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Extracellular enzyme production by *Thermomonospora curvata* grown on bagasse

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SUMMARY

Extracellular enzyme production by the actinomycete, *Thermomonospora curvata*, was characterized during growth at 55 °C on bagasse as sole carbon source. Mycelia adhered to the bagasse fibers during early growth and were released in mature cultures. Extracellular protein reached a maximum on 4% (w/v) bagasse and yielded an electrophoretic profile similar to those produced on purified cellulose. Cellulase production on bagasse exceeded that observed for *T. curvata* on any previously employed substrate. Amylase and pectinase, which were diminished by their instability in culture fluid at growth temperature and by the lack of inducing substrate, were readily inducible by addition of starch or pectin, respectively. Extracellular activities of β-glucosidase and β-xylosidase remained insignificant throughout growth. Xylanase production equaled or exceeded that observed on a variety of other substrates. The combined activity of extracellular enzymes from bagasse-grown *T. curvata* caused a 27% solubilization of the fiber, yielding a mixture of cellooligosaccharides, cellobiose, xylobiose, glucose, xylose, fructose, arabinose and mannitol. Fractionation of concentrated extracellular proteins by size exclusion chromatography yielded single peaks for amylase and pectinase (estimated molecular weights of 58 K and 34 K respectively), while cellulase and xylanase activities were distributed throughout a series of multiple unresolved peaks spanning a molecular weight range of 26–180 K.

INTRODUCTION

Sugar cane is currently being processed at a global annual rate exceeding 7×10^8 metric tons [39]. The dry matter yield (tons per acre) of sugar cane is 2–20 times that of other major US agricultural crops [5]. Bagasse, the fibrous residue after sucrose extraction, consists of 46–49% cellulose, 25–27% hemicellulose and 20–22% lignin [7]. While a portion of the fiber is marketed directly as combustible fuel or animal fodder, the bulk remains currently unused [23]. The high potential yield of fermentable sugars from bagasse has prompted studies employing alkaline extraction [6], acid hydrolysis [26] and mesophilic microbial enzymes [23,24,25]. The use of enzymes from thermophilic microbes offers important advantages over those of mesophiles in large-scale biomass conversion processes [reviewed, 17]. In the following study, bagasse was evaluated as sole carbon source for production of thermostable extracellular enzymes by *Thermomonospora curvata*, an actinomycete which establishes itself as the dominant population during the high temperature composting of a variety of lignocellulosic materials [8,21,29].

MATERIALS AND METHODS

Organism and growth conditions

The actinomycete strain used in this study was identified as *Thermomonospora curvata* by Dr T. Cross (Univ. Bradford, UK). The Thermomonosproaceae is a family of taxonomic convenience which includes strains exhibiting a continuum of identifying characteristics and also many with relatively few similarities [18]. This strain shares some characteristics in common with *Thermomonospora fusca*, but differs as to sugar utilization patterns, growth requirements, morphology and exoenzyme characteristics [41]. At present, the species designation as *curvata* has been retained.

Inocula were prepared from late exponential phase cells grown in 1% cellobiose–mineral salts–vitamin minimal medium [30]. Cells were concentrated about 5-fold by centrifugation and stored in 25% glycerol at −80 °C. Bagasse (obtained from M.A. Patout & Son, Ltd, Jeanerette, LA) was ground to pass a 20-mesh screen in a Wiley mill. Ground fiber was added in concentrations of 0.5–10% (w/v) to 100 ml of minimal medium and autoclaved in 250-ml high-baffled culture flasks (Bellco Glass Inc., Vineland, NJ). For purposes of comparison, ground surgical cotton (Johnson & Johnson, New Brunswick, NJ) with a 99% cellulose dry weight content [31] replaced bagasse as sole carbon source in some experiments. Vitamins (thiamine and biotin, 1 μg ml^{-1}) were added aseptically after cooling. Cultures were started with 1% (v/v) inoculum and shaken on an incline of 30° from vertical at 140 rpm. In some experiments, the repressive effect of soluble sugars on enzyme biosynthesis was tested as follows: culture fluid from a two-day culture growing on 10% bagasse was clarified by centrifugation and deproteinated by ultrafiltration. Sugars were concentrated about 10-fold at 65 °C in a Buchler rotary film evaporator. The sugar concentrate was filter sterilized and added (10%

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v/v) to two-day cultures growing on 3% bagasse. Samples of culture fluid were routinely taken at 10–12 h intervals during growth at 55 °C, clarified by centrifugation (13600 × *g*, 5 min, room temp.) and stored at 4 °C for analyses.

Photomicroscopy

T. curvata culture samples for photomicroscopy were heat-fixed, crystal violet-stained, then photographed in a Zeiss Model I photomicroscope with Kodak Plus-X 35-mm film.

Analyses

Clarified culture fluid samples were assayed for total soluble protein by the method of Bradford [4] using a mixed human albumin/globulin (Sigma Chemical Co., St Louis, MO, catalog no. 540-10) as standard. Reducing sugar was estimated as glucose equivalents by the method of Bernfeld [1].

Units of enzyme activity were expressed as μmol min^{-1}. Amylase (EC 3.2.1.1) was estimated in 2-ml reaction mixtures containing 2% soluble starch (Fisher Scientific, Pittsburgh, PA) in 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer, pH 5.6 and 0.2 ml of clarified culture fluid incubated at 65 °C for 30 min. Cellulase activity was routinely estimated as endoglucanase (EC 3.2.1.4) by reducing sugar liberation from carboxymethyl cellulose (CMC, Hercules, Wilmington, DE, Type 7L1) since exoenzyme activity against CMC and crystalline cellulose was shown to be proportional throughout growth [30]. Reaction mixtures (2 ml) contained 2.5% CMC buffered to pH 6.2 (0.1 M MES) and 10 μl of appropriately diluted enzyme incubated for 10 min at 65 °C. Filter paper cellulase activity was also measured at the same pH and temperature in some samples according to the method of Mandels et al. [16] as modified by Montenecourt and Eveleigh [20]. Pectinase activity was measured as polygalacturonate lyase (EC 4.2.2.2) using polygalacturonic acid as substrate under the conditions previously described [32]. Xylanase (EC 3.2.1.8) was assayed by the release of reducing sugars from 2.5% (w/v) birch xylan in 2-ml reaction mixtures incubated 20 min at 75 °C, pH 8.1. Activities of β-glucosidase and β-xylosidase were estimated by using 20 μl of clarified culture fluid in 1-ml reaction mixtures containing 2 mM concentrations of *p*-nitrophenyl-β-D-glucoside (buffered to pH 5.6 with 0.1 M MES) or *p*-nitrophenyl-β-D-xylopyranoside (buffered to pH 6.1 with 0.1 M MES) respectively. After incubation for 10 min at 53 °C for the glucosidase or 62 °C for the xylosidase, 2 ml of 1.0 M Na_2CO_3 was added to stop the reaction and to enhance the color of the released *p*-nitrophenol. Nitrophenol concentration was measured spectrophotometrically at 400 nm (E_m of 1.81×10^4 M^{-1} cm^{-1}) against reaction mixtures receiving water instead of enzyme. Units were calculated as micromoles of nitrophenol released per min.

Polyacrylamide gel electrophoresis (PAGE)

Extracellular proteins of clarified culture fluids were concentrated approximately 10-fold by ultrafiltration in Amicon (Beverly, MA) YM10 Centricon units. Retentates were washed free of small molecular weight components by three volumes of 0.01 M phosphate buffer, pH 7.2. Washed retentates (20 μl) were applied to non-denaturing 10% polyacrylamide gels (1.5 × 50 × 80 mm) prepared at pH 8.9 essentially according to the method of Laemmli [12] and run at a constant 75 V.

Sugar analysis

A reaction mixture (4 ml) consisting of 3% bagasse in 0.01 M bis-tris propane buffer (pH 7.0) was incubated with 2 mg washed exoprotein for 30 h at 55 °C. The soluble sugar mixture was clarified by centrifugation and filtered through Gelman (Ann Arbor, MI) 0.45-μm pore size Acrodiscs. Sugars were fractionated by high performance liquid chromatography (HPLC) in an LKB 2150 system with a Waters (Milford, MA) model 610 refractive index detector set at 55°. Hydrolysate components were identified and quantitated by comparison of their elution times and integrated peak areas to those of 32 known standards passed through a Bio-Rad (Richmond, CA) Aminex HPX-87C analytical column (300 × 7.8 mm) at a flow rate of 0.6 ml HPCL grade water per min at 85 °C.

Extracellular enzyme fractionation

Proteins of the washed ultrafiltration retentates were fractionated by size exclusion chromatography (SEC); 10-μl aliquots were injected into a Perkin Elmer (Morrisville, NC) HPLC system (model 10 pump and LC100 plotter integrator) with Isco V_4 detector (280 nm) and Phenomenex BioSep-SEC-S3000 300 × 7.5 mm analytical column. Flow rate was 1.0 ml of 0.1 M phosphate buffered saline (0.15 M) at pH 7.2. Column calibration was performed using the molecular weight standards (12–200 K) in the Sigma MW–GF–200 kit.

Statistical analysis

Standard deviations were calculated for all assay methods (except HPLC fractionations) by running six replicate determinations on representative samples. Standard deviations for amylase, β-glucosidase, β-xylosidase, endoglucanase, filter paper activity, pectinase, xylanase, total protein and reducing sugar were ±6.6%, 6.0%, 2.8%, 5.6%, 7.6%, 4.2%, 3.2%, 3.7% and 1.3% respectively. All values shown in the figures are, unless otherwise noted, averages of flasks from two separate experiments.

RESULTS

Within 10 h after inoculation with *T. curvata*, the bagasse fibers were extensively colonized with mycelia (Fig. 1). Many fibers were wrapped with mycelia along their lengths. The fiber ends appeared to support the heaviest colonization, with as many as 5 or 6 mycelia originating from a single tip.

The influence of bagasse fiber concentration on accumulation of extracellular protein and depolymerizing enzymes was determined. The peak soluble protein levels of clarified culture fluids increased in proportion to initial bagasse concentration up to 4% w/v; at that concentration, soluble

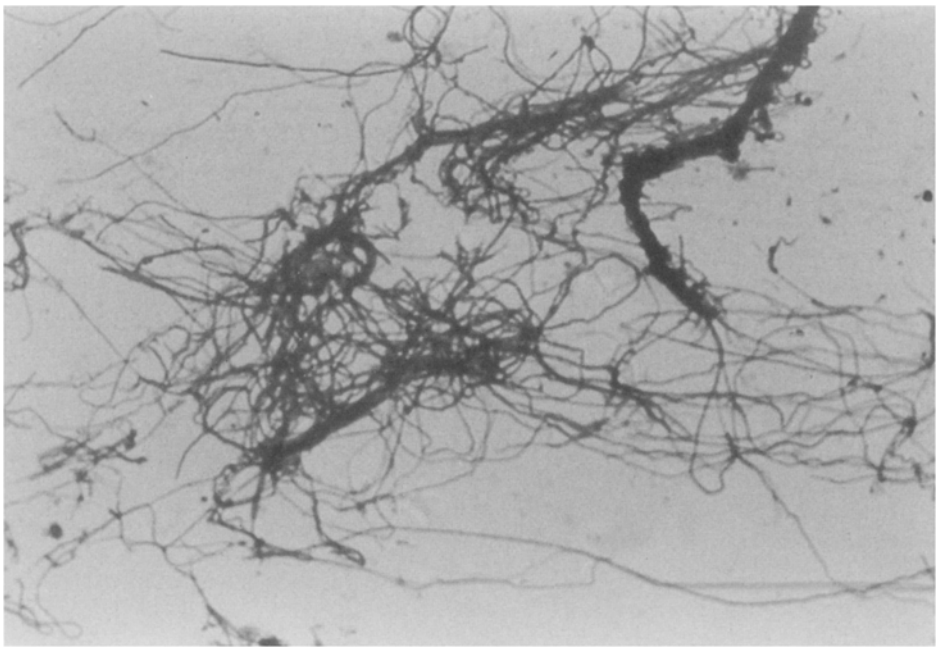

Fig. 1. Colonization of ground bagasse fibers by *T. curvata* mycelia. Note abundance of mycelia emanating from the fiber ends.

protein accumulation approached a maximum by the fifth day of growth (Fig. 2).

The possibility was considered that some of the soluble protein in clarified culture fluid originated from the fibers themselves, either solubilized by the shaking process or released as the result of degradative enzyme activity. To explore this first possibility, a flask containing 4% bagasse was autoclaved in the mineral salts medium, then shaken at 55 °C without inoculation. This passive release of protein into the medium by shaking averaged an apparent 11 μg per ml after 5 days. When subjected to 10-fold concentration by ultrafiltration, the washed retenate did not yield detectable bands on PAGE. The electrophoretic pattern of *T. curvata* proteins produced during growth on bagasse was quite similar (with the exception of two minor bands) to those produced throughout growth on cotton fibers (Fig. 3).

Soluble sugar accumulation was frequently monitored since the extracellular enzyme biosynthesis in this actinomycete is sensitive to cyclic AMP-regulated catabolite repression [42]. Bagasse concentration influenced both the levels of soluble sugar and the time course of its liberation (Fig. 4). Soluble sugars derived from 0.5 or 1.0% bagasse concentrations reached stable levels early in the incubation; this plateau was apparently maintained by non-metabolizable sugars since culture fluid samples did not contain sugars corresponding to any of the 32 chromatographic standards which included all the common sugars known to be utilized by *T. curvata* (detailed data not shown). Bagasse concentrations of 2–5% yielded early surges of soluble sugars, while high concentrations (8–10%) yielded hyperbolic soluble sugar profiles.

These changes in soluble sugar levels apparently influenced

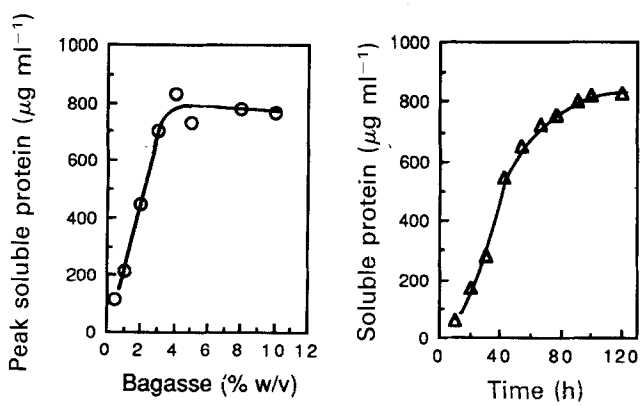

Fig. 2. Influence of bagasse concentration of peak soluble exoprotein accumulation (○); time course of soluble protein accumulation at optimal (4%) bagasse concentration (△).

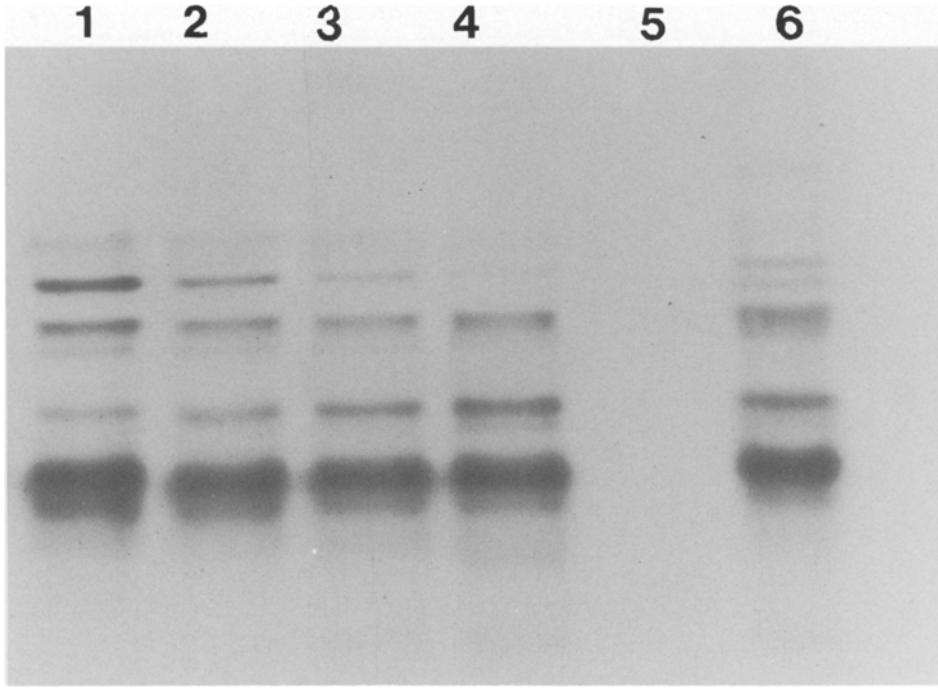

Fig. 3. Electrophoretic comparison of soluble exoprotein ultrafiltration retentates from culture fluids of *T. curvata* grown on cotton fibers or bagasse. Lanes 1–4, exoproteins from cultures grown on cotton for 96, 72, 48 and 24 h respectively; Lanes 5 and 6 are retentates of soluble proteins from uninoculated bagasse medium versus that from a culture grown for 100 h on 3% bagasse respectively.

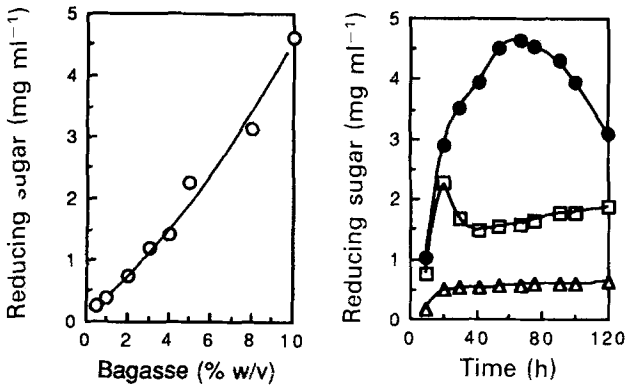

Fig. 4. Influence of bagasse concentration on soluble reducing sugar accumulation (○). Representative time courses of soluble sugar levels at initial bagasse concentrations of 1% (△), 5% (□) and 10% (●).

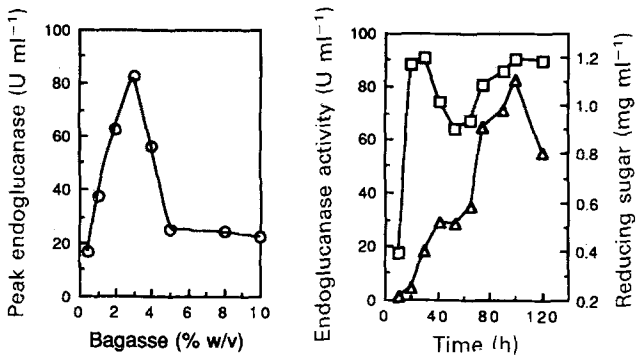

Fig. 5. Influence of bagasse concentration on pea. soluble endoglucanase activity (○). Time courses of soluble sugar (□) and endoglucanase accumulation (△) at the optimal bagasse concentration of 3%.

the rates of extracellular enzyme biosynthesis, particularly in the case of cellulase. Highest endoglucanase levels (82.4 U ml^{-1}, 1.1 filter paper U ml^{-1}) were achieved on 3% bagasse after about 4 days incubation. The initially high reducing sugar release during the first day of growth was followed by a transient repression of endoglucanase biosynthesis (Fig. 5). After some of the sugar was utilized, biosynthesis resumed. A somewhat lesser but similar effect was observed for xylanase biosynthesis which was also induced most effectively (maximum of 30.6 U ml^{-1}) by 3% bagasse and strongly repressed at higher substrate concentrations (Fig. 6). A similar degree of repression was imposed by adding a concentrated ultrafiltrate of sugars from a two-day culture grown on 10% bagasse (detailed data not shown).

Despite the high levels of cellulolytic and xylanolytic activity of cultures grown on 3% bagasse, the extracellular levels of β-glucosidase and β-xylosidase were both less than 0.2 U ml^{-1} throughout the 5-day growth period. The low extracellular β-glucosidase and β-xylosidase levels were apparently not the result of catabolite repression by excess sugar accumulation, since cell-free fluids from cultures grown over the whole range of bagasse concentrations (0.5–10%) had similar activities.

Although bagasse was an excellent inducer of cellulase

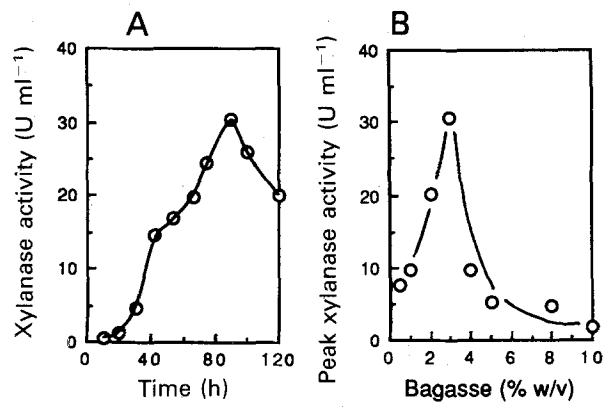

Fig. 6. Time course of xylanase production (A) at the optimal bagasse concentration of 3% (B).

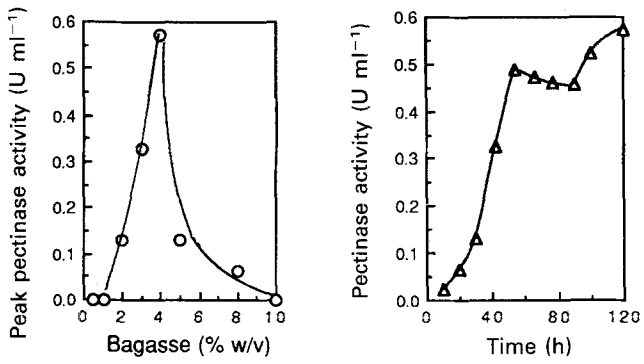

Fig. 7. Influence of bagasse concentration on pectinase production (○). Time course of pectinase activity at the optimal concentration of 4% (△).

and xylanase, it was a relatively poor substrate for pectinase induction in *T. curvata*. The influence of bagasse concentration (Fig. 7) was sharply inflected as it was for cellulase and xylanase, but the maximal observed pectinase activity was only 0.58 U ml^{-1}. Amylase production was also quite low, with a maximum of 1.91 U ml^{-1} at the optimal bagasse concentration of 4% (Fig. 8).

To explore whether low amylase and pectinase activity resulted from catabolite repression, soluble sugar accumulation was maintained at low levels by adding fresh sterile bagasse sequentially (0.5 g daily up to a final concentration of 4%) to a culture started on 1% initial bagasse concentration. Although maximal observed soluble protein release was stimulated about 20%, maximal observed levels of amylase, endogluconase, pectinase and xylanase were decreased 26%, 36%, 4% and 69% respectively, compared to those observed in cultures containing 3% bagasse initially. To test the alternative hypothesis that biosynthesis of amylase and pectinase was limited by inducer availability, *T. curvata* was grown on bagasse for 18 h to establish the base rates of soluble amylase and pectinase biosynthesis. The addition of pectin and starch (100 $\mu g\ ml^{-1}$ concentrations for each) had a marked transient effect on the biosynthesis of their respective enzymes (Fig. 9); these additions had no detectable effect on rates of cellulase or xylanase biosynthesis (detailed data not shown).

Instability under growth conditions also appeared to be a factor in the relatively low amylase and pectinase activities produced in bagasse medium. When culture fluid was removed after 90 h of growth, filter-sterilized, then shaken for another 24 h at 55 °C, half-lives of amylase and pectinase were 31.2 and 2.3 h respectively, while cellulase and xylanase activities were not diminished.

Extracellular enzyme biosynthesis is generally considered to be regulated by induction and catabolite repression [reviewed, 22]. A third control mechanism may be the less well known phenomenon of catabolite inhibition [19] in which the presence of a preferred sugar inhibits the uptake and utilization of all others. Catabolite inhibition would be of particular importance in the induction of depolymerizing extracellular enzymes which require generation of product for induction. It was therefore of interest to identify the sugars released from bagasse by the *T. curvata* enzymes. Incubation of a portion of the ultrafiltration extracellular enzyme retentate with bagasse yielded a complex sugar mixture (equivalent to about 4 mg glucose ml^{-1}) which yielded nine identifiable peaks on HPLC (Fig. 10). Based on the integrated area of each peak, the following sugar concentrations (mg ml^{-1}) were estimated for cellohexaose

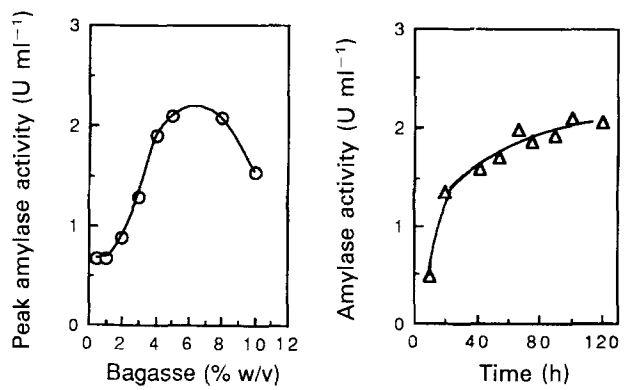

Fig. 8. Influence of bagasse concentration on amylase production (○). Time course of amylase production at the optimal bagasse concentration of 4% (△).

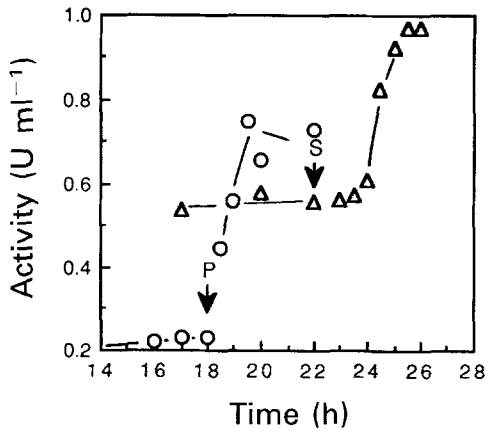

Fig. 9. Induction of pectinase (○) and amylase (△) by the addition (100 $\mu g\ ml^{-1}$) of pectin or starch indicated by P and S respectively.

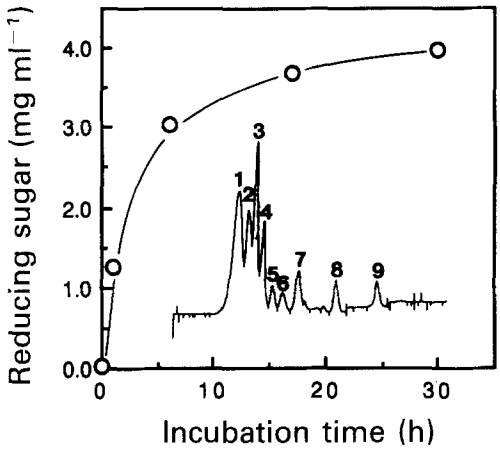

Fig. 10. Solubilization of 3% bagasse by washed concentrated exoenzymes at pH 7.0 (○). Inset depicts HPLC elution pattern as cellohexaose (1), cellotriose (2), cellobiose (3), xylobiose (4), glucose (5), xylose (6), fructose + arabinose (7), mannitol (8) and buffer (9).

(2.12), cellotriose (1.05), cellobiose (1.91), xylobiose (1.20), glucose (0.36), xylose (0.38), fructose + arabinose (0.63) and mannitol (0.39) to yield a total identified soluble sugar yield of 8.04 mg ml^{-1}. This yield represented a 27% conversion of solid fiber to soluble sugar.

In previous studies [15,34], the molecular weights of *T. curvata* exoenzymes were influenced by both culture age and carbon source. Therefore, it was of interest to fractionate the extracellular enzymes concentrated from bagasse culture fluid and compare their native molecular weights with those of the corresponding enzymes produced on purified substrates. SEC-HPLC fractionation of an ultrafiltration retentate yielded a complex protein elution pattern which included discreet peaks for amylase and pectinase and multiple unresolved peaks for both endogluconase and xylanase (Fig. 11). Recoveries of activity after fractionation were 102%, 92%, 64%, and 79% for amylase, endoglucanase, pectinase and xylanase respectively. The molecular weights estimated by SEC-HPLC for the single peaks of amylase and pectinase activity were 58 K and 34 K respectively. The multiple unresolved peaks of endoglucanase and xylanase activity had retention times corresponding to apparent molecular weights of 38 K and 180 K, and 26 K and 58 K, respectively.

DISCUSSION

T. curvata mycelia adhered firmly to the bagasse fiber. This ability may depend on a specific adherence factor contained in its surface cellulosomes [11] since a cellulose affinity substance has been described for the cellulolytic anaerobe, *Clostridium thermocellum* [14]. The ability of a microbe to adhere to cellulose merits attention from both

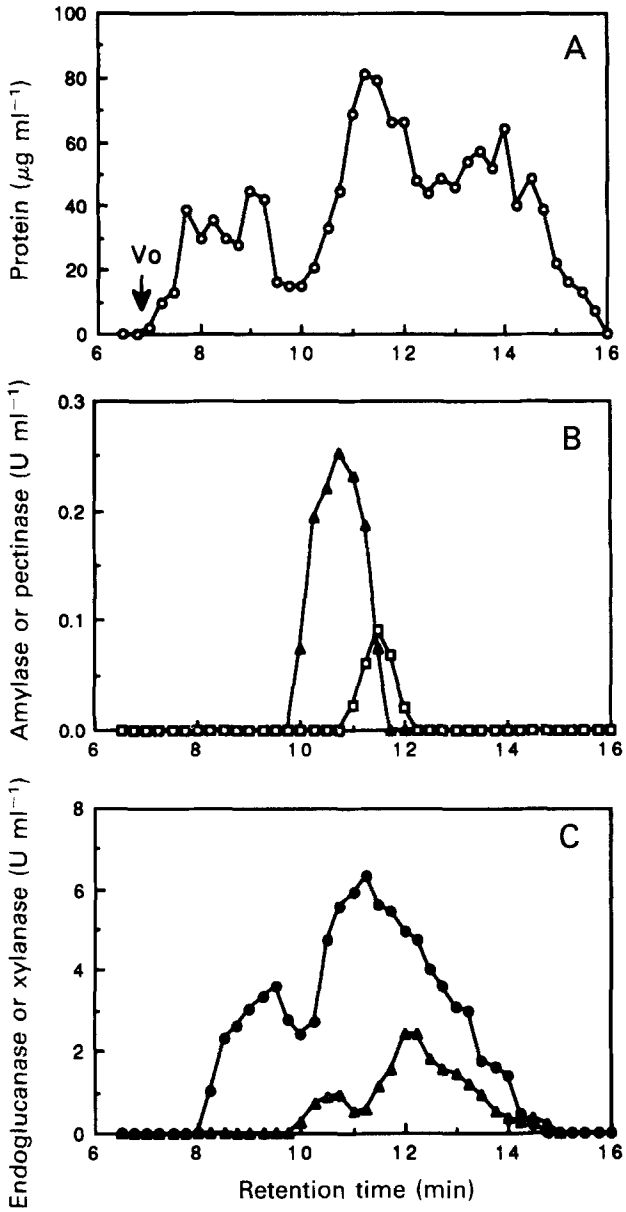

Fig. 11. SEC-HPLC fractionation of washed ultrafiltration retentate exoproteins. (A) Column void volume retention time (V_0) indicated at 6.89 min. Activities indicated for (B) amylase (△), pectinase (□), (C) endoglucanase (●) and xylanase (▲).

basic and applied standpoints. Maintenance of the cell in firm contact with a substrate surface confers competitive advantages in nature [reviewed, 13], while under industrial conditions, microbial adherence to residual substrate solids with the subsequent formation of fiber–mycelial mats facilitates phase separation during exoenzyme recycling and soluble product extraction [27].

T. curvata responded to bagasse in a manner similar to that observed during growth on purified cellulose. Electrophoretic protein patterns from washed ultrafiltration retentates of bagasse-grown and surgical cotton-grown cultures shared most bands in common, suggesting that the actinomycete reacts to bagasse largely as a cellulose source.

This is corroborated by the observation that bagasse supported higher cellulase production by *T. curvata* than any other natural or purified substrate tested [3,31,33,37]. Xylanase (which is co-induced with cellulase, [3]) exceeded levels observed on either purified xylan or cellulose [35]. Furthermore, the low amylase and pectinase levels on bagasse were comparable to those observed earlier on cellulose [32,36]. This reaction to bagasse essentially as a cellulose source may be explained by the sugars released by *T. curvata* enzymes from bagasse; over 60% of the total soluble sugars were cellooligo- or disaccharides. This product mix has a marked influence on the induction of extracellular depolymerizing enzymes. Unlike intracellular enzymes, which are induced by their substrates or derivatives thereof [reviewed, 40], depolymerizing extracellular enzymes are induced by low concentrations of product released by their constitutive levels from their respective substrate polymers in the environment [reviewed, 22]. This mechanism exerts particular control in the case of *T. curvata* in which cellobiose is the preferred sugar. This preference represents an unusual example of catabolite inhibition, a phenomenon first reported by Gaudy et al. [9] who showed that glucose inhibited the uptake and utilization of all other carbohydrates in *Escherichia coli*. This and subsequent studies [19,28] led to the generalization that glucose was the microbial carbon and energy source *par excellence* and was always used first [reviewed, 40]. *T. curvata* is a notable exception to that rule in that cellobiose stops both the uptake and utilization of glucose under all cultural conditions, even in the absence of the energetically advantageous cellobiose phosphorylase [2]. Since cellobiose and higher cellooligosaccharides were the major products of *T. curvata* extracellular enzymes acting on bagasse, their accumulation during growth would assure that only enzymes related to cellulose degradation (or enzymes co-induced with those enzymes) would be induced. In that regard, the low β-glucosidase and β-xylosidase activities in culture fluid during growth on bagasse are reflected in the high cellobiose/glucose and xylobiose/xylose ratios in the product mixture.

The amylase and pectinase concentrated from cell-free culture fluid separated into discreet peaks on SEC-HPLC. The amylase had a molecular weight comparable to that produced earlier by *T. curvata* on purified starch [10]. In contrast, the molecular weight of the pectinase from bagasse was 34 K compared to the 56 K pectinase produced on highly purified pectin [32]; this smaller molecular weight was identical to that of the pectinase induced in *T. curvata* during growth on protein-extracted lucerne fibers [34]. This difference in the pectinase produced on complex plant fiber versus that produced on purified pectin may be a reflection of limited proteolytic modification, since growth of *T. curvata* on plant fiber is accompanied by relatively high extracellular protease activity which can partially degrade the pectinase and alter its kinetic characteristics [34]. It is of some interest in this regard that none of the peaks from SEC-HPLC fractionation of cellulase or xylanase had apparent molecular weights which corresponded to previously determined molecular weights for the corresponding enzymes produced on purified substrates [15,35]. The extensive heterogeneity of the cellulases and xylanases apparent on SEC may also reflect the result of increased protease activity during growth on bagasse.

While bagasse was an excellent inducer of cellulase and xylanase in *T. curvata*, it was a relatively poor substrate, yielding only 27% of its dry weight as soluble sugar after prolonged incubation. Although chemical pretreatments of bagasse and other lignocellulosic materials can greatly increase bioconversion rates [reviewed, 38], the high costs associated with recovery of pre-treatment chemicals make alternatives (such as increased enzyme production and recycling) more appealing in the search for an economically feasible process.

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