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Xylanase Production by *Penicillium canescens* 10-10c in Solid-State Fermentation

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Abstract

Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria. We evaluated the influence of carbon sources, nitrogen sources, and moisture content on xylanase production by *Penicillium canescens* 10-10c in solid-state fermentation. Among agricultural wastes tested (wheat bran, untreated wheat straw, treated wheat straw, beet pulp, and soja meal), untreated wheat straw gave the highest production of xylanase. Optimal initial moisture content for xylanase production was 83%. The addition of 0.4 g of xylan or easily metabolizable sugar, such as glucose and xylose, at a concentration of 2 % to wheat straw enhanced xylanase production. In solid-state fermentation, even at high concentrations of glucose or xylose (10%), catabolic repression was minimized compared to the effect observed in liquid culture. Yeast extract was the best nitrogen source among the nitrogen sources investigated: peptone, ammonium nitrate, sodium nitrate, ammonium chloride, and ammonium sulfate. A combination of yeast extract and peptone as nitrogen sources led to the best xylanase production.

Index Entries: *Penicillium canescens*; xylanase; solid-state fermentation.

Introduction

Xylans are one of the major components in wood and plant materials. Xylan is a collective name of polysaccharides in which in most cases β-1,4-linked D-xylopyranose residues are the main constituents. Depending on their origin, xylans may also contain variable amounts of arabinosyl- and 4-*O*-methylglucuronic acid residues and acetyl groups *(1)*.

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The most important enzymes in the xylan degradation are the endo-1,4-β-xylanases (EC3.2.1.8), which hydrolyze β-1,4-glycosidic linkages between xylopyranose units. The xylanases are of considerable interest as catalysts in various biotechnological applications, such as in bleaching of kraft pulps *(2,3)*, in baking *(4)*, and animal feed *(5)*. A variety of microorganisms, including bacteria, yeast, and filamentous fungi, have been reported to produce xylanolytic enzymes *(6–8)*, with the latter being extensively studied *(9–12)*.

The use of purified xylan as a substrate for bioconversion into xylanases increases the cost of enzyme production. Consequently, for commercial applications, there have been attempts to develop a bioprocess to produce xylanase in high quantities from simple and inexpensive substrates. Abundantly available lignocellulosic residues are an obvious choice as substrates *(10,11,13,14)*.

Among processes used for enzyme production, solid-state fermentation is an attractive one *(15)*. Solid-state fermentation is characterized by the complete or almost complete absence of free liquid. Water, which is essential for microbial activities, is present in an absorbed or complexed form with the solid matrix of the substrate *(16,17)*.

The attraction of solid-state fermentation comes from its simplicity and its closeness to the natural environment for many microorganisms. This method has low capital costs for equipment and operating, high volumetric productivity, lower space requirements, and easier downstream processing compared with that of submerged fermentation *(15,18,19)*. In addition, the reactor equipment is often simple and requires less processing energy than for the corresponding liquid submerged fermentation. The low moisture required to obtain maximum yields of product with fungi excludes in most instances any problem of bacterial contamination *(17,18)*. In some instances, such as the delignification of agricultural residues for increasing their digestibility, solid-state fermentation even offers the chance for a direct applicability of the fermented product *(15)*.

Therefore, the purpose of the present study was to investigate the ability of a *Penicillium canescens* strain to produce xylanase enzyme in solid-state fermentation using easily available different agricultural wastes such as wheat straw, wheat bran, pulp beet, and soja meal.

Materials and Methods

Strain

P. canescens 10-10c was supplied by G. I. Kvesidatse, Institute of Plant Biochemistry, Academy of Sciences, Tbilisi, Georgia.

Fermentation Conditions

For solid-state fermentation, the medium was placed in a 250-mL Erlenmeyer flask containing 5 g of substrate and nutrients (based on 100 mL of liquid medium) plus distilled water to adjust the moisture content. The fermentation medium consisted of: 10 g/L of $Na_2HPO_4 \cdot 2H_2O$, 0.5 g/L of

KCl, and 0.15 g/L of $MgSO_4 \cdot 7H_2O$, plus nitrogen source. Initial experiments were performed using 5 g of different agricultural residues (as carbon sources) and a combination of 0.75 g of yeast extract and 0.2 g of ammonium sulfate (as nitrogen sources). The effect of the nitrogen sources was tested using yeast extract, peptone, ammonium nitrate, sodium nitrate, ammonium chloride, and ammonium sulfate. The concentration of nitrogen sources used was 52.5 mg of N/5 g of wheat straw. In other experiments, yeast extract combined with one of the previous nitrogen sources was used at a concentration of 105 mg of N/5 g of wheat straw. The pH was adjusted to 6.5 before sterilization. The flasks were sterilized at 120°C for 20 min, then inoculated with a 2-mL spore suspension (10^5–10^6 spores/mL).

For submerged cultures, the same medium as just described was employed using 7.5 g/L of yeast extract and 2 g/L of ammonium sulfate, as nitrogen sources. The pH was adjusted to 6.5 before sterilization. Erlenmeyer flasks (250 mL) containing 50 mL of medium were inoculated with a 1-mL spore suspension (10^5–10^6 spores/mL) and incubated at 30°C in a rotatory shaker (120 rpm). At periodic intervals, the filtrate obtained was assayed for enzyme activity.

Enzyme Extraction

The solid-state culture in each flask was picked up at different time intervals. Water containing 0.1% Tween-80 was added to make the volume in flask equivalent to 100 mL. The flask's contents were stirred for 1.5 hours on a magnetic stirrer at laboratory temperature and filtrated. The supernatant filtrates were used as the enzyme source.

Enzyme Assays

Xylanase activity was measured according to Bailey et al. *(20)* using 1% birchwood xylan as substrate; reducing sugars were assayed by a dinitrosalicylic acid method with xylose as the standard *(21)*. One unit of enzyme activity is defined as the amount of sugar (in micromoles) produced per minute of reaction and per milliliter of enzyme solution, in the assay conditions.

Results and Discussion

Effect of Different Lignocellulosic Materials on Xylanase Production

In the first experiments, we examined the effect of different lignocellulosic materials—wheat bran, beet pulp, soja meal, and wheat straw (treated with NaOH and untreated)—as carbon sources on xylanase production. We observed that maximum enzyme activity was obtained by using untreated wheat straw (Table 1). Table 1 shows that the time of cultivation needed to obtain maximum enzyme (7448 U/g) production on this substrate was 12 d. This substrate was used for further studies. Wheat bran, beet pulp, and soja meal culture filtrates exhibited moderate activities. It has been reported that the ratio of cellulose to xylan of the growth

Table 1
Production of Xylanase by *P. canescens* in Solid Cultures on Various Lignocelluloses

Substrate (5 g)	Day	Xylanase (U/g)
Untreated wheat straw	4	2702
	6	3024
	8	3598
	10	5628
	12	7448
	14	6398
Treated wheat straw	4	1134
	6	1792
	8	2282
	10	2954
	12	3332
	14	3192
Wheat bran	4	1470
	6	1582
	8	1414
	10	896
	12	574
	14	294
Pulp beet	4	714
	6	938
	8	1764
	10	1792
	12	1512
	14	1148
Soja meal	4	1400
	6	1932
	8	1832
	10	1610
	12	1372
	14	1274

substrate is important for the production of xylanase *(22)*. Carbohydrates present in untreated wheat straw were by far more effective for xylanase production. Other studies *(23,24)* have also identified the same substrate as being ideally suited for xylanase production in *Aspergillus terreus* and *Thermoascus aurantiacus* cultures. Treatment of wheat straw with alkali caused a 56% reduction in xylanase activity. Removal of the major part of the hemicelluloses may be the reason for this performance. This alkali treatment is also responsible for structural deformity in wheat straw *(13)*.

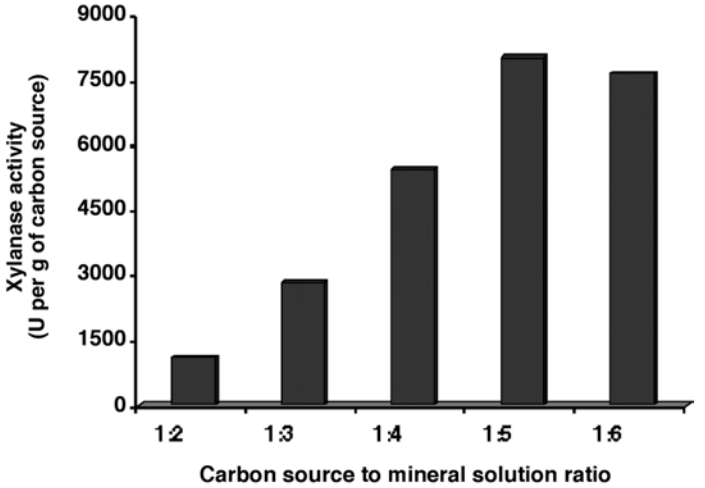

Fig. 1. Effect of carbon source–to–mineral solution ratio on xylanase production by *P. canescens* in solid-state fermentation.

Using untreated wheat straw, higher crystallinity of the substrate or reduced accessibility of hemicellulose apparently increased the induction of xylanase formation. By contrast, pretreatment, which made the substrate more easily accessible and more rapidly depleted, caused a drastic reduction in enzyme production *(25,26)*.

Effect of Moisture Level

To achieve the best conditions to use in solid-state fermentation, the effects of substrate moisture content on xylanase production were examined. Several investigators *(27–29)* have reported that moisture content is a controlling factor for enzyme formation under solid-state fermentation. This effect was therefore studied by changing the wheat straw to mineral solution ratios. Five different ratios were tested. It was taken into consideration that the concentration of soluble medium ingredients was not changed. Figure 1 shows that the ratios 1:5 and 1:6 (initial moisture level above 80%) yielded the highest xylanase production. Maximal activity was attained in the medium with 83% initial moisture content (wheat straw:nutrient solution ratio of 1:5 [v/v]). This could be attributed to faster growth of the organism at higher moisture content and the subsequent early initiation of the enzyme production *(24)*. Many investigators have reported a similar effect of moisture content on xylanase production *(19,30,31)*. Low moisture content is known to decrease the metabolic and enzymatic activity, probably owing to reduced solubility of nutrients from the solid substrate, low swelling, and higher water tension *(24,28)*. Therefore, the importance of moisture level in solid-state fermentation media and its influence on microbial growth and product biosynthesis

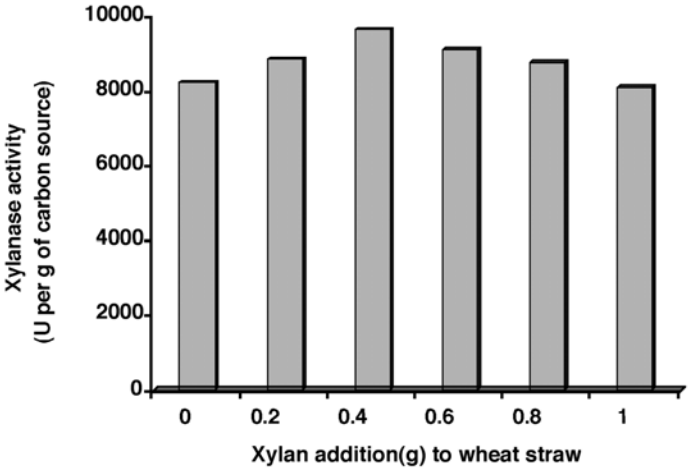

Fig. 2. Effect of addition of xylan to wheat straw on xylanase production by *P. canescens* in solid-state fermentation.

may be attributed to the impact of moisture on the physical properties of the solid substrate. A level of moisture higher than the optimum causes a decrease in porosity, an alteration in substrate particle structure, a gummy texture, a lower oxygen transfer, and an enhancement of the formation of aerial mycelia *(28)*.

Effect of Addition of Xylan

The effect of the addition of xylan on xylanase production was investigated. Our results show that the addition of xylan to the wheat straw medium increased xylanase production (Fig. 2). The highest effect was observed with the addition of 0.4 g of xylan. The addition of small amounts of a purified xylan to complex lignocellulosic substrate has been found to be advantageous and increased considerably the production of xylanase *(19,25)*, since xylan and its derivatives play an important role as inducer of xylanase formation *(10)*.

Effect of Addition of Glucose or Xylose

The expression of xylanases in fungi is subject to regulation by catabolic repression. In submerged fermentation, the accumulation of reducing sugars has been reported to have a negative effect on the production of xylanase *(32,33)*. In our study, in submerged culture, we tested the effect of the addition of 1% of glucose to 1% birchwood xylan on xylanase production. The results shown in Fig. 3 indicate the inducible nature of xylanase production by *P. canescens*. Whereas a very low xylanase activity (0.34 IU/mL) was detected in the medium containing only glucose after

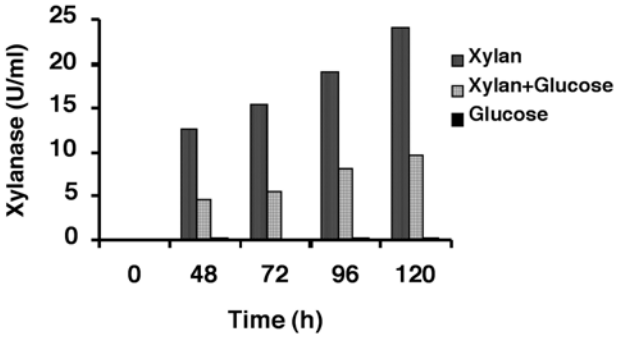

Fig. 3. Induction of xylanase activity on different carbon sources by *P.canescens* in shake-flask culture (120 rpm) at 30°C.

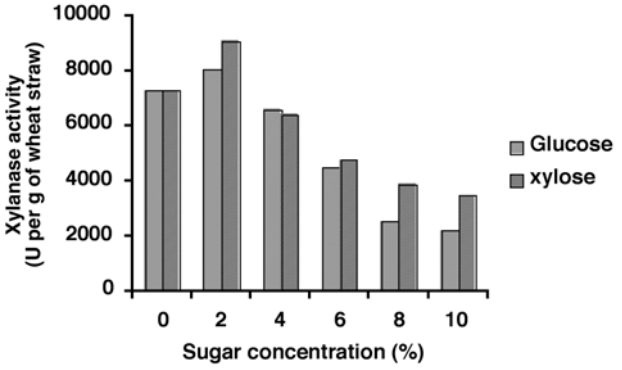

Fig. 4. Effect of glucose and xylose concentrations added to wheat straw on xylanase production by *P. canescens* in solid-state fermentation.

120 h of incubation, in medium containing xylan enzyme activity reached a level of 24.17 IU/mL. The addition of glucose to the medium containing xylan produced catabolic repression of xylanase production. We also tested the effect of increasing concentration of glucose and xylose on xylanase production by *P. canescens* in solid-state fermentation. Basal medium was enriched with different glucose and xylose concentrations. The results shown in Fig. 4 demonstrate that xylanase production increased in the presence of 2% glucose and xylose concentrations. Increases in the glucose or xylose concentrations from 4 to 10% caused a reduction in the production of xylanase. However, no complete catabolic repression was observed even at 10% glucose. Previously, *P. canescens* had been found to grow well and produce high levels of xylanase in a submerged fermentation system using lignocellulosic residues as substrate. The presence of glucose caused severe catabolic repression and decreased xylanase production *(32)*.

The ability of solid-state fermentation to minimize catabolic repression has been reported. Souza et al. *(34)* demonstrated that the production of xylanase by *Aspergillus tamarii* in solid-state fermentation using wheat bran increased with glucose concentration from 0.1 to 1.0%. In addition, the enrichment of the solid-state fermentation with different easily metabolizable sugars such as xylose, fructose, galactose, maltose, and sucrose at 1% (w/w) minimally affected xylanase production. Archana et al. *(28)*, compared xylanase production by *Bacillus licheniformis* A99 in submerged and solid fermentation. They demonstrated that solid-state fermentation served to minimize the repression caused by glucose in contrast to that in submerged fermentation. Glucose, which completely repressed xylanase synthesis in submerged cultivation, was tolerated up to 6% in solid-state fermentation without causing any lowering in enzyme titer while 8 and 10% glucose levels caused minimal repression. The addition of glucose increased pectin esterase and polygalacturonase production by *Aspergillus niger* in the solid-state system, but in submerged fermentation the production was markedly inhibited as reported by Maldonado and Strasser de Saad *(35)*.

Effect of Different Nitrogen Sources

We tested the effect of nitrogen sources on xylanase formation by *P. canescens* in solid-state fermentation. Various inorganic and organic nitrogen compounds were used with a fixed concentration of nitrogen at 52.5 mg of N/5 g of wheat straw. The results shown in Table 2 demonstrate that of the six organic and inorganic nitrogen sources used, yeast extract and peptone were the best for achieving optimal xylanase production by *P. canescens*, 5404 and 3878 U/g, respectively. Our results are in agreement with those reported in the literature in which it was found that fungi-produced highest xylanase activities were obtained with organic nitrogen sources *(36,37)*. Yeast extract has an important role in enzyme synthesis, probably because this complex nitrogen source contains important elements that are necessary for the metabolism of fungus *(14)*. Table 3 indicates that using a combination of yeast extract with one of the other nitrogen sources enhanced xylanase production. The combination of yeast extract with peptone gave the best result (8932 U/g).

Conclusion

One of the factors determining the large-scale use of xylanases will certainly be the cost of xylan-degrading enzyme preparation. The cost of the carbon source, as well as the additional medium components, plays a major role in the economics of xylanase production. The use of the purified inducing substrate xylan for large production scale is far too expensive. Alternatively, less costly carbon sources can be lignocellulosic biomass, which is and will continue to be available in large quantities, as well as residual products from industrial processing of lignocellulose. The results

Table 2
Effect of Nitrogen Sources on Xylanase Production

Nitrogen source	Quantity (g/5 g wheat straw)	Xylanase (U/g)
Yeast extract	0.5	5404
Peptone	0.39	3878
$(NH_4)_2SO_4$	0.24	1820
$NaNO_3$	0.32	2226
NH_4NO_3	0.15	2072
NH_4Cl	0.2	1848

Table 3
Effect of Yeast Extract Combined with Nitrogen Sources on Xylanase Production

Nitrogen source (105 mg/5 g wheat straw)	Xylanase (U/g)
Yeast extract	4088
Yeast extract + peptone	8932
Yeast extract + $(NH_4)_2SO_4$	6720
Yeast extract + $NaNO_3$	6006
Yeast extract + NH_4NO_3	5880
Yeast extract + NH_4Cl	6916

reported herein indicate that *P. canescens* can be cultivated under solid-state fermentation for the production of xylanase using, as carbon source, available agricultural wastes. Untreated wheat straw gave the maximum xylanase activity after 12 d of culture. Catabolic repression of simple sugar such as glucose and xylose was minimized in solid-state fermentation with wheat straw as substrate. Additionally, the results demonstrate the importance of initial moisture above 80% for xylanase production by *P. canescens* in solid-state fermentation. Among nitrogen sources tested, yeast extract supplemented with peptone gave the maximum xylanase production.

Although quantitative comparison of xylanase activities reported in literature is not always possible because no standard enzyme substrate has been adopted yet, the yields of xylanase productivity from *P. canescens* observed in this work were approx 1.5-fold higher than optimum productivities reported in the literature for some microorganism grown in SSF (Table 4).

In conclusion, the fungus *P. canescens*, grown in solid-state fermentation in a simple medium consisting of agricultural byproducts and a low-cost mineral source, proved to be a promising microorganism for xylanase production.

Table 4
Optimum Xylanase Production in Solid-State Fermentation by Filamentous Fungi

Organism	Substrate	Initial moisture content (%)	Cultivation conditions	Activity (IU/g)[a]	Productivity (IU/[Lh])[b]	Reference
Allescheria terrestris	Beet pulp	80	45°C, 3 d	28.3	78.6	*38*
Aspergillus ochraceus	Wheat bran + xylan	60	28°C, 14 d	724	862	*25*
	Wheat straw	60	28°C, 14 d	488	581	
Aspergillus ochraceus NG-13	Wheat straw	75	28°C, 14 d	2120	1580	*39*
	Wheat bran	75	28°C, 14 d	2240	1670	
Aspergillus niger 3T5B8	Wheat bran + cellobiose	60	32°C, 3 d	100.65	559	*40*
Aspergillus awamori	Sugarcane bagasse	92	30°C, 60 h	2500	3333	*14*
Chaetomium cellulolytium ATCC 32319	Wheat straw	50	37°C, 10 d	580	1210	*26*
	Corn stover	50	37°C, 10 d	350	729	
Humicola langinosa	Beet pulp	80	45°C, 3 d	72.6	202	*38*
Penicillium capsulatum	Beet pulp + bran	67	30°C, 9 d	279.9	428	*41*
Penicillium canescens	Wheat straw + xylan	83	30°C, 12 d	9632	5551	This work
Sporotrichum thermophile	Beet pulp	80	45°C, 3 d	27.8	77.2	*38*
Thermoascus aurantiacus	Beet pulp	80	45°C, 3 d	96.7	269	*38*
Thermophilic *Melanocarpus albomyces* IIS-68	Wheat straw	67	45°C, 4 d	756	2620	13
	Holocellulose from rice straw	67	45°C, 4 d	1084	3760	

[a]Maximum activity.
[b]Volumetric productivity; calculation is based on the initial moisture content.

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