2O$	0.07 g
$Na_2MoO_4 \cdot 2H_2O$	0.07 g
Na_3 citrate	4.08 g
NaH_2PO_4	5.75 g
$(NH_4)_2SO_4$	3.67 g
NH_4CL	32.17 g
$CaCl_2 \cdot 2H_2O$	4.08 g

100 cc were added for each gram of glucose per liter final solution. All the continuous flow experiments were made with a glucose concentration of 1.00 g per liter. Ortho phenyl phenol was used at a concentration of 100 mg/l. Reservoire bottles with ortho phenyl phenol were placed on a shaking table to prevent precipitation of the aromatic compounds.

RESULTS

Experiments in Batch Reactors

I. Growth inhibition in batch cultures.

Inhibition of growth has been measured in a series of tubes with decreasing ortho phenyl phenol to measure the threshold concentration of the toxic compounds to survival and growth. A series of test tubes (25 ml) with nutrients and glucose (2.0 g/l) and different concentrations of ortho phenyl phenol have been inoculated with 1 ml inoculum obtained from a batch culture during exponential growth. After four days of incubation (30°C), dry weight of biomass has been measured and some biomass has been streaked on agar plates without xenobiotics to determine whether biomass was viable or not. This experiment (Table I) gives some indication about the sensitivity of the two different strains to "long term" exposure to ortho phenyl phenol, and effect which has been neglected in the experiments made by DuPont (see Introduction).

A third series of identical, but sterile tubes have been exposed to the air for several hours and then incubated for four days (30°C). A contaminant, tollerant to ortho phenyl phenol has been found up to a concentration of 90 mg/l of ortho phenyl phenol. It has been isolated and grown in pure culture on agar plates and has later been used for coculture studies.

II. Estimation of maximum growth in batch cultures

Growth of strains 10 137 and 11 796 has been measured in batch cultures for a preliminary estimation of the maximum growth rate. For both strains a growth rate of 0.10 h^{-1} has been found. This low rate suggests a growth inhibition, eventually caused by its own primary or secondary metabolic products.

Experiments with Continuous Flow Systems

A series of experiments in continuous flow systems have been made. Due to different analytical and systematical problems it was not possible to obtain quantitative data. Therefore some of the objectives could not be reached. The nature of these problems were the different and unknown behavior of these organisms in continuous cultures.

Glucose Analysis

The quantitative measurement of glucose is a key parameter in the analysis of continuous cultures. It provides the values of s_0 resp. 2. The colorimetric enzymatic determination of glucose, No 115 A, produced by Sigma, has been used.

The analysis is based upon the conversion of glucose-6-phosphate, coupled with the subsequent reduction of NADP to NADPH. In the presence of PMS, INTH produced from the reduction of INT by NADPH, is measured colorimetrically at 520 nm.

The amount of INTH formed is directly proportional to the concentration of glucose in the sample. Sigma states 115 that no interfering substances are known except some other hexoses but only in combination with their specific isomerases.

However, in the bulk of reactors with streptomycetes glucose analysis produced values much too great. In most of the measurements the measured glucose concentration exceeded the concentration of the inflow. The only explanation of this error is that streptomycetes produce a soluble compound able to reduce with PMS or INT. It is probably that they are "leaky" for NADPH or an other reducing agent. This phenomenon has been observed in both filtered and unfiltered bulk water, suggesting that this release could be a metabolic product and not a result of lysed cells. Comparison of dry weight biomass and molar concentration of this reducing agent indicates a production up to 80 mMol per gram biomass.

Soluble and Organic Carbon:

Unfortunately, MBL does not contain a facility to measure soluble and total organic carbon. This measurement is a useful tool for a complete carbon mass balance. This measurement would have been beneficial as a control to the specific analysis, such as biomass and glucose.

Measurement of Phenolic Compounds:

Phenolic compounds were analyzed with gas chromatography and thin layer chromatography. In respect to quantitative analysis the results were not very reproducible. But the qualitative analysis especially with TLC was very good.

Operation of Start of the Chemostats:

Before starting up the reactors, the reservoir with the liquid, the reactors, tubing and air filters were autoclaved. The reactors filled with substrate and nutrients, but with xenobiotics, were asptically inoculated. After ca. two days without inflow the biomass reached relatively dense biomass. Then the system was switched from batch to continuous flow (still in absence of xenobiotics). Steady state was assumed after a time t = 4/D. Samples taken at this time were used for the analysis of growth without xenobiotics. After sampling, feed solution was changed to a solution with xenobiotics but without changing the dilution rate allowed the biomass to adapt without additional stress of a toxic shock to the new environment.

Results of Chemostat Experiments:

Growth Rate

The low growth rate of streptomycetes measured in batch cultures (μmax = 0.1 h^{-1}) appears to be the result of growth inhibition due to a metabolic product. Both strains of streptomycetes, 10 137 and 11 796 were not washed out at a dilution rate between 0.11 h^{-1} and 0.16 h^{-1}. Analyses of biomass density indicates growth inhibition. At lower dilution rate the relation of biomass to substrate input was lower than at higher dilution rates.

$D = 0.05$	$\frac{X}{S_0} = 0.01$	(10137)	0.012 (11796)
$D = 0.10$	$\frac{X}{S_0} = 0.02$	(10137)	0.018 (11796)
$D = 0.16$	$\frac{X}{S_0} = 0.0222$	(10137)	0.020 (11796)

Degradation of Orthophenyl Phenol:

Both strains of actinomycetes are able to degrade phenolic compounds. No different phenolic compounds than ortho phenyl phenol could be found, indication that these organisms are able to mineralize this compound. Therefore, cometabolism can be excluded. At a dilution rate D = 0.05 h^{-1} the residual ortho phenyl phenol was about 20 mg/1 for strain 10 137 and 15 mg/1 for strain 11 796. Inflow concentration was for both 100 mg/1. Dry weight of biomass was for the first strain 300 mg/1 and for the second strain 270 mg/1. According to Equation 11 with steady state the removal rate can be calculated:

$$K = \frac{D(A_0 - A)}{X}$$

For strain 10137, K = 13.3×10^{-3} mg removal/mg biomass hour
For strain 11796, K = 15.7×10^{-3} mg removal/mg biomass hour

At D = 0.11 the concentrations were 50 mg/1 resp. 35 mg/1.

Biomass was 127 mg/1 resp. 155 mg/1. The removal rates are:

Strain 10137, $K = 43.3 \times 10^{-3}$ mg removal/mg biomass hour
Strain 11796, $K = 46.1 \times 10^{-3}$ mg removal/mg biomass hour

These rates are not conclusive since the residual concentrations were rather an estimation than a quantitative measurement.

The isolated and tolerant organism alone did not degrade ortho phenyl phenol. But in coculture of the strain 10137 and 11796 with the organism, strain 10137 removed all ortho phenyl phenol, whereas strain 11796 did not show such a high removal efficiency. The experiments with cocultures were made at a dilution rate of $D = 0.08\ h^{-1}$. Consecutively, both cocultures were fed with ortho phenyl phenol only (no glucose). Both cocultures were unstable under these conditions. The tolerant isolate was washed out after $t = 2/D$ and relatively pure cultures of the strains 10137 resp. 11796 were obtained. These organisms were able to maintain a more or less stable culture. But after $t = 6/D$ both were washed out.

CONCLUSION

Since it was not possible to set up complete mass balances and to determine the exact growth kinetics, either with or without xenobiotics, it was not possible to elucidate the mechanisms of degradation of phenolic compounds. However it is possible to derive some qualitative statements. It appears that streptomycetes are able to gain some energy from degrading ortho phenyl phenol. This compound seems to be mineralized and detoxified. Degradation in presence of a different carbon source appears to be facilitated and the data suggest, that the removal rate is a function of the growth rate. Cocultures also might increase the removal efficiency, but it is not evident, what the cause is of this increase.

The experiments with continuous cultures showed clearly, that results obtained in batch cultures can be misleading. It might be, that the biomass in batch systems is exposed to stress and toxic shocks (transfer of inoculum into medium with relatively high concentration of xenobiotics, see Exp. I). In general, the concentrations of ortho phenyl phenol in the continuous systems were much greater than the threshold of toxisity measured in Experiment I.

An interesting finding was the fact, that streptomycetes release a reducing compound, which could be of importance in the degradation of xenobiotic compounds.

This series of experiments with continuous cultures of actinomycetes were a good test for the different methods. It should now be possible to operate continuous cultures with streptomycetes and to obtain a quantitative analysis as well.

TABLE I

concentration of ortho phenyl phenol [mg/1]	Strain 10 137 dry weight [mg]	viability	Strain 11 796 dry weight [mg]	viability
180	5	-	5	-
135	3	-	2	-
90	2	-	4	-
45	3	-	3	-
18	4	-	4	-
13.5	3	-	12	+
9.0	3	-	16	+
4.5	3	-	14	+
1.8	12	+	15	+
0	14	+	13	+