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ORIGINAL PAPER

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An ethanologenic yeast exhibiting unusual metabolism in the fermentation of lignocellulosic hexose sugars

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Abstract Three lignocellulosic substrate mixtures [liquid fraction of acid-catalyzed steam-exploded softwood, softwood spent sulfite liquor (SSL) and hardwood SSL] were separately fermented by the industrially employed SSL-adapted strain Tembec T1 and a natural galactose-assimilating isolate (Y-1528) of *Saccharomyces cerevisiae* to compare fermentative efficacy. Both strains were confirmed as *S. cerevisiae* via molecular genotyping. The performance of strain Y-1528 exceeded that of Tembec T1 on all three substrate mixtures, with complete hexose sugar consumption ranging from 10 to 18 h for Y-1528, vs 24 to 28 h for T1. Furthermore, Y-1528 consumed galactose prior to glucose and mannose, in contrast to Tembec T1, which exhibited catabolite repression of galactose metabolism. Ethanol yields were comparable regardless of the substrate utilized. Strains T1 and Y-1528 were also combined in mixed culture to determine the effects of integrating their distinct metabolic capabilities during defined hexose sugar and SSL fermentations. Sugar consumption in the defined mixture was accelerated, with complete exhaustion of hexose sugars occurring in just over 6 h. Galactose was consumed first, followed by glucose and mannose. Ethanol yields were slightly reduced relative to pure cultures of Y-1528, but normal growth kinetics was not impeded. Sugar consumption in the SSLs was also accelerated, with complete utilization of softwood- and hardwood-derived hexose sugars occurring in 6 and 8 h, respectively. Catabolite repression was absent in both SSL fermentations.

Keywords Yeast · Fermentation · Lignocellulose · Galactose · Ethanol

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Introduction

The effective utilization of lignocellulosic residues for the production of renewable fuels continues to be a primary objective of the biomass-energy community. Ethanol, the predominant fuel obtained from lignocellulosic biomass, can serve as a viable and strategic alternative to a variety of conventional petroleum products for a number of reasons. For example, lignocellulosic feedstocks represent a renewable source of energy, and offer an environmentally benign alternative to traditional petroleum fuel sources [18, 25, 32].

Significant volumes of lignocellulosic residues are potentially available for utilization as a feedstock for liquid fuel production in many regions [25]. However, unique challenges confront aspects of the bioconversion process converting softwood-derived lignocellulose to ethanol. Specifically, it is imperative to consider the nature, distribution and quantity of sugars, and concurrently any inhibitory compounds present in the hemicellulose-rich liquid stream generated from the pretreatment of lignocellulose. The effective fermentation of this hemicellulose-rich, water-soluble mixture is essential to attaining near-theoretical ethanol yield at a reasonable cost. Three hexose sugars, galactose, glucose and mannose, and two pentose sugars, arabinose and xylose, comprise the carbohydrate fraction derived from softwood lignocellulosics [25]. However, the hexose sugars are present in much greater concentrations than the pentose sugars, a situation that contrasts with hardwood-derived feedstocks [34].

Typically, softwood (and hardwood) hemicellulose streams contain naturally occurring and process-induced inhibitory compounds that retard and sometimes inhibit effective fermentation [3, 10, 13, 16, 18, 19, 22]. These compounds and their precursors may be produced from sugar and lignin degradation in the pretreatment stage of the bioconversion process (acid-catalyzed steam explosion), from microbial fermentation, or from the equipment used to process the feedstock [18]. Lignin degradation products and high concentrations of acetic acid have been shown to be especially inhibitory to most unmodified or unadapted fermentative microorganisms [18].

The criteria by which appropriate microorganisms are judged include technical elements, such as their abilities to metabolize all of the sugars present at relatively high concentrations, produce and tolerate (potentially) high ethanol concentrations, generate minimal quantities of non-toxic by-products, employ mechanisms to detoxify or sequester natural or generated inhibitory components, attain sufficient biomass and metabolic activity to perform bioconversion efficiently, and replicate with reasonably short generation times [15, 18, 19]. Thus far, both natural and recombinant yeasts and bacteria have failed to satisfy every technical criterion [15, 18]. For example, thermophilic bacterial species (specifically, *Clostridium* spp., *Thermoanaerobium* spp., and *Thermoanaerobacterium* spp.) have very poor ethanol tolerance, despite having the capacity to generate high ethanol yields [15].

Yeasts are often utilized as industrial fermentative organisms because of their ability to convert sugars to ethanol at near theoretical yields [19, 35]. One such yeast strain, *Saccharomyces cerevisiae* Tembec T1, is an industrially adapted natural yeast strain, which was isolated from a spent sulfite liquor (SSL) stream exiting the Tembec pulp and paper mill in Témiscaming, Québec, Canada. It is recognized as a robust strain with a proven ability to effectively convert lignocellulose-derived substrates to ethanol in the presence of toxic inhibitory compounds.

Another yeast strain, *S. cerevisiae* Y-1528, is a natural isolate obtained from a culture collection that was selected based on its unique capacity to assimilate (take up) galactose (from the extracellular environment). Its galactose fermentation performance was recently ranked best among other screened strains of *S. cerevisiae* from the same culture collection [11]. Galactose is the most recalcitrant of the three main hexose sugars derived from lignocellulose. Higher energy expenditure is necessary for its transport and metabolism, and the utilization of this sugar is generally governed by catabolite repression in microorganisms [5, 17, 21, 26, 27]. Consequently, the capacity to overcome this repression, and thereby utilize galactose as efficiently as glucose and mannose is a highly valued physiological trait in yeasts.

The objectives of this current study were to compare the fermentative performance of *S. cerevisiae* strains Tembec T1 and Y-1528 on three lignocellulose-derived substrate mixtures: the hemicellulose-rich water-soluble fraction derived from the steam explosion of Douglas-fir, softwood and hardwood SSL. All of these substrates are likely to contain inhibitory compounds in addition to hexose and pentose sugars. Secondly, the potentially synergistic performance of mixed cultures of *S. cerevisiae* Tembec T1 and *S. cerevisiae* Y-1528 on defined triple sugar mixtures and SSLs was evaluated. Finally, the taxonomic identities of Tembec T1 and Y-1528 were assessed through molecular genotyping, in order to confirm their previously established phenotypic classification as strains of *S. cerevisiae*.

Materials and methods

Yeast strains and culture media

SSL-adapted *S. cerevisiae* T1 was obtained from Tembec Limited (Témiscaming, Québec, Canada). *S. cerevisiae* Y-1528 was obtained from the USDA Agricultural Research Service (Peoria, Ill.). *S. cerevisiae* BY4742 was obtained from the Wine Research Centre at the University of British Columbia (Vancouver, BC, Canada). Strains were maintained on YPG solid medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose, and 18 g l^{-1} agar), stored at 4°C, and transferred to fresh plates on a bimonthly basis. Cells were grown to high cell density in foam-plugged 250 ml Erlenmeyer flasks containing YPG liquid medium (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, and 10 g l^{-1} glucose) in an orbital shaker for 3 days at 30°C and 200 rpm, with transfer of cells to fresh medium at 24 and 48 h.

Substrates

The Douglas-fir (*Pseudotsuga menziesii*) hemicellulose-rich water-soluble fraction was generated via acid-catalyzed steam explosion (195°C, 4.5 min, 4.5% SO_2) of uniformly chipped wood from a 150-year-old Douglas-fir tree. Steam explosion output was diluted to 15% w/w consistency, filtered, and adjusted to pH 6.0 with sodium hydroxide [25]. Suspended solids were not filtered out. Softwood and hardwood SSL was acquired from Tembec and likewise adjusted to pH 6.0 with sodium hydroxide. Again, suspended solids were not filtered out.

Batch fermentations

Following 3 days growth, cell cultures were harvested, centrifuged (750 *g*, 21°C), and decanted to yield cell pellets. Pellets were then washed three times with sterile deionized water, and subsequently adjusted to a calculated concentration of 80 g dry cell weight (DCW) per liter via standard curves relating 600 nm absorbance to DCW l^{-1} concentration.

Fermentations were performed in rubber-septum-plugged serum bottles containing 40 ml Douglas-fir water-soluble fraction (supplemented with 1.65 g l^{-1} filter-sterilized dibasic ammonium phosphate as nitrogen and phosphorus source), 40 ml softwood or hardwood SSL, or 40 ml YPG liquid medium (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 30 g l^{-1} filter-sterilized galactose, 30 g l^{-1} filter-sterilized glucose, and 30 g l^{-1} filter-sterilized mannose, supplemented with 1.65 g l^{-1} filter-sterilized dibasic ammonium phosphate as nitrogen and phosphorus source) in an orbital shaker for 48 h at 30°C and 125 rpm. The hexose sugar composition of the softwood water-soluble fraction comprised 14 g l^{-1} mannose, 13.7 g l^{-1} glucose, and 3.6 g l^{-1} galactose. The hexose sugar composition of the softwood SSL comprised 13.5 g l^{-1} mannose, 4.3 g l^{-1} glucose, and 3.5 g l^{-1} galactose, while the hardwood SSL contained 6.0 g l^{-1} mannose, 3.3 g l^{-1} glucose, and 1.8 g l^{-1} galactose. Initially, the softwood-derived water-soluble fraction and SSL media were inoculated with pure cultures to achieve an initial cell concentration of 4 g DCW l^{-1}. In the subsequent study, YPG liquid medium and SSL media were inoculated with mixed cultures to achieve an initial cell concentration of 2 g DCW l^{-1} (Tembec T1) and 2 g DCW l^{-1} (Y-1528), for a total of 4 g DCW l^{-1}. Offline sampling was aseptically performed at the time of inoculation and at specific time points thereafter. Aliquots (1 ml) were immediately centrifuged (16,000 *g*) for 4 min at 4°C to yield cell-free supernatants, which were then decanted and frozen at −20°C for separate sugar and ethanol analysis. All fermentation experiments were performed in duplicate with the appropriate negative controls. Furthermore, conditions were duplicated in separate flasks within each experiment.

Sugar and ethanol analysis

High performance liquid chromatography (HPLC) on a DX-600 BioLC chromatograph (Dionex, Sunnyvale, Calif.) was used for sugar determination. Separation was achieved by a CarboPac PA1 anion exchange column (Dionex), and detection was achieved via pulsed amperometry across a gold electrode with the addition of a 200 mM NaOH post-column wash. External standards and experimental samples were appropriately diluted in deionized water, supplemented with fixed volumes of fucose as internal standard, and then filtered through 0.45 μm PVDF (polyvinylidene fluoride) filters prior to injection (20 μl). The column was eluted with deionized water at a flow rate of 1.0 ml min^{-1} for 45 min, 250 mM NaOH for 10 min, and then deionized water for 5 min.

Ethanol determination was achieved by gas chromatography on a 5890 Series II chromatograph with a 6890 autoinjector, splitless injector system, and flame ionization detector (Hewlett Packard, Palo Alto, Calif.). Separation was effected in a 30 m Stabilwax-DA column (internal diameter 0.53 mm) fitted with a 5 m deactivated guard column (Restek, Bellefonte, Pa.). Samples were appropriately diluted in deionized water, supplemented with butan-1-ol as internal standard, and then filtered through 0.45 μm PVDF filters prior to injection (2 μl). An injector temperature of 90°C, a detector temperature of 250°C, and a helium (carrier gas) flow rate of 1.0 ml min^{-1} was employed. The column oven temperature was maintained at 45°C for 6 min, ramped to 230°C at a rate of 20°C min^{-1}, and subsequently maintained at 230°C for 10 min.

Ethanol yields and percent theoretical yields were calculated using the following equations, respectively:

$$Y_{P/S} = [EtOH]_{max} \div [Sugar]_{ini} \quad (1)$$

$$Y_{\%T} = (Y_{P/S} \div 0.51) \times 100 \quad (2)$$

where $Y_{P/S}$ = ethanol yield (g g^{-1}), $[EtOH]_{max}$ = maximum ethanol concentration achieved during fermentation (g l^{-1}), $[Sugar]_{ini}$ = total initial sugar concentration at onset of fermentation (g l^{-1}), $Y_{\%T}$ = percent theoretical yield (%), and 0.51 = theoretical maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g g^{-1}).

Microbial growth analysis

Time-dependent offline sampling was performed aseptically during mixed culture fermentations to yield 1 ml aliquots. Samples were mixed immediately prior to dilution in deionized water, and then subjected to duplicate absorbance determination in a spectrophotometer at 600 nm. Diluted cell-free medium was used to establish background readings and set zero absorbance levels. Values were averaged and corrected for dilution.

Molecular genotyping

Chromosomal DNA from BY4742, Tembec T1, and Y-1528 was isolated in accordance with standard protocols [8]. PCR primers based on conserved regions of fungal rRNA genes and designed to amplify flanking noncoding regions were employed [31, 33], including ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and LR3 (5′-GGTCCGTGTTTCAAGAC-3′). PCR amplification was achieved by combining 0.2 mM dNTP mix (Amersham Biosciences, Baie d'Urfe, Québec, Canada), 1x PCR buffer (Amersham), 1 μM each of forward and reverse primers (ITS1 and ITS4, or ITS3 and LR3), 0.5 μg chromosomal DNA, and 2.5 U *Taq* DNA polymerase (Amersham), in a total volume of 20 μl. The thermocycler program consisted of one cycle of 95°C for 6 min, 35 cycles of 94°C for 20 s, 53°C for 20 s, and 72°C for 1 min, and one cycle of 72°C for 5 min [7]. Chromosomal DNA aliquots from BY4742, Tembec T1, and Y-1528 were separately reacted in

conjunction with the ITS1/ITS4 primer pair and the ITS3/LR3 primer pair, along with negative controls, in duplicate. PCR products were purified with the QiaQuick PCR Purification Kit (Qiagen, Mississauga, ON, Canada), and then divided 5-fold for restriction endonuclease digestion. Restriction digest mixtures consisted of the PCR product, 1 µl restriction endonuclease (*Bsu*RI, *Dra*I, *Eco*RI, *Hinf*I, or *Hin*6I) (Fermentas Life Sciences, Burlington, ON, Canada), 5 µl corresponding to 10× restriction endonuclease buffer (Fermentas), and an appropriate volume of sterile deionized water to total 50 µl. Mixtures were incubated at 37°C for 1 h, dried via a SpeedVac Plus SC210A concentrator (Thermo Savant, Milford, Mass.) to yield DNA precipitate, and resuspended in sterile deionized water. Restriction fragments generated from ITS1/ITS4 and ITS3/LR3 amplicons of BY4742, Tembec T1, and Y-1528 DNA were resolved in high resolution pre-cast 3% agarose gels containing ethidium bromide (Bio-Rad, Mississauga, ON, Canada) in ice-cooled 1× TBE running buffer. Restriction fragments were electrophoresed alongside 1 kb DNA mass ladders at 100 V for 30 min, followed by 80 V for 1 h. Following electrophoresis, DNA banding patterns were visualized under ultraviolet transillumination. Fragment sizes were calculated using regression equations based on DNA mass ladder migration, and patterns then compared to those of the reference strain BY4742 to determine identities.

Results and discussion

Fermentation of the steam-exploded Douglas-fir water-soluble fraction

The ability of Y-1528 to effectively ferment the hemicellulose-rich water-soluble fraction derived from steam-exploded Douglas-fir was compared with T1. Following growth on glucose, Y-1528 consumed all of the hexose sugars in the water-soluble fraction in just over 18 h, while the industrial strain Tembec T1 required between 24 and 48 h to accomplish the same objective (Fig. 1a). More specifically, Y-1528 consumed galactose in 6 h, and glucose and mannose in just over 18 h. The onset of glucose and mannose consumption did not occur until galactose had been almost completely consumed (3 h). In contrast, Tembec T1 consumed glucose and mannose in 9 h, but required between 24 and 48 h to ferment all of the galactose. The onset of galactose consumption did not occur until glucose and mannose had been completely utilized after 9 h, illustrating the normal sequence of metabolic conversion of sugars to ethanol by *S. cerevisiae*. Ethanol yield from Y-1528 (Fig. 1b, Table 1) was slightly higher than that obtained from T1 (92 and 87% of theoretical yield, respectively).

The ability of Y-1528 to tolerate possible naturally occurring and process-induced inhibitory compounds in the softwood-derived water-soluble fraction to at least

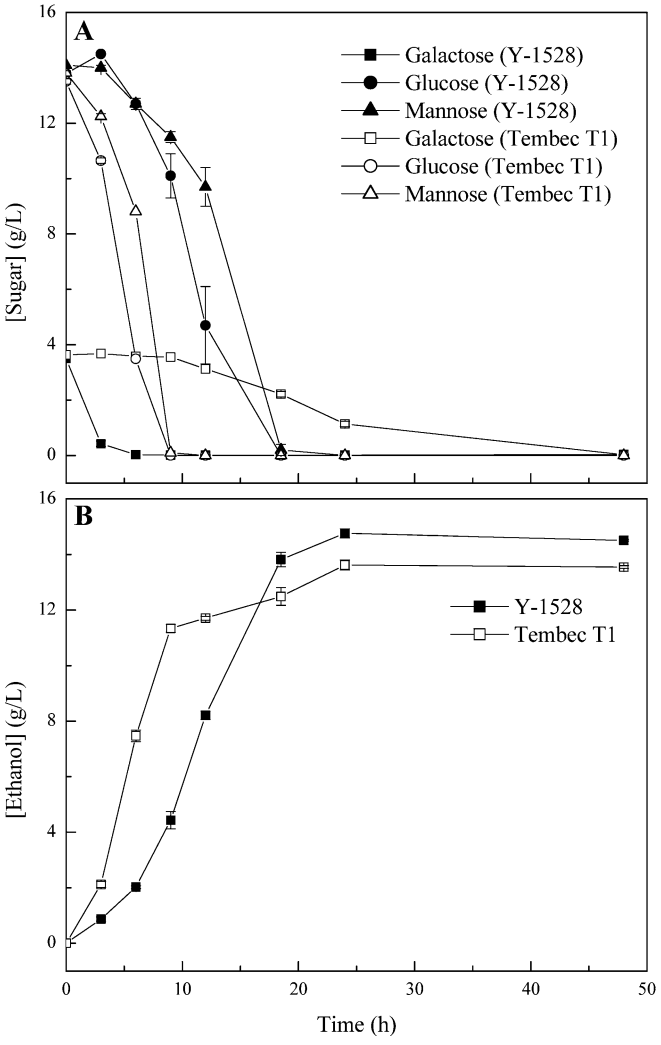

Fig. 1 **a** Consumption of hexose sugars and **b** ethanol production in the hemicellulose-rich water-soluble fraction of steam-exploded Douglas-fir by *Saccharomyces cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1, following growth on glucose. *Vertical bars* Range

the same degree as Tembec T1 was indicated by the complete consumption of all of the hexose sugars in significantly less time, while concurrently exceeding the ethanol yield compared to T1. The rapid consumption of galactose, preceding that of glucose and mannose, reflects the unique metabolic machinery which is currently being extensively characterized [11].

Softwood and hardwood SSL fermentation

The fermentative capacity of Y-1528 in softwood- and hardwood-derived SSLs was assessed and compared to that of Tembec T1. Following growth on glucose, Y-1528 consumed all of the hexose sugars contained in the softwood SSL in less than 10 h, while Tembec T1 required between 24 and 48 h to accomplish the same objective (Fig. 2a). Specifically, Y-1528 consumed galactose in 2 h, glucose in 6 h, and mannose in

Table 1 Maximum ethanol yields (product per unit substrate [$Y_{P/S}$] and percent theoretical [$Y_{\%T}$]) during hexose sugar fermentations by *Saccharomyces cerevisiae* Tembec T1 and/or *S. cerevisiae* Y-1528 (range is indicated). *SSL* Spent sulfite liquor

Substrate	Culture	Ethanol $Y_{P/S}$ (g g^{-1})	Ethanol $Y_{\%T}$ (%)
Softwood-derived water-soluble fraction	Tembec T1	0.44 ± 0.01	87 ± 1
Softwood-derived water-soluble fraction	Y-1528	0.47 ± 0.01	92 ± 1
Softwood SSL	Tembec T1	0.43 ± 0.02	84 ± 4
Softwood SSL	Y-1528	0.41 ± 0.01	81 ± 1
Hardwood SSL	Tembec T1	0.36 ± 0.01	70 ± 1
Hardwood SSL	Y-1528	0.38 ± 0.01	75 ± 1
Defined substrate	Mixed	0.37 ± 0.01	73 ± 1
Softwood SSL	Mixed	0.38 ± 0.01	75 ± 1
Hardwood SSL	Mixed	0.39 ± 0.01	76 ± 1

approximately 10 h. Again, the onset of glucose consumption did not occur until galactose had been completely fermented at the 2-h time point, but mannose consumption was not affected likewise, beginning concurrently with galactose. In contrast, Tembec T1 consumed glucose in 4 h and mannose in less than 6 h, but required between 24 and 48 h to completely consume galactose. The onset of galactose metabolism did not occur until glucose and mannose had been almost completely fermented at the 4-h time point. Ethanol yields (Fig. 3, Table 1) for the Y-1528- and Tembec T1-catalyzed fermentations were similar (roughly 82% of theoretical yield).

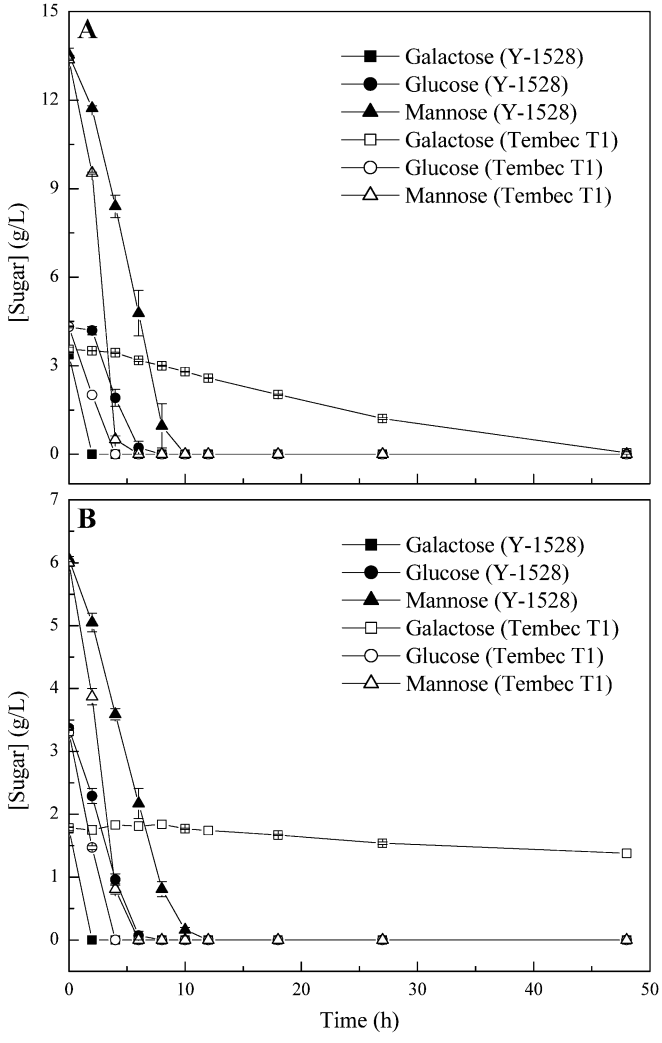

Fig. 2a, b Consumption of hexose sugars present in **a** softwood spent sulfite liquor (SSL) **b** hardwood SSL by *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1, following growth on glucose. *Vertical bars* Range

Following growth on glucose, Y-1528 consumed all of the hexose sugars contained in the hardwood SSL in approximately 10 h, while Tembec T1 required greater than 48 h just to accomplish partial consumption (Fig. 2b). Specifically, Y-1528 consumed galactose in 2 h, glucose in 6 h, and mannose in approximately 10 h. No catabolite repression was evident during fermentation. In contrast, T1 consumed glucose in 4 h and mannose in 6 h, but demonstrated limited fermentation of galactose over 48 h. The exhaustion of glucose and mannose did not result in the onset of significant galactose consumption. Ethanol yield (Fig. 3, Table 1) from Y-1528 was again slightly higher than that obtained with T1 (75 and 70% of theoretical yield, respectively).

The ability of Y-1528 to tolerate naturally occurring and process-induced inhibitory compounds in softwood and hardwood SSLs to at least the same degree as Tembec T1, and effect complete exhaustion of all hexose sugars in a maximum of one-third the time, was a further indication of the strain's capacity for inhibitor resistance. In comparison, defined mixtures of hexose sugars composed of 30 g l^{-1} of galactose, glucose, and mannose, but lacking many of the inhibitory compounds found in SSLs, were also fermented in approximately 10 h by Y-1528 [11]. Similar to the water-soluble fraction derived from steam-exploded Douglas-fir, softwood and hardwood SSLs likely contain a wide variety of inhibitory compounds possessing synergistic potential, including acetic acid, extractives, sugar and lignin degradation products, sulfur-containing reagents and product residues, and equipment-derived metals [15, 18, 23, 24, 28]. The performance of Tembec T1 was clearly distinct from that of Y-1528, with catabolite repression of galactose metabolism observed during both SSL fermentations, and an indication of almost complete inhibition of galactose consumption during hardwood SSL fermentation. Specific inhibitory compounds especially abundant in decomposed hardwood lignocellulose (e.g., furfural and acetic acid) were probably responsible for the strain's inability to utilize galactose, since this

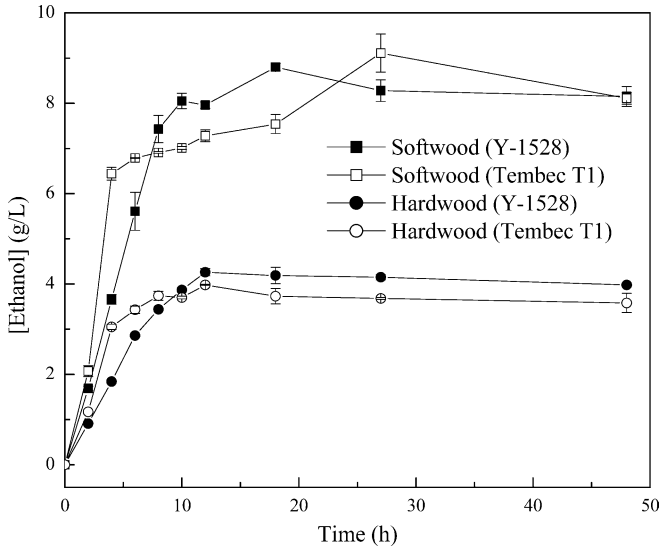

Fig. 3 Ethanol production during softwood and hardwood SSL fermentations by *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1. *Vertical bars* Range

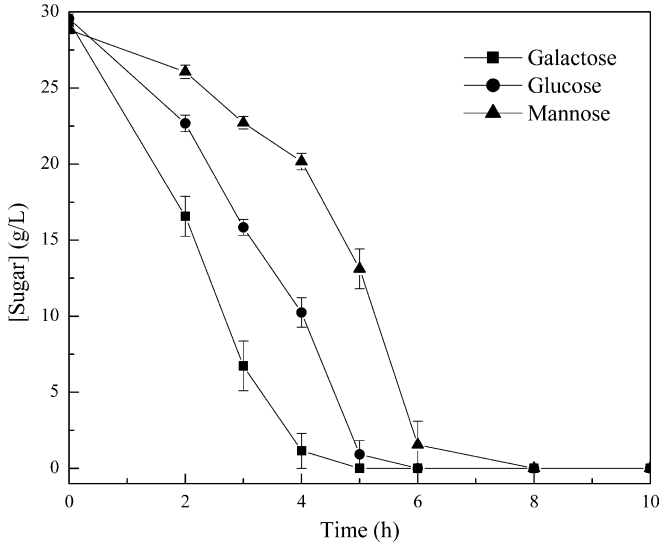

Fig. 4 Sugar consumption during triple sugar fermentation by mixed cultures of *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1, following growth on glucose. *Vertical bars* Range

behavior was absent during softwood SSL fermentation, and because there exists very little redundancy, as well as great complexity, in galactose transport and metabolic pathways, with consequent disproportionate sensitivity to toxic chemicals [5, 17, 20, 21]. The presence of a significant concentration of galactose beyond the 24-h time point has technical and economic implications in terms of residual sugar, which would progressively accumulate in a series of Tembec T1-catalyzed batch fermentations or in continuous fermentation, and cause a substantial increase in the formation of stillage in the distillation processes [32]. The lower ethanol yields during hardwood SSL fermentation were attributed to hardwood-derived inhibitor interference in catabolic sugar degradation, whether effected through hindrance of galactose utilization (in the case of Tembec T1), or through diversion of end-product pathways (in the case of Y-1528, and possibly Tembec T1) [2, 9].

Mixed culture fermentation

The ability of a mixed culture of Tembec T1 and Y-1528 to more effectively ferment triple sugar mixtures than either yeast strain alone was assessed. This represented an attempt to exploit the advantages of each strain, and thus improve sugar consumption and ethanol production. Inocula were prepared separately and combined in equal proportions (as measured by cell densities) into fermentations of defined sugar mixtures and softwood and hardwood SSL. Following growth on glucose, the co-cultured yeast strains consumed all of the hexose sugars in just over 6 h (Fig. 4). Specifically, the strains consumed galactose in just over 4 h, glucose in just over 5 h, and mannose in just over 6 h. No strict catabolite repression was evident in this fermentation. By comparison, Y-1528 alone required almost an extra 2 h to fully consume galactose, an extra 3 h to consume glucose, and an extra 4 h to consume mannose (data not shown). T1 alone consumed glucose and mannose in the same time frame as the co-cultured strains, but required up to an extra 20 h to fully consume galactose, owing to strong catabolite repression in the first 6 h of fermentation (data not shown). Ethanol yield from the co-cultured strains was 73% of theoretical yield (Fig. 5, Table 1), just below the 77% yield achieved by Y-1528 alone [11]. Exponential growth was observed through the 6-h time point, consistent with the gradual and complete exhaustion of all three sugars, and was followed by stationary phase growth through the 26-h time

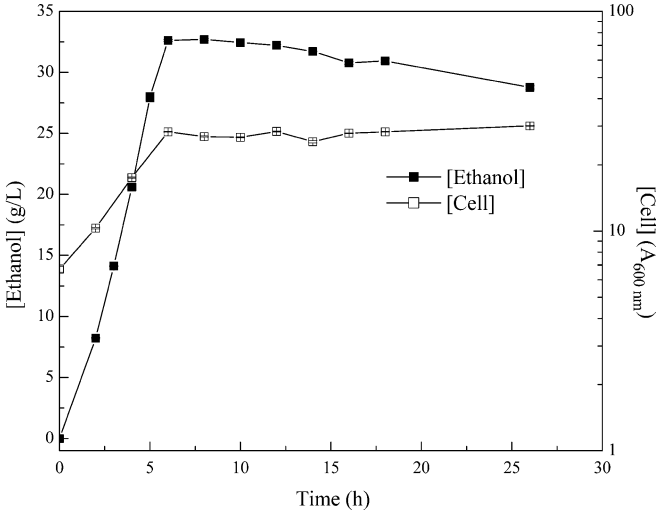

Fig. 5 Ethanol production and microbial growth during triple sugar fermentation by mixed cultures of *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1. *Vertical bars* Range

point as accumulated metabolic products exerted a population-limiting effect (Fig. 5).

The ability of a mixed culture of Tembec T1 and Y-1528 to more effectively ferment SSLs than either yeast strain alone was subsequently assessed. Following growth on glucose, the co-cultured yeast strains consumed all of the hexose sugars in softwood SSL in 6 h (Fig. 6). Specifically, the strains consumed glucose in just over 4 h, and galactose and mannose in 6 h. Ethanol yield from the co-cultured strains was 75% of theoretical yield (Fig. 7, Table 1), slightly below the roughly 82% achieved by Y-1528 or Tembec T1 alone (Fig. 3). Similarly, the co-cultured yeast strains consumed all of the hexose sugars in hardwood SSL in 8 h (Fig. 6), with glucose being consumed in just over 4 h, mannose in 6 h, and galactose in 8 h. Ethanol yield from the co-cultured strains was 76% of theoretical yield (Fig. 7, Table 1), comparable to the 75% achieved by Y-1528 alone and slightly above the 70% achieved by Tembec T1 alone (Fig. 3). No catabolite repression was evident in the fermentation of either liquor. As recorded previously, Y-1528 or Tembec T1 alone required significantly more time to effect complete hexose sugar consumption (Fig. 2).

Several studies have recently been conducted to ascertain the impact of mixed cultures on biomass-to-ethanol processes, with the aim of improving conversion efficiencies [4, 6, 12, 14, 29, 30]. It was thus expected that a mixed culture of Tembec T1 and Y-1528 would rapidly consume a defined triple sugar mixture modeled after lignocellulose-derived hexose sugars by uniting the advantageous metabolic properties of each strain. As predicted, the combination of strains accelerated galactose consumption beyond that achieved by Tembec T1 (and by pure Y-1528, unexpectedly exemplifying synergy), and accelerated glucose and mannose

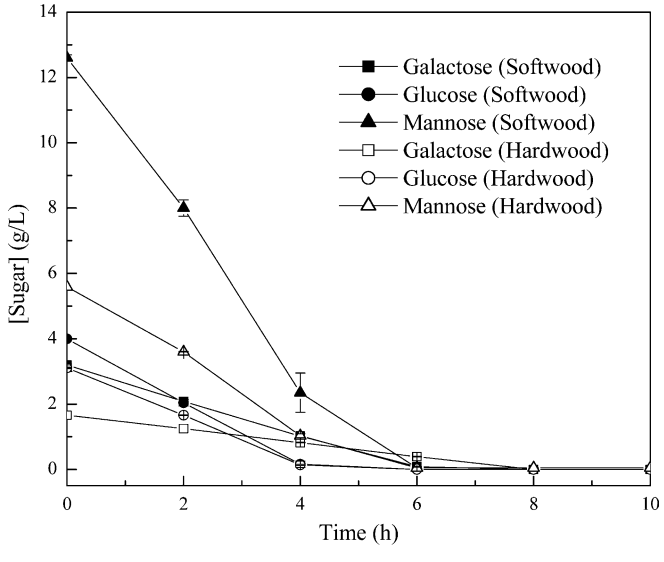

Fig. 6 Hexose sugar consumption during SSL fermentation by mixed cultures of *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1, following growth on glucose. *Vertical bars* Range

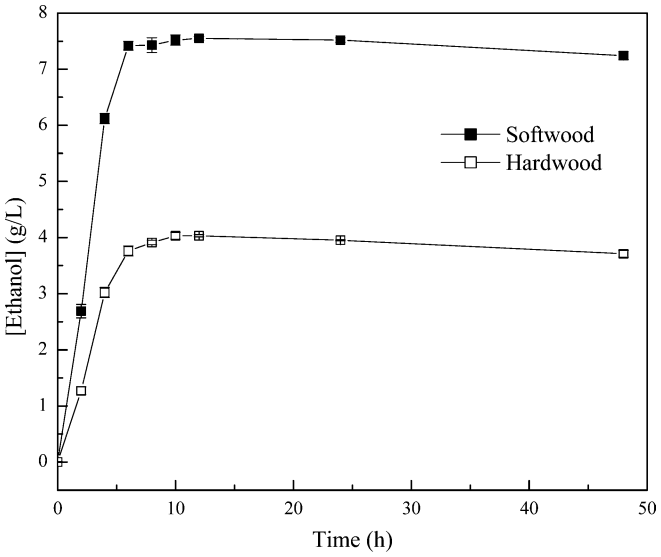

Fig. 7 Ethanol production during softwood and hardwood SSL fermentations by mixed cultures of *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1. *Vertical bars* Range

consumption beyond that achieved by Y-1528. Any potential negative interactions between the respective cell populations did not manifest themselves in poor sugar consumption performance, or in retarded growth. However, ethanol yield was lower, possibly due to metabolic diversion of carbon toward cellular biomass or glycerol [2, 9]. This reallocation may have represented an unidentified detrimental effect resulting from mixed culture competition, and/or from rapid generation and excretion of specific by-products unique to each strain. A number of factors suggested that lignocellulose-derived substrate mixtures would be appropriate media for subsequent simultaneous application of Tembec T1 and Y-1528: the remarkable overall performance improvement witnessed during this mixed culture fermentation, including evidence of a synergistic interaction with respect to galactose consumption; the industrial emphasis on long-term ethanol productivity, rather than yield; and the demonstrated proficiency of both strains, especially Y-1528, during ethanologenic fermentation of the steam-exploded Douglas-fir water-soluble fraction, and softwood and hardwood SSLs.

Consequently, a mixed culture of Tembec T1 and Y-1528 was applied to each SSL. Lignocellulose-derived glucose and mannose were consumed in a substantially shorter time than that required by pure Y-1528, matching pure Tembec T1, but the synergy observed via greatly accelerated galactose consumption in mixed culture fermentations of defined triple sugar mixtures was absent. Galactose was exhausted in much less time than that required by pure Tembec T1, but remained extensively unassimilated past the point at which pure Y-1528 effected complete consumption. This distinct behavior is likely related to the presence of inhibitory compounds in softwood and hardwood SSLs. The presence of these toxic chemical components yielded a ''normal'' or expected

galactose fermentation profile, in which the mixed inoculum (2 g DCW l^{-1} of each strain) catalyzed sugar exhaustion more slowly than a pure culture of the better-performing strain (Y-1528, at 4 g DCW l^{-1}), and faster than a pure culture of the poorer-performing strain (Tembec T1, at 4 g DCW l^{-1}). However, it was noted with interest that in both lignocellulosic media (softwood and hardwood SSLs), the time required for galactose exhaustion was closer to that accorded to pure Y-1528 than to pure Tembec T1, indicating two active and plausible physiological phenomena: the higher intrinsic metabolic capacity for galactose in Y-1528 (already amply demonstrated), and the milder effect of lignocellulose-derived inhibitory compounds on the same strain, at lower specific cell concentration (2 g DCW l^{-1}). Ethanol yields were lower than pure culture yields in the softwood SSL fermentation, likely owing to carbon reallocation (as noted for the mixed culture defined sugar fermentation), but were higher than, or comparable to, pure culture yields in the hardwood SSL fermentation (Table 1). This disparity was difficult to explain, except to suggest that diversion of carbon from ethanol production did not occur as a result of the mixing of two yeast strains in hardwood liquor.

Clearly, these results indicated that the fermentative performance of Y-1528 significantly exceeded that of Tembec T1 on all three lignocellulosic substrate mixtures. In contrast to T1, Y-1528 did not exhibit catabolite repression of galactose metabolism during hexose sugar fermentation. Furthermore, mixed cultures of Y-1528 and T1 accelerated substrate consumption in defined sugar and SSL media, with some evidence of a synergistic interaction between the strains.

Molecular genotyping

In light of the unusual metabolic behavior exhibited by Y-1528, and the absence of molecular analysis of Tembec T1, both strains were subject to genotyping in order to confirm their classical taxonomic classification as *S. cerevisiae*. The conserved rDNA-ITS (ribosomal DNA internal transcribed spacer) region of both strains, as well as that of a reference strain of *S. cerevisiae* (BY4742), was amplified and digested with five restriction endonucleases (*Bsu*RI, *Dra*I, *Eco*RI, *Hinf*I, and *Hin*6I), yielding distinctive type-specific banding patterns (in a cooled, high resolution 3% agarose gel) that aided in identifying both yeasts of interest to the genus and species level (Fig. 8). Fragment sizes were calculated using regression equations based on DNA mass ladder component migration distances and known fragment sizes, and subsequent pattern comparison indicated both strains to be *S. cerevisiae* (Table 2).

Molecular methods of species identification avoid the potential for environmentally mediated fluctuation inherent to phenotypic (morphological and metabolic) characterization. Ribosomal DNA, particularly the ITS region, is known to undergo sufficient evolutionary change to yield variance among species belonging to the same genus, yet typically remain conserved within the said species [7, 31, 33]. Furthermore, the robust nature of PCR-RFLP analysis of conserved regions of genomic DNA in classifying fungi of unknown identity has been demonstrated [7, 31]. The banding patterns obtained from Y-1528 and T1 matched those of *S. cerevisiae* BY4742 (a deletion strain derivative of *S. cerevisiae* S288C [1]), and corresponded to selected patterns gen-

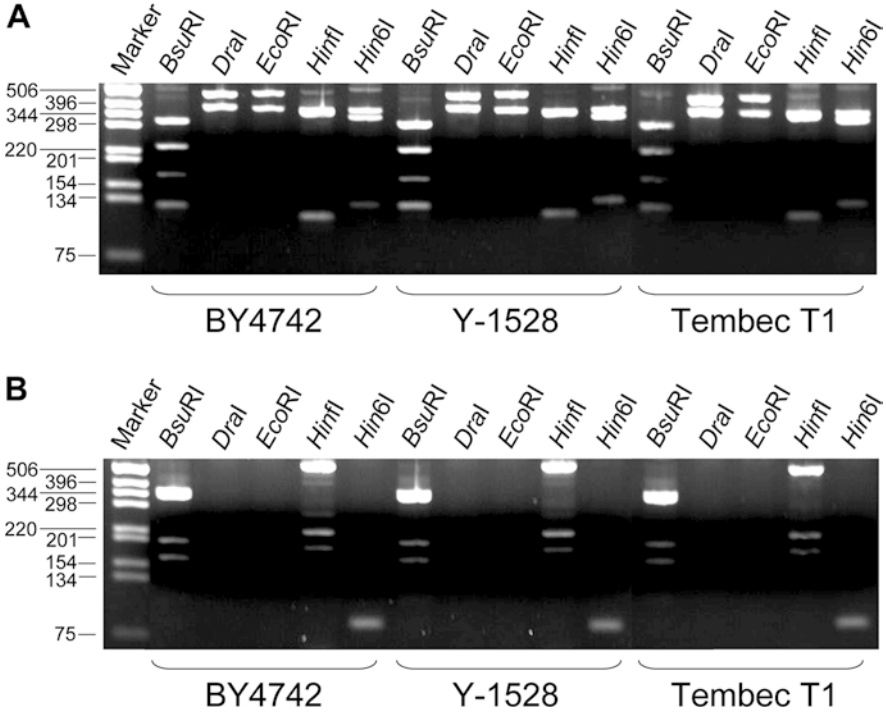

Fig. 8a,b Agarose (3%) gel images of restriction endonuclease fragments of ribosomal DNA amplicons from BY4742, Y-1528, and Tembec T1. Digestion of internal transcribed spacer (ITS)1/ITS4-primed amplicons (**a**) and ITS3/LR3-primed amplicons (**b**) is illustrated. Marker base pair sizes are indicated

Table 2 Restriction fragment patterns by base pair (bp) size, derived from regression equations following amplification of BY4742, Tembec T1, and Y-1528 ribosomal DNA by internal transcribed spacer (ITS)1/ITS4 and ITS3/LR3 primer sets and subsequent endonuclease digestion. Each primer set yielded identical patterns from all three yeast strains

	*Bsu*RI	*Dra*I	*Eco*RI	*Hinf*I	*Hin*6I
ITS1/ITS4					
Band 1 (bp)	321	431	439	351	449
Band 2 (bp)	240	375	367	107	356
Band 3 (bp)	172	–	–	–	330
Band 4 (bp)	123	–	–	–	123
ITS3/LR3					
Band 1 (bp)	334	–	–	458	76
Band 2 (bp)	196	–	–	215	–
Band 3 (bp)	161	–	–	182	–
Band 4 (bp)	–	–	–	–	–

erated from a type strain of *S. cerevisiae* in a recent experiment [7]. These results reaffirmed that the combination of primers (ITS1 and ITS4, or ITS3 and LR3), thermocycling program, and specific restriction endonuclease digestions, as derived from the literature, was effective in permitting discrimination among strains to the species level [7, 31, 33].

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