Master's thesis

L-rhamnose-1-dehydrogenase gene and L-rhamnose catabolism in the yeast *Pichia stipitis*

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Tiivistelmä — Referat — Abstract

The purpose of this work was to identify some of the genes of the catabolic route of L-rhamnose in the yeast *Pichia stipitis*. There are at least two distinctly different pathways for L-rhamnose catabolism. The one described in bacteria has phosphorylated intermediates and the enzymes and the genes of this route have been described. The pathway described in yeast does not have phosphorylated intermediates. The intermediates and the enzymes of this pathway are known but none of the genes have been identified. The work was started by purifying the L-rhamnose dehydrogenase, which oxidates L-rhamnose to rhamnonic acid-γ-lactone. NAD is used as a cofactor in this reaction. A DEAE ion exchange column was used for purification. The active fraction was further purified using a non-denaturing PAGE and the active protein identified by zymogram staining. In the last step the protein was separated in a SDS-PAGE, the protein band trypsinated and analysed by MALDI-TOF MS. This resulted in the identification of the corresponding gene, *RHA1*, which was then, after a codon change, expressed in *Saccharomyces cerevisiae*. Also C- or N-terminal histidine tags were added but as the activity of the enzyme was lost or strongly reduced these were not used. The kinetic properties of the protein were analysed in the cell extract. Substrate specifity was tested with different sugars; L-rhamnose, L-lyxose and L-mannose were oxidated by the enzyme. V_{max} values were 180 nkat/mg, 160 nkat/mg and 72 nkat/mg, respectively. The highest affinity was towards L-rhamnose, the K_m value being 0.9 mM. Lower affinities were obtained with L-lyxose, K_m 4.3 mM, and L-mannose K_m 25 mM. Northern analysis was done to study the transcription of *RHA1* with different carbon sources. Transcription was observed only on L-rhamnose suggesting that *RHA1* expression is L-rhamnose induced. A *RHA1* deletion cassette for *P. stipitis* was constructed but the cassette had integrated randomly and not targeted to delete the *RHA1* gene.

Enzyme assays for L-lactaldehyde dehydrogenase were done similarly to L-rhamnose dehydrogenase assays. NAD is used as a cofactor also in this reaction where L-lactaldehyde is oxidised to L-lactate. The observed enzyme activities were very low and the activity was lost during the purification procedures.

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L-Rhamnose, L-Rhamnose dehydrogenase, EC 1.1.1.174, L-rhamnose catabolism, *Pichia stipitis*, L-rhamnonate, MALDI-TOF MS, L-lactaldehyde, L-lactaldehyde dehydrogenase

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Tiivistelmä — Referat — Abstract

Työn tarkoitus oli identifioida L-ramnoosin kataboliareitin geenejä *Pichia stipitis* hiivassa. Toistaiseksi on löydetty kaksi erilaista L-ramnoosin kataboliareittiä. Bakteereilla esiintyvässä reitissä on fosforyloidut välituotteet. Tämän reitin entsyymit ja niitä koodaavat geenit on kuvattu. Sienillä L-ramnoosin katabolireitin tuotteet ovat fosforyloimattomia. Reitin välituotteet ja entsyymit on kuvattu, mutta yhtään entsyymiä koodaavaa geeniä ei ole identifioitu. Työ aloitettiin puhdistamalla L-ramnoosidehydrogenaasi, joka katalysoi L-ramnoosin hapettumista ramnonihappo-γ-laktoniksi. NAD toimii reaktiossa kofaktorina. DEAE ioninvaihtopylvästä käytettiin entsyymin puhdistukseen. Aktiiviset osat ajettiin ei-denaturoivassa PAGE-geelissä ja zymogram-värjättiin. Proteiini ajettiin SDS-PAGE –geelissä ja trypsinoitiin. Aminohapposekvenssin analysointi tehtiin MALDI-TOF MS laitteella ja vastaava DNA-sekvenssi nimettiin *RHA1*-geeniksi. Kodoninvaihdon jälkeen *RHA1* ekspressoitiin *Saccharomyces cerevisiae* –hiivassa. Proteiinin C- ja N-terminaalisiin päihin lisättiin histidiiniketjut, mutta seurauksena entsyymin aktiivisuus joko katosi tai väheni voimakkaasti. Proteiinin kineettiset ominaisuudet analysoitiin solu-uutteesta. Substraattispesifisyyttä testattiin eri sokereilla. L-Ramnoosin, L-lyksoosin ja L-mannoosin todettiin hapettuvan entsyymin vaikutuksesta. V_{max} arvot olivat vastaavassa järjestyksessä 180 nkat/mg, 160 nkat/mg ja 72 nkat/mg. Suurin affiniteetti oli L-ramnoosilla, jonka K_m arvo oli 0.9 mM. L-Lyksoosin K_m arvo oli 4.3 mM ja L-mannoosin K_m arvo oli 25 mM. Northern-analyysilla tutkittiin eri sokerien vaikutusta *RHA1*:n transkriptioon. Transkriptiota havaittiin vain L-ramnoosilla, joten *RHA1*:n ekspressio on L-ramnoosin indusoimaa. *P. stipitis* –hiivalle tehtiin *RHA1*-deleetiokasetti, mutta sen todettiin integroituneen sattumanvaraisesti eikä se kohdistunut oikein, joten *RHA1*-geenin deleetio epäonnistui.

L-Laktaldehydidehydrogenaasin entsyymianalyysi tehtiin samoin kuin L-ramnoosidehydrogenaasin entsyymianalyysi. NAD toimii kofaktorina myös tässä reaktiossa, jossa L-laktaldehydi hapetetaan L-laktaatiksi. Havaitut entsyymiaktiivisuudet olivat kuitenkin hyvin alhaisia, ja entsyymi inaktivoitui puhdistusprosessissa.

Avainsanat — Nyckelord — Keywords

L-ramnoosi, L-ramnoosidehydrogenaasi, EC 1.1.1.174, L-ramnoosikatabolia, *Pichia stipitis*, L-rhamnonaatti, MALDI-TOF MS, L-laktaldehydi, L-laktaldehydidehydrogenaasi

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List of abbreviations

DDIW	Two times distilled ion changed water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
K_M	The substrate concentration (mol/l) at which an enzyme reaction proceeds at half its maximal rate.
LB	Luria-Bertani
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionisation Time-of-flight Mass Spectrometry
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PGK1 promoter/terminator	Promoter/terminator of the 3-phosphoglycerate kinase of *S. cerevisiae*
RHA1	Gene encoding L-rhamnose dehydrogenase
RNA	Ribonucleic acid
SC	Synthetic complete
SCD	Synthetic complete medium with D-glucose
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOC	recovery broth used after electroporation
TKT promoter	Promoter of the transketolase gene of *P. stipitis*
URA3	Gene encoding the orotidine-5'-phosphate decarboxylase of *S. cerevisiae*
V_{max}	The substrate concentration where constant rate of product formation is achieved.
YNB	Yeast nitrogen base
YPD	Yeast extract peptone with D-glucose
YPX	Yeast extract peptone with D-xylose

X-gal	5-bromo-4-chloro-3-indolyl-β- D-galactorytanoside
XYL promoter/terminator	Promoter/terminator of the xylose reductase gene of *P. stipitis*

1 Introduction

Yeasts have served mankind for several thousands of years. Bread baking and production of alcoholic beverages have exploited the fermentation process by yeasts already a long time before "yeast" was found. The shared path of human and yeast has been food related for most of the history. Only for a couple of decades ago it was understood that yeast are providing also other prospects. First yeast was introduced as an experimental system for molecular biology. Increasing attention has been received as the development of yeast molecular biology has offered new possibilities. Besides being a model organisms for eukaryotic mode of life, yeast are of commercial interest because of the possibilities they offer to biotechnology, agriculture and medicine. Now the public interest is towards biofuels and the enhanced ethanol production.

Yeasts are eukaryotic microbes, a group of fungi. Fungi are divided into different phylas, the *Ascomycota* and *Basidiomycota* being sister groups which are classified as a subkingdom of *Dikarya* (Hibbett et al. 2007). Members of both phylas can grow as yeast or as hyphae, or both. Most of the yeast species are part of the Hemiascomycetes of the *Ascomycota* phyla. The well known yeasts, such as *Saccharomyces cerevisiae* and *Candida albicans*, are part of this group but not all Hemiascomycetes are exclusively yeast and some of the species are even predominately filamentous. Typical to Hemiascomycetes is that they lack fruiting bodies, structures for spore forming (Oliver and Schweizer 1999).

1.1 *Pichia stipitis*

P. stipitis (Pignal 1967) is a haploid, homothallic hemiascomycetous yeast and one of the 91 accepted species of the *Pichia* genus (Kurtzman, Fell 1998). *P. stipitis* was found from the gut of a wood-inhabiting passalid beetle. *P. stipitis* has the highest known native capacity to ferment xylose to ethanol. As xylose is one of the major components of plant lignocellulose, there has been great intrest to study possibilities to use *P. stipitis* for the production of biofuels. *P. stipitis* induces fermentative activity in response to oxygen limitation unlike *Saccharomyces cerevisiae*, which regulates fermentation by sensing the presence of fermentable sugars (Hertz-Fowler and Pain 2007, Jeffries et al. 2007).

The genome of *P. stipitis* CBS 6054 strain was published in March 2007 by Jeffries et al. (2007). The size of the genome is 15.4 Mb and it encodes 5 851 genes. There are eight chromosomes which vary in size from 3.5 to 0.97 Mbp. Comparison with eight other yeast revealed 25 gene families representing 72 proteins that are specific to *P. stipitis*. The closest sequenced relative to *P. stipitis* is the yeast *Debaryomyces hansenii* sharing 151 gene families that are not found in the other genomes. The origin in beetle gut, where the environment has a limited amount of oxygen and the energy source is partially digested wood, explains the numerous genes related to endoglucanase, β-glucosidase, xylanase, mannanase and chitinase (Hertz-Fowler and Pain 2007, Jeffries et al. 2007). *P. stipitis* has an alternative yeast codon usage where CUG codes for leucine instead of serine (Laplaza et al. 2006).

1.2 Production of biofuels by yeast

Biomass covers already about 10% of the world's primary energy demand (Antoni, Zverlov and Schwarz 2007). Rising oil prices, depletion of fossil resources, and environmental challenges are reasons why there has been a growing interest towards biofuels. For now bioethanol is the only microbially produced biofuel, which is produced on an industrial scale. Also biodiesel is produced on industrial scale but microbial processes are presently not involved in its production although use of enzymes and biological systems in transesterification is under development (Antoni, Zverlov and Schwarz 2007). *S. cerevisiae* is used to ferment sugar cane molasses or enzymatically hydrolysed starch from grains to produce ethanol. Plant biomass is a renewable source of energy and the future will hopefully see a technical process using lignocellulosic hydrolysates. For being cost-effective the biofuel industry should be able to exploit feedstocks, such as agricultural residues, wood, municipal solid waste and dedicated energy. These biomasses are composed primarily of cellulose (40–50%), hemicellulose (25–35%) and lignin (15–20%) unlike grain where the major carbohydrate is starch (Gray, Zhao and Emptage 2006). Cellulose has a highly crystalline and compact structure where glucose is linked via β-1,4 glycosidic linkage. This makes cellulose very resistant to microbial degradation. Hemicellulose consists of xylan backbone with various branches of sugars, such as mannose, arabinose and galactose. Ferulic acid ester linkages can covalently link lignin to hemicellulose (Gray, Zhao and Emptage 2006). The main concern for using *S. cerevisiae* for lignocellulosic fermentation is its inability to ferment pentose

sugars, such as xylose and arabinose. Most *S. cerevisiae* strains do not utilize xylose and metabolic engineering is needed to produce strains suitable for biofuel production (Hahn-Hägerdal et al. 2007).

Complete substrate utilization is important in order to make lignocellulosic ethanol processes economically competitive. New cellulases and hemicellulases are being developed. All types of sugars in cellulose and hemicellulose should be converted to ethanol. These sugars are a mixture of hexoses, primarily glucose, and pentoses, mainly xylose. Xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes of *P. stipitis* have been introduced to recombinant *S. cerevisiae* strains (Jeffries 2006). Also a xylose isomerase gene of the anaerobic fungus *Piromyces* sp. E2 has been expressed in *S. cerevisiae* (Kuyper et al. 2003). Both recombinant strains are able to utilize xylose but rates for xylose utilization have been very low.

The strains have to tolerate high sugar and ethanol concentrations in order to decrease the distillation costs. This leads to increased osmolality in the solutions, and thus osmotolerant strains are needed. The fermenting organism must also tolerate inhibitors, which are generated as side products in the hydrolysis processes. These include low molecular weight organic acids, furans and aromatics. Ethanol yield and ethanol productivity are, however, most important factors. Ethanolic fermentation of glucose and starch already reach 90-95 % of the theoretical yield but the yields from other sugars, such as xylose or other pentoses are lower (Hahn-Hägerdal et al. 2007).

The use of ethanol as a fuel is not a new development. Already in the 1860s an ethanol using prototype of a spark ignition engine was designed and ethanol was used as fuel for the T model Ford in the beginning of 1900s. Between 1925 and 1945 ethanol was added to gasoline as an anti-knocking additive. Then ethanol production was abolished due to the low price of gasoline until Brazil decided to start using ethanol again as a fuel in the 1970s (Antoni, Zverlov and Schwarz 2007). However, ethanol is not an ideal fuel because it has lower energy density than gasoline, and it is hygroscopic, which causes problems for storage and distribution (Atsumi, Hanai and Liao 2008). Higher alcohols (C4 or higher) would be better choices as their energy densities are closer to gasoline and they are not hygroscopic. The problem is that no micro-organism has been identified to produce industrially relevant quantities of higher alcohols. Atsumi et al. suggests synthetic approach and metabolic engineering as a solution to produce these higher alcohols. One solution is the utilization of 2-keto acid degradation of the

Ehrlich pathway where amino acids are catabolised to α-keto acids and then decarboxylated to aldehydes (Hazelwood et al. 2008). 2-Keto-acid decarboxylases are important for this alcohol production method and they are common in yeasts, fungi and plants but rare in prokaryotes (Atsumi, Hanai and Liao 2008). Alcohol dehydrogenases can then be used to produce alcohols from aldehydes (Atsumi, Hanai and Liao 2008, Hazelwood et al. 2008). The problem also in this case is that expression of non-native pathways may cause metabolic imbalance and accumulation of heterologous metabolites may cause cytotoxicity (Atsumi, Hanai and Liao 2008).

1.3 Oxidation of hexoses

1.3.1 Embden-Meyerhof-Parnas pathway

Embden-Meyerhof-Parnas pathway, also called glycolytic pathway, is the most commonly used oxidation route for hexoses. As a result of oxidation it forms two pyruvate, two ATP and two NADH molecules from each hexose molecule. Glycolysis can be considered as one of the classic central metabolism pathways together with pentose phosphate pathway and citric acid cycle. It has been found that alternative pathways and abbreviated forms can replace all these pathways.

1.3.2 Entner-Doudoroff pathway

Not all microbes have phosphofructokinase, which is a key enzyme of glycolysis. These microbes use an alternative route for sugar degradation, which is called Entner-Doudoroff pathway. In addition to Embden-Meyerhof-Parnas and pentose phosphate pathways it can be considered as one of three pathways found in nature that provide pyruvate. It is found to be common especially in gram-negative bacteria but it is present in all three phylogenic domains: *Bacteria*, *Archaea* and *Eukaryota* (Peekhaus, Conway 1998). Sometimes different pathways are used for different sugars: *Halococcus* metabolizes glucose via a modified Entner-Doudoroff pathway but fructose is degraded via the Embden-Meyerhof-Parnas pathway. In fact, all possible combinations of classic, alternative and abbreviated metabolic pathways are found together in micro-organisms (Romano and Conway 1996).

The first step of the Entner-Doudoroff pathway is oxidation of glucose-6-phosphate to 6-phosphogluconate as in the pentose phosphate pathway (figure 1). The Entner-Doudoroff pathway has only two reactions different from the glycolytic and

pentose phosphate pathway. 6-phosphogluconate dehydratase forms 2-keto-3-deoxy-6-phosphogluconate (D-erythro-3-deoxy-hexulosonate) which is then cleaved by an aldolase to form pyruvate and glyceraldehyde-3-phosphate. From this on the pathway is similar to the glycolytic pathway. The main differences between these pathways are that in the Entner-Doudoroff pathway one NADPH is formed and the net gain is only one ATP unlike in glycolysis where two ATP and two NADH are formed.

1.3.3 Non-phosphorylated Entner-Doudoroff pathway

There are two known modifications of the Entner-Doudoroff pathway (figure 1). The partially or semi non-phosphorylated pathway is found in halophilic archaebacteria and a few eubacteria. Here glucose is oxidized to gluconate and then phosphorylated to 2-keto-3-deoxy-6-phosphogluconate. From this step on the pathway continues as the normal Entner-Doudoroff pathway. The other modification for Entner-Doudoroff is the non-phosphorylated Entner-Doudoroff pathway where none of the intermediates are phosphorylated. This pathway is used by archaebacterial genera *Thermoplasma*, *Thermoproteus* and *Sulfolobus*. The first steps are as in the partially non-phosphorylated Entner-Doudoroff pathway described above. Then 2-keto-3-deoxygluconate is cleaved to glyceraldehyde and puryvate. Glyceraldehyde is oxidised further to form glycerate and then phosphorylated to form 2-phosphoglycerate. From this point on the pathway continues as the normal Entner-Doudoroff pathway. Enolase and pyruvate kinase are metabolising 2-phosphoglycerate to pyruvate. There is no net synthesis of ATP from the metabolism of the hexose to pyruvate by this pathway. (Lengeler, Drews and Schlegel 1999)

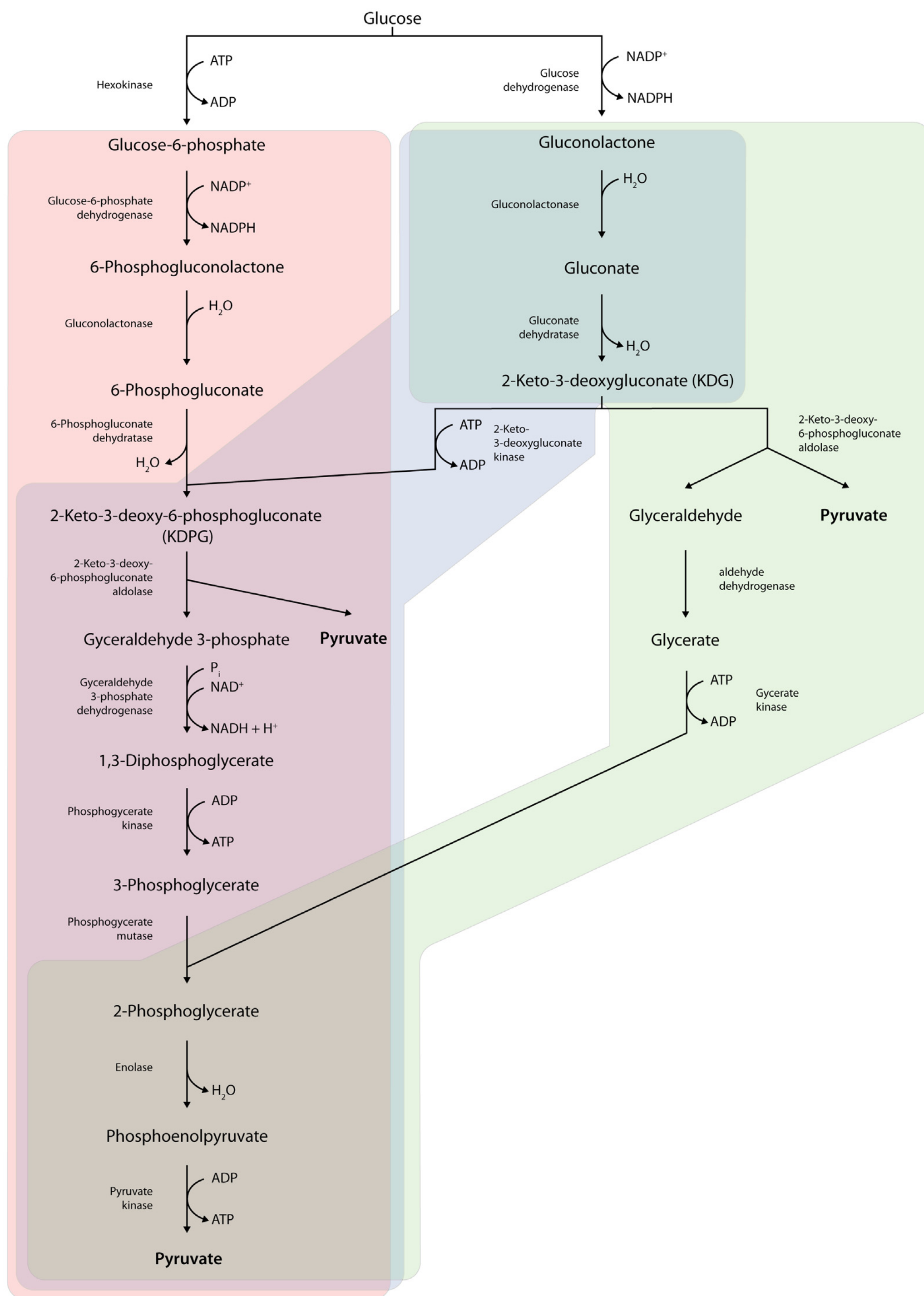

Figure 1. The Entner-Doudoroff pathway on the left (pink), partially non-phosphorylated Entner-Doudoroff pathway in the middle (blue) and the non-phosphorylated Entner-Doudoroff pathway on the right (green).

1.4 L-Rhamnose

L-Rhamnose (L-6-deoxy-mannose) is a deoxy sugar that is enriched in some fractions of plant biomass, such as hemicellulose and pectin. It was also first isolated from a plant called buckthorn (*Rhamnus*) and then named after the plant. L-Rhamnose is also found widely in bacteria as a common component of the cell wall. Different from most other sugars, the L-form of rhamnose is more common in nature than the D-form. Many micro-organisms living on decaying plant material are able to use L-rhamnose as a carbon source. As a 6-deoxy sugar, L-rhamnose is more reduced than the rapidly fermentable sugars like glucose and fructose. The L-rhamnose pathways have been studied since the 1950s.

1.4.1 Biosynthetic pathway of L-rhamnose

The biosynthetic pathway for L-rhamnose has been found only in bacteria and plants. The pathway can be found in both gram-negative and gram-positive bacteria. Bacteria are synthesizing L-rhamnose from glucose-1-phosphate. The intermediates and the encoding genes have been identified. *Pseudomonas aeruginosa* is also known to synthesize the other enantiomer of rhamnose, D-rhamnose from guanosine diphosphate-mannose. L-Rhamnose is a common component of the O-antigen of lipopolysaccharides of gram-negative bacteria as *E.coli*, *Salmonella enterica* and *Shigella flexneri*. A biosynthetic pathway for L-rhamnose has not been identified in humans so there has been interest in it as a potential therapeutic target as drugs inhibiting the enzymes of this pathway are unlikely to interfere with metabolic pathways in humans (Giraud and Naismith 2000).

The first enzyme of the L-rhamnose biosynthetic pathway, RmlA, glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24), converts glucose-1-phosphate to deoxythymidine diphosphate- D-glucose. Then RmlB, dTDP- D-glucose-4,6-dehydratase (EC 4.2.1.46) catalyses the dehydration of deoxythymidine diphosphate- D-glucose to form deoxythymidine diphosphate-4-keto 6-deoxy- D-glucose. The third enzyme, RmlC, deoxythymidine diphosphate-6-deoxy- D-xylo-4-hexulose 3,5 epimerase (EC 5.1.3.13) catalyses double epimerization reaction at positions C3 and C5 forming deoxythymidine diphosphate- L-lyxo-6-deoxy-4-hexulose. Finally RmlD, deoxythymidine diphosphate-6-deoxy- L-lyxo-4-hexulose reductase (EC1.1.1.133) reduces the C4 keto group to hydroxyl and to form the final product deoxythymidine diphosphate- L-rhamnose. Dele-

tion of one or several of *Rml* genes results for example severe colonization defects for *Vibrio cholerae*, inhibits cell-wall polysaccharide synthesis of *Streptococcus mutans* and leads *E. coli* to a loss of serum resistance (Giraud and Naismith 2000).

1.4.2 L-Rhamnose catabolism

There are at least two different pathways for L-rhamnose catabolism. The one described in bacteria, for example in *Escherichia coli*, involves the following intermediates: L-rhamnulose, L-rhamnulose 1-phosphate, dihydroxyacetone phosphate and L-lactaldehyde. The corresponding enzymes for this pathway involving the phosphorylated intermediates include L-rhamnose isomerase (EC 5.3.1.14) (Takagi and Sawada 1964a, Wilson and Ajl 1957a), rhamnulokinase (EC 2.7.1.5) (Takagi and Sawada 1964b, Wilson and Ajl 1957b) and rhamnulose-1-phosphate aldolase (EC 4.1.2.19) (Sawada and Takagi 1964). Depending on the redox conditions, L-lactaldehyde can then be reduced to 1,2-propanediol or oxidized to lactate by lactaldehyde reductase (EC 1.1.1.77) or lactaldehyde dehydrogenase (EC 1.2.1.22). Gene sequences for all these enzymes have been described (Moralejo et al. 1993).

The pathway without phosphorylated intermediates is distinctly different and it has been described in three different yeast species. First it was found by Rigo et al. in the yeast like fungus *Aureobasidium pullulans* (Rigo et al. 1985). In 1984 the pathway involving oxidative intermediates was described also in *P. stipitis* and *Debaryomyces polymorphus* by Twerdochlib et al. (1994). The intermediates in this pathway are L-rhamno-1,4-lactone, L-rhamnoate, L-erythro-3,6-dideoxyhexulosonate, pyruvate and L-lactaldehyde. The corresponding enzymes are NAD-utilizing L-rhamnose-1-dehydrogenase (EC 1.1.1.173), L-rhamno-1,4-lactonase (EC 3.1.1.65), L-rhamnoate dehydratase (EC 4.2.1.90), and L-erythro-3,6-dideoxyhexulosonate aldolase (EC 4.1.2.-). Finally the L-lactaldehyde is oxidized to L-lactate in an NAD-coupled reaction, as in the pathway with the phosphorylated intermediates. The pathway is shown in the figure 2. This pathway has some similarities to the non-phosphorylated Entner-Doudoroff pathway. Common is that the L-erythro-3,6-dideoxy hexulosonic acid, which is similar to the 2-keto-3-deoxygluconate of the Entner-Doudoroff pathway, is split by an aldolase to pyruvate and an aldehyde.

It is known that *S. cerevisiae* does not utilize rhamnose. Van Maris et al. (2006) also reported that *S. cerevisiae* genome does not have genes with a clear homology to genes encoding rhamnose-catabolizing enzymes. As only the genes of the prokaryotic

L-rhamnose route have been known, this would not necessarily mean that there are no homologies to the eukaryotic route. Also the L-rhamnose uptake rate through the plasma membrane of *S. cerevisiae* cells is extremely low. The successful introduction of L-rhamnose pathway to *S. cerevisiae* would require the introduction of the transporter and the enzymes. Engineered strains that can efficiently convert all potentially fermentable substrates of plant biomass hydrolysates are important for enhanced fuel ethanol production.

Van Maris et al. (2006) points out that the introduction of the four enzymes and transporter of the fungal L-rhamnose pathway should enable the conversion of L-rhamnose to equimolar amounts of ethanol, lactaldehyde and CO_2. No ATP would be formed in this pathway. The prokaryotic pathway with the phosphorylated intermediates results in the net generation of two ATP molecules per equimolar amounts of ethanol, L-lactaldehyde and CO_2. This enables the option to select for growth on L-rhamnose but the resulting biomass formation would go at the expense of the ethanol yield. One of the end products of both pathways is L-lactaldehyde, which could be converted to 1,2-propanediol. *S. cerevisiae* already has an enzyme for this reduction, L-lactaldehyde reductase but also one extra NADH per L-lactaldehyde would be required. As a sink for excess reduction equivalents, during fermenting, anaerobic growth, *S. cerevisiae* normally uses glycerol formation for this at the cost of one ATP. This reduces the ethanol yield. Production of 1,2-propanediol might function as an alternative path for excess redox sink and it would actually increase the ethanol yield. Van Maris et al. (2006) came up with the conclusion that bacterial pathway together with a fungal transporter for engineering *S. cerevisiae* would be preferable because this would enable the L-rhamnose selection for growth during the strain construction and long-term industrial cultivation. It is still debatable whether the fungal pathway enabling greater ethanol yield would be better for the end result.

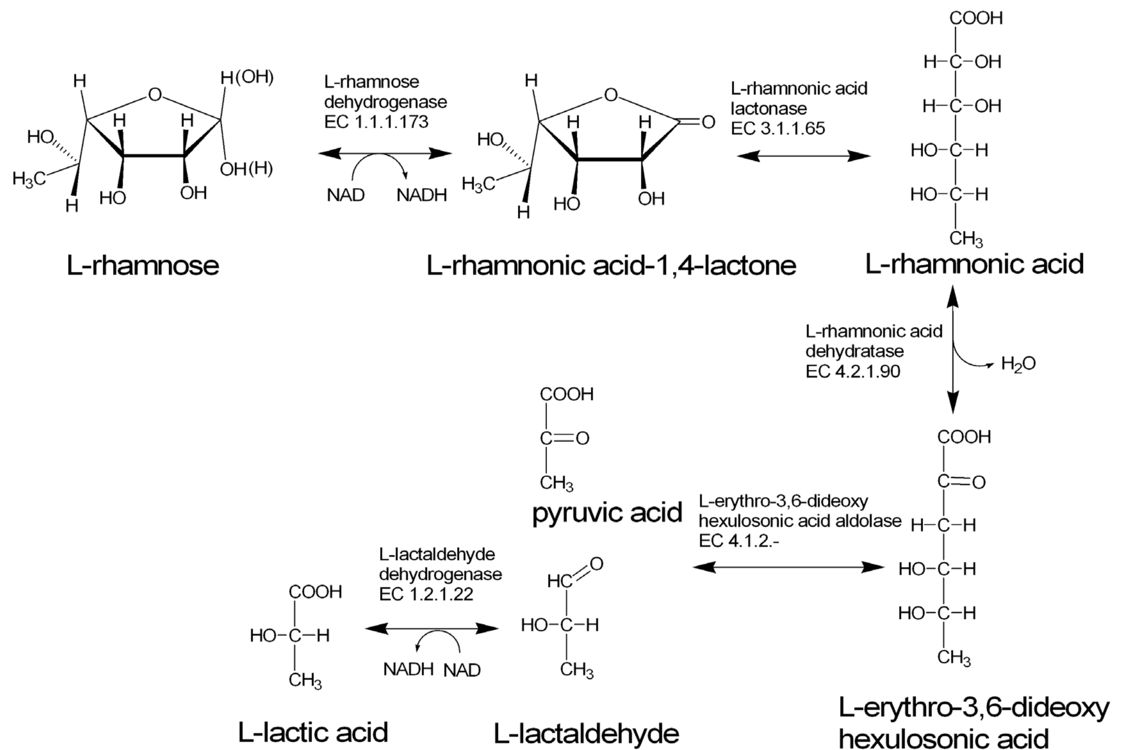

Figure 2. Fungal pathway for L-rhamnose catabolism. The intermediates and enzymes but not the corresponding gene sequences of this pathway have been described previously.

1.5 Catabolic routes similar to the L-rhamnose route

1.5.1 Fungal L-fucose catabolism

L-Fucose is a 6-deoxyhexose like L-rhamnose. The L-fucose pathway (figure 3) in eukaryotic microbes is very similar to the corresponding L-rhamnose pathway. For the yeast like fungus *Aureobasidium pullulans* (synonym to *Pullula pullulans*) this pathway has been described by Guimarães and Veiga (1990). The intermediates in this pathway are L-fucono-1,4-lactone, L-fuconate, 2-keto-3-deoxy- L-fuconate, pyruvate and L-lactaldehyde. The first step of the pathway is catalyzed by an NAD-utilizing L-fucose-dehydrogenase. The second step is spontaneous hydrolysis due to the instability of the L-fuconate-1,4-lactone. Then the L-fucose dehydratase dehydrates L-fuconate to 2-keto-3-deoxy- L-fuconate which is cleaved to pyruvate and L-lactaldehyde. The enzyme activities have been described but the corresponding protein and gene sequences are not known.

1.5.2 Fungal D-galactose catabolism

The most well known pathway for D-galactose catabolism is the Leloir pathway, which has phosphorylated intermediates. There is also another pathway for D-galactose, which has phosphorylated intermediates. It has been called the DeLey-Doudoroff pathway. The DeLey-Doudoroff pathway has a similar route as the Entner-Doudoroff pathway and the end products pyruvate and glyceraldehyde-3-phosphate are identical to the Entner-Doudoroff pathway. As with the L-rhamnose and L-fucose there is also a non-phosphorylated pathway for D-galactose, which has been found in a strain of the fungus *Aspergillus niger* (Elshafei, Abdel-Fatah 2001) (figure 3). The intermediates in this pathway are D-galactonic acid-γ-lactone, D-galactonate, 2-keto-3-deoxy- D-galactonate, pyruvate and D-glyceraldehyde. The involved enzymes are analogous to the enzymes of the L-rhamnose and L-fucose pathways. Also here the corresponding sequences for the proteins are not known.

1.5.3 Fungal D-gluconate catabolism

A fungal D-gluconate pathway has been described in the fungus *Aspergillus niger* (Elzainy, Hassan and Allam 1973) (figure 3). The first step of the pathway is the oxidation of D-gluconate by D-gluconate dehydratase. Resulting 2-keto-3-deoxy- D-gluconate is cleaved by an aldolase to form pyruvate and glyceraldehyde. Also in this case the protein sequences are not known.

1.5.4 Fungal D-galacturonate catabolism

D-Galacturonate is a sugar acid and principal component of pectin. The non-phosphorylated fungal pathway, found first from the mould *Hypocrea jecorina* is also distinctly different from the bacterial pathway (figure 3). The bacterial pathway converts D-galacturonate to pyruvate and D-glyceraldehyde-3-phosphate. The intermediates are D-tagaturonate, D-altronate, 2-keto-3-deoxy-D-gluconate and 2-keto-3-deoxy-D-gluconate-6-phosphate. The enzymes are uronate isomerase, NADH utilizing D-tagaturonate reductase, altronate dehydratase, 2-keto-3-deoxy-D-gluconate kinase and 2-keto-3-deoxy-D-gluconate-6-phosphate aldolase. The fungal pathway is different in that is does not have phosphorylated intermediates and that is has also similarities to the above described pathways. Different to the above described is that this pathway starts from sugar acid. The first step of the fungal pathway is conversion of D-galacturonate to L-galactonate by a reductase. Then dehydratase converts L-galactonate to 2-keto-3-deoxy-L-galactonate, which is cleaved by an aldolase to pyruvate and L-glyceraldehyde. L-glyceraldehyde is then further converted to glycerol by a reductase. Corresponding genes are *gar1* coding D-galacturonate reductase (Kuorelahti et al. 2005), *lgd1* coding L-galactonate dehydratase (Kuorelahti et al. 2006), *lga1* coding 2-keto-3-deoxy-L-galactonate aldolase (Hilditch et al. 2007) and *gld1* coding for L-glyceraldehyde reductase (Liepins et al. 2006).

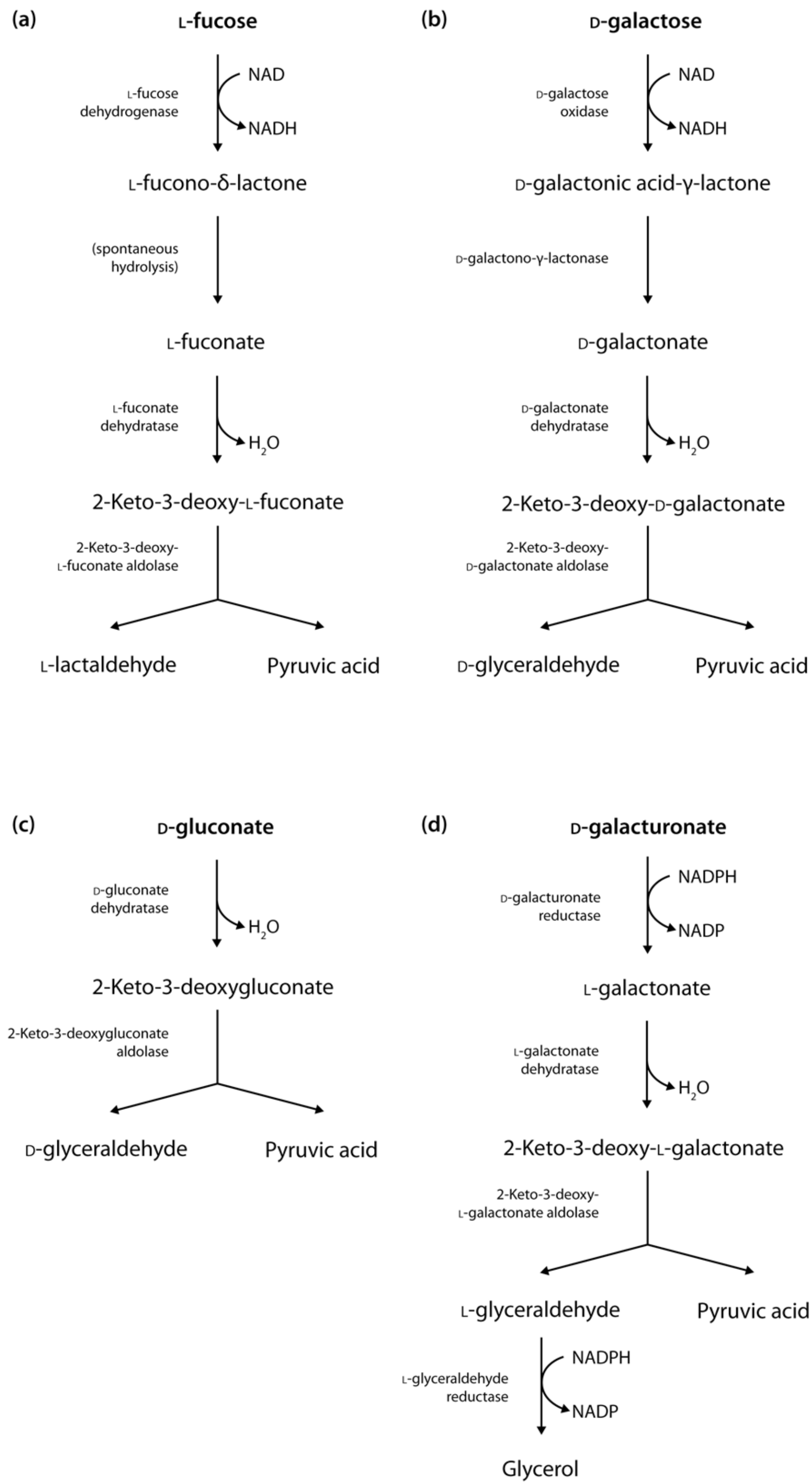

Figure 3. (a) Fungal pathway for L-fucose, (b) Fungal pathway for D-galactose, (c) Fungal pathway for D-gluconate and (d) Fungal pathway for D-galacturonate.

1.6 Aim of the work

The aim of this work was to characterize some of the genes coding the catabolic pathway of L-rhamnose in yeast. As pointed out above, the intermediates and the enzymes of the route are known but the genes of the non-phosphorylated pathway have still not been identified. It is important to know the corresponding gene sequences in order to exploit L-rhamnose pathway in the engineering of *S. cerevisiae* or some other microbes, which are not naturally able to utilize L-rhamnose. Thus the goal was to purify the enzymes in order to identify the corresponding amino acid sequences with MALDI-TOF MS. Then the gene is to be cloned and expressed in *S. cerevisiae* for the kinetic studies. A deletion of the gene is a good method to prove that there are no alternative routes for L-rhamnose catabolism and Northern analysis would give some information about the induction of genes.

2 Materials and Methods

2.1 Yeast and bacterial strains and plasmids

The wild type *P. stipitis* yeast strain CBS 6054 was used for all enzyme assays. Uracil auxotroph *P. stipitis* strain UC7 (H2818) was used for the knock-outs. For transforming *P. stipitis* pJM6 (Yang et al. 1994) based pJML223 and pJML225 plasmids were used. The pJML223 plasmid (p2852) has a *TKT1* promoter and a *XYL1* terminator. The pJML225 (p2853) plasmid has a *XYL1* promoter and terminator. *XYL1* is known to be glucose repressed and *TKT1* is elevated when grown on xylose medium (Jeffries et al. 2007). Xylose was used as a carbon source when these plasmids were used.

The *S. cerevisiae* strain H1346 used in this work is a modification of CEN.PK2 (H1346) strain (VW-1b (MAT**a**) *leu2-3/112*, *ura3-52*, *trp1-289*, *his3-Δ1*, *MAL2-8^C*, *SUC2*) (Stansfield, Stark 2007). This strain was transformed with a multicopy yeast expression vector YEplac195 (p1181) (Verho et al. 2002) containing *URA3* for selection, where the *PGK1* promoter and terminator were introduced. The transformed open reading frames were released as a *Bam*HI fragment and ligated to the *Bgl*II site of the B1181.

For bacterial transformations *E. coli* strain DH5α (F-, *end*A1, *hsd*R17, *rec*A1, *gyr*A96, *rel*A1, ϕ80dΔ*lac*ZM15), XL1-Blue Supercompetent (Stratagene, USA) and TOP10 Electrocomp (Invitrogen, USA) strains were used.

All the plasmids used in the experiments are listed in the table 1 with a short description. Later in the text plasmids are referred to by their p-numbers.

Table 1. Plasmids used in this work. p-number, a short description and a reference.

p-number	Description	Reference
p1181	YEplac195 with *PGK1* promoter and terminator, 2μ, URA3, Amp; 7101bp	VTT lab collection
p2852	JML223, *TKT1* promoter and *XYL1* terminator	VTT lab collection
p2853	JML225, *XYL1* promoter and terminator	VTT lab collection
p3023	*Nat1* wo CTG from GeneArt	This work
p3025	*RDH1* of *P. stipitis* cloned into pCR2.1-TOPO vector	This work

p3026	*Nat1* containing *Bgl*II fragment of B3023 ligated to *Bam*HI sites of B2852	This work
p3027	*Nat1* containing *Bgl*II fragment of B3023 ligated to *Bam*HI sites of B2853	This work
p3069	*Bam*HI cut histidine tagged (C-terminal) *RDH1* fragment ligated to *Bgl*II cut B1181	This work
p3070	*Bam*HI cut histidine tagged (N-terminal) *RDH1* fragment ligated to *Bgl*II cut B1181	This work
p3090	*RDH1* without CTG codon cloned into pCR2.1-TOPO vector	This work
p3132	*RDH1* without CTG codon fragment ligated to *Bgl*II cut B1181	This work

2.2 Media and growth conditions

The nutritious growth medium (YPD) used for *P. stipitis* consisted of 1% Bacto-yeast extract (BD, USA), 2% Bacto-peptone (BD, USA) and 2 % D-glucose (Sigma-Aldrich, Germany). YPX used for *P. stipitis* clones transformed with pJML plasmids was similar to YPD except D-xylose (Sigma-Aldrich, Germany) was used as a carbon source instead of D-glucose. Solid media contained also 2% Bacto-agar (BD, USA). For the knockout transformants 150 μg/ml nourseotrichine (Werner Bio-Agents, Germany) was added.

The synthetic complete (SC) media used for *S. cerevisiae* contained 6.7 g/l YNB without amino acids (BD, USA) and amino acid/nucleotide stock to get following concentrations; 0.1 mM L-adenine; 2 mM L-arginine; 2 mM L-aspartic acid; 0.4 mM L-histidine; 0.2 mM myo-inositol; 4 mM L-isoleucine; 2 mM L-leucine; 0.6 mM L-lysine; 1 mM L-methionine; 0.5 mM L-phenylalanine; 1 mM L-serine; 1 mM L-threonine; 0.4 mM L-tryptophan; 0.2 mM L-tyrosine; 0.2 mM uracil and 1 mM L-valine. Transformats having *URA3* marker for uracil selection were grown on media lacking uracil. SCD-medium was prepared from SC-medium by adding 2 % D-glucose as carbon source.

SOC medium containing 2 % Bacto-tryptone (BD, USA), 0,5 % Bacto-yeast extract (BD, USA), 10 mM NaCl, 2 mM KCl, 10 mM $MgCl_2$, 10 $MgSO_4$ and 0,4 % D-glucose (Sigma-Aldrich, Germany) was used as a recovery broth for *E. coli* cells after electoporation. The *E. coli* cultivations were grown in LB-broth, which consisted of 1 % Bacto-tryptone, 0.5 % Bacto-yeast extract (BD, USA) and 0.5 % NaCl. For the plasmid

selection 100 μg/ml ampicillin (Sigma-Aldrich, Germany) was added. Bacteria were grown in glass test tubes or in 50 ml erlenmeyer flasks at the temperature of +37 °C and shaken by 250 rpm.

2.3 Recombinant DNA techniques

The restriction enzymes *Bam*HI, *Bgl*II, *Hind*III, *Spe*I , *Dpn*I, *Xho*I, *Eco*RI (New England BioLabs, USA), the T4 DNA-ligase (Promega, USA), and the Calf intestinal alkaline phosphatase (Finnzymes, Finland) were used as described in the manuals of the manufacturers. The concentrations used in the PCR-reactions were the ones recommended by the manufacturers. The polymerases used in the PCR-reactions were DyNAzyme EXT (Finnzymes, Finland), which is an optimised mixture of DyNAzyme II DNA Polymerase and a proofreading enzyme, DyNAzyme II (Finnzymes, Finland) and Phusion™ high fidelity polymerase (Finnzymes, Finland) was used for the PCR reactions needed for the deletion cassette. The DNA fragments digested with the restriction enzymes were separated by electrophoresis in 1% agarose gel (SeaKem LE agarose by BMA, USA), and when necessary, DNA fragments were separated from the gel by using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) or MinElute Gel Extraction Kit (QIAGEN GmbH, Germany) when the elution volumes were small. GeneRuler™ 1 kb DNA Ladder (Fermentas, USA) was used as a standard size marker in the agarose gels. TD loading buffer [20 % Ficoll 400 (Amersham Biosciences, USA), 0.0125 % bromophenol blue (Merck, Germany), 0,1 % xylene cyanol ff (Chroma-Gesellschaft, Germany), 250 mM Tris pH 8,0 (MP Biomedicals, USA), 25 mM boric acid (Sigma-Aldrich, Germany), 125 mM EDTA, pH 7,5 (Sigma-Aldrich, Germany), 0,5 % SDS (Sigma-Aldrich, Germany)] was used at 1/5 of the volume to all the DNA samples ran in agarose gel.

All *E. coli* plasmid purifications were done by using QIAprep Spin Miniprep Kit protocol (Qiagen GmbH, Germany). QIAprep Miniprep has been developed for purification of plasmid DNA. Method is based on alkaline lysis of bacterial cells followed by the absorbtion of the DNA onto silica-gel membrane in the high salt concentration (Birnboim and Doly 1979, Vogelstein and Gillespie 1979).

All primers used in this work were manufactured by Sigma-Genosys Ltd. (UK) (Table 2).

Table 2. Primers used for PCR and sequencing reactions: number, name and sequence. Restriction sites for *Bam*HI are underlined.

Number	Name	Sequence (5´to 3´)	Used for
O7892	PsRDHfwdCTG	CTGAAAGAGGAAATTCTCGGACGGTGAAAACAACGTGCTG	CTG codon mutagenesis for *P. stipitis RHA1* gene
O7893	PsRDHrevCTG	GAGCAGGTTGTTTTCACCGTCCGAGATTTCCTCTTTGAG	CTG codon mutagenesis for *P. stipitis RHA1* gene
O7871	PsRDHfwdBamHI	GGATCCATCATGACTGGATTGTTGAATGG	*RHA1* amplifying from the genomic DNA of *P. stipitis*
O7872	PsRDHrevBamHI	GGATCCCTATTGTAAATTGACGAACAATCCTC	*RHA1* amplifying from the genomic DNA of *P. stipitis*
O7885	NAT1fwd	GATCTAAGCTTATGACCACTC	For sequencing
O7886	XYL1rev	CTCTATAAAGCAACCTTCCTG	For sequencing
O7902	RDHdel5fwd	GTAACGCCAGGGTTTTCCCAGTCACGACGACTAGTGGTAGATTGCTACATGTCTC	For constructing the deletion cassette
O7903	RDHdel5rev	TAGCATACAAAACTACTTCTGCATCATATCGATACTGCCAAAATTGGGGC	For constructing the deletion cassette
O7904	RDHdel3fwdXYL1	TAATGTAGAACCAATTAGTGTCTGTGGATCCTTGTATCTTCGTCCATACC	For constructing the deletion cassette
O7905	RDHdel3fwdTKT1	AGATAGTTGGTTGAGTAGCATGAACTCTGGTCTTGTATCTTCGTCCATACC	For constructing the deletion cassette
O7906	RDHdel3rev	GCGGATAACAATTTCACACAGGAAACAGCACTAGTCGGATTTTCCCCCATCTCAC	For constructing the deletion cassette
O7907	hisRDHfwdBamHI	GGATCCATCATGCATCACCATCACCATCACGGTGGAATGACTGGATTGTTGAATGG	Adding the histidine tag to the C-terminal end of the *RHA1* gene
O7996	hisRDHrevBamHI	GGATCCCTAGTGATGGTGATGGTGATGACCTCCTTGTAAATTGACGAACAATCCTC	Adding the histidine tag to the N-terminal end of the *RHA1* gene
O8046	delxylprom	GGAGGAACGCAGACAGAAAC	For constructing the deletion cassette
O8047	deltktprom	CAACTCACGTGCATACCAATC	For constructing the deletion cassette

O8048	delxylterm	CACCTCATCAATTCATTGC	For constructing the deletion cassette
O8049	del5middle	CAGTCGCCATTGATCAACTC	For sequencing
O8050	del3middle	CACCAAGTGAAACTCGCTTG	For sequencing
O8081	delRDHflanktark5	GAAAGCATCGTAAGTCCGTAG	For sequencing
O8082	delRDHflanktark3	CAGCTGCAGTACGATAAGAG	For sequencing
O8083	revNat1	ACCCATCGAGTGCCTCGATG	For sequencing
	M13 Universal primer forwad	GACCGGCAGCAAAATG	For sequencing
	M13 Universal primer reverse	CAGGAAACAGCTATGAC	For sequencing

2.4 Transformations

2.4.1 *E. coli* transformations

E. coli transformations were done by electroporation. 40 µl of competent DH5α *E. coli* cells, stored at –70 °C, were thawed on ice and 2 µl of plasmid DNA was added. For electroporation the GenePulser ™ (BioRad, USA) was used with following settings: 25 μF, 200 Ω and 2.50 kV. The mixture was transferred to an ice-cold 0.2 mm electroporation cuvette (Bio Rad, USA). Immediately after the pulse 1 ml SOC medium was added. The suspension was incubated for one hour at +37 °C and then plated on LB-plates with ampicillin (Sigma-Aldrich, Germany).

For transformations done according to Topo TA Cloning® kit (Invitrogen, USA) the plates were also supplemented with 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactorytanoside). X-gal is used for blue/white screening distinguishing recombinant colonies (white) among non-recombinant ones (blue). The *LacZ* gene of TOPO vector encodes β-galactosidase, which hydrolyzes X-gal and forms an intense blue precipitate and blue colonies. The fragment cloned to the TOPO cloning site interrupts the *LacZ* and colonies remain white.

2.4.2 *S. cerevisiae* transformations

S. cerevisiae yeast transformations were done by using the Gietz Lab Transformation Kit (Molecular Research Reagents Inc.) according to manufacturer's instructions. The transformants were plated on SCD plates lacking uracil and incubated at +30 °C for two to three days.

2.4.3 *P. stipitis* transformations

For *P. stipitis* yeast transformation numerous different methods were tried. For the deletion cassette transformation the method described by Dohmen et al. (1991) was used with the improvements presented by Scruff (2005). PEG 1000 polyethylene glycol was substituted by PEG 4000 (Sigma-Aldrich, Germany) polyethylene glycol.

2.5 Sequencing

All DNA sequencings were done according to the instructions given by Adam et al. (2007) for the Big Dye® sequencing kit (Applied Biosystems, USA) (Platt, Woodhall and George 2007). Capillary electrophoresis was used to analyse the reactions by ABI Prism® 3100 Genetic Analyser (PE/Applied Biosystems, Perkin Elmer, USA).

The resulted *P. stipitis* DNA sequences were compared to sequences from the *P. stipitis* v2.0 database (DOE Joint Genome Institute) using sequence analysis software DNAMAN 4.1 (Lynnon BioSoft, Canada).

2.6 Enzyme purification

P. stipitis CBS 6054 was grown in 500 ml shake flasks containing YNB without amino acids (BD, USA) and 2 % L-rhamnose (Sigma-Aldrich, Germany) as a carbon source. Also controls where 2 % D-glucose or 1% L-rhamnose and 1% D-glucose were used as carbon sources were grown in the same way. Flasks were shaken in +28 °C for two days. After that yeast cells were collected by centrifugation (Hettich AHT 35R) by 3000 g for 10 minutes. Cells were washed and then resuspended into 40 ml of 5 mM sodium phosphate pH 7.0 supplemented with Complete EDTA free protease inhibitor (Roche, Switzerland) according to manufacturer's instructions. The cells were kept on ice. Equal amount of 0.4 mm of diameter glass beads (Sigma-Aldrich, Germany), fresh cell cake and resuspension buffer were added. The cells were broken by shaking them in the

Mini-Beadbeater (Biospec Products) for two times one minute. The mixture was centrifuged in an Eppendorf microcentrifuge at full speed for 20 minutes at +4 °C. The supernatants were collected and desalted in PD-10 Sephandex G-25M gel filtration desalting columns (Amersham Biosciences, USA) according to manufacturer's instructions.

2.6.1 Anion exchange and gradient

Anion exchange chromatography was used for protein purification: Fractogel TSK DEAE–650 (M) ionexchange column material (Merck, Germany) was pipetted into the column so that the volume of the DEAE gel was 10 ml. The column was first washed with 75 ml of 200 mM NaCl, 5 mM sodium phosphate buffer and then with 100 ml of 5 mM sodium phosphate buffer. A peristaltic pump (P-1, Pharmacia) was adjusted to pump 2,5 ml / 2 minutes and a fraction collector (Frac-100, Pharmacia) was used to collect the eluent. The desalted supernatant was loaded to the column and fraction collecting started immediately. Right after the desalted supernatant had gone through the column it was washed with 150 ml 5 mM sodium phosphate buffer, pH 7.0. A gradient was made by adding 100 ml of 5mM sodium phosphate buffer pH 7.0 into the first container and 100 ml 5 mM sodium phosphate, 200 mM NaCl to the second container. The two containers were connected so that they always had an equal volume. To obtain a linear gradient the solution was pumped from the first container which was also mixed with a magnetic stirrer. Fraction collection was done at +4 °C. Finally the column was eluted with 1 M NaCl to get all the rest of the proteins. All fractions were analyzed for the L-rhamnose dehydrogenase activity. The fractions where the enzymatic activity was observed were combined and concentrated using Vivaspin 2 10,000 MWCO PES centrifugation columns (Vivascience Sartorius group, Germany).

2.6.2 Zymogram staining

Zymography is an electrophoretic method used to identify enzymatic activity of enzymes separated in polyacrylamide gels. The concentrated protein samples were ran in the non-denaturing PAGE gel (Bio-Rad 12% Tris-HCl acrylamide gel, Bio-Rad, USA) using a buffer containing 25 mM Tris base (Sigma-Aldrich, Germany) and 192 mM glycine. As a loading buffer for the non-denaturing PAGE a mix of 0.4 M Tris-HCl pH 6.8, 30% glycerol and 0,003% bromophenol blue was added at 1/5 of the volume to the sample. Electrophoresis was done on ice to avoid the system to heat and the proteins to denature. The gel was then stained in a zymogram staining solution containing 0.25

mM nitroblue tetrazolium (Promega, USA), 0.06 mM phenazine methosulfate (Sigma-Aldrich, Germany), 0,5 mM NAD (Sigma-Aldrich, Germany), 200 mM Tris-HCl (pH 8.0), 100 mM L-rhamnose (Sigma-Aldrich, Germany). The only band that appeared during the overnight incubation was cut out and the protein was eluted in 300 μl of buffer [200 mM Tris-HCl (pH 8.0), 0.1 % SDS (Sigma-Aldrich, Germany)] during overnight incubation. The eluted protein was concentrated using Microcon YM-10 (Millipore, USA) column according to manufacturer's instructions. Also the saved DEAE sample not used in the zymogram staining was concentrated in the same way. LSB+β-Me [0.1 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS (Sigma-Aldrich, Germany), 0.02% bromphenol blue (Merck, Germany) and 10% β-mercaptoethanol (Sigma-Aldrich, Germany)] loading buffer was added at 1/4 of the volume to the sample. To denature the proteins both samples were heated for 5 minutes at 95 °C before they were pipetted to the SDS-PAGE gel. SDS-PAGE gel was ran using similar buffer as with the non-denaturing PAGE except also 0.1 % SDS was added.

2.6.3 SDS PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique to separate proteins according to their size (Laemmli 1970). SDS PAGE was used to analyze the concentrated protein samples. Commercial Bio-Rad poly-acrylamidegel (Bio-Rad, ReadyGel, 12% Tris-HCl, Bio-Rad, USA) was used. Bio-Rad Prestained SDS-PAGE standard, Low Range (Bio-Rad, USA), was used as a standard size marker. Tris-glycine-SDS buffer [25 mM Tris, 192 mM glycine and 1% SDS (Sigma-Aldrich, Germany)] was used as a running buffer. After electrophoresis the gel was stained with Coomassie Brilliant Blue R-250 stain solution [500 mg Coomassie Brilliant Blue R-250 Serva Blau R (Serva, Germany), 150 ml ethanol, 50 ml acetic acid, 300 ml H_2O] for one hour to make the protein bands visible. The gel was then washed with destain solution I (40% ethanol, 10 % acetic acid) for two times 15 minutes and with destain solution II (5 % ethanol, 7.5 % acetic acid). The gel was left into the destain solution II for one day and then moved into storage solution containing 4 % glycerol.

2.7 In-gel digestion MALDI-TOF MS

The four proteins observed in the SDS-PAGE gel were cut out and in-gel digested with trypsin and the peptides were extracted essentially according to the method of

Rosenfeld et al. (1992). The samples were desalted using a C-18 matrix (Eppendorf Perfect Pure C-18 Tip). The saturated matrix solution was prepared by dissolving re-crystallized α-cyano-4-hydroxycinnamic acid (CCA, Bruker Daltonics, Germany) in a 50 % acetonitrile in 0.1 % trifluoroacetic acid solution. Equal volumes of purified peptide sample or calibration standard (peptide calibration mixture II, Bruker Daltonics, Germany) were mixed with the saturated matrix solution. 1 μl of this matrix/sample mixture was applied onto the target (Target plate ground steel T F, Bruker Daltonics) and let to dry at room temperature. The peptide masses were then determined by MALDI-TOF MS using a Bruker Autoflex II mass spectrometer. FlexAnalysis software (Bruker Daltronics) was used for the data analysis.

2.8 Site directed mutagenesis kit for changing the CTG codon

Before the *RHAI* was transformed to *S. cerevisiae* H1346 the nucleotides 166-168 of the open reading frame were changed from CTG to TCG with the QuikChange® site directed mutagenesis kit (Stratagene, USA) because of the different codon usage of *P. stipitis* and *S. cerevisiae*. The primers O7892 and O7893 were used for the mutagenesis. The *RHAI* gene was amplified from genomic DNA of *P. stipitis* CBS 6054 using the primers O7871 and O7872. The genomic DNA had been isolated prior to this work at the VTT. The fragment was then cloned into the pCR®2.1-TOPO® vector (Invitrogen, USA). Instructions given by the manufacturer were used for the thermal cycling. The mutagenised plasmids were digested with *Dpn*I restriction enzyme (New England BioLabs, USA) for one hour at +37 °C. XL1-Blue Supercompetent cells (Stratagene, USA) were thawed on ice and aliquoted to 50 μl portions which were used for transformation. 1 μl of *Dpn*I-treated DNA from each sample reaction were mixed with the cells and then incubated for 30 minutes on ice. Transformation mixtures were heat pulsed at +42 °C water bath for 45 seconds and then placed on ice for two minutes. Preheated SOC broth was pipetted on the transformation reactions and they were incubated at +37 °C for one hour prior to the plating on LB media with ampicillin (Sigma-Aldrich, Germany). M13 forward and reverse primers were used for checking the sequences and the resulting plasmid was numbered as p3090.

2.9 Cloning of the *RHAI*

The *RHAI* gene without the CTG codon was amplified by PCR from p3090 using the primers O7871 and O7872. *Bam*HI sites had been added to the primers. The gene was also cloned with primers having C- and N-terminal histidine tags. Six histidines were added at the beginning or at the end of the protein. To introduce the histidine-tag at the N-terminus a coding sequence for MHHHHHHGG was introduced before the original start codon by using primer O7996. To introduce the histidine-tag at the C-terminus the coding sequence for GGHHHHHH was introduced before the stop codon by using primer O7907. The open reading frame was amplified using the following PCR program: an initial denaturation 3 min at 94 °C, a denaturation 30 s at 94 °C, an annealing 30 s at 55 °C and an extension 1 min at 72 °C. These three subsequential steps were carried out 25 times until the final extension 7 min at 72°C. The PCR product was applied to gel electrophoresis and the fragment was extracted from the agarose gel by using QIAquick Gel Extraction Kit (Qiagen, USA). The fragment was then cloned into a pCR®2.1-TOPO® vector (Invitrogen, USA) using TOPO TA Cloning kit (Invitrogen, USA) and transformed to the DH5α *E. coli* strain. *Bam*HI digested open reading frame was then added to the *Bgl*II sites of the p1181 yeast expression vector of *S. cerevisiae* which was then transformed into *S. cerevisiae* strain (H1346). The picture of p1181 with the ligated *RHA1* gene is shown in figure 4.

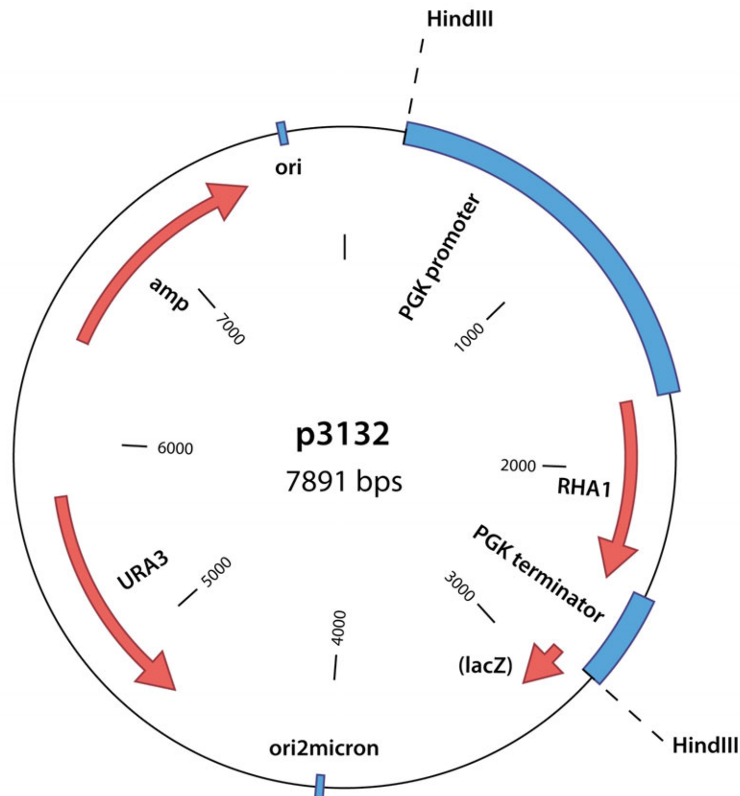

Figure 4. Map of p3132 plasmid for *S. cerevisiae* transformation.

2.10 Enzyme assays

All enzyme assays were done at +30 °C in a Cobas Mira^{+} autosampler analyzer (Roche Diagnostics System, Switzerland). The enzyme activity of the L-rhamnose dehydrogenase was measured in a reaction mixture containing 100 mM TrisCl (pH 8.0) buffer, 1 mM NAD and the crude cell extract or protein preparation. 10 mM L-rhamnose was added to start the reaction. The absorption at the wavelength of 340 nm was used to detect the formation of NADH. To measure the Michaelis-Menten-constants for L-rhamnose and other sugars the NAD concentration was 0.9 mM and the sugar concentrations were varied from 0.4 mM to 40 mM. When measuring the Michaelis-Menten-constants for NAD the L-rhamnose concentration was 60 mM and the NAD concentration was varied from 0.002 mM to 2 mM. To assay the enzyme activity in the reverse direction the crude extract was incubated in 100 mM TrisCl (pH 8.0), 200 μM NADH and 100 mM L-rhamnonate. The disappearance of NADH was followed by measuring the absorbance at 340 nm.

The enzyme activity of L-lactaldehyde dehydrogenase was assayed by using the same measuring conditions. The reaction mixture contained 100 mM TrisCl (pH 6.8-9.5) buffer, 1 mM NAD (Sigma-Aldrich, Germany), 5mM $MgCl_2$ and the crude cell extract or protein preparation. 60 mM DL-lactaldehyde was added to start the reaction.

The Cobas Mira$^+$ was programmed to measure the activity by calculating the change in absorbance over time before (background) and after the addition of the start reagent. The activity was measured from the changes of coenzyme NAD/NADH concentrations and background activity was subtracted. The molar absorptivity (ε) of NADH at the wavelength of 340 nm is 6.22 $mM^{-1}cm^{-1}$. The reaction rate of the enzyme in the volume (v) was calculated as shown in equation 1.

$$v(\frac{nkat}{l}) = \frac{\Delta A}{\varepsilon \cdot l \cdot t} \cdot \frac{V_{total}}{V_{sample}} \cdot 10^9 \, \frac{nmol}{mol} \tag{1}$$

where ΔA = change in absorbance

ε = molar absorptivity

l = length of light path (0.6 cm)

t = time (sec)

V_{total} = total assay volume

V_{sample} = sample volume

The kinetic parameters K_M (Michaelis constant) and V_{max} (the substrate concentration where a constant rate of product formation is achieved) were estimated using the Eadie-Hofstee diagram. The Eadie-Hofstee equation is one of the linearised graphic representation forms of the Michaelis-Menten equation. In the Eadie-Hofstee plot the reaction velocity is plotted as a function of velocity divided by the substrate concentrate ratio (equation 2).

$$v = -K_M \frac{v}{[S]} + V_{\max} \tag{2}$$

where $[S]$ = substrate concentration

v = volumetric rate of reaction

K_M = Michaelis constant for the substrate

V_{max} = the substrate concentration where constant rate of product formation is achieved

The protein amounts of the samples were measured by Cobas Mira$^+$ using the Bradford method (Bradford 1976). 2.0 mg/ml BSA (Bio-Rad, USA) was used as a protein standard. Bio-Rad Protein Assay which contains Coomassie Brilliant Blue G-250 dye in acidic solution was added to the standard and samples were measured at 595 nm wavelength in a spectrophotometer. The absorbance maximum of Coomassie stain changes from 465 nm to 595 nm when it binds to a protein.

The enzymatic activities (nkat/mg protein) were calculated by dividing the maximum volumetric activity (nkat/l) by the protein concentration (mg/l) (equation 3).

$$enzymatic\ activity = \frac{V_{\max}}{c} \qquad (3)$$

2.11 Deletion cassette for *P. stipitis*

Streptomyces noursei gene for nourseothricin acetyltransferase (*nat1*) that gives resistance to nourseothricin (Krugel et al. 1993) was used as an antibiotic resistance marker for *P. stipitis*. The plasmid containing the encoding gene without CUG codons, *Nat1woCUG* (figure 5), was ordered from Geneart (Geneart AG, Germany). The fragment had been cloned into a pGA18 (ampR) vector and it was transformed to DH5α *E. coli* and given a number p3023. The *Nat1woCTG* fragment was cut out using *Bgl*II restriction sites. The *Nat1woCUG* containing *Bgl*II fragment was then ligated into the *Bam*HI digested p2852 and p2853 vectors. DH5α *E. coli* cells were used for transformation.

```
1         ATGACCACTCTTGACGACACGGCTTACCGGTACCGCACCAGTGTCCCGGGGGACGCCGAG
1          M  T  T  L  D  D  T  A  Y  R  Y  R  T  S  V  P  G  D  A  E

61        GCCATCGAGGCACTCGATGGGTCCTTCACCACCGACACCGTCTTCCGCGTCACCGCCACC
21         A  I  E  A  L  D  G  S  F  T  T  D  T  V  F  R  V  T  A  T

121       GGGGACGGCTTCACCTTGCGGGAGGTGCCGGTGGACCCGCCCCTTACCAAGGTGTTCCCC
41         G  D  G  F  T  L  R  E  V  P  V  D  P  P  L  T  K  V  F  P

181       GACGACGAATCGGACGACGAATCGGACGACGGGGAGGACGGCGACCCGGACTCCCGGACG
61         D  D  E  S  D  D  E  S  D  D  G  E  D  G  D  P  D  S  R  T

241       TTCGTCGCGTACGGGGACGACGGCGACTTAGCGGGCTTCGTGGTCGTCTCGTACTCCGGC
81         F  V  A  Y  G  D  D  G  D  L  A  G  F  V  V  V  S  Y  S  G

301       TGGAACCGCCGGCTAACCGTCGAGGACATCGAGGTCGCCCCGGAGCACCGGGGGCACGGG
101        W  N  R  R  L  T  V  E  D  I  E  V  A  P  E  H  R  G  H  G

361       GTCGGGCGCGCGTTGATGGGGCTCGCGACGGAGTTCGCCCGCGAGCGGGGCGCCGGGCAC
121        V  G  R  A  L  M  G  L  A  T  E  F  A  R  E  R  G  A  G  H

421       CTCTGGCTCGAGGTCACCAACGTCAACGCACCGGCGATCCACGCGTACCGGCGGATGGGG
141        L  W  L  E  V  T  N  V  N  A  P  A  I  H  A  Y  R  R  M  G

481       TTCACCCTCTGCGGCCTGGACACCGCCTTGTACGACGGCACCGCCTCGGACGGCGAGCAG
161        F  T  L  C  G  L  D  T  A  L  Y  D  G  T  A  S  D  G  E  Q

541       GCGCTCTACATGAGCATGCCCTGCCCCTAA
181        A  L  Y  M  S  M  P  C  P  *
```

Figure 5. The nucleotide sequence of the *Nat1woCUG* gene, which was used for nourseothrichin resistance in *P. stipitis* and the corresponding protein sequence.

Colot et al. (2006) have described a procedure for high-throughput gene knock-out for filamentious fungi and their method of constructing a plasmid from several fragments with homologous recombination in yeast was used in making the deletion cassette for the *RHA1*. The deletion cassette was designed so that a fragment including *nat1* gene and the *TKT1* or *XYL1* promoter and *XYL1* terminator regions was placed in between 1.0 kb flanks prior and after the *P. stipitis RHA1* open reading frame. The cassette was done according to the instructions (Colot and Collopy) as shown in figure 6. The 5' and 3' flank fragments were amplified separately by PCR from the genomic DNA. 5 forward + 5 reverse (O7902 and O7903) and 3 forward + 3 reverse (O7904 or O7905 and O7906) primer pairs were used for PCR synthesis of the 5' and 3' flank fragments and they also produced additional ends to the flanks homologous to a vector fragment and to the promoter + *nat1* + terminator fragment. Plasmid pRS462 was digested with *Xho*I and *Eco*RI (New England BioLabs, USA) to get a vector containing *URA3* and *amp* for selection. The promoter + *nat1* + terminator fragment was also

amplified by PCR using primer pairs O8046 and O8048 or O8047 and O8048. The four fragments, digested pRS462, promoter + *nat1* + terminator and 1.0 kb 5' and 3' flanks of *RHA1* were then transformed to the *S. cerevisiae* FY834 (H3488) yeast strain that was then grown on SCD plate lacking uracil. The obtained colonies were collected and a plasmid pool was isolated from them as described in 'Knockout Workshop' (Colot and Collopy). The plasmids were transformed to the TOP10 *E. coli* strain that was then grown on LB-ampicillin plates. The deletion cassette with an accurate sequence was chosen after sequencing. The deletion cassette 5' flank + promoter + *nat1* + terminator + 3' flank was obtained by digesting with *Spe*I. This fragment was used to transform *P. stipitis* in order to make a *RHA1* deletion strain.

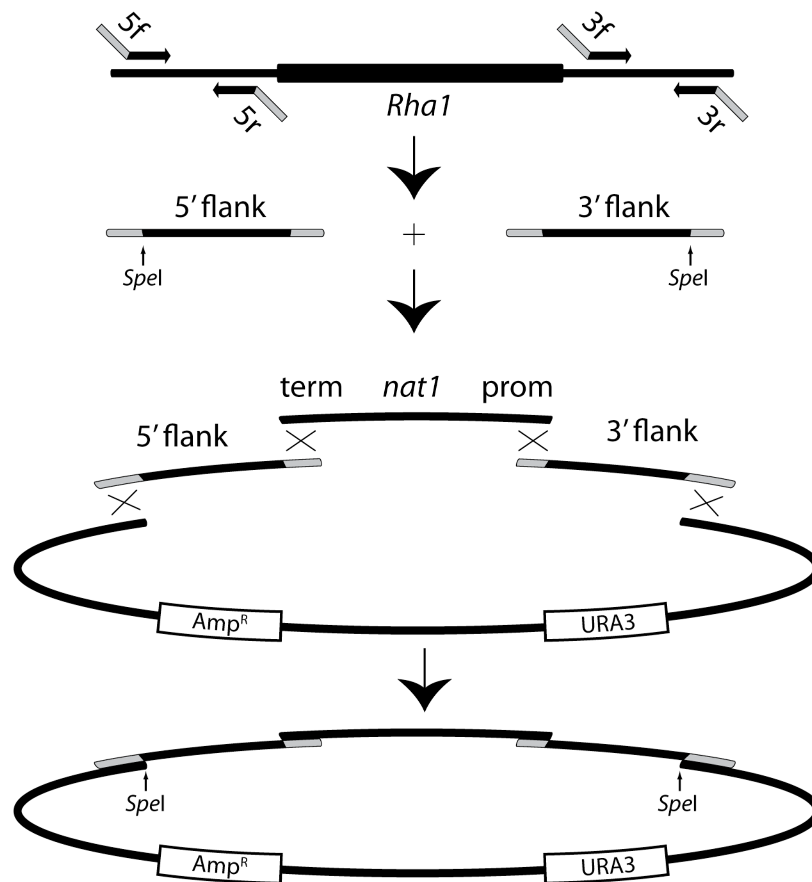

Figure 6. The cassette made to delete *RHA1* gene of *P. stipitis*. Figure is based on the publication of Colot et al (2006).

P. stipitis transformants were plated on YPX medium with 150 μg/ml nourseothricin and incubated at + 30 °C for five days. Colonies were then grown in shake flasks containing YNB without amino acids (BD, USA), amino acid/nucleotide stock and 2 % L-rhamnose as a carbon source. Each colony was also analysed by yeast colony PCR to find out if the deletion fragment was in the right place. Primers pairs O7885 + O8049 and O8083 + O8050 were used. Following PCR program was used: an initial denaturation 3 min at 94 °C, a denaturation 30 s at 94 °C, an annealing 30 s at 50 °C and an extension 2 min at 72 °C. These three sequential steps were carried out 25 times until the final extension 10 min at 72°C. The PCR product was applied to gel electrophoresis.

2.12 Synthesis of L-rhamnoate and L-lactaldehyde

L-Rhamnoate was synthesised by Andreas Petrich (VTT) from L-rhamnose by oxidation with bromine and purified by ion-exchange chromatography as described by Yew et al. (2006). DL-Lactaldehyde was synthesised by Harri Setälä (VTT) using the method first described by Durrwachter et al. (1986) and later modified by Schoevaart (2000). The method is based on the reduction of methylglyoxal 1,1-dimethylacetal. L-Lactaldehyde was synthesised using the method described by Huff and Rudney (1959). It was synthesised by heating ninhydrin with D-threonine. After the addition of $NaHCO_3$ the brown precipitate was removed by filtration. Active carbon Norit A (Norit, USA) was added to remove ninhydrin and the colored products of the reaction. Finally sodium ions and unreacted threonine were removed by Dowex 50 (H^+) (Sigma-Aldrich, Germany).

2.13 Northern analysis

Northern analysis was done using the technique described first by Alwine et al. (Alwine, Kemp and Stark 1977). *P. stipitis* CBS 6054 was grown in YNB medium supplemented with 20 g/l of six different carbon sources: L-rhamnose, D-glucose, maltose, D-galactose, D-xylose and an ethanol/glycerol mixture. The RNA was extracted from the yeast cells with the Trizol reagent kit (Life Technologies Inc., USA) according to the manufacturer's instructions. 5 μg of the total RNA per sample was used in the analysis and the amount was checked by staining with the SYBR Green II RNA gel stain (BMA Biomedicals, Switzerland). 3.7 μl of RNA solution containing 5 μg of RNA was added to 2,7 μl 6 M glyoxal, 8.0 μl dimethylsulfoxide and 1.6 μl 1,2-

dimyristoyl-sn-glycero-3-phosphocholine (DMPC) -NaH_2PO_4 (pH 7.0). Mixtures were incubated at +50 °C for one hour and after cooling to +20 °C samples were loaded to the RNA agarose gel (10 mM DMPC- NaH_2PO_4 (pH 7.0), DMPC-DDIW to get total volume of 350 ml). All equipment and solutions used for RNA work were di-methyl-propyl carbonate (DMPC) (Sigma-Aldrich, Germany) -treated to inactive RNAses.

The RNA gel was blotted over night to the nitrocellulose filter using capillary method and 20x saline-sodium citrate (SSC) solution (860 g NaCl, 440 g trisodium-citratedihydrate, H_2O ad 5000 ml, pH 7.0) as a transfer buffer. After that the RNA was UV cross-linked to the Hybond N membrane (Amersham Biosciences, USA).

10 mg/ml herring sperm DNA was added to the prehybridization mix (500 ml deionised formamide, 100 g dextran sulphate, 1 M NaCl, 1 % SDS (Sigma-Aldrich, Germany), H_2O ad 1000 ml) and mixture was boiled in a water bath for 10 minutes. Pre-hybridization was done in + 42 °C for one hour. As a probe for the hybridization the open reading frame, released as a *Bam*HI from the TOPO vector, was used. The probe was labelled with [α-32P]dCTP (Amersham Biosciences, USA) using the randomly primed DNA labelling kit (Roche, Switzerland). For probe purification a Sephadex G-50 (Amersham Biosciences, USA) gel filtration resin was used. Hybridization incubation was done over night at + 42 °C. After hybridization the filter was washed with 5x, 1x and 0.1x SSPE solutions (20 x SSPE: 870 g NaCl, 138 g $NaH_2PO_4 \bullet H_2O$, 37 g EDTA, H_2O ad 5000 ml). Autoradiography was performed with Typhoon Scanner (Amersham Biosciences, USA) and image processed by Image Quant software (Amersham Biosciences, USA).

3 Results

3.1 Purification of L-rhamnose dehydrogenase

The activity of L-rhamnose dehydrogenase was first measured from the crude extract of the *P. stipitis* CBS 6054 cells. The cells were harvested before the carbon source was utilized. L-Rhamnose dehydrogenase activity of the crude extract of *P. stipitis* cells grown on L-rhamnose was 14 nkat/mg of extracted protein. The activity of the crude extract of the cells grown on D-glucose/ L-rhamnose was 2 nkat/mg. No L-rhamnose dehydrogenase activity was detected in the crude extract of the cells grown on 2% D-glucose. This suggests that the cells used for protein purification should be grown on L-rhamnose. The effect of bivalent cations was tested by leaving out $MgCl_2$ and adding EDTA to chelate the metal ions of the reaction mixture. This did not have any influence on the L-rhamnose dehydrogenase activity.

The purification of the L-rhamnose dehydrogenase included three steps. First the protein extract was eluted with a salt gradient from gradient anion chromatography, DEAE, column. The fractions with the highest activity were identified by the Cobas-Mira$^+$ analyzer. Those fractions were concentrated and used in a native PAGE. In the native PAGE the active enzyme was identified using zymogram staining. Only a single band became visible in this staining. This protein band was cut out then eluted from the gel slice and concentrated. It was then applied to an SDS-PAGE. On the SDS-PAGE also the concentrated active fractions from the DEAE column were loaded. The SDS-PAGE is shown in figure 7. The fractions from the DEAE separation contained about 20 different proteins while after the second purification step including the native PAGE only four proteins were visible in the SDS-PAGE. The estimated sizes of these proteins were 30, 35, 52 and 70 kDa.

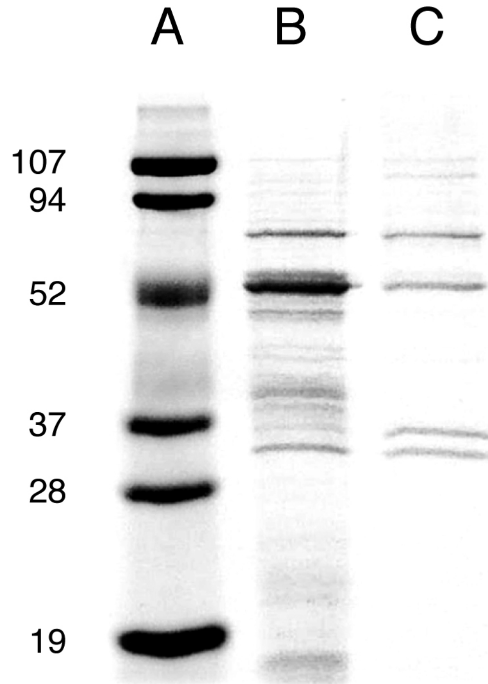

Figure 7. Coomassie Blue stained SDS-PAGE. Lane A contains the molecular mass marker. Sizes in kDa are indicated on left. Lane B shows the active fractions after the DEAE column separation. Lane C contains the protein eluted from the zymogram stained native PAGE.

The DEAE column purified SDS-PAGE results were compared to the zymogram stained native PAGE SDS-PAGE results and their enzyme activities were correlated. As a result, the 30 kDa protein was preliminarily identified as the L-rhamnose dehydrogenase.

3.2 MALDI-TOF MS

The four proteins were trypsinated and their peptide masses were determined by MALDI-TOF MS. On the basis of matching peptide sequences these masses were compared to the genome sequence of *P. stipitis*. The masses 555.247, 900.475, 1199.639, 1761.782, 1872.708 and 2552.586 were identified as tryptic peptides of a protein with the GenBank identifier ABN68405. This protein had been annotated as a putative D-glucose-1-dehydrogenase II. The gene consists of 258 amino acids (figure 8) and its calculated molecular mass is 27.102 Da. The protein belongs to the family of short-chain alcohol dehydrogenases.

```
1         ATGACTGGATTGTTGAATGGAAAGGTGGTTGCAATAACCGGAGGTGTCACTGGTATCGGA
1          M  T  G  L  L  N  G  K  V  V  A  I  T  G  G  V  T  G  I  G

61        CGGGCAATTGCCATAGAAATGGCAAGGAATGGTGCCAAAGTAGTAGTGAACCATTTACCA
21         R  A  I  A  I  E  M  A  R  N  G  A  K  V  V  V  N  H  L  P

121       TCTGAAGAGCAAGCTCAGTTGGCAAAAGAACTCAAAGAGGAAATCCTGGACGGTGAAAAC
41         S  E  E  Q  A  Q  L  A  K  E  L  K  E  E  I  L  D  G  E  N

181       AACGTGCTCACCATTCCAGGGGACATCTCGCTCCCTGAAACAGGCAGAAGGATAGTGGAG
61         N  V  L  T  I  P  G  D  I  S  L  P  E  T  G  R  R  I  V  E

241       CTTGCAGTTGAAAAGTTTGGCGAAATCAATGTGTTCGTTTCCAATGCTGGTGTCTGTGGG
81         L  A  V  E  K  F  G  E  I  N  V  F  V  S  N  A  G  V  C  G

301       TTCAGAGAGTTTCTTGAAATAACTCCCGAAACTTTGTTTCAGACGGTGAATATTAACTTG
101        F  R  E  F  L  E  I  T  P  E  T  L  F  Q  T  V  N  I  N  L

361       AATGGAGCCTTCTTCGCCATCCAAGCAGCTGCACAACAAATGGTCAAGCAGGGCAAAGGA
121        N  G  A  F  F  A  I  Q  A  A  A  Q  Q  M  V  K  Q  G  K  G

421       GGTAGCATTATTGGAATCAGCAGCATCTCTGCCTTGGTTGGAGGAGCACACCAAACCCAT
141        G  S  I  I  G  I  S  S  I  S  A  L  V  G  G  A  H  Q  T  H

481       TACACACCAACTAAAGCTGGAATCTTGTCCTTAATGCAATCTACAGCATGTGCTCTAGGT
161        Y  T  P  T  K  A  G  I  L  S  L  M  Q  S  T  A  C  A  L  G

541       AAGTATGGAATCAGATGCAATGCGATCCTCCCAGGGACAATCAGTACAGCCTTGAACGAA
181        K  Y  G  I  R  C  N  A  I  L  P  G  T  I  S  T  A  L  N  E

601       GAGGACTTGAAAGATCCAGAGAAGAGAAAGTACATGGAAGGGAGAATACCTCTAGGAAGA
201        E  D  L  K  D  P  E  K  R  K  Y  M  E  G  R  I  P  L  G  R

661       GTCGGTGACCCCAAGGATATTGCTGGACCTGCTATCTTCTTGGCAAGTGATATGTCTAAC
221        V  G  D  P  K  D  I  A  G  P  A  I  F  L  A  S  D  M  S  N

721       TACGTTAATGGAGCACAACTACTTGTTGATGGAGGATTGTTCGTCAATTTACAATAG
241        Y  V  N  G  A  Q  L  L  V  D  G  G  L  F  V  N  L  Q  *
```

Figure 8. The nucleotide secuence of the *P. stipitis* L-rhamnose dehydrogenase and the corresponding protein sequence.

3.3 Enzyme activity

To verify that the identified gene was L-rhamnose dehydrogenase the protein was expressed in *S. cerevisiae*. Because of the alternative codon usage of *P. stipitis* where CTG codes for serine instead of leucine the CTG codons had to be changed. The L-rhamnose dehydrogenase contained one CTG codon at bp 166-168 of the open reading frame. By changing the codon to TCG it was ensured that *S. cerevisiae* would express a protein with the same amino acid sequence. The *RHA*1 gene was then ligated into the multicopy plasmid p1181. In order to facilitate the purification the Rha1 protein was also expressed in *S. cerevisiae* with histidine tags. N- and C-terminal histidine tags were introduced by adding the additional nucleotide sequence by PCR. Histidine tag at the N-terminus of the open reading frame of the Rha1 protein did not show any activity. The C-terminally tagged protein showed some activity in the crude extract but the activity observed was reduced by about 80 % when compared to the activity of the non-tagged enzyme. As the histidine tag had such a strong effect on the activity of the L-rhamnose dehydrogenase, the enzyme was not purified. Only the crude extract of the *S. cerevisiae* strain expressing the L-rhamnose dehydrogenase was used for the kinetic characterization.

The L-rhamnose dehydrogenase activity of the crude extract of *S. cerevisiae* H1346 transformants expressing the *RHA1* gene was 180 nkat/mg of extracted protein (figure 9). The crude extract of *S. cerevisiae* cells having the p1181 plasmid without *RHA1* gene did not exhibit any activity. The activity of the L-rhamnose dehydrogenase was also tested with different sugars. D-erythrose, D-allose, D-ribose, D-arabinose, D-tagatose, D-glucose, D-galactose, D-xylose and L-arabinose did not exhibit any activity when used as substrates for the crude extract. With L-rhamnose, L-lyxose and L-mannose, clear activity was observed (figure 9). The structure of L-lyxose and L-mannose is closely related to L-rhamnose (figure 10). The highest activity was seen with L-rhamnose. Also the affinity of the enzyme toward L-rhamnose was the highest, the Michaelis constant K_m was 0.9 mM. Lower affinities were obtained with L-lyxose and L-mannose, the K_m values being 4.3 mM with L-lyxose and 25 mM with L-mannose. The V_{max} and K_m values are seen from the Eadie-Hofstee plots (figure 11).

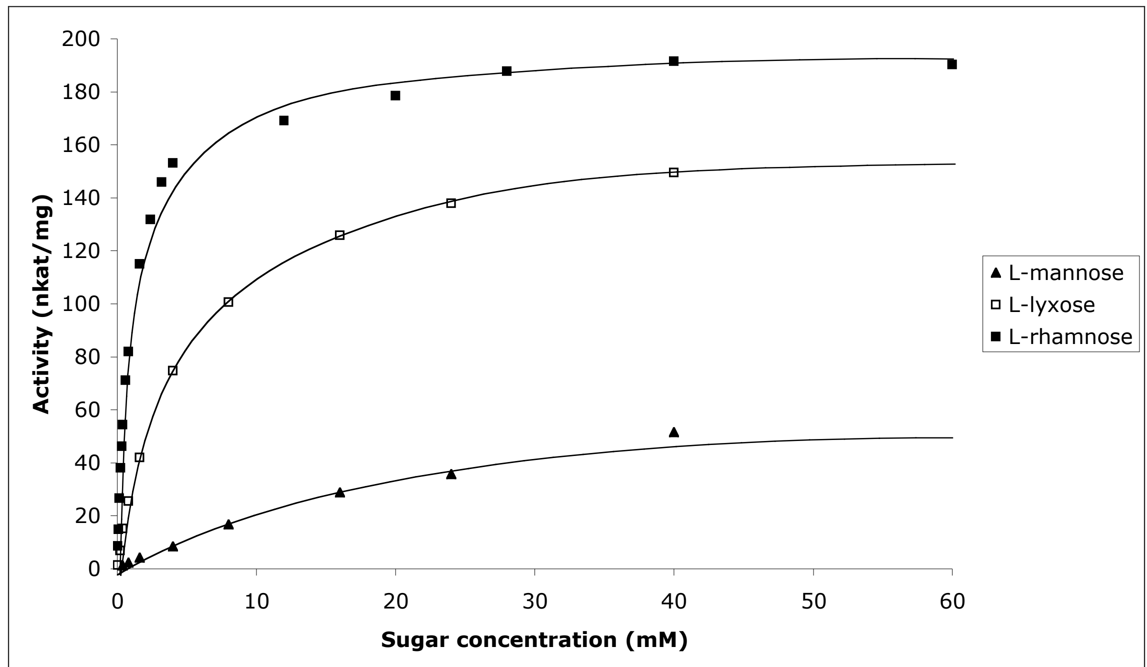

Figure 9. L-rhamnose dehydrogenase activities with different sugar substrates calculated with a Michaelis-Menten kinetic model. The NAD concentration was 1,5 mM and the kinetic properties were measured at pH 8.0.

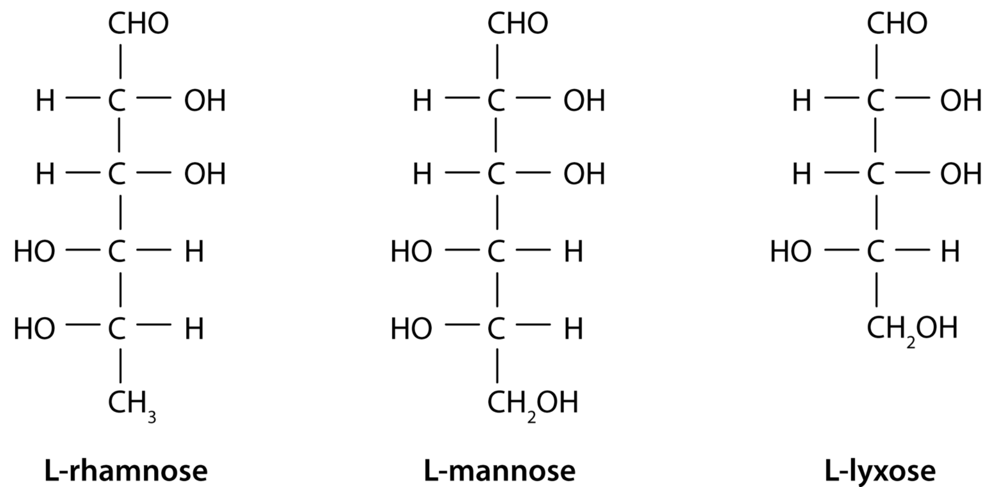

Figure 10. Fischer projection of L-rhamnose, L-lyxose and L-mannose.

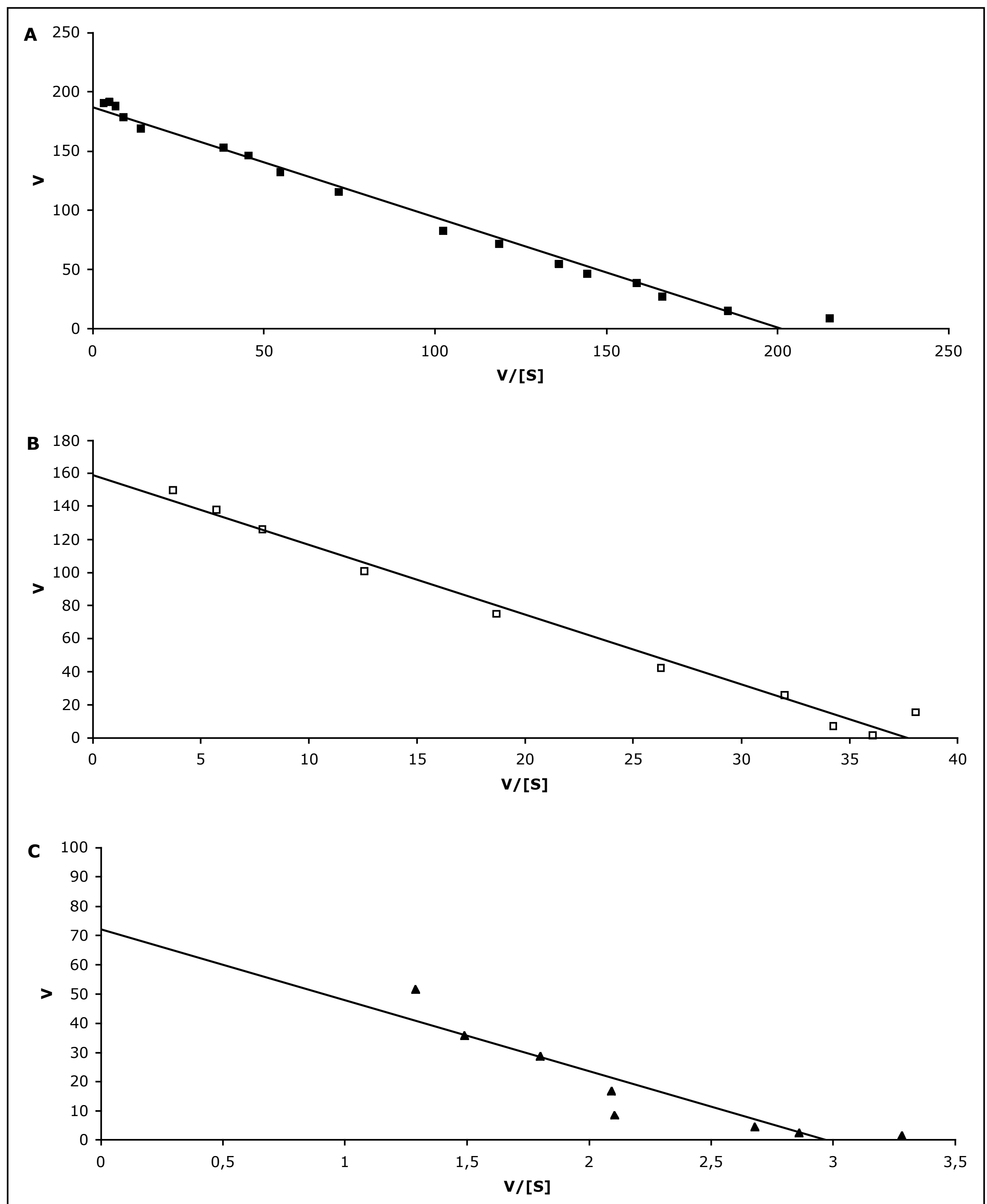

Figure 11. Eadie-Hofstee plots of L-rhamnose (A), L-lyxose (B) and L-mannose (C). The K_m values can be read from the slope and V_{max} values from the y-axis.

The L-rhamnose dehydrogenase activity was measured at different pH varying between 6.8 and 9.5. The highest activity was measured at pH 9.5 as shown in figure 12.

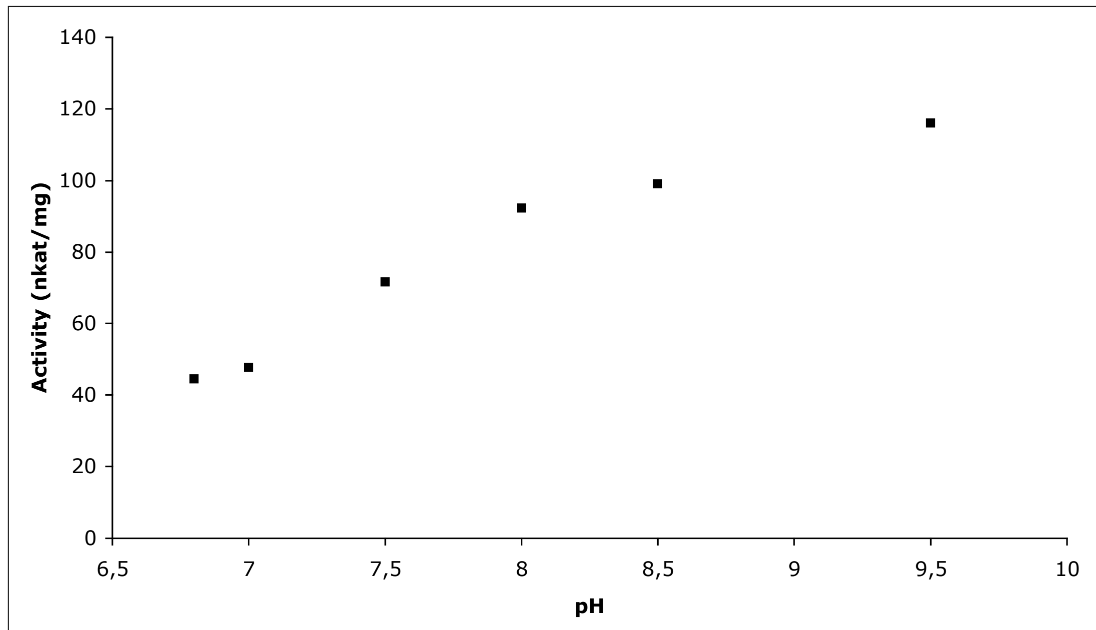

Figure 12. pH dependency of the L-rhamnose dehydrogenase expressed in *S. cerevisiae*.

NADP used as a cofactor instead of NAD did not result in any enzymatic activity. The effect of the NAD concentration to the activity is shown in the figure 13. The highest activity was observed when L-rhamnose was used as a substrate. Calculated by the Michaelis-Menten equation, the maximum velocity of the reaction, V_{max}, was 180 nkat/mg. K_m of the cofactor NAD was 0.2 mM.

Also the reverse direction of the reaction where L-rhamnoate is converted to L-rhamnose was tested. To test the activity NADH was added to the enzyme preparation instead of NAD. No activity was detected.

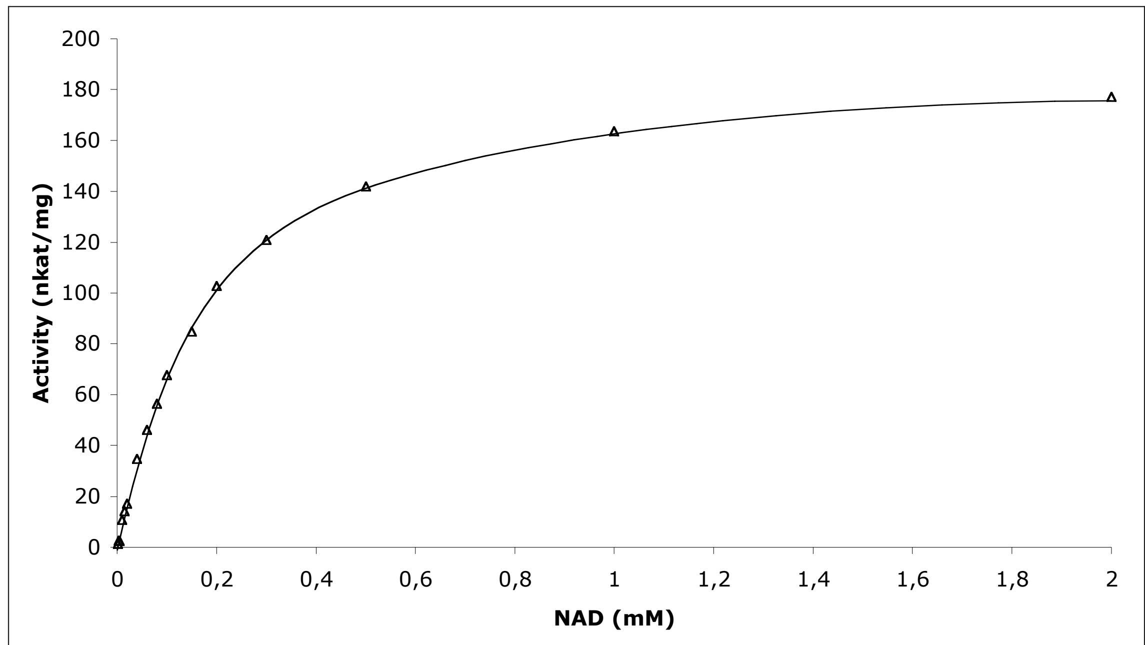

Figure 13. The effect of the NAD concentration to the L-rhamnose dehydrogenase activity calculated with a Michaelis-Menten kinetic model. The L-rhamnose concentration is 60 mM and the kinetic properties were measured at pH 8.0.

3.4 *RHA1* deletion cassette

The deletion cassette was done in order to prove that *RHA1* was essential for L-rhamnose catabolism in the yeast *P. stipitis*. If the knockout strain would not grow on L-rhamnose it would prove that there are no alternative pathways for L-rhamnose catabolism. The *RHA1* deletion cassette was successfully constructed using homologous recombination in the *S. cerevisiae* strain H3488. It was then further amplified in the TOP10 *E. coli* strain. Transformation of the deletion cassette into the *P. stipitis* H2818 strain resulted only in three colonies growing on nourseothricin plate. Each colony was inoculated to the growth medium having L-rhamnose as a carbon source. All three transformants grew on L-rhamnose. The yeast colony PCR which was done for the transformants indicated that the cassette had integrated randomly and not targeted to delete the *RHA1* gene. It seemed that the homologus recombination does not occur very frequently. Also the number of *P. stipitis* transformants was very low so that it seemed very tedious to screen a large number of transformants. The transformation frequency should be first increased to increase the chances to obtain deletions using homologues recombination. Also transposon technique could be used only after the transformation frequency would be much higher.

3.5 Northern blot

The northern blot was used to study the transcription of the *RHA1* gene on different sugars. The *P. stipitis* strain was grown on the six different carbon sources: L-rhamnose, D-glucose, maltose, D-galactose, D-xylose, and a glycerol/ethanol mixture. The RNA stained with CYBR green II, to indicate the total RNA amount is shown in figure 14 and the Northern analysis in figure 15 represents the of *RHA1* transcription. Transcription was observed only on L-rhamnose so it can be concluded that *RHA1* expression is L-rhamnose-induced.

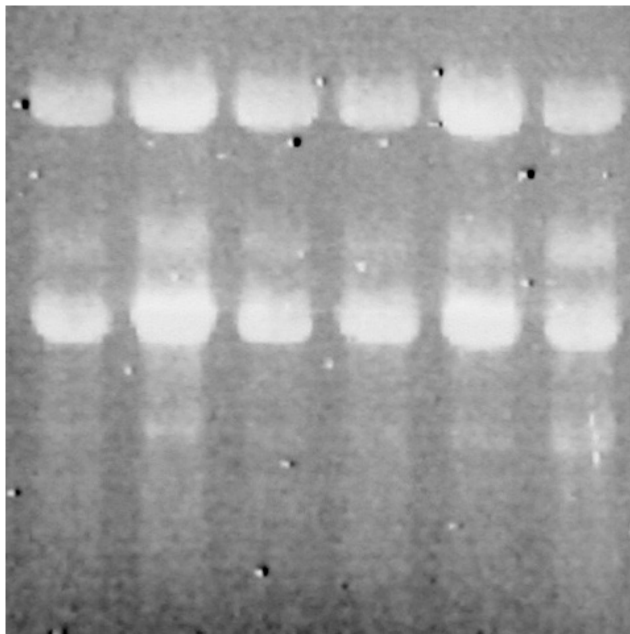

Figure 14. RNA gel stained with SYBR Green II RNA gel stain. Lines from left to right: L-rhamnose, D-glucose, maltose, D-galactose, D-xylose, and a glycerol/ethanol mixture.

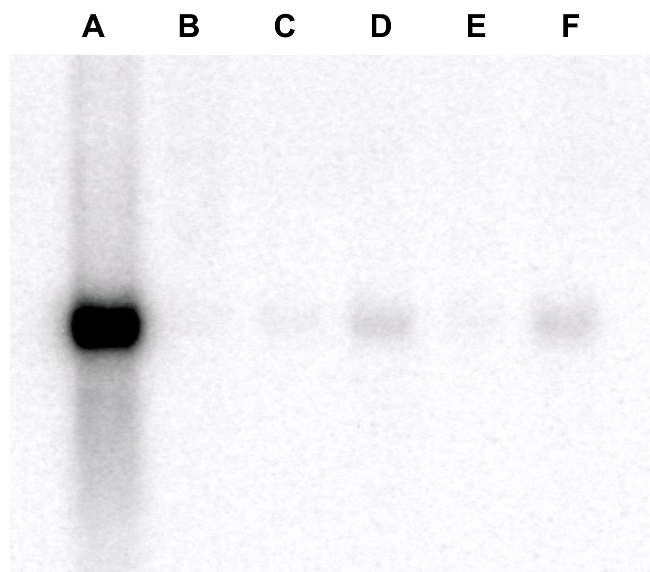

Figure 15. Northern blot analysis of *RHA1* expression. The expression of *RHA1* in *P. stipitis* on six different sugars, from left to right: L-rhamnose (A), D-glucose (B), maltose (C), D-galactose (D), D-xylose (E), and a glycerol/ethanol mixture (F).

3.6 L-Lactaldehyde activity

In order to find the gene coding for the L-lactaldehyde dehydrogenase, similar procedures as with L-rhamnose dehydrogenase were used to purify the protein. *P. stipitis* CBS 6054 cells were grown on L-rhamnose and a crude extract was made as described before. Inspite of the Norit A and Dowex 50 treatments the colour of the synthesised L-lactaldehyde solution was medium brown. It was found out to result a high background absorbance in 340 nm which might be the reason why no activity was detected when using L-lactaldehyde as a substrate. DL-Lactaldehyde was then used for all the activity measurements. From the tested pH values between 6.8-9.5, pH 6.8 was used for measurements as it was observed to result in the highest activity. Bivalent cations or halide ions were necessary as no activity was detected without addition of $MgCl_2$. Still the L-lactaldehyde dehydrogenase activities were very low being only 2.5 nkat/mg of extracted protein. The crude extract of the *P. stipitis* cells grown on D-glucose did not show any activity. The crude extract was partially purified using a DEAE column but L-lactaldehyde dehydrogenase activity was lost during the procedure. The crude extract and purification were done as described earlier (2.6 Enzyme purification). To continue the work to find the corresponding gene for L-lactaldehyde, problems with the low and disappearing activity should be solved first.

4 Discussion

4.1 L-Rhamnose dehydrogenase

There are two known pathways for L-rhamnose catabolism; the one used by prokaryotes which has phosporylated intermediates and the one used by eukaryotes which has the non-phosphorylated intermediates. For the latter pathway only the enzymes and intermediates have been described but none of the corresponding genes have been identified. In this work the gene of the first enzyme in this pathway, L-rhamnose dehydrogenase, was identified. This NAD dependent gene was called *RHA1*.

Rigo et al. reported already in 1985 that L-rhamnose dehydrogenase is found in the yeast like fungus *Aureobasidium pullulans* only when L-rhamnose is used as a carbon source. The same observation was done later in *P. stipitis* by Twerdochlib et al. (1994) and was also confirmed in this study. No activity was detected in *P. stipitis* when the cells were grown on D-glucose. Some activity was present when the cells were grown on a mixture of D-glucose and L-rhamnose and the activity was increased several-fold when L-rhamnose was the only source of carbon. During the purification, the activity appeared as a single peak, indicating that there are no alternative pathways for this step of L-rhamnose catabolism. The purified enzyme was digested with trypsin and the masses of the peptides were identified using MALDI-TOF MS. As the genome sequence of *P. stipitis* has been published the corresponding open reading frame could be identified by the masses of the peptides.

4.1.1 Kinetic properties

To characterize the kinetic properties of the L-rhamnose dehydrogenase *RHA1* was expressed in the heterologus host *S. cerevisiae*. The results showed that *RHA1* indeed coded for an enzyme with this activity. In order to facilitate the purification histidine tags were added to the N- or to the C-terminus but the activity of the tagged proteins was much reduced or there was no activity detected. As this might be an indication that the histidine tag is interfering with the catalytic activity the kinetic properties were measured with the *S. cerevisiae* strain expressing *RHA1* without any tags.

Twerdochlib et al (1994) found out that L-rhamnose dehydrogenase of *P. stipitis* does not produce detectable amounts of L-rhamno-γ-lactone. This suggests that this enzyme produced the more unstable L-rhamno-δ-lactone. The reverse reaction with L-

rhamnoate and NADH was tested in this work. However, no activity was observed, indicating that the enzyme is not active with the linear L-rhamnoate and that at pH 8.0 the lactone is present in too low concentrations for the reverse reaction to happen.

The Rha1 protein was specific to NAD as a cofactor. Besides L-rhamnose also L-lyxose and L-mannose were accepted in the catalytic reaction. The highest activity and affinity were observed with L-rhamnose. As no activity was observed with glucose it can be excluded that the enzyme is a glucose-1-dehydrogenase as suggested in the first annotation based on sequence similarity in NCBI nucleotide blast database.

The findings are similar to the corresponding results with another eukaryotic sugar dehydrogenase, L-fucose dehydrogenase. It was also found to have quite high substrate specificity as only L-galactose besides L-fucose was oxidized by L-fucose dehydrogenase among the ten tested sugars (Conter, Guimaraes and Veiga 1984). It was not tested in this work if the L-fucose would serve as a substrate for the L-rhamnose dehydrogenase but L-fucose dehydrogenase was not active when L-rhamnose was used as a substrate (Conter, Guimaraes and Veiga 1984). C1 to C4 in the three sugars accepted in the reaction share the same configuration. No activity was observed with D-ribose, which has the hydroxyl group at C4 in the opposite configuration. The C1-C4 stereochemical configuration seemed to be essential for recognition by the enzyme as no activity was found on sugars with any other C1-C4 configuration. Also the missing C5, as in D-erythrose, did not serve as a substrate. Based on the results it is impossible to say whether a hydroxyl group is needed at C5 position, as a sugar without a hydroxyl group at C5 was not tested.

According to the NCBI protein blast database the Rha1 belongs to the protein family of short-chain dehydrogenases (SDR). It has the conserved domain of fabG [3-ketoacyl-(acyl-carrier protein) reductase]. Short-chain dehydrogenases / reductases are a large and functionally heterogenous protein family. The sequence identity of the enzymes of the family is only 15-30 % but they display a highly similar α/β folding pattern with a central β-sheet flanked by α-helices (Oppermann et al. 2003). This is typical to the Rossmann-fold, a structural motif which binds nucleotides, most commonly the cofactor NAD. Rha1 has 258 amino acids, which is a very typical length for a SDR enzyme as they normally consist of 250-350 amino acids. SDR enzymes frequently form parts of multi-enzyme complexies or N- or C-terminal transmembrane domains or signal peptides (Oppermann et al. 2003). It could be that also L-rhamnose-dehydro-

genase is part of a cluster of enzymes catalysing consecutive reactions of a metabolic pathway of L-rhamnose. Rha1 also has the glycine-rich T-G-X_3-G-X-G motif typical for SDR enzymes as it is important to maintain the structure of the central β-sheet. This coenzyme binding region has usually a position 12-19 and in Rha1 it has a starting point at position 13 (Figure 6).

Sugar dehydrogenases oxidizing the sugar to a sugar acid are not very common in eukaryotic micro-organisms. The sugar dehydrogenases of eukaryotic microbes belong to very different protein families, although the catalytic reaction is very similar. *Hypocrea jecorina* has an NADP-utilizing D-xylose dehydrogenase which belongs to the GFO/IDH/MOCA protein family (Berghäll et al. 2007). In *S. cerevisiae* NADP-requiring (Kim et al. 1998) and NAD-utilizing D-arabinose dehydrogenases (Amako et al. 2006), which contribute to erythroascorbic acid production, are part of the aldo/keto reductase family. There are also reports of some other eukaryotic sugar dehydrogenases but for these it is not known to which protein family they belong, as the corresponding sequences for the proteins are not known. These include NADP-requiring D-glucose dehydrogenase in *Schizosaccharomyces pombe* (Tsai, Shi and Ye 1995), an NAD-utilizing D-galactose dehydrogenase in *Aspergillus niger* (Elshafei and Abdel-Fatah 2001) and an NADP-requiring D-xylose dehydrogenase in *Pichia quercuum* (Suzuki and Onishi 1973).

4.1.2 *RHA1* deletion cassette

A deletion cassette was made in order to prove that there is only one pathway for the L-rhamnose catabolism in yeast. If the *RHA1* knock-out strain would have been unable to grow on media having only L-rhamnose as a carbon source it would have proven that there is no alternative pathway for L-rhamnose catabolism. A requirement for the generation of such a knock-out strain would have been a high transformation frequency or a transformation method which has a high succeeding rate for homologous recombination.

As always with deletions one should remember the possibility that deletion is lethal. Analyses have revealed that mutations in most eukaryotic genes have little discernable effect. For example only ~20 % of the genes of *S. cerevisiae* are essential for the viability of cells grown in laboratory conditions (Boone, Bussey and Andrews 2007). However, the L-rhamnose pathway is not essential if the cells are grown on some

other carbon source so it could be assumed that also the genes coding for the enzymes of the pathway are not essential.

4.2 L-Lactaldehyde dehydrogenase

It was attempted to purify and clone also the L-lactaldehyde dehydrogenase. Attempts to purify the L-lactaldehyde dehydrogenase were however unsuccessful as the activity was always lost during the purification process. Twerdochlib et al (1994) reported that the activity of L-lactaldehyde dehydrogenase was only one third of the activity of L-rhamnose dehydrogenase. This was confirmed. Using the crude extract of *P. stipitis* cells the activities of L-lactaldehyde dehydrogenase in this work were about one fourth of the L-rhamnose dehydrogenase activity measured. Twerdochlib et al. (1994) used pH 9.0 to measure L-lactaldehyde dehydrogenase, which is different from the observations done in my work. In this work pH 6.8 resulted the highest activity but no pH lower than that was tested. L-Lactaldehyde dehydrogenase from the yeast *S. cerevisiae* has also been purified and characterized (Inoue et al. 1985). The enzyme was found to be most active at pH 6.5 and halide ions such as Cl^- and Br^- remarkably activated the enzyme. Addition of $MgCl_2$ was crucial but no other halide ions or bivalent cations were tested in my work. Mg^{2+} did not have any effect on the lactaldehyde dehydrogenase activity of *S. cerevisiae*. One problem might be that DL-lactaldehyde was used as a substrate as the preparation of L-lactaldehyde was unsuitable for enzyme activity measurements. Inoue et al. (1985) found out that the activity of the enzyme was only 0.2 % when D-lactaldehyde was used instead of L-lactaldehyde.

4.3 Transformation of *P. stipitis*

Transformation of *P. stipitis* was found out to be very problematic since at best only about 20 transformants per transformation were obtained. In another approach it was planned to randomly mutagenise *P. stipitis* with a transposon to create a deletion library. This could have been tested for growth on different carbon sources including L-rhamnose. However, this plan was also abandoned because of the low transformation frequency. In order to create such a library the transformation frequency should have been much higher. In the future it will be important to increase the transformation frequency in order to get knock-out strains or use transposon technique. Klinner et al. (2004) reported that tremendous increase was observed when linearized ends-in vectors

were used instead of circular vectors in *P. stipitis* transformations. Ends-in vectors have a gap in the region with homology to the target gene. Linerized vector was used also in this work when the deletion cassette transformation was made. Also the restriction-enzyme-mediated integration (Riggle and Kumamoto 1998) was found out to enhance the transformation efficiency remarkably. Restriction-enzyme-mediated integration has been developed for nonhomologous ectopic integration of DNA fragments (Riggle and Kumamoto 1998) but also homologous integration can be enhanced in dependence on the chromosomal target region and the restriction enzyme (Klinner and Schäfer 2004). The number of *P. stipitis* transformants when using circular vector was reported to be five per 1 μg DNA versus 188 when using ends-in vector with restriction-enzyme-mediated integration. 18 out of 20 of the latter were found out to be homologous transformants whereas only 1-17 % of transformants with the ends-out vector were homologous (Klinner and Schäfer 2004). Still, even these transformation efficiencies are too small to generate a transposon deletion library but they might be useful for targeted deletion of genes.

4.4 Northern blot

A northern blot analysis was used to get some information about the induction of the *RHA1* gene. The *RHA1* open reading frame was used as a probe on the RNA of *P. stipitis* grown on different carbon sources (Figure 15). *RHA1* was induced only when the yeast was grown on L-rhamnose suggesting that the induction of the L-rhamnose dehydrogenase activity is the result of induction of transcription of *RHA1*.

4.5 Conclusions

The first enzyme of the L-rhamnose catabolic pathway in yeast, L-rhamnose 1-dehydrogenase gene *RHA1* was identified in this work. The enzyme was purified from the yeast *P. stipitis* and the mass of its tryptic peptides determined using MALDI-TOF MS. This enabled the identification of the corresponding gene, *RHA1*. It codes for a protein with 258 amino acids belonging to the protein family of short chain alcohol dehydrogenases. The open reading frame was expressed in *S. cerevisiae*. *P. stipitis* has an alternative codon usage where CUG codes for leucine instead of serine. These codons were changed so that the same amino acid was expressed in *S. cerevisiae*. The NAD-specific enzyme showed the highest activity and affinity with L-rhamnose and a lower activity and affinity with L-mannose and L-lyxose. A Northern analysis revealed that transcription in *P. stipitis* is induced during growth on L-rhamnose but not on other carbon sources.

Further research is needed to find out the corresponding gene sequences of the other enzymes of this pathway. This information is needed in order to transform the pathway to different organisms as *S. cerevisiae*. Information about fungal sugar catabolism is important in order to compose more compelling strains for the biofuel production. Better understanding of fungal catabolic pathways and enzymes related to them is also important for getting more comprehensive picture about fungal phylogenetics.

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