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Study on the Production of a Xylanolytic Complex from *Penicillium canescens 10-10c*

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ABSTRACT

We screened about a hundred microorganisms (including unidentified yeasts, fungi, and bacteria) for their ability to produce xylanolytic enzymes. About 40 of them were hemicellulolytic; among these, we selected *Penicillium canescens 10-10c* for detailed study because of its ability to produce an interesting enzymatic complex in quantity. The xylanase complex was cellulase-free, and had an optimal activity at pH 4.6–5.0 and 55–60°C on birchwood xylan.

The best production was on soya meal and wheat straw; expression of the xylanase was repressed by glucose, xylose, and lactose. The optimization of culture medium and mode (fed-batch) enabled us to improve the production three to four times. The importance of the mixing conditions on the biomass and xylanase production is also reported.

Index Entries: *Penicillium canescens 10-10c*; xylanases; fed-batch culture; hydrodynamic stress.

INTRODUCTION

Hemicellulases are enzymes that specifically degrade hemicelluloses, a heteropolysaccharide principally found in the secondary plant cell wall. This group of enzymes includes xylanases (EC 3.2.1.8 and EC 3.2.1.37). Xylan is the principal component of hemicelluloses from annual plants and hardwoods. Its hydrolysis is accomplished by the concerted action of several enzymes differing in their bond-breaking specificity *(1)*.

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Since lignocellulose is an abundant and renewable resource, the biodegradation of lignocellulose has interested researchers and technologists in many fields *(2)*. Xylanases have found applications in brewing *(3)*, baking *(3,4)*, starch syrups *(3,4)*, and fruit juice *(2,4)* manufacturing, in animal feeding, bioconversion, and biopulping processes *(2,5)*. *Penicillium janthinellum (6,7)*, *Penicillium janthinellum* (BIOURGE) *(8)*, *Penicillium purpurogenum (9,10)*, *Penicillium oxalicum (11)*, *Penicillium capsulatum (12)*, *Penicillium chrysogenum (13)*, *Penicillium herquei (14)*, and *Penicillium funiculosum (15)* are the *Penicillium* sp. most recently reported in literature. Nevertheless, these fungi are less studied than *Trichoderma* sp. or *Aspergillus* sp.

The optimal thermal activity of xylanases secreted by *Penicillium* sp. is between 40 and 50°C (except for the purified xylanase A produced by *P. purpurogenum*, which is mostly active at 70°C). Their optimal pH activity occurs between 3.0 and 5.0 (except for the xylanase A produced by *P. purpurogenum* and the xylanase produced by *P. chrysogenum*, which have their optimal pH at 7.0).

Most publications dealing with xylanases focus on the purification and characterization, whereas much less has been reported on the production, application, and feasibility of production *(16)*. The strain we studied is *Penicillium canescens 10-10c*, a high-level producing mutant of cellulase-free xylanase; in this work, the culture conditions for the production of xylanase by this microorganism are reported.

MATERIALS AND METHODS

Fungus

Penicillium canescens 10-10c was graciously provided by G. I. Kvesitadze, Institute of Plant Biochemistry, Academy of Sciences, Tbilisi, Georgia.

Culture Medium

The culture medium contained the carbon source, either at 3% w/v (wheat straw, soya meal, wheat husk, barley), at 2% w/v (xylan), or at 1% w/v (glucose, lactose, glycerol, saccharose, xylose), and 0.5% w/v yeast extract in mineral salt medium. The wheat straw was partially hydrolyzed with NaOH. Petterson salt medium contained: $NH_4H_2PO_4$ (0.2% w/v), KH_2PO_4 (0.06% w/v), K_2HPO_4 (0.04% w/v), $MgSO_4$ (0.2% w/v). G1 salt medium contained: $Na_2HPO_4 7\,H_2O$ (1.5% w/v), KCl (0.05% w/v), $MgSO_4 7\,H_2O$ (0.015% w/v).

Inoculation

The shake flasks and the fermenters were inoculated with a spore suspension until 10^6 sp/mL was reached. Spores of *P. canescens 10-10c* were produced in a sterile Erlenmeyer containing the following medium:

agar 12 g/L, glucose 2 g/L, and yeast extract 2 g/L. After inoculation and incubation at 28°C for 5 to 7 d, spores were stored at 4°C.

In order to prepare the spore suspension, a sterile magnetic stirrer and a saline solution (NaCl 8 g/L, peptone 1 g/L, and Tween-80 2 mL/L) were transferred into the Erlenmeyer containing spores until the desired spore concentration.

Batch and Fed-Batch Cultures

The fermenter was a 20-L Biolaffite fitted with four baffles, pO_2, pH, and temperature probes. The parameters of the batch culture were 30°C, 300 rpm, and 0.75 vvm (air flow); the volume of medium was 8.5 L; pH was not regulated. One turbine of 0.1-m diameter was placed at height D/3 (D = diameter of the fermenter). For the fed-batch culture, two turbines were used, and the agitation speed varied between 400 and 450 rpm.

The composition of the medium for the batch culture was: wheat straw (3% w/v), soya meal (3% w/v), yeast extract (0.5% w/v) in solution in the G1 salt medium. The fed-batch culture was carried out in three steps:

1. 10 L of medium composed of soya meal (6% w/v), wheat straw (1% w/v), and yeast extract (0.5% w/v) in solution in the G1 mineral salt medium.
2. 4 L of medium composed of yeast extract (1.5% w/v) and the G1 mineral salt medium three times concentrated in a solution of wheat straw.
3. 4 L of medium composed of soya meal (2% w/v), yeast extract (2% w/v), and the G1 mineral salt concentrated twice in a solution of wheat straw.

The solution of wheat straw was obtained after soaking of the partially hydrolyzed wheat straw in water for 2 h and further filtration.

Oxygen and Carbon Dioxide Analyzers

Oxygen and carbon dioxide were analyzed on-line with Servomex OA 570 (Crowborough, England) and Binos (Leybold-Herous, Hanau, Germany) analyzers, respectively. This permitted the calculation of the Kla (on-line), the respiratory quotient (on-line), the oxygen uptake rate (OUR), and the carbon dioxide transfer rate (CTR).

Enzymes Assay

Xylanase assay was carried out according to Bailey et al. *(17)*, using 1% birchwood xylan as substrate; reducing sugars were assayed by the DNS method *(18)* with xylose as standard. One unit of enzyme activity (U) is defined as the amount of sugar (in µmoles) produced per minute of reaction and per mL of enzyme solution, in the assay conditions.

β-xylosidase was assayed as follows: reaction mixture consisting of 1 mL enzyme solution and 1 mL orthonitrophényl-β-D 1-4-xylopyranoside, pH 4.8 (citrate buffer 0.5*N*) was incubated at 50°C for 15 min; after the addition of 1 mL Na_2CO_3 (0.1*M*), absorbance was read at 420 nm.

Cellulase was determined using a reaction mixture consisting of 0.5 mL enzyme solution, 1 mL citrate buffer, pH 4.8 (0.5*N*), and 50 mg Whatman paper no. 1. The reaction mixture was incubated at 50°C for 1 h. Reducing sugars were assayed by the DNS method with glucose as standard. One unit of enzyme activity (U) is defined as the amount of sugar (in μmoles) produced per minute of reaction and per mL of enzyme solution, in the assay conditions.

RESULTS

Optimization of Xylanase Production by *P. canescens 10-10c* in Shake Flask

Influence of Carbon Substrate on Xylanase Production

Carbon substrates were tested to enhance xylanase secretion; this was carried out in shake flasks, at 30°C for 90–120 h. The simple carbohydrates were at 1% (w/v) and the complex carbon substrates at 3% (w/v) in Petterson salt medium complemented with yeast extract. The results are shown in Table 1. The culture filtrate was also tested on various hemicellulosic and cellulosic substrates to detect enzymatic activities.

The course of xylanase production showed that maximal xylanase activity was reached after 90–120 h of culture. Of the 11 substrates tested, the best xylanase activities were obtained with soya meal and wheat straw; these substrates improved the production three to five times when compared to xylan. The xylanase production was repressed by glucose and simple carbohydrates.

Larger pellets of *P. canescens 10-10c* were observed on soluble substrates (glucose, lactose, glycerol) than in media containing insoluble particles (soya meal, wheat straw, barley husk) where no pellets were observed.

The xylanase complex secreted during culture on oatspelt xylan was free of cellulase activity; the β-xylosidase activity reached $3.3\ 10^{-2}$ U/mL, at the time when the production of xylanase was maximum (Table 1).

Influence of Yeast Extract and Mineral Salt Medium on Xylanase Production

The influence of yeast extract on xylanase production was studied. Figure 1 presents xylanase production after growth on oatspelt xylan (2% w/v) in Petterson salt medium with various concentrations of yeast extract. Initial and final pH are also indicated (Fig. 1).

The neutralization of the broth was observed and a better xylanase yield was obtained with the medium complemented with yeast extract;

Table 1
Xylanase Production (U/mL) After Growth (120 h) of *P. canescens 10-10c* on Different Carbon Sources

Carbon source	*P. canescens 10-10c* U/mL
Birchwood xylan	***40-60***
Oatspelt xylan	***80-90***
Soya meal	***260-280***
Wheat straw	***220-250***
Wheat straw + soya meal	***290-310***
Wheat husk	***40-50***
Barley	***60-70***
Xylose	***0***
Lactose	***0***
Saccharose	***0***
Glycerol	***0***
Glucose	***0***
Glucose + xylan	***0***

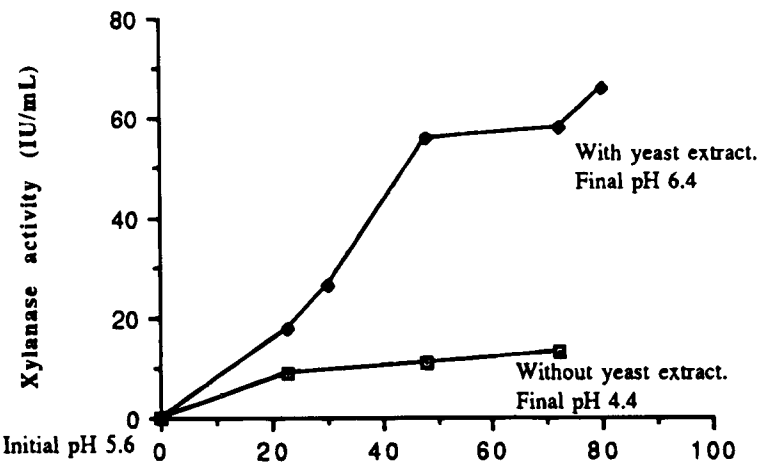

Fig. 1. Time-course of xylanase production during culture of *P. canescens 10-10c* on oatspelt xylan, with (0.5% w/v) or without yeast extract.

Table 2
Xylanase Production (U/mL) Under Different O_2 Transfer Conditions During the Culture of *P. canescens 10-10c*

Substrate	v/V= 1/2.5	v/V = 1/4	v/V = 1/10
Soya meal	*ND*	*260-280*	*480-510*
Soya meal + wheatstraw	*180-200*	*290-310*	*530-560*

ND = not determined.

similar results were obtained after culture on wheat straw, soya meal, and wheat husk.

Cultures on soya meal at several initial pHs (from 4.0–8.0) revealed that initial pH does not influence the productivity. In any case, final pH reached 7.0–7.5.

Xylanase production by *P. canescens 10-10c* was also improved by 20% when G1 salt medium was used instead of Petterson salt medium (data not shown).

Influence of Mixing Conditions on Xylanase Production

P. canescens 10-10c was grown in shake flasks filled with different volumes of medium. In this way, the ratio of "surface in contact with air over volume of liquid" and subsequently gas transfer were modified. In addition, by reducing the volume of liquid in the shake flask, mixing was improved. Table 2 presents the results obtained when the culture volumes were modified. The ratio v/V is the ratio between broth volume (v) and volume of the shake flask (V). The smaller this ratio, the more effective the mixing and the mass transfers.

The result indicated that the mixing operation influenced xylanase production; this was mainly explained by the improvement of oxygen transfer and the subsequently higher biomass yield. Pellets were not observed in any case.

Influence of Substrate Concentrations on Xylanase Production

This experiment was carried out to determine the influence of substrate concentrations on xylanase production; since both the mycelium and the carbon substrate (soya meal and wheat straw) developed a very viscous solution, it seemed important to reduce the viscosity by using less wheat straw or soya meal.

Table 3
Xylanase Production by *P. canescens 10-10c* After 120 h of Growth with Several Substrate Concentrations

% wheat straw	% Yeast extract	% soja meal	U/mL
3	0.5	3	**535**
3	0.5	1	**464**
3	0.2	3	**310**
1	0.5	3	**292**
1	0.5	1	**290**
3	0.2	1	**245**
1	0.2	3	**229**
1	0.2	1	**212**

Table 3 shows the influence of the wheat straw, soya meal, and yeast extract concentrations in the culture medium, on xylanase production by *P. canescens 10-10c* with a G1 salt medium and a v/V ratio of 1:7.5.

Despite higher viscosity of the broth, best results were obtained with the higher substrate concentrations. Wheat straw and yeast extract seemed to have greater influence on the xylanase yield than soya meal, in our assay conditions. Such results could be explained by a greater availability of nutrients.

Optimization of Xylanase Production by *P. canescens 10-10c* in a Lab-Scale Fermenter

Profile of a Batch Culture of P. canescens 10-10c

Figures 2 and 3 show the time-course of xylanase production and the on-line parameters during the growth of *P. canescens 10-10c* in a 2-L fermenter, on wheat straw (3% w/v), soya meal (3% w/v), and yeast extract (0.5% w/v) in G1 salt medium. The agitation speed was 300 rpm, and the aeration rate was 0.75 vvm. pH was not regulated and varied freely between 5.8 and 7.5 during the process. The culture volume was 8.5 L, and the reactor was fitted with a disk-mounted blade turbine (TD4, diameter: 0.1 m).

Spore germination occurred 5–7 h after inoculation; after 20 h of culture, the pO_2 reached 3% of saturation (Fig. 2). In the same time, OUR (mmol/L/h) and CTR (mmol/L/h) were at their maximum (Fig. 3); this coincided with the minimal values of the pO_2. The exponential growing phase seemed to be stopped by oxygen limitation.

The viscosity developed by substrate particles and biomass increased progressively during the growth phase; therefore, oxygen transfer, as shown by the value of Kla (Fig. 2), decreased from 300 to 0 37 h^{-1}.

In a second phase, the respiratory quotient ($Qr = CO_2/O_2$) decreased from 1.0–1.2 down to 0.8 and pO_2 increased gradually. At the same time,

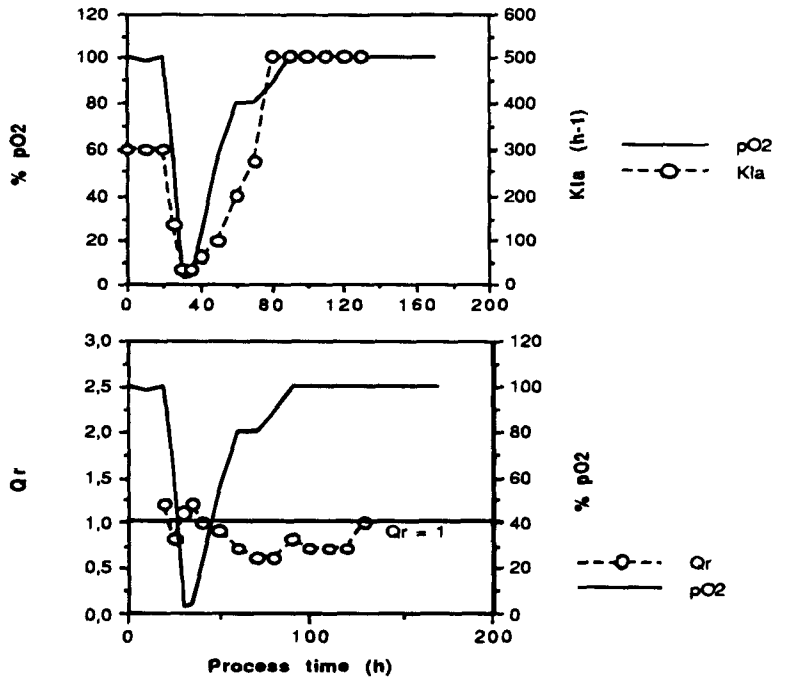

Fig. 2. pO_2, Qr and Kla (global transfer coefficient h^{-1}) evolution during the growth of *P. canescens 10-10c* in a 20/L fermenter on wheat straw (3% w/v), soya meal (3% w/v), and yeast extract (0.5% w/v) in G1 salt medium.

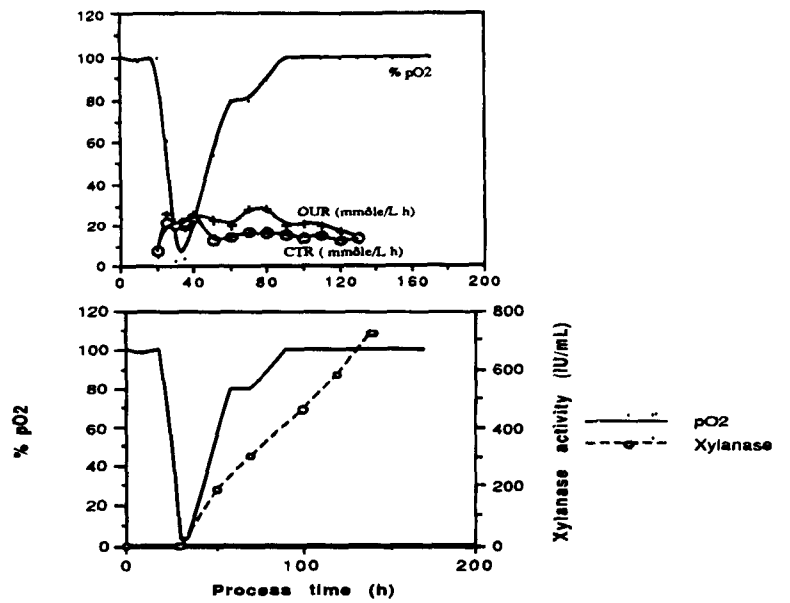

Fig. 3. pO_2, xylanase (U/mL), oxygen uptake rate (OUR mmoles-L/h) and carbon dioxide transfer rate (CTR mmoles-L/h) evolution during the growth of *P. canescens 10-10c* in a 20-L fermenter on wheat straw (3% w/v), soya meal (3% w/v), and yeast extract (0.5% w/v) in G1 salt medium.

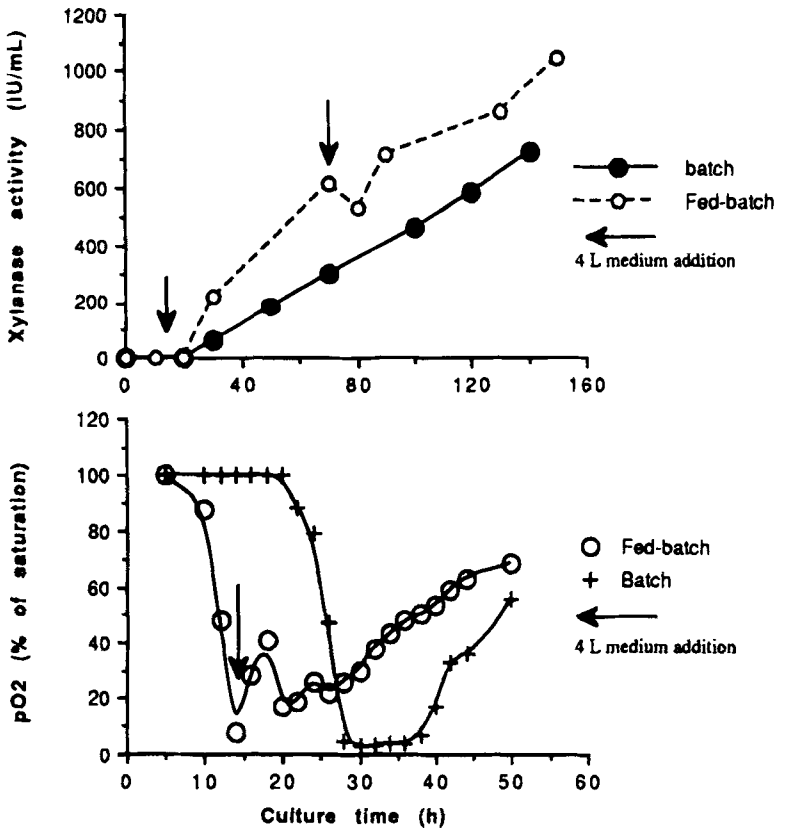

Fig. 4. Evolution of the pO_2 and xylanase production (U/mL) during batch and fed-batch culture of *P. canescens 10-10c*.

xylanase production started. The hyphae became thinner and less branched. The substrate particles were progressively hydrolyzed, leading to lower viscosity and a better mixing of the broth. After 144 h of culture, xylanase production still continued at a constant rate.

Xylanase Production in Batch and Fed-Batch Culture of P. canescens 10-10c

Figure 4 shows the time-course of xylanase production and the pO_2 evolution during batch and fed-batch culture of *P. canescens 10-10c* in a 20-L fermenter.

The best production was obtained with a fed-batch process; xylanase production reached 1050 U/mL in fed-batch culture compared with 700 U/mL in the batch culture.

In the case of the fed-batch culture, 4 L of medium were added after 15 h of culture to dilute the broth and thereby to improve the Kla (data not shown). This addition of fresh medium also reduced total culture time, whereas pO_2 was under 10% of saturation: 2 h in the fed-batch against 11 h in the batch culture (Fig. 4). The second medium addition (after 80 h of culture)

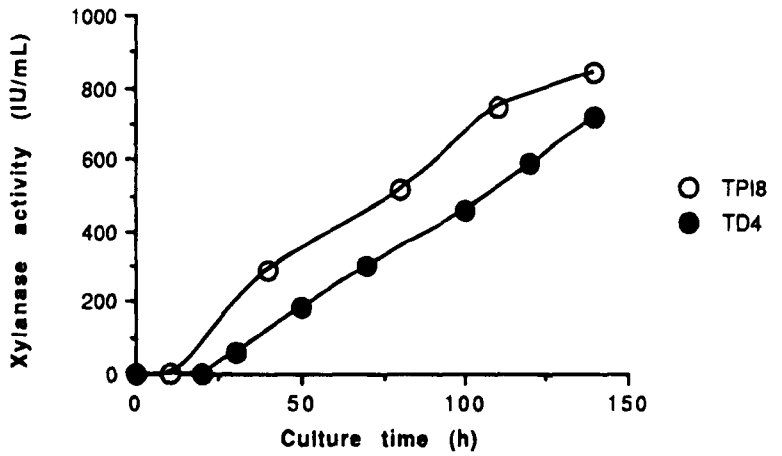

Fig. 5. Evolution of the xylanase production (U/mL) during batch culture of *P. canescens 10-10c* in a 20-L fermenter fitted either with a hub-mounted pitched blade turbine (TP18) or a disk-mounted blade turbine (TD4).

induced an increase in the respiratory activity. Therefore, the pO_2 decreased from 100% down to 50% of saturation, and the OUR and CTR increased proportionally (data not shown).

The use of a seed preculture for the fed-batch inoculation (instead of spores) reduced the lag phase from 20 to 8 h (this was deducted from the pO_2 evolution).

Xylanase Production in Batch Culture with a Hub-Mounted Pitched Blade Turbine (TPI8)

Figure 5 shows the time course of xylanase production and pO_2 evolution during batch culture of *P. canescens 10-10c* in a 20-L fermenter fitted with either a hub-mounted pitched blade turbine (TPI8) or a disk-mounted blade turbine (TD4) (all other culture parameters remaining unchanged).

The batch culture with the TPI8 turbine showed relatively greater xylanase yield (844 U/mL after 144 h of culture time) than the batch with the disk-mounted blade turbine; however, pO_2 evolution was similar in both cases (data not shown).

Influence of Agitation Speed on Biomass Production

Batch cultures in a 6-L fermenter were carried out at different agitation speeds: 450, 600, 900, and 1200 rotations/min (rpm). The biomass (dry matter) was determined after 24 h of culture. The culture medium was composed of 2% glucose, 1% peptone, and 1% yeast extract. Figure 6 presents the results.

Growth of *P. canescens 10-10c* was greatly affected by mixing conditions; biomass after 24 h of culture decreased by almost half at 1200 rpm compared to 450 rpm (3.75 and 7.0 g/L, respectively). Current studies in

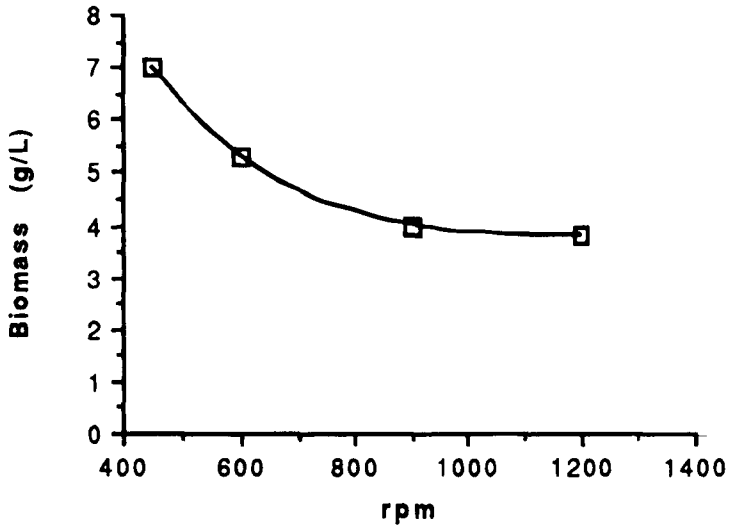

Fig. 6. Biomass (g/L) after 24 h of batch culture at different agitation speeds (450, 600, 900, 1200 rpm) in a 6-L fermenter. Culture medium: 2% glucose, 1% peptone, and 1% yeast extract.

our laboratory confirmed the sensitivity of *P. canescens 10-10c* to hydrodynamic stress and therefore the dependance of the production of xylanase on mixing conditions.

DISCUSSION

Xylanase Production in Shake Flask

P. canescens 10-10c presented several characteristics: the optimum pH and temperature of the xylanase complex were at 4.5–5.0 and 55–60°C respectively; the xylanase was cellulase-free. Protease activity was weak, this is interesting for some applications (for instance, in bread making, since protease liquefies the dough, protease-free xylanases are sought).

Xylanase production by *P. canescens 10-10c* in a shake flask was best on soya meal and wheat straw; these raw materials are inexpensive. Yeast extract increased xylanase yield, and the production increased with high substrate concentrations: the best result was on wheat straw (3% w/v), soya meal (3% w/v), and yeast extract (0.5% w/v) (these substrate concentrations were the highest tested); this result was explained by the better supply to the mycelia with carbon and nitrogen sources. The production level in the shake flask reached 535 U/mL after 120 h of culture. Xylanase production was also improved by 20% when *P. canescens 10-10c* was grown on the G1 salt medium.

Xylanase production by *P. canescens 10-10c* was repressed by simple carbohydrates like glucose, xylose, lactose, or glycerol; the lower yield of

xylanase on xylan or barley could be owing to the amount of xylose (from xylan hydrolysis) and glucose (from starch hydrolysis) in greater quantity than in wheat straw or soya meal.

Carbon sources commonly used for the xylanases production are sugarcane bagasse (*P. janthinellum*) *(6)*, wheat straw (*P. purpurogenum*) *(9)*, corn cobs (*Thermomyces lanuginosus*) *(16,19)*, and xylan and barley husk (*T. lanuginosus*) *(20)*, whereas easily metabolizable sugars (glucose, xylose, sucrose) generally appear to repress xylanases synthesis *(16,21)*; xylose could in some cases reveal an inducing effect *(16)*. Yeast extract (N source) is frequently added in culture medium *(7,16,21)*; soya meal appear to be a very poor inducer of the xylanase production by *T. lanuginosus (16)*.

Another factor that influenced xylanase production by *P. canescens 10-10c* was the agitation conditions; in the shake flask, lower mixing intensities led to lower xylanase yield. Improved oxygen transfer may have induced greater biomass yield and growth rate. Nevertheless, since mixing improves mass transfers, it also modifies the morphology: mycelial or pellet form; pellets were not detected on wheat straw and soya meal in our assay conditions, but were observed on glucose, xylose, lactose, and other soluble substrates. A similar observation was obtained with *T. lanuginosus (21)*. Many factors influence the growth of molds in the form of pellets *(22)*; in this work, the influence of the pH during the culture (from 6.5 to neutral pH when xylanases are produced and from 6.5 to lower pH in other cases), the growth rate and the presence of solid particles are as many parameters that could explain the growth of the mold in the form of pellets or mycelium.

Xylanase Production in Lab-Scale Fermenter

Growth of *P. canescens 10-10c* on wheat straw (3% w/v), soya meal (3% w/v), and yeast extract (0.5% w/v) in a 20-L fermenter developed a high viscosity broth: this led to a drastic decrease of the value of Kla from 300 down to 37 h^{-1}. We concluded that the oxygen transfer was insufficient and was the limiting factor of the culture.

The production of xylanase was not growth-linked; the xylanase accumulation in the medium began after the exponential growth (revealed by the pO_2 evolution) at a lower mycelium growth rate. During xylanase production, the morphology of the hyphae changed: they became thinner and less branched, the respiratory quotient decreased down to 0.8. After 6 d of culture, the production of xylanase still took place, at a constant rate of 6.0 U/mL/h.

Many molds have a nongrowth-associated xylanase production, and it seems that an increase in biomasse would offer a better xylanase yield. Toward this aim, studies are made to improve the oxygen transfer. Since many filamentous microorganisms present a high sensibility to hydrodynamic stress, increasing the agitation speed is not always an appropriate solution, and a current hypothesis to explain lower xylanase production in

fermenter than in shake flask is the lower shear forces that take place in this second case *(6,20,21)*.

In our study, xylanase yield was higher in fermenter than in shake flask; we explain this by a better mixing and oxygen transfer in fermenter. The highest xylanase yield was obtained with the fed-batch culture (1050 U/mL) and the maximum xylanase production rate was 10 U/mL/h just after the first medium addition.

Better yield in fed-batch culture than in batch culture can be attributed to the improved oxygen transfer and to the better supply of the mycelium in substrates. The improvement of the oxygen transfer was owing to the reduction of the broth viscosity subsequently to the addition of medium. That is why the first addition of medium was applied in the first stage of the culture, at a critical moment that revealed change in the metabolism of the mycelium. Such a change in the metabolism was deducted from visual observations (the hyphae became thinner), but also from the respiratory quotient and xylanase accumulation.

The use of a hub-mounted pitched blade turbine (TPI8) instead of a disk-mounted blade turbine (TD4) enabled a better xylanase production rate (7.0 U/mL h against 6.0 U/mL/h, respectively). It seems that lowering shear conditions is beneficial for mycelium and xylanase production. This assumption was confirmed by batch cultures at different agitation speeds: *P. canescens* was affected by hydrodynamic stress. Many authors reported a similar influence of shear forces and hydrodynamic stress on filamentous microorganisms *(23–26)*.

On one hand, the volumetric power dissipation was greater with a TD4 than with a TPI8, all other conditions remaining unchanged. This means that turbulence in the culture broth was greater when the mixing was accomplished by a TD4. Since the power imput could be correlated to shear and to the global mass transfer coefficient (Kla), the use of a TPI8 impeller would have produced lower Kla values; this was not observed. This was attributed to the fact that the Kla values were too low during the TD4 culture to observe a significant decrease of the Kla during the TPI8 culture. Thus, by using a TPI8 impeller, the positive result obtained by less hydrodynamic stress of the mycelia seemed to be more important than the negative effect owing to lower oxygen transfer.

CONCLUSION

Penicillium canescens 10-10c is a high-level xylanase-producing mutant. The xylanase complex is cellulase-free, and has its optimum pH and temperature activity at pH 4.6–5.0 and 55–60°C.

The culture needs an improved oxygen transfer without excessive shear of the hyphae. Solutions must be found to reach these two opposite aims. We propose to modify the turbine design and to use fed-batch culture.

The turbine will be designed to reduce shear stress without loss of oxygen transfer capacity. The fed-batch will be carried out to improve the substrates' supply and the mass transfers.

In our laboratory, further studies will determine the influence of mixing conditions on biomass and xylanase production by *P. canescens 10-10c*.

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