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Optimizing xylanase production by a newly isolated strain CAU44 of the thermophile *Thermomyces lanuginosus*

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Summary

Thermomyces lanuginosus CAU44, a newly isolated thermophilic fungus strain, was used for the production of extracellular xylanase on various lignocellulosic materials under shake flask conditions. High-level production of xylanase by the strain was enhanced by optimizing the type of carbon sources, substrate concentration, particle size and surfactants in the culture medium. The titre of xylanase activity obtained of up to 4156 U ml^{-1} was the highest ever reported.

Introduction

Xylanases (EC 3.2.1.8) have many different industrial applications, including biodegradation of lignocelluloses in animal feed, foods, and textiles, as well as biopulping in the paper and pulp industry (Wong *et al.* 1988). Although xylanases are produced by many microorganisms such as bacteria, fungi and actinomycetes, fungi have received great attention due to the their ability to secrete high level of xylanases into the growth medium. The majority of commercial xylanases are extracellular enzymes produced by mesophilic fungi (Haltrich *et al.* 1996). Since the use of xylanases is related to industrial processes operating at high temperatures, application of thermostable enzymes produced by thermophilic fungi appears to be advantageous (Maheshwari *et al.* 2000). Among thermophilic fungi, *Thermomyces lanuginosus* is one of the best producers of thermostable xylanases from the industrial point of view, mainly due to the fact that it excretes a high level of xylanase into the medium (Singh *et al.* 2003). Some work has already been carried out to study the efficient production of xylanases from this fungus (Gomes *et al.* 1993a, b; Alam *et al.* 1994; Hoq & Deckwer 1995; Puchart *et al.* 1999; Singh *et al.* 2000), and different surfactants (Tween-80) or fatty acids are frequently added to the medium to enhance the yields of xylanases (Gomes *et al.* 1993b; Haltrich *et al.* 1996; Kuhad *et al.* 1998; Ding *et al.* 2004).

T. lanuginosus is reported to be among the best producers of thermostable xylanase in nature. However, differences in xylanase production amongst strains from diverse geographical origin do exist (Hoq & Deckwer 1995; Puchart *et al.* 1999; Singh *et al.* 2003). Hence, the main purpose of the present study was to enhance and accelerate the high-level xylanase production by strain CAU44 of *T. lanuginosus* newly isolated in China. In the present investigation, a successful attempt was made to enhance the xylanase yield from *T. lanuginosus* CAU44 by optimizing the culture conditions. We have achieved a two-fold increase in xylanase production, which is the highest titre of xylanase activity ever reported.

Materials and methods

Materials

Birchwood xylan, beechwood xylan, oat-spelt xylan and carboxymethylcellulose (low viscosity) were purchased from Sigma Chemical Company, St. Louis, MO, USA. The lignocellulosic materials, namely corncobs, corn straw, wheat straw, wheat bran, sugar cane bagasse, rice straw and rice husk, were obtained locally. Corncob xylan was prepared as described previously (Ding *et al.* 2004). Tryptone and yeast extract were products from Oxoid (Basingstoke, Hampshire, England). All other chemicals used were analytical grade reagents unless otherwise stated.

Fungal strains and growth conditions

Thermomyces lanuginosus CAU44 was isolated from the soil samples obtained from earth under decaying tree

fibre layers in Sinkiang Province, China and was identified by the Institute of Microbiology of the Chinese Academy of Sciences (IMCAS). Stock cultures were maintained on potato dextrose-agar (PDA) slopes stored at 4 °C.

For xylanase production, the basal medium of flask culture contained (g l^{-1}): corncob particles, 30; yeast extract, 10; tryptone, 10; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4$, 0.3; $CaCl_2$, 0.3. The initial pH of the medium was adjusted to 6.0 and was not further controlled. The medium was then sterilized at 121 °C for 15 min. An agar block (1.0 cm^2) of an actively growing 5-day-old culture of the strain was used to inoculate the growth medium (100 ml) in 300 ml Erlenmeyer flasks. Triplicate cultures were shaken at 200 rev min^{-1} at 50 °C. After 4 days of cultivation, each culture broth was centrifuged ($10{,}000 \times g$) for 15 min, and the supernatants were examined for xylanase activity.

Enzyme assay and protein determination

Xylanase activity was assayed according to the method of Bailey *et al.* (1992). Reaction mixture containing 0.9 ml of 1.0% (w/v) birchwood xylan and 0.1 ml of a suitably diluted enzyme solution was incubated in 0.05 mol l^{-1}, pH 6.0 citrate-phosphate buffer at 50 °C for 10 min. The reaction was stopped by adding 1 ml DNS (dinitrosalicylic acid). The amount of reducing sugar liberated was determined by the DNS method using xylose (Sigma) as the standard. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute. A similar method was used to assay CMCase by using 1.0% (w/v) carboxymethylcellulose as the substrate and D-glucose as the standard. Protein concentrations were measured by the Lowry method with BSA (bovine serum albumin) as the standard. All assays were conducted in triplicate from three independent samples.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels as described by Laemmli (1970). Protein bands were visualized by Coomassie brilliant blue R-250 staining. The molecular weight standard used was the low molecular weight calibration kit (Amersham): phophorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

Selection of substrates for xylanase production

To investigate the effect of various substrates on the production of xylanases, *T. lanuginosus* CAU44 was cultivated for 4 days on different media with 1.5% of each xylan or 3.0% of each lignocellulosic material as the sole substrate. The initial pH of the medium was adjusted to pH 6.0. Various lignocellulosic materials were chopped in a laboratory hammer mill to a particle size smaller than 0.45 mm (40 mesh), and were used at the same particle size in the experiment.

Effect of substrate (corncob) concentration and particle size on xylanase production

Corncobs were chopped by a chopper into small pieces, dried and ground in a hammer mill. This ground material was then separated by sieves into particles of different sizes and the fraction that passed through the 0.45 mm sieve was used in the medium. Different concentrations of the corncob particles ranging from 0.5 to 6.0% (w/v) were used as corncob substitutents in the basal enzyme production medium. Corncob particles were divided into six fragments by sieving. The 0.45–0.9 mm fragment consisted of particles that passed through the 0.9 mm sieve, but not through the 0.45 mm sieve. The same procedure was used to sort out the <0.125, 0.125–0.3, 0.3–0.45, 0.45–0.9, 0.9–2, and >2 mm fragments.

Effect of surfactants and fatty acids

The effect of different surfactants (Tween-80 and Triton X-100) and fatty acid (olive oil) on xylanase production by *T. lanuginosus* CAU44 was investigated. Different Tween-80 contents ranging from 0 to 3.0% (v/v) were added to media and the enzyme production was monitored.

Results

Xylanase production with different xylans and lignocellulosic materials

The newly isolated *T. lanuginosus* CAU44 was grown on several xylans or lignocellulosic materials to determine the effects of these substrates on the production of xylanases (Table 1). Among the xylans used, corncob xylan was the best carbon source for the xylanase production, and its activity reached the highest value of 3260 U ml^{-1}. Other xylans also resulted in high levels of xylanase activities, which were more than 1500 U ml^{-1}. The use of purified xylan as a substrate for xylanase production is costly and, therefore, the current impetus is focused on the utilization of the more cost-effective lignocellulosic materials. Among the lignocellulosic materials tested as carbon sources, the highest activity (2156 U ml^{-1}) of xylanase was produced on corncobs, whereas much lower levels (25–153 U ml^{-1}) of xylanase activities were produced with corn straw, wheat straw, wheat bran, sugar cane bagasse, rice straw and rice husk (Table 1). Since corncob was by far the most effective for xylanase production, it was thus selected as the substrate for enzyme production in the following experiments.

Table 1. Xylanase activity obtained from different substrates after 4 days of cultivation of *T. lanuginosus* CAU44.

Substrate[a]	Xylanase activity[b] (U ml^{-1})
Birchwood xylan	1620 ± 73
Beechwood xylan	2147 ± 90
Oat-spelt xylan	2385 ± 116
Corncob xylan	3260 ± 128
Corncob particles (0.45–0.9 mm)	2156 ± 68
Corn straw	124 ± 4
Wheat bran	115 ± 5
Wheat straw	53 ± 2
Sugar cane bagasse	153 ± 7
Rice straw	25 ± 1
Rice husk	47 ± 2

[a] 1.5% of each xylan or 3.0% of each lignocellulosic material was used as sole substrate. Amounts of other medium compositions and culture conditions were described in the section of Materials and methods.
[b] Mean ± standard deviation, $n = 3$.

Table 2. Effect of Tween-80 on xylanase production.[a]

Tween-80	Xylanase activity (U ml^{-1}) [b]
Control	2156 ± 68
0.5	2382 ± 72
1.0	2787 ± 85
1.5	3026 ± 96
2.0	3461 ± 104
2.5	2846 ± 88
3.0	2243 ± 73

[a] *T. lanuginosus* CAU44 was cultivated in media containing 4.0% (w/v) of 0.45–0.9 mm corncob plus different Tween-80 contents ranging from 0 to 3.0% (v/v) at 50 °C for 4 days.
[b] Mean ± standard deviation, $n = 3$.

Effect of substrate (corncob) concentration and particle size on xylanase production

The effect of substrate (corncob) concentration was investigated in the range of 1.0–6.0% (w/v) (Figure 1a). The optimal corncob concentration was 4.0% because it produced the highest xylanase activity (2603 U ml^{-1}). Corncobs of different particle sizes were tested in order to determine their effects on xylanase production. It was clear that particle size affected the enzyme production (Figure 1b). The highest activity of 2723 U ml^{-1} xylanase was produced by the corncob of particle size 0.3–0.45 mm whereas lower activities were produced on the corncob of other sizes.

Effect of Tween-80 on xylanase production

The effects of different surfactants (Tween-80 and Triton X-100) and fatty acid (olive oil) on the xylanase production by *T. lanuginosus* CAU44 were investigated. Among these materials, only Tween-80 exerted a marked effect on the xylanase production (data not shown). Different Tween-80 contents ranging from 0 to 3.0% (v/v) were added to the media to study their impact on the enzyme production by *T. lanuginosus* CAU44 (Table 2). With Tween-80 increasing from 0 to 2.0% (v/v), the titre of xylanase activity was increased from 2156 U ml^{-1} to 3461 U ml^{-1}. However, as the content of Tween-80 increased further to 2.5%, the titre of xylanase activity decreased to 2846 U ml^{-1}. Addition of 2.0% (v/v) Tween-80 to the growth medium gave a 1.6-fold increase in the maximum xylanase activity compared with the control.

Effect of culture time on the xylanase production by T. lanuginosus CAU44

The time course of xylanase production by *T. lanuginosus* CAU44 under shaking conditions using the optimized medium (4.0% (w/v) of 0.3–0.45 mm corncob plus 2.0% (v/v) Tween-80) was investigated. The strain produced the highest xylanase activity (4156 U ml^{-1}) when grown on corncob at 50 °C for 4 days (Figure 2). Xylanase activity increased rapidly during the first 4 days but decreased slightly after 4 days. The increase in soluble protein content almost followed the trend of the enzyme activity, where highest soluble protein concentration in culture filtrates was found in cultures with the highest level of xylanase production. During the course of fermentation, a final pH of 8.0 or above was developed on corncob by this strain (data not shown). In this experiment, corncob

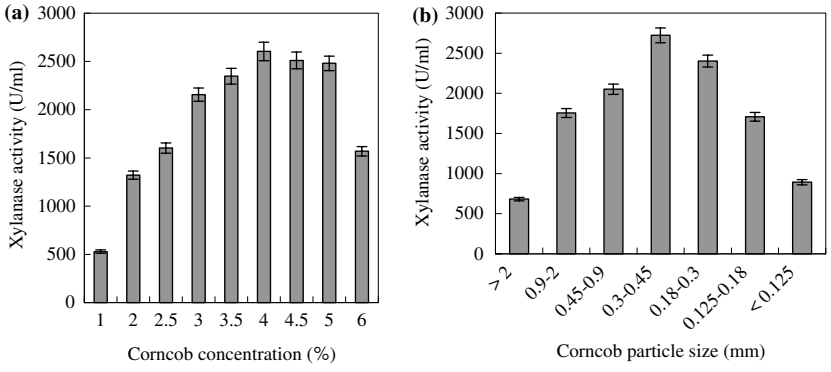

Figure 1. Effect of corncob concentration (a) and particle size (b) on xylanase production by *T. lanuginosus* CAU44. Mean ± standard deviation, $n = 3$.

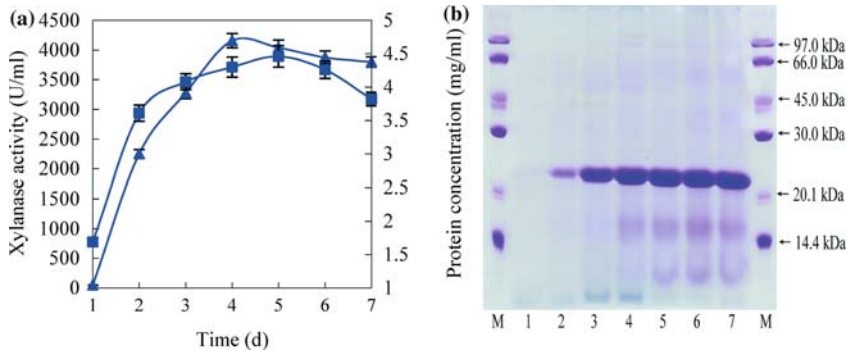

Figure 2. Time-course of xylanase production by *T. lanuginosus* CAU44 cultivated in medium containing 4.0% (w/v) of 0.3–0.45 mm corncob particles and 2.0% (v/v) Tween-80 at 50 °C. Xylanase activity (a): (▲), xylanase activity; (◆), protein content. Mean ± standard deviation, $n = 3$. SDS-PAGE (b): The same volume amounts of samples (15 μl) were analysed by SDS-PAGE on the same gel (12.5% polyacrylamide separating gel). Lanes M, low molecular weight calibration kit (Amersham) ; Lane 1, 1 day; lane 2, 2 days; lane 3, 3 days; lane 4, 4 days; lane 5, 5 days; lane 6, 6 days; lane 7, 7 days.

induced very low levels of cellulolytic activity (equal to or lower than 1.36 U $ml^{-1}$and 0.05 U ml^{-1} for CMCase and Avicelase, respectively). The CMCase and Avicelase activities were marginal (data not shown).

Discussion

Thermomyces lanuginosus is a thermophilic fungus with an exceptional ability to produce extremely high levels of xylanase (Singh *et al.* 2003). A notable variability in the production of xylanase by *T. lanuginosus* strains has been observed (Hoq & Deckwer 1995; Puchart *et al.* 1999). Compared with the level of xylanases produced by different strains of *T. lanuginosus*, the newly isolated strain CAU44 was shown to produce a thermostable xylanase with an activity of up to 4156 U ml^{-1}. This activity was higher than the activity values of 3725 U ml^{-1}, 2840 U ml^{-1} and 2600 U ml^{-1} produced by *T. lanuginosus* strains SSBP (Singh *et al.* 2003), ATCC 46882 (Bennett *et al.* 1998) and ATCC 16455 (Puchart *et al.* 1999) grown on corncobs at 50 °C, respectively. This difference in xylanase production between experiments possibly originated from the strain and culture conditions used. To our knowledge, this is the highest activity level demonstrated by a fungal producer of xylanase reported to date.

One factor that determines the large-scale use of xylanase will certainly be the cost of xylan-degrading enzyme preparation. The cost of carbon source, as well as additional medium components, plays a major role in the economics of xylanase production (Haltrich *et al.* 1996). The carbon source has a marked effect on the xylanase production in *T. lanuginosus* CAU44 (Table 1). The use of the purified xylan in large scale production is far too expensive although high-level xylanase production was obtained by this strain grown on several purified xylans. Lignocellulosic materials, such as corncobs, wheat straw, and sugar cane bagasse are less expensive sources of carbon. *T. lanuginosus* CAU44 grown on corncobs is capable of producing xylanase to a greater extent than some strains of *T. lanuginosus* (Puchart *et al.* 1999; Singh *et al.* 2003). Xylanase formation by *T. lanuginosus* strains is very complex, and is known to be controlled by an induction-repression mechanism. *T. lanuginosus* strains favoured corncobs and exhibited a wide difference of xylanase productivity on corncobs medium (Puchart *et al.* 1999). *T. lanuginosus* strain SSBP yielded the highest xylanases activity (59,600 nkat ml^{-1} or 3569 U ml^{-1}) when growth on coarse corncobs pretreated with autoclaving at 121 °C for 15 min (Singh *et al.* 2000). The necessary pretreatment (autoclaving) of raw material also adds to the cost of production. However, substrate pretreatment exerted only marginal or even negative effect on the level of xylanases formed with many *T. lanuginosus* strains when corncob was used as the inducing substrate (Gomes *et al.* 1993a; Purkarthofer *et al.* 1993).

The highest xylanase level produced by *T. lanuginosus* on corncobs apparently is related to the particulate size of the substrate. Many researchers reported that a particle size of 2–7 mm corncob was optimal, whereas the same substrate used as a fine powder resulted in a significant decrease in xylanase activity (Purkarthofer *et al.* 1993; Bennett *et al.* 1998; Puchart *et al.* 1999; Singh *et al.* 2000). However, the level of xylanase produced by *T. lanuginosus* CAU44 on fine corncobs (0.3–0.45 mm) was approximately three-fold higher than that produced on coarse corncob (> 2 mm) in this study. Further reduction of particle size of corncobs, which were used for the production of the xylanase, from 0.3–0.45 to < 0.18 mm had a negative effect on the enzyme yields obtained. These findings confirm the relevant data reported by others who found that the size of corncob was an important factor for extracellular xylanase production by *T. lanuginosus* (Purkarthofer *et al.* 1993; Singh *et al.* 2003). The observation agrees with the results of Hoq & Deckwer (1995) using *T. lanuginosus* RT9 grown on corncobs, where lower particle sizes resulted in higher xylanase production although coarse corncob is found to be the most effective substrate for xylanase production by many *T. lanuginosus* strains (Puchart *et al.* 1999).

So far, few reports of the effect of Tween-80 on xylanase production by *T. lanuginosus* have appeared. It is found that Tween-80 had little or no influence on xylanase production by *T. lanuginosus* DSM 5826 (Gomes *et al*. 1993b). Another finding of ours indicates that Tween-80 stimulated the xylanase synthesis in *T. lanuginosus* CAU44 and enhanced xylanase production by 1.6-fold. The other surfactants or fatty acid did not have significant effects on xylanase production (data not shown). In previous studies (Haltrich *et al*. 1996; Kuhad *et al*. 1998; Ding *et al*. 2004), xylanase synthesis in certain microorganisms was significantly stimulated by surfactants such as Tween-80. It is assumed that Tween-80 increases the permeability of cell membranes and thus affects the secretion of certain proteins, although the exact mechanisms of action have not been determined (Haltrich *et al*. 1996).

In short, the results reported herein indicate that the new strain CAU44 of *T. lanuginosus* can produce very high-level xylanase using available agricultural wastes, such as corncob particles, as carbon sources under optimized conditions.

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