

Chapter 3

Engineering the *Pichia stipitis* Genome for Fermentation of Hemicellulose Hydrolysates

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Pichia stipitis Pignal (1967) is a hemiascomycetous yeast (Kurtzman, 1990; Melake et al., 1996; Vaughan Martini, 1984), closely related to several yeast endosymbionts of passalid beetles (Nardi et al., 2006) that inhabit and degrade white-rotted hardwood (Suh et al., 2003, 2004). It seems to be well adapted to this environment because the *P. stipitis* genomic sequence reveals numerous features that would enable survival and growth in a wood-inhabiting insect's gut (Nardi et al., 2006). It has the capacity to grow on and ferment xylan (Lee et al., 1986; Ozcan et al., 1991) and to use all of the major sugars found in wood. It both assimilates and ferments cellobiose (Parekh and Wayman, 1986; Parekh et al., 1988). In addition, it has been reported to transform low-molecular-weight lignin-related aromatic aldehydes such as 3,4-dimethoxybenzaldehyde and 3-methoxy-4-hydroxybenzylaldehyde to their corresponding alcohols and acids during aerobic culture (Targonski, 1992).

P. stipitis has been studied mainly for its capacity to ferment D-xylose to ethanol. Xylose is a five-carbon sugar that makes up about 15 to 25% of all hardwoods and agricultural residues (Jeffries and Shi, 1999). Its fermentation is therefore essential for the economic conversion of lignocellulose to ethanol (Gulati et al., 1996; Hinman et al., 1989; Saha et al., 1998). *P. stipitis* can produce up to 57 g of ethanol per liter from xylose (Ferrari et al., 1992) when the pH is maintained between 4.5 and 6 (Slininger et al., 1990). The optimal fermentation temperature is 25 to 26°C for xylose and 30°C for glucose. A maximum volumetric ethanol productivity of 0.9 g of ethanol l⁻¹ · h⁻¹ has been reported (du Preez et al., 1986). The specific ethanol production rate is between 0.15 and 0.17 g of ethanol · g of cells⁻¹ · h⁻¹ on xylose and up to 0.35 g · g⁻¹ · h⁻¹ on glucose (Grootjen et al., 1990). The optimal oxygen transfer rate is 3 to 5 mmol of O₂ · l⁻¹ · h⁻¹ (Guebel et al., 1991). Under oxygen-limited conditions, *P. stipitis* shows a

maximum ethanol yield from xylose of 0.47 g/g (Ligthelm et al., 1988). Ethanol production from acid hydrolysates of wheat straw and eucalyptus wood has been reported with *P. stipitis* at yields of 0.35 to 0.41 g/g (Ferrari et al., 1992; Nigam, 2001b). Fermentation of D-xylose by *P. stipitis* depends on the acetic acid concentration and the availability of oxygen. The volumetric rate of ethanol production is inhibited 50% by acetic acid at concentrations of 0.8 and 13.8 g l⁻¹ at pH 5.1 and 6.5, respectively, under anaerobic conditions (Van Zyl et al., 1991). *P. stipitis* can be adapted to tolerate higher concentrations of acetic acid (Mohandas et al., 1995) and hardwood hydrolysates (Nigam, 2001a).

P. stipitis has been a source of genes for engineering xylose metabolism in *Saccharomyces cerevisiae*—a task that has been undertaken in numerous laboratories around the world (Jeffries and Jin, 2004). Much less effort has gone into engineering *P. stipitis* for improved xylose metabolism. In addition to genes for xylose assimilation, *P. stipitis* possesses the regulatory machinery that enables the efficient conversion of xylose under oxygen-limited conditions. While such engineering is conceivable, alteration of transcriptional regulators and *cis*-acting factors could be very difficult. *P. stipitis* shunts most of its metabolic flux into ethanol and produces very little xylitol, but its xylose fermentation rate is low relative to that of *S. cerevisiae* on glucose. Strain development for improved fermentation rates and higher ethanol tolerance is still needed. Increasing the capacity of *P. stipitis* for rapid xylose fermentation could therefore greatly improve its usefulness in commercial xylose fermentations.

GENETIC SYSTEM

P. stipitis is homothallic, but its ploidy has been difficult to establish (Gupthar, 1994). The high mutational frequency of wild-type strains suggests that they

are haploid. Genome sequencing of *P. stipitis* CBS 6054 did not indicate diploid polymorphisms. Since these strains can also form spores, they are homothallic (Melake et al., 1996). Sporulation can be induced by cultivation under nutritionally poor conditions, and it is possible to increase the ploidy through fusion or mating (Gupthar, 1987; Gupthar and Garnett, 1987). Fusions between *P. stipitis* and *Candida shehatae* can lead to incorporation of *C. shehatae* DNA into the *P. stipitis* genome (Gupthar and Garnett, 1987; Selebano et al., 1993), but fusions between *P. stipitis* and *S. cerevisiae* tend to segregate (Gupthar, 1992). Electrophoretic karyotyping indicated that both *P. stipitis* and its presumptive anamorph have six chromosomes (Passoth et al., 1992), but genome sequencing has shown that two pairs of chromosomes are very similar in size and that the actual number of chromosomes is eight (Jeffries et al., 2007).

GENETIC TRANSFORMATION

The first genetic transformation system for *P. stipitis* described by Ho et al. (1991) was based on the kanamycin resistance gene (Km^r); however, the transformation frequency was very low. Much higher transformation frequencies have been achieved using auxotrophs of *ura3* along with the homologous native *URA3* gene and an autonomous replication sequence, *ARS2* (Yang et al., 1994). This system has been further

extended by disrupting and recovering the *leu2* auxotrophic marker (Lu et al., 1998). Heterologous complementation of *his3* has also been used to transform *P. stipitis* (Morosoli et al., 1993; Piontek et al., 1998).

Orotidine-5'-P decarboxylase (*ura3*) mutants can be obtained by selecting on minimal medium that contains 50 μ g of uracil/ml (or 100 μ g of uridine/ml) along with 1 mg of 5'-fluoroorotic acid (5-FOA)/ml (Boeke et al., 1984). This method has been used successfully with *P. stipitis* to obtain spontaneous mutants from cells that have undergone sporulation, but the technique has not been successful in obtaining mutants from fresh or actively growing cells. Three *P. stipitis ura3* mutants have proven useful. These are TJ-26, PSU1, and UC7 (Fig. 1). Of these, TJ26 and PSU1 are relatively stable point mutations; UC7 is an insertional mutant and is completely stable. All three of these mutants grow relatively poorly—even when the medium is supplemented with uracil—but all three show normal growth when complemented with the native *URA3* gene.

The most useful recipient hosts for genetic manipulation and metabolic engineering are FPL-UC7 (*ura3*), FPL-PLU20 (*ura3*, *leu2* Δ), and FPL-PLU5 (*ura3*, *leu2* Δ). These are completely stable with respect to their selectable markers. These strains also contain a number of mutations that distinguish them from the parental strain, CBS 6054. FPL-061 was obtained following nitrosoguanidine mutagenesis and selection on several different slowly utilized carbon sources in the presence of respiration inhibitors (Jeffries and Livingston, 1992;

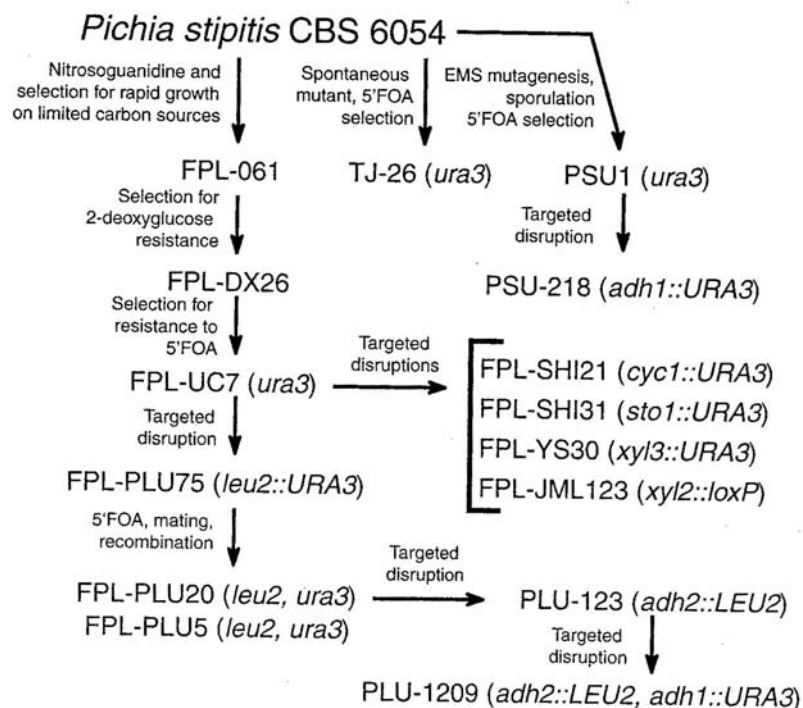


Figure 1. Mutants developed from *P. stipitis* CBS 6054.

Sreenath and Jeffries, 1997). This strain was then selected for resistance to 2-deoxyglucose in order to obtain higher rates of xylose utilization in the presence of glucose (Sreenath and Jeffries, 1999). We then selected for resistance to 5-FOA to obtain the *ura3* strain, UC7, and then used targeted disruption with flanking direct repeats to obtain the PLU20 and PLUS strains (*ura3*, *leu2Δ*) (Lu et al., 1998).

The report of Ho et al. (1991) notwithstanding, drug resistance markers for genetic transformation of *P. stipitis* have been very difficult to identify and use. This is in part because *P. stipitis* uses the nonconventional yeast codon system 12 in which CUG codes for serine rather than leucine (Santos and Tuite, 1995; Sugita and Nakase, 1999). While CUG is not commonly used by *P. stipitis*, it is frequently used in bacterial genes that comprise many drug resistance markers. Modification of the CUG codons in the *Sh ble* selectable marker into other codons specifying leucine enables use of phleomycin D1 (Zeocin) as an antibiotic in selecting for transformation of nonauxotrophic *P. stipitis* strains (Laplaza et al., 2006). In addition to modifying *Sh ble*, Laplaza et al. (2006) modified the CUG codons in Cre recombinase (Guldener et al., 1996) and developed an improved vector with *URA3* flanked by *loxP* sites to enable easy excision and repeated use of the auxotrophic selectable marker.

Genetic transformation techniques for *P. stipitis* generally are similar to those used for *S. cerevisiae* except that transformation frequencies are usually lower, and targeted disruptions are more difficult. Genetic transformation with *URA3* is more efficient than transformation with the modified *Sh ble* marker. Complementation with autonomous vectors is much more efficient than site-specific disruptions. With an ARS-based plasmid, it is possible to obtain transformants with 10 µg of DNA. When carrying out a site-specific disruption using a linearized fragment, it is generally necessary to use at least 500 bp of flanking sequence on each end of the target gene and to use 10 times as much DNA (i.e., approximately 100 µg per transformation mixture). The number of transformants obtained decreases, but the specificity of the targeted disruption increases with increasing length of flanking DNA. For example, with flanking regions of 500 to 1,000 bp, it is generally necessary to screen ~50 to 100 transformants before obtaining a site-specific disruptant. With 1,500 bp, disruptants can be obtained from as few as 30 transformants.

The success of the transformation depends greatly on the viability of the recipient host. It is very important to have a young, vigorously growing culture. This is best accomplished by transferring the culture from a fresh plate into broth the night before the transformation. At the time of harvest for transformation, the cell

Table 1. Fermentation characteristics of three strains of *P. stipitis* grown in shake flasks

Fermentation parameters ^a	CBS 6054 (wild type)	DC7 (<i>ura3</i>)	Shi21 (<i>cyc1</i>)
Y_x (g·g ⁻¹)	0.16	0.17	0.09
Y_p (g·g ⁻¹)	0.41	0.38	0.46
Q_{eth} (g·g ⁻¹ ·h ⁻¹)	0.04	0.03	0.06
Q_{xo} (g·g ⁻¹ ·h ⁻¹)	0.11	0.09	0.13

^a Y_x , cell yield; Y_p , ethanol yield; Q_{eth} , specific ethanol production rate; Q_{xo} , specific xylose consumption rate.

density should not exceed an optical density at 600 nm of 1.0.

We have used targeted disruptions to obtain mutants of beta-isopropylmalate dehydrogenase (*LEU2*) (Lu et al., 1998); cytochrome *c*, *CYC1* (Shi et al., 1999); the alternative oxidase, *STO1* (Shi et al., 2002); alcohol dehydrogenase 1 and 2 (*ADH1* and *ADH2*) (Cho and Jeffries, 1998); D-xylulokinase, *XYL3* (Jin et al., 2002); and xylitol dehydrogenase, *XYL2* (Laplaza et al., 2006) (Fig. 1).

Deletion of cytochrome *c* proved to be a particularly effective means of increasing the specific cell yield of ethanol. *P. stipitis* is a petite-negative yeast in that cells completely deficient in mitochondria are not viable (Alexander and Jeffries, 1990). When *CYC1* is disrupted, however, the cells completely lose their terminal cytochrome oxidase complex and produce ethanol with a 50% higher specific fermentation rate (Shi et al., 1999) (Table 1).

METABOLIC REGULATION

Cultivation conditions can strongly affect expression of fermentative enzymes in *P. stipitis*. Unlike *S. cerevisiae*, which regulates fermentation by sensing the presence of glucose, *P. stipitis* induces fermentative activity in response to oxygen limitation (Klinner et al., 2005; Passoth et al., 1998, 2003). Alcohol dehydrogenase and pyruvate decarboxylase are induced in response to oxygen limitation (Cho and Jeffries, 1998, 1999; Klinner et al., 2005; Passoth et al., 1998, 2003), and transcription of a number of other genes is affected as well.

One of the biggest problems that *P. stipitis* must overcome in converting xylose into ethanol is an imbalance of cofactors that arise during xylose assimilation. *P. stipitis* is a bit unusual in that its enzyme for xylose reductase, Xyl1, accepts either NADH or NADPH as a cofactor. The second enzyme in this pathway, xylitol dehydrogenase, Xyl2, accepts only NAD. Xyl1, however, has a higher affinity for NADPH than for NADH and tends to use it preferentially. The coupling of Xyl1 and Xyl2 activities therefore tends to result in the consumption of NADPH and accumulation of NADH. At the same time,

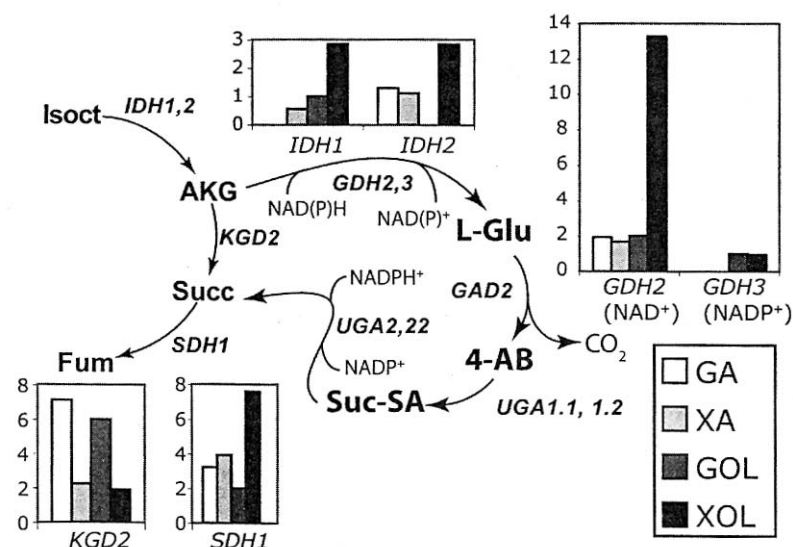


Figure 2. Relative expression of transcripts for the glutamate decarboxylase bypass. Abbreviations: *IDH1* and *IDH2*, isocitrate dehydrogenase 1 and 2; *GDH2*, NAD-specific glutamate dehydrogenase; *GDH3*, NADP-specific glutamate dehydrogenase; *GAD2*, glutamate decarboxylase 2; *UGA1.1* and *UGA1.2*, 4-aminobutyrate aminotransferase; *UGA2* and *UGA22*, succinate semialdehyde dehydrogenase; *KGD2*, 2-ketoglutarate dehydrogenase; Isocit, isocitrate; AKG, 2-keto-glutarate; L-Glu, L-glutamate; 4-AB, 4-aminobutyrate; Suc-SA, succinate semialdehyde; Succ, succinate; Fum, fumarate; GA, glucose aerobic; XA, xylose aerobic; GOL, glucose oxygen limited; XOL, xylose oxygen limited.

excess NADH production (over NAD consumption) arises during oxygen-limited growth because the overall composition of yeast cells is generally more oxidized than the sugar they grow on. Thus, when growing on xylose under oxygen limitation, NADH is present in excess.

P. stipitis compensates for this imbalance in a number of ways. First, it appears to strongly induce expression of NAD-specific glutamate dehydrogenase (*GDH2*) when cells are grown on xylose under oxygen limitation (Fig. 2) (Jeffries et al., 2007). This enzyme consumes NADH to convert 2-keto-glutarate (AKG) into L-glutamate, which can then be decarboxylated by glutamate decarboxylase 2 (*GAD2*) to form 4-aminobutyrate. Transamination by 4-aminobutyrate aminotransferase (*UGA1.1* or *UGA1.2*) converts this into succinate semialdehyde, which is then oxidized by NADP to succinate by succinate semialdehyde dehydrogenase (*UGA2* or *UGA22*). The net effect of this bypass converts NADH into NADPH. 2-Ketoglutarate dehydrogenase (*KGD2*) normally converts AKG into succinate with the generation of NADH, but this enzyme is subject to allosteric inhibition by NADH, so when NADH levels are high during oxygen-limited growth on xylose, *GDH2* is induced and the redox cofactor imbalance can be reduced. The transcript levels of isocitrate dehydrogenase (*IDH1* or *IDH2*) and succinate dehydrogenase (*SDH1*) are higher when cells are growing on xylose under oxygen-limited conditions, but the levels of 2-ketoglutarate are lower, further indicating that the NADH-specific-glutamate dehydrogenase (*GDH2*) is active under these conditions. This by-

pass has been engineered into *S. cerevisiae*, where it has some of the same effect (Grotkjaer, 2005), but it appears to exist naturally in *P. stipitis*.

Another route by which *P. stipitis* appears to use excess reductant when growing on xylose is through the induction of genes for lipid synthesis. Preliminary data based on expressed sequence tags indicate that transcripts for fatty acid synthase (*FAS2*) stearoyl-coenzyme A desaturase (*OLE1*) are induced under oxygen-limiting conditions (Fig. 3).

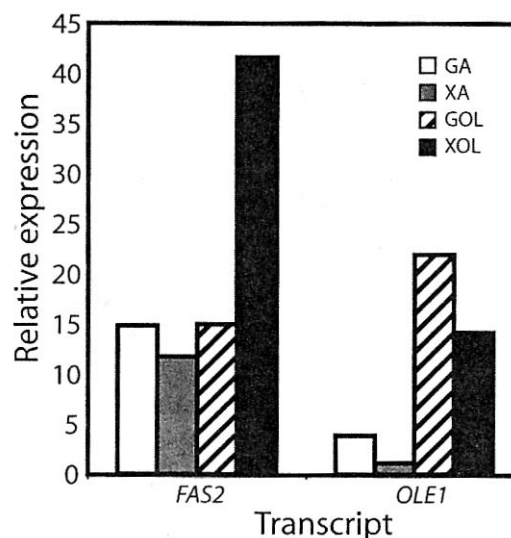


Figure 3. Induction of transcripts for lipid synthesis under oxygen-limiting conditions. Abbreviations: GA, glucose aerobic; XA, xylose aerobic; GOL, glucose oxygen limited; XOL, xylose oxygen limited.

GENOME ORGANIZATION

Xylanase, Cellulase, β -Glucosidase, and Mannanase Utilization

Xylanase production by *P. stipitis* has been recognized since 1991 (Basaran et al., 2000; Ozcan et al., 1991), and the organism has also been transformed with heterologous xylanases to increase xylanase activity (Den Haan and Van Zyl, 2001, 2003; Morosoli et al., 1993; Passoth and Hahn-Hagerdal, 2000). A xylanase gene (*xynA*) cloned from *P. stipitis* NRRL Y-11543 (Basaran et al., 2001) belongs to glycosyl hydrolase family 11. This xylanase gene was not found in the genome of *P. stipitis* CBS 6054, but another xylanase gene (*XYN1*) belonging to glycoside hydrolase family 10 was present (Jeffries et al., 2007). Preliminary expression analyses suggest that the *XYN1* gene is induced when cells are cultivated on xylose.

P. stipitis also appears to possess considerable capacity for assimilation and fermentation of cellulose oligosaccharides. As mentioned earlier, *P. stipitis* can ferment cellobiose (Parekh and Wayman, 1986; Parekh et al., 1988; Sibirny et al., 2003), which is a trait that could be very useful in the simultaneous enzymatic sac-

charification and fermentation of cellulose, but which is also beneficial in the metabolism of partial cellulose hydrolysates formed by thermochemical pretreatments. The genome contains seven β -glucosidases (*BGL1-7*) and three β -(1 $\rightarrow$ 4) endoglucosidases (*BGL1-3*), but what is really striking is the way that they are arranged in the genome. In all but two instances, the β -glucosidase is found immediately adjacent or proximal to one of the three endoglucanases, a sugar transporter, or both (Fig. 4). In each case, the β -glucosidase belongs to glycoside hydrolase family 3 and the endoglucanase to family 5.

Examination of each of these gene clusters shows that the orientation of the β -glucosidase with the endoglucosidase or the sugar transporter is virtually identical in the case of the *BGL1* and *BGL3* clusters on chromosomes 4 and 6, respectively. This indicates that these two clusters probably arose through duplication. Gene orientations in the other clusters are different in each instance, which implies that they did not arise through duplication and that the association of these genes with one another imparts a survival advantage. Further evidence of gene duplication is found in the phylogenetic relationships among the hexose transporters and β -glucosidases in each of these clusters

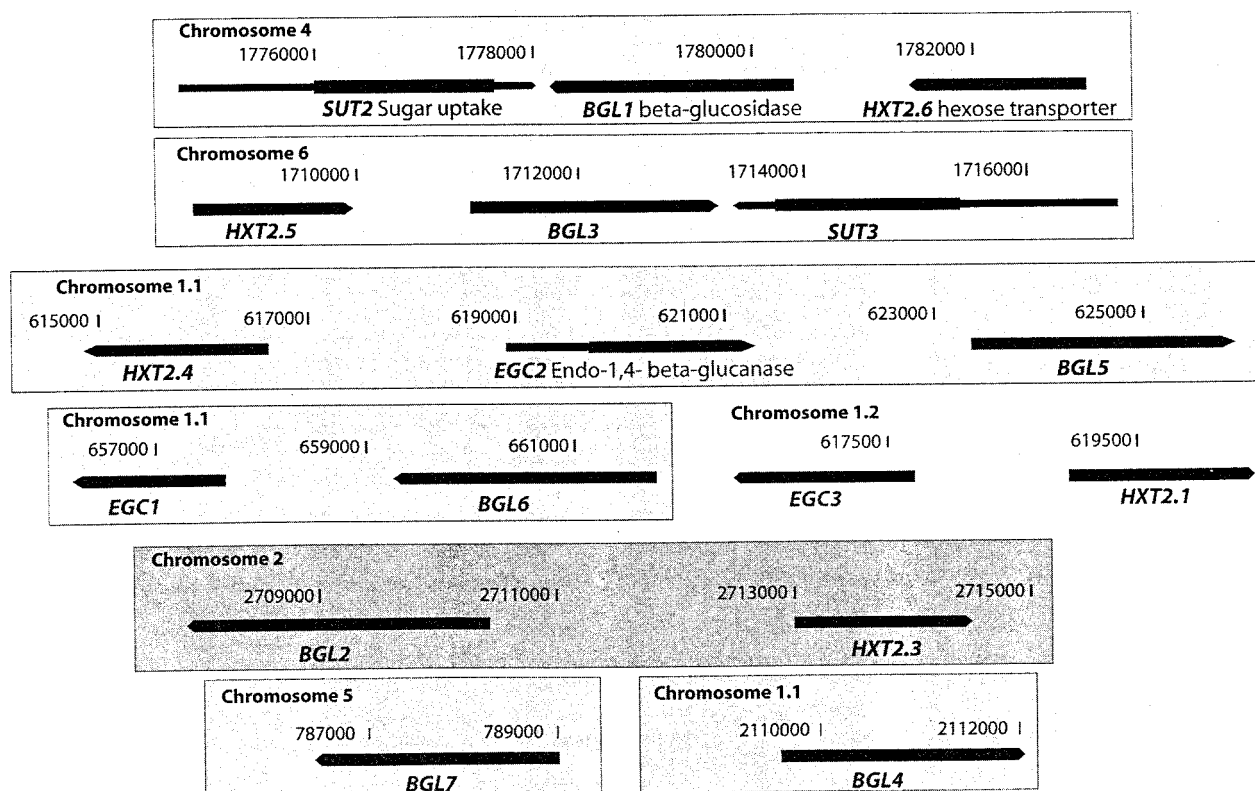


Figure 4. Gene clusters of β -glucosidases and endoglucanases with sugar transporters in the *P. stipitis* genome. Approximate chromosome coordinates are shown. All constructs were derived from the original sequence deposit at the Joint Genome Institute website (<http://genome.jgi-psf.org/Picst3/Picst3.home.html>). The complete genome is also found in GenBank.

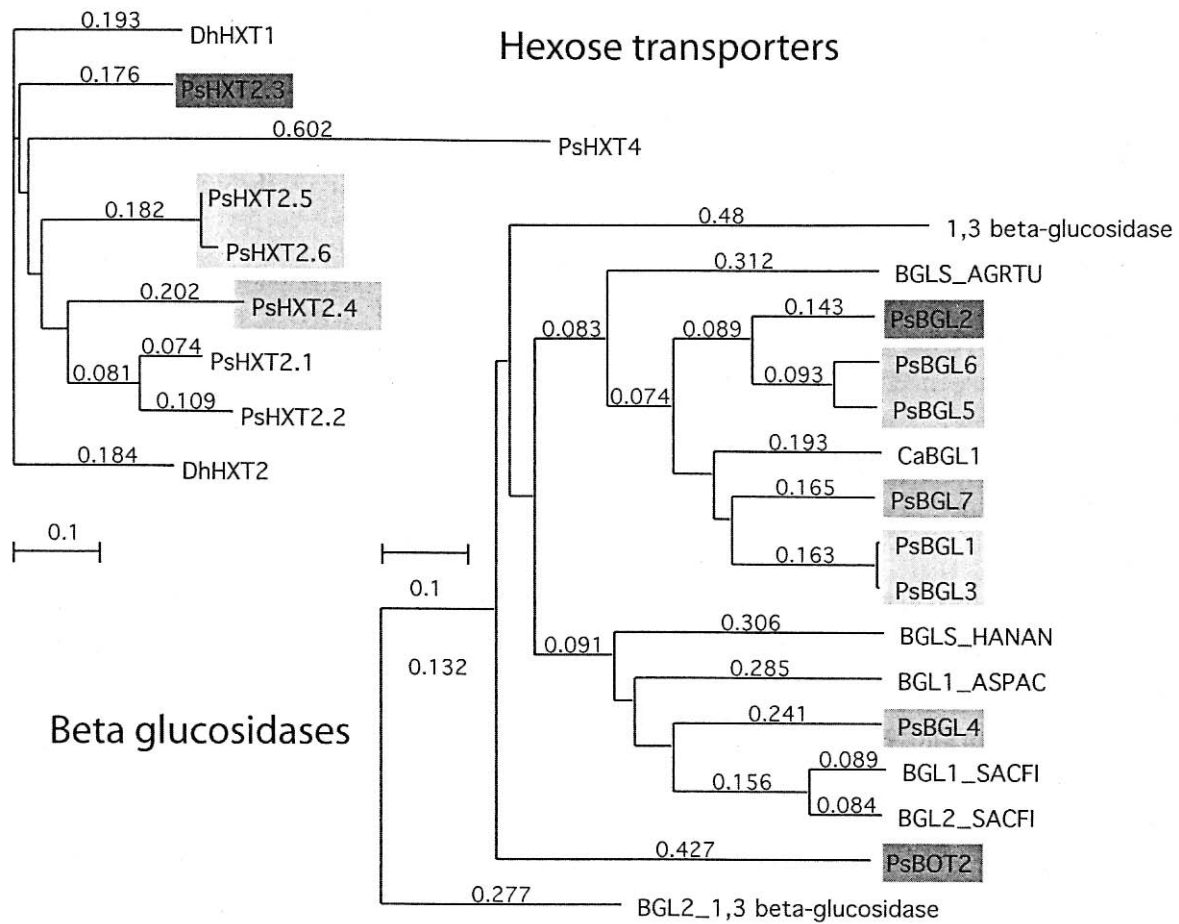


Figure 5. Phylogenetic relationships among hexose transporters and beta-glucosidases found in clusters in *P. stipitis*. Gene names correspond to designations given on the Joint Genome Institute website (see legend to Fig. 4 for URL) and in GenBank. The method used was Neighbor Joining with Best Tree; distances were uncorrected, and gaps were distributed proportionally.

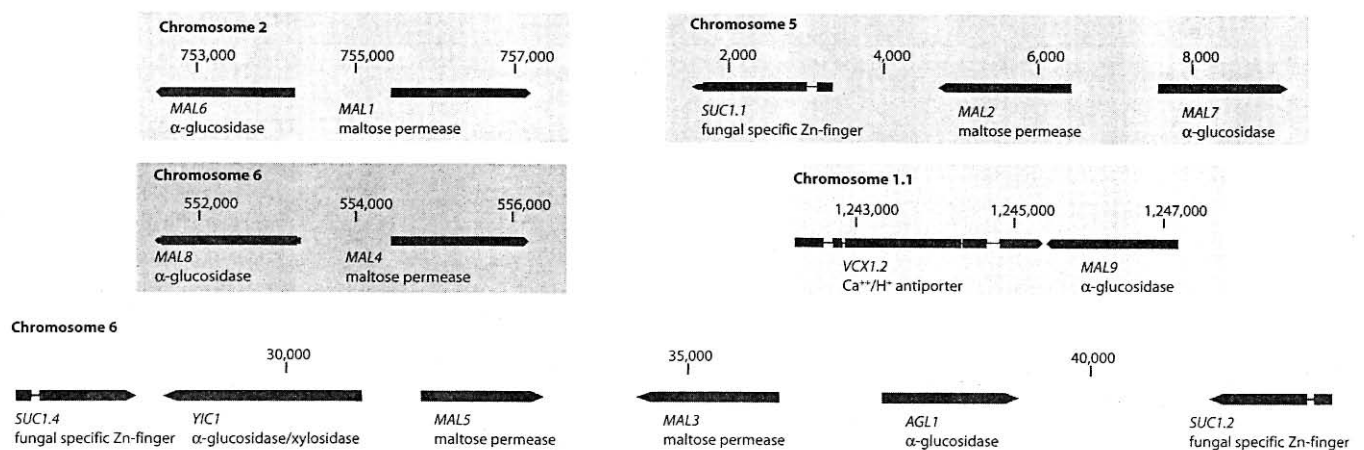


Figure 6. Gene clusters of α -glucosidases with putative maltose permeases in the *P. stipitis* genome. Approximate chromosome coordinates are shown. All constructs were derived from the original sequence deposit at the Joint Genome Institute website (see legend to Fig. 4 for URL). The complete genome is also found in GenBank.

(Fig. 5). *BGL1* and *BGL3*, which are in the paired triplet clusters, are most closely related taxonomically, as are *HXT2.5* and *HXT2.6*. *BGL5* and *BGL6* are closely related as well. They do not share an *HXT* gene in their clusters, but *EGC1* and *EGC2* are more closely related to one another than to *EGC3* (data not shown).

Maltose Utilization

A similar genome organization is observed in the case of α -glucosidases and maltose permeases. In this

case, the genome contains five maltose permeases and five α -glucosidases. In four instances, these are arranged in pairs with each pair containing a permease and a glucosidase in a divergent orientation. In one locus, two permeases and two glucosidase/xylosidase genes are found along with putative fungal-specific Zn finger regulatory proteins (Fig. 6). Phylogenetically, the five maltose permeases (*MAL 1-5*) form a relatively close clade (Fig. 7). The one exception is *MALS*, which is also found associated with *YIC1*, which is the most divergent of all of the α -glucosidases (Fig. 8). In comparing

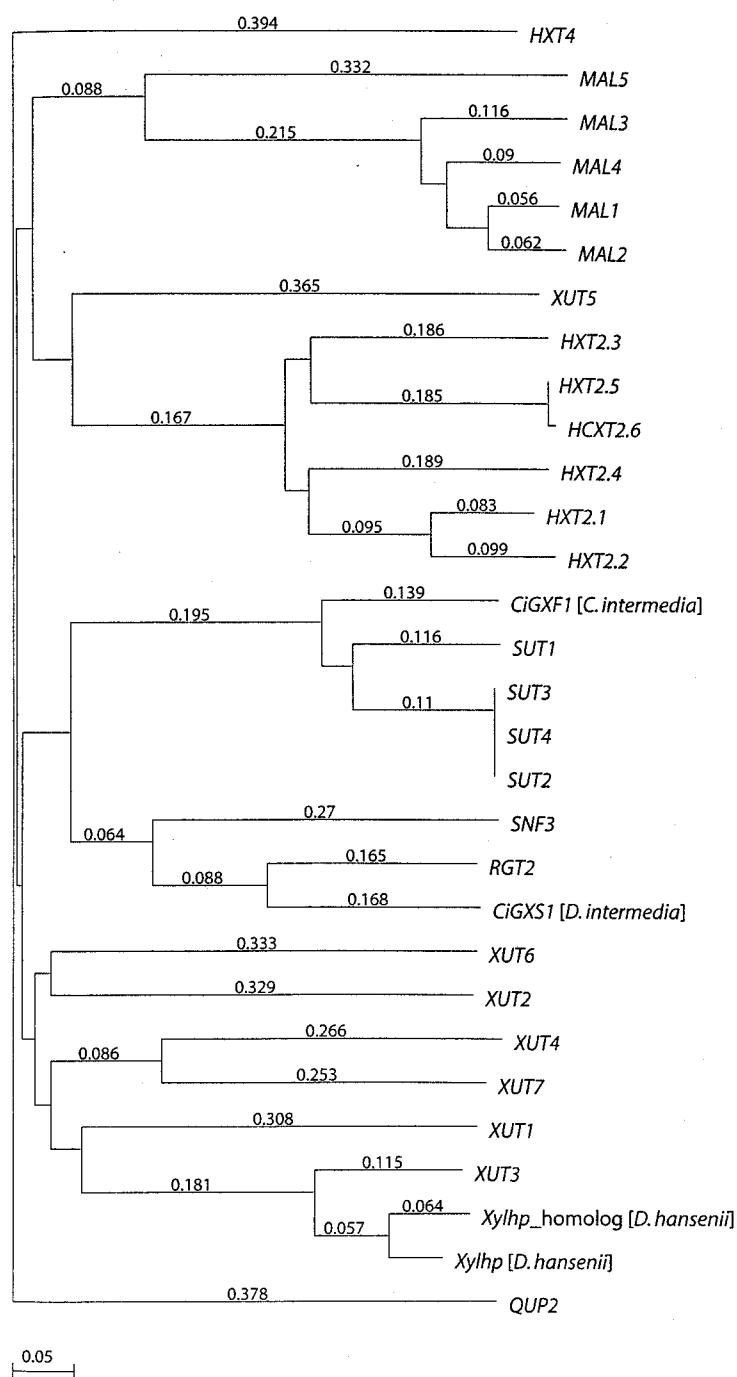


Figure 7. Phylogenetic relationships of putative sugar transporters from *P. stipitis*. Except as noted, all genes are from *P. stipitis*. Gene names correspond to designations given on the Joint Genome Institute website and in GenBank. Sequences for *CiGXF1* and *CiGXS1* are from *C. intermedia*; sequences for *Xylhp* and *Xylhp* homolog are from *D. hansenii*. The method used was Neighbor Joining with Best Tree; distances were uncorrected, and gaps were distributed proportionally.

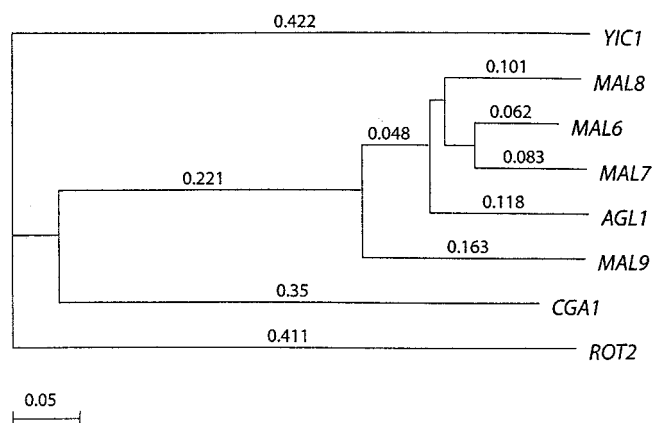


Figure 8. Phylogenetic relationships of α -glucosidases from *P. stipitis*. Gene names correspond to designations given on the Joint Genome Institute website and in GenBank. The method used was Neighbor Joining with Best Tree; distances were uncorrected, and gaps were distributed proportionally.

the relationships among maltose permeases, *MAL1* and *MAL2* are the closest to one another. The α -glucosidases with which they are associated, *MAL6* and *MAL7*, are most closely related (compare Fig. 6, 7, and 8). Likewise, the other paired genes show increasing

divergence, suggesting that these could have arisen through successive duplication.

Galactose and Mannose Utilization

The genome contains three family 2 glycoside hydrolases in three different loci. These are comprised of one β -galactosidase (*LAC4*), one β -mannosidase (*MAN2*), and one β -galactosidase (*BMS1*), the latter of which is immediately adjacent to a putative lactose permease (*LAC3*). *P. stipitis* also has a complete set of genes for galactose metabolism, and many of these are found in a single locus on chromosome 3 (Fig. 9). This is similar to the structure of the galactose gene cluster in *Kluyveromyces lactis* (Webster and Dickson, 1988) and in *S. cerevisiae*, except that in addition to *GAL1*, *GAL7*, and *GAL10*, *P. stipitis* also has *GAL102*, which codes for glucose-4-epimerase.

SUGAR ASSIMILATION PATHWAYS

Efficient sugar uptake (preferably by facilitated diffusion) is very important for fermentation because very little metabolic energy is available from substrate-

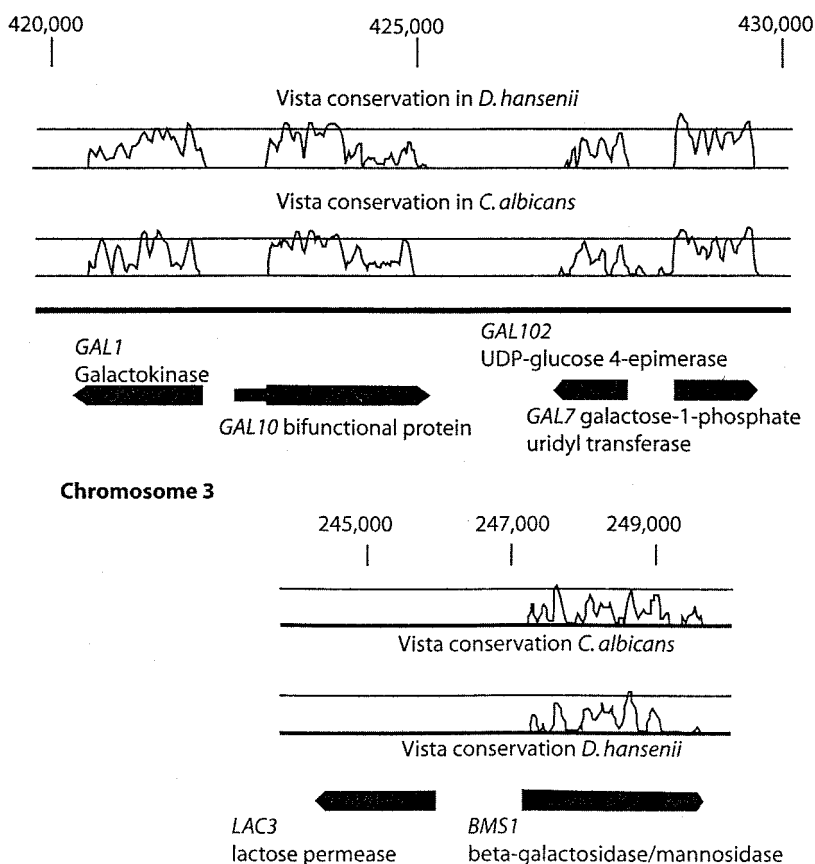


Figure 9. Galactose and lactose gene clusters in *P. stipitis* chromosome 3.

level phosphorylation and the restricted respiration possible under oxygen limitation. Since less metabolic energy is available from the assimilation of xylose than from glucose, this problem is even more critical to xylose fermentation.

Glucose and Xylose Transport

Xylose-specific sugar transporters are critically important for xylose assimilation in the presence of glucose. Glucose and xylose have very similar structures that differ only by the absence of a C6-OH group in xylose. Both sugars are recognized by xylose isomerases, which equilibrate the conversion of xylose or glucose into xylulose or fructose, respectively. In *S. cerevisiae* glucose transporters mediate xylose uptake. Their affinities are, however, much higher for glucose than for xylose, which means that in glucose-xylose mixtures, glucose uptake is almost complete before xylose is assimilated—even in cells that are engineered for xylose metabolism. *P. stipitis* likewise takes up glucose before xylose, but it appears to have several transporters that are relatively specific for xylose.

Thus far, three research groups have described putative xylose transporters from various yeasts. Weierstall et al. (1999) cloned, sequenced, and characterized three sugar uptake genes (*SUT1*, *SUT2*, and *SUT3*) from *P. stipitis* by complementing a glucose transporter-deficient mutant of *S. cerevisiae*, and completion of the *P. stipitis* genome revealed a fourth member of this closely related family (Fig. 7). These four proteins are highly similar in structure. *Sut2* and *Sut3* differ only in a single amino acid. Disruption of *SUT1* resulted in the loss of low-affinity glucose uptake. Leandro et al. (2006) described two glucose/xylose transporters from *Candida intermedia*, and Nobre and Lucas (A. Nobre and C. Lucas, unpublished data) described a putative xylose permease from *Debaryomyces hansenii*.

When these putative xylose transporters are aligned with putative sugar transporters from *P. stipitis* and other related yeasts, a number of proteins cluster together (Fig. 7). The *C. intermedia* glucose/xylose facilitator (GXF1) clusters most closely to the *P. stipitis* *SUT1-4* transporter family. However, the *C. intermedia* putative glucose/xylose symporter (*GXS1*) clusters most closely to a *P. stipitis* protein (*RGT2*) that is similar to a high-affinity glucose sensor. Moreover, the putative xylose transport protein from *D. hansenii*, *Xylhp*, clusters with the *P. stipitis* transporter *XUT3*. Because glucose and xylose are so similar, relatively few changes are necessary to alter substrate affinities; thus, in the absence of functional or regulatory information we cannot draw too many conclusions from these structural homologies between the proteins.

CONCLUSION

The unconventional yeast *P. stipitis* is potentially well suited for the fermentation of xylose and cellulose oligosaccharides from hydrolysates of lignocellulose. It produces ethanol with a relatively high yield and can metabolize all of the common sugars. A genetic system has been developed that includes the auxotrophic markers *URA3* and *LEU3* along with modified forms of the phleomycin D1 resistance marker, *Sh ble*, and the Cre recombinase. This system has been used to disrupt genes for respiration, which has resulted in higher specific fermentation rates. Expression analyses based on expressed sequence tags and gene chips can guide genetic modifications. Many genes in *P. stipitis* are found in functionally related clusters. Further metabolic engineering and strain selection are needed to increase the overall fermentation rate and ethanol tolerance of *P. stipitis* for the commercial bioconversion of hemicellulose hydrolysates.

REFERENCES

- Alexander, M. A., and T. W. Jeffries. 1990. Respiratory efficiency and metabolite partitioning as regulatory phenomena in yeasts. *Enzyme Microb. Technol.* **12**:2-19.
- Basaran, P., N. Basaran, and Y. D. Hang. 2000. Isolation and characterization of *Pichia stipitis* mutants with enhanced xylanase activity. *World J. Microbiol. Biotechnol.* **16**:545-550.
- Basaran, P., Y. D. Hang, N. Basaran, and R. W. Worobo. 2001. Cloning and heterologous expression of xylanase from *Pichia stipitis* in *Escherichia coli*. *J. Appl. Microbiol.* **90**:248-255.
- Boeke, J. D., F. LaCroute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**:345-346.
- Cho, J. Y., and T. W. Jeffries. 1998. *Pichia stipitis* genes for alcohol dehydrogenase with fermentative and respiratory functions. *Appl. Environ. Microbiol.* **64**:1350-1358.
- Cho, J. Y., and T. W. Jeffries. 1999. Transcriptional control of ADH genes in the xylose-fermenting yeast *Pichia stipitis*. *Appl. Environ. Microbiol.* **65**:2363-2368.
- Den Haan, R., and W. H. Van Zyl. 2001. Differential expression of the *Trichoderma reesei* beta-xylanase II (*xyn2*) gene in the xylose-fermenting yeast *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* **57**:521-527.
- Den Haan, R., and W. H. Van Zyl. 2003. Enhanced xylan degradation and utilisation by *Pichia stipitis* overproducing fungal xylanolytic enzymes. *Enzyme Microb. Technol.* **33**:620-628.
- du Preez, J. C., M. Bosch, and B. A. Prior. 1986. Xylose fermentation by *Candida shehatae* and *Pichia stipitis*—effects of pH, temperature and substrate concentration. *Enzyme Microb. Technol.* **8**:360-364.
- Ferrari, M. D., E. Neirrotti, C. Alborno, and E. Saucedo. 1992. Ethanol-production from eucalyptus wood hemicellulose hydrolysate by *Pichia stipitis*. *Biotechnol. Bioeng.* **40**:753-759.
- Grootjen, D. R. J., R. Vanderlans, and K. Luyben. 1990. Effects of the aeration rate on the fermentation of glucose and xylose by *Pichia stipitis* CBS-5773. *Enzyme Microb. Technol.* **12**:20-23.
- Grotkjaer, T., P. Christakopoulos, J. Nielsen, and L. Olsson. 2005. Comparative metabolic network analysis of two xylose fermenting recombinant *Saccharomyces cerevisiae* strains. *Metab. Eng.* **7**:437-444.

- Guebel, D. V., A. Cordenons, B. C. Nudel, and A. M. Giulietti. 1991. Influence of oxygen-transfer rate and media composition on fermentation of D-xylose by *Pichia stipitis* NRRL Y-7124. *J. Ind. Microbiol.* 7:287-291.
- Gulati, M., K. Kohlmann, M. R. Ladisch, R. Hespell, and R. J. Bothast. 1996. Assessment of ethanol production options for corn products. *Bioresour. Technol.* 58:253-264.
- Guldener, D., S. Heck, T. Fiedler, J. Beinhauer, and J. H. Hegemann. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24:2519-2524.
- Gupthar, A. S. 1987. Construction of a series of *Pichia stipitis* strains with increased DNA contents. *Curr. Genet.* 12:605-610.
- Gupthar, A. S. 1992. Segregation of altered parental properties in fusions between *Saccharomyces cerevisiae* and the D-xylose fermenting yeasts *Candida shehatae* and *Pichia stipitis*. *Can. J. Microbiol.* 38:1233-1237.
- Gupthar, A. S. 1994. Theoretical and practical aspects of ploidy estimation in *Pichia stipitis*. *Mycol. Res.* 98:716-718.
- Gupthar, A. S., and H. M. Garnett. 1987. Hybridization of *Pichia stipitis* with its presumptive imperfect partner *Candida shehatae*. *Curr. Genet.* 12:199-204.
- Hinman, N. D., J. D. Wright, W. Hoagland, and C. E. Wyman. 1989. Xylose fermentation—an economic analysis. *Appl. Biochem. Biotechnol.* 20-1:391-401.
- Ho, N. W., D. Petros, and X. X. Deng. 1991. Genetic transformation of xylose-fermenting yeast *Pichia stipitis*. *Appl. Biochem. Biotechnol.* 28-29:369-375.
- Jeffries, T. W., I. V. Grigoriev, J. Grimwood, J. M. Laplaza, A. Aerts, A. Salamov, J. Schmutz, E. Lindquist, P. Dehal, H. Shapiro, Y. S. Jin, V. Passoth, and P. M. Richardson. 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat. Biotechnol.* 25:319-326.
- Jeffries, T. W., and Y. S. Jin. 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl. Microbiol. Biotechnol.* 63:495-509.
- Jeffries, T. W., and P. L. Livingston. June 30, 1992. Xylose fermenting yeast mutants. U.S. patent 5,126,266.
- Jeffries, T. W., and N. Q. Shi. 1999. Genetic engineering for improved xylose fermentation by yeasts. *Adv. Biochem. Eng. Biotechnol.* 65:117-161.
- Jin, Y. S., S. Jones, N. Q. Shi, and T. W. Jeffries. 2002. Molecular cloning of XYL3 (D-xylulokinase) from *Pichia stipitis* and characterization of its physiological function. *Appl. Environ. Microbiol.* 68:1232-1239.
- Klinner, U., S. Fluthgraf, S. Freese, and V. Passoth. 2005. Aerobic induction of respiro-fermentative growth by decreasing oxygen tensions in the respiratory yeast *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* 67:247-253.
- Kurtzman, C. P. 1990. *Candida shehatae*—genetic diversity and phylogenetic relationships with other xylose-fermenting yeasts. *Antonie Leeuwenhoek* 57:215-222.
- Laplaza, J. M., B. R. Torres, Y. S. Jin, and T. W. Jeffries. 2006. Shble and Cre adapted for functional genomics and metabolic engineering of *Pichia stipitis*. *Enzyme Microb. Technol.* 38:741-747.
- Leandro, M. J., P. Goncalves, and I. Spencer-Martins. 2006. Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose-H⁺ symporter. *Biochem. J.* 395:543-549.
- Lee, H., P. Biely, R. K. Latta, M. F. S. Barbosa, and H. Schneider. 1986. Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipitis* strains. *Appl. Environ. Microbiol.* 52:320-324.
- Ligthelm, M. E., B. A. Prior, and J. C. Dupree. 1988. The oxygen requirements of yeasts for the fermentation of D-xylose and D-glucose to ethanol. *Appl. Microbiol. Biotechnol.* 28:63-68.
- Lu, P., B. P. Davis, J. Hendrick, and T. W. Jeffries. 1998. Cloning and disruption of the beta-isopropylmalate dehydrogenase gene (LEU2) of *Pichia stipitis* with URA3 and recovery of the double auxotroph. *Appl. Microbiol. Biotechnol.* 49:141-146.
- Melake, T., V. V. Passoth, and D. Klinner. 1996. Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis*. *Curr. Microbiol.* 33:237-242.
- Mohandas, D. V., D. R. Whelan, and C. J. Panchal. 1995. Development of xylose-fermenting yeasts for ethanol-production at high acetic-acid concentrations. *Appl. Biochem. Biotechnol.* 51-52:307-318.
- Morosoli, R., E. Zalce, and S. Durand. 1993. Secretion of a *Cryptococcus albidus* xylanase in *Pichia stipitis* resulting in a xylan fermenting transformant. *Curr. Genet.* 24:94-99.
- Nardi, J. B., C. M. Bee, L. A. Miller, N. H. Nguyen, S. O. Suh, and M. Blackwell. 2006. Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. *Arthr. Struct. Dev.* 35:57-68.
- Nigam, J. N. 2001a. Development of xylose-fermenting yeast *Pichia stipitis* for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate. *J. Appl. Microbiol.* 90:208-215.
- Nigam, J. N. 2001b. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. *J. Biotechnol.* 87:17-27.
- Ozcan, S., P. Kotter, and M. Ciriacy. 1991. Xylan-hydrolyzing enzymes of the yeast *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* 36:190-195.
- Parekh, S., and M. Wayman. 1986. Fermentation of cellobiose and wood sugars to ethanol by *Candida shehatae* and *Pichia stipitis*. *Biotechnol. Lett.* 8:597-600.
- Parekh, S. R., R. S. Parekh, and M. Wayman. 1988. Fermentation of xylose and cellobiose by *Pichia stipitis* and *Brettanomyces clausenii*. *Appl. Biochem. Biotechnol.* 18:325-338.
- Passoth, V., M. Cohn, B. Schafer, B. Hahn-Hagerdal, and D. Klinner. 2003. Analysis of the hypoxia-induced ADH2 promoter of the respiratory yeast *Pichia stipitis* reveals a new mechanism for sensing of oxygen limitation in yeast. *Yeast* 20:39-51.
- Passoth, V., and B. Hahn-Hagerdal. 2000. Production of a heterologous endo-1,4-beta-xylanase in the yeast *Pichia stipitis* with an O-2-regulated promoter. *Enzyme Microb. Technol.* 26:781-784.
- Passoth, V., M. Hansen, D. Klinner, and C. C. Emeis. 1992. The electrophoretic banding pattern of the chromosomes of *Pichia stipitis* and *Candida shehatae*. *Curr. Genet.* 22:429-431.
- Passoth, V., B. Schafer, B. Liebel, T. Weierstall, and U. Klinner. 1998. Molecular cloning of alcohol dehydrogenase genes of the yeast *Pichia stipitis* and identification of the fermentative ADH. *Yeast* 14:1311-1325.
- Piontek, M., J. Hagedorn, C. P. Hollenberg, G. Gellissen, and A. W. Strasser. 1998. Two novel gene expression systems based on the yeasts *Schwanniomyces occidentalis* and *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* 50:331-338.
- Saha, B. C., B. S. Dien, and R. J. Bothast. 1998. Fuel ethanol production from corn fiber—current status and technical prospects. *Appl. Biochem. Biotechnol.* 70-72:115-125.
- Santos, M. A. S., and M. F. Tuite. 1995. The CUG codon is decoded in-vivo as serine and not leucine in *Candida albicans*. *Nucleic Acids Res.* 23:1481-1486.
- Selebano, E. T., R. Govinden, D. Pillay, B. Pillay, and A. S. Gupthar. 1993. Genomic comparisons among parental and fusant strains of *Candida shehatae* and *Pichia stipitis*. *Curr. Genet.* 23:468-471.
- Shi, N. Q., J. Cruz, F. Sherman, and T. W. Jeffries. 2002. SHAM-sensitive alternative respiration in the xylose-metabolizing yeast *Pichia stipitis*. *Yeast* 19:1203-1220.
- Shi, N. Q., B. Davis, F. Sherman, J. Cruz, and T. W. Jeffries. 1999. Disruption of the cytochrome c gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. *Yeast* 15:1021-1030.
- Sibirny, A. A., O. B. Ryabova, O. M. Chmi, V. Sibirny, Z. Kotylak, and D. Grabek. 2003. Xylose and cellobiose fermentation to etha-

- nol by the thermotolerant methylotrophic yeast *Hansenula polymorpha* and by xylose fermenting yeast *Pichia stipitis*. *Yeast* **20**:S219-S219.
- Slininger, P. J., R. J. Bothast, M. R. Ladisch, and M. R. Okos.** 1990. Optimum pH and temperature conditions for xylose fermentation by *Pichia stipitis*. *Biotechnol. Bioeng.* **35**:727-731.
- Sreenath, H. K., and T. W. Jeffries.** 1999. 2-Deoxyglucose as a selective agent for derepressed mutants of *Pichia stipitis*. *Appl. Biochem. Biotechnol.* **77-79**:211-222.
- Sreenath, H. K., and T. W. Jeffries.** 1997. Diminished respiratory growth and enhanced assimilative sugar uptake result in higher specific fermentation rates by the mutant *Pichia stipitis* FPL-061. *Appl. Biochem. Biotechnol.* **63-65**:109-116.
- Sugita, T., and T. Nakase.** 1999. Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus *Candida*. *Syst. Appl. Microbiol.* **22**:79-86.
- Suh, S. O., C. J. Marshall, J. V. McHugh, and M. Blackwell.** 2003. Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. *Mol. Ecol.* **12**:3137-3145.
- Suh, S. O., M. M. White, N. H. Nguyen, and M. Blackwell.** 2004. The status and characterization of *Enteroramus dimorbus*: a xylose-fermenting yeast attached to the gut of beetles. *Mycologia* **96**:756-760.
- Targonski, Z.** 1992. Biotransformation of lignin-related aromatic-compounds by *Pichia stipitis* Pignal. *Zentbl. Mikrobiol.* **147**:244-249.
- Van Zyl, C., B. A. Prior, and J. C. Du Preez.** 1991. Acetic-acid inhibition of D-xylose fermentation by *Pichia stipitis*. *Enzyme Microb. Technol.* **13**:82-86.
- Vaughan Martini, A. E.** 1984. Comparazione dei genomi del lievito *Pichia stipitis* e de alcune specie imperfette affini. *Ann. Fac. Agr. Univ. Perugia* **38B**:331-335.
- Webster, T. D., and R. C. Dickson.** 1988. The organization and transcription of the galactose gene cluster of *Kluyveromyces lactis*. *Nucleic Acids Res.* **16**:8011-8028.
- Weierstall, T., C. P. Hollenberg, and E. Boles.** 1999. Cloning and characterization of three genes (*SUT1-3*) encoding glucose transporters of the yeast *Pichia stipitis*. *Mol. Microbiol.* **31**:871-883.
- Yang, V. W., J. A. Marks, B. P. Davis, and T. W. Jeffries.** 1994. High-efficiency transformation of *Pichia stipitis* based on its *URA3* gene and a homologous autonomous replication sequence, *ARS2*. *Appl. Environ. Microbiol.* **60**:4245-4254.

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