0273-2289/93/3940—0509$03.40

Kinetics of Growth and Catechol Production by *Bacillus stearothermophilus* BR321

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ABSTRACT

A continuous bioprocess for the specialty chemical catechol has been developed using *Bacillus stearothermophilus* BR321. The *meta* pathway for phenol degradation has been blocked at the catechol 2,3-dioxygenase enzyme using transposon mutagenesis. As a result, the organism grows on a dilute growth medium supplemented with phenol and accumulates catechol. In batch culture, catechol concentrations up to 1 m*M* were obtained. A kinetic model has been developed to describe batch growth for different initial phenol concentrations. In continuous culture conducted at a diluation rate of 0.12 hr^{-1}, steady states were obtained for feed phenol concentrations up to 0.24 m*M*, resulting in catechol concentrations up to 0.08 m*M*. Above this phenol concentration, oscillatory dynamics were observed.

Index Entries: *Bacillus stearothermophilus;* catechol; phenol; kinetics; thermophile.

NOMENCLATURE

X: cell concentration (cells/100 μL)
μ: specific growth rate (h^{-1})
P: phenol concentration (m*M*)
C: catechol concentration (m*M*)
S: substrate concentration (g/L)
$y_{s/x}$: substrate yield coefficient ([g substrate·100 μL]/[L·cells])

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$y_{c/p}$: catechol yield (mol catechol produced/mol phenol used)
m: maintenance coefficient ([g substrate·100 μL]/[L·h·cells])
k_p: specific phenol degradation rate ([m*M* phenol·100 μL]/[h·cells])
k_c, n: catechol inhibition constants
D: dilution rate (h^{-1})

INTRODUCTION

Catechol, also known as pyrocatechol or 1,2-dihydroxybenzene or 1,2-benzenediol, is a specialty chemical used industrially for fur dyeing, leather tanning, photographic applications, and as a polymer intermediate (*1*). It is currently produced by phenol hydroxylation using hydrogen peroxide, and costs $2.80/lb (*2*). Annual production of catechol is around 20,000 tonnes (*1*).

Bacillus stearothermophilus strain BR219, a phenol-resistant thermophile, degrades phenol to carbon dioxide and water via the *meta* pathway (*3,4*). Addition of tetracycline blocks this pathway by inhibiting catechol 2,3-dioxygenase, the second enzyme in the pathway, resulting in catechol accumulation (*5*). *Bacillus stearothermophilus* BR321, the strain used in this study, is a mutant developed from BR219 that contains transposon Tn916 stably inserted in the catechol 2,3-dioxygenase gene. This mutation prevents the formation of active catechol 2,3-dioxygenase and thus causes catechol accumulation (*6*). This article reports the growth and catechol-production properties of *Bacillus stearothermophilus* BR321 in batch and continuous culture.

ANALYTICAL TECHNIQUES

Cell concentration was measured by colony counts, cell dry weight, or turbidity. The dry weight assay has been described previously (*7*). In the colony-count assay, a known volume of cell broth was spread on LB plates, after a suitable dilution, to give 30–300 colonies/plate. The plates were then incubated in an oven at 55°C (*8*). The resulting colonies were counted, and the data were expressed as cells (colony-forming units)/100 μL of sample. If catechol was not present in the reactor, the simpler turbidity assay was used. In this assay, the optical density (OD) at 650 nm was measured vs a sterile medium blank using a Perkin Elmer (Norwalk, CT) Lambda 3A spectrophotometer. When catechol is present, the brown color of the catechol oxidation products interferes with the assay. Phenol and catechol were assayed by the colorimetric, 4-amino antipyrene methods as described by Martin (*9*) and Irie et al. (*10*), respectively.

CULTURE CONDITIONS

Batch experiments were conducted in 250-mL Bellco (Vineland, NJ) flasks with stainless-steel caps that allow gas exchange, while maintaining sterility. An Aquatherm water bath shaker (New Brunswick, Edison, NJ) was used to maintain the temperature at 55°C and provide agitation at 200 rpm. A dilute medium (DP) (*11*) that contained (per liter) 0.5 g K_2HPO_4, 1 g NH_4Cl, 0.2 g $MgSO_4$, 0.2 g yeast extract, 0.1 g casamino acids, 1 mL trace element solution (*12*), and the desired amount of phenol was used in these experiments.

Continuous-culture experiments on DP medium were conducted in a 1.3-L New Brunswick Bioflo II fermentor. The impeller rate was 100 rpm, and the air flow rate was 3 L/min. Dilution rate was held steady at 0.12 h^{-1}, and the feed phenol concentration was varied from 0 to 0.36 m*M*. A 5% inoculum of log phase *B. stearothermophilus* BR321 culture grown on LB-Broth (*13*) that contained (per liter) 10 g tryptone, 5 g yeast extract, and 5 g NaCl, was used in both batch and continuous cultures. Additional details of the fermentation techniques and growth media have been given previously (*7*).

RESULTS

Batch Culture

Batch growth experiments were conducted at phenol concentrations of 0, 0.8, 1.52, and 2.39 m*M* to study the kinetics of BR321, and to determine the effect of phenol concentration on growth. Figures 1, 2, and 3 show the time profiles of growth, phenol concentration, and catechol production for the different cases. As seen in Fig. 1, cell growth peaked approx 8 h after inoculation, during which time the number of cells increased by a factor of about 3. Phenol concentration did not significantly affect the rate of growth and maximum cell concentration in the range of concentrations tested. Based on the data shown in Figs. 2 and 3, the molar catechol yields were 0.634, 0.745, and 0.652 mol catechol produced/mol phenol consumed for initial phenol concentrations of 0.802, 1.52, and 2.39 m*M*, respectively.

Assuming that phenol degradation occurs only by the *meta* pathway and that catechol 2,3-dioxygenase production is entirely disabled by the transposon insertion, the number of moles of catechol produced should equal the number of moles phenol consumed. Thus, the molar catechol yield values were less than expected. This discrepancy may be the result of the autoxidation of catechol, as evidenced by the increasing brown color of the flasks with time. The amount of catechol unaccounted for increased

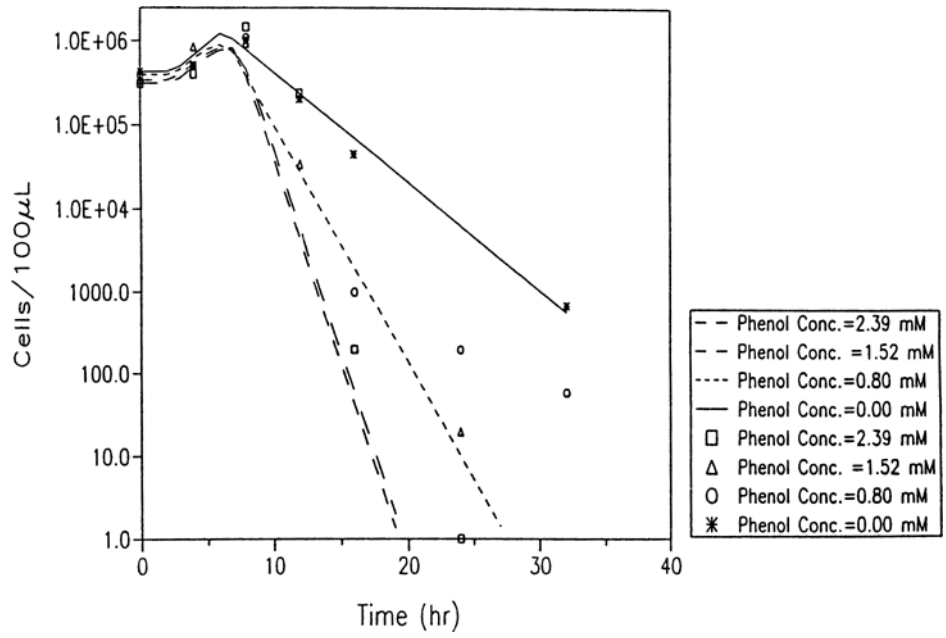

Fig. 1. Cell concentration vs time in batch culture. —— Phenol Conc. = 2.39 mM; -- Phenol Conc. = 1.52 mM; ---- Phenol Conc, = 0.80 mM; — Phenol Conc. = 0.00 mM; □ Phenol Conc. = 2.39 mM; △ Phenol Conc. = 1.52 mM; ○ Phenol Conc. = 0.80 mM; * Phenol Conc. = 0.00 mM.

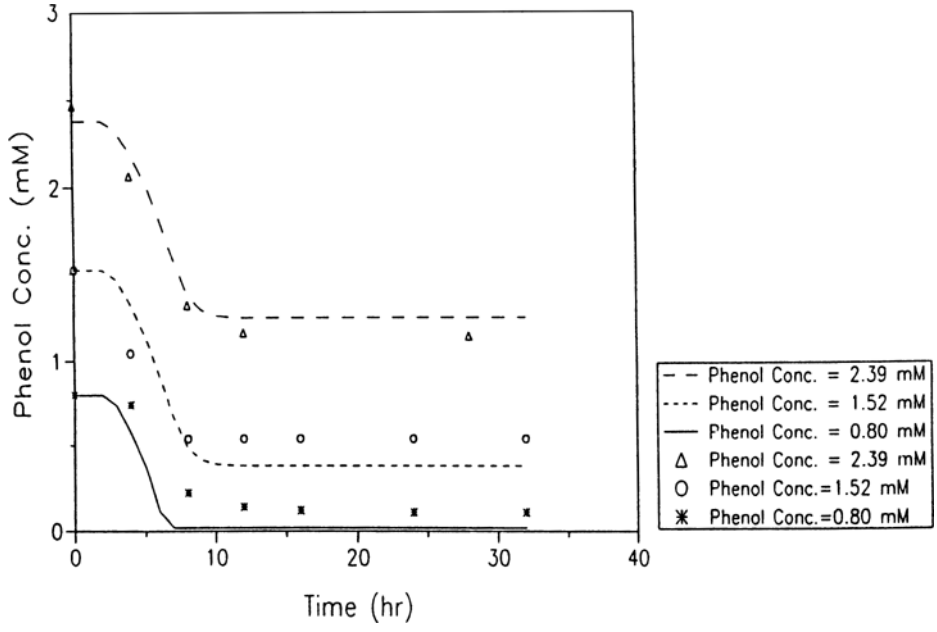

Fig. 2. Phenol concentration vs time in batch culture. —— Phenol Conc. = 2.39 mM; ---- Phenol Conc. = 1.52 mM; — Phenol Conc. = 0.80 mM; △ Phenol Conc. = 2.39 mM; ○ Phenol Conc. = 1.52 mM; * Phenol Conc. = 0.80 mM.

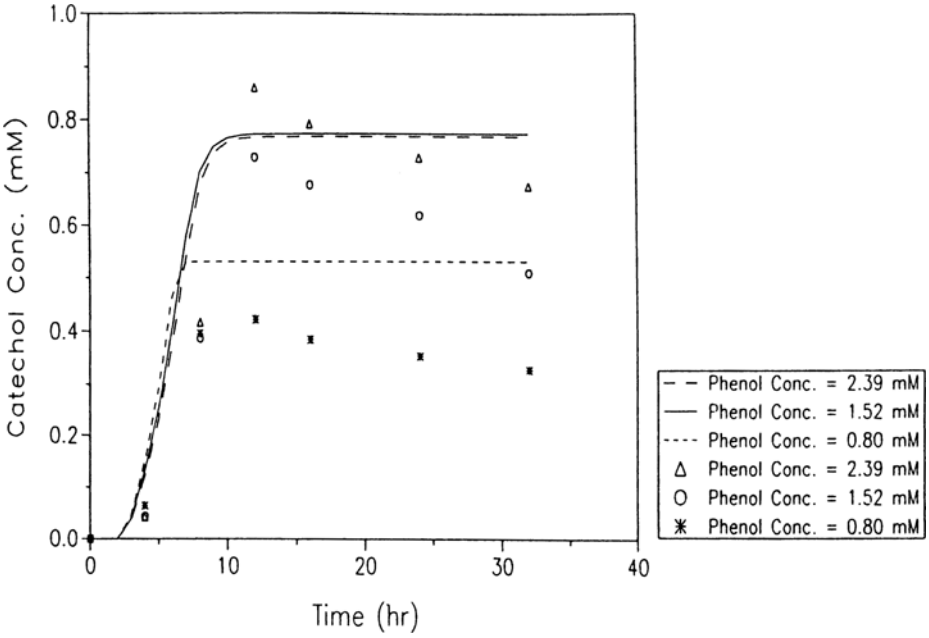

Fig. 3. Catechol concentration vs time in batch culture. —— Phenol Conc. = 2.39 mM; — Phenol Conc. = 1.52 mM; ---- Phenol Conc. = 0.80 mM; △ Phenol Conc. = 2.39 mM; ○ Phenol Conc. = 1.52 mM; * Phenol Conc. = 0.80 mM.

with increasing phenol concentrations, but the molar yield of catechol remained almost the same. The maximum catechol concentration achieved was 0.86 m*M*. In another experiment (data not shown) using 2.5 m*M* phenol, a catechol concentration of 1 m*M* was achieved.

In all four cases, the viable cell concentration peaked 8 h after inoculation and then declined rapidly. Thus, onset of the death phase is thought to be owing to substrate depletion after 8 h. However, the rate of cell death increased with increasing catechol concentration, suggesting that catechol, or a catechol autoxidation product, is toxic to the cells.

Batch Model Development

The batch growth kinetics of *B. stearothermophilus* BR321 were relatively unaffected by phenol concentration in the range of 0 to 2.4 m*M* during the first 8 h. Thus, it was assumed that the specific growth rate was constant and independent of the phenol concentration during the growth phase. The complex nature of the carbon substrates used in these experiments (yeast extract and casamino acids) made it impractical to measure a "substrate concentration" experimentally. It was therefore assumed that all substrates could be lumped together. The rate of cell death was assumed to be a function of the catechol concentration.

The model consists of two sets of equations: one for the growth phase and another for the death phase. Substrate uptake and bioconversion of

phenol to catechol are assumed to occur only in the growth phase. Conversely, when the substrate concentration drops to zero, growth ceases, and death begins.

Batch Model Equations

Growth phase:

$$(dX / dt) = \mu X - k_c C^n X \quad (1)$$

$$(dS / dt) = - y_{s/x} \mu X - mX \quad (2)$$

$$(dP / dt) = - k_p X \quad (3)$$

$$(dC / dt) = - y_{c/p} (dP / dt) \quad (4)$$

Death phase:

$$(dX / dt) = - k_d X - k_c C^n X \quad (5)$$

$$(dS / dt) = 0 \quad (6)$$

$$(dC / dt) = 0 \quad (7)$$

$$(dP / dt) = 0 \quad (8)$$

The values of the model constants were determined from the data given in Figs. 1, 2, and 3, and are given in Table 1. The specific growth rate (μ) was determined from turbidity data (not shown) for the case where no phenol was added. The specific cell-death-rate constant (k_d) was evaluated as the slope of the death-phase region of Fig. 1 for the case where no phenol was added. The coefficients of linearity (*14*) in both these fits were higher than 95%. The cell death rates increased as the catechol concentration in the reactor increased, suggesting that catechol toxicity played an important role in accelerating cell death. The difference in cell death rates, between the cases where there was no catechol produced and where catechol was produced, was attributed to catechol toxicity. The catechol-toxicity constants (k_c and n) were calculated by plotting this difference in the cell death rates (log scale) vs the catechol concentration in the reactor, as shown in Fig. 4.

The value of $y_{s/x}$ was evaluated as the concentration of carbon substrate added to the medium divided by the change in the cell concentration. Cell maintenance was assumed to be 5% of the total substrate consumed. As a first approximation, the specific phenol-degradation coefficient (k_p) was evaluated as the rate of change in phenol concentration divided by the maximum cell concentration. The value of k_p was then adjusted slightly to optimize the agreement between the data, given in Fig. 2, and the model.

The model was solved using the IMSL (International Mathematical and Statistical Library, Houston, TX) subroutine "IVPRK." This subroutine uses a combination of the fifth- and sixth-order Runge-Kutta-Verner methods to solve simultaneous differential equations.

Table 1
Model Constants

μ (h^{-1})	0.32
k_d (h^{-1})	0.3
k_c ($mM^{-2.3}$ h^{-1})	0.63
n	2.3
$y_{x/s}$ ([g substrate·100 μL)/(L·cells])	2.4×10^{-7}
k_p ([mM phenol·100 μL]/[h·cells])	3×10^{-7}
m ([g substrate·100 μL]/[L·h·cells])	1.2×10^{-8}
y_{cp} (mol catechol/mol phenol)	0.677

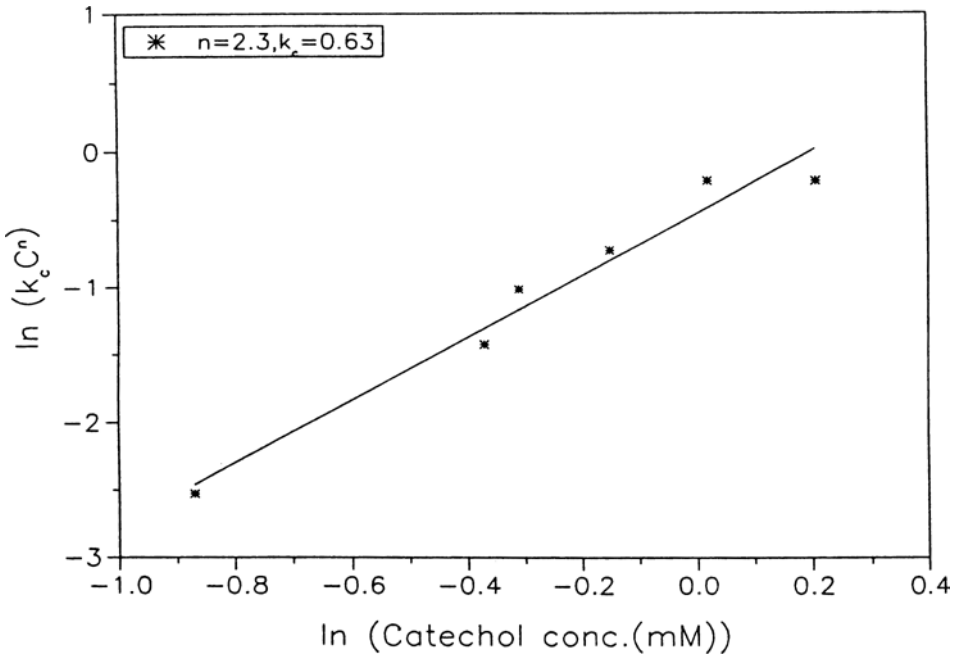

Fig. 4. Determination of catechol inhibition constants.

Batch Model Results

The model predictions are shown along with the experimental data in Figs. 1, 2, and 3. There is good quantitative agreement between the model predictions and the experimental data for cell growth and phenol degradation. The average molar catechol yield was used to evaluate the catechol concentration, and the model predictions are shown in Fig. 3.

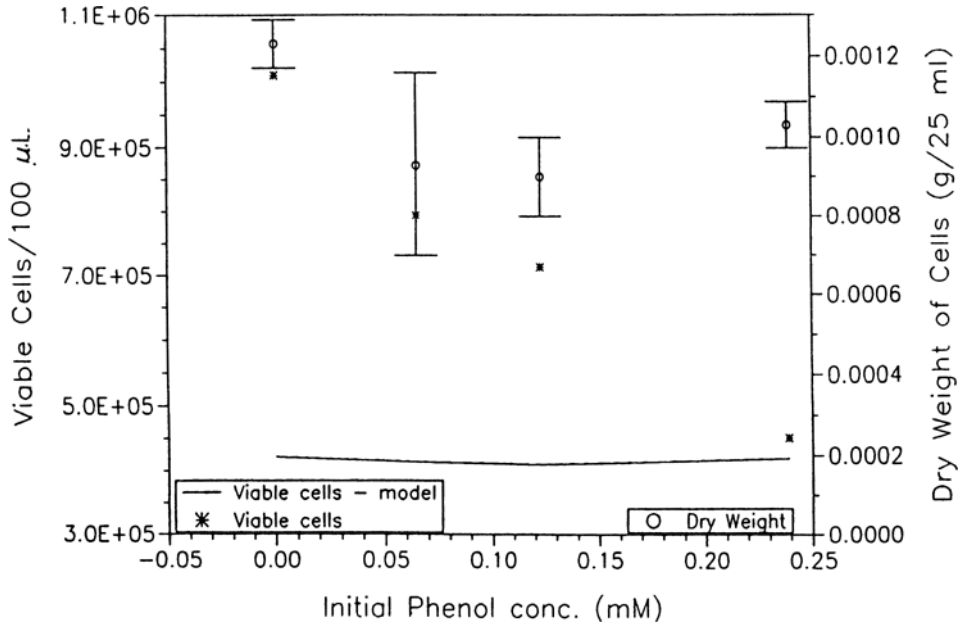

Fig. 5. Steady-state cell concentration vs feed phenol concentration in continuous culture.

Continuous Culture

The main objective of the continuous-culture experiments was to evaluate the feasibility of using this organism for producing catechol continuously. The steady-state cell dry-weight concentration and viability are plotted as a function of the feed phenol concentration in Fig. 5. The steady-state catechol concentration is plotted as a function of the feed phenol concentration in Fig. 6. The maximum steady-state catechol concentration achieved was 0.08 m*M*. When the feed phenol concentration was changed from 0.24 to 0.36 m*M*, the catechol concentration began to oscillate, as shown in Fig. 7. This experiment was run for more than 50 residence times, and during this time, the catechol concentration went through three cycles. The period of the oscillations increased from one cycle to the next. Also, there was an increasing lag period from one cycle to the next (i.e., 10 h between the first two cycles and 100 h between the next two cycles). The maximum catechol concentration achieved in this experiment was 0.11 m*M*. The reactor broth turned increasingly brown as the feed phenol concentration was increased, suggesting increasing concentrations of catechol-oxidation products.

Continuous Culture Model

The mathematical model developed from batch experiments was extended to the continuous process by including a flow term in each of the

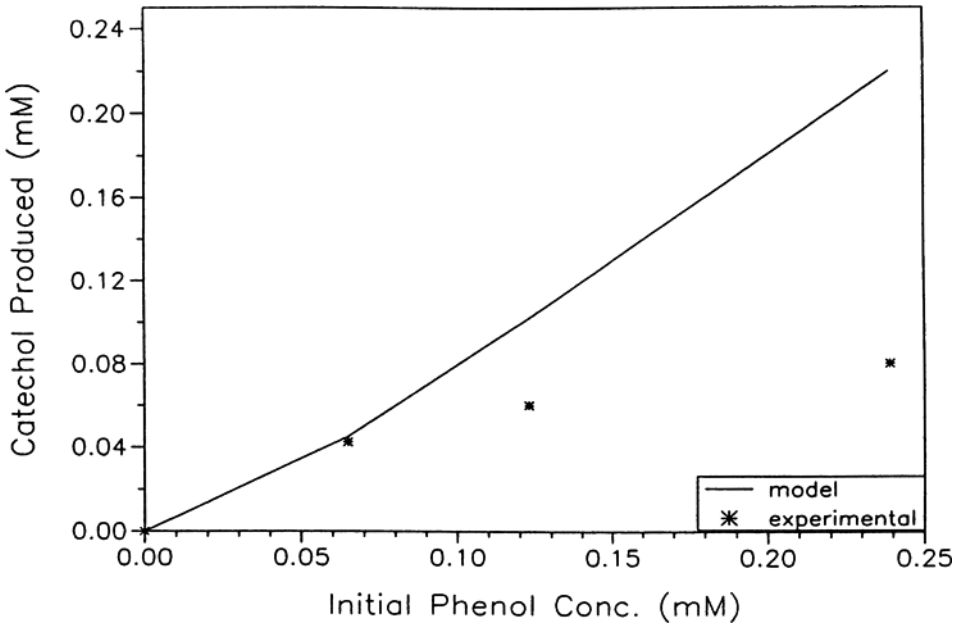

Fig. 6. Catechol concentration vs feed phenol concentration in continuous culture.

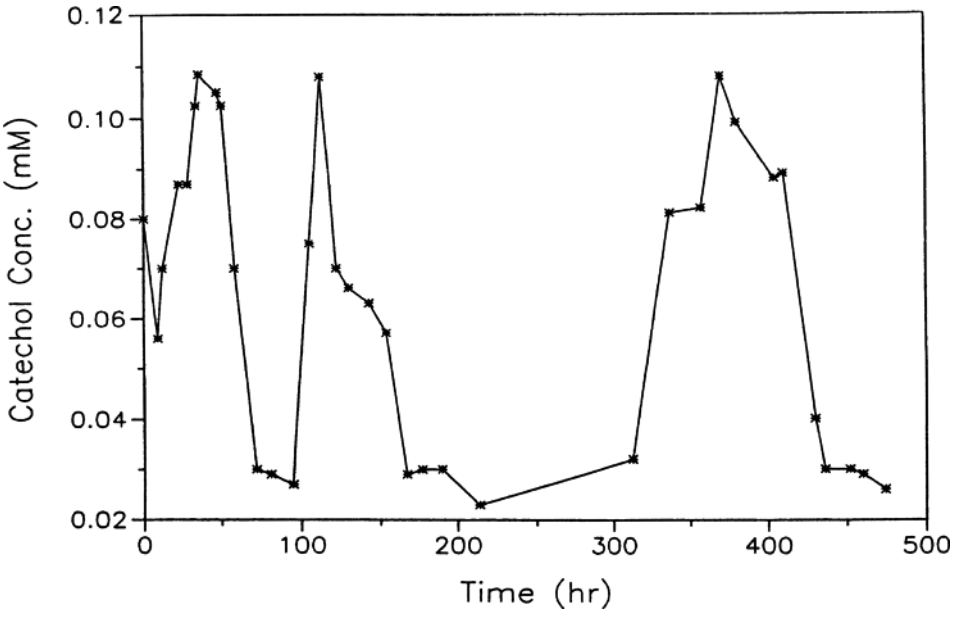

Fig. 7. Unsteady-state growth of BR321-feed phenol concentration= 0.36 m*M*.

differential equations. The model results for cell and catechol concentrations are shown, along with the experimental data, in Figs. 5 and 6, respectively.

DISCUSSION

The highest catechol concentrations were achieved in batch culture. These concentrations, up to 1 m*M*, appear to be toxic to the cells, as shown by the increasing cell death rate associated with increasing catechol concentrations. Autoxidation products of catechol may also be toxic. Partial oxidation of catechol is evidenced by the appearance of a brown color after exposure of catechol to air. This autoxidation may explain the unexpected deviation from a molor equivalence between the amount of phenol consumed and catechol produced.

Like its parent stain, *Bacillus stearothermophilus* BR321 is phenol-tolerant. It can grow rapidly (0.3 h^{-1}) in 2.4 m*M* phenol. No inhibition of cell growth was observed for phenol concentrations up to 2.4 m*M*.

The simple mathematical model developed for the batch experiments reasonably predicts growth and phenol consumption. However, the model overpredicts catechol production when the theoretical, rather than the experimental, catechol yield was used. It is not clear why the catechol yield is lower than expected. Potential explanations are that a portion of the phenol is being metabolized by a different pathway, that the transposon mutation is not entirely stable, and that extracellular catechol-degradation reactions (oxidation and polymerization) occur at a significant rate. Further research is needed in this area. The ability to measure the substrate concentration could also enhance the understanding of this bioprocess.

The continuous-culture experiments demonstrated the ability of *B. stearothermophilus* BR321 to produce catechol continuously, although at lower concentrations. As the feed phenol concentration increased, the cell viability decreased, presumably because of catechol toxicity. At the same time, the cell dry weight remained approximately constant, suggesting that the total amount of cell mass produced is limited by the amount of carbon substrate in the feed.

When applied to the continuous-culture data, the mathematical model developed from the batch data underpredicted the cell concentration. This disagreement is not surprising. The continuous experiments were conducted in a New Brunswick Bioflo II fermentor, which has better control, mixing properties, and aeration than the Bellco flasks used for the batch experiments. In addition, the model is simple in nature, and does not account for saturation kinetics with respect to the carbon substrate, catechol autoxidation, or the inhibitory effect of catechol on the rates of cell growth and phenol uptake. There is even evidence (not shown) that the oxidation byproducts may be more toxic to the cells than the catechol itself.

The oscillatory behavior observed at higher feed phenol concentrations is common for continuous culture in which the product is inhibitory (*15,16*). This behavior is thought to arise from intracellular metabolic regulation processes that impart time lags into the process dynamics (*17*). In addition, this organism is prone to rapid death in the absence of substrate, as shown in Fig. 1. This tendency could also lead to oscillations in continuous culture and could help explain the high fraction of dead cells obtained for high-feed phenol concentrations in Fig. 7.

These results bear on the feasibility of an industrial catechol fermentation. Because of its high tolerance to phenol and elevated growth temperatures, the risk of microbial contamination is less than for other fermentations. Thus, less expensive fermentation equipment might be feasible for continuous catechol fermentations. However, the low product concentration and the tendency to oscillate make continuous operation problematic. The use of a continuous separation process to remove the catechol selectively as it is produced could greatly enhance the reactor productivity. Such a separation process could be based on solvent extraction or adsorption onto a solid phase. The high growth rates and ability to grow on dilute, inexpensive media seem to indicate that this process may become a suitable alternative for catechol production.

CONCLUSIONS

Bacillus stearothermophilus BR321 grows rapidly (0.3 h^{-1}) on a dilute (DP) medium and converts phenol to the specialty chemical catechol. Catechol concentrations as high as 1 m*M* were achieved in batch culture. Cell death followed immediately after the growth phase. Catechol autoxidation products may also affect the kinetics of this organism. A simple mathematical model was developed to predict the rates of cell growth, death, and catechol production in batch culture. Cell death was attributed to both a lack of substrate and catechol toxicity. The model fit the batch data well, but did not agree with the continuous-culture results. Catechol yields were about 30% less than theoretical.

Continuous-culture experiments conducted at a dilution rate of 0.12 h^{-1} showed continuous, steady-state catechol production up to a feed phenol concentration of 0.24 m*M*. Above this concentration, oscillatory dynamics were observed.

ACKNOWLEDGMENTS

We would like to thank P. J. Oriel and M. R. Natarajan for providing the microorganism used in this study. The funding provided by the Michigan State University Crop and Food Bioprocessing Center is also appreciated.

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