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Full Length Research Paper

A comparative study of *Thermomyces lanuginosus* strains on thermostable xylanase production

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Strains of *Thermomyces lanuginosus* could be differentiated into two groups based on their ability to produce xylanase using xylan or xylose as sole of carbon source. One group of these strains produced high xylanase activity either in the medium using xylan or xylose as a sole of carbon source. The xylanase production by *T. lanuginosus* ATCC 44008, THKU-11, and THKU-25, which were the representative members of this group, increased when xylose was added to the xylan medium. In contrast, there was another group that produced high xylanase activity only in the xylan medium. Addition of xylose to the xylan medium resulted decreasing of xylanase production in *T. lanuginosus* ATCC 46882, TISTR 3465 and THKU-85 that belonged to this group. The finding indicated that induction and repression mechanism of xylose on xylanase expression was different among the strains of *T. lanuginosus*. In addition, phylogenetic analysis obtained from random amplified polymorphic DNA (RAPD) pattern using primer UBC 241 pointed to greater diversity of high and low xylanase producing strains using xylose as a carbon source.

Key words: *Thermomyces lanuginosus*, RAPD, thermostable xylanase.

INTRODUCTION

β-Xylanase (Endo 1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is one of the important enzymes involved in xylan degradation working synergistically with other disbranching enzymes (Yasui et al., 1984; Hrmova et al., 1991; Ghosh et al., 1994). Numerous bacteria, streptomycetes and fungi can degrade xylan by producing a range of enzymes. *Thermomyces lanuginosus* is a potent thermophilic strain, which produces a single cellulose free endo-β-xylanase (Kitpreechavanich et al., 1984; Anand et al., 1990; Singh et al., 2000a, 2003). Xylan, xylose, xylobiose and non-metabolizable β-xylosides known to be common inducers of xylanase produced by this fungus (Purkarthofer and Steiner, 1995). In a previous study, xylanase of *Humicola lanuginosa* (Griffon and Maublanc) Bunce which was deposited at MIRCEN Bangkok culture collection, Thailand with name *T. lanuginosus* TISTR 3645, was produced at high level when xylan was used as a carbon source whereas very low activity was obtained with xylose as a carbon source; its activity was at similar level to that of glucose, galactose, cellobiose and arabinose et al., 2001). Hoq et al. (1994) also reported that low xylanase production of *T. lanuginosus* RT9 was found when use xylose was used as a carbon source. In contrast to those, *T. lanuginosus* DSM5826 (Purkarthofer and Striner, 1995) and DSM 10635 (Xiong et al., 2004) produced high levels xylanase with xylose as a carbon source. It is still doubtful that this fungus may

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possess different induction-repression systems. Several strains of *T. lanuginosus* were previously extensively isolated and screened for high xylanase production (Khucharoenphaisan and Kitpreechavanich, 2004).

In this study, the ability on xylanase production in the medium using ether xylan or xylose by different strains of *T. lanuginosus*, which were mostly-locally isolated, was studied. The effect of xylose on xylanase synthesis by the growing cell of selected isolates of *T. lanuginosus* was investigated to explain the induction-repression mechanisms. In addition, RAPD analysis proved to be a suitable method to distinguish between the high and low xylanase producing *T. lanuginosus* (Singh et al., 2000b). Moreover, determination of their differentiation among the strains inducing and repressing xylanase production by xylose using RAPD technique was also investigated.

MATERIALS AND METHODS

Microorganism and cultivation

Eighty-seven locally isolated *T. lanuginosus* strains which were identified according to the manual of fungal taxonomy (Cooney and Emerson, 1964; Domsch et al., 1993; Salar et al., 2007). Some of these were confirmed by their internal transcribed spacer (ITS) regions sequence (Boonlue et al., 2003). *T. lanuginosus* ATCC 46882 and ATCC 44008 purchased from The American Type Culture Collection (ATCC) and *T. lanuginosus* TISTR 3465 deposited at MIRCEN Bangkok culture collection, Thailand were also used in this study. The basal medium for xylanase production consisted of (per liter of distilled water) 2.0 g KH_2PO_4, 0.3 g $MgSO_4$.$7H_2O$, 0.3 g $CaCl_2$, 5.0 g peptone, 3.0 g yeast extract; and 10 g carbon source. The initial pH of medium was adjusted to 6.0, and autoclaved for 20 min at 121°C. Two agar blocks (2 mm) from 3-day-old colony of each *T. lanuginosus* strain grown on yeast glucose agar plate was inoculated into 15 ml cultured medium in 50 ml of Erlenmeyer flasks and then incubated continuously on a reciprocal shaker at 120 rpm (JEIO TECH, model BS-30) and 45°C.

Effects of xylan and xylose on xylanase production by strains of *T. lanuginosus*

Each strain was grown in the medium using either oat spelt xylan or xylose as carbon source for 5 days. The clear supernatant obtained from filtration of culture broth through Whatman No. 1 filter paper (Whatman International Ltd., USA) was used for determination of xylanase activity. Six selected strains of *T. lanuginosus* were grown in the medium containing xylan or xylose at concentration of 0.1, 0.5 and 1% as a carbon source.

Effect of xylose addition on xylanase production by selected strains of *T. lanuginosus*

The selected strains of *T. lanuginosus* ATCC 44008, ATCC 46882, TISTR 3465, THKU-11, THKU-25, and THKU-85 were grown in the medium containing 1% xylan as a carbon source. Then, syrup of xylose or glucose was added to the 3rd day xylan grown culture to make up 10 g/l and further incubated. The supernatants of each culture were periodically sampled for determination of β-xylanase activity.

These strains were also grown in the medium containing 1% xylan as a carbon source. Then, syrup of xylose was added to the 3rd day xylan grown culture to make up 1, 2.5, 5, 10, 20 g/l and further shaken at 120 rpm and incubated at 45°C for 5 days. Each culture was sampled for determination of xylanase activity.

Determination of β-xylanase activity

β-Xylanase was assayed by determination of reducing sugars liberated from oat spelt xylan when incubated with the diluted enzyme solutions at pH 6.0 and 50°C for 10 min. The reducing sugars were measured using the DNS reagent (3,5-dinitrosalisylic acid) (Miller, 1959). One unit of β-xylanase activity was defined as the amount of enzyme that produced 1 µmole of xylose in 1 min.

Random amplified polymorphic DNA (RAPD) analysis

Genomic DNA from *T. lanuginosus* strains was isolated using the method of Boonlue et al. (2003). Three primers used in this study were UBC 235(5'-CTGAGGCAAA-3), UBC 241(5'-GCCCGACGCG-3) and UBC 280 (5'-CTGGGAGTGG-3). A standard PCR procedure was used to amplify genomic DNA (Williams et al., 1990). The PCR reaction volume of 10 ml contained 0.3 mM primer, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 50 ng of genomic DNA, separate 0.2 U of Blend Taq Plus DNA polymerase (Life Science Department, Toyobo Co., LTD, Japan) and recombinant Taq DNA polymerase (Life Science Department, Toyobo Co., LTD, Japan) and 3.0 -3.5 mM $MgCl_2$ in 1X PCR buffer. Reaction were performed using an automated thermocycler (Takara PCR Thermal Cycler Dice). PCR was performed with initial denaturation for 2 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 34°C, and elongation for 2 min at 68°C (for Blend Taq Plus DNA polymerase) or 72°C (for recombinant Taq DNA polymerase). A final elongation step of 5 min at 72°C was included. PCR products were analyzed by electrophoresis in 2% agarose gels consisting 0.0005 mg/ml of ethidium bromide, in 0.5X Tris-borate-EDTA (TBE) and banded patterns were analyzed using the Gel Documentation 2000 System (Bio-Rad, US).

Dendogram construction

Polymorphic bands were identified using electrophoresis imaging. The bands were scored as present (1) or absent (0). The pair-wise evolutionary distances are given by generating the distance matrix. Pair of samples were chosen and clustered with the minimum distance between two samples. The branching point was positioned at a distance of: number of distance/number of sample = distance of branch. Then these two samples were joined as one new node. Distance from these nodes to other node were calculated by using the equation (Opperdoes, 1997):

$$d(c,k) = \frac{d(i,k)+d(j,k)}{2} ; c = \{i,j\}$$

Following the first clustering, i and j are considered as a single cluster, and the new distance matrix was calculate as mention above. Column and line in the distance matrix that contains first 2 samples were erased and the calculation was then repeated for the last one row and column.

RESULTS AND DISCUSSION

Effects of xylan or xylose on xylanase production by different strains of *T. lanuginosus*

Xylanase production by 90 strains of *T. lanuginosus* in medium using either oat spelt xylan and xylose as an energy source and carbon source was diverse depending on the strains. It was found that 18 isolates of *T. lanuginosus* produced xylanase activity in a range of 100 - 134 units/ml in the xylan medium whereas 8 isolates produced xylanase activity in a range of 69.1 - 95.5 units/ml in the xylose medium as shown in Table 1. There was a group of isolates producing high xylanase either in the xylan or xylose medium. The representative isolates of this group were THKU-11, THKU-25 and ATCC 44008. In contrast to this group, there was another group of isolates that were able to produce high xylanase only in the xylan medium but less activity was detected in the xylose medium. The representative isolates were TISTR 3465, THKU-85 and ATCC 46882. The representative isolates of each group were cultivated in the medium containing either oat spelt xylan or xylose as a carbon sources at concentrations of 0.1, 0.5 and 1%. It was found that xylanase production by all the isolates were positively affected by xylan. The activities increased when xylan concentration was increased (Table 2). A little xylanase activity was produced by strains of TISTR 3465, THKU-85 and ATCC 46882 in xylose medium regardless of xylose concentration.

T. lanuginosus has been reported to be good producer of xylanase when grown on xylan (Singh et al., 2003). Moreover, the significant differentiation on xylanase production levels occurred when each strain was grown in the medium having xylan and corncob as a carbon source (Puchart et al., 1999; Singh et al., 2000b). Singh et al. (2000c) reported that xylanase levels produced by *T. lanuginosus* SSBP using xylose as a carbon source were lesser than using corncob.

Effect of xylose addition on xylanase production by the selected strains of *T. lanuginosus*

Xylanase production by the selected isolates of *T. lanuginosus* rapidly increased within 4-day cultivation and then became slightly constant. Addition of xylose or glucose to the 3rd day of xylan-grown culture of *T. lanuginosus* TISTR 3465, THKU-85 and ATCC 46882 decreased xylanase production as shown in Figure 1A-C. TISTR 3465 was the strain that xylose had the strongest repression effect on xylanase production. This was correlation to very low xylanase activity when the culture grew on xylose medium. In contrast, addition of xylose to the xylan-grown cultures of THKU-11, THKU-25 and ATCC 44008 did not show any repression effect on xylanase formation whereas addition of glucose deeply decreased the activity (Figure 1D - F). Addition of xylose into xylan-grown cultures at the 3rd day cultivation of TISTR 3465, THKU-85 and ATCC 46882 effectively decreased β-xylanase production as concentrations of xylose were increased (Figure 2). This finding was similar to the report of Hoq et al. (1994) who observed low xylanase expression of *T. lanuginosus* RT9 in the xylose medium. Nevertheless, strains THKU-11, THKU-25 and ATCC 44008 xylanase gradually increased when the added xylose increased. This indicated that xylanase formation by these strains might be induced by xylose (Figure 2). This indicates that no catabolic repression caused by xylose occurred. However, regulation of xylanase production from *T. lanuginosus* DSM5826 and DSM 10635 were induced by the presence of xylose as previously reported by Purkarthofer and Striner (1995) and Xiong et al. (2004). The rate of xylose uptake into the cell may be of consideration on the effect of xylose on the regulation of xylanase synthesis.

Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis of the selected *T. lanuginosus* strains using Blend Taq Plus DNA polymerase and primer UBC 241 distinguished the relationships among the strains into seven groups and three different clusters as shown in Figure 3. Interestingly, the dendogram was able to distinguish between high-level xylanase producing strains and low-level xylanase producing strains cultured in the medium using xylose as a sole of carbon source. For cluster A, *T. lanuginosus* strains THKU-56, ATCC 44008, THKU-30, THKU-33, THKU-49, THKU-86 and THKU-2 except THKU-77 that produced moderate amount of xylanase activity were apparently closely related. While cluster B, strains THKU-25, THKU-11, THKU-9 and THKU-4 that produced xylanase of 69.2, 95.5, 69.1 and 67.8 U/ml, respectively, also showed a close relationship (the exception is strain TISTR 3465 which also produced low level of xylanase). This indicated that RAPD analysis have ambiguous separation. In contrast to those, the dendogram could not distinguished between each cluster of strains producing high and low level of xylanase in the xylan medium (Figure 3). Singh et al. (2000b) reported that RAPD analysis with primer UBC 241 and *Taq* DNA polymerase could distinguish between eight *T. lanuginosus* that produced high and low xylanase by using corncob as a carbon source. Saldanha et al. (2007) compared RAPD pattern using 8 primers; OPW2, OPW4, OPW5, OPW12, OPW14, OPX9, OPX13, OPX14 (Operon Technologies, Alameda, USA) to differentiate the laccase production by *Botryosphaeria* isolates. The profiles showed a strong differentiation between the groups. Groups I and II showed high levels of production of laccase ranging from 0.02 to 0.06 U/ml (non–induced)

Table 1. Xylanase production by *T. lanuginosus* strains in medium using either xylan or xylose as a sole of carbon sources at 5th day cultivation.

Strain	Xylanase activity (U/ml)[a]	
	Xylan medium	Xylose medium
ATCC 44008	100	40.7
ATCC 46882	100	6.4
TISTR 3465	95	0.2
THKU-2	102	53.0
THKU-4	101	67.8
THKU-5	106	58.4
THKU-6	105	77.1
THKU-8	108	67.9
THKU-9	51	69.1
THKU-10	103	73.9
THKU-11	107	95.5
THKU-12	106	34.4
THKU-21	100	66.8
THKU-22	101	74.6
THKU-25	102	69.2
THKU-26	101	63.0
THKU-27	95	78.0
THKU-28	89	70.1
THKU-30	28	32.6
THKU-33	96	28.5
THKU-49	57	44.4
THKU-56	56	36.3
THKU-77	60	64.3
THKU-83	112	9.0
THKU-84	106	68.0
THKU-85	76	8.1
THKU-86	134	48.8
THKU-88	113	9.1

[a]Mean value of triple assays

Table 2. Xylanase production by *T. lanuginosus* strains on the medium using either xylan or xylose as a carbon sources at various concentrations at 5th day cultivation.

Strain	Xylanase activity (U/ml)[a]					
	Xylan concentration			Xylose concentration		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
ATCC 44008	43.4	68.9	100.2	0.0	26.7	40.7
THKU-11	42.4	72.4	107.4	0.0	24.9	95.0
THKU-25	44.5	61.8	102.0	0.0	11.9	69.0
ATCC 46882	58.5	86.7	99.9	0.0	5.1	6.4
TISTR 3465	34.4	53.8	94.7	0.0	0.0	0.2
THKU-85	43.4	68.9	100.2	0.0	2.7	8.1

[a]Mean value of triple assays

and 1.04 to 5.30 U/ml (induced), while Group III showed significant lower production levels of the enzyme. The RAPD banding pattern produced by primer OPA-11 (CAATCGCCGT) was polymorphic between low and high xylanase producing strains of *Penicillium* sp. (Bakri et al., 2007). This observation may prove useful in the further

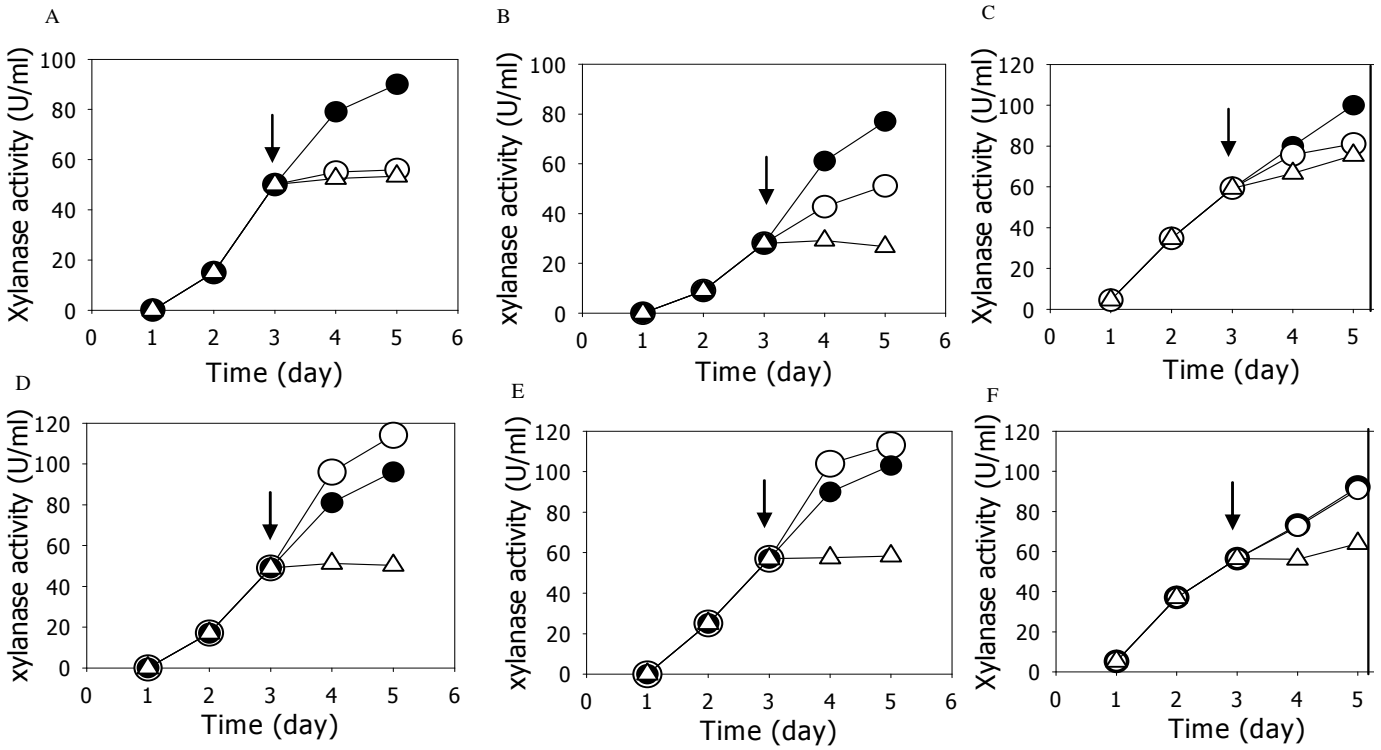

Figure 1. Effect of xylose or glucose addition to the 3rd day xylan-grown culture on the xylanase production by *T. lanuginosus* strains at 5-day cultivation of TISTR 3465 (A), THKU-85 (B), ATCC 46882 (C), THKU-11 (D), THKU-25 (E), and ATCC 44008 (F). Xylan grown culture (●), 1% xylose added to xylan-grown culture (○), 1% glucose added to xylan-grown culture (△). Arrow indicated the addition of xylose or glucose.

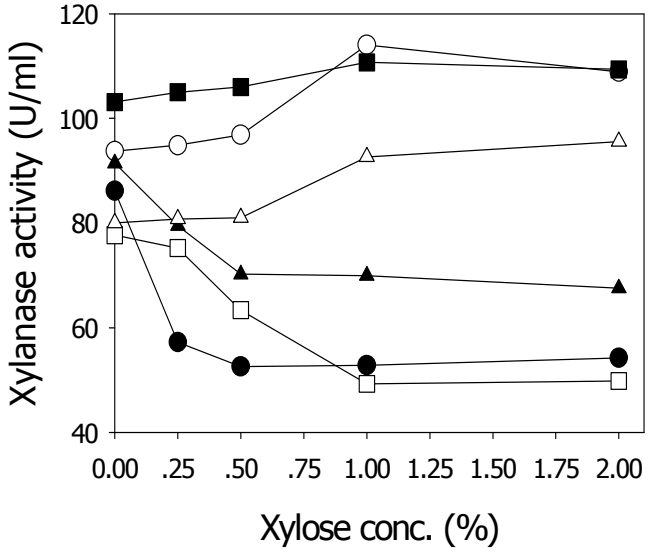

Figure 2. Effect of addition of xylose at various concentration to the 3rd day xylan-grown culture on the xylanase production by *T. lanuginosus* strains at 5-day cultivation of TISTR 3465 (●), THKU-85 (□), ATCC 46882 (▲), THKU-11 (○), THKU-25 (■) and ATCC 44008 (△) at 5-day cultivation.

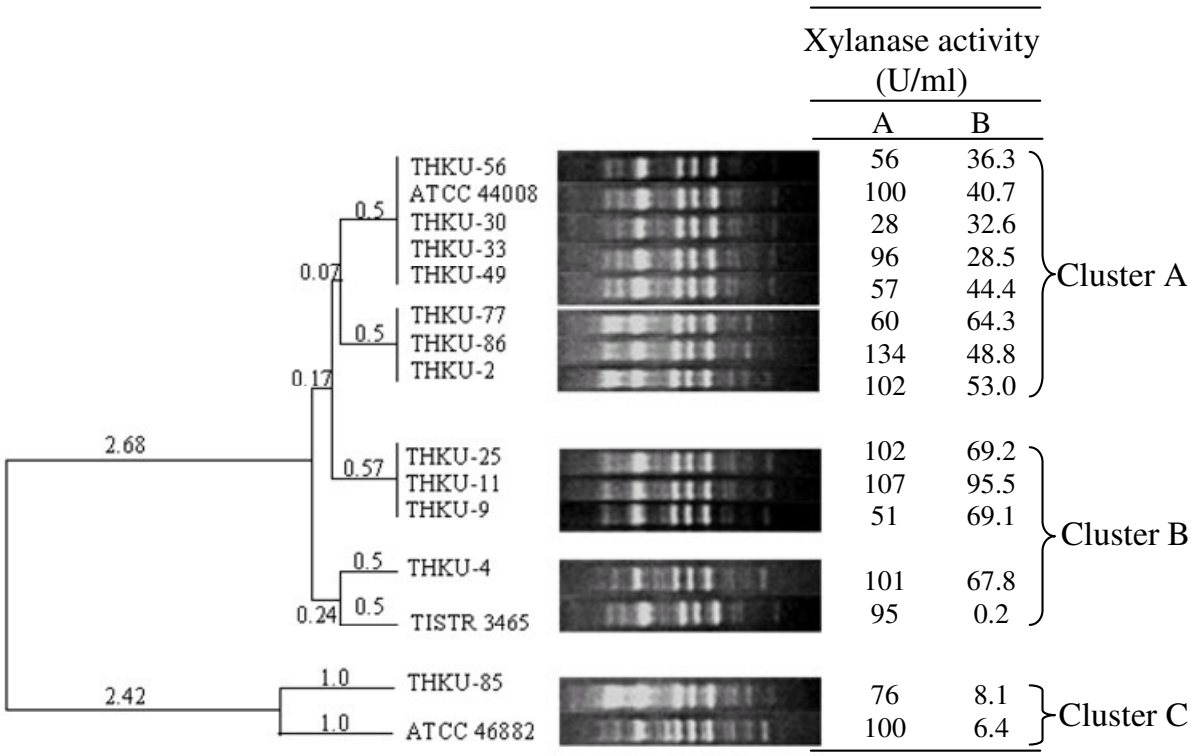

Figure 3. Dendogram indicating relationships of *T. lanuginosus* strains obtained with the primer UBC 241 with xylanase activity obtained from 5-day cultivation using xylan (A) and xylose (B) as a sole of carbon source.

characterization of *T. lanuginosus* strains that are hyperproducers of xylanase.

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