CHARACTERIZATION OF AN ETHANOLOGENIC YEAST EXHIBITING ATYPICAL GALACTOSE METABOLISM

by

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Abstract

In the near future, biomass-derived energy is predicted to substantially complement that generated from petroleum. However, certain types of biomass employed as substrates in the microorganism-mediated production of renewable fuel ethanol contain significant amounts of the recalcitrant hexose sugar galactose. The consumption of galactose in hexose sugar-fermenting yeasts is often delayed with respect to other sugars, such as glucose and mannose, because of an intrinsic preference for carbon sources requiring less energy in the preparatory reactions preceding glycolysis. This work comprised the search for, and characterization of an ethanologenic yeast capable of efficiently assimilating galactose.

Screening experiments conducted with wild-type *Saccharomyces cerevisiae* strains identified one isolate (Y-1528) exhibiting exceptionally fast galactose fermentation. The absence of conventional glucose repression, including a preference for galactose as carbon source and notable delays in the utilization of glucose and mannose, was demonstrated in mixed sugar fermentations. Endogenous extracellular glucose was observed during double sugar fermentations of galactose and mannose. This glucose was traced to supplied galactose by radioisotope labeling, suggesting involvement of UDP-galactose 4-epimerase in the responsible reaction mechanism(s). Sub-cellular fractionation was employed in an attempt to ascertain enzyme localization in Y-1528.

Fermentations of lignocellulosic substrate mixtures by Y-1528 illustrated better performance than that accomplished by a reference yeast strain, and again showed a preference for galactose. Mixed cultures of Y-1528 and the same reference strain demonstrated accelerated hexose sugar consumption, and no detrimental effects from competition, during synthetic and lignocellulosic substrate fermentations. Glucose repression was absent in mixed culture fermentations.

Fermentations of synthetic sugar mixtures augmented with lignocellulosic inhibitory compounds showed Y-1528 to have better performance than a reference yeast strain, despite a global detrimental effect relative to inhibitor-free fermentations. Cell recycle batch fermentations of spent sulfite liquor illustrated the toxic effect of the hardwood variant, as well as a net loss of performance from all strains tested.

Y-1528 was taxonomically confirmed as *S. cerevisiae*. UDP-galactose 4epimerase chromatographic purification was unsuccessful, but a partial sequence of the enzyme, showing complete identity with type sequence, was obtained by electrophoretic separation, liquid chromatography, and mass spectrometry. A significantly mutated UDP-galactose 4-epimerase gene was successfully sequenced.

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Co-Authorship Statement

This thesis contains four manuscripts which were written with the intent of publication in peer-reviewed journals. The manuscripts comprising chapters 2, 3, and 4 have all been published. The manuscript comprising chapter 5 will be submitted for publication. The contributions to the work by each author are as follows:

Chapter 2: Jeffrey Keating helped identify the research opportunity, guided the design of the research, performed the research and data analyses, and prepared the manuscript. Jamie Robinson assisted in the design of the research. Rodney Bothast and John Saddler helped identify the research opportunity. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

Chapter 3: Jeffrey Keating helped identify the research opportunity, guided the design of the research, performed the research and data analyses, and prepared the manuscript. Jamie Robinson assisted in the design of the research. Michael Cotta and John Saddler helped identify the research opportunity. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

Chapter 4: Jeffrey Keating helped identify the research opportunity, guided the design of the research, performed the research and data analyses, and prepared the manuscript. Chris Panganiban assisted in performing the research. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

Chapter 5: Jeffrey Keating helped identify the research opportunity, guided the design of the research, performed the research and data analyses, and prepared the manuscript. Alex Berlin and Thomas Canam assisted in performing the research. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

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Chapter 1 - Introduction

Background

The effective utilization of lignocellulosic residues derived from global forestry activities as a feedstock for the production of renewable fuel continues to be a primary objective of the biomass-energy industry. Ethanol serves as a viable and strategic alternative to a variety of conventional petroleum products for a number of reasons: lignocellulose feedstocks are abundant in many regions of the world; geopolitical concerns regarding security of fuel supply are addressed and rectified; the toxicity of ethanol and its combustion by-products is significantly reduced relative to petroleum; the combustion of ethanol originating from lignocellulosic materials provides for no net atmospheric release of carbon dioxide, due to the respiration of CO₂ during forest growth and maturation; the industrial and commercial infrastructure already exists for processing and distribution of organic liquid fuels; political will, financial incentives, and future legislation have the potential to substantially impact the competitive advantages of lignocellulose-derived ethanol over other energy sources, and most importantly they represent a renewable source of energy [12,17,23].

Softwood Bioconversion

In Canada, softwood trees represent 66% of the stocked forest area [6]. More importantly, Canadian softwood volume in the nation's stocked forests comprises 77% of total wood volume, and in British Columbia, 94% of said volume [6]. Consequently, lignocellulosic residues available for utilization as a feedstock for liquid fuel production in Canada, and in particular British Columbia, are predominantly a product of softwood harvest and processing [17].

In this regional context, distinctive challenges must be surmounted in converting softwood-derived lignocellulose residues to ethanol. The nature, distribution and quantity of sugars must be considered, as do the inhibitory compounds specific to softwood-sourced process streams. The monosaccharide fraction of liquid softwood hydrolysate contains three hexose sugars (galactose, glucose, and mannose) and two pentose sugars (arabinose and xylose). However, the hexose sugars are predominant, as opposed to the prevalent pentose sugars in hardwood-derived feedstocks. Despite the preference for hexose sugars exhibited by the traditional microorganisms employed in sugar fermentation, the mixture of galactose, glucose, and mannose presents a

metabolic obstacle to the efficient derivation of ethanol. This can be traced to catabolite repression, mostly among sugar fermenting yeasts, but also among certain bacteria, in which substrates are fermented sequentially according to the energy expenditure required in intracellular transport and metabolism [5,11,15]. For example, catabolite repression is most notable during galactose utilization as it lags markedly behind glucose and mannose consumption in the yeast *Saccharomyces cerevisiae*.

Additionally, liquid softwood hydrolysates possess intrinsic and process-derived inhibitory compounds that hinder effective fermentation [12]. These chemicals and their precursors may be produced from wood fibre degradation during pre-treatment, from the fermentative reactions themselves, and/or from bioconversion vessels [12]. This is an especially significant issue if, as expected for reasons of practicality, economy, and convenience, numerous bark-containing softwood residues constitute the lignocellulosic feedstock entering the process [4].

Pre-treatment stage

Pre-treatment is a required stage in the bioconversion of lignocellulosic feedstocks (Fig. 1.1), as the original form of the material exists as a tightly bound matrix of cellulose, hemicellulose, and lignin [10,12,23,24]. Therefore, the bioconversion of some of these materials to monomeric components, and then to ethanol, necessitates the solubilization of most of the hemicellulose fraction and the transformation of the remaining cellulose to a form amenable to subsequent acid- or enzyme-catalyzed hydrolysis [10,12,23,24,26]. There are numerous physical, chemical, and biological methods of pre-treatment, including the following: ball milling, freeze explosion, pressurized hot water saturation, high temperature dilute acid or low temperature concentrated acid immersion, acid-catalyzed steam explosion, alkaline (sodium hydroxide) hydrolysis, ammonia fibre explosion, wet oxidation, nonionic surfactant (or other organic solvent) dissolution, and fungal inoculation [10,12,23,24,26]. Every option has advantages and disadvantages, related to operational and reagent costs, toxic substance derivation, and efficiency [12,23]. It is known that mechanical methods are energy-intensive and thus inefficient, while chemical methods inadvertently degrade sensitive feedstock components that could potentially enhance the recovery of final In contrast, biological methods add complexity and require volumes of ethanol. extended completion times [24]. Optimally, a pre-treatment regime should yield reactive cellulose matrices and all potentially derived hemicellulose sugars in a monomeric form,

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Fig. 1.1. Lignocellulosic biomass conversion, showing material components, process stages, and biological inputs, along with ultimate ethanol recovery. Process stages, though combinable, are shown separately for illustrative purposes.

prevent hexose and pentose sugar degradation, minimize the production of inhibitory compounds affecting fermentation, produce little or no solid waste residue, and rely on simple physical and chemical principles. Furthermore, it must effectively convert large batches of residue of inconsistent dimensions and low moisture content, as well as employ inexpensive and moderately sized equipment [10,26]. Reality dictates that acetic acid (in hardwood hemicellulose more than softwood hemicellulose), free extractives (terpenes, alcohols, tannins, and other compounds), sugar degradation products (furfural, 5-hydroxymethylfurfural, 4-oxopentanoic acid, methanoic acid, and humic compounds), lignin degradation products (substituted aromatic and polycyclic compounds), pre-treatment reagent residues, and/or equipment-derived metals (iron, chromium, copper, and nickel) will be generated, regardless of method [10,12].

The pre-treatment methods that utilize biomass-sourced and externally supplied acids, whether at high temperature, low temperature, or in conjunction with steam, effectively hydrolyze hemicellulose, and successfully prepare cellulose fibres for subsequent acid- or enzyme-catalyzed hydrolysis [4,10,12,22,26]. In particular, strong acids partially or wholly hydrolyze cellulose as well as hemicellulose, minimizing the need for a hydrolysis stage [12]. However, inhibitory compounds, the identities of which stem from the specific nature of the feedstock material, are also liberated during the course of these chemical reactions, necessitating a detoxification protocol or equivalent strategy of improved tolerance before bioconversion proceeds [4,12,26]. At industrial scales, processes involving these methods may incorporate water recirculation in order to reduce freshwater consumption, and would consequently suffer more of the deleterious effects of potent inhibitor concentrations after many cycles [12,26]. Nonetheless, acid pre-treatment methods are heavily favoured in several technoeconomic models of bioconversion [4,10,12,22,26]. Continuous flow-through processes, present in many models, more effectively depolymerize hemicellulose and degrade fewer sugar molecules than batch operations, and so are adopted as part of acid pre-treatment regimes [10]. Conversely, the extremely low sugar concentrations that result from continuous processes are a notable disadvantage, and indicate the need for water abstraction prior to fermentation [10].

An experimental counter-current flow-through reactor is presently in development, and is capable of highly efficient acid hydrolysis of cellulose and hemicellulose [10,26]. This reactor requires only 0.07% sulfuric acid for effective

catalysis (a far lower concentration than that used in most other acid pre-treatments of softwood lignocellulose), physically shrinks the biomass feedstock, and yields relatively high sugar concentrations, thus avoiding the over-diluted output stream of traditional continuous processes [10]. Enzymatic hydrolysis of the cellulose remaining after pre-treatment may not be necessary if most of the crystalline structural polysaccharide is broken down to glucose in the counter-current reactor [10]. If the costs of inhibitor detoxification are contained or otherwise afforded through the financial returns of a cellulase-free process, the novel reactor could be employed in lignocellulose bioconversion until such time that enzymes or microorganisms become fully capable of every required stage of biomass-to-ethanol processing [10].

Acid-catalyzed steam explosion is an established and popular pre-treatment method in the bioconversion of several fibre types to various chemical products, including the physico-chemical transformation of principal wood components into monomeric, oligomeric, and polymeric derivatives of hemicellulose, cellulose, and (to a lesser extent) lignin [2,14,16,20,24]. Acid-impregnated (usually softwood) or virgin (usually hardwood) wood chips are compressed with high-pressure steam under specified temperature, pressure, and time conditions, typically ranging from 170 to 280°C, 1.2 to 1.7 MPa, and 10 seconds to 20 minutes, respectively, depending on the desired effect [14,24]. A subsequent and sudden decompression to normal atmospheric pressure releases the exploded material, which is partially hydrolyzed and effectively ruptured into liquid and solid streams [2,14,16,24]. The liquid stream contains water-soluble lignin monomers and oligomers, hemicellulose-derived sugars, and possibly some products of cellulose hydrolysis (such as glucose), while the solid stream contains polymeric cellulose and lignin loosely bound together [2,14,16,24]. Furthermore, the feedstock particle size is reduced, the cellulose-lignin matrix is more highly exposed (de-fibrillated) and consequently more porous to aqueous solvents, and cellulose crystallinity and polymer length are decreased [14,16,24]. The combination of temperature, residence time, and acid concentration (on a weight/weight basis) determines pre-treatment severity, and is summarized in equation 1:

 $\log R_o = t \cdot e^{(T-100)/14.75} - \log (pH)$

(1)

where R_o = reaction ordinate (severity factor), t = time (minutes), and T = temperature (°C) [2,14,24]. Higher reaction rates and greater feedstock changes are observed at higher severities ($R_o \approx 3.6$ -4.0). For example, in softwoods, hemicellulose

is almost completely solubilized and significantly degraded to inhibitory compounds, while the cellulose is moderately hydrolyzed [2,14,24]. At medium severities ($R_o \cong 3.3$ -3.5), hemicellulose decomposes to monomers with only a few remaining oligomers, and cellulose is slightly hydrolyzed [2,14,24]. At lower severities ($R_o \cong 3.0$ -3.2), hemicellulose breaks down to a mixture of oligomers and monomers, and cellulose hydrolysis is limited [2,14,24]. The optimum achievement in lignocellulose bioconversion is to balance hemicellulose monomer recovery with readily hydrolyzable cellulose recovery, therefore ensuring efficient enzymatic hydrolysis and fermentation following pre-treatment [14,16,24].

The water wash that follows the initial pre-treatment is performed to dilute residual reagents bound to the solid fraction and recover loosely associated soluble sugars, actions which concurrently boost hydrolysis efficiency and fermentation yields [24]. Subsequently, oxidative alkaline delignification is required to fractionate solid-phase cellulose and lignin [16,24]. Currently, very few applications exist for the lignin residues obtained in this process step, and the material is accordingly treated as low-value waste fuel [26]. Attempts are made to recover delignification chemicals, so as to yield some enduring value in further process cycles [12].

Hydrolysis stage

The hydrolysis of cellulose may be accomplished via acids or enzymes, though the latter (more selective) method is preferred [10,12,26]. Despite being slow and costly, cellulase complexes do not induce metal corrosion, and do not degrade monomeric sugars to inhibitory compounds, as do the acid alternatives [12,24]. In a purely sequential process, complete saccharification is followed by the separation of glucose from other components of the hydrolytic mixture, in order to facilitate sugar fermentation [10,12,24]. Today, the simultaneous saccharification and fermentation (SSF) process dominates research foci, and commercial bioconversion implementation, for critical economic and technical reasons [10,26].

The history of enzymatic cellulose hydrolysis is long, and marked with progressive discoveries surrounding the recalcitrant substrate and its vulnerability to biological catalysis [10]. Cellulose hinders fast solubilization by means of a resolutely insoluble and complex structure, and requires a host of synergistic enzymes to achieve full hydrolytic degradation, including endoglucanase, exoglucanase, and β -glucosidase (also known as cellobiase) [10,23]. These unique activities are contained within an

enzyme complex collectively referred to as cellulase, derived from various natural fungi and bacteria [23]. Most cellulase complexes are obtained from Trichoderma reesei, and though readily available, remain extremely expensive [10,12,23]. Research activities have targeted the cost of cellulase complexes through investigations of improved production yields, enzyme recycling, enzyme immobilization, employment of bacterial sources (*i.e.*, Klebsiella oxytoca), and horizontal gene transfer of appropriate deoxyribonucleic acid sequences into versatile and robust microorganisms and plants [10,12,23]. For example, a highly active endoglucanase from Acidothermus cellulolyticus was recently introduced to and expressed in tobacco and potatoes, thus providing a common and readily available supply of the enzyme [10]. Cellulase genes have also been established in Lactobacillus species, non-traditional hosts for the expression of hydrolytic enzymes [10]. Other research bodies and industrial entities, such as Novozymes and Genencor International (both sponsored by the United States Department of Energy), have attempted and continue to attempt to achieve increased thermostability, improved specific binding, and increased specific activity of cellulase complexes, especially those secreted from Trichoderma reesei, Thermobifida fusca, Cellulomonas fimi, Humicola insolens, Pichia pastoris, and Clostridium beijerinckii [10]. The ideal cellulase complex does not exist in a single organism, but is represented in a mixture derived from a diverse collection of fungi and bacteria [10]. If this mixture is suitably exploited, the genes responsible may be integrated into one source in the future, possibly one that can withstand inhospitable environments with ease, and simultaneously convert the products of an ideal cellulase complex (having hemicellulase activity) to ethanol [10,24,26]. This achievement would be recognized as direct microbial conversion (DMC), an elusive goal of many years of pursuit [10,24,26]. Anaerobic, thermophilic *Clostridium* species are regarded as the best DMC candidates at this time, but ethanol yields, and tolerances to ethanol and hydrolysate components, need substantial improvement [26].

The end-product inhibition inherent to batch operation cellulose hydrolysis has made SSF an attractive option for the efficient and continuous removal of glucose from the enzyme system [12,23,24,26]. SSF enhances the hydrolysis rate, reduces the enzyme loading requirement, (indirectly) improves ethanol yields, and lowers the risk of microbial contamination [12,24,26]. However, the disparate temperatures involved in saccharification and fermentation (approximately 45°C and 30°C for *T. reesei* cellulase

and mesophilic *S. cerevisiae*, respectively), the effect of ethanol and other microbial metabolites on cellulase structure and function, the performance-related response of fermentative microorganisms to foreign proteins, and the difficulty in separating cell mass from solid residue for the purpose of cell recycling represent uncertainties yet to be adequately addressed [12,23,26]. A non-isothermal SSF process is one solution to the problem of environmental discrepancies between optimal hydrolysis and fermentation conditions [23].

Fermentation stage

Fermentation entails the employment of a microbial species to metabolize a substrate or substrates, and yield a product or products. In the context of softwood bioconversion, hexose and pentose sugars derived from cellulose and hemicellulose degradation are converted to ethanol [10,12,24,26]. This process may be isolated from the other stages of bioconversion, or integrated with cellulose hydrolysis under an SSF regime, a situation similar to fed-batch fermentation [26]. In either case, cellular biomass and potentially valuable by-products (CO₂, organic acids, organic alcohols, 1,2-propanediol, and aromatic compounds) can be generated to varying degrees [10,24,26]. The subsequent distillation process generates stillage at a rate of 10-15 L per litre of ethanol, representing another source of potentially constructive output material [23,26].

Yeasts and bacteria are the fermentative machinery, and are usually selected on the basis of the nature of the substrate(s) and the desired product(s) [24]. The introduction of pentose catabolism genes to hexose-fermenting microorganisms is a favoured approach when converting lignocellulosic sugar monomers to ethanol Recombinant bacteria, specifically of the species Escherichia coli, [10,12,23]. Zymomonas mobilis, Klebsiella oxytoca, and Lactobacillus spp., and genetically modified Saccharomyces cerevisiae and Pichia stipitis yeast strains, are frequently chosen to ferment hardwood feedstocks, owing to the high concentration of recalcitrant pentose sugars (xylose and arabinose) in these substrates [10,12,23,26]. Two industrial participants, Celunol and BlueFire Ethanol, employ genetically modified E. coli and Z. mobilis, respectively, to ferment hexose and pentose sugars released from rice straw lignocellulose during their industrial bioconversion operations [10]. However, recombinant microorganisms do not survive for long periods in industrial settings, as the environmental conditions are relatively uncontrolled and not ideal from a microbial fermentation perspective [12,23].

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The effective fermentation of wood sugars derived from the hydrolysis of lignocellulosic residues is a difficult achievement for many natural strains of yeasts and bacteria. Specifically, the presence of a mixture of hexose sugars, pentose sugars, and synergistic and/or inhibitory compounds in wood hydrolysates imparts a significant metabolic challenge to microorganisms [12]. Lignin degradation products and acetic acid are especially inhibitory to most unmodified microorganisms [12]. Furthermore, specific fermentation conditions that constitute a feasible industrial approach must be met.

Microorganisms are assessed for their abilities to efficiently metabolize wood sugars (galactose, glucose, mannose, arabinose, and xylose) over a wide concentration range, generate and tolerate ethanol, produce few undesired by-products, maintain genetic stability (including plasmids and chromosomal insertions), detoxify or sequester inhibitory hydrolysate components, and quickly replicate [10,12]. These technical criteria have not yet been satisfied by unmodified or recombinant microorganisms; even thermophilic bacteria, normally very versatile and diverse, exhibit problems in the context of ethanologenic fermentation [10,12].

Additional criteria include process variables, such as competitive fitness (the ability to survive and thrive in the presence of other microorganisms competing for the same resources), amenability to frequent handling (including tolerance to rapid environmental changes), culture manageability during growth and fermentation (comprising flocculation behaviour, gas production, and other factors), versatility between batch, fed-batch, continuous, and immobilized culture, shear stress tolerance, temperature and pH tolerance, conversion efficiency, and safety to humans and the natural environment (including minimal aerosol generation) [12,24].

The final criteria are of critical importance to the overall feasibility of the biomassto-ethanol conversion project. Industrial elements, such as microbial hardiness, process efficiency, cost, scale-up analysis, waste generation and utilization, safety, product marketability, and public relations must be considered at a relatively early stage in research and development [12].

Ethanol recovery and use

Ethanol is a two-carbon monohydric primary alcohol of formula CH₃CH₂OH. This compound, having both hydrophilic and lipophilic character, is soluble in water as well as organic solvents. Ethanol forms a binary azeotrope in aqueous mixtures at a

concentration of 95% v/v, with a boiling point (78.2°C) below that of absolute ethanol (78.5°C), and must therefore be supplemented with a third component to effect efficient distillation [18]. A two-phase tripartite azeotrope consisting of ethanol, water, and toluene is often composed to break the binary azeotrope of ethanol and water: the ethanol and toluene partition to an organic phase, while the water forms a solitary aqueous phase [18]. Primary distillation is then employed to boil off the ethanol-toluene mixture, which is subsequently distilled to yield absolute alcohol. Alternatively, a water-adsorbing molecular sieve is employed in secondary distillation, subsequent to the receipt of the 95% v/v binary azeotrope in primary distillation [23]. With a melting point of -117.3° C, absolute ethanol is an appropriate solvent for conditions in which fluidity must be maintained below the freezing point of water.

Ethanol is used in alcoholic beverage manufacture, solvent applications, acetaldehyde, acetic acid, and ethylene synthesis, transportation fuel, biological sample preservation, thermometer fluid, and disinfection [18].

Yeast Metabolism

Yeasts can be broadly classified as unicellular fungi, belonging to the domain Eukarya, kingdom Fungi, phylum Ascomycota, class Saccharomycetes, and order Saccharomycetales. Yeast cells retain eukaryotic features such as larger size, plasma membrane sterols, membranous organelles, vacuoles, 80S ribosomes, mitochondria, and nucleoli [9]. As fungi, yeasts rely upon chemoorganotrophic nutrition, and possess chitin (a polymer of N-acetylglucosamine) within their semi-rigid cell walls.

Yeast cells in particular occupy a select range of habitats, including terrestrial, marine, and aquatic environments, most of which are characteristically abundant in sugars [9]. This can be traced to rather conventional nutritional requirements. Furthermore, symbiotic relationships with animals, plants, bacteria, and other fungi frequently define the existence of yeast cells, a strategy also linked to nutritional access [9]. Metabolically, yeasts are remarkably versatile, with capacities for aerobic, anaerobic, microaerophilic, and aerotolerant growth.

Yeast cells are capable of living independently and often take on spherical, oval, or cylindrical morphology. Cell division takes place via budding or fission, and sexual reproduction, a process inherent to certain yeast species, occurs with the fusion of two cells. As a consequence of morphological and physiological diversity, taxonomic classification of the yeasts is currently based on these properties [9]. In addition to the most commonly employed species, *Saccharomyces cerevisiae*, many other families and genera exist.

Yeasts as industrial fermentative organisms

Yeasts are frequently utilized as fermentative organisms because of their exceptional ability to convert sugars to ethanol at near theoretical yields [3]. Moreover, some yeasts (for example, various species of Saccharomyces, Kluyveromyces, and Candida) have intrinsically high sugar and ethanol tolerances because of niche acclimation, which makes their selection for alcoholic fermentation favorable [3]. Other advantages of yeast fermentations include minimal generation of undesired metabolic by-products (due to well-controlled glycolysis), high inhibitor tolerance in the case of complex substrate catabolism, reasonably stable genetic composition (horizontal gene transfer rates are less than those in bacteria), unique extracellular growth-permitting pH range (generally 4 to 6 for most yeasts, in contrast to bacteria, which usually grow and ferment effectively between pH 5 and 9), high fermentation efficiency (following initial rapid growth, maintenance requirements for yeast cells become proportionally greater with a corresponding decrease in the amount of carbon allocated to new cells), and short generation times (as compared with animal, plant, and insect cell cultivation) [3,9]. Finally, some yeast taxa such as Saccharomyces are able to flocculate and are therefore easily filtered out of culture broth, a useful feature for cell recycle fermentations and product recovery.

Practical reasons for selecting yeast species for industrial fermentations include hardiness (yeasts are amenable to frequent manipulation and handling without suffering losses in viability or fermentative capacity), shear stress tolerance (attributable to the yeast cell wall), safety and acceptability (most common fermentative yeasts pose less of a threat to human and environmental health than corresponding bacterial species and are more willingly accepted by the non-scientific public), and industrial experience (yeasts have been employed in alcoholic fermentations for thousands of years, and as such, are familiar and relatively inexpensive biocatalysts) [3,9].

Central metabolic pathways

The central metabolic pathways of fermentative yeasts all comprise variations of glycolysis (the Embden-Meyerhof-Parnas, or EMP, pathway). This pathway consists of

three stages: preparatory reactions, in which a sugar substrate is phosphorylated twice (consuming two ATP molecules) and then converted into two moles of triose phosphate (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, the former of which may be converted to the latter or reduced and dephosphorylated to form glycerol); oxidation, in which two moles of glyceraldehyde-3-phosphate (assuming glycerol is not formed) are oxidized and phosphorylated simultaneously, and subsequently dephosphorylated twice over multiple reactions to form four ATP and two moles of pyruvate; fermentation product reactions, in which two moles of pyruvate are reduced to two moles of ethanol with concurrent release of two moles of carbon dioxide and the regeneration of NAD⁺ [9]. Together, the EMP pathway employs 17 enzymes to accomplish the net generation of two ATP molecules and two fermentation products (Fig. 1.2).

Whereas *Saccharomyces*, *Kluyveromyces*, and *Candida* primarily produce ethanol and carbon dioxide along with minor amounts of glycerol, the genera *Pichia*, *Kloeckera*, *Torulopsis*, and *Schizosaccharomyces* are predisposed to forming minor metabolic by-products such as acetate, acetaldehyde, lactate, succinate, and 2,3butanediol, in addition to the ethanol and carbon dioxide [3,19]. All of these minor byproducts are derivatives of pyruvate that have been incompletely or improperly reduced, in many circumstances failing to drive NAD⁺ regeneration. In contrast, glycerol regenerates NAD⁺ and serves as an osmotic regulator for the cell [9]. Most fermentative yeasts generate fusel oils (high molecular weight alcohols) during the sugar-to-ethanol conversion as a consequence of extreme extracellular conditions and/or protein-supplemented feedstock [3].

Genetically modified metabolic pathways

The unlimited potential for genetic modification of fermentative yeast metabolism can be directly attributed to the body of extensive research conducted on *Saccharomyces cerevisiae*. Historical familiarity with this organism prompted early scientific endeavors into its morphology, biochemistry, and genetics that continue to this day. Consequently, tools for the molecular manipulation of *S. cerevisiae* and similar yeasts have been developed and repeatedly refined to the extent that these species are some of the most easily modified on earth. The two foci representing the majority of recent research in metabolic engineering of yeasts include extending the substrate range to approach and eventually assume the nutritional versatility of bacteria such as



Fig. 1.2. The ethanologenic fermentative Embden-Meyerhof-Parnas pathway (glycolysis), showing the consumption of glucose, the synthesis of ATP, and the production of compounds reduced from pyruvate. Enzymes are not shown. Fructose bisphosphate yields two triose phosphate compounds in the actual pathway (only one is shown).

Escherichia coli, and isolating metabolic product formation to wholly comprise ethanol and carbon dioxide [12]. However, diversification of the glycolytic pathway is not limited to these two foci, and frequently being industrially motivated, reflects a host of novel applications of fermentative metabolism.

Fermentative function of ethanol

The production of ethanol during alcoholic fermentations is secondary to the regeneration of NAD⁺ [9]. The key intermediate of substrate-level phosphorylation, pyruvate, is first decarboxylated to acetaldehyde and then acts as an electron acceptor in order to oxidize NADH back to NAD⁺. Consequently, ethanol is produced. The compound itself is of no value to the microorganism performing anaerobic glycolysis. and hence accumulates unchanged in the cytoplasm and surrounding culture medium (a concentration gradient across the plasma membrane is not established because of free diffusion of ethanol) [3]. Along with the depletion of substrate(s), the accumulation of ethanol during yeast fermentations reduces the risk of microbial contamination, but retards further growth. The density and specific heat of the culture medium are decreased, while the volatility is increased with the production of ethanol (to an extent dependent on medium volume, inoculum size, and available substrate), leading to higher rates of evaporation and higher susceptibility to thermal input and output. Finally, the water activity of the culture medium is decreased by the dissolution of ethanol in water, imparting a requirement for intracellular compatible solutes (glycerol in fermentative yeasts) [9].

Yeast glucose metabolism

Glucose is the most rapidly fermented nutritional substrate among conventional strains of *Saccharomyces cerevisiae*, and is therefore the preferred source of energy and carbon for growth and maintenance [8,15]. It is unrivalled in regulating growth and development, as well as metabolic processes, both through signal transduction and direct interaction with cellular components [15]. In particular, glucose represses the expression of genes required to catabolize slowly fermented sugars, thereby ensuring immediate and full allocation of resources to the acquisition of easily obtained nutrient material [8].

The first and most limiting step of glucose metabolism is plasma membrane transport [15]. Uptake occurs through facilitated diffusion, a passive process that assists glucose down a concentration gradient [15]. *Saccharomyces cerevisiae* has the

largest major facilitator superfamily (MFS) of transporter proteins of any known organism, consisting of Hxt1-17, Rgt2, Snf3, and GAL2 [8,15]. The Hxt gene family encodes hexose transporters, of which seven (Hxt1-7) are known to functionally mediate glucose uptake [8,15]. Without any of these seven proteins, growth on glucose and other rapidly fermented sugars is inhibited. In contrast, the expression of any one of these seven proteins permits growth [15]. No single transporter is essential for growth on glucose, indicating a redundant system; this is manifested in two uptake regimes, one constitutively functional and having a low affinity for sugar, the other repressed by significant glucose concentrations and having a high affinity for sugar [15]. These regimes explain the vigorous growth observed in a broad micromolar to molar range of glucose concentrations [15]. In fact, though the rate of glucose transport remains constant regardless of extracellular glucose concentration, the amount of available sugar does prescribe the co-expression of certain transporters, the unique interactions of which determine affinity for glucose [15]. This is accomplished through regulation of Hxt gene expression, and via catabolite inactivation (accelerated protein degradation) of inappropriate Hxt proteins [8,15]. Hxt2, Hxt6, and Hxt7 encode highaffinity transporters, whereas Hxt1, Hxt3, and Hxt4 encode low-affinity transporters [15]. Incidentally, the Hxt8-17 genes encode proteins either unable to transport glucose, or too weakly expressed to function effectively [15].

Rgt2 and *Snf3* encode similar regulatory plasma membrane proteins that sense extracellular glucose, and concomitantly signal the differential intracellular transcription of *Hxt* genes [8,15]. Both proteins are expressed at very low levels, but function uniquely: Rgt2 is a low-affinity, constitutively expressed glucose sensor, while Snf3 is a high-affinity glucose sensor, repressed by Mig1 binding protein at high glucose concentrations [8,15].

Hxt1 is induced only by high concentrations of glucose, whereas *Hxt2* and *Hxt4* are induced only by low concentrations of the sugar [8,15]. *Hxt3*, however, is induced by glucose regardless of sugar concentration, and represents the most versatile transporter gene in the MFS [8,15]. Three synergistic regulatory pathways dictate the relative expression levels of *Hxt1-4*. A repressor protein encoded by *Rgt1* inhibits *Hxt* gene family transcription in the absence of glucose [15]. However, in the presence of glucose, Rgt2 and Snf3 sense high and low glucose concentrations, respectively, and signal a repressor protein encoded by *Grr1* to inhibit Rgt1 function [15]. Interestingly,

Hxt1 requires the activation of the high glucose-induced Hog1 mitogen-activated protein kinase pathway in concert with Grr1-mediated inhibition of Rgt1 function to be transcribed [21]. In contrast, high glucose concentrations block the production of a repressor protein encoded by *Snf1*, permitting Mig1 to freely bind to and repress *Hxt2* and *Hxt4*, superseding any effect of Grr1-mediated inhibition of Rgt1 function [8,15].

Hxt6 and *Hxt7* are uniquely repressed by Snf3 at high glucose concentrations, but are not significantly induced otherwise, suggesting a high level of basal expression [15]. This odd function of Snf3 contradicts its alleged repression by Mig1 at high glucose concentrations, and remains to be more satisfactorily explained.

Limited information regarding the expression levels of *Hxt5* and *Hxt8-17* is available. Each of these putative hexose transporters is expressed at low levels, except for *Hxt5* and *Hxt13* [15]. *Hxt9*, *Hxt11*, and *Hxt12* expression is not regulated by glucose, whereas *Hxt10*, *Hxt16*, and *Hxt17* expression is repressed by glucose [15]. *Hxt5*, *Hxt8*, and *Hxt13-15* expression is induced at low glucose concentrations and repressed at high sugar levels, and the *Hxt5* and *Hxt13* genes in particular are regulated in a manner very similar (but not identical) to *Hxt2* and *Hxt4* [15]. Notably, some of these genes may not actually encode glucose transporters, since only *Hxt8* and *Hxt11* have been experimentally associated with such true function [15].

The uptake of glucose is followed by phosphorylation via hexokinase 1 (gene Hxk1), hexokinase 2 (gene Hxk2), or glucokinase (gene Glk1) [8,15]. Hxk2 is the primary phosphorylation enzyme at high glucose levels, while Hxk1 and Glk1 predominate at low glucose concentrations and in the presence of other substrates [8,15]. Mig1 may be involved in glucose-mediated repression of Hxk1 and Glk1 [8].

Yeast galactose metabolism and mixed sugar fermentation

Galactose metabolism in *Saccharomyces cerevisiae* is a particularly energy demanding process. It is dependent upon a succession of enzymes, including galactose permease, galactokinase, hexose-1-phosphate uridylyltransferase, and UDP-glucose-4-epimerase (Fig. 1.3) [11,13]. These enzymes, excepting the sugar transport protein galactose permease (gene *GAL2*), comprise the Leloir pathway, a critical and complex set of pre-glycolytic reactions. Furthermore, regulatory elements exercise a high level of control over the utilization of the sugar [11,13].

The Leloir pathway begins with the phosphorylation of galactose at the anomeric carbon, catalyzed by galactokinase (gene *GAL1*), yielding galactose-1-phosphate



Fig. 1.3. The pre-glycolytic reactions of galactose and glucose, comparing metabolic intermediates and enzyme participation in the transformation to glucose-6-phosphate.

[11,13]. Galactose-1-phosphate is then converted to glucose-1-phosphate, via the combined catalysis of hexose-1-phosphate uridylyltransferase (gene *GAL7*) and UDP-glucose-4-epimerase (gene *GAL10*, from which a bifunctional protein is translated, the other function being galactose mutarotase) [11,13]. Incidentally, this reaction yields transient intermediates, in the form of nucleotide diphosphate sugars. GAL7 transfers a UDP moiety to galactose-1-phosphate, yielding galactose-1-UDP, which subsequently undergoes epimerization to glucose-1-UDP, via GAL10 epimerase [11,13]. Glucose-1-UDP is then transformed into glucose-1-phosphate, with the transfer of the UDP moiety back into a recirculating biochemical pool, by GAL7 [11,13]. Finally, glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase (gene *GAL5*), a non-Leloir pathway enzyme [11,13].

The regulation of galactose metabolism employs a dual control principle. Critical enzyme production is induced in the presence of galactose, and repressed in the presence of glucose [5,13,15]. Two regulatory genes, *GAL3* and *GAL4*, are involved, through protein expression, in the induction of structural galactose metabolism genes: *GAL4* encodes an upstream transcriptional activator, which attaches itself to nucleotide sequences just prior to the coding region; *GAL3* encodes a galactose-sensitive binding protein, which inserts itself into the constitutively repressing GAL4/GAL80 complex, thereby releasing the GAL4 activation domain [5,13,15]. *GAL80* encodes a GAL4-binding protein, which prevents GAL4-mediated activation of structural genes when appropriately bound [5,13,15]. A relatively newly discovered gene, GAL6, encodes a protein that exerts a negative impact similar to GAL80 [13].

Further investigation of *GAL3*, *GAL4*, and *GAL80* has revealed intriguing interactions among the regulatory and structural galactose metabolism genes. A loss-of-function mutation in *GAL3* greatly retards the timing of *GAL* induction, but does not affect galactose fermentation once the yeast have adapted to the new substrate [25]. GAL3 is thus involved only in accelerating the initiation of induction and plays no part in actually maintaining transcription of the structural galactose genes. In contrast, GAL4 is phosphorylated following the initiation of induction, subsequently losing affinity to (or immobilizing) GAL80, such that the transcription of *GAL1*, *GAL7*, and *GAL10* is maintained [25]. The two-step *GAL* induction process of establishment and maintenance is thereby effected by GAL3 and GAL4, respectively (Fig. 1.4).

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Fig. 1.4. Structural galactose metabolism gene regulation by induction, showing lack thereof, establishment, and maintenance.

Galactose and ATP have been shown to be necessary for the stable binding of GAL3 to GAL80. GAL3 undergoes a reversible conformational change upon initial association with intracellular galactose, but only in the presence of the cofactor ATP, allowing binding to the GAL4/GAL80 complex [25]. The GAL4 activation domain is consequently revealed, either by allosteric realignment or dissociation from GAL80, and structural gene transcription begins. GAL3 thus acts as both a galactose sensor and signal transducer, the latter activity ceasing, via GAL3/GAL80 dissociation, upon withdrawal of galactose [25]. The withdrawal of galactose also witnesses the dephosphorylation of GAL4, resulting in cessation of structural gene transcription [25].

Glucose-mediated repression of galactose metabolism involves Mig1, a regulatory gene that encodes a binding protein, and possibly Hxk2, which encodes a protein that can act not only to phosphorylate glucose but as a protein kinase [5,8,13,15]. Mig1 is considered the key transcription factor in arbitrating the cellular preference for glucose. In the presence of glucose, cytosolic Mig1 is dephosphorylated and localizes to the nucleus, where it binds with Ssn6 and Tup1 (Fig. 1.5) [5,13]. The tripartite Mig1/Ssn6/Tup1 complex binds to the promoters of GAL1, GAL7, GAL10 and GAL4. preventing expression of galactokinase, the hexose-1-phosphate uridylyltransferase, UDP-glucose-4-epimerase, and most importantly, the structural galactose metabolism gene transcriptional activator, resulting in redundant repression of the Leloir pathway genes [5,11,13]. Surprisingly, the amounts of Mig1, Ssn6, and Tup1 are not regulated by glucose via signal transduction. Instead, Mig1 is rendered inactive by cytosolic localization and phosphorylation by Snf1 in the absence of glucose [5]. Glucose, in addition to decreasing GAL2 transcription and competing with galactose for plasma membrane transport via the residual transcribed protein, also mediates the inactivation of the permease, effecting its internalization via endocytosis, and its subsequent vacuolar degradation [5,11,15]. The absence of plasma membranebound GAL2 causes poor growth on galactose [5,15]. A direct effect of glucose is evident in GAL3 repression, which manifests in permanently bound GAL4/GAL80 [5].

With regard to sugar metabolism energetics, galactose presents an interesting case. In steady-state cultivation experiments of aerobically grown *Saccharomyces cerevisiae*, 3-4 fold lower levels of glucose-6-phosphate and fructose-6-phosphate can be observed during galactose-limited growth as compared to glucose-limited growth [13]. Moreover, there is only a 2.5-fold increase in the specific galactose uptake rate

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Fig. 1.5. A schematic demonstrating the central role of Mig1 in the glucose-mediated repression of galactose metabolism. Note: a comprehensive sequence of events involving glucose in the repressed galactose pathway is not shown.

after the addition of a galactose pulse to galactose-cultivated yeast, approximately half of that noted in the specific glucose uptake rate after the addition of a glucose pulse to glucose-cultivated yeast [13]. Despite appearances of equal plausibility, flux control in a galactose-limited culture is preferably ascribed to elements of the Leloir pathway, rather than GAL2-mediated sugar transport, because the specific galactose uptake rate observed following a galactose pulse is far slower than the maximum specific velocity calculated from *in vitro* studies of the high-affinity kinetics of GAL2 common to galactose-limited cultivation [13].

GAL1 may be indirectly linked to one aspect of flux control, since under steadystate galactose-limited growth it functions at a fraction of its *in vitro* maximum specific velocity [13]. This inefficiency is due to low free ATP concentrations, a possible consequence of the galactose-influenced ATP-dependent interaction of GAL3 and GAL80, and one that is absent in glucose-limited growth [15,25]. The deficit of intracellular ATP limits biosynthetic reaction capacity and slows the entire cellular metabolism.

Another constituent of the Leloir pathway in *Saccharomyces cerevisiae*, GAL7 (hexose-1-phosphate uridylyltransferase), has a very poor affinity for its substrate, galactose-1-phosphate [13]. Consequently, the concentration of galactose-1-phosphate in the cytoplasm often accumulates to toxic levels during galactose metabolism, and thus may inadvertently act as a substrate for inositol monophosphatase (IMPase), an enzyme engaged in signal transduction processes [13]. IMPase, establishing a cycle perpetuated by GAL1, and causing a stall in preparatory reactions prior to glycolysis, converts galactose-1-phosphate back to galactose [13]. This phenomenon, in combination with signal transduction process interference, inflicts a severe physiological liability on yeast cells metabolizing galactose.

The imperfection inherent to the Leloir metabolic pathway, the disruption to signal transduction, and the energy deficit unite to rationalize the overwhelming preference for glucose, rather than galactose, as an energy and carbon source in *S. cerevisiae*.

Galactose and glucose have opposite hydroxyl group orientations at the 4-prime carbon [13]. Yeast hexose transport and phosphorylation systems classically exhibit high fidelity in discriminating between dissimilar substituent configurations around this carbon atom [11]. In contrast, glucose and mannose, stereochemically distinct at the 2-

prime carbon, share a part in one common, constitutively expressed system during uptake and metabolism [11]. Glucose uptake is characterized by rapid plasma membrane transport through a variety of transporter proteins, and one phosphorylation step prior to glycolysis [13]. In contrast, galactose utilizes a cell signal-sensitive, indiscriminate, and labile transporter protein, and an intricate sequence of phosphorylation, group transfer, and epimerization in preparation for glycolysis [5,11,13,15]. The transport systems for galactose and glucose are not independent, however [11]. The galactose and glucose transport proteins, GAL2 and the Hxt series, respectively, share greater than 60% sequence similarity [11,15]. In certain S. cerevisiae strains, galactose is a very strong inhibitor of glucose and mannose utilization, under conditions of prior acclimation to galactose [11]. This rare inhibition could be employed by yeasts to stop the effect of glucose and mannose on galactose utilization; if galactose is the primary sugar in a given environment, small amounts of glucose and/or mannose transported across the plasma membrane could slow or shut down galactose utilization and therefore reduce competitive fitness [11]. More commonly, as occurs in almost all yeasts, glucose and mannose interfere with galactose utilization [11]. Consequently, a relationship exists between either the transport reactions of pre-glycolytic galactose and glucose metabolism, or the phosphorylation reactions of the same stage [11]. Hexokinase (genes Hxk1 and Hxk2), glucokinase (gene Glk1), and galactokinase are functionally specific to their respective substrates, negating the possibility of interactions during phosphorylation [11,13]. Moreover, D-fucose, which is not phosphorylated by galactokinase, but is transported by galactose permease, acts like galactose in inhibiting glucose and mannose utilization [11]. These observable facts prove that a relationship exists in the transport functions of galactose and glucose [11]. This coincides with the competition between galactose and glucose for plasma membrane transport via GAL2 [5].

UDP-glucose-4-epimerase, a crucial Leloir pathway enzyme, transforms galactose-1-UDP to glucose-1-UDP by stereochemically inverting the hydroxyl group on the 4-prime sugar carbon [1,11,13]. As far as is known, all C-4 epimerases are intracellular [1,7]. However, this property is not firmly decreed, as enzymes of all types are occasionally discovered in new species, strains and niches, and afterward structurally, proximally, or mechanistically characterized as novel and unique [1].

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Research Objectives

The research program explored aspects of the fermentation process inherent to the bioconversion of softwood residues to fuel ethanol. The focus was on mixed sugar fermentation and the ability of microorganisms to overcome catabolite repression, and thus effectively and simultaneously ferment all hexose sugars present in a softwoodderived lignocellulose matrix. Conventional microorganisms employed in the fermentation of hexose sugar mixtures utilize glucose and mannose prior to galactose in order to achieve fast growth and biomass accumulation with minimal resource allocation and energy expenditure [5,11,15]. It was hypothesized that naturally evolved yeasts in specific environments may have undergone regulatory system modification in order to accommodate unconventional sugar utilization patterns, while maintaining resource efficiency and growth kinetics suitable to those particular niches. In concert with elements of process optimization, the acquisition, screening, characterization, and employment of one or more of these yeasts in liquid softwood hydrolysate fermentations may supplant the need for the creation and use of genetically modified microorganisms. Ultimately, high yield ethanol production by unmodified, robust, safe, and efficient yeast cultures in a cost-effective, simple, and environmentally sound process is desired.

The conducted research incorporated the following objectives:

1) To screen natural yeast strains pre-determined to assimilate galactose for the ability to ferment galactose-supplemented synthetic culture medium.

2) To comparatively assess the performance of spent sulfite liquor-adapted and laboratory reference strains of *Saccharomyces cerevisiae* (Tembec T1 and BY4742) and the naturally occurring yeast strains (having potentially improved galactose utilization patterns) in mixed synthetic sugar and wood-derived substrate fermentations, including a consideration of the effect of particular inhibitory compounds.

3) To determine the general function and destination of sugar carbon and energy in the selected yeast through the relation of biomass accumulation, growth pattern, substrate consumption, and ethanol production.

4) To characterize the mechanism(s) responsible for any altered galactose utilization behaviour in the selected yeast, and explore opportunities for enzyme and gene identification, isolation, and production/cloning.
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Chapter 2 - Characterization of a unique ethanologenic yeast capable of fermenting galactose

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Introduction

Currently, many biotechnological processes employ mixed sugars derived from agricultural waste, which exemplifies an abundant and readily accessible carbon source [1–4], as substrates for bioconversion to fuel-grade ethanol. The predominant polysaccharides, oligosaccharides, and monosaccharides in starch- and cellulose-containing plant materials are comprised of pentose and hexose sugars, which typically co-exist as compositionally diverse heteropolymers [4,5].

Although traditional microorganisms employed in ethanologenic sugar fermentation exhibit preferences for hexose sugars, the mixture of galactose, glucose, and mannose presents a metabolic obstacle to the efficient derivation of ethanol. This can be traced to catabolite repression, mostly among sugar fermenting yeasts, but also among certain bacteria, in which substrates are fermented sequentially according to the energy expenditure required for transport and metabolism [3,6–9]. For example, galactose utilization markedly lags behind glucose and mannose consumption in the yeast *Saccharomyces cerevisiae* because of catabolite repression [6].

Galactose metabolism is an energy-intensive, inductive process in Saccharomyces cerevisiae, requiring the synthesis of numerous enzymes, including galactose permease (GAL2), galactokinase, hexose-1-phosphate uridvlvltransferase. and UDP-glucose-4-epimerase [7,10,11]. With the exception of GAL2, which is involved in transporting galactose across the yeast cell plasma membrane and into the cytoplasm, all of the enzymes are constituents of the Leloir pathway, a complex set of metabolic reactions evolved to prepare galactose for classic glycolytic catabolism [7,11,12]. Furthermore, elaborate regulatory mechanisms have evolved to exert fine control over the utilization of galactose [3,7,10,11,13].

Galactose and glucose differ stereochemically at the 4-carbon, having opposite hydroxyl group orientations [11]. Interestingly, yeast hexose transport and phosphorylation systems are (in almost all cases) precise in differentiating between dissimilar substituent configurations around the same 4-carbon [7]. Glucose and mannose, differing only at the 2-carbon, utilize one common constitutive system to enter yeast cells and undergo metabolism [7]. The catabolism of glucose is limited to fast, simple plasma membrane transport through numerous transporter proteins, and a single phosphorylation step before the initiation of glycolysis [11,14]. In contrast,

galactose utilizes one inducible, repressible, non-discriminating, and fragile transporter protein, and a complex sequence of phosphorylation, group transfer, and epimerization before the initiation of glycolysis [6–8,11]. Despite these differences, the transport systems for galactose and glucose do not act independently [7]. The mediators involved in galactose and glucose transport, GAL2 and a series of Hxt proteins, respectively, are part of the same functional family, sharing greater than 60% sequence similarity [7,8].

UDP-glucose-4-epimerase (EC 5.1.3.2), part of a series of crucial Leloir pathway enzymes, converts galactose-1-UDP to glucose-1-UDP, using an epimerization (stereochemical inversion) mechanism centred on the 4-carbon [7,11,15,16]. The scientific literature lists all known C-4 epimerases as localized to the intracellular environment of bacterial, yeast, plant, and animal cells [15,17]. Interestingly, epimerases in particular are easily evolved, since the simple cleavage and restoration of any bond at a stereogenic carbon represents the catalytic basis of these enzymes, and all that is additionally required is nonstereospecificity during catalysis [16,18].

The objective of this study was to screen a group of natural yeast strains for exceptional metabolic performance on galactose, to test any such strain in industrially relevant mixed hexose sugar fermentations, and to characterize said strain in order to reveal unique properties and elaborate upon potential novel mechanisms of galactose assimilation and catabolism.

Materials and Methods

Yeast strains and culture media

Spent sulfite liquor-adapted *Saccharomyces cerevisiae* T1 was obtained from Tembec Limited (Témiscaming, Que., Canada). *Saccharomyces cerevisiae* NRRL Y-1347, Y-1528, Y-965, Y-562, and Y-567 were received from the Agricultural Research Service, US Department of Agriculture, Peoria, IL.

Enzymes

UDP-galactose 4'-epimerase (specific activity ≥ 10 units mg⁻¹) was obtained from Calbiochem-Novabiochem, San Diego, CA. Phosphoglucose isomerase (EC 5.3.1.9; specific activity 595 units mg⁻¹ protein) was obtained from Sigma–Aldrich, St. Louis, MO. Zymolyase 20T (specific activity 20,000 units g⁻¹) was obtained from ICN Biomedicals, Irvine, CA.

Culture conditions

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, Difco, Becton Dickinson, MD) at 4°C and transferred to fresh plates on a bimonthly basis. Cells were grown to high cell density (culminating in average 600 nm absorbance values of approximately 10) in foam-plugged 250 mL Erlenmeyer flasks containing YP-sugar liquid media (10 g L⁻¹ yeast extract and 10 g L⁻¹ peptone, supplemented with either 10 g L⁻¹ galactose, glucose, or mannose) in an orbital shaker for 3 days at 30°C and 200 rpm, with concurrent transfer to fresh medium performed every 24 h.

Batch fermentations

Following 3 days of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile deionized water and subsequently adjusted to a calculated concentration of 48 g dry cell weight (DCW) per liter on a spectrophotometer (Milton Roy, Rochester, NY) via standard curves relating 600 nm absorbance to DCW L⁻¹ concentration.

Fermentations were performed in either foam-plugged 125 mL Erlenmeyer flasks or rubber septum-plugged serum bottles containing 40 mL YP-sugar liquid media (unless otherwise noted, 10 g L⁻¹ yeast extract and 10 g L⁻¹ peptone, supplemented with 30 g L⁻¹ filter-sterilized galactose, glucose, and/or mannose as well as 1.65 g L⁻¹ filter-sterilized dibasic ammonium phosphate as nitrogen and phosphorus source) in an orbital shaker for 24–48 h at 30°C and 125 rpm. Media were inoculated to achieve an initial cell concentration of 2.4 g DCW L⁻¹. Offline sampling was aseptically performed at the time of inoculation and at specific time points thereafter. One milliliter aliquots were immediately centrifuged (14,000 rpm) for 4 min at 4°C to yield cell-free supernatants, which were then decanted and frozen at -20°C for separate sugar, ethanol, and glycerol analysis. All fermentation experiments were performed in duplicate with the appropriate negative controls. Furthermore, conditions were duplicated in separate flasks within each experiment.

Sugar, ethanol, and glycerol analysis

High performance liquid chromatography on a DX-600 BioLC chromatograph (Dionex Corporation, Sunnyvale, CA) was used for sugar determination. Separation was achieved by an anion exchange column (CarboPac PA1, Dionex Corporation), and detection was carried out 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 μ m polyvinylidene fluoride (PVDF) filters prior to injection (20 μ L). 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 during each run.

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, CA). Separation was effected in a 30 m Stabilwax-DA column (internal diameter 0.53 mm) fit with a 5 m deactivated guard column (Restek Corporation, Bellefonte, PA). Samples were appropriately diluted in deionized water, supplemented with butan-1-ol as internal standard, and then filtered through 0.45 μ m PVDF filters prior to injection (2 μ L). 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:

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

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

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).

High performance liquid chromatography on a DX-300 chromatograph (Dionex Corporation) was used for glycerol determination. Separation was achieved by an anion exchange column (CarboPac MA1, Dionex Corporation), and detection was carried out via pulsed amperometry across a gold electrode. Samples were appropriately diluted in deionized water, supplemented with erythritol as internal standard, and then filtered through 0.45 μ m PVDF filters prior to injection (25 μ L). The column was eluted with 200 mM NaOH at a flow rate of 0.40 mL min⁻¹ for 14 min, 480 mM NaOH for 40 min, and then 200 mM NaOH for 14 min during each run.

(2)

¹⁴C radioactivity analysis

Radioactive isotope-labeled sugars were separated and detected with the same equipment and methods as unlabeled sugars. The separated fractions were collected in an FC204 fraction collector (Gilson, Middleton, WI) at 1-min intervals, supplemented with excess scintillation cocktail, and analyzed in an LS 6000 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Background radiation was ascertained and subtracted from all collected activity data.

Biomass analysis

Time-dependent offline sampling was aseptically performed during fermentations to yield one milliliter aliquots. Samples were mixed immediately prior to dilution in deionized water, and then subject to duplicate absorbance determination in a spectrophotometer at 600 nm. Diluted cell-free media were used to establish background readings and set zero absorbance levels. Values were averaged and corrected for dilution, and then converted to DCW L⁻¹ using equations relating the two parameters.

Sub-cellular fractionation

Galactose-acclimated cells were collected from 80 mL of actively growing culture by centrifugation at $12,000 \times g$ for 1 min at 0°C. Ten milliliters of the supernatant, representing the extracellular fraction, were immediately collected and stored at 4°C. The remaining supernatant was discarded and the cell pellet was re-suspended in icecold phosphate-buffered saline (PBS). Cells were again recovered by centrifugation at $12,000 \times q$ for 1 min at 0°C. The cell pellet was then re-suspended in stabilizing buffer A (1 M sorbitol, 10 mM MgCl₂, 2 mM DTT, 50 mM pH 7.8 potassium phosphate, and 0.575 mM PMSF) at 21°C, and incubated for exactly 10 min at 30°C. Cells were subsequently recovered by centrifugation at $12,000 \times g$ for 1 min at 0°C, re-suspended in stabilizing buffer B (1 M sorbitol, 10 mM MgCl₂, 2 mM DTT, 25 mM pH 7.8 potassium phosphate, 0.575 mM PMSF, and 25 mM pH 5.5 sodium succinate), and incubated for 2 min at 30°C. The suspension was then supplemented with 10 mL of 50 mg/mL Zymolyase 20T and incubated for 15 min at 30°C. At 15 min, two 1 mL aliguots of the suspension were removed and examined under phase-contrast microscopy, one aliquot treated with CA-630 non-ionic detergent and the other untreated, to confirm protoplast formation and susceptibility to lysis. Incubation was subsequently continued for 30 min.

Protoplasts were then collected by centrifugation at 500 \times *g* for 10 min at 4°C. The supernatant, representing the cell wall and periplasmic fraction, was carefully collected and stored at 4°C. The protoplast pellet was re-suspended in 8 mL of no-salt lysis buffer, consisting of 50 mM HEPES, 0.575 mM PMSF, 1% CA-630 non-ionic detergent, and 1 µg/mL aprotinin. The suspension was then stored for 30 min at 0°C and supplemented with 10 mL of 20 mM pH 7.2 Tris–HCl buffer.

The suspension was subsequently centrifuged at $1000 \times g$ for 5 min at 4°C, yielding a pellet containing nuclei, which was stored at 4°C. The supernatant was centrifuged at 9500 × g for 10 min at 4°C, yielding a pellet containing mitochondria, which was also stored at 4°C. The supernatant was again centrifuged at 14,000 × g for 25 min at 4°C, yielding a pellet containing plasma membrane. This pellet was washed twice with 1 mL of 20 mM pH 7.2 Tris–HCl buffer to remove any cytosolic contaminants, and then re-suspended in 1 mL of the same buffer and stored at 4°C. The supernatant, containing cytosolic components, was stored at 4°C.

Each fraction was subsequently desalted through a PD-10 column (Amersham Biosciences, Baie d'Urfé, Que., Canada) to remove low molecular weight substances, using pH 7.4 elution buffer (containing 50 mM HEPES·KOH, 5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, and 0.1% bovine serum albumin). Protein content of the desalted fractions was measured in triplicate via absorbance at 280 nm on a GeneQuant *pro* spectrophotometer (Biochrom, Cambridge, England).

Epimerase enzyme assay

Duplicate 400 μ L aliquots of nuclear, mitochondrial, cytosolic, plasma membrane, cell wall and periplasmic, and extracellular fractions were separately mixed with 61.3 μ L of 10 mg/mL UDP-galactose (solvated in 20 mM pH 7.2 Tris–HCl buffer), 60 μ L of 100 mg/mL NAD⁺ (solvated in 20 mM pH 7.2 Tris–HCl buffer), and sufficient volumes of 100 mM pH 8.6 NaOH·glycine buffer to total 1 mL. These mixtures, along with negative controls (deficient in any sub-cellular fractions), positive controls (substituting 200 μ L of 0.2 μ g/mL commercial UDP-galactose 4'-epimerase for sub-cellular fractions), and standards (deficient in any sub-cellular fractions but supplemented with 61.3 μ L of 10 mg/mL UDP-glucose), were subsequently incubated for 40 min at 27°C [19].

Marker enzyme assay

Duplicate 1.15 mL aliquots of nuclear, mitochondrial, cytosolic, plasma membrane, cell wall and periplasmic, and extracellular fractions were separately mixed with 0.05 mL of 100 mM fructose-6-phosphate, 0.05 mL of 100 mM MgCl₂, and sufficient volumes of 250 mM pH 7.4 glycylglycine buffer to total 1.5 mL. These mixtures, along with negative controls (deficient in any sub-cellular fractions), positive controls (substituting 0.05 mL of 0.5 unit/mL commercial phosphoglucose isomerase for sub-cellular fractions), and standards (deficient in any sub-cellular fractions but supplemented with 0.05 mL of 100 mM glucose-6-phosphate), were subsequently incubated for 5 min at 25°C [20].

Chromatographic nucleotide sugar analysis

Nucleotide sugar determination was accomplished through high performance liquid chromatography on a Summit LC chromatograph (Dionex Corporation). Separation was achieved with a Zorbax Rx-C8 reversed-phase analytical column (Agilent Technologies, Palo Alto, CA), and detection was facilitated with UV absorbance at 264 nm [21]. Standards and experimental samples were diluted 1:25 in deionized water, and then filtered through 0.45 µm PVDF filters prior to injection (20 µL). The column was eluted with a gradient of 50 mM pH 7.0 potassium phosphate buffer (supplemented with 2.5 mM tetrabutylammonium hydrogen sulfate) and 100 mM pH 7.0 potassium phosphate buffer mixed with acetonitrile (1:1, supplemented with 2.5 mM tetrabutylammonium hydrogen sulfate) at a flow rate of 1.0 mL min⁻¹. The elution consisted of 98% phosphate buffer and 2% acetonitrile/phosphate buffer graded to 65% phosphate buffer and 35% acetonitrile/phosphate buffer over 29 min, followed by reverse grading to 98% phosphate buffer and 2% acetonitrile/phosphate buffer between 29 and 30 min. A 15-min wash with 98% phosphate buffer and 2% acetonitrile/phosphate buffer concluded each 45-min run.

Chromatographic sugar phosphate analysis

Sugar phosphates were determined by high performance liquid chromatography on a DX-600 BioLC chromatograph (Dionex Corporation). Separation was achieved by an anion exchange column (CarboPac PA1, Dionex Corporation), and detection was carried out via pulsed amperometry across a gold electrode with the addition of a 200 mM NaOH post-column wash. Standards and experimental samples were diluted 1:2 in deionized water, and filtered through 0.45 µm PVDF filters prior to injection (20 µL). The column was eluted at 1.0 mL min⁻¹ over 20 min with a gradient of 100 mM sodium acetate to 200 mM sodium acetate in 100 mM NaOH, followed by accelerated grading to 500 mM sodium acetate in 100 mM NaOH between 20 and 30 min.

Results and Discussion

Screening of yeast strains

Yeast strains known to assimilate galactose (as indicated by gas accumulation in Durham tubes on different carbon sources) were obtained, and screened for their capacity to consume and utilize galactose as a primary carbon source (Fig. 2.1). Concurrently, they were compared against a presently employed industrial strain of *S. cerevisiae* (Tembec T1) used in the production of fuel-grade ethanol from lignocellulose-derived sugars. The five natural yeast strains exhibited varying responses to galactose, following acclimation to glucose. *S. cerevisiae* Y-1528 demonstrated exceptional behavior, completely consuming galactose within 6 h, whereas the four other strains and Tembec T1 required a minimum of 10 and a maximum of 24 h each to equally utilize all of the available sugar (Fig. 2.1). In contrast to the galactose consumption patterns of the remaining strains, the performance of Y-1528 displayed no evidence of a lag in utilization due to acclimation to a new sugar environment.

Furthermore, Y-1528 grew very well in additional fermentations of 100 and 225 g L^{-1} autoclave-sterilized galactose, following acclimation to galactose. Complete sugar consumption occurred in the 100 g L^{-1} galactose culture in 24 h, yielding significant quantities of ethanol (26.70 g L^{-1} , or 53% of theoretical yield) and glycerol (3.2 g L^{-1}). Over 68% of the available sugar was consumed in the 225 g L^{-1} galactose culture in 48 h, yielding 55% of theoretical ethanol (62.96 g L^{-1}) and 7.9 g L^{-1} glycerol. However, negligible sugar consumption occurred in the 300 g L^{-1} galactose in 48 h, yielding only 2% of theoretical ethanol (3.09 g L^{-1}) and 1.7 g L^{-1} glycerol.

Ecologically established mutant strains of microbial species often exhibit distinct metabolic features that make them useful in biotechnological applications, in addition to their critical role in natural biochemical transformations. Therefore, it was proposed that yeasts isolated from specific environments might have altered regulatory systems that permit unconventional sugar utilization patterns well suited to particular niches. In conventional yeasts, the regulation of galactose metabolism employs a dual control



Fig. 2.1. Galactose consumption by five natural strains of *S. cerevisiae* (Y-1347, Y-1528, Y-965, Y-562, Y-567) and one industrial strain of *S. cerevisiae* (Tembec T1), following acclimation to glucose. Range is indicated by vertical bars.

principle. Critical enzyme production is induced in the presence of galactose, and repressed in the presence of glucose [1,3,6,8,9,11].

It was evident that Y-1528 was unique among the screened strains, likely employing a novel mechanism to transport and metabolize galactose. Based on the absence of lag time in galactose utilization, it appeared that a constitutively expressed system was involved. However, the nature of this mechanism could not be discerned on the basis of single sugar batch fermentations, except to observe that it was a retained feature independent of acclimation to other hexose sugars (glucose, in the case of these screening experiments, as well as during the course of agar plate strain maintenance), and that it was not a consequence of laboratory strain modification (in contrast to Hxk2 deletion strains, which co-consume galactose and glucose, and *Mig1Mig2* deletion strains, which show reduced lag in the presence of galactose and glucose) [1,22]. A concurrent investigation of the taxonomic identity of Y-1528 via molecular genotyping confirmed its classification as Saccharomyces cerevisiae. showing no divergence among rRNA genes conserved to the species level (Keating et al., 2003; submitted). This strain was consequently chosen for further study in a mixed sugar fermentation experiment. The other strains (Y-1347, Y-965, Y-562, Y-567, and Tembec T1) performed as expected, requiring 4–6 h to induce gene expression and synthesize galactose transport and metabolism enzymes before effecting significant reductions in sugar levels.

The ability of Y-1528 to withstand very high concentrations of galactose, in the presence of thermally generated inhibitory compounds derived from autoclave treatment, and to nevertheless effect significant sugar consumption with concomitant ethanol and glycerol production, was indicative of exceptional osmotolerance and inhibitor resistance. This performance suggested that this yeast was harvested from a natural environment rich in galactose, and one in which the concurrent presence of sugar decomposition products selected for the resilience found in this strain.

Y-1528 mixed sugar fermentation – acclimation effects

Single sugar fermentations demonstrated that consumption rates were affected by acclimation conditions. Galactose, glucose, and mannose were independently utilized at the fastest rate by cells acclimated to glucose and mannose, and at a slower rate by cells acclimated to galactose (Fig. 2.2). However, ethanol yields did not differ significantly based on acclimation conditions (Table 2.1). Interestingly, galactose-



Fig. 2.2. Sugar consumption during single sugar (galactose [Gal], glucose [Glc], or mannose [Man]) fermentation by *S. cerevisiae* Y-1528; (A) following acclimation to galactose; (B) following acclimation to glucose; (C) following acclimation to mannose. Range is indicated by vertical bars.

	EtOH $Y_{P/S}$ (g g ⁻¹)	EtOH Y _{%T} (%)	$[Glycerol]_{max}$ (g L ⁻¹)	[Dry cell] _{max} (g L^{-1})
Gal (Gal-acclimated)	0.38 ± 0.00	75 ± 1	1.8 ± 0.0	3.9 ± 0.2
Gal (Glc-acclimated)	0.36 ± 0.01	70 ± 1	2.5 ± 0.1	3.9 ± 0.0
Gal (Man-acclimated)	0.36 ± 0.00	71 ± 1	2.5 ± 0.1	3.6 ± 0.0
Glc (Gal-acclimated)	0.39 ± 0.00	77 ± 1	1.3 ± 0.1	4.3 ± 0.1
Glc (Glc-acclimated)	0.40 ± 0.00	79 ± 0	1.5 ± 0.0	4.4 ± 0.0
Glc (Man-acclimated)	0.41 ± 0.00	81 ± 0	1.7 ± 0.0	4.0 ± 0.1
Man (Gal-acclimated)	0.42 ± 0.00	82 ± 0	0.7 ± 0.0	4.5 ± 0.0
Man (Glc-acclimated)	0.39 ± 0.00	76 ± 0	0.6 ± 0.0	5.0 ± 0.2
Man (Man-acclimated)	0.38 ± 0.00	75 ± 0	0.9 ± 0.0	4.3 ± 0.1

Table 2.1. Maximum ethanol yields (product per unit substrate $[Y_{P/S}]$ and percent theoretical $[Y_{\%T}]$), glycerol production, and biomass accumulation during single sugar fermentation by *S. cerevisiae* Y-1528 – range is indicated.

acclimated cells yielded the lowest, and mannose-acclimated cells the highest, glycerol production (Table 2.1). Biomass quantification indicated that mannose-acclimated cells yielded lower dry cell concentrations than either galactose- or glucose-acclimated cells (Table 2.1).

Double sugar fermentations indicated that consumption rates were also affected by acclimation conditions. Galactose and glucose were utilized at the fastest rate by cells acclimated to glucose, slightly slower by cells acclimated to mannose, and slowest by cells acclimated to galactose (Table 2.2). Galactose and mannose were utilized at the fastest rate by cells acclimated to glucose, and much slower by cells acclimated to galactose or mannose (Table 2.2). Glucose and mannose were utilized at the fastest rate by cells acclimated to glucose, slower by cells acclimated to mannose, and slowest by cells acclimated to glucose, slower by cells acclimated to mannose, and slowest by cells acclimated to glucose, slower by cells acclimated to mannose, and slowest by cells acclimated to galactose (Table 2.2). Surprisingly, ethanol yields did not differ significantly based on acclimation conditions (Table 2.3). Galactose-acclimated cells yielded lower glycerol production than glucose- or mannose-acclimated cells (Table 2.3). However, biomass data indicated that mannose-acclimated cells yielded lower dry cell concentrations than either galactose- or glucose-acclimated cells (Table 2.3), similar to the single sugar fermentation.

Triple sugar fermentations indicated that consumption rates, ethanol yields, glycerol production, and dry cell concentrations were not affected by acclimation conditions (Fig. 2.3, Table 2.4).

The trend toward more rapid galactose utilization by cells acclimated to glucose and mannose, rather than galactose, challenges conventional explanation. The pathways of galactose metabolism should have been repressed in those cells acclimated to glucose and mannose, and galactose consumption consequently retarded in those environments. Galactose metabolism requires greater energy than glucose and mannose metabolism, as well as an enzyme cohort that is normally repressed in the presence of glucose, making this inductive process an expensive undertaking in traditional strains of *Saccharomyces cerevisiae* [6–8,11]. Similarly, the trend in double sugar fermentations toward more rapid sugar utilization by cells acclimated to glucose, and to a lesser extent mannose, rather than galactose, suggests that sugar consumption patterns are less dependent on the stresses of adaptation to new carbon sources and more dependent on an undetermined factor. Curiously, triple sugar fermentation consumption trends were unaffected by acclimation to the three different

Time (h)	[Gal] (g L ⁻¹)	$[Glc] (gL^{-1})$	[Gal] (g L ⁻¹)	[Man] (gL^{-1})	$[Glc] (gL^{-1})$	[Man] (g L ⁻¹)
Gal-acclimate	d					
0	29.8 ± 0.4	29.7 ± 0.3	29.7 ± 0.2	$29.0~\pm~0.3$	30.6 ± 0.0	29.4 ± 0.4
2	29.5 ± 0.1	30.5 ± 0.1	28.0 ± 0.0	27.9 ± 0.1	$29.3~\pm~0.1$	28.8 ± 0.2
4	$28.6~\pm~0.2$	$30.1\ \pm\ 0.2$	28.9 ± 0.3	28.9 ± 0.1	$28.2~\pm~0.5$	$28.5\ \pm\ 0.1$
6	26.6 ± 0.2	29.0 ± 0.1	28.7 ± 0.2	$29.2~\pm~0.1$	23.9 ± 0.2	$27.7~\pm~0.1$
8	$21.4~\pm~0.3$	$25.1~\pm~0.7$	26.3 ± 0.5	$27.7~\pm~0.4$	$9.5~\pm~0.6$	$23.2\ \pm\ 0.3$
10	12.2 ± 1.1	$19.7~\pm~0.2$	24.8 ± 0.1	27.8 ± 0.2	0.1 ± 0.1	7.9 ± 1.2
24	$0.0~\pm~0.0$	$0.0~\pm~0.0$	0.0 ± 0.0	$0.0~\pm~0.0$	$0.0~\pm~0.0$	0.0 ± 0.0
Glc-acclimate	d					
0	29.8 ± 0.2	29.9 ± 0.4	30.3 ± 0.1	29.7 ± 0.2	31.1 ± 0.2	30.0 ± 0.3
2	$23.6~\pm~0.1$	29.9 ± 0.3	26.4 ± 0.3	29.0 ± 0.3	25.0 ± 0.2	27.8 ± 0.1
4	11.4 ± 0.5	26.4 ± 0.1	$18.2~\pm~0.3$	$28.1~\pm~0.2$	9.4 ± 0.1	21.5 ± 0.5
6	0.0 ± 0.0	5.9 ± 0.7	0.8 ± 0.4	24.9 ± 0.4	$0.0~\pm~0.0$	3.0 ± 0.4
8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.4 ± 1.8	$0.0~\pm~0.0$	0.0 ± 0.0
10	$0.0\ \pm\ 0.0$	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$0.0~\pm~0.0$	0.0 ± 0.0
24	0.0 ± 0.0	$0.0\ \pm\ 0.0$	$0.0~\pm~0.0$	$0.0~\pm~0.0$	$0.0~\pm~0.0$	$0.0\ \pm\ 0.0$
Man-acclimat	ed					
0	29.5 ± 0.5	29.6 ± 0.5	30.2 ± 0.3	29.5 ± 0.4	30.9 ± 0.3	29.8 ± 0.3
2	27.7 ± 0.3	30.2 ± 0.1	29.2 ± 0.1	29.6 ± 0.1	27.0 ± 0.1	28.2 ± 0.0
4	23.3 ± 0.9	28.7 ± 0.0	28.3 ± 0.2	28.9 ± 0.1	17.2 ± 0.2	24.8 ± 0.2
6	14.5 ± 2.1	24.8 ± 1.0	28.0 ± 0.1	29.3 ± 0.2	1.2 ± 0.2	15.2 ± 0.4
8	1.6 ± 1.4	$10.6~\pm~2.8$	25.5 ± 0.5	27.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10	$0.0~\pm~0.0$	0.0 ± 0.0	23.6 ± 1.3	26.8 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$0.0~\pm~0.0$	0.0 ± 0.0	$0.0\ \pm\ 0.0$

Table 2.2. Remaining sugar concentrations during double sugar (galactose [Gal] and glucose [Glc], galactose [Gal] and mannose [Man], or glucose [Glc] and mannose [Man]) fermentation by *S. cerevisiae* Y-1528 – range is indicated.

	EtOH $Y_{P/S}$ (g g ⁻¹)	EtOH 1/157 (%)	$[Glycerol]_{max}$ (g L ⁻¹)	[Dry cell] _{max} (gL ⁻¹)
Gal + Glc (Gal-acclimated)	0.36 ± 0.00	70 ± 0	1.5 ± 0.0	5.3 ± 0.0
Gal + Glc (Glc-acclimated)	0.38 ± 0.00	74 ± 0	3.3 ± 0.0	5.4 ± 0.0
Gal + Glc (Man-acclimated)	0.37 ± 0.00	73 ± 0	3.0 ± 0.1	4.7 ± 0.0
Gal + Man (Gal-acclimated)	0.39 ± 0.00	77 ± 0	1.4 ± 0.1	5.7 ± 0.0
Gal + Man (Glc-acclimated)	0.36 ± 0.01	71 ± 1	3.3 ± 0.1	5.6 ± 0.0
Gal + Man (Man-acclimated)	0.37 ± 0.00	73 ± 0	2.6 ± 0.0	4.9 ± 0.1
Glc + Man (Gal-acclimated)	0.40 ± 0.00	78 ± 1	1.0 ± 0.0	5.6 ± 0.0
Glc + Man (Glc-acclimated)	0.40 ± 0.00	79 ± 1	2.4 ± 0.0	5.5 ± 0.0
Glc + Man (Man-acclimated)	0.37 ± 0.00	73 ± 1	$2.6~\pm~0.0$	5.1 ± 0.1

Table 2.3. Maximum ethanol yields (product per unit substrate $[Y_{P/S}]$ and percent theoretical $[Y_{\%T}]$), glycerol production, and biomass accumulation during double sugar fermentation by *S. cerevisiae* Y-1528 – range is indicated.



Fig. 2.3. Sugar consumption during triple sugar (galactose [Gal], glucose [Glc], and mannose [Man]) fermentation by *S. cerevisiae* Y-1528; (A) following acclimation to galactose; (B) following acclimation to glucose; (C) following acclimation to mannose. Range is indicated by vertical bars.

	EtOH $Y_{P/S}$ (g g ⁻¹)	EtOH Y _{%T} (%)	$[Glycerol]_{max}$ (gL ⁻¹)	[Dry cell] _{max} (gL ⁻¹)
Gal + Glc + Man (Gal-acclimated)	0.39 ± 0.00	76 ± 1	4.3 ± 0.1	11.7 ± 0.4
Gal + Glc + Man (Glc-acclimated)	0.39 ± 0.00	77 ± 1	4.4 ± 0.0	11.2 ± 0.0
Gal + Glc + Man (Man-acclimated)	0.39 ± 0.00	77 ± 1	4.5 ± 0.0	12.1 ± 0.6

Table 2.4. Maximum ethanol yields (product per unit substrate $[Y_{P/S}]$ and percent theoretical $[Y_{\%T}]$), glycerol production, and biomass accumulation during triple sugar fermentation by *S. cerevisiae* Y-1528 – range is indicated.

hexose sugars, and as a result it is hypothesized that if one specific sugar is the trigger for faster or slower utilization, the concurrent presence of the other two sugars might mask such patterns.

Consistent throughout single and double sugar fermentations was higher glycerol production from glucose- and mannose-acclimated cells than from galactose-acclimated cells. The respective glycerol production may reflect the rate of glycolysis occurring in each culture, as observed in sugar consumption trends, but does not account for similar ethanol yields in single and double sugar fermentations. The relatively high glycerol production from mannose-acclimated cells is in direct opposition to biomass data, which indicate lower dry cell concentrations. This may be indicative of divergent carbon allocation, toward catabolic rather than anabolic cellular processes.

Y-1528 mixed sugar fermentation – substrate effects

Single sugar fermentations demonstrated that each single sugar was completely consumed within 4–6 h (Fig. 2.2). Ethanol yields from galactose were lower than those from glucose and mannose (Table 2.1). Glycerol production, in contrast, was evidently higher in galactose fermentations, lower in glucose fermentations, and lower still in mannose fermentations (Table 2.1). Biomass data indicated that dry cell concentrations were highest in mannose fermentations, lower in glucose fermentations, and lower still in galactose fermentations (Table 2.1).

Double sugar fermentations indicated that galactose was consumed more rapidly than glucose and mannose, and glucose was consumed more rapidly than mannose (Table 2.2). Unexpectedly, endogenous glucose formation was detected in galactose and mannose fermentations, following the onset of sugar consumption, despite the absence of the sugar in the initial substrate mixture (Fig. 2.4). The choice of acclimation sugar did not affect the production of this extracellular glucose. Ethanol yields from all three combinations of sugars were similar (Table 2.3). However, glycerol production was consistently higher in galactose and glucose fermentations than in glucose and mannose fermentations (Table 2.3). Biomass data demonstrated that there were no significant differences in dry cell concentrations between each of the double sugar fermentations (Table 2.3).

Triple sugar fermentations (Fig. 2.3) demonstrated that galactose was consumed most rapidly (within 6 h), glucose less so (within 8 h), and mannose least rapidly (within 10 h). Ethanol yields, glycerol production, and biomass data are shown in Table 2.4.



Fig. 2.4. Time-lapse chromatogram of endogenous glucose (Glc) formation during double sugar (galactose [Gal] and mannose [Man]) fermentation by *S. cerevisiae* Y-1528.

The rapid consumption of single sugars, including galactose, without evidence of a lag phase indicated that Y-1528 was not subject to conventional metabolic preference for energetically favorable substrates. However, the lower ethanol yield achieved following galactose fermentation suggested that carbon allocation during glycolysis was altered. In concert with higher glycerol production and lower dry cell concentration, these data imply an enzymatic mechanism that, in shifting away from anabolic processes, either generates more secreted catabolic intermediates than comparable glucose or mannose metabolism, or sufficiently changes the intracellular redox state to necessitate excess production of glycerol [23,24]. Also noted was an inverse correlation between glycerol and biomass production, which reinforced the suggested re-allocation of carbon according to substrate.

The more rapid exhaustion of galactose in double sugar fermentations indicated that conventional patterns of diauxic growth were not followed in Y-1528 cultures. During the course of galactose and mannose fermentations, the surprising appearance of glucose in the culture medium yielded a clue to the exceptional abilities of this yeast strain. Furthermore, during triple sugar fermentations this yeast strain's characteristic consumption patterns in the simultaneous presence of galactose, glucose, and mannose also demonstrated the absence of conventional diauxic growth patterns, with galactose exhaustion preceding that of glucose and mannose. These results further emphasized unconventional substrate preferences, signaling a need to discern the mechanistic background of galactose metabolism in Y-1528. Importantly, complete consumption of all three sugars occurred within 10 h, indicating that, despite altered sugar preferences, the strain was capable of rapidly exhausting each available substrate through high capacity glycolytic flux.

¹⁴C radiolabeled mixed sugar fermentation

To address the formation of endogenous glucose during galactose and mannose double sugar fermentations, radioactive tracers were added to determine the source of the unexpected sugar. The first fermentation consisted of [1-¹⁴C] galactose in combination with unlabeled mannose, and the second consisted of [1-¹⁴C] mannose in combination with unlabeled galactose. The labeled galactose fermentation indicated that galactose was the carbon source for the endogenously formed glucose, whereas the labeled mannose fermentation indicated no such source (Fig. 2.5). It was therefore evident that a C-4 epimerase, rather than a C-2 epimerase, was responsible for the



Fig. 2.5. Radioactivity during radioisotope-labeled double sugar (galactose [Gal] and mannose [Man]) fermentation by *S. cerevisiae* Y-1528. (A) $[1-^{14}C]$ galactose and $[1-^{12}C]$ mannose. (B) $[1-^{12}C]$ galactose and $[1-^{14}C]$ mannose. Range is indicated by vertical bars.

formation of glucose.

The determination of the presence of a C-4 epimerase, rather than a C-2 epimerase, indicated that the substrate galactose was responsible for the endogenous formation of glucose during double sugar fermentations of galactose and mannose. Though C-4 epimerases are prevalent in a wide range of living organisms, catalyzing the stereochemical conversion of galactose to glucose via a variety of mechanisms, their presence in *Saccharomyces cerevisiae* has not been implicated in changing the nature of carbon catabolite repression in mixed sugar environments. Therefore, the central role of this C-4 epimerase in the unusual fermentative performance of Y-1528 suggested that the enzyme was either structurally unique, localized to a sub-cellular region or regions in which the observed behavior became possible, or mechanistically atypical in the nonstereospecific isomerisation of galactose. The subsequent experiment conducted to begin characterization of the C-4 epimerase was chosen on the working assumption of conventional mechanisms of stereochemical conversion, and designed to establish enzyme localization.

Sub-cellular fractionation

To determine the localization of C-4 epimerase enzyme activity, six isolated cell fractions were generated from Y-1528 and the "normal" strain Tembec T1: extracellular, cell wall and periplasmic, plasma membrane, cytosolic, mitochondrial, and nuclear. Each fraction, once desalted in elution buffer and confirmed to contain protein via UV light absorbance at 280 nm (data not shown), was independently added to a C-4 epimerase enzyme assay as well as to a marker enzyme (phosphoglucose isomerase) The assay aliquots were then chromatographically analyzed for nucleotide assay. sugars and phosphate sugars, respectively. Significant epimerase activity was apparent in the cytosolic, cell wall and periplasmic, plasma membrane, mitochondrial, and nuclear fractions of Y-1528, and absent in the extracellular fraction (Fig. 2.6A). However, the marker enzyme assay showed small amounts of phosphoglucose isomerase activity in all fractions that exhibited epimerase activity, indicating cytosolic contamination of the cell wall and periplasmic, plasma membrane, mitochondrial, and nuclear fractions (data not shown). Nevertheless, phosphoglucose isomerase activity was shown to be highest in the cytosolic fraction. Epimerase activity was absent in all of the fractions of Tembec T1 (Fig. 2.6B), despite evidence of cytosolic contamination of



Fig. 2.6. UDP-glucose-4-epimerase activity in isolated nuclear, mitochondrial, cytosolic, plasma membrane, periplasmic/cell wall, and extracellular fractions of *S. cerevisiae*, as measured via UV light absorbance of UDP-glucose product. (A) Y-1528. (B) Tembec T1.

the cell wall and periplasmic, plasma membrane, mitochondrial, and nuclear fractions (data not shown).

The successful adaptation and development of methods to assay epimerase activity and detect the products of such catalysis implied typical mechanisms of epimerization in Y-1528 [15,16]. However, the localization of the C-4 epimerase in Y-1528 and Tembec T1 proved difficult to ascertain, with experimental evidence pointing toward cytosolic contamination of all fractions except the extracellular. Clarification of the sub-cellular distribution of the epimerase might be possible through the application of immunolocalization and electron microscopy, following high pressure freezing and freeze-substitution with fixatives dissolved in organic solvents [25,26]. To account for other causes of the unusual behavior of Y-1528 in batch culture, the molecular structure and function of the GAL10 gene and epimerase protein will be investigated through isolation, purification, and sequencing protocols. Interestingly, the Tembec T1 fractions did not exhibit epimerase activity, despite significant protein cohorts, suggesting delayed induction of galactose metabolism genes in this strain and providing contrast to the immediate activation of the Leloir pathway and associated enzymes in Y-1528. The role of galactose catabolon regulation in Y-1528 will therefore be a target of future studies.

Summary

A wild-type microorganism with an enhanced capacity for the assimilation and fermentation of galactose would address an enduring challenge in the bioconversion of sugar-constituted substrates to ethanol, since current candidate microbes either exhibit significantly delayed and slow galactose metabolism (both in the presence and absence of other hexose sugars), or must be genetically modified to better ferment this recalcitrant sugar. A *Saccharomyces cerevisiae* strain (Y-1528) obtained from culture collection was selected on the basis of exceptional fermentative performance on galactose from among other wild-type strains of the same species. Y-1528 exhibited high osmotolerance and tolerance of inhibitory compounds produced by autoclaving growth media, and fermented galactose without a lag phase and prior to glucose and mannose in mixed sugar fermentations, even when previously acclimated to glucose or mannose. Furthermore, extracellular glucose appeared during co-fermentations of galactose and mannose, produced via a UDP-glucose-4-epimerase. Experiments to localize this epimerase proved difficult, but served to illustrate (on a molecular scale)

notable differences between Y-1528 and Tembec T1 in the metabolism of galactose. Y-1528, while interesting with regard to fundamental mechanisms of sugar metabolism, also satisfied some of the criteria for a suitable bioconversion microorganism. It quickly fermented all three hexose sugars, tolerated high sugar concentrations, and was not substantially affected by thermally generated inhibitory compounds.

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Chapter 3 - An ethanologenic yeast exhibiting unusual metabolism in the fermentation of lignocellulosic hexose sugars

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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, III.). *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% SO₂) 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

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 μ m PVDF (polyvinylidene fluoride) filters prior to injection (20 μ L). 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 μ m PVDF filters prior to injection (2 μ L). 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:

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

$$Y_{\%T} = (Y_{P/S} \div 0.51) \times 100$$
(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'Urfé, 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 Qia-Quick 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 (BsuRI, Dral, EcoRI, Hinfl, or Hin6I) (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. 3.1A). 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



Fig. 3.1. (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.

after 9 h, illustrating the normal sequence of metabolic conversion of sugars to ethanol by *S. cerevisiae*. Ethanol yield from Y-1528 (Fig. 3.1B, Table 3.1) 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 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 Specifically, Y-1528 consumed galactose in 2 h, glucose in 6 h, and (Fig. 3.2A). mannose in 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. 3.3, Table 3.1) for the Y-1528- and Tembec T1-catalyzed fermentations were similar (roughly 82% of theoretical yield).

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. 3.2B). 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. 3.3, Table 3.1) from Y-1528 was

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	

Table 3.1. 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.



Fig. 3.2. 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.



Fig. 3.3. Ethanol production during softwood and hardwood SSL fermentations by *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1. *Vertical bars*: Range.

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 onethird 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, sulfurcontaining 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 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. 3.4). 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 (Fig. 2.4). 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. 3.5, Table 3.1), 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 point as accumulated metabolic products exerted a population-limiting effect (Fig. 3.5).

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. 3.6). 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. 3.7, Table 3.1), slightly below the roughly 82% achieved by Y-1528 or Tembec T1 alone (Fig. 3.3). Similarly, the co-cultured yeast strains consumed all of the hexose sugars in hardwood SSL in 8 h (Fig. 3.6), 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. 3.7, Table 3.1), comparable to the 75% achieved by Y-1528 alone and slightly above the 70% achieved by Tembec T1 alone (Fig. 3.3). No catabolite repression was evident in the



Fig. 3.4. 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.



Fig. 3.5. 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.



Fig. 3.6. 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. 3.7. Ethanol production during softwood and hardwood SSL fermentations by mixed cultures of *S. cerevisiae* Y-1528 and *S. cerevisiae* Tembec T1. *Vertical bars*: Range.

fermentation of either liquor. As recorded previously, Y-1528 or Tembec T1 alone required significantly more time to effect complete hexose sugar consumption (Fig. 3.2).

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 As predicted, the combination of strains accelerated galactose of each strain. consumption beyond that achieved by Tembec T1 (and by pure Y-1528, unexpectedly exemplifying synergy), and accelerated glucose and mannose 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 3.1). 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. 3.8). Fragment sizes were calculated using regression equations based on DNA mass ladder component migration distances and known fragment sizes, and subsequent pattern



Fig. 3.8. 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.

comparison indicated both strains to be S. cerevisiae (Table 3.2).

methods of species Molecular 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 generated 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].

	Bsu RI	DraI	EcoRI	Hinfl	Hin6I
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)					

Table 3.2. 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.

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Chapter 4 - Tolerance and adaptation of ethanologenic yeasts to lignocellulosic inhibitory compounds

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Introduction

Energy obtained from plant biomass represents a viable alternative to petroleum fuels in meeting increasing global growth and sustenance demands. Bioethanol is an important modern adjunct to petroleum because it is renewable, can make use of fast rotation plants, produces fewer emissions than fossil fuels, generates no net carbon dioxide, and is compatible with current fuel distribution infrastructure [33,46,51].

Plant-derived bioethanol can be generated from starch- or lignocellulose-rich materials. Lignocellulose-derived feedstocks, in particular, are abundant and embody a poorly utilized resource [42]. Despite these benefits, by-products liberated during processing commonly retard the downstream hydrolytic treatments and fermentation employed in generating bioethanol [27,36].

The bio-processing of lignocellulose inevitably involves a form of chemical treatment: the two most common methods are dilute-acid hydrolysis and acid-catalyzed steam explosion [11]. One unintended effect of these non-selective approaches is the generation of numerous lignocellulosic degradation products and reaction vessel artifacts [4,24,37]. The contribution of organic compounds produced by lignocellulose degradation, such as furans, weak acids, and phenolic derivatives to fermentation inhibition is significant [4,26,36], and the proportion of each depends on the specific lignocellulosic feedstock undergoing hydrolysis. For example, softwoods (SWs) generally contain more lignin than hardwoods (HWs), while HW hemicellulose is highly acetylated [37,46]. Common inhibitory compounds from pentose and hexose sugar degradation include the furans, furfural and hydroxymethylfurfural (HMF), and weak acids such as acetic acid, formic acid, and levulinic acid [37].

In anaerobic environments, most ethanologenic microorganisms (*e.g.*, *Saccharomyces cerevisiae* (*S. cerevisiae*)) reduce furans to their corresponding alcohol moieties as a means of detoxification [28,37]. However, furans at high concentrations exert an inhibitory effect, interfering with glycolytic enzymes and macromolecule synthesis [2,3,23].

The hydrolysis of HW hemicellulose (and to a lesser extent SW hemicellulose) produces acetic acid, which is relatively abundant with respect to other lignocellulosederived weak acids and has cytotoxic effects at elevated concentrations [25,26,39]. Based on evidence that acetic acid (pK_a 4.76) in an undissociated form is lipophilic, and that its conjugate base (acetate) is lipophobic, this inhibitory compound can migrate across cellular membranes during fermentation and undergo dissociation at physiological pH, leading to acetate entrapment, intracellular proton accumulation, consequent cytosol acidification, and retardation of metabolic processes [28,37].

Spent sulfite liquor (SSL) is a by-product of pulp mills that employ an acid sulfite process to generate purified cellulose for paper manufacture. It contains hemicellulose-derived sugars at a typical concentration of 3–4%, sugar degradation products (such as furfural and HMF), weak acids (primarily acetic acid), lignosulfonates, and assorted salts [13,45]. Consequently, SSL, a low-cost feedstock similar to other lignocellulosic hydrolysates in chemical profile, is widely considered and utilized as a substrate for the production of bioethanol [13,14,49].

A long-standing interest of the alcoholic fermentation industry is increasing ethanol productivity, while concurrently minimizing costs [5,47]. One development is cell recycle batch fermentation (CRBF), which involves the harvest of cellular biomass upon substrate exhaustion, and its immediate reuse as inoculum in fresh substrate [16,44]. Consecutive cycles of this Mellé-Boinot process have been shown to increase ethanol productivity, yield, and tolerance, as well as lower the net cost related to inoculum production and fermentation vessel operation [16,19,20,41]. At the cellular level, substrate carbon is no longer required for extensive biomass accrual with each cycle, and reduced lag phases are observed.

The objectives of this study were to assess the effects of three inhibitory compounds (furfural, HMF, and acetic acid) on the fermentative performance of two notable yeast strains, *S. cerevisiae* Tembec T1 and *S. cerevisiae* Y-1528 [18], and to determine the sugar consumption patterns and ethanologenic capacities of these strains in CRBFs of SSL.

Materials and Methods

Yeast strains, culture media, and substrates

S. cerevisiae T1 and Y-1528 were obtained from Tembec Limited (Témiscaming, Québec, Canada) and the USDA Agricultural Research Service (Peoria, IL), respectively. Strains were maintained on YPG solid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 18 g/L 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 yeast extract, 10 g/L peptone, and 10 g/L 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.

Softwood and HW SSL was acquired from Tembec Limited and adjusted to pH 6.0 with sodium hydroxide. Suspended solids were not removed by filtration.

Inhibitor-supplemented and cell recycle 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 concentration of 100 g dry cell weight (DCW) per liter by relating 600 nm absorbance to DCW/L concentration.

Inhibitor-supplemented fermentations were performed in foam-plugged 125 mL Erlenmeyer flasks containing 50 mL YPG liquid medium (10 g/L yeast extract, 20 g/L peptone, and 30 g/L each of filter-sterilized galactose, glucose, and mannose) augmented with 0, 0.8, or 1.6 g/L filter-sterilized furfural, 0, 3.0, or 4.0 g/L filter-sterilized HMF, or 0, 5.0, 10.0, or 15.0 g/L filter-sterilized acetic acid. The acetic acid-augmented media were adjusted to pH 6.0 prior to inoculation. Media were inoculated with either Tembec T1 or Y-1528 to achieve an initial cell concentration of 4 g DCW/L. Flasks were incubated in an orbital shaker for 49 h at 30°C and 150 rpm. Offline sampling was performed aseptically and 1 mL aliquots 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 sugar, ethanol, and inhibitory compound analyses.

Complex (rather than defined) media were employed in inhibitor-supplemented fermentations in order to emulate industrial conditions as much as possible, and to ensure that any undetermined auxotrophic properties of Tembec T1 or Y-1528 would not manifest in physiological changes due to the absence of nutrients. Inhibitor concentrations were selected to broadly encompass levels in most lignocellulosic hydrolysates, including SSL.

CRBFs were performed in foam-plugged 125 mL Erlenmeyer flasks containing 50 mL SW or HW SSL. The hexose sugar composition of the SW SSL comprised 13.3 g/L mannose, 5.0 g/L glucose, and 3.2 g/L galactose, while the HW SSL contained 6.8 g/L mannose, 3.2 g/L glucose, and 1.7 g/L galactose. The SW SSL contained approximately 0.05 g/L furfural, 0.16 g/L HMF, and 10 g/L acetic acid, while the HW SSL contained approximately 0.18 g/L furfural, 0.11 g/L HMF, and 15 g/L acetic acid.

To accurately simulate conditions at SSL-fermenting pulp mills, in which wood feedstocks frequently change in response to demand for different paper products [13], fermentation substrates were alternated with each cycle according to the following three schemes:

- (1) SW (Cycle 1) \rightarrow HW (Cycle 2) \rightarrow SW (Cycle 3)
- (2) SW (Cycle 1) \rightarrow SW (Cycle 2) \rightarrow HW (Cycle 3)
- (3) HW (Cycle 1) \rightarrow HW (Cycle 2) \rightarrow SW (Cycle 3)

Initially, substrates were inoculated with either Tembec T1 or Y-1528 to achieve a starting cell concentration of 4 g DCW/L. At the conclusion of each cycle (46 h in an orbital shaker at 30°C and 150 rpm), cells were harvested, adjusted to a calculated concentration of 100 g DCW/L via spectrophotometer-derived standard curves, and then added to fresh substrate to (again) achieve an initial cell concentration of 4 g DCW/L. Offline sampling and supernatant preparation (for sugar, ethanol, and glycerol analyses) was accomplished using the same procedures employed in inhibitor-supplemented fermentations. All fermentation experiments were performed in duplicate with the appropriate negative controls. Furthermore, biological duplicates in separate flasks were performed within each experiment.

Chemical analyses

High performance liquid chromatography (HPLC) on a DX-600 BioLC chromatograph (Dionex, Sunnyvale, CA) was used for sugar determination. Separation was achieved on 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 supplemented with fucose as internal standard, and then filtered through 0.45 μ m PVDF (polyvinylidene fluoride) filters prior to injection (20 μ L). The column was eluted with deionized water at a flow rate of 1.0 mL/min.

Ethanol and acetic acid quantification 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, CA). 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 supplemented with butan-1-ol as internal standard, and then filtered through 0.45 μ m PVDF filters prior to injection (2 μ L). An injector temperature of 90°C, a detector temperature of 250°C, and a helium (carrier

gas) flow rate of 1.0 mL/min 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, and subsequently maintained at 230°C for 10 min.

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

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

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

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

Glycerol determination was achieved by HPLC using a CarboPac MA1 anion exchange column (Dionex), and detection was achieved via pulsed amperometry across a gold electrode. External standards and experimental samples were supplemented with erythritol as internal standard, and then filtered through 0.45 μ m PVDF filters prior to injection (25 μ L). The column was eluted with 200 mM NaOH at a flow rate of 0.40 mL/min for 14 min, 480 mM NaOH for 40 min, and then 200 mM NaOH for 14 min.

Furfural and HMF were quantified on a Summit HPLC chromatograph (Dionex). Separation was achieved by a LiChrospher RP18 reversed phase column (Varian Instruments, Walnut Creek, CA), and detection was achieved at 280 nm. External standards and experimental samples were supplemented with catechol as internal standard, and then filtered through 0.45 μm PVDF filters prior to injection (25 μL). The column was heated to 60°C and eluted at a flow rate of 0.5 mL/min, employing a ternary gradient of 7.4 mM phosphoric acid (eluent A), acetonitrile (eluent B), and a 4:3:3 mixture of 7.4 mM phosphoric acid, methanol, and acetonitrile (eluent C). The elution consisted of a 20 min transition from 95% eluent A and 5% eluent C to 50% eluent A and 50% eluent C, followed by further grading to 100% eluent C over 4 min and a subsequent 1 min-hold, and then a 1 min transition from 100% eluent C to 50% eluent B. A 1-min hold at 100% eluent B was followed by a 1-min transition back to 95% eluent A and 5% eluent C and a 10 min re-equilibration to end the run.

Results and Discussion

Furfural-supplemented fermentation

The fermentation of furfural-augmented YPG medium yielded interesting results. Sugar consumption data showed that Y-1528 exhausted all of the hexose sugars in 6 h in the presence of approximately 0.8 g/L furfural (matching the rate observed in the furfural-free reference fermentation), and in 8 h in the presence of approximately 1.6 g/L furfural (Fig. 4.1A). Tembec T1 showed correspondingly slower consumption patterns, exhausting all of the hexose sugars in 8 h (reference), just over 8 h (0.8 g/L furfural), and 11 h (1.6 g/L furfural) (Fig. 4.1A).

Both Y-1528 and Tembec T1 generated similar concentrations of ethanol in the absence of furfural and in the presence of 0.8 g/L and 1.6 g/L furfural. Ethanol productivity in Y-1528 was markedly lower at the highest concentration of furfural (Fig. 4.1B), as expected with a longer timeframe of complete hexose sugar exhaustion. Ethanol productivity in Tembec T1 matched trends observed in sugar consumption, with a small decrease in the presence of 0.8 g/L furfural and a more significant reduction at 1.6 g/L furfural (Fig. 4.1B).

Furfural consumption data showed that Y-1528 effected complete degradation of the inhibitory compound in 4 h when presented with an initial concentration of 0.8 g/L, and in just over 2 h when presented with an initial concentration of 1.6 g/L (Fig. 4.1C). Tembec T1 showed a similar consumption pattern at 0.8 g/L furfural, but demonstrated a distinct 2-h lag prior to degradation when presented with 1.6 g/L, requiring just over 4 h from the onset of fermentation to eliminate the inhibitory compound (Fig. 4.1C).

Previous research has demonstrated that concentrations of furfural up to 2 g/L mildly reduce the rate of glucose consumption in *S. cerevisiae*-catalyzed fermentations, an effect that can be attributed to the inhibition of glycolytic enzymes [2,3]. Similarly, the furfural concentrations in these experiments had a small, but proportionally significant effect on consumption of galactose, glucose, and mannose. The more rapid hexose sugar consumption by Y-1528 (with respect to Tembec T1) in the reference fermentation and at both furfural concentrations was expected, and is ascribed to more efficient sugar transport and/or metabolism [17].

The absence of effect on ethanol yield suggests the possibility that the carbon normally diverted from the glycolytic intermediate dihydroxyacetone phosphate toward glycerol production was instead available for ethanol production, compensating for any



Figure 4.1. Hexose sugar consumption (A), ethanol production (B), and furfural consumption (C) in furfural-augmented YPG medium by *S. cerevisiae* strains Y-1528 and Tembec T1, following growth on glucose. Range is indicated by vertical bars.

inhibition of alcohol dehydrogenase (or other glycolytic enzyme) activity [37,39,50]. The detrimental effect on ethanol productivity can be traced to the hindrance of glycolytic enzyme function, which slows the rate of product evolution [3,22,30].

The substantially higher furfural consumption rate accompanying a higher initial furfural concentration in the Y-1528-catalyzed fermentations signifies cellular demand for regenerated NAD⁺, which is produced through the conversion of furfural to furfuryl alcohol. These results suggest that the demand for regenerated NAD⁺ is being met primarily by rapid furfural reduction (detoxification), and is reinforced by a slower rate of ethanol production at the highest initial furfural concentration (1.6 g/L) [38]. Furthermore, assuming intracellular NADH is present in excess, a higher initial furfural concentration (*e.g.*, 1.6 g/L) will result in a higher furfural consumption rate because more substrate is available for alcohol dehydrogenase-driven catalysis [37]. The 2-h lag in furfural consumption (but not in sugar consumption or ethanol production) exhibited by Tembec T1 in the presence of 1.6 g/L suggests a slow onset of detoxification through reduction of the inhibitor to furfuryl alcohol.

HMF-supplemented fermentation

Sugar consumption rates demonstrated that both Y-1528 and Tembec T1 exhausted all of the hexose sugars in 6 h in the reference fermentation. However, Y-1528 only required 8 h in the presence of approximately 3.0 g/L HMF, and just over 8 h in the presence of approximately 4.0 g/L HMF, while Tembec T1 comparatively required over 8 h (3.0 g/L HMF), and between 8 and 10 h (4.0 g/L HMF) (Fig. 4.2A).

Ethanol production data showed that Y-1528 generated a much higher concentration in the absence of HMF than in the presence of 3.0 g/L and 4.0 g/L HMF (99%, 89%, and 88% of theoretical yield, respectively). Ethanol productivity was substantially lower at both concentrations of HMF (Fig. 4.2B), as expected with longer timeframes of complete hexose sugar exhaustion. Likewise, Tembec T1 produced a higher concentration in the absence of HMF than in the presence of 3.0 g/L and 4.0 g/L HMF (99%, 90%, and 87% of theoretical yield, respectively). Ethanol productivity matched trends observed in sugar consumption, with a marked decrease in the presence of 3.0 g/L HMF and a further reduction in the presence of 4.0 g/L HMF (Fig. 4.2B).

HMF consumption data showed that Y-1528 could completely degrade the HMF in 4 and 6 h when presented with initial concentrations of 3.0 g/L and 4.0 g/L,



Figure 4.2. Hexose sugar consumption (A), ethanol production (B), and HMF consumption (C) in HMF-augmented YPG medium by *S. cerevisiae* strains Y-1528 and Tembec T1, following growth on glucose. Range is indicated by vertical bars.

respectively. Tembec T1, in contrast, showed an extended consumption pattern when presented with initial HMF concentrations of 3.0 and 4.0 g/L, effecting complete degradation in approximately 7 and 8 h, respectively (Fig. 4.2C).

As with furfural, HMF had a proportionately unfavorable effect on the rate of hexose sugar consumption. However, at the higher concentrations employed (3.0 and 4.0 g/L *vs.* 0.8 and 1.6 g/L), HMF demonstrated lower toxicity than the five-carbon inhibitor, concurring with previous findings [22,43,48]. Tembec T1 fermentations were affected to a greater extent by HMF than those catalyzed by Y-1528, with comparatively slower rates of sugar consumption at each inhibitor concentration. Since the detoxification of HMF is accomplished by its reduction to HMF alcohol, and this reduction is a function of alcohol dehydrogenase activity [48], a higher efficiency ADH is implicated in Y-1528.

The significant effect on ethanol yield in both of these fermentations was intriguing and distinct, because unlike furfural, the cellular detoxification response to HMF is associated with NADPH, a cofactor involved in anabolic processes [50]. As such, the reduction of HMF does not regenerate NAD⁺, and thus carbon is allocated to glycerol production (to produce NAD⁺ and thus maintain overall redox balance) [37]. The inhibition of glycolytic enzymes by furans [2] and the consequential decrease in ethanol yield would therefore not be compensated by increased carbon availability. The approximate 10% loss in ethanol yield observed with the addition of HMF supports this assertion. The negative effect on ethanol productivity, as previously observed under similar experimental conditions [22], can be traced to glycolytic enzyme inhibition, since HMF and furfural share identities as furanoic aldehydes and clear evidence exists of the latter's physiological impact on glycolysis [2]. The marginally higher ethanol productivity exhibited by Y-1528 at both HMF concentrations (but not in the reference fermentation) is likely a function of the respective rates of hexose sugar consumption.

Contrasting furfural, HMF consumption appeared to be dose-dependent, with the time to complete degradation proportional to the initial concentration. In requiring the cofactor NADPH for reduction/detoxification, HMF affects macromolecule (*i.e.*, amino acid and nucleotide) synthesis, but does not provide for the regeneration of NAD⁺ made scarce by catabolism [50]. Hence, an increased consumption rate accompanying a higher initial concentration of the inhibitor, as observed in the furfural-augmented fermentation, would not benefit the cellular redox balance by compensating for reduced

NAD⁺ output via ethanol and glycerol. Interestingly, the rate of degradation of HMF was not severely retarded with respect to that of furfural, in opposition to extensive evidence of such a phenomenon in previously reported *S. cerevisiae*-catalyzed batch fermentations [22,48]. Thus, Y-1528 and Tembec T1 are apparently better adapted to detoxify HMF than many other strains of *S. cerevisiae*, and the faster consumption rate exhibited by Y-1528 reflects more efficient detoxification through the ADH-catalyzed reduction of HMF to HMF alcohol. This enhanced metabolic efficiency may be attributed to the environment in which Y-1528 evolved preceding isolation, a subject of recent speculation [17].

Acetic acid-supplemented fermentation

In the presence of 5.0 g/L, 10.0 g/L, and 15.0 g/L acetic acid, Y-1528 exhausted all of the hexose sugars in 8 h, a comparable rate to the acetic acid-free reference fermentation (Fig. 4.3A). The rates of sugar consumption in the first 2 h of fermentation showed some variance, with the higher levels of acetic acid slightly retarding uptake and metabolism, but rate convergence was soon established. Tembec T1 showed similar but not identical consumption patterns, exhausting all of the hexose sugars in 8 h (reference, 5.0 g/L, and 10.0 g/L), and between 8 and 10 h (15.0 g/L) (Fig. 4.3A).

Ethanol production data showed that Y-1528 generated increasing yields in the presence of 0 g/L, 5.0 g/L, and 10.0 g/L acetic acid (72%, 78%, and 81% of theoretical yield, respectively), and a substantially lower concentration in the presence of 15.0 g/L acetic acid (60% of theoretical yield). Tembec T1 produced a higher ethanol concentration at 5.0 g/L acetic acid, but then lower yields in the presence of 10.0 g/L and 15.0 g/L acetic acid (73%, 79%, 70%, and 58% of theoretical yield, respectively). As with Y-1528, ethanol productivity was initially lower at higher levels of acetic acid, but production rates eventually became similar (Fig. 4.3B).

Acetic acid data showed that both Y-1528 and Tembec T1 did not effect consumption of the compound in the presence of 5.0 g/L, 10.0 g/L, or 15.0 g/L (Fig. 4.3C).

The apparent lack of effect on the rate of sugar consumption by Y-1528 and Tembec T1 was surprising, especially with acetic acid concentrations of 10.0 g/L and 15.0 g/L, since experimental evidence has indicated inhibition of cellular processes in *S. cerevisiae* at concentrations ranging from 0.5 to 9.0 g/L [25]. In more recent experiments [29], minimal media supplemented with 0.5 g/L showed reduced rates of



Figure 4.3. Hexose sugar consumption (A), ethanol production (B), and acetic acid consumption (C) in acetic acid-augmented YPG medium by *S. cerevisiae* strains Y-1528 and Tembec T1, following growth on glucose. Range is indicated by vertical bars.

glucose consumption, and with increasing concentrations of acetic acid (beyond 1.0 g/L) slowed sugar exhaustion even further. The toxicity of acetic acid in most instances has been attributed to its transition across the plasma membrane and subsequent dissociation into lipophobic acetate and protons in the pH-neutral intracellular environment [22,40]. The simultaneous hexose sugar utilization by Y-1528 [17,18] and flocculating behavior of Tembec T1 provide unique, but equally applicable rationales for the observed minimal inhibition of sugar consumption in the presence of acetic acid. The slightly faster sugar consumption rate exhibited by Y-1528 (relative to Tembec T1) at the highest concentration of acetic acid indicates a more enduring capacity for resisting the detrimental impact of this inhibitor as exposure levels rise.

The ethanol yield trend observed in these experiments is reinforced by previous research, in which *S. cerevisiae* catalyzed increasing yields in the presence of up to 6–10 g/L acetic acid, and decreasing yields at concentrations beyond [22,39]. The higher concentrations of acetic acid (*e.g.*, 15.0 g/L, in the case of Y-1528 and Tembec T1) may cause markedly lower ethanol yields, despite little effect on sugar consumption rates, because of the inhibition of certain glycolytic enzymes by intracellular acidification and acetate accumulation, and unspecified toxicity as effected by extracellular anions [8,37,39].

The absence of acetic acid consumption has been observed in previous research [10,26], and may implicate extracellular anions as the active mediator of the observed effect on ethanol yields, and to a lesser extent, ethanol production. Endogenous acetate production was not detected from either yeast strain in inhibitor-free reference fermentations (data not shown), even though evidence exists for the generation of minor amounts in various other strains of *S. cerevisiae* [29].

Cell recycle batch fermentation

Sugar consumption data from Scheme (1) indicated that Y-1528 exhausted all of the SW-derived hexose sugars in 4 h in Cycle 1, all of the HW-derived hexose sugars in 24 h in Cycle 2, and all of the SW-derived hexose sugars in 12 h in Cycle 3 (Table 4.1). Closer inspection of the data showed the most striking performance penalty to have occurred in the rate of galactose consumption, with a loss of preference for this sugar following Cycle 1 (data not shown).

Ethanol production data from Scheme (1) showed that Y-1528 generated 73% of theoretical yield from the SW substrate in Cycle 1, 63% from the HW substrate in Cycle
Scheme (1)	Culture	[Sugar] _{initial} (g/L)	[Sugar] _{residual} (g/L)	<i>t</i> ([EtOH] _{max}) (h)
SW	Y-1528	20.59 ± 0.17	0.00 ± 0.00	4
HW	Y-1528	10.45 ± 0.32	1.99 ± 0.01	8
SW	Y-1528	20.74 ± 0.23	0.23 ± 0.01	12
SW	Tembec T1	22.02 ± 0.04	0.00 ± 0.00	4
HW	Tembec T1	10.28 ± 0.15	2.14 ± 0.01	8
SW	Tembec T1	21.56 ± 0.41	0.23 ± 0.01	12
Scheme (2)	Culture	[Sugar] _{initial} (g/L)	[Sugar] _{residual} (g/L)	$t([EtOH]_{max})$ (h)
SW	Y-1528	21.37 ± 0.03	0.00 ± 0.00	4
SW	Y-1528	20.99 ± 0.06	0.45 ± 0.04	12
HW	Y-1528	10.22 ± 0.18	0.23 ± 0.02	12
SW	Tembec T1	22.07 ± 0.09	0.00 ± 0.00	4
SW	Tembec T1	21.29 ± 0.31	0.40 ± 0.01	12
HW	Tembec T1	10.64 ± 0.13	0.36 ± 0.09	12
Scheme (3)	Culture	[Sugar] _{initial} (g/L)	[Sugar] _{residual} (g/L)	$t([EtOH]_{max})(h)$
HW	Y-1528	11.54 ± 0.77	0.00 ± 0.00	8
HW	Y-1528	10.40 ± 0.14	6.91 ± 0.02	12
SW	Y-1528	20.76 ± 0.51	15.96 ± 0.05	12
HW	Tembec T1	11.60 ± 0.05	0.00 ± 0.00	12
HW	Tembec T1	10.68 ± 0.18	5.05 ± 0.16	12
SW	Tembec T1	22.01 ± 0.17	4.52 ± 0.73	12

Table 4.1. Initial [hexose sugar] and residual [hexose sugar] at t([EtOH]_{max}) during cell recycle batch fermentations of softwood (SW) and hardwood (HW) spent sulfite liquor (SSL) by *Saccharomyces cerevisiae* (*S. cerevisiae*) strains Y-1528 and Tembec T1. Range is indicated.

2, and 67% from the SW substrate in Cycle 3 (Table 4.2). Ethanol productivity matched sugar consumption trends. Interestingly, glycerol production data showed that in response to cell recycle, Y-1528 generated increasing concentrations of this solute, from a maximum of 0.04 g/g (product per unit substrate) in Cycle 1, to 0.08 g/g and 0.10 g/g in Cycles 2 and 3, respectively (Table 4.2).

Sugar consumption data from Scheme (1) showed that Tembec T1, in a manner almost identical to Y-1528, exhausted all of the SW-derived hexose sugars in 4 h in Cycle 1, all of the HW-derived hexose sugars in 24 h in Cycle 2, and all of the SWderived hexose sugars in 12 h in Cycle 3 (Table 4.1). Mannose, glucose, and galactose consumption rates were negatively impacted, with progressive severity, by the switch to a HW substrate in Cycle 2 (data not shown). A partial recovery was witnessed with the re-introduction of SW SSL in Cycle 3, leading to glucose and mannose exhaustion in 8 h, and galactose exhaustion in 12.

Ethanol production data from Scheme (1) showed that Tembec T1 generated 67% of theoretical yield from both the SW and HW substrates in Cycles 1 and 2, and 62% from the SW substrate in Cycle 3 (Table 4.2). Ethanol productivity again matched sugar consumption trends. Glycerol production data showed that Tembec T1, like Y-1528, generated increasing concentrations of this compound, from a maximum of 0.05 g/g in Cycle 1, to 0.08 g/g and 0.10 g/g in Cycles 2 and 3, respectively (Table 4.2).

Sugar consumption data from Scheme (2) showed that Y-1528 and Tembec T1 exhausted all of the SW-derived hexose sugars in 4 h in Cycle 1, all of the SW-derived hexose sugars in 12 h in Cycle 2, and all of the HW-derived hexose sugars in 8–12 h in Cycle 3 (Table 4.1). The rate of galactose consumption slowed considerably from Cycle 1 to Cycle 2, with complete exhaustion of the sugar taking in excess of 12 h versus 2 h prior to cell recycle (data not shown). Glucose and mannose consumption were likewise affected by the second iteration of SW SSL substrate, though not to the same extent as galactose depletion (data not shown).

Ethanol production data from Scheme (2) showed that Y-1528 generated 71% of theoretical yield from the SW substrate in Cycle 1, 64% from the SW substrate in Cycle 2, and 71% from the HW substrate in Cycle 3 (Table 4.2). Tembec T1 generated 67% of theoretical yield from the SW substrate in Cycle 1, 71% from the SW substrate in Cycle 2, and 70% from the HW substrate in Cycle 3 (Table 4.2). Ethanol productivity again corresponded to sugar consumption trends. Glycerol production data showed

Scheme (1)	Culture	EtOH $Y_{P/S}$ (g/g)	EtOH $Y_{\%T}$ (%)	Glycerol $Y_{P/S}(g/g)$
SW	Y-1528	0.37±0.01	73±1	0.04 ± 0.01
HW	Y-1528	0.32 ± 0.01	63 ± 1	0.08 ± 0.01
SW	Y-1528	0.34 ± 0.01	67 ± 1	0.10 ± 0.01
SW	Tembec T1	0.34 ± 0.01	67 ± 1	0.05 ± 0.01
HW	Tembec T1	0.34 ± 0.01	67 ± 1	0.08 ± 0.01
SW	Tembec T1	0.31 ± 0.01	62 ± 2	0.10 ± 0.01
Scheme (2)	Culture	EtOH $Y_{P/S}$ (g/g)	EtOH $Y_{\%T}$ (%)	Glycerol Y _{P/S} (g/g)
SW	Y-1528	0.36±0.01	71±1	0.05 ± 0.01
SW	Y-1528	0.33 ± 0.01	64 ± 1	0.06 ± 0.01
HW	Y-1528	0.36 ± 0.01	71 ± 1	0.11 ± 0.01
SW	Tembec T1	0.34 ± 0.01	67 ± 1	0.04 ± 0.01
SW	Tembec T1	0.36 ± 0.02	71 ± 4	0.06 ± 0.01
HW	Tembec T1	0.36 ± 0.01	70±1	0.08 ± 0.01
Scheme (3)	Culture	EtOH $Y_{P/S}$ (g/g)	EtOH $Y_{\%T}$ (%)	Glycerol Y _{P/S} (g/g)
HW	Y-1528	0.31 ± 0.01	62 ± 1	0.08 ± 0.01
HW	Y-1528	0.14 ± 0.01	28 ± 1	0.04 ± 0.01
SW	Y-1528	0.03 ± 0.01	5 ± 1	0.07 ± 0.01
HW	Tembec T1	0.31 ± 0.01	61 ± 1	0.08 ± 0.01
HW	Tembec T1	0.14 ± 0.01	28 ± 3	0.08 ± 0.01
SW	Tembec T1	0.22 ± 0.02	43 ± 3	0.08 ± 0.01

Table 4.2. Maximum ethanol yields (product per unit substrate $[Y_{P/S}]$ and percent theoretical $[Y_{\%T}]$) and glycerol yields (product per unit substrate $[Y_{P/S}]$) during cell recycle batch fermentations of softwood (SW) and hardwood (HW) spent sulfite liquor (SSL) by *Saccharomyces cerevisiae* (*S. cerevisiae*) strains Y-1528 and Tembec T1. Range is indicated.

that Y-1528 generated significantly more of this solute in Cycle 3 (a maximum of 0.11 g/g) than in Cycles 1 and 2 (maxima of 0.05 g/g and 0.06 g/g, respectively) (Table 4.2). Tembec T1 generated progressively more glycerol with each cycle (maxima of 0.04 g/g, 0.06 g/g, and 0.08 g/g in Cycles 1, 2, and 3, respectively) (Table 4.2).

Sugar consumption data from Scheme (3) showed that Y-1528 exhausted all of the HW-derived hexose sugars in 4 h in Cycle 1, and then failed to consume all of the HW- and SW-derived hexose sugars over the monitored duration of 46 h in Cycles 2 and 3 (1.9 g/L and 2.9 g/L residual sugar, respectively) (Table 4.1). As expected, the rate of galactose depletion was most seriously affected, transitioning from rapid exhaustion in 2 h in Cycle 1 to no observed consumption in Cycle 2 (1.6 g/L present at the onset and conclusion of incubation), and only fractional consumption in the first 4 h of Cycle 3 (2.2 g/L residual sugar) (data not shown). The rates of glucose and mannose depletion were also significantly retarded.

Ethanol production data from Scheme (3) showed that Y-1528 generated 62% of theoretical yield from the HW substrate in Cycle 1, 28% from the HW substrate in Cycle 2, and 5% from the SW substrate in Cycle 3 (Table 4.2). Glycerol production data showed maxima of 0.08 g/g, 0.04 g/g, and 0.07 g/g in Cycles 1, 2, and 3, respectively (Table 4.2).

Sugar consumption data from Scheme (3) demonstrated that Tembec T1 exhausted all of the HW-derived hexose sugars in just over 6 h in Cycle 1, failed to consume all of the HW-derived hexose sugars over the monitored duration of 46 h in Cycle 2 (1.3 g/L residual sugar), and depleted all of the SW-derived hexose sugars in 24 h in Cycle 3 (Table 4.1). Similarly, the rate of galactose consumption was significantly reduced from Cycle 1 to Cycle 2, with residual sugar present after 46 h (1.0 g/L) versus depletion in 2 h prior to cell recycle (data not shown). Galactose was fully consumed in 24 h in Cycle 3, with very little uptake in the first 12 h, likely due to catabolite repression. The rates of glucose and mannose depletion were likewise affected by the second iteration of HW substrate, with exhaustion taking four times as long, while the introduction of SW SSL in Cycle 3 relieved the stressed metabolism to a great extent, after taking into account the amount of available fermentable sugar (data not shown).

Ethanol production data from Scheme (3) showed that Tembec T1 generated 61% of theoretical yield from the HW substrate in Cycle 1, 28% from the HW substrate

in Cycle 2, and 43% from the SW substrate in Cycle 3 (Table 4.2). Glycerol production data showed the generation of equal concentrations of this solute (maxima of 0.08 g/g) in all three cycles (Table 4.2).

The decrease in Y-1528's galactose consumption rate in Scheme (1) was somewhat expected, as galactose transport and metabolism is especially sensitive to chemically induced perturbations, owing to relatively high complexity and inadequate redundancy in the pathway [9,31,34,35]. The switch to a HW substrate in Cycle 2, with the attendant change in the identity, quantity, and ratio of inhibitory compounds seemed to cause catabolite repression of galactose metabolism, and the subsequent switch back to a SW substrate (Cycle 3) slightly relieved this shift, leading to glucose and mannose exhaustion in 8 h and galactose exhaustion in 12. The overall pattern of hexose sugar consumption, taking into account that the HW SSL contained approximately half the fermentable sugar, indicates that an inhibitory compound(s) is specifically abundant in processed HW (furfural, acetic acid, and syringyl lignin degradation products). In contrast, sugar consumption seems to be less affected by the cycling of HW and SW SSL. Since furfural and acetic acid (at similar or greater concentrations) had little independent effect upon sugar consumption rates in the inhibitor-supplemented fermentations, syringyl lignin-derived degradation products and synergistic toxicity from multiple inhibitors may be influencing the slowing of metabolism associated between Cycle 1 and Cycle 2. Low molecular weight phenolic derivatives of HW lignin are particularly toxic to microorganisms, especially S. cerevisiae [1,7,21], acting via biological membrane disruption and causing subsequent physiological disturbances [12]. Additionally, syringyl lignin residues exhibit greater thermolability than gualacyl units, enhancing the potential toxicity of degraded HW, regardless of attendant functional groups [15]. Synergistic toxicity resulting from the interaction of predominant inhibitory components of HW SSL, which may further slow metabolism from Cycle 1 to Cycle 2, has been demonstrated with furfural and acetic acid [39] and with furfural and syringyl lignin degradation products [32]. Nevertheless, there was a net loss of performance from Cycle 1 to Cycle 3, suggesting that Y-1528 did not adapt well to rapidly changing lignocellulosic substrates.

The achieved ethanol yields in Scheme (1) are consistent with the observed impact of the particular sequence of substrates on sugar consumption. Since furfural alone had no detrimental effect on ethanol yield in inhibitor-supplemented fermentations catalyzed by Y-1528, and acetic acid itself was observed to reduce ethanol yield in inhibitor-supplemented fermentations at the concentration found in HW SSL (15 g/L), it is likely that it may have contributed to the yield depression. The slight yield increase from Cycle 2 to Cycle 3 illustrated that a less toxic environment (*i.e.*, SW SSL) permitted Y-1528 to recover some of its ethanologenic capacity. Increased glycerol synthesis, in concert with reduced ethanol production, represents a physiological reaction to an unbalanced intracellular redox state, which can be traced to altered metabolism [6].

With Tembec T1, the net loss of performance from Cycle 1 to Cycle 3 of Scheme (1) was unexpected, as the strain was initially isolated and propagated from a pulp and paper mill SSL stream. However, in light of this reduced performance, it is important to note that Tembec T1 (and Y-1528) preserved a capacity for sugar consumption in a rapidly changing toxic substrate environment. The achieved ethanol yields in Scheme (1) demonstrate subtle differences between this strain and Y-1528, with evidence of a lesser impact in the transition to HW SSL in Cycle 2 and a loss of ethanologenic capacity in the subsequent switch to SW SSL in Cycle 3.

The Scheme (2) transition to a HW substrate in Cycle 3 illustrated a similar phenomenon to that observed in Scheme (1). The apparent absence of adaptation to the SW substrate from Cycle 1 to Cycle 2 suggests either the transgression of inhibitor exposure limits efficacy and results in a consequential loss of tolerance in both strains, or cell death. However, the dissimilar effect of cell recycling on individual sugar consumption rates implies inhibitor-mediated metabolic toxicity as opposed to the selection of resistant cells in the overall yeast population.

The ethanol yields achieved by Y-1528 in Scheme (2) were surprising, as it is evident that the second iteration of SW substrate had a more dramatic impact than the subsequent transition to HW SSL in Cycle 3, in which a recovery to the yield obtained prior to cell recycle was effected. The 7% loss in ethanol yield from Cycle 1 to Cycle 2 confirmed the trend observed in Scheme (2) sugar consumption patterns, wherein inhibitory compound exposure limits were purported to have been exceeded through two consecutive incubations in SW SSL, resulting in hampered metabolism. However, the switch to a HW substrate in Cycle 3, and the concomitant improvement in ethanologenesis contrasts with the accumulated observations of detrimental consequences to fermentation in this particular lignocellulosic mixture. The yields achieved by Tembec T1 indicate a mild improvement in ethanologenic capacity via cell

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recycle, demonstrating a unique adaptive response despite retarded sugar metabolism and increased glycerol production.

In Scheme (3), adaptation to the HW substrate was absent in Cycle 2, reinforcing the trend observed in Scheme (2), in which Y-1528 did not adapt to the SW substrate in the switch from Cycle 1 to Cycle 2. Again, the disparate effect upon individual sugar consumption rates suggests specific, inhibitor-mediated hindrance of sugar metabolism. The transition to a SW substrate in Cycle 3 was accompanied by a marginal recovery in sugar consumption rates, as demonstrated by glucose and mannose exhaustion at rates similar to those observed in Cycle 2, despite significantly higher fermentable sugar content. This recovery did not extend to the rate of galactose depletion, reflecting the sensitivity of this metabolic pathway to chemical perturbation.

The achieved ethanol yields in Scheme (3) illustrate a remarkable loss of ethanologenic capacity in Y-1528 upon sustained exposure to inhibitory compounds in SSL, reinforced by the strain's inability to fully consume all of the fermentable sugars over the course of incubation following cell recycle, and notwithstanding decreasing, then increasing glycerol production. As the HW SSL substrate has been shown to be particularly toxic to both yeast strains, a 34% decrease in ethanol yield from Cycle 1 to Cycle 2 was not unexpected, but the additional 23% yield loss from Cycle 2 to Cycle 3 was somewhat intriguing, as the marginal increase in sugar consumption rate accompanying the transition to a SW substrate was thought to indicate metabolic recovery.

The ethanol yields achieved by Tembec T1 in Scheme (3) mirrored those of Y-1528 in the first two cycles, and then substantially diverged in the last cycle. This ethanologenic response to the particular sequence of substrates in Scheme (3) was more consistent with the trends observed in sugar consumption for both strains, in which the HW SSL exerted a stronger toxic effect than the SW SSL on metabolic efficacy. Predictably, the lowest ethanol yield in this instance corresponded to the only incidence of incomplete sugar exhaustion, the second iteration of HW substrate.

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Chapter 5 - Molecular characterization of UDP-galactose 4-epimerase protein and associated genes of the Leloir pathway in a unique ethanologenic yeast

A version of this chapter will be submitted for publication. Keating JD, Berlin A, Canam T, Mansfield SD (2007) Molecular characterization of UDP-galactose 4-epimerase protein and associated genes of the Leloir pathway in a unique ethanologenic yeast. 46 pp.

Introduction

The exploitation of petroleum reserves has enabled modern industrialization, consumer product diversification, and global mobility [48]. However, depletion of hydrocarbon sources, in conjunction with substantial increases in oil consumption rates, is presenting a significant challenge to current raw material and energy supply [30,48]. Furthermore, the sporadic geographic location of petroleum deposits complicates situations of economic dependency and the forecasting of future energy provisions [48]. Above all else, the petrochemical carbon cycle is unbalanced: fossil oil is formed over millions of years, while its depletion is on the scale of decades. The rapid transformation of encapsulated solid and liquid carbon to gaseous (atmospheric) forms is leading to global climate change [24,48].

In contrast, the biomass carbon cycle nearly balances the formation and depletion processes. Renewable biomass, of which over 90% of natural production is in the form of lignocellulose, represents an abundant, inexpensive, and locally available feedstock for conversion to carbonaceous fuel [17,30,48]. However, in some cases the fermentable feedstock (*i.e.*, softwood) contains the recalcitrant hexose sugar galactose in low quantities [51], which has negligible effect on ethanol yield during fermentation, but a considerable impact on productivity in industrially typical fed-batch and continuous biomass-to-ethanol processes. Thus, a microorganism able to ferment galactose with equal (or greater) efficiency to other hexose sugars is desired.

The discovery of such a yeast strain, capable also of performing exceptionally well in lignocellulose-derived, inhibitor-rich substrates, has been documented [26,27,28]. However, during mixed sugar fermentation studies, this strain (*S. cerevisiae* Y-1528) displayed unusual sugar consumption patterns consistent with a modified galactose metabolism pathway [26]. Galactose metabolism in type strains of *S. cerevisiae* is governed by a specific sugar transport enzyme, and subsequently the pre-glycolytic Leloir pathway [3,35]. β -D-galactose is first transported into the cell by galactose permease (GAL2) and epimerized to α -D-galactose by galactose mutarotase (bifunctional GAL10). α -D-galactose is then phosphorylated by galactokinase (GAL1), yielding galactose-1-phosphate. Galactose-1-phosphate is converted to UDP-galactose by galactose to galactose-1-phosphate, producing UDP-galactose and glucose-1-

phosphate. Fulfilling the cycle, UDP-galactose is epimerized to UDP-glucose by UDP-glactose 4-epimerase (bifunctional GAL10) [20,32,34].

GAL10 has been implicated in the unusual galactose metabolism exhibited by the Y-1528 strain during mixed sugar fermentation [26]. GAL10 from a conventional strain of *S. cerevisiae* has been purified and analyzed [12,45], presenting an opportunity to ascertain and study potential differences in this enzyme in Y-1528 with a yeast displaying traditional fermentation profiles. Indeed, in normal *S. cerevisiae* strains provided with galactose, GAL10 is expressed in clear excess of metabolic requirements, facilitating its isolation [49]. Further consideration of the enhanced fermentation activity shown by Y-1528 has also provoked interest in the associated genes encoding GAL1, GAL2, and GAL7. These genes, along with *GAL10*, have been fully sequenced in type strains of *S. cerevisiae* [9,25] and thus offer a comparative basis for a molecular investigation of the structural galactose metabolism genes in Y-1528.

The objectives of this study were to isolate and characterize the putatively unique *GAL10* gene in *S. cerevisiae* Y-1528.

Materials and Methods

Yeast strains and culture media

SSL-adapted *S. cerevisiae* T1 was obtained from Tembec Limited (Témiscaming, QC, Canada). *S. cerevisiae* Y-1528 was obtained from the USDA Agricultural Research Service (Peoria, IL, USA). *S. cerevisiae* BY4742 was obtained from the Wine Research Centre at the University of British Columbia (Vancouver, BC, Canada). All strains were maintained on YPG solid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 18 g/L agar), stored at 4°C, and transferred to fresh plates on a bimonthly basis. Cells were grown to high cell density in foam-plugged 500 mL Erlenmeyer flasks containing YP-Gal or YPG liquid medium (10 g/L yeast extract, 10 g/L peptone, and 30 g/L galactose or glucose) in an orbital shaker for 3 days at 30°C and 225 rpm, with transfer of cells to fresh medium at 24 and 48 h.

Enzymes

UDP-galactose 4-epimerase (specific activity ≥ 10 units/mg) was obtained from Calbiochem-Novabiochem, San Diego, CA. Lyophilized enzyme was re-suspended in 100 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.0.

Preparation of crude cellular protein extract

High cell density cultures were harvested in the exponential growth phase (t = 3)h), timed to coincide with the active depletion of galactose in Y-1528-catalyzed single sugar fermentations [26], and then centrifuged (750 g, 4° C) to yield supernatant and cell pellets. If required, the supernatant was subject to an extracellular enzyme assay (detailed below); otherwise it was not characterized in depth. Cell pellets were washed three times with sterile deionized water and vortexed to create a thick cell paste. A minimal amount of ice-cold deionized water was added to allow decanting. All the following steps were conducted in a 4°C cold room. The cell paste was transferred to a syringe and then dispersed drop-wise into sufficient liquid nitrogen to permit submersion and immediate freezing. Frozen cell aggregations (strings and spherical clumps) were placed in a stainless steel Waring blender and disintegrated (fractured) via three successive two-minute high-speed bursts, yielding a fine frozen yeast powder. The yeast powder was then added to twice the original cell paste volume of filter-sterilized, ice-cold protease inhibition storage buffer (20 mM Tris CI, pH 7.5, 10% v/v glycerol, 100 mM KCI, 0.1 mM disodium EDTA, 0.01 mM EGTA, 1 mM DTT, 1 mM PMSF, and 0.1 mM NaHSO₃), gently mixed, and centrifuged (5000 g, 4° C). The supernatant, comprising a crude cellular protein extract, was stored at 4°C for a short period prior to further manipulation.

Extracellular enzyme assay and chemical analysis

Galactose-grown Y-1528 culture supernatant was incubated for 3 hours at 30°C and 225 rpm with the addition of an equal volume of 6% w/v filter-sterilized galactose (yielding a final concentration of 3% w/v galactose) or sterile deionized water (mimicking basal sugar levels from fermentation). Samples were aseptically collected for sugar analysis at the start and end of incubation.

High performance liquid chromatography (HPLC) on a DX-600 BioLC chromatograph (Dionex, Sunnyvale, CA, USA) was used for sugar determination. Separation was achieved on 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 fucose as internal standard, and then filtered through 0.45 μ m PVDF (polyvinylidene fluoride)

filters prior to injection (20 μ L). The column was eluted with deionized water at a flow rate of 1.0 mL/min.

Protein purification

The crude cellular protein extracts from galactose-grown BY4742, Tembec T1, and Y-1528 were subject to quantification of protein concentration, epimerase activity, constituent molecular mass distribution, and constituent isoelectric point distribution, as were sample mixtures in all subsequent stages of purification.

Protein concentration was assessed spectrophotometrically (in triplicate) via UV light absorption at 280 nm, and calculated by correlation to bovine serum albumin (BSA) calibration curves. Sample buffer was used to establish background absorption and set zero absorbance baselines. Values were averaged, and if necessary, corrected for dilution.

Epimerase activity was assessed via chromatographic measurement of substrate depletion and product formation following all incubation assays. Duplicate 100 μ L aliquots of sample were mixed with 61.3 μ L of 10 mg/mL UDP-galactose (solvated in 20 mM pH 7.2 Tris–HCl buffer), 60 μ L of 100 mg/mL NAD⁺ (solvated in 20 mM pH 7.2 Tris–HCl buffer), and sufficient volumes of 100 mM pH 8.6 NaOH glycine buffer to total 1 mL. These mixtures, along with negative controls (deficient in protein-containing sample), positive controls (substituting 200 μ L of 0.2 μ g/mL commercial UDP-galactose 4-epimerase for protein-containing sample), and standards (deficient in protein-containing sample but supplemented with 61.3 μ L of 10 mg/mL UDP-glucose), were subsequently incubated for 60 min at 27°C [39]. Reactions were terminated with the addition of 100 μ L of 1 M HCI [16].

Nucleotide sugar determination was accomplished through high performance liquid chromatography on a Summit LC chromatograph (Dionex). Separation was achieved with a Zorbax Rx-C8 reversed-phase analytical column (Agilent Technologies, Palo Alto, CA), and detection was facilitated with UV absorbance at 264 nm [33]. Standards and experimental samples were diluted 1:25 in deionized water, and then filtered through 0.45 μ m PVDF filters prior to injection (20 μ L). The column was eluted with a gradient of 50 mM pH 7.0 potassium phosphate buffer (supplemented with 2.5 mM tetrabutylammonium hydrogen sulfate) and 100 mM pH 7.0 potassium phosphate buffer mixed with acetonitrile (1:1, supplemented with 2.5 mM tetrabutylammonium

hydrogen sulfate) at a flow rate of 1.0 mL/min. The elution consisted of 98% phosphate buffer and 2% acetonitrile/phosphate buffer graded to 65% phosphate buffer and 35% acetonitrile/phosphate buffer over 29 min, followed by reverse grading to 98% phosphate buffer and 2% acetonitrile/phosphate buffer between 29 and 30 min. A 15-min wash with 98% phosphate buffer and 2% acetonitrile/phosphate buffer concluded each 45-min run.

Constituent molecular mass distribution was determined via denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Sample protein concentration was adjusted to 0.5, 1.0, and 2.0 mg/mL prior to sample buffer treatment and gel loading. Proteins were resolved in pre-cast Criterion XT Bis-Tris 4-12% gradient polyacrylamide gels in 1× XT MOPS running buffer (Bio-Rad, Mississauga, ON, Canada). Proteins were electrophoresed alongside Precision Plus protein standards (Bio-Rad) at 200 V for 55 min. Following electrophoresis, the separated proteins were stained with SYPRO Ruby solution (Bio-Rad) and visualized under UV transillumination.

Constituent isoelectric point distribution was determined via isoelectric focusing (IEF). Sample protein concentration was adjusted to a range of 1 to 8 mg/mL prior to centrifugation (16,250 *g*, 4°C) and gel loading. Proteins were resolved in pre-cast PhastGel IEF 3-9 homogeneous polyacrylamide gels containing 2-6% carrier ampholyte (Amersham Biosciences, Baie d'Urfé, QC, Canada). Proteins were electrophoresed alongside IEF standards (Bio-Rad) under pre-programmed temperature and voltage conditions in a Pharmacia LKB PhastSystem (Amersham Biosciences). Following electrophoresis, resolved proteins were fixed in 40% methanol, 10% trichloroacetic acid, stained with SYPRO Ruby solution (Bio-Rad), and visualized by UV transillumination.

Progressive centrifugal filtration of the crude cellular protein extract was conducted at 4°C and comprised macro-clarification through 8 μm glass microfibre filters, micro-clarification through 0.22 μm PVDF filters, and size exclusion through 50 kDa cut-off Amicon Ultra-15 regenerated cellulose membranes (Millipore, Mississauga, ON, Canada). This combined process facilitated the removal of most low molecular mass proteins and buffer solutes, and concurrently concentrated proteins with mass greater than 50 kDa.

Gel filtration chromatography (GFC) of the > 50 kDa cellular protein cohort was conducted with a custom-assembled BioLogic DuoFlow/Maximizer fast protein liquid chromatograph (Bio-Rad), and employed a HiLoad 16/60 Superdex 75 dextran-agarose preparative column (Amersham Biosciences) to effect separation. Detection was achieved with UV absorbance at 280 nm. Standards consisted of cytochrome c (12 kDa), lysozyme (14 kDa), cellobiohydrolase I (56 kDa), and bovine serum albumin (66 kDa), and along with experimental samples were diluted 1:4 in GFC buffer (20 mM Tris·CI, 1 mM disodium EDTA, 5 mM DTT, pH 7.4), then filtered through 0.45 μ m PVDF filters prior to injection (1.2 mL per run). The column was eluted isocratically with GFC buffer at a flow rate of 1.0 mL/min. Fraction collection (at 2.5 mL/tube) was initiated at 40 min and concluded at 75 min, representing all size fractions > 70 kDa. Fractions showing the bulk of protein (> 70 kDa) were collected twice in consecutive runs and pooled. The pooled (500 μ L) fractions were centrifugally concentrated in 10 kDa cut-off Amicon Ultrafree-MC microconcentrator tubes (Millipore) to yield 100 μ L for assays.

Chromatofocusing (CF) of epimerase-active pooled GFC fractions was conducted with the aforementioned BioLogic DuoFlow/Maximizer FPLC, and employed a Mono P 5/200 GL polystyrene/divinyl benzene preparative column with mixed quaternary and tertiary amine ion exchangers (Amersham Biosciences) to achieve separation. Detection was achieved with UV absorbance at 280 nm and online pH measurement. Experimental samples were washed three times into starting buffer (25 mM Bis-Tris, adjusted to pH 7.1 with a saturated solution of iminodiacetic acid) using 10 kDa cut-off centrifugal microconcentrator tubes, and then diluted to 2 mg/mL protein with the same buffer prior to injection (0.5 mL per run). The column was equilibrated with the starting buffer, and then eluted with Polybuffer 74 (Amersham Biosciences) and 2 M NaCl. Prior to use, the Polybuffer 74 was diluted 1:10 in deionized water and adjusted to pH 4.0 with a saturated solution of iminodiacetic acid, or diluted 1:7 in deionized water and adjusted to pH 3.0 with a saturated solution of iminodiacetic acid. Equilibration occurred with 100% starting buffer at a flow rate of 1.0 mL/min for 5 min. and was followed by sample injection. The elution consisted of 100% Polybuffer 74 at 1.0 mL/min for 60 min, then 100% 2 M NaCl at 0.5 mL/min for 40 min. Column reequilibration occurred with 100% starting buffer at 1.0 mL/min for 40 min. Fraction collection in glass tubes was initiated upon sample injection and concluded at the end of the run, each tube collecting 1.0 mL. Fractions showing protein in the pH range 5.5 to 6.0 were to be selected twice in consecutive runs and pooled if necessary, with 500 μ L of each fraction centrifugally concentrated in 10 kDa cut-off microconcentrator tubes to yield 100 μ L for assays.

Anion exchange chromatography (AEC) of epimerase-active pooled GFC fractions was again conducted with the aforementioned BioLogic DuoFlow/Maximizer FPLC, and employed either a Mono P 5/200 GL column or HiPrep 16/10 Q Sepharose FF preparative column with quaternary ammonium anion exchangers (Amersham Biosciences) to effect separation. Detection was achieved with UV absorbance at 280 nm and online pH measurement. Experimental samples were washed three times into starting buffer (20 mM Tris CI, pH 7.1) using 10 kDa cut-off centrifugal microconcentrator tubes, and then diluted to 3 mg/mL (Mono P column) or 1 mg/mL protein (Q Sepharose column) with the same buffer prior to injection (0.5 mL per run). The columns were equilibrated with the starting buffer, and then eluted with starting buffer, 20 mM Tris-CI (pH 7.1) mixed with 1 or 2 M NaCI (buffer B), and 1 or 2 M NaCI. Mono P (or Q Sepharose) column equilibration occurred with 100% starting buffer at a flow rate of 1.0 (or 5.0) mL/min for 4 min, and was followed by sample injection. The elution consisted of 100% starting buffer at 1.0 (or 5.0) mL/min for 8 min, 100% starting buffer graded to 100% buffer B at 1.0 (or 5.0) mL/min over 40 min, 100% buffer B at 1.0 (or 5.0) mL/min for 8 min, 100% starting buffer at 1.0 (or 5.0) mL/min for 8 min, then 100% NaCl at 0.5 (or 2.5) mL/min for 16 min. Column re-equilibration occurred with 100% starting buffer at 1.0 (or 5.0) mL/min for 8 min. Fraction collection in glass tubes was initiated upon sample injection and concluded at the end of the run, where each tube collected 1.0 (Mono P) or 2.0 mL (Q Sepharose). Fractions showing protein were to be selected twice in consecutive runs and pooled if necessary, with 500 µL of each fraction centrifugally concentrated in 10 kDa cut-off microconcentrator tubes to yield 100 μ L for assays.

Gene amplification, cloning, and sequencing

Genomic DNA from BY4742, Tembec T1, and Y-1528 was isolated using a FastDNA Kit (Qbiogene, Montréal, QC, Canada), and involved harvest of cell pellets from 1 mL YP-Gal culture in the exponential growth phase. Cells were re-suspended in 100 μ L sterile deionized water, and the suspensions supplemented with 1 mL cell lysis solution (CLS-Y, Qbiogene). Suspensions were then added to Lysing Matrix D (Qbiogene) and the cells contained therein disrupted at setting 5.0 (5 m/s) for 30 s using the FastPrep Instrument (Qbiogene). Tubes containing the disrupted cells were centrifuged for 5 min (14,000 *g*, 4°C), yielding 600 μ L supernatant, to which 600 μ L Binding Matrix (Qbiogene) was added. These solutions were gently mixed by inversion

and allowed to stand for 5 min at room temperature. Solutions were then centrifuged for 1 min (14,000 g, 4°C) and the supernatants discarded. Pellets were re-suspended in 500 μ L salt-ethanol wash solution (SEWS-M, Qbiogene) and centrifuged for 1 min (14,000 g, 4°C). Supernatants were discarded and the pellets centrifuged again for 1 min (14,000 g, 4°C) to remove all liquid. Pellets were re-suspended in 100 μ L DNA elution solution (DES, Qbiogene), incubated for 3 min at room temperature, and then centrifuged for 1 min (14,000 g, 4°C). Finally, supernatants were transferred to fresh tubes. DNA concentration and purity was measured in triplicate by 260 nm UV absorbance and 260/280 nm UV absorbance ratio, respectively, on a GeneQuant *pro* spectrophotometer (Biochrom, Cambridge, UK). DNA was stored at -20°C.

Yeast RNA was isolated in accordance with the following protocol. A cell pellet was harvested from 1 mL YP-Gal culture in the exponential growth phase, immediately supplemented with 1 mL TRIZOL (Invitrogen, Burlington, ON, Canada), and incubated for 5 min at room temperature. The solution was then vortexed gently, supplemented with 200 μ L chloroform, and shaken vigorously for 15 s. Following 3 min incubation at room temperature, the mixture was centrifuged for 15 min (12,000 *g*, 4°C) and the resulting aqueous phase transferred to a fresh tube. 2-propanol (500 μ L) was added and the mixture incubated for 10 min at room temperature. The tube was then centrifuged for 10 min (12,000 *g*, 4°C), the supernatant discarded, and 1 mL 75% ethanol added. The tube was again centrifuged for 30 min, re-suspended in RNase-free sterile deionized water, and incubated for 10 min at 60°C. RNA concentration and purity was measured in triplicate by 260 nm UV absorbance and 260/280 nm UV absorbance ratio, respectively, on a GeneQuant *pro* spectrophotometer. RNA was stored at -80°C.

RNA from Y-1528 was rendered DNA-free by the following protocol. RNA (10 μ g) was supplemented with 5 μ L TURBO 10× buffer (Ambion, Streetsville, ON, Canada), diluted to 44 μ L with diethylpyrocarbonate-treated water, and treated with 1 μ L TURBO DNase (Ambion). This solution was incubated for 30 min at 37°C, subsequently supplemented with 5 μ L DNase Inactivation Reagent (Ambion), vortexed briefly, and allowed to stand for 2 min at room temperature. The mixture was then centrifuged for 2 min (10,000 *g*, 4°C) and the supernatant transferred to a fresh tube. RNA concentration

and purity was measured in triplicate by 260 nm UV absorbance and 260/280 nm UV absorbance ratio, respectively, on a GeneQuant *pro* spectrophotometer. RNA was stored at -80°C.

The 3' RACE reaction was performed on 1 μ g DNA-free RNA by adding the following reagents from the FirstChoice RLM-RACE Kit (Ambion): 3 μ L nuclease-free water, 4 μ L dNTP mix, 2 μ L 3' RACE adapter, 2 μ L 10× RT buffer, 1 μ L RNase inhibitor, and 1 μ L M-MLV reverse transcriptase. This mixture was shaken gently, centrifuged briefly, and then incubated for 1 h at 42°C. The resulting DNA was stored at -20°C for subsequent use in 3' RACE-PCR.

PCR primers were based on genomic DNA sequences and followed accepted rules regarding robust design (*e.g.*, the need to avoid primer self-complementarity). The gene sequences for galactokinase (*GAL1*, 1587 bp), galactose permease (*GAL2*, 1725 bp), galactose-1-phosphate uridyltransferase (*GAL7*, 1101 bp), and UDP-galactose 4-epimerase (*GAL10*, 2100 bp), as well as their respective upstream and downstream regions [9,25], were used to derive non-degenerate ssDNA oligonucleotide primers. The gene sequences for *GAL1*, *GAL2*, *GAL7*, and *GAL10*, in combination with BLAST sequence alignments [2,9,25], were used to derive degenerate primers. Additionally, amino acid codons and the *GAL10* sequence [9] were used to derive non-degenerate and degenerate primers from the 2-dimensional LC-MS/MS-sourced peptide fragment of GAL10. Primer sequences based on conserved regions of fungal rRNA genes (for putative molecular confirmation of species identity) were obtained from previous work [50]. Non-degenerate and degenerate galactose structural gene primers, as well as fungal rRNA gene primers, are listed in Table 5.1.

PCR amplification was achieved by combining 80 pmol dNTP mix (Amersham Biosciences), 1× PCR buffer (Amersham Biosciences), 8 pmol each of forward and reverse primers (Table 5.2), 50, 100, 200, 300, or 500 ng genomic DNA, 2.5 U Tag DNA polymerase (Amersham Biosciences), and (optionally) 12.5% v/v dimethylsulfoxide, in a total volume of 20 µL. The thermocycler program consisted of one cycle of 94°C for 3 min, 35 or 40 cycles of 94°C for 30 s, 40, 45, 47, 50, 52, 53, 55. 56, or 57°C for 40 s, and 72°C for 2 min, and one cycle of 72°C for 8 min. All PCR amplifications, along with negative and positive controls (the latter represented by BY4742 and Tembec T1 genomic DNA), were performed in duplicate.

Non-Degenerate Galactose Structural Gene Primers						
GAL1F	5'-ATGACTAAATCTCATTCAGAAGA-3'					
GAL1R	5'-TTATAATTCATATAGACAGCTGC-3'					
GAL2F	5'-ATGGCAGTTGAGGAGAACAA-3'					
GAL2R	5'-TTATTCTAGCATGGCCTTGT-3'					
GAL7F	5'-ATGACTGCTGAAGAATTTGA-3'					
GAL7R	5'-TTACAGTCTTTGTAGATAATGAA-3'					
GAL10F	5'-ATGACAGCTCAGTTACAAAG-3'					
GAL10R	5'-TCAGGAAAATCTGTAGACAA-3'					
UTRF	5'-TGGTGGTAATGCCATGTA-3'					
UTRB	5'-TACAGCAAATCTATGTGT-3'					
GAL10P1F	5'-GTAGGTGAATCTACACAA-3'					
GAL10P1R	5'-TTGTGTAGATTCACCTAC-3'					
Degenerate Galactose Structural (Gene Primers					
DGAL1F	5'-GTIATTITACCTGAGTTCAA-3'					
DGAL1R	5'-TCTTTTACCTTTTCTATGTTG-3'					
DGAL2F	5'-TACTGTACIAAITACGG-3'					
DGAL2R	5'-TTAGTTTCIGGAACIAAGAA-3'					
DGAL7F	5'-GAAGAATTTGATTTTC-3'					
DGAL7R	5'-CCATIGAITATGGGAAACT-3'					
DGAL10F1	5'-TGTTTTIGTIACIGGTGGTG-3'					
DGAL10R1	5'-GTAGTCCATTTCCATAAATC-3'					
DGAL10F2	5'-TTTGCIGITTTIAAIGCTGT-3'					
DGAL10R2	5'-TCAGGAAAATCTGTAGA-3'					
DGAL10P2F	5'-GTIGGIGAATCIACICAA-3'					
DGAL10P2R	5'-TTGIGTIGATTCICCIAC-3'					
DGAL10P3F	5'-GTIGGIGAGTCIACICAA-3'					
DGAL10P3R	5'-TTGIGTIGACTCICCIAC-3'					
DGAL10P4F	5'-GTIGGIGAATCIACICAG-3'					
DGAL10P4R	5'-CTGIGTIGATTCICCIAC-3'					
DGAL10P5F	5'-GTIGGIGAGTCIACICAG-3'					
DGAL10P5R	5'-CTGIGTIGACTCICCIAC-3'					
Fungal rRNA Gene Primers						
ITS4	5'- TCCTCCGCTTATTGATATGC-3'					
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'					

Table 5.1. PCR primers employed in GAL and rRNA gene amplification.

Table 5.2.	Forward	and	reverse	PCR	primer	pairs	employed	in	GAL	and	rRNA	gene
amplification	า.											

Target Gene(s)	Primer Pairs
GAL1	GAL1F + GAL1R
GAL2	GAL2F + GAL2R
GAL7	GAL7F + GAL7R
GAL10	GAL10F + GAL10R
GAL1	DGAL1F + DGAL1R
GAL2	DGAL2F + DGAL2R
GAL7	DGAL7F + DGAL7R
GAL10	DGAL10F1 + DGAL10R1
GAL10	DGAL10F1 + DGAL10R2
GAL10	DGAL10F2 + DGAL10R1
GAL10	DGAL10F2 + DGAL10R2
ITS1, 5.8S, ITS2, and 25S	ITS4 + ITS5
GAL10	UTRF + UTRB
GAL10	UTRF + GAL10P1R
GAL10	UTRB + GAL10P1F
GAL10	UTRF + DGAL10P2R
GAL10	UTRB + DGAL10P2F
GAL10	UTRF + DGAL10P3R
GAL10	UTRB + DGAL10P3F
GAL10	UTRF + DGAL10P4R
GAL10	UTRB + DGAL10P4F
GAL10	UTRF + DGAL10P5R
GAL10	UTRB + DGAL10P5F
GAL10	GAL10F + GAL10P1R
GAL10	GAL10R + GAL10P1F

.

PCR products were resolved in 0.8% agarose gels containing ethidium bromide (Bio-Rad) in 1× TAE running buffer. Amplicons were electrophoresed alongside 1 kb DNA mass ladders at 120 V for 20 min. Following electrophoresis, DNA bands were visualized under UV transillumination. Band sizes were estimated based on DNA mass ladder migration.

For direct sequencing, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada). The QIAquick Gel Extraction Kit (Qiagen) was employed to extract gel-embedded DNA for PCR amplification using amplicon bands as template. Cloning of PCR products into bacterial plasmids (in preparation for sequencing) was accomplished with the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA purification was achieved with the QIAprep Spin MiniPrep Kit (Qiagen). Sequencing of PCR products was performed on automated sequencers (Applied Biosystems, Foster City, CA, USA). Raw sequence data were analyzed via ClustalW and BLAST [2,8]. Translated sequence data were analyzed via BLAST, ProtParam, SignalP, and WoLF PSORT [2,4,14,22].

Protein sequencing

The crude cellular protein extracts from galactose-grown BY4742, galactosegrown Tembec T1, galactose-grown Y-1528, and glucose-grown Y-1528, as well as extracellular supernatant from galactose-grown Y-1528, were subject to progressive centrifugal filtration and 1-dimensional electrophoretic analysis (SDS-PAGE). Filtration was conducted at 4°C and comprised centrifugation for 15 min (3,000 *g*), collection of supernatant, and then size exclusion separation through 10 kDa cut-off microconcentrator tubes, removing most low molecular mass components and concentrating proteins with mass > 10 kDa. SDS-PAGE was performed as in the protein purification experiment, with various volumes of unfiltered (30 μ L) and filtered (15, 30 μ L) samples loaded into the 4-12% gradient polyacrylamide gel.

The band of interest in the specified lane containing galactose-grown Y-1528 sample, along with the equivalent spot (by molecular mass) in the lane containing glucose-grown Y-1528 sample, was excised from the gel, chemically isolated, subject to tryptic digestion, and then analyzed via 2-dimensional LC-MS/MS (University of Victoria – Genome British Columbia Proteomics Centre, Victoria, BC, Canada). Peptide fragment sequence matching was accomplished with Mascot and BLAST [2,37].

Results and Discussion

UDP-galactose 4-epimerase purification

The extracellular supernatant of galactose-grown Y-1528 culture showed no conversion of either basal or exogenous galactose to glucose over 3 hours of incubation (data not shown). The conditions for the assay, mimicking sugar fermentation, were limited to specified temperature, time, and agitation, and had no provision for exogenous enzyme co-factors, as the evolution of endogenous glucose in culture medium supplemented only with other hexose sugars occurred in a similar fermentation environment [26]. Therefore, the appearance of endogenous glucose, regardless of whether it might necessitate the extracellular presence of a series of enzymes constituting the Leloir pathway [20], or, hypothetically, a modified galactose metabolism pathway comprising a single exported enzyme (multifunctional or employing a unique galactose-to-glucose conversion mechanism), can be stated to not require exogenous co-factors. As such, it can be tentatively concluded from this experiment that the unique mechanism by which galactose is rapidly assimilated and extracellular glucose produced does not involve exported sugar metabolizing enzymes. This result supports the contention that yeasts, which are generally limited to the uptake and metabolism of simple, soluble nutrients, have no ecological or competitive basis for exporting valuable catabolic enzymes (in contrast to mycelial fungi, which often degrade extracellular biopolymers such as starch, hemicellulose, and/or cellulose to obtain carbon and energy).

The crude cellular protein extract from Y-1528, having undergone progressive centrifugal filtration, showed activity during epimerase assays (Fig. 5.1). The crude cellular protein extracts from BY4742 and Tembec T1, which were subject to the same growth and treatments, also showed activitý during epimerase assays (data not shown). Positive controls containing commercially acquired UDP-galactose 4-epimerase confirmed the suitability of assay conditions, negative controls showed the absence of catalytic reactions, and standards permitted detection of, and discrimination between substrate and product. Additionally, protein concentration measurement indicated the presence of significant amounts of protein following all stages of filtration (denoting low protein binding by the various membrane materials comprising the filters). SDS-PAGE evaluation showed proteins spanning a wide range of molecular masses after 8 and 0.22 μ m filtration and a lesser cohort of < 50 kDa proteins following 50 kDa filtration,



Fig. 5.1. UDP-galactose 4-epimerase activity in (A) 8 μ m filtered, (B) 0.22 μ m filtered, and (C) 50 kDa filtered cellular protein extracts of *S. cerevisiae* Y-1528, as measured by HPLC separation and 264 nm UV light absorbance detection of UDP-glucose product.

while IEF revealed a consistently broad spectrum of isoelectric points among the protein assemblage throughout each stage (data not shown).

Evidence of UDP-galactose 4-epimerase activity in Y-1528 affirms the existence of at least one component enzyme of the Leloir pathway. The conversion of the intermediate metabolite UDP-galactose to UDP-glucose suggests that the other known pre-glycolytic preparatory reactions might also occur in this strain, despite the abnormal preference for galactose in environments containing a variety of hexose sugars and the surprising appearance of extracellular glucose during mixed sugar fermentation [26]. However, the presence and activity of conventional GAL1 [46], GAL2 [41], and GAL7 [11] in Y-1528 would need to be assessed through individual protein purification schemes in order to verify an intact Leloir pathway.

Four GFC fractions of the filtered cellular protein extract of Y-1528 showed activity during epimerase assays (Fig. 5.2A). Two fractions of the protein extract of BY4742 (Fig. 5.2B), and six fractions of the protein extract of Tembec T1 also showed activity during epimerase assays (Fig. 5.2C). Once again, positive controls containing commercially acquired UDP-galactose 4-epimerase confirmed the suitability of assay conditions (Fig. 5.2A), negative controls showed the absence of catalytic reactions, and standards permitted detection of and discrimination between substrate and product. Protein concentration measurement in the active fractions indicated the presence of lesser but still appreciable amounts of protein following GFC (signifying the separation of the total protein cohort on the basis of molecular mass). Similarly, SDS-PAGE showed high molecular mass proteins in the range of 70 to 150 kDa, and IEF revealed a broad spectrum of isoelectric points among the protein assemblage (data not shown).

Continued evidence of UDP-galactose 4-epimerase activity in the collected GFC fractions of Y-1528 filtered cellular protein extract implied that the enzyme in this strain had a molecular mass > 70 kDa, befitting its expected size of 78,195 Da [21]. Having successfully performed partial purification of the Y-1528 GAL10 by size exclusion, subsequent isolation efforts focused on separating the remaining protein by isoelectric point. The UDP-galactose 4-epimerase of *S. cerevisiae* is known to have an isoelectric point of 5.84 [21], and other yeast proteins displaying the molecular mass range of 70 to 150 kDa have pl values spanning 4.06 to 9.92 (Tagldent, Swiss Institute of Bioinformatics) [13]. Chromatofocusing on a Mono P column was selected to effect separation, potentially resolving proteins with as little difference as 0.02 pH units in







Fig. 5.2. UDP-galactose 4-epimerase activity in GFC fractions of filtered cellular protein extracts from (A) *S. cerevisiae* Y-1528, (B) *S. cerevisiae* BY4742, and (C) *S. cerevisiae* Tembec T1, as measured by HPLC separation and 264 nm UV light absorbance detection of UDP-glucose product.

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isoelectric point. The risk of protein denaturation (destruction of secondary and tertiary structure by charge modification) and consequent loss of catalytic activity due to focusing of the epimerase to its pl value was acknowledged, with the intent to subsequently elicit refolding via immediate buffer exchange. In the event of permanent denaturation, final purification and sequencing would nevertheless be pursued via online 1-dimensional liquid chromatography (1-D LC) and electro-spray ionization (ESI) or matrix-assisted laser-desorption ionization – time-of-flight (MALDI-TOF) mass spectrometry [18,36].

The application of epimerase-active Y-1528 GFC fractions to the Mono P chromatofocusing column resulted in unusually strong binding of protein to the stationary phase. Online monitoring of column output via UV absorbance at 280 nm showed the absence of protein elution over the entire Polybuffer-mediated pH range specified by the program method (7.1 to 4.0) (data not shown). Only the introduction of 2 M NaCl (a column cleaning and ion exchanger renewal agent) to the column was able to displace non-separated bound protein. Interestingly, the subsequent application of epimerase-active BY4742 and Tembec T1 GFC fractions to the same column, employing the same program method, resulted in successful separation of protein on the basis of pl, confirming protocol integrity (data not shown). A second attempt to separate the active Y-1528 GFC fractions, employing a more robust program method (Polybuffer 74 diluted 1:7 in deionized water *vs.* the original 1:10, and adjusted to pH 3.0 (rather than 4.0) with iminodiacetic acid), again resulted in the absence of protein elution over the entire Polybuffer-mediated pH range. 2 M NaCl was required to displace non-separated bound protein.

An extensive review of the literature revealed no evidence of recalcitrant protein binding during chromatofocusing experiments. Furthermore, the prospect of an unspecified physicochemical interaction between proteins in the mobile phase and the stationary phase matrix, causing strong and lasting binding, was discounted on account of the non-reactive, non-carbohydrate-based polystyrene/divinyl benzene resin present in the Mono P column. The contrasting observations of the Y-1528 and Tembec T1 protein cohorts suggested an unusual, difficult to discern, and as yet unidentified property unique to the former strain. Without addition of urea, glycerol, or nonionic/zwitterionic detergents to maintain or enhance solubility, the protein cohort in active Y-1528 GFC fractions may have aggregated while initially resident in the chromatofocusing column and therefore been unable to elute across the Polybuffermediated pH gradient [23]. Alternatively, as chromatofocusing is a separation mode based on differential states of electronic charge distribution as a function of pH, it is possible the mixture of distinctive proteins in the active Y-1528 GFC fractions formed a unified cluster possessing column binding capacity at multiple pH values, preventing selective elution of individual components across a linear gradient. Having been unsuccessful with chromatofocusing as a method of further purification of active GFC fractions, anion exchange chromatography was attempted. The Mono P column was retained for this new separation mode and complemented by an isocratic pH profile and ionic strength gradient mediated by NaCI.

The application of epimerase-active Y-1528 GFC fractions to the Mono P column with anion exchange buffers (starting buffer, buffer B mixed with 1 M NaCl) once more resulted in unusually strong binding of the protein cohort to the stationary phase. Online UV absorbance detection showed the absence of protein elution over the entire ionic strength range specified by the program method (data not shown). Non-separated bound protein was only eluted with the introduction of 1 M NaCl to the column. Epimerase-active BY4742 and Tembec T1 GFC fractions were again successfully separated on the basis of the relative ionic binding strength of individual proteins (data not shown). A subsequent attempt to separate the active Y-1528 GFC fractions utilizing buffer B mixed with 2 M NaCl also failed for the same apparent reason as before (data not shown), requiring 2 M NaCl to displace non-separated bound protein.

To consider the effect of column chemistry on the abnormal behaviour of the active Y-1528 GFC fractions during anion exchange chromatography, a Q Sepharose column was substituted for the Mono P. The program method was unchanged, employing starting buffer and buffer B mixed with 2 M NaCl. Strong, enduring binding of protein to the stationary phase was again observed (data not shown), requiring 2 M NaCl for elution.

As with chromatofocusing, the recalcitrant binding of active Y-1528 GFC fractions to both anion exchange columns was puzzling. In the case of the Q Sepharose column, the stationary phase matrix comprised a carbohydrate base (highly cross-linked agarose), which may have facilitated an unspecified physicochemical interaction with carbohydrate-modifying enzymes in the protein cohort (yeasts are rich in such catalysts [40]). However, that the filtered cellular protein extract of Y-1528 did

not undergo an unspecified physicochemical interaction with the GFC column (whose stationary phase matrix comprised cross-linked agarose and dextran) reduced the likelihood of such an occurrence in the later stages of purification. Additionally, parallel binding to a column whose matrix was non-carbohydrate-based (Mono P) suggested a mechanism independent of mobile/stationary phase interaction. Since chromatofocusing and anion exchange chromatography employ similar separation modes based on electronic charge and the distribution of said charges, column binding hypotheses related to protein ionization in Y-1528 are entirely plausible and worthy of future study.

Due to the recurring difficulties noted above, progression to final purification and sequencing was halted.

UDP-galactose 4-epimerase sequencing

The transcription and subsequent translation of *S. cerevisiae* structural galactose metabolism genes, including *GAL10*, is regulated via glucose-mediated repression, respiratory carbon source-mediated derepression, and galactose-mediated induction [42]. Y-1528 cultures grown in galactose- *vs.* glucose-augmented media, but otherwise treated identically, thus provided for differential protein expression of the target gene. In order to ascertain the presence of any unique protein(s) within the galactose-grown Y-1528 supernatant, 1-dimensional electrophoresis of unfiltered and filtered cellular protein extracts was performed. The reported molecular mass (78,195 kDa) of UDP-galactose 4-epimerase in type strains of *S. cerevisiae* [21], in concert with experimental evidence obtained during the attempted Y-1528 protein purification suggesting an enzyme with size > 70 kDa, provided a target range in which to search the separated supernatant. A distinct and unique protein band appeared in the galactose-grown Y-1528 filtered cellular protein broth with the expected molecular mass (Fig. 5.3), emphasized by the same band's absence in the adjacent lane containing the glucose-grown Y-1528 filtered cellular protein suspension.

Comparative analysis of the size-restricted protein cohorts by 2-dimensional LC-MS/MS revealed, among many differentially expressed unique proteins in the narrow molecular mass range centred on 78 kDa, the presence of UDP-galactose 4-epimerase in the galactose-grown Y-1528 supernatant (Table 5.3). One peptide fragment with the residues AVGESTQIPLR had 100% amino acid sequence identity with the known *S. cerevisiae* GAL10 sequence (n=699) from n=96 to n=106, representing 1.6% of the total



Fig. 5.3. Polyacrylamide (4-12% gradient) gel image of unfiltered and filtered cellular protein extracts from galactose-grown BY4742, galactose-grown Tembec T1, and galactose- and glucose-grown Y-1528, as well as extracellular supernatant from galactose-grown Y-1528. The protein band of interest in the lane containing filtered cellular protein extract from galactose-grown Y-1528 is highlighted. Selected marker protein sizes are indicated. Bgal = galactose-grown BY4742, Tgal = galactose-grown Tembec T1, Ygal = galactose-grown Y-1528, Yglc = glucose-grown Y-1528, Ext = extracellular supernatant.

Table 5.3. Differentially expressed unique proteins present in the molecular mass range subject to 2-D LC-MS/MS analysis, and their nominal sizes [21].

Galactose-Grow	vn Y-1528	
HSC82	Chaperonin (81 kDa)	
HSP82	Heat shock protein 90 (81 kDa)	
GPH1	Glycogen phosphorylase (103 kDa)	
GAL10	UDP-galactose 4-epimerase (78 kDa)	
ADE2	Phosphoribosylamino-imidazole-carboxylase (62 kDa)	
Glucose-Grown	Y-1528	
HIS4	Phosphoribosyl-AMP cyclohydrolase (88 kDa)	
APE2	Aminopeptidase yscll (106 kDa)	
PRT1	Translation initiation factor 3 subunit (88 kDa)	
GLN4	Glutamyl-tRNA synthetase (93 kDa)	_
protein. The absence of matching peptide fragments derived from the other 98.4% of the total GAL10 amino acid sequence suggested substantial mutation(s) in these regions of the protein, underlined by the much greater protein sequence coverage by peptide fragments generated from other proteins in the size-restricted supernatant.

The other differentially expressed unique proteins in the molecular mass range selected for analysis (Table 5.3) offer some insight to the cellular processes active in galactose-grown vs. glucose-grown Y-1528. The HSP90 family chaperonin HSC82 (EC 3.6.4.9) and HSP90 family heat shock protein HSP82 (EC 3.6.4.10) are frequently required for regulatory and signalling protein activation [38]. The expression of these proteins in galactose-grown Y-1528 may be related to induction of the strongly regulated galactose metabolism genes, or to the direction of the products of such genes to specific cellular locales. Interestingly, a previous investigation of UDP-galactose 4epimerase in Y-1528 considered unusual localization of the enzyme to explain the fermentation behaviour of the strain, but due to methodological difficulties could not determine the sub-cellular fraction(s) in which catalytic activity was taking place [26]. The glycogen phosphorylase GPH1 (EC 2.4.1.1) is expressed under stress in nutrientlimited environments, and, together with an accessory enzyme, catalyzes the conversion of glycogen (a storage carbohydrate) to glucose [10,44]. The expression of this protein in galactose-grown Y-1528 may reflect the strain's rapid catabolism of galactose and consequent need to substitute another carbon source for cellular processes once the depletion point of the primary sugar approaches or is reached. In addition, the production of glucose via GPH1 may be related to the appearance of endogenous extracellular glucose during mixed sugar fermentations by Y-1528 [26]. If glycogen synthase and glycogen phosphorylase (GSY2 and GPH1, respectively), or GPH1 alone, are expressed at abnormally high levels in this strain, and/or if expression occurs far prior to complete galactose exhaustion, it is possible that excess glucose evolves inside the cell and the sugar passively diffuses, via hexose transporters, to the extracellular environment [5]. Phosphoribosylamino-imidazole-carboxylase ADE2 (EC 4.1.1.21) is expressed in the absence of adenine, as well as during amino acid starvation, and is involved in *de novo* biosynthesis of the purine nucleotides adenine and guanine [15]. Phosphoribosyl-AMP cyclohydrolase HIS4 (EC 3.5.4.19), likewise, is expressed under amino acid starvation conditions, and is required for histidine biosynthesis [1,19]. The expression of these proteins in galactose- and glucose-grown

Y-1528, respectively, is likely related to the rapid metabolic flux occurring in both cultures and the resultant depletion of critical precursors to biological macromolecules (nucleic acids, proteins, and energy compounds). However, the energy (ATP) requirements of galactose-grown Y-1528 may be higher than those of glucose-grown Y-1528, as indicated by ADE2 expression. This is certainly plausible, since galactose metabolism is energetically more demanding than glucose metabolism [34]. The aminopeptidase APE2 (EC 3.4.11.1) catalyzes the splicing of externally supplied leucine-containing dipeptides into component amino acids, thus deriving leucine plus one other residue [47]. The expression of this protein in glucose-grown Y-1528 may reflect a deficit in particular polypeptide synthesis precursors, and may be traced to greater production of leucine-rich proteins than in galactose-augmented cultures. The structural protein PRT1 forms a core subunit of translation initiation factor 3 [29], and the glutamyl-tRNA synthetase GLN4 (EC 6.1.1.17) synthesizes glutamyl-tRNA [31]. Both are essential for protein biosynthesis under any growth conditions (including galactose augmentation), and thus their expression in glucose-grown Y-1528 is not exclusive.

Concurrent 1-dimensional electrophoresis of filtered extracellular supernatant from galactose-grown Y-1528 demonstrated the absence of significant amounts of protein and no distinct band corresponding to putative UDP-galactose 4-epimerase near the molecular mass ascribed to it (78 kDa). This observation supported evidence presented earlier that galactose metabolism enzymes do not appear to be exported in Y-1528, and therefore play no extracellular role in the rapid assimilation of galactose and accumulation of extracellular glucose during fermentation.

The UDP-galactose 4-epimerase peptide fragment derived from 2-dimensional LC-MS/MS was used to design the non-degenerate primers GAL10P1F and GAL10P1R, as well as the degenerate primers DGAL10P2F and DGAL10P2R, DGAL10P3F and DGAL10P3R, DGAL10P4F and DGAL10P4R, and DGAL10P5F and DGAL10P5R. Each of these numbered primer pairs comprised oligonucleotide sequences in reverse complement. Additional non-degenerate primers (UTRF and UTRB) were designed from genomic DNA sequences in the untranslated regions flanking both the 5' and 3' ends of *GAL10*.

Leloir pathway and associated gene sequencing

An assessment of species identity was performed shortly after initiating PCR amplification of the structural galactose metabolism genes in *S. cerevisiae* (*GAL1*, *GAL2*, *GAL7*, and *GAL10*). This genotyping comprised amplification of sequences found in the conserved rDNA-ITS region of the yeast genome [50], was intended to rationalize the experimental approach and support previous data [27], and involved Y-1528, BY4742 (a reference strain derived from *S. cerevisiae* S288C, the template for the yeast genome sequencing project [6]), and Tembec T1 (an industrially adapted *S. cerevisiae* strain isolated from pulp and paper mill spent sulfite liquor). PCR components and conditions consisted of the ITS4 and ITS5 primers, 100 ng Protocol 1 genomic DNA, 35 amplification cycles, and a 57°C annealing temperature. BY4742, Tembec T1, and Y-1528 amplicons (Fig. 5.4, lanes 1, 4, and 5, respectively) resulting from amplification of 762 bp sequences (spanning the entire ITS1, 5.8S, and ITS2 ribosomal rRNA genes, as well as part of the 25S ribosomal rRNA gene) were directly sequenced.

Y-1528 had 98.4% and 98.2% identity with BY4742 and Tembec T1, respectively, in the terminal 3' end of the ITS1 region (85 bp) and the entirety of the 5.8S, ITS2, and 25S regions spanned by the primers (Fig. 5.5). This level of molecular identity in the rDNA-ITS region strongly suggests that Y-1528 is a species of *Saccharomyces* (Fig. 5.6) [7]. Experimental approaches to amplify the galactose metabolism genes in Y-1528 were thus validated.

The first attempt to amplify *GAL1*, *GAL2*, *GAL7*, and *GAL10* employed the GAL1F and GAL1R, GAL2F and GAL2R, GAL7F and GAL7R, and GAL10F and GAL10R primers, respectively. Each of these primer pairs represented oligomeric DNA sequences at the 3' and 5' termini of the target genes, and were intended to encompass entire gene sequences upon PCR-mediated extension. PCR conditions comprised 40 amplification cycles and a 56°C annealing temperature, and utilized 200 ng genomic DNA as template. Gel electrophoresis of the PCR output showed distinct single bands of amplified BY4742 and Tembec T1 genomic DNA from all of the galactose structural gene primers (Fig. 5.7), setting the stage for cloning and sequencing of the reference strains' galactose structural genes. BY4742 and Tembec T1 galactose structural gene sequences were confirmed to match those of type strains of *S. cerevisiae*. However, there was no evidence of amplification of Y-1528 template sequences.



Fig. 5.4. Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from BY4742, Tembec T1, and Y-1528. Lanes 1 (BY4742), 4 (Tembec T1), and 5 (Y-1528) illustrate amplicons from rDNA-ITS regions. Selected marker base pair sizes are indicated.



Fig. 5.5. Aligned rDNA sequences of *S. cerevisiae* BY4742 ("B"), *S. cerevisiae* Tembec T1 ("T"), and *S. cerevisiae* Y-1528 ("Y").



Fig. 5.6. Fast minimum evolution algorithm-generated dendrogram classifying Y-1528 among various yeast species via **rDN**A sequences.



Fig. 5.7. Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from BY4742, Tembec T1, and Y-1528. Amplicons from putative galactose structural gene sequences are shown. The four lanes in each set correspond sequentially to BY4742, Tembec T1, Y-1528, and negative control. Selected marker base pair sizes are indicated.

Employing different PCR conditions (40 amplification cycles and a 55°C annealing temperature) and using 300 ng genomic DNA and GAL10-specific primers, multiple bands of amplified Y-1528 genomic DNA were achieved (Fig. 5.8), while GAL1, GAL2, and GAL7 were not visible following amplification with their respective primers. One of these bands had the expected size (~2000 bp), and was subsequently gelexcised, purified, and cloned. A colony PCR was performed to screen for positive colonies, employing M13 primers (whose sequences are integrated into the cloning vector plasmid) as well as the GAL10 primers. In order to verify that the isolated fragment in the cloning vector was indeed GAL10, one of the positive colonies was grown in liquid culture and its plasmid DNA harvested and subject to a PCR using the degenerate primers designed from the 2-dimensional LC-MS/MS-derived UDPgalactose 4-epimerase peptide fragment (DGAL10P2F/R. DGAL10P3F/R. DGAL10P4F/R, and DGAL10P5F/R) in conjunction with the GAL10F and GAL10R primers. In a convincing illustration, distinct bands of the expected sizes (~300 bp and \sim 1800 bp) were observed in most of the gel lanes (Fig. 5.9). Consequently, these plasmid-borne amplicons were sequenced, yielding the entire Y-1528 GAL10 sequence (Fig. 5.10). The 3' terminus of the gene (20 bp) was re-sequenced via a 3' RACE-PCR to correct any nucleotides incorrectly dictated by the original 3' primer and hence accurately reflect the actual template sequence. This specialized PCR involved 35 amplification cycles and a 50°C annealing temperature, and utilized 300 ng of 3' RACE DNA as template. The DGAL10P3F and 3' Outer (Ambion) primers were used to effect amplification. Sequence analysis showed that the Y-1528 GAL10 had 77% identity with type strains of S. cerevisiae.

The Y-1528 *GAL10* sequence was translated to an amino acid sequence (Fig. 5.11) and aligned in BLAST. GAL10 had 83% amino acid sequence identity with type strains of *S. cerevisiae*, indicating significant mutation, but demonstrated a conserved epimerase catalytic residue (Tyr-163) [45]. Thus, it is highly probable that the epimerization reaction catalyzed by the Y-1528 UDP-galactose 4-epimerase proceeds by the conventional mechanism outlined in the literature [20]. Interestingly, a comparative analysis of the Y-1528 and *S. cerevisiae* type strain GAL10 sequences via ProtParam predicted a substantially lower isoelectric point (5.30 *vs.* 5.66), as well as five more negatively charged residues (89 *vs.* 84) and three fewer positively charged residues (68 *vs.* 71) in the Y-1528 protein. A second analysis via SignalP predicted that



Fig. 5.8. Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from Y-1528. Amplicons from the putative *GAL10* sequence are shown. Lanes 2 and 3 correspond to negative control and Y-1528, respectively. Selected marker base pair sizes are indicated.



Fig. 5.9. Agarose (0.8%) gel image of PCR-amplified cloning vector plasmid insert DNA sequences originally from Y-1528. Amplicons from the putative *GAL10* sequence are shown. Lanes 1-4 sequentially illustrate the results of GAL10F and DGAL10P2R, GAL10F and DGAL10P3R, GAL10F and DGAL10P4R, and GAL10F and DGAL10P5R primer-mediated amplification. Lanes 5-8 sequentially illustrate the results of DGAL10P2F and GAL10R, DGAL10P3F and GAL10R, DGAL10P4F and GAL10R, and DGAL10P5F and GAL10R primer-mediated amplification. Selected marker base pair sizes are indicated.

	1	10	20	30	40	50	60	70	80	90	100	110	120	130
JK SC Consensus	ATGACI Atgaci Atgaci	AGCTCAGTT AGCTCAGTT AGCTCAGTT	ACAAAGTCAA Acaaagtgaa Acaaagtgaa	GTITCTECARI RGTACTTCTRI agTaCTcCaRI	INNECGTTT Inntegtt Innecgttt	TAGTTACAGGT TGGTTACAGGT Tagttacaggt	IGGTGCTGGT IGGTGCTGGA IGGTGCTGGA	IACATTGGTTI IACATTGGTTI IACATTGGTTI	CACACACTG Cacacactg Cacacactg	ITGTGGAATTG IGGTAGAGCTA IgGTaGAacTa	ATTGAGAATGG Attgagartgg Attgagartgg	GTACGAGTGT Atatgactgt atacgactgt	GTTGTTGTA GTTGTTGCT GTTGTTGCa	GATAATC Gataacc Gataacc
	131	140	150	160	170	180	190	200	210	220	230	240	250	260
JK.	ICTOR		TRIGATICIG			ARCTARGCATO		TATAAGGT		GATAGAGAAG	GTCTGGACAAT		GCCACGATA BATATARA	
Consensus	TeTCa	ATTCaaCg	TATGATICIG	TaGCcRGGTT	GRaalcII	aACCAAGCATO	ReATTCCaTI	CTATAAGGT	IGAccTgTG	GAcaGAaAAG	GTCTGGAaAAg	GITTTCARR	aacAcaAaA	TCGACTC
	261	270	280	290	300	310	320	330	340	350	360	370	380	390
JK SC	TGTGA GGTAA	TICACTITG	CTGGTTTAAA CTGGTTTAAA	GGCAGTTGGTI GGCTGTAGGTI	GAGTETACT Gratetaca	CARATCCCACT Craatcccccct	TAGETATTA IGAGATACTA	CATAATAACI ICACAATAACI	RTCCTTGGTF RTTTTGGGA	CETTEGITII Ctetcettii	GTTAGAATTGA ATTAGAGTTAA	TGCRGCAGTE TGCRACAATE	CGGTGTCTC Carcgtttc	CARAFTT
Lonsensus	391	400	410	420	430	440	450	460	470	480	490	500	510	520
JK	GTTTT		AGCCRCGGTT	TATEGTEATE	GACTAGAT	TTCCARATACO	ATTCCRATTC	CAGAAGAGTI	STCCACTGG	TCCARCTART	CCTTATEGTAR	CACCABATAT	ACTATCORG	RARATCT
SC Cons ens us		TCATCTTC	TGCTRCTGTC aGCcRCgGTc	TATGGTGATG	CTACGAGAT Cyacyagat	Teccaratae	ATTCCAATe	CHGHHGHATI CAGAAGAaTI	GICLETIAG	GCCARCTART	CCgTATGGTaA	CACCARATAC	aCcATcGAG	HIHILI HAaATCT
	521	530	540	550	000111000	5/0	560	530		610 CCCTTCOTCC			640 	630
JK SC Consensus	TGAAT	GATCTTTAC GATCTTTAC GATCTTTAC	ANTAGEGACA Antagegaca Antagegaca	HGGHGHGHGHGG AARAAAGTTG(AaaAaAGTTG(GARGTTTGC GargetttgC GargetttgC	CATCTIGCETT TATCTIGCETT gatctigcett	INCTICNACCO Intitancco Incticnacco	ANTIGGIGC Antiggigc Antiggigc	CATCCATC	IGGATTAATCG IGGATTAATCG IGGATTAATCG	GAGAAGATCCA GAGAAGATCCG GAGAAGATCCA	CTAGGTATAC CTAGGTATAC	Caracaatt Caracaatt Caracaatt	IGT TOCC IGT TOCC IGT TOCC
	651	660	670	680	690	700	710	720	730	740	750	760	770	780
JK SC Consensus	ATATA Atata Atata Atata	EGGETCAAG Eggetcaag Eggetcaag	TCGCTGTTGG TRGCTGTTGG TaGCTGTTGG	GAGACGTGARI TAGGCGCGAGI gAGaCGcGAal	HARCTTTTT HECTTTRC HACTTTAC	ATCTTTGGTGF ATCTTCGGAGF ATCTTcGGaGF	ICGATTATGAT ICGATTATGAT ICGATTATGAT	AGCAGAGATE TCCAGAGATE accagagate	GGTACACCTA GGTACCCCCGA GGTACCCCCGA	NTCAGAGATTA NTCAGEGATTA NTCAGEGATTA	CATTCACGTTG TATCCACGTAG CATCCACGTAG	TTG <mark>ACT</mark> TAGC TTGATCTAGC TTG <mark>Acc</mark> TAGC	CAAGGGTCA AAAAGGTCA AAAAGGTCA	TATTGCT TATTGCA TATTGCa
	781	790	800	810	820	830	840	85 0	860	870	880	890	900	910
JK SC Consensus		GAAGTACTTI GCAATACCT GaAaTACcT	GGATGCTTAT AGAGGCCTAC aGAgGCcTAC	AATCAACAAA AATGAAAAATGA AATCAAAAAA	NAGGTETET NAGGTETET NAGGTETET	GT <mark>RGG</mark> GRGTGG GTCGTGRGTGG GTaGgGRGTGG	AATTTGGGTT AACTTGGGTT AACTTGGGTT	ICTGGTARGG(ICCGGTARAG(ICCGGTARAG(GETETAC <mark>GG</mark> Getetacagi Getetacagi	ITTTTGAAGTT ITTTTGAAGTT ITTTTGAAGTT	TACCACGCATT TATCATGCATT TACCACGCATT	CTGCAAGGCT CTGCAAAGCT CTGCARaGCT	TCGGGAATT TCTGGTATT TCgGGaATT	GACCTTC GATCTTC GACCTTC
	911	920	930	940	950	960	97 0	98 0	99 0	1000	1010	1020	1030	1040
JK SC Consensus	CRTATI CRTAC	GAAGTCACT HAAGTTACG HAGTCAC	GGTAGAAGAG GGCAGAAGAG GGCAGAAGAG GGCAGAAGAG	CCGGTGATGTT CAGGTGATGTT Caggtgatgtt	TTGAATTT TTGAACTT TTGAACTT	GACAGCCAAAC GacagctaaaC GacagccaaaC	CRGATAGAGO CRGATAGEGO CRGATAGEGO	TGAGAATGAA CAAACGCGAA	ATTABAATGO ACTGAAATGO ACTGAAATGO	CAGACEGAAT CAGACEGAGT Gaagacegaat	TGCARGTRGAN TGCAGGTTGAA TGCAGGTTGAA	GATTCTTGTA Gactcctgca Gactcctgca	AGGACTTAT Aggatttat Aggacttat	GGAAATG GGAAATG GGAAATG
	1041	1050	1060	1070	1080	1090	1100	1110	1120	1130	1140	1150	1160	1170
JK SC Consensus	GECCA GECCA GACTA	CTGARAATC CTGAGAATC CTGAGAATC	CCTTTGGATA CTTTTGGTTA CCTTTGGTTA	CCAACTTAAG CCAGTTAAGG CCAGTTAAGG	GTGTTGAA GTGTCGAG GTGTCGAG	TCGARGTTTGC GCCAGATTTTC eCcRaattteC	TACCGAAGAT	ATGCGTTATE ATGCGTTATE ATGCGTTATE	GATGCTAGA Gatgcaaga Gacgcaaga Gacgcaaga	TTGTCACCAT	TGGCGCTGGTA TGGTGCCGGCA TGGcGCcGGcA	CCAGATTECA CCAGATTECA CCAGATTECA	AGCTACTAT AGCCACGTT AGCCACGTT	TGCGAAC TGCCAAT TGCCAAC
	1171	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
JK SC Consensue	TTRGGT TTGGGG		TGTGGATTIG	RAGG FTGATGO RAAG TGAACGO RAAG TGAACGO	GACARTCTG GACARTCAG	TEGTTCTTGGT TTGTTCTTGGC	TATGACAGTO TATGARARTO	ARAGC GGG TH	ATTTGAACCO ATTTGAATCO ATTTGAACCO	TGATAGTGCT	TATGTTGGGGG TATATAGGCGC TATATAGGCGC	TACCATTGGT Cacgatcggc	AGGTATGCA AGGTATGCT AGGTATGCT	NATCGTA NATCGTA NATCGTA
consensus	1301	1310	1320	1330	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430
JK SC Consensus		HGGGTAAA HGGGTAAG HGGGTAAA	TTTERCETAR TTTRGTTTATE	ATGGTAATGAG SCARCAAAGAG acaacAAaGAG	TATCARTT TATCAGTT TATCAGTT	GACTGICANIA RACCGITANIA ARCCGICANIA	ATGGAATCAA ACGGCGTTAA AcgGaatcAA	ITGETARTERI ITGEGRATERI ITGEGRATERI	IAGTAGTGTT IAGTAGTATC IAGTAGTATC	GGATCTTTCC GGTTCTTTCC GGATCTTTCC	ATGTGAAGCGT ACAGAAAAAGA AcagaAAaaGa	TTTTGGGAC	CTATTGTCC CCATCATTC CCATCATCC	AGRACCC AGRATCC NARATCC
	1431	1440	1450	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550	1560
JK SC Consensus	TTCAR TTCAR TTCAR	GGACGTCT GGATGTTT GGACGTCT	TTACTGCTGA TTACCGCCGA TTACcGCcGA	NTACATGCTCT GTACATGCTG ATACATGCTG	TTGACAAA TAGATAAT Tagacaaa	GGACAGAATTC GAGAAGGACAC GaaaAGaAcaC	CGAATTTCCT CGAATTTCCA CGAATTTCCA	GGAGATCTCI GGTGATCTA GGaGATCTA	I TGGTAACGE I TGGTAACCE I TGGTAACCE	TTCAGTATAC	ATTGGATGTTA TGTGAACGTTG agTGaAcGTTa	ICGAAAAAAAG CCCBAAAAAAG CC BA AAAAAG	TTTGGTAAT TTTGGAAAT TTTGGaAAT	IGAGTAT GGTATAT 2GaaTAT
	1561	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660	1670	168 0	1690
JK SC	AAGGG1 AAAGG1 BAAGG1	GACCITGT	TTCTGGTGAA TCCTGGTGAA	GCTACACCAG GCGACGCCAA GCGACGCCAA		CTARCCATACT CARATCATAGT	TATTICAACT	TGAATAAACO TGAACAAGCO	TCRCGRGGF RTRTGGRGF	CACTATTGAG CACTATTGAG			TCTARTARG TCARAARA	
consensus	1691	1700	1710	1720	1730	1740	1750	1760	1770	1790	1790	1800	1810	1820
ЖL	ATGT G	ACAAAAAT	GITATICCAA	CGGGTAAAGTT	INT T GAGAG	AGATATTOCTA		TCARRAGECE	ACAGTACTE	GGTTCTGATG	AACCCCAATAT	GATCAGICIT	TCGTCGTTG	ncgaa ga
SC Cons e nsus	ATGTC	Jacaaaaa Jacaaaaaa Jacaaaaaa	aTGATTCCTA aTgATTCCaA	CGGGTARTATO CGGGTARaaTo	CETCGATAG CatcGAgAG	AGANATTOCTA Agaaattocta	ICCTTTRACTO ICCTTTAAcTO	T HCAHAGECE TaaAAAGCCE	IRCGGICTTF IRCaGIacTF	IGGCCCCAAAA IGGccCcaAaa	ATCCCCAGTTI AaCCCCCAaTaTI	GATCagTcTT	TCGTcGTgG	icghnan Icghnan
	1821	1830	1840	1850	1860	1070	1880	1890	1900	191 0	1920	1930	1940	1950
JK SC Cons en sus		IGRATARGCI IGCCAAGTCI IGaaaAagCi	AGATCANCAC Nantcantac Rantcantac Rantcancac	GTTGAATAAC(TCTAAACAAT(gcTaAACAAC(gcTaAACAAC(GAATTGA <mark>AA</mark> Gaattgac <mark>g</mark> Gaattga _{aa}	ACTGTTGTARA CTTATTGTCAR actaTTGTaAR	INGETTTCCAT IGGETTTTCAT INGETTTCCAT	CCRGATTCGF CCCGATTCCF CCaGATTCcF	ATATCGCTT ATATACAT ATATCaCat ATATCaCat	TGGAGGTTCT TAGAAGTTTT TagAaGTTcT	AACTACAGAACI RAGTACAGAGCI AActaCagaaci	CRACETATER Cracetater Cracetater	GTTGTRTAC ATTTTATACI ATTgTRTAC	IGGTGAT GGTGAT GGTGAT
	1951	1960	1970	1980	1990	2000	2010	2020	2030	2040	2050	2060	2070	2080
JK SC Cons e nsus		TCCGCTGG TCTGCTGG TCcGCTGG	TTTCACTGCA TTACGAAGCA TTaCaaaGCA	RGACRAGGTTT RGACRAGGTTT RGACRAGGTTT	ICGCTGTCG ITGCAATTG ICGCaaTcG	ARCCTGGTAGA Agcctggtaga Agcctggtaga	TATATIGATG TACATIGATG TACATIGATG	CCGTANATCA CTATCAATCA CCATAAATCA	IAGAGGAATO IAGAGAACTO IAGAGAAATO	GAGGGATAAC GAAAGATTGT GAaaGATaac	GTAATTIIGAA GTAACCIIGAA GTAACCIIGAA	SCGCGGTGAA NAACGGTGAA saaCGGTGAA	AGTTATGGC Acttacggg Acttacggc	iccaaga iccaaga iccaaga
	2081	2090	2100											
JK SC Consensus	TTGTCT TTGTCT TTGTCT	IACAGGTTT IACAGATTT IACAGATTT	TCTTAR TCCTGR TCcTar											

Fig. 5.10. Aligned nucleotide sequences of *S. cerevisiae* Y-1528 *GAL10* ("JK"), *S. cerevisiae* type strain *GAL10* ("SC"), and fungal *GAL10* equivalents ("Consensus").



Fig. 5.11. Aligned amino acid sequences of *S. cerevisiae* Y-1528 GAL10 ("JK"), *S. cerevisiae* type strain GAL10 ("SC"), and fungal GAL10 equivalents ("Consensus").

the Y-1528 GAL10 is a non-secretory protein, in accordance with the predicted absence of a signal peptide directing post-translational transport. WoLF PSORT analysis predicted cytosolic localization of both the Y-1528 and *S. cerevisiae* type strain GAL10.

The next set of PCR, targeting Y-1528 *GAL1*, *GAL2*, and *GAL7* sequences, used 500 ng genomic DNA. PCR conditions comprised 40 amplification cycles and a 56°C annealing temperature. Gel electrophoresis of the PCR output showed a slightly subdued single band of amplified Y-1528 genomic DNA from the *GAL7* primers (data not shown). Though this amplicon had the expected size (~1100 bp), repeated attempts to clone the product failed, as did attempts to have it directly sequenced.

The last attempt to amplify Y-1528 *GAL1*, *GAL2*, and *GAL7* sequences without PCR additives or the employment of degenerate primers again used 40 amplification cycles and a 56°C annealing temperature in concert with 500 ng template, or a 47°C annealing temperature in concert with 100 ng template. Gel electrophoresis of the 56°C PCR output showed faint single bands of amplified Y-1528 genomic DNA from the *GAL1* and *GAL7* primers (Fig. 5.12A). Moreover, the 47°C PCR output illustrated faint single bands of amplified Y-1528 genomic DNA from the *GAL2* and *GAL7* primers (Fig. 5.12A). Though these amplicons were below expected size, they were the sole amplified products following the PCR, and so were subject to attempted cloning and sequencing. Repeated attempts to clone the *GAL1*, *GAL2*, and *GAL7* PCR products failed.

The addition of dimethylsulfoxide (DMSO) to the PCR can facilitate amplification of template DNA with stable secondary structure [43]. DMSO deconstructs secondary structure and makes more amenable the melting of double-stranded DNA into single strands for polymerase-mediated primer extension. PCR conditions comprised 40 amplification cycles and an annealing temperature of 47°C, and utilized 100 ng genomic DNA in the presence of 12.5% v/v DMSO. Gel electrophoresis of the PCR output showed a faint band of amplified Y-1528 genomic DNA from the *GAL7* primers (Fig. 5.12B). However, this amplicon was not cloned and sequenced, since it was of unexpectedly small size and prior attempts with a similar amplicon (Fig. 5.12A) were unsuccessful.

Acknowledging the lack of success in amplifying *GAL1*, *GAL2*, and *GAL7* via non-degenerate priming, the degenerate primers DGAL1F and DGAL1R, DGAL2F and DGAL2R, and DGAL7F and DGAL7R were employed, respectively, to account for



Fig. 5.12. (A) Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from Y-1528. Lanes 2-7 show the results of 500 ng template and an annealing temperature of 56°C, and lanes 8-13 100 ng template and an annealing temperature of 47°C. Amplicons from putative *GAL1*, *GAL2*, and *GAL7* sequences are shown. The two lanes in each set correspond sequentially to Y-1528 and negative control. Selected marker base pair sizes are indicated. (B) Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from BY4742 and Y-1528. Amplicons from putative *GAL1*, *GAL2*, and *GAL7* sequences are shown. The three lanes in each set correspond sequentially to BY4742, Y-1528, and negative control. Selected marker base pair sizes are indicated.

potential nucleotide mutations in Y-1528 *GAL* sequences. An example of the degenerate primer design process is shown in Fig. 5.13. The necessity of designing from conserved sequences (to minimize the number of inosine nucleotides and thus maintain specificity as much as possible) meant that theoretical amplicons would only encompass part of the target gene sequence in most cases. The primer pairs were located at the following target gene coordinates:

DGAL1F (bp 25) and DGAL1R (bp 1472) \rightarrow 1448 bp amplicon DGAL2F (bp 676) and DGAL2R (bp 1568) \rightarrow 893 bp amplicon DGAL7F (bp 10) and DGAL7R (bp 883) \rightarrow 874 bp amplicon

In one attempt with degenerate primers, 200 ng genomic DNA was used as template for the *GAL1*, *GAL2*, and *GAL7* primers in PCR comprising 35 amplification cycles and an annealing temperature of 40°C. Gel electrophoresis of the PCR output showed a single faint band (of expected size) of amplified Y-1528 genomic DNA from the *GAL1* primers, which incidentally mirrored a more distinct band of amplified BY4742 genomic DNA (Fig. 5.14). Though this amplicon was of very low concentration, it was decided to attempt cloning and sequencing.

The PCR product from the *GAL1* primers did not undergo stable cloning, and generated a 72 bp insert in the cloning vector plasmid (a fraction of the anticipated 1448 bp). Sequence analysis showed that the insert did not match *GAL1* sequences from either BY4742 or Tembec T1, and when aligned in BLAST, had 93% sequence identity (among 82% of the query) with the uncharacterized *S. cerevisiae* open reading frame YDL133W on chromosome IV. This demonstrated that non-specific primer-template binding occurred, highlighted by the residence of *GAL1* on chromosome II in type strains of *S. cerevisiae* [9].

Due to recurring difficulties, further attempts to sequence the *GAL1*, *GAL2*, and *GAL7* genes in Y-1528 were temporarily halted. It is highly probable that these gene sequences in this strain are significantly divergent from type, and will require complex and specialized PCR techniques, as well as successful cloning, for full characterization.

Structural galactose metabolism genes and proteins in Y-1528

Synthesizing outcomes from the attempted UDP-galactose 4-epimerase purification and Leloir pathway and associated gene sequencing, it is patently clear that *S. cerevisiae* Y-1528 distinguishes itself from other strains of the same species. Owing to a multifaceted adaptation to galactose-rich carbon sources in its original environment,



Fig. 5.13. An illustration of the DGAL10F2 degenerate primer. The 2100 bp GAL10 shows the relative positions and extension directions of the DGAL10F1, DGAL10F2, DGAL10R1, and DGAL10R2 primers. Relatively conserved sequences of 6 diverse organisms, including *S. cerevisiae*, which were used to derive the primer sequence, are displayed. A = adenine, C = cytosine, G = guanine, I = inosine, T = thymine. Asterisks indicate locations of base pair variation between species into which inosine was designed.



Fig. 5.14. Agarose (0.8%) gel image of PCR-amplified genomic DNA sequences from BY4742 and Y-1528. Amplicons from putative *GAL1*, *GAL2*, and *GAL7* sequences are shown. The three lanes in each set correspond sequentially to BY4742, Y-1528, and negative control. Selected marker base pair sizes are indicated.

this strain (in all probability) possesses sequence-modified *GAL1*, *GAL2*, and *GAL7*. Experimental results have clearly shown that *GAL10*, and by extension the UDP-galactose 4-epimerase, bear sufficient sequence variation from type to facilitate remarkable substrate versatility during fermentation and possibly permit unusual compartmentalization of the enzyme within the cell via a heretofore unknown signalling motif. Highly efficient and/or multiple catalytic reaction centres, in combination with distinctive secondary and tertiary structure, may be responsible for the exceptional metabolic flux exhibited in the presence of galactose. The sequences of the nominal regulatory genes *GAL3*, *GAL4*, *GAL80*, and others newly discovered, as well as the functionality of their respective encoded proteins [42], may also be significantly altered. Future investigations of Y-1528 at the molecular level should take into account these theories and hypotheses, as new and potentially applicable metabolic paradigms are likely to be discovered.

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Chapter 6 - Conclusions and recommendations for future work

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Conclusions

The characterization of the *Saccharomyces cerevisiae* Y-1528 yeast strain comprised the assessment of its galactose assimilation capacity, its fermentation performance in lignocellulose-derived substrates and industrially relevant regimes, its anabolic and catabolic properties in the presence of simple and complex media, and the mechanisms underlying its enhanced fermentation activity at the molecular level. These investigations employed a variety of methodologies with general applicability to laboratory, pilot plant, and industrial scale operations, and, in all circumstances, also demonstrated the performance of Y-1528 in relation to reference strains of *S. cerevisiae*.

From a global perspective, the characterization and subsequent utilization of such strains in the conversion of biomass-derived sugars to ethanol is pertinent and timely. As known petroleum supplies steadily deplete due to rapid extraction and expenditure, and the rate and cost of discovery of new oil reserves descends and ascends, respectively, the need to supplement and ultimately replace deep geological energy deposits becomes critically apparent. There also exists an environmental advantage to the derivation of energy from surface biomass over the exploitation of petroleum-based resources. Interestingly, certain biomass components contain a relevant percentage of galactose, which is fermented with particular vigour by Y-1528.

S. cerevisiae Y-1528 was found to possess exceptional galactose assimilation capacity. It was also capable of simultaneously maintaining competitive consumption rates of the other predominant hexose sugars, glucose and mannose. This is an important trait, since most industrial scale fermentations operate on a continuous basis, and the accumulation of any substrate will detrimentally affect productivity, and may eventually cause a change in the physical properties of the culture medium and subsequently trigger inhibition. Y-1528 was also observed to generally perform as well as, and in certain cases better than an industrially-adapted reference strain of *S. cerevisiae* on lignocellulosic substrates such as steam-exploded liquid fractions of softwood biomass and softwood and hardwood spent sulfite liquor (SSL). Y-1528 readily tolerated the coexistence of the industrially adapted *S. cerevisiae* strain in synthetic and lignocellulosic substrates, suffering no reduction in fermentation capacity and in fact performing synergistically to achieve excellent productivity. These intrinsic characteristics of Y-1528 make it eminently applicable to industrial environments where biomass is fermented to ethanol, as diverse lignocellulosic substrates will likely be the

primary feedstock over the course of operations. Furthermore, in large scale fermentations, mixed cultures will develop periodically due to the inability to maintain strict environmental controls, and may be deliberately utilized to effect simultaneous pentose sugar consumption.

Despite some performance degradation compared to substrates without inhibitory compounds, the efficacy of Y-1528 was found to exceed that of the industrially-adapted S. cerevisiae strain in fermentations of synthetic sugar mixtures supplemented with inhibitors commonly found in processed lignocellulosic biomass. The maintenance of metabolic function when confronted with the toxicity inherent to depolymerized lignocellulosic substrates is a valued trait in yeast strains potentially employed in industrial fermentations, and the ability of Y-1528 to perform better than a strain isolated directly from such an inhibitory biomass-derived mixture shows its prospective versatility. However, the implementation of multiple (SSL) substrate cell recycle batch fermentations demonstrated poor adaptation on the part of both strains, although ethanologenic capacity was preserved. This illustrates a cautionary lesson in putting into service a yeast strain that has not been exposed to the full spectrum of competitive and biochemical pressures present in semi-natural and natural environments, and moreover the fragility of previously adapted yeast strains when repeatedly challenged with new substrates. Though industrial utility is a prime consideration in selecting a particular microorganism for fermentation applications, it is of great importance to also contemplate its endurance, knowing that, in nature, evolutionary fitness is never the permanent domain of a single yeast or bacterial strain. It may be imprudent to rely on one specific yeast isolate for ethanologenic fermentations involving a steady stream of different lignocellulosic or other complex substrates.

In light of the unusual preference for galactose exhibited by Y-1528, its taxonomic classification as *S. cerevisiae* was twice confirmed by phylogenetic analyses. The observation of endogenous glucose accumulation in the extracellular environment during mixed sugar fermentations was a further sign of abnormal metabolism, and prompted a comprehensive investigation of the molecular mechanisms responsible for such traits. The cellular compartmentalization of the activity of a target galactose metabolism enzyme, UDP-galactose 4-epimerase, remains undetermined notwithstanding an experimental attempt to have it ascertained, but the sequence of said protein and its corresponding gene were resolved, permitting the prediction of

certain biochemical characteristics of the enzyme. However, the remaining structural galactose metabolism genes (*GAL1*, *GAL2*, *GAL7*) remain unresolved despite a robust effort. The substantial mutation in the Y-1528 UDP-galactose 4-epimerase, in conjunction with probable differences in the other galactose metabolism enzymes of this yeast strain clearly manifest in exceptional substrate utilization patterns and suggest novel catalytic capacity. The full molecular characterization of these proteins will offer significant insight to natural biodiversity. More importantly, this unique set of genes has great genetic potential as an inducer and enhancer of desired metabolic reactions in other organisms.

Recommendations for Future Work

The primary obstacles encountered in this work related to molecular characterization of galactose metabolism in Y-1528. As such, the following suggestions concern altered approaches to obtaining gene sequences, purified enzymes, and sub-cellular localization data.

The employment of RACE (rapid amplification of cDNA ends) or RAGE (rapid amplification of genomic ends) PCR will significantly increase the probability of accurately amplifying *GAL1*, *GAL2*, and *GAL7* by attaching a known oligonucleotide extension to one or the other end of these gene sequences, permitting the use of one primer essentially guaranteed to bind to template.

If these target genes are successfully amplified, cloned, and sequenced, their translation to protein sequences may reveal unique regulatory, signalling, or catalytic domains. If discovered, these motifs may further explain the fermentation properties of Y-1528 (rapid and preferred galactose utilization, extracellular accumulation of endogenous glucose), and could present hypotheses to be tested on purified enzyme samples.

Effective non-destructive chromatographic and electrophoretic separation modes should also be employed to obtain purified galactose metabolism enzymes, in order that substrate, inhibition, kinetics, and catalysis studies can be performed.

The sub-cellular localization of the galactose metabolism enzymes of Y-1528 can be realized once sequence data are generated. Peptide antibodies linked to visualization markers can be synthesized and applied to yeast cells for histochemical analysis. Lastly, cloned galactose metabolism genes from Y-1528 can also be expressed in microbial expression systems and subsequently integrated into other organisms. The consequent enhanced galactose metabolism may prove useful in numerous industrial applications where this sugar or its derivatives serve as substrate or intermediate.

Appendix 1 - Physiological characteristics of USDA-acquired natural yeast strains

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The contents of Appendix 1 have been removed due to copyright restrictions. The information removed is tabulated data describing the culture collection identification number, accession date, original substrate, taxonomic identity, method of identification, biochemistry, metabolism, and physiology of each of the USDA-acquired yeast strains employed in the conduct of thesis-related laboratory experiments. These data were obtained in personal communications with Cletus Kurtzman, Supervisory Microbiologist at the Agricultural Research Service Culture Collection, USDA National Center for Agricultural Utilization Research, Peoria, Illinois, USA.