Appl Biochem Biotechnol (2011) 164:1023–1036
DOI 10.1007/s12010-011-9191-5

Heterologous Expression of Transaldolase Gene *Tal* from *Saccharomyces cerevisiae* in *Fusarium oxysporum* for Enhanced Bioethanol Production

Jin-Xia Fan · Xiao-Xue Yang · Jin-Zhu Song · Xiao-Mei Huang · Zhong-Xiang Cheng · Lin Yao · Olivia S. Juba · Qing Liang · Qian Yang · Margaret Odeph · Yan Sun · Yun Wang

Received: 15 July 2010 / Accepted: 31 January 2011 /
Published online: 12 March 2011

Abstract The filamentous fungus *Fusarium oxysporum* is known for its ability to ferment xylose-producing ethanol. However, efficiency of xylose utilization and ethanol yield was low. In this study, the transaldolase gene from *Saccharomyces cerevisiae* has been successfully expressed in *F. oxysporum* by an *Agrobacterium tumefaciens*-mediated transformation method. The enzymatic activity of the recombinant fungus (cs28pCAM-Sctal4) was 0.195 times higher than that of the wild-type strain (cs28). The recombinant strain also exhibited a 28.83% increase in ethanol yield on xylose media compared to the parental strain. Enhanced ethanol production and a reduction in the biomass were observed during xylose fermentation. Ethanol yield from rice straw by simultaneous saccharification and fermentation with cs28pCAM-Sctal4 was 0.25 $g\,g^{-1}$ of rice straw. The transgenic strain of *F. oxysporum* cs28pCAM-Sctal4 might therefore have potential applications in industrial bioenergy production.

Keywords Transaldolase · *Fusarium oxysporum* · Xylose fermentation · Bioethanol

Introduction

Fermentation of lignocellulosic biomass is an attractive alternative for the production of bioethanol. D-xylose is the main pentose sugar found in the hemicellulose fraction of lignocellulosic materials. However, the most recognized alcohol fermentation microorganism, *Saccharomyces cerevisiae*, is unable to ferment xylose and other pentose sugars. *Fusarium oxysporum*, a filamentous fungus, can ferment glucose, xylose, and cellulose and has been regarded as a good source of ethanol [1, 2], especially owing to its ability to produce many cellulolytic and xylanolytic enzymes [3–8] and carry out a one-step

J.-X. Fan · X.-X. Yang · J.-Z. Song (✉) · X.-M. Huang · Z.-X. Cheng · L. Yao · O. S. Juba · Q. Liang · Q. Yang (✉) · M. Odeph · Y. Sun · Y. Wang
Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China
e-mail: sjz@hit.edu.cn
e-mail: qianyanghit@yahoo.com

fermentation process. However, the efficiency of ethanol production and xylose consumption of *F. oxsporum* is lower.

The first step of xylose catabolism in *F. oxysporum* involves its conversion to xylulose. D-xylulose is then phosphorylated to form D-xylulose-5-phosphate. The following steps appear to use a combination of the pentose phosphate pathway (PPP) and the Embden–Meyerhof–Parnas pathway and lead to ethanol production (Fig. 1). In a previous study [2], sedoheptulose-7-phosphate (S7P) accumulated during oxygen-limited xylose fermentation. Accumulation of S7P affects the PPP, resulting in reduction of xylose consumption and acetic acid production. The accumulation of S7P might be attributed to a limitation in the transaldolase (TAL) reaction or competition for glyceraldehyde-3-phosphate (G3P) between the PPP and glycolysis, which results in slow xylulose fermentation [9].

Transaldolase (D-sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphateglycerone-transferase, EC 2.2.1.2) is an enzyme of the non-oxidative PPP. It catalyzes the reversible transfer of a three-carbon ketol unit from S7P to G3P to form erythrose 4-phosphate (E4P) and fructose 6-phosphate. This enzyme, together with transketolase, links the PPP to

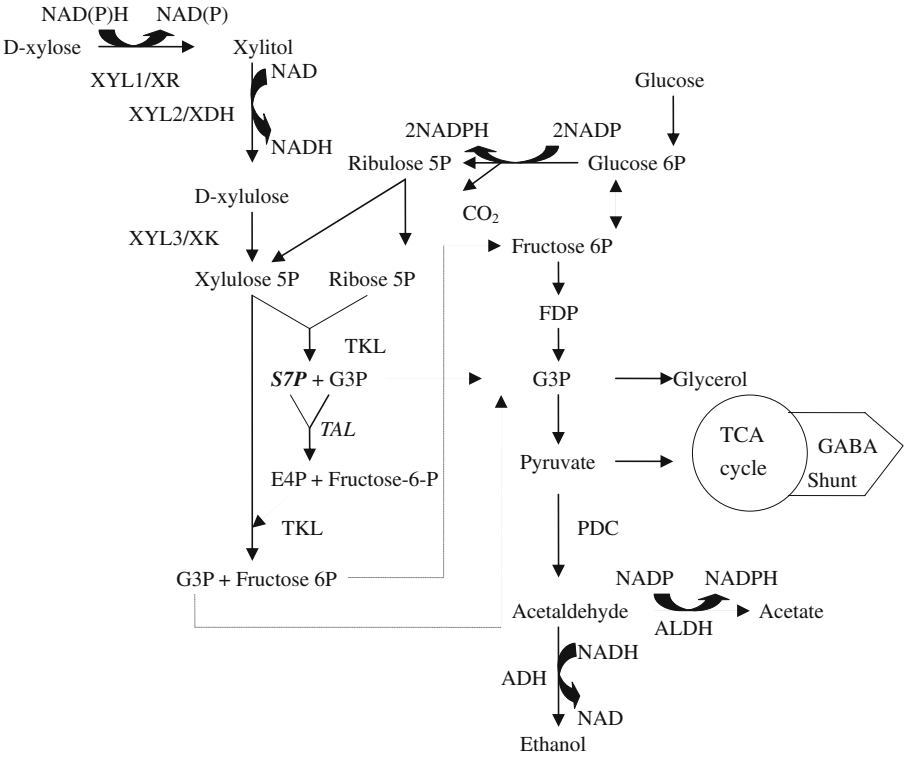

Fig. 1 Metabolic pathways for glucose and xylose in *F. oxysporum*. *XR* xylose reductase, *XDH* xylitol dehydrogenase, *XK* xylulokinase; *xylulose 5P* xylulose-5-phosphate, *ribulose 5P* ribulose-5-phosphate, *ribose 5P* ribose-5-phosphate, *TKL* transketolase, *TAL* transaldolase, *G3P* glyceraldehydes-3-phosphate, *S7P* sedoheptulose-7-phosphate, *E4P* erythrose-4-phosphate, *fructose 6P* fructose-6-phophate, *glucose 6P* glucose-6-phosphate, *FDP* fructose diphosphate, *PDC* pyruvate dehydrogenase, *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, *TCA* tricarboxylic acid cycle, *GABA shunt* γ-amino-n-butyric acid shunt

glycolysis. TAL has been identified in a wide range of microorganisms and *tal* genes have been cloned from yeast [10, 11], archaea [12], bacteria [13], and fungi [14].

Previous findings indicate that overexpression of the inherent *tal* gene (*sctal1*) of *S. cerevisiae* in a xylose reductase and xylitol dehydrogenase-containing strain, improved aerobic growth on xylose considerably [15]. *Zymomonas mobilis* harboring TAL is able to grow on xylose and efficiently ferment it to ethanol [16]. Taking these findings into account, heterologous expression of *tal* in *F. oxysporum* would probably improve the ethanol yield when using xylose as a substrate.

In this study, the TAL gene (*sctal*) from *S. cerevisiae* was expressed in *F. oxysporum* using an *Agrobacterium tumefaciens*-mediated method. We determined that the ethanol yield of the recombinant strain was enhanced by using xylose as a substrate. Furthermore, the simultaneous saccharification and fermentation (SSF) of pretreated rice straw by the transformant *F. oxysporum* cs28pCAM-Sctal4 was investigated.

Materials and Methods

Microorganism Strains, Plasmids, and Growth Conditions

F. oxysporum strain cs28 was isolated from 2-year old corn stalks in the Pingfang region of Heilongjiang Province (China). It was stored in tubes on PXA medium (potato 200 gl^{-1}, xylose 20 gl^{-1}, and agar 20 g l^{-1}). For the production of conidia, fungal cultures were grown on PXA medium at 28°C for 7 days. *A. tumefaciens* strain AGL-1 (AGL0 recA:: bla pTiBo542ΔT-region Mop^{+} Cb^{R}) [17] was grown at 28°C in YEP liquid media (10 gl^{-1} peptone, 10 gl^{-1} yeast extract, 5 gl^{-1} NaCl, pH 7.0) containing streptomycin (100 mg l^{-1}) and rifampicin (10 mg l^{-1}), then 15% (v/v^{-1}) sterile glycerol was added and the organism was stored at −80°C. *Agrobacterium* transformation was conducted in liquid and solid induction media (IM) [18]. *S. cerevisiae* strain H158 was stored in our laboratory and grown in YPD medium (10 gl^{-1} yeast extract, 20 gl^{-1} peptone, 20 gl^{-1} glucose) at 28°C.

Plasmids used in this study included pCAMBIA-1301 [19], pBI121 [20] and pUC18 (TaKaRa, Japan). *Escherichia coli* Top10 strain was used for plasmid construction and propagation and was maintained in Luria–Bertani medium at 37°C.

Construction of the Binary Vector pCAM-Sctal and Fungus Transformation

The *sctal* gene was polymerase chain reaction (PCR) amplified from *S. cerevisiae* strain H158 genome using primers P1: 5′-CGCGGATCCATGTCTGAACCAGCTTCAAAA-3′ (*Bam*HI site is underlined) and P2: 5′-GGAGCTCTTAAGCGGTAACTTTCTTTT-3′ (*Sac*I site is underlined). Initially, pBI121 and pUC18 were digested with *EcoR*I and *Pst*I, a 3.0 kb fragment from pBI121 was ligated into pUC18, resulting in pUC-CaMV-GUS-NOS. pUC-CaMV-GUS-NOS vector was digested with *Bam*HI and *Sac*I, and the 3.8 kb fragment was ligated to the *sctal* PCR product and transformed into *E. coli*. Transformant was verified and designated pUC-CaMV-Sctal-NOS. Using primers P3: 5′-AACTG CAGGTCCCCAGATTAGCCTTTTCAA-3′ (*Pst*I site is underlined) and P4: 5′-ACATGCATGCCCGATCTAGTAACATAGA TGACACC-3′ (*Sph*I site is underlined) the *sctal* gene expression cassette was amplified by PCR using pUC-CaMV-Sctal-NOS as the template. The *sctal* gene expression cassette was ligated with the *Pst*I and *Sph*I linearized pCAMBIA-1301 vector, resulting in the recombinant pCAM-Sctal.

The recombinant plasmid (pCAM-Sctal) and a control vector (pCAMBIA-1301) were transformed into *F. oxysporum* strain cs28 by an *A. tumefaciens*-mediated method, as described by Mullins et al. [21]. Seven-day-old spores were harvested with sterile distilled water (dH_2O) and filtered through sterile Miracloth (Calbiochem, La Jolla, CA, USA). *A. tumefaciens* strain AGL-1 was streaked from glycerol stocks onto YEB medium (1.4% agar), containing the appropriate antibiotics for selection, and grown at 28°C. A single colony was used to inoculate each liquid culture. Bacterial cells were grown in 2 ml of minimal medium with 50 μg ml^{-1} kanamycin for 48 h, at 28°C, shaking at 250 rpm. The cells were diluted to an OD_{660} of 0.15 in 5 ml of IM containing 200 μM acetosyringone (AS) (Aldrich Chemical, Milwaukee, WI, USA) and grown at 28°C for 6 h to reach and OD_{660} of 0.25. A 100 μl aliquot was mixed with 100 μl of the conidia suspension (10^6 cells per ml^{-1}; in this mixture the fungal cell/bacterial cell ratio was 1:250). A 200 μl aliquot of this mixture was plated onto filter papers placed onto IM with 200 μM AS. After 2 days growth, the filter papers were transferred to PXA medium containing 50 μg ml^{-1} hygromycin B (Roche, Germany) and 300 μg ml^{-1} cefotaxime to kill *A. tumefaciens*. Putative transformants, visible 5–6 days later, were transferred to PXA medium containing 50 μg ml^{-1} hygromycin B.

Phylogenetic Studies

To compare the sequences of TAL family members in filamentous fungi and yeast, we used the protein sequence of eight filamentous fungal species: *Penicillium marneffei*, *Aspergillus oryzae*, *F. oxysporum*, *Gibberella zeae*, *Aspergillus fumigatus*, *Magnaporthe grisea*, *Neurospora crassa*, and *Aspergillus terreus*; and six kinds of yeast: *S. cerevisiae*, *Saccharomyces castellii*, *Saccharomyces kluyveri*, *Saccharomyces bayanus*, *Kluyveromyces lactis*, and *Candida glabrata*. A phylogenetic study was performed using the amino acid sequences of the TAL proteins from the filamentous fungi and yeast from NCBI (http://www.ncbi.nlm.nih.gov/). The amino acid sequences were aligned using the Clustal W program [22]. A phylogenetic tree was constructed using the minimum evolution method with a heuristic search by MEGA version 4.1 [23] and visualized with TreeView X [24].

Detection of *Sctal* Integration in *F. oxysporum*

To determine whether the *sctal* gene was integrated into the *F. oxysporum* genome in the transformants, Southern blot hybridization was performed. Genomic DNA (5 μg) was digested with *Hin*dIII, separated on a 1% agarose gel, and then blotted onto a nylon membrane. Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) were used for probe preparation and detection of the *sctal* gene. The *sctal* gene was amplified from the plasmid pCAM-Sctal, and labeled with digoxigenin. The blotted membrane was prehybridized at 65°C in a digoxigenin Easy Hyb solution, and then hybridized in the same solution supplemented with the digoxigenin-labeled *sctal* probe overnight. The membrane was washed with 2×SSC and 0.1% sodium dodecyl sulphate (SDS) at room temperature, followed by 0.1×SSC and 0.1% SDS at 68°C. After washing the membrane with a solution containing 0.1 M maleic acid and 0.15 M NaCl, pH 7.5, the hybridized signal was detected using the chemiluminescent substrate CDP-STAR in the detection kit (Roche, Germany), according to the manufacturer's instructions.

Real Time RT-PCR

Relative quantification of sc*tal* expression was achieved by real time RT-PCR. Total RNA was extracted, respectively from different transformants with Trizol (Invitrogen, USA) and treated with RNAse-free DNAse (Promega). Quality and integrity of total RNA were determined by running 1 μl of total RNA in a 1% agar gel, and the quantity was tested by a NanoDropTm 1000 spectrophotometer (Thermo FisherScientific, USA). The reverse transcription reaction was conducted in 20 μl reaction system according to the manufacturer's protocol (TaKaRa). Gene specific primers (SF: 5′-GCCAAGCAAC-CAACTTACGC-3′and SA: 5′-GGTGGAGACTCTGCCTGGAAC-3′) were designed according to the corresponding sequence of *sctal*. Partial of 18 s rRNA gene was amplified with primers (18sF: 5′-AGCGAGACCGCCACTAGATTT-3′ and 18sA: 5′-TCATTTCAACCCTCAAGCCC-3′) as a control. PCR reaction was done in a 20 μl volume contained 10.0 μl 2×SYBR® Premix Ex Taq™, 50× ROX Reference Dye II 0.4 μl (TaKaRa), 0.2 μM of each primer and 2 μl template (10× diluted cDNA). The real-time PCR was performed according to the manufacturer's instruction (TaKaRa) under the following condition: 30 s pre-denaturation at 95°C, 5 s denaturation at 95°C, 34 s collection fluorescence at 60°C, 40 cycles. All reactions, including non-template controls, were carried out in three times with three independent biological replicates. The real-time qRT-PCRs were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Dissociation curves were generated for each reaction to ensure specific amplification. Threshold values were generated from the ABI PRISM 7500 Software Tool (Applied Biosystems, Foster City, CA, USA). Relative quantification of *sctal* gene towards 18 S rRNA was performed using the SDS software (Applied Biosystems, Foster City, CA, USA) and comparative Ct (2-ΔΔCt) value method [25]. A probability (P) value equal or less than 0.01 was used to determine the significance of difference between different strains.

Genetic Stability of Transformants

To test the mitotic stability of the *sctal* gene in *F. oxysporum* transformants, spores from the transformants were inoculated into 3 ml of PX medium (xylose, 20 gl^{-1}; potato 200 gl^{-1}) grown at 28°C and 120 rpm. After 4 days of growth, 50 μl of each culture were plated on PXA containing 50 μl ml^{-1} hygromycin B as well as unsupplemented PXA. Mitotic stability was also tested on solid cultures by transferring mycelium of each transformant to one edge of a PXA plate. Once the growth reached the opposite side of the plate, mycelium from the growing edge were transferred to PXA containing 50 μl ml^{-1} hygromycin B as well as unsupplemented PXA.

SDS-PAGE and Enzymatic Activity Assays of Recombinant TAL

The *F. oxysporum* transformant cs28pCAM-Sctal4, control transformant cs28pCAM and wild-type strain cs28 were inoculated in fermentation media (yeast extract 2 gl^{-1}; $NaNO_3$ 10 gl^{-1}; $MgSO_4$ 0.2 gl^{-1}; KH_2PO_4 2 gl^{-1}; $CaCl_2$ 0.1 gl^{-1}; xylose 20 gl^{-1}; pH 6.0) for 96 h at 30 °C, respectively. The total proteins of biomass were extracted by TCA/acetone method by Wang et al. [26]. After adding 2× loading buffer, the solvents were boiled for 5 min, centrifuged for 10 min at 8,000 rpm and loaded into the slab gel of 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Transaldolase activity was measured using Soderberg's procedure [12] with some modification. The 1 ml reaction mixture, consisting of 50 mM imidazole (pH 7.5), 5 mM fructose-6-phosphate, 100 μM erythrose-4-phosphate, 100 μM NADH, and five units each of the auxiliary enzymes triose phosphate isomerase and glycerol phosphate dehydrogenase (Sigma-Aldrich), was each run in a standard 1 cm path length quartz cuvette at 45°C. Absorbance at 340 nm was determined by spectrophotometer (Thermo FisherScientific, USA). One unit of activity was defined as the amount of enzyme that converted 1 μmol of NADH into NAD per min at 45°C. The protein concentration was determined according to Bradford's procedure [27], with bovine serum albumin as the standard.

Xylose Fermentation Condition and Analysis Methods

F. oxysporum strain cs28 and transformant cs28pCAM-Sctal4 were cultured in PX medium at 30°C and 140 rpm for 24 h. Xylose (30 gl^{-1}) media was prepared separately. The compositions of the fermentation media (g l^{-1}) besides the sugars were: yeast extract, 2; $NaNO_3$, 15; $MgSO_4$, 0.2; KH_2PO_4, 3; $CaCl_2$, 0.2; pH 6.0. Fermentation was performed in sterile 100-ml Erlenmeyer flasks in a constant temperature incubator at 30°C. Each Erlenmeyer flask contained 50 ml of sugar media and 5 ml of inocula. All of the media were autoclaved at 121°C for 20 min before use. Samples of the culture were taken at different time intervals.

The dried biomass was measured from 50 ml of cell suspension. The suspension was centrifuged, and the cell pellet was washed twice with water and dried overnight at 105°C. The first supernatant was analyzed for ethanol and acetic acid by gas chromatography. The ethanol and acetic acid concentration was measured by GC-4890 (Agilent, USA) gas chromatography with an Inwax (Agilent) capillary column and a flame ionization detector. The chromatogram was run at an oven temperature of 70°C and an injection temperature of 200°C using N_2 as the carrier gas and H_2 as the flaming gas. The concentrations of xylose were determined using the 3,5-dinitrosalicylic acid (DNS) method [28]. The analysis of variance was performed using SAS 9.0 software.

SSF Condition

Rice straw was ground using a hammer mill through a 2 mm grid. This straw was slurred in 0.5% sulfuric acid solution. The mixtures were autoclaved at 121°C for 15 min. The solid fraction was washed five times with tap water at 50 °C, filtered and stored at 4°C prior to SSF. Twenty grams of pretreated rice straw was added to 150 ml conical flasks containing 50 ml nutrient salt medium ($MgSO_4$-$7H_2O$ 0.2 g, $NaNO_3$ 10 g, K_2HPO_4 2 g, $FeSO_4$-$7H_2O$ 0.02 g, $CaCl_2$-$2H_2O$ 0. 2 g, yeast extract 2 g, distilled water 1 l) and autoclaved (20 min, 121°C). Then the preparation was 10% (*v*/*v*) inoculated (conidia of a inoculating loop were cultured 3 days in seed culture medium and was 10% (*v*/*v*) inoculated in the culture medium for rice straw SSF experiment) and incubated at 30°C, 200 rev min^{-1} for 4 days, then the flasks were incubated in a constant temperature incubator at 30°C for 3 days.

Cellulolytic and Hemicellulolytic Enzyme Activities Assay

The β-glucosidase activity (pNPGase) was determined by measuring the p-nitrophenol (pNP) that is derived from the hydrolysis of p-nitrophenyl-β-D-glucose (pNPG) [29] with some modifications. The reaction mixtures consisted of 1 ml of 5 mM pNPG in acetate buffer (pH 5.0), and 0.5 ml of cultured supernatant after centrifugation. They were incubated at. After incubation at 50°C for 30 min, 1.0 mL of 1.0 M sodium carbonate was

added to the mixture and the amount of pNP liberated was estimated from the absorbance at 420 nm. One unit of pNPGase activity was defined as the amount of enzyme required for releasing total reducing sugar equivalent to 1 mmol pNP/min.

Assays for crude CMCase and xylanase were performed using 0.5% sodium carboxyl methyl cellulose (CMC-Na) and 0.5% birch wood xylan (Sigma) in 0.1 M acetate buffer, pH 5.0, respectively. The reaction mixture, consisting of 1 ml CMC-Na or 1 ml birch wood xylan as the substrate and a 0.5 ml of cultured supernatant after centrifugation, was incubated at 50°C for 30 min, supplemented with 0.5 ml of DNS, and boiled for 10 min. After cooling, the reduced sugars released in response to CMCase and xylanase activity were measured at 540 nm [30]. One unit of CMCase or xylanase activity was defined as the amount of enzyme required for releasing total reduced sugar equivalent to 1 mmol glucose per minute.

The Avicelase activity was determined by measured by the Fujii procedure [29] with some modifications. A sample solution (0.5 mL) was added to 1 mL of an Avicel suspension (5 g of Avicel was equilibrated in 1 L of a 0.1 M acetate buffer (pH 5.0) for 24 h) in an L-shaped test tube. The test tube was incubated, with vibration, at 50°C for 60 min. The reducing sugar in the liquid was measured by the same procedure as used for CMC activity measurement above.

Results

TAL Phylogenetic Analysis

Based on the amino acid sequences of TAL from different organisms, a phylogenetic tree was constructed using MEGA version 4.1 (Fig. 2). The phylogenetic tree showed two

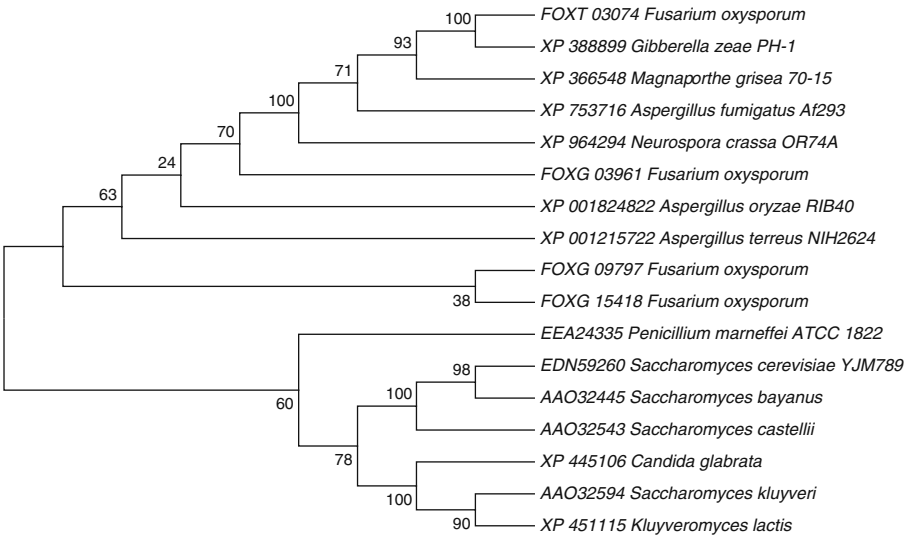

Fig. 2 Phylogenetic tree of transaldolase proteins from different fungi. All of the protein sequences were obtained from the GenBank database and Broad Institute (Harvard and MIT, Boston, USA). Minimum evolution bootstrap values greater than 50% were given. The homologues of transaldolases were identified by BLAST search, and the protein sequences found were then aligned using CLUSTAL W software. The tree was generated from the aligned region with bootstrapping

distinct groups: TALs from yeast formed one group, excluding the TAL from *P. marneffei* ATCC1822, and TALs from filamentous fungi formed a separate group. In the recently released genome sequence of *F. oxysporum* (Broad Institute of Harvard and MIT, http://www.broad.mit.edu), four genes were annotated as transaldolases, namely foxg_09797.2, foxg_15418.2, foxg_03961.2 and foxg_03074.2. The TAL amino acid sequences from *F. oxysporum* and *S. cerevisiae* were aligned using the ClustalX program. The similarity between the TAL sequences from these two organisms was found to be 25%, 32%, 39%, and 64%, respectively, indicating that there was generally a low level of homology between TAL sequences in these two organisms.

Sensitivity and Mitotic Stability of *F. oxysporum* Strain cs28 and the Transformants cs28pCAM-Sctal to Hygromycin

A major pre-requisite for using hygromycin B resistance as a selective marker in fungal transformation is the sensitivity of the host strain (*F. oxysporum* cs28) to this drug. To test this, either spores of cs28 or mycelia were inoculated onto agar plates containing different concentrations of hygromycin. As shown in Table 1, growth of *F. oxysporum* strain cs28 was inhibited at hygromycin concentrations higher than 50 μg ml^{-1}.

To detect if the transforming *sctal* gene was stably inherited during vegetative growth, four randomly picked transformants were grown for 1 week on PXA medium without hygromycin, before being transferred back to PXA medium containing hygromycin. Growth without selection pressure did not affect the level of hygromycin resistance for any of the transformants, which illustrated that the integrated *sctal* gene was mitotically stable. Furthermore, the transformants had a high level of resistance to hygromycin (100 μg ml^{-1}; Table 1).

Integration and Heterologous Expression of the *Sctal* Gene in *F. oxysporum*

To determine the *sctal* integration, genomic Southern blot analysis was performed using the digoxigenin-labeled *sctal* probe. The transformants (cs28pCAM-Sctal1, cs28pCAM-Sctal2, cs28pCAM-Sctal3, and cs28pCAM-Sctal4) showed hybridizing bands of different sizes, indicating that the T-DNA was integrated at random chromosomal locations, mostly as single copy inserts (Fig. 3).

Table 1 Sensitivity of wild-type and putative transformants of *F. oxysporum* on hygromycin medium

Strains	Hygromycin concentration (μg ml^{-1})				
	0	25	50	75	100
cs28	+	+	−	−	−
cs28pCAM-Sctal1	+	+	+	+	+
cs28pCAM-Sctal2	+	+	+	+	+
cs28pCAM-Sctal3	+	+	+	+	+
cs28pCAM-Sctal4	+	+	+	+	+

Samples were incubated for seven days; *cs28* wild-type strain; *cs28pCAM-Sctal1, cs28pCAM-Sctal2, cs28pCAM-Sctal3, and cs28pCAM-Sctal4* putative transformants with plasmid (pCAM-Stal); (+) growth, (−) no growth

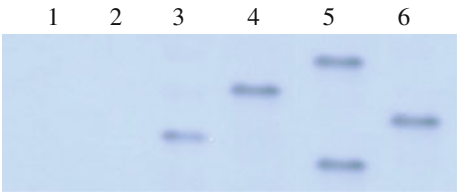

Fig. 3 Southern blot analysis of genomic DNA from transformants. Genomic DNA isolated from four randomly picked transformants was digested with *Hind*III, electrophoresed, blotted onto a nylon membrane and probed with a *sctal* gene fragment. *1* cs28, *2* cs28pCAM, *3–6* cs28pCAM-Sctal1, cs28pCAM-Sctal2, cs28pCAM-Sctal3, cs28pCAM-Sctal4

To demonstrate the presence of the RNA transcript during expression of the *sctal* gene, the four stable transformants were analyzed by real-time RT-PCR. As shown in Fig. 4, the expression levels of *sctal* in cs28 and control transformant cs28pCAM were not observed. The expression level of transformant cs28pCAM-Sctal4 was the highest in all transfomants. The transformant cs28pCAM-Sctal4 was used to further study.

SDS-PAGE analysis was performed to determine whether the SCTAL protein had been synthesized in transformant cs28pCAM-Sctal4. Compared with the wild-type strain *F. oxysporum* cs28 and the control transformant cs28pCAM, the transformant cs28pCAM-Sctal4 showed a clearly visible protein band with a molecular mass of approximately 35 kDa in the SDS-PAGE gel (Fig. 5), indicating that SCTAL protein had been successfully synthesized in the recombinant *F. oxysporum* cs28pCAM-Sctal4.

The recombinant TAL activity of the transgenic cs28pCAM-Sctal4 showed a peak value at 36 h. After 36 h, the enzyme activity decreased (Fig. 6). Moreover, the enzyme activity of transformant cs28pCAM-Sctal4 was 0.195 times higher than the wild-type strain cs28. This significant difference in enzyme activity between (df=2, f=38.25 (p<0.01)) indicated that *sctal* had been expressed in the *F. oxysporum* transformant.

Xylose Fermentation

To determine the effect of overexpressing the *sctal* gene in *F. oxysporum*, the transformant cs28pCAM-Sctal4 was fermented on xylose medium in shaken flasks under oxygen-limited conditions. A significant difference was observed between the weight of the biomass of the cs28pCAM-Sctal4 transformant and that of the cs28 wild-type strain in xylose medium (p<

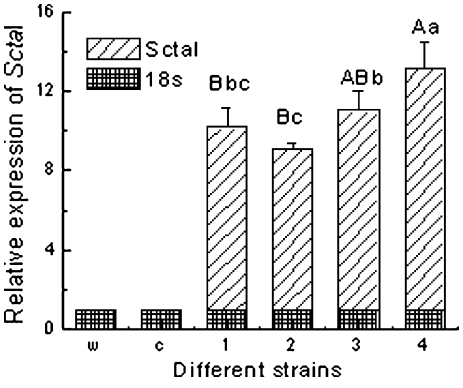

Fig. 4 Real-time quantitative PCR analysis of *sctal* transcripts in the fungal transformants. *Sctal* expression values were normalized against those of 18 s rRNA which was set to *1* *w*, cs28; *c*, cs28pCAM; *1–4*, cs28pCAM-Sctal1, cs28pCAM-Sctal2, cs28pCAM-Sctal3, cs28pCAM-Sctal4. Each value represents the mean±SD from three independent replicates. *Different letters above the columns* indicate a significant difference determined by Duncan's multiple comparisons test (P<0.01)

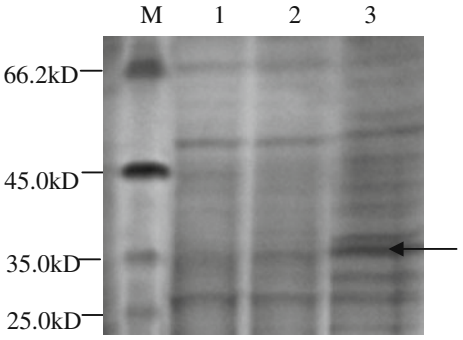

Fig. 5 SDS-PAGE analysis of recombinant TAL. *M* protein marker; *1*, *2*, *3* intracellular total protein of wild-type strain cs28, control transformant cs28pCAM and transformant cs28pCAM-Sctal4, respectively. The *arrow* shows the position of recombinant TAL

0.01; Table 2). The biomass of the transformant cs28pCAM-Sctal4 was decreased compared with wild strain cs28.

The ethanol yield was significantly different between the cs28pCAM-Sctal4 transformant (0.414 gg^{-1}) and the wild-type strain cs28 (0.321 gg^{-1}) using xylose (df=2, f=226.81 ($p<0.01$)). The ethanol yields of cs28pCAM-Sctal4 improved by 28.83% on xylose fermentation (Table 2), and ethanol yield toward the theoretical vale was 81%. A significant difference in acetic acid yields between the transformant cs28pCAM-Sctal4 and cs28 was observed (df=2, f=126.65 ($p<0.01$)). The acetic acid yields of cs28pCAM-Sctal4 increased by 16.22% (Table 2).

SSF Dynamics of Recombinant Fungus

The SSF experiment was conducted using the recombinant fungus cs28pCAM-Sctal4 strain. A type growth associated enzyme production time course was found during the growth phase. The cellulolytic and hemicellulytic enzyme activities of the recombinant fungus cs28pCAM-Sctal4 were higher than that of the wild type cs28 as shown in Fig. 7.

Ethanol production of cs28pCAM-Sctal4 was somewhat increased in comparison with the wild type strain cs28. Similarly, acetic acid yield also improved during the SSF process

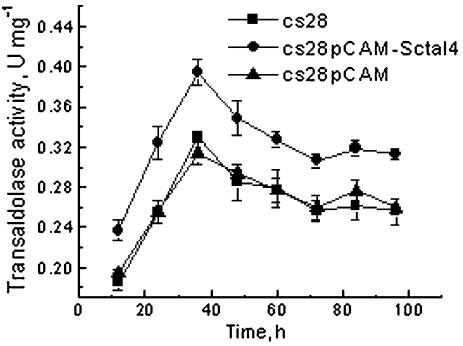

Fig. 6 Transaldolase activity of the recombinant fungus. Biomass of the recombinant fungus was used for measuring transaldolase activity. The enzyme storage buffer was substituted for enzyme solution as a control. The transformants were cultured on xylose at 30°C for 12–96 h, at 12 h intervals. The enzyme activity at different times was measured. *Filled square* transaldolase activity of wild strain cs28, *filled circle* transaldolase activity of cs28pCAM-Sctal4, *filled triangle* transaldolase activity of cs28pCAM. Data represent the mean±standard deviation of triplicate individual experiments

Table 2 Ethanol, acetic acid production, and biomass from xylose by recombinant fungus

Strains	Ethanol yield (g g^{-1})	% Theoretical yield	Acetic acid yield (g g^{-1})	Dried biomass (g g^{-1})
cs28	0.32147	63	0.232	0.286
cs28pCAM-Sctal4	0.41415	81	0.27	0.269

as shown in Fig. 8. The ethanol and acetic acid yields were 0.25 and 0.16 g g^{-1} of rice straw, respectively.

Discussion

F. oxysporum is a promising microorganism that is capable of a direct conversion of cellulose to ethanol. Besides cellulases, xylanases produced by *F. oxysporum* have been also characterized; moreover it produces high β-glucosidase and α-L-arabinofuranosidase activity during hydrolysis [1, 31, 32]. The production of cellulolytic and xylanolytic enzymes by *F. oxysporum* grown on different lignocelluloses substrates like corn stover [33], rice chaff [34], sugar beet pulp [35], sugarcane bagasse, and sawdust [36] has been reported. In the same way, the capability of this microorganism to transform both hexoses and pentoses into ethanol has been established [2, 37]. However, ethanol production was lower and byproducts like xylitol and acetic acid may also been formed in the fermentation process [38]. The genetic engineering method could be applied to *F. oxysporum* for improvement of ethanol yield. *A.*

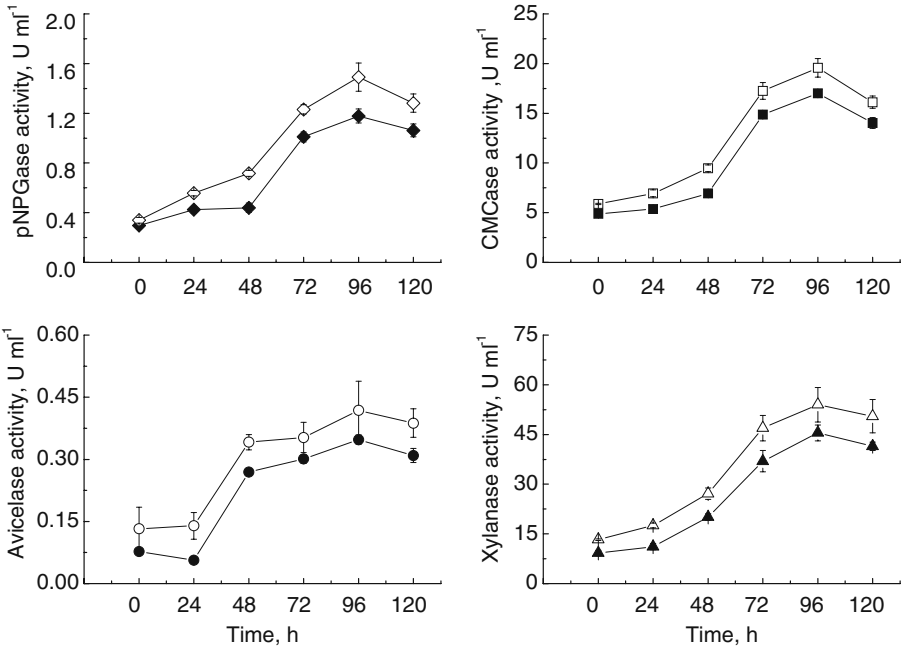

Fig. 7 Time course of cellulase and xylanase production for cs28pCAM-Sctal4 (*open symbols*) and cs28 (*closed symbols*) in batch cultures using 20 g l^{-1} pretreated rice straw as carbon source

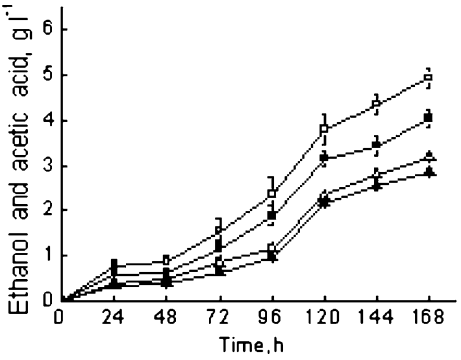

Fig. 8 Time courses of ethanol and acetic acid production of cs28pCAM-Sctal4 (*open symbols*) and cs28 (*closed symbols*) during the SSF. *Empty square* ethanol, *empty triangle* acetic acid. Data represent the mean±standard deviation of triplicate individual experiments

tumefaciens was recently developed for filamentous fungi transformation, such as *Fusarium circinatum*, *Mycosphaerella grominicola*, and *Aspergillus awamori* [18, 39, 40]. The molecular analysis of transformants demonstrated the random integration of single copy of the T-DNA into the *M. grominicola* and *A. awamori* [39, 40] genome and transformation resulted in hygromycin B-resistant clones, which were mitotically stable [41]. The same phenomenon has previously been observed for transformants of *F. oxysporum* (Fig. 3; Table 1). The cauliflower mosaic virus CaMV35S promoter is a constitutive promoter involved in regulation of gene expression when the host grows. However, the metabolites tend to slow down as the strain ages. Transaldolase gene is similarly a constitutive gene in *F. oxysporum*. So, the TAL enzyme activity from the transformant would show the same tendency as that of the wild-type strain(Fig. 6). This CaMV35S promoter has most commonly been used in plant transformation studies [42]. It has also conferred strong gene expression in different fungi, for example, *Ganoderma lucidum* and *Pleurotus citrinopileatus* [43]. In this study, the CaMV35S promoter played a strong role to sc*tal* expression in *F. oxysporum* (Figs. 4, 5, 6). This confirmed that the CaMV35S promoter could function effectively in dicot plant and fungi to promote gene expression.

Meinander et al. [44] reported a significant decrease in the biomass on glucose medium after overexpression of *sctal* in *S. cerevisiae*. We also found that the biomass of cs28pCAM-Sctal4 was lower than that of the cs28 on xylose medium. Overexpression of *tal* in *S. cerevisiae* did not improve the ethanol yield in S103-TAL strain, xylose was used for growth and xylitol formation, but not for ethanol production [15]. In this study, the ethanol yield from xylose fermentation improved by 28.83% in the transformant cs28pCAM-Sctal4 (Table 2). Although overexpression of the *sctal* gene in *F. oxysporum* improved ethanol production, the yield of acetic acid also increased (16.22%; Table 2). This result suggested that the balance of the redox cofactor flux in *F. oxysporum* had been disturbed. A previous study on *F. oxysporum* growth on acetate revealed that acetate consumption could significantly increase the pool of NADH [45].

SSF eliminates the need for complete hydrolysis of carbon substrates prior of the fermentation. In the SSF process, enzymatic hydrolysis, cell growth, and microbial production occur simultaneously. A direct benefit of the SSF is a decrease in the inhibition caused by mono or disaccharide accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs. In addition, the presence of ethanol in the culture medium leads to reduced potential for microbial contamination. *F. oxysporum* DSM 841 produced ethanol and acetic acid with the yield of 0.17 and 0.25 $g g^{-1}$ of Avicel during SSF process, respectively [46]. In this study, the production ethanol and of transformant (0.25 $g\ g^{-1}$; Fig. 8) were higher than that of *F. oxysporum* DSM 841.

In this research, the ethanol production of transformants cs28pCAM-Sctal4 was higher than that of *F. oxysporum* cs28, but the improvement was still low. The main reasons for this might be that the expression levels of *sctal* in *F. oxysporum* was low and acetic acid yield was enhanced during the fermentation process (Table 2 and Fig. 8). To increase the ethanol yield and decrease by-product (acetic acid) formation, the target gene that specifically limits acetic acid production should be investigated further and inverse metabolic engineering technology could potentially be employed. This proposal will be studied in our future work.

In summary, the overexpression of the *tal* gene from *S. cerevisiae* in *F. oxysporum* could improved ethanol production and xylose consumption. Ethanol could also be produced by SSF from diluted acid pretreated rice straw. The recombinant fungus cs28pCAM-Sctal4 could be useful for industrial bioethanol production and application.

Acknowledgments This research was funded by National 863 Research Program (2006AA10Z424) and National Key Technology R&D Program of China during the eleventh Five-Year Plan period (2006BAD07A01).

References

1. Panagiotou, G., Christakopoulos, P., & Olsson, L. (2005). *Enzyme and Microbial Technology, 36*, 693–699. doi:10.1016/j.enzmictec.2004.12.029.
2. Panagiotou, G., Christakopoulos, P., Villas-Boasa, S. G., & Olsson, L. (2005). *Enzyme and Microbial Technology, 36*, 100–106. doi:10.1016/j.enzmictec.2004.07.009.
3. Esperanza, G. G., Isabel, M., & Roncero, G. (2001). *Current Genetics, 40*, 268–275. doi:10.1007/s00294-001-0260-0.
4. Ruiz-Roldán, M. C., Pietro, A. D., & Huertas-González, M. D. (1999). *Molecular & General Genetics, 261*, 530–536. doi:10.1007/s004380050997.
5. Christakopoulos, P., Goodenough, P. W., & Kekos, D. (1994). *European Journal of Biochemistry, 224*, 378–385.
6. Christakopoulos, P., Kekos, D., & Macris, B. J. (1995). *Journal of Biotechnology, 39*, 85–93. doi:10.1016/0168-1656(94)00147-5.
7. Christakopoulos, P., Mamma, D., & Nerinckx, W. (1995). *Archives of Biochemistry and Biophysics, 316*, 428–33.
8. Gianni, P., Dimitris, K., Basil, J. M., & Christakopoulos, P. (2003). *Industrial Crops and Products, 18*, 37–45. doi:10.1016/S0926-6690(03)00018-9.
9. Senac, T., & Hahn-Hägerdal, B. (1991). *Applied and Environmental Microbiology, 57*, 1701–1706.
10. Schaaff, I., Hohmann, S., & Zimmermann, F. K. (1990). *European Journal of Biochemistry, 188*, 597–603.
11. Fiki, A. E., Metabteb, G. E., Bellebna, C., Wartmann, T., Bode, R., Gellissen, G., et al. (2007). *Applied Microbiology and Biotechnology, 74*, 1292–1299. doi:10.1007/s00253-006-0785-8.
12. Soderberg, T., & Alver, R. C. (2004). *Archaea, 1*, 255–262.
13. Sprenger, G. A., Schörken, U., Sprenger, G., & Sahm, H. (1995). *Journal of Bacteriology, 177*, 5930–5936.
14. Pel, H. J., Winde, J. H., Archer, D. B., Dyer, P. S., Hofmann, G., Schaap, P. J., et al. (2007). *Nature Biotechnology, 25*, 221–231. doi:10.1038/nbt1282.
15. Walfridsson, M., Hallborn, J., Penttlä, M., Keränen, S., & Hahn-hägerdal, B. (1995). *Applied and Environmental Microbiology, 61*, 4184–4190.
16. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., & Picataggio, S. (1995). *Science, 267*, 240–243. doi:10.1126/science.267.5195.240.
17. Lazo, G. R., Stein, P. A., & Ludwig, R. A. (1991). *Nature Biotechnology, 9*, 963–967. doi:10.1038/nbt1091-963.
18. Covert, S. F., Kapoor, P., Lee, M. H., Briley, A., & Nairn, C. J. (2001). *Mycological Research, 105*, 259–264. doi:10.1017/S0953756201003872.
19. Hajdukiewicz, P., Svab, Z., & Maliga, P. (1994). *Plant Molecular Biology, 25*, 989–994.
20. Chen, P. Y., Wang, C. K., Song, S. C., & To, K. Y. (2003). *Molecular Breeding, 11*, 287–293. doi:10.1023/A:1023475710642.

21. Mullins, E. D., Chen, X., Romaine, P., Raina, R., Geiser, D. M., & Kang, S. (2001). *Phytopathology, 91*, 173–180.
22. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). *Nucleic Acids Research, 22*, 4673–4680.
23. Kumar, S., Dudley, J., Nei, M., & Tamura, K. (2008). *Briefing in Bioinformatics, 9*, 299–306. doi:10.1093/bib/bbn017.
24. Page, R. D. M. (1996). *Computer Application in the Bioscience, 12*, 357–358.
25. Livak, K. J., & Schmittgen, T. D. (2001). *Methods, 25*, 402–408. doi:10.1006/meth.2001.1262.
26. Wang, W., Tai, F. J., & Chen, S. N. (2008). *Journal of Separation Science, 31*, 2032–2039. doi:10.1002/jssc.200800087.
27. Bradforda, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
28. Zhao, L., Zhang, X., & Tan, T. (2008). *Biomass and Bioenergy*. doi:10.1016/j.biombioe.2008.02.011.
29. Fujii, M., Homma, T., Ooshima, K., & Taniguchi, M. (1991). *Applied Biochemistry and Biotechnology, 28–29*, 145–156. doi:10.1007/BF02922596.
30. Huang, X. M., Yang, Q., Liu, Z. H., Fan, J. X., & Chen, X. L. (2010). *Applied Biochemistry and Biotechnology, 162*, 103–115. doi:10.1007/s12010-009-8700-2.
31. Christakopoulos, P., Mamma, D., Kekos, D., & Macris, B. J. (1999). *World Journal of Microbiology & Biotechnology, 15*, 443–446. doi:10.1023/A:1008936204368.
32. Christakopoulos, P., Katapodis, P., Hatzinikolaou, D. G., Kekos, D., & Macris, B. J. (2000). *Applied Biochemistry and Biotechnology, 87*, 127–133. doi:10.1385/ABAB:87:2:127.
33. Panagiotou, G., Kekos, D., Macris, B. J., & Christakopoulos, P. (2003). *Industrial Crops and Products, 18*, 37–45. doi:10.1016/S0926-6690(03)00018-9.
34. Tao, S., Peng, L., Beihui, L., Deming, L., & Zuohu, L. (1998). *Bioprocess Engineering, 18*, 379–381. doi:10.1007/PL00008997.
35. Cheilas, T., Stoupis, T., Christakopoulos, P., Katapodis, P., Mamma, D., Hatzinikolau, D. G., et al. (2000). *Process Biochemistry, 35*, 557–561. doi:10.1016/S0032-9592(99)00103-X.
36. Pavarina, E. C., & Durrant, L. R. (2002). *Applied Biochemisty and Biotechnology, 98–100*, 663–677. doi:10.1385/ABAB:98-100:1-9:663.
37. Panagiotou, G., Villas-Bôas, S. G., Christakopoulos, P., Nielsen, J., & Olsson, L. (2005). *Journal of Biotechnology, 115*, 425–434. doi:10.1016/j.jbiotec.2004.09.011.
38. Panagiotou, G., & Christakopoulos, P. (2004). *Journal of Bioscience and Bioengineering, 97*, 299–304. doi:10.1016/S1389-1723(04)70209-1.
39. Gouka, R. J., Gerk, C., Hooykaas, J. J., Wouter, C. T., & Groot, J. A. (1999). *Nature Biotechnology, 17*, 598–601. doi:10.1038/9915.
40. Zwiers, L. H., & Waard, M. A. D. (2001). *Current Genetics, 39*, 388–393.
41. Hanif, M., Pardo, A. G., Gorfer, M., & Raudaskoski, M. (2002). *Current Genetics, 41*, 183–188. doi:10.1007/s00294-002-0297-8.
42. Kern, M. F., Maraschin, S. F., Vom Endt, D., Schrank, A., Vainstein, M. H., & Pasquali, G. (2010). *Applied Biochemistry and Biotechnology, 160*. doi:10.1007/s12010-009-8701-1.
43. Sun, L., Cai, H., Xu, W., Hu, Y., & Lin, Z. (2002). *Molecular Biotechnology, 20*, 239–244. doi:10.1385/MB:20:3:239.
44. Meinander, N. Q., Boels, I., & Hahn-Hägerdal, B. (1999). *Bioresource Technology, 68*, 79–87.
45. Panagiotou, G., Pachidou, F., Petroutsos, D., Olsson, L., & Christakopoulos, P. (2008). *Bioresource Technology, 99*, 7397–7401. doi:10.1016/j.biortech.2008.01.017.
46. Kumar, P. K. R., Singh, A., & Schügerl, K. (1991). *Applied Microbiology and Biotechnology, 34*, 570–575. doi:10.1007/BF00167900.