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Preliminary studies on the endo-xylanase and acetyl esterase of *Clostridium thermohydrosulfuricum* (JW 102)

I M. Ezeonu*, A. N. Moneke, and S. K.C. Obi
Department of Microbiology, University of Nigeria, Nsukka, Nigeria.

Abstract
Clostridium thermohydrosulfuricum (JW102) produced low levels of xylanase in mineral medium containing yeast extract and beechwood xylan. Production of xylanase was highest at growth temperatures of 62 – 64°C. Both xylan and xylose supported acetyl esterase production. Xylose, as sole carbon source, supported the production of slightly more acetyl esterase than xylan, while enzyme produced in the presence of xylan was relatively more stable in the fermentation medium, maintaining over 80% of peak activity after 20 hours. Acetyl esterase activity was highest at assay pH of 6.5. At pH 5.5 and 6.0, enzyme activity was, respectively, 5% and 22% of that recorded at pH 6.5.

Key words: Hemicellulases, *Clostridium thermohydrosulfuricum*, xylanase, acetyl esterase, beeechwood xylan.

Introduction

Plant biomass is essentially lignocellulose. Lignocelluloses are complex polymers of cellulose, hemicelluloses and lignin, and represent the most abundant renewable organic matter on earth (Okeke and Obi, 1995; Gawande and Kamat, 1999; Gupta *et al.*, 2000). Xylan is a major component of hemicelluloses and makes up about 30% of the dry weight of the cell walls of monocotyledons (Wong *et al.*, 1988). Structurally, it consists of a backbone of 1,4-linked β-D-xylopyranosyl residues and side chains of L-arabinofuranose, D-glucuronic or 4-O-methyl-D-glucuroonic acid. Acetylation at O-3 or O-2 position is a common feature of the xylans of many wood species.

Complete breakdown of xylans requires the synergistic action of such xylanolytic enzymes as endo-1,4-β-xylanase (EC3.2.1.8), β-xylosidase (EC3.2.1.37), α-glucuronidase (EC3.2.1), α-L-

*Corresponding author

arabinofuranosidase (EC3.2.1.55), acetyl esterase (EC3.1.1.6) and has recommended itself for biobleaching and bioprocessing of pulps and the treatment of effluents from paper industries (Garg *et al.*, 1998; Gupta *et al.*, 2000).

In recent years, there have been reports on xylanases from moulds (Puchart *et al.*, 1999), yeasts (Liu *et al.*, 1998), actinomycetes (Fernandez *et al.*, 1998), and bacteria (Gessesse and Mamo, 1998; Inagaki *et al.*, 1998). Most of these published works on xylanases relate to aerobic microorganisms, leaving the anaerobes somewhat underreported. However, notable attempts at correcting this apparent imbalance have been made by Wiegel *et al.* (1985), Shao *et al.* (1995) and De Blois and Wiegel (1995).

In this paper, we report the results of preliminary studies on some hemicellulases of *Clostridium thermohydrosulfuricum* (JW 102), an obligate thermophilic anaerobe.

Methods

Organism and cultivation conditions

Clostridium thermohydrosulfuricum was obtained from Prof. Juergen Wiegel of the Department of Microbiology, University of Georgia, Athens, GA, USA. The organism was grown in a standard mineral medium constituting (g l^{-1}): $Na_2HPO_4.12H_2O$, 9; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.2; NH_4Cl, 1.0; ferric ammonium citrate, 0.005; $CaCl_2.2H_2O$, 0.010 and trace elements including (mg l^{-1}): $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $CoCl_2.6H_2O$, 20; $CuCl_2.6H_2O$, 0.79; $NiCl_2.6H_2O$, 2; and $Na_2MoO_4.2H_2O$, 3. The pH was adjusted to 6.9 and then made anaerobic using the Hungate technique (Brant, 1972), which involved bringing the medium to a boil and gassing with a mixture of 5% H_2 and 95% N_2 for 15 mins. While still maintaining the gassing procedure, the solution was dispensed in 10 ml portions into pregassed Hungate tubes using a pregassed serological pipette. Thereafter, the tubes were sealed with butyl rubber stoppers and crimped before autoclaving at 121°C for 15 minutes.

Evaluation of yeast extract and beechwood xylan as substrates for C. thermohydrosulfuricum

A set of mineral medium supplemented with 0.2% yeast extract and another with yeast extract and 0.5% beechwood xylan were each inoculated with 10% of 48h old culture of *C. thermohydrosulfuricum* (JW 102). The cultures were incubated at 60°C and cell counts (microscopic) were carried out at given time intervals.

Effect of temperature on xylanase production by C. thermohydrosulfuricum

Sterile mineral medium containing 0.2% yeast extract and 0.5% beechwood xylan (pH 6.9) was inoculated with 10% inoculum of 48h old culture of *C. thermohydrosulfuricum* (JW 102) and incubated at temperatures ranging from 60°C to 70°C in a temperature gradient incubator. At specified time intervals, the cultures were assayed for xylanase activity. Enzyme activities were determined from culture supernatants obtained by centrifugation at 5,000 x g for 30 minutes.

Effect of beechwood xylan, and xylose on acetyl esterase production

Acetyl esterase production was studied under two conditions. In one condition, the mineral medium was supplemented with 0.2% yeast extract and 0.5% beechwood xylan (hereafter referred to as xylan medium); and in the second condition, the medium was supplemented with 0.2% yeast extract

and 0.5% xylose (hereafter referred to as xylose medium). Each medium (pH 6.9) was inoculated with 1.0 x 10^7 cells/ml of a 48h old culture of *C. thermohydrosulfuricum* (JW 102) and incubated at 60°C. Enzyme activity, cell counts (microscopic) and pH changes of the cultures were determined at given time intervals.

In these studies, all solutions of yeast extract, beechwood xylan and xylose used as mineral medium supplements were made anoxic by the Hungate technique and sterilized by autoclaving at 121°C, 15 mins.

Effect of pH on acetyl esterase activity

Mineral medium with 0.2% yeast extract and 0.5% beechwood xylan (pH 6.9), was inoculated with 1.5 x 10^7 cells/ml *C. thermohydrosulfuricum* (JW 102) and incubated at 60°C for 42 hours. Thereafter, the culture supernatant obtained as described previously was assayed for acetyl esterase activity. To determine the optimum pH for activity, the enzyme was assayed at pH 5.5, 6.0 and 6.5.

Enzyme assays

Xylanase

Cultures were assayed for xylanase activity using the parahydroxy benzoic acid hydrazide (PAHBAH) assay method. To this end, 0.4ml of the enzyme (culture supernatant) was mixed with 0.4ml of 0.6% beechwood xylan solution containing 0.05% NaN_3 (pH, 6.4) and placed immediately in an ice bath; 0.1ml of this mixture was immediately pipetted into a test tube and placed on ice. The remaining substrate / supernatant mixture was then kept in a water bath and maintained at 60°C for 15 minutes. Thereafter, 0.1ml of the reaction mixture was pipetted into a test tube followed by the addition of 0.4ml distilled water and 1.5ml PAHBAH reagent. The tube was heated in a boiling water bath for 10 minutes after which the absorbance at 405nm was measured. Assays were conducted in triplicates and an uninoculated mineral medium was used as control. Enzyme activity (μmol/ml/min) was read from a xylose standard curve.

Acetyl esterase

Acetyl esterase activity was determined according to Methods in Enzymology (Johnson *et al.*, 1988) using p-nitrophenylacetate in potassium phosphate buffer (pH 5.5; 6.0; and 6.5) as substrate. The enzyme sample (0.1 ml culture supernatant) was incubated with 0.9ml of substrate at 60°C and absorbance at 405nm was measured at given time intervals. The enzyme activity was read from a standard curve of para-nitrophenol.

Results and Discussion

Growth, and xylanase production by C. thermohydrosulfuricum

C. thermohydrosulfuricum (JW 102) was grown in mineral medium with yeast extract and in mineral medium containing yeast extract and beechwood xylan. The medium with yeast extract alone supported slightly better growth of the organism than medium containing xylan (Fig. 1).

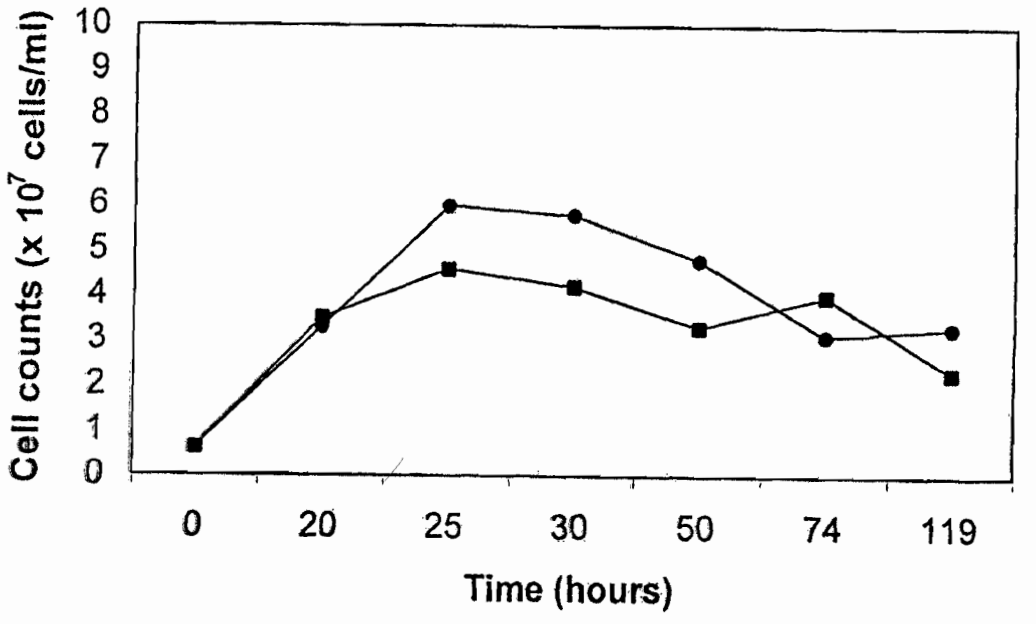

Fig.1: Growth of *C. thermohydrosulfuricum* in mineral medium with yeast extract (●) and mineral medium with yeast extract and 0.5% beechwood xylan (■).

When grown at temperatures ranging from 60 to 70°C, enzyme production was strongest (4.0 nmol/ml/min) at 64°C (Table 1).

Table 1. Effect of incubation temperature on xylanase production by *C. thermohydrosulfuricum*

Incubation temperature (°C)	Xylanase activity (nmol/ml/min)
60	1.03
62	3.5
64	4.0
66	2.6
68	1.8
70	not detectable

The low levels of enzyme recorded under the experimental culture conditions may be due to a number of reasons: The working strain of *C. thermohydrosulfuricum* (JW 102) may be an intrinsically weak xylanase producer, a situation which could arise if the organism is inbued with low copy number of the xylanase gene; and if the strength of the transcriptional promoter is

suboptimal. Native promoters in different genes are known to vary considerably in RNA polymerase binding strength (Brock *et al.*, 1994). Strong promoters are critical for strong gene expression. Xylanases, like many catabolic enzymes of bacteria are inducible rather than constitutive and exist in multiple and distinct forms. Their production in microorganisms requires the presence of their specific substrates and inducers among which are beechwood, birchwood larch, and oat spelt xylans with the latter being the most effective in thermophilic anaerobes (Wiegel, 1985; De Blois and Wiegel, 1995). The difference in induction performance of these various xylans may not be unconnected with the structural and compositional variability associated with xylans from different sources (Shao *et al.*, 1995).

In this study, beechwood xylan at 0.5% (w/v) served as both carbon source and inducer and resulted in low xylanase production by *C. thermohydrosulfuricum* (JW 102). De Blois and Wiegel (1995) showed that in addition to right choice of inducer, the right concentration of the inducer is critical to high enzyme production. This was demonstrated with oat spelt xylan, which at 0.05% (w/v) resulted in strong induction of xylanase in *Clostridium* sp. EPP 100 but repressed the enzyme at higher inducer levels. It is therefore conceivable that the very high concentration of beechwood xylan (higher by a factor of 10) may have negatively impacted on xylanase production by *C. thermohydrosulfuricum* (JW 102).

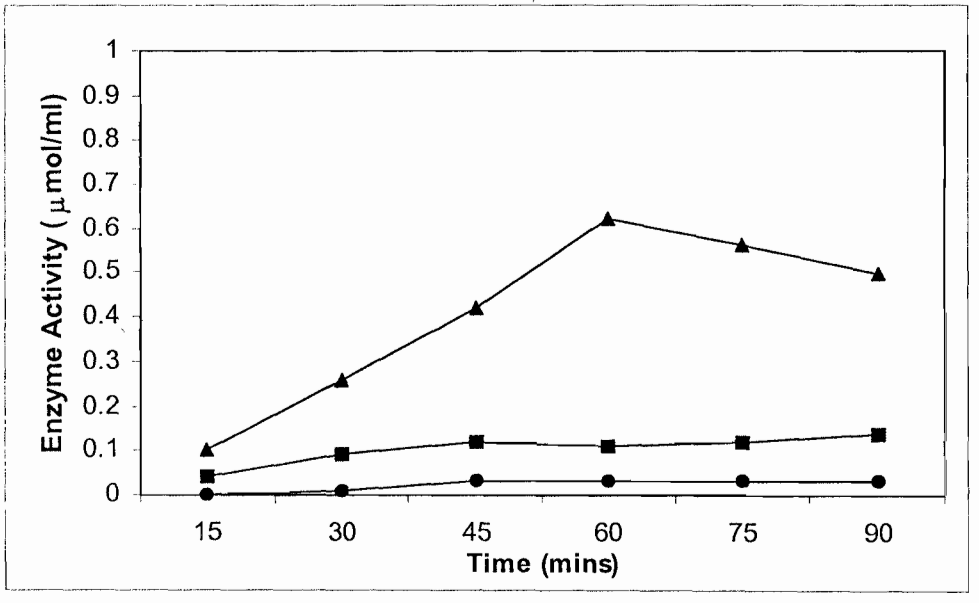

Fig. 2. Activity of acetyl esterase of *C. thermohydrosulfuricum* (JW 102), at pH 5.5 (●), pH 6.0 (■) and pH 6.5 (▲).

Production and activity of acetyl esterase

Assay of culture supernatants showed that C. thermohydrosulfuricum (JW 102) produced acetyl esterase. The enzyme had its highest activity at pH 6.5. At this pH, the measured activity followed a linear curve, rising steadily until a reaction time of 60 minutes when the highest activity was recorded. Thereafter, the activity dropped. At pH 5.5 and 6.0, activity levels were, respectively, 5% and 22% of that at pH 6.5 (Fig. 2).

Information on the pH activity optima of microbial acetyl esterases is scanty. However, Poutanen and Sundberg (1988) reported a pH optimum of 5.5 for Trichoderma reesei while 7.0 and 7.5 were recorded for acetyl esterase I and II respectively of Thermoanaerobacterium sp. (JW/SL – YS 485) by Shao and Wiegel (1995).

Acetyl esterase production on xylan medium and on xylose medium

In xylan medium, both enzyme activity and cell count peaked at 40h. Thereafter, the cell count dropped steadily until the end of the investigation. Enzyme activity, on its part, dropped slightly at 50h and remained at that level at 70h before further decrease. The pH changed only slightly (6.9 – 5.9) throughout the investigation (Fig. 3).

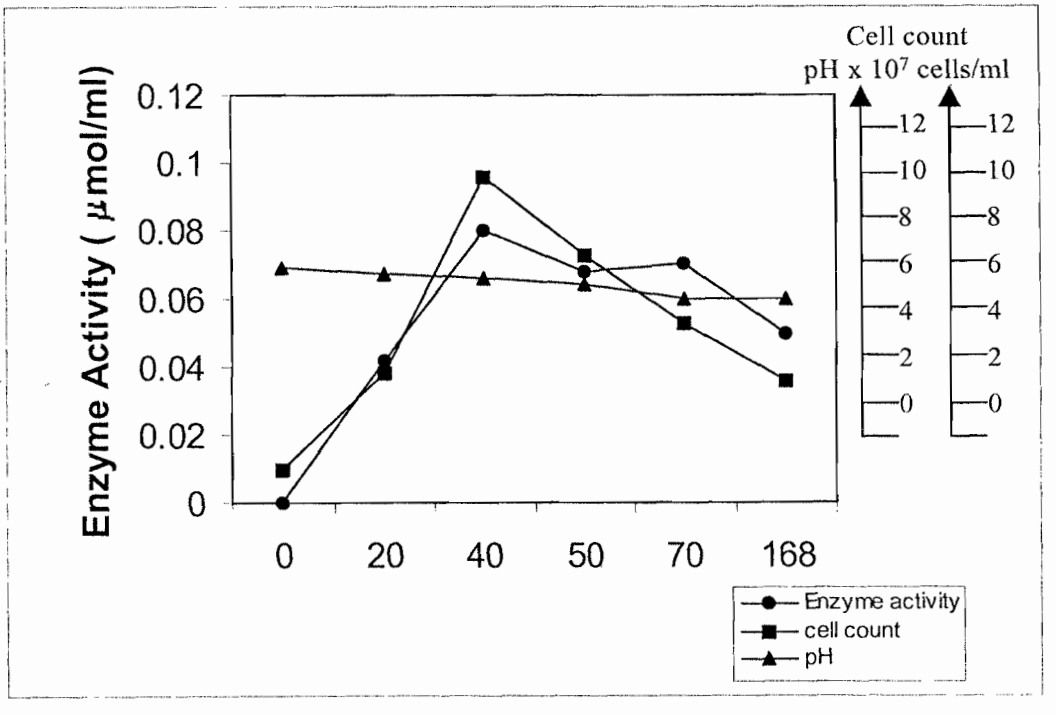

Fig. 3. Time course of acetyl esterase production in mineral medium plus xylan by C. thermohydrosulfuricum (JW 102).

In xylose medium, on the other hand, the cell count achieved an early peak at 20h and remained at this level at 40h, after which it started dropping. Enzyme production was also at its maximum (0.1 μmol/ml) at 40h. At 50h, however, a sharp decrease in both cell count and enzyme level was recorded. The loss of activity of the enzyme in xylose medium corresponded to the drop in pH of the medium to less than 6 (Fig.4).

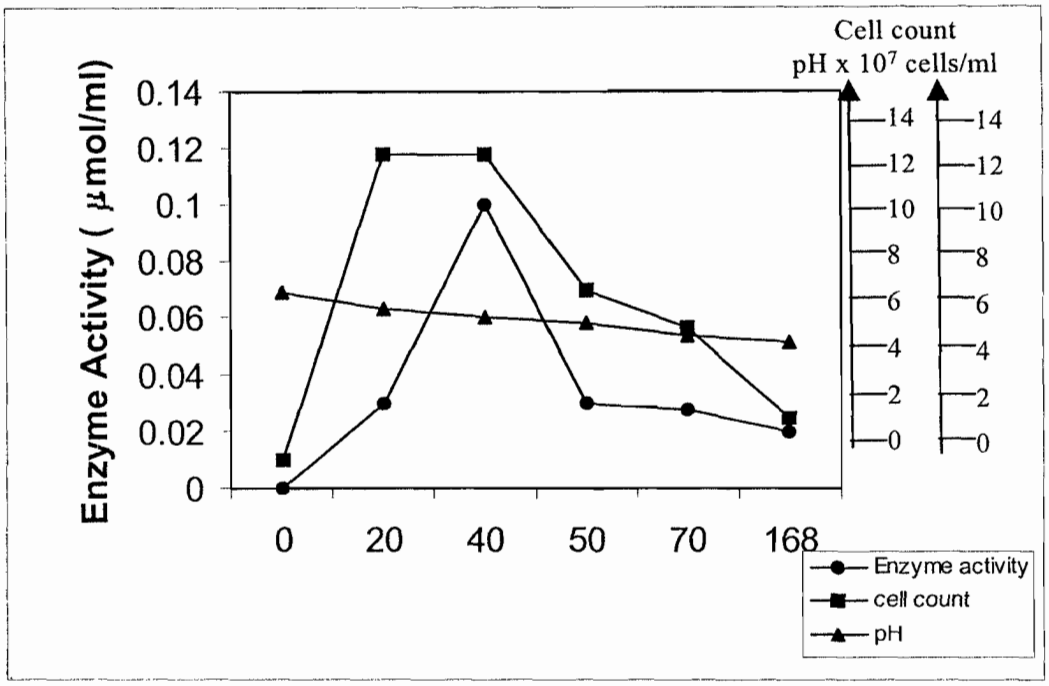

Fig. 4. Time course of acetyl esterase production in mineral medium plus xylose by C. thermohydrosulfuricum (JW 102).

These results suggest that at pH 6, and below, acetyl esterase production is suboptimal in Clostridium thermohydrosulfuricum (JW 102) and that the growth of the organism on beechwood xylan is not accompanied by massive release of organic acids into the medium, hence the little change in pH.

On the whole, it is not possible at this stage, to pass a verdict on the promise or otherwise of C. thermohydrosulfuricum (JW 102) as a source of high yields of xylanase and acetyl esterase. Further studies with such proven strong inducers as oat spelt xylan, cellobiose, xylose and lactose (De Blois and Wiegel, 1995; Singh et al., 2000) are required.

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