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Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated bioprocessing (CBP) of lignocellulosics

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ABSTRACT

In an effort to increase ethanol productivity during the consolidated bioprocessing (CBP) of lignocellulosics by *Fusarium oxysporum*, we attempted the constitutive homologous overexpression of one of the key process enzymes, namely an endo-xylanase. The endo-β-1,4-xylanase 2 gene was incorporated into the *F. oxysporum* genome under the regulation of the *gpdA* promoter of *Aspergillus nidulans*. The transformation was effected through *Agrobacterium tumefaciens* and resulted in 12 transformants, two of which were selected for further study due to their high extracellular xylanase activities under normally repressing conditions (glucose as sole carbon source). During natural induction conditions (growth on xylan) though, the extracellular enzyme levels of the transformants were only marginally higher (5–10%) compared to the wild type despite the significantly stronger xylanase 2 mRNA signals. SDS-PAGE verified enzyme assay results that there was no intracellular xylanase 2 accumulation in the transformants, suggesting the potential regulation in a post transcriptional or translational level. The fermentative performance of the transformants was evaluated and compared to that of the wild type in simple CBP systems using either corn cob or wheat bran as sole carbon sources. Both transformants produced approximately 60% more ethanol compared to the wild type on corn cob, while for wheat bran this picture was repeated for only one of them. This result is attributed to the high extracellular xylanase activities in the transformants' fermentation broths that were maintained 2–2.5-fold higher compared to the wild type.

1. Introduction

While the demand for petroleum products increases, it is certain that oil productivity will reach to a maximum in the next decades (Kerr, 2007). On the other hand, renewable energy sources, like solar and wind, mainly due to the lack of storage capacity and reliable energy flow, have to be outreached before they are widely used (Heal, 2010).

Liquid biofuels (biodiesel and bioethanol) are considered successful replacements for gasoline, diesel and jet fuel. Bioethanol is commercially produced mainly through yeast fermentation of free sugars, such as sucrose from sugar cane in Brazil (Marris, 2006) or hydrolysed starch–corn seed hydrolysate in US (Sanderson, 2006). The increasing criticism on the sustainability of these first-generation biofuels has raised attention to the second-generation biofuels, mainly cellulosic and hemicellulosic ethanol, whose production is based on the non-edible biomass of the crops and has the potential to provide benefits such as consuming waste residues and making use of abandoned land (Sims et al., 2010). However, the main technological impediment to a more widespread utilization of (hemi)cellulosic materials for ethanol production is the absence of a low cost technology for overcoming their recalcitrance against hydrolysis (Zaldivar et al., 2001). The production of cellulo/xylanolytic enzymes, biomass hydrolysis and fermentation of the resulting sugars to ethanol in a one step process by a single microorganism or a consortium of different organisms, would aid to overcome the aforementioned problems. Such systems, that define the third generation biofuels, are often called consolidated bioprocessing (CBP) systems and introduce a large cost reduction (Lynd et al., 2002; Carere et al., 2008).

Fusarium oxysporum is among the few microbial species that have the enzymatic system to break down cellulose and hemicellulose while simultaneously ferment the corresponding hexoses and pentoses to ethanol, performing a single step ethanol production from agricultural and forestry residues (Linko et al., 1984; Christakopoulos et al., 1989; Singh and Kumar, 1991; Singh et al., 1992; Panagiotou et al., 2005b). The wild type strain F3 of *F. oxysporum* has been extensively studied towards this direction, and its metabolism, especially during pentose fermentation, has been thoroughly evaluated (Panagiotou and Christakopoulos, 2004;

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Panagiotou et al., 2005a, 2006). In recent studies, the strain has been shown to effectively produce a complete system of hydrolytic enzymes when grown on various agro-industrial ligno-cellulosic byproducts, such as dry citrus peels, corn cob and brewer's spent grain and simultaneously ferment the corresponding oligosaccharides to ethanol with promising yields (Mamma et al., 2008; Xiros et al., 2008a; Xiros and Christakopoulos, 2009). In all these studies, the hydrolysis of the lignocellulosic material has been proven as the major bottleneck on the productivity of the overall bioconversion process. The corresponding hydrolases, mainly cellulases and xylanases are inducible enzymes and their efficient production in the fermentation medium is a time consuming step. Homologous overexpression of one or more of these enzymes under constitutive control, could provide a higher break down rate of the (hemi)cellulosic biomass and thus increase the supply of sugars to the ethanol production pathway. As a proof of principle for this approach, we decided to overexpress into *F. oxysporum* F3, one of its major xylanases, namely endo-β-1,4-endoxylanase 2, and evaluate the performance of the corresponding transformant strains in simple lab scale consolidated bioprocessing experiments using corn cob and wheat bran as sole carbon sources.

2. Materials and methods

2.1. Strains, plasmids and reagents

The wild type strain F3 of *F. oxysporum* was used throughout the study (Christakopoulos et al., 1989). The strain AGL1 of *Agrobacterium tumefaciens* (Lazo et al., 1991) was obtained from Dr. K. Haralampidis (Department of Biology, University of Athens, Greece). Plasmid pBluescript SK II was obtained from Stratagene (now Agilent Technologies), while plasmid pBHt1 (Mullins et al., 2001) was a kind gift from Dr. S. Kang (Department of Plant Pathology, Pennsylvania State University, USA). Plasmids were propagated in *Escherichia coli* strain DH5α. For plasmid isolation and purification the Macherey – Nagel GmbH & Co. KG (Dueren, Germany), Nucleospin® Plasmid DNA Purification kit (Cat. No. 740 588.50) was used, according to the instructions of the manufacturer. All reagents were obtained from either Sigma or Applichem and were of the highest analytical purity available. Restriction enzymes were from Takara Bio Europe (Saint Germain en Laye, France). Fine grinded (<1 mm) corn cob [42% cellulose, 38% hemicelluloses, 8% lignin, w/w] and wheat bran [12% cellulose, 35% hemicelluloses, 29% starch, 11% protein, 5% lignin, w/w] were obtained from the Agricultural University of Athens and Loulis Mills S.A. (Pireas, Greece), respectively, and supplemented the mineral medium (see below) without any pretreatment.

2.2. Media and growth conditions

A. tumefaciens and *E. coli* strains were cultivated in standard Luria-Bertani (LB) broth and LB agar (LBA) media and maintained in 50% LB–50% glycerol at −80 °C. *F. oxysporum* F3 was maintained in Potato Dextrose Agar (PDA - Applichem, Germany) plates or slants at 4 °C.

Transformation media were prepared according to Mullins et al. (2001) with some modifications (personal communication with Dr. S. Kang). Minimal medium (MM) for *A. tumefaciens* cultures prior to *F. oxysporum* transformation consisted of ($g\,L^{-1}$): K_2HPO_4, 2; KH_2PO_4, 1.45; $MgSO_4{\cdot}7H_2O$, 0.6; NH_4NO_3, 0.5; NaCl, 0.3; $CaCl_2{\cdot}2H_2O$, 0.01; $FeSO_4$, 0.001; glucose, 2; Spore elements soln, $5\,mL\,L^{-1}$ ($100\,mg\,L^{-1}$ of each: $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, H_3BO_3, $MnSO_4{\cdot}H_2O$ and $Na_2MoO_4{\cdot}2H_2O$). Induction medium (IM) had the composition of MM plus $5\,g\,L^{-1}$ glycerol, 0.2 mM acetosyringone and $40\,mL\,L^{-1}$ of 1 M MES (pH 5.3). Growth medium (FM) for selection and further cultivation of the transformed strains of *F. oxysporum* consisted of ($g\,L^{-1}$): $NaNO_3$, 3; KCl, 0.3; $MgSO_4{\cdot}7H_2O$, 0.3; KH_2PO_4, 0.3; glucose, 10; Hutner's trace elements soln, $2\,mL\,L^{-1}$ and hygromycin, $50\,mg\,L^{-1}$ when required. Solid media additionally contained $15\,g\,L^{-1}$ Bacteriological Agar (Oxoid).

Basal growth medium (BGM) for liquid aerobic cultures of *F. oxysporum* strains (transformed and wild-type) consisted of ($g\,L^{-1}$): $NaH_2PO_4{\cdot}2H_2O$, 7; $Na_2HPO_4{\cdot}2H_2O$, 9.5; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 10; $MgSO_4{\cdot}7H_2O$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.3 (initial pH was adjusted to the value of 6). Sterilization was effected at 121 °C for 20 min. Carbon source (glucose, birchwood xylan, corn cob or wheat bran) supplemented BGM as required and was sterilized dry. The liquid aerobic cultures were inoculated at 5% (v/v) with a spore suspension prepared by adding 15 mL deionized sterile water, containing 100 μL Tween 80, into a 7 day old PDA slant. Cultures were incubated at 29 °C in an orbital shaker at 220 rpm.

Lab scale consolidated bioprocessing experiments were performed as follows: The aerobic (biomass and enzyme production) stage was performed as described above for 3 days in 250 mL Erlenmeyer flasks with 50 mL of working volume. Following mycelial growth, the content of each aerobic flask was aseptically transferred in a rubber stoppered 60 mL flask, containing the calculated amount of dry sterilized corn cob or wheat bran ($40\,g\,L^{-1}$ final concentration of solid). The flasks were sealed and incubated at 29 °C and 130 rpm (fermentative stage). Small aliquots (0.1–0.3 mL) were drawn daily through a syringe. Samples were centrifuged (12,000 rpm, 10 min, 4 °C) and the supernatant was used for the determination of ethanol concentration.

2.3. Xylanase gene isolation and plasmid construction

F. oxysporum F3 genomic DNA, was isolated from liquid cultures in FM (without hygromycin) following 24 h growth at 25 °C and 150 rpm. Mycelia were harvested, frozen in liquid nitrogen and genomic DNA was isolated as described by Sambrook et al. (1989). The xylanase 2 gene was isolated from the *F. oxysporum* F3 genomic DNA using the following primers:

5′-TAGCGGCCGCATGGTTTCCTTCACCTCTCTCC-3′
5′-CGACTAGTTTACTGGGAGACAGTCATGCTG-3′

This procedure also introduced *Not*I and *Spe*I restriction sites (underlined) in the flanks of the xylanase 2 gene. Xylanase 2 gene was cloned between the *gpdA* promoter of *A. nidulans* (Punt et al., 1990) and the *trpC* terminator of *A. nidulans* into the polylinker site of plasmid pBluescript II SK. The primers designed to isolate the above sequences were:

5′-ATTCCGCGGCCGGTGACTCTTTCTGGC-3′
3′-TAGCGGCCGCGGGAAGATGAATATACTGAAGATGG-5′
for the *gpdA* promoter (*Sac*II and *Not*I sites underlined) and
5′-CCACTAGTCCATGTCAACAAGAATAAAACGC-3′
3′-ATACCCGGGCGAGTGGAGATGTGGAGTGG-5′
for the *trpC* terminator (*Spe*I and *Sma*I sites underlined).

The *gpdA-xyl2-trpC* construct from pBluescript II SK was isolated by PCR and introduced into *Sma*I digested plasmid pBHt1 yielding plasmid pBHtxyl Successful insertion was verified by restriction analysis.

2.4. Transformations

Plasmid pBHtxyl was used to transform *A. tumefaciens* strain AGL-1 as previously described (Mullins et al., 2001). The transformed *Agrobacterium* strain was cultivated in MM, supplemented with $50\,\mu g\,mL^{-1}$ kanamycin at 28 °C for two days. A certain amount from this culture was used as inoculum for IM medium, supplemented with the same kanamycin concentration, in order to obtain an initial OD_{600} equal to 0.15 ± 0.02. The IM medium culture was

allowed to grow for 6 h at 28 °C and 200 rpm. One hundred microliters of the IM culture were mixed with an equal volume of *F. oxysporum* F3 spore suspension (approx. 10^5 spores). The mixture was applied on Whatman membranes (Whatman Cat. #7141 104, 47 mm diameter 0.45 μm pore size) and placed on solid IM, supplemented with kanamycin (50 μg mL^{-1}). Following two day incubation at 25 °C, the membrane was transferred to solid FM, supplemented with kanamycin, cefotaxim 20 mM and hygromycin 50 μg mL^{-1}. Transformants were isolated after 7 days of incubation at 25 °C.

2.5. DNA and RNA manipulations

For Southern hybridization, genomic DNA was digested by *Eco*RI overnight and fractionated on a 1% agarose gel. The DNA was then transferred to a nitrocellulose membrane and hybridized overnight at 65 °C with ^{32}P-labeled probes (Sambrook et al., 1989). The probes were prepared by random primer labeling of the xylanase 2 coding region of *F. oxysporum*. The membrane was washed at 65 °C, successively in 2×, 2×, 1× SSC buffer containing 0.1% SDS for 30 min each and then exposed to X-ray film at -80 °C for 3 h. For RNA isolation, mycelium was collected from liquid cultures in BGM with glucose or xylan as sole carbon source, frozen in liquid nitrogen and RNA was extracted according to Sambrook et al. (1989). For northern analysis, total RNA was fractionated in 1% agarose, 10 mM orthophosphate buffer and transferred to nitrocellulose membrane (Sambrook et al., 1989). Hybridization conditions were as described in Southern analysis.

2.6. Electrophoresis

SDS-PAGE analysis was performed in culture supernatants and mycelium extracts obtained from aerobic cultures of the wild type and transformant strains in either glucose or xylan. For mycelium extracts preparation, filtered biomass was washed several times with deionized water and disrupted in liquid nitrogen using a mortar and pestle. Upon nitrogen evaporation, disrupted biomass was resuspended in potassium phosphate buffer (pH 6.5, 10 mM) and centrifuged (25,000 × *g*, 15 min, 4 °C) to obtain a clear intracellular extract. Electrophoresis was conducted according to Laemmli (Laemmli, 1970) on a 10% polyacrylamide gel. Gels were stained by silver staining. Equal protein loading in each lane was performed by appropriate sample dilution and verified by protein estimation (Bradford, 1976).

2.7. Enzyme assays

For the determination of total endo-xylanolytic, endo-cellulolytic and amylase activities, 50 μL of properly diluted enzyme sample was added into 950 μL of 2% (w/v) either birchwood xylan (Cat. No. X0502), carboxy-methyl cellulose (Cat. No. C5678), or soluble starch (Cat. No. S9765), respectively (all from Sigma Chemical Co.) in 100 mM potassium phosphate buffer at pH 7 for xylanase, and pH 6 for cellulase and amylase. The mixture was incubated at 40 °C for 15 or 30 min (linear range) under continuous stirring (800 rpm) in a Thermo-Shaker (BOECO, Germany). Following incubation, sample tubes were centrifuged (4 °C, 5 min, 12,000 rpm) and total reducing sugars concentration was determined in the supernatant with the DiNitro-Salycilic acid (DNS) method (Miller, 1959) using an appropriate calibration curve constructed with xylose (for xylanase) or glucose (for cellulase and amylase). A boiled sample (15 min, 100 °C), undergone the same procedure, was always used as blank. Activity was expressed in nkat mL^{-1} (Bailey et al., 1992).

2.8. Ethanol determination

Ethanol concentration was determined through Gas Chromatography on a Perkin-Elmer chromatography system, through FID detection using a Supelcowax-10 column (60 m × 0.53 mm, d_f = 2 μm). Helium at 10 psi was used as carrier gas. The temperature program applied, consisted of 5 min at 40 °C followed by a ramp of 5 °C/min up to 200 °C. 1 μL samples were introduced into the system using an on column injector at 250 °C. Quantification was performed from the corresponding peak areas using a calibration curve prepared from various aqueous ethanol standards.

3. Results and discussion

3.1. Transformation

Previous work on *F. oxysporum* F3 xylanases has resulted in the purification and characterization of four enzymes with clear endo-xylanolytic character (Table 1). Using the available amino-terminal sequences from the corresponding references as templates, we screened the *F. oxysporum* genome database (Broad Institute, MIT) through *blastp* software. Two out of the three available aminoacid sequences were identified as endo-xylanases with high degree of similarity (18 out of 20 aminoacids for xylanase 2 and 22 out of 24 aminoacids for xylanase C). Based on the higher pH stability, the high affinity for xylan and the broader substrate spectrum, as expressed from the measured relative enzyme activities on two different xylans used in xylanase assays—namely birchwood and oat spelt (Table 1), we chose to proceed with xylanase 2 for the rest of our work. The corresponding gene was isolated through PCR from *F. oxysporum* F3 genomic DNA, placed between the *gpdA* promoter and the *trpC* terminator of *A. nidulans* and inserted into plasmid pBHt1 yielding plasmid pBHtxyl The latter was used for *A. tumefaciens* mediated *F. oxysporum* F3 transformation.

The transformation resulted in twelve strains (Fx1–Fx5 and Fx7–Fx13) with hygromycin resistance. All were examined for their extracellular xylanase activity in aerobic cultures in BGM with 20 g L^{-1} glucose as sole carbon and energy source (Fig. 1). Among them, three strains, Fx10, Fx11 and Fx13, revealed the highest extracellular enzyme activity. As expected, wild type strain F3 produced only barely detectable traces of xylanase activity, while for strains Fx10 and Fx13 maximum enzyme activities were up to 250 nkat mL^{-1} and 230 nkat mL^{-1}, after two days of incubation, respectively (Fig. 1). This result shows that the constitutive expression of the endo-β-1,4-xylanase 2 was successful and the transformed stains can produce xylanase under natural repressing/non-inducing conditions—glucose as sole carbon source (Calero-Nieto et al., 2007). Southern blot analysis for the wild-type

Table 1
Physicochemical characteristics of the xylanases from *F. oxysporum* F3.

Reference	MW (kDa)	pH_{opt}	T_{opt} (°C)	pH_{stab}	K_M^{app} (mg L^{-1})	Birch/Oat[a]	*Blastp* result (aa similarity)
Christakopoulos et al. (1996b)	21	6	60	4–10	0.41	0.76	FOXG_09638.2 (18/20) endo-1,4-β-xylanase 2 precursor
Christakopoulos et al. (1996b)	24	6	55	7–9	0.37	0.48	FOXG_09688.2 (19/26) conserved hypothetical protein
Christakopoulos et al. (1997)	38	7	40–50	7–9	0.8	0.37	FOXG_17421.2 (22/24) endo-1,4-β-xylanase C precursor
Christakopoulos et al. (1996a)	60	7.5	50	6–8	11.1	0.55	No AA sequence available

[a] Relative activity on birchwood xylan compared to oat-spelt xylan.

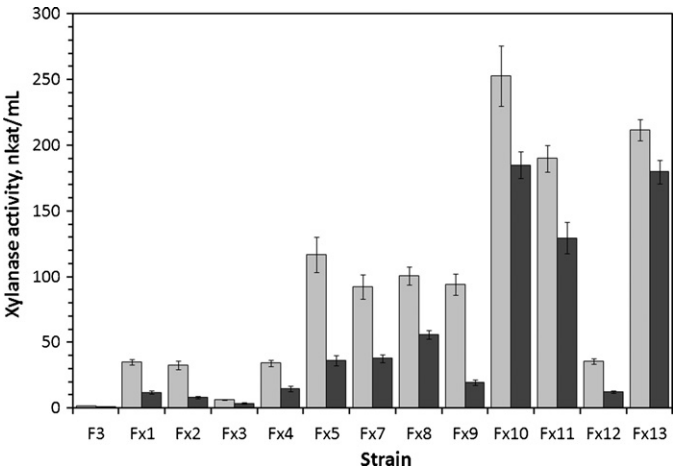

Fig. 1. Extracellular xylanase activities in liquid cultures with glucose as sole carbon source of the wild type (F3) and transformant strains (Fx1–Fx13). Cultivation time: Light grey bars, 2 days; Dark grey bars, 3 days. Data represent the mean of triplicates and error bars correspond to the SD among the three independent flasks.

and the three transformants that showed the highest activities in the previous experiment, revealed that the transformation resulted in at least two additional copies of the xylanase 2 gene compared to the wild-type (Fig. 2). In addition, transformants Fxy10 and Fxy11 had the same blot pattern. The less intense band that is present in all lanes could, most probably, correspond to the xylanase 1 gene (FOXG_15742.2, Broad Institute, MIT) which has high similarity with xylanase 2 gene (data not shown).

3.2. Regulation of xylanase expression

Regulation of xylanase 2 transcription under different induction conditions was evaluated by studying the detailed time course of xylanase activity in parallel aerobic BGM cultures on glucose and xylan, combined with northern analysis (Fig. 3). In glucose cultures (Fig. 3A), no xylanase 2 mRNA was detected in the wild type strain (F3) and as a result, only traces of xylanase activity were detected in the extracellular liquid. Both transformants yielded very strong xylanase 2 mRNA signals and began to produce the enzyme already from the first hours of the culture. Xylanase production closely followed growth and reached a maximum after 2 days, corresponding well with the mRNA data. A more complicated pattern was observed during growth on xylan though (Fig. 3B).

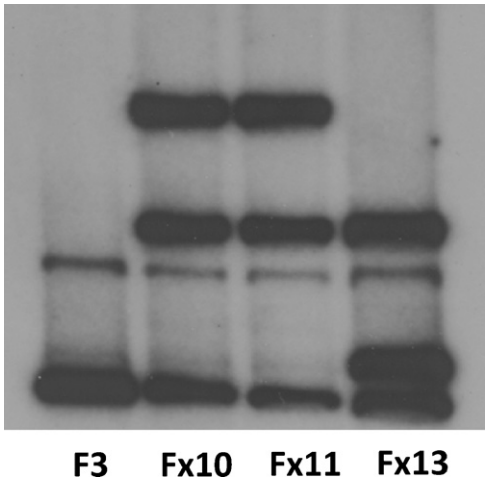

Fig. 2. Southern blot analysis for xylanase 2 gene of the wild type *F. oxysporum* strain F3 and the three transformants with the highest xylanolytic activity.

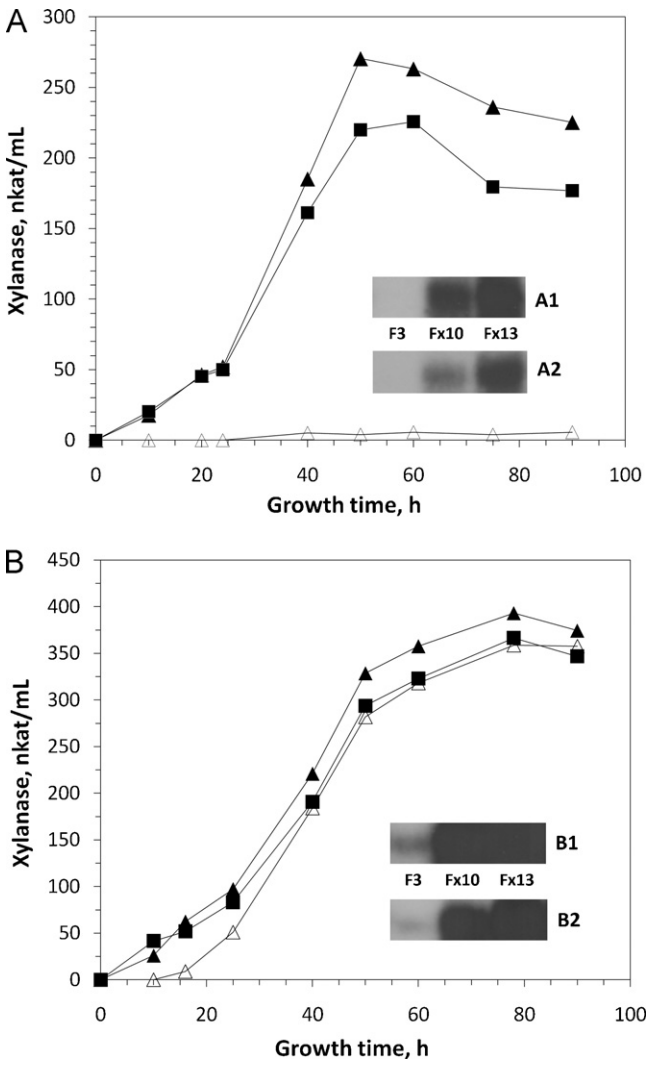

Fig. 3. Extracellular xylanase activity in liquid cultures on BGM with 10 g L^{-1} glucose (A) and 10 g L^{-1} xylan (B). △, wild type *F. oxysporum* F3; ■, strain Fx10; ▲, strain Fx13. All cultures were performed in triplicate flasks. Data represent the mean of the three flasks. For all data points SD ranged from 2.2 to 11.6%. Corresponding northern analysis of the xylanase 2 gene under different induction conditions. A1 and A2: Glucose cultures at 14 and 36 h, respectively. B1 and B2: Xylan cultures at 14 and 36 h, respectively.

Xylanase 2 mRNA was clearly detected in the wild type strain F3 at 14 h of growth and fainted significantly after 36 h. At equal loading and exposure conditions, the corresponding signal in the transformants was extremely strong indicating a much stronger constitutive expression by the *gpdA* promoter compared to the natural xylanase 2 promoter. Xylanase production was delayed compared to glucose cultures reaching a maximum at approximately 75 h of growth with the final levels being approximately 40% higher than those in glucose cultures. The latter can be attributed to the fact that *F. oxysporum* has at least four different xylanases (including xylanase 2) and the presence of xylan as sole carbon source induces the expression of the corresponding genes both in transformants and wild type strain (Calero-Nieto et al., 2007). The fact though, that the *gpdA* promoter is constitutively activated in a manner independent to xylan presence is verified by the observation that in the transformant cultures, xylanase activity was clearly detected already from the first hours post-inoculation compared to the wild type (Fig. 3B). One intriguing point that still remains is that in xylan cultures the maximum measured extracellular xylanase levels in the wild type strain are only marginally lower than those

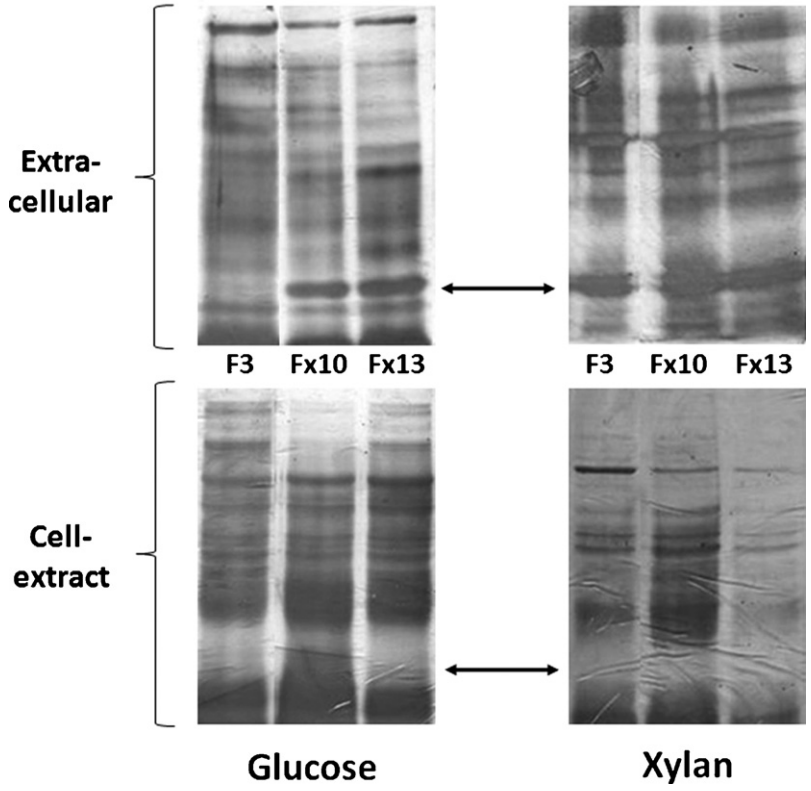

Fig. 4. SDS-Page analysis of the extracellular proteins and the cell bound protein extract in glucose and xylan cultures. The arrow represents the MW value that corresponds to xylanase 2.

of the transformants, a fact that does not agree with the big differences in the corresponding mRNA levels. This could indicate the regulation of xylanase production in a post transcriptional or translational level or a probable saturation of the excretory mechanism. The latter was examined through SDS-PAGE analysis of the culture supernatants and the corresponding intracellular extracts (Fig. 4). A protein band that corresponds to xylanase 2 molecular weight clearly appears in the supernatants of the transformants' glucose cultures and in the supernatants of all cultures on xylan. This band is absent in the culture supernatant of the wild type strain grown on glucose as well as in all intracellular extracts. In addition, only traces of xylanase activity were detected in these intracellular extracts. These observations overrule any intracellular xylanase 2 accumulation in the transformed strains under inducing conditions (xylan) that could result from oversaturation of the excreting mechanism.

3.3. Fermentative performance

At the final part of our work we examined the validity of our assumption that the homologous over-expression of a major hydrolytic enzyme, such as xylanase, could enhance the fermentative performance of *F. oxysporum* during the CBP of lignocellulosics. For this reason we performed a series of simple lab scale CBP experiments using the two selected transformants (Fx10 and Fx13) and the wild type *F. oxysporum* strain (F3) with corn cob or wheat bran as sole carbon sources at an initial concentration of 40 (g solid load) L^{-1} (see Section 2). Both agricultural by-products are produced in significant quantities worldwide and are considered as promising lignocellulosic sources for enzyme and ethanol production (Latif and Rajoka, 2001; De Carvalho Lima et al., 2002; Palmarola-Adrados et al., 2005; Xiros and Christakopoulos, 2009). Fig. 5 shows the time course of ethanol production during the fermentative part of the process, while Fig. 6 summarizes the extracellular endo-xylanase, endo-glucanase and amylase activities measured at the beginning (end of aerobic stage) and close to the end of the alcoholic fermentation.

With corn cob, both transformants performed significantly better with respect to ethanol productivity compared to the wild type. More specifically, the maximum ethanol concentrations obtained were 2.68 and 2.85 g L^{-1} for transformants Fx10 and Fx13, respectively, while the corresponding value for the wild type strain F3 was 1.90 g L^{-1}. It is noteworthy that for strain Fx13 ethanol levels leveled off 5 days earlier (Fig. 5A). The constitutive xylanase expression in the two transformants, resulted in more than double enzyme activity levels in the corresponding culture supernatants compared to the wild type strain (Fig. 6A). At the end of the aerobic stage (Day 1 of the anaerobic fermentative stage), when growth and hydrolases' production has completed, xylanase activities reached 420 and 500 nkat mL^{-1} for the transformants Fx10 and Fx13, respectively, while the corresponding xylanase activity for the wild type was 215 nkat mL^{-1}. These levels remained practically unchanged till the end of the fermentation. The more than double xylanase levels in the transformants' cultures most probably facilitated xylose supply towards the fermentative pathway resulting in approximately 50% higher ethanol concentrations at the end of the anaerobic stage (Fig. 5A). Transformation did not seem to negatively affect the expression of the total endoglucanase and amylase activities under these conditions, but the relative activities for both enzymes were lower compared to xylanase for all strains.

When wheat bran was used as a sole carbon source, the pattern revealed was somewhat different. The performance of transformant strain Fx10 did not differentiate significantly from that of the wild type. Ethanol concentrations in the cultures of both these strains (F3 and Fx10) leveled off equally after 9 days of fermentation (Fig. 5B). On the contrary, transformant strain Fx13 continued to produce ethanol for additional 4 days and leveled at ethanol concentrations 60% higher (8.83 g L^{-1}) than the other two strains (~5.5 g L^{-1}). Wheat bran is known as a more efficient enzyme

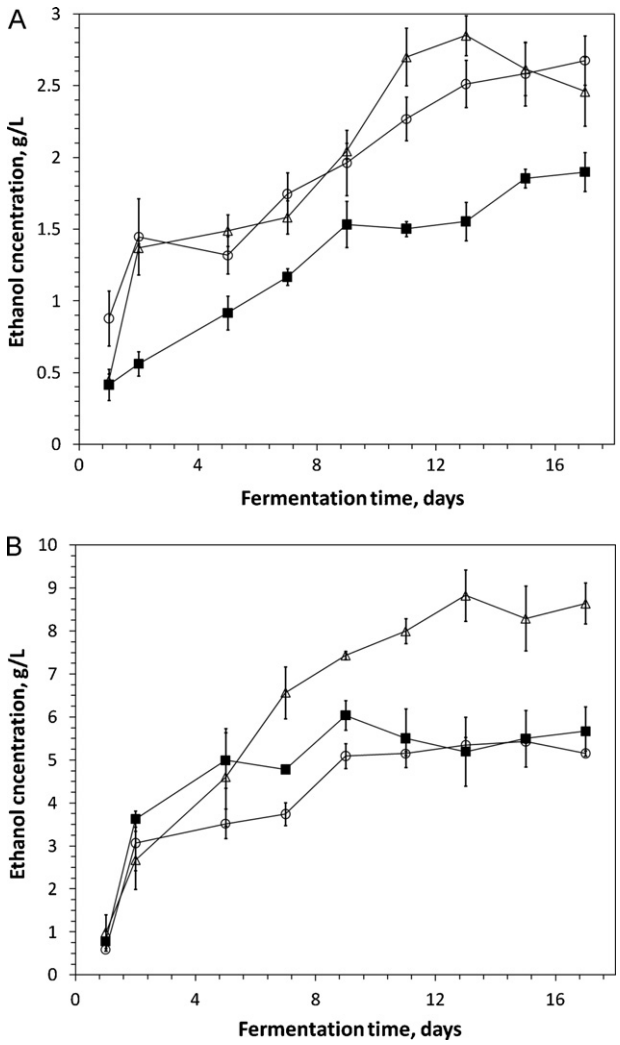

Fig. 5. Ethanol concentration during the anaerobic (fermentative) part of the consolidated bioprocessing of corn cob (A) and wheat bran (B) by the wild type (■), and Fx10 (○) and Fx13 (△) transformant strains. The fermentations were conducted in triplicate and error bars correspond to the SD among the three independent flasks.

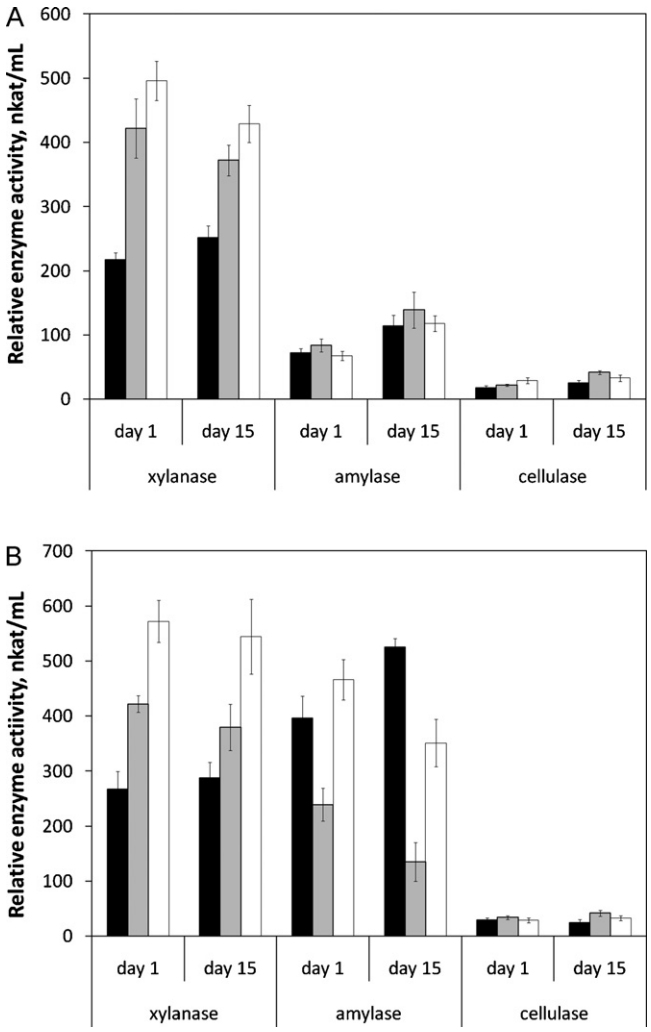

Fig. 6. Enzyme activities during the anaerobic (fermentative) part of the consolidated bioprocessing of corn cob (A) and wheat bran (B) by the wild type and transformant *F. oxysporum* strains. Black bars, wild type F3; Grey bars, transformant strain Fx10; White bars, transformant strain Fx13. The fermentations were conducted in triplicate and error bars correspond to the SD among the three independent flasks.

inducer compared to corn cob (De Souza et al., 2006; Sun et al., 2008) and in our case it seems that there was a significant level of interaction among the natural enzyme induction pathways and the constitutive xylanase expression in the transformants. In contrast to corn cob, there was a significant difference in xylanase activities and between the transformants as well. Extracellular xylanase total levels in the fermentation broth after the end of the aerobic stage were 270, 420 and 580 nkat mL^{-1} for the wild type, Fx10 and Fx13 strains, respectively (Fig. 6B) and remained practically at the same levels until the end of the fermentation. In addition, since starch represents almost one third of the dry mass of wheat bran (29%, w/w, according to the wheat bran supplier) its presence induced the production of significant amounts of amylase (almost 5-fold higher compared to corn cob) providing additional glucose for fermentation. In the transformant strain Fx10, it seems that the amylase production pathway was probably negatively affected by the transformation and the corresponding amylase levels were much lower even than those of the wild type strain (Fig. 6B). This fact explains the differences in ethanol production among the two transformants during CBP on wheat bran.

Table 2 presents the CBP performance characteristics of the strains used in this work, within the framework of an extensive literature summary on substrate/microorganism configurations during CBP of lignocellulosics. Although one can argue that it is difficult to find a common ground for comparison, due to the variable recalcitrance of the substrates tested and the variety of process optimization steps performed by the different research groups, it is possible to draw some conclusions concerning the current status of CBP research. The first five rows (#1 to #5) in Table 2, represent strain evaluation studies with most of the strains used, being genetically engineered. In these studies though, the substrate used was either pure cellulose or pure xylan, neither of which represents a realistic substrate option for CBP. Rows #6 to #10 describe process optimization studies on realistic substrates using wild type microbial strains.

As far as substrate conversion is concerned, higher yields are generally reported with the use of pure cellulose or hemicellulose as sole carbon sources. This is an expected result, since these substrates are directly available to the cellulases and hemicellulases produced during the aerobic stage of CPB and consequently, the monosaccharide supply for the fermentative part is generally increased. As a result, the corresponding yields and productivities are also higher. Of particular importance, is the very recent study of Shin et al. (2010) where a co-culture of two *E. coli* strains, engineered to express several xylanolytic enzymes was able to

Table 2
Literature summary of performance characteristics during consolidated bioprocessing of lignocellulosics.

#	Substrate	Microorganism(s)	Conversion[a] ($g\,kg^{-1}$)	Yield[b] (% of theoretical)	Productivity[c] ($mg\,kg\,h^{-1}$)
1	Microcrystalline cellulose	*Clostridium thermocellum*, wild type	165	32.0	375
2	H_3PO_4 swollen cellulose	*Saccharomyces cerevisiae*, genetically engineered	100	19.4	520
3	Carboxy Methyl Cellulose	*Cellvibrio japonicus*, genetically engineered	4	0.7	73
4	Cellulose 123	*F. oxysporum* wild type F3	350	67.8	2121
5	Birchwood xylan	Genetically engineered binary *E. coli* co-culture	284	53.8	11833
6	*Napiergrass* powder	*Klebsiella oxytoca*, wild type	46	15.8	311
7	Bean curd refuge	Co-culture of wild type *Thermoanaerobacterium* sp. and *Geobacillus* sp.	124	42.0	2870
8	Alkali pretreated brewer's spent grain	*Neurospora crassa*, wild type	74	26.4	1446
9	Alkali pretreated brewer's spent grain	*F. oxysporum* wild type F3	63	23.0	839
10	Alkali pretreated brewer's spent grain	*F. oxysporum* wild type F3	107	38.3	2196
11	Corn cob	*F. oxysporum* wild type F3	48	11.5	146
12	Corn cob	*F. oxysporum*, transformant Fx13	71	17.1	286
13	Wheat bran	*F. oxysporum* wild type F3	138	34.7	754
14	Wheat bran	*F. oxysporum*, transformant Fx13	222	56.0	936

[a]Conversion was determined as the mass of ethanol produced per unit mass of lignocellulosic material loaded in the fermentation vessel.
[b]Percent theoretical yield was determined based on the maximum ethanol yield that corresponds to the total carbohydrate content (cellulose, hemicelluloses, starch, oligosaccharides) of the material used.
[c]Productivity is expressed as mass of ethanol produced per unit mass of total carbohydrates per unit time, at the point of maximum ethanol concentration.
1. Xu et al. (2010), optimized process with respect pH and *T*, bioreactor.
2. Den Haan et al. (2007), *S. cerevisiae* engineered to incorporate endoglucanase and β-glucosidase, no process optimization.
3. Gardner and Keating (2010), no process optimization.
4. Panagiotou et al. (2005b), optimized for pH, *T* and aeration levels, bioreactor.
5. Shin et al. (2010), partially optimized with respect to the induction time (IPTG) of the various xylanase genes.
6. Lin et al. (2010), optimized process with respect to pH, *T*, time and yeast extract concentration, shake flasks.
7. Miyazaki et al. (2008), partially optimized with respect to pH, shake flasks.
8. Xiros et al. (2008b), optimized process, with respect to substrate pretreatment, enzyme production in a solid state aerobic stage and microaeration conditions in bioreactor (fermentative stage).
9. Xiros et al. (2008a), optimized process, with respect to substrate pretreatment, enzyme production in a solid state aerobic stage and microaeration conditions in bioreactor (fermentative stage).
10. Xiros and Christakopoulos (2009), optimized process, with respect to substrate pretreatment, enzyme production in a submerged state aerobic stage and microaeration conditions in bioreactor (fermentative stage).
11–14. Present work, no process optimization.

transform birchwood xylan into ethanol at a yield equal to 54% of the theoretical in only 24 h, in a partially optimized process. Of course the system has to be evaluated with real life complex substrates where a complete enzyme system for lignocellulose hydrolysis is required. The wild type strain of *F. oxysporum* that was used in the present study has also been evaluated with very good results on pure cellulose in a bioreactor study optimized with respect to pH, *T* and aeration levels (Panagiotou et al., 2005b).

Of equal and more practical importance are the studies dealing with the CBP of agricultural byproducts. The recalcitrance of the corresponding substrates results in decreased ethanol conversions in the range of 10% per weight of the solid load. Theoretical ethanol yields range considerably from 12% to 56% and a similar variation is being observed for the reported productivities. The highest productivity was reported for bean curd refuge as substrate, using a *Thermoanaerobacterium* sp. and *Geobacillus* sp. co-culture (Miyazaki et al., 2008) at an optimized process in shake flasks. The corresponding value though, is rather overestimated since bean curd refuge contains a significant amount of free sugars (12%, w/w of its dry mass) that are readily fermented prior to cellulose and hemicelluloses (Miyazaki et al., 2008). *F. oxysporum* wild type F3, has been used in the majority of studies involving CBP of lignocellulosics using alkali pretreated brewer's spent grain (BSG). The process was optimized in bioreactors with respect to enzyme production and microaeration conditions. The approach proposed in this work significantly enhanced the CBP performance of *F. oxysporum* using corn cob or wheat bran as sole carbon source during the process. The overexpression of xylanase 2 in the transformant strain Fx13 has resulted in a 48, 49 and 95% increase in conversion, yield and productivity, respectively, when corn cob was used as sole carbon source. Although the actual values of the above process parameters are relatively low, it has to be taken into account that this is the first report on the sole use of this highly recalcitrant lignocellulosic material in CBP and that we have attempted no optimization on the process, since the main scope of our work is to compare the performance between the wild type and the transformant. In addition, when wheat bran was used as sole carbon source the transformant Fx13 revealed the highest conversion and yield ($222\,g\,kg^{-1}$ and 56%, respectively) among the literature data and relatively high productivity ($936\,mg\,kg\,h^{-1}$), improving the performance characteristics of the wild type strain by 60, 61 and 25%, respectively.

Taking into account the results of our work as well as those summarized in Table 2, we may conclude that consolidated bioprocessing appears to be an attractive alternative for the production of ethanol from lignocellulosics. Its main advantage is the simplicity introduced by the combination of the enzyme production, biomass hydrolysis and fermentation steps, all in one vessel, a fact that definitely reduces both installation and operational costs. On the other hand, it seems to be quite difficult to identify a microbial strain that produces a complete range of highly active hydrolases and is simultaneously capable to ferment all types of simple sugars produced (glucose, xylose, arabinose, mannose, etc.) at acceptable rates (Lynd et al., 2002, 2005). Many genetic engineering approaches have tried to overcome this problem by successfully introducing cellulase and hemicellulase genes into natural ethanol producers such as *Zymonomas mobilis* or *Saccharomyces cerivisiae* (Den Haan et al., 2007; Van Zyl et al., 2007; Linger et al., 2010). When it comes to recalcitrant lignocellulosic biomass though, this approach requires the introduction of a large number of genes required for complete biomass hydrolysis (Stephanopoulos, 2007). For this reason, we believe that a wild type microorganism that is a natural biomass decomposer and is also able to ferment a wide range of simple sugars represents a better starting point for genetic manipulations towards CBP applications. In this work we provided proof of

principle for this approach, since the homologous overexpression of a major hydrolytic enzyme (xylanase) in such a strain—namely the wild type *F. oxysporum* F3, resulted in transformants with significantly improved performance characteristics during CBP.

4. Conclusions

In this study, *F. oxysporum* transformants constitutively expressing the endo-β-1,4-xylanase 2 were developed, in an effort to increase ethanol productivity, during the CBP of complex agricultural byproducts. Transformation was effected though *A. tumefaciens* and several transformants were obtained producing xylanase 2 mRNA under naturally repressing conditions, as well as significantly higher xylanase mRNA levels than the wild type, under naturally inducing conditions. Two of the transformants were selected and tested in simple CBP experiments using either corn cob or wheat bran. Both performed better than the wild type with respect to ethanol productivity with corn cob, while on wheat bran the transformation proved beneficial for only one of the strains. Although the corresponding CBP experiments were not optimized, our results clearly represent a proof of principle, that the constitutive homologous overexpression of key hydrolytic enzymes may enhance the overall ethanol productivity during the CBP of lignocellulosics.

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