World Journal of Microbiology & Biotechnology **20**: 629–632, 2004.
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Production of cellulase by *Clostridium papyrosolvens* CFR-703

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Received 17 February 2003; accepted 17 February 2004

Keywords: anaerobic, cellulase, *Clostridium papyrosolvens*, fermentation, production

Summary

Clostridium papyrosolvens producing filter paperase, carboxymethyl cellulase and cellobiase under anaerobic cultivation conditions at 35 °C is described. Higher activities of filter paperase and carboxymethylcellulases were assayed in 48 h culture filtrate, while maximum cellobiase accumulated in the culture broth at 72 h. Filter paperase, carboxymethylcellulase and cellobiase activities were optimum at 35 °C and pH values of 7.0, 6.5 and 7.5 respectively. Cultivation of the strain in 1000 ml Hungate bottles with 1% cellulose at pH 6.5 and 35 °C produced carboxymethyl cellulase, filter paperase and cellobiase activities of 45, 35 and 20 IU/ml respectively.

Introduction

Lignocellulosic wastes are the largest group of wastes present on this planet causing environmental pollution (Rani & Nand 2000). Cellulases are enzymes which hydrolyse the β-1,4-glycosidic linkages of cellulose. They fall into 13 of the 82-glycoside hydrolase families identified by sequence analysis, and are traditionally divided into endoglucanases (E.C. 3.2.1.4) and cellobiohydrolases (E.C. 3.2.1.91) (Schulein 2000). Cellulolytic microorganisms produce a wide variety of different catalytic and non-catalytic enzyme modules, which form the cellulases and act synergistically at their substrates (Bayer *et al*. 1998a, b).

Attempts have been made to develop processes for cellulase production using fungal cultures such as *Neocallimastix frontalis* (Mountfort & Asher 1985), *Trichoderma reesei* (Ilmen *et al*. 1997, Mattinen *et al*. 1997), *Penicillium pinophilum* and *Phanerochaete chrysosporium* (Henricksson *et al*. 1999), *Thermomyces chryosporium, Humicola insolvens* (Schulein 1997) and *Aspergillus oryzae* (Takashima *et al*. 1998).

Bacterial cellulases have not been extensively studied, except for some reports on *Bacteroides succinogenes* (Lewis *et al*. 1988), *Clostridium thermocellum* (Mori 1992), *Clostridium thermocopraie* (Jin & Toda 1989) and *Acetovibrio cellulolyticus* (Mackenzie *et al*. 1985) and *Cellulomonas fimi* (Tull & Whithers 1994). The bacterial cellulases have very high activities against crystalline celluloses like cotton or Avicel (Johnson *et al*. 1981) and are also more thermostable in comparison to fungal cellulases.

Since *Clostridium papyrosolvens* was identified as an effective cellulase producer, studies on cultivation conditions for optimum production of the enzyme were carried out and the results are discussed in this paper.

Materials and methods

Bacterial strain and cultural conditions

The culture used in this study was a strain of *Clostridium papyrosolvens* isolated at CFTRI, Mysore (Sharmila *et al*. 2001). The organism was grown in Lewis medium under anaerobic conditions at 35 °C.

Chemicals

Cellulose, cellobiose, carboxymethylcellulose, Avicel were obtained from Sigma Chemical Co., USA. All other chemicals were of reagent grade obtained from Qualigens, India.

Enzyme assay

Filter paperase, carboxymethylcellulase and cellobiase were assayed according to the procedure described earlier (Sharmila *et al*. 1998; Rani & Nand 2001). Enzyme activities were expressed as units. One unit of enzyme corresponded to 1 μmol of glucose released min^{-1} by 1 ml of the culture broth.

Table 1. Production of cellulase by *Clostridium papyrosolvens* using different agricultural residues as substrates.

Substrate (0.5%)	Enzyme activities of different components of cellulase (IU/ml)		
	CMCase, 48 h	Cellobiase, 72 h	Fpase, 48 h
Rice bran	21	17	17
Wheat bran	30	25	13
Coconut coir	35	21	11
Jute fibre	17	15	7
Paddy straw	19	11	5
Maize stalk	25	7	3
Jower stalk	13	5	9
Filter paper (Whatman No. 1)	9	16	6
Absorbent cotton	5	30	15

Pre-treatment of lignocellulosic materials

Agricultural wastes (200-mesh size) were used at 3% concentration as sources of carbon (see Table 1).

Results and discussion

Effect of incubation time on cellulase production

The highest yields of carboxymethylcellulase and filter paperase were obtained after 48 h, whereas cellobiase gave maximum yield after 72 h of incubation. Maximum production of filter paperase and carboxymethylcellulase were obtained after 96–120 h in *Streptomyces albaduncus* (Harchand & Singh 1997). Production of cellulases at comparatively earlier stages of fermentation for the identification of *C. papyrosolvens* suggested the usefulness of this strain for enzyme production.

Effect of pH on cellulase production

The optimum pH for filter paperase, carboxymethylcellulase and cellobiase activities were found to be 7.0, 6.5 and 7.5, respectively (Figure l). Similar observations were made for *Thermoactinomyces* (Hagerdal & Harriel 1979) and *Nectria catalinensis* (Pardo & Forchiassin 1998). pH values of 5.2 and 5.6 were found to be optimum for the production of endoglucanase and

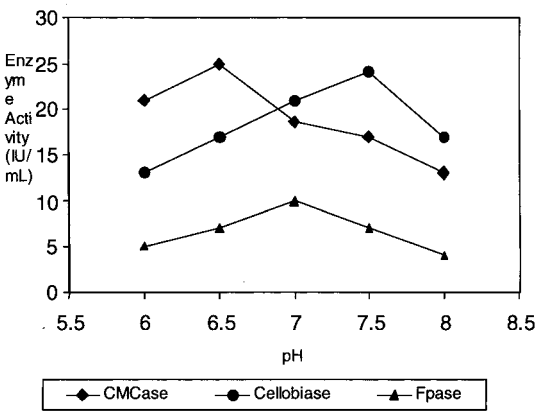

Figure. 1. Effect of PH on enzyme production by *Clostridium papyrosolvens* CFR-703.

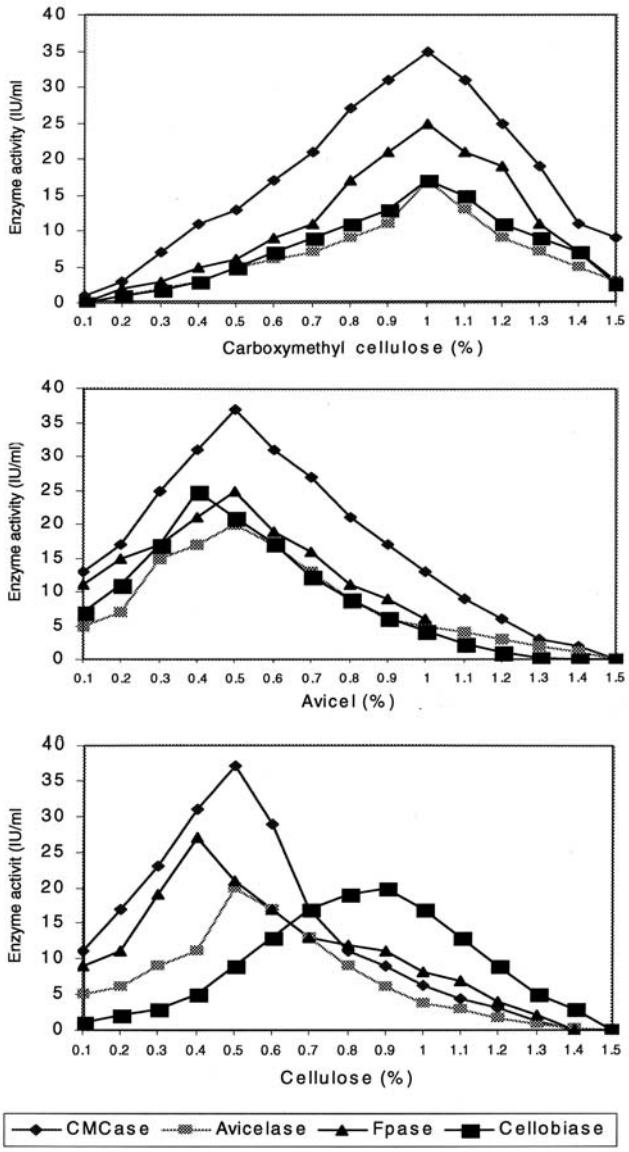

Figure. 2. Effect of various concentrations of carboxymethylcellulose, avicel and cellulose on enzyme production by *Clostridium papyrosolvens* CFR-703.

cellobiase of *Clostridium acetobutylicum* (Song *et al.* 1985).

Effect of temperature on cellulase production

Filter paperase, carboxymethylcellulase and cellobiase showed maximum activities at 35 °C. While *Clostridium celerecrescens* (Malek *et al.* 1988) showed a similar trend as observed in this study, *Clostridium thermocellum* (Showale & Sadana 1978) showed the maximum activity at 70 °C.

Effect of agricultural wastes on cellulase production

Table 1 shows the production of cellulase using different types of agricultural wastes. The highest carboxymethylcellulase activity was achieved with coconut coir. Maximum activity of filter paperase, cellobiase were observed with rice bran followed by wheat bran.

Cellulase production during growth on various carbon sources

Maximum activities of filter paperase, Avicelase and carboxymethylcellulase were recorded with 0.5% cellulose, while maximum activity of cellobiase was observed with 1% cellulose (Figure 2). Similar observations were made for *Pseudomonas* sp., *Streptomyces* sp., *Bacillus* sp., *Cytophaga* sp., *Serratia* sp., (Doi *et al.* 1998) and *Bacteroides succinogens* (Lewis *et al.* 1988). Addition of carboxymethylcellulose in the cultivation medium did not affect the enzyme yields as did other carbon sources such as cellulose and Avicel.

Effect of nitrogen sources on cellulase production

The effect of different nitrogen compounds on cellulase production by *Clostridium papyrosolvens* was investigated with 0.3% nitrogen content using appropriate carbon sources (Table 2). Of the organic and inorganic nitrogen sources used, the highest yields of all the three components of cellulases were obtained with yeast extract. The present results showed lower cellulase activity with inorganic nitrogen apparently, suggesting reduced utilization of inorganic nitrogen by anaerobic bacteria.

Effect of metals on cellulase production

Metal ions have been shown to influence enzyme production by microorganisms in culture (Rani & Nand 2000). Ferrous ion was found to induce the maximum activity of carboxymethylcellulase, whereas manganese gave the maximum activities of filter paperase and cellobiase. Silver, mercury and copper were found to inhibitory (data not shown) (Table 3).

Scale-up studies on cellulase production

Studies were conducted using the optimized fermentation variables for cellulase production by *C. papyrosolvens* in a series of 1 l Hungate bottles under anaerobic

Table 2. Effect of nitrogen sources on cellulase production.

Nitrogen source (0.3%)	Enzyme activity of different components of cellulase (IU/ml)		
	CMCase, 48 h	Cellobiase, 72 h	Fpase, 48 h
$(NH_4)_2SO_4$	21	5	2
$(NH_4)_2HPO_4$	27	13	11
$(NH_4)_2NO_3$	9	11	3
NH_4Cl	17	7	5
$NaNO_3$	30	17	15
Yeast extract	39	23	19
Peptone	14	15	6
Urea	11	15	7

Table 3. Effect of Metal ions on cellulase production by *Clostridium papyrosolvens.*

Metal ion (10 mM)	Enzyme activity of different components of cellulase (IU/ml)		
	CMCase, 48 h	Cellobiase, 72 h	Fpase, 48 h
Zn^{2+}	21	5	21
Fe^{2+}	41	9	14
Co^{2+}	9	11	9
Mn^{2+}	17	15	7
Hg^{2+}	3	2	1
Ag^{+}	5	1	2
Ca^{2+}	33	17	17
Mg^{2+}	27	27	25

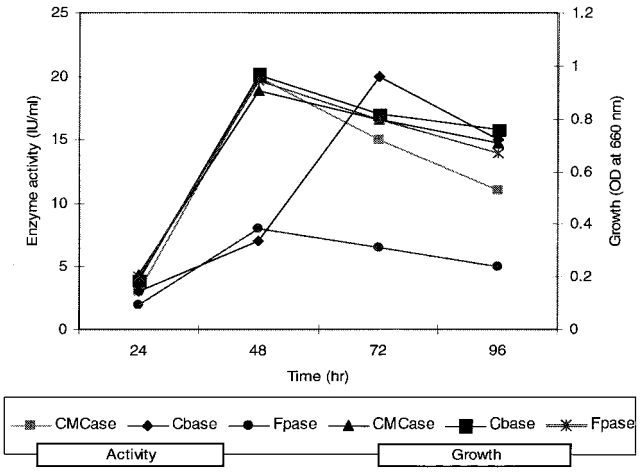

Figure. 3. Kinetics of scaled-up enzyme production by *Clostridium papyrosolvens* CFR-703.

cultivation conditions (Figure 3). Essentially similar results were observed to those of the small-scale cultures.

Conclusion

Results of the present studies suggested the use of *C. papyrosolvens* for cellulase production in shorter periods of time with a cheap medium for enzyme production. There are very few reports in literature on the standardization of fermentation variables using anaerobic bacteria. Hence, these results add significance for the possible exploration of this organism for the production of industrial enzymes.

Acknowledgements

Authors thank Dr. V. Prakesh, Director, CFTRI, Mysore for keen interest in the work. Financial support by Ministry of Non-Conventional Energy Sources (MNES), Government of India, New Delhi is gratefully acknowledged.

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