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Optimization of Culture Medium and Conditions for Penicillin Acylase Production by *Streptomyces lavendulae* ATCC 13664

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Abstract

The culture medium for *Streptomyces lavendulae* ATCC 13664 was optimized on a shake-flask scale by using a statistical factorial design for enhanced production of penicillin acylase. This extracellular enzyme recently has been reported to be a penicillin K acylase, presenting also high hydrolytic activity against penicillin V and other natural aliphatic penicillins such as penicillin K, penicillin F, and penicillin dihydroF. The factorial design indicated that the main factors that positively affect penicillin acylase production by *S. lavendulae* were the concentration of yeast extract and the presence of oligoelements in the fermentation medium, whereas the presence of olive oil in the medium had no effect on enzyme production. An initial concentration of 2.5% (w/v) yeast extract and 3 μg/mL of $CuSO_4 \cdot 5H_2O$ was found to be best for acylase production. In such optimized culture medium, fermentation of the microorganism yielded 289 IU/L of enzyme in 72 h when employing a volume medium/volume flask ratio of 0.4 and a 300-rpm shaking speed. The presence of copper, alone and in combination with other metals, stimulated biomass as well as penicillin acylase production. The time course of penicillin acylase production was also studied in the optimized medium and conditions. Enzyme production showed catabolite repression by different carbon sources such as glucose, lactose, citrate, glycerol, and glycine.

Index Entries: *Streptomyces lavendulae*; penicillin acylase; fermentation; medium optimization; factorial design.

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Introduction

Penicillin acylases (EC 3.5.1.11) are used in industry to catalyze the hydrolysis of naturally occurring penicillin G and V to produce the β-lactam nucleus 6-aminopenicillanic acid (6-APA), which is then chemically or enzymatically converted into other semisynthetic antibiotics *(1)*. Penicillin G acylases are mainly involved in the worldwide enzymatic production of 6-APA, whereas penicillin V acylases account for only 12% of the worldwide production. Nevertheless, an industrial process using penicillin V, as the precursor for 6-APA, could be more advantageous for several reasons. On the one hand, penicillin V shows higher stability in aqueous solutions at lower pH during extraction from the fermented broth. This enhanced stability of penicillin V may also lead to greater yield of 6-APA because the enzymatic reaction is performed in an aqueous medium. On the other hand, penicillin V acylases reach higher levels of conversion by using a higher substrate concentration compared with penicillin G acylases, and their broader optimal pH reduces the buffering requirement during hydrolysis *(2)*.

Many microorganisms are involved in the extracellular or intracellular production of penicillin V acylases *(2)*. *Streptomyces lavendulae* is an actinomycete that was formerly described to produce an extracellular penicillin V acylase, and a few years ago it was reported that this enzyme had good hydrolytic activity toward natural aliphatic penicillins such as penicillin K, penicillin dihydroF, and penicillin F *(3)*. Such novel hydrolytic capability is quite interesting in industry, because those mentioned aliphatic penicillins represent approx 2% of the total penicillin content in the production of penicillin G and penicillin V by fermentation and create problems in the final crystallization of 6-APA. Recently, it has been proposed that this enzyme be classified as a penicillin K acylase because its highest activity was found toward substrates with octanoyl as the acyl chain *(4,5)*. As reported in a previous work, the fermentation medium used to obtain penicillin acylase from *S. lavendulae* was skim milk as the sole nutrient source. However, this medium had several drawbacks: it was very complex and expensive, and long incubation times were required *(3)*.

The aim of the present work was to design a simple and cheap production medium to obtain penicillin acylase by using *S. lavendulae* in short fermentation times. A statistical factorial design of experiments was used to determine the main effects of the different components of the medium, and the possible interaction of these components. This methodology has been extensively used for optimization of the production of other microbial enzymes with different biotechnological interest *(6–14)*. From the results obtained from the factorial design, we optimized the composition of the medium and the fermentation conditions. The influence of different carbon sources on penicillin acylase production was also studied.

Materials and Methods

Microorganism and Preparation of Inoculum

S. lavendulae subsp. *lavendulae* (ATCC 13664) was grown and maintained on agar plates for 4 d at 29°C and stored at 4°C according to Torres et al. *(3)*. Inoculum was prepared in all cases by transferring a loopful of cells from the agar plates to 250-mL Erlenmeyer flasks containing the production medium.

Analytical Methods

After 72 h of fermentation, samples were centrifuged at 14,000*g*, and the sedimented material was collected and dried overnight at 50°C to determine cellular concentrations. The supernatant was used to determine both enzyme activity and protein concentration. Acylase activity was determined by measuring the 6-APA released during the hydrolysis of penicillin V by the *p*-dimethyl-aminobenzaldehyde method according to the methodology described by Torres et al. *(3)*. One enzyme activity international unit is defined as the amount of enzyme producing 1 μ*M*/min of 6-APA under the assay conditions. Protein concentration was determined according to the Bradford method *(15)*, employing bovine serum albumin as the standard.

Experimental Design

A complete factorial design was chosen to study the effects and interactions of three factors or variables, each varied at three levels that also included three center points to determine experimental error *(16)*. The crucial factors (components in the medium) involved in the study were concentration of yeast extract, concentration of olive oil, and concentration of a solution of oligoelements. Each liter of the solution of oligoelements contained 35 g of $MgSO_4$, 2 g of $CaCO_3$, 5.4 g of $FeCl_3 \cdot 6H_2O$, 1.44 g of $ZnSO_4 \cdot 7H_2O$, 1.11 g of $MnSO_4 \cdot H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.062 g of H_3BO_3, 0.49 g of $NaMoO_4 \cdot 2H_2O$, and 0.28 g of $CoCl_2 \cdot 6H_2O$. Oligoelements were dissolved in deionized water by adding concentrated HCl, and the final solution was sterilized by filtration prior to its use. Table 1 provides the factors and levels at which the experiments were carried out. Low and high factor settings were coded as –1 and +1, and the center point was coded as 0. The response chosen was the production of penicillin acylase after 72 h of fermentation. The dependence of the response on the experimental variables was expressed as the following equation of first degree:

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ij} x_{ij} \quad (1)$$

in which Y is the response; b_0 is the mean value of the response; b_i is the principal effect of a factor, x_i; and b_{ij} is the interaction between two factors, x_i and x_j. All experiments were carried out in an orbital shaker incubator at 20°C and 250 rpm. Cultures were grown aerobically under submerged

Table 1
Variables and Levels of Experimental Design

Independent variable	Name of variable	Level –1	Level 0	Level +1
x_A (% [w/v])	Yeast extract	0.5	1.0	1.5
x_B (% [v/v])	Olive oil	0.0	0.5	1.0
x_C (% [v/v])	Solution of oligoelements	0.0	0.2	0.5

conditions in 250-mL Erlenmeyer flasks containing the components of the experimental design dissolved in 100 mL of 20 m*M* potassium phosphate, pH 7.0. The results of the experimental design were analyzed with a Statgraphics plus 5.0 program (Statistical Graphics). Taking into account the most important variables detected by the experimental design, we studied the effect of the amount of each component in the medium in order to enhance enzyme production. All experiments were carried out in the same conditions as previously mentioned. In our study, fermentations during 72 h were carried out in duplicate, and all the results had a standard error within 5% of the mean values.

Results and Discussion

We carried out eight experiments that were the result of the combination of the variables at the high (+1) and low (–1) level (experiments 1–8), and three experiments in the center-point conditions (experiments 9–11). The response was penicillin acylase production (Y), and the results are summarized in Table 2. Coefficients of the full model (Eq. 1) were evaluated by regression analysis and tested for their significance (Tables 3 and 4). The nonsignificant coefficients were eliminated on the basis of p values after examining the coefficients, and the model was finally refined. It was observed that the main effects that positively affect penicillin acylase production by *S. lavendulae* were yeast extract concentration (b_A) and oligoelement concentration (b_C) in the fermentation medium. The interaction between yeast extract and oligoelements (b_{AC}) could have a positive effect on penicillin acylase production, but statistical analysis revealed that this interaction and the other ones (b_{AB}, b_{BC}) were not significant. On the other hand, enzyme production was not significantly affected by the presence of olive oil in the medium. The final response equation to predict penicillin acylase production after eliminating the insignificant terms was as follows:

$$Y = 61.91 + 82.25x_A + 25.75x_C \quad (2)$$

A coefficient of determination (R^2) value of 0.97 (Table 4) showed that the equation is highly reliable. The model was found to be adequate for prediction within the range of variables employed. Thus, we found that a very simple and cheap medium was adequate for the production of penicil-

Table 2
Experimental Matrix Factorial Design

Experiment	Variable x_A	x_B	x_C	Y (IU/L)
1	+1	–1	–1	89
2	–1	–1	–1	19
3	+1	–1	+1	153
4	+1	+1	+1	106
5	+1	+1	–1	78
6	–1	+1	–1	24
7	–1	+1	+1	25
8	–1	–1	+1	29
9	0	0	0	53
10	0	0	0	46
11	0	0	0	59

Table 3
Estimated Effects
for Penicillin Acylase Production
Using a 2^3 Factorial Design[a]

Coefficient	Value
b_0	61.91 ± 3.46
b_A	82.25 ± 8.13
b_B	–14.25 ± 8.13
b_C	25.75 ± 8.13
b_{AB}	–14.75 ± 8.13
b_{AC}	20.25 ± 8.13
b_{BC}	–11.25 ± 8.13

[a]Standard errors are based on the total error with 4 df.

Table 4
Analysis of Variance Using a 2^3 Factorial Design

Source	Sum of squares	df	Mean square	F ratio	p Value
b_A	13,530.1	1	13,530.1	102.47	0.0005
b_B	406.125	1	406.125	3.08	0.1543
b_C	1326.13	1	1326.13	10.04	0.0339
b_{AB}	435.125	1	435.125	3.30	0.1437
b_{AC}	820.125	1	820.125	6.21	0.0673
b_{BC}	253.125	1	253.125	1.92	0.2384
Total error	528.159	4	132.04		
Total (corrected)	17,298.9	10			
R^2 = 96.9469%					
Standard error of estimate = 11.4909					
Mean absolute error = 5.04132					
Durbin-Watson statistic = 0.911184 (p = 0.0811)					

lin acylase by *S. lavendulae*. Such a medium was composed of a 1.5% (w/v) concentration of yeast extract and a 0.5% (v/v) concentration of the solution of oligoelements dissolved in 20 m*M* potassium phosphate, pH 7.0. Enzyme production (170 IU/L) with this medium was slightly lower than that described by Torres et al. *(3)* employing skim milk (178 IU/L) as the nutrient source. Nevertheless, the new medium required only 72 h of fermentation, instead of 120 h, and it was obviously cheaper than the one composed of skim milk.

Optimization of Medium for Enhanced Penicillin Acylase Production

Taking into account the main variables detected by the experimental design, we studied the effect of the concentration of each component in the medium in order to obtain higher levels of penicillin acylase production. Thus, we combined different concentrations of yeast extract and oligoelements (Table 5) under the conditions described under Methods. Cell growth, measured as dry weight, was increased when yeast extract content was increased in the media (media 2, 3, 7, 12, and 13). On the other hand, an increase in the concentration of oligoelements in the medium had no influence on cell growth, which was approximately constant (6 g/L) at a fixed amount of yeast extract (media 4–11). Extracellular protein production had a behavior similar to that observed for cell growth. We observed that enzyme production was clearly enhanced (248 IU/L) when the medium contained a 2.5% (w/v) concentration of yeast extract and a 1.25% (v/v) concentration of the solution of oligoelements.

By using this basal medium (medium 8; *see* Table 5), we studied the effects of aeration and shaking speed on penicillin acylase production (Table 6). We confirmed that penicillin acylase production was even higher (289 IU/L) at a ratio of volume of medium/volume of Erlenmeyer flask of 0.4 and a shaking speed of 300 rpm than that obtained for the basal medium (248 IU/L). Under these conditions, cell growth was higher (8.8 g/L), and a relatively lower extracellular protein production led to an increase in the specific activity (0.41 IU/mg) of the supernatant compared to other conditions.

Effect of Carbon Source on Enzyme Production

S. lavendulae was grown in the presence of various carbon sources to determine their effect on the production of penicillin acylase. In these experiments, the optimized basal medium was supplemented with 1% (w/v) glucose, lactose, citrate, glycerol, or glycine, and fermentation was carried out at the optimized conditions. In all cases, we observed a clear catabolite repression (Table 7). Penicillin acylase production was inhibited in the presence of glucose and glycine, whereas the repression was lower in the presence of lactose. A similar repression by glucose was observed in the production of penicillin V acylases from *Beijerinckia indica* var *penicillanicum (17)* and *Chainia*, a sclerotial *Streptomyces (18)*. However,

Table 5
Effect of Composition of Medium on Penicillin Acylase Production by *S. lavendulae*

Medium	Yeast extract (% [w/v])	Oligoelements (% [v/v])	Enzymatic activity (IU/L)	Protein (mg/L)	Dry weight (g/L)	pH
1 (control)	1.5	0.50	156	596	4.6	8.5
2	1.5	1.00	149	675	4.9	8.1
3	2.0	1.00	187	803	5.1	8.0
4	2.5	0.50	188	782	6.0	8.3
5	2.5	0.75	210	790	5.8	8.2
6	2.5	0.87	236	789	5.9	8.1
7	2.5	1.00	228	829	5.8	8.1
8	2.5	1.25	248	843	6.0	8.0
9	2.5	1.50	242	850	6.0	8.0
10	2.5	1.75	221	847	6.1	8.0
11	2.5	2.00	153	908	5.8	7.9
12	3.5	1.00	210	861	6.6	8.0
13	4.5	1.00	217	865	7.0	8.0
14	4.5	2.00	190	964	7.2	7.9

Table 6
Effects of Aeration and Agitation on Penicillin Acylase Production by *S. lavendulae*[a]

Ratio of medium/ Erlenmeyer flask	Shaking (rpm)	Enzymatic activity (IU/L)	Protein (mg/L)	Dry weight (g/L)	pH
1.0	250	96	652	3.6	7.6
0.4	200	210	499	4.5	7.9
0.4	250	248	843	6.0	8.0
0.4	300	289	706	8.8	8.4
0.4	350	167	694	8.0	8.6
0.2	250	171	973	7.4	8.4
0.1	250	71	978	7.4	8.5

[a]All experiments were carried out in medium 8 (described in Table 5).

Table 7
Effects of Carbon Source on Penicillin Acylase Production by *S. lavendulae*[a]

Carbon source (1% [w/v])	Enzymatic activity (IU/L)	Protein (mg/L)	Dry weight (g/L)	pH
Nil	289	706	8.8	8.4
Glucose	78	621	10.2	7.0
Lactose	209	713	8.3	8.3
Citrate	106	259	3.1	8.0
Glycerol	190	767	10.8	7.0
Glycine	80	169	5.3	8.4

[a]All experiments were carried out in medium 8 (described in Table 5).

Table 8
Effect of Trace Elements on Penicillin Acylase Production by *S. lavendulae*[a]

Absence of trace element in medium	Enzymatic activity (IU/L)	Protein (mg/L)	Dry weight (g/L)	pH
Nil	289	706	8.8	8.4
Mo	306	638	8.8	8.4
Cu	110	166	7.1	8.3
B	296	638	8.2	8.3
Co	272	638	8.7	8.4
Ca	278	705	8.8	8.4
Mg	297	675	7.8	8.5
Zn	285	613	8.2	8.5
Mn	296	654	8.6	8.4
Fe	236	611	9.2	8.2

[a]All experiments were carried out in medium 8 (described in Table 5).

production of other penicillin acylases is not repressed by glucose, such as penicillin V acylases from *Bacillus sphaericus* and *Erwinia aroideae (19,20)*. On the other hand, citrate had an inhibitory effect on enzyme production, although its stimulating effect in penicillin acylase synthesis by *Pleurotus ostreatus* has been reported *(21)*.

Effect of Metals on Enzyme Production

We studied the effect of the absence of each metal in the solution of oligoelements on enzyme production in order to simplify the components of the basal medium. We maintained the same concentration of all other oligoelements in the solution but eliminated one of them. Cell growth and penicillin acylase activity in the supernatant was studied after 72 h of fermentation at the optimized conditions (0.4 volume ratio and 300-rpm shaking speed), as shown in Table 8. We observed that the presence of copper had a stimulating effect on cell growth as well as on enzyme production.

In fact, copper is an essential trace element that stimulates cellular differentiation in *Streptomyces (22,23)*, contributing to microbial culture growth. As an example, the addition of copper has been reported to enhance the accumulation of biomass in cultures of similar microorganisms such as *Streptomyces tendae (24)*. In addition, an increase in cell growth of *S. lavendulae* was accompanied by an enhanced production of a dark-colored pigment, which could be related to the synthesis of melanin to the medium. In fact, melanin is commonly produced by several fungi and some bacteria, mainly actinomycetes, in their cells and/or culture media *(25)*. In *Streptomyces*, the production of melanin is widespread among different species. Similarly to higher organisms, melanin pigment can be biosynthesized using L-tyrosine as a precursor through the action of tyrosinase, an ubiquitous enzyme belonging to the group of polyphenol oxidases *(26,27)*. Tyrosinase is a copper-containing monooxygenase that catalyzes both the *O*-hydroxylation of monophenols and the oxidation of *O*-diphenols to *O*-quinones that can be further polymerized to render melanin. Thus, the presence of copper in the fermentation medium of *S. lavendulae* could also contribute to the activation and secretion of tyrosinase that would be involved in melanin biosynthesis. In fact, *Streptomyces* structural genes that encode for tyrosinase and a conserved protein essential for the expression of melanin belong to the same polycistronic operon. Such protein plays the dual role of regulating copper incorporation and promoting the secretion of apotyrosinase in *Streptomyces (28)*. Nevertheless, the apparent critical role of copper in the biosynthesis of penicillin acylase and its relationship to melanin biosynthesis has not been reported yet.

Time Course of Enzyme Production With Optimized Medium and Conditions

Figure 1 shows the time course of growth of *S. lavendulae* and penicillin acylase production using 2.5% (w/v) yeast extract and 1.5% (v/v) solution of oligoelements at the optimal fermentation conditions (0.4 volume ratio and 300-rpm shaking speed). The formation of microbial biomass occurred after 24 h of cultivation, reaching a maximum at 72 h, after which the cell mass declined significantly. Enzyme production, slightly lagged with cell growth from the beginning, increased after 48 h of fermentation, reached its maximum at 72 h, and then declined with cell growth. This fact is probably related to autolysis of the cells and breakdown of the enzyme owing to the release of intracellular proteases. The results confirmed that penicillin acylase must be considered as an enzyme involved in secondary metabolism. From the data in Fig. 1, we could calculate the rate of *S. lavendulae* cell growth ($0.105 \pm 0.01\ h^{-1}$) and the rate of penicillin acylase production ($0.4605 \pm 0.007\ h^{-1}$). The maximum yield of enzyme production was reached after 72 h of fermentation. At this time, penicillin acylase production was 289 IU/L, which corresponds to 32.9 IU/g of dry cell weight. This result was similar to that obtained using 1.5% (v/v) solution of oligoelements

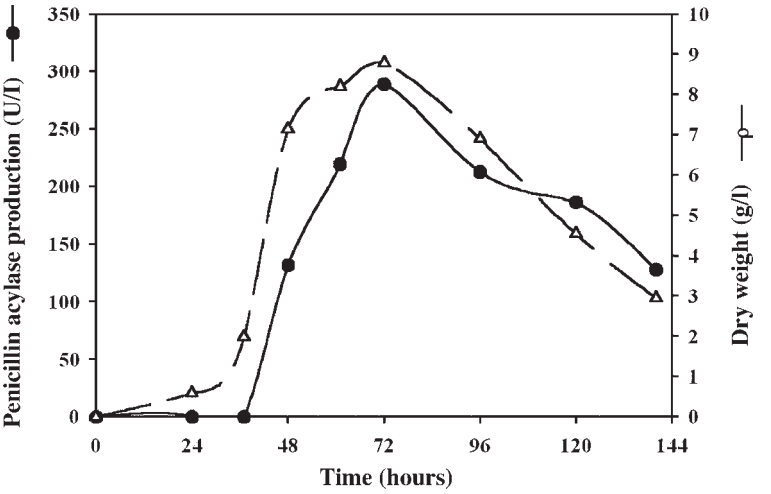

Fig. 1. Time course of cell growth of *S. lavendulae* and penicillin acylase production at 20°C using 2.5% (w/v) yeast extract and 1.5% (v/v) solution of oligoelements at optimal fermentation conditions (volume of medium/volume of flask ratio of 0.4 and shaking speed of 300 rpm). At the indicated times, cell dry weight (△) and enzyme activity (●) of the supernatant were determined. The data shown are averages of duplicate assays with the standard error within 5% of the mean values.

with only copper sulfate in its composition (which corresponded to a final concentration of 3 µg/mL of $CuSO_4 \cdot 5H_2O$ in the medium) at the same time and conditions. Our results were comparable with those for the production of other penicillin acylases such as penicillin V acylases from *B. indica* var *penicillanicum* (89.7 IU/g of dry cell weight) *(17)* and *P. ostreatus* (12 IU/g of dry cell weight) *(21)*.

Conclusion

We established a very simple fermentation medium for higher penicillin acylase production by *S. lavendulae* than previously reported *(3)*. The effect of every component of the medium—yeast extract, olive oil, and oligoelements—was studied by factorial design of experiments. We observed that the presence of olive oil did not increase enzyme production although it has been reported to be beneficial in the production of secondary metabolites by other *Streptomyces (29,30)*. The addition of 2.5% (w/v) yeast extract in 20 m*M* potassium phosphate, pH 7.0, and copper supplementation (3 µg/mL of $CuSO_4 \cdot 5H_2O$) were adequate for high penicillin acylase production at a short fermentation time (72 h). In such optimized culture medium, fermentation of the microorganism at 20°C yielded 289 IU/L in 72 h when employing a volume of medium/volume of flask ratio of 0.4 and a shaking speed of 300 rpm. As reported previously, this novel β-lactam acylase is very interesting for the antibiotic industry because it is able to hydrolyze those natural aliphatic penicillins that represent approx 2% of

the total penicillin content in the production of penicillin G and penicillin V by fermentation and create problems in the final crystallization of 6-APA. In addition, this enzyme has shown an enhanced stability and activity in the presence of organic solvents *(31–33)*, and it has been successfully immobilized to the epoxy-activated support Eupergit C™ *(34–36)*. Because of the mentioned operational and economic reasons, introduction of this enzyme as an immobilized biocatalyst in industrial processes involving the production of 6-APA from penicillin V should be highly considered in the future.

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