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Inoculum Studies in Production of Penicillin G Acylase by *Bacillus megaterium* ATCC 14945

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Abstract

This article reports studies concerning the production of penicillin G acylase (PGA) by *Bacillus megaterium*. This enzyme has industrial use in the hydrolysis of penicillin G to obtain 6-aminopenicillanic acid, an essential intermediate for the production of semisynthetic β-lactam antibiotics. Although most microorganisms produce the enzyme intracellularly, *B. megaterium* provides extracellular PGA. The enzyme production by microorganisms involves several steps, resulting in a many operational variables to be studied. The study of the inoculum is an important step to be accomplished, before addressing other issues such as culture optimization and downstream processing. In this study, using a standard inoculum as reference, several runs were performed aiming at the definition of operational conditions in the PGA production. Cell concentration and PGA activity in the production medium were measured after 24, 48, and 72 h of the beginning of the production phase. This study encompasses the duration of the inoculum germination phase and the concentration of cells used to startup the germination. Based on these results, PGA productivity during the production phase was maximized. The selected values for these variables were 1.5×10^7 spores/mL of germination medium, germination during 24 h, and 72 h for the production phase.

Index Entries: Penicillin G acylase; *Bacillus megaterium*; inoculum preparation.

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Introduction

The production of semisynthetic β-lactam antibiotics from penicillin is based on the precursor 6-aminopenicillanic acid (6-APA), which is produced after the enzymatic hydrolysis of penicillin G. The enzyme penicillin amido hydrolase (EC 3.5.1.11, also known as penicillin G acilase [PGA]) catalyzes this reaction. Most of the industrial interest regarding this enzyme derives from its acil-transfer capacity. The major product of commercial interest catalyzed by PGA is 6-APA, whose worldwide production grew from 5500 t in 1989 to 7000 t in the 2000 *(1)*.

PGA may be obtained from several microorganisms. Most of them are bacteria, but also some fungi and yeasts produce it *(2–4)*. Some known bacteria are *Escherichia coli*, *Proteus rettgeri*, *Alcaligenes faecalis*, *Arthrobacter viscosus*, *Bacillus megaterium*, and species of *Pseudomonas* and *Micrococcus (5–6)*. Enzymes from different microorganisms can present different characteristics. PGA from *E. coli* is a 90,000 mol wt, dimeric enzyme. Its smallest subunit is responsible for the recognition of the substrate (the phenylacetyl moiety), including configuration changes on the second subunit that exposes the active center (a terminal serine). PGA from *B. megaterium* is not very well characterized in the literature. Illanes et al. *(7)* report a mol wt of 120,000 for this enzyme. Savidge and Cole *(8)* report different values for kinetic parameters of PGA produced by several strains of *E. coli* and *B. megaterium*. It can be inferred from those values that the catalytic role of these enzymes is not tightly related to their primary structure.

The advantages of producing extracellular enzymes are very well known: considerable savings in downstream processing costs may be achieved. Most of the aforementioned microorganisms are not able to excrete the enzyme into the culture medium. Two exceptions are reported in the literature: *A. viscosus (9–10)* and *B. megaterium (2,7,11–12)*. The present study focused on the optimization of the inoculum for the cultivation of *B. megaterium*. This microorganism is stored in the form of endospores, and, therefore, the optimization of their germination and propagation is important to reduce the lag phase in the bioreactor. During the productive phase, an inductor (phenylacetic acid) is added to the medium, and it is desirable that the microorganism be already adapted at this point, beginning the production of PGA in the shortest possible time.

This article intends to define operational conditions for the phase of inoculum preparation (here called germination phase), aiming at the reduction of cultivation time spans. Assays were performed to check the role that two variables have on the overall productivity of the process: initial concentrations of spores and duration of the germination step.

Materials and Methods

Materials

Cheese whey was from Sigma (St. Louis, MO) and commercial casein was from Riedel (Riedel-de Haën, Germany). Enzyme Alcalase® was

donated by Novo Nordisk A/S (Bagsvaerd, Denmark). Phenylacetic acid was from Carlo Erba (Divisione Chimica Industriale, Milano, Italy) and penicillin-G potassium salt from Paraquímica S/A (SP, Brazil). Antifoam TH-201-S was donated by Union Carbide do Brasil, Itatiba (SP, Brazil). Other reactants were of analytical grade and from different manufacturers.

Microorganism

The strain *B. megaterium* ATCC 14945 was purchased from Fundação Tropical (Campinas, SP, Brazil) and kept in a refrigerator, in slant flasks. The preparation of a batch culture of spores began with the propagation of the stock culture to tubes containing agar nutrient medium, which were kept at 30°C for 7 d. The spores were then transferred to Roux flasks containing agar nutrient medium, using a 0.9% NaCl salt solution, and kept at 30°C for 7 d more. Then a 20% glycerol solution was added, and 3 mL of the suspension, containing approx 10^8 spores/mL, was transferred to cryovials and kept at –50°C. Two batches of spores were used during this experiment.

Culture Media

The germination medium was composed of40.0 g/L of hydrolyzed casein (enzymatically, with Alcalase), 7.0 g/L of cheese whey, and 0.5 mL/L of antifoam at pH 8.0. The production medium contained in 51.3 g/L of hydrolyzed casein, 19.6 g/L of cheese whey, 4.1 g/L of calcium carbonate, 2.7 g/L of phenylacetic acid, and 0.5 mL/L of antifoam at pH 8.0. When using the second batch of spores, a salt solution was added with the following composition: 75.3 Mg/L of $MgCl_2 \cdot 6H_2O$, 55.1 mg/L of $CaCl_2 \cdot 6H_2O$, 5.3. mg/L of Na_2SO_4, 14.5 mg/L of $FeCl_3 \cdot 6H_2O$, 1.21 mg/L of $CoCl_2 \cdot 6H_2O$, 1.07 mg/L of $MnCl_2 \cdot 4H_2O$, 0.8 mg/L of $CuCl_2 \cdot 2H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and 0.063 mg/L of $ZnCl_2$.

Cultivation Conditions

At the beginning of the germination phase, Erlenmeyer flasks were inoculated with spores suspension (from the cryogenic vials) resulting in initial concentrations of 8.5×10^6, 1.5×10^7, and 2.5×10^7 spores/mL medium and incubated for 24, 48, or 72 h at 30°C in a rotary shaker (New Brunswick) at 300 rpm. The seed medium represented 10% of the total volume of the enzyme production medium. The production phase was also proceeded for 72 h at 30°C in a rotary shaker at 300 rpm, and for each inoculum concentration and germination time. The inductor, phenylacetic acid, was added after 8 h of cultivation. At the end of the assay, the samples were centrifuged at 14,000*g* for 20 min at 4°C. The solid material was collected and dried to determine cellular concentrations. The supernatant was used to conduct further analyses.

Analytical Methods

The enzymatic activity ofPGA was determined by hydrolysis of 0.2 *M* penicillin G, at pH 8.0 (with 10 m*M* phosphate buffer) and 37°C. The 6-APA

Table 1
PGA and Biomass Production for
Germination of 8.5×10^6 Spores/mL of Medium

	Production phase (h)					
	24		48		72	
Germination phase (h)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)
24	46	4.1	88	5.2	115	5.2
48	52	4.4	110	5.2	89	5.3
72[a]	47 ± 7	3.6 ± 0.4	96 ± 10	5.7 ± 0.5	106 ± 10	5.4 ± 0.5

[a]Triplicate.

produced was measured spectrophotometrically at 415 nm, after reaction with dimethylaminebenzaldehyde, according to Balasingham et al. *(13)*. One activity unit (IU) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of 6-APA from penicillin G within 1 min under these experimental conditions. Initial velocities were taken (linear phase of the curve) and a large excess of substrate in relation to the enzyme concentration was used (zero-order reaction).

Biomass concentration was determined as dry wt of solids, after 24 h at 60°C.

Results and Discussion

Table 1 gives the results of PGA production. The initial concentration of spores was fixed (8.5×10^6 spores/mL), and different germination times and production phase lengths were tested, in order to check whether values previously reported (*[14]*: 72 h for germination) provided the maximum enzyme activity. Germination of spores is very fast (about 2 h) *(15)*. Additional incubation is intended to increase cell mass before production phase (when phenylacetic acid, inductor, is added).

From Table 1 it is possible to conclude that the best results were achieved when 24 h was used for germination, followed by a production phase of 72 h. Germination runs longer than 24 h did not improve significantly the final enzyme productivity. After running the production phase for 24 h, the supernatant displayed enzyme activity equal to 50 IU/L, no matter what its germination time span. Small variations in cell mass that could be observed from one assay to another apparently did not interfere with the production of enzyme. Therefore, a reduction in the duration of the germination phase could be achieved without affecting the overall productivity of the process. Table 2 provides the calculated productivity and yield of product with respect to biomass (the specific productivity) for 8.5×10^6 spores/mL of medium at the beginning of the germination step.

Table 2
Productivity and Yield for 8.5×10^6 Spores/mL of Medium

Run duration (h) ($t_{germination} + t_{production}$)	PGA final activity (IU/L)	Productivity (UI/[L·h])	Product/biomass yield (UI/g_{cell})
(24 + 24) = 48	46	1.0	11
(24 + 48) = 72	88	1.2	17
(24 + 72) = 96	115	1.2	22
(48 + 24) = 72	52	0.7	12
(48 + 48) = 96	110	1.1	21
(48 + 72) = 120	89	0.7	17
(72 + 24) = 96	47	0.5	13
(72 + 48) = 120	96	0.8	17
(72 + 72) = 144	106	0.7	20

Table 3

PGA and Biomass Production for
Germination of 2.5×10^7 Spores/mL of Medium

	Production phase (h)					
	24		48		72	
Germination phase (h)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)
24	64	4.3	88	4.8	89	4.6
48	61	4.2	88	4.8	96	5.2
72	59	3.6	89	5.3	106	5.6

A second set of assays (Table 3) was designed to check these results with respect to the duration of the runs, and to verify whether a higher load of spores (2.5×10^7 spores/mL) would significantly improve the productivity. Table 3 shows that enzyme concentration after 48 h of the beginning of the production phase was essentially the same, irrespective of the germination time span. The slightly higher cell mass that is observed when the germination was carried out for 72 h does not reflect higher enzyme activity; in other words, the yield is lower in this assay. Based on these results, we may conclude that, for our case, a 24-h germination run followed by 72 h of cultivation was the optimal procedure to increment PGA productivity and yield.

Regarding the load of spores, it is noticeable that a higher initial concentration of spores (Table 3) did not lead to higher biomass or higher enzyme production, at least for our work conditions. Therefore, to simplify the experimental procedure, the suspension volume of the spores was fixed at 3 mL (volume contained in the cryogenic vials), which results in an initial concentration of 1.5×10^7 spores/mL of germination medium.

Table 4
Productivity and Yield for 2.5×10^7 Spores/ mL of Medium

Run duration (h) ($t_{germination} + t_{production}$)	PGA final activity (IU/L)	Productivity (UI/[L·h])	Product/biomass yield (UI/g_{cell})
(24 + 24) = 48	64	1.3	15
(24 + 48) = 72	88	1.2	18
(24 + 72) = 96	89	0.9	20
(48 + 24) = 72	61	0.9	15
(48 + 48) = 96	88	0.9	18
(48 + 72) = 120	96	0.8	18
(72 + 24) = 96	59	0.6	17
(72 + 48) = 120	89	0.8	17
(72 + 72) = 144	106	0.7	19

Table 5
PGA and Biomass Production of Spores from
Lot #2 and Germination of 1.5×10^7 Spores/mL of Medium

	Production phase (h)					
	24		48		72	
Germination phase (h)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)	PGA (IU/L)	Cells (g/L)
24	71	6.1	71	6.4	90	6.2
48	51	5.5	81	7.2	92	6.2

Table 4 displays the calculated productivity and yield of product with respect to biomass (the specific productivity) for 2.5×10^7 spores/mL of medium. Table 4 shows that the yield for this combination (24 + 72 h) was also maximized. Productivity and yield were slightly lower than for the concentration of 8.5×10^6 spores/mL but still within a 10% margin of error.

It is a common feature in bioreaction engineering that the optimum conditions may vary significantly when different sets of cryogenically conserved microorganisms are inoculated in the bioreactor. To check the reported conclusions, a second set of spores was used in similar experiments. The results obtained for lot #2 of *B. megaterium* ATCC 14945 spores are displayed in Table 5 and Fig. 1, for 24 and 48 h of germination and the same production time spans used formerly.

The presented results confirm the previous ones obtained using the first set of spores. There was no increase in enzyme concentration after 72 h of the production phase, when germination phase time was increased from 24 to 48 h. The productivity obtained using the second set of spores

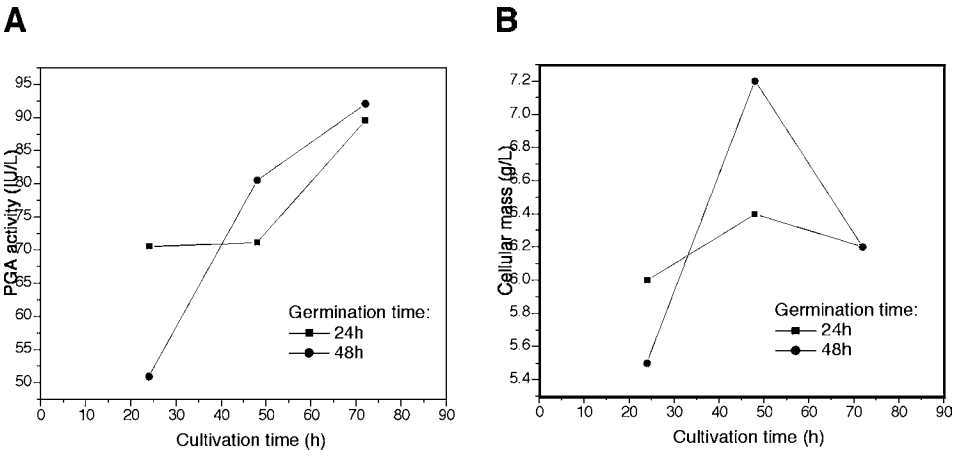

Fig. 1 **(A)** PGA activity and **(B)** cellular mass of spores from lot #2 and germination of 1.5×10^7 spores/mL of medium.

was indeed lower than the former one. Nevertheless, the mix of salts described in Materials and Methods *(7)* was able to restore the strain productivity within reasonable levels. The oscillation of the productivity was acceptable, if one takes into account the experimental errors as well as the unavoidable variations that occur when different lots of inoculum are used.

Conclusions

After testing different strategies to prepare the inoculum of *B. megaterium* to produce PGA, our conclusion is that 24 h is enough for the microorganism to germinate adequately. The preliminary results, in a shaker, indicate that the production phase should endure 72 h, in order to reach the highest overall productivity of the cultivation in a bioreactor.

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