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ORIGINAL PAPER

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Performance of a recombinant strain of *Streptomyces lividans* for bioconversion of penicillin G to deacetoxycephalosporin G

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Abstract We examined the performance of *Streptomyces lividans* strain W25 containing a hybrid expandase (deacetoxycephalosporin C synthase; DAOCS) gene, obtained by in vivo recombination between the expandase genes of *S. clavuligerus* and *Nocardia lactamdurans* for resting-cell bioconversion of penicillin G to deacetoxycephalosporin G. Strain W25 carried out a much more effective level of bioconversion than the previously used strain, *S. clavuligerus* NP1. The two strains also differed in the concentrations of $FeSO_4$ and α-ketoglutarate giving maximal activity. Whereas NP1 preferred 1.8 mM $FeSO_4$ and 1.3 mM α-ketoglutarate, recombinant W25 performed best at 0.45 mM $FeSO_4$ and 1.9 mM α-ketoglutarate.

Keywords Antibiotics · Bioconversions · Deacetoxycephalosporin · Expandase · Directed evolution · Penicillin

Introduction

Semisynthetic cephalosporins constitute the largest selling group of antibiotics worldwide. Many of these medically important compounds are made chemically from 7-aminodeacetoxycephalosporanic acid. At present, this intermediate is made by synthetic ring-expansion of the inexpensive penicillin G molecule to form deacetoxycephalosporin G (DAOG), followed by enzymatic removal of the phenylacetate side-chain. The synthetic ring-expansion step [4] is an expensive, multistepped and polluting series of reactions. We hope to replace it with an environmentally compatible enzymatic method employing the expandase (deacetoxycephalosporin C synthase) of the cephamycin C biosynthetic pathway in *Streptomyces clavuligerus*. The natural substrate of this dioxygenase is the commercially unavailable penicillin N molecule and, under normal reaction conditions, the enzyme failed to attack penicillin G [14]. However, we demonstrated that, by modifying the reaction conditions, a low level of activity could be detected with extracts or resting cells of *S. clavuligerus* [5]. Modification of growth conditions stimulated the bioconversion [8], which could be carried out by free or immobilized resting cells [6]. However, the extent of bioconversion was still very low and work with cell-free extracts [1] showed that this was due to inactivation of the expandase by components of the reaction mixture, i.e., Fe^{2+} in combination with ascorbate or α-ketoglutarate. We were recently able to increase the yield of DAOG in the cellular bioconversion somewhat by eliminating agitation during the reaction and by adding an alkane [9, 10] and catalase to the reaction mixture [11]. A further advance was made by construction of strain W25 of *S. lividans* containing a hybrid expandase [2]. The strain was made by the technique of directed evolution, i.e, by in vivo homeologous recombination of expandase genes from *S. clavuligerus* NRRL 3585 and *Nocardia lactamdurans* placed in tandem in a plasmid construct. A visual method was used to detect recombinant clones and one such clone appeared to produce an increased level of DAOG. Optimization of the performance of recombinant *S. lividans* strain W25 is the subject of this communication.

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Materials and methods

The culture used previously was *S. clavuligerus* strain NP1 (ATCC 700751) which produces only a trace of cephalosporins [15] and thus does not carry over detectable levels of such antibiotics with the washed cells into the reaction vessel. The standard procedure established for strain NP1 was as follows. The seed culture was established by inoculation of 50 ml of MST medium [12] plus thiostrepton in a 250-ml triple-baffled flask with 100 μl of a 20% glycerol stock culture previously stored at −80 °C. This medium contained (per liter): 30 g trypticase soy broth without dextrose (BBL Becton Dickinson, Cockeysville, Md.), 1% soluble starch, 90 mM 3-(*N*-morpholino)propane sulfonic acid buffer (MOPS; USB, Cleveland, Ohio) and 5 mg of thiostrepton; and the pH was adjusted to 7.0 before autoclaving the medium. Incubation was at 30 °C on a shaker at 220 rpm for 48 h. The seed (10 ml) was added to 100 ml of MT2E medium plus 5 mg thiostrepton/l in a 500-ml triple-baffled flask. MT2E medium is a modification of MST in which 2% ethanol replaces the starch [8]; and the ethanol was added to the rest of the medium after autoclaving it and allowing it to cool. The growth flask was incubated under the same conditions as above but only for 24 h. Cells were harvested by centrifugation at 10,000 *g* for 10 min at 4 °C, washed twice with cold double-distilled (dd) water and resuspended in 12.5 ml of dd water. For *S. clavuligerus* strain NP1, the procedure was modified by withholding thiostrepton from all the media.

The standard bioconversion reaction was conducted in a 250-ml Erlenmeyer flask containing 10 ml of the following reaction mixture: 0.05 M MOPS buffer (pH 6.5), 1.28 mM α-ketoglutaric acid, 4.0 mM ascorbic acid, 1.8 mM $FeSO_4 \cdot 7H_2O$, 8.0 mM KCl, 8.0 mM $MgSO_4 \cdot 7H_2O$, 5 ml of hexane, 20 mg of penicillin G and 4.0 ml of washed cells. The pH was adjusted to 6.5 with 0.5 N KOH. The order of addition of the components was as previously described [16]. When catalase was used in the reaction mixture (1 mg/ml), it was Catalase C-40 (Sigma, St. Louis, Mo.), a thymol-free preparation from bovine liver containing 10,700 units/mg solids and 17,300 units/mg protein. The reaction mixture was incubated statically at 30 °C for periods up to 48 h. The reaction began when penicillin G was added. The concentration of product DAOG was estimated at various times by the paper disk–agar diffusion assay as previously described [5], using DAOG as standard and penicillinase to destroy the unused penicillin G. *Escherichia coli* strain Ess, a β-lactam-supersensitive mutant [13] was used as the assay organism.

Results and discussion

Superiority of recombinant strain

The construction of the *S. lividans* strain W25 and other recombinant strains was described by Adrio et al. [2]. We found that the best converter of the group was W25. When *S. lividans* W25 was compared with *S. clavuligerus* NP1 under a number of different growth and incubation conditions, we found W25 to be superior in performance. For example, in two such head-to-head comparative experiments, NP1 produced DAOG at 12.8 mg/l and 17.2 mg/l at 5 h, whereas strain W25 yielded 62 mg/l and 70 mg/l.

Response to growth conditions

We previously observed that *S. clavuligerus* NP1 performs better when cells are harvested at 1 day, rather than at 2 days [9]. The same was observed with the hybrid strain. In this experiment, production of DAOG by strain W25 at 5 h was 25 mg/l using a 2-day inoculum and 88 mg/l with a 1-day seed.

Previous studies on *S. clavuligerus* NP1 showed that the growth medium that yielded the most active cells was MT2E [8], a medium in which 2% ethanol was substituted for the 1% starch in MST medium. For *S. lividans* W25, we tested a large number of media by varying the carbon source in the medium of Jensen et al. [12], i.e., 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, or 6.0% ethanol, 1% or 2% methanol, or 1% starch plus 0.0, 0.5, 1.0, 2.0, or 2.5% ethanol. Of these, the best conversion was obtained with cells grown in MT4E medium, i.e., the original MST medium minus starch and containing 4% ethanol (Fig. 1).

Response to changes in the composition of the bioconversion reaction mixture and incubation conditions

S. clavuligerus NP1 is positively affected in bioconversion extent by elimination of agitation and addition of alkanes [9, 10] and catalase [11] to the reaction mixture. The negative effect of shaking was also observed with *S. lividans* W25. With shaking, DAOG production at 5 h was 64 mg/l; and under static conditions, it was 106 mg/l. The positive effect of 50%(v/v) hexane on the performance of W25 was observed as follows: no hexane gave 64 mg DAOG/l at 5 h and 76 mg/l at 24 h, hexane addition gave 88 mg/l at 5 h and 134 mg/l at 24 h. Catalase at 1 mg/ml (10,000 units/ml) also stimulated conversion by strain W25. In the absence of catalase, DAOG formation was 96 mg/l at 5 h and 132 mg/l at 24 h; and, with catalase, the titers were 128 mg/l and 240 mg/l, respectively. In accordance with Smith et al. [17], who reported that yeast extract contains a component capable of destroying hydrogen peroxide, we found that catalase could be replaced by yeast extract at 0.1 mg/ml (data not shown).

A favorable pH for bioconversion by *S. clavuligerus* NP1 is 6.5 [3]. We found the same to be true for *S. lividans* W25 (Fig. 2). With regard to temperature, *S. lividans* W25 performed better at 20–25 °C (Fig. 3) than at 28 °C, the latter being previously used for *S. clavuligerus* NP1.

A lowering of the iron concentration was favorable for the recombinant strain. Whereas *S. clavuligerus* NP1 performed best at 1.8 mM $FeSO_4$ [5], *S. lividans* W25 preferred 0.45 mM (Fig. 4). An opposite finding was made with α-ketoglutarate: *S. clavuligerus* NP1 performed best at 1.3 mM [5], whereas *S. lividans* W25 preferred the somewhat higher concentration of 1.9 mM. The hybrid strain performed best at an ascorbate concentration of 4–6 mM. A previous study with *S. clavuligerus* NP1 showed a similar optimum ascorbate concentration, i.e., 4–8 mM [5]. A statistical study of $FeSO_4$, ascorbate and α-ketoglutarate concentrations using the hybrid strain confirmed that the optimal

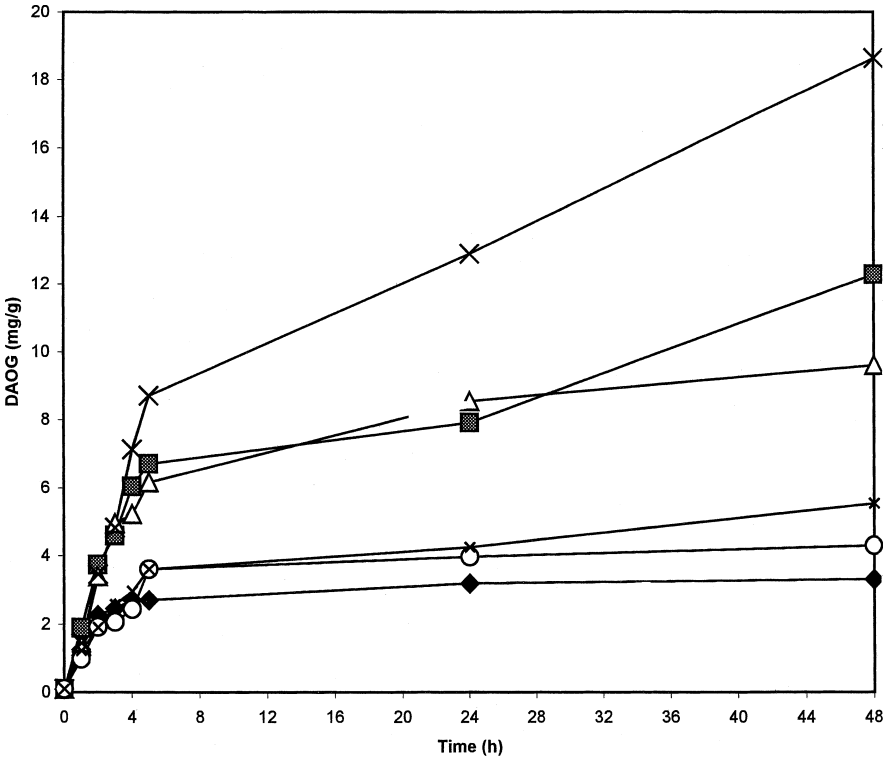

Fig. 1 Effect of growth medium on the specific production of deacetoxycephalosporin G (*DAOG*). Conditions: growth for 1 day, followed by reaction at 28 °C , pH 6.5, with 50% hexane, Fe(II) at 1.8 mM, α-ketoglutarate at 1.28 mM and ascorbate at 4 mM, with no agitation and no catalase. Growth media (see Materials and methods): *black diamonds* MT, *dark squares* MT2E, *white triangles* MT3E, *large crosses* MT4E, *white circles* MT1M, *small crosses* MT2M. *E* Ethanol, *M* methanol

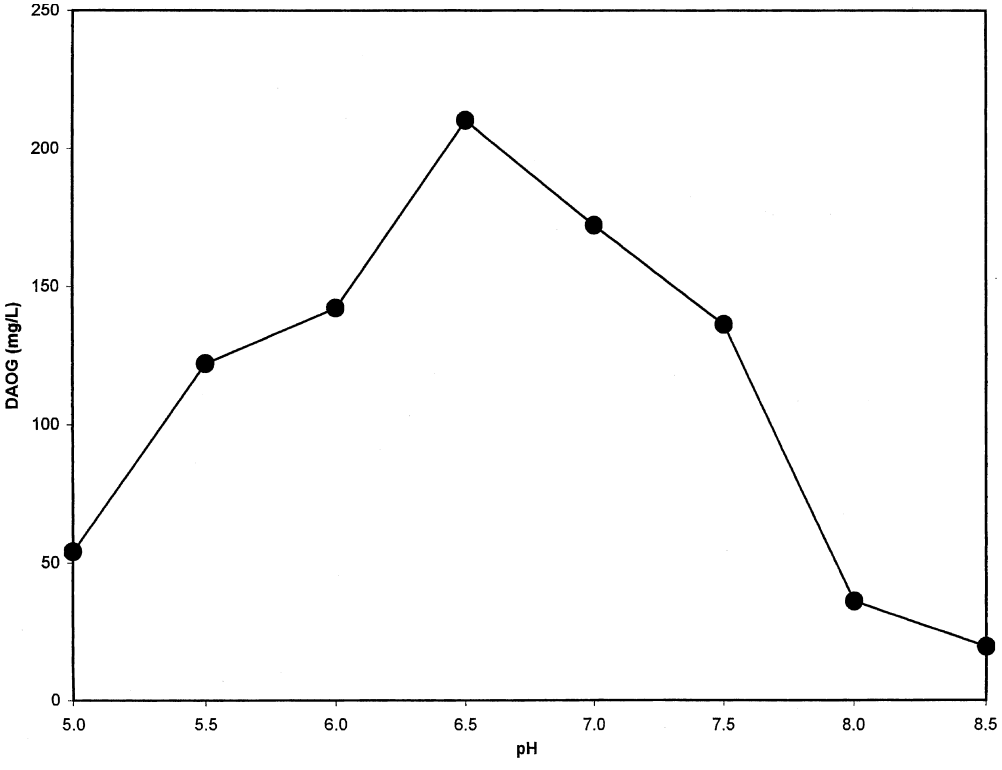

Fig. 2 Effect of pH on the volumetric production of DAOG by cells of *Streptomyces lividans* W25. Conditions: growth in medium MT4E for 1 day, followed by reaction at 28 °C for 48 h with 50% hexane, Fe(II) at 1.8 mM, α-ketoglutarate at 1.28 mM and ascorbate at 4 mM, with no agitation and no catalase. *L* Liters

concentrations for bioconversion were 0.45 mM $FeSO_4$, 1.92 mM α-ketoglutarate and 4.0 mM ascorbate (data not shown). The $FeSO_4$ concentration was the most important and ascorbate the least of the three factors.

The use of directed evolution, a blind approach to protein engineering, is proving to be an excellent means of developing stable, highly active microbial oxidative enzymes for biotechnological applications. In this

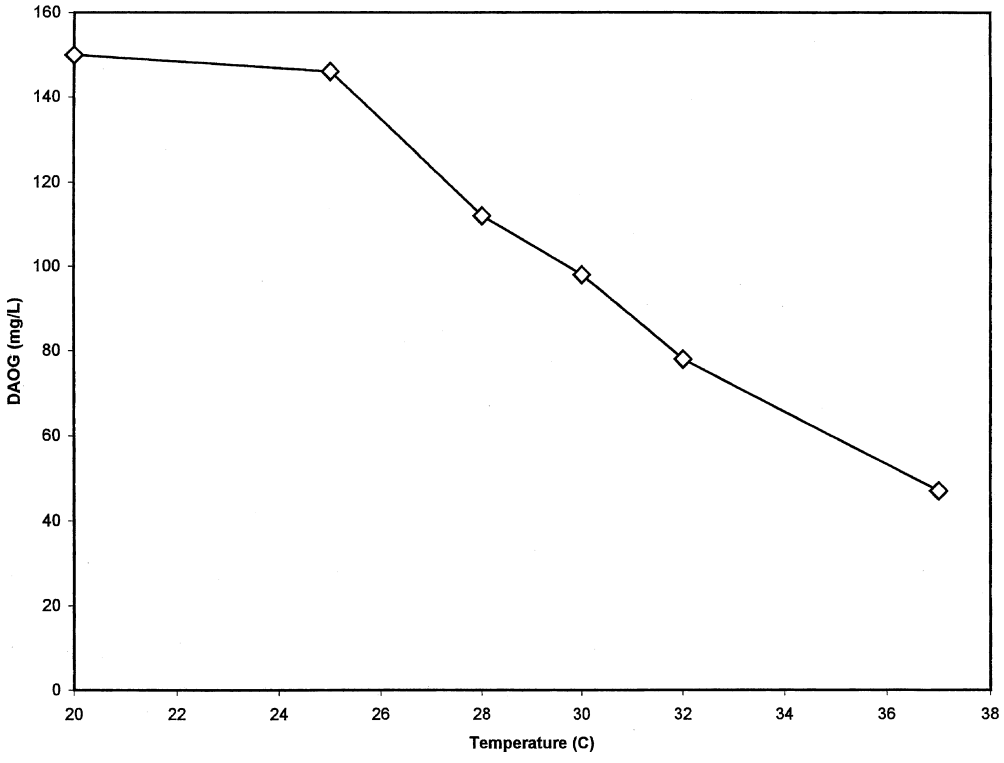

Fig. 3 Effect of temperature on the volumetric production of DAOG by cells of *Streptomyces lividans* W25. Conditions: growth in medium MT4E for 1 day, followed by reaction at pH 6.5 for 48 h with 50% hexane, Fe(II) at 1.8 mM, α-ketoglutarate at 1.28 mM and ascorbate at 4 mM, with no agitation and no catalase

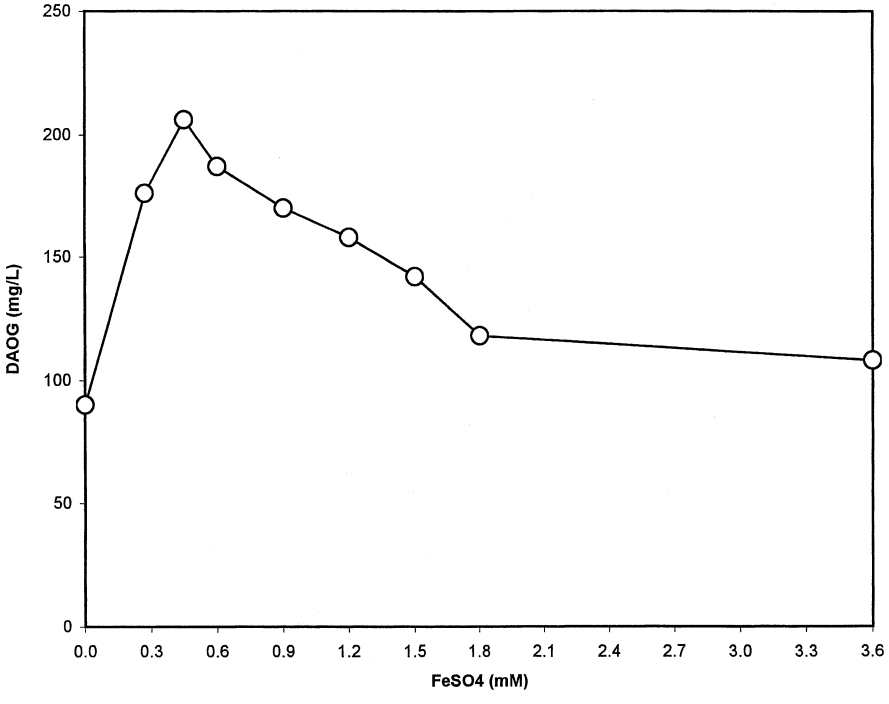

Fig. 4 Effect of Fe(II) concentration on the volumetric production of DAOG by cells of *Streptomyces lividans* W25. Conditions: growth in medium MT4E for 1 day, followed by reaction at 28 °C and pH 6.5, for 48 h with 50% hexane, α-ketoglutarate at 1.28 mM and ascorbate at 4 mM, with no agitation and no catalase

contribution, we show that the recombinant strain *S. lividans* W25, containing a hybrid expandase gene originating from *S. clavuligerus* and *N. lactamdurans*, is superior to the previously used *S. clavuligerus* NP1 strain [7] for bioconversion of the inexpensive penicillin G to the expensive cephalosporin intermediate DAOG. Some similarities were found in the conditions favoring bioconversion by the two strains. These are the 1-day growth duration to prepare cells, static incubation of the reaction mixture, addition of hexane and catalase, a pH of 6.5 and an ascorbate concentration of 4.0 mM. However, the recombinant strain preferred a lower

reaction temperature, a lower $FeSO_4$ concentration and a higher level of α-ketoglutarate. We hypothesize that the four-fold lower iron concentration preferred by the recombinant strain is probably the main reason for the improved bioconversion. This is based on the action of iron not as the cofactor in the biological oxidation reaction but, more importantly, as an inactivator of the expandase [1].

We anticipate that further improvements in this important bioconversion can still be made by additional molecular genetic and biochemical modifications, so that it will become an economically relevant alternative to the chemical ring expansion [4].

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