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Production of cellulases by a wild strain of *Gliocladium virens*: optimization of the fermentation medium and partial characterization of the enzymes*

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Summary. The medium composition was optimized on a shake-flask scale for production of cellulases by *Gliocladium virens* using the Graeco-Latin square technique. With the optimized medium 0.33 units of filter paper (FP)-cellulase, 1.52 units of β-glucosidase, and 30.45 units of xylanase were produced per millilitre of culture filtrate at 120 h of fermentation. In a laboratory fermentor the lag phase was much reduced and 0.25 units of FP-cellulase, 0.77 units of β-glucosidase, and 24.04 units of xylanase were obtained per millilitre of culture filtrate at 39 h. Characterization of the enzymes with respect to pH and temperature optima, and pH and heat stabilities indicated that cellulases and xylanase of *G. virens* have properties comparable to those of *Trichoderma reesei* and some other fungi.

Introduction

Many microorganisms, mostly fungi, degrade cellulosic and hemicellulosic materials and produce a complete set of cellulases for the hydrolysis of cellulose and hemicellulose to respective sugars (Enari and Markkanen 1977; Bisaria and Ghose 1981; Coughlan 1985).

Among the fungi, *T. reesei* and its mutants are the best producers of extracellular cellulases. Although *T. reesei* and its mutants enable the production of large amounts of cellulases, the economic saccharification of raw materials has not yet been achieved due to several factors such as high enzyme production cost, costly pretreatment of lignocellulosics, low specific activity of cellulases, and inactivation of the cellulases at high temperature (50° C) (Esterbauer et al. 1983a; Mandels 1985; Steiner et al. 1987a). Hence, emphasis is still placed on the development of new strains and mutants of microbes with high specific cellulase activities and better physico-chemical properties than those currently obtained.

It has been shown that cellulase production by *T. reesei* and other microbes is influenced by several factors such as types and concentrations of cellulosic substrates, organic and inorganic nitrogen sources, macro and trace elements, and fermentation conditions, e.g. age, type and amount of inoculum, pH, temperature, aeration, and stirring (Enari and Markkanen 1977; Mandels and Andreotti 1978; Desrochers et al. 1981; Duff 1987; Steiner et al. 1987b).

Although *G. virens* has been reported to possess a complete set of cellulase complexes, which is secreted into the culture media (Erbeznik et al. 1986; Gomes et al. 1989) and is able to degrade cellulosics and lignocellulosics (Todorović et al. 1987), very little is known about its nutrient requirements and culture conditions for optimal production of cellulases. The aim of the present work was to optimize the fermentation media and culture conditions for cellulase production by *G. virens* and to examine the enzymatic properties of the culture filtrate and crude enzyme preparation.

Materials and methods

Microorganism. Gliocladium virens Miller et al., was isolated from decomposed jute stacks at the Jute Research Institute, Dhaka, Bangladesh, during the rainy season of 1986. The fungus has been identified by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The fungus was grown on

* Dedicated to Prof. Dr. R. M. Lafferty on the occasion of his 60th birthday

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malt extract agar or potato dextrose agar at 28°-30° C for 2-4 days and then stored at 4° C until use. Stock cultures were transferred to fresh medium every 5-6 weeks and incubated under the same conditions. The strain is deposited in our institute under the stock number BT 2170.

Media. Initially, the basic mineral medium of Mandels and Reese (1957), without $CaCl_2 \cdot 2H_2O$, was used. It was prepared using tap water and contained (g/l): cellulose (Avicel or sulphite pulp), 10.0; meat peptone, 2.0; KH_2PO_4, 15.0. The initial pH of the medium was 5.5. High amounts of KH_2PO_4 maintained the pH during fermentation.

Inoculum and shake culture experiments. A piece of malt extract agar (1 cm^2) with an actively growing 3-4-day-old colony of *G. virens* was used as inoculum in shake cultures. Shake cultures were carried out in 300-ml erlenmeyer flasks containing 100 or 150 ml medium. The inoculated flasks were shaken continuously on an orbital shaker at 200 rpm and 30° C for 6 days. The culture was filtered through a glass-fibre filter and the filtrate was used for the assays of enzymes, soluble protein and reducing sugars.

Bioprocess. Fermentations were carried out in a 5-l stirred-tank bioreactor (Biostat-V, Braun Melsungen, FRG) with a 3-l working volume. The temperature was maintained at 30° C and the pH was controlled with 5% NH_4OH and 5% H_2SO_4. The aeration rate was 0.5 vvm during the whole fermentation period. The stirrer speed was 300 rpm for the first 20 h and then decreased to 200 rpm for the rest of the fermentation period. Silicon SE-2 (10%) (Wacker-Chemie, Munich, FRG) was used as an antifoam agent. The inoculum was 1-2-day-old shake-flask preculture (250 ml). One fermentation was carried out using 25 cm^2 agar blocks with a 4-day-old colony of *G. virens* as the inoculum.

Optimization procedure. Optimization of the fermentation medium was carried out using the Graeco-Latin square technique (Auden et al. 1967) for types and concentrations of cellulose, peptone and inorganic nitrogen, and for KH_2PO_4. The optima were determined by summing up the results obtained with three respective media for one type or concentration of a particular ingredient.

Pretreatment of lignocellulosic raw materials. Raw materials such as wheat straw, jute fibre and jute stick were cut into small pieces. These chopped raw materials, barley husk and rice husk were steamed at 189°-190° C in an autoclave for 10 min, dried overnight at 100° C and ground in a mill to pass through a 0.5 mm screen.

Enzyme assays. Filter paper cellulase activity was determined using filter paper (Whatman No. 1) as described by Mandels et al. (1976). Xylanase activity was determined by estimating xylose liberated from birch hemicellulose (Lenzing AG, Lenzing, Austria) according to Esterbauer et al. (1983b). One unit of FP-cellulase and xylanase activity was defined as 1 µmol glucose or xylose equivalents released per min. β-Glucosidase was assayed by the method of Herr et al. (1978) using *p*-nitrophenyl-β-D-glucoside (Sigma, St. Louis, MO, USA). One unit of enzyme activity was expressed as the amount of enzyme which liberates 1 µmol *p*-nitrophenol/min.

Protein determination. Soluble protein in culture filtrate was precipitated with two volumes of acetone, redissolved in 0.01 *M* NaOH and estimated by the method of Lowry et al. (1951).

Reducing sugar. Reducing sugar production by enzymatic hydrolysis and free reducing sugar in the culture broth was quantified by the method of Miller (1959).

Total dry matter (TDM). This was determined by filtering a definite volume of culture broth through a pre-weighed glass-fibre filter, washing several times with distilled water, drying the filter cake at 105°-110° C for 24 h, and reweighing.

Results and discussion

Optimization of the fermentation media

The effects of three different cellulosic substrates, three different organic nitrogen sources (peptones), four different inorganic nitrogen sources and three different concentrations of urea on cellulase production by *G. virens* were studied in order to select the optimal medium components. The experiment, arranged in Graeco-Latin square design, consisted of nine different media and each type or concentration of the ten chosen ingredients occurred in three media (Table 1). A preliminary experiment for maintaining the pH value between 5 and 6 during fermentation and for enhanced cellulase production showed that the optimum concentration of KH_2PO_4 was 1% (data not shown), which was used in all subsequent shake cultures.

Filter paper (FP)-cellulase and β-glucosidase activities produced in nine different media are shown in Table 1. Maximum FP-cellulase (0.27 units) and β-glucosidase (0.68 units) were produced in medium 2 while minimum FP-cellulase (0.07 units) and β-glucosidase (0.02 units) were produced in medium 1. From the summed FP-cellulase (0.63 units in media 2, 5 and 8) and β-glucosidase (1.58 units in media 3, 6 and 9) activities it appeared that the best carbon source for FP-cellulase production is sulphite pulp and for β-glucosidase production is wheat straw. However, when enzyme production was calculated per gram of carbohydrate content, wheat straw appeared to be the best carbon source for both the enzymes, perhaps because of its heterogenous composition. The best organic nitrogen source was bactopeptone (in media 4, 5 and 6) which probably has better essential amino acid composition for the growth of *G. virens* and for cellulase production. Among the inorganic nitrogen sources $(NH_4)_2HPO_4$ (in media 2, 4 and 9) was found to be the best. In a further experiment using 1% wheat straw, 0.2% bactopeptone, 0.06% urea, 0.03% $MgSO_4$, 1% KH_2PO_4, 0.1% Tween-80 and 0.1% trace element solutions, it was confirmed

Table 1. Design for selection of medium ingredients for *Gliocladium virens* culture using Graeco-Latin square and enzyme activities obtained after 6 days shake culture

Ingredient type [% (w/v)]	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7	Medium 8	Medium 9
Avicel	1.00%			1.00%			1.00%		
Sulphite pulp		1.00%			1.00%			1.00%	
Wheat straw			1.00%			1.00%			1.00%
Meat peptone	0.20%	0.20%	0.20%						
Bactopeptone				0.20%	0.20%	0.20%			
Soya peptone							0.20%	0.20%	0.20%
$(NH_4)_2SO_4$	0.15%					0.15%		0.15%	
$(NH_4)_2HPO_4$		0.15%		0.15%					0.15%
KNO_3			0.15%		0.15%		0.15%		
Urea 0.00%	0.00%				0.00%				0.00%
Urea 0.03%			0.03%	0.03%			0.03%		
Urea 0.06%		0.06%				0.06%	0.06%		
Enzyme	Results (IU/ml)								
FP-Cellulase	0.07	0.27	0.21	0.24	0.19	0.18	0.22	0.17	0.17
β-Glucosidase	0.02	0.68	0.54	0.63	0.33	0.52	0.59	0.37	0.52

In addition, each medium contained: 0.03% $MgSO_4$, 1.00% KH_2PO_4, 0.10% (v/v) Tween-80, 0.10% (v/v) trace element solution I ($MnSO_4$, 1.6 g/l; $ZnSO_4 \cdot 7H_2O$, 3.45 g/l; $CoCl_2 \cdot 6H_2O$, 2.0 g/l) and 0.10% (v/v) trace element solution II ($FeSO_4 \cdot 7H_2O$, 5.0 g/l; FP = filter paper

that of the three ammonium salts e.g. $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $(NH_4)H_2PO_4$ (all 0.14%), $(NH_4)_2HPO_4$ was the best inorganic nitrogen source (data not shown). It was also confirmed that the presence of urea enhances cellulase production by *G. virens*.

For cellulase production, different concentrations of steamed wheat straw (0.5%–3.0%), bactopeptone (0.15%–1.0%), $(NH_4)_2HPO_4$ (0.1%–0.25%) and urea (0.06%–1.0%) were used in shake cultures of *G. virens*. Several experiments were performed according to the Graeco-Latin square technique and the design of only the final experiment and the enzyme activities in all nine media are shown in Table 2. Maximum FP-cellulase (0.27 units) and β-glucosidase (2.22 units) activi-

Table 2. Design for optimization of ingredient composition for culture medium of *G. virens* using Graeco-Latin square and enzyme activities obtained after 6 days shake culture

Ingredient type [% (w/v)]	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7	Medium 8	Medium 9
Wheat straw 1.00%	1.00%			1.00%			1.00%		
Wheat straw 1.50%		1.50%			1.50%			1.50%	
Wheat straw 2.00%			2.00%			2.00%			2.00%
Bactopeptone 0.20%	0.20%	0.20%	0.20%						
Bactopeptone 0.30%				0.30%	0.30%	0.30%			
Bactopeptone 0.40%							0.40%	0.40%	0.40%
$(NH_4)_2HPO_4$ 0.14%	0.14%					0.14%		0.14%	
$(NH_4)_2HPO_4$ 0.15%		0.15%		0.15%					0.15%
$(NH_4)_2HPO_4$ 0.16%			0.16%		0.16%		0.16%		
Urea 0.06%	0.06%				0.06%				0.06%
Urea 0.07%			0.07%	0.07%				0.07%	
Urea 0.08%		0.08%				0.08%	0.08%		
Enzyme	Results (IU/ml)								
FP-Cellulase	0.18	0.24	0.26	0.17	0.22	0.27	0.13	0.23	0.26
β-Glucosidase	1.01	1.76	2.08	1.06	1.65	2.22	0.81	1.80	2.18

In addition, each medium contained components as indicated under Table 1

ties were produced in medium 6 while minimum FP-cellulase (0.18 units) and β-glucosidase (1.01 units) activities were produced in medium 1. Summation of the enzyme activities led to the conclusion that optimum concentrations of wheat straw (in media 3, 6 and 9), bactopeptone (in media 1, 2 and 3), $(NH_4)_2HPO_4$ (in media 1, 6 and 8) and urea (in media 1, 5 and 9) were 2.0%, 0.2%, 0.14% and 0.06% respectively for optimum production of cellulases by *G. virens*. Greater difference in the summed enzyme activities for different concentrations of wheat straw indicated that substrate concentrations had more influence on cellulase production, while little difference in the summed enzyme activities for different concentrations of other nutrients indicated that the concentrations of these nutrients used were not limiting cellulase production.

Cellulose and peptone were required for the production of cellulases, but high concentrations of cellulose (above 1.5% sulphite pulp and 2% wheat straw) and peptone (above 0.4%) were inhibitory to fungal growth and enzyme production. The decrease in activity may be attributed to adsorption of the enzymes to the substrates (Steiner et al. 1988) and/or to the increase in pH (6.8–7.0) due to high substrate and peptone concentrations.

Shake cultures

Figure 1 shows a typical time course of shake-flask experiment with *G. virens* using the optimized medium. Enzyme synthesis commenced within 24 h of incubation, increased rapidly and reached a peak at day 4. Thereafter enzyme production remained unchanged or decreased slightly. The pH values, initially adjusted to 5.5, did not decrease during the growth phase of the fungus; on the contrary, they increased slightly after day 1 of incubation and then decreased on day 2. On day 3 the mycelium autolysed and the enzymes were secreted into the medium with a rise in pH. A 5–6 day period was determined as the optimal time for the shake culture and production of cellulases and xylanase by *G. virens* on steamed wheat straw.

Effects of raw materials on cellulase production

In addition to the substrates used in the optimization experiments, the effects of cellulosic materials on cellulase production were compared in

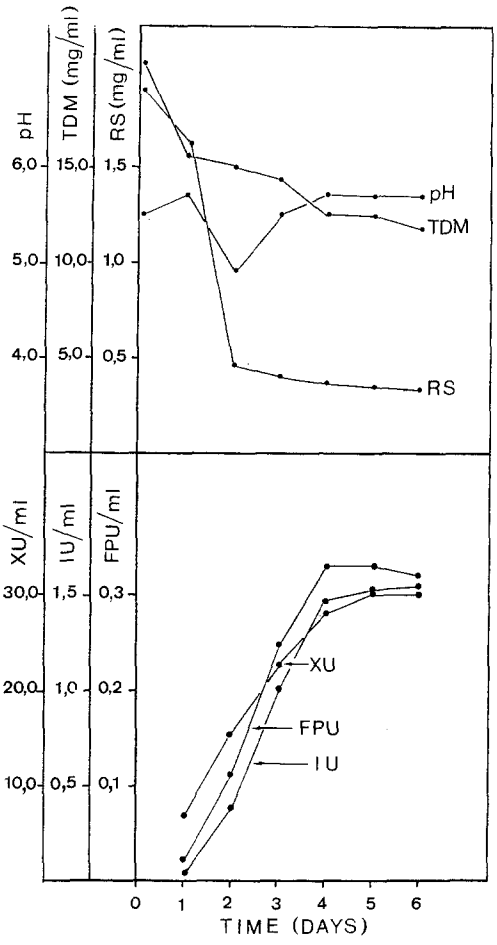

Fig. 1. Shake cultures of *Gliocladium virens* using the optimized medium. Six duplicate cultures, each 150 ml, were set up and at the indicated times duplicate cultures were used for estimating FPU (filter paper cellulase units), IU (β-glucosidase units), XU (xylanase units), TDM (total dry matter), RS (total reducing sugar) and pH values

shake cultures using the optimized medium (Table 3). The best yields of FP-cellulase and β-glucosidase per gram of carbohydrate were obtained with steamed barley husk. Cellulase yields with other lignocellulosic materials, e.g. wheat straw, jute stick, jute fibre and newspaper were also encouraging compared to the yields with pure and expensive celluloses, e.g. Avicel and sulphite pulp. These led to the assumption that cellulase yield is not primarily determined by the sugar composition or lignin content of the raw materials. Since the relative proportion of individual sugars as well as lignin content was not altered by steaming, it has been proposed that physical alteration of the lignin, increase in surface and pore sizes, partial decrystallization of the celluloses, removal of the acetyl group from hemicellulose, depolymerization of hemicellulose, etc. by steaming

Table 3. Effects of different lignocellulosic raw materials and celluloses on production of cellulases by *G. virens* in shake cultures[a]

Substrates	FPU/ml	IU/ml	Soluble protein (mg/ml)	Reducing sugar (mg/ml)	TDM (mg/ml)	Final pH
Wheat straw (steamed)	0.16	0.67	0.39	0.24	5.35	6.04
Barley husk (steamed)	0.24	0.67	0.58	0.21	6.50	6.07
Rice husk (steamed)	0.05	0.16	0.14	0.01	7.16	6.29
Jute fibre (steamed)	0.13	0.56	0.24	0.02	9.43	6.23
Jute stick (steamed)	0.14	0.57	0.35	0.05	7.57	6.17
Newspaper (untreated)	0.13	0.49	0.26	0	8.53	6.25
Sulphite pulp	0.26	0.74	0.84	0.04	6.50	5.69
Avicel	0.21	0.69	0.56	0.01	6.48	5.76

[a] Medium composition: 1% substrate, 0.2% bactopeptone, 0.14% $(NH_4)_2HPO_4$, 0.06% urea and other components as indicated under Table 1. Abbreviations: FPU, filter paper cellulase units; IU, β-glucosidase units; TDM, total dry matter

results in better degradability of the raw materials and production of cellulases (Fan et al. 1980; Doppelbauer et al. 1987).

Bioprocess experiment

A typical batch fermentation time-course is shown in Fig. 2. The lag phase was reduced in duration compared to that which existed in shake-flask scale cultures. The production of FP-cellulase, β-glucosidase and xylanase occurred quite early. Maximum FP-cellulase (0.25 units/ml) was produced at 39 h when 0.77 units β-glucosidase and 24.04 units xylanase/ml culture filtrate were produced. After this, production of β-glucosidase and xylanase continued to increase very slowly. It was noted that NH_3 consumption and decrease in TDM (lignocellulose + mycelia) were most rapid up to 24 h after which NH_3 consumption and decrease in TDM became very slow or stopped, indicating the stationary phase of fungal growth and slow utilization of lignocellulosic materials. Perhaps, the presence of free sugars (0.52 g/l), generation of some inhibitors or deficiency of some nutrients in the medium resulted in the retardation of fungal growth and decreased utilization of cellulose.

β-Glucosidase activity of the cellulase complex produced in the fermentor was reduced in comparison to that achieved in shake flasks. The ratio of β-glucosidase to FP-cellulase in the fermentor was 4.0 as compared to a ratio of 4.6 which was achieved in shake flasks (Fig. 1). For *T. reesei* MCG 77, a hyper-FP-cellulase producer, the ratio has been reported to be 0.44–0.83 using steamed wheat straw (Doppelbauer et al. 1987).

Partial characterization of the enzymes

FP-Cellulase, β-glucosidase and xylanase components of the enzyme complex were characterized with respect to pH and temperature optima, and pH and temperature stabilities. Crude enzyme was prepared by precipitating the clear culture fil-

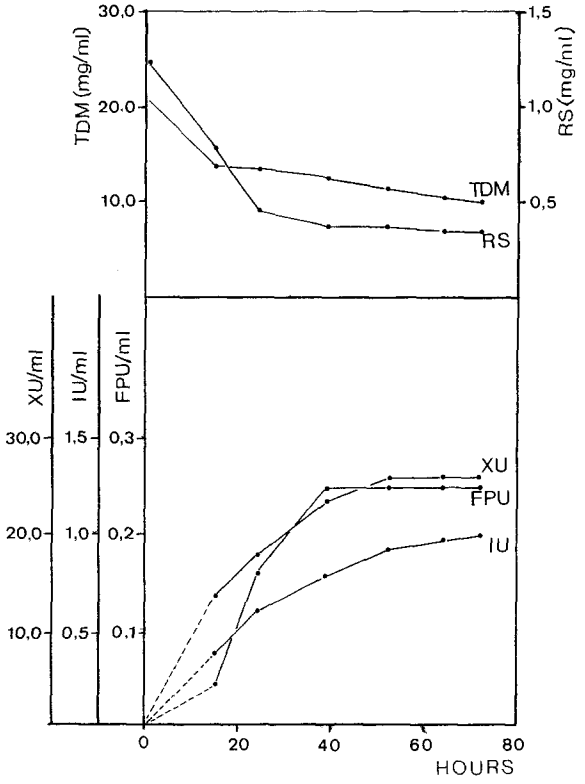

Fig. 2. Time course of fermentation of *G. virens* using the optimized medium. Up to 20 h the pH was controlled at 5.6 and then at 5.0. Preculture age was 1 day. At the indicated times samples were withdrawn and RS, pH values, TDM, FPU, IU and XU were determined

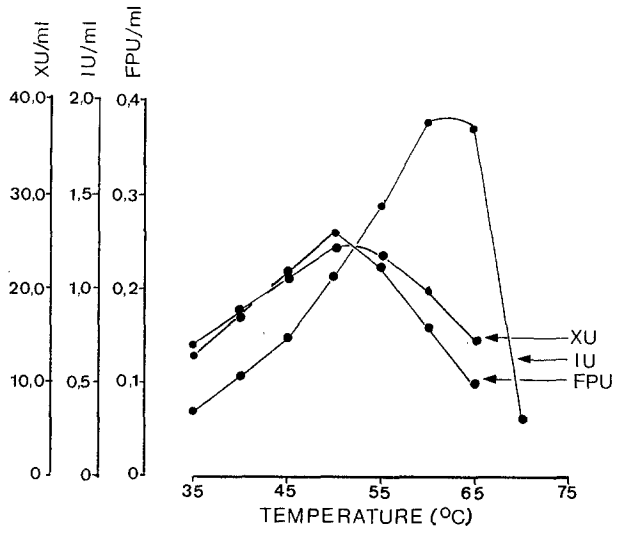

Fig. 3. Temperature optima of FPU, IU and XU activities of *G. virens*

trate (cooled to 4° C) with three volumes of ethanol (cooled to −18° C), standing overnight at −18° C, harvesting the precipitate by centrifugation and freeze-drying.

The temperature optima for both FP-cellulase and xylanase were found to be 50° C while that for β-glucosidase was found to be between 60° C and 65° C using the standard assay conditions as described in Materials and methods (Fig. 3). The pH optima for FP-cellulase, β-glucosidase and xylanase were found to be 5.0, 4.8–5.0 and 5.2 respectively under the standard assay conditions (Fig. 4). The pH and temperature optima for *G. virens* cellulases and xylanase are comparable to those for *T. reesei* and other fungal pure or crude cellulases and xylanases (reviewed by Coughlan 1985; Poutanen 1988).

The long-term heat stability of all three enzymes was studied at 40°, 45°, 50° and 55° C. Crude enzyme powder was dissolved in citrate buffer, pH 4.8, and incubated for 0–72 h at the indicated temperatures. Samples were withdrawn at different times and the residual enzyme activity was assayed using standard conditions. Heat stabilities of FP-cellulase, β-glucosidase and xylanase are shown in Fig. 5a–c. At 40° and 45° C β-glucosidase was the most stable enzyme, followed by FP-cellulase and xylanase, whereas at 50° and 55° C FP-cellulase was the most stable enzyme, retaining about 47%–58% activity in 72 h. Both xylanase and β-glucosidase were rapidly inactivated at 50° and 55° C. These results disagree with those of Todorović et al. (1987) who reported that FP-cellulase of *G. virens* retains only 18%–28% activity and xylanase retains 60%–70% activity during the course of only 5 h heating at 50° C. The FP-cellulase of our wild strain of *G. virens* shows similar heat stability to that of *T. reesei* (Reese and Mandels 1980).

The long-term pH stabilities of FP-cellulase, β-glucosidase and xylanase activities are shown in Fig. 6a–c. Crude enzyme, dissolved in citrate (pH 2.5–5.5) and phosphate buffers (pH 6.0–8.0), was incubated at 30° C for 0–96 h and the residual enzyme activities were measured at the times indicated. The FP-cellulase remained most stable between pH 4.5 and 5.5, retaining 81%–82% activity in 96 h while β-glucosidase remained most stable between pH 4.0 and 7.0 retaining 95%–100% activity. Xylanase was most stable between pH 3.5 and 6.5 retaining 82%–93% activity. Alkaline (pH 7.5–

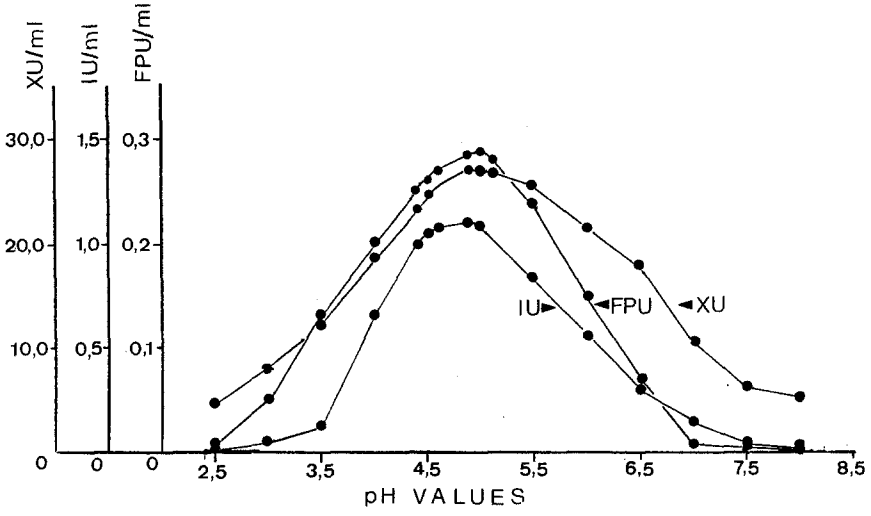

Fig. 4. The pH optima of FPU, IU and XU activities of *G. virens*

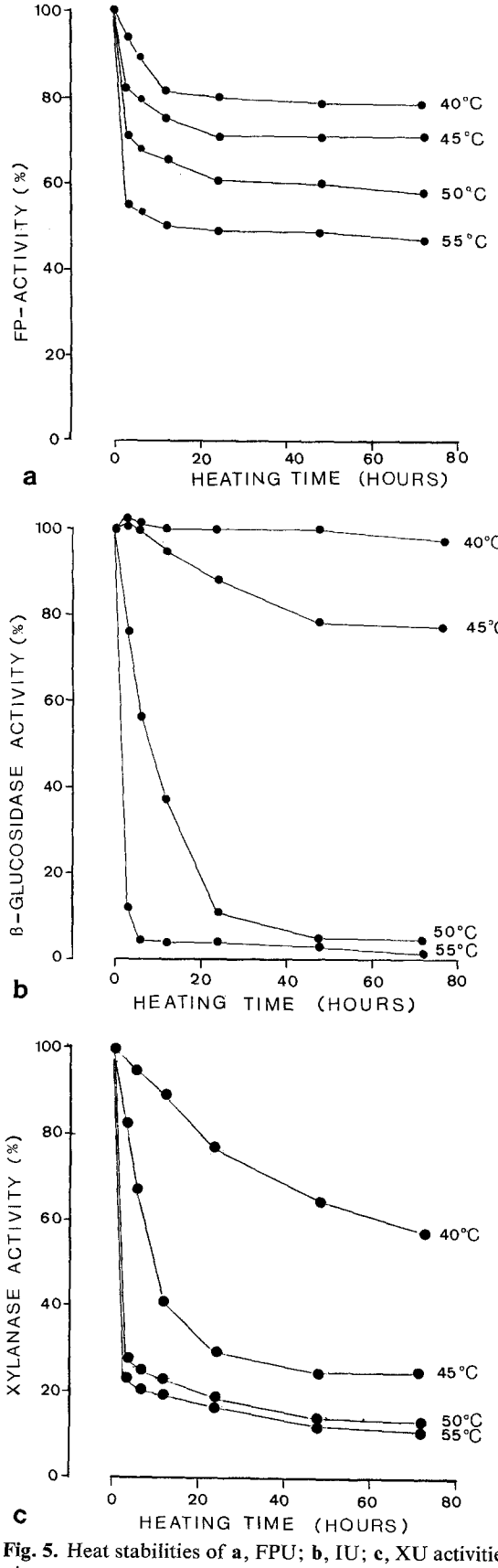

Fig. 5. Heat stabilities of **a**, FPU; **b**, IU; **c**, XU activities of *G. virens*

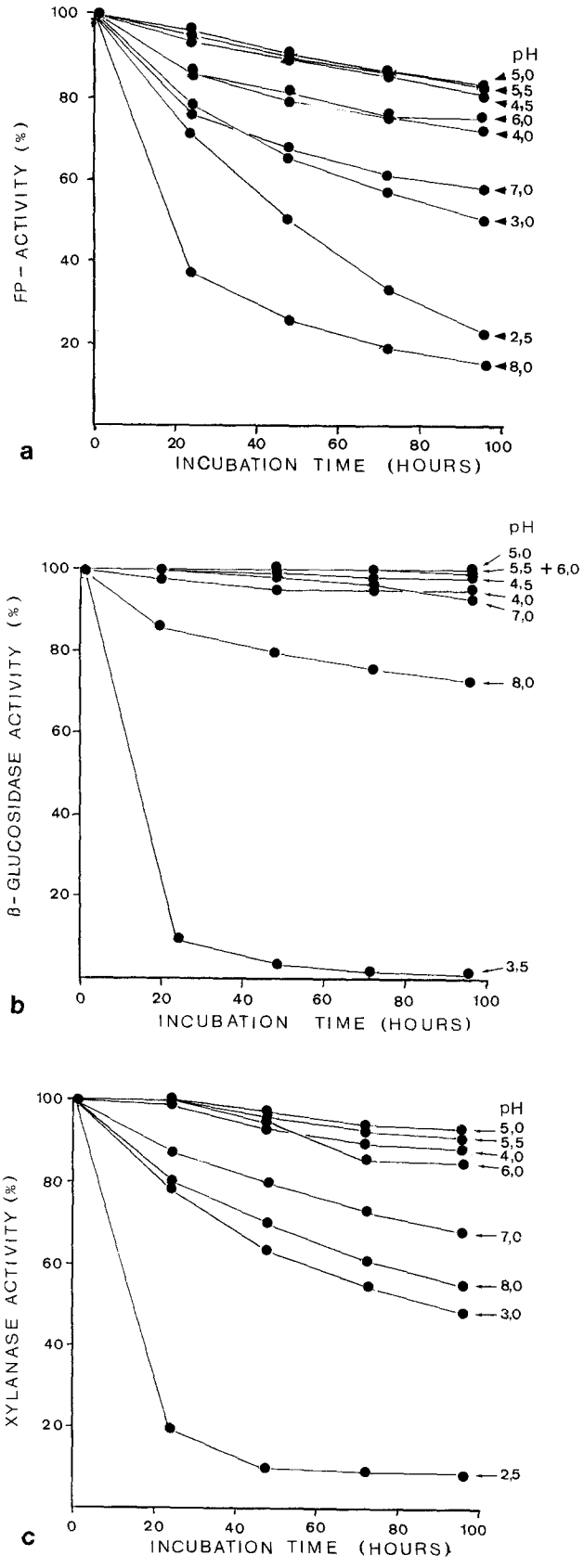

Fig. 6. The pH stabilities of **a**, FPU; **b**, IU; **c**, XU activities of *G. virens*

8.0) as well as acidic (pH 3.5 and below) conditions caused rapid inactivation of the enzymes. Considering the pH and heat stabilities of the enzymes it can be concluded that all three enzymes are most stable at 45° C and pH 4.5–5.5.

In our view, *G. virens* deserves further attention as it produces a complete set of cellulase complexes, which is essential for rapid hydrolysis of lignocellulosic materials. Moreover, it is a fast-growing fungus with a short lag phase compared to *T. reesei*, it produces good amounts of β-glucosidase and xylanase, and the stabilities of the enzymes are comparable to those of *T. reesei* enzymes. The low FP-cellulase production by *G. virens* limits its applicability. However, further development of fermentation conditions and/or mutation of the wild strain might improve cellulase production.

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