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Study of Different Media for Production of Penicillin G Acylase from *Bacillus megaterium* ATCC 14945

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Abstract

In this study, several fermentation media were tested for the production of penicillin G acylase (PGA) using *Bacillus megaterium*. The carbon sources studied were glucose and lactose. The nitrogen sources studied were enzymatic casein hydrolysates produced with proteases of different specificities. The replacement of glucose with cheese whey and the addition of free amino acids in the PGA production were also tested. The results showed a strong correlation between the nitrogen source and enzyme yield and the presence of glucose repression. The highest enzyme concentration achieved was 138 IU/L using casein hydrolyzed with 0.6 L of Alcalase® and cheese whey.

Index Entries: *Bacillus megaterium*; penicillin G acylase; fermentation; medium optimization; enzymatic casein hydrolysates; cheese whey.

Introduction

Penicillin G acylase (PGA) is one of the most important industrial enzymes. Its main application is the hydrolysis of penicillin G to obtain 6-aminopenicillanic acid (6-APA), a key compound for the production of semisynthetic antibiotics such as amoxicillin and ampicillin. More than 60% of the 6-APA produced worldwide uses the enzymatic route. The estimated global production of 6-APA for the year 2000 is 7000 t/yr *(1)*, and the total operating cost of 6-APA is 52.57 (US$)/kg. The enzyme represents approx 7% of total cost *(2)*.

Most of studies found in the literature on PGA production refer to the intracellular expression of the enzyme by *Escherichia coli (2–5)*. *Bacillus megaterium* can produce the extracellular enzyme, facilitating its purification

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and consequently decreasing downstream and final product cost. Despite the potential advantages of the extracellular enzyme, few studies related to *B. megaterium* PGA are available in the literature, possibly because information on its production is protected by industrial secrecy.

These studies state that the production of PGA by *B. megaterium* is induced by phenylacetic acid *(1,6–8)*, but high concentrations of this acid inhibit the microorganism's growth. The enzyme expressed by this microorganism is specific for penicillin G *(1)*. The need for specific amino acids, such as histidine, is also reported *(7)*. Studies on defined (using histidine as the nitrogen source) and complex media (using casein hydrolysate as the nitrogen source) showed best results in a complex medium without glucose and an apparent dependence of the enzyme excretion on phosphate levels *(1)*. In one of the most recent works *(8)*, a statistical optimization was used to determine the best medium composition for *B. megaterium* PGA production. Glucose was the carbon source and enzymatically hydrolyzed casein was the nitrogen source.

Cheese whey is the principal liquid byproduct of the dairy industry, with an estimated global production of 145 million/yr *(9)*. About 40% of this byproduct is usually discarded without treatment, causing serious environmental damage because of its high biological oxygen demand: 40,000–50,000 mg O_2/L *(10)*. From this standpoint, besides the potential advantages of cheese whey lactose as a carbon source to improve PGA production, its use would contribute to decreased water contamination.

The objective of this study was to produce penicillin G acylase using *B. megaterium*. The influence of medium composition on *B. megaterium* PGA production was studied, after changing carbon and nitrogen sources. Glucose was compared to analytical grade lactose and to dry and fresh cheese whey, which contains about 50.0 g of lactose/L. Several nitrogen sources were studied. They were obtained from casein, hydrolyzed using proteases with distinct specificity. The addition of specific amino acids was also studied.

Materials and Methods

Materials

Glucose, lactose, antifoam Solvitol LV (Pluronic PE6100), arginine, asparagine, proline, and histidine were supplied by Synth S/A; phosphates and other mineral salts were from Merck; phenylacetic acid was from Carlo Erba, from Synth SIA; and penicillin G potassium salt was from Paraquimica S/A (SP, Brazil). Dried and fresh cheese whey were donated by Nestle S/A (Brazil) and Cooperativa de Laticínios São Carlos (SP, Brazil), respectively. Dry cheese whey contained 9.0 g/L of protein and 47.6 g/L of lactose, and fresh cheese contained 5.3 g/L of protein and 47.6 g/L of lactose. Commercial hydrolyzed casein was purchased from Difco and casein from Riedel. The enzymes Neutrase® (0.5 L) and Alcalase® (0.6 L) were donated by Novo Industria do Brasil.

Microorganism: Maintenance and Propagation

B. megaterium ATCC 14945 was purchased from Fundação Tropical (Campinas, SP, Brazil) and maintained on 2% agar slants containing 3 g/L of meat extract, 5 g/L of peptone, and 1.5 g/L of glucose. Cells were allowed to grow for 7 d at 30°C.

Fermentation Conditions and Media Composition

Preparation of Inoculum

Erlenmeyer flasks containing two different media were incubated for 72 h and 30°C in shaker at 300 rpm (New Brunswick Scientific Co., NJ). The first medium consisted of enzymatically hydrolyzed casein (40 g/L), glucose (5 g/L), and antifoam (0.5 mL/L). In the second medium, glucose was replaced with cheese whey (7 g/L). The seed medium represented 10% of the total volume of the fermentation medium for enzyme production.

Enzyme Production

Erlenmeyer flasks containing production medium and inoculum were incubated for 72 h at 300 rpm and 30°C. The medium composition was based on two different media reported in the literature, changing the carbon source and/or nitrogen source. Medium A was designed for carbon limitation *(1)*. It was used in runs 1 and 3 and contained 75.3 mg/L of $MgCl_2 \cdot 6H_2O$, 55.1 mg/L of $CaCl_2 \cdot 6H_2O$, 53.3 mg/L of $Na_2 \cdot SO_4$, 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 $CuCl_2 \cdot 2H_2O$, 0.68 mg/L of $Na_2MoO_4 \cdot 2H_2O$, and 0.063 mg/L of $ZnCl_2$. The level of phosphate (at KH_2PO_4/Na_2HPO_4 ratio to obtain pH 7.0) was 32.3 g/L. The nitrogen source was 3.4 g/L of commercial casein hydrolysate and the carbon source was 0.7 g/L of glucose. In runs 4 and 5, medium A was used to replace commercial casein hydrolysate by the same amount of casein hydrolyzed using Neutrase (run 4) and glucose by 1.3 g/L of dry cheese whey (run 5). Medium B *(8)* was used in runs 2 and 6–13 and always contained 4.0 g/L of $CaCO_3$. As the carbon source, medium B contained 7.9 g/L of glucose (run 2) or cheese whey (runs 6–12), as specified in Tables 1–4. As the nitrogen source, medium B contained 51.0 g/L of casein hydrolyzed using different proteases, as specified in Table 3. In run 7, 9.6 g/L of free amino acids (arginine, proline, histidine, and asparagine) was added to medium B. The inducer, phenylacetic acid, was added after 10 h of fermentation, 1.5 g/L in medium A and 2.1 g/L in medium B. Fresh cheese whey was used only in run 13.

Casein Hydrolysis

Hydrolysis of 8% casein was carried out batchwise in a Metrohn pH-stat, model Titrino, using a 0.5 *M* NaOH solution to keep pH constant. The temperature (50°C) and pH used in the assays are the optimum values recommended by Novo Industri: pH 7.0, 7.5, and 8.0 for 0.5 L of Neutrase, 0.5 L of Neutrase + 0.6 L of Alcalase, and 0.6 L of Alcalase, respectively. The mass relation between casein and enzyme used in the experiments was 50:1.

After fermentation, the samples were centrifuged at 14,000*g* for 20 min at 4°C. The sedimented material was collected and dried to determine cellular concentrations. The supernatant was used to carry out further analyses.

Analytical Methods

Enzyme activity was determined by hydrolysis of 0.2 *M* penicillin G, at pH 8.0 (10 m*M* phosphate buffer) and 37°C. The 6-APA produced was measured spectrophotometrically at 415 nm, after reaction with dimethylaminebenzaldehyde, according to Balasingham et al. *(3)*. Samples were taken every minute and the initial velocity of the reaction was calculated. Appropriate enzyme concentration in the reaction mixture was used to ensure that the maximum velocity of the reaction was measured. The cell-associated activity was measured in the supernatant after rupture of cells. The microorganism's cells were broken using small glass beads in a refrigerated centrifuge. 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 experimental conditions.

The protein concentration was determined using the Lowry method, modified by the Hartree *(11)* and Bradford *(12)* methods. The protein contents in cheese whey was determined using the modified Lowry method *(11)*. To verify the presence of protease activity in the PGA production by *B. megaterium*, 5 mL of the supernatant was added to 20 mL of casein solution (8% weight basis), in a pH-stat, at 30°C and pH 7.0, and the consumption of a 0.5 *M* NaOH solution was followed.

The method of Somogyi *(13)* was used to determine lactose, whereas glucose was measured using the enzymatic method (glucose-oxidase) from Merck-RJ Br *(14)*.

Results and Discussion

In runs 1 and 2, two different media reported in the literature *(1,8)* were used to produce PGA by *B. megaterium* ATCC 14945. Although Illanes et al. *(1)* and Hojo *(8)* had obtained enzymatic activity values of 160 and 192 IU/L, respectively, the results of these runs showed that the microorganism grew but that no enzyme activity was detected using either medium A or medium B. In run 3, which reproduces the composition of run 1, the values of enzyme activity (in the supernatant and associated to the cells) and the values of glucose, lactose, and protein concentrations were monitored at different fermentation times. These results are shown in Fig. 1 and confirm microorganism growth and no enzyme production. All fermentation conditions described by Illanes et al. *(1)* were reproduced, including inoculum size. The reported initial cell concentration in the production medium *(1)* was similar to the one determined in run 3. Illanes et al. *(1)* detected the highest enzyme activity at around 60 h of fermentation, 40 h after glucose depletion. The results presented in Fig. 1 show that

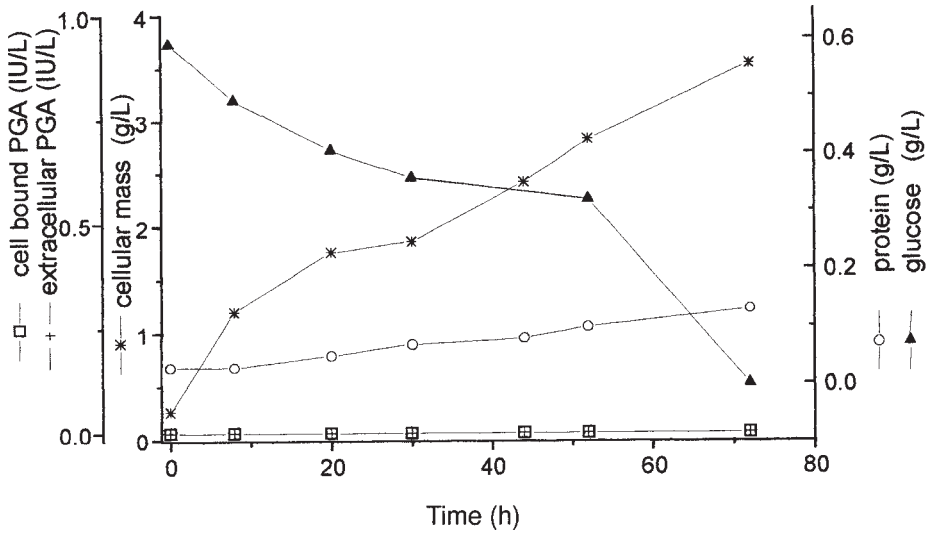

Fig. 1. Several variables monitored at different fermentation times on run 3. A reproduction of one of the reported media to produce PGA by *B. megaterium (1)*.

Table 1
Results for Final Carbon Source Consumption, Biomass Concentration, and PGA Production by *B. megaterium*[a]

Run	$HCas_0$[b] (g/L)	$ChWe_{d0}$[b] (g/L)	Amino acids (g/L)	Time (h)	Glucose (g/L)	Lactose (g/L)	Cells (g/L)	PGA activity (IU/L)
4(A)	3.4 ($HCas_{NE}$)	—	—	0	0.6	—	0.65	0
				72	0.0	—	4.4	5.03
5(A)	3.4 ($HCas_{com}$)	1.3	—	0	—	0.90	0.69	0
				72	—	0.75	5.4	0
6(B)	51.0 ($HCas_{NE}$)	19.6	—	0	—	14.30	0.63	0
				72	—	11.60	6.6	45.6
7(B)	51.0 ($HCas_{NE}$)	19.6	9.6	0	—	10.40	0.8	0
				72	—	7.20	8.0	81.7

[a]In each run, either the carbon source or the nitrogen source was changed. $HCas_{com}$, commercial casein hydrolysate (Difco); $HCas_{NE}$, casein hydrolyzed using Neutrase; $ChWe_{d0}$, initial dry cheese whey concentration in the production medium; $HCas_0$, initial casein hydrolysate concentration in the production medium. (A), medium A is used, changing either carbon or nitrogen source; (B), medium B is used, changing either carbon or nitrogen source.

[b]Variables values in $t = 0$ h.

a longer time was necessary for glucose depletion in run 3, with a slower growth of the microorganism, when compared to reported results *(1)*. Perhaps a fermentation time longer than 72 h could lead to the same PGA production than the one achieved by Illanes et al. *(1)*. Nevertheless, they reported enzyme activity of about 60 IU/L before 20 h, time for glucose depletion in their work, whereas there was no sign of PGA in the whole period of 72 h of fermentation in run 3.

In view of this, we decided not to repeat this run. The increase in protein concentration with fermentation time indicates the production of proteins other than penicillin G acylase. Several enzymes are known to be secreted by *B. megaterium*, including α-amylase, β-amylase, neutral protease, β-glucanase, megacins (the phospholipase Meg A), glucanotransferase, and chitosanases *(15)*. Therefore, some other enzyme could be responsible for the detected increase in protein in the supernatant, but this aspect was not elucidated in our study. The obtained results in these three first runs showed that despite the fact that all the refereed microorganisms were ATCC 14945, the strain used in this study showed a different behavior from those reported in the literature *(1,8)*. At this point, an exploratory work was begun.

The first attempt to find an operational condition to produce PGA by *B. megaterium* was to change the carbon and nitrogen sources in media A and B. Media compositions of runs 4–7 are reproductions of media A and B, in which commercial casein hydrolysate was replaced by casein hydrolyzed by Neutrase and glucose was replaced by cheese whey. In run 7, free amino acids were added to the medium. Table 1 shows the results obtained in these runs. In run 4, commercial casein hydrolysate was replaced by casein hydrolyzed by Neutrase. The different specificities of the proteases results on peptides with different molecular weights and structures. Thus, the same amount of hydrolyzed casein may contain different amounts of more easily assimilable nitrogen source. In runs 5(A) and 6(B), glucose was replaced by lactose present in cheese whey *(16)*. The results of these runs showed that enzyme production is related to the concentration of nitrogen source if a low level of glucose concentration is used. When 3.4 g/L of commercial casein hydrolysate (Difco) was replaced by the same low amount of casein hydrolyzed using Neutrase, the presence of 5 IU/L of PGA in the fermentation medium was already observed. In run 6, a higher concentration of casein hydrolyzed using Neutrase was used (51.0 g/L). The results of this run show a significant production of the enzyme (45.6 IU/L). Results of run 7 show a still higher enzyme activity (81.7 IU/L) when free amino acids were added to the medium. The need for specific amino acids has already been reported in the literature *(7)*. The results of run 6 also indicate that PGA production could be repressed by glucose at levels used in run 2 (7.9 g/L). The only difference between run 2 and 6 was the use of glucose instead of cheese whey.

Although a low PGA activity was obtained in run 4 (5 IU/L) when compared to the one reported in Illanes et al. *(1)*, this result shows that the specificity of the protease used to hydrolyze casein is a significant variable. Changing the protease is a cheaper way of increasing the concentration of assimilable nitrogen than by adding free amino acids. Thus, different casein hydrolysates were produced and tested as the nitrogen source for PGA production. Figure 2 shows casein hydrolysis using different proteases, and Table 2 lists the respective values of protein concentration, determined by the modified Lowry and Bradford methods. The influence of these different hydrolysates on *B. megaterium* PGA production were studied in

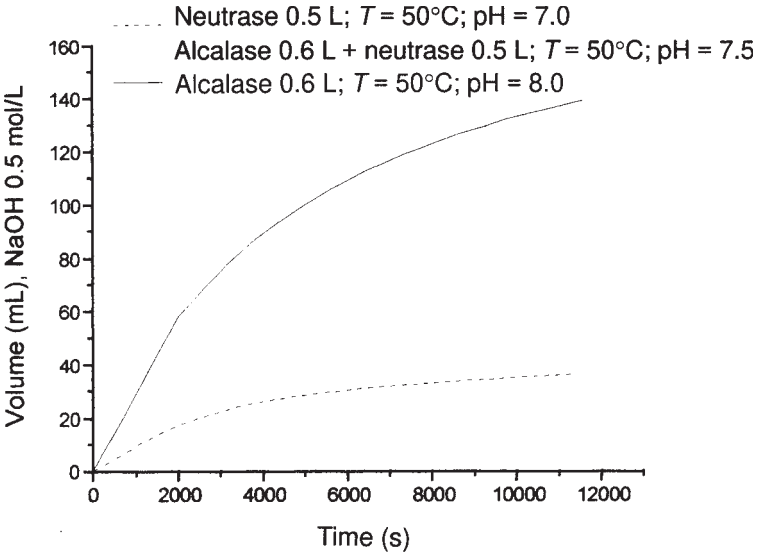

Fig. 2. Hydrolysis of casein using different proteases (substrate, 8%; enzyme-substrate ratio 1/50).

Table 2
Protein Concentration Before and After Hydrolysis of 8% Casein, Using Different Proteases and Determined Using Two Analytical Methods

Casein	Protein (g/L) (Bradford method)	Protein (g/L) (Lowry method)
Before hydrolysis	55.30	79.7
Hydrolyzed by Alcalase	0.46	88.0
Hydrolyzed by Alcalase + Neutrase	0.56	86.0
Hydrolyzed by Neutrase	2.33	75.9

runs 8–10, the results of which are shown in Table 3. It can be observed in Fig. 2 that the highest degree of hydrolysis is achieved using Alcalase, which was confirmed by the results presented in Table 2, showing the protein contents determined using both the modified Lowry and the Bradford methods. The latter method can only measure oligopeptides larger than 5000 Daltons. Therefore, although the hydrolysates show similar Lowry protein concentrations, it can be observed that the one produced with Alcalase shows the smallest protein concentration. Table 3 shows that the highest PGA activity is achieved using Alcalase as protease, which indicates that the presence of a more easily metabolizable nitrogen source favored enzyme production.

The next step was the investigation of the role of cheese whey on PGA production. The main metabolizable components of cheese whey are lactose and protein. The proteins present in cheese whey cannot be metabolized without previous hydrolysis. Therefore, unless *B. megaterium*

Table 3
Influence of Specificity of Protease Used
to Obtain Casein Hydrolysates on Production of PGA by *B. megaterium*

Run[a]	Time (h)	Cells (g/L)	PGA activity (IU/L)	Protein (g/L)[b]	Protein (g/L)[c]	Lactose (g/L)
8(B)	0	0.27	—	0.97	75.06	14.94
HCas Alcalase	72	—	108.95	1.14	54.65	13.53
9(B)	0	0.40	—	0.89	72.42	14.94
HCas Neutrase/Alcalase	72	—	79.37	0.91	52.67	13.13
10(B)	0	0.54	—	1.83	87.57	12.30
HCas Neutrase	72	3.76	69.03	1.98	67.15	4.46

[a]HCas, casein hydrolysate; (B), medium B is used, changing either carbon or nitrogen source.
[b]Determined using the Bradford method.
[c]Determined using the modified Lowry method.

Table 4
Effect of Replacement of Dry Cheese Whey
with Analytical Grade Lactose and Fresh Cheese Whey
on Production of PGA by *B. megaterium*

Run[a]	Time (h)	Cells (g/L)	PGA activity (IU/L)	Protein (g/L)[b]	Protein (g/L)[c]	Lactose (g/L)
11(B) = run 8	0	0.20	—	0.72	82.74	11.77
$ChWe_d$	72	3.05	102.00	1.05	66.06	9.09
12(B)	0	0.22	—	0.35	79.66	9.37
Lactose	72	—	60.22	0.47	59.47	6.09
13(B)	0	1.56	—	1.07	73.74	12.26
$ChWe_F$	72	—	138.00	1.15	59.47	11.29

[a]$ChWe_d$, dry cheese whey; $ChWe_f$, fresh cheese whey.
[b]Determined using the Bradford method.
[c]Determined using the modified Lowry method.

produces an extracellular protease, cheese whey will be simply a carbon source. Protease activity in the fermentation medium was checked and protease production was never detected, under the experimental conditions we used. Therefore, the replacement of cheese whey by analytical grade lactose should lead to the same production of the enzyme. Table 4 shows the results of this test. Run 11 is a replicate of run 8. In run 12, cheese whey was replaced by lactose. In run 13, dry cheese whey was replaced by fresh cheese whey, to verify whether the drying process may cause loss of cheese whey nutrients such as vitamins. The results presented in Tables 3 and 4 show that the same enzyme activity that was achieved in run 8 was obtained in run 11, which confirms the results obtained earlier. In run 12, an impressive decrease in enzyme activity is observed after the replacement of cheese whey by lactose, indicating the important contribution of

other nutrients present in cheese whey for PGA production. This result is confirmed by run 13, in which the use of fresh cheese whey led to an even better production of PGA. The improvement observed using fresh cheese whey may be caused by the presence of higher concentrations of the key nutrient(s). This unidentified nutrient(s) may suffer partial degradation during the cheese whey drying process.

The maximum value of enzyme activity achieved in the present study (138 IU/L) is close to those reported by Illanes et al. *(1)* and Hojo *(8)*. However, further studies to improve *B. megaterium* PGA production are still in progress in our laboratory. It has been suggested that penicillin acylase is involved in the degradation of phenylacetylated compounds for the generation of phenyl acetic acid, which may be used as a carbon source when the bacterium is in its free-living mode *(17)*. Nevertheless, the regulatory mechanisms for PGA expression are not well established because the natural function of the enzyme is still unclear *(18)*. Therefore, further exploratory optimizations of the medium are necessary.

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