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GROWTH AND ENDOGLUCANASE ACTIVITY OF *ACETIVIBRIO CELLULOLYTICUS* GROWN IN THREE DIFFERENT CELLULOSIC SUBSTRATES

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ABSTRACT

The growth kinetics of *Acetivibrio cellulolyticus* grown in medium containing different carbon sources (cellobiose, amorphous or crystalline cellulose) was investigated. The specific growth rate was higher in cellobiose fed cultures than in the presence of the other two substrates. Endoglucanase production was greater in cultures grown on amorphous cellulose; enzyme activity increased during the stationary phase in cultures grown on crystalline cellulose.

Key words: *Acetivibrio cellulolyticus*, endoglucanase activity, cellulose

INTRODUCTION

Acetivibrio cellulolyticus is a mesophilic anaerobic bacterium able to degrade a wide variety of cellulosic materials (15) by producing enzymes capable of acting on native cellulose. Only a few organisms have the ability to hydrolyse native cellulose, such as *Clostridium cellobioparum* and *Ruminococcus albus* (6) isolated from soil and rumen, respectively. *A. cellulolyticus* plays an important role in the transformation of sewage sludge, which presents a cellulose content of around 13-15% (12).

Both the physiology and cellulolytic activity of aerobic and anaerobic bacteria and fungi have become subjects of studies because of the great interest on bioconversion of cellulose to fuels and chemical feedstocks (10). Anaerobic cellulolytic microorganisms are of great significance for large scale industrial applications; however, appropriate methods of cultivation are still at an early stage of development. Special attention should be given to biodigestion processes that aim at the fast transformation of cellulose rich pollutants.

The biological hydrolysis of cellulose is dependent upon a group of enzymes - endoglucanase, exoglucanase and β-glucosidase - which act synergistically on the substrates (2), generating low molecular weight reducing sugars (15).

The growth of *A. cellulolyticus* in the presence of different substrates has not been properly characterized yet, as well as the carbon sources that may provide both maximum cell protein and endoglucanase production.

Despite the fact that pure cultures do not mirror all the complex interactions taking place in a natural community, the potential action of certain microorganisms can only be verified in *in vitro* systems. The aim of the present investigation was to study the growth of a strain of *A. cellulolyticus*

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isolated from an established sewage sludge culture, in medium containing three different carbon and energy sources, establishing its growth phases and specific growth rates. Endoglucanase activity curves were also analysed under these conditions.

MATERIALS AND METHODS

The microorganism used was *A. cellulolyticus* (ATCC 33288) isolated and characterized by Patel *et al* (11). Cells were grown in synthetic medium previously described by Hatt and Gantt (3) and modified by lowering the concentration of $Na_2S \cdot 9H_2O$ (added as reducing agent) to 0.025 $g.L^{-1}$. This medium was prepared under an atmosphere of 80% N_2 - 20% CO_2. A volume of medium free of both carbon source and reducing solution was poured into a 1000 ml serum vial containing 3 $g.L^{-1}$ of either amorphous cellulose powder (Sigma C-6288) or filter paper (Whatman Nº 1). Prior to being reduced and autoclaved at 121ºC for 20 minutes, the pH of the medium was adjusted to 7.6. When D-(+)-cellobiose (Sigma C-7252) was used as substrate, the solution was filter sterilized using 0.22 µm pore size filters (Millipore) and added to the autoclaved medium, to give a final concentration of 3.0 $g.L^{-1}$. A 72 h culture grown in medium containing the same substrate tested in the assay was used as inoculum (10%, v/v). Each assay started from a lyophilised culture. The final volume of the culture was 350 ml. The headspace of the vials contained 80% N_2 – 20% CO_2. Cultures were incubated at 37ºC in a rotary shaker at 200 rpm. Periodically, a 10 ml sample was removed from the growing culture and centrifuged for 20 min at 39,000 x g. The cell pellet was washed twice with distilled water and cell protein content was then measured by the method of Lowry (7), in order to monitor bacterial growth; bovine serum albumin (Sigma A-4378) was used as standard.

Endoglucanase activity was assayed in culture supernatants. Quantitation of endoglucanase activity was done by determining the amount of reducing sugar generated from carboxymethylcellulose (8). Briefly, 0.5 ml of culture supernatant was incubated with 0.5 ml of 0.05M citrate buffer, pH 4.8, containing 1% carboxymethylcellulose sodium salt (CMC, Sigma C-4888), 10 mM DL-dithiothreitol (Sigma D-0632) and 0.02% (w/v) NaN_3. The assay was performed aerobically at 37ºC for 30 minutes. Reducing sugars were assayed colorimetrically using dinitrosalicylic acid reagent (9). Specific growth rates were determined using a software devised for that purpose (18). With respect to culture preservation, two methods were employed: freeze drying (17) modified for anaerobic bacteria and weekly subculture in fresh media. To determine the best substrate for subculturing of *A. cellulolyticus,* two cultures were maintained during 77 days. The cells were weekly subcultured in medium containing either cellulose or cellobiose and cell protein content was checked on each occasion.

RESULTS AND DISCUSSION

Based on the growth curves obtained for the three substrates studied (Fig.1), it was possible to determine the most appropriated one for growth of *A. cellulolyticus.* A clear difference was observed among the lag phases, which were longer in the cellulose containing cultures (29 h and 17 h for amorphous and crystalline cellulose, respectively) as compared to that grown on cellobiose (7h). Different results in the literature show that a lag phase may or may not be present when *A. cellulolyticus* grows on cellobiose, even considering an inoculum of 72 h (11, 15). Patel and Mackenzie (10) obtained growth curves where the lag phase extended for 8 h, a feature that was confirmed by this paper.

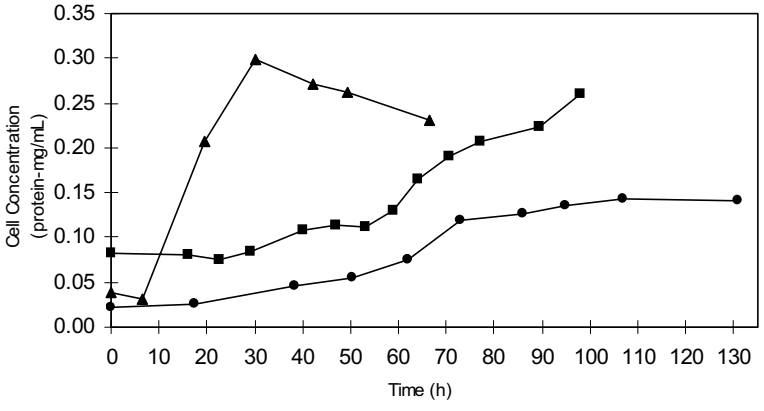

Figure 1. *A. cellulolyticus* growth curves obtained in the presence of cellobiose (▲) amorphous cellulose (■) and crystalline cellulose (●) expressed through the cellular protein concentration (average of three assays).

In the present study, the specific growth rate of *A. cellulolyticus* grown on cellobiose (0.130 h^{-1}) was 4.4 times higher than that detected when using either amorphous or crystalline cellulose as substrate (0.027 h^{-1} and 0.026 h^{-1}, respectively). A value of 0.170 h^{-1} was found by Patel and Mackenzie (10) for cultures of this microorganism in medium containing cellobiose. The low growth rates obtained with cellulose as substrate could be explained by the fact that its complex structure impose a constraint on solubilization (10). Cellulases are not characterized by fast hydrolysis of the substrate (20) and therefore do not promote a swift availability of soluble sugar; this, in turn, may affect bacterial proliferation. Although growth was slower in the presence of celluloses, the highest protein content obtained for amorphous cellulose (0.24 $mg.ml^{-1}$) was similar to that observed for cellobiose (0.27 $mg.ml^{-1}$), which establishes a very long exponential phase (Fig. 1). Cultures grown on crystalline cellulose had about 40% less protein than those grown on amorphous cellulose. Saddler and Khan (15) obtained different results, since their cultures grown on crystalline cellulose (avicel) and on cellobiose reached maximum protein values of 0.22 $mg.ml^{-1}$ and 0.20 $mg.ml^{-1}$, respectively. Saddler *et al.* (14) obtained lower values than these: 0.072 $mg.ml^{-1}$ for cellobiose, 0.060 $mg.ml^{-1}$ for amorphous cellulose, 0.035 $mg.ml^{-1}$ for crystalline cellulose and 0.084 $mg.ml^{-1}$ for avicel. Therefore, taken together, the data in the literature show that the cell protein content of the cellulolytic populations may differ considerably under similar culture conditions. On the other hand, it should be noted that the values obtained for cellulose cultures are probably underestimated because of the adsorption of the cells in insoluble substrate (1).

Culture preservation by sub-culturing (data not shown) showed that cellobiose does not sustain *A. cellulolyticus* viability for long periods of time. Cellulose, on the other hand, was a good substrate for this purpose. Despite having established which was the best substrate for preservation by subculturing, all the experiments started with freeze dryed *A. cellulolyticus* to ensure that the microorganisms were obtained from the same source.

Enzyme activity curves obtained for the three substrates studied indicated the most appropriated source to produce endoglucanase.

Fig. 2a shows that endoglucanase activity in cultures grown on cellobiose was detected during the exponential phase, that is, earlier than on both crystalline and amorphous cellulose, probably because growth in the presence of that sugar also took place earlier. Cultures on amorphous cellulose presented enzymatic activity during the lag phase (Fig. 1). Contrary to cellobiose fed cultures, endoglucanase activity in cultures grown on cellulose was always on the increase until the end of the tests. Fig. 2b shows a trend towards stability of specific activity during the stationary phase in the presence of amorphous cellulose, indicating that a greater enzymatic production does happen during the exponential growth phase. However, differing from other cultures, a raise in endoglucanase activity at the end of the exponential phase was observed in cultures grown on crystalline cellulose. Similar results were obtained for enzyme specific activity suggesting that the bacterial population continued to produce endoglucanase even during the stationary phase (almost half of the total measured activity). When analyzing the growth phases at which extracellular bacterial enzymes are released, Priest (13) noticed that, generally, there is little or no enzymatic secretion during the lag phase, except when they act on substrates that are their main or sole carbon and energy source. This was observed in the present study, where cultures grown on amorphous cellulose were active during the lag phase. The production of endoglucanase during the stationary phase when bacteria were grown on crystalline cellulose was an exception.

Endoglucanase activity of cultures grown on amorphous cellulose was 2.8 times higher than that of cultures grown on cellobiose (Fig 2a). The specific activity when using amorphous cellulose was 3.6 times higher compared to cellobiose containing cultures (Fig 2b). These results indicate that the low cell content observed on amorphous cellulose was able to produce the higher endoglucanase activity. Maximum endoglucanase activity measured in supernatants of cultures grown on crystaline cellulose was 1.2 times higher than that of cultures grown on cellobiose. Similar results were obtained by Saddler and Khan (15). Endoglucanase activity in culture supernatants of *A. cellulolyticus* grown on amorphous cellulose was 2.4 times higher than that detected when medium contained crystalline cellulose. These values do not agree with other data on endoglucanase activity in cultures grown on crystalline cellulose, which was reported to be about 1.4 times (14) or 1.1 times (16) higher than that on amorphous cellulose; also, in such studies, the specific activities were the same for both

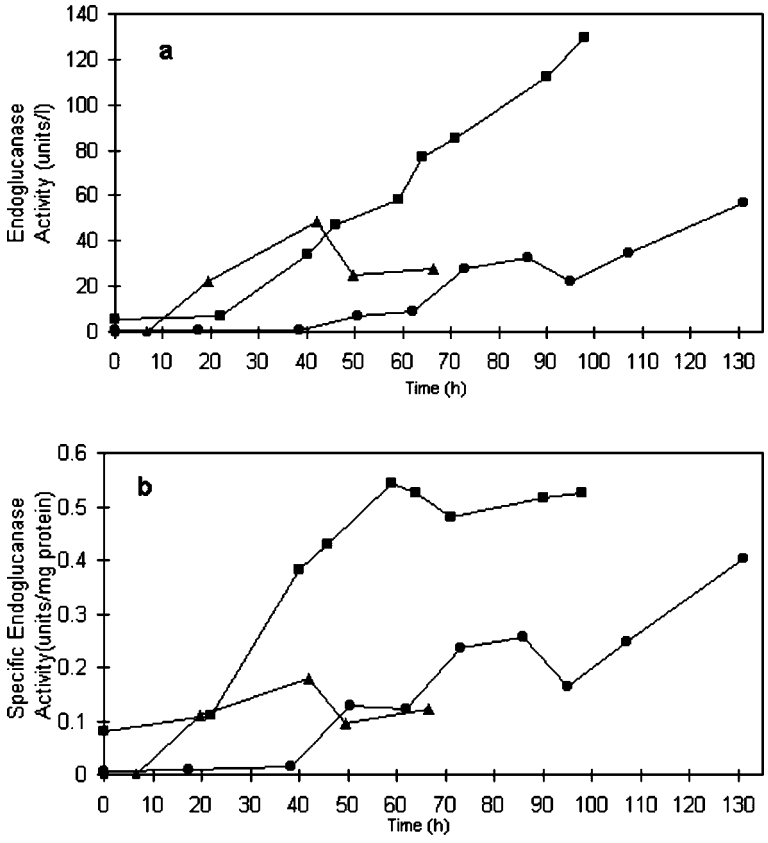

Figure 2. Endoglucanase activity curves of *A. cellulolyticus* grown on cellobiose (▲) amorphous cellulose (■) and crystalline cellulose (●)in culture supernatant (a) and specific activity (b) (the results represent the average of three assays).

substrates. Likely, the results presented in this report show similar maximum values for endoglucanase specific activity in both cultures grown on amorphous and on crystalline cellulose; however the curves showed that the enzyme production was delayed in cultures grown on crystalline cellulose.

Endoglucanase production in cultures grown on amorphous cellulose was, in this study, always higher compared to cultures grown on cellobiose and crystalline cellulose (Fig. 2a), indicating that amorphous cellulose is the best substrate for its production.

Taking together the results obtained with the different substrates presently used, it might be possible to infer some features of *A. cellulolyticus* endoglucanase regulation. As with other cellulolytic microorganisms, *A. cellulolyticus* endoglucanase synthesis could be viewed as an adaptive phenomenon. Up to now, no metabolic inducer has been described for this enzyme in *A. cellulolyticus*. In *Clostridium termocellum*, phylogenetically related to *A. cellulolyticus* (5), substrates that are metabolized more readily may lead to repressed cellulase synthesis and enzyme induction may only be triggered by low substrate concentration (4). This is confirmed by the present study, which shows that, when using cellulose as substrate, *A. cellulolyticus* growth rate was low but enzyme production was higher. The cellobiose concentration presently used (3.0 $g.L^{-1}$) was very close to that employed by Stoppok *et al.* (19) for growth of *Cellulomonas uda* (2.9 $g.L^{-1}$) and the results obtained were also similar, that is, a lower production of endoglucanase was found in the presence of cellobiose as compared to cultures grown on amorphous cellulose. The authors suggested inhibition of endoglucanase activity by a high cellobiose concentration and also proposed a mechanism of induction of enzyme production.

The results presented herein indicate that cellulose is the best carbon source for endoglucanase production as well as preservation of *A. cellulolyticus*. Nevertheless, greater amounts of cell protein were produced at a fast rate when cellobiose was used as carbon source.

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RESUMO

Crescimento e atividade de endoglicanase de *Acetivibrio cellulolyticus* cultivado em três diferentes substratos celulósicos

O estudo da cinética de crescimento de *Acetivibrio cellulolyticus* cultivado em três diferentes substratos celulósicos (celulose amorfa, celulose cristalina e celobiose) revelou que em celobiose, a velocidade específica de crescimento foi maior que nos dois tipos de celulose. A atividade de endoglicanase foi maior em celulose amorfa; em celulose cristalina a atividade enzimática, aumentou durante a fase estacionária.

Palavras-chave: *Acetivibrio cellulolyticus*, atividade de endoglicanase, celulose

REFERENCES

1. Breuil, C.; Patel; G.B. Effect of pH and oxygen on growth and viability of *Acetivibrio cellulolyticus. J. Gen. Microbiol.* 125: 41-46, 1981.
2. Gilbert, H.J.; Hazlewood, G. Bacterial cellulases and xilanase. *J. Gen. Microbiol.,* 139: 187- 194, 1993.
3. Hatt, M.; Gantt, M.J. (Eds.) ATCC Media Handbook, American Type Culture Collection, Rockville, Maryland, 1984.
4. Johnson, E.A.; Bouchot, F.; Demain, A. L. Saccharification of complex cellulosic substrates by cellulase system from *Clostridium thermocellum*. *Appl. Envion. Microbiol*. 43: 1125-1132, 1982.
5. Leschine, S. B. Cellulose degradation in anaerobic environments*. Annu. Rev. Microbio*l, 49: 399-426, 1995.
6. Ljungdahl, L.G.; Eriksson, K. Ecology of microbiol cellulose degradation. *Adv. Microb. Ecol.* 8: 237-299, 1985.
7. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275, 1951.
8. Mandels, M.; Andreotti, R.; Roche, C. Measurement of saccharifying cellulase. John Willey and Sons, New York. *Biotechnol. Bioeng. Symp*. 6: 17-34, 1976.
9. Miller, G.L. Use of dinitrosalysilic acid reagent for determination of reducing sugar. *Anal. Chem.* 3l: 426-428, 1959.
10. Patel, G.B.; Mackenzie, C.R. Metabolism of *Acetivibrio cellulolyticus* during optimized growth on glucose, cellobiose and cellulose. *Envir. J. Appl. Microbiol*. *Biotechnol.* 16: 212-218, 1982.
11. Patel, G.B.; Khan, A.W.; Agnew B.J.; Colvin, J.R. Isolation and characterization of an anaerobic, cellulolytic microorganism, *Acetivibrio cellulolyticus* gen. nov., sp. nov. *Inter. J. Syst. Bacteriol.* 30: l79-l85, 1980.
12. Peres, C.S.; Sanchez, C.R.; Matumoto, C.; Shmidell, W. Anaerobic biodegradability of the organic components of municipal solid wastes (OFMSW). *Wat, Sci. Tech.* 25:285-293, 1992.
13. Priest, F.G. Extracellular enzyme synthesis in the Genus *Bacillus*. *Bacteriol. Rev.* 41: 711-53, 1977.
14. Saddler, J. N.; Khan, A . W.; Martin, S.M. Regulation of cellulase synthesis in *Acetivibrio cellulolyticus*. *Microbios*, 28: 97-106, 1980.
15. Saddler, J.N.; Khan, A.W. Cellulase production by *Acetivibrio cellulolyticus. Can. J. Microbiol.* 26: 760-765, 1980.
16. Saddler, J.N.; Khan, A.W. Celluloytic enzyme system of *Acetivibrio cellulolyticus. Can. J. Microbiol*. 27: 288-294, 1981.
17. Silva, L.F.; Gomes, J.G.C.; Oliveira, M.S.; Alterthum, F. Freeze-drying of industrial yeast strains: influence of growth conditions cooling rates and suspending media on the viability of recovered cells. *Rev. Microbiol*., 23: 117-122, 1992.
18. Simões, D.A. Logiciel de Lissage, 1994 (software)
19. Stoppok, W.; Rapp, P.; Wagner, F. Formation, location, and regulation of endo-1,4-β-glucanase and -β-glucosidases from *Cellulomonas uda. Appl. Environ. Microbiol*. 44: 44-53, 1982.
20. Teunissen, M. J.; Kort, G.V.M.; Op Den Camp, H.J.M.; Huis In't Veld, J.H.J. Productuion of cellulolytic and xylanolytic enzymes during growth of the anaerobic fungus *Piromyces* sp. on different substrates. *J. Gen. Microbiol.* 138: 1657-64, 1992.