Culture Conditions For Endo-β-Glucanase Production By *Paecilomyces Species*

J. C. Okolo[1] and S. K. C. Obi[2]
[1]Department of Industrial Microbiology, Federal University of Technology, P.M.B.1526, Owerri
[2]Department of Microbiology, University of Nigeria, Nsukka

Corresponding author: J. C. Okolo, Department of Industrial Microbiology, Federal University of Technology, P.M.B.1526, Owerri, Imo State, Nigeria

Abstract

Production of endoglucanase (E C 3.2.1.4) by *Paecilomyces* sp on different cellulose substrates, nitrogen sources, surfactants, pH, temperature and aeration was studied. Optimal enzyme production was obtained in a mineral salt medium containing ammonium sulphate (0.5%w/v) and Rice hull (5.0% w/v) at an initial pH of 5.0 at 30^{0}C under shake flask condition. Addition of surfactants reduced enzyme production. Time course for endoglucanase production by the organism showed that enzyme production followed the same pattern as the extracellular protein synthesis and increased with the exponential growth phase. The highest enzyme production (6.4 Uml $^{-1}$) was obtained on the eight day of incubation at a culture pH of 4.5.

Keywords: *Paecilomyces* sp, Endoglucanase, Rice hull.

Introduction

Cellulose is a glucose polymer which comprises about two-third of the total carbohydrate content of most woody plants and about half of the total carbohydrate content for herbaceous plants (Lynd,1996). Its hydrolysis would help alleviate the shortage of food and feeds, help solve waste disposal problems and diminish enormous dependence on fossil fuels by providing a convenient and renewable source of energy (Kumakura 1997). Endo-β-glucanase or 1,4-β-D-glucan-4-glucanohydrolase (E.C 3.2.1.4) is one of the major enzymes involved in cellulose hydrolysis. It is responsible for decreasing the degree of cellulose polymerisation by internally cleaving cellulose chain at relatively amorphous regions (Lynd *et al.*, 2002). Its production has been demonstrated in a number of organisms notably, *Trichoderma reesei* (Medve *et al.*, 1998) and *Penicillium pinophilum* (Brown *et al.*, 1987). In this paper, induction of endo- β -glucanase of *Paecilomyces* sp by different cellulose substrates and the effects of different sources of nitrogen and surfactants, pH, temperature and aeration on enzyme production were studied.

Materials and Methods

Micro-organism: The organism was isolated from soil and identified as *Paecilomyces* sp by the Mycology division, North Carolina Memorial Hospital, University of North Carolina, USA. Stock cultures were maintained on Potato dextrose Agar (PDA) slants and stored at 4^{0}C.

Media and culture method: A basal salt medium (BSM) with composition similar to that developed by Mandels and Weber (1969) for the production of cellulase by *Trichoderma reesei* was used. The medium consisted of the following (g/l^{-1}): KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; and trace elements (mg/l $^{-1}$): $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.56; $ZnSO_4.7H_2O$, 1.4; and $CoCl_2$, 2.0. The effects of different sources and concentrations of nitrogen, carbon and surfactant as well as initial pH, temperature and aeration on enzyme production were studied. The variables

Table1: Effects of nitrogen and cellulose sources on endoglucanase production

Nitrogen source (0.27 %w/v)	Final pH	Endoglucanase activity (U/ml)	Cellulose source (1 %w/v)	Final pH	Endoglucanase activity (U/ml)
Bean meal	5.6 ± 0.2	0.10 ± 0.06	Avicel	5.6 ± 0.3	0.04 ± 0.04
Cow blood meal	6.2 ± 0.0	0.28 ± 0.04	Corn cob	5.4 ± 0.2	0.90 ± 0.31
Cotton seed meal	5.7 ± 0.1	0.24 ± 0.05	NaOH-treated rice straw	5.1 ± 0.3	0.26 ± 0.08
Melon meal	5.2 ± 0.3	0.26 ± 0.04	Rice hull	5.4 ± 0.1	1.88 ± 0.11
Soy bean meal	5.6 ± 0.2	0.22 ± 0.05	Untreated rice straw	5.5 ± 0	0.18 ± 0.06
Proteose peptone	5.8 ± 0.1	0.08 ± 0.00	Wood shavings	5.3 ± 0.3	0.08 ± 0.02
$NaNO_3$	6.0 ± 0.4	0.16 ± 0.06			
$(NH_4)_2SO_4$	5.6 ± 0.0	0.48 ± 0.02			

Media composition for Nitrogen source:BSM + 1.0% (w/v) Avicel + 0.2% (v/v)Tween 80. pH 6.0.
Media composition for Cellulose source: BSM + 0.5% (w/v) $(NH_4)_2SO_4$ + 0.2% (v/v) Tween 80.pH 6.0.

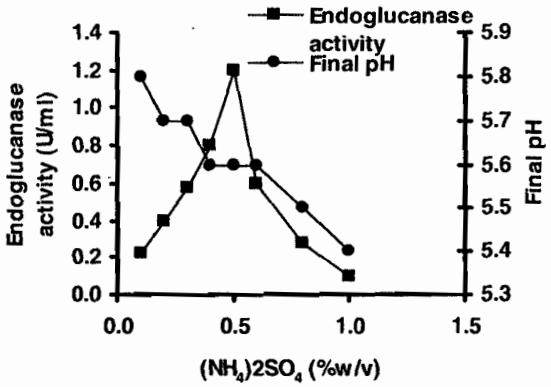

Fig.1: Effects of ammonium sulphate on endoglucanase activity

were studied sequentially first as sources and then as concentrations. The crude cellulose substrates were ground to a fine mesh (*ca* lmm diameter) with the Wiley's mill. Sodium hydroxide treated rice straw was prepared by boiling rice straw in 4% 1N NaOH solution for 15min.

Four agar plugs (1.6 cm diameter) of a 96h PDA culture of the organism grown at 35°C was inoculated into 250 ml Erhlenmeyer flasks containing 30ml medium. Fermentation lasted for four days at 30°C on a rotary incubator (150 rpm). The supernatant obtained after centrifugation (3000 rpm, 15 min, 4°C) was used as the enzyme source.

Time course for the production of endo-β-glucanase was studied at 150rpm and 30°C for 10 days. Growth was monitored by plate count method on PDA supplemented with 1.5%(w/v) sorbose to restrict colony size.

Protein estimation was by the method of Lowry *et al.* (1951) using Bovine Serum Albumen as a protein standard.

Enzyme assay: Endo-β-glucanase was assayed according to the method of Stewart and Leatherwood (1976). The reaction mixture consisted of 1ml of 1.0% (w/v) carboxy-methyl-cellulose (CMC) in 0.2M sodium acetate buffer and 1ml of culture supernatant. The mixture was incubated at 50°C for 30min and the reaction stopped by the addition of 2ml of 3,5 dinitrosalicylic acid reagent (Bernfeld, 1955). The tubes were covered and heated for 5min in a boiling water bath and cooled thereafter. Following the addition of 20ml of distilled water, absorbance was measured in a Pye Unicam SP8 –100 spectrophotometer at 540nm. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of reducing sugar expressed as glucose per minute under the assay condition. Each experiment was carried out in triplicate and results analysed statistically where appropriate. Students t-test was used to determine means which were significantly different ($P< 0.05$)

Results and Discussion

The highest yield of endo-β-glucanase (0.48 Uml^{-1}) was observed in the sample

Table 2: Effects of surfactants and initial pH on endoglucanase production

Surfactant (0.2%v/v)	Final pH	Endoglucanase activity (U/ml	Initial pH	Final pH	Endoglucanase activity (U/ml
None	5.0 ± 0.1	4.30 ± 0.04	3.0	3.1 ± 0.1	5.28 ± 0.09
Sodium dodecyl sulphate	5.4 ± 0.2	0.00 ± 0.00	4.0	4.4 ± 0.2	5.24 ± 0.12
Triton-X-100	5.1 ± 0.2	2.96 ± 0.11	5.0	4.4 ± 0.1	5.30 ± 0.02
Tween- 80	5.1 ± 0.0	3.26 ± 0.05	6.0	5.4 ± 0.0	4.40 ± 0.11
			7.0	6.0± 0.2	4.28 ± 0.10
			8.0	6.2 ± 0.2	1.40 ± 0.05
			9.0	7.6 ± 0.4	0.32 ± 0.50
			10.0	8.0 ± 0.3	0.18 ± 0.23

Media composition for initial pH: BSM + 0.5% w/v $(NH_4)_2SO_4$ + 5.0% (w/v) Rice hull. pH 6.0.
Media composition for surfactants: BSM + 0.5% (w/v) $(NH_4)_2SO_4$ + 5.0% (w/v) Rice hull. pH 6.0.

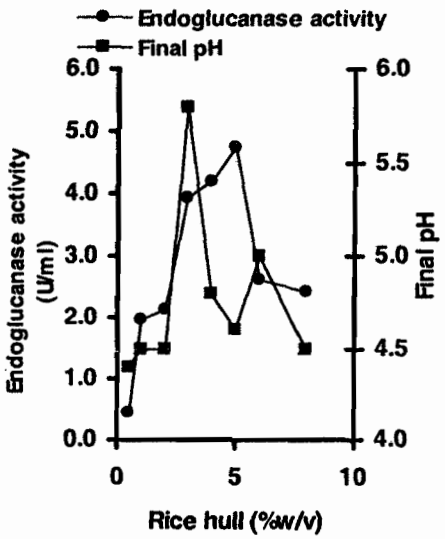

Fig. 2: Effects of rice hull on endoglucanase production

containing $(NH_4)_2SO_4$ while the lowest (0.08 Uml^{-1}) was in the sample containing protease peptone as the nitrogen sources (Table 1). Enzyme production increased with increasing concentrations of $(NH_4)_2SO_4$ until a peak was attained at 0.5%(w/v) $(NH_4)_2SO_4$ (Fig. 1) The preference of the inorganic nitrogen source to the organic nitrogen sources for enzyme production could be due to the ease of assimilation of the inorganic nitrogen by the organism. Nitrogen in $(NH_4)_2SO_4$ is present in the reduced form which can be easily utilized by the organism for cell synthesis and other metabolic activities (Paul and Clark, 1989). A similar observation was reported for the synthesis of endo-β-glucanase by *Sporotrichum thermophile* (Coutts and Smith, 1976).

Greater endo-β-glucanase production was obtained from the crude cellulosic substrate, rice hull, than the pure avicel and treatment of rice straw with NaOH did not enhance enzyme production (Table 1). This is because cellulase biosynthesis is induced by cellulose and inhibited by glucose, the end product of cellulose hydrolysis (Lynd *et al.*, 2002). The inaccessibility of the crude cellulose substrate, rice hull, to hydrolysis must have led to enhanced enzyme induction. The pure cellulose on the contrary could have been easily hydrolysed to the inhibiting glucose by the organism. Brown *et al.* (1987) however reported greater yields of endo-β-glucanase by *Penicillium pinophilum* NTGIII/6 on purified solka floc than milled barley straw. This contrast might have resulted from the unequal production of other enzymes in the cellulase complex, especially β-glucosidase. Endo-β-glucanase production by *Paecilomyces* sp increased with increasing concentration of rice hull until a peak (4.74Uml^{-1}) was attained at a concentration of 5.0% (w/v) (Fig. 2).

Despite many reports on other species of the stimulatory effects of surfactants on cellulase enzyme production (Okeke and Obi, 1993; Pushalkar *et al.* 1995) due probably to increased membrane permeability (Reese and Maguire, 1971), endo-β-glucanase production by *Paecilomyces* sp was inhibited by surfactants (Table 2). This could be because surfactants increased the permeability of the cell membrane of *Paecilomyces* sp and caused the release of other intracellular components which adversely affected enzyme production. Pardo *et al.* (2000), also observed an inhibition of endo-β- glucanase production in *Thecotheus pelletieri* upon surfactant addition.

Table 3: Effects of temperature and aeration on endoglucanase production

Temperature (°C)	Static		Shake Flask	
	Final pH	Endoglucanase activity (U/ml)	Final pH	Endoglucanase activity (U/ml)
30	4.9 ± 0.2	2.14 ± 0.28	4.9± 0.4	5.94 ± 0.64
35	5.2 ± 0.4	0.62 ± 0.04	4.8± 0.5	5.32 ± 0.16
40	4.5 ± 0.3	0.48 ± 0.12	5.0± 0.2	0.60 ± 0.40
45	5.3 ± 0.2	0.36 ± 0.04	4.9± 0.3	0.38 ± 0.39

Media composition: BSM + 0.5% w/v $(NH_4)_2 SO_4$ + 5.0% (w/v) Rice hull. pH 5.0.

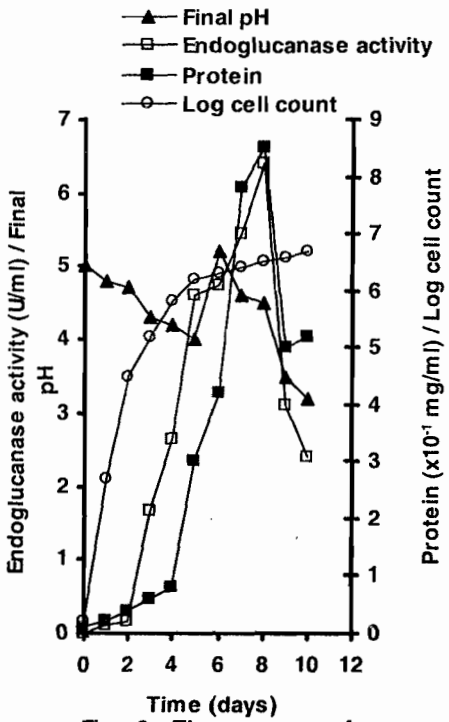

Fig. 3: Time course for endoglucanase production

Optimum enzyme yield was obtained with an initial culture pH of 5.0, at 30°C under shake flask condition (Table 2).

The inability to maximally produce enzyme at higher temperatures (Table 3) might indicate the unsuitability of the organism for high temperature industrial applications, however the crude or purified extracellular enzyme might be thermostable. Shaking disperses the toxic metabolic products of the organism thus reducing the per cell concentration of the metabolite in the growth medium. A similar observation was made for *T. pelletieri* (Pardo *et al*., 2000).

The dynamics of endo-β-glucanase production by *Paecilomyces* sp (Fig.3) shows that enzyme production follows the same pattern as extracellular protein synthesis and increased with the exponential growth phase into the stationary phase. The highest enzyme yield (6.4 Uml $^{-1}$) was obtained on the eighth day of incubation at a culture pH of 4.5. The sharp decrease in enzyme production as the stationary phase advanced could be due to the production of secondary metabolites, which are inhibitory to enzyme synthesis.

References

Bernfeld, P. 1955. Amylase alpha / beta. *Methods in enzymology 1.* 149 - 158.

Brown, A. J., Collins, S. and Wood, T. 1987. Development of a medium for high cellulase, xylanase and β-glucosidase production by a mutant stain NTGIII/6 of a cellulolytic fungus *Penicillium pinophilum. Enzyme Microbial Technology 9*: 355- 359

Coutts, A. and Smith, R. 1976. Factors influencing production of cellulase from *Sporotrichum thermophile. Applied and Environmental Microbiology. 31*: 819-824.

Kumakura, M. 1997. Preparation of immobilized cellulase beads and their application to hydrolysis of cellulosic materials. *Process Biochemistry. 32:* 555-559.

Lowry, O., Rosenbrough, N., Farr, N. and Randall, J. 1951 Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry. 193*:265-275.

Lynd, L. R. 1996. Overview and evaluation of fuel ethanol from cellulosic biomass :Technology, Economics,

the Environment, and Policy. *Ann. Rev. Energy and Environ. 21*: 403-465.

Lynd, L. R., Weimer, P. J., Zyl, W. and Pretorius, S. 2002. Microbial cellulase utilisation: Fundamentals and Biotechnology. *Microbiol. and Mol. Biol. Rev 66:* 506-577.

Mandels, M. and Weber, J. 1969. The production of cellulases. *Advances in chemical series 95*: 391 – 414.

Medve, J., Lee, K. D. and Tjerneld, F. 1998. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endo-glucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol Bioeng 59*: 621 – 634.

Okeke, B. and Obi, S. 1993. Production of cellulolytic and xylanolytic enzymes by an *Arthgrapis* sp. *World J. Microbiol. Biotechnol. 9:* 345 – 349.

Pardo, A. G., Obertello, M. and Forchiassin, F. 2000. Cellulose and xylan degrading enzymes in *Thecotheus pelletieri. Revista Argentina de Microbiologia.32:* 255-258.

Paul, E. A. and Clark, F. C. 1989. *Soil microbiology and biochemistry.* Academic Press Ltd London .p7.

Pushalkar, S., Rao, K. and Menon, K. 1995. Production of β-glucosidase by *Aspergillus terreus. Curr. Microbiol. 30*: 255-258.

Reese, E. and Maguire, A. 1971. Increase in cellulase yields by addition of surfactants to cellobiose cultures of *Trichoderma viride. Developments in Industrial Microbiology. 12*: 212-224.

Stewart, J. and Leatherwood, J. 1976. Depressed synthesis of cellulases by *Cellulomonas. Journal of Bacteriology 128:* 609 –615.