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Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid-state fermentation

W. Grajek

Institute of Food Technology of Plant Origin, Academy of Agriculture,
60-624 Poznan, ul. Wojska Polskiego 31, Poland, and
Laboratoire Associé à la Chaire de Biotechnologie de l'INA-PG
INRA, 17 rue Sully, F-21034 Dijon, France

Summary. Six thermophilic fungi were examined for their ability to produce cellulolytic enzymes in liquid (LF) and solid-state fermentation (SSF). The best cellulase activities were achieved by *Thermoascus aurantiacus* and *Sporotrichum thermophile*. Taking into consideration that solid-state medium obtained from 100 g of dry sugar-beet pulp occupies about 1 l of fermentor volume equivalent to 1 l of LF, it was confirmed that enzyme productivity per unit volume from both fungi was greater in SSF than in LF. The cellulase system obtained by SSF with *T. aurantiacus* contained 1.322 IU/l of exo-β-D-glucanase, 53.269 IU/l of endo-β-D-glucanase and 8.974 IU/l of β-D-glucosidase. The thermal and pH characteristics of cellulases from solid-state fermentation of *T. aurantiacus* and *S. thermophile* are described.

Introduction

The thermophilic fungi are among the numerous microorganisms which are capable of producing extracellular hydrolases. This group of microorganisms is very interesting for large-scale production of enzymes in solid-state fermentation (SSF).

One of the technical problems in SSF is the maintenance of temperature at optimal level during the whole cultivation period. The use of thermophilic strains can be an effective solution to this problem. Preliminary studies on the properties of some enzymes produced by thermophilic fungi showed that many enzymes from these organisms have inherently superior temperature stability than enzymes of mesophilic strains.

Offprint requests to: W. Grajek

In the literature there is a relatively large amount of information on the production of cellulases by thermophilic fungi in submerged fermentation (LF) (Aranjo et al. 1983; Coutts and Smith 1976; Shaker et al. 1984; Stewart and Parry 1981; Trivedi and Rao 1979; Wase et al. 1985a, b), but there is no information on the production of cellulases in SSF.

This paper reports the results of research on the production of cellulases by the strains of six species of thermophilic fungi, in submerged and solid-state fermentations.

Materials and methods

Microorganisms. The strains of thermophilic fungi used were isolated from self-heating wood chip piles and identified as *Mucor pusillus* (Lindt), *Thermoascus aurantiacus* (Miehe), *Sporotrichum thermophile* (Apinis), *Humicola lanuginosa* (Griffon and Maublanc), *Allescheria terrestris* (Apinis) and thermotolerant *Aspergillus fumigatus* (Fresenius).

The cultures were maintained on yeast-starch agar (Emerson 1941) at 45°C and stored at 10°C.

Inoculum. The 5-days-old cultures were transferred into 100 ml Mandels and Weber (1969) liquid medium with 10 g of soluble starch as a carbon source. Initial pH of the media was adjusted for each strain respectively: *A. fumigatus* pH 3.5, and the other fungi pH 5.0. After 3 to 5 days of incubation the cultures were homogenized by aseptical shaking with glass beads and used to inoculate the culture media.

Culture conditions. Liquid fermentation (LF) in Mandels and Weber (1969) mineral medium containing 1% of substrate was performed as follows: 0.8 g powdered sugar-beet pulp and 0.2 g of Solka floc BW-300 cellulose (Brown and Co, NH, USA) were suspended in 100 ml of mineral medium, placed in a 500-ml conical flask and sterilized at 115°C for 60 min. Urea was sterilized by membrane filtration. Initial pH of media was adjusted as described above.

Each flask was inoculated with 10 ml of the inoculum. The cultures were incubated on a rotary shaker at their respective temperatures for 72 h.

The broth after cultivation was used for enzyme studies.

Solid-state fermentations (SSF) utilized a stationary layer within 500 ml Erlenmeyer flasks placed in a culture chamber. A mixture of 8 g of dry sugar-beet pulp and 2 g of Solka floc cellulose was added to the flask, moistened with 20 ml of water and sterilized at 115° C for 60 min. The mineral salt solution without urea, was autoclaved to 4-fold concentration. Urea was sterilized at 4-fold concentration by membrane filtration. After sterilization, each flask was supplemented with 10 ml of salt solution and seeded with 10 ml of inoculum. The final moisture content was about 80% w/w. The culture flasks were incubated at 45° C for 3 to 5 days. The moisture content was controlled at 12 h intervals.

Enzyme recovery. The culture broth from LF was centrifuged and the supernatants were used for enzyme assay. The solid-state cultures were suspended in 100 ml of cold deionized water and placed on a shaker for 1 h. The suspension was filtered through a nylon cloth, and then centrifuged. The filtrates obtained were used for enzyme studies.

Enzyme studies. The activities of 1,4-β-D-glucan 4-glucanohydrolase (endo 1,4-β-D-glucanase, CMCase, EC 3.2.1.4) towards carboxymethyl-cellulose, and 1,4-β-D-glucan cellobiohydrolase (exo-1,4-β-D-glucanase, FPA, EC 3.2.1.91) towards filter paper Whatman n° 1 were determined according to the method of Mandels and Weber (1969). β-D-glucoside glucohydrolase (β-glucosidase, EC 3.2.1.21) activities were assayed using p-nitrophenyl-β-D-glucopyranoside by the method of Herr (1979). The reducing sugars released in the hydrolysis reaction were measured by the dinitrosalicylic acid method as described by Miller (1959). Enzyme activities were expressed as international units equivalent to micromoles of glucose or p-nitrophenol released per minute of reaction and calculated per ml of liquid fermentation filtrates. In experiments concerning the temperature and pH profile characteristics, the enzyme assays were performed at 55° to 80° C and a pH of 3.0 to 7.5, using McIlvaine buffer.

Results and discussion

The results of the fermentations, which are presented in Table 1, showed that there is distinct influence of the culture method on the production of enzymes by fungi examined. Among the strains used, the highest cellulolytic activity was produced by *Thermoascus aurantiacus* and *Sporotrichum thermophile* grown in solid-state fermentation, and *T. aurantiacus, S. thermophile* and *Allescheria terrestris* in liquid cultures. The comparison of results obtained in LF and SSF is difficult, because of different units of expression of enzyme activity. To eliminate this difficulty it is proposed to present the results in relation to activities obtained per volume of culture.

Such presentation of enzyme activities provides a better orientation in practical productivities of fermentation. Therefore, in Table 1, the enzyme activities obtained from one litre of culture volume are presented. It was confirmed that 100 g of dry pulp, moistened with mineral solution up to 75—80%, occupies about 1 l of fermentation volume. On this basis it was assumed that the solid medium prepared from 100 g of dry pulp was equivalent to 1 l of liquid medium.

It was found that all strains examined, except *A. terrestris*, obtained higher enzyme activities per unit of culture volume in solid-state fermentations. In many cases it was observed that the differences were extremely high with advantages to SSF. Thus, the exo-β-glucanase activities of *T. aurantiacus* and *S. thermophile* were estimated as 280 and 1.322 IU/l, and 660—1215 IU/l in LF and SSF, respectively. In solid-state fermentation with *T. aurantiacus*, it was found that productivities of endo-β-glucanases and β-D-glucosidase were 9-fold higher than in submerged cultures. The results for the cultivation of *A. terrestris*, where higher activities of endo-β-glucosidase and β-D-glucosidase were observed in submerged culture, were an exception.

In general, the highest differences, depending on culture methods, appeared in productivity of β-D-glucosidase; they were much smaller in productivities of exo-β-glucosidase. It can be also confirmed that the production of cellulolytic enzymes in solid-state fermentation can be more economic than the production using submerged

Table 1. Yield of the cellulase production by thermophilic fungi in liquid (LF) and solid-state fermentation (SSF)

Microorganisms	Enzymatic activities expressed as IU/l of culture volume					
	exo-β-D-glucanase		endo-β-D-glucanase		β-D-glucosidase	
	LF	SSF	LF	SSF	LF	SSF
Aspergillus fumigatus	50	67	70	370	1	54
Allescheria terrestris	60	84	1460	302	1242	50
Humicola lanuginosa	50	50	30	167	6	4676
Mucor pusillus	10	71	30	332	2	2361
Sporotrichum thermophile	660	1215	2460	10914	873	1215
Thermoascus aurantiacus	280	1322	560	53260	937	8974

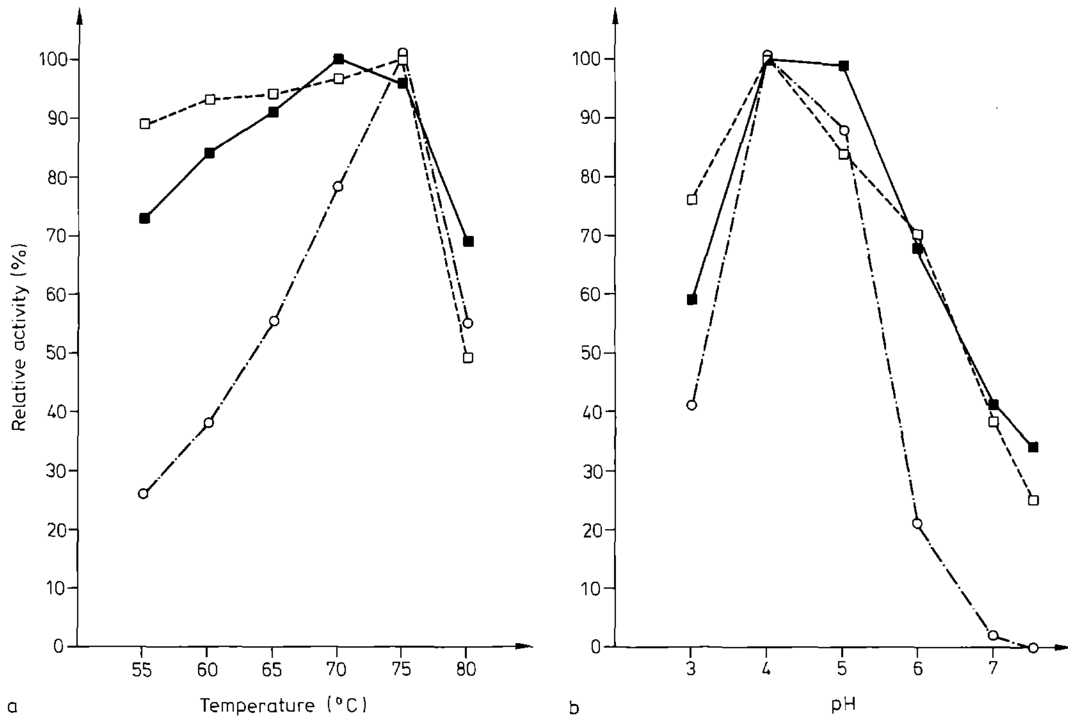

Fig. 1. pH and temperature profiles of exo-β-D-glucanase (□), endo-β-D-glucanase (△) and β-D-glucosidase (×) produced by *T. aurantiacus* in SSF

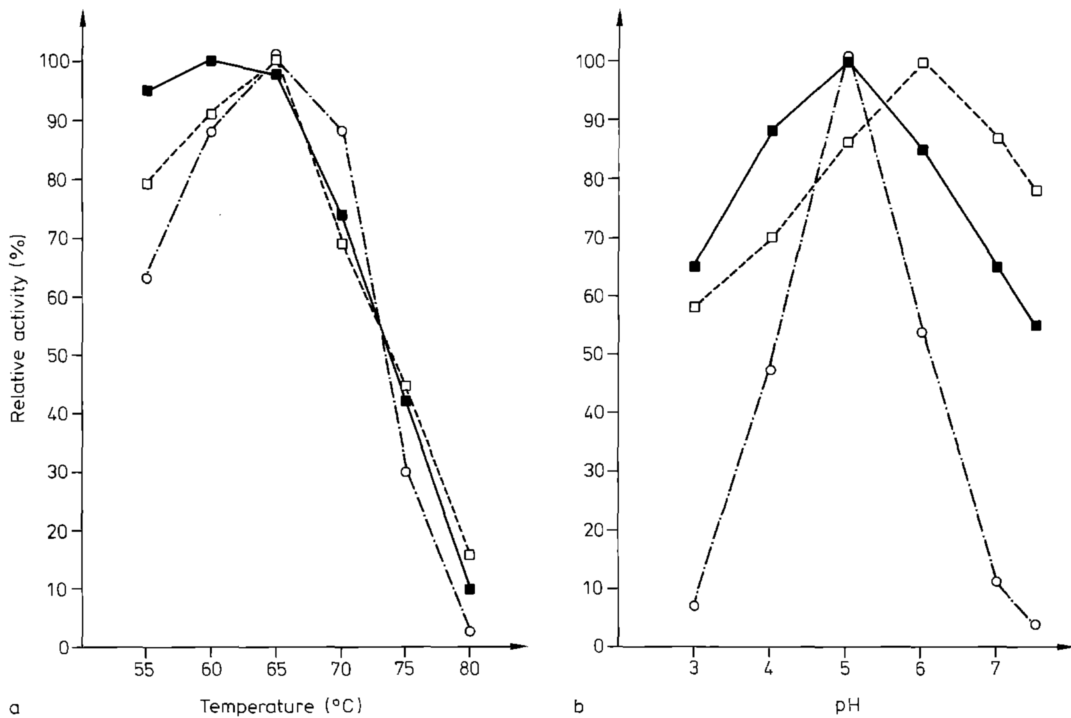

Fig. 2. pH and temperature profiles of exo-β-D-glucanase (□), endo-β-D-glucanase (△) and β-D-glucosidase (×) produced by *S. thermophile* in SSF

technique. Thus, the production of enzymes and single-cell protein in one process can be an attractive perspective in biotechnology.

The investigation carried out previously (Grajek 1986) showed that *T. aurantiacus* and *S. thermophile* are able to enrich the sugar-beet pulp with crude protein up to 21—23% of dry matter in solid-state fermentation. Taking into consideration these data and results obtained in this work it was decided to investigate the pH and temperature characteristics of enzymes from SSF of *T. aurantiacus* and *S. thermophile*. The pH and temperature profiles are presented in Fig. 1 and 2. It was observed that the enzymes of *T. aurantiacus* showed an higher thermal stability than the enzymes of *S. thermophile*. The both strains produced the cellulases with an optimum for their activities at pH 4.5 to 5.5.

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