APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1980, p. 320-326
0099-2240/80/02-0320/07$02.00/0
Vol. 39, No. 2

Biological Process for Converting Naphthalene to *cis*-1,2-Dihydroxy-1,2-Dihydronaphthalene

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A biological process for converting naphthalene to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (DHD) catalyzed by *Pseudomonas putida* strain 119 was optimized in flask experiments. These studies revealed the following: (i) *P. putida* 119 can propagate efficiently and produce DHD when supplied one of several carbon sources and naphthalene; (ii) maximum DHD production by *P. putida* 119 occurs in logarithmic-growth-phase cells and decreases at various rates in the stationary growth phase, depending upon the carbon source used; (iii) several analogs of salicylic acid can be used as effective inducers of naphthalene metabolism in *P. putida* cells growing on glucose; and (iv) the addition of chemical surfactants to naphthalene-cell (*P. putida* 119) mixtures stimulates DHD production.

The metabolism of naphthalene in microbial systems has been reported frequently in the last 20 years (2-9, 13, 17, 18), and very early a metabolic pathway was postulated for dissimilation of naphthalene into the central metabolic routes of *Pseudomonas* and other unidentified species (5). The first product of naphthalene degradation, 1,2-dihydro-1,2-dihydroxynaphthalene (Fig. 1), was originally postulated as having a *trans* configuration, but later was indisputably proven to have a *cis* configuration in several *Pseudomonas* species (8, 9).

This compound is an important precursor leading to the production of a commodity pesticide. The batch flask studies described here involve the production of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (DHD) from naphthalene by a mutant strain of *Pseudomas putida* (3, 4). Since the mutant accumulates DHD (8, 9) due to lack of the corresponding dehydrogenase activity (11), these studies centered around the search for alternative carbon and energy sources allowing high DHD production. In addition, methods to improve mass transfer of naphthalene were studied. Finally, salicylic acid has been observed to induce naphthalene metabolism in succinate-grown cells (1, 14, 15), but it became important to find more effective and less costly inducer chemicals for naphthalene metabolism. A method reportedly for the production of the *trans* isomer of DHD from naphthalene by a species of *Nocardia* has been previously described (W. H. Wegner, U.S. patent 3,755,080, August 1973).

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MATERIALS AND METHODS

Organism. *P. putida* strain 119 was provided by D. T. Gibson, University of Texas at Austin.

Chemicals. Reagent-grade naphthalene was obtained from Fisher Scientific Co. Reagent-grade sodium dibasic and monobasic phosphates, ammonium sulfate, potassium chloride, dextrose, and sodium succinate were purchased from Matheson, Coleman and Bell Co. Reagent-grade sodium salicylate, manganese chloride ($2H_2O$), and anhydrous calcium chloride were purchased from Allied Chemical Co. Reagent-grade anhydrous magnesium sulfate was purchased from Mallinckrodt. Ferrous sulfate ($7H_2O$) was purchased from Baker Chemical, and 2-hydroxybenzyl alcohol was purchased from ICN. Shell Neodol 25-3A was purchased from Sika Chemical Corp., and Tergitol NP-14 was obtained from Union Carbide Corp. 2-Aminophenol and 5-chlorosalicylic acid were purchased from Eastman Chemical Products, Inc., and 3,5-dichlorosalicylic acid was purchased from K & K Laboratories, Inc. The 5-chlorosalicylic acid was recrystallized from aqueous ethanol.

DHD assay. The microbial production of DHD was assayed spectrophotometrically as described previously (4; L. A. Kapicak and D. P. Cox, manuscript in preparation).

Growth medium. To 1 liter of distilled water was added: $NaH_2PO_4 \cdot H_2O$, 3.86 g; $Na_2HPO_4 \cdot 7H_2O$, 10.22 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4$, 0.05 g; KCl, 0.05 g; $FeSO_4$, 0.1 mg; $MnCl_2 \cdot 2H_2O$, 0.5 mg; and $CaCl_2$, 27.5 mg. The following were sterilized and added separately to the growth medium: glucose, 5 g; naphthalene, 2 g; and sodium salicylate, 0.2 g.

Cell growth. Growth of the culture was estimated by measuring light scattering of culture broth samples in a 1-cm cuvette at 525 nm in a Coleman model 55 single-beam spectrophotometer and by dry gravimetric determinations.

Culture conditions. (i) Inoculum source. *P. putida* strain 119 stored in liquid nitrogen was used as a

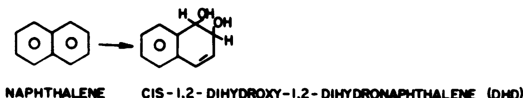

FIG. 1. *Degradation of naphthalene.*

source of inoculum. Frequently, strain 119 from liquid nitrogen was subcultured to glucose agar plates, and isolated colonies from these plates were used as inoculum for flasks. Due to the unstable nature of this strain, growth on plates was used as an inoculum source for only 1 week.

(ii) Batch flask technique. *P. putida* strain 119 was aseptically introduced into sterile 500-ml Erlenmeyer flasks containing 100 ml of minimal medium with inducer minus naphthalene. The flask and contents were incubated on a rotary shaker at 30°C and 200 rpm. Growth was observed for 24 h, and then cells were washed three times in physiological saline. The cells were then added to each flask for DHD production to bring the initial absorbance at 525 nm to about 0.2. These flasks, containing identical medium plus 2.0 g of naphthalene per liter, were incubated as before. All batch flask studies were performed in duplicate. When necessary, cells were grown in 2-liter Erlenmeyer flasks in 500 ml of medium in an identical manner.

(iii) Growth of *P. putida* 119 in 14-liter fermentors: growth procedure. A 500-ml volume of *P. putida* 119 culture was added aseptically to 10 liters of sterile medium in a 14-liter Virtis fermentation vessel. The parameters monitored were pH, dissolved oxygen, temperature, air flow, and agitation.

Sample removal and analysis. Samples were removed by opening a sample line and closing the air exhaust line, which forced rapid sample removal from a point 2 to 3 inches (about 5 to 7.5 cm) above the bottom of the growth vessel.

(iv) Concentrated cell-naphthalene emulsion experiment: preparation of inoculum for batch flask study. Inocula were grown in 100 ml of growth medium containing 5 g of yeast extract (Difco) per liter for approximately 24 h. This culture was transferred to two 2-liter flasks, each containing 500 ml of medium. These flasks contained identical medium supplemented with 2 g of naphthalene per liter. After 16 h, the entire flask contents were aseptically transferred to a sterile 14-liter fermentor. The fermentor contained 10 liters of the same medium and was incubated under the following conditions: temperature, 30°C; pH range, 7.05 to 7.35; agitation rate, 900; dissolved-oxygen range, 1 to 8 mg/liter; air flow rate, 4 liters/min (0.4 volume of air/volume of medium per min).

During the 24-h growth period, the cell mass grew from an initial absorbance at 525 nm of 0.26 to 3.0. At this time, 1.5 liters of culture (2.3 g dry weight equivalent) was harvested in a refrigerated centrifuge. The supernatant fluid was discarded, and the cells were suspended in 100 ml of the appropriate naphthalene-emulsion test medium described below.

Preparation of naphthalene emulsions. The 5% naphthalene suspensions were prepared by placing 200 ml of growth medium (without glucose or mineral salts solution), 10 g of naphthalene, and 1 ml each of Tergitol NP-14 (Union Carbide) and Shell Neodol 25-3A (a nonionic and anionic surfactant, respectively) in a Waring blender and mixing for 5 min.

Shake flask compositions and incubation conditions. Reagents were added to the shake flasks containing the emulsified naphthalene mixture and cells. The final compositions are listed in Tables 1 and 2. Each flask was incubated at 400 rpm on a rotary shaker at 30°C for 24 h. Samples were removed for DHD assay as described below.

Assay procedure. Five-milliliter samples were removed from each flask at intervals and centrifuged at 10,000 rpm. The supernatant fluid was diluted and assayed for DHD.

RESULTS

Determination of replicability of batch flask studies. Initial experiments were performed to determine the replicability of batch flask studies. A culture of *P. putida* 119 was grown in minimal medium for 24 h in the usual manner. The culture was then diluted 10-fold into fresh medium containing inducer (sodium salicylate) and incubated as before. This culture,

TABLE 1. *Medium components used in first high-biomass-naphthalene emulsion study*

Flask no.	Component[a]				
	Biomass[b]	5% Naphthalene	1% Succinate	0.5% NP-14	0.5% 25-3A
1	+	−	−	−	−
2	+	+	−	−	−
4	+	+	+	−	−
5	+	+	+	+	+
6	+	+	−	+	+
7	+	−	−	+	+
8	−	+	−	+	+

[a] +, Added to 100 ml of minimal medium; −, not added.
[b] ~23 mg/ml, dry weight.

TABLE 2. *Medium components used in second high-biomass-naphthalene emulsion study*

Flask no.	Component[a]				
	Biomass[b]	5% Naphthalene	1% Succinate	1% NP-14	25-3A (%)
1	+	−	−	−	−
2	+	+	−	−	−
3	+	+	+	−	−
4	+	+	−	+	0.5
5	+	+	+	+	0.5
6	+	+	+	+	1
7	+	−	−	+	0.5
8	−	+	−	+	0.5

[a] See Table 1.
[b] ~36 mg/ml, dry weight.

being induced for naphthalene metabolism, was used to inoculate eight 500-ml Erlenmeyer flasks containing a minimal medium supplemented with salicylate, naphthalene, and sodium gluconate. The eight flasks were incubated as previously described and monitored for growth and DHD production.

After an initial lag period, the logarithmic growth phase began and continued for 4 to 6 h (Fig. 2). The mean growth rate calculated from data of the eight replicate flasks was 0.36 generations/h. Measurable DHD production from naphthalene began with the onset of logarithmic growth and continued at a decreasing rate throughout the stationary phase to a final mean level of 1.7 to 1.8 g/liter. In calculating the results, we observed a mean variation of less than 0.1 in both growth and DHD production data. Greater variance occurred in the turbidity data than in the DHD concentration data, particularly during the stationary growth phase.

Effects of growth and DHD production with various carbon sources. We sought carbon sources on which *P. putida* could grow rapidly and simultaneously exhibit high DHD production from naphthalene. Since species of *Pseudomonas* are known to grow well on certain low-molecular-weight organic acids (16), several were included in this study. Carbon sources tested included the sodium salts of lactate, succinate, gluconate, and citrate. Ethanol, glucose, and corn syrup were also tested, the latter providing a mixture of glucose, maltose, and higher-molecular-weight polysaccharides. Sucrose (and molasses) were not utilized for growth by *P. putida* 119.

When corn syrup, gluconate, and glucose were used as carbon sources, cell growth rates of *P.*

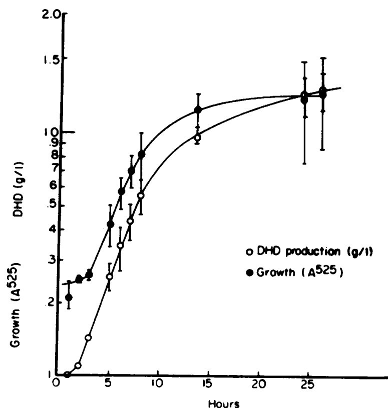

FIG. 2. *Mean growth curve and DHD production of eight replicate cultures of P. putida 119 grown on gluconate-naphthalene medium.* A^{525}, *absorbance at 525 nm.*

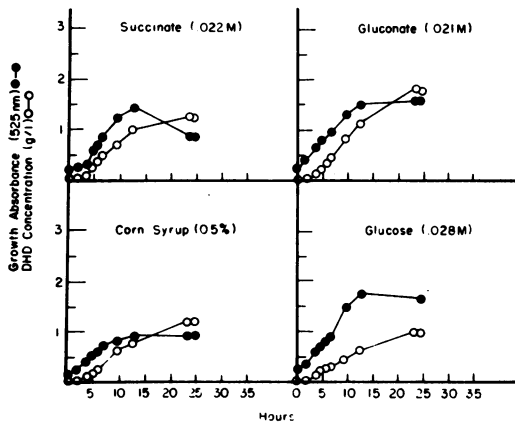

FIG. 3. *Growth and DHD production by P. putida 119 when grown in shake flasks with succinate, gluconate, corn syrup, and glucose as carbon sources.*

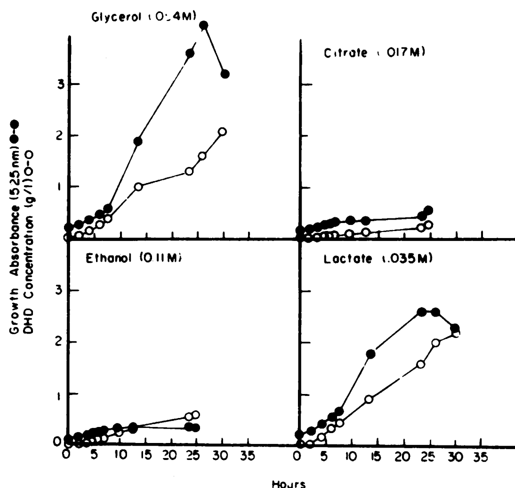

FIG. 4. *Growth and DHD production by P. putida 119 when grown in shake flasks with glycerol, citrate, ethanol, and lactate as carbon sources.*

putida were similar (Fig. 3 and 4). Cell growth was poorest on citrate and ethanol.

DHD production by these cells varied, depending on the carbon sources provided. Cells grew poorly on ethanol but exhibited the highest DHD production per unit cell. Cells grown on either succinate or corn syrup exhibited somewhat lower production per cell but higher than for 0.5% glucose-grown cells. The fact that corn syrup-grown cells produced more DHD than glucose-grown cells here was related to a glucose concentration effect described elsewhere (4).

DHD productivity rates were logarithmic in all batch flask studies during logarithmic growth regardless of the growth substrate. The specific activities of DHD production continued upward at a declining rate during the stationary phase.

These conditions were observed when *P. putida* 119 was grown on all carbon sources except glucose used at concentrations of 0.5% and higher (4).

Comparison of salicylic acid with analogs as inducers of naphthalene metabolism. The effectiveness of several analogs was compared with that of salicylic acid in inducing DHD production from naphthalene when cells were growing on glucose. As is evident from the growth curves, the presence of each chemical did not significantly affect the growth rates over the initial 12-h period, although the final growth yields were lower in flasks containing highest inducer levels (Fig. 5). DHD production during the logarithmic growth phase was significantly induced by salicylate at both concentrations tested. However, neither 2-hydroxybenzyl alcohol nor 2-aminobenzoate induced DHD production significantly above the controls.

Another structural analog, 2-aminophenol, stimulated DHD production during stationary but not logarithmic growth (Fig. 5). The stimulation by 2-aminophenol appeared to be equally effective at as low as 5 mg/liter (Fig. 6). No attempt was made to identify the mechanism of the stimulating effect produced by 2-aminophenol, but we suspect a naphthalene transport facilitation or a glucose-sensitive naphthalene induction mechanism.

Cells were grown as previously described in the presence of two chlorinated analogs of salicylic acid. The inducing effect of 50-mg/liter concentrations of 5-chlorosalicylate and 3,5-dichlorosalicylate was compared with that of equal concentrations of salicylate (Fig. 7). No significant effect on growth by any of the analogs was observed. The DHD production by all cultures was similar (about 1.2 g/liter in 24 h). With

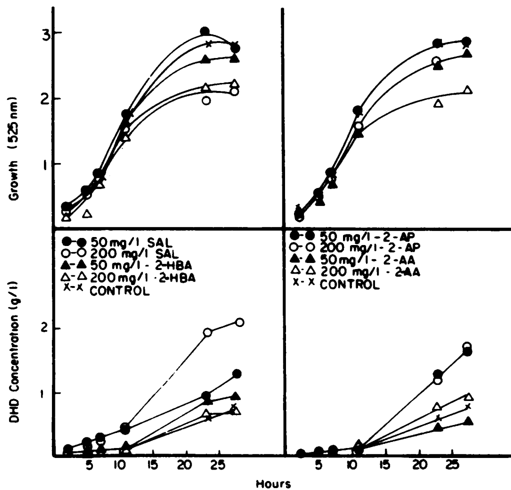

FIG. 5. *Growth and DHD production by P. putida 119 in glucose-naphthalene medium with four different naphthalene oxygenase inducers. Abbreviations: SAL, salicylate; 2-HBA, 2-hydroxybenzyl alcohol; 2-AP, 2-aminophenol; 2-AA, 2-aminobenzoate.*

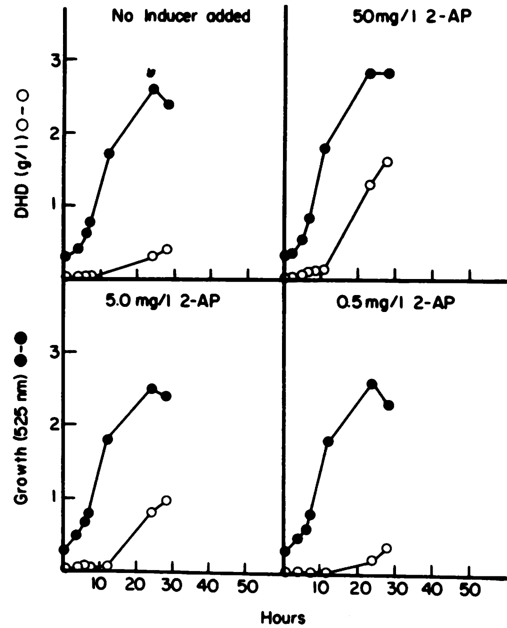

FIG. 6. *Growth and DHD production by P. putida 119 when grown on glucose-naphthalene medium with various concentrations of 2-aminophenol (2-AP) as naphthalene oxygenase inducer.*

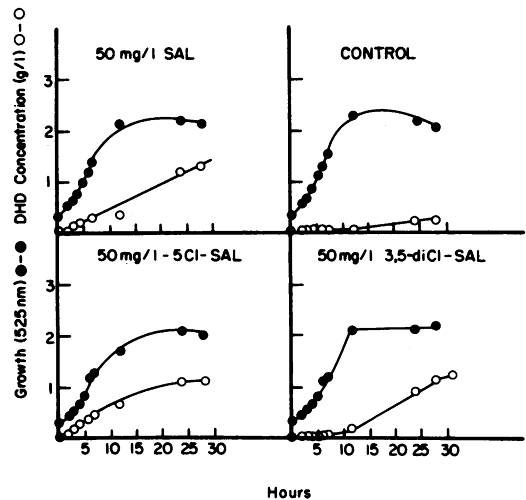

FIG. 7. *Growth and DHD production by P. putida 119 when grown on glucose-naphthalene medium with various salicylate analogs as naphthalene oxygenase inducers. Abbreviations: SAL, salicylate; 5-Cl-SAL, 5-chlorosalicylate; 3,5-diCl-SAL, 3,5-dichlorosalicylate.*

3,5-dichlorosalicylic acid, the induction effect was analogous to that observed with 2-aminophenol. No DHD production was observed during logarithmic growth, but did begin during the stationary phase.

DHD inhibition of growth of *P. putida* 119. *P. putida* cultures were routinely observed to slow in growth rate and DHD production regardless of medium composition as DHD concentration reached about 0.5 g/liter. Experiments were conducted to determine whether DHD was, in fact, inhibiting either cell growth or DHD production. Cells were grown in glucose minimal medium (no naphthalene) which contained various amounts of exogenously added DHD. Both the growth rates and the extent of growth were found to be adversely affected (Fig. 8). At added DHD concentrations of 0.5 g/liter, the growth rate was reduced about 75% compared with controls. Flasks containing 1 and 2 g of DHD supplements per liter exhibited even lower growth rates. No significant cell growth was observed at concentrations higher than 5 g/liter. In a subsequent experiment, cells growing on glucose minimal medium containing naphthalene were allowed to grow through two doublings when DHD was added to determine its effect during logarithmic growth. Control flasks containing neither naphthalene nor DHD grew to the highest biomass levels observed and exhibited cell growth yields comparable to reported literature values for *Pseudomonas* species (10, 12) (Fig. 9). Cells grown in presence of 2 g of naphthalene per liter exhibited fairly comparable growth rates through the initial two doublings, but exhibited much lower final growth yields as DHD production commenced (Fig. 9). The cultures receiving exogenous DHD added during the logarithmic growth phase stopped growing for an additional 30-h incubation period. No additional DHD production (or loss) was observed except in the naphthalene control flasks.

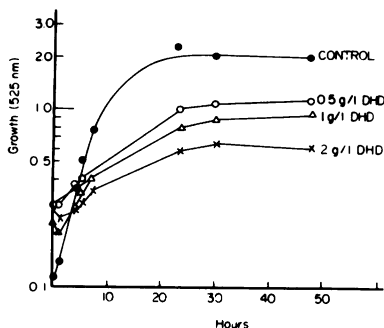

FIG. 8. *Effect of added DHD on growth of P. putida 119 in glucose medium.*

Effects of addition of naphthalene-solubilizing agents on DHD production by *P. putida* 119. A critical concern in this fermentation process was to increase naphthalene mass transfer rates. Since this chemical is soluble in water only to 30 mg/liter at 25°C, we tried to increase the yields of DHD by using surfactants in a batch flask study performed with concentrated aqueous suspensions of cell mass and naphthalene. The naphthalene was blended in surfactant-containing mixtures and added to concentrations of 50 g/liter in growth medium. The biomass was obtained from a batch fermentor culture grown over 24 h and concentrated 15-fold (Tables 1 and 2). Mineral salts were not added to the medium in order to minimize growth. Since DHD concentrations in these experiments were calculated on the basis

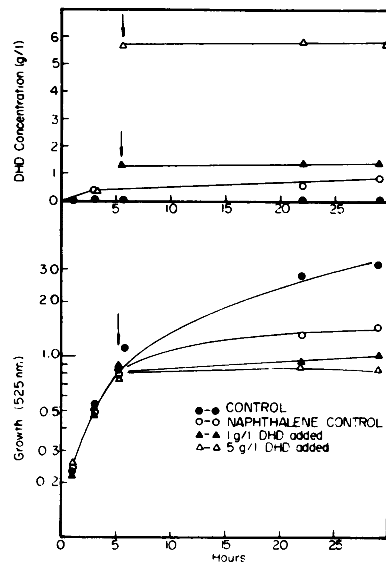

FIG. 9. *Effect of DHD added to logarithmically growing P. putida 119 on growth and DHD production. DHD was added to each flask at the times indicated (↓).*

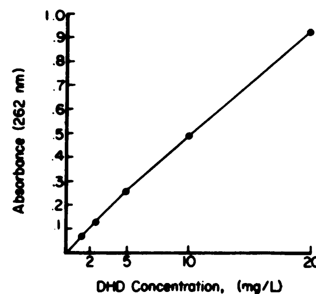

FIG. 10. *Standard curve for DHD absorbance at 262 nm in aqueous solution.*

of absorbance at 262 nm (Fig. 10), we had to use several control flasks to minimize background absorbance. The highest value from these determinations was subtracted when DHD concentrations were calculated. DHD production was stimulated in the flasks containing biomass-naphthalene-surfactant mixtures much more than in those without surfactant (Fig. 11). DHD was produced at a linear rate to 5 g/liter in 24 h in flasks 5 and 6. The highest concentration in 46 h was estimated to be about 8 g/liter in flask 5 and 6 g/liter in flask 6. The culture containing no surfactant but supplemented with succinate (flask 4) yielded 2.7 g of DHD per liter in 46 h, whereas an identical culture minus the carbon source (flask 2) produced 1.4 g/liter in the same time period. The highest apparent production rate calculated from these data was 0.3 g/liter per h in flask 4 during the initial 8-h period.

A repeat study corroborated that naphthalene-surfactant-succinate mixtures containing biomass (Fig. 12) were most productive. The best DHD production rate (0.47 g/liter per h) was higher than previously observed, but biomass levels were 1.5 times higher than in the first experiment. Two surfactant mixture combinations were tested in which a 2:1 ratio of nonionic to anionic surfactant was compared with a 1:1 ratio of the same; little difference was observed.

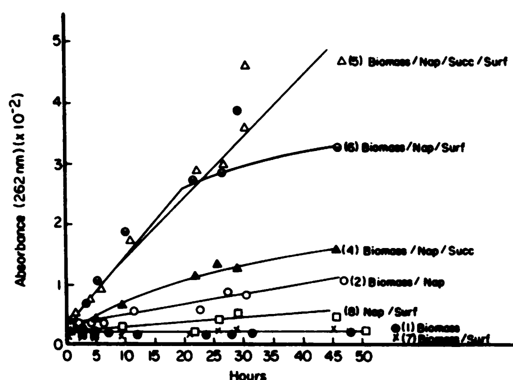

FIG. 11. *DHD production (absorbance) by resting-cell suspensions of P. putida 119 in seven media. Abbreviations: Nap, naphthalene; Succ, succinate; Surf, surfactant.*

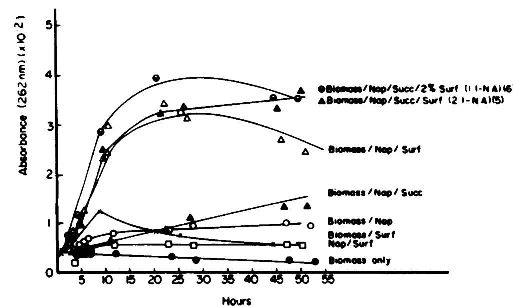

FIG. 12. *DHD production (absorbance) by resting-cell suspensions of P. putida 119 in eight media as indicated on the figure and in the text. Abbreviations as in Fig. 11.*

DISCUSSION

A wide variety of carbon sources were tested and found suitable for a fermentation process in which *P. putida* converts naphthalene to DHD. Glucose, in high concentrations, adversely affected naphthalene conversion rates, although in low-glucose medium, high DHD productivity was previously observed (4). Glucose (i.e., corn syrup) remains an ideal substrate from a cost standpoint, but other carbon sources including gluconate and lactate, not causing catabolite repression of naphthalene metabolism, are also suitable.

Salicylate and chlorinated analogs induced naphthalene metabolism in this *P. putida* mutant during growth in glucose minimal medium, whereas 2-hydroxybenzyl alcohol and 2-aminobenzoate did not. The inducing effect of salicylate was observed to be concentration dependent. The presence of 3,5-dichlorosalicylate or 2-aminophenol stimulated naphthalene metabolism in *P. putida* grown on glucose at the onset of stationary growth, indicating some unidentified synergistic effect. A more thorough study of this glucose-sensitive stimulation is needed. Since 2-aminophenol was equally effective in stimulating naphthalene metabolism at concentrations from 5 to 200 mg/liter, it appears to be an attractive candidate for use in any proposed large-scale fermentation.

Since the purpose of this work was to identify an inducing compound effective at low concentrations in a glucose medium, higher concentrations of 2-aminobenzoate or 2-hydroxybenzyl alcohol were not used. Their inability to stimulate naphthalene metabolism in glucose-grown *P. putida* contradicts results obtained elsewhere, but may indicate inducer concentration or growth substrate effects (14, 15).

The results of the naphthalene-surfactant experiments indicated that these methods for improving naphthalene mass transfer are effective. DHD production rates were doubled, and final yields were increased from four- to sevenfold when surfactants were used. These data also suggest that logarithmic-growth-phase cells are not required for high DHD production, as indicated in the shake flask studies.

The decrease in DHD production as seen in all of the batch cultures in stationary growth

phase was caused in part by product inhibition. Production rates usually began to decline as DHD concentrations reached about 0.5 g/liter regardless of the growth substrate used. When added exogenously, DHD caused a substantial drop in growth rates and DHD production. Unpublished oxygen uptake studies with naphthalene-grown cells (not *P. putida* 119) demonstrated that DHD could significantly inhibit naphthalene oxidation when the ratio of soluble concentration of DHD to naphthalene was greater than 20:1. This ratio was approximated when DHD concentrations reached about 0.6 g/liter. More refined studies with enzyme preparations from *P. putida* 119 are needed to determine the effect of DHD on naphthalene oxygenase activity.

Future studies leading to the optimization of this process should involve naphthalene mass transfer. An obvious approach to resolving the inducer and end-product inhibition problems would be a search for constitutive naphthalene-metabolizing strains and DHD-resistant mutants.

Continuous glucose-limited fermentations provide one method for reducing both catabolite repression and end-product inhibition. Preliminary results of such experiments in our laboratory have already been reported (3).

ACKNOWLEDGMENTS

We express our appreciation to D. T. Gibson for providing the culture but, more importantly, for his patient and invaluable counsel to the program.

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