Microbial Production of 4,4'-Dihydroxybiphenyl: Biphenyl Hydroxylation by Fungi

ROBERT D. SCHWARTZ,[†] * A. L. WILLIAMS, AND D. B. HUTCHINSON

Union Carbide Corp., Research and Development Department, South Charleston, West Virginia 25303

Of 15 species of fungi examined for their ability to hydroxylate biphenyl, 10 produced 4-hydroxybiphenyl. Seven of the 10 also produced 4,4'-dihydroxybiphenyl. The most efficient strains, *Absidia pseudocylindrospora* NRRL 2770 and *Absidia* sp. NRRL 1341, were more closely examined to determine their growth characteristics and the kinetics of biphenyl hydroxylation in batch fermentation. In the absence of biphenyl, *A. pseudocylindrospora* 2770 and *Absidia* sp. 1341 grew about as rapidly and efficiently in a defined glucose minimal medium as in a complex medium. Substrate yield coefficients for glucose in both media were 0.4 to 0.5 g of biomass per g of glucose, and the specific growth rate was about 0.17 h⁻¹ (doubling time, about 4 h). In this unoptimized system, 10 to 15 g of biomass per liter (dry weight) could be produced, using a defined salt solution and glucose as sole carbon and energy source. In the presence of biphenyl, growth was inhibited, more so for strain 1341 than for strain 2770. However, the specific activity for biphenyl hydroxylation (milligrams of biphenol per gram of biomass) was about 3.5 times greater for strain 1341. Furthermore, biphenyl hydroxylation appeared to require the presence of an oxidizable carbon and energy source (and perhaps growth) to proceed and, at least for strain 1341, hydroxylation seemed to be more efficient in the complex medium.

One of the monomers used in the synthesis of the Union Carbide sulfonebiphenol engineering polymer Radel is 4,4'-dihydroxybiphenyl. An alternative to the chemical synthesis of this biphenol is the sequential microbial hydroxylation of biphenyl to 4-hydroxybiphenyl and then to 4,4'-dihydroxybiphenyl. Although much work has been done on halogenated biphenyls (1, 6, 8), relatively little has been reported on the microbial hydroxylation of biphenyl itself. Many bacteria have been isolated that can grow on biphenyl (3, 4, 9, 10). The initial reaction is catalyzed by a dioxygenase, and the primary oxidation product is a *cis*-dihydrodiol, 2,3-dihydro-2,3-dihydroxybiphenyl (3, 4, 9).

Some fungi will hydroxylate biphenyl in the four position, although none have been reported to grow on it. This reaction is presumably catalyzed by a monooxygenase, the primary oxidation product being an epoxide (5, 13, 14).

Until quite recently, only two fungi, *Cunninghamella blakesleeana* ATCC 8688a and *C. bainieri* ATCC 9244, had been reported to produce 4,4'-dihydroxybiphenyl (13). While this report was in preparation, Dodge et al. also reported on several species of fungi that could hydroxylate biphenyl and produce 4,4'-dihydroxybiphenyl (5).

We initiated a screen to ascertain the technical feasibility of producing 4,4'-dihydroxybiphenyl via biphenyl hydroxylation. Fifteen species of fungi were examined. We report on the successful hydroxylation of biphenyl to 4,4'-dihydroxybiphenyl and the discovery of at least one genus not previously shown capable of this reaction (U.S. patent 4,153,509, May 1979).

MATERIALS AND METHODS

Microorganisms. The following fungi were obtained from the National Regional Research Laboratory, Peoria, Ill.: *Absidia pseudocylindrospora* 2770, *A. ramosa* 1332, *A. glauca* 1324, *Absidia* sp. 1341, *A. spinosa* 1347, *Cunninghamella blakesleeana* 1369, *C. echinulata* 1386, *C. elegans* 1392, *C. elegans* 1393, *Mucor rouxii* 1430, *M. bacilliformis* 2346, *M. pusillus* 2543, *Aspergillus ochraceus* 398, *A. niger* 599, and *Rhizopus stolonifer* 1477. Cultures were maintained on Sabouraud agar slants and stored in liquid nitrogen as mycelial blends in 20% glycerol.

Media. Five types of media were used and contained, per liter of deionized water: (i) Sabouraud broth (10 g of neopeptone, 20 g of glucose, pH 5.7); (ii) Sabouraud agar (10 g of neopeptone, 40 g of maltose, 15 g of agar, pH 5.6); (iii) Smith-Rosazza (S-R) broth (5 g of soytone, 20 g of glucose, 5 g of yeast extract, 5 g of NaCl, 5 g of K₂HPO₄, pH 7.0); (iv) Czapek-Dox (C-D) broth (3 g of NaNO₃, 1 g of K₂HPO₄, 0.5 g of MgSO₄, 0.5 g of KCl, 0.01 g of FeSO₄, pH 7.0); (v) P₁ minimal medium (11). The latter two media were supplemented with glucose as specified below.

Sterilization was accomplished by autoclaving at 15

[†] Present address: Stauffer Chemical Co., Richmond, CA 94804.

lb for 15 min. For P₁ and C-D minimal media, glucose was sterilized separately and added as the carbon and energy source (2 or 3%, wt/vol, as stated). Note that S-R and Sabouraud media are complex media; S-R medium contains soytone and yeast extract, and Sabouraud medium contains neopeptone. P₁ is a defined high-phosphate medium containing many trace elements and ammonia as nitrogen source; C-D is a defined low-phosphate medium containing few trace elements and nitrate as nitrogen source.

Biphenyl, when present, was added as dry crystals at the concentration of 3.5 g/liter. This concentration of biphenyl was arbitrarily chosen and is far in excess of biphenyl solubility in water (9.6 mg/liter at 25°C).

Growth conditions. Shake flask experiments were conducted in 500-ml baffled flasks containing 100 ml of medium. Inocula were prepared by growing the culture in one or more flasks in the same medium to be used in the experiment, usually for 2 to 4 days, followed by blending the entire contents in a sterile Waring blender. Next, an appropriate volume (3 to 5 ml) was transferred by pipette to each experimental flask. For each day's assays, the entire contents of one flask were processed. Incubation was in an NBS G-25 shaker (New Brunswick Scientific Co., Edison, N.J.) at 28 to 30°C and 250 rpm.

For concentrated mycelium studies, after 4 to 6 days of growth (except where noted), the mycelia were concentrated by filtration through Whatman no. 1 filter paper, transferred to a 500-ml shake flask, and suspended in an appropriate volume of the same medium in which they were grown. The concentration factor was usually two- to four-fold.

Sample preparation. At the times indicated, one shake flask was removed from the shaker and the pH was measured. The entire contents were then blended, and the volume was measured. For biomass determinations, two 5-ml portions of the blend were transferred by pipette to tared centrifuge tubes. The cell pellet obtained by centrifuging for 15 min at 15,000 rpm (Sorvall RC5) was washed with water, centrifuged again, and dried at 100°C to constant weight (~24 h). The supernatant from the first centrifugation was used for the glucose assay. The remaining blend was acidified with HCl to pH 1 to 2, allowed to stand at room temperature for 15 min, and extracted with ethyl acetate (1 part of ethyl acetate/5 parts of blend) by blending; then the phases were separated by centrifugation, and the volume of ethyl acetate recovered from the blend was measured. This extract was used for the thin-layer chromatography (TLC) and gas chromatography (GC) biphenol assays.

Glucose assay. Glucose concentrations were estimated by the hexokinase/glucose 6-phosphate dehydrogenase assay (Calbiochem, La Jolla, Calif.). The method is rapid, specific for glucose, and sensitive to about 4 mg of glucose per 100 ml (0.004%); it is based on the increase in absorbance at 340 nm caused by the reduction of the cofactor nicotinamide adenine dinucleotide phosphate.

Biphenol assays. The presence of hydroxylated biphenyls was determined by TLC (qualitative) and GC (quantitative) by comparing *R_f* values and retention times, respectively, of the unknown products with those of authentic samples. The absolute identity of

the fermentation products was determined by physicochemical methods (GC-mass spectroscopy, infrared, nuclear magnetic resonance). The details will be reported elsewhere.

TLC assay. Sil-G25 UV₂₅₄ silica gel plates (20 by 20 cm; Brinkmann Instruments Inc., Westbury, N.J.) were used. Ten microliters of the ethyl acetate extracts was spotted at the origin, dried, and eluted with chloroform/acetone (80:20, vol/vol) for 60 min. Product spots were visualized under ultraviolet light and with Gibbs reagent (5). A standard mixture was run with each plate. The *R_f* values of interest are: biphenyl, 0.79; 4-hydroxybiphenyl, 0.51; and 4,4'-dihydroxybiphenyl, 0.38.

GC assays. Two GC assays were developed. The first resolved the 3-hydroxybiphenyl from the 4-hydroxybiphenyl, but required sample derivatization. The second method could not resolve the 3-hydroxybiphenyl from the 4-hydroxybiphenyl, but did not require derivatization. (Note that little 3-hydroxybiphenyl is expected in the fungal system, based on the biochemistry of the reaction [5, 9]). The compounds listed in Table 1 were purchased from Aldrich Chemical Co. (Milwaukee, Wis.) or RFR Corp. (Hope, R.I.).

Method 1. The trifluoroacetoxy derivatives of the hydroxylated biphenyls were prepared as follows: to 0.5 ml of ethyl acetate extract containing 1-hexadecene (see below) was added 5 μ l of triethylamine followed by the slow addition of 0.5 ml of trifluoroacetic anhydride and waiting 2 min for the reaction to be completed. The assay was performed with a Hewlett-Packard model 5830-A gas chromatograph equipped with a flame ionization detector. Injection port and detector temperatures were 220 and 250°C, respectively. The column was 10 feet by 1/8 inch (about 3 m by 0.3 cm) stainless steel packed with 10% Apiezon L on 60/80 Chromosorb W (acid washed, silanated). An isothermal temperature of 170°C was maintained, and the carrier gas flow was 37 ml of helium per min. The phenols were quantitated by measuring the ratio of the phenol peak area to the peak area of 1-hexadecene. The latter was added to the ethyl acetate before extraction and used as an internal standard (36.8 μ g of 1-hexadecene per ml of ethyl acetate).

Method 2. The same gas chromatograph and flame

TABLE 1. GC retention times for the compounds of interest by each of the methods developed

Compound	Retention time (min)	
	Method 1 ^a	Method 2 ^b
2,2'-Dihydroxybiphenyl	3.6	8.0
2-Hydroxybiphenyl	5.8	5.4
Biphenyl	10.1	3.5
3-Hydroxybiphenyl	12.1	— ^c
4-Hydroxybiphenyl	13.6	8.8
1,5-Dihydroxynaphthalene	—	12.0
1-Hexadecene	18.7	—
4,4'-Dihydroxybiphenyl	20.6	16.2

^a Trifluoroacetoxy derivatives; 10% Apiezon L on 60/80 Chromosorb W.

^b Underivatized; Tenax GC 60/80 mesh.

^c —, Not resolved from 4-hydroxybiphenyl.

ionization detector were used. Injection port and detector temperatures were 175 and 325°C, respectively. Dual temperature-compensating glass columns (1.8 m by 4 mm) packed with Tenax GC 60/80 mesh, and temperature programming, starting at 240°C and increasing at the rate of 5°/min to a maximum of 300°C followed by isothermal operation for 6.5 min, were used. The carrier gas flow was 20 ml of helium per min. To quantitate the phenols, equal volumes of the ethyl acetate extract (without 1-hexadecene) and a solution containing 1,5-dihydroxynaphthalene as internal standard (210 mg per ml of ethyl acetate) were mixed, 1 to 5 µl was injected, and the ratio of the phenol peak area to the peak area of 1,5-dihydroxynaphthalene was measured.

For both GC assays, standard curves were constructed for 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl. To ensure quantitative recovery of the phenols from fermentation broth, known amounts of the products were added to broth, extracted with ethyl acetate, and quantitatively recovered. Table 1 shows the GC retention times for each of the methods.

RESULTS

Table 2 summarizes biphenyl hydroxylation by growing and concentrated growing fungal cultures in complex media (S-R and Sabouraud). The concentrations shown for the hydroxybiphenyls were not necessarily found at the same time during the course of an experiment. Early experiments indicated that, with most growing cultures, biphenyl hydroxylation was inhibited if the biphenyl was added at the time of inoculation. Therefore, for the screen, biphenyl was added to the growing cultures no less than 17 h after inoculation. For the concentrated growing

cultures, biphenyl was added at the time of concentration.

The following cultures failed to hydroxylate biphenyl: *M. rouxii* 1430; *M. bacilliformis* 2346; *M. pusillus* 2543; *A. ochraceus* 398; *R. stolonifer* 1477.

Of the 15 cultures examined, 10 hydroxylated biphenyl to 4-hydroxybiphenyl. Seven of the 10 also produced 4,4'-dihydroxybiphenyl. The most efficient strains were *A. pseudocylindrospora* 2770 and *Absidia* sp. 1341. More strains were positive in S-R medium (pH 7.0) than in Sabouraud medium (pH 5.7).

The time course of biphenyl hydroxylation by *Absidia* sp. 1341 is shown in Fig. 1 for growing and concentrated growing mycelia in S-R medium. In both cases, the concentration of 4,4'-dihydroxybiphenyl exceeded that of 4-hydroxybiphenyl, and the product concentration was apparently increasing when the experiment was terminated. This relationship was not observed for all organisms examined. The maximum conversion observed was with *Absidia* sp. 1341 concentrated growing mycelia: 1.8% for 4-hydroxybiphenyl and 2.2% for 4,4'-dihydroxybiphenyl based on the initial amount of biphenyl present, 3.5 mg/ml.

A. pseudocylindrospora 2770 and *Absidia* sp. 1341 were more closely examined to determine their growth characteristics, the kinetics of biphenyl hydroxylation, and the effect of biphenyl and its oxidation products on growth in batch fermentations.

As an economic analysis (Union Carbide

TABLE 2. Biphenyl hydroxylation by mycelial fungi

Strain	Highest concn of biphenyl hydroxylation products (µg/ml)							
	Growing mycelia				Concentrated growing mycelia			
	4-Hydroxybiphenyl		4,4'-Dihydroxybiphenyl		4-Hydroxybiphenyl		4,4'-Dihydroxybiphenyl	
	S-R	Sabouraud	S-R	Sabouraud	S-R	Sabouraud	S-R	Sabouraud
<i>Absidia pseudocylindrospora</i> 2770 ^a	45.8	54.4	57.0	23.6	38.8	13.8	24.1	30.8
<i>A. ramosa</i> 1332 ^a	0	0	0	0	10.4	0	0	0
<i>A. glauca</i> 1324	17.8	ND ^b	13.5	ND	17.1	ND	10.3	ND
<i>Absidia</i> sp. 1341	37.8	ND	61.6	ND	63.9	ND	75.6	ND
<i>A. spinosa</i> 1347	15.5	ND	0	ND	5.9	ND	9.5	ND
<i>Cunninghamella blakesleeana</i> 1369 ^a	9.8	6.4	6.2	0	30.4	13.4	5.0	1.9
<i>C. echinulata</i> 1386 ^a	20.3	37.5	3.9	0	19.2	12.0	1.0	1.8
<i>C. elegans</i> 1392 ^a	5.8	2.6	0	0	7.8	7.8	1.1	0
<i>C. elegans</i> 1393 ^a	0	0	0	0	11.6	ND	0	ND
<i>Aspergillus niger</i> 599	2.7	ND	0	ND	0	ND	0	ND

^a Examined during 18-day incubation (growing mycelia) or 14-day incubation (concentrated mycelia) in the presence of biphenyl. The other strains were incubated for 10 days (growing mycelia) and 7 days (concentrated mycelia) in the presence of biphenyl.

^b ND, Not done.

Corp., unpublished data) has shown that an economically viable process will require a defined medium (i.e., salts plus carbon and energy source; no yeast extract, soy, etc.), these strains were examined in P₁ and C-D media. S-R medium was included for comparison. Table 3 summarizes the results from several growth experiments in the presence and absence of biphenyl. The substrate yield coefficient (Y_s) was calculated on the initial glucose concentration, although S-R contains yeast extract and soytone, which contribute to the biomass. Note that for these experiments the biphenyl was added at the time of inoculation. In the absence of biphenyl, both strains grew well. On S-R and P₁ media, the extent of growth and efficiency of glucose utilization were comparable, i.e., 12 to 17

g/liter and Y_s of 0.44 to 0.54. Both parameters were less on C-D medium, i.e., 11 and 12 g/liter and 0.31 and 0.36 for 2770 and 1341, respectively. In the presence of biphenyl, growth of both strains was inhibited. The extent of inhibition was generally greater for 1341 than 2770, the exception being C-D medium, where 1341 outgrew 2770. In the presence or absence of biphenyl, growth appeared to occur faster on S-R than either of the minimal media. The extent of this difference in rates may be more apparent than real, as will be shown below.

Shake flask experiments aimed at more carefully elucidating the kinetics of growth and biphenyl hydroxylation are shown in Fig. 2 and 3. For both strains, S-R and P₁-3% glucose media were used in the presence and absence of biphenyl. Data for each day were obtained by harvesting the entire contents of one flask. Dry weight, glucose consumption, pH, and biphenol production were monitored. Biphenyl was added at time of inoculation.

For 2770 growing on S-R medium, the maximum biomass level, about 12 g/liter, was observed in 1 to 3 days (Fig. 2). No significant difference was observed in the presence or absence of biphenyl, and the glucose was consumed by day 3. The Y_s for glucose was 0.4. The major difference occurred in pH. In the absence of biphenyl, the pH decreased to 3 and remained between 3 and 3.5. In the presence of biphenyl, the pH decreased to 6 and then drifted back toward neutrality. Biphenyl was hydroxylated, essentially, only when glucose was present, or perhaps only when the fungus was actively growing. The biphenol yield was 10 to 12 mg of 4-hydroxybiphenyl and 6 to 8 mg of 4,4'-dihydroxybiphenyl per liter. About 0.5% of the added biphenyl (3.5 g/liter) was transformed to bi-

FIG. 1. Biphenyl hydroxylation versus time. *Absidia* sp. 1341, S-R medium, 3.5 mg of biphenyl/ml. (A) Mycelium grown 6 days before biphenyl addition. (B) Mycelium grown 9 days and concentrated twofold before biphenyl addition. Symbols: ■, 4-hydroxybiphenyl; ●, 4,4'-dihydroxybiphenyl; ▲, 4-hydroxybiphenyl plus 4,4'-dihydroxybiphenyl.

TABLE 3. Biomass yield for *A. pseudocylindrospora* 2770 and *Absidia* sp. 1341 on complex media (S-R) and glucose minimal media (P₁ and C-D) in the presence (added at time of inoculation) and absence of biphenyl

Expt	Medium	Strain 2770				Strain 1341			
		Inoculum (g/liter)	Time sampled ^a	Yield (g/liter)	Y_s^b (g of cells/g of glucose)	Inoculum (g/liter)	Time sampled	Yield (g/liter)	Y_s (g of cells/g of glucose)
1	S-R	8.5	18 h	17.3	0.44	4.8	18 h	15.6	0.54
	S-R + biphenyl	8.5	18 h	15.6	0.36	4.8	18 h	11.7	0.36
2	S-R	2.5	2 days	12.3	0.49	2.5	2 days	12.3	0.49
	S-R + biphenyl	2.5	2 days	9.9	0.37	2.5	2 days	4.4	0.10
3	P ₁ -3% glucose	2.0	4 days	15.2	0.44	2.2	4 days	15.9	0.46
	+ biphenyl	2.0	13 days	9.7	0.26	2.2	13 days	7.6	0.18
4	C-D-3% glucose	1.8	4 days	11.0	0.31	1.6	13 days	12.3	0.36
	+ biphenyl	1.8	4 days	7.3	0.18	1.6	13 days	8.7	0.24

^a Time that maximum dry weight was observed.

^b Yield - inoculum/initial glucose concentration.

FIG. 2. *A. pseudocylindrospora* 2770 growth and biphenyl hydroxylation. Shake flasks, S-R medium. Control: dry weight (●—●), pH (●—●), glucose (●—●). Plus biphenyl: dry weight (▲—▲), pH (▲—▲), glucose (▲—▲), 4-hydroxybiphenyl (○—○), 4,4'-dihydroxybiphenyl (●—●).

FIG. 3. *A. pseudocylindrospora* 1341 growth and biphenyl hydroxylation. Shake flasks, S-R medium. Control: dry weight (●—●), pH (●—●), glucose (●—●). Plus biphenyl: dry weight (▲—▲), pH (▲—▲), glucose (▲—▲), 4-hydroxybiphenyl (○—○), 4,4'-dihydroxybiphenyl (●—●).

phenols and, based on the maximum biomass observed, about 1.5 mg of biphenyl was transformed per g of biomass.

On P₁-3% glucose medium, the rates of growth and glucose consumption were less than on S-R. Some inhibition in rate was observed in the

presence of biphenyl. Biomass reached a maximum on day 3 of 14 g/liter, commensurate with the exhaustion of glucose. The Y_s was 0.35. The change in pH was similar to that in S-R; i.e., in the absence of biphenyl, the pH decreased to 3 and remained between 3 and 3.8; in the presence of biphenyl, the pH decreased to 4.5 and then drifted back to 6. The biphenol yields and rates were also similar to those in S-R.

The results of a similar experiment are shown in Fig. 3 for *Absidia* sp. 1341. On S-R medium, in the absence of biphenyl, the maximum biomass level of 12.5 g/liter was reached in 1 day and the glucose was consumed by day 2. The Y_s for glucose was 0.45, and the pH decreased to about 4 and remained there. In the presence of biphenyl, growth was almost completely inhibited. However, the pH decreased to about 6 and remained between 6 and 6.5, and more than 75% of the glucose was apparently consumed by day 2, followed by its reappearance. 4-Hydroxybiphenyl production continued through day 6, reaching about 16 mg/liter; 4,4'-dihydroxybiphenyl reached a maximum of about 12 mg/liter by day 3. About 0.8% of the added biphenyl was hydroxylated and, based on a maximum biomass level of 5 g/liter, about 5.6 mg of biphenyl was transformed per g of biomass.

On P₁-3% glucose minimal medium, in the absence of biphenyl, a biomass concentration of 16 g/liter was reached after 1 day. The rate of glucose consumption was less than on S-R, being exhausted by day 6 and resulting in a Y_s of 0.37. The pH decreased to about 4 and then rose to 5.5 by day 13. In the presence of biphenyl, not only was growth inhibited, but the biomass lysed. On day 3, the level was 60% of that present at time zero; on day 14, it was 40%. The pH remained essentially unchanged. By day 6 more than 80% of the glucose was apparently consumed, followed by its reappearance. Net glucose consumption was about 16% (5 g/liter), which accounts for the slight increase in biomass observed on day 1, i.e. 2 g/liter. Biphenol production reached 6 and 5 mg/liter for 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl, respectively.

A spontaneous variant of *Absidia* sp. 1341 was isolated that could grow in the presence of biphenyl (*Absidia* sp. 1341R). Not unexpectedly, this variant did not hydroxylate biphenyl and may owe its tolerance to its ability to exclude biphenyl.

To determine which of the compounds of interest was the most inhibitory, the three strains (1341, 1341R, and 2770) were incubated in S-R medium containing biphenyl, 4-hydroxybiphenyl, or 4,4'-dihydroxybiphenyl. Strains 2770 and 1341R grew in the presence of 3.5 g of

biphenyl per liter; strains 2770, 1341R, and 1341 grew in the presence of 0.5 g of 4,4'-dihydroxybiphenyl per liter. However, the three strains failed to grow in the presence of 4-hydroxybiphenyl at concentrations exceeding 35 mg/liter. Hence, the intermediate product appears to be the most toxic. Of further interest is the observation that although the biphenyl and 4,4'-dihydroxybiphenyl had low solubilities in our media (about 12 and 65 mg/liter, respectively), the 4-hydroxybiphenyl was virtually insoluble.

DISCUSSION

These investigations demonstrate that a fungal biotransformation of biphenyl to 4,4'-dihydroxybiphenyl is a technically feasible route to this polymer intermediate.

Although microbial biphenyl hydroxylation is infrequently reported in the literature, our limited search using complex media resulted in 10 of 15 strains (representing three of five genera) capable of hydroxylating biphenyl at the four position. Furthermore, seven of the strains (representing two genera) produced 4,4'-dihydroxybiphenyl. One of the genera, *Absidia*, had not previously been reported capable of this reaction and was the most efficient of the genera tested. The ability of fungi to perform this transformation may not be as limited as the literature suggests, but merely reflect the lack of a thorough investigation.

Hydroxylation at the two or three position was not observed. 2- and 3-hydroxybiphenyl are not expected to be formed in significant amounts and, if present, may have been below our limits of detection. Recently, Dodge et al. (5) reported that these compounds are produced by several species of fungi hydroxylating biphenyl.

Two strains, *A. pseudocylindrospora* 2770 and *Absidia* sp. 1341, were selected for closer examination. Both have been shown capable of biphenyl hydroxylation in defined glucose minimal medium. For an economically viable process this is important, since growth in complex media would be more costly than in a simple, defined salt solution.

Comparing growth in S-R, P₁, and C-D media, we conclude that both 2770 and 1341 can be grown about as well in a defined minimal medium with glucose as sole carbon and energy source as in a complex medium. In this unoptimized system, a high biomass can be generated (15 g/liter) in a reasonable period of time (1 to 4 days), and glucose is used efficiently ($Y_p = 0.4$ to 0.5). The poorer growth observed in the simplest medium tested, C-D, may result from the difference in nitrogen source (ammonia versus nitrate), lower phosphate concentration, or lack of some trace element.

The results indicate that 2770 performs about as well in S-R complex medium as in P₁ defined medium, both in the presence and absence of biphenyl. The major difference occurs in pH. We hypothesize that this pH behavior is caused by biphenyl inducing the release of basic proteins. That biphenyl hydroxylation is growth related or requires an oxidizable carbon and energy source is suggested by the halt in biphenol production at or shortly after glucose exhaustion.

In the absence of biphenyl, 1341 behaves similarly to 2770 in both media. However, in the presence of biphenyl, growth is dramatically inhibited in both media. No simple explanation is available for the disappearance and reappearance of the bulk of the glucose. In S-R medium, the initial decrease in pH to 6, coupled with little or no growth and about 60% net glucose consumption, suggests that the fungus, although unable to grow, is metabolically active. Furthermore, biphenol production was much greater in S-R than P₁. Some component of the yeast extract or soy in the S-R medium may afford a level of protection to 1341, enabling the organism to maintain metabolic activity and oxidize (detoxify?) the biphenyl.

On a unit biomass basis, 1341 in S-R medium was about 3.5 times more efficient in biphenyl oxidation than 2770 in S-R or P₁, an indication of species differences.

Another indication of species difference is the inhibition and lysis observed in the presence of biphenyl with 1341 and the resistance of 2770. Neither is unexpected. Biphenyl and biphenyl derivatives have been used as fungicides and are known to cause swelling and bursting of sensitive strains. Likewise, biphenyl-resistant fungi have been isolated (7). Furthermore, we isolated a strain of 1341 that grows well in the presence of biphenyl. Unfortunately, indications are that this particular culture does not hydroxylate biphenyl.

The biphenol yields are much higher in the screen. Although biomass concentrations were not determined in the screen, the cultures were grown or grown and concentrated before biphenyl addition. The results suggest that the time of addition of biphenyl is important or that the biomass concentration in the screen, before biphenyl addition, was greater than in the subsequent experiments.

The low yields could result from the presence of phenol oxidase (resulting in phenol coupling) or glutathione transferase (resulting in soluble glutathione conjugates). We have observed phenol coupling with these cultures with phenol as substrate. Dodge et al. (5) have evidence for the formation of glucuronide or sulfate conju-

gates, using other fungi hydroxylating biphenyl. These are potential problems that will have to be overcome or opportunities for alternative microbial routes to 4,4'-dihydroxybiphenyl. For example, in vivo or in vitro phenol coupling, catalyzed by a phenol oxidase and using phenol as substrate, may be an attractive route to 4,4'-dihydroxybiphenyl.

The intermediate product, 4-hydroxybiphenyl, although insoluble in our media, is the most inhibitory when compared with biphenyl and 4,4'-dihydroxybiphenyl. We choose not to speculate on the reasons underlying this observation. However, we are attempting to isolate 4-hydroxybiphenyl-resistant strains. Alternatively, the inhibition may be removed by conducting the fermentation in the presence of a water-insoluble solvent that solubilizes the 4-hydroxybiphenyl, without harming the cells (12).

The efficiency, product concentration, and rate of the transformation are low. Whether these can be increased for the productivities required in a commercial process is uncertain.

ACKNOWLEDGMENT

We thank L. A. Kapicak for contributions in helping to develop the TLC and GC assays.

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