ENZYME SYSTEMS FROM THE THERMOPHILIC FUNGUS *TALAROMYCES EMERSONII* FOR SUGAR BEET BIOCONVERSION

Sara Fernandes,[a+] Patrick G Murray,[a+] and Maria G. Tuohy[a*]

The thermostable enzyme systems produced by the thermophilic ascomycete fungus *Talaromyces emersonii* cultivated on various carbon sources were investigated for the production of high value products from sugar beet. A broad range of enzymatic activities relevant to cellulose, hemicellulose, and pectin hydrolysis were identified in *T. emersonii* culture filtrates. In hydrolysis experiments conducted at 71ºC, the enzyme cocktails generated sugar-rich syrups from untreated sugar beet plants. Maximal levels of sugar beet hydrolysis were obtained with *T. emersonii* enzyme cocktails induced with sorghum/ beet pulp (68%) and sugar beet plant (56%). The principle monosaccharides released were glucose, xylose, and arabinose with minor amounts of galactose and galacturonic acid. Northern analysis of RNA isolated from *T. emersonii* when sugar beet plants were used as the sole carbon inducing source showed that genes required for polysaccharide hydrolysis and five carbon monosaccharide metabolism were co-ordinately expressed.

Keywords: Sugar beet; Hydrolysis; Bioconversion; Talaromyces emersonii

Contact Information: a: Molecular Glycobiotechnology Group, Biochemistry Department, National University of Ireland, Galway, Ireland. [*]*Corresponding Author:maria.tuohy@nuigalway.ie*
[+] *Both authors contributed equally to this work.*

INTRODUCTION

Conversion of biomass to bioethanol is of global importance. Energy production is undergoing a dynamic period of change, driven by increased awareness of the requirement for alternatives to fossil fuels as sole energy sources. Biomass is perhaps the most important renewable energy source in terms of technical and economic feasibility (Ragauskas et al. 2006). It is a local resource that can contribute to the diversification of energy supply and can therefore be considered a major future energy source. Consequently, production of fuel ethanol from renewable lignocellulosic materials has been extensively studied in recent decades (Aristidou and Penttila 2000). Lignocellulosic biomasses represents a diverse and versatile energy source that is widely available from agro-based industries. Biomass comprises types of residues such as beet pulp and bagasse from sugar processing mills, cotton stalks, rice husks, wood industry residues, short rotation energy crops, and forest plantations. Biomass represents a rich source of complex carbohydrates, which can be converted to simple sugars for fermentation to biofuel. Ethanol is traditionally produced by glucose fermenting yeasts; therefore sugar cane and sugar beets, which contain a substantial amount of glucose-containing carbohydrates, could constitute one of the main biomass sources of worldwide ethanol production. However, both the cellulose and hemicellulose fractions of biomass must be utilised to make bioethanol production economically feasible (Wyman 2003).

Sugar beet is an attractive substrate for a biotechnological strategy involving enzyme-catalysed conversion of its complex sugar polymers to high value simple sugar feed stocks. On a zero moisture basis sugar beet contains 78% carbohydrate, consisting of approximately 22-30% cellulose, 24-32% hemicellulose, and 24-32% pectin. Sugar beet has a low lignin content (3-4%), and the degree of crystallinity of the cellulose present is also lower. These factors can limit the action of certain cellulases (Coughlan et al. 1985).

In nature, microorganisms, such as fungi, are the architects of plant biomass hydrolysis. Microorganisms secrete a variety of highly efficient enzyme systems to generate a readily metabolisable carbon source for survival. Enzymatic hydrolysis of complex lignocellulosic material requires multi-component enzyme systems that include cellulases, hemicellulases, and pectin-degrading activities, with different substrate specificities (Tabka et al. 2006). Enzymes, as they are produced by living systems, are expensive to obtain and thus add significantly to production costs in industry. *Talaromyces emersonii* is a thermophilic filamentous fungus that inhabits the soil, decaying masses of plant material in piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for its development (Stolk 1972). *T. emersonii* produces thermostable, highly specific enzymes that work efficiently and have excellent long-term storage properties (Moloney et al. 1983, 1985; Coughlan and Ljungdahl 1988). Adding to the cost-effectiveness of using this fungus as an enzyme factory is the fact that it produces the enzymes of interest extracellularly, secreting them into the growth media, making downstream harvesting of enzymes relatively easy and straightforward. *T. emersonii* thermostable enzyme systems required for cellulose hemicellulose and pectin degradation have been described and characterized previously (McCarthy et al. 2003, 2005; Murray et al. 2001, 2004; Tuohy et al. 1993; Tuohy and Coughlan 1992). High activity temperatures allow shorter reaction times, decreased viscosity of hydrolysate mixtures, and elimination of microbial contamination.

This paper examines the feasibility for using thermostable hydrolytic enzyme blends produced by *T. emersonii* when various carbon sources are used as inducers for bioconversion of untreated sugar beet plants (including tops and stalks) to fermentable sugar syrups. We also examine sugar beet plants as an inducers of genes required for the five-carbon metabolism that is essential for improvement of ethanol-producing microorganisms in the conversion of hydrolysate syrups for the production of bioethanol.

EXPERIMENTAL

Materials

Unless otherwise stated, all reagents and chemicals were of molecular biology grade and were purchased from Sigma Aldrich (Dublin, Ireland).

Fungal Strain and Enzyme Production

T. emersonii (laboratory stock) was routinely subcultured, at 45^{o}C, on Sabouraud Dextrose Agar (SDA) for 2-4 days. Liquid cultures of *Talaromyces emersonii* were

grown at 45°C, pH 4.5, in the mineral salts/inducing medium as described earlier (Moloney et al. 1983). Glucose "starter" cultures were prepared by aseptically inoculating sterile medium containing 2.0% (w/v) glucose (generally 100 ml glucose supplemented nutrient medium per 250 ml Erlenmeyer flask), with 3-4 1 cm^2 pieces of mycelial mat taken from the outer edges of actively growing agar-plate cultures. Glucose starter cultures were grown as described above for 24 h and used to inoculate larger volumes of medium containing 2% glucose, which were grown for a further 24 h. These cultures were then used to seed 2 L flasks containing various inducers and combinations thereof, sorghum, beet pulp, wheat bran, rye flakes, tea leaves, and sugar beet as described elsewhere (Tuohy and Coughlan 1992). The cultures were allowed to grow as described previously for 120 h, after which the liquid filtrate was collected by filtration through fine grade muslin, and centrifuged at 6000g for 30 min. at 4°C. Crude extracts were stored at 4°C or 20°C until required further use. Mycelia were washed with cold sterile distilled water and stored at –70°C.

Enzyme Activity Assays

Enzyme activities, unless otherwise stated, were determined at 50ºC in 50 mM ammonium acetate buffer pH 5.0. The hydrolysis of CM-cellulose (6% (w/v); 10 min), barley β-glucan (1% (w/v); 10 min), oats spelt xylan (1% (w/v); 5 min), pectic galactan (1% (w/v); 10 min), debranched arabinan (1% (w/v); 5 min), 10 min), polygalacturonic acid (1% (w/v); 15 min), laminarin (1% (w/v); 15 min), citrus pectin (1% (w/v); 15 min), and rhamnogalacturonan (1% (w/v); 15 min); 15 min) were measured as reducing sugars released by the DNS method (Miller 1959), using appropriate standards. Total cellulase activity was determined, using Whatman No 1 filter paper as the assay substrate (Wood and Bhat 1988). Exo-acting enzyme activities were measured using the appropriate 1 mM 4-nitrophenyl-α or β-Glycosides as substrate (Murray et al. 2004). Increase in absorbance at 410 nm on release of the Nitrophenolate ion, following incubation of the enzyme with the appropriate substrate for 15 min at 50°C (the stopping reagent was sodium carbonate) was monitored spectrophotometrically. All assays were preformed in triplicate and results presented as the mean value. The protein content of crude extracellular extracts was determined by the Bensadoun and Weinstein modification of the method of Lowry and co-workers (Bensadoun and Weinstein 1976; Lowry et al. 1951) using Bovine Serum Albumin (BSA, fraction V) as a standard.

Sugar Beet Hydrolysis Experiments

Total sugar beet plant (including tops and stalks) was dried for 24 h at 70°C and homogenised in a blender to a fine powder to increase surface area for hydrolysis. Dried sugar beet (1 g) was incubated with enzyme cocktails (0.5 mg total protein) in 50 mM ammonium acetate buffer pH 4.5 at 71ºC in a total volume of 10 ml for 12 h in a shaking incubator (100 rpm). Samples were then centrifuged at 13000 rpm for 15 min and supernatant removed. Pellet was treated again under identical conditions for a further 12 h to see if further levels of sugars could be released. Hydrolysis reactions were terminated by boiling. Samples were stored at -20ºC for further analysis. The total sugar beet percentage hydrolysis values are calculated from a 78% carbohydrate content in sugar beet on a zero moisture basis (Coughlan et al. 1985).

Sugar Analysis

Total soluble carbohydrates were analysed by high performance ion exchange chromatography on an ICS-3000 Ion Chromatography System from Dionex Corporation, Sunnyvale California USA. Products of hydrolysis and standard sugars were fractionated on a CarboPac PA-10 column using a decreasing 200-18 mM NaOH gradient operating at a flow rate of 1 ml/min at ambient temperature. An ED40 electrochemical detector, in the integrated amperometry mode, facilitated detection of eluting product peaks. Data were collected and processed using Chromelean™ Version 6.70 software. Products were positively identified by spiking samples with standard sugars.

RNA Extraction and Northern Analysis

RNA from fungal cells was extracted according to the methods outlined by Chomozynski and Sacchi (1987), and Northern blotting was performed according to established techniques (Sambrook et al. 1989). Expression analyses were conducted using digoxygenin (Dig) labelled probes amplified from *T. emersonii* chromosomal DNA, using degenerate primers designed against existing carbohydrase and five-carbon metabolic gene sequences present in the National Centre Biotechnology Information database. Detection was carried out using the Digoxygenin detection kit from Roche Applied Science, Burgess Hill, West Sussex, UK.

RESULTS AND DISCUSSION

Enzyme Production

Talaromyces emersonii was grown on five different inducing substrates for 120h. The results in Figure 1 (A-C) showed that different batteries of key polysaccharide-hydrolysing enzyme activities were produced and that cellulase, hemicellulase, and pectinase production is dependent on the inducing substrate. All the key enzymes required for cellulose degradation, cellobiohydrolase I and II (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), endoglucanase (EC 3.2.1.4), were produced on all the substrates. The highest level of filter paper activity (4.36 IU/ml) was exhibited when a combination of sorghum and beet pulp (cocktail 1) was used as the inducing carbon source (Fig. 1 (A)). In addition, Cocktail 1 contained the highest levels of CMCase (2.3 IU/ml) and β-glucosidase (1.8 IU/ml) activities (Fig. 1 (A)). The natural sugar beet pathogen *Sclerotuim rolfsii* produced cellulase activities, with maximal CMCase and β-glucosidase activities of approximately 4 U/ml and 0.7 U/ml, respectively (Moussa and Tharwat 2007).

T. emersonii produced a broad range of hemicellulose-degrading enzymes (Fig. 1 (B)) and substrates rich in hemicellulose influenced enzyme production. Hemicellulase activities were maximal in cocktails 1, 4, and 5. Cocktails 1 and 4 contained the highest levels of xylanase (10.92 IU/ml and 11.22 IU/ml) and β-1,3-(4)-glucanase (12.46 IU/ml and 15.60 IU/ml), while cocktail 5 displayed the highest levels of β-xylosidase, α-galactosidase, and α-arabinofuranosidase (10.74, 11.48 and 15.59 IU/ml, respectively). Similar studies using *Thermomyces lanuginosus* strain (IMI 158749) revealed higher levels of main-chain endo-acting xylanase activity but much lower levels of side-chain

exo-acting β-xylosidase activity when induced by xylan (Puchart et al. 1999) than those produced by *T. emersonii* in this study.

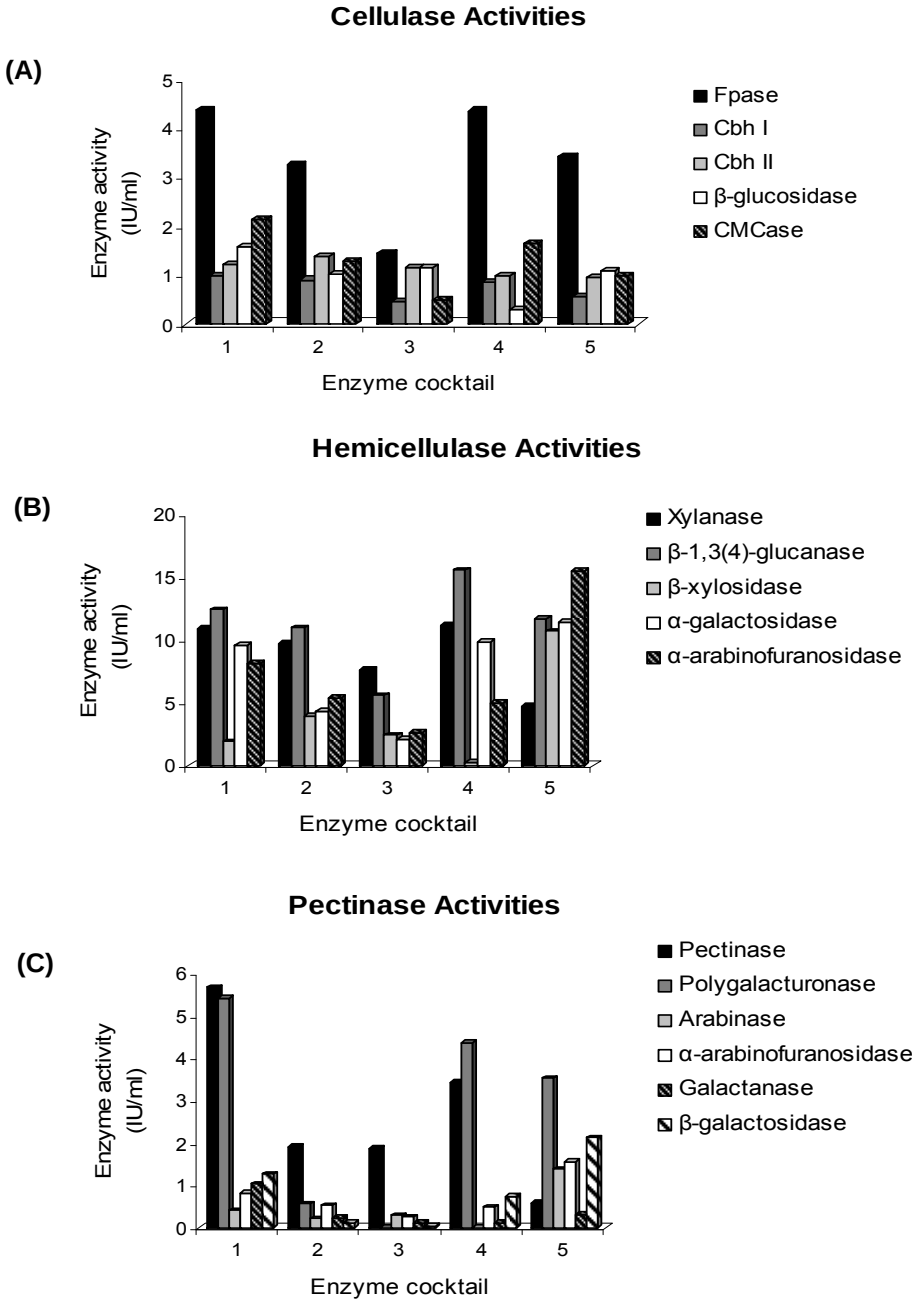

Fig. 1. Enzyme activities produced by *T. emersonii* cultured in sorghum / beet pulp (1), wheat bran / beet pulp (2), rye flakes / wheat bran (3), beet pulp / tea leaves (4), and sugar beet (5). Levels of enzymes responsible for cellulose, hemicellulose, and pectin degradation are represented by (A), (B), and (C), respectively.

Sugar beet is a well-known potential source of pectins (24-32% pectic poly-saccharides) (Coughlan et al. 1985). Therefore, the main pectin-degrading enzymes were found in the cocktails where sugar beet or sugar beet pulp was present as a carbon source (Fig. 1 (C)). Highest levels of pectinase and polygalacturonase activities (5.66 IU/ml and 5.42 IU/ml) were detected in cocktail 1, while cocktail 5 contained the highest arabinase (1.38 IU/ml), α-arabinofuranosidase (1.58 IU/ml), and β-galactosidase (2.12 IU/ml) levels. The enzymatic levels reported in other studies, using different microorganisms and various lignocellulosic substrates, indicate that enzymatic levels obtained in this study were comparable and in some cases superior to those cited in the literature.

Hydrolysis Studies

The efficiency of enzymatic hydrolysis depends on the diversity of enzymes that are required to break down the substrates to soluble sugars. Because cocktail 1 and 5 contained the highest levels of a broad range of cellulase, hemicellulase, and pectinase activities, the performance of these cocktails was investigated in terms of relative amounts of sugar released from sugar beet. Previously, the optimum pH value for saccharification of beet pulp was found to be 4.8, while the optimal temperature of a *T. emersonii* enzyme system was found to be 50-80ºC (Considine et al. 1988; Moloney et al. 1984). Therefore, sugar beet plant, with no chemical pre-treatment, was incubated with cocktail 1 and 5 for 24 h at 71ºC, pH 4.5. Initial hydrolysis, after 12 h of incubation, yielded a high concentration of sucrose as well as glucose, xylose, but relatively little arabinose in hydrolysates. The high amount of sucrose released is in agreement with studies conducted by Ogbonna *et al*. (2001); sugar beet juice has been shown to contain 165 g of sucrose/kg sugar beet. Sucrose can be used as a fermentation substrate for production of ethanol (Atiyeh and Duvnjak 2002), i.e. all components of the sugar beet plant can be utilized rather than selected fractions. The high levels of sucrose in this study in initial sugar beet extract may affect arabinose release. It has been reported previously that concentrations of sucrose can reduce the concentration of water, which may lower the rate of hydrolysis by enzymes responsible for arabinose release (Dixon and Webb 1964). In order to investigate if sucrose was affecting the amount of arabinose released, sugar beet hydrolysate was centrifuged to remove residual sucrose and re-treated under identical conditions for a further 12 h. The main monosaccharides produced were arabinose, glucose, xylose, and a small amount of polygalacturonic acid. The polygalacturonases present in the enzymatic systems released little galacturonic acid, probably due to the lack of other pectinolytic accessory activities such as pectin methylesterase, pectin acetylesterase, or pectin lyase (Micard et al. 1996). The amount of monosaccharides released from 1 g of sugar beet was: 255.5 mg of glucose, 47.0 mg of galactose, 108.1 mg of xylose, 103.4 mg of arabinose, and 22.19 mg of galacturonic acid, when sugar beet was incubated with cocktail 1. With cocktail 5, the amount of each monosaccharide was as follows: 213.7 of glucose, 24.3 mg of galactose, 112.6 mg of xylose, 40.3 mg arabinose, and 42.85 mg of galacturonic acid (Table 1). The different amounts of arabinose released by the two cocktails may be due to lower levels of other enzymes in cocktail 5 responsible for the breakdown of arabinan polymers, or the substrate specificity of the arabinose-releasing enzymes in cocktail 5. The yields of total sugars obtained using both cocktails were comparable to those released by enzymatic

hydrolysis of sugar beet pulp (Coughlan 1985; Spagnuolo et al. 1997), with the exception that xylose levels were higher in this study. However, Morroccan sugar beet roots have a higher percentage of xylose in the hemicelluloses isolated from this source (Fares et al. 2004). The difference between our results and those reported earlier could be due to the fact that the relative abundance and distribution of each of the individual components in the plant material varies according to the season of harvest, maturity, and local climatic conditions, or the expression of different xylanolytic enzymes.

Because sugar beet must be considered a complex substrate, it can be assumed that monosaccharide production is due to degradation of cellulosic, hemicellulosic, and pectic components. As both cocktails efficiently degraded the sugar beet plant polymers, this demonstrates the potential of the *T. emersonii* enzymes to generate monosaccharide-rich hydrolysates. The enzyme cocktails 1 and 5 catalysed 68 and 56% hydrolysis, respectively, of sugar beet after 24 h incubation at 71ºC and pH 4.5. Increasing the temperature of hydrolysis to higher values, with the same enzyme dosage and pH, did not result in any further increase in sugar beet hydrolysis (data not shown).

Table 1. Monosaccharides Yields (mg) from Sugar Beet Plants Hydrolysed with Cocktails 1 and 5.

Monosaccharides (mg)	Cocktail 1	Cocktail 5
Glucose	255.5	213.7
Galactose	47.0	24.3
Xylose	108.1	112.6
Arabinose	103.4	40.3
Galacturonic acid	22.19	42.85

Northern Analysis of *T. emersonii* Gene Expression Relevant to Sugar Beet Hydrolysis and Five-carbon Metabolism

To obtain more information regarding the observed induction of cellulose- and hemicellulose-degrading enzymes, Northern analysis studies were conducted. To produce enzyme cocktails with optimal hydrolytic potential, it is important to understand how the fungus regulates carbohydrase induction and repression. The expression of four key cellulase-encoding genes, *cbh1* (encoding cellobiohydrolase I), *cbh2* (encoding cellobiohydrolase II), *eg1* (encoding endoglucanase I), and *cel3a* (encoding β-glucosidase cel3a), as well as two key xylanase encoding genes; *bxl1* (which encodes β-xylosidase I) and *xyn* (encoding xylanase) during cultivation of *T. emersonii* on sorghum / beet pulp and sugar beet, at various time points is represented in Figure 3 (A). Initial transcripts of target hydrolases were low but increased to a maximum at 72 h and 100 h when *T. emersonii* was cultured on sorghum / beet pulp and sugar beet respectively. This temporal induction can be explained by the presence of small amounts of free glucose present in the sugar beet plant prior to hydrolysis (HPLC analysis data not shown). Free glucose inhibits transcription of hydrolytic enzymes (Kubicek and Penttilä 1998) and would be metabolised first before induction of transcription of hydrolytic genes, resulting in later production of the enzymes involved in polymers degradation.

Because the main sugars released from the enzymatic hydrolysis of sugar beet in this study were glucose, xylose, and arabinose, the economic utilisation of sugar beet hydrolysate syrups for the production of bioethanol requires simultaneous fermentation of both hexose and pentose sugars under large-scale industrial conditions. Several industrial microorganisms can readily utilize hexose sugars; however, efficient pentose-utilizing organisms are less common. For example, the yeast *Saccharomyces cerevisiae*, one of the most well-characterized organisms capable of producing ethanol from hexose sugars, does not naturally ferment pentoses (Jeffries 2006). It has been recognised that genetic engineering of naturally fermenting microorganisms such as *S. cerevisiae* is required for transport and efficient bioconversion of pentose sugars to bioethanol. Pathways for pentose sugars metabolism (Figure 2) are thus relevant for microorganisms living on decaying plant material as well as in biotechnology when cheap raw materials such plant hydrolysates are to be fermented to ethanol.

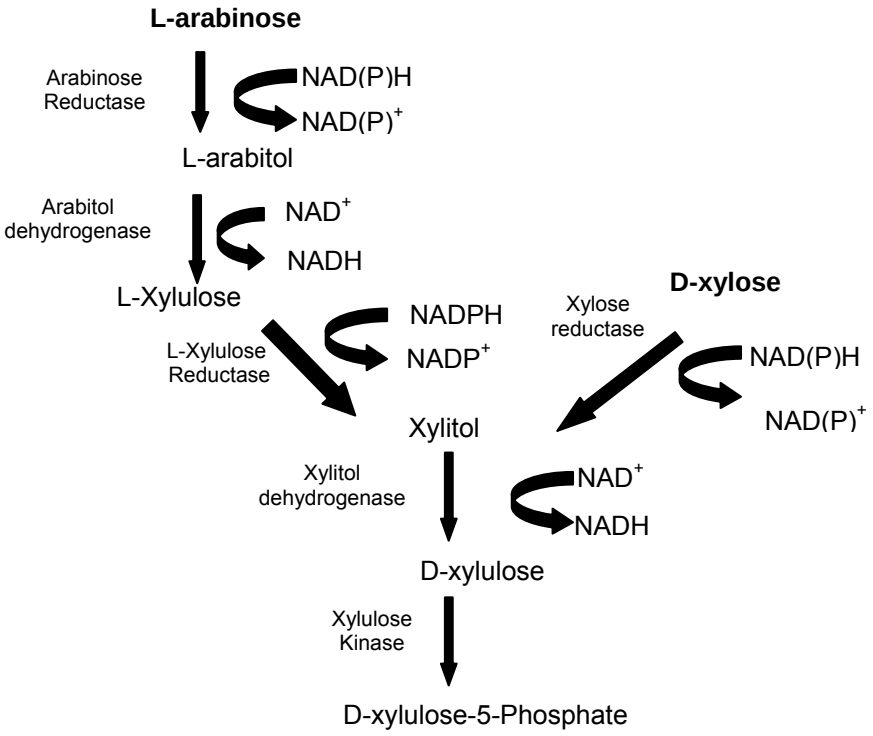

Fig. 2. Fungal pentose pathway

As *T. emersonii* grows equally well on five- and six-carbon sugars as sole carbon sources, the expression of genes relevant to pentose metabolism was investigated when sugar beet was used as carbon source. As illustrated in Fig. 3 (B), the sugar beet plant is an inducer of five-carbon metabolism, since genes encoding xylose reductase (*XR*), xylitol dehydrogenase (*XDH*), L-arabinitol dehydrogenase (*ADH*), L-xylulose reductase (*LXR*), and xylulose kinase (*XK*) were induced by sugar beet after 36 h of growth.

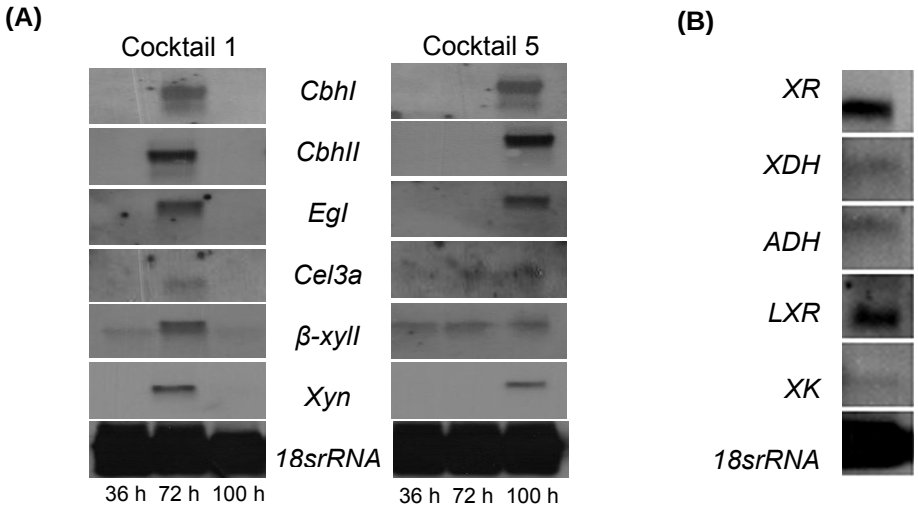

Fig. 3. (A) Northern analysis of *T. emersonii* cellulase and xylanase gene expression after 36, 72, and 100 h of growth in sorghum / beet pulp (cocktail 1) and sugar beet (cocktail 5); (B) expression of genes involved in pentose metabolism induced by sugar beet after 36 h.

Novel technologies for the production of alternative fuels, including the engineering of yeasts for commercial fermentation of pentose sugars to ethanol, are receiving increased attention. Therefore, isolation of genes from microbial sources such as *T. emersonii* capable of efficiently metabolising pentose sugars is very important to improve biotransformation of sugar rich syrups generated from sugar beet, through genetic engineering of naturally fermenting microorganisms such as *S. cerevisiae*.

Presently, due to the importance of biomass ethanol to combat increased crude oil costs, there is a significant interest in biomass-degrading enzymes. Nevertheless, there is a need to decrease enzyme production costs and to produce new biocatalysts showing higher temperature stability. Preliminary investigations of enzyme systems produced by *Talaromyces emersonii* in this work demonstrate increased temperature activities relative to enzyme systems from other fungal sources and should lead to an overall decrease in the cost of enzymatic monosaccharide production from complex biomass sources. Further studies on characterisation of the individual enzymatic components of the enzyme systems should lead to more efficient substrate specific enzyme cocktails for biomass hydrolysis.

CONCLUSIONS

1. The thermostability and economical production of *T. emersonii* enzyme cocktails demonstrated in this study indicate the potential of this organism to rival the established fungal producers of biotechnological enzymes, i.e. species of *Aspergillus*, *Penicillium*, and *Trichoderma*.
2. Expression analysis of enzymes required for biomass conversion in combination with the enzyme screening study conducted has clearly demonstrated the ability of *T. emersonii* to produce an array of hydrolytic activities required for efficient

bioconversion of plant material to high-value products such as simple monosaccharide sugars. The hydrolytic potential of the enzyme systems was also evaluated, and the significant percentage values for conversion of sugar beet polymers to simple sugars indicate that sugar beet in combination with *T. emersonii* enzyme systems has potential in a biotechnology strategy to generate sugar-rich feedstocks.

3. Apart from being an attractive substrate for a bioethanol production strategy, sugar beet also induced all genes relevant to five-carbon metabolism in *T. emersonii*. The screen conducted provides evidence for a coordinated expression of the hydrolytic and metabolic genes required for efficient utilisation of a complex biomass source by this fungus.

ACKNOWLEDGMENTS

This work was funded by Enterprise Ireland and supported under the National development Plan 2000-2006, which was part funded by the European Regional development fund.

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Article submitted: November 15, 2007; Peer-review completed: June 9, 2008; Revised version received and accepted: July 30, 2008; Published Aug. 1, 2008.