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Partial Characterization of an Inducible Amylase Produced by *Pseudomonas cellulosa*

Scientific Note

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ABSTRACT

The products secreted by *Pseudomonas cellulosa* were examined to determine if this bacterium could be used in a fluidized-bed bioreactor system to produce enzymes for converting waste cellulose to chemical feedstocks. *P. cellulosa* grows on a minimal salts medium supplemented with starch. *P. cellulosa* was found to secrete an amylase that was not produced when grown on media where cellulose was the only substrate. Therefore, the amylase produced by this bacterium is inducible, whereas production of cellulase was found to be constitutive. The amylase was found to have an apparent molecular weight of approx 97,000 Daltons. The activity of the amylase was found to be highest at alkaline pH values from 7.0 to 8.0, which is similar to the optimal pH for the cellulase.

Index Entries: Cellulase; amylase; *Pseudomonas fluorescens subsp. cellulosa, Pseudomonas cellulosa.*

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INTRODUCTION

A wide variety of bacterial producers of cellulase have been described, including a bacterium that has been previously called *Pseudomonas fluorescens var. cellulosa* (*1*). Recently, it has been suggested that this bacterium had few characteristics similar to *P. fluorescens*, and it was suggested that it be called *P. cellulosa* (2). *P. cellulosa* has a number of characteristics that make it attractive to produce cellulase in a fluidized-bed bioreactor. However, the full metabolic potential of this bacterium must be explored, so that bioreactor conditions and downstream recovery of materials can be optimized. Recently, we noted that a zone of starch hydrolysis was found surrounding colonies of *P. cellulosa* when grown on starch-containing medium (Dees et al., 1994, 16th Symposium on Biotechnology for Fuels and Biochemicals). This suggested that this bacterium secreted an amylase into the culture medium. We further examined the production of amylase and partially characterized it. We also re-examined the production of cellulase when *P. cellulase* was grown in media with and without cellulose.

MATERIALS AND METHODS

Source of Microorganism

The cellulase-degrading bacterium was obtained from the NCIMB repository. The microorganisms catalog number NCIMB 10462 are listed as *Pseudomonas* species.

Culture Conditions

NCIMB 10462 was maintained on solid media consisting of M9 liquid medium (*1*) to which 15 g/L agarose had been added along with 0.1% carboxymethyl cellulose (CMC) as the sole carbon source or 0.01% (w/v) soluble corn starch. Liquid media were also prepared in a similar fashion, but without agar. Cellulosic media consisted of an M9 salt solution as previously described (3). Agar was added to 15 g/L for solid media along with soluble cellulosic components (e.g., carboxymethyl cellulose, CMC) to 0.1% (w/v). Cellulosic liquid medium consisted of the M9 salts solution to which strips of Whatman No. 1 filter or newspaper had been added. Alternatively, cellulose-containing medium was made by adding Avicell powder (0.1% w/v) to M9 salt solution. Corn starch was added (0.1% w/v) to M9 medium to make the starch liquid medium.

Amylase and CMCase Activity

Amylase activity was determined by a diffusion assay. The starch hydrolysis assay was performed on plates composed of 0.1% (w/v) corn starch dispersed in 1.5% agar. Culture supernatants were added to wells cut into the starch agar plate, and the plates were incubated for 18 h at 37°C. Starch plates were stained by flooding them with Gram's iodine solution for 15 min at room temperature. Iodine was removed from the plates by washing them with distilled water. Amylase activity was determined by observing the zone diameter of hydrolyzed areas around the wells. CMCase activity was determined using a similar agar diffusion assay with 0.1% (w/v) CMC in agar plates. Plates were stained with 0.1 mg/mL Congo Red dye for 15 min at room temperature. Plates were washed with a 1*M* NaCl solution.

Electrophoretic Procedures

Radioimmunoprecipitates were examined using standard nondenaturing gel electrophoretic procedures. Polyacrylamide minigels used were a 4–20% linear gradient from a commercial source (Bio-Rad Inc., Richmond, CA). Electrophoretically separate proteins were visualized using a commercially available silver-stain method (BioRad Inc.). Silver-stain conditions were similar to those described by the manufacturer with the exception that all gels were incubated at room temperature for 24 h in 50% methanol:water (v/v) and then distilled water for 2 h prior staining.

Zymograms were used to determine the approximate molecular weight of the amylase. Zymograms were performed on indicator gels composed of 0.1% (w/v) cornstarch or 0.1% CMC (w/v) in 2% (w/v) agarose similar to CMCase activity zymograms previously described (*4*). The zymograms were obtained using procedures similar to those described previously (*4*) with the exception that electrophoretically separated proteins were electroblotted into the enzyme indicator medium using a commercial transfer unit (Bio-Rad Inc.). Transfer conditions were similar to those described by the manufacturer of the transfer unit.

RESULTS

Figure 1 shows that enzymatic degradation of starch is found only in the supernatants of cultures that have starch. Amylase activity was retained by both 10k and 30K filters, but passed through a 100k filter. No amylase activity was detectible in culture supernatants from a liquid CMC culture

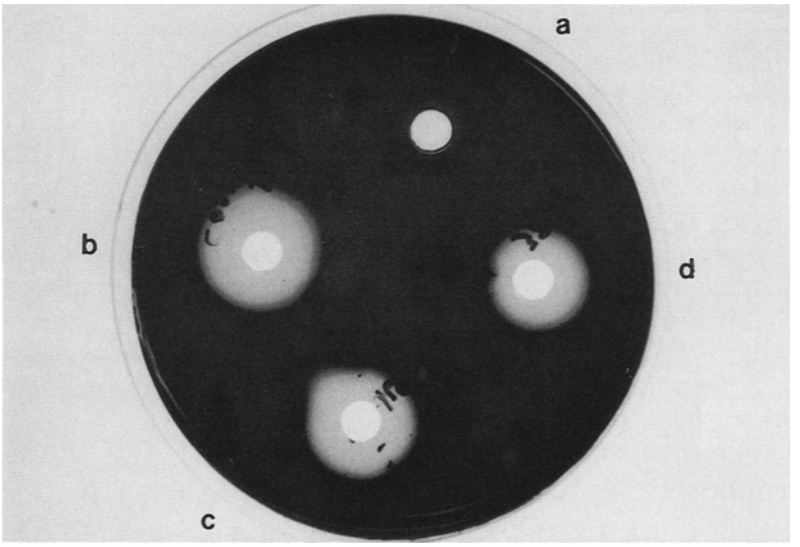

Fig. 1. Clear zones around the wells show areas where starch has been hydrolyzed (iodine stain). Amylase activity is found in supernatant from *P. cellulosa* grown in M9-starch medium (b), but not in supernatant from M9-Avicell (a). Amylase activity was found in the filtrate of a 100-kDa filter (c), but not in the retentate (data not shown). Amylase activity was retained by a 30-kDa filter (d), but not in the filtrate (data not shown).

medium (Fig. 1). In contrast, Fig. 2 shows that the CMCase activity found in culture supernatants is similar in spent CMC medium and starch medium. Figure 3A shows that nine to ten silver-stained proteins in starch medium supernatant can be visualized on nondenaturing electrophoretic gels. The zymogram in Fig. 3B shows that the only band capable of hydrolyzing starch has an apparent mol wt of approx 97,000 Daltons. No hydrolysis of starch was observed by any protein from culture supernatants of the bacterium grown on CMC medium (Fig. 3 lane a). Many other proteins were found to have CMCase activity, including ones with apparent mol wt of 23, 29, 50–52, and 105 kDa (zymogram not shown).

Figure 4 shows that the optimal pH activity for CMCase is approx 7.2–7.4. Similarly, the optimal amylase activity appears to occur at the same pH range as shown by the CMCases (Fig. 5).

DISCUSSION

Most recently, interest in bioconverting cellulosic waste has centered on the use of fungi or thermophilic bacteria to produce cellulase. However, *P.*

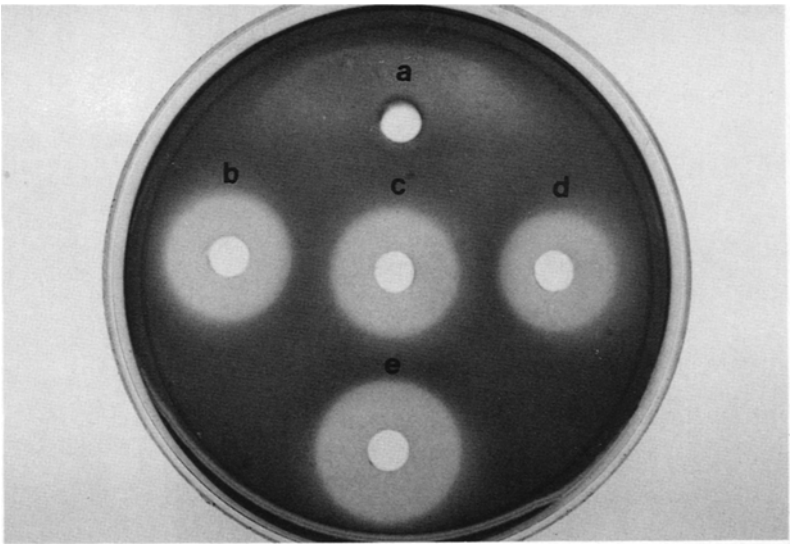

Fig. 2. Clear zones around the wells cut in a CMC indicator plate show areas where CMC has been hydrolyzed (Congo Red staining). CMCase activity is present in the supernatants of *P. cellulosa* grown in M9-Avicell (b), Trypticase Soy Broth (c), M9-filter paper (d), or M9-starch medium (e). No hydrolysis is found around the control well, which contains M9 medium (a).

cellulosa has a number of characteristics that make it especially attractive for use in continuous-flow bioreactor systems. For example, *P. cellulosa* does not secrete a proteinase that might decrease enzyme recovery or contaminate cellulase or amylase preparations (2). Additionally, cellulase production by this bacterium is constitutive (Fig. 1), whereas the amylase can be induced (Fig.2). Therefore, this bacterium can be used to produce cellulase without contamination by amylase. Since cellulase production is constitutive, expensive inducers of cellulase (e.g., sophorose) will not be required (5). Starch can be used as an inexpensive maintenance or growth medium for bacteria incorporated into a bioreactor matrix. *P. cellulosa* also grows best (Dees et al., 1994, 16th Symposium on Biotechnology for Fuels and Chemicals) and produces cellulases that are highly active in weakly alkaline environments (Fig. 4). The amylase activity also appears to be optimal at pH values of 7.2–7.4 (Fig. 5). Since the cellulase and amylase pH optima were estimated by a diffusion assay, effects of the pH change on the two enzyme substrates are unknown. This might affect the rate of diffusion into the indicator plate medium, which could affect the results obtained. However, the pH optima for the two enzymes is consistent with the optimal pH for growth of this organism (Dees et al., 1994, 16th Symposium on Biotechnology for Fuels and Chemicals).

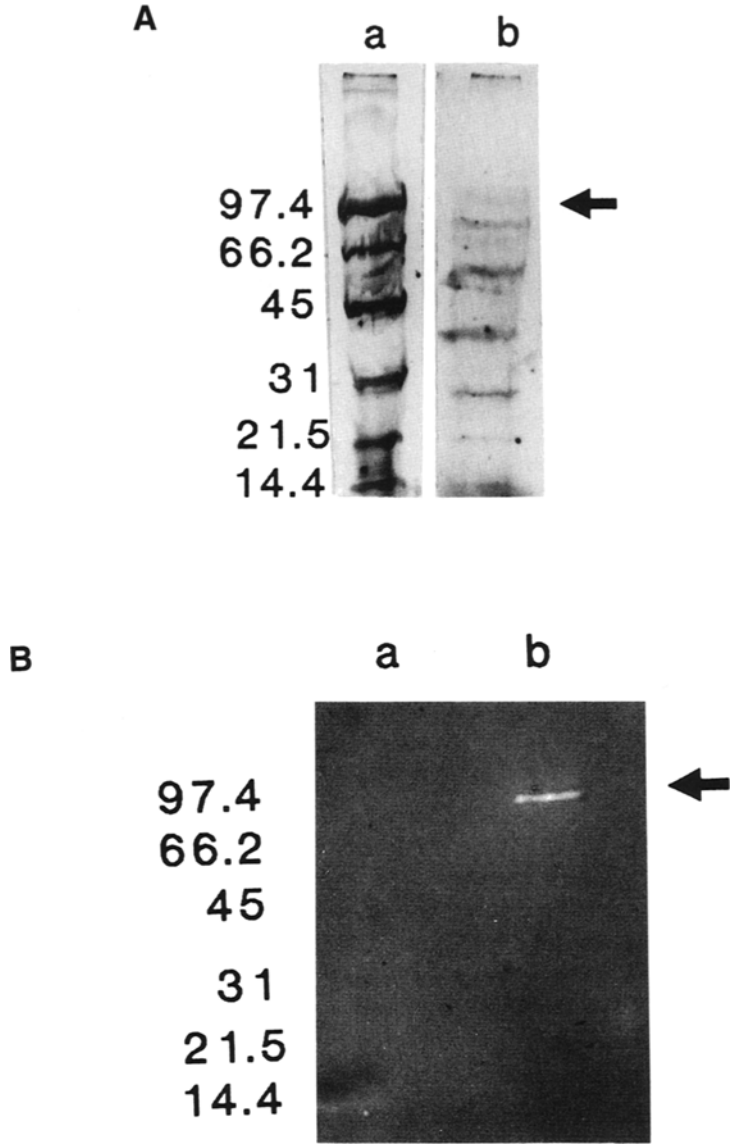

Fig. 3. (**A**) Approximately 9–11 proteins can be identified in the culture supernatant of *P. cellulosa* grown in M9-starch medium (a). The arrow points to a protein thought to be the amylase. All other bands exhibit CMCase activity when tested using the Zymogram method (data not shown). (**B**) A starch Zymogram performed on electrophoresed culture supernatants from *P. cellulosa* grown on M9-starch medium. A clearing zone of starch hydrolysis (arrow) corresponds to a protein with an approximate mol wt of 97,000 Daltons.

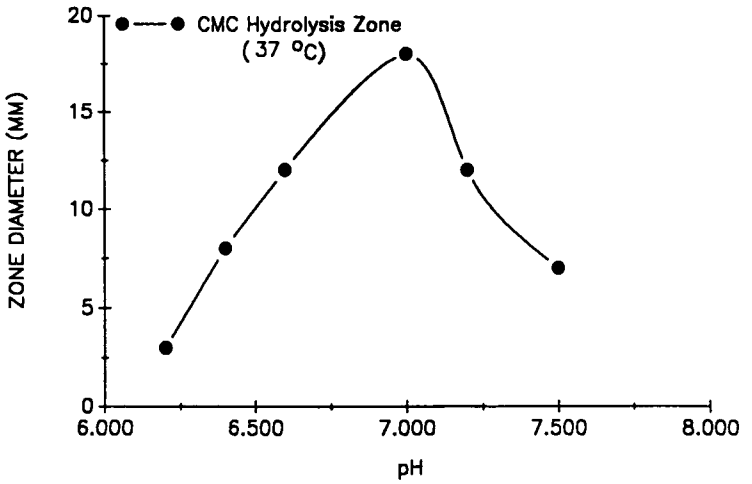

Fig. 4. The largest zones of CMC hydrolysis were found in indicator plates with a pH of approx 7.0–7.1.

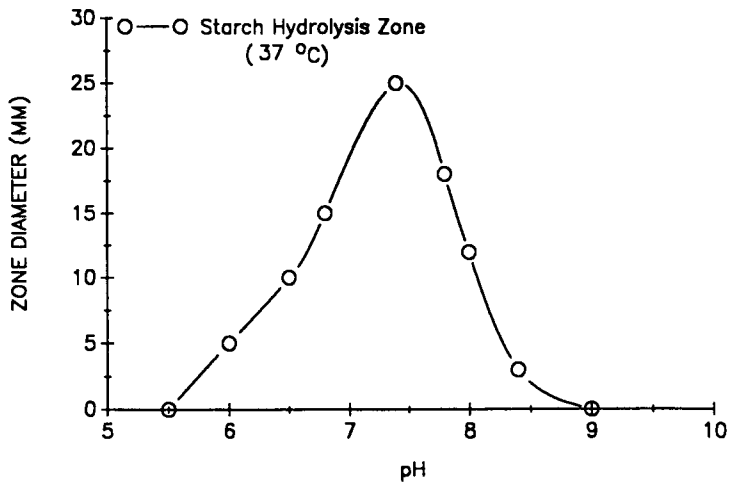

Fig. 5. The largest clearing zones representing starch hydrolysis were found on indicator plates with a pH of approx 7.1–7.2.

Therefore, the results obtained using the diffusion-based assays should be estimates of enzyme performance at different pH values.

P. cellulosa is an attractive candidate for production of amylase or cellulase under mildly alkaline conditions. Further study is required to characterize more fully enzymes produced by this bacterium, so that conditions can be optimized for production and downstream recovery of product from continuous-flow bioreactor systems.

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