Xylanase production by a hyperxylanolytic mutant of *Fusarium oxysporum*

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Mutagenesis of Fusarium oxysporum *DSM 841 enhanced the activity of xylanase and β-xylosidase enzymes by more than threefold. Wild-strain spores were subjected to UV or N-methyl-N'-nitro-N-nitrosoguanidine treatments. The hyperxylanolytic mutant (NTG-19) secretes high levels of xylanolytic enzymes on commercial xylan as well as several agricultural residues, of which wheat bran supported maximum enzyme yields. Addition of 0.2% olive oil, an inexpensive source of fatty acids, resulted in a further 25% increase in enzyme secretion.*

Keywords: *Fusarium oxysporum;* mutagenesis; mutant; xylanase; agricultural residues

Introduction

Xylan, a major component of hemicellulose, can be enzymatically hydrolyzed to xylose by xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37). Xylanase from fungi and bacteria has been studied extensively for the conversion of waste biopolymers to xylose, which can be used as a fermentation substrate for the production of single-cell protein, xylitol, ethanol and other solvents, and organic acids.[1–4] Xylanases have also gained increasing attention as an aid to chemical pulp bleaching.[5–7] To reach commercial feasibility, enzyme production must be increased by either introducing more potent strains or by inducing mutants secreting higher levels of xylanases. In this article, isolation and xylanase production of a hyperproducing mutant of *Fusarium oxysporum* is reported.

Materials and methods

Microorganism

The test organism used in these experiments was *F. oxysporum* DSM 841, obtained from K. Schügerl (Institute für Technische Chemie, Hannover, Germany). It was maintained on yeast extract malt agar medium.[8]

Medium

Basal medium containing peptone (0.3%), KH_2PO_4 (0.2%), $MgSO_4$ (0.1%), $CaCl_2$ (0.1%), and yeast extract (0.3%) was supplemented with carbon source unless stated otherwise. The pH of the medium was adjusted to 5.0 before sterilization. Solid medium was prepared by addition of 2% agar to the liquid medium before heat sterilization.

Mutagenesis

Mutagenesis was performed on spores from 4- to 6-day-old fungal colonies. The spore suspension (approximately 10^5–10^6 ml^{-1}) was prepared in normal saline (0.85%) containing 0.01% Tween-80.

Mutagenesis with ultraviolet (UV) irradiation was carried out using a UV tube (6 W). The 5% survivors were grown by spread plating 0.1 ml of treated spores on solid medium containing 1% xylan and 0.1% sodium tauroglycocholate (colony growth restrictor) and incubating at 30°C. Rapid evaluation of the mutants after colony formation was achieved after overnight incubation of the plates at 50°C.

Chemical mutagenesis was performed on UV mutants by exposing them to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The spore suspension (10^5–10^6 ml^{-1}) was treated with NTG (100 μg ml^{-1}) solution for 30–60 min. The mutant spores were washed with saline water and plated as described above. Mutants forming large clear zones around their colonies were selected.

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Table 1 Xylanase and β-xylosidase yields of *Fusarium oxysporum* DSM 841 and its mutant strains on commercial xylan and various natural agricultural residues in shaker flasks

Substrate	Xylanase (IU ml^{-1})			β-Xylosidase (IU ml^{-1})		
	DSM 841	UV-11	NTG-19	DSM 841	UV-11	NTG-19
Xylan	11.3[d]	19.1[d]	35.6[d]	3.9	5.2	9.6
Rice straw	5.1[a]	ND	14.9[a]	1.7	ND	3.4
Sugarcane bagasse	4.4[b]	ND	11.3[b]	1.0	ND	2.6
Wheat bran	7.3[c]	ND	22.8[c]	2.8	ND	6.2
Wheat straw	6.1[a]	ND	15.0[a]	1.7	ND	4.1

Substrate concentration, 1%; incubation time, 144 h for wild type and 120 h for mutants. Visible growth: a, Moderate; b, moderate to dense; c, dense; d, best growth on xylan. Values are the means of three replicates
Abbreviation: ND, not determined

Xylanase production by Fusarium oxysporum *mutants*

Mutants were tested for production of xylanase in submerged culture in a liquid basal medium containing larchwood xylan as carbon source. Erlenmeyer flasks (500 ml) containing 100 ml of medium were inoculated with 0.4 ml of spore suspension (10^5–10^6 spores ml^{-1}) and incubated at 30°C with shaking (250 rpm). Samples were taken at 24-h intervals, and xylanase and β-xylosidase activity measured. Each experiment was performed three times.

Analytical methods

Xylanase and β-xylosidase activities were estimated by measuring xylose and *p*-nitrophenol liberated from larchwood xylan and *p*-nitrophenyl-β-D-xylopyranoside, respectively.[9] One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of product min^{-1} ml^{-1} of enzyme filtrate under the assay conditions (15 min, 50°C).

Results and discussion

Fusarium oxysporum DSM 841 is a fast-growing microorganism. It produces whitish aerial mycelium in 24 h on solid medium and starts producing conidia after 48 h of the incubation at 30°C.

The mutants developed in this investigation were chosen on the basis of their ability to hydrolyze xylan. The screening was based on the ratio of diameter of visible growth of the colony to that of degraded xylan clearing zone as an index of xylanolytic activity. The lower the ratio, the higher the activity.

Several UV mutants isolated were tested in the liquid medium with xylan as sole carbon source to evaluate their xylanase production. *Table 1* compares the growth pattern and the enzyme activities of the parent strain DSM-841 and those of the two mutants UV-11 and NTG-19, when grown on different carbon sources in shaker flasks. It can be seen that the xylanase and β-xylosidase activities of mutants UV-11 and NTG-19 increased by as much as two- to threefold. The mutant strains required an incubation period of 5 days, whereas it was 6 days for the parent strain. The mutant strain NTG-19 was further investigated in detail for xylanase production using natural agricultural residues in shaker flasks.

The use of purified xylan as substrate is uneconomical for large-scale production of xylanases; therefore, several natural agricultural residues were tested as substrates. *Table 1* shows the xylanase production in shaker flasks, using basal medium containing either rice straw, wheat straw, sugarcane bagasse, or wheat bran as sole carbon source at a concentration of 1%. Wheat bran was the most suitable substrate for both xylanase and β-xylosidase enzymes. Wheat bran was used for further studies, not only for the

Table 2 Influence of glucose and xylose on xylanase production by mutant strain *Fusarium oxysporum* NTG-19

Carbon source	Xylanase (IU ml^{-1})[a]				
	Fermentation time (h)				
	24	48	72	96	120
1% Xylan	2.5	10.2	16.7	25.1	35.0
1% Xylan + 1% glucose	0	2.7	12.2	20.4	29.3
1% Xylose	3.5	9.9	14.0	23.0	30.0

[a]Values are the means of three replicate experiments

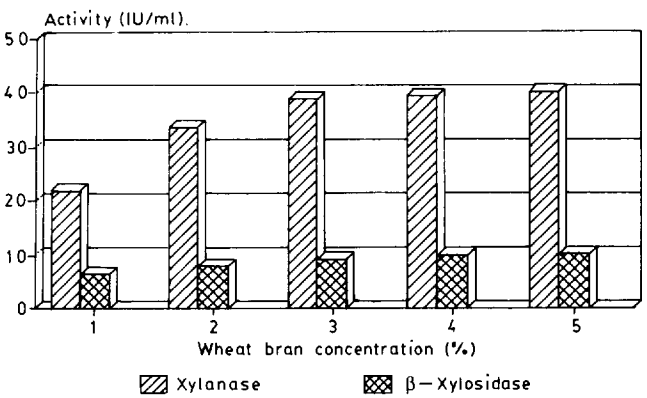

Figure 1 Xylanase and β-xylosidase production by *F. oxysporum* NTG-19 with different concentrations of wheat bran as sole carbon and nitrogen source. Incubation period was 120 h

Table 3 Effect of surfactant and fatty acids on xylanase secretion by mutant strain *Fusarium oxysporum* NTG-19 using wheat bran (3%) as sole carbon and nitrogen sources[a]

Addition	Concentration (%)	Xylanase (IU ml^{-1})	β-Xylosidase (IU ml^{-1})
None	—	38.8	9.3
Tween-80	0.1	40.8	10.8
	0.2	44.2	11.5
	0.3	43.5	11.8
Olive oil	0.1	44.7	11.9
	0.2	53.0	13.4
	0.3	53.0	13.9

[a]Incubation time, 120 h. Values are the means of three replicate experiments

higher enzyme yields, but also because no physical or chemical pretreatment of the substrate was required. Furthermore, wheat bran contained about 26% protein, which permitted the elimination of peptone and yeast extract, the expensive ingredients of the medium. The wheat bran was used as the main carbon and nitrogen source in subsequent experiments.

Addition of 1% glucose to the basal medium containing xylan caused apparent catabolite repression of xylanase production that started only after 72 h; the derepression of the enzyme might be because of exhaustion of sugar (*Table 2*). In contrast, 1% xylose added to the basal medium yielded extracellular xylanase levels comparable to those obtained on xylan alone. This is in agreement with other work showing that the regulation of xylanase expression is not influenced by xylose[10] and may be because xylose is not the main end product of the action of this enzyme[11,12]; therefore, it may not inhibit enzyme production.

The effect of increasing wheat bran concentration on xylanase production in the modified medium (devoid of peptone and yeast extract) is shown in *Figure 1*. Enzyme activity increased up to 3% substrate concentration and no significant increase was observed thereafter. The removal of peptone and yeast extract from the medium did not affect enzyme activity at all.

The use of surfactants and fatty acids is well documented to increase the production of hydrolytic enzymes.[13,14] Such compounds probably increase the permeability of cell membranes and affect the secretion of certain proteins. Enzyme activities from *F. oxysporum* NTG-19 were considerably increased by the addition of Tween-80 or olive oil (*Table 3*). Addition of 0.2% olive oil (an excellent inexpensive source of oleic acid) in the medium resulted in maximum enzyme activity, yielding up to 53 IU ml^{-1}.

In conclusion, a mutant strain with clearly improved xylanase and β-xylosidase activities has been produced from *F. oxysporum* DSM 841. Further studies on optimizing the overproduction of extracellular xylanase by *F. oxysporum* NTG-19 are underway.

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