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Effect of Solvents on Bioconversion of Penicillin G to Deacetoxycephalosporin G

QIANG GAO† and ARNOLD L. DEMAIN*

Biology Department, Massachusetts Institute of Technology,
Cambridge, MA 02139, U.S.A.

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The bioconversion of penicillin G, an inexpensive substrate, to the valuable intermediate for semisynthetic cephalosporin production, deacetoxycephalosporin G (DAOG), had been recently shown to be increased by eliminating agitation and adding decane. The present work examining other solvents shows that all alkanes tested are equivalent to decane in activity but that other solvents are either inhibitory or less active than alkanes. Optimum conditions of pH and temperature for the alkane system are not very different from the previously used aqueous system.

The current method of producing cephalosporin intermediates from penicillins for synthesis of semisynthetic cephalosporins is a chemical process[1] which involves many steps, is expensive and generates polluting materials. We are attempting to develop an environmentally-friendly biological process for expanding the thiazolidine ring of penicillin G into the dihydrothiazine ring of deacetoxycephalosporin G (DAOG)[2~5]. DAOG can be easily converted enzymatically into 7-aminodeacetoxycephalosporanic acid (7-ADCA), an important feed stock for production of many semisynthetic cephalosporins. Semisynthetic cephalosporins are the leading group of antibiotics with a total market of 11 billion dollars per year[6].

Unfortunately, the degree of conversion of penicillin G into DAOG is very low due to the inactivation of the deacetoxycephalosporin C synthetase (DAOCS; expandase) during the bioconversion by the cofactors of the reaction, *i.e.*, Fe^{2+} plus α-ketoglutarate or plus ascorbate[7]. We have been exploring means of retarding the inactivation and have recently found that elimination of agitation and addition of decane stimulates the degree of conversion[8].

Our rationale for testing decane was the knowledge that water-immiscible solvents are usually less toxic to cells than water-miscible solvents, often increase enzyme activity and stability and prevent the hydrolysis of the substrate or product[9~11]. Since they are also known to decrease toxicity of the substrate and/or product, we thought that they might also decrease the inactivation of the expandase by Fe^{2+} plus ascorbate or Fe^{2+} plus α-ketoglutarate. We favored the alkanes because they are relatively biocompatable, having log P (octanol-water partition coefficient) values over 3.0. Log P values for some alkanes are as follows: cyclohexane 3.2, hexane 3.5, heptane 4.0, octane 4.5, hexadecane 8.7. Such high log P values are favorable for activity and stability of cells and enzymes[12]. When compared to other solvents for enzyme catalyzed reactions, alkanes are often best[13~15]. Decane was tested first because it had been reported to have minimum deleterious effects on microbial viability[16]. The present work examines other solvents including a number of additional alkanes.

Materials and Methods

Microorganism, Medium and Culture Conditions

The microorganism used was *S. clavuligerus* NP1, a mutant strain that produces only a trace of cephamycins[17]. It is useful since it does not carry over detectable cephalosporins into the bioconversion reaction. Inocula were obtained by growth in 250 ml baffled Erlenmeyer flasks containing 40 ml of MST medium[2] for 48 hours at 30°C and 220 rpm. Four ml of this seed were transferred

* Corresponding author: ademain@drew.edu
† Present address: Department of Chemical Engineering, University of Colorado, Boulder, CO 80309-0424

Fig. 1. DAOG production in the presence of 32% (v/v) concentrations of decane (●), hexane (○), benzene (▲), butanol (■) and DMSO (■).

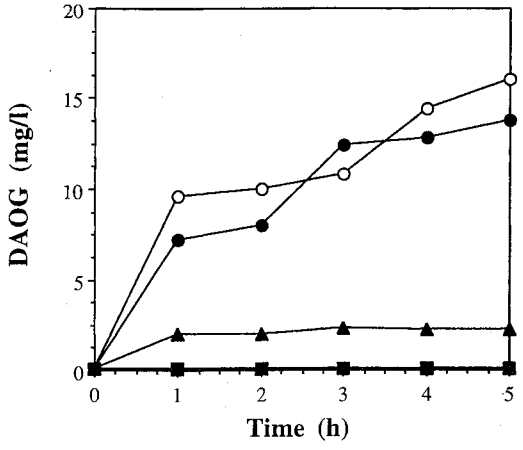
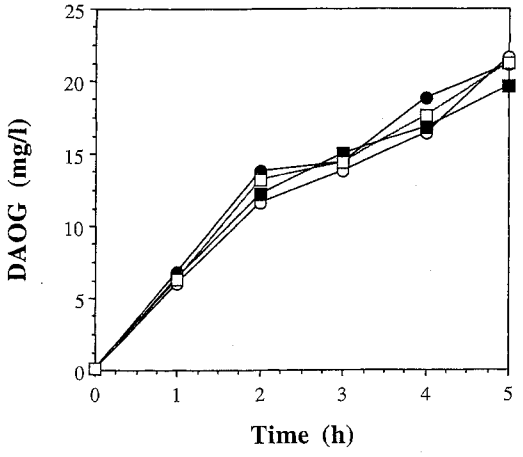


Fig. 2. Stimulation of DAOG production by 32% concentrations of decane (□), octane (●), dodecane (○) and hexadecane (■).



into each of a number of 500 ml baffled flasks containing 80 ml of MT2E medium[3] containing 3% Trypticase Soy Broth without Dextrose (BBL Cockeysville, MD), 2% ethanol and 90 mM MOPS buffer. The ethanol was added just before inoculation. The pH of the rest of the medium was adjusted to 7.0 before autoclaving. Cells were grown at 30°C at 250 rpm for 24 hours. Mycelia were pooled, washed twice and finally resuspended into 10 ml of distilled water per original flask. Four ml of the cell suspension were used in each ring-expanding biocoversion. The rest of the suspension was stored at −20°C up to 4 weeks for later use in the biocoversion reaction.

Ring-expanding Bioconversion

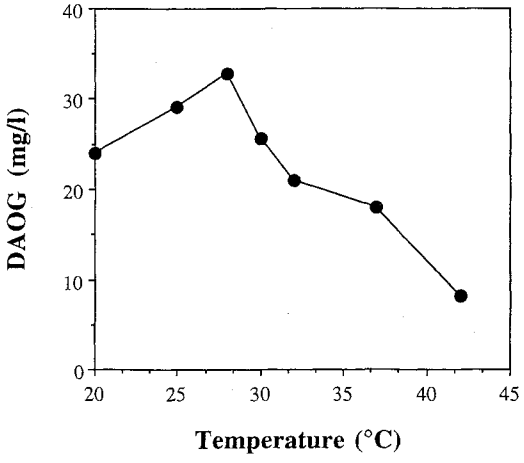
The control biotransformation mixture contained 1.8 mM $FeSO_4$, 1.28 mM α-ketoglutarate, 4 mM ascorbate, 8 mM $MgSO_4 \cdot 7H_2O$, 8 mM KCl, 5.6 mM penicillin G (2 mg/ml), 3.2 or 5 ml decane and 50 mM MOPS (pH 6.5) in a final volume made up to 10 ml with distilled water in 250 ml baffled Erlenmeyer flasks. The pH was adjusted to 6.5 with KOH. The order of addition of components was as established earlier[18]. Incubation was static[8] at 30°C. Samples were collected at zero time and at hourly intervals up to 5 hours. Reactions were stopped by mixing 0.5 ml of the reaction mixture with 0.5 ml methanol, and centrifuging to remove cells. Product (DAOG) concentration was determined on the supernatants with the paper disc-agar diffusion bioassay using *Escherichia coli* strain Ess (β-lactam supersensitive mutant) with 50,000 IU of penicillinase (Difco Bactopenase concentrate) per ml of LB agar[2]. DAOG was used as standard. The DAOG was made up in 32% or 50% decane, depending on the concentration in the reaction mixture. The addition of decane to standard DAOG was done since the zone diameter produced by DAOG was slightly enhanced by decane.

Results

The positive effects of eliminating agitation during the reaction and adding 32% decane were found in our previous study[8]. A series of other solvents were compared to decane at the 32% concentration. It was found that the reaction was totally inhibited by *n*-butanol, DMSO, dioxane, isopropyl alcohol and cyclohexanol, and almost completely by benzene. Some of these data are shown in Fig. 1. Solvents that were as effective as decane were hexane (Fig. 1), heptane, octane, dodecane and hexadecane (Fig. 2). Solvents that were active but less active than the above alkanes were *p*-xylene, butylacetate, and octanol-1 (data not shown). The conversion was only slightly improved by increasing the alkane concentration up to 53% concentration (data not shown).

We decided to recheck other conditions of the alkane-stimulated bioconversion. For this work, 50% hexane was used. Variation in pH from 5.5 to 8.5 showed that the reaction was totally inhibited at pH 8.5, seriously inhibited by pH 8.0 and slightly inhibited at pH 7.5 (data not shown).

Fig. 3. Effect of temperature on DAOG production in the presence of 50% hexane.



pH 5.5, 6.0, 6.5 and 7.0 yielded equivalent activity indicating a plateau in bioconversion between pH 5.5 and 7.

Examination of temperature effects was done between 20°C and 42°C (Fig. 3). The reaction was seriously inhibited at 42°C and moderately inhibited at 37°C. Fairly good conversions were observed at 20°C to 30°C with an optimum at 28°C.

Discussion

Applications of enzymes and cells have been broadened by the use of organic solvents replacing part or all of the water in a reaction mixture[19]. Reactions that do not occur in water can be catalyzed, enzymes can be made more stable, they can exhibit "molecular memory", and can experience changes in selectivity including substrate-, stereo-, regio- and chemo-selectivity. Stability enhancement was the reason that we first tested organic solvents and found success with decane[8]. In the present study, the stimulation of the bioconversion of penicillin G to DAOG was observed with all alkanes tested. *i.e.* hexane, heptane, octane, decane, dodecane and hexadecane. No other solvents were as effective, with many having serious inhibitory effects. One possible reason for the alkane effect is the much higher solubility of oxygen in alkanes than in water. The alkane may act as an oxygen sponge, controlling the steady state level of oxygen in the aqueous medium. Thus, there could be a reduction of the oxygen concentration in the aqueous phase. Since oxygen is required for both the reaction and the inactivation of the expandase enmzyme, oxygen limitation might affect the inactivation more seriously than the bioconversion reaction. This would act in a manner similar to the beneficial effect of eliminating agitation of the reaction tubes[8].

The optimum conditions in the alkane system was found to be a pH range of 5.5 to 7.0 and a temperature of 28°C. Previously, we had used pH 6.5[4] and a temperature of 30°C[2]. Thus the alkane system, despite its greater activity, does not have very different pH and temperature requirements that the completely aqueous system.

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