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Oxidation of n-Alkanes: Growth of *Pseudomonas putida*

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Summary. The induction of alkane hydroxylase activity was investigated in two strains of *Pseudomonas putida* with a view to the production of primary alcohols. n-Nonanol production rates (16.0 μmol/g dry wt/h) with an alcohol dehydrogenase negative mutant *P. putida* PpS173 were considerably lower than might be expected from the growth of a wild type on n-alkane. Production of cells by fed-batch culture on n-nonane, with a specific alkane hydroxylase activity of 3.9 mmol/g/h, was considered most suitable for isolation of the alkane hydroxylase.

Introduction

Microorganisms are able to break down aliphatic and aromatic hydrocarbons by the stereospecific introduction of one or more hydroxyl groups. The use of microbial hydroxylases as catalysts in industrial reactors depends on the successful development of methods for their large-scale production and isolation. The terminal hydroxylation of n-alkanes is an example of such a reaction that is also difficult to accomplish by chemical substitution. However, there are few detailed reports in which the optimisation of the enzymatic production of an n-alkane transformation product is the goal. Some exceptions are the production of long chain dicarboxylic acids (Uchio and Shiio 1972) and the transformation of alk-l-enes to the corresponding epoxide (Schwartz and McCoy 1977).

The initial stages of n-alkane assimilation in *Pseudomonas putida* involve oxidation to the corresponding fatty acid via the primary alcohol and aldehyde. The alkane hydroxylase complex responsible for the hydroxylation to the primary alcohol has been well characterised (McKenna and Coon 1970; Ueda et al. 1972; Lode and Coon 1973; Benson et al. 1977). Whole-cell alkane oxidising activity is inducible in *Pseudomonas* and other n-alkane-utilising bacteria. *In P. aeruginosa* 473 and *P. putida* strains the n-alkane substrate and a variety of non-metabolisable compounds are inducers; the level of induced activity is also influenced by the carbon source for growth (van Eyk and Bartels 1968; Grund et al. 1975). Characterisation of the structural genes for the initial stages of n-alkane assimilation led to the mutation and selection of strains unable to metabolise the primary alcohol and, therefore, to grow on the n-alkane (Benson and Shapiro 1976). Here we describe experiments to investigate suitable methods for the production of *P. putida* containing a high level of alkane hydroxylase for the production of primary alcohols.

Materials and Methods

Microorganisms and Fermentation Media

Pseudomonas putida PpG6 (OCT^+) was kindly provided by Dr. J. Johnston, University of Illinois, Illinois, USA.

P. putida PpS173 (*alcA*$^-$ (CAM-OCT *alk*$^+$ *alcO*$^-$)) unable to grow on alkanes and primary alcohols, was kindly provided by Dr J. Shapiro, University of Chicago, Chicago, USA.

Cultures were maintained at 4 °C on nutrient agar slopes. Fermentation medium, made up in deionised water, consisted of 3 g/l NH_4Cl (2 g/l NH_4Cl for *P. putida* PpS173), 4 g/l KH_2PO_4, 6 g/l $Na_2HPO_4 \cdot 2H_2O$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l yeast extract (Bovril Ltd., Burton on Trent), 10 ml/l of a mineral salt solution containing 100 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $ZnSO_4 \cdot 7H_2O$, 10 mg/l $CuSO_4 \cdot 5H_2O$, 4 mg/l H_3BO_3, 4 mg/l $MnSO_4 \cdot 7H_2O$ and 3 mg/l $Na_2MoO_4 \cdot 2H_2O$. Details of the carbon source and inducer are given with each experiment. 0.1 ml/l polypropylene glycol 2000 (Shell Chemicals UK Ltd.) was used for antifoam.

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Table 1. Induction of the alkane hydroxylase of *Pseudomonas putida* PpS173 during growth on different carbon sources

Carbon source	μmax (1/h)	Cell yield (g/g)	Specific activity (μmol nonan-1-ol/g/h)	Volumetric activity (μmol nonan-1-ol/l/h)
sodium pyruvate[a]	0.41	0.37	16.0	59.4
sodium malonate[a]	0.46	0.18	12.5	22.7
lactic acid[a]	0.63	0.49	0.0	0.0
glycerol[a]	0.31	0.50	6.1	30.5
glycerol[b]	0.28	0.43	6.9	29.7

In all instances a 60 ml inoculum was added to 3 l of fermentation medium containing 10 g/l carbon source; the agitator speed was 600 rpm and the aeration rate 0.33 v/v/min. Inducer was added during the exponential phase to a final concentration of:
[a] 5 mM-diethoxymethane
[b] 18.7 mM-n-nonane

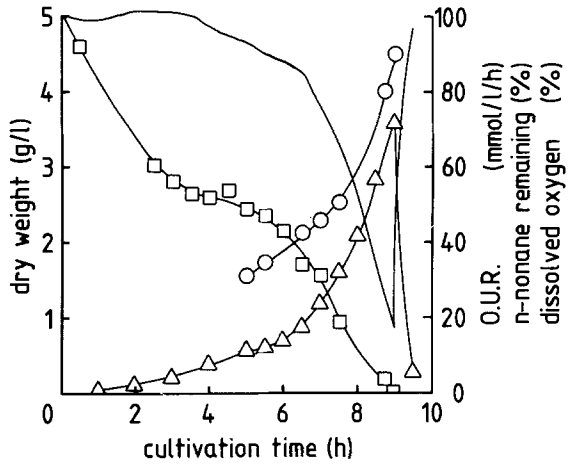

Fig. 1. Batch culture of *Pseudomonas putida* PpG6 on 2% (v/v) n-nonane. A 60 ml inoculum grown on the fermentation medium was used. The agitator speed was 790 rpm and the aeration rate 0.33 v/v/min. –○–○– cell dry weight; –△–△– oxygen uptake rate; –□–□– n-nonane; —— D.O.T.

Batch and Fed-Batch Cultures

Cultures were grown at 30 °C in a 5 litre baffled vessel containing 3 l of sterile medium, adjusted to and maintained at pH 6.8 by addition of 2M-HNO_3 or 2M-NaOH. Sterile air was supplied through a ring sparger sited below an eight-bladed vaned disc impeller.

Details of time of addition and amount of inducer, or n-alkane in fed-batch cultures, are given with each fermentation.

Analytical Methods

Culture samples were centrifuged and the cells (0.5 g packed wt) resuspended in 2 ml phosphate buffer, pH 7.0 (I = 0.1), containing chloramphenicol (0.25 mg/ml), fermentation carbon source (5 mg/ml) and trace salts ($MgSO_4$ and mineral salts as specified for fermentation media).

Whole-cell alkane hydroxylase activity was measured at 30 °C in shaken sealed tubes by one of two methods, depending on the microorganism used:

(i) the reaction was started by the addition of 10 μl n-nonane. After 1 h the tubes were cooled, the contents extracted with 10 ml ice-cold diethyl ether and analysed for nonan-l-ol by GLC.

(ii) the reaction was started by the addition of 20 μl n-nonane. After 0.5 h the tubes were cooled, 25 μl n-decane added as an internal standard, the contents extracted with propionic acid (Manfredini and Wang 1972) and analysed for n-nonane by GLC.

Specific activities are expressed as either μmol nonan-l-ol produced or mmol n-nonane consumed/g dry wt/h.

Where appropriate, culture samples were analysed for n-nonane by extraction with propionic acid and measurement by GLC.

Results

The Production of nonan-1-ol by an Alcohol Dehydrogenase Negative Mutant

Pseudomonas putida PpS173 is unable to grow on n-alkanes or primary alcohols. Hence, expression of alkane hydroxylase activity in this strain requires induction during growth on a non-repressing carbon source. Results for the fermentation of this strain on different carbon substrates with induction by diethoxymethane and n-nonane are shown in Table 1. The lower cell yield on glycerol for n-nonane induction was a reflection of the addition of n-nonane perturbing cell growth.

These rates of nonan-1-ol production by *P. putida* PpS173 are much lower than the alkane hydroxylation rate that might be expected for the wild type growing on n-nonane.

Batch Culture of P. putida PpG6 on n-nonane

Determination of the n-alkane utilisation rate during growth on n-nonane is complicated by the volatility of this hydrocarbon. The apparent rate of n-nonane assimilation as determined from the liquid concentration of n-

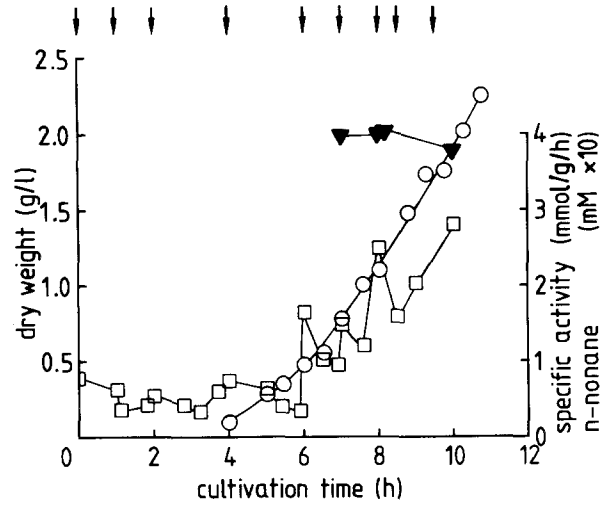

Fig. 2. Fed-batch culture of *Pseudomonas putida* PpG6 on n-nonane. The 60 ml inoculum was grown on the fermentation medium in the presence of n-nonane. The agitator speed and aeration rate were varied to prevent excessive alkane loss and oxygen limitation. —o—o— cell dry weight; —□—□— n-nonane; —▼—▼— specific activity; → addition of 25.3 mmol n-nonane

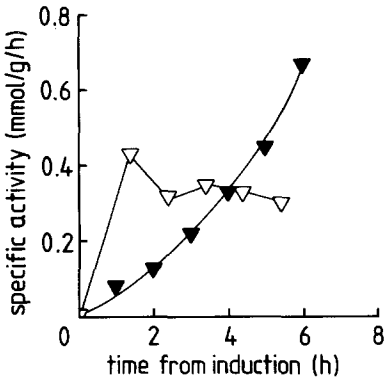

Fig. 3. Induction of the alkane hydroxylase of *Pseudomonas putida* PpG6 during growth on 1% (v/v) glycerol. The 60 ml inoculum was grown on the fermentation medium in the absence of inducer. The agitator speed was 600 rpm and the aeration rate 0.33 v/v/min. —▽—▽— addition of 28.1 mmol n-nonane; —▼—▼— addition of diethoxymethane to a final concentration; of 5 mM

nonane is influenced by n-nonane stripping from the culture. Figure 1 shows the rapid loss of the n-alkane from the vessel during the initial stages of batch culture of *P. putida* PpG6 on n-nonane. Assuming a cell yield of 1.0 (g/g), the rate of n-nonane assimilation is equal to the specific growth rate. The specific n-nonane assimilation rate, calculated from the specific growth rate on this basis, is 4.6 mmol/g dry wt/h.

Fed-batch Culture of P. putida PpG6 on n-nonane

The results from the batch culture grown on n-nonane indicate a high alkane hydroxylase activity. To minimise alkane loss and improve cell yields, cultures were grown with intermittent feeding of n-nonane (Fig. 2). n-Nonane additions were made with an increasing frequency as the fermentation progressed to prevent alkane limitation.

During the fermentation the D.O.T. did not fall below 50% air saturation. The specific activity, as measured during the later part of the experiment, was almost constant at a high level (3.9 mmol/g dry wt/h). In later production runs higher cell concentrations (13 g dry wt/l) and yields were achieved and cells were harvested with a similar specific activity (3.9 mmol/g/h).

Induction of the Alkane Hydroxylase of P. putida PpG6 During Growth on Glycerol

Induction of the wild type during growth on glycerol was investigated as an alternative to growth on n-alkane. The pattern of induction during exponential growth on glycerol was dependent on the inducer used (Fig. 3). After the addition of n-nonane there was a rapid initial rise in n-alkane oxidising activity. The culture activity then remained constant at 1.5 mmol/l/h and the resulting decline in specific activity was not affected by the addition of a second batch of inducer 3.2 h later. The response to diethoxymethane was initially lower but resulted in a higher level of activity that was not maximal 5.5 h after induction.

Induction by n-nonane of the Alkane Hydroxylase of P. putida PpG6 Following Growth on Other Carbon Sources

When cells were induced with n-nonane while growing on glycerol, alkane hydroxylase synthesis ceased when the specific activity had only risen to about 0.4 mmol/g dry wt/h (Fig. 3). Further experiments were done in which n-nonane induction followed the completion of growth on glycerol, glucose or citrate. In some cases the specific activity reached 0.8–1.4 mmol/g/h but the fluctuation of values with time made harvesting of active cells rather uncertain.

Discussion

As our objective was to produce cells able to convert n-alkanes to primary alcohols the use of the alcohol dehydrogenase mutant, *Pseudomonas putida* PpS173, initially seemed attractive. The result with sodium pyruvate

was comparable to that (a n-nonane hydroxylation rate of 27.2 μmol/h/g) reported by Benson and Shapiro (1976) taking into account their use of n-octane as an inducer. We had hoped to improve on this activity but the results, even on other carbon sources, were disappointingly low.

The rates of n-alkane assimilation with *P. putida* PpG6, grown by different methods, were far greater than the maximum rate of nonan-1-ol production by *P. putida* PpS173. Batch culture of *P. putida* PpG6 on n-nonane was not pursued due to the difficulty in overcoming alkane loss from the vessel, and served only to indicate possible rates of alkane hydroxylation. Higher cell yields were obtained with fed-batch cultures and specific activities were comparable to those derived from the batch culture.

The result of n-nonane induction of *P. putida* PpG6 during exponential growth on glycerol suggests different roles for diethoxymethane and n-nonane in the induction of the alkane hydroxylase system. van Eyk and Bartels (1968) showed that the suspension of uninduced cells in a buffer containing malonate and n-hexane, resulted in a rapid increase followed by a gradual decrease in the ability to oxidise n-alkanes. However, this is the only other reference to this phenomenon.

Cells induced after the completion of growth on glycerol have been used in fractionation studies. The uncertainly of final specific activities with these cultures and the higher activities achieved by fed-batch culture on n-nonane proved this latter method most suitable for the production of material for the isolation of an active cell-free alkane hydroxylase.

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