

Cephalosporin C production and genetic improvement of the fungus *Acremonium chrysogenum* based on morphological mutant isolation*

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ABSTRACT

C-10 strain of the fungus *Acremonium chrysogenum* produced about 1.5 g/l of cephalosporin C, a yield that was much lower than expected according to the literature. This discrepancy, confirmed by exhaustive tests, showed that C-10 strain lost its high cephalosporin C production ability, probably due to genetic events. Strain improvement, based on isolation of morphologically compact mutant colonies, confirmed that this is a useful procedure for isolation of strains with increased titer. About 2% of tested colonies produced significantly more cephalosporin C than the original strain.

INTRODUCTION

DNA recombinant techniques have been used to improve cephalosporin production in *Acremonium chrysogenum* (Samson *et al.*, 1985, 1987; Skatrud *et al.*, 1987; Chapman *et al.*, 1987; Baldwin *et al.*, 1990; Hoskins *et al.*, 1990; Smith *et al.*, 1992). These techniques have some advantages when compared to classical procedures. For example, mutations can be directed, and thus drastically reduce the number of isolates that must be screened to obtain an improved strain (Skatrud *et al.*, 1989).

However, classical techniques are still extensively used to improve fungi. According to Lein (1983), although non-recombinant DNA approaches do not have the elegance of modern ones, fermentation problems are diverse in nature and generally involve multigene characteristics. In these cases, non-recombinant DNA methodologies are still used for the improvement of fungal polygenetic traits. Moreover, the application of classical techniques permits a more appropriate use of recombinant DNA procedures.

Acremonium lacks most of the structures and biological features of *Aspergilli* and *Penicillia* (Peberdy, 1987). On the other hand, parasexual events are useful and have been applied to this asexual fungus (Nüesch *et al.*, 1973; Ball and Hamlyn, 1978; Hamlyn *et al.*, 1985). Random selection is also a powerful tool and is utilized for genetic improvement of this fungus (Trilli *et al.*, 1978; Smith, 1985).

An alternative strategy to random selection is rational selection, that consists in isolating improved mutants from different selective lines. Chang and Elander (1979) reported effective methods for the direct

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selection of improved variants of *A. chrysogenum*. In one of these lines, the authors studied three types of morphological mutants. They observed that those which formed smaller compact colonies on a chemically defined medium showed the largest proportion of improved isolates (about 4%).

The aim of the present work was to improve cephalosporin C production of *A. chrysogenum* C-10 strain in shake-flask conditions.

MATERIAL AND METHODS

Microorganisms

C-10 strain of *Acremonium chrysogenum* (ATCC 48272) and *Alcaligenes faecalis* (ATCC 8750).

Media

Chemically defined medium (CDM) (in g/l): 20.0 sucrose, 3.0 NaNO₃, 0.5 K₂HPO₄, 0.5 KH₂PO₄, 0.5 KCl, 0.5 MgSO₄·7H₂O, 0.01 FeSO₄·7H₂O, 15.0 agar. The pH was adjusted to 7.2 with 1.0 N NaOH and sterilized at 121°C for 15 min. Complete medium (CM): the CDM plus (g/l): 4.0 yeast extract (Difco), and 4.0 bacto-peptone (Difco).

Seed medium (SM) (in g/l): 40 soluble starch, 30 corn steep liquor, 10 soybean meal, 20 soybean oil, 3.0 CaCO₃, 1.0 (NH₄)₂SO₄. The pH was adjusted to 7.0 with 1.0 N NaOH and sterilized at 121°C for 15 min.

Fermentation medium (FM) (in g/l): 36 sucrose, 27 glucose, 20 yeast extract (Difco), 24 soybean oil, 7.5 CaSO₄, 10 CaCO₃, 8.0 (NH₄)₂SO₄, 3.0 D,L-methionine. The pH was adjusted to 6.4 with 1.0 N NaOH and sterilized at 121°C for 15 min.

Tryptic soy broth (TSB): 30 g/l of TSB (Difco). The pH was adjusted to 7.3 with 1.0 N NaOH or 1.0 N HCl and sterilized at 121°C for 15 min. Tryptic soy agar (TSA) was prepared by adding 8 g/l or 15 g/l of agar (Difco) to TSB medium.

Stock cultures of *A. chrysogenum* were maintained on CM slants at 5°C. *A. faecalis* was cultured on TSB at 37°C and maintained on TSA slants at 5°C, or lyophilized.

Growth conditions

The incubation temperature was 25°C in all cases. Shaker conditions were 250 rpm for the following times: seed, two days, C-10 fermentation, seven days, and mutant fermentation, six days.

Fermentation of C-10 strain

Mycelia of 7-day old CM slant cultures were suspended in 6 ml saline with a pipette. The suspensions were transferred to test tubes containing glass beads and homogenized for one min in a vortex. Suspensions of 2.5 ml were transferred to 500-ml Erlenmeyer flasks containing 50 ml SM. After incubation, 1.6 ml SM medium was transferred to a 500-ml Erlenmeyer containing 40 ml FM. Three flasks were used to test cephalosporin production every 24 h for seven days.

Analytical methods

Growth was measured by centrifugation (Pan et al., 1982). Sugar reduction was determined by the DNS method (Milles, 1959), and cephalosporin C was determined by HPLC after filtration through a 0.20 µm filter (Millipore). Analyses were carried out in a Varian (9010 and 9050) chromatograph with a Lichrospher RP-18 column (Merck) using a 254-nm detector. The mobile phase was 0.03% KH₂PO₄ (p/v).

Random selection

Suspensions prepared as described above were irradiated with short wave UV light in order to obtain 1 and 5% survival, and then, diluted to 50-100 colony-forming units (c.f.u.)/ml. Aliquots of 0.1 ml were spread on 10-cm diameter petri dishes containing 20 ml CM. After a 5-day incubation period, the plates were replicated and turned upside down. Then, 1.5 ml chloroform was added to the lid of each plate, and after 30 min the plates were kept open for 15 min. Then, 10 ml TSA medium containing 0.8% agar and a high concentration (10⁸ cells/ml) of the cephalosporin sensitive bacterium, *A. faecalis*, were poured over the colonies. After overnight incubation, the inhibition haloes were measured and the equivalent concentration of cephalosporin C obtained by comparison to a standard assay. The colonies that showed production higher than the population average were considered as possible improved isolates and were tested using the same methods described for the C-10 strain. After the tests, a new classification was made and the mutants with a cephalosporin C production superior to the C-10 population average were kept for further tests.

Rational selection

Isolation of compact colonies: suspensions prepared and irradiated as described above were diluted

and spread on petri dishes containing CDM. After a 15-day incubation period, small compact colonies were isolated.

Fermentation tests: the compact mutants were tested in the same way as described above for cephalosporin C production in the shaker. Those with increased production were kept for further tests.

Fermentation tests of mutants with superior production of cephalosporin C

The fermentations were carried out as described for the C-10 strain. Six repetitions were carried out for each strain.

Statistical analysis

Comparisons between each mutant and the original strain were performed by the Kolmogorov-Smirnov non-parametrical procedure (Siegel, 1975). In addition, the Dunnet procedure (Steel and Torrie, 1960) was used for variance analysis.

RESULTS AND DISCUSSION

C-10 Production

C-10 is known to be a high cephalosporin C-producing strain. Shen *et al.* (1986) report a production of 18 g/l in shaker fermentations using a complex medium. Rowlands (Panlabs, Inc.) stated that under the same conditions C-10 strain can produce between 10 and 13 g/l of cephalosporin C (personal communication). We made exhaustive attempts to reach the mentioned level. These included tests of different media and fermentation conditions (data not shown). However, the maximum production obtained was 1.5 g/l of cephalosporin C (Figure 1). This low production was first observed in the culture acquired from ATCC and confirmed in the culture kindly provided by Rowlands. It is possible that the C-10 strain lost its high cephalosporin C production ability due to genetic events. MacDonald (1968) and Ball (1973) isolated derivatives from industrial strains of *Penicillium chrysogenum* with reduced penicillin production. They explained the production decrease as a possible result of mutations that occurred during storage. The latter authors emphasized the lack of data in the literature concerning the occurrence of these variations. Also, Skatrud and Queener (1989), Smith *et al.* (1991), and Walz and Kück (1991), using different methods,

reported that industrial strains of *A. chrysogenum* have chromosome patterns clearly different from the original ones. This indicates that chromosome translocation and other aberrations occurred during the process of genetic improvement of those strains. As C-10 is an industrial strain, it is likely that its chromosomes have some translocations and that during storage a rearrangement could have occurred which abruptly decreased the production level of the strain.

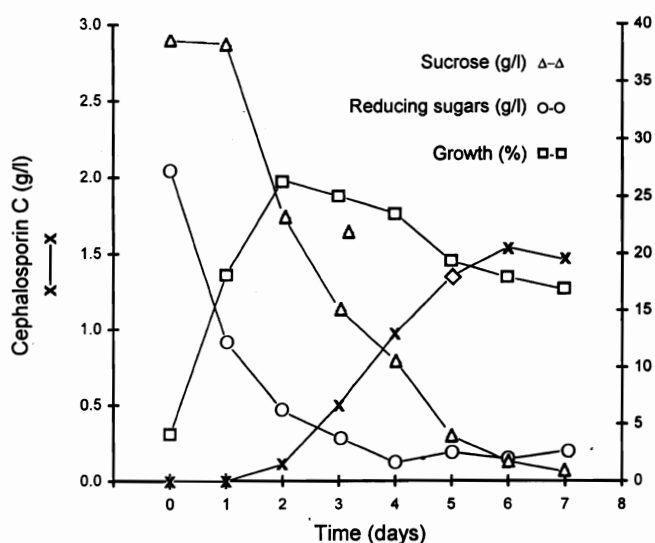


Figure 1 - Growth, sugar consumption and cephalosporin C production by C-10 strain of *Acremonium chrysogenum* during a 7-day shaking-flask fermentation.

Random improvement

Our best efforts produced an average of 1.5 g/l of cephalosporin C in the C-10 strain. Genetic improvement for this antibiotic production was carried out utilizing the media and conditions that permitted the highest yields. Random selection was first carried out on solid medium (Table I and Figure 2). The diameters of the inhibition haloes were measured and compared to a standard curve in order to obtain the equivalent cephalosporin C concentration. The yields expressed in this case did not come from a directed assay. Mutated populations grown on solid media showed a wide range of titers. In a study of *Aspergillus nidulans*, Simpson and Caten (1979) observed that the range of titers increased in mutated populations relative to those of the control population. They also adopted a statistical procedure to classify the survivors of unchanged cultures, vs. titer-increasing and titer-decreasing mutants. By this method, survivors whose log titer exceeded or fell short of the untreated parental

strain by 1.96 standard deviations or more were defined as increasing ('+') or decreasing ('-'), respectively. All survivors with log titers within the range parental log titer \pm 1.96 standard deviations were considered unchanged.

Superior mutants were those whose titers exceeded the C-10 strain average by 1.96 standard deviations. In the case of solid medium production, the average and standard deviation were used, and only those isolates whose titer was superior to 0.24 g/l were considered to be superior (Figure 2). These 54 isolates and C-10 strain had their cephalosporin C production tested in the shaker (Figures 3 and 4). Statistical analysis, using Kolmogorov-Smirnov test, showed that there were no significant differences between the two distributions. This means that C-10 and superior isolates can be considered as coming from the same population, though R-79, R-818 and R-1191, had higher cephalosporin C titers than C-10.

Table I - Cephalosporin C titer of random isolates of *Acremonium chrysogenum* after a 5-day incubation period.

Cephalosporin C (g/l)	Number of isolates	Cephalosporin C (g/l)	Number of isolates
0.00-0.02	9	0.14-0.16	271
0.02-0.04	37	0.16-0.18	286
0.04-0.06	116	0.18-0.20	180
0.06-0.08	143	0.20-0.22	124
0.08-0.10	171	0.22-0.24	87
0.10-0.12	212	0.24-0.26	46
0.12-0.14	298	0.24-0.28	8
Mean = 0.1396		SD = 0.0532	

SD = Standard deviation.

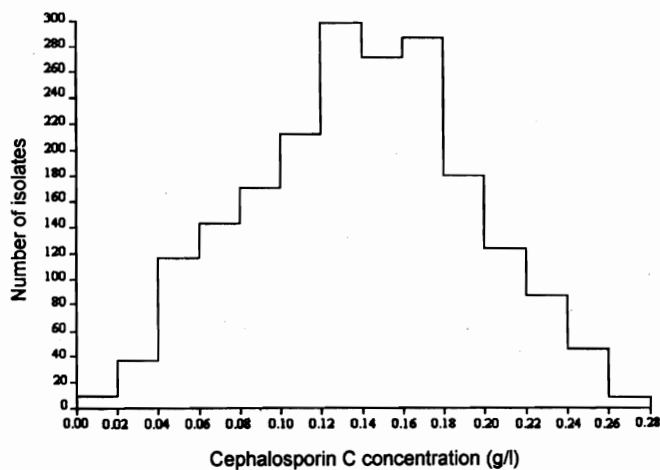


Figure 2 - Cephalosporin C yield of random isolates of *Acremonium chrysogenum* in solid medium.

Rational improvement

Ninety-eight isolates, showing compact colonies, were grown on chemically defined medium and were obtained at a frequency of 2.17×10^{-3} . They were tested in the shaker (Figure 5). There were no statistical differences between morphological isolates and C-10 strain distributions (Kolmogorov-Smirnov test) (Figure 4). However, the classification method described above indicated three compact mutants, M-5, M-76 and M-90, as superior isolates.

Test of superior isolates

Cephalosporin C production by superior random mutants (R-79, R-818 and R-1191) and superior mutants isolated by the rational improvement method (M-5, M-76 and M-90) was tested again and compared to the C-10 strain. Statistical analysis showed significant

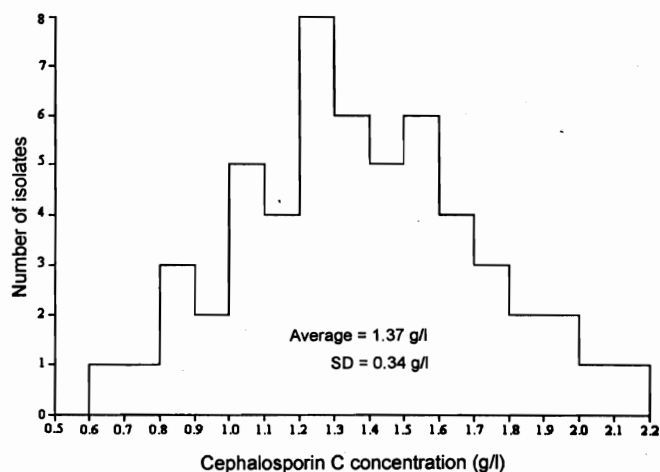


Figure 3 - Cephalosporin C yield of random isolates of *Acremonium chrysogenum* in the shaker.

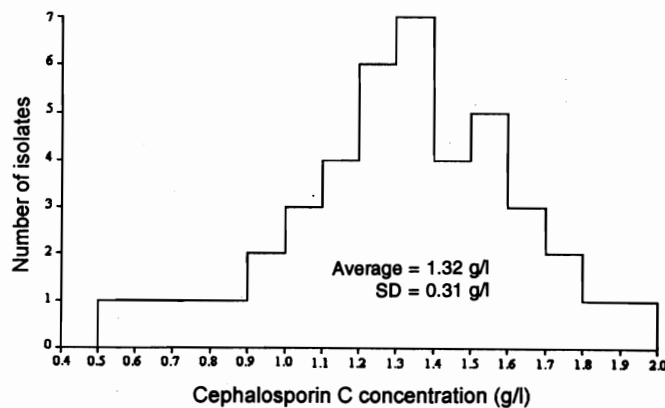


Figure 4 - Cephalosporin C yield of C-10 strain of *Acremonium chrysogenum* in the shaker.

differences among the strains (Table II). In addition, Dunnet test demonstrated that isolates M-5 and M-76 produced significantly more cephalosporin C.

No improved random strains were isolated from the 1988 samples tested. Chang and Elander (1979) tested 2000 colonies and isolated four with improved titer. This discrepancy could be explained by differences in selection procedures and the strict system we adopted for classifying the superior isolates. Using rational selection, we isolated two improved mutants among the 98 compact colonies tested (2% frequency). These results confirm that rational selection in *A. chrysogenum*, using compact colonies, is an appropriate method to isolate mutants with improved cephalosporin C production.

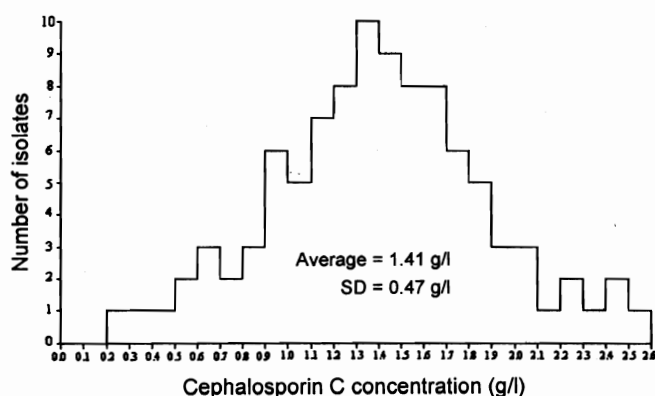


Figure 5 - Cephalosporin C yield of morphological mutants of *Acremonium chrysogenum* in the shaker.

Table II - Production of cephalosporin C (g/l) by C-10 strain of *Acremonium chrysogenum* and isolates.

C-10	R-79	R-818	R-1191	M-5	M-76	M-90
0.82	1.27	1.08	0.82	1.17	1.23	1.26
0.90	1.41	1.13	1.00	1.61	1.89	1.55
1.15	1.46	1.13	1.25	1.91	2.03	1.55
1.33	1.64	1.32	1.39	2.15	2.45	1.87
1.49	1.85	1.47	1.58	2.20	2.53	2.01
1.68	1.87	1.82	1.66	2.41	2.72	2.30
Average						
1.23	1.58	1.32	1.28	1.91	2.14	1.76

Analysis of variance

Source	d.f.	SS	MS	F
Strains	6	4.37	0.73	5.21*
Residual	35	5.02	0.14	

C.V. = 29.8%

*Significant at 1% level (Dunnet test: 0.60).

Improved isolates were tested for cephalosporin C production in highly aerobic conditions, 30 months after being stored. No reduction of production was detected. Cephalosporin C production was also tested on media with cobalt (10 to 100 µg/ml) and the production levels were the same. These results indicate that, although reduced in size, the compact isolates had no alteration of cytochromes, mainly cytochrome oxydase, that would have increase in activity in the presence of oligoelements such as cobalt.

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RESUMO

A linhagem de *Acremonium chrysogenum* designada C-10 produziu em torno de 1,5 g/l de cefalosporina C, quantidade bem inferior à citada na literatura. Esta discrepância, confirmada através de exaustivos testes, provavelmente se deve a algum evento genético. O melhoramento genético utilizando mutantes morfológicos que apresentam crescimento reduzido em meio quimicamente definido confirmou ser este um método clássico apropriado para o isolamento de linhagens melhoradas. Cerca de 2% dos mutantes ensaiados apresentaram produção superior à da linhagem C-10 original.

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