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Decreased production of *para*-hydroxypenicillin V in penicillin V fermentations

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SUMMARY

Penicillin V (phenoxymethyl penicillin) is produced by industrial strains of *Penicillium chrysogenum* in the presence of phenoxyacetic acid (POAc), a side-chain precursor for the penicillin V molecule. The wild-type strain of *P. chrysogenum* produces an undesirable penicillin byproduct, *para*-hydroxypenicillin V (*p*-OH penicillin V), in addition to penicillin V, via *para*-hydroxylation of POAc and subsequent incorporation of the *p*-OH phenoxyacetic acid into the penicillin molecule. Most of the *p*-OH penicillin V is produced late in cycle when the POAc concentration in the medium is nearly depleted. The level of *p*-OH penicillin V produced by the control strain ranges up to 10–15% of the total penicillins produced. 3-Phenoxypropionic acid and *p*-bromophenylacetic acid partially inhibit the formation of *p*-OH penicillin V with a minimal effect on penicillin V productivity. Mutants deficient in their ability to hydroxylate POAc were found to produce lower levels of *p*-OH penicillin V. Multi-step mutation and screening, starting with the wild-type strain, have culminated in isolation of mutants which produce *p*-OH penicillin V as 1% of the total penicillins with no adverse effect on penicillin V productivity.

INTRODUCTION

Penicillins are produced by *Penicillium chrysogenum* through two enzymatic steps (following primary metabolite formation) culminating in the formation of a cyclic *β*-lactam antibiotic, isopenicillin N [5]. Penicillin V (phenoxymethylpenicillin) is synthesized through transacylation of isopenicillin N or 6-aminopenicillanic acid (6-APA) in the presence of activated phenoxyacetic acid, a side-chain precursor for the penicillin V molecule [5], (Fig. 1). Industrial strains of *P. chrysogenum* often produce an undesirable metabolite, *p*-hydroxy penicillin V (*p*-OH penicillin V), in addition to penicillin V in the presence of phenoxyacetic acid [4]. The maximum levels of *p*-OH penicillin V vary from 10–15% of the total penicillins, depending on strains and processes. It is generally believed that *p*-OH penicillin V is produced through *para* -hydroxylation of phenoxyacetic acid (POAc), followed by incorporation of *para*-hydroxy phenoxyacetic acid (*p*-OH POAc) into the penicillin molecule, rather than through *para*-hydroxylation of the preexisting penicillin V molecule [4], (Fig. 1).

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The presence of *p*-OH penicillin V in penicillin V fermentation broth leads to low product purity for penicillin V. Furthermore, when penicillin V is used as a starting material for the manufacture of 7-aminodeacetoxy-cephalosporanic acid (7-ADCA), an important intermediate for semisynthetic cephalosporins, the yield of 7-ADCA is greatly reduced if *p*-OH penicillin V is present as an impurity in significant amounts (unpublished data).

This paper reports on a concerted program focused on reducing the *p*-OH penicillin V levels in the penicillin V fermentation, either by addition of specific chemical inhibitors to fermentation broths or by mutation and screening for strains having reduced ability to produce *p*-OH penicillin V.

MATERIALS AND METHODS

Organisms

The strains of *Penicillium chrysogenum* used in this study are those used for the industrial production of penicillin V at Bristol-Myers Squibb Company. Strain No. 1020 was derived from a continuous strain improvement program selecting for superior penicillin V productivity. Strain No. 1020, which forms compact colonies on agar media and is prototrophic, produces a significant amount of *p*-OH penicillin V (12–15%) in addition to penicillin V in a variety of fermentation media.

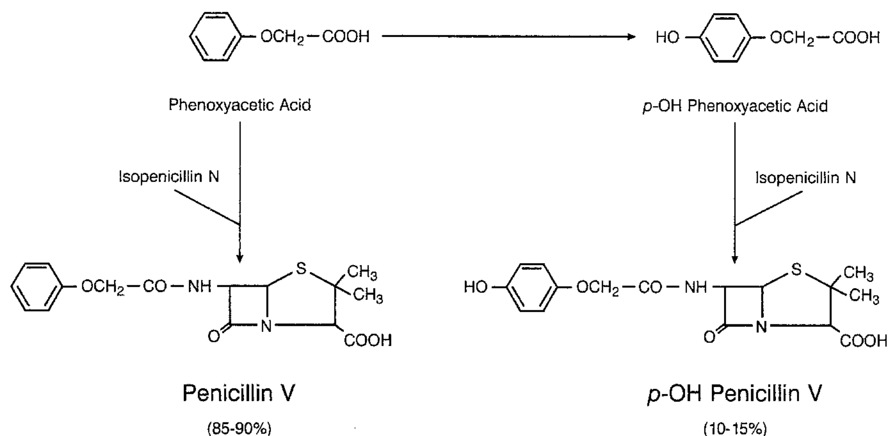

Fig. 1. Possible pathway for the formation of *p*-OH penicillin V.

Strain 1020 is considered to be of wild-type phenotype with respect to its ability to produce *p*-OH penicillin V.

Culture conditions and media

Penicillium strains were maintained on the sporulation agar (SA) medium as described by Chang et al. [3]. The agar plug method described by Chang and Elander [2] was used for prescreening of colonies deficient in ability to hydroxylate POAc. The medium used for preparation of agar plugs was the sporulation agar supplemented with 0.5% POAc. A cylindrical agar plug (6 x 4 mm) inoculated with spores from a 7–10-day colony was incubated at 24 °C in a moist chamber. After 7 days incubation, each plug was transferred to a test tube (15 x 25 mm) and extracted with 2 ml distilled water. *p*-OH POAc concentrations in the water extract were determined by a colorimetric method described in Analytical procedures.

Culture media and conditions for penicillin V fermentation have been described [3]. For the screening of low *p*-OH penicillin V-producing strains in shake flasks, the concentration of POAc in the fermentation medium was reduced to 1%. All fermentation flasks were incubated at 24 °C on a gyrotory shaker operating at 235 rpm (75 mm stroke) for 7–9 days.

Analytical procedures

The concentrations of penicillin V, POAc, *p*-OH penicillin V, and *p*-OH POAc in fermentation broths were determined by high performance liquid chromatography (HPLC) as described by Chang et al. [3]. For semi-quantitative determination of *p*-OH POAc in solution, a colorimetric method for measuring phenol was used [6]. The method entails converting phenol or phenolic compounds, in the aqueous presence of an oxidant in ammonia buffer, into the corresponding quinone-imine, which reacts with aminopyrine to give an N-substituted quinone-imine (red color). 1 ml of ammonium acetate buffer (0.5 M, pH 8.0), 1 ml of 1% aqueous solution of aminopyrine, and 1 ml of 2% solution of potassium ferricyanide [$K_3Fe(CN)_6$] was added to 1 ml of an aqueous solution of phenol or *p*-OH POAc. Absorption of the mixture at 500 nm was determined following incubation at ambient temperature for 30 min.

Mutagenic treatment

The procedure for mutagenic treatment has been described [3]. The irradiated spore suspension was diluted and plated out on a sporulation agar. After 10 days of incubation at 25 °C, the colonies were either replica-plated for isolation of mutants sensitive to inhibitors or transferred to agar plugs for the detection of mutants having reduced ability to hydroxylate POAc.

RESULTS

Kinetics of p-OH penicillin V formation

When the wild-type strain (No. 1020) of *Penicillium chrysogenum* was cultivated in medium containing penicillin V (0.1–2%) in the absence of POAc, the concentration of penicillin V remained unchanged and no *p*-OH penicillin V was detected in broths following 7 days of incubation. This result suggested that hydroxylation of penicillin V does not occur under normal fermentation conditions. When the strain was incubated in media containing 1.5% POAc, penicillin V was produced rapidly after 24 h with concurrent depletion of POAc from the media (Fig. 2). Initially, no significant production of *p*-OH penicillin V was observed. As the concentration of POAc in the media decreased to below 2 mg/ml, the amount of *p*-OH penicillin V increased sharply to as high as 12–15% of the total penicillins produced (the sum of penicillin V

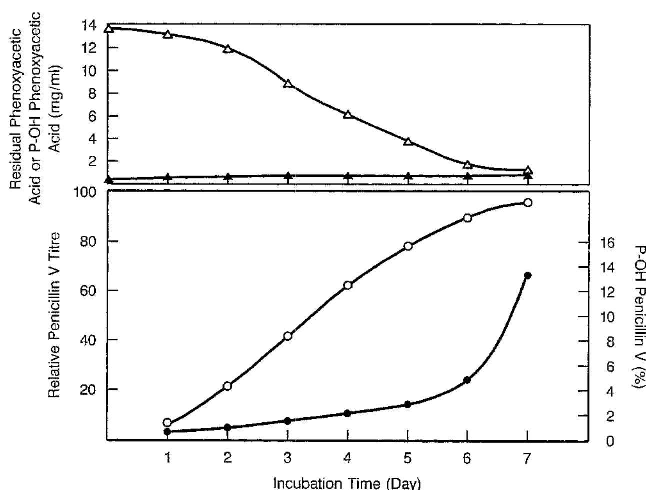

Fig. 2. Time course of penicillin V and *p*-OH penicillin V production in shake flasks by the control strain No. 1020: ○, relative penicillin V titer; ●, % *p*-OH penicillin V; △, residual phenoxyacetic acid; ▲, residual *p*-OH phenoxy acetic acid.

and *p*-OH penicillin V). However, the concentration of the *p*-OH POAc in the medium increased only slightly throughout the fermentation (Fig. 2). The high rate of *p*-OH penicillin V production late in cycle (6th–7th day) suggested that *p*-OH POAc can be incorporated into penicillin (forming *p*-OH penicillin V) when the POAc level was low (Fig. 1).

Inhibition of p-OH penicillin V formation by chemical additives

Fifty-five chemical compounds which represent various structural analogues of POAc and phenylacetic acid (PAA) were screened for their ability to inhibit-*p*-OH penicillin V formation in shake flask fermentations. Each of the compounds was added in three concentrations (0.01, 0.025, and 0.05%) at 0 h and incubated for 7 days. Most compounds were either too toxic, thus inhibiting growth, or had no effect on production of penicillin V or *p*-OH penicillin V at the concentrations used. The results of the screening with a few selected compounds are summarized in Table 1. Note that only 3-phenoxypropionic acid (3-POPA) and *p*-bromophenylacetic acid (*p*-BrPAA) were found to inhibit the formation of *p*-OH penicillin V at concentrations which exert only a limited inhibition of mycelial growth (data not shown) and penicillin V production. However, because of the incomplete inhibition of *p*-OH penicillin V formation, i.e., 50% of the control, by these two compounds and the relatively high concentration required to achieve inhibition, it was impractical to use them in large-scale fermentations.

TABLE 1

Effect of various chemical compounds on penicillin V and *p*-OH penicillin V production in shake flasks

Additive	Concentration [a] (%)	Relative titers [b]	
		Penicillin V	*p*-OH penicillin V
None	–	100	100
p-aminophenylacetic acid	0.05	102	95
p-bromophenylacetic acid	0.01	98	88
	0.025	92	69
	0.05	89	54
p-chlorophenylacetic acid	0.05	95	92
p-nitrophenylacetic acid	0.05	60	82
p-bromophenoxyacetic acid	0.05	92	87
p-chlorophenoxyacetic acid	0.05	92	82
p-nitrophenoxyacetic acid	0.05	82	78
3-phenylpropionic acid	0.05	46	72
3-phenoxypropionic acid	0.01	102	81
	0.025	97	50
	0.05	92	42
4-phenylbutyric acid	0.05	95	82
4-phenoxybutyric acid	0.05	92	80

[a] Each compound was filter sterilized and added to the fermentation medium prior to inoculation.
[b] 7th-day titers.

Development of low p-OH penicillin V strains by mutation and screening

UV was used as a mutagen for the development of low *p*-OH penicillin V producing strains. Two types of screening were employed for the initial survey of mutagen-treated colonies prior to shake flask evaluation. One method involved the isolation of mutants with increased sensitivity to 3-POPA or *p*-BrPAA. Treated colonies were replicated onto an agar medium containing sublethal concentrations of 3-POPA or *p*-BrPAA (0.01%). Colonies showing poor or no growth on inhibitor-containing medium were isolated and tested in shake flasks for penicillin V productivity and *p*-OH penicillin V levels. Among 20 3-POPA sensitive mutants and 12 *p*-BrPAA sensitive mutants, none were found to produce reduced level of *p*-OH penicillin V with normal penicillin V titers (data not shown).

Another approach for mutant screening involved the isolation of mutants with reduced ability to hydroxylate POAc using the agar plug method. Mutated colonies grown on agar plugs containing POAc were screened for their ability to produce phenolic compounds which can be detected by a colorimetric assay. The colonies yielding the lowest concentrations of phenolic compounds (*p*-OH POAc) were collected and evaluated for penicillin V and *p*-OH penicillin V titers in a fermentation medium containing a low concentration (1%) of POAc precursor.

Starting with the control strain No. 1020 which produced normal penicillin V titers and *p*-OH penicillin V at 12% of total penicillins, a mutant strain No. 1187 producing normal penicillin V titers with a *p*-OH penicillin V level at 8% of the total penicillins was obtained, Strain No. 1187 was then used for the next round of mutation and screening, giving rise to another strain (No. 1289) producing 5% *p*-OH penicillin V. Through such repeated sequential mutation and screening, two strains, No. 1411 and No. 1497, producing *p*-OH penicillin V at 1% or lower of total penicillins but with normal penicillin V titers were finally obtained. The sequence of mutational steps and the characteristics of various mutants obtained are summarized in Table 2. The time course of penicillin V and *p*-OH penicillin V production for the control and one of the mutants, No. 1411, are shown in Figs. 2 and 3, respectively.

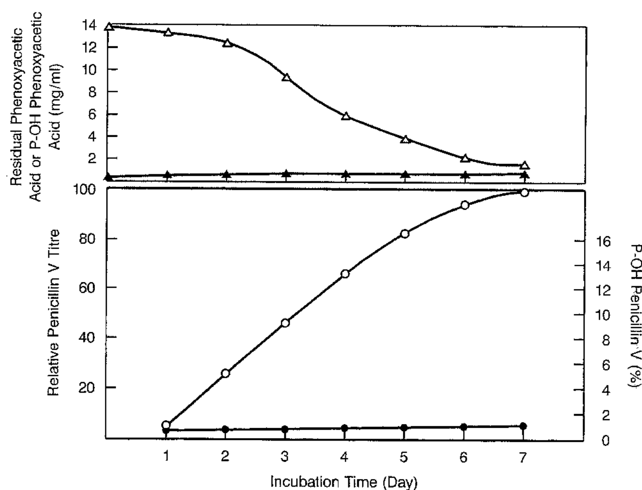

Fig. 3. Time course of penicillin V and *p*-OH penicillin V production in shake flask by the mutant strain No. 1411: ○, relative penicillin V titer; ●, % *p*-OH penicillin V; △, residual phenoxyacetic acid; ▲, residual *p*-OH phenoxy acetic acid.

DISCUSSION

In the penicillin G fermentation, phenylacetic acid (PAA) is used as a precursor for the side chain of penicillin G molecule. It has been reported that PAA can be hydroxylated by *P. chrysogenum* into *o*-OH PAA, *m*-OH PAA, and *p*-OH PAA [4]. However, little of the hydroxylated PAA is incorporated into penicillin, thereby

TABLE 2

Sequential derivation of low *p*-OH penicillin V strains

Strain No.	Progenitor	Relative pen. V titers [a]	Relative *p*-OH pen. V titers [a]	% *p*-OH pen. V [b]
1020 (control)	–	100	100.0	12.0
1187	1020	95	65.0	8.2
1289	1187	97	42.0	5.2
1391	1289	100	25.0	3.0
1411	1391	105	7.5	0.9
1497	1391	102	8.3	1.0

[a] 7th-day titers.

[b] As percent of total penicillins produced (the sum of penicillin V and *p*-OH penicillin V).

resulting in a waste of expensive PAA. In the penicillin V fermentation, POAc is hydroxylated primarily at the *para*-position, resulting in the formation of the *p*-OH penicillin V molecule (unpublished data). Our objectives in reducing *p*-OH penicillin V levels in penicillin V fermentation were aimed at improving precursor utilization efficiency as well as final product purity.

In this paper we studied the kinetics of *p*-OH penicillin V formation and found that most of the *p*-OH penicillin V was produced late in the fermentation cycle when the precursor (POAc) concentration was nearly depleted (<2 mg/ml). Our results are consistent with the belief that preexisting penicillin V molecules are not hydroxylated to *p*-OH penicillin V to any appreciable extent.

The high rate of *p*-OH penicillin V production late in the cycle suggests that *p*-OH POAc is incorporated as efficiently as POAc into the penicillin molecule when the POAc concentration is low. The fact that little *p*-OH penicillin V was formed at conditions of high POAc concentrations is important since it means that no significant amount of *p*-OH penicillin V will be produced unless the POAc concentration becomes limiting (<2 mg/ml). Thus, the use of a condition (a low POAc-containing medium) in which the high *p*-OH penicillin V characteristics of a strain could express is essential for the successful detection of low *p*-OH penicillin V strains.

Among the many structural analogues of POAc and PAA examined for their effect on the formation of *p*-OH penicillin V, only 3-POPA and *p*-BrPAA were found effective in inhibiting *p*-OH penicillin V formation at sublethal concentrations (Table 1). However, their use was not justified on an industrial scale because of the high concentrations (0.05%) required to achieve the rather limited inhibitory effect.

Mutants of *P. chrysogenum* that were rendered more sensitive to the inhibitory effect of 3-POPA and *p*-BrPAA were isolated and tested for their ability to produce penicillin V and *p*-OH penicillin V. None were found to produce reduced levels of *p*-OH penicillin V in the absence of the inhibitors. The rationale for this increased sensitivity approach was derived from citric acid fermentation using strains of *Candida lipolytica* [1]. The wild-type strain of *C. lipolytica* produced a significant amount of isocitric acid (10%), an undesirable by-product, in addition to citric acid. Fluoroacetic acid (FAA) was found to inhibit the formation of isocitrate by inhibiting the enzyme aconitrate hydratase, which converts citric acid to isocitric acid. Mutants which were rendered more sensitive to FAA were isolated and found to produce lower levels of isocitrate in the absence of FAA.

Another screening approach using the agar plug method focused at reducing the ability of the organism to hydroxylate POAc was successful in generating low *p*-OH penicillin V strains. Initial efforts were directed toward isolating mutants with reduced ability to hydroxylate POAc with no regard for their ability to produce penicillin V. Starting with the wild-type strain (No. 1020) which produced *p*-OH penicillin V at a level of 12% of total penicillins, and through repeated rounds of mutational screening, strains (No. 1411 and No. 1497) producing *p*-OH penicillin V at only 1% of total penicillins but with normal penicillin V titers were obtained (Table 2). The fact that multiple steps of mutation and screening were needed to reduce *p*-OH penicillin V to negligibly low levels may suggest that multiple genes may be involved in the hydroxylation of the POAc precursor.

It is of interest to note that even though the low *p*-OH penicillin V mutants produced negligible amounts of *p*-OH POAc, their ability to hydroxylate PAA (to *p*-OH PAA, *m*-OH PAA, and *o*-OH PAA) remained unchanged (data not shown). These results are consistent with those reported by Lein [4] who reported that mutants deficient in the ability to hydroxylate PAA show no significant change in their ability to hydroxylate POAc. Thus, the enzymes that hydroxylate POAc could be different from those that hydroxylate PAA. Our efforts to demonstrate a cell-free enzyme system for hydroxylation of POAc have been unsuccessful thus far. It is possible that the enzyme(s) may be extremely labile or could be extremely sensitive to the normal procedures used for enzyme extraction.

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