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Short contribution

Selection of hypercellulolytic derepressed mutants of *Cellulomonas* sp.

F. Alea, H. Rodriguez, and A. Enriquez

Microbiology Department, Biotechnology Division, Cuban Research Institute for Sugar Cane By-Products. P. O. Box 4026, C. Postal 11000, Havana, Cuba

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Summary. Mutants from *Cellulomonas* sp.IIbc were obtained by combined treatment of UV light and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The selection criterion for the screening of catabolite-repression-resistant mutants was based on the formation of clear zones around the bacterial colonies in medium containing 0.5% Walseth cellulose and 0.5% glucose. Mutants produced not only clear zones in significantly lower times than the parent strain, but also exhibited higher specific growth rates and cellulolytic activity when grown on bagasse pith. The cellulase-derepressed character of the mutants was demonstrated by the presence of cellulolytic activity in cultures grown in the presence of high levels of glucose. These results raise the possibility of enhancing the productivity of bacterial degradation of lignocellulosic substrates for single cell protein production.

Introduction

In the production of single cell protein (SCP) from lignocellulosic materials the low cell productivity of the fermentation process is one of the present problems to be overcome. The low growth rate of the available strains, among others factors, influences the low cell productivity. Many genetic improvements have been performed to obtain mutants resistant to catabolite repression or end-product inhibition, which seem to be the elements responsible for slow cellulose degradation (Choi et al. 1978; Hagget et al. 1978; Stewart and Leatherwood 1976).

The objective of this work was to search for mutants from a *Cellulomonas* sp. resistant to catabolite repression with increased cellulolytic activity, thus allowing the possibility of more efficient degradation of lignocellulosic substrates for SCP production.

Materials and methods

Strain and cultivation conditions. Cellulomonas sp. strain IIbc was used (Enríquez 1981). Shake cultures were carried out in 500-ml erlenmeyer flasks modified by the addition of a lateral tube, using an optimized (MO) saline medium (Rodríguez et al. 1983) and 1% pretreated sugar-cane bagasse pith at 32° C and 100 rpm. The sugar-cane bagasse pith was pre-treated with NaOH (20% NaOH/pith proportion) for 1 h at 80° C.

Growth was monitored by measuring the absorbance at 600 nm after decanting the medium for 10 min into the lateral tube for sedimentation of the bagasse particles.

Fermentor cultivations were carried out in a 5-l (effective volume) fermentor, under approximately optimal growth conditions (pH 6.5, 32° C and 1.5 v/v/min air-flow rate, Enríquez 1981), in a fermentor (MF) medium (Rodríguez et al. 1983). Dry biomass and substrate consumption were determined as described previously (Alea et al. 1988).

Induction and isolation of mutants. For induction of mutants with UV light, the bacterial culture was irradiated at 20 erg/mm per second for 160 s, corresponding to a 1% survival level. The induction of mutants with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was based on the procedure described by Adelberg et al. (1965).

The selection criterion for the screening of catabolite repression-resistant mutants was based in the formation of clear zones around bacterial colonies in the selection medium (Alea et al. 1988), in the presence of glucose.

Enzymatic activity determinations. Whole culture samples were used for these determinations. The carboxymethylcellulase (CMCase) and filter paper (FP) activity were assayed as described by Rodríguez and Volfová (1984). The activity was expressed in international units (IU: moles of glucose released per millilitre per minute) and the specific activity was expressed in IU per optical density (OD) units of the culture. The xylanase activity was determined according to the technique described by Rickard and Laughlin (1980).

Regulation experiment. In order to establish the possible derepressed character of the cellulolytic systems of the mutants, strains were cultivated in 2-l capacity erlenmeyer flasks containing 200 ml MO medium with 1% pretreated bagasse pith and 0.5% glucose or xylose (in separate flasks). At time intervals, samples were taken and the OD of the cultures and the CMCase and xylanase activities were determined.

Offprint requests to: F. Alea

Results and discussion

From about 30000 screened colonies from the UV mutagenic treatments, five colonies were isolated that exhibited clear zones in shorter times than the parent strain on selection medium. From their growth on MO medium with 1% sugar-cane bagasse pith two mutants, designated M-2 and M-13, which showed a diauxic pattern of growth on this substrate (Enríquez 1981) were selected for further experiments. Mutant M-13 showed a higher specific growth rate in comparison to the parent strain (IIbc) in both exponential growth phases (0.199 and 0.112 h^{-1} versus 0.120 and 0.096 h^{-1} for the IIbc strain) while the M-2 mutant only yielded a higher growth rate than IIbc in the first growth phase (0.165 h^{-1}), according to Student's *t* test, at 95% reliability.

The mutant M-13 produced higher levels of CMCase activity than mutant M-2 and the parental strain (Student's *t* test, 95% reliability) in both phases of growth (4.4 and 4.7 IU/OD $\times 10^{-2}$ for M-13, versus 0.9 and 3.0 IU/OD $\times 10^{-2}$ for the parent strain) in agreement with their growth characteristics. Besides this, mutant M-2 showed higher enzymatic activity than the wild-type strain in the first growth phase (2.4 IU/OD $\times 10^{-2}$ for M-2, versus 0.9 IU/OD $\times 10^{-2}$ for strain IIbc), coinciding with its higher specific growth rate in this phase.

Maximum CMCase activity yielded by mutant M-13 was 0.15 IU/ml. This value represents about ten times the maximum CMCase activity reported by Langsford et al. (1984) for *C. fimi,* and about six times that produced by a recombinant *Escherichia coli* bearing cloned *Cellulomonas* cellulase genes (Owolabi et al. 1988).

Figure 1 shows the CMCase activity of mutant M-13 compared to the IIbc strain, both cultivated in a 5-l fermentor. This mutant yielded higher CMCase activity with respect to the parent strain (IIbc) throughout the culture, corresponding to the results obtained in the shaker runs.

With regard to xylanase activity, mutant M-13 produced 31.8 IU/OD $\times 10^{-2}$ in the first growth phase compared to 27.6 IU/OD $\times 10^{-2}$ produced by the IIbc strain, and 25.8 IU/OD $\times 10^{-2}$ by the M-2 strain. The main attack on the bagasse hemicellulose takes place in this first growth phase (Enríquez 1981; Rodríguez et al. 1985). The kinetics of xylanase production in the fermentor showed higher activity within the first few hours of growth, compared to the IIbc strain (Fig. 2), thus corresponding with the shake flask results.

A second mutagenesis was on mutant M-13 using NG as a chemical mutagen with similar selection methods. From about 40000 colonies, four presumptive mutants were isolated. One of them, mutant M-32, showed a slightly higher specific growth rate in the second growth phase compared to the parent strain (0.123 h^{-1} versus 0.112 h^{-1} for the M-13 strain), while the rest of the putative mutants showed similar results in all parameters evaluated.

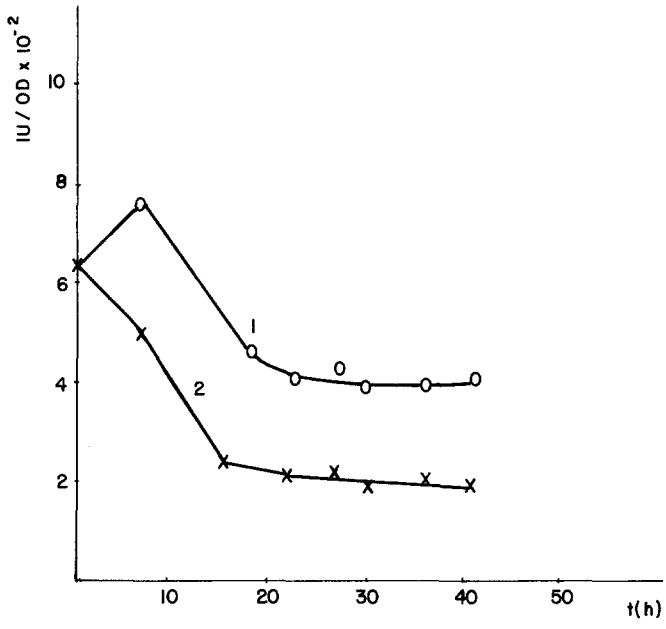

Fig. 1. Carboxymethylcellulase (CMCase) activity of the M-13 and IIbc strains in a 5-l fermentor on a fermentor (MF) medium plus sugar-cane bagasse pith: *1,* mutant M-13; *2,* strain IIbc. IU, international units; OD, optical density

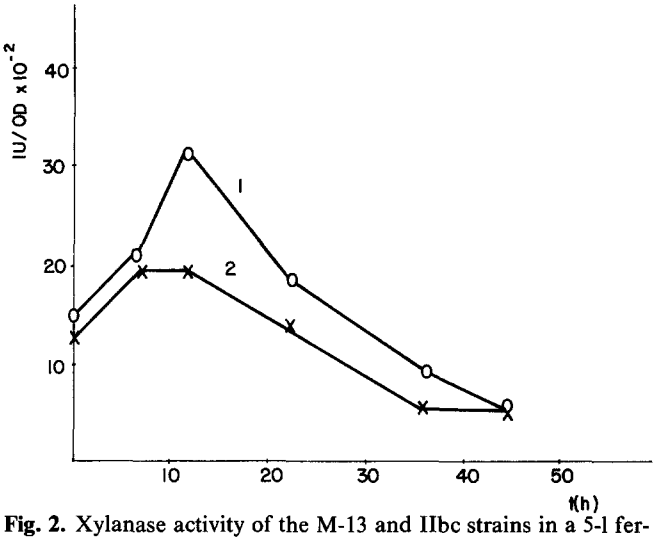

Fig. 2. Xylanase activity of the M-13 and IIbc strains in a 5-l fermentor on MF medium plus sugar-cane bagasse pith: *1,* mutant M-13; *2,* strain IIbc

Catabolite-repression-resistant mutants may include hyperenzyme-producing strains (Demain 1972). The *Cellulomonas* cellulase complex is repressed by several catabolites; mutants resistant to cellulase-catabolite repression should be detectable by observing their cellulolytic activity when grown on cellulosic substrates in the presence of these catabolites. The results of the regulation experiment performed with the new mutant M-32 are presented in Figs. 3 and 4.

Very low cellulolytic activity was found in the wild-type culture in the presence of high levels of glucose or

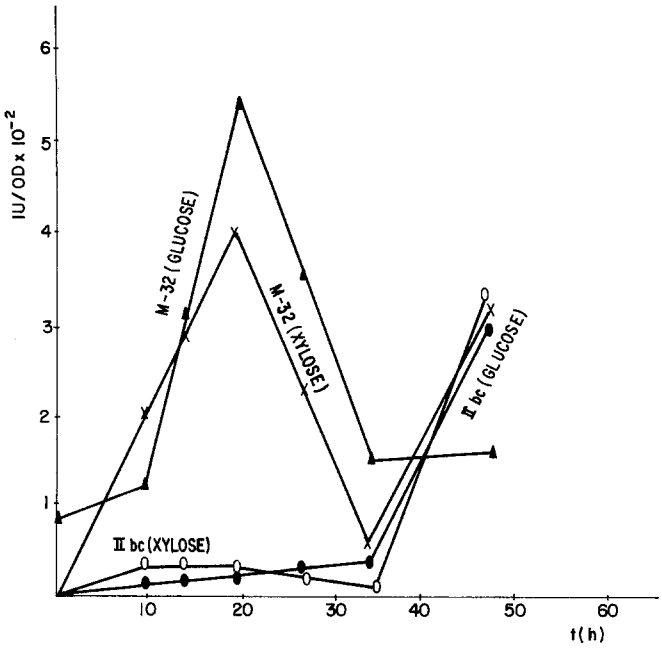

Fig. 3. CMCase activity of mutant M-32 and the wild-type (IIbc) in an optimized (MO) medium plus sugar-cane bagasse pith cultures, in the presence of glucose or xylose

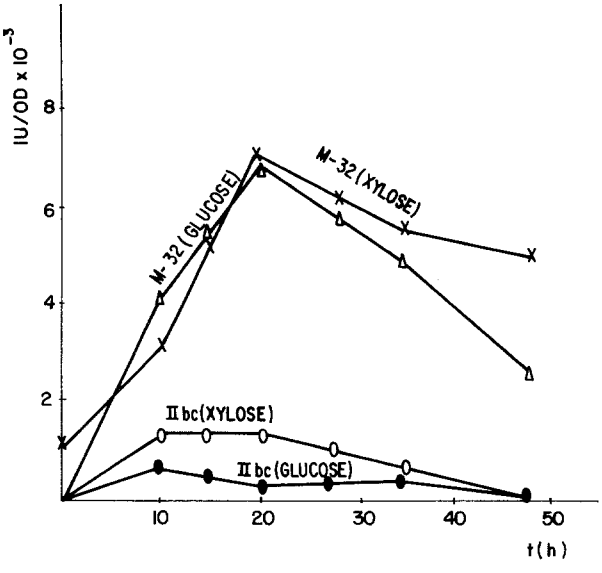

Fig. 4. FP activity of mutant M-32 and the wild-type (IIbc) in MO medium plus sugar-cane bagsasse pith cultures, in the presence of glucose or xylose

xylose. When the sugar concentration decreased to values below 0.3% (this level was reached at 48 h of cultivation), the enzyme activity increased sharply. This result agrees with other studies of cellulase repression in bacteria by high reducing sugar levels (Saddler and Khan 1981; Stewart and Leatherwood 1976). The repression of cellulases by xylose in the IIbc strain is consistent with previous reports on the interrelationship between xylanase and cellulase systems (Hrmová et al. 1986; Rapp and Wagner 1986). In contrast to the IIbc strain, M-32 yielded high cellulolytic activity even in the presence of high glucose or xylose levels, i.e., on initial cultivation (Figs. 3 and 4). Therefore, its derepressed character for both sugars was demonstrated. It seems that the mutants were derepressed with regard to glucose and xylose. This mutation allowed them to produce a higher level of both cellulolytic and also xylanolytic enzymes, and, correspondingly, to exhibit a higher growth rate on the lignocellulosic substrate.

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