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Microbial Hydrocarbon Co-oxidation

II. Use of Ion-Exchange Resins

R. L. RAYMOND, V. W. JAMISON, AND J. O. HUDSON
Sun Oil Company, Marcus Hook, Pennsylvania 19061

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Anion-exchange resins, a weakly basic polystyrene-polyamine type and a macroreticular type, IR-45 and IRA-93, respectively, were shown to significantly increase yields of acidic products in co-oxidation systems. *p*-Toluic, 2,3-dihydroxy-*p*-toluic, and α,α-cis,cis dimethylmuconic acids, resulting from the oxidation of *p*-xylene by three cultures of *Nocardia*, accumulated on the resin in shaken flasks or agar plates during the cultivation. Final product concentration increased with increasing resin concentration. Mineral balances were not affected if the resin was properly conditioned before use.

Ion-exchange resins have found wide application in analytical chemistry for the recovery and purification of many types of compounds (1). In commercial processes, the removal of trace impurities and recovery of certain pharmaceuticals have been reported. Humphrey and Raymond (2) have recently described a number of microbial hydrocarbon oxidations in which yields were substantially increased by the addition of anion-exchange resins. Tone et al. (4) reported that higher concentrations of salicylic acid could be obtained in a naphthalene fermentation by *Pseudomonas aeruginosa* if an anion-exchange resin was present. They attributed the increased yields to removal of the salicylic acid which was inhibitory to the culture.

In a previous report, we observed that the acidic products resulting from *p*-xylene oxidation by isolates of *Nocardia* in shaken flasks were at the 0.5 to 1.5 g/liter level or lower (3). We also observed that a number of factors, such as hydrocarbon concentration, time of addition of the co-oxidizable substrate, and *p*H level, resulted in highly variable results. At the plate and shaken flask level, we have always been plagued by a lack of reproducibility in co-oxidation experiments. This report describes the effects that anion-exchange resins have on the concentration of products during the oxidation of aromatic hydrocarbons.

MATERIALS AND METHODS

Microorganisms. *N. corallina* A-6, *N. salmonicolor* A-100, and *N. corallina* V-49 were isolated by the soil plating technique with hydrocarbons as carbon sources (3; V. W. Jamison, R. L. Raymond, and J. O. Hudson, Abstr. 154th Natl. Meeting Am. Chem. Soc., Chicago, 1967). These cultures were maintained on mineral agar slants containing filter paper discs impregnated with *n*-hexadecane.

Media and culture conditions. Resins prepared as described below were added to a mineral salts medium, and the fermentation was carried out in baffled shaken flasks on an incubator (model G-27; New Brunswick Scientific Co., New Brunswick, N.J.) shaker at 30 C as previously described (3). Agar-resin plates were prepared by adding 2 ml of the wet resin to a petri dish followed by 20 ml of mineral agar.

Types and preparation of the ion-exchange resin. A weakly basic, polystyrene-polyamine type, a macroreticular type and a strongly basic quaternary-ammonium-polystyrene type, IR-45, IRA-93, and IRA-400 (Rohm and Haas Co., Philadelphia, Pa.), respectively, were prepared for fermentations as follows.

The resins were exchanged with 4% NaOH, washed with water, and exchanged with a 5% phosphate solution made of Na_2HPO_4 and KH_2PO_4 to give a final *p*H of 7.0. After a thorough washing with distilled water, small batches of IR-45 and IRA-400 were sterilized by boiling for 1 hr on each of 2 successive days. IRA-93 was sterilized in an autoclave at 252 F (122.2 C) for 10 min. After a final wash with sterile distilled water, the resin was ready to be added to the cultivation medium.

Analysis of product. A 1-ml sample of resin was removed with a wide-mouth pipette, washed with water, and brought to a boil in 50 ml of 1.3 N HCl-methanol. Concentrations of products were determined directly on these extracts by measuring their absorption in the ultraviolet region with a Beckman DK2A at peaks appropriate for the specific compounds under consideration. Products remaining in the broth were determined by diethyl ether extraction as previously described (3). Isolation of unknown compounds from resin was accomplished by exchange with 4% NaOH, acidification with HCl, and extraction with diethyl ether.

The resin from agar plates was treated in a similar

manner after separation from the agar by boiling in water.

RESULTS AND DISCUSSION

Effect of resin type, concentration, and composition in shaken flasks. Of several ion-exchange resins tried, only two, IR-45 and IRA-93, were sufficiently nontoxic to the cultures under investigation to be used at fairly high concentrations. Preliminary tests indicated that IRA-400 was not quite as satisfactory as IR-45, and it was more difficult to remove the products when using it. Therefore, it was not included in further studies. Table 1 presents data for the oxidation of *p*-xylene in shaken flasks by cultures A-6 and A-100 in the presence of three concentrations of IR-45. Under the conditions of the experiment, the resin demonstrated a very marked improvement in the product accumulation. Its presence did not significantly alter the ratio of 2,3-dihydroxy-*p*-toluic acid (DHPT) to *p*-toluic acid (PTA).

Products resulting from the intermittent addition of resin to the same fermentation are shown in Table 2. Control flasks without resin additions reached a maximum product concentration of 0.4 g/liter (0.1 g of DHPT, 0.3 g of PTA) on the 7th day.

Further evaluation of anion-exchange resins indicated that an equilibrium could be established more quickly with the aromatic organic acids by use of the macroreticular types. It was also determined that IRA-93 was more resistant to attrition than the gel-type, IR-45, a necessary requirement for use in high-speed stirred fermentors.

TABLE 1. *Effect of IR-45 resin concentration on PTA and DHPT production from p-xylene by N. corallina A-6 and N. salmonicolor A-100*[a]

Concn of resin (ml/liter)	A-6 products		A-100 products	
	Amt (g/liter)	Per cent DHPT	Amt (g/liter)	Per cent DHPT
0	0.1	0	0.1	0
70	3.8	21	2.0	25
150	4.5	18	3.0	20
300	5.5	22	5.3	28

[a] Basal mineral salts medium (100 ml) containing 0.2% urea, 0.5% phosphate (*p*H 7.0) in 500-ml shaken flasks. Cultures were grown for 6 days on *n*-hexadecane before *p*-xylene additions. Total hydrocarbons added incrementally over a 13-day period were: *p*-xylene, 14 g/liter; *n*-hexadecane, 3 g/liter. Incubation was carried out on a gyratory shaker at 30 C.

The first lot of IRA-93 obtained from the manufacturer gave satisfactory results up to concentrations of 200 ml/liter. A concentration of 300 ml/liter was completely inhibitory to both A-6 and A-100 cultures. The second lot obtained was more toxic than the first and required extensive washing and conditioning to make it usable at low concentrations. The effect of IRA-93 concentration on *p*-xylene oxidation to DHPT and PTA by *N. corallina* A-6 is shown in Table 3. Product which remained free in the fermentation broth rarely exceeded 10% of the total product of the system at the *p*H employed in these experiments.

Effect of mineral concentration. Because of the exchange with anions in the medium, several

TABLE 2. *Effect of intermittent addition of IR-45 resin on PTA and DHPT production from p-xylene by N. corallina A-6*[a]

Time	Resin	*p*-Xylene	*n*-Hexadecane	PTA	DHPT
days	*ml*	*ml*	*ml*	*g/liter*	*g/liter*
0	0	0	0.1		
1	0	0	0.2		
2	1	0.18	0.02		
3	1	0.18	0.02		
4	1	0.18	0.02		
5	0	0	0	0.4	0.6
6	0	0	0		
7	1	0.18	0.02	1.7	0.6
8	0	0.36	0.04	2.2	1.3
9	3	0.36	0.04	2.8	1.9
10	4	0.72	0.08		
11	0	0.18	0.02		
12	2	0.36	0.04	9.2	1.5
13	0	0.36	0.04		
14	8	0.36	0.04	15.4	1.3

[a] Medium and physical conditions as shown in Table 1. After 48 hr hydrocarbons were added as a 90% *p*-xylene-10% *n*-hexadecane mixture.

TABLE 3. *Effect of IRA-93 resin concentration on PTA and DHPT production by N. corallina A-6*[a]

Concn of resin	*n*-Hexadecane	*p*-Xylene	Product free in broth		Product on resin	
			Amt (g/liter)	Per cent DHPT	Amt (g/liter)	Per-cent DHPT
ml/liter	*ml*	*ml*				
50	0.5	0.6	0.15	21	1.9	23
100	0.5	0.8	0.14	29	2.3	39
200	0.45	0.65	0.13	24	3.4	31

[a] Medium and physical conditions as shown in Table 1.

TABLE 4. *Effect of mineral concentration on the production of PTA and DHPT from p-xylene by N. salmonicolor A-100 in IRA-93 resin systems*[a]

System	Mineral salt concn	Nitrogen	Product in broth				Product on resin	
			With resin		Without resin			
			Amt (g/liter)	Per cent DHPT	Amt (g/liter)	Per cent DHPT	Amt (g/liter)	Per cent DHPT
1	1×	0.2% Urea	0.31	11.4	0.14	26.4	3.2	37.5
2	2×	0.2% Urea	0.19	16.9	0.06	24.0	1.9	36.8
3	5×	0.2% Urea	0.41	29.4	0.11	24.5	3.2	31.2
4	1×	0.1% $(NH_4)_2SO_4$	0.10	12.4	0.21	28.4	1.7	29.4
5	1×	0.3% $(NH_4)_2SO_4$	0.15	28.8	0.07	34.5	2.2	50.0
6	1×	0.3% $(NH_4)_2SO_4$	0.49	9.1	0.07	52.0	3.7	29.8

[a] Shaken flasks (500 ml) with 10 ml of resin and sufficient mineral salts medium to make 100 ml. All systems contained 0.5% PO_4^{3-}, with the exception of no. 6 which had 0.1% PO_4^{3-} and 1.0% $CaCO_3$. Total incubation time was 120 hr at 30 C, with *n*-hexadecane and *p*-xylene added incrementally as previously described.

TABLE 5. *Effect of IR-45 anion exchange resin on the production of acidic products from p-xylene by N. corallina V-49 in agar plates*

System[a]	Growth	Product (mg/plate)[b]		
		Replicate 1	Replicate 2	Replicate 3
Control	+++[c]	9	9	9
IR-45 resin (2 ml)	+++	64	55	70

[a] Mineral agar plates were inverted over a filter paper disc in a lid containing ~100 mg of *n*-hexadecane incubated for 10 days in a vacuum oven at 30 C with an air atmosphere saturated with *p*-xylene.

[b] Mixture of α,α-dimethyl-cis,cis-muconic acid and PTA.

[c] Heavy confluent.

concentrations of minerals were investigated to determine whether the resin had an adverse effect on the balance. Table 4 shows that no apparent changes resulted from increasing the minor constituents by a factor of 5 in systems 1 to 3. In the same table, comparison of system 1 with systems 4 and 5 shows that $(NH_4)_2SO_4$ was not as effective a nitrogen source as urea in the presence of resin. Since this could be the result of a build-up of phosphate resulting from the SO_4^{2-} exchange or of competition for sites between PO_4^{3-}, SO_4^{2-}, and organic acid, substituting $CaCO_3$ for phosphate, as shown in system 6 (Table 4), appeared to substantiate the toxic effect of the phosphate. In our routine testing of new co-oxidation systems, the initial phosphate concentration in the medium was 0.1% or lower when a phosphate-exchanged resin was used. We also found that in some systems it was advantageous to use chloride-exchanged resin.

Agar-resin systems. In our earlier studies of co-oxidation, it was an extremely time-consuming and laborious task to carry out enough shaken flask experiments to evaluate a culture. We found that the use of resins in agar plates greatly facilitates these evaluations. Table 5 demonstrates the reproducibility of this method and the significant quantity of product found on each plate. In many instances, sufficient material could be isolated from one plate for characterization. An added advantage of the plate system is the freedom from cells and the residual hydrocarbon used for growth. Before recovery of the resin, we routinely scraped the cells from the plate surface with a microscope slide.

The use of anion-exchange resins in hydrocarbon fermentations probably serves a number of functions which contribute to the increased yields. These functions are: (i) *p*H control, (ii) controlled feeding of the hydrocarbon substrate, (iii) removal of product to prevent further oxidation, and (iv) removal of products which tend to repress the desired reaction.

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