# Penicillin Acylase-Catalyzed Solid-State Ampicillin Synthesis

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Dedicated to Professor Roger Sheldon on the occasion of his 60th birthday to honour his outstanding contributions to organic synthesis and catalysis.

**Abstract:** The ability of immobilized penicillin acylase from *E. coli* to retain a remarkable catalytic activity in solid-state systems has been demonstrated. Stabilization of immobilized penicillin acylase by inorganic salt hydrates allowed us to exploit nearly the whole catalytic activity of the enzyme at a very low water content. Using this technique, enzymatic synthesis of ampicillin in solid-state systems was performed with high yields (up to 70% starting from equimolar mixture of reagents) and rates comparable to the corresponding values in homogeneous solutions and heterogeneous systems, "aqueous solution-precipitate". Peculiarities of the enzymatic solid-state acyl transfer process, such as absence of the clear-cut maximum on the ampicillin accumulation curves and dependence of the synthetic efficiency on the enzyme loading, have been observed. The space-time yield of solid-state enzymatic ampicillin synthesis was shown to be up to ten times higher compared to the homogeneous solutions and heterogeneous "aqueous solution-precipitate" systems.

**Keywords:** antibiotics; enzymes; salt hydrates; solid-state systems, stabilization

## Introduction

Enzymatic conversions in heterogeneous aqueous systems have nowadays become a field for extensive investigations. Exploitation of such systems, especially for synthetic purposes, could have remarkable advantages: high space-time yield due to operation with high concentrations of reactants during the whole reaction process, possibility to shift thermodynamic equilibria, the absence of hazardous organic solvents, ecological advantages, etc. Special cases among these systems are the reactions in low-water media, or so-called solid-state enzymatic reactions. At the moment, the ability of several enzymes, such as α-chymotrypsin,[1,2] proteinase K, subtilisin, papain,[3] thermolysin,[4] and penicillin acylase (PA)[5] to catalyze synthetic reactions in nearly solid-state conditions at a very low water content has been demonstrated. Moreover, biocatalytic conversions in such high-condensed medium in some cases showed better reaction rates and equilibrium yields compared to conventional enzymatic processes.[3,6,7,8] It was shown also that solid-state enzymatic reactions possess a number of specific features, such as dependence of the reaction rate and synthetic efficiency on the physico-mechanical properties of the reactants and their composition,[9] the presence of the inorganic salts and buffer components,[10] decreased enzyme stability at the low water content,[4,5] all of which have to be optimized for the development of a practical biocatalytic process.

In this work, we report our results on the penicillin acylase-catalyzed solid-state ampicillin synthesis at low water content without adding any organic solvents. It is to be noticed that, despite the number of publications on the enzymatic synthesis in solid-state systems reviewed above, most of them are dealing with thermodynamically controlled condensations. The targeted synthesis of ampicillin represents an example of kinetically controlled enzymatic acyl transfer from the activated acyl donor to a nucleophile [11,12] in a solid-state system. We believe that our observations concerning the stabilization of immobilized enzyme preparation and regularities of the enzymatic acyl transfer at low water content can encourage further studies and practical applications of the penicillin acylase-catalyzed solid-state processes.

## Results and Discussion

### Stabilization of the Immobilized Penicillin Acylase by Inorganic Salt Hydrates

At first, the possibility to use immobilized penicillin acylase in systems with low water content was studied. For that we investigated the stability of enzyme preparations under the conditions of solid state synthesis. Figure 1 shows, that under these experimental conditions, immobilized penicillin acylase was substan-

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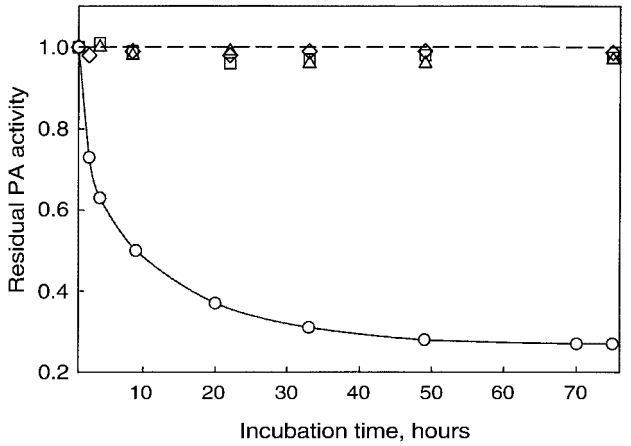


**Figure 1.** Stability of immobilized penicillin acylase: (○) = immobilized enzyme (the moist preparation), (Δ) = in a mixture with $CH_3COONa \cdot 3\,H_2O$, (◇) = in a mixture with $Na_2SO_4 \cdot 10\,H_2O$, (□) = in a mixture with $MgSO_4 \cdot 7\,H_2O$. In all cases, 30% w/w of salt hydrate was used, $a_w = 0.8$. Lines just indicate trends.

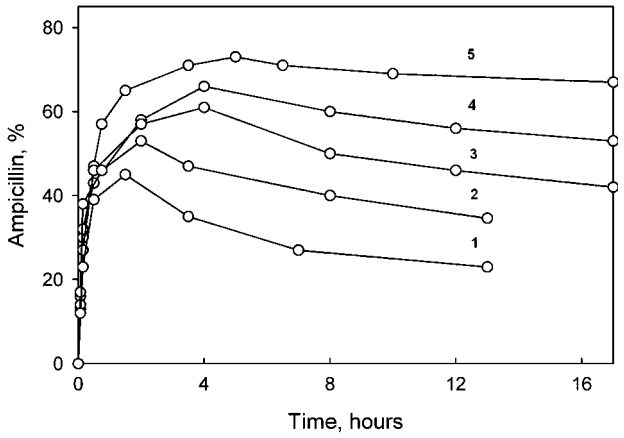


**Figure 2.** Ampicillin accumulation curves in the penicillin acylase-catalyzed solid-state synthesis. Reaction conditions: (1) = 35, (2) = 30, (3) = 20, (4) = 15, and (5) = 10% w/w of initial water content, 1.4 mmol of 6-APA, 1.4 mmol of D-PGM, 0.5 mmol of $Na_2SO_4 \cdot 10\,H_2O$, 6 nmol of immobilized PA, $a_w = 0.8$.

tially inactivated (after 50 h of incubation it retained only 27% of the initial catalytic activity). In our previous studies [5] we have found, that even at 3–5% water content the immobilized penicillin acylase can retain up to 45% of the initial activity and a remarkable inactivation was observed only when the water content dropped below a certain "critical" level (approximately 20–25% w/w), which seems to be required to maintain the catalytically active conformation of the enzyme molecules (so called "minimal hydrated shell"). Therefore, we studied the possibility to stabilize penicillin acylase by adding salt hydrates to the reaction system. It is known[1,10] that presence of salts and salt hydrates can remarkably decrease the minimal amount of water molecules needed to maintain the active conformation of the enzyme. As shown in Figure 1, incubation of the moist preparations of immobilized PA in a mixture with different salt hydrates allowed us to maintain nearly the whole catalytic activity of the enzyme during at least 80 hours (under the same conditions, but without salt hydrates, PA retained only 25% of its activity).

## Penicillin Acylase-Catalyzed Solid State Ampicillin Synthesis

As was mentioned in introduction, a number of factors, such as physicomechanical properties, initial ratio of the reactants, quantity and distribution of water in the reaction medium, presence of buffers, salts and salt hydrates, and even the way of mixing, can play a crucial role in the whole reaction process. Therefore, we have tested a number of reaction systems in order to develop an effective and reliable procedure for the solid-state enzymatic ampicillin synthesis (under a "solid-state" system we mean one in which physicochemical properties cannot be described in terms of liquids or suspensions, i.e., pastes or moist powders). It was noticed that both productivity and rate of solid-state synthesis depend on how the reaction mixture was originated. We have observed that the best reproducible results can be achieved when the solid-state reaction systems were made as follows: the equimolar mixture of reagents (6-APA and D-PGM) was adjusted to the required pH value and dried at room temperature; then, this pretreated mixture was carefully crushed and the obtained fine powder was mixed with the preparation of immobilized PA and a salt hydrate, containing the necessary amount of water. Starting reaction mixtures in most cases represented wet powders instead of sticky or rubbery pastes, which can be observed when the water is added directly to the dry mixture of reactants (see also[9]). Using this technique, the ampicillin synthesis, catalyzed by immobilized PA, was performed in solid-state systems containing various amounts of water. It was observed that solid-state enzymatic synthesis of ampicillin can effectively proceed in systems containing 5–30% w/w of water, with high yields (up to 70% in an equimolar mixture of 6-APA and acyl donor) and at a reaction rate comparable to those obtained in saturated homogeneous solutions or heterogeneous systems "aqueous solutions-precipitate" (see Figure 2 and Table 1). The initial ampicillin accumulation rates were only slightly dependent on the water content (Figure 3). These observations support the suggestion[13] that, in solid-state systems, enzymatic reactions proceed through the saturated or supersaturated solution of reactants, dissolved in a microscopic aqueous phase.

**Table 1.** The productivity of PA-catalyzed ampicillin synthesis in homogeneous solutions, heterogeneous "aqueous solution-precipitate", and solid-state systems.

| Entry | Initial system state | Initial amounts of reactants[a], 6-APA:D-PGM, [mmol] | Amount of PA active sites [nmol] | Amount of PA active sites per 1 g of initial reactants mixture [nmol] | Initial synthesis rate, [μmol/min] | STY[d] [mol/(L × h)] |
|---|---|---|---|---|---|---|
| 1[b] | Homogeneous, saturated with 6-APA | 3:5 | 30 | 20 | 38 | 0.1 |
| 2[c] | Heterogeneous with 6-APA precipitate | 6:9 | 28 | 10 | 64 | 0.23 |
| 3 | Solid-state (water 10% w/w) | 1.4:1.4 | 6 | 11 | 42 | 1.7 |

[a] For the entries 1 and 2 reaction volume was 10 mL.
[b] Ref.[14].
[c] Ref.[16].
[d] Space-time yield.

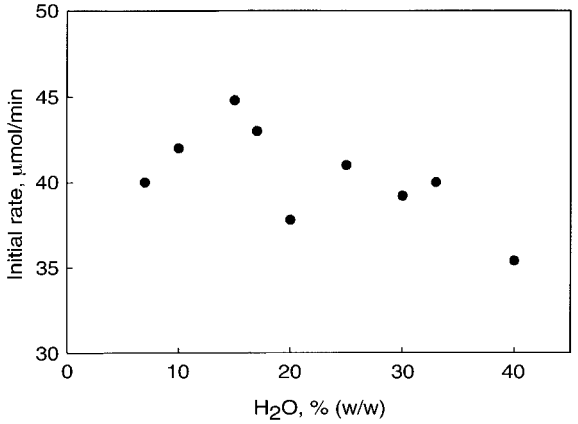


**Figure 3.** Dependence of the initial synthesis rates of PA-catalyzed ampicillin synthesis on the initial water content. Reaction conditions: 1.4 mmol of 6-APA, 1.4 mmol of D-PGM, 0.5 mmol of $Na_2SO_4 \cdot 10\ H_2O$, 6 nmol of immobilized PA, $a_w = 0.8$.

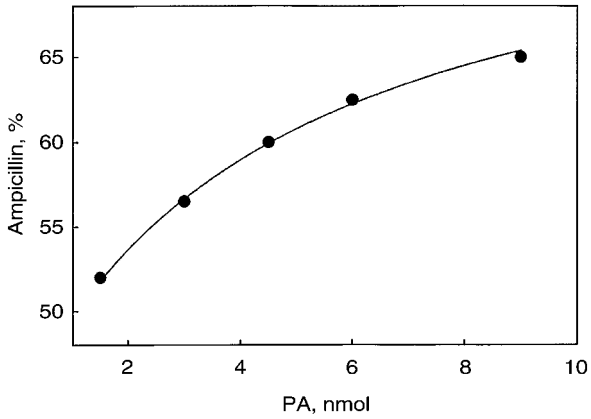


**Figure 4.** Dependence of the maximum ampicillin yield on the enzyme loading. Reaction conditions: initial water content = 15% w/w, 1.4 mmol of 6-APA, 1.4 mmol of D-PGM, 0.5 mmol of $Na_2SO_4 \cdot 10\ H_2O$, $a_w = 0.8$. Solid line just indicates trends.

## Peculiarities of the Enzymatic Solid State Synthesis

Nevertheless, enzymatic synthesis in the solid-state demonstrated a number of specific features, discriminating this class of reactions from homogeneous and heterogeneous enzymatic conversions. Particularly, one can see (Figure 2 curves 4,5), that at very low water content ampicillin accumulation curves do not exhibit a clear-cut maximum, inherent to the enzymatic acyl transfer reactions in aqueous medium (including quite concentrated heterogeneous "aqueous solution-precipitate" systems[14]) due to the secondary hydrolysis of the target product by penicillin acylase. Then, the maximum ampicillin yield was found to be dependent on the enzyme loading (Figure 4). As we have shown above, immobilized penicillin acylase, stabilized by salt hydrates, retains nearly its total catalytic activity under the conditions of solid-state enzymatic synthesis. Hence, these effects cannot be explained by enzyme inactivation in the course of the reaction and most probably should be ascribed to the mass transfer limitations, which dictate the peculiarities of the enzymatic synthesis in the low-water systems. Our observations on the solid-state ampicillin synthesis in differently originated solid-state systems (see discussion above) also demonstrate the important role of the distribution of the free water and enzyme in the reaction mixture. In general, the best results were obtained in the reaction mixtures with the most isotropic physicomechanical properties (i.e., when all reactants were distributed homogeneously in the reaction volume).

Probably the major advantage of the solid-state systems is the principal possibility to perform enzymatic conversion in a very limited reaction volume. In Table 1 we compared penicillin acylase-catalyzed ampicillin synthesis in three different systems: saturated homogeneous solutions, heterogeneous systems "aqueous solution-precipitate", and solid-state systems. One can see that, at the same enzyme loading, solid-state systems provide an efficiency (in terms of space-time yield) almost twenty times higher compared to saturated homogeneous solutions and more than seven times higher compared to heterogeneous biocatalytic systems "aqueous solution-precipitate".

## Conclusions

The possibility to perform effective penicillin acylase-catalyzed acyl transfer to the nucleophile in the systems at very low water content was demonstrated for the first time. Both reaction rate and maximum ampicillin yield were shown to be dependent on the water content, enzyme loading, and the physicomechanical properties of the reaction mixtures. The suggested method for the stabilization of immobilized enzyme preparations by inorganic salt hydrates allowed us to achieve a high effectivity of ampicillin synthesis from the equimolar substrate mixture and this makes the solid-state biocatalytic processes attractive for the further development.

## Experimental Section

### Reagents

The Eupergit C-immobilized penicillin acylase was from Bristol-Myers-Squibb (USA). The activity of the moist preparation was 30 U/mg. Concentration of the active sites in immobilized penicillin acylase, determined by titration with phenylmethylsulfonyl fluoride (PMSF),[15] was 30 nmol per 1 g of moist enzyme preparation. Benzylpenicillin and 6-aminopenicillanic acid (6-APA) were from Fluka (Switzerland), ampicillin, D-(−)-phenylglycine (D-PG), and D-(−)-phenylglycine methyl ester (D-PGM) were from DSM (The Netherlands). Other chemicals and buffer components were from Merck, Darmstadt, Germany. Organic solvents were of analytical grade.

### Analysis

Samples were analyzed by HPLC using an Altex 110A pump, a $4.6 \times 150$ mm Nucleosil C-18 column, an LKB 2138 S UV detector at 214 nm and an LKB 2221 integrator. The eluent was prepared by adjusting the pH of a 0.68 g $L^{-1}$ solution of $KH_2PO_4$ in acetonitrile/water (30:70 v/v) containing 0.68 g $L^{-1}$ sodium dodecyl sulfate to 3.0 with phosphoric acid. The flow rate was 1.0 mL $min^{-1}$.

### Studies on Stability of Immobilized Penicillin Acylase at the Low Water Content

To study the properties of immobilized penicillin acylase under the conditions of solid-phase reactions, 100–200 mg of the moist preparation, placed onto a slide, were kept in a closed vessel with fixed water activity at room temperature and the residual catalytic activity was determined in the samples taken at intervals. The pressure of water vapor was maintained at a fixed level using sulfuric acid solutions of various concentrations. The thermodynamic water activity ($a_w$) was determined as P/Ps, where P is the pressure of water vapor over the acid solution and Ps is the pressure of saturated water vapor at a given temperature. The residual activity of penicillin acylase preparations was determined from the initial rate of the enzymatic hydrolysis of benzylpenicillin as described earlier.[5] The content of water in the enzyme preparations was determined gravimetrically.

### Stabilization of Penicillin Acylase Preparations by Salt Hydrates

To study an effect of salt hydrates on the stability of PA preparations, a weighed amount of the immobilized enzyme was kept in a closed vessel with fixed water activity at room temperature in the mixture with $Na_2SO_4 \cdot 10\ H_2O$, $MgSO_4 \cdot 7\ H_2O$ or $NaCH_3COO \cdot 3\ H_2O$ (up to 30% w/w). The residual activity of penicillin acylase preparations in the course of this incubation was determined as described above.

### Ampicillin Synthesis in the Solid-State Systems

Preparation of the reaction mixture: the equimolar solution of reagents (0.1 M of 6-APA and 0.1 M of D-PGM) in 0.01 M phosphate buffer was adjusted to pH 6.5 using a 1 M KOH and dried in a closed desiccator ($a_w = 0.4$) at room temperature. Then the mixture of reagents, containing less than 3% w/w of water, was crushed and the obtained fine powder was used to mix with enzyme preparation. Enzymatic synthesis was started by mixing a 20–200 μg of immobilized PA (as a wet powder alone or mixed with corresponding salt hydrate), with the dry powder of the reagents. The only source of water in the reaction mixture was water added with the moist enzyme preparation. All reactions were performed in the desiccator at a fixed water activity and room temperature. The content of water in the reaction system was determined gravimetrically. Samples of the reaction mixture were prepared by diluting an aliquot of the mixture in eluent to stop the enzymatic reaction, and analyzed by HPLC.

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