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Effect of xylan and rice husk on feruloyl esterase activity in *Penicillium rubens*

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ABSTRACT

Objective. Analyzed the effect of xylan and rice husk as sole sources of carbon on the FAE activity and expression of *faeA* and *faeB* genes of *Penicillium rubens* (Wisconsin 54-1255), in submerged fermentation. **Materials and methods.** The fermentations were carried out for 24, 48, and 72 h (28°C/250 rpm), in flasks with a modified Sakamoto medium. FAE activity was determined using the synthetic substrate Ethyl 4-hydroxy-3-methoxycinnamate. The transcription of *faeA* and *faeB* was analyzed by RT-PCR. The PCR products were resolved by electrophoresis and analyzed by densitometry. **Results.** The analysis of gene expression showed that the use of xylan had a positive effect on the expression of both genes. The highest transcriptional activity of both *faeA* and *faeB* was detected at 48 h. On the other hand, rice husk showed negative results. The data obtained when the FAE activity was determined, supported the results observed in the gene expression analysis. **Conclusion.** Different from xylan the rice husk did not demonstrate an inducer effect on the feruloyl esterase activity in *P. rubens*.

Keywords: Catabolite repression; enzyme activity; gene expression; fermentation; *Penicillium*; transcriptional activation (*Sources: MeSH, NLM, CAB*).

RESUMEN

Objetivo. Analizar el efecto del xilano y la cascarilla de arroz como únicas fuentes de carbono, sobre la actividad feruloil esterasa y la expresión de los genes *faeA* y *faeB* de *Penicillium rubens* (Wisconsin 54-1255), en fermentación sumergida. **Materiales y métodos.** Las fermentaciones se realizaron durante 24, 48 y 72 h (28°C/250 rpm), en matraces con medio Sakamoto modificado. La actividad FAE se determinó usando el sustrato sintético Etil 4-hidroxi-3-metoxicinamato. La transcripción de *faeA* y *faeB* fue determinada por RT-PCR. Los productos de PCR fueron resueltos por electroforesis en gel y se analizaron por densitometría. **Resultados.** El análisis de la expresión génica evidenció que el uso del xilano tuvo un efecto positivo en la expresión de ambos genes. La mayor actividad transcripcional, tanto de *faeA* como de *faeB*, se observó a las 48 h. Por su parte, la cascarilla de

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arroz produjo resultados negativos. Los datos obtenidos al determinar la actividad feruloil esterasa, respaldaron los resultados observados en el análisis de expresión génica. **Conclusión.** A diferencia del xilano, la cascarilla de arroz no demostró un efecto inductor sobre la actividad feruloil esterasa en *P. rubens*.

Palabras clave: Represión catabólica; actividad enzimática; expresión génica; fermentación; *Penicillium*; activación transcripcional (*Fuentes: MeSH, NLM, CAB*).

INTRODUCTION

Feruloyl esterases (EC. 3.1.1.73) belong to the carboxylic ester hydrolases and are the key enzymes in the ester bonds excision between the hydroxycinnamic acid and the galactose or arabinoxylans in hemicellulose. This group of esterases is also known for its acronym: FAE (Ferulic acid esterases). The hydrolysis of the ester bonds in plant cell walls using FAE enzymes, allows polysaccharides and different phenolic compounds such as hydroxycinnamic acids to be released easily (1). Hydroxycinnamic acids are high-value chemicals due to their various biological properties, of which ferulic acid is the most abundant and widely distributed (2). Many studies have reported on the applications of ferulic acid in the food, biomedical, and pharmaceutical industries due to their antioxidant, antimicrobial, antiviral, anticarcinogenic, and anti-inflammatory properties (2,3). Thus, the use of FAEs enzymes is very important in different biotechnological processes such as enzymatic hydrolysis for the bioethanol production, the biological delignification of plants for the paper production, the hydroxycinnamic acid esterification as well as the digestibility and recovery increase of vegetable residues derived from agroindustry (1,3).

The feruloyl esterase production from microorganisms and its biotechnology application has been an object of investigation in the last years. Currently, filamentous fungi are the microorganism group most employed in the searching and production of these enzymes. However, the amount of fungal sequences deposited in the databases is still small (4). In some cases, the enzymatic activity is reported and even the enzymes are purified without knowing the sequences of the genes that encode them. This is the *Penicillium rubens* situation (classified before as *P. chrysogenum*) one of the most valuable fungi for the biotechnology industry (5). Fortunately, the public character of the fungal genomes has facilitated the search for coding sequences of feruloyl esterases through genome mining. After having a search based

on similarity in the *P. rubens* (Wisconsin 54-1255) genome, two coding sequences feruloyl esterases were found and denominated *faeA* and *faeB*. It was found that *faeA* contains an open reading frame (ORF-Pc20g07010) of 933 bp, with a single intron (89 bp), which codes for a 280 amino acids protein called PrFaeA (CAP86030). On the other hand, *faeB* includes an open reading frame (ORF-Pc12g08300) of 1643 pb, with a 58 pb intron, that encodes for a 527 amino acids protein denominated PrFaeB (CAP80457). Both fungal proteins were bioinformatically verified, determining that they belong to type A and B FAEs, respectively.

Taking into account that the husk is one of the by-products generated in a large quantity during the rice production and rich in hemicelluloses and phenolic compounds (6) it has been considered to evaluate its use as a substrate in the production of FAEs enzymes by *P. rubens* (Wisconsin 54-1255) under submerged fermentation conditions. Thus, this work aimed to analyze the rice husk effect on the enzymatic activity feruloyl esterase and the transcription of the coding genes for feruloyl esterases PrFaeA and PrFaeB, in comparison with the xylan use as an only carbon source.

MATERIALS AND METHODS

Fermentation. The culture medium described by Sakamoto et al (10), with modifications (NH₄NO₃: 2 gl⁻¹; K₂HPO₄: 1 gl⁻¹; MgSO₄·7 H₂O: 0.5 gl⁻¹; KCl: 0.5 gl⁻¹; FeSO₄: 0.01 gl⁻¹; Soybean peptone: 1 gl⁻¹; rice husk: 20 gl⁻¹; distilled water) was taken into account. The rice husk was subjected to a grinding and screening pretreatment to be subsequently subjected to autoclave solubilization by thermal steam treatment. The medium was called RHS (rice-husk-sakamoto). To prepare the second culture medium, the rice husk was replaced by xylan (2%). This medium was denominated XS (xylan-sakamoto). For the determination of enzymatic activity, the fermentations were carried out for

24, 48, and 72 h, at 28°C and 250 rpm, in 250 ml flasks with 50 ml of medium. In order to obtain the enzyme extracts, the content of each flask was processed as described by Sakamoto et al (7). The amount of protein present in each extract was measured using the Bradford method. The FAE activity was determined by using the synthetic substrate Ethyl 4-hydroxy-3-methoxycinnamate (Sigma-Aldrich) following the procedure suggested by Sakamoto et al (7). For these experiments, U is described as the amount of enzyme that is capable of releasing 1 µmol of AF in a time of 1 minute.

Analysis of gene expression. Fermentations were carried out for 72 h, to 28°C and 250 rpm, in 500 ml flasks with 100 ml of medium. Mycelium samples were taken from each medium at the 24 h, 48 h, and 72 h. The DNA and RNA extractions were performed as indicated by Gil-Durán et al (8). The transcription of *faeA*, *faeB*, and *actA* was analyzed by RT-PCR. The cDNA synthesis was carried out as indicated by García-Rico et al (9). The PCR amplification was done following these conditions: 5 min at 94°C; 30 s at 94°C; 30 s at 58°C; 30 s at 72°C (35 cycles) and 10 min at 72°C. The oligonucleotides used are listed in Table 1. The PCR products were resolved by electrophoresis. Taking into account the size of the amplicons, the GeneRuler 50 bp DNA Ladder (ThermoFisher) was used as a molecular weight marker. For the positive control, *P. rubens* genomic DNA was used as a template, while for the negative control no template was used. The densitometry analysis was performed using the "MYImageAnalysis" software (ThermoFischer) as indicated by Torrent et al (10). The normalizing gene was *actA*.

Table 1. Primers used in RT-PCR analysis.

Target gene	Primer name	Sequence (5'→3')	Length (pb)
<i>faeA</i>	FaeA-Fw	CTCGCTCCATTTGACACCTT	100
	FaeA-Rv	ACAAGTGACTCGACCTGATCT	
<i>faeB</i>	FaeB-Fw	ACCGCCAACCTGAGGTAATC	101
	FaeB-Rv	CTTGTCGAAGCCCTCCTCAT	
<i>actA</i>	ActA-Fw	TCCCCATCTACGAGGGTTTCT	100
	ActA-Rv	CGTCCACGCAGGATCTTCAT	

RESULTS

The analysis of enzymatic activity (FAE activity) from the culture media RHS and XS, produced results that contrast each other. On the one

hand, it was observed that FAE activity in the RHS medium was null, while in the XS medium enzymatic activity was detected at all times tested. The use of xylan as the sole source of carbon induced an FAE activity whose point of the maximum activity was observed at 48 h, at which time it doubled the activity detected at 24 h (Figure 1). Enzymatic activity at 72 h ranged around 90% of what was observed at 48 h.

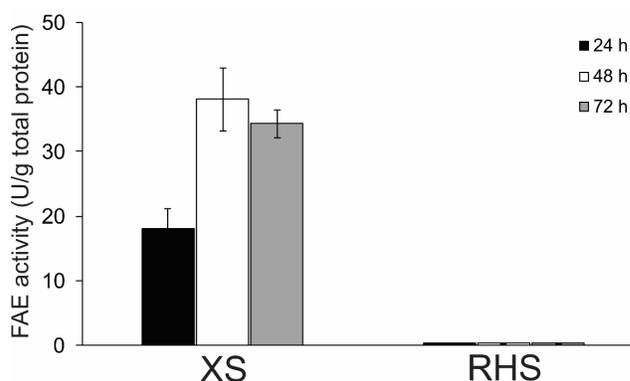


Figure 1. FAE activity of *P. rubens* enzyme extracts.

The data obtained for 24 h, 48 h, and 72 h of submerged fermentation in XS and RHS media are shown. Error bars represent the standard deviation of two replicas from two independent experiments ($p < 0.05$). (U) Unit: the amount of enzyme that is capable of releasing 1 µmol of AF in a time of 1 minute.

Similarly, the gene expression analysis indicated that, unlike rice husk, the use of xylan showed an inductor effect on gene expression of *faeA* and *faeB*. When the products of RT-PCR were resolved by electrophoresis, the amplicon of the expected size (100 bp) was only observed in the fermentation with XS medium (Figure 2). In the XS medium, the transcriptional activity of *faeA* was detected from 24 h to 72 h of fermentation, while for *faeB* the expression was clearly observed only after 48 h of culture (Figure 2B).

Analysis by densitometry showed that *faeA* reached its maximum expression level at 48 h (Figure 3). At 72 h, the relative transcription level in the mycelium remained around 70% in comparison with the transcription levels of *actA*, used as a normalizing gene due to its constitutive character. Different from *faeA*, the *faeB* expression was not detected at 24 h. However, the highest transcriptional activity was observed at 48 h. After 72 h, the *faeB* relative expression decreased to less than half of what was observed at 48 h (Figure 3).

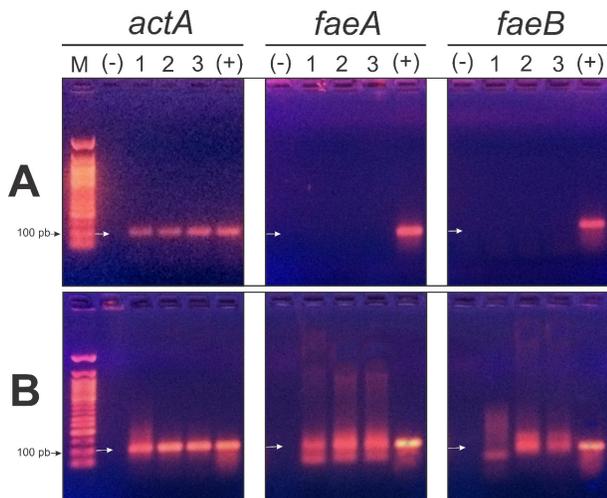


Figure 2. Profile expression of *faeA* and *faeB* with xylan and rice husk as only sources of carbon.

The RT-PCR products resolved in agarose gel (2%) are shown. **A:** Sakamoto medium modified with rice husk (RHS). **B:** Medium with xylan (XS). It is pointed for each gene, from left to right: negative control (-), 24 h (1), 48 h (2), 72 h (3), and positive control (+). M: molecular weight marker (GeneRuler 50 bp DNA Ladder). The arrow indicates the amplification of a 100 bp fragment. Positive control: the template was genomic DNA. Negative control: no template was used.

For both genes, the highest levels of expression were detected at 48 h fermentation, in accordance with what was observed in the enzyme activity tests. On the other hand, the rice husk produced opposite results (Figures 1 and 2). No transcriptional activity was detected in tested times for either of the two genes. Thus, the modified Sakamoto medium with rice husk did not work as an inducer of feruloyl esterase activity in *P. rubens*.

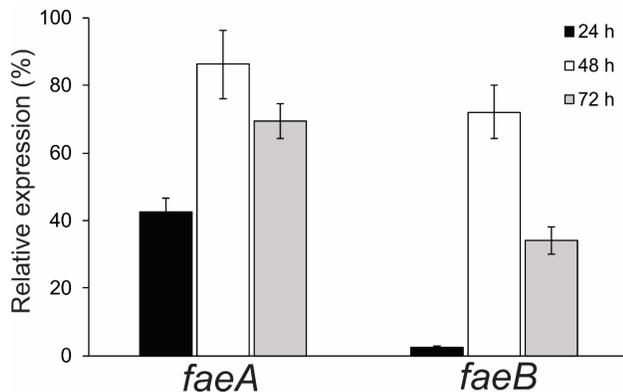


Figure 3. Effect of xylan as carbon source on transcription of *faeA* and *faeB* in *P. rubens*.

In the figure 3, shows the data obtained for 24 h, 48 h, and 72 h of submerged fermentation in XS medium is shown. The levels of *faeA* and *faeB* transcripts were quantified by densitometry. The normalizing gene was *actA*. The *faeA/actA* and *faeB/actA* ratios were expressed as a percentage (10).

DISCUSSION

In filamentous fungi, genes encoding feruloyl esterases are regulated by catabolite repression. This negative control is mediated by *CreA* through repression of *XlnR*, a transcriptional activator of xylanolytic genes (11). Our results showed that xylan had a greater inductor effect on *faeA* than on *faeB*, during the whole fermentation. This difference was important at 24 h (Figure. 2). In *Aspergillus niger*, sugars such as xylose and arabinose differentially induced the expression of *faeA* instead *faeB*, after a few hours of growth (12). The above agrees with what was observed in *P. rubens*, especially if we consider that the main product of the hydrolysis of xylan is xylose. Additionally, the xylose concentration has an impact on gene expression. At low concentrations the xylose work as an inducer and at high concentrations as a repressor, in a mechanism *CreA*-mediated (13). This behavior would explain the decrease that was observed in *faeA* transcription after 48 h since it could obey the increase xylose, due to hydrolytic activity. Moreover, the *faeB* gene is induced by the presence of aromatic compounds such as ferulic acid, in *A. niger* (4). Thus, in the presence of complex polysaccharides, the expression of *faeA* gene led to the release of these specific aromatic compounds, stimulating the *faeB* expression. Furthermore, in this work, we report the null effect of rice husk on the transcription of genes coding for feruloyl esterase enzymes. In natural substrates, the hemicelluloses are chemically very variable. In particular, arabinoxylans from cereal husks are described as the most lignified, with the greatest diversity of side chains, and sometimes insoluble in water (14). Therefore, it can be assumed that some components such as xylan and ferulic acid may be inaccessible to operate as inducers on gene expression. Finally, the possible generation of inhibitor compounds during the rice husk pretreatment should be considered. In steam pretreatment, the partial degradation of hemicellulose can lead to the generation of toxic compounds such as furan derivatives (such as furfural and 5-hydroxymethylfurfural) affecting enzyme activity (15).

Conflict of interest

The authors declare that there is no conflict of interest.

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