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Propylene oxide production from propylene by immobilized whole cells of *Methylosinus* sp. CRL 31 in a gas-solid bioreactor

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Summary. Methanotrophic bacteria have been shown to oxidize gaseous alkenes to the corresponding epoxides utilizing an $NADH_2$-dependent methane monooxygenase. A cell paste of methane-grown methylotrophs was coated on porous glass beads. The production of propylene oxide from propylene was performed in a gas-solid bioreactor to ensure continuous production and removal of product epoxide from the microenvironment of the biocatalyst. The amount of propylene oxide produced before cofactor regeneration was between 120–145 μmoles/20 mg cells in about 10 h depending on the microbial strains used. The conversion rate for propylene was 2.7%. Regeneration of cofactor $NADH_2$ was performed in the bioreactor with the vapor of a cosubstrate, methanol.

Introduction

The epoxidation of alkenes (Colby et al. 1977; Higgins et al. 1979; Hou et al. 1979a; Patel et al. 1979) and the hydroxylation of alkanes (Patel et al. 1980b; Hou et al. 1981b) by methylotrophic bacteria in both whole cells and cell-free systems have been reported. Methane monooxygenase requires a cofactor, $NADH_2$, for catalyzing both the epoxidation of alkenes and the hydroxylation of alkanes. Obligate methanotrophs cannot utilize compounds with carbon-carbon bonds. The product epoxides accumulate extracellularly. In whole cell systems, the epoxidation of alkenes and the hydroxylation of alkanes do not require an exogeneous supply of cofactor, $NADH_2$ (Hou et al. 1979a). When the cofactor is depleted, the epoxidation or hydroxylation stops. Previously, we reported the stimulation of epoxidation by methane metabolites in resting cell suspensions of methanotrophic bacteria (Hou et al. 1980b). The reason for the stimulation of epoxidation in the in vivo system was postulated to be the provision of additional reducing power, or the reduced form of NAD^+, for the methane monooxygenase system.

Recently, we have identified and purified many dehydrogenases from methylotrophic microorganisms: methanol dehydrogenase (Patel et al. 1978), aldehyde dehydrogenase (Patel et al. 1980a), formaldehyde reductase (Hou et al. 1982a), formate dehydrogenase (Hou et al. 1982b), secondary alcohol dehydrogenase (Hou et al. 1979b; 1981a), and propanediol dehydrogenase (Hou et al. 1983). We have also demonstrated the epoxidation of alkenes by cell-free soluble methane monooxygenase from a facultative methylotroph, *Methylobacterium* sp. (Patel et al. 1982), and an obligate methanotroph, *Methylosinus* sp. CRL 31 (Hou et al. 1982c). The epoxidation of propylene by this soluble methane monooxygenase system using $NADH_2$ which was regenerated/generated by dehydrogenases and their substrates was also demonstrated (Hou et al. 1982c). Figure 1 shows the cofactor regeneration scheme for the production of propylene oxide.

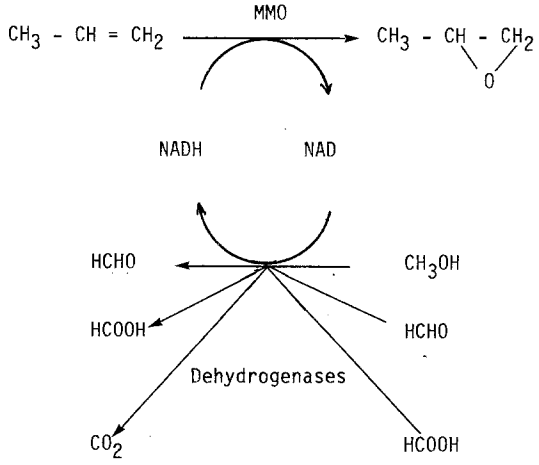

Fig. 1. Cofactor regeneration scheme for the production of propylene oxide

In recent years, this epoxidation of alkenes by bacteria has received considerable attention because of its possible industrial application (Higgins et al. 1979; Hou et al. 1980a; Dalton 1980). A method employing immobilized enzymes for the production of epoxide was reported (Parkinson 1980). However, because of the complicated enzyme isolation procedures necessary, and the instability of methane monooxygenase in in vitro systems, the use of whole cells seems more feasible. In addition, the cofactor regeneration can be more easily achieved in whole cell systems. Two major problems in the biotechnological process for the production of epoxides are the regeneration of cofactor and the build-up of reaction product around the biocatalyst. This paper describes a gas-solid heterogeneous bioreactor for the production of propylene oxide from propylene, which ensures continuous production and removal of product epoxide from the microenvironment of the biocatalyst. Regeneration of cofactor $NADH_2$ was performed in the bioreactor with a co-substrate, methanol.

Materials and methods

Methylosinus sp. CRL 31 was isolated from a soil sample from the Bayway Refinery (Linden, NJ) by an enrichment culture technique using methane and air (1 : 1 v/v) as described previously (Hou et al. 1979a). The organisms were maintained at 30° C on mineral salts agar plates in a dessicator under an atmosphere of methane and air (1 : 1 v/v), with methane being the sole carbon and energy source. Organisms were grown on methane (10% methane, 15% carbon dioxide, and 75% air mixture) at 30° C in batch culture on mineral salt medium (Foster and Davis 1966) in a 30-l fermentor (New Brunswick Scientific Co., Edison, NJ). Cells were washed twice with 0.05 M potassium phosphate buffer pH 7.0. Product propylene oxide was assayed with gas liquid chromatography as described previously (Hou et al. 1979a). Porous glass beads 2 mm diameter were purchased from SGA Scientific Inc., Bloomfield, N.J.

Results and discussion

Different whole-cell immobilization methods were studied. Covalent binding of the cells to long ligands of a macro molecule or polyacrylamide entrapment of cells all resulted in loss of propylene epoxidation activity. This is possibly due to the alteration of the membrane structure or the creation of a diffusion barrier for gaseous substrates by polyacrylamide. Finally, a simple gas-solid heterogeneous bioreactor system was successfully demonstrated for the continuous production of propylene oxide. Cell paste of methane-grown *Methylosinus* sp. CRL 31 in 0.05 M potassium phosphate buffer pH 7.0 was coated on porous glass beads (2 mm diameter). Cells (about 20 mg cell protein) were physically adhered in a thin layer on these glass beads (about 7 ml) and were packed in a glass reactor (15 cm × 1 cm Ø) *1* (Fig. 2). The bioreactor was equipped with a jacket for circulating water to control reaction temperature.

The mixture of gaseous substrates (a mixture of propylene and oxygen 1 : 1, v/v) was introduced through a water bottle *6* (which was maintained at 40° C) to pick up moisture and then into the bottom of the gas-solid bioreactor *1*. The temperature of the bioreactor was kept at 40° C (above the boiling point of product propylene oxide, 35° C). The product

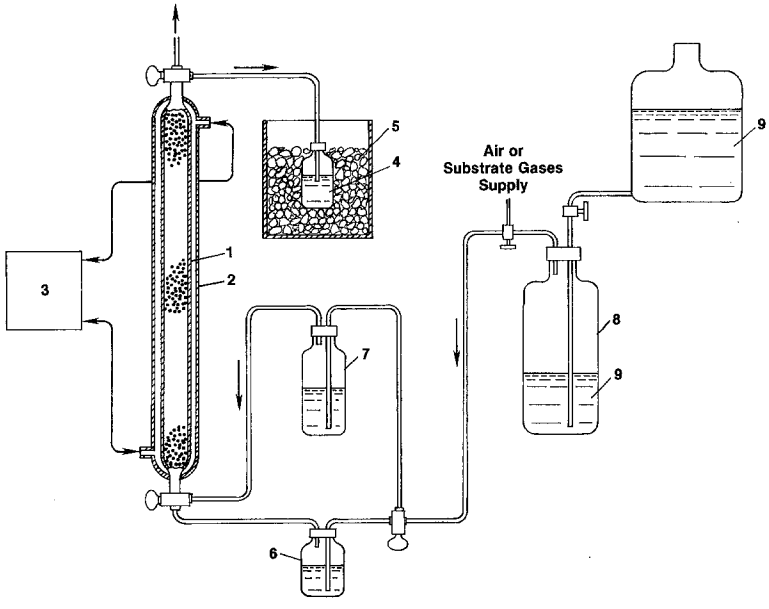

Fig. 2. Schematic diagram of the gas-solid bioreactor system with biocatalyst regeneration capacity. Cell paste of methane-grown *Methylosinus* sp. CRL 31 was coated on glass beads and packed in the bioreactor. *1* bioreactor; *2* jacket; *3* temperature control device; *4* liquid for product recovery; *5* ice; *6* water at 40° C; *7* methanol for biocatalyst regeneration; *8* gaseous substrate mixture; *9* water

propylene oxide was recovered by cooling using ice as the coolant *4*.

The gaseous phase of the bioreactor was evacuated and then was filled with the gaseous substrate mixture. The gas mixture was then introduced continuously into the bioreactor at a flow rate of about 0.5 ml/min. The relative humidity inside the bioreactor was maintained at about 70%. The production of propylene oxide was found to be at a constant rate of 18 μmoles/h for the first 7 h (Fig. 3). The conversion rate for propylene was 2.7%. After 7 h of continuous operation, the rate of propylene oxide production slowed down, possibly due to the depletion of endogeneous reducing power (cofactor $NADH_2$). After 10 h of operation, propylene oxide production essentially stopped. In batch experiments using the gas-liquid system described previously (Hou et al. 1979a) the same cell suspension produced propylene oxide at 1.5 μmoles/h/mg protein for 2 h. After 2 h the reaction stopped. Some stimulation of propylene oxide production by the addition of methanol was also observed (Hou et al. 1980b).

In situ regeneration of the biocatalyst in the bioreactor was conducted after 12 h of operation using methanol as the regeneration substrate. The substrate gas mixture inside the jar *8* was replaced with air. The air was forced to pass through a methanol bottle *7* (maintained at 40° C) and then into the bioreactor, carrying methanol vapor with it. Therefore there was no washoff of cells. The regeneration of the biocatalyst was continued for 30 min at 3 ml/min flow rate. At the end of the regeneration, the substrate mixture (propylene and oxygen) was reintroduced. The production of propylene oxide immediately resumed at a constant rate of 12 μmoles/h. After an additional 6 h of operation, the reaction rate slowed down again, indicating the need for additional cofactor regeneration. Cofactor regeneration was performed once again. Although, the resumption of propylene oxide production was detected, the rate was sharply reduced. Microbial cells of other methanotrophic bacteria were also tested in this heterogeneous bioreactor. Data shown in Table 1 indicated that this bioreactor can be applied to the cells of other methanotrophs.

A major problem in the biotechnological process is the toxicity of the reaction product for the biocatalyst. To avoid epoxide accumulation in the microenvironment of the biocatalyst, a rapid removal of this product is essential. This has been accomplished by maintaining the temperature of the gas-solid bioreactor at greater than 40° C. In addition, the amount of water used in our gas-solid bioreactor is significantly less in comparison to the conventional gas-liquid bioreactor system. Other experimental conditions used here were chosen from those used in our previous papers. Although we have not optimized the reaction conditions for this heterogeneous gas-solid bioreactor with a particular methanotrophic strain, this paper demonstrates the feasibility of using this new type of bioreactor for the production of chemicals from gaseous substrates.

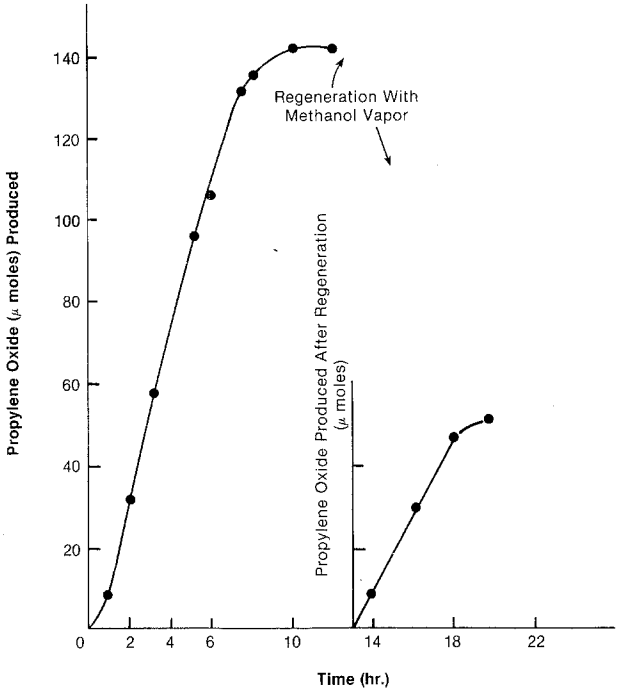

Fig. 3. Production of propylene oxide from propylene by cells of methanegrown *Methylosinus* sp. CRL 31 packed in a gas-solid bioreactor and the regeneration of the biocatalyst. Twenty mg cell paste was coated on glass beads and was packed in the bioreactor. The flow rate of gaseous substrates supplied was 0.5 ml/min. Product propylene oxide was assayed by gas chromatography. Regeneration of the biocatalyst was conducted after 12 h of operation

Table 1. Production of propylene oxide from propylene in a gas-solid bioreactor

Microbes	Propylene oxide produced (μmol/20 mg cells)		
	Initial	1st regeneration	2nd regeneration
Methylosinus sp. CRL 31	145	50	18
Methylococus sp. CRL M1	120	18	6
Methylosinus trichosporium OB3b	135	40	10

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