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Growth Kinetics of *Bacillus stearothermophilus* BR219

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ABSTRACT

Bacillus stearothermophilus BR219, a phenol-resistant thermophile, can convert phenol to the specialty chemical catechol. The growth kinetics of this organism were studied in batch, continuous, and immobilized-cell culture. Batch growth was insensitive to pH between 6.0 and 8.0, but little growth occurred at 5.5. In continuous culture on a dilute medium supplemented with 10 m*M* phenol, several steady states were achieved between dilution rates of 0.25 and 1.3 h^{-1}. Phenol degradation was found to be uncoupled from growth. Immobilized cells grew rapidly in a rich medium, but cell viability plummeted following a switch to a dilute medium supplemented with 5 m*M* phenol.

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

INTRODUCTION

Thermophilic microorganisms are able to catalyze a variety of economically important chemical reactions and offer several advantages over their mesophilic counterparts for bioprocessing (*1,2*). In addition to the inherently higher diffusion and reaction rates that occur at elevated temperatures, it is theorized that the increased resistance to thermal degradation required for thermophily may also result in increased resistance to chemical denaturation (*3*). Thus, thermophiles may play an important future role in bioconversion or biodegradation of chemicals at concentrations generally toxic to mesophiles.

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Bacillus stearothermophilus BR219, originally isolated from river sediment, exhibits extreme phenol tolerance, growing well on 15 m*M* (1.4 g/L) phenol (*4*). It harbors a 60 kb plasmid coding for phenol degradation via the *meta* pathway. The addition of 5 μg/mL tetracycline blocks this pathway by selectively inhibiting catechol 2,3 dioxygenase, resulting in catechol accumulation in the medium (*5*). Catechol (*o*-dihydroxybenzene) is a specialty chemical ($2.50 per pound) currently produced from *o*-chlorophenol, a relatively expensive raw material. An alternative biological process using this strain to produce catechol from inexpensive phenol has recently been patented (*6*). This paper reports the growth kinetics of *B. stearothermophilus* BR219 in batch, chemostat, and immobilized-cell culture.

METHODS

Growth Media

Fermentations were carried out using either a rich nutrient medium (L broth) containing per liter: 10 g tryptone, 5 g yeast extract, and 5 g NaCl, or a dilute (DP) medium (*7*) containing 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 (*8*), and the desired amount of concentrated phenol stock solution (88% by volume). For both media, the pH was adjusted to 7.2 prior to autoclaving. The L broth gives rapid growth to high cell densities but represses the phenol-degradation pathway. The DP medium sustains rapid cell growth to low cell densities, does not repress the pathway, and supplies the reducing equivalents needed by the NADH-linked phenol hydroxylase for bioconversion of phenol to catechol (*4*).

Analytical Methods

Cell concentration was measured by turbidity, dry weight, or staining with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). In the turbidity assay, the optical density (OD) of the sample was measured at 660 nm vs an uninoculated medium blank using a Perkin Elmer Lambda 3A spectrophotometer. Samples were quantitatively diluted with uninoculated medium to an OD value less than 1.2, if necessary. In the dry-weight assay, a 50 mL sample was volumetrically withdrawn and centrifuged at 5900*g* for 8 min at 4°C. An aliquot of the supernatant was refrigerated at 4°C for later phenol assay, and the remainder was discarded. The cells were resuspended in an equal volume of distilled water and then centrifuged again as before. The supernatant was discarded, and the cell pellet was quantitatively rinsed into a pre-weighed drying pan. The sample was dried in an 85°C oven to a constant weight. The dry-weight cell concentration was taken as the difference between the final dry weight and the weight of the empty pan divided by the volume of the liquid sam-

ple. In the continuous-culture studies, the turbidity and dry-weight data are correlated by the calibration factor 0.142 g dry wt/L·ODU. The standard deviation of this value is relatively high (0.0145 g/L·ODU), owing to imprecision in the dry-weight assay at the low cell concentrations supported by the DP medium.

The INT assay is based on the intracellular reduction of the colorless INT salt to the intensely red INT formazan. The reaction is indicative of an active electron-transport system; thus, metabolically inactive cells do not give a response. Further details of the INT method are given by Worden (*9*). To perform the assay, two gel beads with entrapped cells were removed from the reactor and incubated in 1 mL of 0.1% INT solution at 55°C for 30 min. The beads were then minced and extracted with 3 mL acetone. The extractant was diluted by a factor of 3 with acetone, and its optical density was read at 490 nm against an acetone blank. Phenol was assayed by the colorimetric, 4-aminoantipyrene method (*10*).

Thermostable Gel Beads

Cells were immobilized in a thermostable gel matrix composed of a 2:1 ratio of 1% Gelrite (Kelco Division of Merck & Co., Inc) and 2% alginic acid (Sigma #A-2033) by weight. Water was steam-sterilized and then used to make the 2% alginate and 1% Gelrite stock solutions. The Gelrite solution was boiled for 2 min, mixed with the alginate solution, and then allowed to sit overnight at room temperature. Forty milliliters of late-log-phase *B. stearothermophilus* culture were centrifuged, and the cells were resuspended in 150 mL of liquid gel. Beads 3 mm in diameter were formed by dripping the gel into a sterile, 3% $MgCl_2$ hardening solution at room temperature, and allowing 15 min for proper bead hardening.

Culture Conditions

All experiments were conducted at 55°C. This temperature was found to be optimal for catechol production from phenol (*5*).

Batch growth experiments on L broth were conducted in a 1.3 L New Brunswick Bioflo II fermenter, using automatic addition of 1 N NaOH to control pH. The impeller rate was 300 rpm, and the air flow rate was 3 L/min. A 5% inoculum of log-phase culture grown on L broth was used.

Continuous culture experiments were carried out in the same reactor using DP medium with 10 m*M* phenol. The reactor was inoculated (5%) with log-phase cells grown on L broth. Once growth was visibly apparent, continuous feeding was initiated. No pH control was needed, because of the relatively low cell concentrations. The maximum pH change was 0.2 pH units.

Immobilized-cell experiments were conducted in a 250 mL Erlenmeyer flask. Approximately 100 beads were placed into 150 mL of modified L broth (1 g/L of NaCl was replaced by 1 g/L of $MgCl_2 \cdot 6H_2O$ to maintain

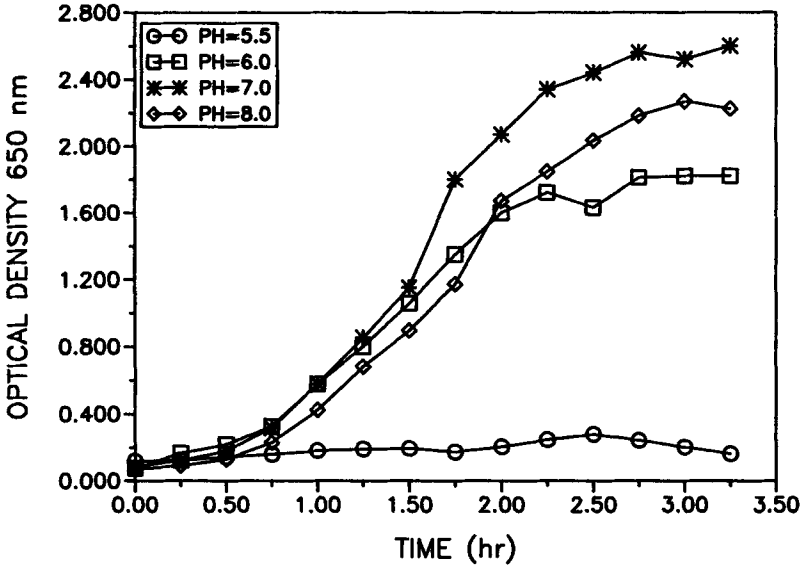

Fig. 1. Effect of pH on batch growth in L broth.

long-term bead integrity). Temperature control and mixing were provided by a water-bath shaker at 200 rpm. After 4 h of cell growth, the L broth was replaced by DP medium with 5 m*M* phenol, and the incubation was continued. Periodically, bead samples were taken and analyzed for viable cells using the INT method.

RESULTS

Batch Culture

The batch growth kinetics of *B. stearothermophilus* BR219 in L broth are shown in Fig. 1 for several pH values. The initial growth rates were relatively unaffected by pH in the range of 6.0 to 8.0, but the cell yield was highest at a pH of 7.0. The specific growth rate, determined by the slope of a semilogarithmic plot of OD vs time, reached a maximum value of 2.0 h^{-1} early in the fermentation and then declined steadily. Growth was almost negligible at a pH of 5.5.

Because the aqueous solubility of oxygen decreases as temperature increases, oxygen availability is a likely rate-limiting factor in fast-growing, aerobic, thermophilic fermentations. The oxygen concentration in equilibrium with air at 55°C is 5.9 mg/L, compared with 7.3 mg/L at 35°C (*11*). In an attempt to avoid oxygen limitation, relatively high impeller and air flow rates were used in these experiments. In addition, the oxygen concentration was monitored continuously with an oxygen probe (data not shown). The oxygen concentration dropped considerably as the cell concentration increased, but was never less than 10% air saturation (0.6

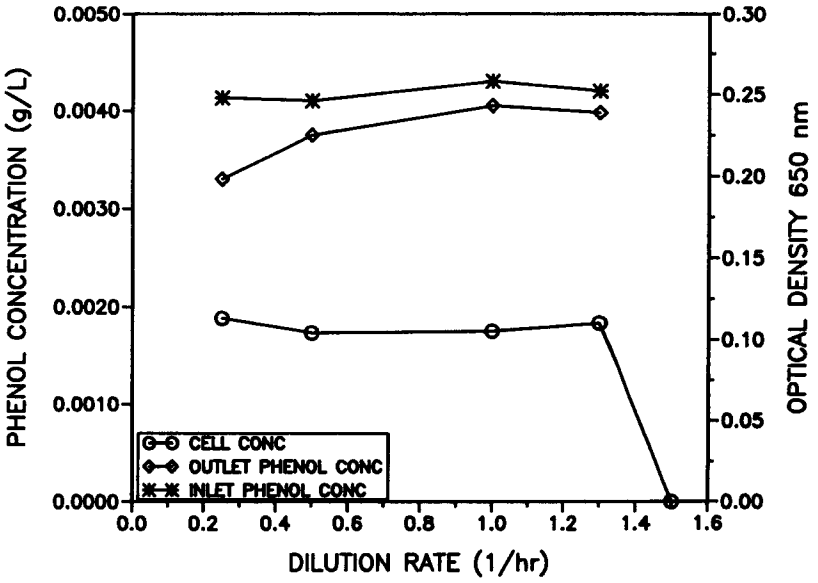

Fig. 2. Steady-state phenol and cell concentrations for continuous growth in DP medium supplemented with 10 m*M* phenol.

mg/L). The Monod constant for oxygen (K_o) for this strain is not known. However, K_o values tabulated by Atkinson and Mavituna all lie between 1×10^{-4} and 0.1 mg/L (*12*). Thus, significant oxygen limitation in these batch experiments is unlikely, but cannot be entirely ruled out.

Continuous Culture

The steady-state cell and phenol concentrations for continuous culture on DP medium with 10 m*M* phenol are shown in Fig. 2 as a function of dilution rate (D). The cell concentration was approximately constant between dilution rates of 0.25 and 1.3 h^{-1}, whereas the fractional phenol degradation varied from 5% at a dilution rate of 1.3 h^{-1} to 20% at a dilution rate of 0.25 h^{-1}. At the higher dilution rates, the bioreactor was found to be marginally stable. Small perturbations, such as minor adjustments in the flow rate, occasionally induced sudden lag phases that lasted up to several hours. Thus, the reactor had to be monitored closely following such changes. The onset of a lag phase at a dilution rate of 1.3 h^{-1} would result in 95% of the cells being washed out in slightly over 2 h (3 residence times). When a lag phase was observed, the pump was switched off until growth was again apparent.

The specific growth rate (μ) and the specific phenol-degradation rate (q_p) express the rates of cell-mass production and phenol degradation per unit mass of cells. At steady state, $\mu = D$, and q_p is given by the following equation

$$q_p = D(P_{in} - P_{out})/X$$

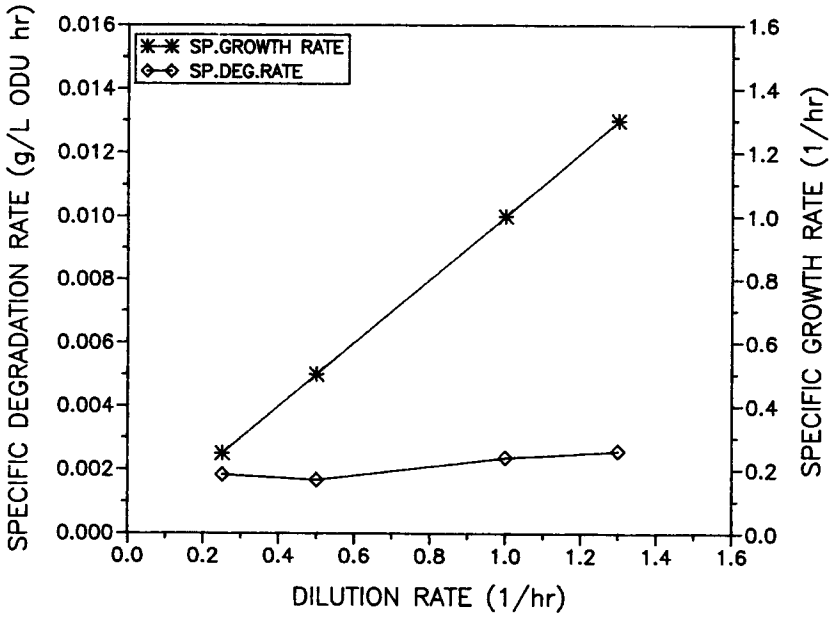

Fig. 3. Steady-state specific growth and phenol-degradation rates for continuous growth in DP medium supplemented with 10 m*M* phenol.

where P_{in} and P_{out} are the inlet and outlet phenol concentrations, and X is the cell concentration. Figure 3 shows μ and q_p as a function of the dilution rate. The markedly different slopes illustrate that phenol degradation was uncoupled from growth under these conditions.

Immobilized-Cell Culture

The dynamics of viable-cell concentration within the thermostable gel beads are shown in Fig. 4. Rapid cell growth occurred during hours 1–4, during which the beads were incubated in L broth. Beads stained with INT and cut in half were intensely colored throughout the bead. However, after the medium was switched to DP with 5 m*M* phenol at hour 5, more than 95% of the INT response was lost within 6 h. During this time, INT-stained beads clearly showed an expanding core region devoid of color, surrounded by a colored region. Finally, after hour 11, the INT reading stabilized at a low level, and stained beads showed only a faint surface layer of red color and a clear interior.

DISCUSSION

Several features of growth and phenol degradation by *B. stearothermophius* BR219 at 55°C have been demonstrated in these experiments. Like

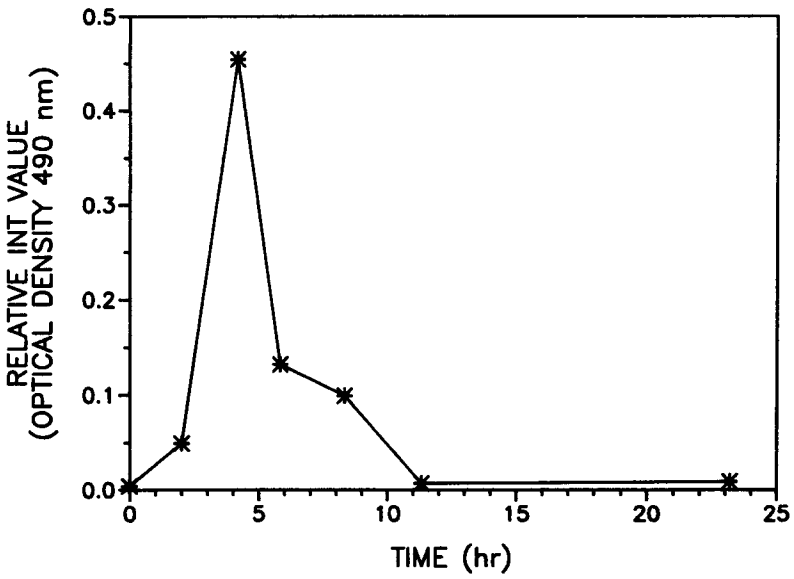

Fig. 4. Dynamics of immobilized cells as indicated by INT staining.

many thermophiles, this strain is capable of extremely high specific growth rates (2.0 h^{-1} at 55°C). In addition, it exhibits extreme phenol tolerance; it can grow on 15 m*M* phenol (*4*), which, to our knowledge, is the highest published microbial tolerance to phenol. This strain can also sustain high steady-state specific growth rates (1.3 h^{-1}) in the presence of 9.5 m*M* phenol. In a previous experiment, steady state was achieved at dilution rates as high as 2.0 h^{-1} using the same medium (DP with 10 m*M* phenol) after a longer period of adaptation (data not shown). This high level of phenol tolerance is consistent with the theory that thermostable enzymes often have increased resistance to chemical denaturation.

Phenol degradation in this strain was shown to be uncoupled from growth. Over a fivefold variation in μ, q_P was relatively constant, varying only 45%. Research is currently underway to place the genes coding for the *meta*-pathway enzymes under the control of a different promoter (*13*).

The small increases in q_P noted at higher dilution rate may have been the result of gradual adaptation of the culture to growth on phenol, rather than a true dependence of q_P on μ. The steady states were achieved sequentially from lowest to highest dilution rates, and continuous culture is commonly used as a tool for improving growth properties on inhibitory substrates (*14*).

Although the INT assay can provide useful information difficult to obtain with other methods, interpretation of the data is not always straight forward. For one strain of *B. stearothermophilus*, INT color was found to be directly proportional to cell concentration during both the log and stationary phases of a batch growth cycle (unpublished data). However, for

Pseudomonas alcaligenes, there was some deviation from a direct proportionality (*9*); thus, in some cases, the INT response may vary with the metabolic state of the cell. Insufficient calibration data are currently available to translate INT measurements into active-cell-mass concentration data for immobilized cells of *B. stearothermophilus* BR219.

The thermostable gel support matrix supported rapid growth of entrapped *B. stearothermophilus* BR219 cells on rich nutrient broth. The sudden decrease in viable cell concentration following the switch to a dilute medium supplemented with phenol may have been caused by insufficient nutrient levels. A similar trend has been observed during batch growth of suspended cells of this strain on DP medium. Further research is thus needed to sustain cells in the stationary phase while not repressing the phenol-degradation pathway.

CONCLUSIONS

Bacillus stearothermophilus grows rapidly at 55°C on both a rich nutrient medium and a dilute medium supplemented with phenol, achieving specific growth rates as high at 2.0 h^{-1}. It is extremely phenol-tolerant, capable of steady-state specific growth rates greater than 1.0 h^{-1} in the presence of 9.5 m*M* phenol. The specific phenol degradation rate is relatively unaffected by growth rate in continuous culture, varying only 45% over a fivefold variation in specific growth rate. The combination of rapid growth rates, extreme phenol tolerance, and *meta* pathway, make this organism well suited for bioconversion processes, such as catechol production from phenol, and biodegradation of aromatics in wastewater-treatment processes.

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