BACTERIAL UTILIZATION OF LIGNANS

I. METABOLISM OF α-CONIDENDRIN

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Lignins are complex methoxylated polymers which are extremely resistant to microbial action (Phillips, 1934; Norman, 1936; Waksman, 1946). Although *in situ* lignins are attacked by some soil organisms and certain basidiomycetes, (Phillips *et al.*, 1930; Tenney and Waksman, 1930; Waksman and Hutchings, 1936; ZoBell and Stadler, 1940; Fischer, 1953) the isolated lignins appear to be more resistant to microbial attack than the naturally occurring compounds. The literature review of Gottlieb and Pelczar (1951) lists several inherent weaknesses in most reported data, especially (a) failure to recognize that trace nutrients in the lignin substrate might be supporting microbial growth and (b) use of nonspecific methods to measure lignin utilization.

Adams and Ledingham (1942) studied the capacities of several wood rotting organisms to grow in sulfite liquor and sodium lignosulfonate. Pelczar *et al.* (1950) apparently were the first to use Brauns' (1939) "native lignin" in microbiological investigations. Raynaud *et al.* (1955) indicated that phenol lignin was metabolized by pseudomonads. However they have recently observed that the loss of lignin in culture media was due to its adsorption on bacterial cells and not to utilization by the organisms (Raynaud *et al.*, 1956).

According to Erdtman (1955) and Haworth (1941), lignans, a family of less complex methoxylated aromatic compounds of known molecular composition are structurally related to lignins in that both are formed by dehydrogenation of $C_6 \cdot C_3$ groups, such as the guaiacyl-propane units. Konetzka *et al.* (1952) reported that a species of the genus *Flavobacterium* can use the lignan, α-conidendrin, as its sole carbon source.

Because of the structural relationship between lignins and lignans it seems possible that organisms capable of utilizing lignans might be subsequently adapted to dissimilate isolated lignins and lignins in wood processing wastes. The purpose of this study was to investigate the capacities of bacteria to degrade α-conidendrin by using organisms isolated from natural sources, to assay the potentials of some adaptation techniques in increasing the rate of metabolism of this compound, and to study the metabolic pathway of its dissimilation. Attempts were also made, using enrichment methods, to isolate organisms capable of degrading commercially extracted lignins.

MATERIALS AND METHODS

Culture enrichment and isolation techniques. Sources of natural materials included (a) manure, decaying wood, soil, aged sawdust, forest litter, composts, and cattail deposits from Ohio and Kentucky; (b) mud and river water from Appleton, Wisconsin Rapids, and Little Chute, Wisconsin; and (c) wood processing waste, largely crude commercial lignin, which had been exposed to weathering for 6 to 7 years in the Springfield, Oregon, area.

The synthetic basal medium for enrichment cultures contained 0.1 to 1.0 per cent of α-conidendrin as the sole carbon source. Other components, such as salts, growth factors, and nitrogen sources were added to determine their effect on the efficiency of bacteria to metabolize α-conidendrin. The medium for setting up enrichment cultures to isolate α-conidendrin utilizing microorganisms was the nitrate containing mineral salts medium used by Konetzka *et al.* (1952). Basal medium was sterilized by autoclaving at 121 C for 20 min. Solutions of iron and calcium salts were sterilized by filtration. The α-conidendrin was prepared in 1.0, 5.0, and 10.0 per cent suspensions and sterilized with ethylene oxide (Wilson and Bruno, 1950). Media were prepared for use by aseptically combining appropriate amounts of components

followed by pH adjustment with sterile reagents. Growth of fungi present in original sample material was inhibited by adding 100 μg of actidione per ml of medium.

Two methods of incubation in liquid media were used: (a) replicate 250-ml flasks containing 40 ml of α-conidendrin basal medium were incubated at room temperature with shaking or aeration and at 35 and 45 C without shaking; (b) enrichment cultures in 500- to 5000-ml volumes of media, containing varying concentrations of α-conidendrin were incubated at room temperature with aeration and shaking. Periodic subcultures were made in fresh portions of the same medium. Evidence of growth at the expense of α-conidendrin was obtained by microscopic examination and agar plate counts, at successively increasing incubation intervals, to determine whether significant increases in the number of bacteria had occurred.

For enrichment on solid media, α-conidendrin agar pour or streak plates were inoculated with cultures previously enriched in liquid media and also with portions of material from the original sample source. Representative colonies, and particularly colonies producing large, clear peripheral zones or color changes, were isolated and transferred to α-conidendrin agar slants.

Analytical methods. At appropriate intervals during incubation, culture volumes were restored to the original 40 ml to compensate for evaporation. For assay of remaining α-conidendrin, solids were separated by centrifugation at 10,000 rpm and the supernatant filtered through a Whatman no. 50 filter paper. Supernatant solutions were analyzed for: (a) ultraviolet adsorption at 280 mμ to determine levels of aromatic degradation products in solution and (b) the presence of methoxyl groups (Clark, 1932) which are indicative of remaining α-conidendrin. The pH of the supernatants was also determined. The solid portions of cultures were washed several times with distilled water, dried, weighed, ground in a mortar, and redried. After determining the weight of remaining total solids, the amount of metabolized α-conidendrin was determined by analyzing the solid portion for methoxyl groups and bacterial nitrogen. The supernatant and solid portion of cultures were examined in triplicate at intervals of 2, 5, 10, 15, and 20 days.

Chromatographic analysis. For determination of phenolic acids by chromatography, solutions were acidified, treated with NaCl, and extracted with ether. The ether fractions were analyzed by unidimensional descending paper partition chromatography for the presence of aromatic acids as possible degradation products (Evans *et al.*, 1949; Bate-Smith and Westall, 1950; Armstrong *et al.*, 1956; Lederer and Lederer, 1957), using butanol-ethanol-water (4:1:5), isopropanol-aqueous ammonia-water (8:1:1), and butanol-acetic acid-water (4:1:5) as solvent systems. Chromatograms were developed in a 20 C constant temperature room for 18 hr, dried overnight in a chromatograph oven at 110 C. The spraying agents were a 0.2 per cent aqueous solution of *p*-nitrobenzene-diazonium fluoborate followed by 5.0 per cent aqueous solution of Na_2CO_3.

To detect the presence of aliphatic keto acids, the following chromatographic methods were applied: (a) a developing system of toluene-acetic acid-water (20:1:12) followed by exposure to ammonia vapor. The position of the acid on the chromatogram was made visible by dipping in Nessler's solution (Liberman *et al.*, 1951); (b) a solvent composed of *n*-butanol-formic acid (19:1) saturated with water and sprayed with an aqueous mixture of 0.1 per cent semicarbazide hydrochloride and 0.15 per cent sodium acetate (Magasanik and Umbarger, 1950).

RESULTS AND DISCUSSION

Isolation, identification and selection procedures. Various enrichment culture techniques and screening procedures produced organisms from all environments sampled which were capable of degrading α-conidendrin under experimental conditions. The enrichment culture methods of Winogradsky and Beijerinck (Waksman, 1932) selected strains which, in comparison to their parent cultures, had increased capacities to degrade the substrate. Strains with increased capacity to utilize progressively higher concentrations of α-conidendrin were isolated by the gradient plate technique of Szybalski and Bryson (1952). In most cases enrichment culture methods yielded mixed cultures consisting of species common to all sample sources. All active organisms isolated were gram-negative bacilli, most of which were motile.

Physiological and morphological studies and infrared spectrophotometric analysis (Riddle *et al.*, 1956) of whole cell suspensions have shown the mixed cultures to contain species belonging to the genera *Pseudomonas*, *Flavobacterium*, and *Achromobacter*. The presence of pseudomonad

TABLE 1

*Relationship of sample source and ability of mixed cultures to degrade α-conidendrin**

Mixed Culture Designation	Source of Samples	No. of Samples Examined	No. of Samples Yielding Active Cultures	Dominant Organism
22a	Lignin waste, Weyerhauser Co., Springfield, Ore.	5	5	*Pseudomonas* sp. *Flavobacterium* sp.
16b	Water, Consolidated W. P. & P. G., Wisconsin Rapids, Wisc.	3	3	*Pseudomonas* sp. *Flavobacterium* sp.
20a	Mud, Fox River, Appleton, Wisc.	4	2	*Pseudomonas* sp. *Flavobacterium* sp.
5	Decaying wood, Cowan Lake, Ohio	3	3	*Pseudomonas* sp. *Flavobacterium* sp. *Achromobacter* sp.
3	Manure, Hamilton County, Ohio	4	3	*Pseudomonas* sp.
14	Forest litter, Sky Bridge, Ky.	4	2	*Pseudomonas* sp.

* Arranged in order of decreasing capacity to degrade α-conidendrin.

strains in mixed cultures was confirmed by use of the cytochrome oxidase test (Gaby and Hadley, 1957).

The optimum incubation temperature for metabolic activity of these organisms was between 25 and 28 C (room temperature). Fair growth occurred at 20 C, whereas growth was poor at 35 and 45 C. Quantitative studies to determine the extent of utilization were limited to mixed and pure cultures isolated from liquid media containing 0.5 per cent α-conidendrin and incubated at room temperature. The ability of pure cultures to degrade the substrate, immediately after their isolation, was generally less than that of the mixed cultures from which they were derived.

The relationship between the source of samples from which mixed cultures were obtained and their efficiency in dissimilating α-conidendrin as shown by representative data in table 1 appears to be significant. Of the 16 mixed cultures investigated, those obtained from areas which receive paper mill effluents and from aged lignin deposits were most efficient. Active cultures were not isolated from all samples from each source. In many instances only a certain percentage of the samples examined yielded cultures which degraded or could be adapted to the degradation of the substrate.

Addition of vitamin B_{12} to the medium increased the rate, but not the total percentage of degradation. Of all inorganic nitrogen sources used, $NaNO_3$ appeared to be the most effective for increasing the rate and percentage of degradation of α-conidendrin. Trace elements had little or no effect.

There was a progressive increase in viscosity of the culture fluids as incubation progressed and this was accompanied by a change in color of the medium from white to yellow to pinkish brown. Growth response, as measured by plate counts, increased sharply until the 10th day of incubation, after which it reached a plateau and then gradually decreased.

Quantitative determinations. There was a gradual change in the pH of the medium from pH 7.0 to 8.2 during incubation. Representative data which reflect the changes resulting from growth of pure and mixed cultures are presented in table 2. Results obtained by means of 5 analytical methods either individually or collectively indicate that significant amounts of α-conidendrin were degraded. From 9.14 to 72.79 mg of the original 200 mg of the substrate remained after 20 days in media inoculated with mixed cultures. The cell crop of mixed cultures, including the weight of insoluble by-products, ranged from 6.73 to 15.61 mg and 16.53 to 43.02 mg after respective incubation periods of 2 and 20 days. The methoxyl content, which was originally 17.47 per cent, decreased to 0.79 per cent during the experiment. The pure cultures

TABLE 2

Summary of changes produced in α-conidendrin media by mixed and pure cultures

Mixed Cultures	Control	2 Day Incubation			20 Day Incubation		
		22a	14	Mean of 16 cultures	22a	14	Mean of 16 cultures
Wt residual solids (mg)	200	157.17	192.29	175.77	52.16	89.32	70.72
Methoxyl (%) in total residual solids	17.47	12.36	16.21	14.43	0.80	6.37	3.46
Wt α-conidendrin (mg)	200	141.56	185.56	165.31	9.14	72.79	39.59
Bacterial wt (mg)		15.61	6.73	10.49	43.02	16.53	30.75
Ultraviolet adsorption (280 mμ) supernatant	0.149	0.422	0.117	0.246	1.330	0.574	0.861
Pure Cultures		22a-1	1-3		22a-1	1-3	
Wt residual solids (mg)	200	154.36	195.21	178.48	59.73	96.18	78.37
Methoxyl (%) in total residual solids	17.47	12.13	16.40	14.60	1.37	7.38	4.31
Wt α-conidendrin (mg)	200	138.93	187.75	167.39	15.66	84.51	49.23
Bacterial wt (mg)		15.43	7.46	11.13	44.07	12.67	29.20
Ultraviolet adsorption (280 mμ) supernatant	0.149	0.424	0.111	0.220	1.206	0.544	0.805

showed similar activity. After incubation for 20 days, from 15.66 to 84.51 mg of α-conidendrin remained. The cell weight of pure cultures including the weight of insoluble by-products varied from 7.46 to 15.43 mg and 12.67 to 44.07 mg after incubation periods of 2 and 20 days, respectively. The methoxyl content decreased to 1.37 per cent after incubating for 20 days. There was a progressive increase in the amount of aromatic material in the supernatant solution as determined by adsorption of light at 280 mμ. This indicates an accumulation of metabolic products resulting from the dissimilative activities of both pure and mixed cultures.

Sixteen mixed and 17 pure cultures were examined to determine their relative efficiency to metabolize the lignan. Table 3 shows the capacities of representative cultures to degrade the compound. The percentages are based on the weight of substrate remaining after incubation. α-Conidendrin metabolized by mixed cultures ranged from 53.23 to 88.42 per cent and 63.61 to 95.43 per cent after incubation for 10 and 20 days, respectively. The amount utilized by pure cultures representing the genera *Pseudomonas*, *Flavobacterium*, and *Achromobacter* ranged from 43.38 to 88.52 per cent and 57.75 to 92.17 per cent after respective incubation periods of 10 and 20 days.

Determination of aromatic materials in the supernatant of liquid cultures, methoxyl content of residual solids, cell weight, and calculated percentages of the lignan degraded by the most and least active pure cultures are compared in figures 1 and 2. The rate of dissimilation increased rapidly from the 2nd through the 10th day, following which it was appreciably reduced and finally reached a plateau. There was a progressive increase in the amount of aromatic metabolic products coincident with the disappearance of the original material. The growth rate of pure cultures reached a peak on the 10th day of incubation. The cell crop of the most active pure culture on the 10th day was 52.22 mg, whereas that of the least active was 20.87 mg.

In general, the results show that mixed cultures were relatively more efficient than pure cultures in their ability to utilize α-conidendrin as a sole carbon source. Of the pure cultures studied, species of *Pseudomonas* were the most efficient and most numerous, whereas the species of *Flavobacterium* and *Achromobacter* were less effective.

TABLE 3

Percentage degradation of α-conidendrin by pure and mixed cultures

Pure Cultures	Organism	Incubation Time in Days		
		2	10	20
22a-2	*Flavobacterium* sp.	28.13	84.74	90.35
16a-2	*Pseudomonas* sp.	25.78	80.78	87.89
1-3	*Achromobacter* sp.	6.13	43.38	57.75
1-1	*Pseudomonas* sp.	10.71	52.01	67.24
Mixed Cultures				
22a	*Pseudomonas* sp. *Flavobacterium* sp.	29.22	88.42	95.43
16b	*Pseudomonas* sp. *Flavobacterium* sp.	26.42	81.38	90.49
3	*Pseudomonas* sp.	8.50	55.09	66.75
14	*Pseudomonas* sp.	7.22	53.23	63.61

Chromatographic analysis of ether extracts of the supernatant from liquid media inoculated with pseudomonad cultures indicated the presence of vanillic, *p*-hydroxybenzoic, and protocatechuic acids as degradation products. Vanillic acid was demonstrated in the early stages of incubation, whereas the other acids were detected much later. Unidimensional descending chromatograms of the ether extracts were developed in parallel with a mixture of 0.4 per cent solutions of vanillic, *p*-hydroxybenzoic, and protocatechuic acids. The R_f values of the unknown spots from the ether extracts coincided very closely with those obtained with the known acids and with results reported in the literature. Color reactions produced with the spray reagent were: vanillic acid, bluish green; *p*-hydroxybenzoic acid, reddish purple; protocatechuic acid, yellow; and α-conidendrin, light brown. Traces of a keto acid, tentatively identified as β-ketoadipic acid, have been demonstrated chromatographically after prolonged incubation in α-conidendrin media. The Nessler's reagent brought out faint traces of the acid as small orange spots with the same position and color as those of known β-ketoadipic acid. The semicarbazones of the unknown and of β-ketoadipic acid appeared as dark shadows, with the same R_f values, on a faintly fluorescing paper when

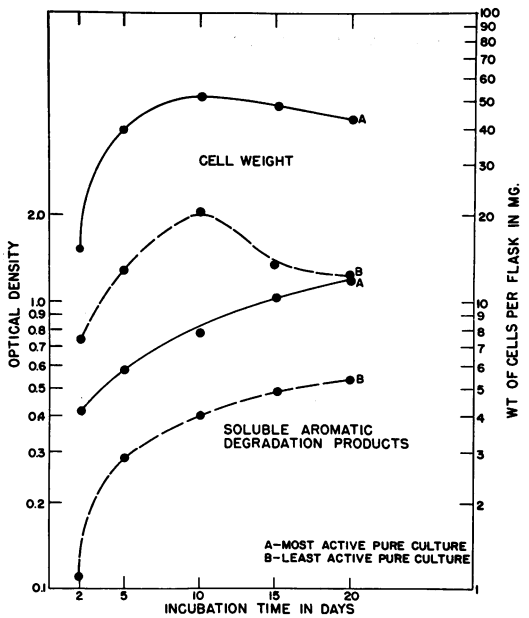

Figure 1. The presence of aromatic materials by the adsorption of light at 280 mμ and calculation of cell weight from the methoxyl content and total weight of residual solids.

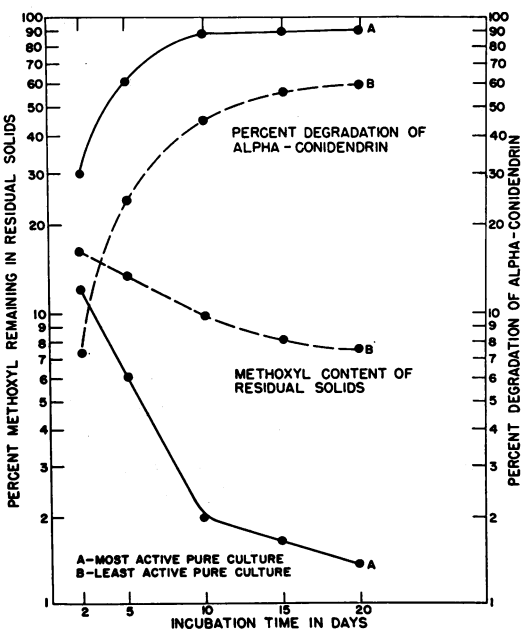

Figure 2. Methoxyl content of residual solids and percentage degradation of α-conidendrin.

TABLE 4

Chromatographic behavior of phenolic and aliphatic acids detected in α-conidendrin media inoculated with pseudomonad strains

Intermediate Products of α-Conidendrin Degradation	R_f' Values	R_f Values					
	Butanol-ethanol-water	Isopropanol-aqueous NH_3-water		Butanol-acetic acid-water		Toluene-acetic acid-water	Butanol-formic acid-water
	SEC*	SEC	Armstrong†	SEC	Bate-Smith‡	SEC	SEC
α-Conidendrin	0.91	0.54	—	0.87	—	—	—
Phenolic acids							
Vanillic	0.65	0.26	0.25	0.89	0.92	—	—
p-Hydroxybenzoic	0.79	0.23	0.23	0.91	0.90	—	—
Protocatechuic	0.55	0.06	0.06	0.82	0.85	—	—
Aliphatic acid							
β-Ketoadipic	—	—	—	—	—	0.61	0.43

* Authors values for unknown spots of extracts from media and of known acids.

† Values of Armstrong *et al.* (1956).

‡ Values of Bate-Smith (1950).

illuminated with ultraviolet light. The R_f values of unknown spots and of representative known phenolic acids and β-ketoadipic acid together with those reported by other workers are presented in table 4. It will be noted that the positions of vanillic and *p*-hydroxybenzoic acids were reversed from those found by Bate-Smith. Further work on the isolation and quantitative determination of β-ketoadipic acid is necessary to definitely establish its identity.

The organisms adapted to the utilization of α-conidendrin dissimilated vanillic, *p*-hydroxybenzoic, and protocatechuic acids when grown on chemically defined media with each of these acids as a sole carbon source. Cultures freshly isolated from a natural source by streaking on α-conidendrin agar when incubated in a medium with either vanillic, *p*-hydroxybenzoic, or protocatechuic acid as the only carbon source showed turbidimetric measurements indicating a limited amount of growth. However, when the same culture was subjected to many successive transfers in a liquid α-conidendrin medium, and was then inoculated in a medium with any one of the above acids as the sole carbon source, the amount of growth increased from 40- to 60-fold over that obtained in the same acids before the culture was adapted. The efficiency to utilize α-conidendrin or any of these acids was rapidly lost if the cultures were subsequently grown on tryptose, brain heart infusion, or yeast extract media. Chromatographic analysis of the mineral vanillic acid media inoculated with α-conidendrin dissimilating pseudomonad strains indicated the presence of *p*-hydroxybenzoic, protocatechuic, and β-ketoadipic acids as intermediates. The presence of protocatechuic and β-ketoadipic acids was also demonstrated chromatographically in *p*-hydroxybenzoic acid defined media inoculated with the above organisms.

Results obtained in growth response studies with α-conidendrin adapted cultures, when subsequently grown in media in which each of the above acids was the only carbon source, and results obtained by chromatographic analysis tend to indicate that the metabolic pathway in the dissimilation proceeds from α-conidendrin → vanillic acid → *p*-hydroxybenzoic acid → protocatechuic acid → ketoadipic acid. Data on the pathway of bacterial dissimilation of this compound agree with the findings of Konetzka *et al.* (1957), whose studies revealed that a *Flavobacterium* sp. utilized α-conidendrin via the following intermediates: conidendric acid → vanillic acid → *p*-hydroxybenzoic acid → protocatechuic acid.

The anthrone test for mono-, di-, and polysaccharides established that a polysaccharide was present in the brown viscous fluid from α-conidendrin cultures. Before use as a substrate, α-conidendrin was purified by repeated washing in acetone and precipitation by the addition of

water, and was shown to be carbohydrate free.

Representative mixed and pure cultures were unable to dissimilate commercial lignin residues such as Polyfon H, Indulin A, Orzan A, and Orzan S when grown in aerated and agitated liquid media containing these compounds as energy sources. Chromatographic and spectrophotometric analysis of supernatant solutions from liquid cultures incubated at room temperature and 35 C did not show the presence of any lignin intermediates. When media were incubated under anaerobic conditions at 35 and 55 C, there was likewise no evidence of utilization of these materials. The organisms metabolized traces of carbohydrates present as impurities in lignin materials but did not dissimilate these complex polymers.

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SUMMARY

By means of enrichment culture techniques, α-conidendrin dissimilating bacteria have been isolated from widely separated geographical locations. Sample sources included manure, soil, decaying wood, and forest litter as well as mud and water from below paper mill effluent outfalls. Progressive selection procedures further increased the efficiency of the isolates to degrade the lignan.

Although active genera were common to all sample sources, pseudomonad strains were most numerous and most active. Species of the genera *Flavobacterium* and *Achromobacter* capable of degrading α-conidendrin were also frequently present. Mixed cultures appeared to be more efficient than pure cultures.

Adaptation studies, together with chromatographic and spectrophotometric analyses of the degradation products, indicate that the metabolic pathway in the dissimilation of α-conidendrin by pseudomonad strains may proceed from α-conidendrin → vanillic acid → *p*-hydroxybenzoic acid → protocatechuic acid → ketoadipic acid.

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