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One-pot conversion of cephalosporin C by using an optimized two-enzyme process

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Abstract

The main industrial process for producing 7-amino cephalosporanic acid (7-ACA), a precursor of semi-synthetic cephalosporin antibiotics, is the two-step enzymatic route based on D-amino acid oxidase and glutaryl acylase working in separate reactors. Taking advantage of the recently developed variants of cephalosporin C acylase from *Pseudomonas* N176 (named VAC) and the optimized recombinant overproduction of the two enzymes, we set up a one-pot system to directly convert cephalosporin C into 7-ACA. We optimized the process by identifying the most favorable operational conditions, substrate, and enzyme concentrations. Among the VAC variants employed, our results indicated that HS-HS-F72βR VAC is the best choice because of the high activity on both substrates (glutaryl-7-ACA and cephalosporin C) and the absence of substrate and product inhibition effects. Under optimized conditions and by adding further aliquots of the biocatalysts, > 98% of cephalosporin C was converted, yielding 7-ACA as the main reaction (oxo-7-ACA was below the detection level and glutaryl-7-ACA was < 1 mM). At the 20-mL bioconversion scale, approx. 81 mg of 7-ACA are produced in 41 hours from 15 mM cephalosporin C. The low cost of the one-pot enzymatic production of 7-ACA is a main advantage of the proposed method, that is further strengthened by the good purity of the final product.

Introduction

β-Lactam antibiotics are the most widely employed antibiotics in clinical practice, having already served mankind for over 70 years. The β-lactam antibiotics represent ~65% of the world's antibiotic market, reaching over $20 billion dollars per year [1]. More than 50 marketed cephalosporins (≈ 200 tons per year, for a market of $10 billion) are produced: two-thirds of semi-synthetic cephalosporins are synthesized using the starting scaffold 7-amino cephalosporanic acid (7-ACA). Since the "cephem" nucleus possesses two major sites that can be modified (the 7-amino and the C3 acetoxy), new and effective cephalosporins continue to be developed: the third-generation semi-synthetic cephalosporins have remarkable potency against a wide range of bacteria, including *Pseudomonas aeruginosa*, a pathogen that does not respond to several antibiotics [2]. Due to the contaminant reagents present in the conventional chemical route used to convert the mature antibiotic cephalosporin C (CephC) into 7-ACA and the high consumption of energy, biocatalytic approaches to producing semi-synthetic cephalosporins have been studied during the last few decades [3-7]. There are several advantages to producing 7-ACA from cephalosporin C by using an enzymatic process over a chemical one, which are mainly related to the quality of the final product [8]. Nowadays, the unique enzymatic route applied industrially consists in a two-pot process that employs D-amino acid oxidase (DAAO, EC 1.4.3.3) and glutaryl-7-aminocephalosporanic acid acylase (GA, EC 3.5.1.93) [9-11]. The flavoenzyme DAAO oxidizes the D-α-amino adipyl moiety of CephC to give α-ketoadipyl-7-ACA, which is converted to Gl-7-ACA nonenzymatically; this latter compound is susceptible to enzymatic attack by various acylases (see Scheme 1). The original two-step process developed by Boehringer Mannheim used two reactors containing the enzymes immobilized on an organic and inorganic polymer, respectively, and yielded 90% conversion of 75 mM CephC into 7-ACA [10]. The main drawback of this enzymatic process is the complexity of using two separate reactors, which was mandatory due to differences in the operational conditions and stability of the two enzymes.

While this approach has matured through long-term research and practice, manufacturers continue to seek novel technologies that simplify the reaction process and reduce production costs. With this aim, some investigators have focused great efforts on a one-step protocol in which CephC is converted directly into 7-ACA by a true CephC acylase [8, 12-14], as well as on setting up a conversion performed by simultaneous action of DAAO and GA [4, 7]. Previous attempts seeking a single enzyme to directly convert CephC into 7-ACA for industrial application have not been successful yet. Concerning the simultaneous action of DAAO and GA for 7-ACA production in a single reactor, the main problems are the presence of H_2O_2 during the reaction process (produced by the DAAO reaction and required to push decarboxylation of oxo-7-ACA into Gl-7-ACA, see Scheme 1), which can inactivate the enzymes being employed, especially DAAO [7], and the substrate/product inhibition effects observed with GA [8].

With regard to DAAO biocatalysis, the catalytic properties of three available DAAOs relevant for CephC bioconversion, namely, a mammalian DAAO and two others from yeast (*Rhodotorula gracilis*, RgDAAO, and *Trigonopsis variabilis*, TvDAAO) were investigated [15]. The enzymes from microorganisms appear to be far more suitable catalysts for bioconversion. RgDAAO, which shows a broader pH activity and a higher activity at saturating oxygen concentrations, appears to be a suitable catalyst for industrial application [16], also because their recombinant production in *E. coli* is inexpensive [17, 18]. Concerning the acylase activity, we recently produced by protein engineering a number of GAs from *Pseudomonas* N176 (named VAC) that could hydrolyze both CephC and Gl-7-ACA [14, 19-21]. The kinetic properties of these VAC variants differ and they are also produced in *E. coli* at high level (and low cost) [14, 22].

In the present work, we set up a one-pot system to convert CephC directly into 7-ACA by the synergistic action of recombinant RgDAAO and VAC variants.

Experimental

Materials

CephC, 7-ACA, and Gl-7-ACA were kindly supplied by Antibioticos S.p.A (Rodano, Italy). All other chemicals were of analytical grade and were used as received.

Commercial glutaryl acylase (GAR) was a gift from Recordati S.p.A (Opera, MI, Italy); its specific activity on Gl-7-ACA is 3.6 U/mg protein. Recombinant VAC proteins (wild-type, H57βS-H70βS, H57βS-H70βS-F72βR, and H57βS-H70βS-L154βY VACs) were obtained as stated in [14]. From 1 L of fermentation broth, 55, 77, 103, and 35 mg of wild-type, H57βS-H70βS, H57βS-H70βS-F72βR, and H57βS-H70βS-L154βY were produced; the recombinant VAC variants were purified by HiTrap chelating chromatography (GE Healthcare, Little Chalfont, UK) with a specific activity of 0.7, 3.0, 1.0, and 2.8 U/mg protein on CephC as a substrate, respectively. The final enzyme preparations were equilibrated in 20 mM potassium phosphate buffer, pH 8.0. The amount of protein was estimated by absorbance at 280 nm using a molar extinction coefficient of 110 mM^{-1} cm^{-1}.

D-Amino acid oxidase from *Rhodotorula gracilis* (RgDAAO) was produced as stated in [17, 18]. From 1 L of fermentation broth, 45 mg of RgDAAO were produced with a specific activity of 98 U/mg protein on CephC as a substrate.

Catalase from beef liver (CAT) was purchased from Roche (Basel, Switzerland); its specific activity is 65,000 U/mg protein on H_2O_2 as a substrate.

Determination of acylase activity

The standard activity assay was based on the hydrolysis of Gl-7-ACA or CephC to 7-ACA and the subsequent formation of a yellow Schiff's base (with a maximum of absorbance at 415 nm, ε = 0.635 mM^{-1} cm^{-1}) by the reaction of *p*-4-dimethylaminobenzaldehyde (pDMAB) and the primary

amino group of 7-ACA [19, 23]. One unit of acylase is defined as the amount of enzyme that converts 1 μmol of substrate per minute under the assay conditions. In detail: 0.1 mL of enzyme was mixed with 0.1 mL of 1% [w/v] Gl-7-ACA or 2% [w/v] CephC at pH 8.0 and incubated for 10 min at 25 °C. The reaction was stopped by adding 0.6 mL of 20% acetic acid, and then 0.133 mL of 0.5% [w/v] *p*DMAB (dissolved in methanol) was added and the mixture incubated for another 10 min at 25 °C, before measuring the absorbance at 415 nm.

Determination of catalase activity

Catalase (CAT) activity was determined spectrophotometrically by monitoring the decomposition of H_2O_2 and measuring the change in absorbance at 240 nm [24]: 0.97 mL of 0.036% H_2O_2 (w/w) in 50 mM potassium phosphate buffer pH 7.0 were incubated with 0.03 mL of enzyme solution. Measurements were carried out at 25 °C. One catalase unit was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute.

Determination of DAAO activity

DAAO activity was assayed with an oxygen electrode (Clark type) at pH 8.0 with 50 mM CephC as substrate at air oxygen saturation ($[O_2]$ = 0.253 mM). One DAAO unit is defined as the amount of enzyme that converts 1 μmol of substrate per minute at 25 °C [17].

Biocatalyst's stability at 25 °C

The stability of DAAO, GAR, or VAC was determined by incubating the enzymes at 1 mg/mL protein concentration (RgDAAO), 15 mg/mL protein concentration (GAR), and 7-14 mg/mL protein concentration (VAC variants) in 20 mM potassium phosphate buffer, pH 8.0, at 25 °C. At fixed times, the residual activity of the enzymes was measured as described above.

Bioconversion of CephC to 7-ACA

The reaction mixture contained 15-50 mM of CephC dissolved in 100 mM potassium phosphate buffer, pH 8.0, and the enzymes at a 4.5 kU/L (in a final volume of 2 mL). Reaction mixtures were incubated at 25 °C under shaking and aliquots were drawn at different times for HPLC analysis (Jasco, Cremello, Italy). Samples were separated on a C8-Aquapore RP-300 column 7μ (250 x 4.6 mm; Life Technologies) using 25 mM KH_2PO_4, pH 3.0, and 5% acetonitrile as elution buffer [19]. The flow rate of the eluant was 1 mL min^{-1} and analytes were detected at 254 nm.

Results and discussion

Characterization of the catalysts

Kinetic properties. The kinetic parameters for RgDAAO, GAR, and different VAC variants on CephC and Gl-7-ACA as substrate were determined at pH 8.0 and 25 °C [14, 15, 19, 20]: RgDAAO and GAR are only active on CephC and Gl-7-ACA, respectively, while all VAC variants are active on both substrates (see Table 1). In particular, the H57βS-H70βS (HS-HS), HS-HS-F72βR, and HS-HS-L154βY VAC variants show a higher activity than wild-type VAC on CephC (up to 4-fold) [12]. In contrast, the HS-HS-F72βR VAC variant is the most active acylase on Gl-7-ACA. Wild-type and HS-HS-F72βR VAC variants suffered from a substrate inhibition effect at increasing Gl-7-ACA concentrations, showing a K_i of 21 and 6.3 mM, respectively, that might affect bioconversion in a reactor containing the acylase activity only. Furthermore, RgDAAO was also inhibited by Gl-7-ACA (mixed-type, Table 1) [15]. This clearly means that, in the two-step enzymatic CephC bioconversion operated in two separated reactors, the inhibition effect could affect the conversion yield of the first step. However, these inhibitory effects should be mitigated in a one-pot bioconversion where RgDAAO and VAC operate together since Gl-7-ACA should not accumulate to a significant extent.

A further important aspect to be considered in the CephC bioconversion is the inhibition effect due to the final product (7-ACA). As reported in Table 1, wild-type VAC is strongly inhibited by 7-ACA, with a K_i of 3 mM, whereas none of the VAC variants tested showed any product

inhibition effect. Furthermore, RgDAAO is also inhibited by 7-ACA in noncompetitive mode (K_i = 23.6 mM): a high concentration of the final product, which is only obtained when the full process is performed in a single bioreactor and employing a ≥ 25mM CephC concentration, could affect completion of the bioconversion process by inhibiting the flavooxidase [15].

Definition of experimental conditions for the one-pot process. In order to study how protein stability affects the bioconversion time course, we compared the time course of enzyme activity when incubated at 25 °C in 20 mM phosphate buffer, pH 8.0, and in the absence of the substrate (Fig. 1). After 7 h of incubation, no significant decrease in activity was observed in the wild-type VAC, GAR, and RgDAAO , while 81%, 75%, and 60% of the initial activity values were found for the HS-HS-L154βY, HS-HS, and HS-HS-F72βR VAC variants, respectively. A further main operative factor that affects the conversion of CephC into 7-ACA is the pH value. In a previous study [19] we reported that wild-type and HS-HS VAC variants show a similar pH dependence of activity on Gl-7-ACA in the pH range 5.0–9.0: a 40% increase in activity was evident when shifting the pH from 8.0 to 9.0. On the other hand, the activity of the wild-type and HS-HS VAC variant on CephC measured at pH 9.0 is fourfold higher than at pH 8.0 for wild-type as well as for all of the HS-HS VAC variants. GAR showed a broad pH optimum between 7.0 and 9.5 [25] while for RgDAAO an increase in activity starting from pH 6.0, with a plateau in the pH range 8.0-10.0, was apparent [15].

Concerning the compounds present in the bioconversion reaction, the stability of CephC, Gl-7-ACA, and 7-ACA in solution decreases at pH values higher than 8.0 and all these chemicals gradually decompose at temperatures above 30 °C (The Merck Index 1996) [26]. Therefore, as an optimal compromise, all subsequent experiments were conducted at 25 °C and pH 8.0, conditions at which < 8% of CephC degradation was observed after 24 hours of incubation (data not shown).

One-pot conversion of cephalosporin C

With the final aim of identifying the best reaction conditions for a single-pot conversion of CephC, 4.5 kU of VAC wild-type and RgDAAO per liter were employed simultaneously at pH 8.0 and 25 °C, in the presence of 50 mM CephC, as commonly used in industrial bioconversion [10, 19]. The bioconversion time course was followed and the reaction products quantified using HPLC chromatography (the retention times of the different products were: 3.5 min for 7-ACA, 4.5 min for CephC, 6.5 min for oxo-7-ACA, and 12.5 min for Gl-7-ACA; see Fig. 2).

At first, the time course of conversion by each single enzyme (4.5 kU/L, 50 mM substrate, pH 8.0 and 25 °C) was investigated. As reported in Fig. 3A, wild-type VAC yields a significantly higher conversion of Gl-7-ACA than of CephC: in both cases, a plateau is observed at ≥ 5 hours, corresponding to ~ 60% and ~ 18% conversion, respectively. Under the same conditions, 4.5 kU/L of RgDAAO converted ~ 60% of CephC in 6 hours: during the reaction time course a significant amount of the oxo-7-ACA intermediate accumulated, this decreasing the yield in terms of Gl-7-ACA production (Fig. 3B). Doubling the amount of RgDAAO did not result in a full conversion of the 50 mM CephC substrate: the conversion stopped at ~ 80% of substrate deamination (Fig. 3B). Since RgDAAO seemed to preserve a large part of its activity after 6-8 hours of incubation (see above), this result indicates product inhibition of the flavoenzyme.

Subsequently, the two enzymes were used in one-pot and in a soluble form. By adding 4.5 kU/L of both RgDAAO and wild-type VAC, 50 mM CephC was fully converted in ~ 7 hours although only 30 mM 7-ACA was produced (Fig. 4A). HPLC analysis did not show any significant Gl-7-ACA accumulation over time (≤ 2%), which was instead observed after adding H_2O_2 (0.05 mmol) after 6 hours of reaction. This result suggests that an oxo-7-ACA intermediate is produced which can be fully decarboxylated to Gl-7-ACA after exogenously adding hydrogen peroxide (Fig. 4A). The same biotransformation in the presence of RgDAAO was then performed by replacing wild-type VAC with the HS-HS, HS-HS-F72βR, and HS-HS-L154βY VAC variants or with commercial glutaryl acylase GAR. Similarly to wild-type VAC, CephC was largely converted in 7 hours by the VAC variants in all cases (residual concentration was ≤ 30%) and 7-ACA

concentration reached 25-50% of the initial substrate concentration (the highest production was observed for the HS-HS-F72βR variant, whose time course closely resembled the conversion observed with wild-type VAC; compare Fig. 4A and 4C). Furthermore, the lowest CephC transformation was apparent for GAR (Fig. 4E). The time courses reported in Fig. 4A-E for the various VAC variants differ significantly in terms of accumulation of oxo-7-ACA and Gl-7-ACA intermediates: this indicates that, in all cases, the first step catalyzed by RgDAAO was faster than the second one due to the acylase activity, also because part of the CephC is simultaneously converted to 7-ACA by VAC variants.

Substrate concentration. A main cost associated with the production of 7-ACA has to do with purification from other components of the reaction mixture. In order to increase the CephC transformation yield and enrich 7-ACA in the final product, the substrate concentration was reduced, seeking in this way to avoid the inhibition effects that could have been present at 50 mM CephC; see above. By decreasing CephC concentration at 25 and 15 mM, an increase in the percentage of 7-ACA production is apparent for wild-type VAC, reaching 10 mM of the final product (67% yield) when the lowest substrate concentration was used (Fig. 3C). Analogously, RgDAAO fully deaminated CephC, yielding a ≥ 90% of conversion in terms of Gl-7-ACA production at 15 mM substrate concentration in 5 hours (see Fig. 3D).

The effect of decreasing the substrate concentration in the one-pot-two-enzyme conversion process was then investigated. By using wild-type VAC and RgDAAO simultaneously, the 7-ACA production increased up to 85% of the substrate concentration (Fig. 4F): at both 15 and 25 mM CephC the amount of reaction intermediates was ≤ 2 mM. For all the tested enzyme mixtures, the highest conversion yield (expressed as percentage of 7-ACA produced with respect to the starting CephC concentration) was apparent when lowering the substrate concentration (see Fig. 4F-L). The best results were observed for wild-type and HS-HS-F72βR VAC. Interestingly, doubling the amount of RgDAAO and wild-type VAC (9 kU/L) at 25 mM CephC did not increase the amount of 7-ACA produced, the concentration reaching a plateau at ~ 20 mM after 3 hours of reaction (data

not shown). This result further supports our conclusion that the main factor limiting full conversion of CephC is product inhibition while the enzyme's stability in the reaction mixture is not a main concern.

As reported in Table 2, bioconversions starting from 25 mM of CephC reached a higher percentage of 7-ACA production than those obtained starting from 50 mM of CephC (85% vs. 55%) due to a lower production of by-products and a faster CephC conversion.

Influence of H_2O_2. As shown in Scheme 1, the first step of CephC bioconversion involves two reactions: the enzyme-catalyzed oxidative deamination of CephC to oxo-7-ACA and its spontaneous conversion to Gl-7-ACA by H_2O_2 released in the first reaction. In order to avoid an accumulation of oxo-7-ACA, H_2O_2 solution was exogenously added to the reaction mixture. Here, the rate of adding H_2O_2 must be strictly controlled because an excess can promote the decomposition of the cephem nucleus and also inactivate the employed enzymes [26]. Accordingly, H_2O_2 solution was added at a rate of 0.01 mmol h^{-1} in the conversion of 50 mM CephC: a strong decrease in oxo-7-ACA was observed (compare Fig. 5A and B). In fact, at the end of the reaction performed in the presence of H_2O_2, a 0.5% of oxo-7-ACA was observed (vs. 21.8% in the absence of H_2O_2, Table 2). In this way, the reaction was pushed to yield product formation, which resulted in a higher 7-ACA production (65.8% vs. 54.9%). Nevertheless, a higher accumulation of Gl-7-ACA (reaching 21% of the reaction products) was observed. Such an effect was also apparent using RgDAAO and HS-HS-F72βR VAC (data not shown), suggesting that it was mainly due to VAC inhibition from the added H_2O_2. Interestingly, in both cases CephC conversion was faster, indicating that a lower accumulation of oxo-7-ACA resulted in a lower inhibition of RgDAAO.

Indeed, the amount of H_2O_2 produced by RgDAAO reaction is clearly not sufficient to fully decarboxylate the oxo-7-ACA intermediate but it could affect the conversion yield because of enzyme inactivation. RgDAAO, wild-type VAC, and catalase (CAT) were thus employed simultaneously in the conversion of 50 mM CephC to 7-ACA in one reactor (RgDAAO:VAC:CAT ratio was 1:1:10 in terms of enzyme units). As shown in Fig. 5C and Table 2, a 22% oxo-7-ACA

accumulation was observed, and only 3.1% of Gl-7-ACA was present in solution. This result clearly shows that in the one-pot reaction the amount of H_2O_2 is a crucial factor, affecting both the nonenzymatic conversion of oxo-7-ACA into Gl-7-ACA and the next enzymatic conversion to 7-ACA by H_2O_2 inhibition of acylase.

Biocatalyst selection. On the basis of the kinetic, physical, and chemical properties shown before, we aimed to identify the best combination of enzymes and substrate concentration to convert CephC into 7-ACA in one-pot using a rotator mixer and the enzymes in the free form. The highest 7-ACA production, starting from 25 mM of CephC as substrate, was observed for the one-pot reaction carried out with the HS-HS-F72βR VAC variant as biocatalyst (88% of 7-ACA was achieved) due to a faster conversion of CephC (i.e., after 2 h of incubation, 9% of CephC was unreacted as compared to 19%, 37%, and 45% observed for the wild-type, HS-HS, and HS-HS-L154βY VAC variants, respectively; see Table 2). The accumulation of Gl-7-ACA was similar to that observed using wild-type VAC (~ 3%) and up to 7.5-fold lower than for the HS-HS and HS-HS-L154βY VAC variants. Furthermore, with the HS-HS-F72βR VAC variant there was a lower oxo-7-ACA accumulation after 7 hours of reaction (5.6-fold lower than wild-type VAC). Owing to the low 7-ACA production (see Table 2) the commercial GAR was discarded. Similarly, a low 7-ACA yield (~ 45%) was also obtained in the one-step bioconversion performed with RgDAAO and the HS-HS or HS-HS-L154βY VAC variants. This result is mainly due to a lower specific activity on Gl-7-ACA in comparison to HS-HS-F72βR VAC variant (up to 10-fold), although a 2-fold higher activity was observed on CephC (see Table 1).

Based on this evidence and the results reported in Table 2 and Fig. 4, the conversion of 15 mM CephC (the substrate concentration yielding the highest conversion into 7-ACA) was performed at a higher scale (20 mL of reaction) using 0.45 kU/L of VAC and RgDAAO and adding additional identical amounts of the two biocatalysts after 15 and 24 h of reaction when the reaction slowed down probably because of a partial inactivation of VAC (which is more apparent for the HS-HS-F72βR VAC variant, see Fig. 1). As depicted in Fig. 6A, after 41 hours of reaction using

RgDAAO and HS-HS-F72βR VAC ≥ 98% of CephC was converted, yielding 7-ACA as the main product: oxo-7-ACA was below the detection limit and Gl-7-ACA was < 1 mM, this indicating that exogenous hydrogen peroxide was not required. A similar time course was also apparent with wild-type VAC, although a residual amount of CephC was apparent (Fig. 6B).

Conclusions

It is of utmost importance that the 7-ACA enzymatically produced from CephC is of good quality, the starting molecule for synthesis of novel cephalosporin derivatives under mild and "green" conditions. A number of different approaches have been proposed based on the use of various DAAO and glutaryl acylases (both natural and engineered enzymes), for a review, see [10, 11, 27]. In this work we investigated the use of the recombinant flavoenzyme from *R. gracilis*, the most useful DAAO for biocatalysis [16], and a number of engineered acylase variants active on both CephC and Gl-7-ACA [14, 19, 20] in a one-pot process. The final aim was to simplify the reaction process by employing a single reactor and to improve the 7-ACA yield and purity. RgDAAO is sensitive to Gl-7-ACA inhibition (K_i = 11.8 mM, Table 1): the simultaneous presence of RgDAAO and VAC enzymes was thought to decrease the product inhibition of the first enzymatic step because Gl-7-ACA accumulation, is prevented by acylase conversion, thus acting as "scavenger" of the DAAO inhibitor. Indeed, the use of VAC as a glutaryl acylase which is also active on CephC (Table 1), facilitates CephC conversion.

The choice of optimum values for different process parameters – temperature, pH, substrate concentration, and best VAC variant employed – was considered with the view of obtaining a maximum yield of 7-ACA and a minimum presence of CephC and by-products: the conversion rate at the established optimum parameters (pH 8.0 and 25 °C) depends mainly on the ratio between the enzymatic activity and substrate concentration used: 4.5 kU/L on 15 mM of CephC allowed the highest conversion of CephC. Concerning H_2O_2, it seems to play a double, opposite effect: the amount produced by RgDAAO reaction is not sufficient to push the full decarboxylation of oxo-7-

ACA (Fig. 5). On the other hand, when it was exogenously added, the amount of the oxo-7-ACA intermediate dropped (0.5% vs. 21.8% in the absence of H_2O_2, Fig. 5A and B) but it was paralleled by an accumulation of Gl-7-ACA because of VAC inactivation. The addition of catalase, as an H_2O_2 scavenger, resulted in a large accumulation of the oxo-7-ACA intermediate which did not disappear at longer reaction time since it is not a substrate of acylase (Fig. 5). Accordingly, in the optimized process we avoided adding H_2O_2.

Among the VAC variants employed, our results indicate that the HS-HS-F72βR VAC is the best choice for the one-pot CephC bioconversion (see Fig. 6A). This result correlated with the higher enzymatic activity on both substrates (Gl-7-ACA and CephC) of this VAC variant in comparison to the wild-type VAC (see Table 1).

For a number of the CephC bioconversion time courses (see Fig. 3 and 4), it was apparent that a large part of the yield was produced during the initial stage of the conversion process (> 70% of 7-ACA was obtained within 2 hours), while the remnant conversion required longer times. This observation points to a product inhibition effect since: i) no enzyme inactivation was observed during the time course of reaction; and ii) the highest 7-ACA production was apparent at lower substrate concentration. Under optimized conditions (i.e., 15 mM CephC, 0.45 kU/L of RgDAAO and HS-HS-F72βR VAC and further addition of the biocatalysts at 15 and 24 hours of conversion), > 98% of CephC was converted, yielding 7-ACA as the main reaction product after 41 hours of reaction. Under these conditions, oxo-7-ACA was below the HPLC detection level and Gl-7-ACA was < 1 mM (Fig. 6). Our results compare positively with those previously obtained using coimmobilized DAAO and GA (85% conversion of 12 mM CephC and < 34% of 7-ACA production) [4] or when catalase was also coimmobilized (full conversion of 40 mM CephC and 80% production of 7-ACA) [7] as well as when the two proteins were fused in a single polypeptide chain (100% conversion of 12 mM CephC and ≤ 80% of 7-ACA production) [27] or when permeabilized *P. pastoris* cells expressing DAAO were mixed with immobilized GA (full conversion of 25 mM

CephC and 76% or 90.9% 7-ACA production depending on whether cells that coexpressed catalase were used or not) [26, 28].

VAC HS-HS-F72βR is produced as a recombinant enzyme in *E. coli* with an overall yield of 103 mg of pure protein per liter of fermentation broth and a specific activity of 1.6 and 52.9 U/mg protein on CephC and Gl-7-ACA as substrate, respectively [14]. Similarly, wild-type RgDAAO is produced in *E. coli* at a level of 45 mg of pure enzyme per liter with a specific activity on CephC of 98 U/mg protein [18]. At the 20 mL bioconversion scale, as reported in Fig. 6, approx 81 mg of 7-ACA are produced in 41 hours from 15 mM CephC. Owing to the low cost of enzyme production and the use of the enzymes in a single reactor, the cost associated with the one-pot enzymatic production of 7-ACA is low: this is a main advantage that is further strengthened by the good purity of the final product. The immobilization of the two biocatalysts should offer opportunity to improve the stability and reusability, further strengthening the use of the one-pot procedure.

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Scheme 1. Two-step enzymatic route (right) and one-pot bioconversion (left) of the natural antibiotic CephC into 7-ACA by DAAO and acylase. The one-pot process was performed using the recombinant RgDAAO and a number of variants of VAC.

Figure 1. Time course of stability of the employed biocatalysts when incubated at 25 °C in 20 mM phosphate buffer, pH 8.0: (●) VAC wild-type, (■) HS-HS, (▲) HS-HS-F72βR, (♦) HS-HS-L154βY VAC enzymes, (◢) GAR and (◣) RgDAAO. The reported values are the average of three experiments; where not shown, the error bars are smaller than the symbol used.

Figure 2. HPLC chromatogram of the reaction mixture during the bioconversion of the natural antibiotic CephC into 7-ACA. Retention time of the different products: 3.5 min for 7-ACA, 4.5 min for CephC, 6.5 min for oxo-7-ACA, and 12.5 min for Gl-7-ACA. Separation conditions: C8-Aquapore RP-300 column 7μ (250 x 4.6 mm; Life Technologies); 25 mM KH_2PO_4, pH 3.0, and 5% acetonitrile as elution buffer; flow rate 1 mL min^{-1}; absorbance detection at 254 nm.

Figure 3. Bioconversion of CephC or Gl-7-ACA by wild-type VAC or RgDAAO. A) Time course of conversion of 50 mM CephC or Gl-7-ACA as the starting substrate by 4.5 kU/L wild-type VAC. Symbols used: (●) CephC, (■) 7-ACA obtained from CephC, (◊) Gl-7-ACA, (□) 7-ACA obtained from Gl-7-ACA. C) Conversion of 25 (filled symbols) or 15 mM (open symbols) CephC by 4.5 kU/L wild-type VAC: (●, ○) CephC, (▲, Δ) oxo-7-ACA, (♦, ◊) Gl-7-ACA. B) Time course of conversion of 50 mM CephC by 9 kU/L (open symbols) or 4.5 kU/L (filled symbols) of RgDAAO: (●, ○) CephC, (▲, Δ) oxo-7-ACA, (♦, ◊) Gl-7-ACA. D) Conversion of 25 (filled symbols) or 15 mM (open symbols) CephC by 4.5 kU/L RgDAAO: (●, ○) CephC, (▲, Δ) oxo-7-ACA, (■, □) 7-ACA. Conditions: 100 mM potassium phosphate buffer, pH 8.0, at 25 °C under shaking. The reported values are the average of three experiments; for all measurements the error bars are smaller than the symbol used.

Figure 4. Time course of CephC conversion by one-pot reaction performed using RgDAAO and various acylases (A-E) Reactions performed at 50 mM CephC concentration. F-L) Reactions performed at 25 mM (filled symbols) or 15 mM (open symbols) CephC concentration. A, F) Wild-type VAC. Arrow in panel A indicates the addition of an aliquot of 0.05 mmol of H_2O_2. B, G) HS-HS VAC variant; C, H) HS-HS-F72βR VAC variant; D, I) HS-HS-L154βY VAC variant; E, L) GAR. Conditions: 4.5 kU/L of enzyme, in 100 mM potassium phosphate buffer, pH 8.0, at 25 °C under shaking. Symbol used: (●, ○) CephC, (▲, Δ) Oxo-7-ACA, (■, □) 7-ACA, (♦, ◊) Gl-7-ACA. The reported values are the average of three experiments; for all measurements the error bars are smaller than the symbol used.

Figure 5. Effect of H_2O_2 on the conversion of CephC by RgDAAO and wild-type VAC. A) Reaction in the absence of exogenous H_2O_2; B) the same reaction after adding 0.01 mmol H_2O_2 every hour; C) same reaction as in panel A and in the presence of 45 kU/L of catalase. Conditions: 4.5 kU/L of each enzyme, in 100 mM potassium phosphate buffer, pH 8.0, at 25 °C under shaking. Symbol used: (●), CephC; (▲), Oxo-7-ACA; (♦), Gl-7-ACA; (■), 7-ACA. The reported values are the average of three experiments; for all measurements the error bars are smaller than the symbol used.

Figure 6. Bioconversion of 15 mM CephC at 20 mL reaction scale. A) Reaction performed using 0.45 kU/L of RgDAAO and 0.45 kU/L of HS-HS-F72βR VAC. B) Reaction performed using 0.45 kU/L of RgDAAO and 0.45 kU/L of wild-type VAC. Arrows indicate the addition of a further aliquot of the two enzymes. Conditions: 100 mM potassium phosphate, pH 8.0, 25 °C, under shaking. Symbol used: (●), CephC; (▲), Oxo-7-ACA; (■), 7-ACA; (♦), Gl-7-ACA. The reported values are the average of three experiments; for all measurements the error bars are smaller than the symbol used.

Table 1. Comparison of known kinetic properties of RgDAAO, GAR, and VAC variants on CephC and Gl-7-ACA as substrate.

	CephC				Gl-7-ACA				7-ACA
	V_{max} (U/mg)	K_m (mM)	V_{max} / K_m	K_i (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} / K_m	K_i (mM)	K_i (mM)
RgDAAO[(a)]	110 ± 10	4.0 ± 0.5	27.5	-	-	-	-	11.8	23.6
GAR[(b)]	-	-	-	-	3.6 ± 0.2	3.3 ± 0.6	1.1	-	-
VAC Wild-type[(b)]	0.7 ± 0.1	9.5 ± 0.3	0.08	-	36.4 ± 2.7	1.5 ± 0.2	24.3	21	3[(c)]
VAC H57βS-H70βS (HS-HS)[(b)]	3.0 ± 0.1	12.2 ± 0.9	0.24	-	4.8 ± 0.1	6.9 ± 0.7	0.70	-	-
VAC HS-HS-F72βR[(d)]	1.6 ± 0.06	16.4 ± 2.3	0.10	-	52.9 ± 2.3	2.0 ± 0.2	26.4	6.3	-
VAC HS-HS-L154βY[(d)]	3.3 ± 0.15	4.5 ± 0.7	0.73	-	7.9 ± 0.8	2.0 ± 0.6	3.95	-	-

a 15

b 20

c 19

d 14

Table 2. Enzymatic bioconversion of CephC to 7-ACA employing 4.5 kU/L of both RgDAAO and acylase enzymes. The amount of each reaction component was determined by HPLC analysis at 25 °C and pH 8.0 after 7 hours of bioconversion. The values determined after 120 min are reported in parentheses.

	CephC		Oxo-7-ACA[a]		Gl-7-ACA		7-ACA	
	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)
15 mM CephC								
VAC wt	4.9	32.6 (47.0)	0.0	0.0 (0.0)	--	--	10.1	67.2 (52.6)
RgDAAO	0.3	1.9 (43.6)	1.1	7.1 (3.1)	13.7	91.0 (53.4)	--	--
RgDAAO + VAC wt	0.2	1.5 (31.8)	1.1	7.1 (0.1)	0.7	4.6 (1.7)	12.9	86.6 (66.3)
RgDAAO + VAC HS-HS	0.9	5.7 (31.4)	2.2	15.0 (14.1)	2.0	13.5 (10.1)	9.9	65.9 (44.5)
RgDAAO + VAC HS-HS-F72βR	0.9	6.5 (30.7)	0.1	0.4 (0.8)	0.0	0.0 (0.0)	13.9	93.1 (67.0)
RgDAAO + VAC HS-HS-L154βY	1.2	8.3 (32.7)	1.3	8.6 (7.6)	3.1	20.9 (14.9)	9.3	62.2 (44.8)
RgDAAO + GAR	1.0	6.6 (33.0)	0.0	0.0 (0.0)	1.6	10.6 (1.3)	11.8	78.4 (60.6)
25 mM CephC								
VAC wt	14.1	56.3 (72.8)	4.9	19.8 (11.2)	--	--	6.0	23.8 (16.0)
RgDAAO	2.5	10.0 (48.0)	2.0	7.9 (11.2)	20.6	82.2 (40.8)	--	--
RgDAAO + VAC wt	1.5	6.1 (19.2)	1.7	6.9 (7.2)	0.5	1.9 (1.2)	21.3	85.0 (72.4)
RgDAAO + VAC HS-HS	2.3	9.3 (37.2)	5.5	21.9 (13.2)	6.1	24.5 (13.2)	11.1	44.3 (36.4)
RgDAAO + VAC HS-HS-F72βR	1.9	7.4 (8.8)	0.3	1.4 (13.6)	0.8	3.1 (4.0)	22.0	88.1 (73.6)
RgDAAO + VAC HS-HS-L154βY	3.9	15.7 (44.4)	2.3	9.3 (8.0)	7.1	28.5 (16.4)	11.6	46.4 (31.2)
RgDAAO + GAR	5.2	20.7 (64.1)	0.0	0.0 (0.0)	3.3	13.3 (0.0)	16.6	66.2 (35.9)
50 mM CephC								
VAC wt	41.1	82.2 (91.6)	2.2	4.4 (0.2)	--	--	6.7	13.4 (8.2)
RgDAAO	20.4	40.8 (73.0)	8.5	17.0 (13.0)	21.1	42.2 (14.0)	--	--
RgDAAO + VAC wt	1.7	3.5 (54.6)	10.9	21.8 (8.0)	9.9	19.8 (0.0)	27.5	54.9 (37.4)
RgDAAO + VAC HS-HS	11.4	22.7 (67.2)	3.9	7.9 (3.4)	22.1	44.2 (9.2)	12.6	25.2 (20.2)
RgDAAO + VAC HS-HS-F72βR	8.5	17.0 (65.8)	12.5	24.9 (0.0)	2.8	5.6 (0.2)	26.2	52.4 (34.0)
RgDAAO + VAC HS-HS-L154βY	13.2	26.4 (65.0)	10.4	20.8 (11.6)	13.6	27.2 (3.8)	12.8	25.5 (19.6)

RgDAAO + GAR	16.2	32.5 (70.4)	9.1	18.2 (6.4)	6.2	12.4 (1.2)	18.5	36.9 (22.0)
Influence of H_2O_2[b]								
RgDAAO + VAC wt	1.7	3.5 (54.6)	10.9	21.8 (8.0)	9.9	19.8 (0.0)	27.5	54.9 (37.4)
RgDAAO + VAC wt + H_2O_2	6.3	12.6 (43.0)	0.3	0.5 (11.6)	10.5	21.0 (3.2)	32.9	65.8 (42.2)
RgDAAO + VAC wt + CAT	4.3	8.6 (46.6)	11.0	22.1 (5.0)	1.6	3.1 (0.0)	33.1	66.2 (48.4)

[a] The oxo-7-ACA content was roughly estimated since it represents the difference between the content of the reaction components mentioned in the table.

[b] In presence of 50 mM CephC.

Scheme 1

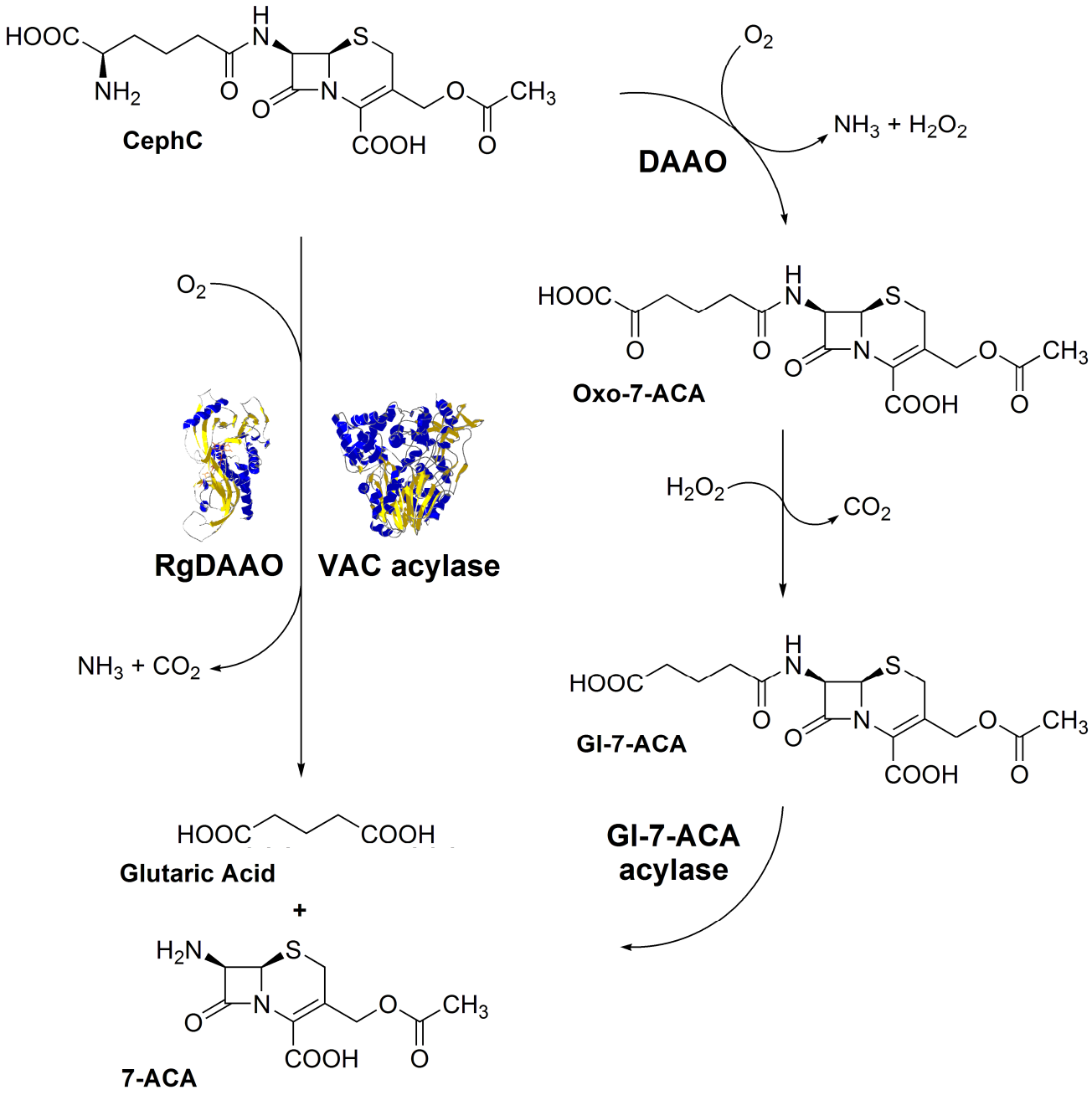
CephC
DAAO
O_2
$NH_3 + H_2O_2$
Oxo-7-ACA
H_2O_2
CO_2
GI-7-ACA
GI-7-ACA acylase
RgDAAO
VAC acylase
$NH_3 + CO_2$
Glutaric Acid
+
7-ACA

Figure 1

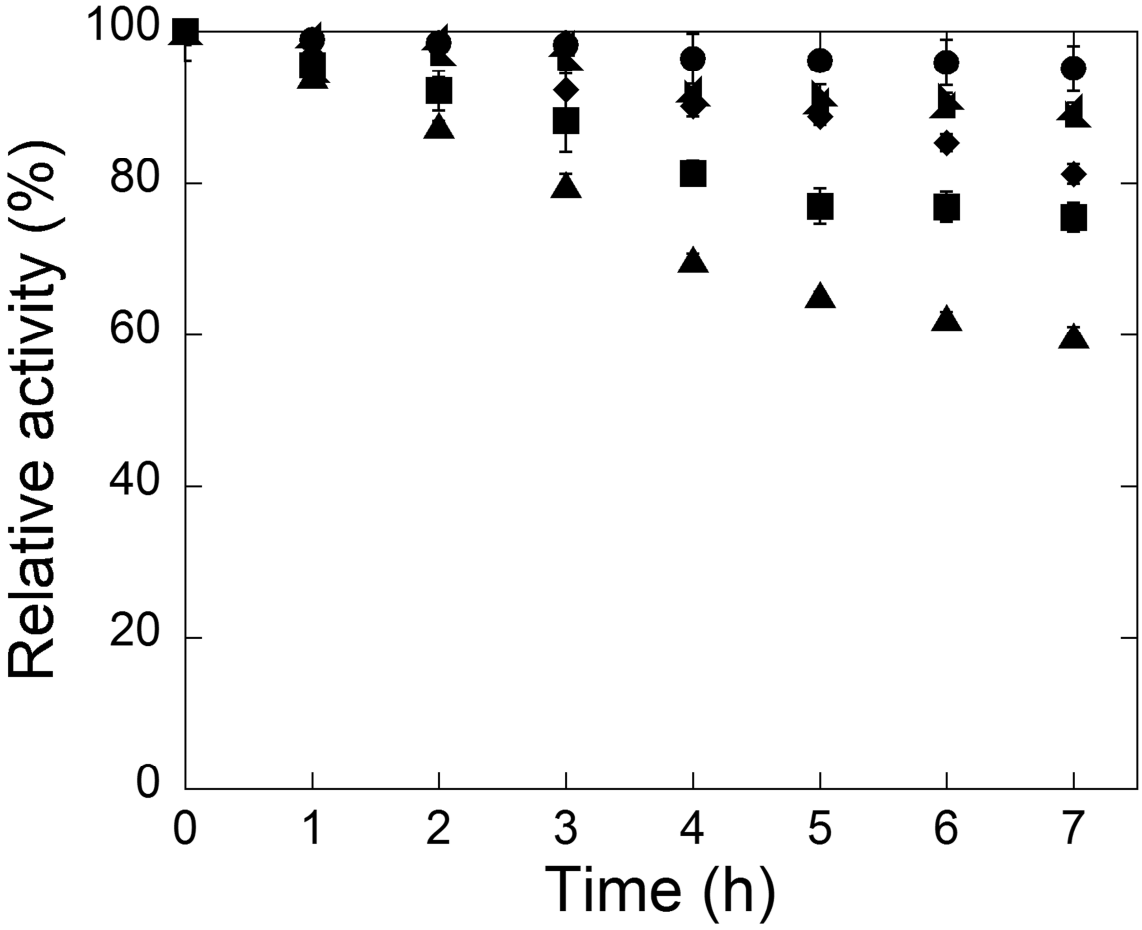

Figure 2

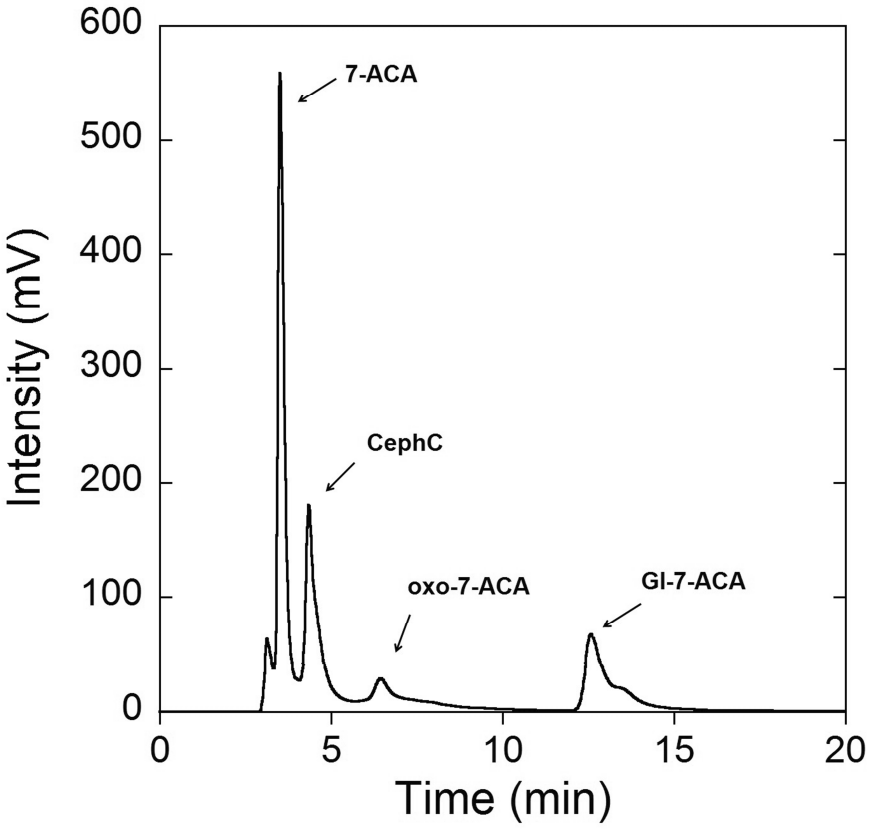
600
500
400
300
200
100
0
Intensity (mV)
0
5
10
15
20
Time (min)
7-ACA
CephC
oxo-7-ACA
Gl-7-ACA

Figure 3

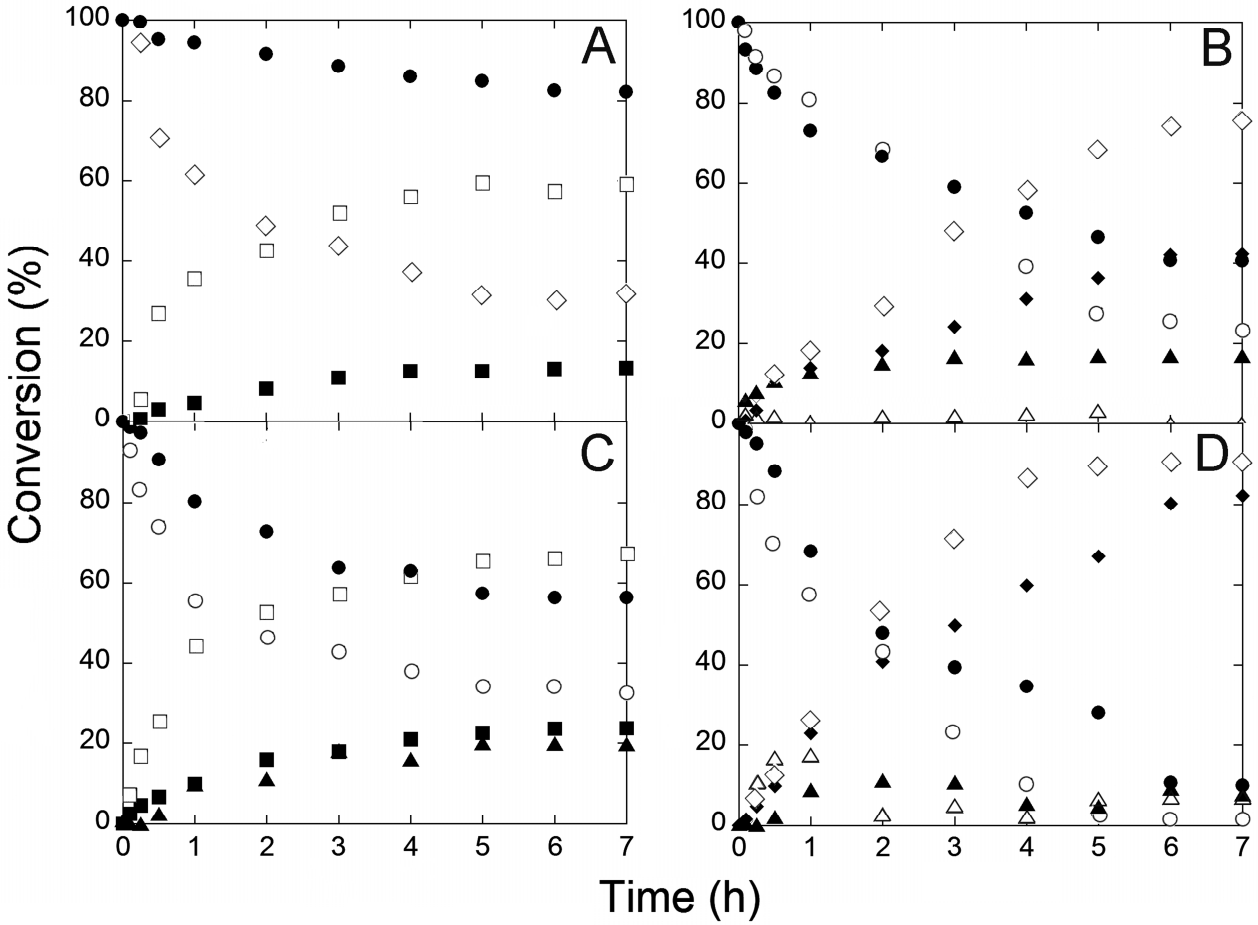

Figure 4

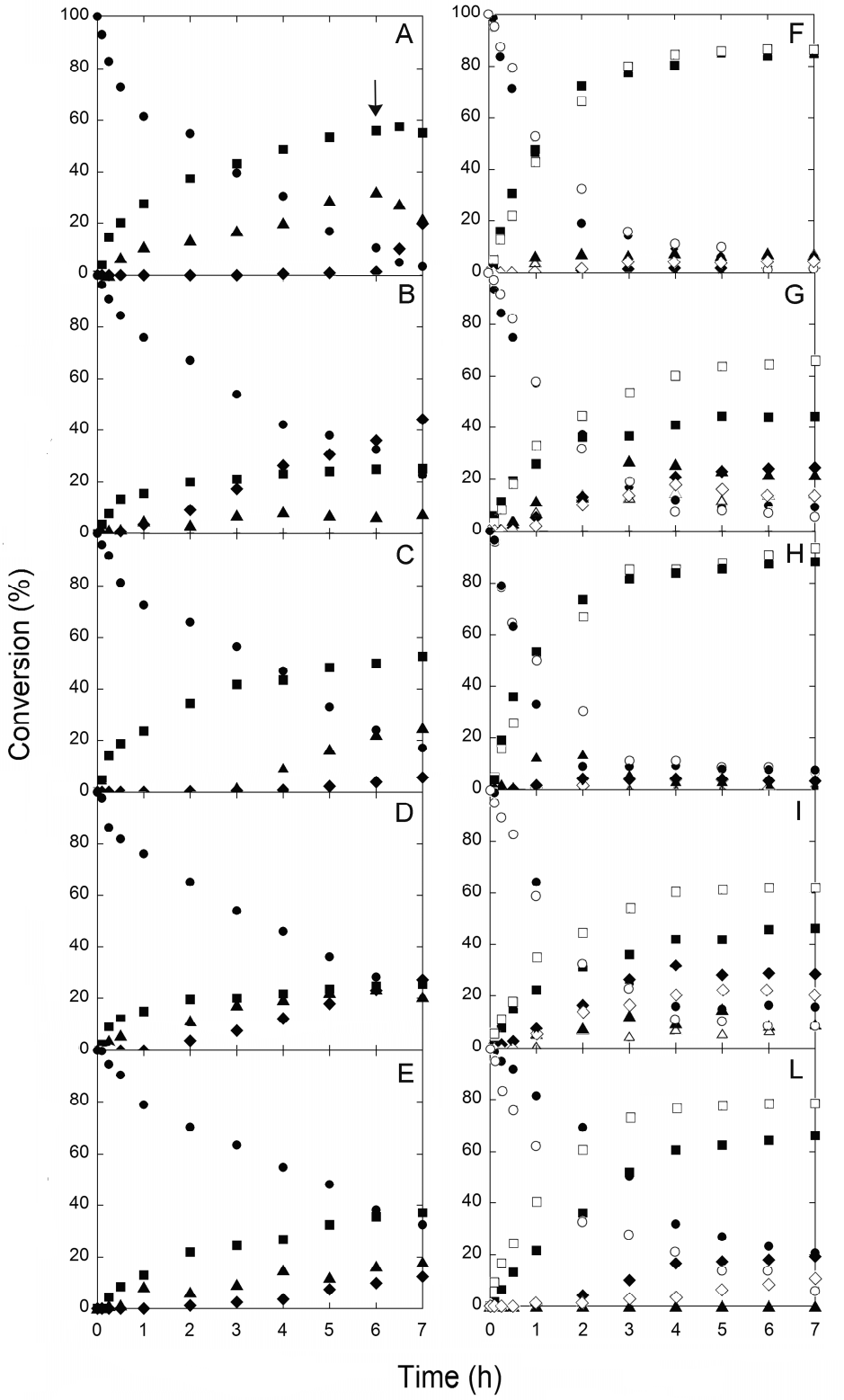

Figure 5

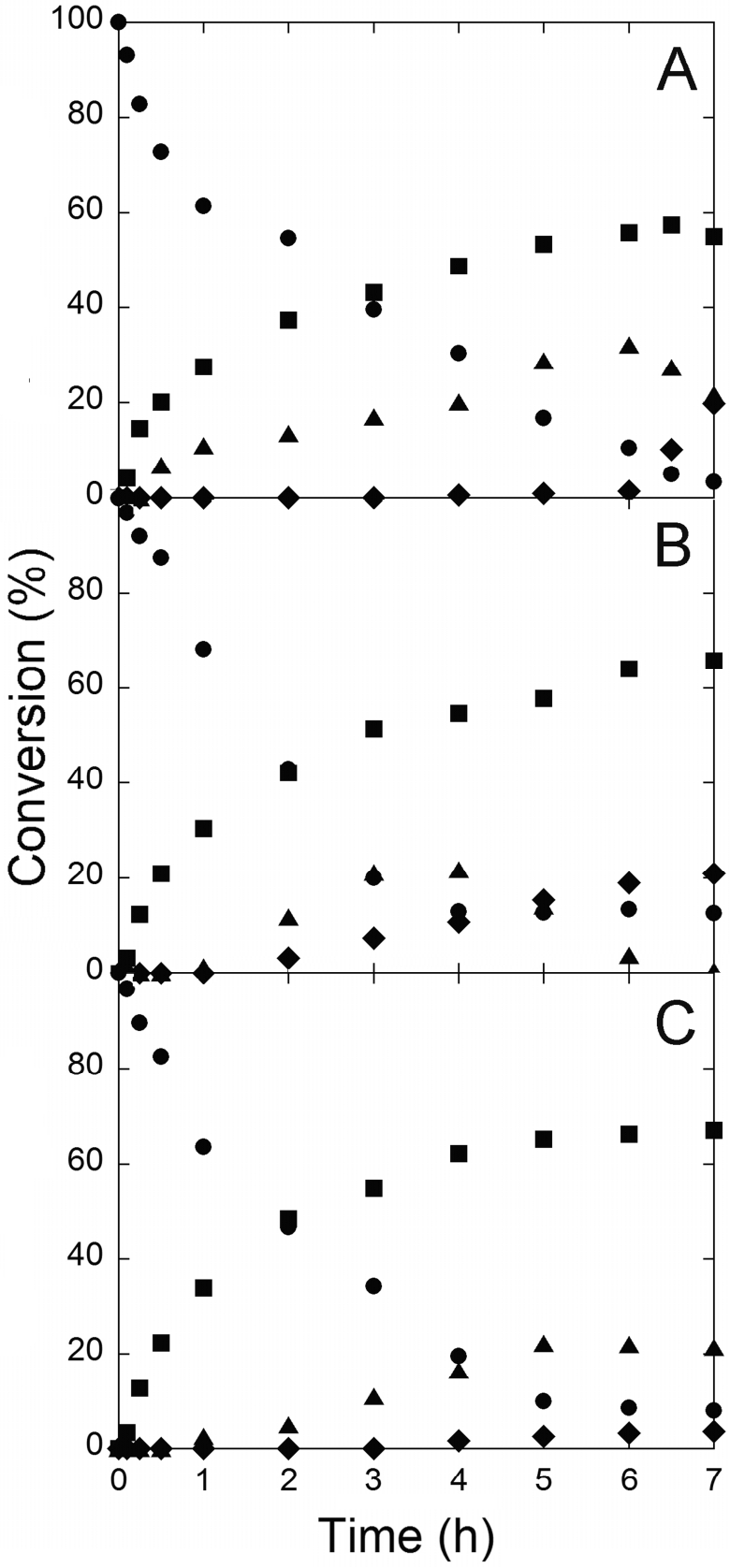

Figure 6