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Expression and evaluation of enzymes required for the hydrolysis of galactomannan

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Abstract The cost-effective production of bioethanol from lignocellulose requires the complete conversion of plant biomass, which contains up to 30 % mannan. To ensure utilisation of galactomannan during consolidated bioprocessing, heterologous production of mannan-degrading enzymes in fungal hosts was explored. The *Aspergillus aculeatus* endo-β-mannanase (*Man1*) and *Talaromyces emersonii* α-galactosidase (*Agal*) genes were expressed in *Saccharomyces cerevisiae* Y294, and the *Aspergillus niger* β-mannosidase (*cMndA*) and synthetic *Cellvibrio mixtus* β-mannosidase (*Man5A*) genes in *A. niger*. Maximum enzyme activity for Man1 (374 nkat ml^{-1}, pH 5.47), Agal (135 nkat ml^{-1}, pH 2.37), cMndA (12 nkat ml^{-1}, pH 3.40) and Man5A (8 nkat ml^{-1}, pH 3.40) was observed between 60 and 70 °C. Co-expression of the *Man1* and *Agal* genes in *S. cerevisiae* Y294[Agal-Man1] reduced the extracellular activity relative to individual expression of the respective genes. However, the combined action of crude Man1, Agal and Man5A enzyme preparations significantly decreased the viscosity of galactomannan in locust bean gum, confirming hydrolysis thereof. Furthermore, when complemented with exogenous Man5A, *S. cerevisiae* Y294[Agal-Man1] produced 56 % of the theoretical ethanol yield, corresponding to a 66 % carbohydrate conversion, on 5 g l^{-1} mannose and 10 g l^{-1} locust bean gum.

Keywords *Aspergillus niger* · *Saccharomyces cerevisiae* · β-Mannanase · β-Mannosidase · α-Galactosidase; galactomannan

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Introduction

The abundance and sustainable production of lignocellulosic plant material offers an attractive alternative to the use of fossil fuel reserves for the production of transport fuels [13]. However, the cost-effective production of biofuels from lignocellulosics requires the complete hydrolysis of the plant material, which typically consists of 40–45 % cellulose, 25–50 % hemicellulose and 10–40 % lignin [16]. Mannan and xylan constitute the major hemicellulosic components. Softwoods contain 15–20 % (w/w) mannan with some spruce species containing up to 25 % [19]. Legume seeds can contain more than 30 % mannan per dry weight [6].

Full utilisation of plant biomass requires effective hydrolysis of the different polysaccharides to fermentable sugars. *Saccharomyces cerevisiae* is effective for bioethanol production from simple sugars, including glucose and mannose [11], but does not produce the enzymes required for galactomannan hydrolysis. If both mannan hydrolysis and fermentation can be performed by a single organism (e.g. a *S. cerevisiae* strain expressing the required enzymes), it will make a significant contribution to the development of strains for consolidated bioprocessing (CBP) of lignocellulosic plant material. Successful expression of a variety of recombinant cellulases and hemicellulases in *S. cerevisiae* has demonstrated the potential of CBP for bioethanol production from cellulosic feedstock [27], but effective enzymes for mannan hydrolysis have been elusive.

Mannan is classified as glucomannan, galactomannan and galactoglucomannan depending on its composition. Complete hydrolysis of mannan requires the combined effort of several enzymes, such as β-mannanases (1,4-β-D-mannan mannohydrolases, EC 3.2.1.78), β-mannosidases (1,4-β-D-mannopyranoside hydrolases, EC 3.2.1.25), α-galactosidases

(1,6-α-galactoside galactohydrolases, EC 3.2.1.22), β-glucosidases (1,4-β-D-glucoside glucohydrolases, EC 3.2.1.21) and acetyl-mannan esterases (EC 3.1.1.6) [16]. The endo-β-1,4-mannanase cleaves the β-1,4-mannopyranosyl linkages in the mannan backbone, producing oligosaccharides of varying lengths [26]. Hydrolysis of the oligomannans is performed by β-mannosidase, releasing mannose units [16]. In turn, α-galactosidases, β-glucosidases and acetyl-mannan esterases catalyse the removal of galactose, glucose and acetic acid, respectively, from the mannan chain [15, 16].

Enzymes involved in mannan hydrolysis have been identified and characterised in various fungi, in particular in *Aspergillus* strains [28]. Aspergilli are versatile organisms with the ability to grow on inexpensive substrates, such as agricultural waste [3] and are known for the production of various hydrolytic enzymes. A number of enzymes involved in mannan degradation have been cloned and expressed in foreign hosts [28], with expression of the *Aspergillus aculeatus Man1* in *Yarrowia lipolytica* [21] yielding 26139 nkat ml^{-1} in fed batch cultivation.

In this study, a recombinant *S. cerevisiae* strain was constructed to degrade mannan through expression of the *A. aculeatus* β-mannanase (*Man1*, GH5) and *Talaromyces emersonii* α-galactosidase (*Agal,* GH27). The *A. niger* and *Cellvibrio mixtus* β-mannosidases (*cMndA* (GH2) and *Man5A* (GH5), respectively) were expressed as functional enzymes in *A. niger* D15 and evaluated for mannan hydrolysis in combination with Man1 and Agal. The Man5A and Agal exhibit activity on oligo and polymeric substrates, making them the ideal candidates for plant biomass hydrolysis [9, 24]. The enzymes were partially characterised and evaluated on locust bean gum as model substrate. The *S. cerevisiae* Y294[Agal-Man1] strain demonstrated the conversion of mannan to ethanol under oxygen-limited conditions in the presence of exogenous β-mannosidase. In principle, the use of the recombinant strain could potentially reduce the need for adding external enzymes.

Materials and methods

Media and cultivation

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). *Escherichia coli* DH5α strains were cultured at 37 °C in Terrific Broth (12 g l^{-1} tryptone, 24 g l^{-1} yeast extract, 4 ml l^{-1} glycerol, 0.1 M phosphate buffer) containing 100 μg ml^{-1} ampicillin [22].

The *S. cerevisiae* Y294 host strain was maintained on YPD agar plates (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} glucose) and transformants were selected and maintained on SC^{-URA} agar plates containing 6.7 g l^{-1} yeast nitrogen base without amino acids [Difco Laboratories], 20 g l^{-1} glucose and yeast synthetic drop-out medium supplements [Sigma-Aldrich (Germany)]. Aerobic cultivation of *S.cerevisiae* strains was performed on a rotary shaker (200 rpm) at 30 °C in 125 ml Erlenmeyer flasks containing 25 ml double strength SC^{-URA} medium (2 × SC^{-URA}), 13.4 g l^{-1} yeast nitrogen base without amino acids [Difco Laboratories], 20 g l^{-1} glucose and supplemented with yeast synthetic drop-out medium supplements.

The *A. niger* D15 host strain was maintained on spore plates [18]. For heterologous protein expression, the *A. niger* fungal strains were cultivated in double strength minimal media (2 × MM, with 100 g l^{-1}glucose, lacking uridine) [20].

Strains and plasmids

The relevant yeast, fungal and bacterial genotypes and plasmids used in this study are listed in Table 1 and the final vector constructs are shown in Fig. 1.

DNA manipulations and plasmid construction

Standard protocols were followed for DNA manipulation [22] with enzymes for restriction digests and ligations sourced from Roche applied science (Germany) and used as recommended by the supplier. The *Agal* (Accession nr EU106878) and *Man1* (Accession nr L35487) genes were subcloned from pMA-RQ-Agal (Table 1) and pBluescript-Man1 [23] and cloned onto yBBH1, resulting in yBBH1-Agal and yBBH1-Man1, respectively (Fig. 1a). The $ENO1_P$-*Agal*-$ENO1_T$ cassette was excised from yBBH1-Agal with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of yBBH1-Man1, yielding yBBH1-Agal-Man1 (Fig. 1b). The synthetically designed *C. mixtus* β-mannosidase (*Man5A*) (Accession nr AY526725) was codon-optimised (GeneArt®) for expression in *S. cerevisiae* and the truncated *Man5A* gene (without a secretion signal) was cloned in frame with the *XYNSEC* coding region onto pGTP2, generating pGTP2-Man5A (Fig. 1c).

The cDNA and genomic copy of the mannosidase gene of *A. niger* (designated *cMndA* and *gMndA*, respectively) were cloned from the *A. niger* 10864 strain. The strain was grown in minimal media for 72 h, the mycelia harvested and total nucleic acid isolated according to La Grange et al. [14]. Total cDNA was generated using the RevertAid™ H Minus First Strand cDNA Synthesis Kit and OligoT primer (Fermentas) and used for amplification of the 2.8-kb *cMndA* gene (Accession nr XM_001394595) using the GeneAmp® PCR system 9700 (Applied biosystems), TaKaRa™ Ex Taq™ Polymerase (TaKaRa Bio Inc.) and primers AnmndA-R (5′-TAGGCGCGCCTGCGAATGCTA

Table 1 Strains and plasmids used in this study

Yeast strains		
S. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	ATCC 201160
S. cerevisiae Y294[BBH1]	*URA3 $ENO1_P$-$ENO1_T$*	[17]
S. cerevisiae Y294[BBH4]	*URA3 $ENO1_P$-XYNSEC-$ENO1_T$*	[17]
S. cerevisiae Y294[Agal-Man1]	*URA3 $ENO1_P$-Agal-$ENO1_T$*; *$ENO1_P$-Man1-$ENO1_T$*	This study
S. cerevisiae Y294[Man1]	*URA3 $ENO1_P$-Man1-$ENO1_T$*	This study
S. cerevisiae Y294[Agal]	*URA3 $ENO1_P$-Agal-$ENO1_T$*	This study
Fungal strains		
A. niger 10864	Wild type	ATCC 10864
A. niger D15#26	*cspA1 pyrG1 prtT13 phmA* (non-acidifying)	[12]
A. niger D15[GTP2]	*$pyrG^+$ gpd_P-$glaA_T$*	This laboratory
A. niger D15[Man5A]	*$pyrG^+$ gpd_P-Man5A-$glaA_T$*	This study
A. niger D15[MndA]	*$pyrG^+$ gpd_P-MndA-$glaA_T$*	This study
A. niger D15[cMndA]	*$pyrG^+$ gpd_P-cMndA-$glaA_T$*	This study
Bacterial strains		
E. coli DH5α	*fhuA2Δ(argF-lacZ)U169 phoAV44Φ80 Δ(lacZ) M15gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	[22]
Plasmids		
yBBH1	*bla URA3 $ENO1_P$-$ENO1_T$*	[17]
yBBH4	*bla URA3 $ENO1_P$-XYNSEC-$ENO1_T$*	[17]
yBBH1-Man1	*bla URA3 $ENO1_P$-Man1-$ENO1_T$*	This laboratory
yBBH1-Agal-Man1	*bla URA3 $ENO1_P$-Agal-$ENO1_T$*; *$ENO1_P$-Man1-$ENO1_T$*	This study
yBBH1-Agal	*bla URA3 $ENO1_P$-Agal-$ENO1_T$*	This study
yBBH4-XYNSEC-Man5A	*bla URA3 $ENO1_P$-XYNSEC-Man5A-$ENO1_T$*	This study
pGTP2	*bla gpd_P-$glaA_T$*; *$pyrG_P$-pyrG-$pyrG_T$*	This laboratory
pGTP2-gMndA	*bla gpd_P-gMndA-$glaA_T$*; *$pyrG_P$-pyrG-$pyrG_T$*	This study
pGTP2-cMndA	*bla gpd_P-cMndA-$glaA_T$*; *$pyrG_P$-pyrG-$pyrG_T$*	This laboratory
pGTP2-MndA	*bla gpd_P-MndA-$glaA_T$*; *$pyrG_P$-pyrG-$pyrG_T$*	This study
pGTP2-Man5A	*bla gpd_P-Man5A-$glaA_T$*; *$pyrG_P$-pyrG-$pyrG_T$*	This study
pBluescript-Man1	*bla Man1*	[23]
pMA-RQ-Man5A	*bla Man5A*	GeneArt
pMA-RQ-Agal	*bla Agal*	GeneArt

TTGATAAT-3′) and AnmndA-L (5′-GCTTAATTAACCCTTCTAGCTGTA CGC-3′). The PCR product was digested with *Pac*I and *Asc*I and cloned into the corresponding sites of pGTP2, yielding pGTP2-cMndA (Fig. 1c). Similarly, the gMndA was amplified from the total nucleic acids and cloned onto pGTP2, yielding pGTP2-gMndA.

Bacterial and fungal transformations

Recombinant plasmids were transformed into chemically competent *Escherichia coli* DH5α cells, followed by selection on LB-Amp agar plates containing 100 μg ml^{-1} ampicillin [22]. Electro-competent *S. cerevisiae* Y294 cells [8] were transformed with plasmids yBBH1-Agal, yBBH1-Man1, yBBH1-Agal-Man1 and yBBH1, followed by selection on SC^{-URA} agar plates. Spheroplasts of the *A. niger* D15 host strain [18] were transformed with plasmids pGTP2-cMndA, pGTP2-Man5A and pGTP2. The *A. niger* D15 transformants were selection for on minimal medium agar plates lacking uridine. The *S. cerevisiae* Y294[BBH1] and *A. niger* D15[GTP2] strains served as reference strains.

Enzyme characterisation

All substrates (except OBR-mannan) and standards for enzyme activity measurements were sourced from Sigma-Aldrich (Germany). The presence of extracellular β-mannanase activity was confirmed on OBR-mannan

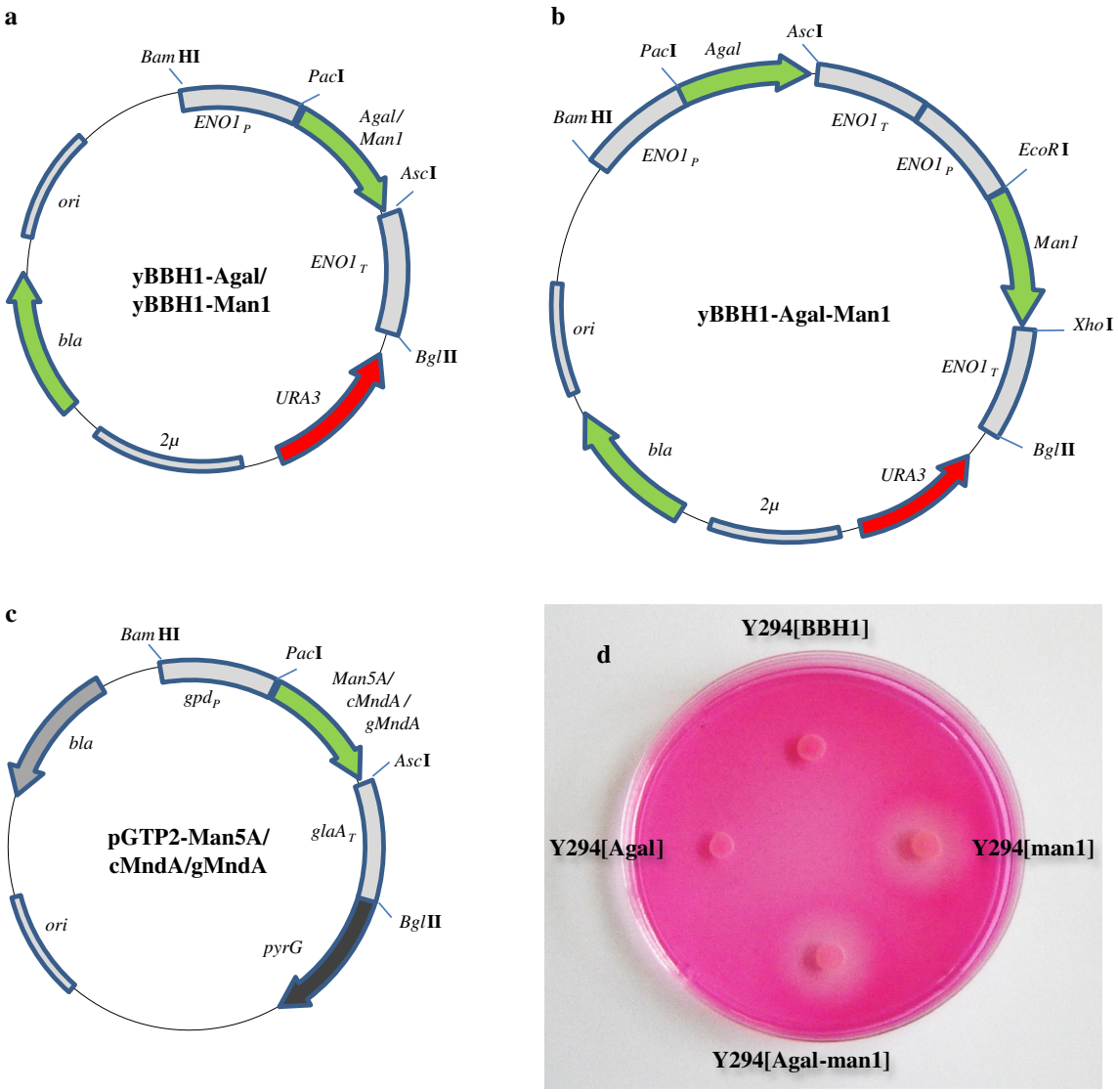

Fig. 1 Schematic representation of the final vector constructs expressing **a** either *Aga1* or *Man1*; **b** both *Aga1* and *Man1*; and **c** either *Man5A*, *gMndA* or *cMndA*. **d** Recombinant *S. cerevisiae* Y294[Man1] and *S. cerevisiae* Y294[Agal-Man1] strains displaying extracellular β-mannanase activity on SC^{-URA} agar plates containing 0.5 % (w v) OBR-mannan after 24 h of incubation at 30 °C

plates, i.e. SC^{-URA} plates with 0.5 % (w/v) OBR-mannan [5]. The recombinant *S. cerevisiae* strains were transferred (spotted) onto the OBR-mannan plates and secretion of β-mannanase was indicated by a clear zone around the colony after cultivation at 30 °C for 24 h.

The recombinant *S. cerevisiae* and *A. niger* strains were inoculated at a final concentration of 1×10^6 cells/spores ml^{-1} in $2 \times SC^{-URA}$ and $2 \times$ MM, respectively. Supernatant was harvested at regular intervals and used to determine the extracellular enzyme activity. The β-mannanase activity was determined using the reducing sugar assay [4] using 0.5 % (w/v) locust bean gum as substrate and mannose as standard. The colorimetric changes were measured spectrophotometrically at 540 nm with an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA), and β-mannanase activity was expressed in nkat ml^{-1}, where 1 katal equals 1 mol of mannose released per second.

The β-mannosidase and α-galactosidase activities were determined using 2 mM of *p*-nitrophenyl β-D-mannopyranoside (*p*NPM) and 2 mM *p*-nitrophenyl α-galactopyranoside (*p*NPGal), respectively, as substrates in 50 mM citrate buffer (pH 3.4). The supernatant and substrate (50 µl each) were incubated for 5 min at 50 °C and the reactions terminated by the addition of 200 µl of 1 M sodium carbonate. Colorimetric changes were measured at 400 nm on a microtitre plate reader using *p*-nitrophenol (*p*NP) as the standard. Enzyme activities were expressed in nkat ml^{-1}, where 1 katal equals 1 mol of *p*NP released per second.

The pH and temperature optima of Man1, cMndA, Man5A and Agal in the fungal supernatants were determined (as described above) using their respective substrates in 50 mM citrate phosphate buffer at pH values ranging from 1.72 to 7.20. The temperature optimum was determined by performing the assays at temperatures ranging from 30 to 80 °C.

Locust bean gum rheology

The recombinant Man1, Agal and Man5A enzymes were added at a final concentration of 2 nkat ml^{-1} to a 0.5 % (w/v) locust bean gum solution prepared in 50 mM citrate buffer at pH 5. Viscosity measurements were performed in triplicate on a Physica MCR 501 (Anton Paar, Germany) using a double-gap configuration and heating at 50 °C with a Peltier system (C-PTD200). Flow curves were analysed using the Rheoplus software based on measurements taken at regular intervals over 10 min at a shear rate of 61.9 s.

Fermentation studies

Pre-cultures of *S. cerevisiae* Y294[BBH1] and *S. cerevisiae* Y294[Agal-Man1] were prepared overnight in 2 × SC^{-URA} medium and inoculated at a final concentration of 50 g l^{-1} in 2 × SC^{-URA} medium containing 5 g l^{-1} glucose and 10 g l^{-1} locust bean gum. Ampicillin (100 µg ml^{-1}) and streptomycin (15 µg ml^{-1}) were added to inhibit bacterial contamination. Lyophilised β-mannosidase, Man5A, was added to a final concentration of 300 mg l^{-1} (7.5 nkat ml^{-1}). Agitation and incubation were done on a magnetic multi-stirrer at 30 °C, with regular sampling with a syringe needle pierced through a rubber stopper.

Ethanol, glycerol, acetic acid, mannose and glucose concentrations were quantified with high-performance liquid chromatography (HPLC) using a surveyor plus liquid chromatograph (Thermo scientific) with a refractive index detector. The compounds were separated on a Rezex RHM Monosaccharide 7.8 × 300 mm column (00H0132-K0, Phenomenex) at 60 °C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml min^{-1}.

Results

Strain construction and functional expression of recombinant genes

The *S. cerevisiae* Y294 strain was used as host for the heterologous production of the *A. aculeatus* β-mannanase (Man1) and *T. emersonii* α-galactosidase (Agal), expressed from the multi-copy, episomal vector yBBH1 under control of the constitutive *S. cerevisiae* enolase I gene (*ENO1*) promoter and terminator sequences (Fig. 1a, b). The 1 403-bp synthetic *C. mixtus* β-mannosidase *Man5A* gene displayed 100 % homology to the *Man5A* gene [9]. The *A. niger gMndA* fragment had a 96.86 % DNA homology with the published gene sequence [1], but contained three introns (237–354; 434–487; and 720–775) as opposed to the two introns reported by Ademark et al. [1]. In addition, a 3-bp deletion was identified at position 197 in c*MndA*. However, activity was evident on *p*-nitrophenyl β-D-mannopyranoside (*p*NPM).

The *cMndA* (with its native secretion signal sequence) and *Man5A* (with the *XYNSEC* secretion signal) were subcloned into the *A. niger* expression vector, pGTP2, under control of the constitutive *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and *A. awamori* glucoamylase terminator ($glaA_T$) sequences (Fig. 1c). The final plasmid constructs were transformed into *S. cerevisiae* Y294 (plasmids yBBH1-Agal, yBBH1-Man1, yBBH1-Agal-Man1 and yBBH1) and *A. niger* D15 (plasmids pGTP2-cMndA, pGTP2-Man5A and pGTP2).

Partial characterisation of recombinant enzymes

A clear zone around *S. cerevisiae* Y294[Man1] and *S. cerevisiae* Y294[Agal-Man1] transformants on OBR-mannan plates (Fig. 1d) confirmed endo-mannanase activity. As expected, no zones were observed for *S. cerevisiae* Y294[BBH1] (reference strain) and *S. cerevisiae* Y294[Agal], which lacked endo-mannanase genes.

Diluted supernatant containing recombinant MndA and Man5A displayed maximum activity at pH 3.40, whereas Man1 peaked at pH 5.47 and Agal at pH 2.37 when incubated for 30 min at 50 °C (Fig. 2a). Optimal activity for Agal was observed at 60 and at 70 °C for Man5A, MndA and Man1 (Fig. 2b) when incubated for 30 min at their respective optimal pH values.

Using the predetermined optimal conditions for the enzymes, maximum β-mannosidase activity for *A. niger* D15[cMndA] (11.6 nkat ml^{-1}) was observed after 156 h (Fig. 3a), which was 1.5-fold higher than *A. niger* D15[Man5A]. The α-galactosidase activity for *S. cerevisiae* Y294[Agal] reached 131.6 nkat ml^{-1} after 48 h, i.e. 2.8-fold higher than *S. cerevisiae* Y294[Agal-Man1] (Fig. 3b). The *S. cerevisiae* Y294[Man1] strain also produced a 1.2-fold higher endo-mannanase activity (374.17 nkat ml^{-1}) than the co-expressing *S. cerevisiae* Y294[Agal-Man1] strain (326.67 nkat ml^{-1}) at 36 h (Fig. 3c).

Mannanases decrease the viscosity of locust bean gum

A gradual decrease in viscosity of galactomannan in 0.5 % locust bean gum was observed for the reference sample due to the influence of temperature and sheering by the

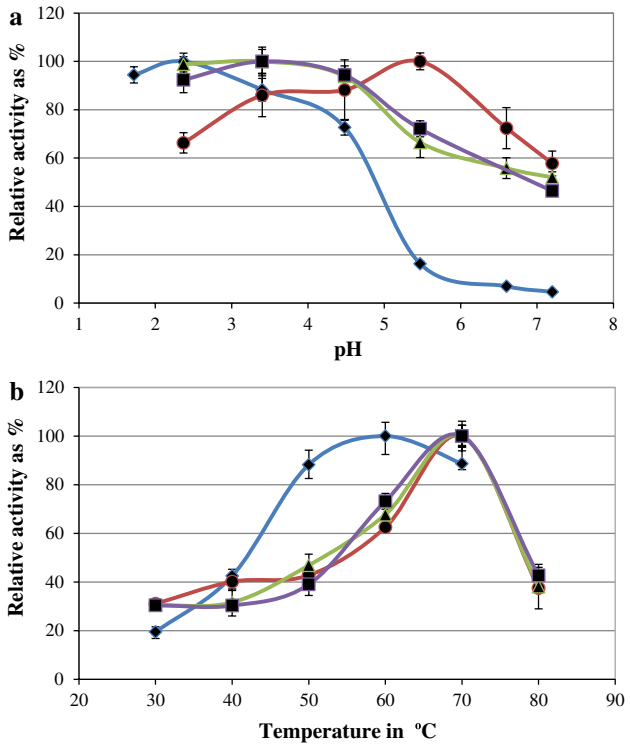

Fig. 2 The effect of **a** pH and **b** temperature on the relative activity of the (*filled square*) cMndA, (*filled triangle*) Man5A, (*filled circle*) Man1 and (*filled diamond*) Agal determined using their respective substrates. *Error bars* indicate the standard deviation from the mean value

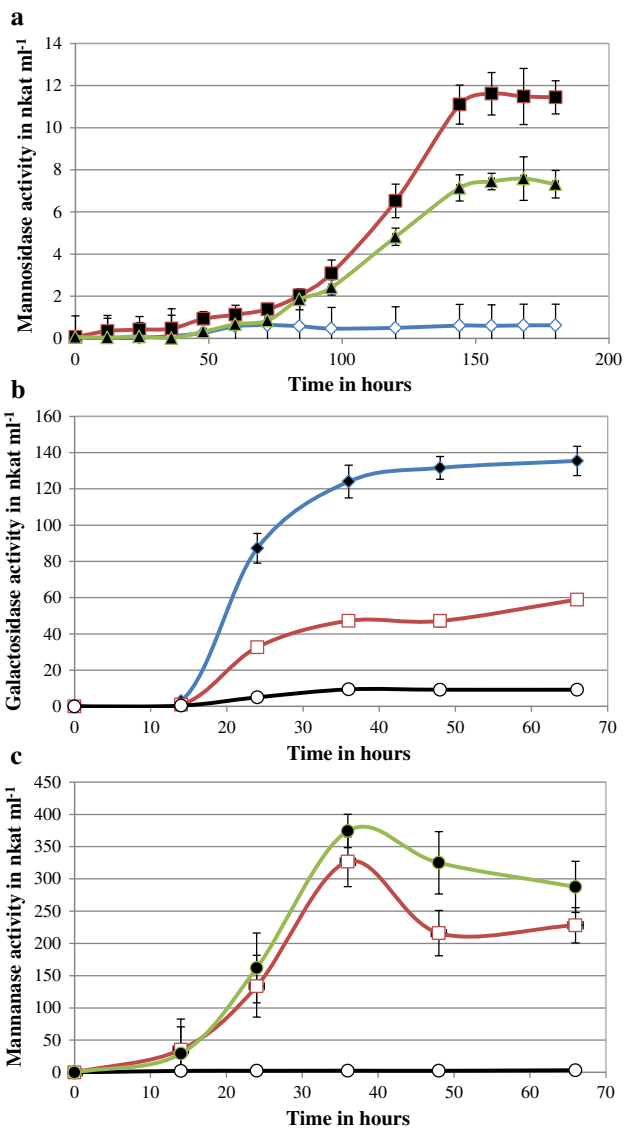

Fig. 3 **a** Extracellular mannosidase activity in (*filled square*) *A. niger* D15[cMndA], (*filled triangle*) *A. niger* D15[Man5A] and (*opened diamond*) *A. niger* D15[GTP2]. **b** Extracellular galactosidase and **c** endo-mannanase activity in (*opened circle*) *S. cerevisiae* Y294[BBH1], (*filled circle*) *S. cerevisiae* Y294[Man1], (*filled diamond*) *S. cerevisiae* Y294[Agal] and (*opened square*) *S. cerevisiae* Y294[Agal-Man1]. *Error bars* indicate the standard deviation from the mean value

apparatus. When the rheology values for the enzyme treatments were normalised accordingly, little change in viscosity occurred in the presence of crude supernatant from *S. cerevisiae* Y294[Agal] (Fig. 4). Crude extracts containing recombinant mannosidase (Man5A) had a significant influence on the viscosity of the substrate, with an even stronger impact from the mannanase (Man1). The combined crude extracts containing Man1, Agal and Man5A proved to be the most effective for liquefaction of the mannan in locust bean gum, with almost complete liquefaction after 4 min.

The *S. cerevisiae* Y294[BBH] and *S. cerevisiae* Y294[Agal-Man1] strains were monitored for ethanol production under oxygen-limited conditions using mannose and locust bean gum as carbohydrate source. After 6 days of fermentations, the mannose production and utilisation reached equilibrium (Fig 5). After 12 days of fermentation in SC^{-URA} medium, the *S. cerevisiae* Y294[BBH] and *S. cerevisiae* Y294[Agal-Man1] strains produced a maximum of 0.103 and 4.56 g l^{-1} ethanol, respectively (Fig. 5). Given the presence of 5 g l^{-1} mannose and 10 g l^{-1} locust bean gum in the growth medium, this implies that *S. cerevisiae* Y294[Agal-Man1] produced 56 % of the theoretical ethanol yield and obtained a 66 % conversion of the available carbohydrate to ethanol (Table 2).

Discussion

Since cellulose and starch are by far the most abundant polysaccharides in nature, research on bioethanol production from plant biomass has concentrated on the utilisation of these two substrates. However, mannan can constitute up to 30 % of the plant biomass [6] and can thus not be ignored if the ethanol yield derived from plant biomass needs to be maximised. However, hydrolysis of mannan requires the action of a number of enzymes, including β-mannanases,

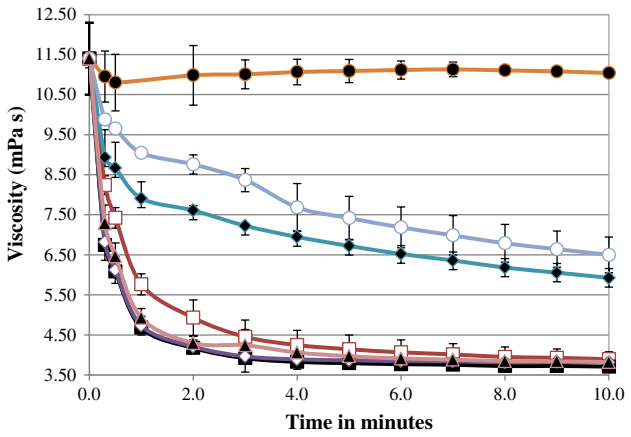

Fig. 4 Analysis of the hydrolytic effect of the recombinant enzymes on the viscosity of 0.5 % (w/v) locust bean gum. The (*filled circle*) Agal, (*opened square*) Man1 and (*opened circle*) Man5A were evaluated individually and in different combinations: (*filled diamond*) Agal and Man5A; (*opened diamond*) Man1 and Man5A; (*filled triangle*) Man1 and Agal; and (*filled square*) Man1, Agal and Man5A. Values had been normalised to take into account the effect of the temperature and shearing on the viscosity. *Error bars* indicate the standard deviation from the mean value

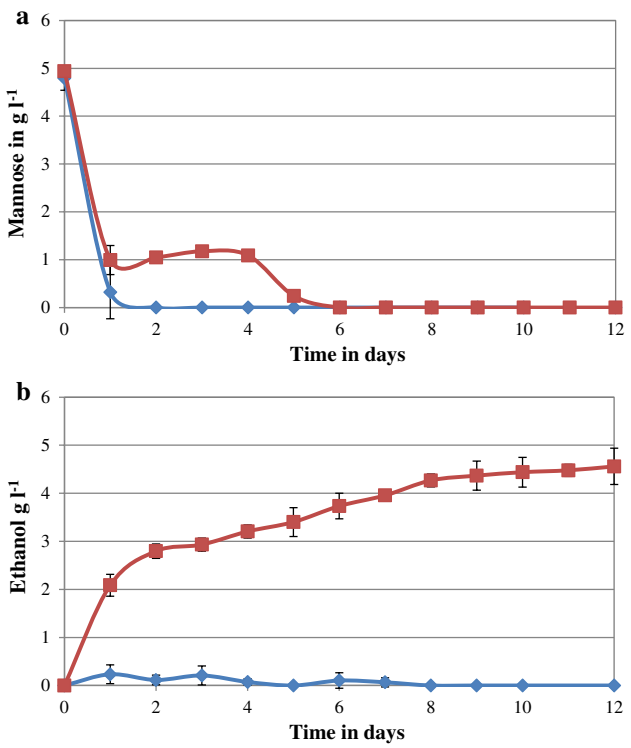

Fig. 5 The **a** mannose consumption and **b** ethanol production by (*filled diamond*) *S. cerevisiae* Y294[BBH1] and (*filled square*) *S. cerevisiae* Y294[Agal-Man1] were monitored under oxygen-limited conditions in double strength SC^{-URA} media (10 g l^{-1} locust bean gum and 5 g l^{-1} mannose). The cultures had been supplemented with 100 mg of lyophilised Man5A at time 0. Values represent the mean of three repeats and *error bars* represent the standard deviation

β-mannosidases, α-galactosidases, β-glucosidases and acetyl-mannan esterases, which are not part of the native *S. cerevisiae* secretome.

To construct yeast strains capable of degrading and/or utilising mannan, a number of fungal enzymes were targeted for heterologous expression. The *A. aculeatus Man1* gene was previously expressed in *S. cerevisiae* using the *PGK1* promoter and terminator expression cassette [23], whilst the *T. emersonii Agal* gene was expressed in *Pichia pastoris* [24]. In the present study, the *Man1* and *Agal* genes were subcloned onto the yBBH1 vector and expressed in *S. cerevisiae*. The *A. niger* MndA [1, 2, 10] and *C. mixtus* Man5A [7, 9] β-mannosidase enzymes have previously been characterised and *Man5A* expressed in *E. coli* [7, 9]. The strategy requires the addition of the mannosidase externally. Therefore, the *cMndA* and *Man5A* had been expressed using an *A. niger* strain known for its high levels of expression [20, 21].

Both MndA and Man5A displayed optimum activity at pH 3.4 and 70 °C (Fig. 2). The results for MndA fall within the range previously reported [2], but the optimum pH for Man5A falls well below the optimal pH 7.0 reported by Dias et al. [9]. The Man1 and Agal displayed optimum activity at 70 °C and pH 5.47, and 80 °C and pH 2.37, respectively (Fig. 2). Setati et al. [23] reported 50 °C and pH 3 as optima for the Man1 expressed in *S. cerevisiae*, whereas Similä et al. [24] reported 70 °C and pH 4.5 as optimal for Agal expression in *P. pastoris*. The deviation in optimal conditions may be ascribed to the different host strains and media composition, which could impact glycosylation patterns and thus the characteristics of the enzymes [25].

All the recombinant enzymes were partially characterised in terms of enzyme activity, pH and temperature preferences as well as their effect on the viscosity of galactomannans (locust bean gum). The *A. niger* D15[cMndA] strain produced more β-mannosidase activity than *A. niger* D15[Man5A] (Fig. 3a), but these values are significantly lower than those previously reported [1, 2]. This may be due to the high copy numbers (up to 25) of the vectors used by Ademark et al. [1] and the absence of 3 bp in the cDNA copy of MndA in the present study, which may have led to sub-optimal protein folding or functionality.

The extracellular α-galactosidase and β-mannanase activities from *S. cerevisiae* Y294[Agal], *S. cerevisiae* Y294[Man1] and *S. cerevisiae* Y294[Agal-Man1] on locust bean gum were higher than those that were co-expressed (Fig. 3b, c). The maximum α-galactosidase activity from *S. cerevisiae* Y294[Agal] was 2.3-fold higher than from *S. cerevisiae* Y294[Agal-Man1], whilst the maximum β-mannanase activity from *S. cerevisiae* Y294[Man1] was 1.1-fold higher than from *S. cerevisiae* Y294[Agal-Man1]. The yBBH1-Agal-Man1 plasmid is significantly

Table 2 Conversion of locust bean gum to ethanol and by-products by different recombinant *S. cerevisiae* strains

Substrate/product (g l^{-1})	*S. cerevisiae* Y294 [BBH]			*S. cerevisiae* Y294 [Man1-Agal] + Man5A		
	Day 0	Day 6	Day 12	Day 0	Day 6	Day 12
Substrate						
Locust bean gum	10.0	10.0	10.0	10.0	10.0	10.0
Mannose	5.0	5.0	5.0	5.0	5.0	5.0
Hexose equivalent[a]	16.1	16.1	16.1	16.1	16.1	16.1
Products						
Glucose	0	0	0	0.32	0	0
Mannose	4.79			4.94		
Glycerol	0	0	0	0	0.182	
Acetic acid	0	0.82	0.005	0	0	1.65
Ethanol	0	0.103	0	0	3.73	4.56
CO_2^b	0.00	0.10	0.00	0.00	3.57	4.36
Total carbon	4.79	1.02	0.01	5.26	7.49	10.57
Substrate-to-product conversion	30 %	6 %	0 %	33 %	47 %	66 %
Ethanol (% of theoretical yield)	0 %	1 %	0 %	0 %	45 %	56 %

[a] For determination of total hexose sugar equivalent, the consumption of H_2O during hydrolysis of each hexose moiety in the LBG was considered

[b] CO_2 produced, based on ethanol concentrations (equimolecular quantities produced)

larger than yBBH1-Agal and yBBH1-Man1, which could result in a lower copy numbers and thus lower protein levels. The maximum Agal activity observed in this study (135 nkat ml^{-1}) was slightly more than the 116 nkat ml^{-1} reported for its expression in *P. pastoris* [24].

The recombinant Man1 and Man5A significantly reduced the viscosity of locust bean gum, whereas Agal1 had no impact on galactomannan (Fig. 4). Since no endo-mannanase activity was detected in the Man5A crude extract (in agreement with [9]), the reduced viscosity was probably due to the release of mannose from the non-reducing end of the mannan chain, resulting in a gradual decrease in molecular weight. The addition of Agal to either Man1 or Man5A further reduced the viscosity of the locust bean gum, which was attributed to the removal of galactose side chains by Agal and thus exposed more possible sites of attack for Man1 and Man5A. The combination of Man1 and Man5A amplified the decrease in viscosity, probably due to the production of shorter oligosaccharides. However, a combination of all three enzymes (Man1, Agal and Man5A) did not significantly reduced viscosity more than that observed for the two-way combinations. It is plausible that the increased mannose and galactose levels could have resulted in feedback inhibition of the enzymes.

When Man5A was added, *S. cerevisiae* Y294[Agal-Man1] produced 5-fold more ethanol than the control strain on 5 g l^{-1} mannose and 10 g l^{-1} locust bean gum (Fig. 5). The 66 % carbohydrate conversion was lower than expected, but could be ascribed to possible exhaustion of the β-mannosidase Man5A enzyme, which was added once-off at the beginning of the fermentation. Little mannose was detected throughout the fermentation, indicating that the mannose was consumed immediately upon release. The mannose production and consumption reached equilibrium at day 6.

To our knowledge, this is the first report on ethanol production by *S. cerevisiae* using mannan as a carbohydrate source. The *T. emersonii* α-galactosidase was successfully expressed by itself and in combination with a mannanase in *S. cerevisiae*. The *A. niger* strain provided an effective alternative expression system for *cMndA* and *Man5A*, representing the first report on *Man5A* expression in *A. niger*. The combination of β-mannanase and either β-mannosidase or α-galactosidase indicated a significant reduction in the viscosity of galactomannan when compared with the individual enzymes.

For consolidated bioprocessing of mannan, the ideal scenario would be to express a mannanase, α-galactosidase and β-mannosidase (and β-glucosidase for galactogluco mannan or glucomannan) in a single host (e.g. *S. cerevisiae*) for continuous enzyme production, thus allowing complete hydrolysis of mannan and simultaneous fermentation of the sugar components. However, this would require the expression of a functional β-mannosidase in *S. cerevisiae*, which has been unsuccessful to date.

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