The influence of an amino acid permease on diacetyl production during beer fermentation

vorgelegt von

M.Sc. Biotechnology

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aus Kerala, Indien

von der Fakultät III - Prozesswissenschaften

der Technischen Universität Berlin

zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften

- Dr.-Ing. -

genehmigte Dissertation

Promotionsausschuss:

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Tag der wissenschaftlichen Aussprache: 05.12.2013

Berlin 2014
D 83

O LORD, how many are Your works! In wisdom You have made them all.
Psalm 104:24

Acknowledgements

I express my gratitude to everyone who has contributed toward helping me achieve my professional goals. I couldn't have come this far with my innate abilities alone, but because I have had the opportunity to work with some wonderful people who contributed much to my life with knowledge, support and motivation.

First of all I thank God Almighty for my life, health and strength needed to come this far.

I express my sincere thanks to Prof. Dr. Ulf Stahl for giving me the valuable opportunity to carry out my doctoral research in the Department of Applied and Molecular Microbiology, TU Berlin. I thank him in particular for his guidance, support and kindness throughout my research period.

I would also like to extend my deepest gratitude to Prof. Dr. Christine Lang for her excellent supervision and for sharing her expertise without which I could not have finished this work.

Special thanks to Dr. Anne Karradt for every help that was extended and for many valuable discussions throughout my doctoral studies.

I am also thankful to all my colleagues in the Department of Applied and Molecular Microbiology for their support and conducive working environment they created around me: Isil Baki, Sandra Dounia, Eric Lorenz, Markus Schöner, Dr. Martin Hageböck, Dr. Martin Senz, Rita Waggad, Roslin Bensmann, Katja Glowacz, Andrea Bayer, Dr. Lysann Strack, Dr. Rena Leisegang, Franziska Wanka, Markus Fiedler, Andreas Albin, Sebastian Riedel, Birgit Bernhardt, Birgit Baumann and Susanne Engelhardt.

A special thanks to the Department for Brewing and Beverage Science & Applications (VLB): Kurt Marshall, Kolja Gigla, Patricia Diniz and Veronica Menzel for allowing me to use their facilities and for all the help I received to successfully complete my work.

I also want to thank Dr. Thomas Volkmer at the Institute of Biology, Humboldt University for all his help during radioactivity measurements.

Finally, I would like to express my profound gratitude to my parents for their unconditional love and support. Many thanks to all my friends who have supported me during my time in Berlin.

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Abbreviations

AHAS	Acetoydroxyacid synthase
BAP	Branched chain amino acid permease
BCAA	Branched-chain amino acid
bp	base pair(s)
BSA	Bovine serum albumin
CDS	Coding Sequence
CGH	comparative genomic hybridisation
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deeoxynucleotide triphosphate set
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetic acid
g	gram
G418	Geneticine
GAP1	General amino acid permease
Gap1p	General amino acid permease-protein
GC-ECD	Gas chromatography - Electron capture detector
h	hour
HRP	Horseradish peroxidase
i.e.	Id est (that is)
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
kDa	kilo Dalton
KmR	kanamycin resistance
LB	Luria-Bertani
M, mM	Molar, Millimolar
mm	millimeter
MEBAK	Mitteleuropäische Brautechnische Analysenkommission
min	minute
MW	Molecular weight
nm	nanometer
nt	nucleotide
OD	Optical density
ON	Over night
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoeresis

PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl flourid
m RNA	messenger Ribonucleic acid
rpm	Rotations per minute
RT	room temperature
S. bayanus	*Saccharomyces bayanus*
S. carlsbergensis	*Saccharomyces carlsbergensis*
S. cerevisiae	*Saccharomyces cerevisiae*
Sc-type	*Saccharomyces cerevisiae* type
Non-Sc-type	non-*Saccharomyces cerevisiae* type
S. pastorianus	*Saccharomyces pastorianus*
s	second
SGD	*Saccharomyces* Genome Database
SDS	Sodium dodecyl sulphate
TDA	Transcriptome data analysis
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
V	Voltage
VDK	Vicinal diketone
VLB	Versuchs-und Lehranstalt fuer Brauerei in Berlin
Vol	Volume
w/v	weight/volume
wt	wild type
YED	Yeast Glucose medium
YEPD	Yeast Peptone Glucose medium

Theoretical Section

1. INTRODUCTION I

1.1 Nitrogen assimilation in yeast

Nitrogen availablity is an essential factor needed for growth and various metabolic activities in yeast. It utilizes nitrogen in the form of ammonium salts, amino acids and small peptides (dipeptides and tripeptides) which are found e.g. in brewing wort (Cruz, 2002). These assimilated nitrogen sources are then converted to ammonia, glutamate and glutamine which play a vital role in the formation of higher alcohols, organic acids, esters and diketones that are responsible for the characteristic flavour profiles of beer (Parkes, 2002).

Wort consists of 19 essential amino acids and small peptides which are collectively known as free amino nitrogen (FAN) (Lekkas, 2005). Its concentration greatly determines the quality and efficiency of the beer fermentation. However excess of FAN is quite disadvantageous as it may result in high concentrations of fusel alcohols in beer. Therefore for a standard malt-wort with 12 °P, the ideal FAN concentration ranges between 200 – 240 mg/l (Eßlinger, 2009). Once assimilated, amino acids pass via the transaminase system in yeast where the amino groups are removed and are utilized as building blocks for the synthesis of various amino acids and proteins.

Although wort contains a wide range of 30 distinct nitrogen sources, not all of them support the growth of yeast to the same extent. For this reason, the uptake of amino acids in yeast is a highly regulated process through various amino acid permeases whose transcriptional control takes place either by nitrogen catabolite repression (NCR) or by SPS plasma membrane amino acid sensor system (Ssy1p-Ptr3p-Ssy5) (Crépin, 2012). Through these mechanisms, yeasts select preferred nitrogen compounds that support fast growth with doubling times of 2 h (asparagine, glutamine, and ammonium) or minor preferred ones leading to doubling times < 3 h (aspartate, alanine, serine, arginine, glutamate, phenylalanine and valine) over non-preferred ones that support slower growth with doubling time > 4 h (leucine, isoleucine, methionine, threonine, tryptophan, and tyrosine) (Godard, 2007). Those amino acids that support fast growth are consumed early compared to specific permeases under SPS mediated control mechanism. On the other hand, those amino acid sources that support slow growth are consumed at a later stage under the control of NCR mechanism.

Based on the order of uptake of the amino acids into yeast, they are classified into four groups as listed in Table 1 (Eßlinger, 2009).

Table 1: Classification of amino acids based on their order of uptake into yeast

Classification	Quality of nitrogen source	Amino acids
Group A	Very good	glutamine, glutamate, asparagines, aspartate, serine, threonine, lysine
Group B	Good	valine, methionine, leucine, isoleucine, histidine
Group C	Poor	glycine, phenylalanine, tyrosine, tryptophan, alanine
Group D	Poor/least preferred	proline.

1.2 Nitrogen Catabolite Repression (NCR)

To prevent the uptake of non-preferred nitrogen sources at the start of fermentation, yeast uses the nitrogen catabolite repression mechanism. The molecular mechanism includes sensing of the available nitrogen sources and induction of the required systems while repressing the unfavourable systems. NCR enables transcriptional activity of amino acid permeases involved in the uptake of amino acids that are poor nitrogen sources to be repressed as long as preferred nitrogen sources (Table 1) are available (Schure, 2000).

NCR-mediated transcriptional repression is modulated by the activity of four DNA-binding GATA transcription factors namely *GLN3* (Glutamine metabolism), *GAT1* (Transcriptional activator with GATA-1-type zinc finger DNA-binding motif), *DAL80* (Degradation of Allantoin), and *GZF3* (Gata Zinc Finger protein) (Hofman-Bang, 1999). While *GLN3* and *GAT1* are transcriptional activators *DAL80*, and *GZF3* are repressors of Gln3p- and Gat1p- mediated transcription. Under conditions of poor nitrogen source availability, Gln3p and Gat1p accumulate in the nucleus leading to the activation of NCR controlled gene transcription. But in the presence of good nitrogen source, these transcriptional activators are restricted to the cytoplasm where they interact with Ure2p (a Gln3 inhibitor) causing a rapid decrease in the expression of genes encoding and transport systems required for uptake and degradation of poorly used nitrogen sources (Cunningham, 2000).

Furthermore, the activation of *GLN3* and *GAT1* transcription factors is controlled by their interactions with TOR (target of rapamycin) proteins (Bertram, 2002). During nitrogen starvation, TOR proteins are inhibited by rapamycin proteins resulting in the dephosphorylation and accumulation of the Gln3p and Gat1p in the nucleus. On the other hand upon availability of good nitrogen sources, interaction of TOR protein with Gln3p causes its phosphorylation and retention in the cytoplasm. All these interactions work together to control the expression of the NCR genes.

The various amino acid transporters involved in the uptake of non-preferential nitrogen sources, that are subjected to NCR control are unspecific permeases like Gap1p (general amino acid permease) and Agp1p (affinity glutamine permease), high specific permeases like Put4p (proline permease) and Mep1p, Mep2p, and Mep3p (ammonium permeases). Once induced, the amino acid permeases are localized at the plasma membrane and are involved in the active transport of the available poor nitrogen sources into yeast (Deed, 2011).

1.3 SPS amino acid sensor system (Ssy1p-Ptr3p-Ssy5)

Yeast detects available amino acids in the medium using the SPS-sensor complex (Ssy1-Ptr3-Ssy5) situated in the plasma membrane of yeast (Crépin, 2012). Ssy1 is the amino acid sensor on the plasma membrane that transmits intracellular signals to activate the amino acid permease genes involved in the transport of the respective amino acids. Ssy1 devoid of transport activity works together with two other intracellular proteins Ptr3 and Ssy5 and transmits signals causing the activation of transcription factors Stp1, Stp2 and Uga35/Dal81. The expression of these transcription factors is regulated by the yeast amino-acid sensor independent (ASI) complex (Asi1-Asi2-Asi3) which is involved in preventing illegitimate expression of genes in the absence of amino acid signalling (Forsberg, 2001).

When activated, these transcription factors bind to SPS-sensor regulated promoters and induce transcription of amino acid permeases. The amino acid transporters are then transferred to the plasma membrane using Shr3 (a membrane-localized chaperone) resulting in increased amino acid uptake (Ljungdahl, 1992). However when there is nitrogen-depletion in the medium, the SPS-sensor signals for localization of transcription factors Stp1 and Stp2 to the cytosol which in turn results in the repression of amino acid permeases controlled by the SPS-sensor complex. This repression of the SPS-regulated genes is followed by activation of NCR-sensitive genes which is reversible by re-addition of good nitrogen sources to the medium (Lei, 2013).

Various amino-acid permeases that are activated by SPS-signalling mechanism includes branched chain amino acid permeases (Bap2p and Bap3p), the high-affinity glutamine transporter Gnp1p, the tyrosine and tryptophan permeases Tat1p and Tat2p, the dicarboxylic amino acid permease Dip5p, and the high-affinity methionine permease Mup1p. In general, permeases involved in the uptake of amino acids that are taken up during the early stages of fermentation (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe) are encoded by genes that are subjected to Ssy1p-mediated regulation.

1.4 Amino acid permeases in yeast

Amino acid permeases in yeasts include a family of 23 members (Nelissen, 1997), (Didion, 1998). These amino acid permeases are accommodated in the yeast plasma membrane whereby a wide range of amino acids are transported into the cells.

Based on their regulation the family of amino acid permeases in yeast fall into two different classes. The expression of most of the permeases are constitutive where as some of them are regulated by sensing the availability of nutrients. Most permeases are involved in the import of specific amino acids while some import a broad range of substrates. The list of various amino acid permeases in yeast along with their function are provided in Table 2.

Table 2: Amino acid permeases and their transport function in yeast. (Yeast Transport Protein database: YTPdb) (André, 1995)

Regulation mechanism	Gene	Description/function
Nitrogen catabolite repression (NCR)	*GAP1* YKR039W	General amino acid permease
	CAN1 YEL063C	Arginine permease
	DAL5 YJR152W	Allantoate permease
	MEP2 YNL142W	Ammonium permease
	UGA4 YDL210W	GABA permease - also involved in delta-aminolevulinate transport
	PUT4 YOR348C	Proline permease
Ssy1-Ptr3-Ssy5 (SPS)	*HIP1* YGR191W	Histidine permease
	DIP5 YPL265W	Glutamate and aspartate permease
	LYP1 YNL268W	Lysine permease
	AGP1 YCL025C	Broad-specificity amino-acid permease - inductible by most neutral amino acids
	GNP1 YDR508C	Broad-specificity amino-acid permease
	TAT1 YBR069C	Tyrosine and Tryptophan Amino acid Transporter
	TAT2 YOL020W	Tryptophan Amino acid Transporter
	BAP2 YBR068C	Branched-chain Amino acid Permease
	BAP3 YDR046C	Branched-chain Amino acid Permease (paralog of BAP2)
	SSY1 YDR160W	Permease-like sensor of external amino acids
	MMP1 YLL061W	S-methylmethionine permease
	SAM3 YPL274W	S-adenosylmethionine permease
	AGP2 YBR132C	Carnitine permease
	MUP1 YGR055W	High-affinity methionine permease
	MUP3 YHL036W	Low-affinity methionine permease
	HNM1 YGL077C	Choline permease
	BIO5 YNR056C	7-keto 8-aminopelargonic acid permease
	TPO1 YLL028W	Vacuolar polyamine-H+ antiporter
	DTR1 YBR180W	bisformyl dityrosine-H+ antiporter of the plasma membrane involved in excretion of bisformyl dityrosine to the maturing spore wall
	SIT1 YEL065W	Transporter of the bacterial siderophore ferrioxamine B
	ENB1 YOL158C	Transporter of the siderophore enterobactin
	ARN2 YHL047C	Transporter of the fungal siderophore triacetylfusarinine C
	ARN1 YHL040C	Transporter of ferrirubin, ferrirhodin and other ferrichromes

1.5 Higher alcohols in beverage flavour production

Flavour production in yeast is a combination of yeast activity during brewing process and wort composition (Piddocke, 2010). Various yeast metabolites including aromatic and aliphatic alcohols, esters, organic acids and sulphur compounds make up more than 200 flavour components identified in beer. Most of the aromatic compounds have a major

effect on the beverage flavour even when present in low quantities due to their low flavour threshold. An overview of the important metabolites formed during beer fermentation is shown in Figure 1.

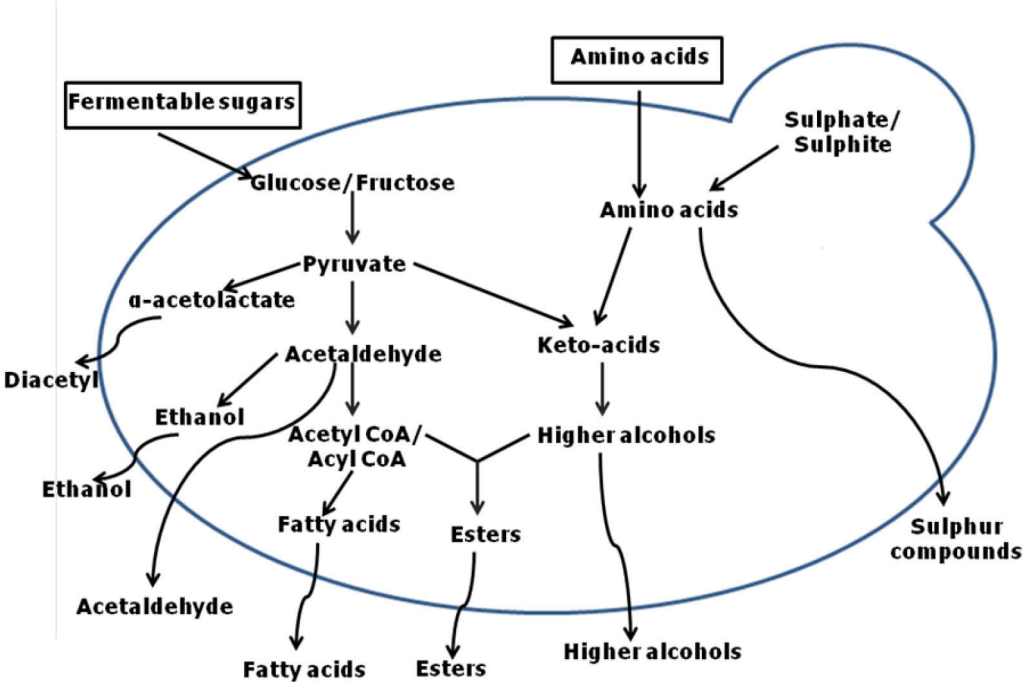


Figure 1: Metabolic pathways in brewers' yeast leading to flavour production

Yeasts obtain their supply of amino acid nutrients from malt. These amino acids are firstly absorbed into the cell and are utilized through transamination reactions. Through this process yeasts remove the amino group from amino acids that are absorbed at a faster rate and utilize their amino groups to synthesize essential amino acids (e.g. amino acids that are absorbed slowly) by attaching the corresponding organic acids to them. The remaining organic acids are converted into aldehydes through loss of a CO_2 residue (decarboxylation) and are ultimately converted to higher alcohols (also called fusel alcohols). This is known as the catabolic (Ehrlich) route to higher alcohol formation. They can also be synthesized from carbohydrates through similar decarboxylation and reduction reactions on the anabolic route of higher alcohol formation. Some higher alcohols are also obtained by reduction of aldehydes and ketones in wort (Hazelwood, 2008).

Production of higher alcohols following transamination and reduction reactions imparts various flavours to beer. The fusel alcohols namely propanol, isobutanol, 2-methylbutanol, 3-methyl butanol are significant in imparting distinct flavours to beer. However, among these compounds, 3-methyl butanol is the largest contributor to the flavour while n-propanol is the smallest (Pietruszka, 2010).

Optimal amino acid concentrations are highly essential for appropriate higher alcohol concentrations in beer. Surplus or shortage of a particular amino acid could lead to

inhibition or over-production of their corresponding alcohols respectively. Also excess supply of amino acids (FAN) leads to increased levels of higher alcohols in beer.

Amino acids are involved in the formation of fusel acids and fusel alcohols including aromatic amino acids (phenylalanine, tyrosine, and trytophan), branched-chain amino acids (leucine, valine, and isoleucine), and the sulphur-containing amino acid (methionine). Fusel alcohols produce esters by their reaction with Acetyl-CoA producing fruity flavour in beer. They also have significant influences on alcoholic and solvent-like aroma. The relation of the higher alcohol and the amino acid from which it is formed and the resultant flavour produced in beer is listed in Table 3.

Table 3: Higher alcohols and their corresponding amino acids involved in flavour compound production

Amino acids	Higher alcohols	Flavour/Aroma
Leucine	Isoamyl alcohol	Bitter
Isoleucine	Amyl alcohol	Alcohol,vinous
Valine	2-Methylpropanal	Alcohol
Phenylalanine	2-Phenylethanol	Rose, perfume
Tryptophan	Tryptophol	Almonds
Tyrosine	Tyrosol	Bitter
Metheonine	Methionol	Cooked vegetable
Isoleucine	2-methylbutanol	Alcohol
Leucine	3-methylbutanol	Fusel, pungent
Threonine	Propanol	Alcohol

1.6 Esters, organic acids, ketones and aldehydes in the formation of flavour-active compounds

Several by-products of amino acid metabolism in yeast yield different flavours in beer. These flavour-active esters are formed by the condensation reaction between either acetyl/acyl-CoA and higher alcohols or ethanol. The formation of these esters in yeast confers fruity-flowery flavour to beer. Some examples of different flavours produced by ester compounds include ethyl-acetate with acetone (solvent-like) flavour, iso-amyl-acetate with banana (fruity) flavour, phenylethyl acetate with roses, honey, apple flavours, ethyl caproate and ethyl caprylate with apple flavour.

Organic acids are mainly obtained from wort while the rest is synthesised by yeast. These organic acids confer a sour taste to beer. Among several organic acids in beer, those

which are important for beer flavour include isovaleric acid (old hops flavour), caprylic and caproic (goat-like odour), phenylacetic acid (astringent flavour).

Among all ketones produced, vicinal diketones are of major importance to beer flavour. Diacetyl due to its low flavour threshold (0.1-0.15 ppm) offers an unpleasant buttery flavour to beer. Diacetyl is synthesized by the spontaneous decarboxylation reaction of α-acetolactate, a by-product in the valine bioynthesis pathway.

Acetaldehyde, another significant flavour compound in beer is an intermediate during alcohol formation and amino acid metabolism. Aldehydes in beer are largely derived from wort and are also produced by yeast from oxo-acid pools both via anabolic process (carbon source) and the Ehrlich pathway (amino acids). The presence of acetaldehyde produces fruity flavours (green apples, pumpkin) in beer. Typical flavour producing compounds in beer are listed in Table 4 (Meilgaard, 1979).

Table 4: Flavour compounds produced by yeasts during beer fermentation

Flavour	Compounds
Body	Polysaccharides (Dextrins)
Sour, acidic	Lactic acid, Acetic acid
Bitter	Iso-alpha acids
Sulphury	Dimethyl Sulfide (DMS)
Sulphudic	Hydrogen sulfide (rotten or boiled egg like)
Cooked vegetable	Dialkyl sulfides, DMS, Butyl mercapton, Ethyl mercapton
Metallic	Ferrous iron and some organic compounds
Salty	Sodium chloride, Magnesium sulphate, other mineral salts
Papery (cardboard)	Aldehyde, 2-trans-nonenal
Buttery	Diacetyl
Phenolic	Chlorophenols
Caramel,burnt	Melanoidins
Resinous,grassy	Aldehyde (hexanal)
Solvent-like	Ethyl acetate and other esters/fusel alcohols
Estery,fruity	Ethyl acetate, Ethyl caprylate, Ethyl caproate
Floral,hoppy	Phenethanol
Husky, grainy	aromatic aldehyde cyclopentyl methanol (husky)
Sweet	Dextrins

1.7 Influence of amino acid permeases on beer flavour

Different nitrogen sources including ammonium, amino acids, and di- and tripeptides play an important role in influencing beer flavour production. However amino acids represent the major source of the assimilable nitrogen in wort. The final concentrations of higher alcohols and ethyl ester or acetate ester derivatives are therefore dependent on the uptake efficiency of the corresponding amino acid and the sugar utilization rate. As seen previously, yeasts have a well regulated amino acid transport system involved in the uptake of various nitrogen sources required for its growth. The uptake of amino acids is highly regulated through mechanisms like nitrogen catabolite repression (NCR) and SPS systems. Different specific and general amino acid permeases involved in the uptake of amino acids are listed in Table 2.

Now we will focus on amino acid permeases significant in beer flavour production. Beer flavour is influenced in two ways: (i) Order for nitrogen source consumption, (ii) availability of nitrogen sources.

Amino acids are taken up into yeast by the Ehrlich pathway. After the initial transamination reaction, the excess α-keto acids are converted into fusel alcohols or fusel acids and excreted into the medium. The accumulation of a particular by-product in the beer is dependent on the uptake order and rate of the corresponding amino acid. For example, the ready assimilation of glutamate in yeast results in a fifth flavour, Umami. Also amino acids like valine, leucine, isoleucine, methionine, and phenylalanine are continuously assimilated via the Ehrlich pathway throughout the course of the fermentation resulting in fusel oil accumulation in beer.

Another means of influencing beer flavour is through addition of particular amino acids to the wort. For example, supplementation of the medium with valine and isoleucine results in reduced levels of VDK (vicinal diketone) production during fermentation (Krogerus K., 2013). Higher availability of the amino acids results in enhanced uptake of the particular amino acid leading to feedback inhibition in the amino acid biosynthesis pathway of valine and isoleucine, thereby reducing VDK levels. But increased assimilation could also lead to enhanced utilization of the particular amino acid thereby influencing the levels of flavour compound production. For example, addition of certain amino acids like alanine, proline, valine, leucine and isoleucine resulted in higher concentrations aliphatic alcohols and esters (ethyl acetate, isoamyl acetate, n-propanol, isobutanol and amyl alcohols) (Engan, 1970).

1.8 Amino acid transporters involved in major flavour producing compounds during beer fermentation

In yeast, various amino acid permeases are involved in the transport of amino acids across the plasma membrane with different affinities, specificities, capacities and regulations. Those amino acid permeases that are involved in the transport of various amino acids that contribute to important flavour in beer are described below.

1.8.1 Branched-chain amino acid permeases (Bap2p, Bap3p) influences vicinal diketone and fusel alcohol levels

Bap2p, Bap3p are amino acid permeases involved in the uptake of leucine, isoleucine and valine (branched-chain amino acids). The expression of these permeases is under the control of the plasma membrane Ssy1-Ptr3-Ssy5 (SPS) sensor. Previous studies showed that the transcription of *BAP2* is greatly induced in the presence of leucine leading to the increase in branched-chain amino acid uptake. However this induction by leucine was only effective in the case of cer-*BAP2* (*Saccharomyces cerevisiae BAP2* gene) and not for Lg-*BAP2* (lager part in the brewing yeast).

The increased levels of uptake of the branched chain amino acids will have positive effects on flavour production. When cells have sufficient valine uptake, diacetyl levels are reduced due to feedback inhibition in valine biosynthesis pathway. Similarly reduced levels of 2,3-pentanedione was achieved upon increased uptake of isoleucine. Overexpression of this leucine transporter also increased isoamyl alcohol production leading to increased bitterness flavour in beer. Similarly, the production of 2-methylbutanoate esters (fruity flavour) was increased upon increased uptake of isoleucine (Perez, 2002). Also addition of valine significantly increased production of the expected corresponding alcohol and ester (2-methylpropanol and 2- methylpropanoic acid ethyl ester) (Roze, 2010).

1.8.2 Control of higher alcohol and ester production using tyrosine and tryptophan amino acid transporters (Tat1p and Tat2p)

In addition to ethanol, several higher alcohols are synthesized during beer fermentation and contribute most significantly to alcoholic flavour and warm mouth-feel. The formation of these higher alcohols is maximized during amino acid starvation (low FAN levels), unfavourable to beer flavour. Control of higher alcohol formation can therefore be controlled by uptake efficiency of the corresponding amino acid and the sugar utilization rate.

The tryptophan amino acid transporters, Tat1p and Tat2p mediate high affinity uptake of aromatic amino acids tyrosine, tryptophan and phenylalanine. They are also involved in

the low affinity transport of valine, leucine, isoleucine and histidine. The expression of these permeases is under the control of the amino acid sensor Ssy1p-mediated regulation.

Increase in the uptake of these aromatic amino acids should have its effect on the production of the corresponding fusel alcohols. For example, increased uptake tryptophan can lead to increased production of tryptophol (almond flavour). Similar effect could be observed upon increase in theronine and phenylalanine uptake, producing higher alcohol and flowery flavours respectively.

1.8.3 Sulphur compounds production and methionine and cysteine transporters (Mup1p and Yct1p)

Sulphur is essential for yeasts in the formation of amino acids, proteins and Coenzyme A. The presence of sulphur compounds in beer produces dramatic effects on its flavour. Sulphur compounds are produced from sulphate, sulphite and sulphide ions present in the wort. During fermentation the yeasts produce hydrogen sulfide (H_2S), which when present in lower levels gives the desirable flavour of pale lager beers. However at higher concentrations, H_2S gives rise to the rotten egg smell responsible for the skunky odor in bad beer.

Another compound responsible for off-flavour in beer is Dimethyl sulfide (DMS) which when present in high concentrations (>100 µg/L) imparts a cooked sweet corn flavour to beer. Trans-2-nonenal is another compound associated with unfavourable papery and cardboard-like flavour in beer. However when *s*ulphur dioxide present in beer reversibly reacts with trans-2-nonenal*,* it produces other flavour-inactive compounds thereby reducing its adverse flavour impact. Hence increased SO_2 production is desirable during fermentation.

Sulphur containing amino acids like cysteine and methionine are very important in beverages as they are responsible for aromatic structure of beer and wine. Yeasts transport cysteine and methionine using high affinity permeases like Mup1p (methionine and cysteine transporter) and Yct1p (cysteine transporter). The expression of these nitrogen permease genes is subjected to SPS-regulation mechanism. The sulfate compounds are taken up into yeast and used in the biosynthesis of methionine, and cysteine resulting in the release of off-flavour by-products like H_2S in the process. Defective uptake of cysteine and methionine by the respective amino acid permeases (also due to lack of sufficient nutrients in the wort), may affect yeast growth and also result in excess production of sulfur compounds.

1.8.4 Nitrogen assimilation through ammonia, glutamate and glutamine transporters

Nitrogen utilization in yeast involves assimilation of the three key compounds: ammonia, glutamate and glutamine. Glutamate and glutamine provide nitrogen to the cells for the synthesis of amino acids and proteins. Previous research shows that glutamate and glutamine are the major donors of nitrogen in both yeasts and bacteria (Reitzer and Magasanik, 1987).

The assimilation of various nitrogen compounds gives rise to higher glutamate and glutamine levels in the cells. The nitrogen compounds that are transported into yeast via permeases are utilized for the biosynthesis of various amino acids and/or converted into ammonium and glutamate. Glutamine is synthesized from glutamate and ammonium condensation reactions using glutamine synthetase (*GNL1*). The ammonium, glutamate, and glutamine together form the hub of nitrogen metabolism, yielding several flavour compounds in beer. Accumulation of glutamate results in the umami flavour in beer. Likewise, ammonia produces caramelization in beer as it reacts with wort sugars (Maillard reaction) giving rise to a burnt flavour in the beverage.

The specific permeases that are involved in the transport of these nitrogen compounds are therefore responsible for the various flavour compounds produced. Since glutamate and glutamine are considerd as good nitrogen sources, their accumulation in the cell will result in the down regulation of NCR-sensitive genes. *GNP1* is the high-affinity glutamine permease which also transports Leu, Ser, Thr, Cys, Met and Asn. The expression of these permeases is modulated by the Ssy1p-Ptr3p-Ssy5p (SPS) sensor of extracellular amino acids.

DIP5 (Dicarboxylic amino acid permease) mediates high-affinity and high-capacity transport of L-glutamate and L-aspartate. This permease is also involved in the transport of Gln, Asn, Ser, Ala, and Gly. *MEP1* and *MEP2* are the ammonium permeases involved in the transport only ammonium ($NH4^+$). The expression of this permease is under the nitrogen catabolite repression regulation. Due to this reason, the high level transcription of *MEP2* leading to ammonium assimilation during the early hours of fermentation takes place only upon low concentration of glutamine, a key component of NCR regulation.

1.8.5 Beer flavour during nitrogen limitation conditions

The quality of the flavour compounds produced can be greatly influenced by the availability of nitrogen sources in the wort, which in turn activates the transporter genes to take up amino acids that support good growth. However, the permeases of amino acids subjected to NCR will be derepressed when the good sources (glutamate, ammonium) are depleted and only poor nitrogen sources are available. These permeases

include general amino acid permease Gap1p (transporting all naturally occurring amino acids) and Put4p (transporting proline) (Vandenbol, 1989; Jauniaux, 1990).

During limited availability of good nitrogen sources, the poor nitrogen sources are utilized for obtaining the required amino acids which could in turn lead to stress–related off flavours in the beverage (Fairbairn, 2012). For example, low concentrations of valine and leucine inhibit formation of isoamyl acetate. Also the uptake of amino acids is related to the formation of hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) formation.

The general amino acid permease (Gap1p), which is said to be the major transporter of arginine, senses amino acid substrates to transport all available nitrogen sources into the cell during conditions of nitrogen starvation. *GAP1* is transcriptionally regulated by the available nitrogen source and is under the control of nitrogen catabolite repression mechanism. Gap1p regulation is complex taking place both transcriptionally and post-translationally. In the presence of good nitrogen sources (glutamate or glutamine) the amino acid transport activity of Gap1p is low whereas their activity in the presence of poor nitrogen sources (proline or arginine) is high.

Similarly proline permease (*PUT4*) is required for high-affinity transport of proline. Although proline is the least-preferred nitrogen source for yeast and is not normally taken up during fermentation, it is the most abundant source of nitrogen wort and must (Huang, 2000). During the unavailability of good nitrogen source, yeasts degrade proline into glutamate through the proline utilization pathway.

Other permeases that are constitutively expressed in the presence of the particular amino acids include Hip1p (histidine transporter), Can1p (arginine transporter), Lyp1p (lysine transporter) and Tat2p (tryptophan transporter) (Cooper, 1982; Fink, 1985; Hoffmann, 1985; Sychrova, 1993; Schmidt, 1994).

1.9 Conclusion

Sufficient FAN amounts in the wort are necessary to promote adequate growth and a good flavour profile during alcoholic fermentations. Previous studies have revealed that, different nitrogen combinations can produce variations in aroma outcomes which are strain dependent. Another important factor that alters flavour production is the timing of nitrogen addition (Beltran, 2004). Certain flavour compounds are dependent on the addition of nitrogen source while certain others are independent of their addition. For example, higher FAN concentrations in the wort produced higher amounts of isoamyl acetate (Hashimoto, 2012). The uptake of various amino acids through the regulations of their amino acid permeases can therefore be said to have a strong influence on flavour compound production.

The availability of certain amino groups (e.g. branched chain amino acids) significantly alters the production of higher alcohols or aroma compounds like diethyl succinate (fruity/sweet ester). Furthermore, the timing of nitrogen source addition seems to favour different pathways of aroma compound formation. During initial fermentation the anabolic formation of aroma compounds is favoured leading to the uptake of the preferred nitrogen sources resulting in higher concentrations of related esters and fatty acids.

The order of assimilation of nitrogen substrates depends on the availability of nitrogen compounds and on the strain used. The utilization of the nitrogen sources are also dependent on the amino acid permeases whose expression levels may vary between different strains. For example, the lager brewing strains are polyploid in nature and are known to show interspecies differences in their phenotype (flavour production) due to copy number variations, single nucleotide polymorphism, variations in gene activation in response to environmental stress etc. As a result, the extent to which different strains are able to activate amino acid uptake and catabolism could largely vary, which leads to variations in their flavour profile production.

Practical Section

2. Introduction II

2.1 Optimization of brewing yeasts for brewing applications

Fermentation is the cumulative effect of the growth of yeast on wort which enables utilization of glucose in the glycolytic pathway to pyruvate which is then converted via acetaldehyde to ethanol and carbon dioxide. The first implementation of pure cultures of Brewing yeast in beer fermentation was in 1883 in the Carlsberg Brewery Laboratories.

Selection of brewing yeasts with optimal fermentation characteristics is of great interest to the brewers. Brewers' yeast strain optimisation may lead to a more efficient beer production process resulting in beer with improved quality. In this regard, several engineering strategies like rational and inverse metabolic engineering, evolutionary engineering and global transcription machinery engineering are employed (Cakar, 2012). Among these, metabolic engineering strategies (rational and inverse metabolic engineering) are crucial tools for generation of strains with strongly improved phenotypes.

Metabolic engineering helps analyse and modify metabolic pathways by controlling gene expression in yeast (Bailey, 1991). While rational metabolic engineering is based on available information about the pathways, enzymes and their regulation, inverse metabolic engineering intends to improve an organism by investigating a heterologous organism possessing the desired phenotype and identifying the genetic basis for differing values of the trait (Nevoigt, 2008).

Recent technological advances in transcriptomics, proteomics, metabolomics etc help identify differences at various molecular levels and discover targets for metabolic engineering (Gill, 2003; Bro, 2004). On the whole, a combination of all available methods is used for strain development. The genetic modification leading to the desired phenotype should be easily transferable to other relevant host strains.

2.2 Brewing yeasts

Brewing yeasts are categorized into two main types i.e. ale and lager yeasts, based on their fermentation characteristics. While ale yeasts are top fermenting yeasts whose fermentation temperature is in the range of 20 to 25 °C, lager yeasts ferment at lower temperatures of 8-14 °C. The two yeasts differ in the production of different flavour compounds. Lager yeasts produce more sulphur compounds but less fruity esters than ale yeasts during primary fermentation. Also due to higher fermentation temperatures, the diacetyl production during ale fermentation is higher than in lager fermentation but the reduction happens much quicker. As a result, the aging process in the case of ale

fermentation is shorter than that of lager fermentation lasting up to six weeks at low temperatures (around 0 °C). Lager beers comprise 90 % of the worldwide beer production while production of ale beers is mostly on the British Isles (Kodama, 2006).

Ale yeast strains are said to be polyploid and closely related to laboratory strains of *S. cerevisiae* but lager yeast strains are allopolyploid hybrids of *S. cerevisiae* and other *Saccharomyces* yeasts. Ale yeasts are classified as the "top-fermenting" type, *Saccharomyces cerevisiae* and lager yeasts are the "bottom-fermenting" type also called as *S. carlsbergensis*, *S. uvarum* and *S. cerevisiae* (Hui, 2004).

Classification of brewers' yeast was carried out mainly based on its ability to assimilate certain substrates, its colony and cell morphology and its mode of reproduction. The budding characteristics of the lager and ale yeasts differ. Lager yeasts separate into mother daughter cells soon after budding. However, in ale yeasts, the cells remain together when they bud again resulting in a small complex cell cluster. Also at the end of fermentation, ale yeasts rise to the top of the fermentation tank while the lager yeasts collect on the bottom of the tank.

Using various technological advancements such as rDNA technology, DNA re-association methods etc, it was found that *S. carlbergensis* showed high homology of 53 % to *S. cerevisiae* and 72 % to *S. bayanus* and 93 % homologous in genome constitution to *S. pastorianus* (Vaughan-Martini, 1985, 1987).

Furthermore the amplified fragment length polymorphism (AFLP) pattern of ale yeasts and lab yeast S288c and the two-dimensional gel electrophoresis of their proteomes showed that ale yeasts were closely related to the laboratory yeast strains (93.7 % homology) compared to the lager yeasts (74.6 % homology) (Azumi, 2001; Kobi, 2004)

2.3 Production of vicinal diketones

During fermentation, yeast metabolizes substrates into various products and by-products which have a considerable effect on the aroma profile and taste of the resulting beer. Yeast multiplies its mass during fermentation utilizing amino acids and other nutrients. For example, minerals are essential for stabilization of the yeast enzyme systems. Also apart from proteins, lipids are important components of the cell wall and are also needed for the uptake of nutrients and are synthesized from molecular oxygen and acetyl coenzyme A.

Diacetyl and pentanedione which contain two ketone (oxo-) groups on adjacent (vicinal) carbon atoms are together referred to as vicinal diketones (VDK) (Figure 2). These two metabolites are obtained as by-products during beer fermentation when their oxo-hydroxy precursors (α-acetolactate for diacetyl and α–ketobutyrate for 2,3 pentanedione) are produced in excess and pass through the yeast cell membrane into the beer. Diacetyl

is produced early in the fermentation and then most of it is reabsorbed by the yeast and reduced to flavourless compounds later on. Yeast strains differ in their diacetyl reduction ability.

Valine and isoleucine biosynthesis in yeast is localized in the mitochondria. The precursors (alpha-acetolactate and alpha–ketobutyrate) when in beer undergo spontaneous, non-enzymatic oxidative decarboxylation to yield the respective diketones. These diketones are reabsorbed into the yeast cell where they are reduced to the corresponding mono-alcohols and di-alcohols (acetoin, 2,3-butanediol and 2,3-pentanediol) with less intensity in taste and aroma.

Diacetyl which has a flavour that resembles butterscotch has a taste threshold 10 times lower than pentanedione with a sweet honey-perfume smell (Kallmeyer, 2004). Since most yeast strains make more diacetyl than pentanedione and because of its lower flavour threshold (0.15 mg/L for diacetyl and 0.90mg/L for pentandione), diacetyl is more significant than pentanedione.

Diacetyl is reduced to acceptable levels during maturation. Conversion of alpha-acetolactate to diacetyl is slow and is the rate-limiting step during maturation process. Brewers are therefore interested to obtain yeast strains with reduced diacetyl production so as to shorten the maturation time, thereby making the beer production a cost effective process.

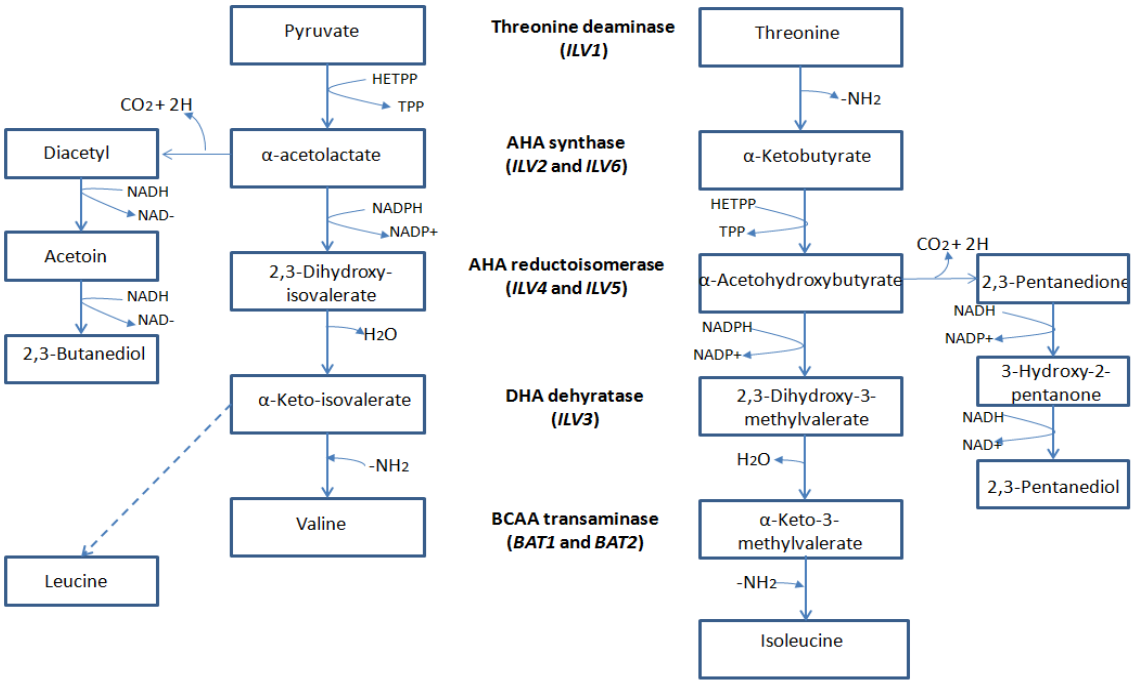


Figure 2: Biosynthesis and degradation of branched-chain amino acids (valine, leucine and isoleucine) in yeast and some related metabolites.

In recent years, a wide range of strategies were used for obtaining low diacetyl producing strains using genetic engineering approach. Use of low storage temperatures, limiting

wort oxygen content and avoiding bacterial contaminations also help reduce diacetyl content in the final beer.

Various strategies in genetic engineering adopted to minimize diacetyl formation include:

(i) Reduction or disruption of the activity of the gene encoding acetohydroxyacid synthase (AHAS) enzyme that catalyzes the formation of α-acetolactate from pyruvate.

(ii) Increase of metabolic flux in the valine production pathway (increased conversion of α-acetolactate).

(iii) Elimination of diacetyl formation from its precursor α-acletolactate,

To reduce diacetyl based on blocking the formation of its precursor α-acetolactate, the activity of acetohydroxyacid synthase (AHAS) responsible for the formation of α-acetolactate, was blocked. This was done either by completely or partially deleting the *ILV2* gene (Gjermansen, 1988; Falco, 1985). Although deletion of *ILV2* reduced diacetyl content in beer, the deletion strain encountered valine deficiency since the uptake of extracellular valine from the medium was not sufficient for growth (Kiellandt-Brandt, 1995).

Furthermore, overexpression of *ILV5* gene encoding acetohydroxyacid reductoisomerase was found to significantly reduce diacetyl (Mithieux, 1995). By increasing the activity of acetohydroxyacid reductoisomerase, the metabolic flux toward valine production is increased eventually resulting in a feedback inhibition when sufficient valine is synthesized.

The prevention of formation of diacetyl from its precursor α-acetolactate, was carried out by the introduction of heterogeneous α-acetolactate decarboxylase into green beer or expressed in brewers' yeast. This enzyme catalyzes the direct conversion of α-acetolactate to acetoin, thereby eliminating diacetyl formation.

Much research has been carried out in the past years to understand diacetyl formation and reduction during beer fermentation due to interest in shorter fermentation time. Although recent developments in science enabled reduction of diacetyl through genetic manipulations, most of it remains incompatible with the German beer purity law (Donalies, 2008). However the use of "self-cloned" yeast strains carrying no heterogeneous DNA in them are more easily accepted in food and beverage (Akada, 2002).

2.4 General Amino Acid Permease (Gap1p)

Nutrients are known to induce signalling cascades that control growth, in particular during conditions of nutrient starvation wherein yeast cells arrest growth and enter stationary phase. *GAP1* gene coding for the general amino acid permease is a unique transporter of a wide range of amino acids including all naturally occurring amino acids as well as several amino acid analogs when the cells are deprived of good nitrogen sources in the growth medium. In addition to its role as a transporter molecule, Gap1p also functions as a receptor for activation of protein kinase A (PKA) in response to amino acids via the fermentable growth medium-induced (FGM) pathway (Kriel, 2010). The activity of Gap1p is known to be down-regulated in response to the availability of good nitrogen sources. As in the case of other transporter proteins involved in signalling, Gap1p is subjected to a complex regulatory control. The tight regulation of Gap1p in response to amino acid content in the environment is said to be carried out by the signalling function of Gap1p.

2.4.1 Gap1p-an amino acid transceptor

The expression of *GAP1* gene is regulated by nitrogen catabolite repression (NCR) wherein gene expression is controlled by the available nitrogen sources in the medium. Upon induction, *GAP1* accumulates at the plasma membrane and acts as an amino acid scavenger for transporting low levels of external amino acids under nitrogen-limited conditions. The transport activity of Gap1p enables the uptake of broad range of amino acids and related compounds including non-metabolizable amino acids such as D-amino acids (Magasanik, 2002). The physiological purpose of expression of an amino acid transporter like Gap1p with high substrate promiscuity is better understood in its role as a transceptor. Besides amino acid transport, the major role of Gap1p is to detect all potential nitrogen sources in the medium, in order to activate the cellular machinery required for initiation of protein synthesis and fermentation (Kriel, 2011).

In an environment with a mixture of metabolizable and non-metabolizable amino acid substrates, to avoid competitive inhibition by the latter group yeast cells initiate cellular machinery to support good growth. Upon depletion of preferred nitrogen sources, the PKA pathway and cellular growth machinery is down regulated causing yeast cells to enter into the stationary phase (Garrett, 2008) (Figure 3 a). However, re-addition of preferred nitrogen sources into the medium causes reactivation of PKA pathway and resumption of fermentative growth and the down-regulation of stationary-phase characteristics (Figure 3 b). This amino acid-induced activation of PKA pathway is shown to be mediated by the receptor function of Gap1p (Kimpe, 2012). Upon re-addition of amino acids, Gap1p is also subjected to ubiquitination, a signal that triggers endocytic internalization of membrane transport proteins leading to vacuolar degradation. On the

whole the complex regulatory mechanism of Gap1p could be the result of protection of cells against overstimulation of PKA pathway. The Gap1p activates PKA in response to amino acids via the so-called fermentable growth medium-induced (FGM) pathway. The FGM pathway induction requires all essential nutrients like fermentable carbon source and a complete growth medium.

When the cells enter the stationary phase, the PKA activity (responsible for the control of various cellular metabolism, stress resistance and proliferation) is low (Thevelein, 1999). As a result, there is decreased expression of various cellular proteins, increase in reserve carbohydrates, resistance to stress and growth arrest.

Other nutrient transporters in yeast and fungi are known to be more strongly expressed at the plasma membrane when their substrate is present in limiting levels and to undergo rapid endocytic internalization upon addition of substrate. Other tranceptors that undergo similar controls on their intracellular trafficking as Gap1p include Pho84 transceptors, Fur4 uracil permease (Galan JM, 1996),the metal ion transporters Smf1 (Eguez, 2004), Zrt1 (Gitan, 2000), Ftr1 (Felice, 2005), Ctr1 (Liu, 2007), Alr1 (Graschopf, 2001), and the siderophore transporters Arn1 (Kim, 2002) and Sit1 (Erpapazoglou, 2008).

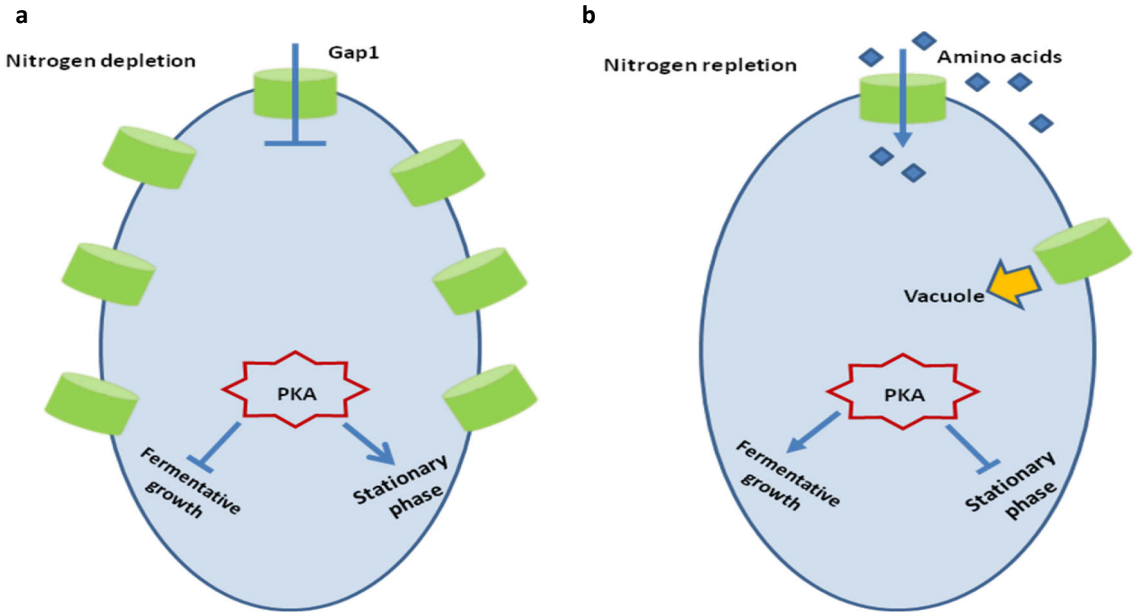


Figure 3: The signalling function and intracellular sorting of the Gap1 permease in yeast according to availability of amino acids in the medium.

a) Upon nitrogen-starved conditions, Gap1p accumulates at the plasma membrane leading to transport of available amino acids into the cell and signals the down regulation of PKA (protein kinase A) pathway thereby preparing the cells to enter the stationary growth phase.

b) Availability of amino acids causes Gap1p sorting to the vacuole for degradation and activation of PKA pathway, signalling the cells toward fermentative growth.

2.4.2 *GAP1* regulation

The intracellular trafficking pathway of the Gap1p transceptor is highly complex and is largely influenced by nitrogen supply (Figure 4). The synthesis of Gap1p occurs upon the binding of the transcription factors Gln3p and Nil1p (key regulators of NCR gene expression) to the target GATA sequences under poor nitrogen conditions. While Gln3p-dependent transcription of *GAP1* is said to be repressed when high concentrations of ammonia are present in the medium, Nil1p-dependent transcription is repressed by high glutamate concentrations (Stanbrough, 1996). Under conditions of nitrogen depletion, Gln3 and Nil1 localize to the nucleus and bind to GATA sequences found within the promoter region of *GAP1* thereby initiating transcription. The newly synthesized Gap1p is transported to endoplasmic reticulum (ER) to enable protein folding and then to the trans-Golgi network (TGN) to be sorted either to the plasma membrane under nitrogen-depleted conditions or degraded at the vacuole/lysosome under nitrogen-rich conditions. The stabilization of Gap1p at the plasma membrane is said to occur using the TORC1-regulated protein kinase Npr1 (Merhi, 2012).

The transcription of *GAP1* is also inhibited by TORC1 complex which regulates several amino acid permeases. Upon availability of good nitrogen sources, TORC1 maintains several highly specific amino acid permeases but as TORC1 is inhibited either through nutrient deprivation or treatment with rapamycin, the Gap1p is involved in the active transport of amino acids.

Studies on Gap1p showed that the activity of the permease does not always correlate with its mRNA abundance or protein expression thereby showing that *GAP1* is highly controlled both at the transcriptional level and post-translational level (Stanbrough, 1995). The process of endocytosis of Gap1p followed by vacuolar degradation involves Gap1p ubiquitination on two lysine residues in the cytosolic N terminus (Figure 4). The Gap1p which is localized to the plasma membrane is sorted to the vacuole by the Rsp5-Bul1/2 ubiquitin ligase complex. The ubiquitinated Gap1p is then delivered to the multivesicular body (MVB), where it is either delivered to the vacuole for degradation or recycled back to the plasma membrane for transport activity (Risinger, 2008).

Gap1p moving from Golgi apparatus to the plasma membrane is also affected by the presence of external amino acids causing it to be deviated to the MVB for degradation at the vacuole/lysosome. However this recycling from MVB-to-TGN could depend on levels of internal amino acids like glutamate and glutamine as well. Prior to the endocytosis of Gap1p into MVB and proteolytic degradation in the vacuole, the ubiquitin molecule is removed via Doa4, the deubiquitinating enzyme (Kriel, 2011).

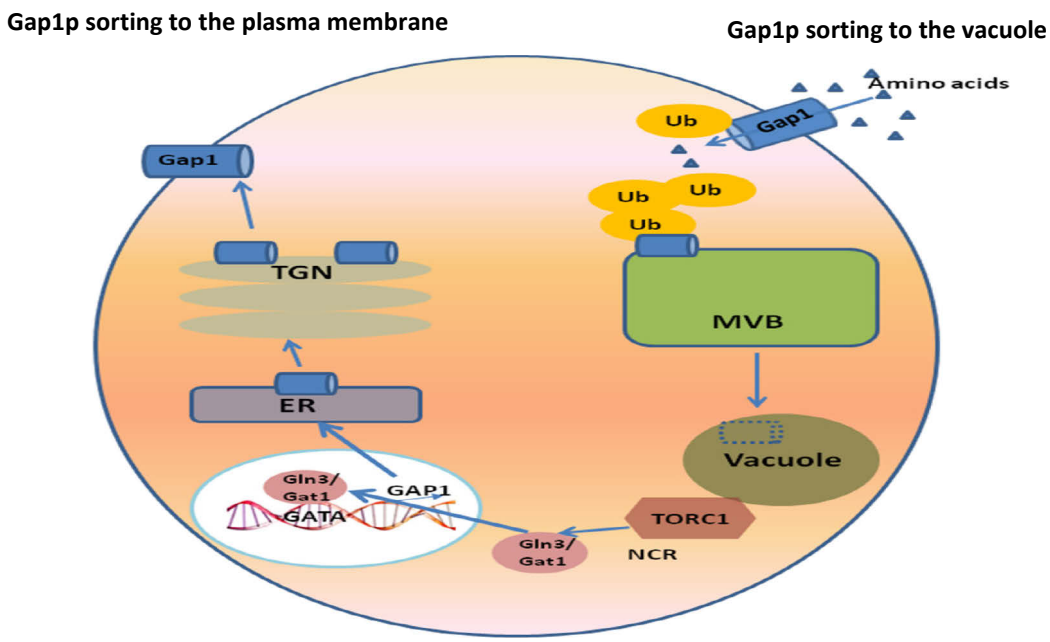


Figure 4: Regulation of the sorting of Gap1 permease in yeast

TGN: trans-Golgi network, MVB: multivesicular body, ER: Endoplasmic reticulum, Ub: ubiquitin, Gln3/Gat1: Transcriptional activators, NCR: Nitrogen catabolite repression, TORC1: Target of rapamycin complex 1.

2.4.3 Intracellular Regions of Yeast Gap1 Permease

The *GAP1* gene maps to yeast chromosome XI. Gap1 permease belongs to the amino acid-polyamine-organocation (APC) superfamily of amino acid transporters (Paulsen, 1998). Previous studies showed that impediment in Gap1p activity was not due to their non-expression. There are multiple intracellular regions within Gap1p that are essential for their secretion, transport activity, signalling and down-regulation (ubiquitination) (Merhi, 2011). Additionally amino acid side chains are important for determining substrate preference and form part of the amino acid binding site (Regenberg, 2001).

Gap1p has 602 amino acid residues that are arranged in 12 transmembrane domains (TMD) flanked by cytosol-facing N- and C-terminal tails (Figure 5). The two ubiquitination acceptor residues that are located on the N-terminus are marked in dark green (lysines K9 and K16). The adjacent 20-35 residues with dark green frame are also essential for ubiquitination. Additionally the light grey shaded residues that are located both in the N-terminus and within the middle internal cytosolic loop are essential for transport acitivity of the permease. The light blue shaded domains in the intracellular loops are considered important for the exit of Gap1p from the endoplasmic reticulum. The dark grey region located in the third cytosolic loop play an important role in Gap1p sorting. Also the yellow and red shaded amino acid-binding residues located within the transmembrane domains are essential for both transport and signalling. Other additional residues (green) that are located either within TMDs or in the loops, are essential for transport of specific amino acid substrates. The C-terminus is essential for recycling and sorting of Gap1p to the

plasma membrane. The purple frame containing the di-leucine motif and the purple shaded glutamate residues play an important role in Gap1p endocytosis (Hein, 1997). Deletion of the last 11 amino acid residues in the C-terminus affects the ubiquitination and endocytosis processes and causes constitutive over activation of the PKA pathway (Donaton, 2003). Other residues framed in light blue that contain Sec23/Sec24 COPII recognition motif also play an important role in ER exit (Malkus, 2002; Soetens, 2001).

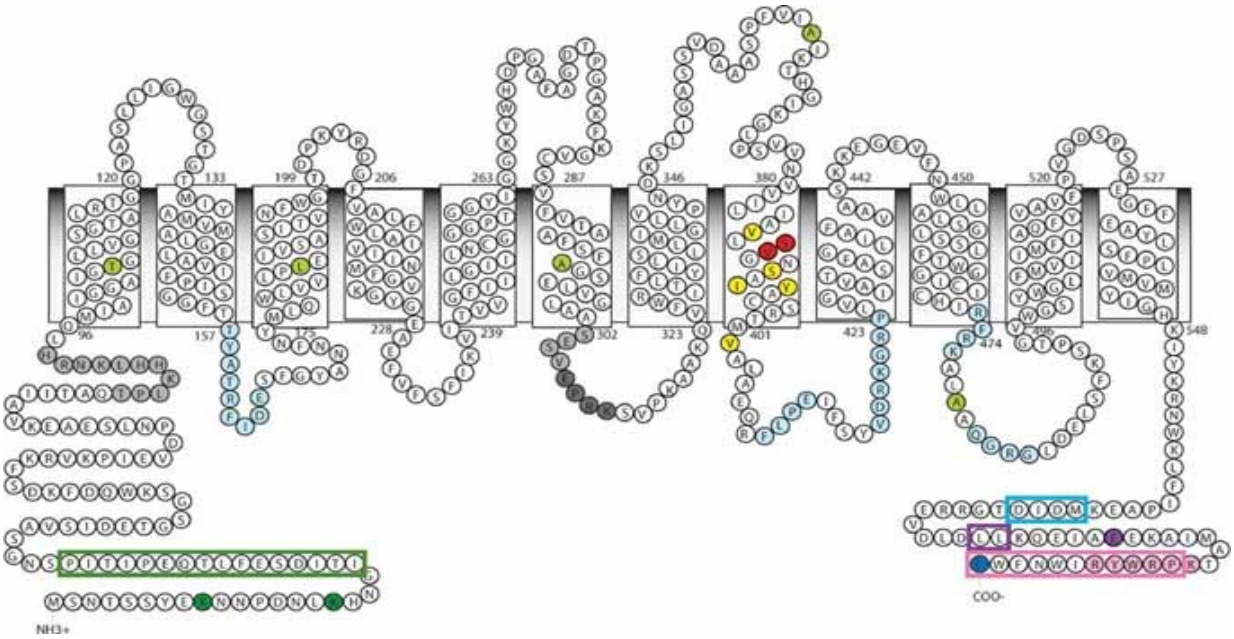


Figure 5: Structural features of the Gap1 transceptor essential for its transport, signalling and down regulation (Kriel, 2011).

2.5 Transcriptome data analysis and the hypothesis

Many strains show phenotypic and genotypic variations that facilitate their adaptation to different environments (Dugar, 2013). DNA microarrays typically compare two biological samples and present the results of those gene-by-gene comparisons as the logarithm base two of the ratio of the measured expression levels for the two samples (Sharov, 2004). This powerful tool can thus be used to analyze thousands of genes under various conditions to identify gene candidates that can be manipulated to improve a desired phenotype (Wittmann, 2012).

Transcriptional expression patterns of the yeast whole genome during beer fermentation were carried out to look for genetic differences between strains possessing interesting traits relevant to brewing. Six lager brewing strains which showed significant differences in diacetyl production were analyzed. For the microarray-based comparative transcriptome analysis, cells harvested from the 30 L main fermentation when the apparent extract reached values of 8 % and 6 % were used. The two time points were selected because higher genetic differences within the strains between the logarithmic growth phase (Apparent extract = 8 %) and the transition to stationary phase (Apparent

extract = 6 %) were expected. Transcriptome analysis was performed using the microarray chip technology with *S. cerevisiae* (Strack, 2009).

To determine genes that are relevant to the differences in diacetyl production, the transcripts of the six selected yeasts were analyzed. The aim of this work was to look for novel genes that are differentially expressed under brewing conditions among different strains differing in their diacetyl production.

Among the detectable genes showing strong or weak activities, several genes with significant differences between the low diacetyl producing Strain B and high diacetyl producing Strain D were registered. *GAP1* coding for general amino acid permease was short-listed among several genes showing highest differences in their mRNA expression. The identification of *GAP1* as a potential candidate was carried out by Schilling (Organobalance GmbH, unpublished). From the TDA, low diacetyl producing strains like Strain B, Strain F and Strain E had lower *GAP1* mRNA abundance compared to the high diacetyl producers like Strain D, Strain A and Strain C (Figure 6). *GAP1* is known to function as an amino acid transporter and sensor (tranceptor) since the availability of amino acids for the cell influences diacetyl production. *GAP1* was therefore identified as a potential candidate that influences diacetyl production in lager brewing yeast based both on its function and differential expression among brewing yeasts.

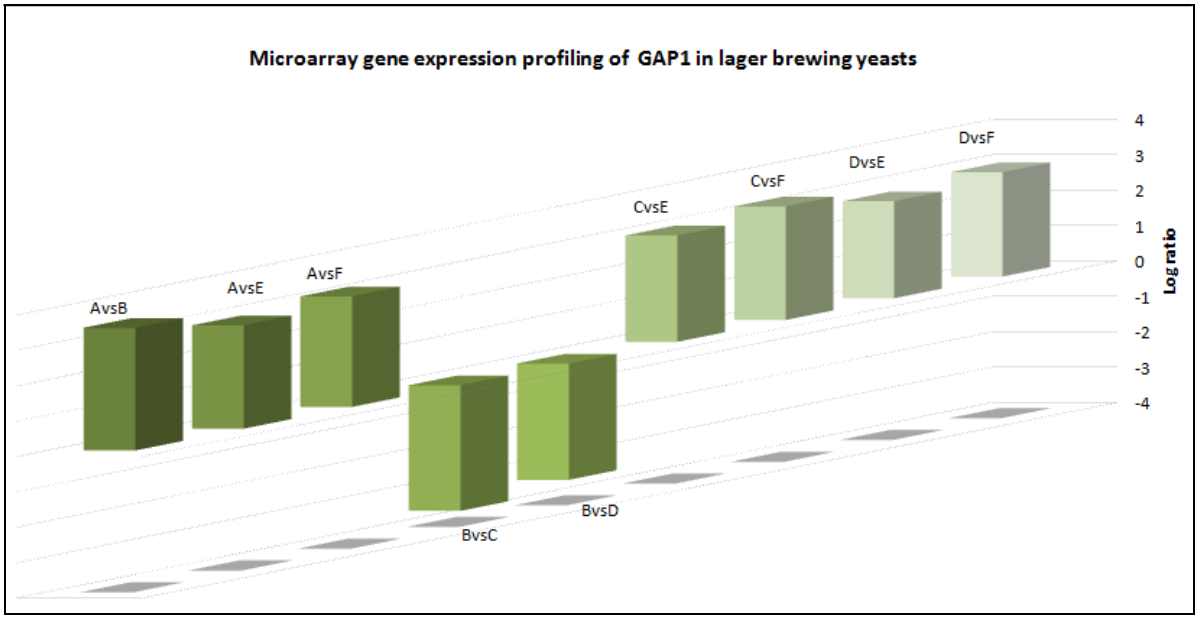


Figure 6: Microarray gene expression profiling of *GAP1* in Lager brewing yeast strains

Beer fermentation was carried out using six bottom fermenting brewers' yeast strains (Strains A, B, C, D, E and F). For the transcriptome analysis, the cells were harvested during the primary fermentation at extract concentrations 8 % and 6 %. Three samples from three independent fermentations of each yeast strains were compared. The above data showing differences in *GAP1* mRNA expression was obtained from the transcriptome data analysis (Strack, 2009).

2.6 Hypothesis and verification strategy

The composition of chromosome in different lager brewers' yeast strains could be quite diverse (Kodama, 2006). This denotes that the gene copy number of certain chromosomes could vary between strains (Nakao Y., 2009). Differences in mRNA abundance between strains can be caused by differences in copy number, promoter activity or mRNA stability (Nevoigt, 2008).

The microarray-based transcriptome analyses of six lager brewing strains under brewing conditions showed that *GAP1*, an amino acid transporter in yeast, was highly expressed in high diacetyl producing strains compared to low diacetyl producers. Based on these observations and with the available knowledge that differences at the genetic level within brewing strains could lead to their phenotypic differences, a hypothesis was deduced. The formulated hypothesis states that there is a positive correlation between Gap1p activity and diacetyl production in yeast i.e. strains with higher Gap1p amounts produce more diacetyl than those with lower Gap1p amounts.

Accordingly, the research hypothesis $\mathbf{H_1: Diacetyl_{highGap1p} > Diacetyl_{lowGap1p}}$

And conversely, the null hypothesis $\mathbf{H_0: Diacetyl_{highGap1p} \leq Diacetyl_{lowGap1p}}$

where $Diacetyl_{highGap1p}$ stands for diacetyl production in strains with high Gap1p amounts, and $Diacetyl_{lowGap1p}$ for diacetyl production in strains with low Gap1p amounts.

2.7 Experimental design for hypothesis testing

In the attempt to prove the hypothesis, the questions that will be addressed in this thesis are:

- Do these differences in *GAP1* mRNA abundance among different brewing strains still exist at the protein level and their activity?
- Does increase in Gap1p amounts in lager brewers' yeast strains lead to a corresponding increase in diacetyl levels?
- What is the role of the amino acid permease in influencing diacetyl production?
- Can difference in *GAP1* mRNA abundance in lager yeast be traced back to variations in gene copy number?

In order to answer these questions in the best possible way, the following experimental design was used (Table 5).

Table 5: Study of influence of *GAP1* on diacetyl levels in yeast strains: Hypothesis testing

Task	Wildtype/Transformants with differences in GAP1	Analyses	Significance
Verification of TDA in industrial brewing yeasts	•Strain D (High diacetyl producer) •Strain C (Medium diacetyl producer) •Strain F (Low diacetyl producer) •Strain B (Low diacetyl producer)	Immunodetection of Gap1p	Measurement of differences in GAP1 protein amounts among wild-type brewing strains
		Gap1p activity measurement in Nitrogen repression medium and in wort	Measurement of differences in GAP1 activity during maximum Gap1p expression and during beer fermentation conditions
Hypothesis testing in *S. cerevisiae* model strain	•GAP1 overexpression strain using self-replicating plasmid •*Δgap1* BY4741 •*Wild-type BY4741*	Immunoblot analysis	Measurement of differences in GAP1 protein amounts
		Diacetyl production in N repression medium	Analysis of difference in diacetyl production between strains
Hypothesis testing in Lager brewing strain C	•GAP1 overexpression strain using self-replicating plasmid •Wild-type strain with empty plasmid	Immunoblot analysis	Measurement of differences in GAP1 protein amounts
		Activity measurements	Measurement of differences in GAP1 activity during maximum Gap1p expression and during beer fermentation conditions
		Diacetyl production	Analysis of difference in diacetyl production between strains
Hypothesis testing in Lager brewing strain F	•GAP1 overexpression strain using self-replicating plasmid •Wild-type strain with empty plasmid	Immunoblot analysis	Measurement of differences in GAP1 protein amounts
		Activity measurements	Measurement of differences in GAP1 activity during maximum Gap1p expression and during beer fermentation conditions
		Diacetyl production	Analysis of difference in diacetyl production between strains

3. Materials and Methods

3.1 Laboratory equipment

Autoclave	Varioklav 500 EV (H+P Labortechnik, Oberschleissheim)
Balance	Feinwaage basic, L2200P, 1231MP & Typ 1907 (Sartorius, Göttingen)
Blotting apparatus	Semi-dry blotter (Hölzel, Wörth)
Centrifuge	Sorvall RC-5B /Dupont, Bad Homburg) Microrapid K (Hettich, Tuttlingen) Centrifuge Speed-Vac (Savant, Hickville, USA)
Clean bench	Uvub1200 Uniflow (UniEquip, Martinsried)
Electrophoresis chamber	Mini Sub & Wide Mini Sub™ Cell, Mini Protean (BioRad, Munich) Protein-Gel apparatus (Biotec Fischer, Reiskirchen)
Gel Documentation	Gel Logic 212 PRO (Carestream Molecular Imaging)
Microscope	Leitz, Wetzlar
PCR machine	MyCycler™thermal cycler (Biorad), PCR Thermocycler Biometra Tpersonal (Biometra, Germany)
Pipettes	Pipetman P10, P20, P200, P1000 (Gilson, France)
pH meter	Labor-pH-Meter 766 (Knick, Germany)
Water bath	Grant LTD; Thermomix 1460 BBraun, Melsungen
Spectrophotometer	Uvikon 860, Kontron Instruments
Vortexer	Vortex-Genie 2 (USA Scientific)
Refractometer	HRT32: Manual Hand-held Refractometer (Krüss, Germany)
Incubator	Biometra OV1, Biometra Göttingen
Distillation unit	PARNAS WAGNER "MAKRO" (VLB LaboTech, Berlin)
X-ray cassettes	KodakX-Omantic with intensifying screen (Kodak, Berlin)
Sonicator	Braunsonic 300S, BBraun, Melsungen

3.2 Chemicals and reagents

Agarose	Biozym LE Agarose, Biozym Scientific GmbH
Antibodies	Primary antibody: Gap 1 rabbit polyclonal Antibody (200 µg/ml), Santa Cruz Biotechnology Inc. CA, USA
	Secondary antibody: Swine Anti-Rabbit Immunoglobulins/HRP, Dako
Enzymes	Restriction endonucleases (Roche Diagnostics, Mannheim; New England Biolabs; Fermentas, DNAseI (Amersham); Zymolyase, Protease, RNAse (Gibco BLR), Shrimp Alkaline Phosphatase (Fermentas)
Kits	BioRad Protein-Assay (BioRad, Munich), PCR purification kit, Gel-extraction kit, Plasmid Miniprep kit (Analytik Jena AG, Germany)
Protein Markers	Unstained Protein Molecular Weight Marker (Fermentas), PageRuler™ Prestained Protein Ladder (Fermentas)
Midori Green DNA stain	Labpro Scientific
Nucleic acids	GeneRuler ™ DNA Ladder Mix (Fermentas), 100bp Plus DNA ladder (Bioneer, Seoul)
Oligonucleotides	Metabion, Berlin; Eurofins MWG Operon
PCR-reagents	Phusion® High-Fidelity DNA Polymerase (NEB Inc.) DreamTaq Green (Thermo Scientific) KAPAHiFi™ Polymerase (PEQLAB Biotechnologie)

Biochemicals/Chemicals	Ampicillin, Geneticin (Gibco Invitrogen, Karlsruhe), Agar-Agar, Yeast extract, Glucose, Peptone, Tryptone, EDTA, Tris, Chloroform, Glycerol, Isopropanol, Phenol, Herring Sperm (Promega), Calcium chloride, Potassium chloride, Calcium acetate, Magnesium chloride, Manganese chloride, Potassium acetate, Rubidium chloride, Sodium chloride, Tween 20, TBST, L-Citrulline, SDS, acrylamide/bisacrylamide, TEMED, APS, Milk powder, Ethanol, Coomassie Brilliant Blue R250, MES, Nonidet P40, PMSF (Roche)
Radiolabeled Amino acid	Citrulline, L-[Ureido-^{14}C]

All chemicals used in this study are mentioned in the respective techniques along with their amounts used. These chemicals were purchased from Sigma-Aldrich subsidiaries (Germany), Merck KGaA (Germany), Carl Roth GmbH + Co. KG (Germany), BD Biosciences (Germany), Fermentas (Germany), Novagen (Germany), Megazyme (Ireland) and Bio-Rad Laboratories Inc. (USA).

3.3 Strains used in the study

Strain	Lab name	Genotype	Source or reference
S. cerevisiae			
BY4741		*MAT*a *his*3Δ*1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf, Germany
BY4741-*Δgap1*		*MAT*a *his*3Δ*1 leu2Δ0 met15Δ0 ura3Δ0*; YKR039w::kanMX4	Euroscarf, Germany
BY4741- p416*GAP1*	BY4741 *GAP1* OE	BY4741 with GAP1 overexpression using p416*TEF6* plasmid	This study
S. carlsbergensis			
Sc-06168 H06	B	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin
Sc-06165 1000-22	C	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin
Sa-06167 Sc. DN	D	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin

Sa-06143 W34/70	F	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin
Sc-06165-p416TEF6*GAP1*	C p416*GAP1*	Bottom fermenting yeast with *GAP1* overexpression using single copy plasmid	This work
Sc-06165-YEpTKmTEF6*GAP1*	C YEpT*GAP1*	Bottom fermenting yeast with GAP1 overexpression using multi-copy plasmid	This work
Sc-06165-YEpTKmr	C YEpTKmr	Bottom fermenting yeast with empty plasmid	This work
Sa-06143-YEpTKmTEF6*GAP1*	F YEpT*GAP1*	Bottom fermenting yeast with *GAP1* overexpression using multi-copy plasmid	This work
Sa-06143-YEpTKmr	F YEpTKmr	Bottom fermenting yeast with empty plasmid	This work
Escherichia coli	DH5α	*fhuA2 Δ(argF-lacZ); U169; phoA; glnV44; Φ80; Δ(lacZ)M15; gyrA96; recA1; relA1; endA1; thi-1; hsdR17*	Meselson, 1968

3.4 Oligonucleotides

The sequence region in blue represents the primer recognition site; the recognition site of restriction enzymes are marked in red; Flanking ends that are homologous to the region upstream and downstream of the sequence to be replaced are marked in green.

Reference	Oligo name	Sequence (5' to 3')	Application
P1	Fw-*GAP1*	GCG TCT AGA AAA ATG AGT AAT ACT TCT TCG TA	Amplifying *GAP1* from genomic DNA
P2	Rv-*GAP1*	GCC ATC GAT TTA ACA CCA GAA ATT CCA GAT	
P3	Fw-*GAP1* p416 HR	CAT TAG AAA GAA AGC ATA GCA ATC TAA TCT AAG TTT TCT AGA AAA ATG AGT AAT ACT TCT TCG TAC	Cloning *GAP1* by replacing yECitrine in p416TEF6 via homologous recombination
P4	Rv-*GAP1* p416 HR	ATC GAC AAA GGA AAA GGG GCC TGT CTC GAG GTC GAC GGT ATC GAT TTA ACA CCA GAA ATT CCA GAT	
P5	Fw-p416 *GAP1*	GCT CAT TAG AAA GAA AGC ATA GC	Primers for sequencing *GAP1* after integration into p416
P6	Rv-p416 *GAP1*	CAA AGG AAA AGG GGC CTG TC	
P7	Fw- TEF6 *GAP1* YEpTKm HR	ATG TCT GCC CCT AAG AAG ATC GTC GTT TTG CCA GGT GAC CAC GTT ATA GCT TCA AAA TGT TTC TAC TCC	Cloning TEF6-*GAP1*-Cyc by replacing LEU2 in YEpTKm via homologous recombination
P8	Rv- Cyc YEpTKm HR	TTA AGC AAG GAT TTT CTT AAC TTC TTC GGC GAC AGCATC ACC GAC CCG AGC GTC CCA AAA CCT TC	
P9	Fw-TEF6 *GAP1*	GCG GAT ATC ATA GCT TCA AAA TGT TTC TAC TCC	Diagnostic PCR to verify integration of TEF6-*GAP1*-Cyc inside YEpTKm
P10	Rv-Cyc	GCG GAT ATC CGA GCG TCC CAA AAC CTT C	
P11	Fw-YEpTKm	GGT CTA GAG ATC TGT TTA GCT TGC C	Diagnostic PCR to verify uptake of YEpTKm into brewers' yeast
P12	Rv-YEpTKm	CTC ATC GAG CAT CAA ATG AAA CTG C	

3.5 Plasmids

p416TEF6*yECitrine*	Derivative of p416 *TEF1* containing yECitrine gene under the control of *TEF6*-promoter (Nevoigt *et. al.*, 2006)
p416TEF6*GAP1*	Derivative of p416 *TEF6* yECitrine containing *GAP1* gene under the control of *TEF6*-promoter (this work)
p416Kmr	Derivative of p416 *TEF1* minus yECitrine-*TEF6* (this work)
YEpTKmr	Derivative of YEplac161 containing kanamycin gene under the control of *TEF1*-promoter (kindly provided by Prof. Dr.Nevoigt, Jacobs University)

YEpTKmTEF6*GAP1*CycT	Derivative of YEpTKm containing the expression cassette TEF6-*GAP1*-Cyc minus *LEU2* (this work)

3.6 Media compostition

YPD medium	1 % Yeast extract; 2 % Peptone; 2 % Glucose; pH 6.3
YED medium	1 % Yeast extract; 2 % Glucose; pH 6.3
LB medium Ampicillin	1 % Tryptone; 0.5 % Yeast-Extract; 0.5 % NaCl; pH 7.4 100 mg/ml
SOB medium	2 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract, 8.6 mM NaCl, 2.5 mM KCl
SOC medium (50x)	1 M Glucose, 0.5 M $MgSO_4$, 0.5 M $MgCl_2$
TB I	10 mM $CaCl_2$, 30 mM Potassium acetate (pH 5.8), 50 mM MnCl, 100 mM $RbCl_2$, 15 % (v/v) Glycerol, 10 mM MOPS (pH 7.0)
TB II	10 mM $RbCl_2$, 75 mM $CaCl_2$, 15 % (v/v) Glycerol
WMIX (1 L)	10 g Na-Glutamate; 75 mg Inosit; 250 mg $MgCl_2$ * 6 H_2O; 100 mg $CaCl_2$ * 2 H_2O; 550 mg $MgSO_4$ * 7 H_2O; 1 ml 1000x Trace elements solution; 4 ml 250 x Vitamin solution; 1g Casamino acids; 100ml 20 % Glucose (2 %)
Trace elements (1000x)	0.175 g $ZnSO_4$ * $7H_2O$; 0.05 g $FeSO_4$ * $4H_2O$; 0.01 g $CuSO_4$ * 5 H_2O; 0.01 g $MnCl_2$ * 4 H_2O; 0.01 g NaMoO * 2 H_2O for 100 ml
Vitamin solution: (250x)	0.25 g Nicotinic acid; 0.625 g Pyridoxine; 0.25 g Thiamine; 0.0625 g Biotin; 1.25 g Ca-Pantothenate for 100 ml
Medium supplements for BY4741 strain	Leucine (0.04 g/l); Histidine-HCl (0.1 g/l); Methionine (0.08 g/l); Uracil (0.04 g/l)
WMIX-Proline	0.1 % Proline is added to WMIX (minus Na-Glutamate) medium
Wort	11.5 °P (Standard wort) kindly provided by pilot brewery,VLB

Solid media were prepared using 1.5 % Agar (Biozym ME Agarose) to the liquid media.

3.7 Buffers and Solutions

Yeast Transformation

TE-Buffer (10x)	0.1 M Tris-HCl pH 7.0; 0.01 M EDTA pH 8,0
Trafo-Solution I	0.1 M Lithium-Acetate*2H_2O in 1x TE buffer
Trafo- Solution II	0.1 M Lithium-Acetat*2H2O; 1x TE buffer with 50 % PEG 4000

DNA Gel electrophoresis

TAE Buffer:	20 mM Sodium acetate; 40 mM Tris; 2 mM EDTA; with glacial acetic acid pH 8,3
Stopper Solution(4x)	60 % (w/v) Sucrose; 20 mM EDTA; 0.025 % (w/v) Bromphenol blue

SDS-PAGE und Blotting

Stacking gel	0.4 ml of 40 % acrylamide/bisacrylamide (29:1), 1.0 ml of 0.5 M Tris (pH 6.8), 40 µl of 10 % SDS, 30 µl of 10 % APS, 3 µl of TEMED, 2.56 ml dist. Water
Separating gel	2 ml of 40 % acrylamide/bisacrylamide (29:1), 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10 % SDS, 100 µl of 10 % APS, 10 µl of TEMED, 5.4 ml dist. Water
Transfer buffer (1x)	3.03 g/L Tris; 14.4 g/L Glycine; 100 ml/L Methanol
TBST	100 mM Tris/HCl (pH 7.5); 150 mM NaCl; 0.1 % Tween 20
Blocking solution	5 % Milk powder in TBST
Running buffer (10x)	250 mM Tris;1.92 M Glycine; 1 % SDS
Sample buffer (2x)	100 mM Tris-HCl, pH 6.8; 4 mM EDTA; 4 % SDS; 20 % Glycerol; 0.02 % Bromphenol blue; 2 % 2-Mercaptoethanol

Phosphate buffered saline (PBS) buffer	150 mM NaCl, 8.4 mM Sodium phosphate dibasic (Na_2HPO_4), 1.6 mM Potassium phosphate monobasic (KH_2PO_4)
Zymolyase buffer (Yeast-Miniprep)	
Buffer	10 mM tri-Na-Citrate, 1 M Sorbit, 10 mM EDTA pH7.5 for enzyme activity of Zymolyase

Enzyme	40 µl of 1 M DTT (stored at -20 °C) and 2 mg Zymolyase (stored at 4°C) are added to 4 ml of the buffer prior to usage.

3.8 Molecular biology methods

3.8.1 Polymerase Chain Reaction and Agarose gel electrophoersis

PCR (polymerase chain reaction) is an *in vitro* technique to produce large copies of a specific DNA sequence in a short time. To perform a PCR, a 50 µl reaction mixture containing the target DNA sequence or colonies picked from agar plates, 1 µl of 2.5 mM dNTP mix, 5 µl of 10 x reaction buffer, 1 µl each of the 10 pmol/µl primers (forward and reverse) and 1-2 U Phusion High Fidelity DNA polymerase or Dream Taq polymerase were used. The reaction mix was then placed into the Thermocycler programed to run according to the following conditions: 98 °C, 10 min (initial denaturation); 25 x [98 °C, 10 s; T_m (annealing temperature), 30 s (-0.30 °C/cycle); 72 °C, 30 s/kb] 72 °C, 10 min (final extension); 4 °C, hold.
The PCR products were checked using agarose gel electrophoresis on a 1 % agarose gel in 0.5 x TAE buffer mixed with Midori Green DNA stain (5 µl Midori Green in 100 ml gel). The DNA samples were mixed with loading buffer and gels were run at 80 V for 40 min. After the run, the separated DNA was viewed and recorded using a gel documentation system.

3.8.2 DNA sequencing

Cloned gene constructs were sequenced using the dideoxy chain termination method developed by Sanger (Sanger, 1977). Sequencing was performed by the group of Dr. Martin Meixner at Humboldt University, Berlin.

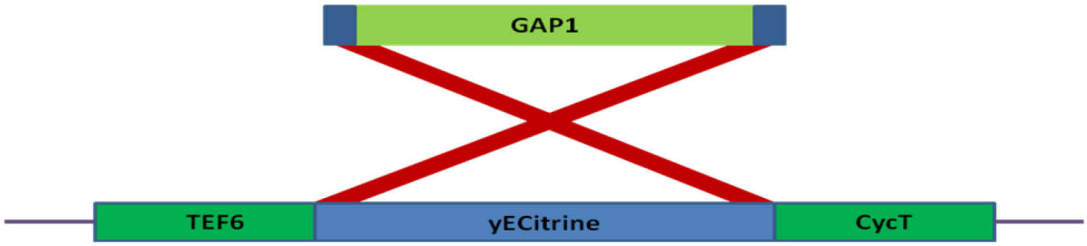
3.8.3 Yeast transformation and homologous in vivo plasmid recombination

3.8.3.1 Construction of p416TEF6*GAP1*

Traditional plasmid construction method involves restriction digests and *in vitro* ligation reactions. An alternative method is *in vivo* plasmid construction by homologous recombination in yeast (Andersen, 2011). This method takes advantage of the highly efficient homologous recombination system in yeast. The steps in the yeast-mediated ligation involved linearization of the vector of interest, p416*TEF6*yECitrine (plasmid map in appendix: Figure C) using *Mfe* I found inside the yECitrine gene. The linearized vector was dephosphorylated to avoid religation. This vector was then co-transformed into yeast along with the *GAP1* PCR fragment carrying the 45 bp flanking ends homologous to the

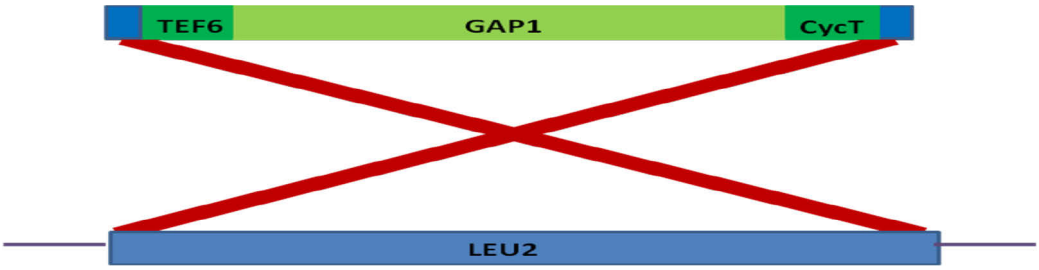
target locus inside the vector. *GAP1* gene coding for General Amino acid Permease was amplified by colony PCR using primers P3 and P4 that bind upstream and downstream of *GAP1* coding sequence in the *S. cerevisiae* genomic DNA.

The primers P3 and P4 (T_m= 55 °C) were designed to replace the reporter gene yECitrine with *GAP1* to be expressed under the influence of a strong *TEF6* promoter. The transformation into BY4741 (host) in which the homologous recombination occurred was carried out using the Li-Ac yeast transformation method. The positives clones were selected on YED-G418 agar plates (300 µg/ml) and the verification of *GAP1* integration into the plasmid was carried out by diagnostic-colony PCR using primers P5 and P6 (T_m=51 °C). The plasmid is isolated from the positive clones and retransformed into competent *E. coli* cells to obtain higher plasmid concentration. The plasmid was re-isolated using *E .coli* plasmid miniprep and verified by restriction analysis using *Xba* I and *EcoR* I and subsequent sequencing with primers P5 and P6.



3.8.3.2 Construction of YEpTKmTEF6*GAP1*CycT

The high-copy self replicating plasmid for the constitutive expression of *GAP1* was constructed in the same manner as p416TEF6*GAP1* via homologous recombination in yeast. The TEF6*GAP1*CycT fragment was amplified by PCR with primers P7 and P8 (T_m= 56 °C) using p416 TEF6 *GAP1* as the template. The primers contained 45 bp each of flanking DNA that is identical in sequence to the *LEU2* gene which was the targeted locus for gene insertion. YEpTKmr linearization was carried out with EcoRV found within the *LEU2* gene. The re-ligation of the plasmid is minimized by phosphatase treatment. The replacement of *LEU2* with the TEF6*GAP1*CycT construct took place in BY4741 strain by homologous recombination. The positive clones were selected on YED-G418 plates(300 µg/ml) and verification of the insertion of the gene of interest was carried out by colony PCR using primers P9 and P10 (T_m=56 °C). The plasmid was further verified by restriction with *BamH* I and subsequent sequencing with primers P9 and P10.



3.8.4 Transformation of Yeast by Lithium Acetate/Single-Stranded Carrier DNA/Polyethylene Glycol Method

The introduction of exogenous DNA into yeast by transformation was carried out by lithium acetate/PEG method (Gietz, 1998). An overnight preculture in YPD medium was used to inoculate a 50 ml main culture to a start OD_{600} of 0.3 and was incubated with shaking at 160 rpm for 4 h until the cells reach an OD_{600} of 0.8-1.0. The cells were then placed on ice for 30 min and 30 ml of the culture was cetrifuged at 863 g for 2 min. The harvested cells were washed in ice cold water and in 0.1 M LiAc (Trafo-Solution I). The cells were resuspended in 100-200 µl of Trafo-Solution I and were thus made ready for transformation. The transformation mix contained: 40 µl of competent cells, 10 µl of 10 mg/ml Single-Stranded Carrier DNA (Salmon Sperm DNA), 230 µl Trafo-Solution II, 0.5-5 µg DNA. The transformation mix was incubated for 30 min at 28 °C followed by addition of 30 µl DMSO. The transformation was carried out for 1 h at 42 °C or 37 °C in the case of temperature sensitive strains. The cells were suspended in YED medium and incubated overnight at 28 °C which enabled regeneration of the dominant kanMX marker. When using the KanMX selectable marker, the positive clones were selected on the following concentrations of Geneticin (G418) as in the table below. The YED-G418 plates were incubated in the dark at 28 °C for 3-4 days to select the transformed clones.

Strains	G418 concentration (µg/ml)		
	Initial selection on YED agar	Subsequent plating on YED agar	Liquid medium
BY4741	300	350-400	200
Strain C	20	50-100	50
Strain F	15	50-100	50

3.8.5 Preparation of *E. coli* competent cells using Rubidium chloride method (Hanahan, 1983)

This method is based upon Rubidium chloride method and is reported to give better transformation efficiencies than the $CaCl_2$ procedure (Cohen, 1972). 0.5 ml of an overnight preculture of *E.coli* was added to the SOB medium containing 49 ml SOB medium and 1 ml 50x SOC. The inoculated culture was incubated at 37 °C with shaking at 250 rpm until an OD_{600} of 0.3-0.5 was reached. All successive steps had to be carried out on ice at 4 °C. The culture was cooled on ice for 15-30 min and centrifuged at 1075 g

for 5 min. The pellet was resuspended in 16 ml TB I and incubated on ice for 15 min followed by centrifugation at 746 g for 5 min. The pellet was resuspended in 2-4 ml TB II. The competent cells were aliquoted and subjected to rapid freezing in liquid Nitrogen and stored at -70 °C.

3.8.6 Transformation of *Escherichia coli* competent cells

The competent *E. coli* cells were initially thawed on ice and then mixed with the DNA (10-20 µl ligation mix or 50-100 ng plasmid DNA) to be transformed. This mix was incubated on ice for 30 min and subjected to heat shock for 2 min at 42 °C. Following the heat shock treatment, the transformed cells were placed on ice for 5 min followed by addition of 800 µl LB-medium (without antibiotic) and incubated at 37 °C for 45-60 min with shaking at 200 rpm. The transformation mix was then plated on LB-Amp plates with appropriate dilutions and incubated overnight at 37 °C.

3.8.7 Gel elution

Gel elution enables isolation of a desired fragment of intact DNA from an agarose gel by effectively removing primers, primer dimers, dNTPs, enzymes and salts from PCR and other reaction mixtures. Subsequently, the DNA was extracted using a innuPREP Gel Extraction Kit (Analytik Jena AG) according to manufacturer's instruction.

3.8.8 Plasmid isolation from yeast (*S. cerevisiae*) and *E. coli*

For the isolation of plasmid from transformed yeasts, 2 ml each of 48 h cultured yeast strains were centrifuged at 5000 × g for 7 min, the supernatants were discarded and the pellets were resuspended in 300 µl zymolyase buffer and incubated at 37 °C for 60 min with slow shaking. Then 200 µl of glass beads were added and the suspension was vortexed for 10 min, and then centrifuged for one minute at 12,000xg. 300 µl of the aqueous phase was transferred into new Eppendorf tubes and the plasmid isolation was carried out with a innuPREP Plasmid Mini Kit Plus (Analytik Jena AG) without the addition of the initial resuspension buffer.

For the purification of plasmid DNA from *E. coli*, 5 ml LB medium with 100 µg/ml ampicillin were inoculated with cells from a plate or a frozen culture and incubated overnight at 37 °C and 200 rpm. The fully grown culture was then centrifuged at 5000xg and purified according to the manufacturer's protocol with a innuPREP Plasmid Mini Kit Plus (Analytik Jena AG).

3.8.9. Restriction and dephosphorylation

For restriction of plasmid in a 20-50 µl reaction mix, 1/10 10x Reaction buffer, 2 U Restriction enzyme/µg DNA were mixed incubated at 37 ° C for at least two hours.

Treatment with alkaline phosphatase was mainly used when a blunt-end or a single restriction enzyme was used. 1 µg DNA was restricted with 5 µl 10x alkaline phosphatase buffer and 1 µl shrimp alkaline phosphatase (Fermentas) was added to a total volume of 50 µl. The DNA was then purified using a innuPREP PCRpure Kit (Analytik Jena AG) according to the manufacturer's instructions.

3.8.10. Ligation

T4 DNA ligase catalyzes the formation of phosphodiester bonds between neighboring 3'-hydroxyl and 5'-phosphate ends in double-stranded DNA (Harada, 1993). In a 20 µl ligation mix, 1/10 10x ligase buffer and 1 µl of T4 ligase (Fermentas) was mixed with 100 ng restricted plasmid. The ligation was carried out for 2 h at room temperature or incubated overnight at 14 °C.

3.9 Immunodetection of Gap1p in yeast using *GAP1* antibody

3.9.1 Preparation of crude protein extract from yeast

An overnight culture of yeast in WMIX medium was washed and transferred for 2 h into WMIX Proline for better Gap1p expression. Cells were cooled on ice for 30 min, harvested and washed with ice-cold water. The cells were then resuspended in ice-cold extraction buffer (50 mM NaCl, 20 mM Tris, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM EGTA) supplemented with 0.1% Nonidet P40 and protease inhibitors phenylmethylsulphonyl fluoride (PMSF) and pepstatin A. For preparation of crude extracts, vigorous vortexing of the cell suspension with 0.5 mm glass beads was carried out (3x for 15 s each with regular cooling on ice). The cooled extracts were cleared by centrifugation at 13,000 r.p.m. for 5 min and the supernatant was centrifuged again for 30 min at 13,000 r.p.m. to obtain crude protein extract.

3.9.2 Preparation of membrane-enriched extracts

Yeast cells grown in WMIX-Proline medium were dissolved in digestion buffer (500 µl Phosphate buffered saline buffer + 2 mM PMSF) and were broken open with glass beads. The suspension was centrifuged at 14,000 g, 30 min at 4 °C. Supernatant (cytosolic proteins) was then discarded and pellet containing membrane proteins was solubilized in 400 µl Solubilization buffer (1% Triton X-100 and 1mM PMSF in PBS) by shaking at 900 rpm for 40 min. The suspension was then centrifuged at 4000 rpm for 5 min at 20 °C. Supernatant containing membrane proteins could be stored at -20 °C.

3.9.3 Bradford assay for protein determination

Protein quantification was carried out by BioRad protein assay according to Bradford method of protein determination (Bradford, 1976).

3.9.4 SDS-PAGE

The protein samples were analyzed on a 12% gel in Laemmli's system (Laemmli, 1970). The protein samples were mixed with 2 x sample buffer and incubated at 37 °C for 15 min to completely dissolve the SDS. Gels were run initially at 80 V until the proteins pass through the stacking layer and then increased to 120 V, stained with Coomassie staining solution (0.25 % Coomassie blue G250 in 10 % acetic acid and destained in 10 % acetic acid.

3.9.5 Protein detection on Western Blot

After protein separation on SDS-PAGE, the proteins were transferred to a PVDF blotting membrane. The SDS-gel, the PVDF membrane and 8 pieces of Whattmann blotting papers were equilibrated in the transfer buffer for 10 min. The blotting was carried out on a Semi Dry Blotting Apparatus by applying 56 mA for 1.5 h. For hybridization, the filters were firstly washed with TBST buffer (twice for 15 min) followed by blocking of filters with blocking buffer (5% skimmed milk in TBST buffer). The filters were washed again with TBST buffer (twice for 15 min) and incubated O.N. at 4 °C with primary antibody (1:500 dilution in TBST) specific for detection Gap1 in *S. cerevisiae*. After washing with TBST buffer (twice for 15 min) the membranes were incubated for 1 h at RT with secondary antibody (horseradish peroxidase-conjugated swine anti-rabbit 1:1000 dilution in TBST). After washing with TBST buffer (three times for 15 min), membrane was incubated for detection with Pierce ECL Western Blotting Substrate (Thermo Scientific) and exposed to an X-ray film and developed according to manufacturer's instructions.

3.10 Determination of L-citrulline transport

3.10.1 Gap1p activity of cells grown nitrogen repressed medium (Donaton, 2003)

For the determination of Gap1p activity, nitrogen starved-yeast cells that were cultivated in WMIX Proline medium for 24 h were cooled on ice for 30 min and harvested. The cells were then washed twice with 25 mM MES/KOH buffer (pH 6) and resuspended in fresh WMIX Proline medium with 4 % glucose (to provide cells with energy for the import). The final cell density was adjusted to 80 mg ml^{-1} (wet weight). 3 samples of 500 µl cell suspension were taken to determine the total protein concentration by Bradford assay. For the import assay, 50 µl of this cell suspension was prewarmed at 28 °C for 10 min. 50 µl of 2 x L-Citrulline containing 5 mM unlabelled + [^{14}C]- L-citrulline to obtain a specific radioactivity of 10 Bq $nmol^{-1}$ was added to the cells. After 10 s, 10 ml of cold water was added to stop the import. The cells were then filtered through a glass microfibre filter (Whatman GF/C, retention particle size 1.2 mm) pre-wetted with an L-citrulline solution (2.5 mM) and immediately washed twice with 20 ml of ice-cold water.

For each determination, three samples and 2 blanks (without cells) were taken. The filters were stored at -20 °C prior to measurement. The measurement of radiation on the filter was counted in a liquid scintillation counter (Beckman Coulter LS6500).

3.10.2 Gap1p activity of cells under brewing conditions

For the determination of Gap1p activity in cells under brewing conditions, cells were harvested when the apparent extract content reached 6%. To avoid competitive inhibition of citrulline transport by other amino acids in the wort (e.g. glutamine), cells grown on wort were harvested, washed twice in 25 mM MES/KOH (pH 6), and transferred to preheated WMIX Proline medium containing 4% glucose just before the transport assay. (Craene, 2001). The addition of glucose provides the cells with energy required for the amino acid transport. The import assay was carried out in the same manner as described in Section 3.10.1.

3.10.3 Calculations of [^{14}C]- L-Citrulline and L- Citrulline mix

[^{14}C]- L-Citrulline (stock)

Specific activity	55 mCi/m mol
Concentration	0.1 m Ci/ml =3.7 MBq/ml

Hence, to obtain a specific activity of 10 Bq/nmol in 100 µl 2x L-Citrulline (5 mM) 1.5 µl of [^{14}C]- L-Citrulline (stock) was added.

3.11 Cultivation of *E. coli* and yeast cultures

The cultivation of *E. coli* was carried out in 100 ml Erlenmeyer flasks at 37 °C and 200 rpm in LB medium on a rotary shaker. For the selection of plasmid-carrying strains, 100 µg/ml ampicillin was used.

Yeast cultivation was carried out either in the complex YPD medium or in the minimal WMIX medium which is a modification of WMVIII medium (Lang, 1995). Yeast precultures were grown at 28 °C and 150 rpm in 100 ml Erlenmeyer flasks for 48 h and main cultures at 28 °C and 150 rpm in 250 ml baffled flasks on a rotary shaker.

3.12 Lab scale brewing

Brewing trials were conducted in 2 L Schott flasks fitted with membrane screw caps to enable gas permeability. 1.2 L standard wort with an original gravity of 11.5 °P (kindly provided by the Studienbrauerei, VLB) was filled into each flask. Propogation of the preculture is carried out at 26 °C with shaking at 130 rpm until a pitching rate of 10^7 cells/ml could be achieved. 50 µg/ml of G418 was added to the preculture to provide selection pressure for the brewing yeast transformants. The cells were acclimatized for

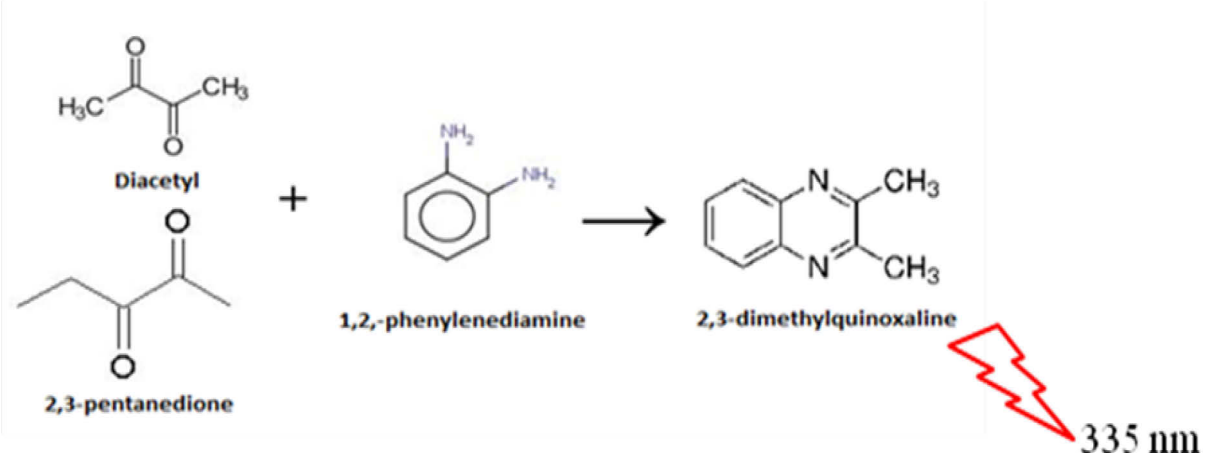
one day to the fermentation temperature of 20 °C. The cells were harvested and washed once in fresh wort to remove all traces of G418. 15 million cells were pitched into each flask for primary fermentation. The primary fermentation was carried out for 7 days with samples taken everyday for the determination of apparent extract concentration, pH and cell number. Diacetyl was measured using spectrophotometric method (Section 3.14).

3.13 Fermentation in EBC columns

The fermentations were carried out in 12 modified EBC fermentation tubes of stainless steel with a capacity of 4 L . Using these tubes, comparison of yeast strains under the same experimental conditions were carried out in parallel. The fermentations were carried out for 7 days at a temperature of 14 °C and a pressure of 0.8 atm. The pre-culture propogation and sampling was carried out in the same manner as described in Section 3.12.

3.14 Spectrophotometric determination of vicinal diketones (Diacetyl and 2,3-Pentanedione) (MEBAK, 2002)

The photometric method is a quick and well established method for the determination of vicinal diketones and preferred over expensive gas chromatographic methods. However the method does not permit preferable differentiation between diacetyl and pentanedione. The principle of this method involves the reaction between diacetyl and/or 2,3-pentanedione and 1,2-phenylenediamine to form 2,3-dimethylquinoxaline which exhibits a specific absorption at 335 nm.



In order to measure the total diacetyl content (the sum of free diacetyl and its precursor, α-acetolactate) in the beer, the sample is heated at 70 °C for 90 min. For the measurement, 100 g of non-decarbonated beer sample was added into a pre-heated distillation appliance. A few drops of anti-foam emulsion was added to the beer to control excessive foaming during the boiling step. The steam supply was adjusted to obtain ~25 ml distillate in 2 min. The distillate was distributed into two 25 ml EM flasks with 10 ml of

the collected distillate in each flask (main value, blank value). 0.5 mL of 1,2-phenylenediamine is added to the sample, mixed and incubated in dark for 30 min. The reaction is stopped by the addition of 2 mL 4 N HCl. To the blank value, 2.5 mL of 4 N HCl was added. The extinction of the main value against blank at 335 nm was measured within 20 min. The measurement range of this method is 0-1 mg/kg.

Calibration standards of diacetyl were prepared by diluting a stock solution of 10 ppm diacetyl in beer. Standards were prepared in the range of 0 to 1.2 ppm and the corresponding absorbance values at 335 nm were determined (Figure 7). The values for the unknown samples were determined by comparison to the standard curve.

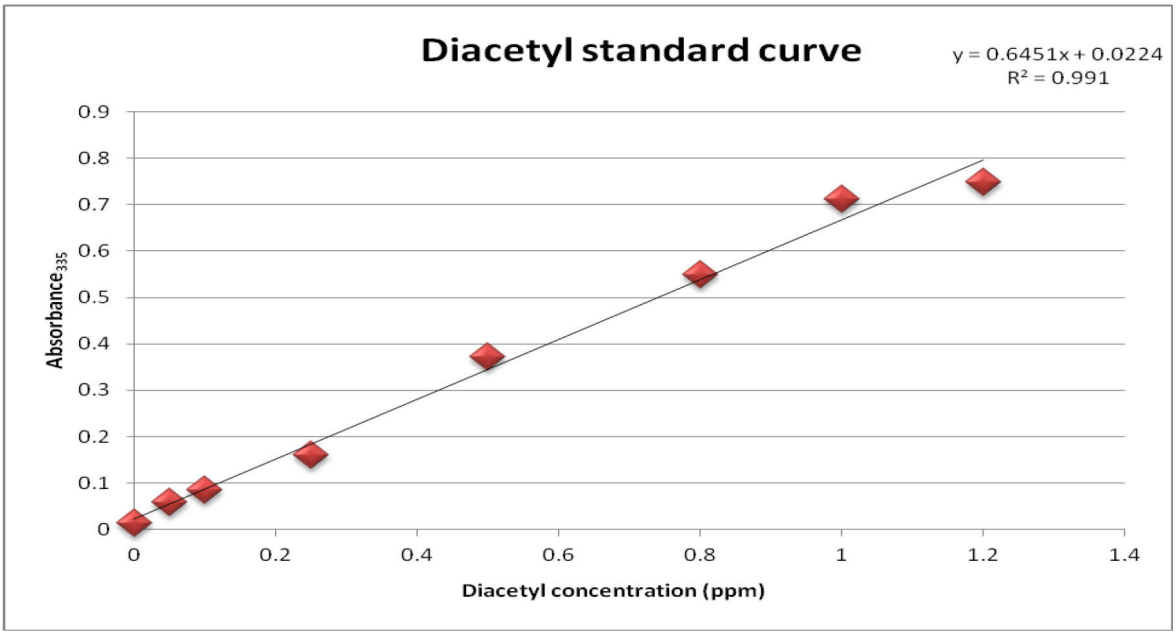


Figure 7: Diacetyl calibration curve: determination of diacetyl concentration using Spectrophotometric method

4. Results

4.1 Detection of Gap1 proteins in bottom fermenting brewers' yeast strains

4.1.1 Protein sequence alignment of the *Saccharomyces cerevisiae* (Sc-) and non-*Saccharomyces cerevisiae* (non-Sc) type-Gap1p

Lager brewers' yeasts contain two versions of many genes (Sc-type and non-Sc-type) (Winde, 2003). The coding DNA sequence (CDS) of the pure non-*S. cerevisiae* part of the lager brewers' yeast (WH 34/70) was kindly provided by Dr. Kodama (personal communication). A nucleotide sequence alignment carried out between Sc-*GAP1* and non-Sc-G*AP1* showed that their CDS had the same length with an identity of 83 %.

The Gap1 antibody used for the detection of Gap1p in yeast (Gap 1 Antibody (Y-40): sc-98846), binds to the epitope corresponding to the first 40 amino acid residues at the N-terminus of Gap1p of *S. cerevisiae*. To check whether the antibody specific for binding to the Sc-Gap1p could immunodetect non-Sc-Gap1p as well, the investigation of homology in the Gap1p sequences between lager brewers' yeast WH 34/70 and *S. cerevisiae* was carried out. The amino acid sequence of Gap1p of non-Sc origin showed 89 % identity with the sequence of Sc-Gap1p. However considering only the first 40 amino acid residues, the binding site of the Gap1 antibody, an identity of 97.5 % was observed (Figure 8). It could therefore be assumed that antibody binding to the non-Sc-Gap1p is highly probable due to close sequence identity with Sc-Gap1p.

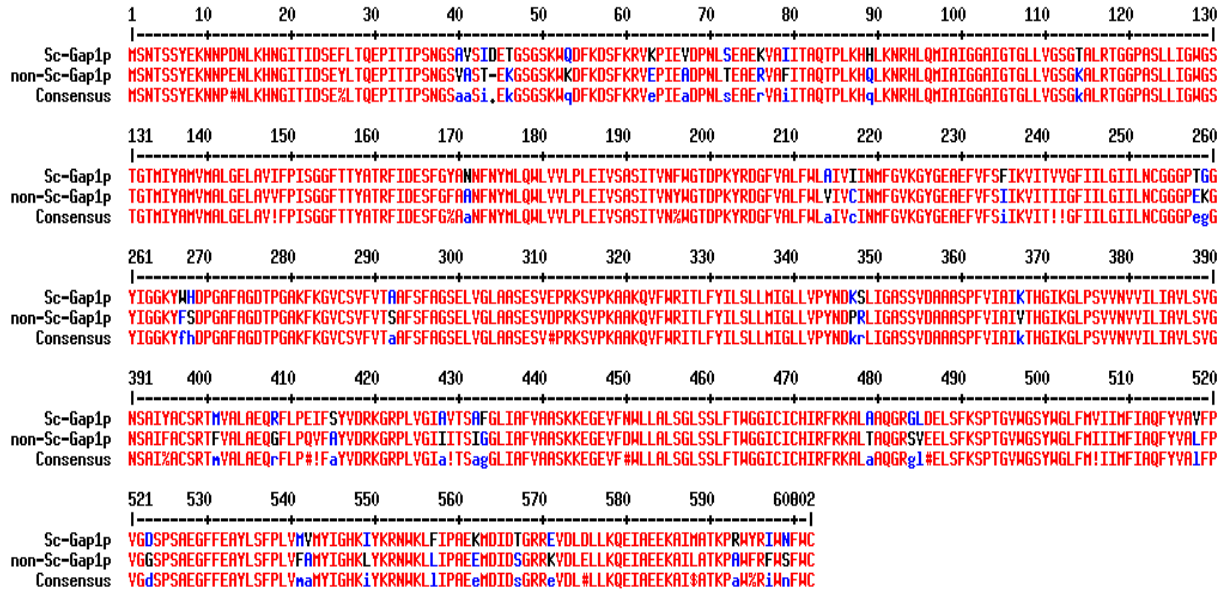


Figure 8: Amino acid sequence alignment between Sc-Gap1 and non-Sc-Gap1

Highly conserved residues are represented in red colour and in uppercase letters in the consensus line while the weakly conserved residues appear in blue colour and in lowercase letters. Other residues appear in black colour. A position with no conserved residue is represented by a dot in the consensus line.

4.1.2 Gap1 antibody detection of Gap1p from *S. bayanus*

To further confirm the binding of the Gap1 antibody to Gap1p of non-Sc origin, immunoblot analysis of the total protein extracts of *S. bayanus* (the second parent of the lager brewers' yeast) and a bottom fermenting yeast Sc-06165 (Strain C) was carried out. The protein extracts were prepared from cultures grown in WMIX-Proline medium for better Gap1p expression. 3 µg of the total protein extract was loaded on the SDS gel for Immunoblot analysis. A Coomassie-stained gel was used to verify that the amounts of protein fraction loaded in each lane were the same. A clear band corresponding to ~70 kDa was obtained with both strains thereby confirming the hybridization of the *GAP1* antibody to both the Sc- and non-Sc-Gap1p. There is a slightly higher loading of the *S. bayanus* protein fraction compared to Lager yeast which is the reason for a stronger protein band in *S. bayanus* (Figure 9). This result is now further evidence that the detection of non-Sc-Gap1p by the polyclonal antibody raised in rabbits against *S. cerevisiae* Gap1p is possible.

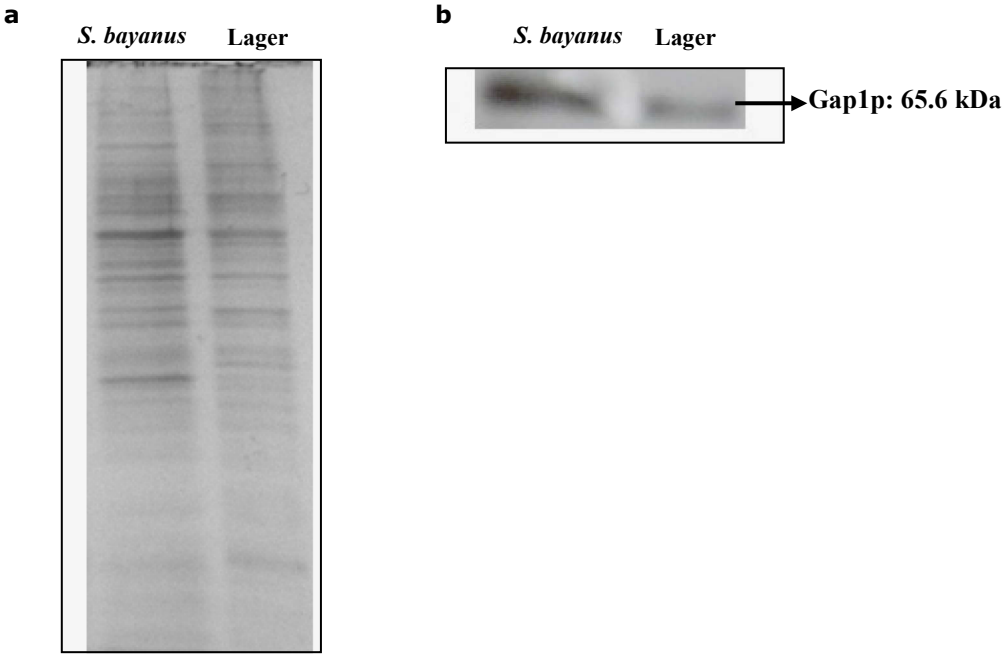


Figure 9: (a) SDS gel stained with coomassie and (b) Immunoblot analysis of Gap1 antibody binding to both Sc- and non-Sc-Gap1p

Immunoblot analysis was used to confirm the binding of *GAP1* antibody in *S. bayanus* and lager brewing yeast (Strain C). Whole cell lysates were prepared and protein concentration was determined by Bradford protein assay. Aliquots containing 3 µg protein fractions were separated by sodium dodecylsulfat polyacrylamide gel (12 %) electrophoresis (SDS-PAGE) at 120 V for 1h in Tris-glycin. Gap1p detection was carried out using *GAP1*(Y-40) polyclonal antibody (1:500); Primary antibodies were detected with horseradish peroxidase–conjugated polyclonal Swine Anti Rabbit (SAR)-IgG secondary antibody (1:1000) followed by detection by enhanced chemoluminescence.

4.2 Analysis and quantification of the expression of Gap1p among Brewers' yeast strains as detected by the microarray data

4.2.1 Immunoblot detection of endogenous *GAP1* gene expression in Brewers' yeast strains under conditions of Nitrogen starvation

In the attempt to verify the hypothesis (Sections 2.5-2.7), the question if there was a concordance between Gap1 protein and transcript levels had to be addressed. Gap1 protein levels are known to be largely influenced by post-transcriptional and post-translational processing. During growth on proline or urea as the sole nitrogen source, an accumulation of highly active and stable form of Gap1 protein occurs at the plasma membrane (Grenson, 1983). Higher Gap1p expression is achieved by transferring yeasts from medium containing glutamate to the medium with proline for 2 h. (Lauwers, 2007). The presence of Gap1p in the membrane fraction of the cell extract is detected by Immunoblotting. To ensure that the same amount of protein fraction is loaded in each lane, a Coomassie-stained SDS-gel was used. The experiments have been carried out at least thrice, and proved reproducible. The result of one representative experiment is shown below (Figure 10). The amount of protein was determined densitometrically using ImageJ software.

The sample in lane 1 that contains the protein fraction of Brewers' yeast Strain B grown in nitrogen rich medium, showed lower protein content in comparison with other samples. All other amounts of protein samples loaded on the SDS gel (lanes 2-6) were comparable (Figure 10 a).

The Gap1p signal detected in the proline-grown wild-type strains showed an intense band corresponding to ~70 kDa. The cells grown in nitrogen rich medium showed weaker Gap1p signal, which is barely detectable in the Immunoblot compared with those grown in nitrogen-starved medium. Activation/derepression of Gap1p is observed in Strain B wild-type after transferring the cells into nitrogen starvation medium for 2 h. of the signal intensity of Gap1p varies among all strains used in this experiment. From the above result it is seen that Strain D shows the strongest Gap1 protein signal subsequently followed by Strain C, Strain F and Stain B (Figure 10 b).

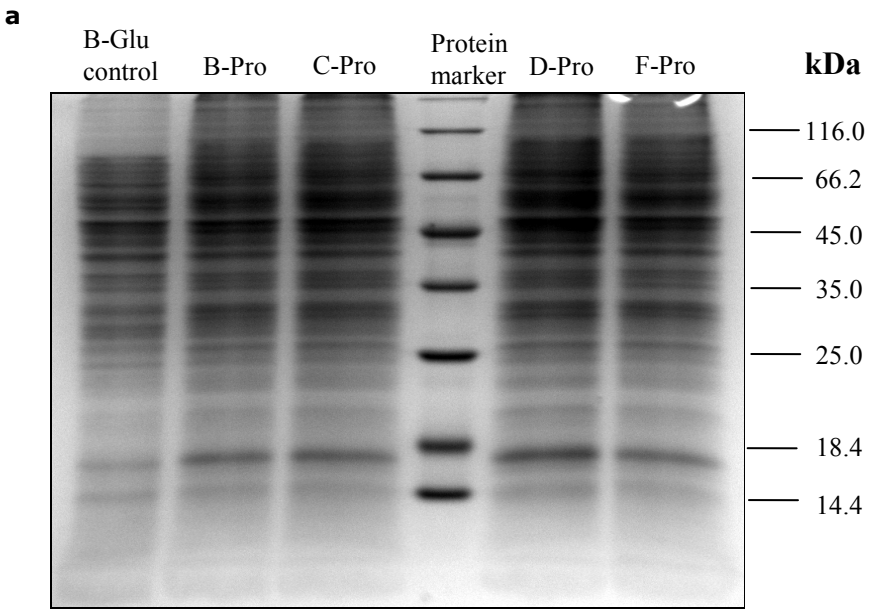


Figure 10 a: Coomassie-stained SDS gel showing membrane-enriched fraction of different commercial Brewers' yeast strains

Protein concentration of the membrane fraction was determined by Bradford protein assay. 10 µg of membrane fraction was loaded per lane. Coomassie staining of SDS gel was performed to ensure loading equal amounts of protein in each lane. Lane 1 contains Brewers' yeast (Strain B) grown in WMIX with glutamate as its sole N source; remaining lanes carry protein fraction of Brewers' yeast obtained after 2 hours of transfer to WMIX-Proline medium (B/C/D/F: Pro). Unstained Protein Molecular Weight Marker (Fermentas) was used.

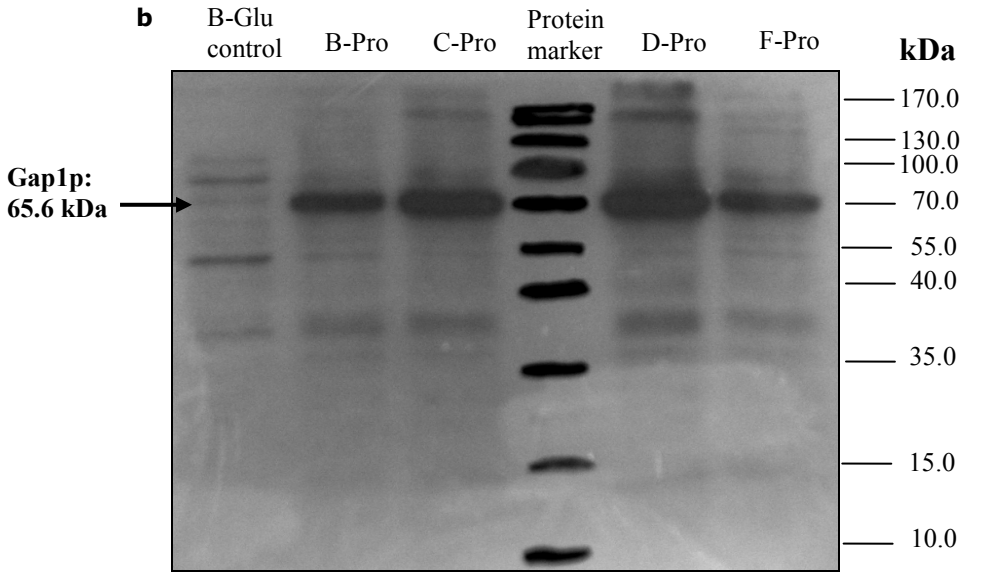


Figure 10 b: Immunoblot showing differences in Gap1p expression among brewers' yeast strains with differences in diacetyl production

Gap1 detection was carried out using *GAP1*(Y-40) polyclonal antibody raised in rabbit (1:500); Primary antibodies were detected with horseradish peroxidase–conjugated Polyclonal Swine Anti Rabbit (SAR)-IgG secondary antibody (1:1000) followed by detection using enhanced chemoluminescence. Prestained Protein Ladder (Fermentas) was used to determine protein size.

4.2.2 Gap1 permease activity measurements under conditions of Nitrogen starvation

Gap1p activity was determined by measuring incorporation of 2.5 mM [^{14}C]-labeled citrulline, a Gap1 permease-specific substrate, as described by (Donaton, 2003). When Gap1 synthesis is induced in yeast, the permease is targeted to the plasma membrane (Andre´,2006). The question if the measured differences in the Gap1 protein expression among brewers' yeast wild type strains lead to differences in the permease activity can be conclusively answered by measuring their permease activity.

In order to determine the differences in activity between these strains under conditions of maximum Gap1p expression, all strains were grown in nitrogen repressed medium (WMIX Proline). The background radiation is measured by addition of [^{14}C]-labeled citrulline without addition of cells to the filter, and subtracted from all experimental results.

Table 6: Comparison four brewers' yeast strains pertaining differences in Gap1p protein amounts and activity

Strains compared	Gap1p protein abundance (fold)	Gap1p activity increase (fold)
D Vs C	1.4	1.5
D Vs F	2	2
C Vs F	1.4	1.3
C Vs B	1.5	4
D Vs B	2.5	6

Strain C, the medium diacetyl producer showed a 1.5-fold lower activity than the high diacetyl producer Strain D, but had a 1.3-fold and a four-fold higher activity in comparison with low diacetyl producing Strains F and B respectively. Likewise, Strain D had a two- and a six-fold higher activity than Strains F and B respectively (Figure 11 and Table 6). These results indicate that the permease activity varies among brewers' yeast strains. The results also show a strong correlation to the transcriptome data and collectively indicate that brewers' yeast strains that differ in their diacetyl production show corresponding differences in their Gap1 permease activity.

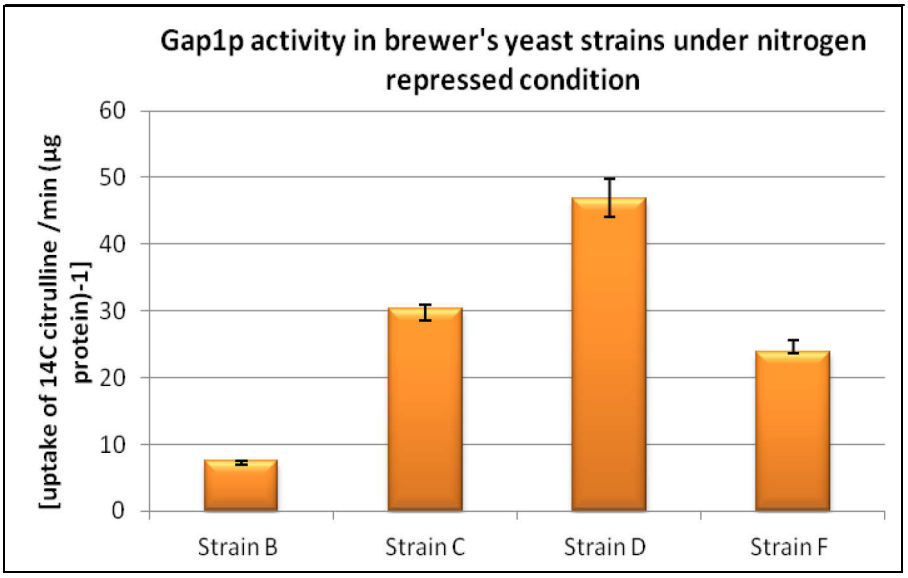


Figure 11: Transport of 2.5 mM L-citrulline in the wild-type brewers' yeast strains

Cells growing on minimal medium containing 2 % glucose and 0.1 % proline were harvested, washed with MES/KOH buffer (25 mM, pH 6.0) and suspended in fresh minimal medium containing 4 % glucose. Gap1p activity measured after addition of 5 mM unlabelled L-citrulline + [^{14}C]- L-citrulline to obtain an end concentration of 2.5 mM L-citrulline to cells starved of nitrogen on a glucose-containing medium. Normalization of protein concentration is carried out using Bradford assay.

4.2.3 Correlation between diacetyl production, Gap1p amounts and Gap1p activity in different Brewers' yeast strains

The aim of the study was to analyse the genetic basis for the differences in their diacetyl levels. The six brewers' yeast strains (Strains B, C, D, F, A and E) were globally analysed for mRNA abundance using whole yeast DNA microarrays (Strack, 2009). *GAP1* coding for General amino acid permease was found to be differentially expressed among these strains. The transcriptome data analysis revealed that high diacetyl producing strains like D, A and C have higher transcript abundance of *GAP1* compared with the low diacetyl producing strains like F, E and B. The formulated hypothesis that there is a positive correlation between *GAP1* and diacetyl production had to be verified. Hence measurements of Gap1p protein levels (Section 4.2.1) and Gap1p activity (Section 4.2.2) were carried out in four brewing strains with differences in their diacetyl levels.

Gap1p is subjected to regulatory processes occurring after mRNA is synthesized - that is, post-transcriptional and post translational regulations. These regulations occur in controlling steady-state protein abundance (Vogel, 2012). From the results obtained with the Immunoblot, strains with higher *GAP1* transcript abundance have been shown to correlate with a greater abundance of protein and those with lower transcript abundance had lower protein abundance in them (see Figure 10).

Furthermore, comparison of the Gap1p amounts and activity showed that there is a tight correlation between protein amounts and protein activity with the exception as seen in Strain B (see Figure 11 / Table 6). Comparing the Gap1 protein abundance between different strains, it is seen that, Strain D has a 1.5-fold increase compared with Strain C where as a 2-fold increase in comparison with Strain F. The same level of increase can also be seen in the protein activity of these strains. Similarly Strain C shows a 1.3-fold increase in both its protein abundance and activity compared with Strain F. In contrast, Strain B which showed a 1.5-fold and a 2.5-fold lower protein levels compared with Strains C and D respectively had a considerable decrease of 4 and 6-fold activity in these strains. This could indicate that Strain B has lower amounts of active Gap1 proteins leading to lower protein activity. In general, it could be said that Gap1p accumulated at the plasma membrane which is detected by Immunoblot are proved to be active proteins. Hereby, the differences in the Gap1p both at the protein and activity level among brewers' yeast exhibiting differences in diacetyl production, have been verified (Figure 12). This knowledge should act as a basis to improve the understanding of the complex interactions between the expression of the amino acid permease and its effect on diacetyl production.

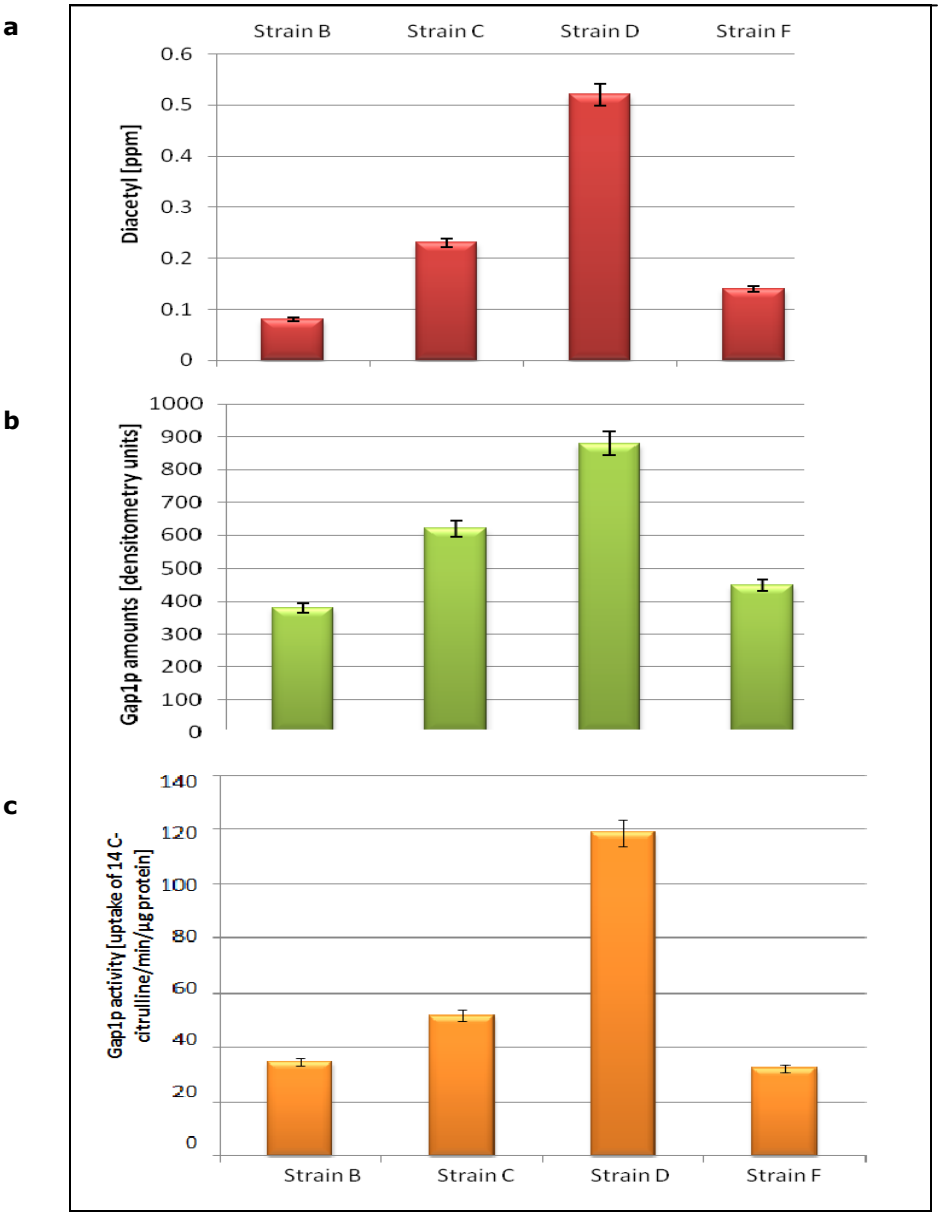


Figure 12: Correlation between diacetyl production, Gap1p amounts and Gap1p activity in different Brewers' yeast strains

(a) Average diacetyl production in different brewers' yeast strains. Fermentations were carried out in 30 L cylinder-conical tanks at 11 °C, Wort: 11.5 °P. Diacetyl measurements were carried out using Vicinal Diketone-GC/ECD-Headspace-Method (Strack, 2009).

(b) Immunoblot carried out with *GAP1* antibody for detection of Gap1p from Lager brewing yeast strains grown in WMIX-Proline medium. Gap1p amounts were calculated using Density Plot from Immunoblot. Values shown are the average values from at least three independent experiments.

(c) Gap1 activity (uptake of [^{14}C] -citrulline .min^{-1}·mg prot.$^{-1}$) was assayed in cells grown on WMIX-Proline medium by measuring incorporation of [^{14}C] citrulline (2.5 mM).

4.3 *Saccharomyces cerevisiae* model: Verification of hypothesis

Saccharomyces cerevisiae as a model organism toward the study of influence of *GAP1* on diacetyl production

4.3.1 Engineering of a CEN/ARS based shuttle vector for the expression of *GAP1* gene in yeast

Several lines of evidence indicate that brewers' yeast showing differences in diacetyl amounts have different Gap1p amounts. To determine the role of *GAP1* in influencing diacetyl levels, an autonomous shuttle vector p416 was selected into which the *GAP1* gene was cloned. The expression of *GAP1* is under the control of the strong and constitutive TEF6 promoter which is suitable for use in yeasts. The CEN/ARS plasmid p416TEF6*GAP1* used to overexpress the *GAP1* gene controlled by the TEF6 promoter is a derivative of the plasmid p416TEF6*yECitrine* (Nevoigt, 2006). The construction of p416TEF6*GAP1* was carried out by replacement of the reporter protein yECitrine with the coding sequence of *GAP1* gene through homologous recombination in lab strain BY4741. The positive clones that grew on YPD-G418 (300 µg/ml) selection plates were further verified by plasmid isolation and restriction analysis using *Xba*I and *EcoR*I (Figure 13). The original plasmid (p416TEF6*yECitrine*) was used as a negative control in this restriction analysis. *Xba*I restricts the plasmid at positions 4802 and 2866 bps respectively whereas *EcoR*I restricts only inside the *GAP1* gene. As a result, p416TEF6*GAP1* plasmid would result in 3 fragments of sizes 6.2, 1.9 and 0.68 kbps respectively. But those plasmids in which the *yECitrine* gene has not been replaced due to plasmid re-ligation yields 2 fragments of sizes 6.2 and 1.9 kbps respectively. It is noteworthy to mention that a success rate of 70 % was obtained with this method.

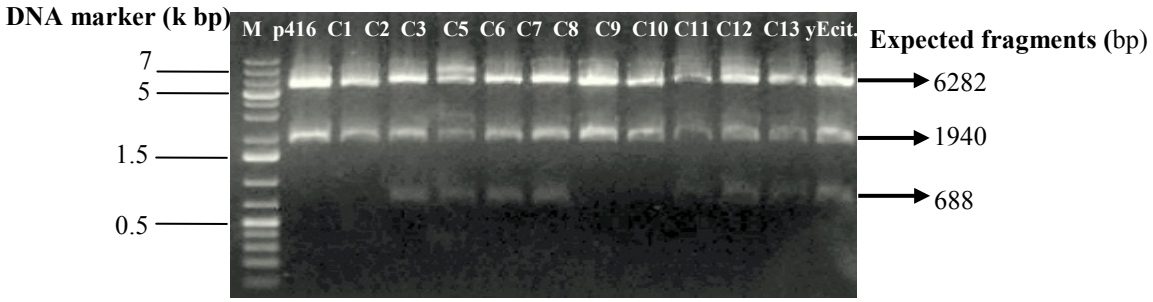


Figure 13: Restriction analysis of isolated p416TEF6*GAP1* plasmid to verify *GAP1* integration by homologous recombination in yeast

Plasmids after restriction with *Xba*I and *EcoR*I that yielded 6.2, 1.9 and 0.68 kbps respectively carried *GAP1*; the original plasmid carrying the *yECitrine* gene will result in 2 fragments of sizes 6.2 and 1.9 kbps.

4.3.2 Sequencing of the yeast shuttle vector p416TEF6*GAP1*

Since the *GAP1* gene was amplified by PCR from the yeast genome, to rule out any possibilities of mismatches occurring in the gene sequence, 1834 bp of the CDS of *GAP1* was sequenced. The sequencing was carried out using primers P5 and P6 that bind upstream and downstream of the *GAP1* gene. The analysis of the sequence data revealed that no mismatches were added to the gene sequence through PCR. The gene was also found to be inserted in the correct orientation. The sequence alignment is attached to the Appendix (Figure A).

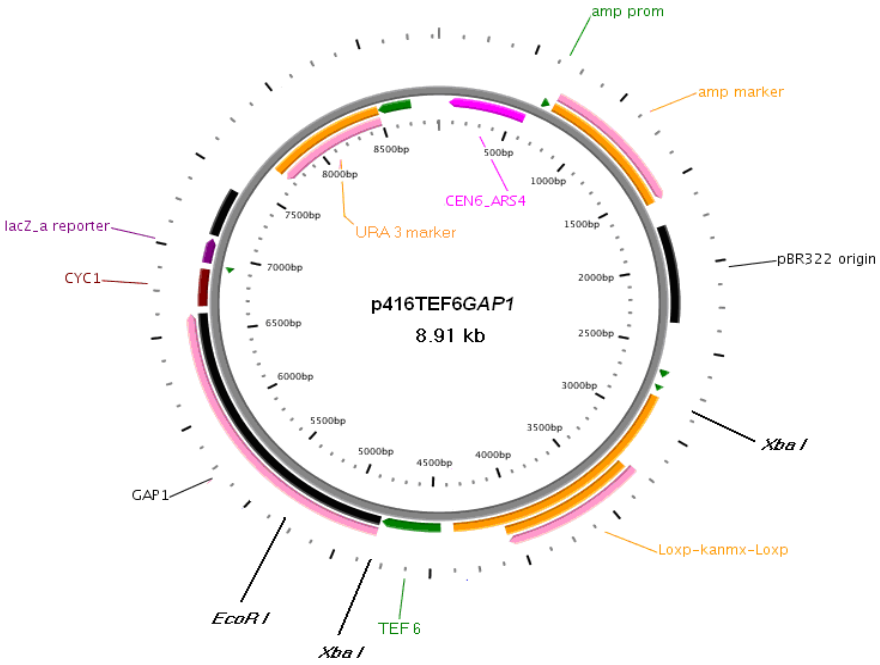


Figure 14: Plasmid map of p416TEF6*GAP1*

GAP1 expression vector was constructed by replacing the yECitrine gene originally present in the p416 vector. The vector p416 is a centromeric plasmid containing the ampicillin resistance gene, the *URA3* gene and the constitutive TEF6 promoter. The kanmx resistance marker which confers resistance against Geneticin (G418) antibiotic enables its use in both *S. cerevisiae* haploid strains and Brewers' yeast strains.

4.3.3 Construction of empty plasmid p416kmr

The empty plasmid for the control transformation was constructed by deleting the reporter gene *yECitrine* and the TEF6 promoter from the original plasmid (p416TEF6 *yECitrine*) using *Xho*I followed by re-ligation of the plasmid. The religated vector was then transformed and propagated in *E. coli* and the plasmids were re-isolated from selected clones and verified by restriction analysis. *Xba*I and *Xho*I restricts p416TEF6*yECitrine* resulting in bands corresponding to 5140, 1453, 741 and 483 bps respectively. However in the p416kmr plasmid the smaller bands (741 and 483 bps) that restricts inside the *yECitrine*-TEF6 region is absent and thereby results only in two bands of sizes 5140 bp and 1453 bp respectively (Figure 15).

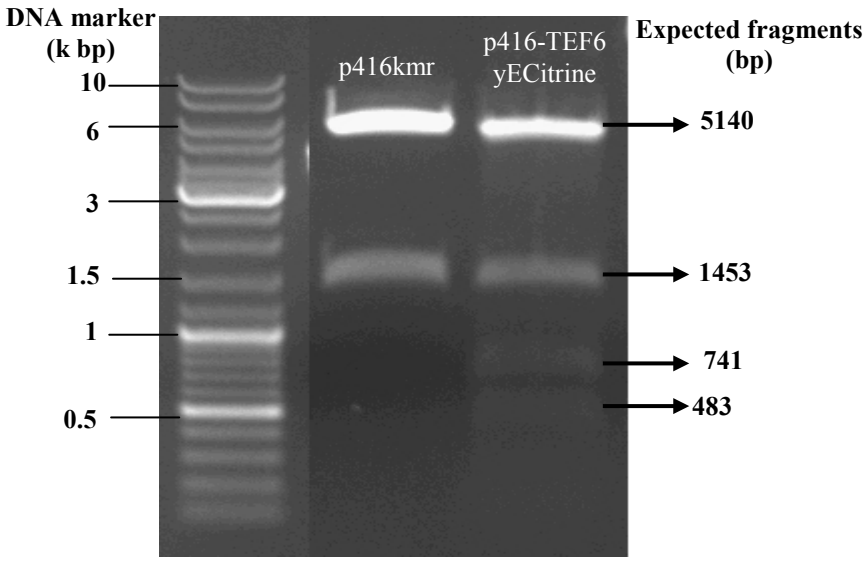


Figure 15: Restriction analysis of isolated p416kmr plasmid

Restriction of the empty plasmid p416kmr with *Xba*I and *XhoI* yields 2 fragments of sizes 5.14 and 1.4 respectively; the original plasmid carrying the yECitrine gene results in 4 fragments of sizes 5.14, 1.4, 0.74 and 0.48 kbps respectively.

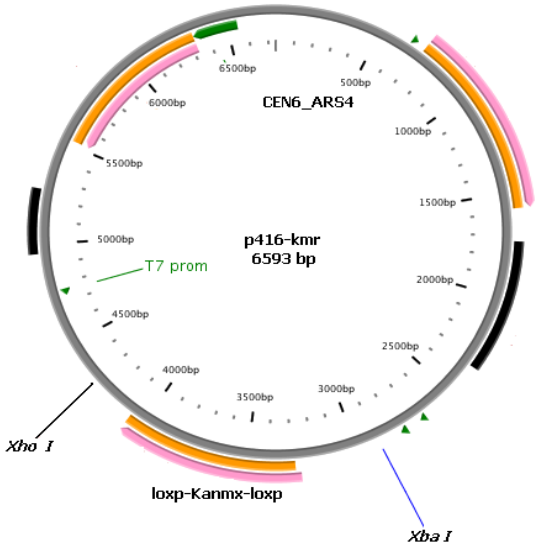


Figure 16: Plasmid map of the empty plasmid, p416kmr

An empty plasmid for control transformation in Brewers' yeast was constructed by removing the *yECitrine* gene and TEF6 promoter from the p416 vector. The kanmx resistance marker which confers resistance against Geneticin (G418) antibiotic enables its use in both *S. cerevisiae* haploid strains and Brewers' yeast strains.

4.3.4 Differences in Gap1p in *Saccharomyces cerevisiae* model organism

Lab strain BY4741 was used as the model organism to obtain strains with varying Gap1p levels. The three strains used in this study were wild-type, *Δgap1*, *GAP1* OE (BY4741 carrying p416TEF6*GAP1*). These strains were used with the end aim of comparing their diacetyl levels. The overexpression strain (*GAP1* OE) used in this study was generated by transforming the wild-type BY4741 strain with p416TEF6*GAP1*, a CEN-based expression

plasmid under the control of a strong TEF6 promoter. The *Δgap1* was obtained from the Euroscarf collection.

The Gap1p amounts in the strains were checked by Immunoblot analysis. The growth of cells in nitrogen starved WMIX-Proline medium resulted in highest Gap1p expression. The SDS-PAGE gel shows comparable protein fraction loaded in each lane (Figure 17 a). The Immunoblot revealed that the *Δgap1* strain clearly produces no Gap1p. Increased amounts of Gap1p in the *GAP1* overexpression strain in comparison with the wild-type strain (Figure 17 b). The overexpression of Gap1p achieved using a low copy plasmid is modest but significant. The above result proves that strains with differences in their Gap1p amounts have been obtained. These strains with varying Gap1p levels could in turn be used for measuring their diacetyl production to verify the hypothesis.

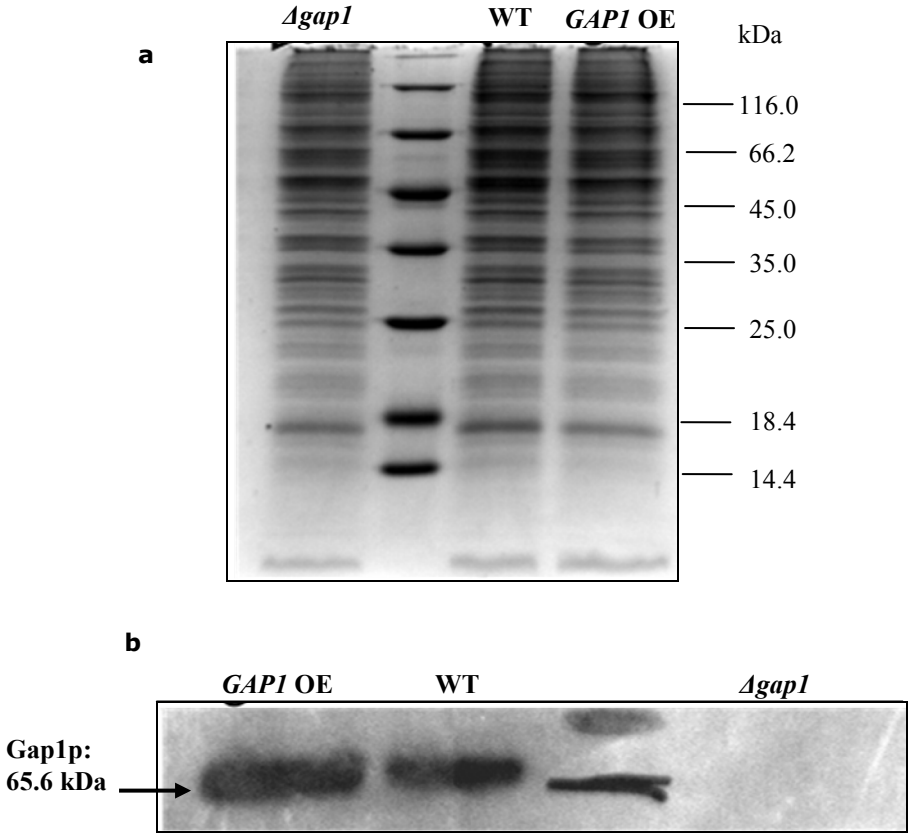


Figure 17: Expression of *GAP1* in *S. cerevisiae* BY4741 strains: SDS-PAGE (17 a) and Immunoblot (17 b)

BY4741 strain *Δgap1* with no Gap1p expression, wild type strain and BY4741 transformed with vector p416TEF6*GAP1* for Gap1p overexpression (*GAP1* OE) were grown in nitrogen repressed WMIX-Proline medium. 10 µg of membrane-enriched protein fraction was loaded in each lane of the SDS gel. Coomassie staining of SDS-PAGE gel was performed to ensure that equal amounts of protein was loaded in each lane. Primary antibodies were detected with horseradish peroxidase–conjugated polyclonal Swine Anti Rabbit (SAR)-IgG secondary antibody followed by enhanced chemiluminescence.

4.3.5 Higher levels of *GAP1* in yeast increases diacetyl production

Influence of *GAP1* on diacetyl production can be best studied by gene mutation and overexpression. This study was carried out in the *S. cerevisiae* BY4741 strain for the following reasons:

1. Industrial brewers' yeast strains are polyploid and for this reason one part of these functional studies have been carried out in haploid laboratory-derived strain otherwise unsuitable for industrial production purposes.
2. Availability of *GAP1* deletion strain (Euroscarf deletion strain collection)

Due to various auxotrophies in the cell, lab strain BY4741 is generally incapable of fermenting and so these strains have been tested for their diacetyl production in synthetic medium. All strains were therefore grown in WMIX-Proline medium in which the highest difference in Gap1p levels between strains could be obtained. Consequences of *GAP1* deletion and overexpression on yeast growth physiology was also studied to show that differences in diacetyl levels in the strains are indeed due to differences in their Gap1p levels (Figure 18).

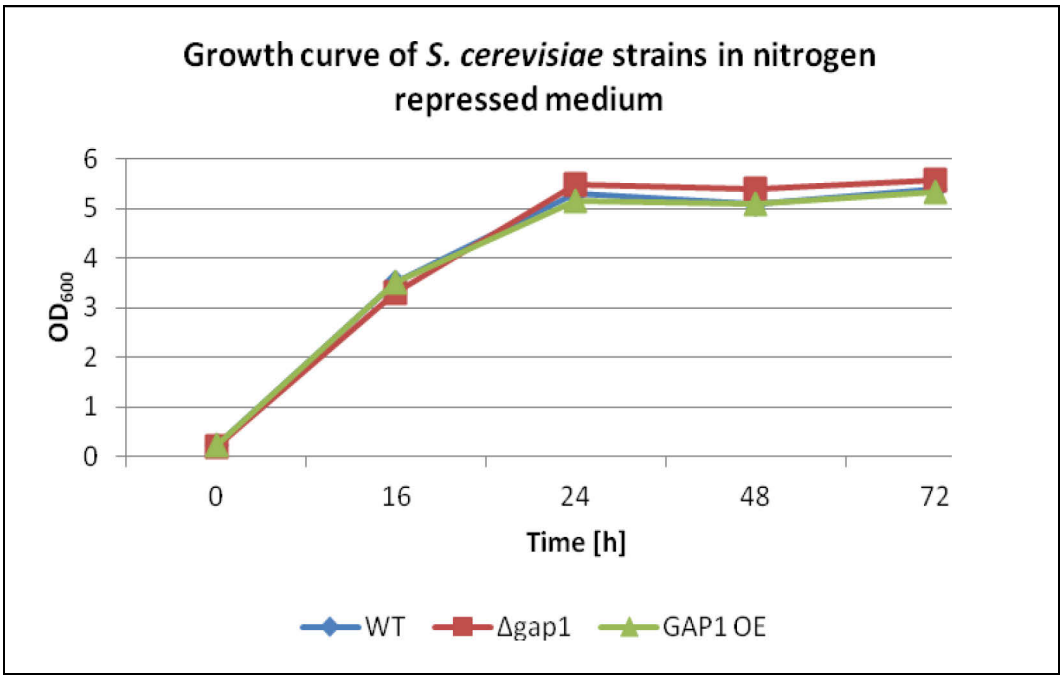


Figure 18: Growth curve of *S. cerevisiae* BY4741 strains

Cells were cultivated in shake flasks in WMIX-Proline medium at 28 °C for 72 h at 200 rpm. Growth curve was obtained by measuring the optical density of the culture at 600 nm using appropriate dilutions. Values shown are the average values from at least three independent experiments.

Diacetyl levels were examined in *Δgap1* and in *GAP1* overexpression strains of *S. cerevisiae* and were compared to that of the wild type strain. The diacetyl synthesis

rate (16-24 h) is highest in the *GAP1* overexpression strain followed by the wild-type and then by the *Δgap1* strains. However, the rate of degradation or reabsorption of diacetyl (48-72 h) is similar in all three strains. The overall diacetyl production of the three strains throughout the fermentation process showed that the *Δgap1* mutant showed a 20 % lower diacetyl level in comparison to the WT and a 50 % lower diacetyl production in comparison to the *GAP1* OE strain. On the other hand, the diacetyl production in *GAP1* OE was increased by 30 % in comparison to the WT (Figure 19). These results prove to be a good indicator that influencing Gap1p levels indeed leads to changes in diacetyl levels.

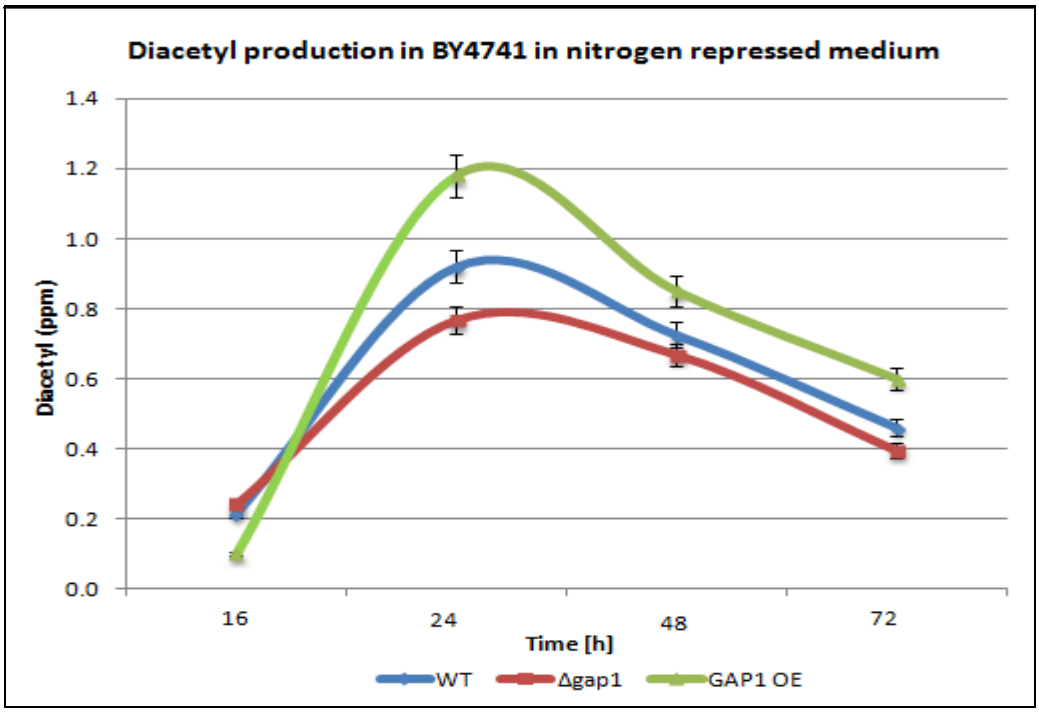


Figure 19: Extracellular diacetyl profiles of *S. cerevisiae* strains grown in nitrogen repressed WMIX medium with proline as the sole nitrogen source to enable maximum Gap1p expression

Strains were grown in shake flasks in WMIX-Proline medium at 28 °C for 72 h with shaking 200 rpm. Diacetyl content of each of the strains shown are representative of the average values from at least three independent experiments. The respective mean valuesvand standard deviations of three parallel diacetyl determinations are shown.

4.3.6 Correlation between diacetyl production and Gap1p amounts in *S. cerevisiae* BY4741 strain

For the study of influence of *GAP1* on diacetyl production, the *S. cerevisiae* BY4741 strain is predicted to be a good model to extrapolate from. Through Inverse Metabolic Engineering approach which is a powerful framework for engineering cellular phenotypes (Gill, 2003), *GAP1* gene was repressed or overexpressed in the strain until the desired phenotype is obtained. For this purpose the diacetyl levels in the wild-type, *Δgap1* and *GAP1* OE BY4741 strains have been studied.

The densitometric analysis of the immunblot of Gap1 protein showed that while the *Δgap1* strain had no expression of the permease, the *GAP1* OE strain showed a 1.5-fold higher Gap1p amounts than the wild type strain (Figure 20). This difference in the levels of permease expression seemed to have an effect on the diacetyl production in these strains. Consequently, the diacetyl production in the *Δgap1* strain was found to be 20 % lower than the wild-type strain whereas a 30 % higher diacetyl was produced in the *GAP1* OE strain compared to wild-type. These results could strongly suggest that the transporting and or signaling function of *GAP1* plays an important role in the amino acid uptake which in turn brings an effect on diacetyl production in the cells.

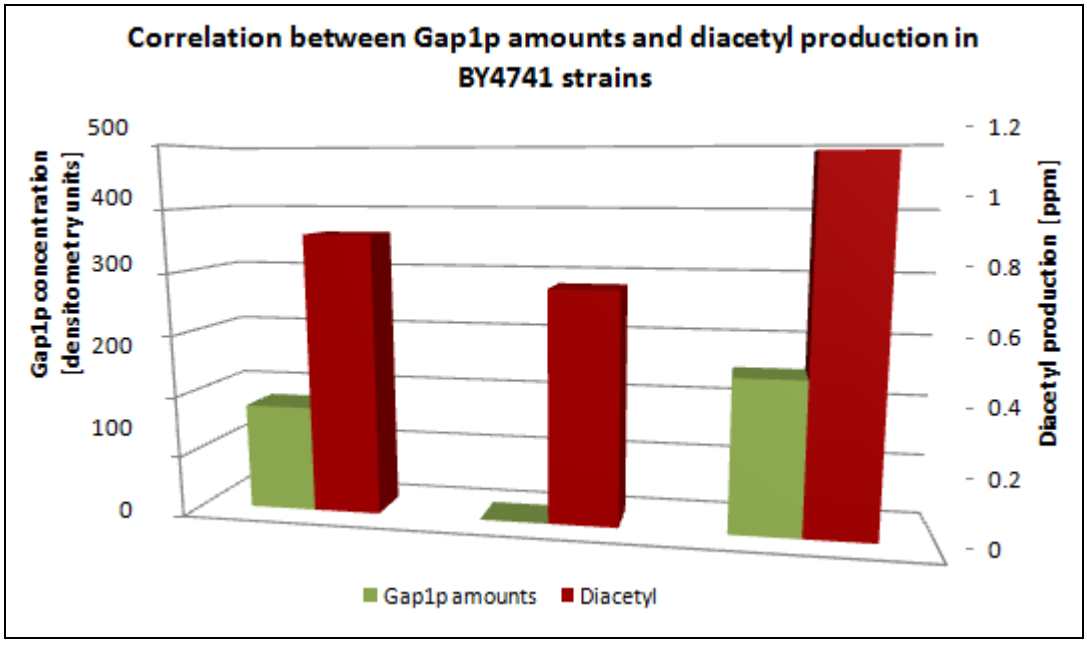


Figure 20: Diacetyl production during log phase in *S. cerevisiae* strains and corresponding differences in Gap1p amounts

Average diacetyl production in *S. cerevisiae* strains grown in WMIX-Proline medium at 28 °C for 72 h. Gap1p amounts were calculated using Density Plot from Immunoblot.

4.4 Brewers' yeast strain I: Verification of hypothesis

Saccharomyces carlsbergensis- Sc-06165 (Strain C) as a model organism toward study of influence of *GAP1* on diacetyl production

4.4.1 Transformation of p416TEF6*GAP1* and p416kmr into Brewers' yeast Strain C

Yeast transformations were carried out according to the LiAc-polyethylene glycol method. Positive clones were selected by initially plating them on 20 µg/ml G418-YED followed by plating them on 50 and 100 µg/ml G418-YED plates successively. Plasmid uptake into the Brewers' yeast was verified by plasmid isolation and verification by restriction analysis. p416TEF6*GAP1* was restricted using *Xba*I to obtain two fragments of sizes 6973 and 1936 bps respectively (Figure 21 a). The restriction of p416kmr plasmid was carried out using *Xba*I and *Xho*I to 2 fragments of sizes 5140 bp and 1453 bp (Figure 21 b).

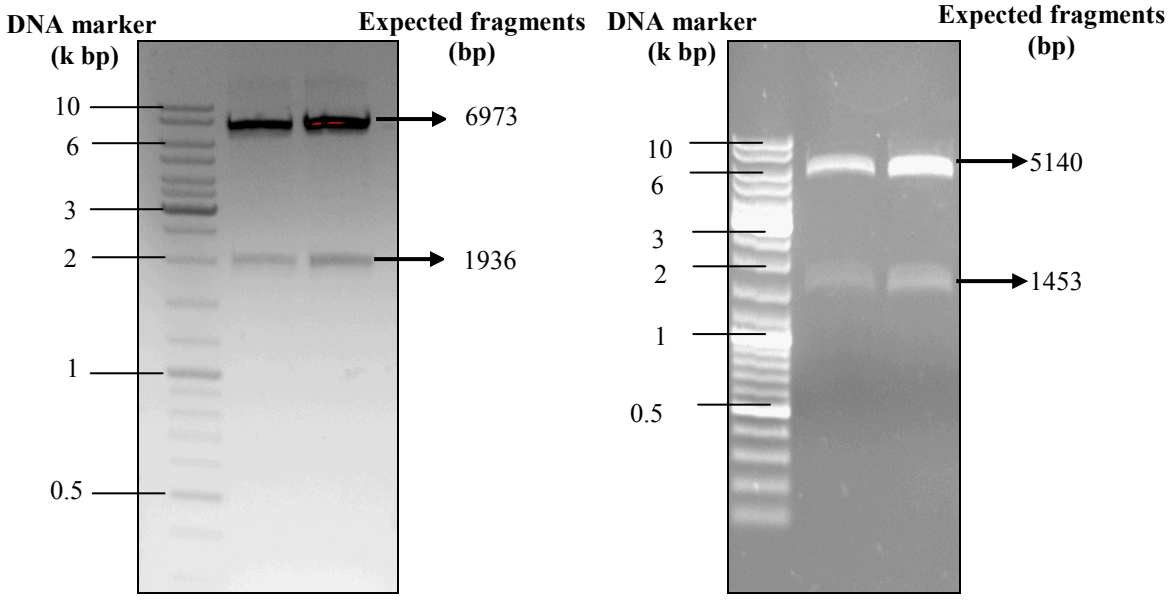


Figure 21: Verification of the uptake of the plasmids p416TEF6*GAP1* and p416kmr into the respective Strain C transformants

(a) Restriction analysis of p416TEF6*GAP1* isolated from Strain C clone using yeast DNA miniprep. Complete double digestion of DNA was obtained with *Xba*I yields fragments of 1936 bp and 6973 bp.

(b) Restriction analysis of p416kmr isolated from Strain C clone using yeast DNA miniprep. A double digest using *Xba*I and *Xho*I yield fragments of sizes 5140 bp and 1453 bp.

4.4.2 High-copy plasmid for *GAP1* overexpression in brewers' yeast

In order to study the behaviour of the *GAP1*-overexpressing brewing strains under brewing conditions, the maximum overexpression of *GAP1* was required. This was achieved by using a 2-micron plasmid maintained stably in cells at high copy number. The YEp yeast episomal plasmid vectors replicate autonomously because of the presence of a segment of the yeast 2 µm plasmid that serves as an origin of replication (2 µm ori). The 2 µm ori is responsible for the high copy-number and high frequency of transformation of YEp vectors (Schneiter, 2004). The plasmid YEpTKmTEF6*GAP1* used to overexpress the *GAP1* gene controlled by the TEF6 promoter is a derivative of the plasmid YEpTkmr. YEpTKmTEF6*GAP1* was constructed by replacing the *LEU2* selection marker with *GAP1*-TEF6-Cyc1T through homologous recombination inside lab strain BY4741. The positive clones that grew on YPD-G418 (300 µg/ml) selection plates were further verified by colony PCR. Colonies were replated on YPD-G418 (350 µg/ml) plates prior to verifying the right clones via colony PCR. The primers P9 and P10 used for the diagnostic PCR binds upstream and downstream of the protein-coding gene resulting in a product size of 2.51 kbp (Figure 22).

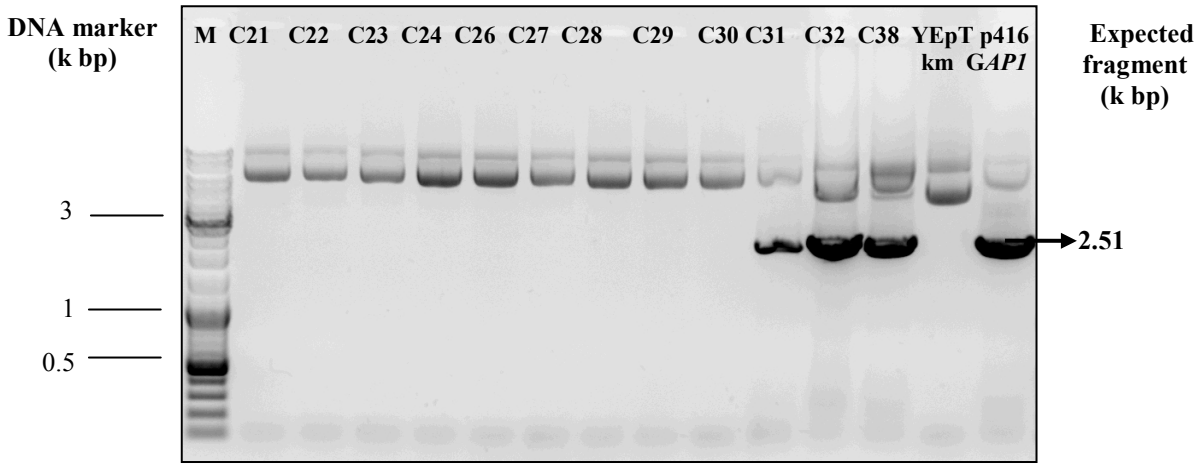


Figure 22: Colony PCR results used for identification of clones in which TEF6-*GAP1*-Cyc1T has been successfully integrated into YEpTkmr plasmid

Positive control: p416*GAP1*TEF6CYc1T plasmid; negative control: empty plasmid YEpTkmr. Primers P9 and P10 used for diagnostic PCR gives a 2.51 kbp fragment for plasmids carrying the TEF6-*GAP1*-Cyc1T fragment.

The positive clones identified via colony PCR were sequenced using primers P9 and P10. Sequencing of the constructs was performed and the results revealed that the replacement of the *LEU2* gene by TEF6-*GAP1*-Cyc1T was successful and no mismatches were observed. The empty plasmid YEpTKm was used as the control (Appendix: Figure B).

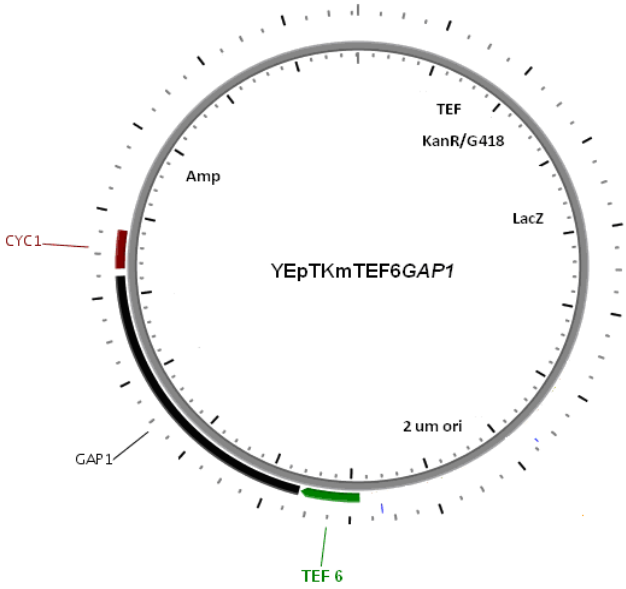


Figure 23: Plasmid map of the constructed YEpTKmTEF6*GAP1*

The self-replicating plasmid comprises of *GAP1* from the *S. cerevisiae*, a strong constitutive TEF6 promoter and CyC1 terminator gene. Plasmid p416TEF6*GAP1* was used as the template for obtaining the 2.51 kb long *GAP1*-TEF6-Cyc1T fragment which was used to replace the *LEU2* gene in the original YEpTkmr plasmid via homologous recombination in yeast.

4.4.3 Transformation of YEpTKmTEF6*GAP1* and YEpTkmr (high copy) plasmids into Brewers' yeast

The *GAP1* expression plasmid was introduced into the yeast host through Li-Ac/PEG transformation method. Transformation of YEpTkmTEF6*GAP1* into Strain F, a low diacetyl producer and Strain C, a medium diacetyl producer was purported to obtain Gap1p overexpression. The same strains were also transformed with an empty YEpTkmr plasmid as a control for studying changes in diacetyl production of the strains when subjected to Gap1p overexpression. The transformed strains were selected on YED-G418 plates and verified using colony PCR using primers P9 and P10. The forward and reverse primers bind upstream and downstream of TEF6 and CyC1T respectively, resulting in amplicons of size 2.51 kb (Figure 24 a, b). The same strains were also transformed with an empty YEpTkmr plasmid and the plasmid uptake was verified by colony PCR using primers P11 and P12 that bind upstream and downstream of TEF-Kanmx respectively (gel picture not shown).

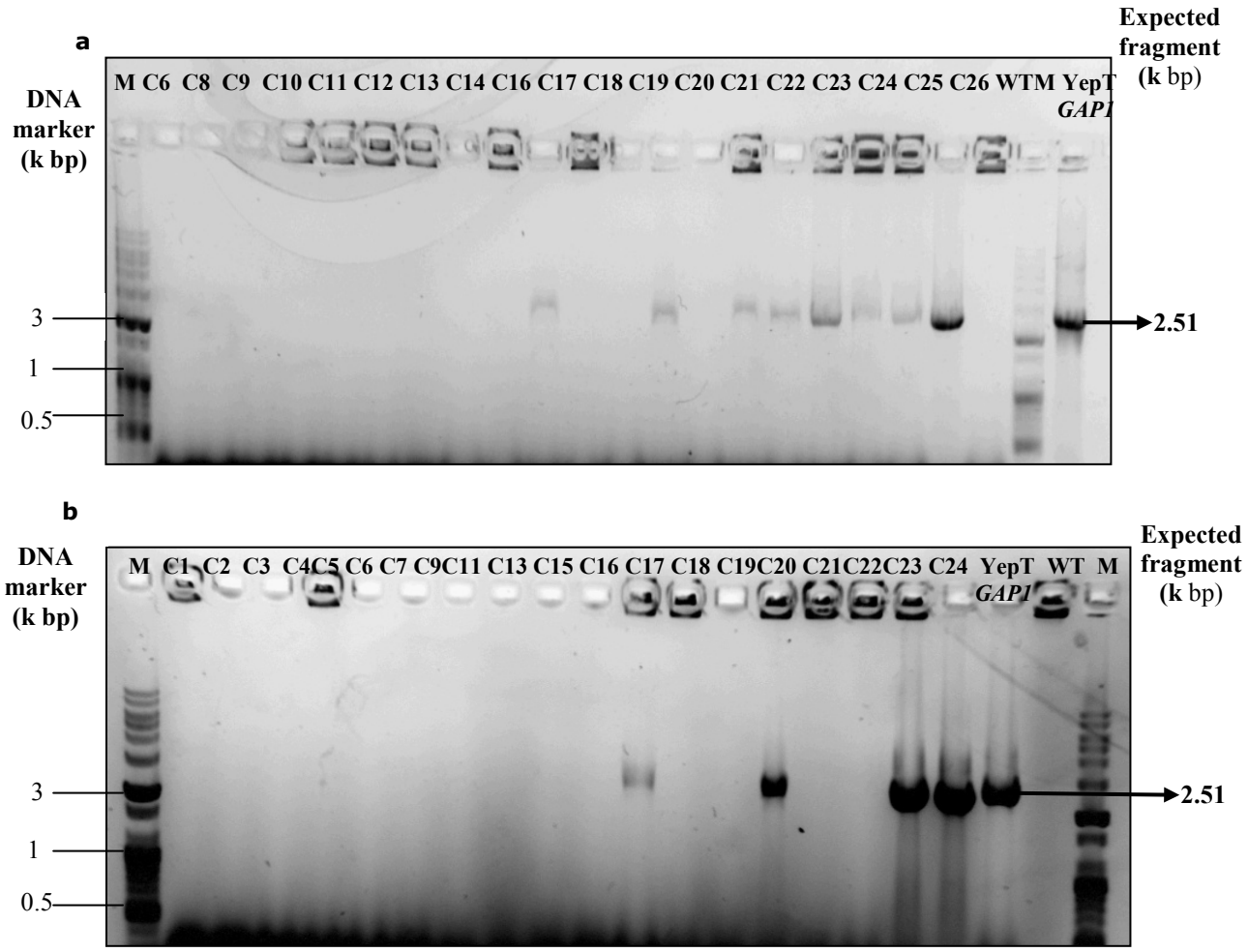


Figure 24: Colony PCR results used for identification of Strain C (a) and Strain F (b) clones with successful uptake of YEpTKmTEF6*GAP1*

Positive control: YEpTKmTEF6*GAP1* plasmid; negative control: Respective wild-type Strains C and F. Primers P9 and P10 used for diagnostic PCR gives a 2.51 kbp fragment for plasmids carrying the TEF6-*GAP1*-CyC1T fragment.

4.4.4 Overexpression of the *GAP1* gene in the Brewers' yeast Strain C

Owing to the higher-ploidy nature of industrial strains, the deletion of the *GAP1* gene for the functional studies was considered to be laborious. Therefore the strategy designed to further verify the hypothesis involved conversion of a low diacetyl producing strain into a high diacetyl producer by overexpressing the *GAP1* gene in the low producer. The expression of *GAP1* gene was evaluated in the Strain C wild type and Strain C *GAP1* transformants (overexpression with both single copy and multicopy plasmids) by protein Immunoblot. The use of a multicopy plasmid to overexpress *GAP1* gene under the influence of a strong TEF promoter enabled maximum expression of Gap1p in brewers' yeasts. The evidence for the same could be seen by an intense Gap1 signal on Western blot. In the case of wild type cells expressing native Gap1, lower Gap1 protein amounts were detected. Gap1 overexpression achieved by the low copy plasmid is modest in comparison to that achieved by the high copy plasmid (Figure 25 b).

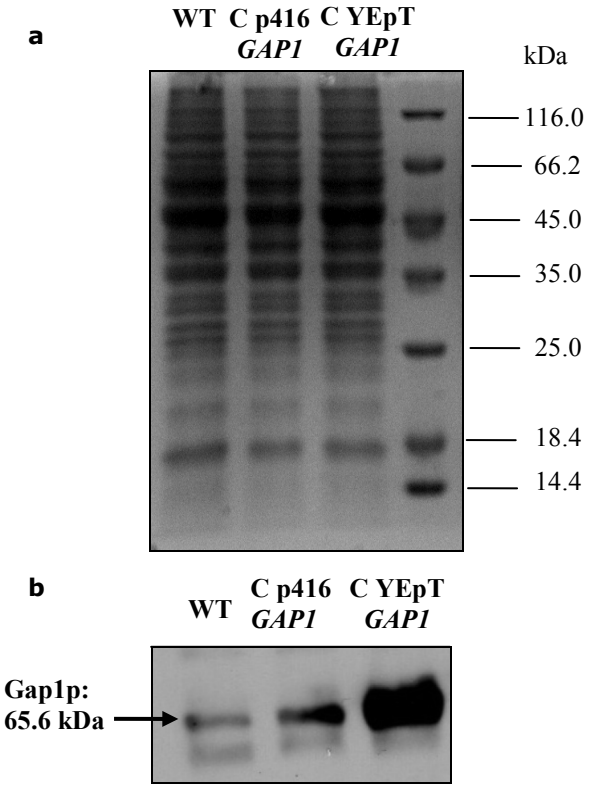


Figure 25: Detection of Gap1p in Strain C wild-type and Strain C transformants: SDS-Gel (25 a) and Immunoblot (25 b)

Gap1p was immunodetected in whole protein fractions of wild-type and *GAP1* transformant strains each carrying a single copy CEN plasmid and a multi-copy 2µm plasmid for Gap1p overexpression. 10 µg of total protein fraction of Strain C wild type and transformants was loaded per lane of the SDS gel. Coomassie stained SDS-PAGE with protein fractions of Strain C wild-type and Strain C transformants was carried out to ensure loading of equal protein amounts in each lane. Primary antibodies were detected with horseradish peroxidase–conjugated polyclonal Swine Anti Rabbit (SAR)-IgG secondary antibody followed by enhanced chemiluminescence.

4.4.5 Constitutive expression of *GAP1* using a self-replicating multi copy plasmid increases the permease activity among Brewers' yeast strains

To determine the physiological consequences of unregulated Gap1p activity on diacetyl production in brewers' yeast, *GAP1* was expressed under TEF6 promoter for constitutive protein expression. Gap1p is known to have its maximum expression under limited nitrogen conditions. So in order to verify overexpression of Gap1p in Strain C-YEpT*GAP1* in comparison to wild-type Strain C, cells were grown in nitrogen starved, glucose-containing medium. Transport of 2.5 mM L-citrulline was measured in the brewers' yeast strains.

Gap1p activity in Strain C YEpT*GAP1* overexpression was found to be three-fold times higher than the wild-type strain (Figure 26). This increase in activity in the transformant strain seems to be in accordance to the five-fold increase in Gap1 protein amounts in overexpression strain as detected by the Immunoblot (Figure 25). The overexpression of

Gap1p using a 2 µm plasmid, under a constitutive promoter has clearly led to copious production of Gap1p proteins that are active transporters on the plasma membrane.

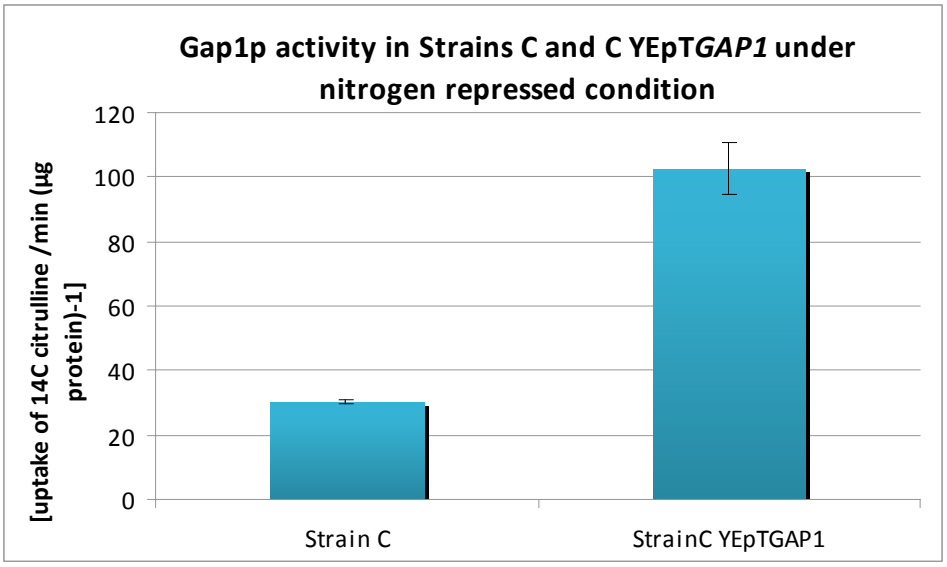


Figure 26: Overexpression of *GAP1* permease activity in Lager brewing Strain C.

Cells of Strain C wild-type and Strain C YEpT*GAP1* (carrying the vector YEpTkmTEF6*GAP1*) grown for 24 h on minimal medium containing 2 % glucose and 0.1 % proline. Cells were harvested, washed with MES/KOH buffer (25 mM, pH 6), and suspended in fresh minimal medium containing 4 % glucose. Import assay was performed by addition of 2.5 mM [^{14}C]-L-citrulline to cells. Protein concentrations of the strains were measured using Bradford assay and the transport rate is expressed as uptake of ^{14}C-L- citrulline per min per microgram protein.

4.4.6 Fermentation of brewers yeast Strain C with *GAP1* overexpression resulted in higher levels of diacetyl

Brewers yeast Strain C was transformed with the single copy p416TEF6*GAP1* plasmid to obtain higher *GAP1* expression (Figure 27). The wild-type and the transformant (C p416*GAP1*) strains were analyzed under brewing conditions. The fermentation behaviour such as cell number, pH and apparent extract consumption of the C WT and the C p416*GAP1* strains were continuously monitored to ensure comparable phenotypic behaviour among the strains (Figure 28).

During the first part of fermentation yeast multiply rapidly whereas during the second part of fermentation the cell concentrations in the suspension decrease due to the flocculation behavior of brewing yeasts. The breaking down of sugars produces alcohol. Therefore, the complete consumption of the fermentable sugars (up to an apparent extract of concentration of 3-2 %) is essential for the good taste of the beer. The remaining sugars are dextrins, which cannot be utilized by the yeast.

At the beginning of the fermentation (Days 1-2), the diacetyl synthesis rate in the Strain C p416*GAP1* was higher than that of wild-type strain. After Day 2 there is no further increase in the diacetyl levels in both strains, but instead has a similar rate of diacetyl

reabsorption. As a result, toward the end of the fermentation (Day 6) both *GAP1* overexpression strain and wild-type strain have same amounts of diacetyl. The use of the single-copy p416 plasmid for *GAP1* overexpression resulted in a modest increase in Gap1p amounts in the Strain C p416*GAP1*. However this overexpression was sufficient to produce a 1.2 fold increase in the overall diacetyl amounts in the *GAP1* overexpression strain compared with the wild type strain (Figure 29).

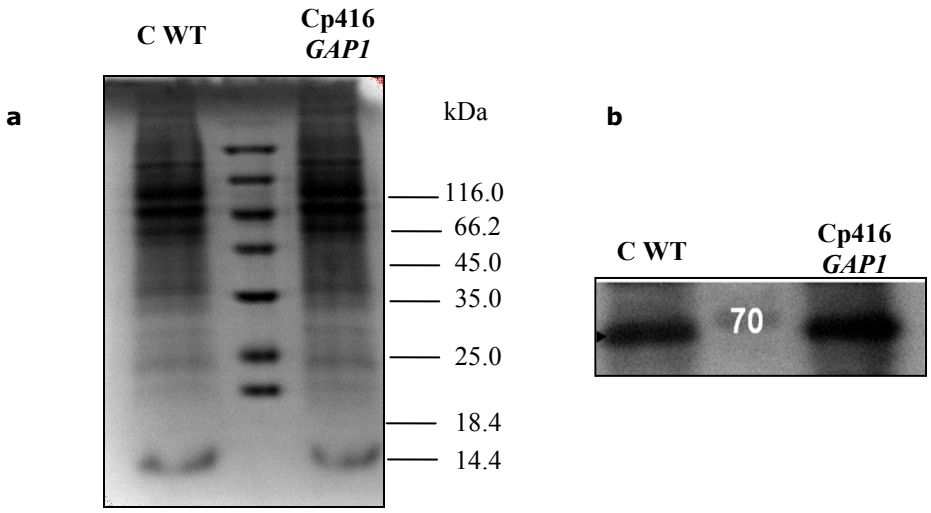


Figure 27: Expression of Gap1p in Strain C wild-type and Strain Cp416*GAP1*: SDS-PAGE (27 a) and Immunoblot (27 b)

Gap1p was immunodetected in whole protein fractions of wild-type and transformant strains. 10 µg protein fractions were loaded on each lane. Gap1 antibody (Santa Cruz Biotechnology, Inc.) was used for detection.

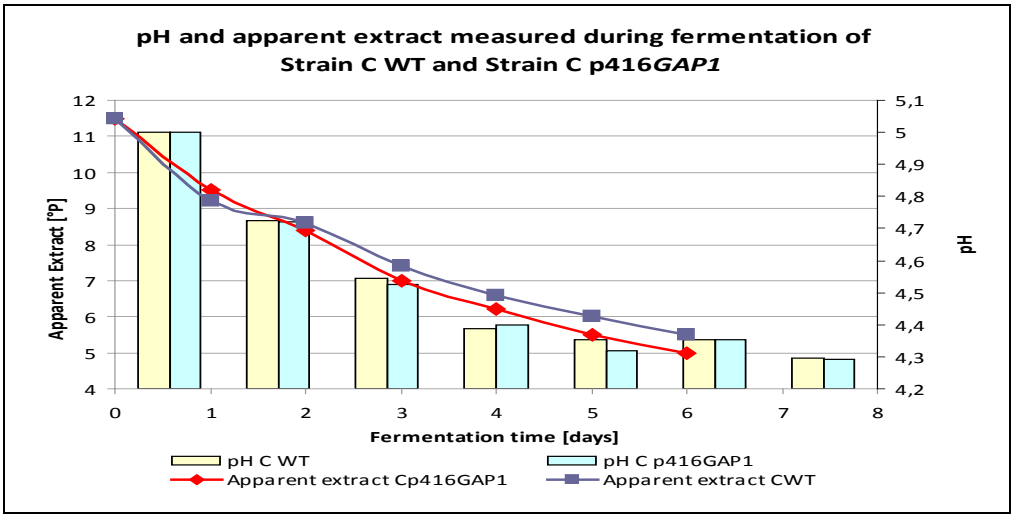


Figure 28: Time courses of apparent extract and pH measurements during the fermentation of wild-type and transformant lager brewers' yeast strains

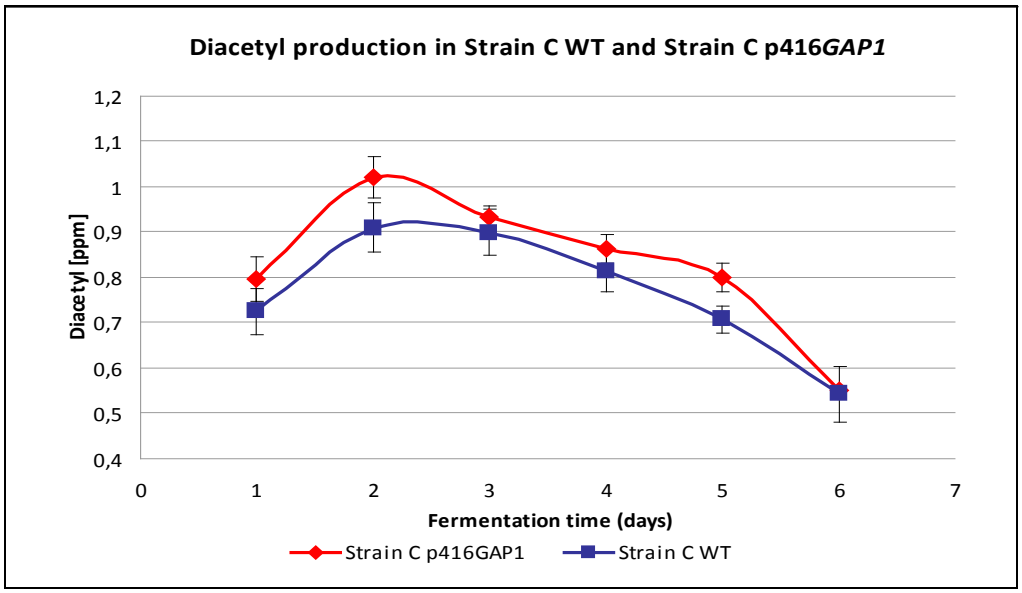


Figure 29: Diacetyl production by Strain C p416*GAP1* in comparison to the wild type Strain C

Fermentation was carried out in 5-litre EBC columns under industrially relevant brewing conditions (14 °C, 11.5 °P brewers' wort). Diacetyl concentration during fermentation was measured using spectrophotometric method.

This result serves to indicate that influencing Gap1p levels indeed leads to changes in diacetyl levels. Due to strong post translational regulation of *GAP1* under enriched nitrogen conditions, a copious increase in Gap1p amounts might be necessary to further verify the positive correlation between Gap1p expression level and diacetyl levels. Hence Brewers' yeast Strain C was transformed with the multi copy YEpTKmTEF6*GAP1* plasmid to obtain maximum *GAP1* expression. Both amounts and activity of Strain C YEpT*GAP1* and Strain C YEpTkmr were checked under nitrogen repressed conditions. The overexpression strain showed a five-fold increase in Gap1 protein amounts and a three-fold increase in its activity compared to the wild type strain under nitrogen repressed conditions (Figures 24, 25). However this difference in activity between the strains could be reduced further when they are grown in nitrogen rich wort. This might occur due to strong post-translational regulations in brewing yeasts because of which newly synthesized Gap1p are subjected to ubiquitination and subsequent sorting to the vacuole in order to maintain steady protein levels.

The fermentation behaviour of Strain C YEpT*GAP1* and Strain C YEpTkmr such as pH and apparent extract consumption were comparable (Figure 30). Diacetyl levels checked using the above strains fermented under simulated brewing conditions showed that Strain C *GAP1* showed an overall increase in diacetyl production by 1.6-fold compared to wild type strain carrying empty plasmid (Figure 31). Although this increase in the diacetyl level is mediocre, this might be in direct correlation to the increase in Gap1p activity in Strain C YEpT*GAP1* in comparison with Strain C YEpTkmr in the wort during the brewing

process. This could be further verified by studying the Gap1p activity in these strains under brewing conditions.

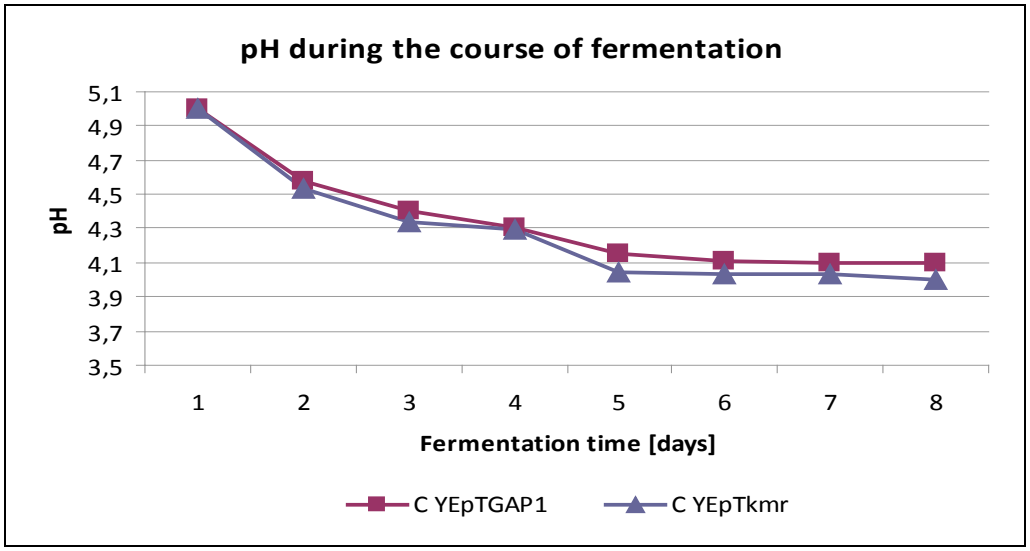


Figure 30: Measured pH values during the main fermentation of Strain C with empty plasmid and Strain C with YEpTkmTEF6*GAP1* plasmid

Fermentation carried out in 2 L flasks under industrially relevant brewing conditions (T = 20 °C, wort: 11.5 %). Shown above is the course of fermentation of at least three fermentations carried out for each strain.

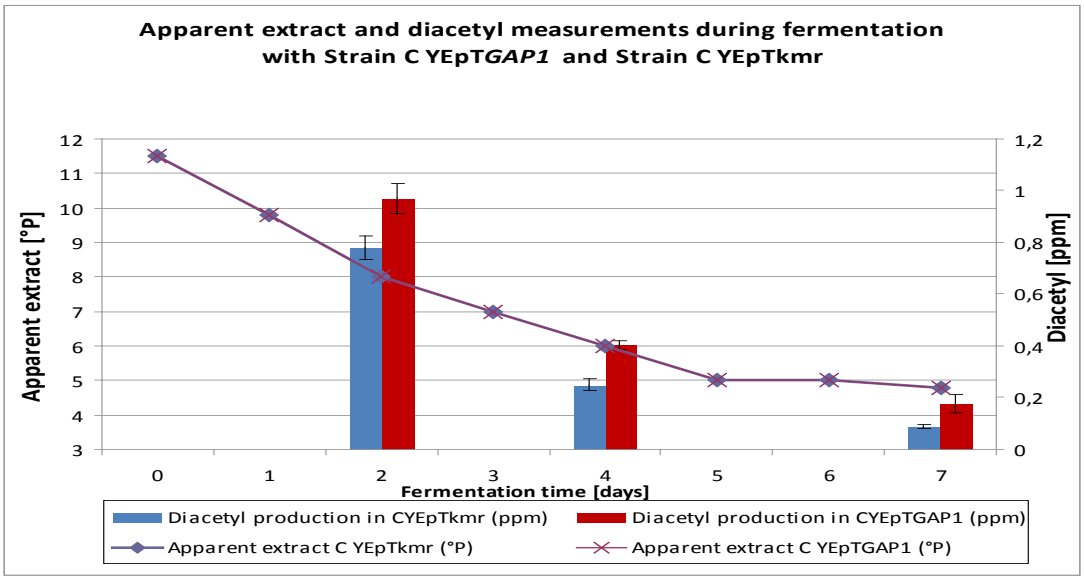


Figure 31: Diacetyl production by Strain C YEpTkmTEF6*GAP1* in comparison to Strain C YEpTkmr

Fermentation was carried out in 2-litre flasks under industrially relevant brewing conditions (20 °C, 11.5 °P brewers' wort). Time courses of apparent extract during the fermentation were measured. Diacetyl concentration during fermentation was measured using spectrophotometric method. All measurements were carried out in triplicates.

4.4.7 Correlation between diacetyl production, Gap1p amounts and Gap1p activity in Strain C YEpT*GAP1* and Strain C YEpTkmr

For further evidence of support to the hypothesis that *GAP1* plays a significant role in influencing diacetyl production during the primary fermentation, Strain C, a renowned German brewing yeast strain was used as the next model organism. The aim of the work was to ascertain if Gap1p overexpression in Strain C results in enhanced diacetyl production.

For the constitutive gene expression of *GAP1* under strong TEF6 promoter, a CEN plasmid and a 2 µm plasmid carrying *GAP1* were transformed into Strain C. Comparison of Gap1p expression in the wild-type and overexpression strains was performed using Immunoblot and activity studies using cells grown in nitrogen repressed medium.

Gap1p overexpression using the CEN plasmid resulted in a two-fold increase in protein amounts compared to wild-type strain. On the other hand the Gap1p expression using the 2 µm plasmid increased by five-fold in Strain C YEpT*GAP1* compared to the wild-type strain and two-fold compared to Strain C p416*GAP1* (Figure 32 a). Correspondingly a three-fold increase in the Gap1p activity was measured in the Strain C YEpT*GAP1* compared with wild-type. Although the activity of Strain C p416*GAP1* was not measured based on the activity measurement of Strain C YEpT*GAP1* it can be assumed that increase in Gap1p amounts produced an equivalent increase in its activity (Figure 32 b).

The measurement of the diacetyl production in these strains under brewing conditions showed that the overall diacetyl production in Strain C increased by 14 % using the CEN plasmid for *GAP1* overexpression whereas a 40 % increase was achieved using the 2 µm plasmid (Figure 32 c). All these results clearly point to the evidence that increase in the activity of Gap1p in the cell produces corresponding increase in diacetyl levels.

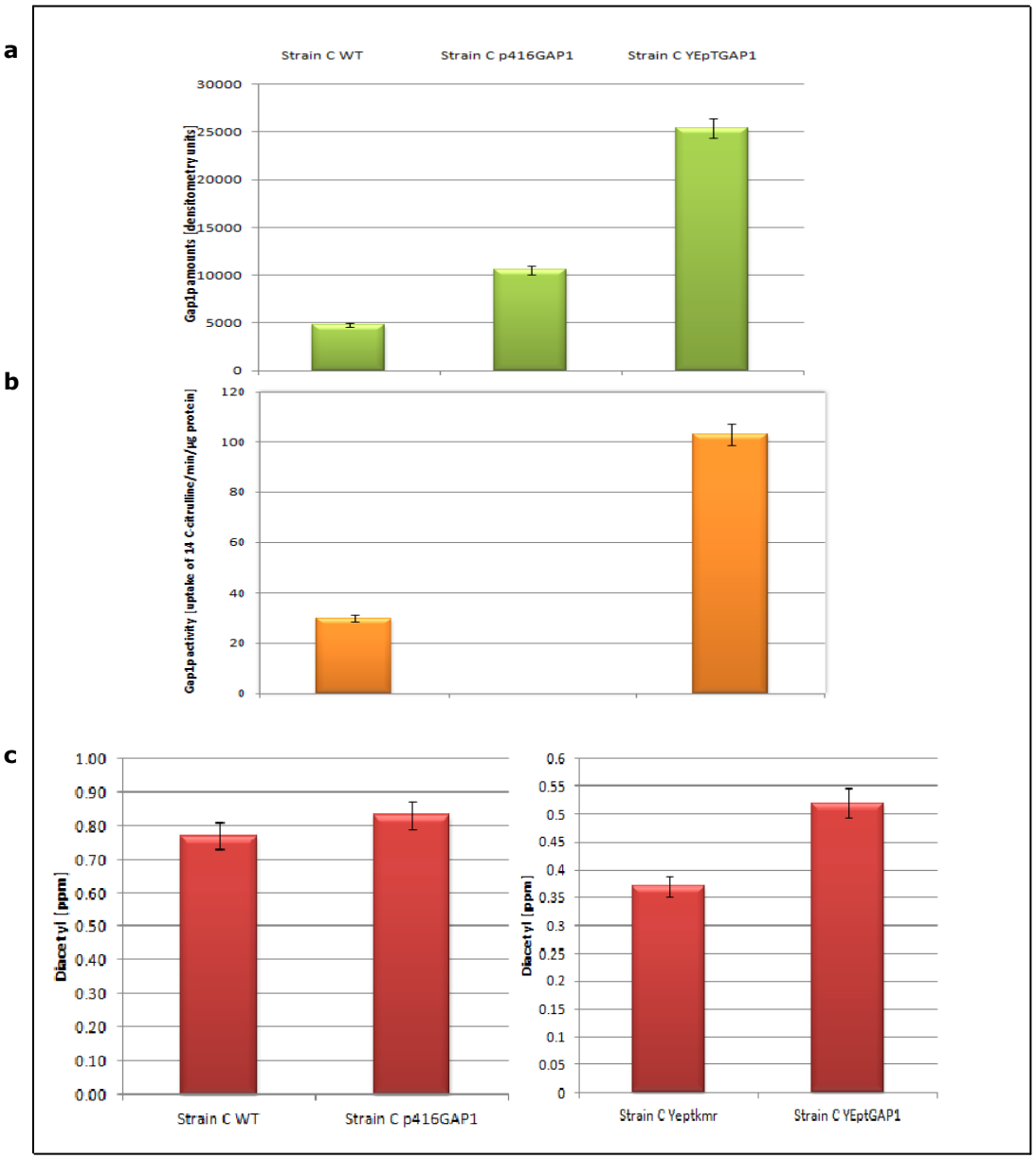


Figure 32: Correlation between diacetyl production, Gap1p amounts and Gap1p activity in wildype and Gap1p overexpression strains of lager Brewing Strain C

(a) Immunoblot carried out with *GAP1* antibody for detection of Gap1p from lager Brewing Strain C wild-type and transformants grown in WMIX-Proline medium. Gap1p amounts were calculated using Density Plot from Immunoblot. Values shown are the average values from at least three independent experiments.

(b) Gap1 activity (uptake of ^{14} C-citrulline $min^{-1} \cdot mg\ prot.^{-1}$) was assayed in cells grown on WMIX-Proline medium by measuring incorporation of [^{14}C] citrulline (2.5 mM).

(c) Overall diacetyl levels in Strain C wild-type with empty plasmid, Strain C with p416TEF6*GAP1* and Strain C with YEpTkmTEF6*GAP1* plasmid. Fermentations for Strain C-WT and Strain C p416*GAP1*was carried out in 5-litre EBC columns (14 °C, 11.5 °P brewers' wort). Fermentations for Strain C-YEpTkmr and Strain C p416*GAP1*was were carried out in 2 L flasks at 20 °C, Wort: 11.5 °P. Diacetyl measurements were carried out using spectrophotometric method.

4.5 Brewers' yeast strain II: Verification of hypothesis

Saccharomyces carlsbergensis- W34/70 (Strain F) as a model organism toward study of influence of *GAP1* on diacetyl level

4.5.1 Overexpression of the *GAP1* gene in the Brewers' yeast Strain F

Western blot analysis of total protein extract of Strain F wild-type and *GAP1* overexpression constructs (clone 17, clone 23, clone 24, clone 25) are shown below (Figure 33). Gap1 overexpression is well pronounced in all the transformants in comparison to wild type strain. In particular the overexpression in clones 17 and 25 are outstanding. These clones are chosen for further experimental verification of the hypothesis.

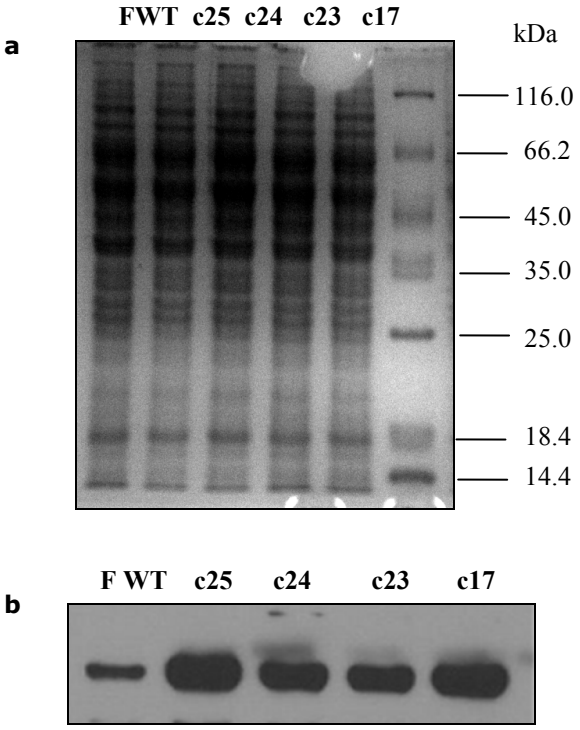


Figure 33: Detection of Gap1p in Strain F wild-type and Strain F transformants: SDS-PAGE (33 a) and Immunoblot (33 b)

Brewers' yeast Strain F was transformed with the multicopy vector YEpTkmTEF6*GAP1* to obtain transformants with higher expression of *GAP1*. Whole protein fractions of wild-type and *GAP1* transformant strains that were identified as positive using colony PCR were checked for their Gap1 expression. 10 µg of whole protein fraction of each strain was loaded per lane of the SDS gel. Immunodetection was carried out by Gap1p antibody.

Gap1p activity in Strain F with Gap1p overexpression leads to a 2.5-fold increase in its activity in comparison to the wild-type strain (Figure 34). This increase in activity in the transformant strain seems to be proportional to the three-fold increase in Gap1 protein amounts in overexpression strain as detected by the Immunoblot.

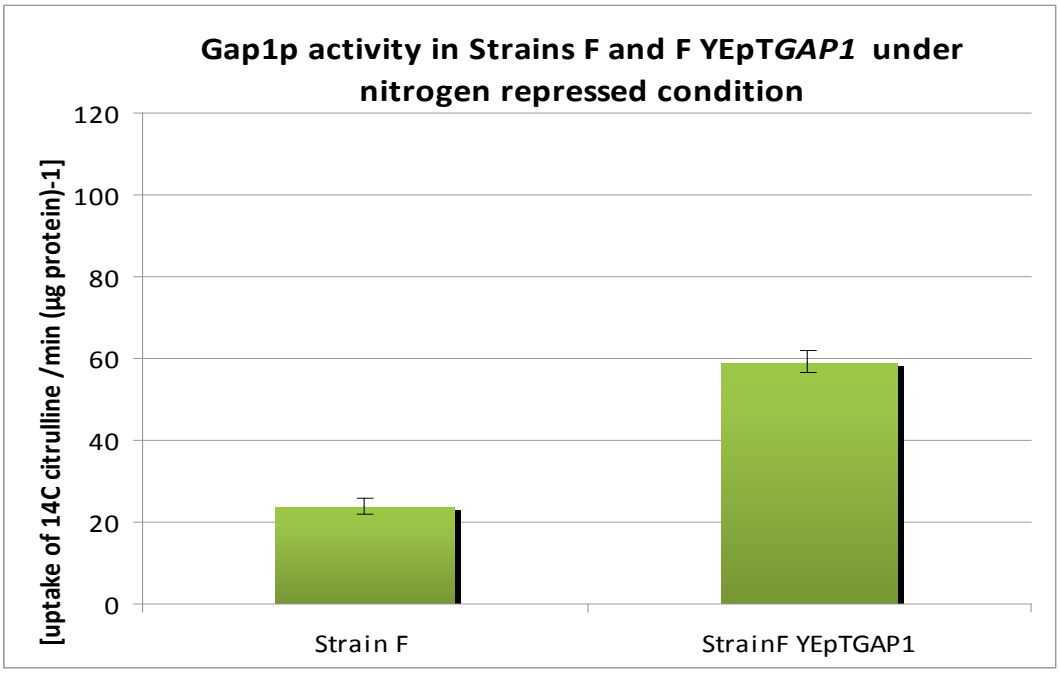


Figure 34: Overexpression of *GAP1* permease activity in Lager brewing Strain F

Cells of Strain F wild-type and Strain F YEpT*GAP1* (carrying the vector YEpTkmTEF6*GAP1*) grown on WMIX-Proline medium for maxiumum Gap1p expression. Gap1p activity was measured by addition of 2.5 mM [^{14}C]-L-citrulline to cells and the transport rate is expressed as uptake of ^{14}C-L- citrulline per min per microgram protein.

4.5.2 Diacetyl levels significantly increased in *GAP1* overexpression brewers' yeast Strain F

The second brewers' yeast model to validate the hypothesis is Strain F, a low diacetyl producing strain. The Strain F is transformed with the self replicating plasmid YEpTKmTEF6*GAP1* for maximal Gap1p expression. The protein level of *GAP1* measured in Strain F YEpT*GAP1* under nitrogen repressed conditions showed a three-fold increase in protein amounts than the wild type strain. Also a 150 % increase in the Gap1p activity was observed in Strain F YEpT*GAP1* than in Strain F YEpTkmr under poor nitrogen conditions. However when the strains were grown in wort under brewing conditions, this difference in their activity could be lower due to post translational regulations of the permease.

These strains were fermented for 7 days under brewing conditions until the apparent extract reduced from 11.5 °P to 4 °P. Fermentation behaviour of the two strains such as pH and apparent extract consumption were comparable (Figure 35). Diacetyl measurements of the two strains showed that Strain F YEpT*GAP1* had a phenomenal three-fold increase in its diacetyl production as compared to Strain F YEpTkmr (Figure 36). This significant increase in diacetyl levels in Strain F YEpT*GAP1* could be attributed

to the fact that Strain F does not undergo such a strong post translational regulation unlike Strain C. Due to *GAP1* overexpression, considerable amounts of active Gap1p are present which could indirectly influence diacetyl levels in this strain.

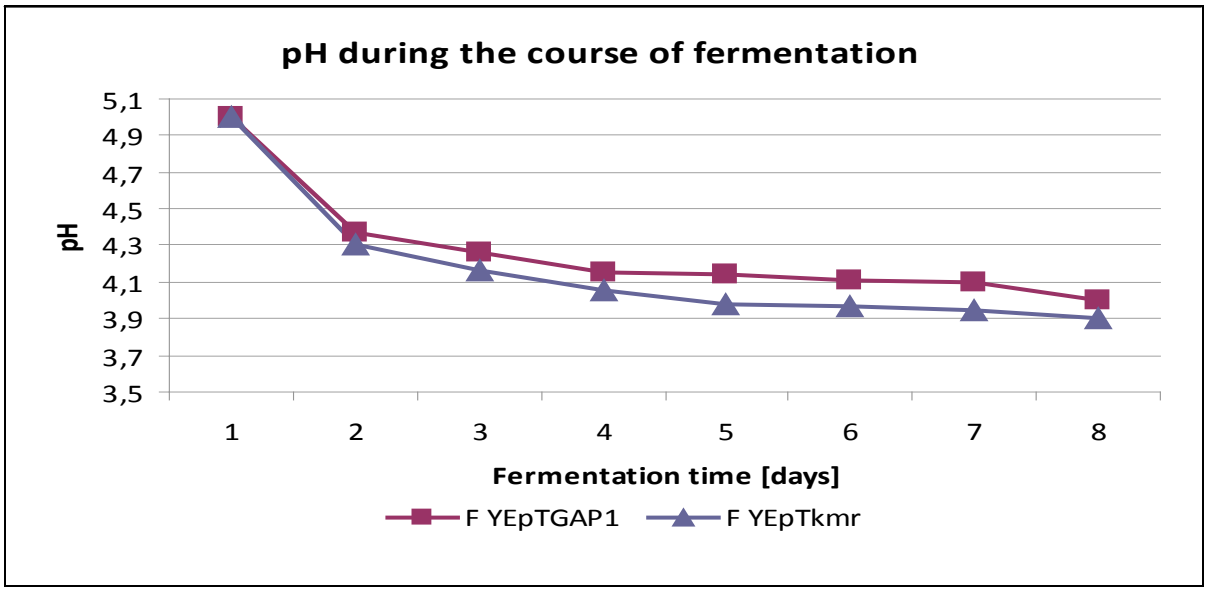


Figure 35: Measured pH values during the main fermentation of Strain F with empty plasmid and Strain F with YEpTkmTEF6*GAP1*

Fermentation carried out in 2 L flasks under industrially relevant brewing conditions (T = 20 °C, wort: 11.5 %).

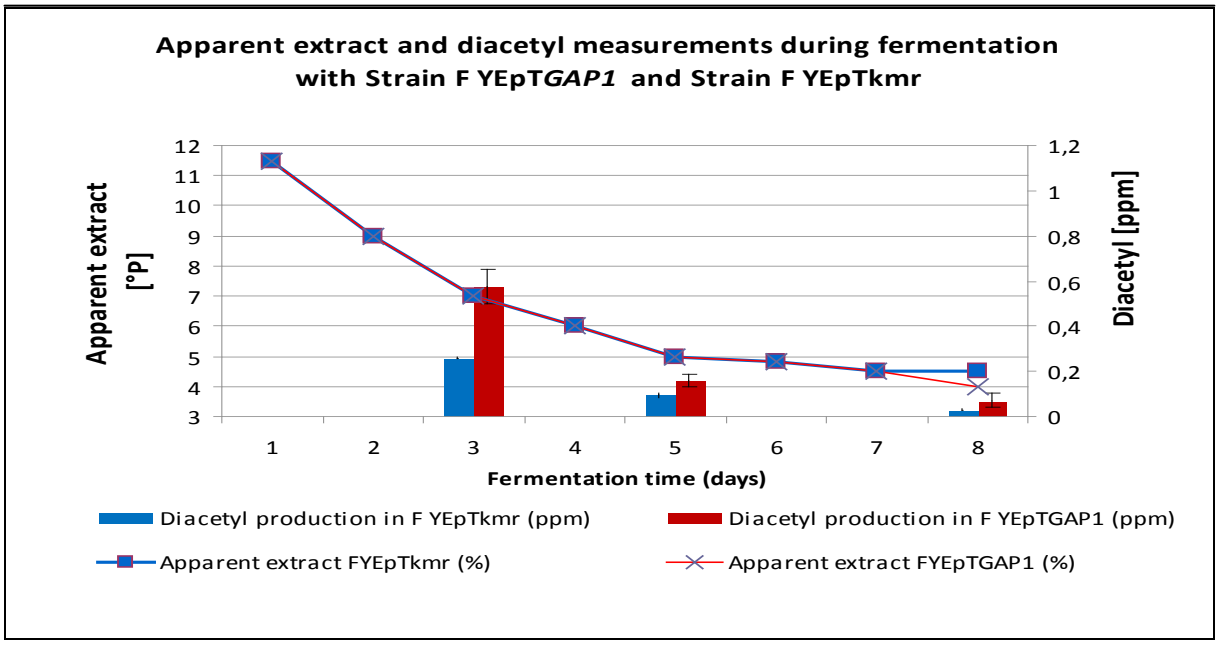


Figure 36: Diacetyl production by Strain F YEpT*GAP1* in comparison to Strain F YEpTkmr

Fermentation was carried out in 2 L scale under industrially relevant brewing conditions (20 °C, 11.5 °P brewers' wort). Time courses of apparent extract during the fermentation were measured. Diacetyl concentration during fermentation was measured using spectrophotometric method. All measurements were carried out in triplicates.

4.5.3 Correlation between diacetyl production, Gap1p amounts and Gap1p activity in Strain F YEpT*GAP1* and Strain F YEpTkmr

Influence of *GAP1* on diacetyl levels was further checked by overexpressing Gap1p in Strain F, widely used yeast strain in German brewing which is known to be a low diacetyl producer. High expression of *GAP1* was achieved in Strain F using the YEpTKmTEF6*GAP1* plasmid with a 2µ origin of replication. The Immunoblot analysis comparing the Strain F wild-type and Strain F YEpT*GAP1* showed that the Gap1p amounts in the latter are increased by four-fold (Figure 37 a). Likewise, the Gap1p activity in the strain is increased 2.5-fold after Gap1p overexpression (Figure 37 b). Fermentation of these strains under brewing conditions showed that Gap1p overexpression led to a remarkable 200 % increase in the diacetyl production (Figure 37 c). These results demonstrate unequivocal proof of concept for the role of *GAP1* in influencing diacetyl production in yeast.

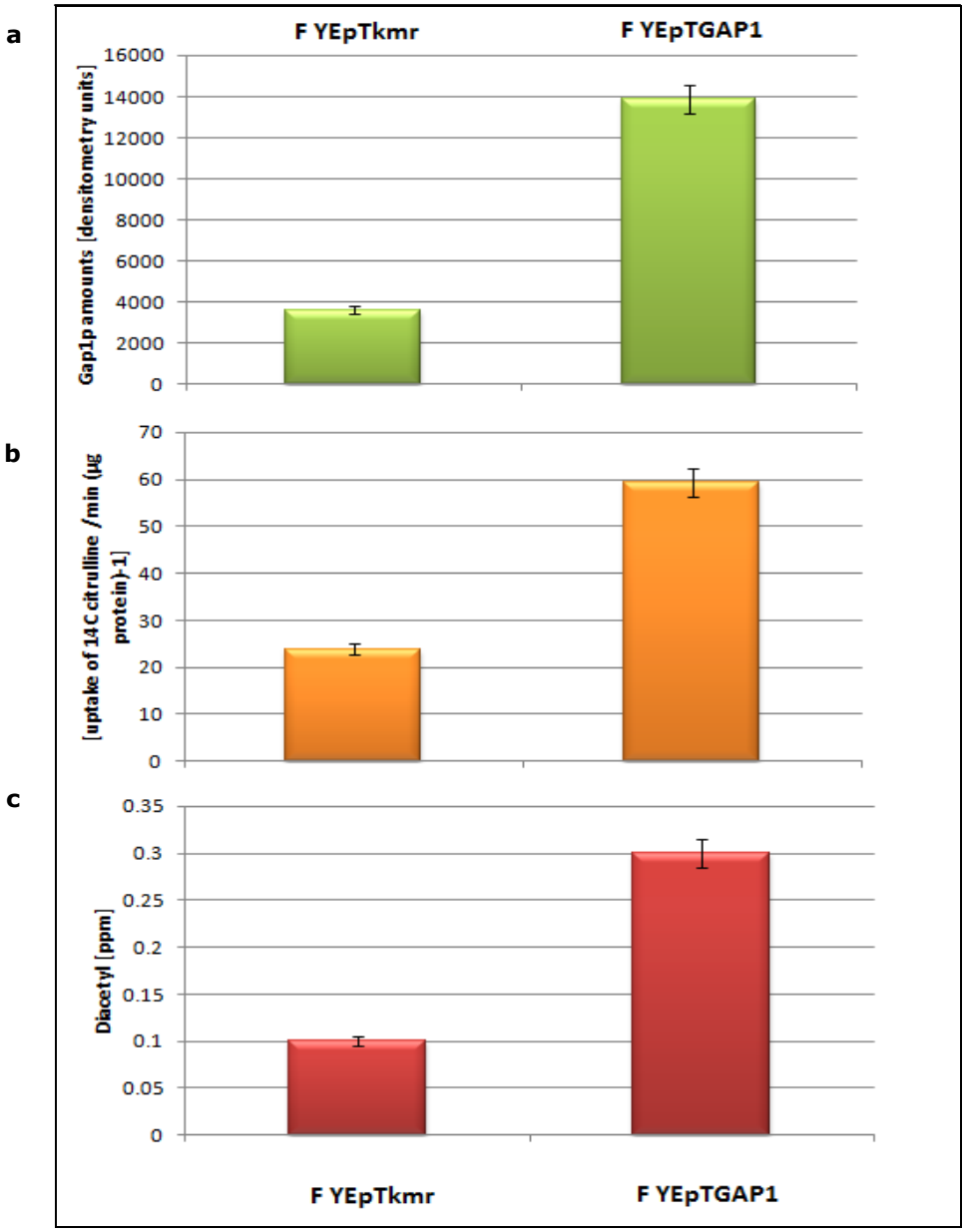


Figure 37: Correlation between diacetyl production, Gap1p amounts and Gap1p activity in wild type and Gap1p overexpression strains of lager Brewing Strain F

(a) Immunoblot carried out with *GAP1* antibody for detection of Gap1p from lager Brewing Strain F wild-type and transformant with 2µm plasmid for *GAP1* overexpression grown in WMIX-Proline medium. Gap1p amounts were calculated using Density Plot from Immunoblot. Values shown are the average values from at least three independent experiments.

(b) Gap1 activity (uptake of ^{14} C-citrulline $min^{-1} \cdot mg\ prot.^{-1}$) was assayed in cells grown on WMIX-Proline medium by measuring incorporation of [^{14}C] citrulline (2.5 mM).

(c) Average diacetyl production in Strain F wild-type with empty plasmid and Strain F *GAP1*. Fermentations were carried out in 2 L flasks at 20 °C, Wort: 11.5 °P. Diacetyl measurements were carried out using Spectrophotometric Method.

4.6 Gap1p expression under brewing conditions

4.6.1 Gap1p activity measured under brewing conditions correlates with diacetyl levels in different brewing strains

The role of *GAP1* gene in regulating diacetyl levels in brewing yeasts was studied in strains that showed differences in their Gap1p expression levels. The strains used in this part of the study include four wildtype brewing strains, C, D, B and F and two *GAP1* overexpression strains, C-YEpT*GAP1* and F-YEpT*GAP1*. Cells were harvested at an apparent extract content of 6 % of the main fermentation where all six strains were in the early stationary phase and showed comparable cell densities. This time point was chosen in order to look for differences in the Gap1p expression level between yeast strains during their transition to stationary phase. The activity of the amino acid transporter in such cells was measured using the uptake assay based on L-citrulline into the cells by Gap1p. Corresponding samples were taken for diacetyl measurements to connect diacetyl level with the transporter activity.

Cells grown in wort rich in nitrogen are expected to have reduced Gap1p activity. However during the fermentation process, the cells are said to evolve from a nitrogen-repressed situation at the beginning of the fermentation to a nitrogen-derepressed situation as the nitrogen is consumed (Beltran, 2004).

A tight correlation was found between the Gap1p permease activity and diacetyl levels among all strains (Figure 38). Strain D, known for its high diacetyl levels was found to also have the highest Gap1p activity. Strain D showed a three-fold higher Gap1p activity in comparison with Strain B, a low diacetyl producer. A corresponding four-fold increase in diacetyl was seen in Strain D than in Strain B.

This correlation was further emphasized in Strain F where a 1.7-fold increase was seen in both diacetyl levels and Gap1p activity of the Gap1p overexpression strain compared to Strain F with empty plasmid. The same pattern was also observed in Strain C in which both diacetyl levels and Gap1p activity was increased by 1.2-fold in the Gap1p overexpression strain compared to its wild type strain carrying empty plasmid. Interestingly at AE = 6 %, Strain C and Strain F showed similar levels of Gap1p activity and diacetyl levels.

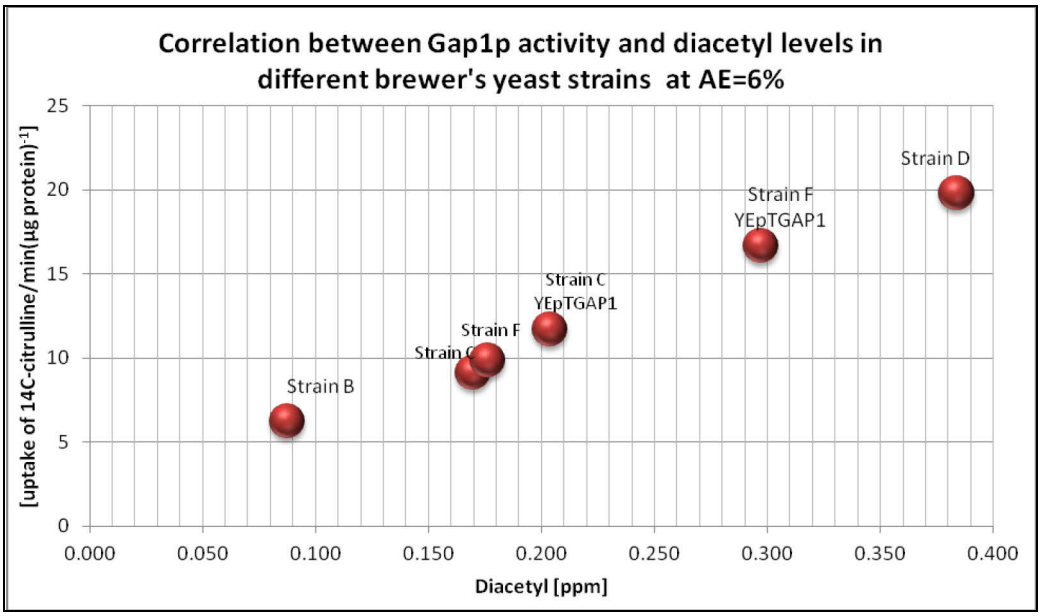


Figure 38: Correlation between *GAP1* permease activity and diacetyl production in Lager brewing strains under brewing conditions

Lager brewing yeast wild-type and *GAP1* overexpression strains (carrying the vector YEpTkmTEF6*GAP1*) were fermented under industrially relevant brewing conditions (20 °C, 11.5 °P brewers' wort). Samples were taken for *GAP1* activity and diacetyl measurements when the apparent extract concentration was 6 % (Day 4). For the permease assay, cells were harvested, washed with MES/KOH buffer (25 mM, pH 6), and suspended in fresh minimal medium containing 4 % glucose. Import assay was performed by addition of 2.5 mM [^{14}C]-L-citrulline to cells. Protein concentrations of the strains were measured using Bradford assay and the transport rate is expressed as uptake of ^{14}C-L- citrulline per min per microgram protein. All activity measurements were subtracted from the background radioactivity measured using Δgap1 BY4741 strain as the negative control. Samples were taken in parallel for diacetyl measurements at apparent extract concentration was 6 %. Diacetyl concentration was measured using spectrophotometric method. All measurements were carried out in triplicates.

4.6.2 Influence of nitrogen content on Gap1p activity: a comparison of Gap1p activity in wort and in nitrogen-repressed medium

The protein activity of *GAP1* permease was analysed during alcoholic fermentation. Gap1p is regulated both transcriptionally and post-translationally by the nitrogen source in the growth medium. As a result, *GAP1* could have been repressed in the first hours after the inoculation in the wort. But during the course of the fermentation, *GAP1* is activated when ammonia depletion occurs (Beltran, 2004). Gap1p activity was measured during beer fermentation in cells harvested upon reaching an apparent extract concentration of 6 %. All activity measurements were subtracted from the background radioactivity measured using *Δgap1* BY4741 strain as the negative control. The Gap1p activity thus measured by the rate of [^{14}C]-citrulline uptake was detected when the cells reached the end of the log/early stationary phase. This could point out that as the wort N content decreases, Gap1p sorting to the plasma membrane occurs, thereby exhibiting higher Gap1p activity. However the Gap1p activity measured using cells grown in WMIX

proline medium for 24 h was found to be higher than in wort. The differences in the Gap1p activity between different Brewers' yeast strains were still detectable in wort (Figure 39). Stains D and F showed a two-fold higher activity in WMIX-Proline than in wort whereas Gap1p activity of Strain B seems to be almost unaltered in both mediums. Strain C on the other hand exhibited a three-fold increase in activity in WMIX compared to wort, showing that there is a higher rate of degradation of Gap1p in Strain C in nitrogen-rich medium compared to other strains. In the case of Strains C and F with *GAP1* overexpression (using YEpTkmTEF6*GAP1*), similar patterns of regulation were observed as in the WT cells. Strain F YEpT*GAP1* showed a three-fold increase in activity in WMIX than in wort while Strain C YEpT*GAP1* showed an eight-fold increase. This pronounced decrease in Strain C YEpT*GAP1* activity in wort suggests that in Strain C, increased sorting of Gap1p to the vacuole occurs despite increase in protein amounts through constitutive expression of *GAP1*.

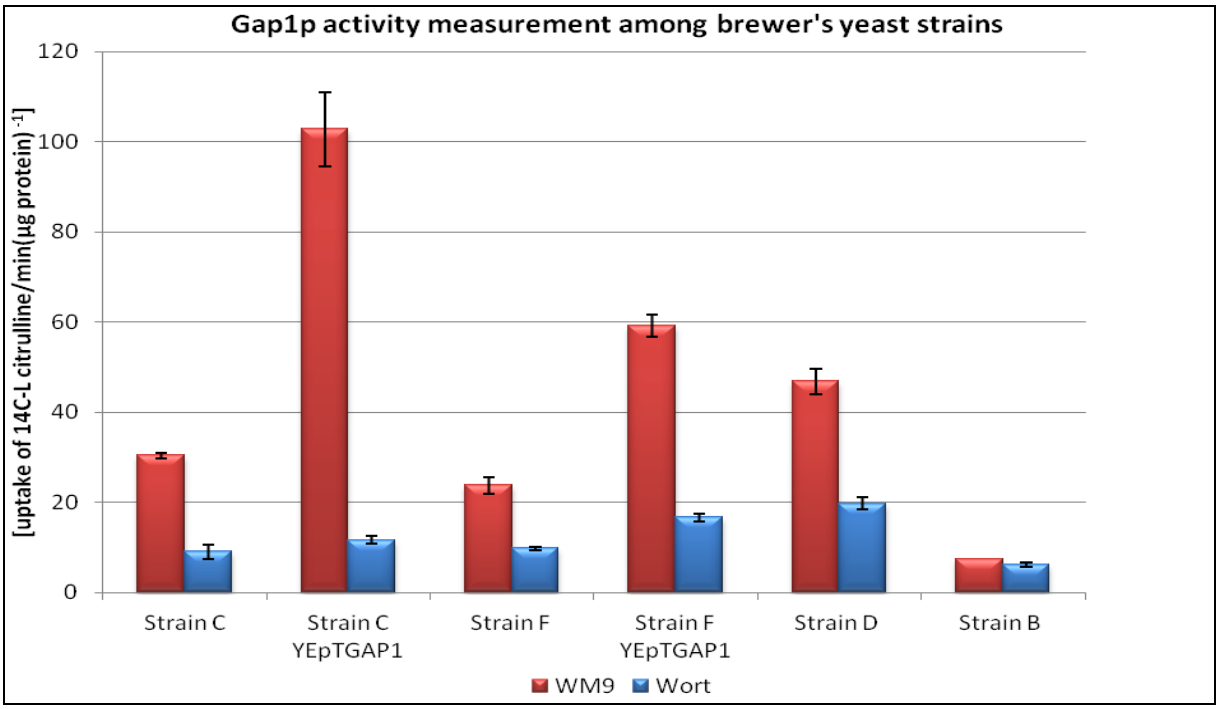


Figure 39: Comparison of *GAP1* permease activity in Lager brewing strains under Nitrogen repressed medium and in brewing wort

For obtaining maximum *GAP1* activity, Brewers' yeast wild-type and transformants with *GAP1* overexpression were grown for 24 h on minimal medium containing 2 % glucose and 0.1 % proline. Like wise for activity measurements under brewing conditions, cells grown in wort were harvested when the wort apparent extract was 6 %. Cells were washed with MES/KOH buffer (25 mM, pH 6), and suspended in fresh minimal medium containing 4 % glucose. Transport rate of the permease was measured as uptake of ^{14}C-L- citrulline per normalized to the protein concentration for each strain.

5. Discussion

Transcriptome data analysis was performed in order to identify potential gene candidates that could influence diacetyl production during brewing process. Using the transcription data a potential candidate gene was identified for follow-up work at the protein level (Schilling, unpublished data). The aim of this thesis was to verify and understand the differences in the mRNA abundance of the *GAP1* gene found between six lager brewers' yeast strains that showed differences in their diacetyl production. Additionally, the project focused on influencing the phenotype (diacetyl production) of the brewing strains by altering gene expression of *GAP1* in different yeast strains. A good understanding of the regulation of this high-range amino acid permease during the brewing process could provide the link between diacetyl production and Gap1 protein variation among brewing strains.

5.1 Verification of transcriptome data: Low diacetyl producers show lower activity of Gap1p than high diacetyl producers

Nature provides copious examples of genetic variations within yeast strains which in turn shape the individual phenotypes (Rockman, 2006). To have a better understanding of these genetic differences and their related phenotypic changes, yeast whole genome microarray hybridization was carried out (Strack, 2009).

The genetic differences present among the yeast strains were measured between the logarithmic growth phase (Apparent extract= 8 %) and the transition to stationary phase (Apparent extract= 6 %) during beer fermentation. The strains used for the transcriptome data analysis were bottom-fermenting brewing yeasts which showed differences in their fermentation behaviour. For example, Strain D differed significantly in its sugar utilization compared with other strains. The diacetyl production in this strain was also found to be the highest. In contrast, Strain B flocculated much earlier than other strains, resulting in an incomplete sugar utilisation. However, the diacetyl production of Strain B by the end of the main fermentation was below the beer diacetyl taste-threshold of 0.1 mg/ml (Strack, 2009; Duong T., 2009). These variations in phenotypes among different strains could be associated with altering gene expression (Treusch, 2013).

Variations in the expression levels of several genes stood out significantly from each other, and might influence the desired phenotype. Upon considering the differentially expressed genes between the high diacetyl producer, Strain D and the low diacetyl producer, Strain B, some novel genes were shortlisted (Schilling, unpublished data). Among them were *GAP1* (General amino acid permease), *AQY2* (Aquaporins, mediates water channel activity), *CWH43* (sensor/transporter protein involved in cell wall

biogenesis), *AQR1* (Plasma membrane multidrug transporter), *ERV46* (involved in the membrane fusion stage of transport), *TOM22* (imports mitochondrially directed proteins), *SEO1* (Putative permease), and *OXA1* (Mitochondrial inner membrane insertase) (Strack, 2009). However, considering the gene function, *GAP1* which codes for General amino acid permease was chosen as a potential candidate as it might play a role in regulating amino acid uptake into yeast thereby influencing diacetyl levels during beer fermentation. Also, the transcriptome data revealed that lager brewing strains with higher diacetyl production showed higher levels of *GAP1* transcripts than the low diacetyl producers. Generally in yeast there is a tight association between mRNA abundance and protein abundance. However, these correlations might largely vary for the same genes across different individuals (Straub, 2011). The reason for the variations between gene expression and protein levels is that several processes such as protein stability, transcription and translation rate are involved in the making of a functional protein. Since *GAP1* is subjected to tight regulations at the post-transcriptional and post-translational level, congruity between mRNA and protein expression was checked.

For the study of Gap1 protein amounts, four strains (used for TDA) showing differences in diacetyl production were strategically chosen. The four strains used were Strain D (a high diacetyl producer), Strain C (a medium diacetyl producer), Strain F (a low diacetyl producer) and Strain B (a low diacetyl producer). The other two strains (A and E) were omitted from the immunoblot analysis for the following reasons. Strain A, whose diacetyl production is higher than C but lower than D is genetically related to Strain C. So it could be assumed that their behavioural pattern is the same. The exclusion of Strain E on the other hand followed the fact that it has similar levels of diacetyl production as Strains B and F.

By growing yeast in a medium containing proline as its sole nitrogen source, *GAP1* is activated and accumulates at the plasma membrane (Hoshikawa, 2003). The strong inducibility of *GAP1* expression depending on substrate availability could be verified by growing the cells initially in minimal medium with good nitrogen source (glutamate) and shifting them into proline medium for 2 h.

The result from the immunoblot shows the maximum expression of Gap1p in each strain at a given time. The differences in the amounts of Gap1p that were detected by the immunoblot technique (Figure 10 B) confirm the interspecies variability in gene expression as discussed by Straub (Straub, 2011). The Gap1 protein level in each strain with low-abundance transcripts tend to have low-abundance proteins and likewise is true for strains with high-abundance transcripts. However, since Gap1p is known to undergo strong post-transcriptional regulations, a direct correlation between mRNA levels and protein levels cannot be expected. Interestingly, the increase in the Gap1 protein amounts in the brewers' strains was proportional to the diacetyl production of the

respective strains (Figure 12). In other words, the order of increase of both diacetyl production and Gap1 protein amounts in the strains are as follows: Strain D>Strain C>Strain F>Strain B. Thus we can go one step further and say that lager brewing strains that are low diacetyl producers (like Strains F and B) have relatively lower amounts of Gap1 proteins in comparison with high diacetyl producing strains (like Strains D and C).

Under the influence of proline in the medium, the Gap1p that accumulates at the plasma membrane are known to be highly active (Grenson, 1983 ; Omura, 2005). As in the case of Gap1 protein amount determination by immunoblot, the four strains used for activity determination were also grown in poor nitrogen medium (WMIX-Proline) to enable maximum Gap1p expression in each strain. Moreover, under these conditions the immunoblot and activity measurement results could be compared since they were both carried out in the nitrogen-repressed medium (Figures 12 B, 12 C).

The Gap1p activity measured by the uptake rate of Gap1 permease - specific substrate, radiolabeled citrulline, gives a clear picture of the amount of stable and active Gap1 permease that are located at the plasma membrane to transport available amino acids into the cell.

The activity of the permease differs between all the four strains used in the experiment as seen in the case of their protein amounts. As a result, Strain D showed highest Gap1p activity sequentially followed by Strains C, F and B (Figure 11). The differences in the activity between the strains were found to be highly significant yielding a p-value = 0.0036 (with the significance level set to $p<0.05$). These results show that, under conditions of poor nitrogen availability, different strains produce different Gap1p amounts which are translated into highly abundant and active proteins. As a result of the differences in the Gap1p amounts among different brewers' yeast strains, there is a corresponding variation in the Gap1p activity. Therefore with reference to its protein amounts, the Gap1p activity among different brewers' yeast strains is the same. The correlations seen between the Gap1p amounts and activity imply that differences in the *GAP1* transcripts (based on transcriptome data) among brewing strains produced differences in their Gap1 protein activity. Therefore, we conclude that strains with high diacetyl production showed higher Gap1 activity than low diacetyl producers.

Previous studies have shown that genetic variation in a gene's activity could be due to variation in the gene itself or due to variation in an unlinked locus (Rockman, 2006; Haldane, 1932). Lager brewing yeasts are known to be polyploid, aneuploid, or alloploid (Jespersen, 1999) showing differences in the copy number of certain chromosomes in their genetic set-up (Kodama, 2006). Therefore, these differences in the Gap1p amounts might clearly point out to the possible variations in the *GAP1* gene copy-number in each

strain. Other possibilities might be point mutations leading to variations in their promoter strengths, strain-specific regulations of Gap1p etc.

While looking closely at the Gap1p protein amounts and activity of the brewers' yeast strains, we can see that Strains F and B which are both low diacetyl producers have nearly comparable Gap1 protein abundance, with Strain F having 1.2-fold higher protein amounts than Strain B. However looking at their Gap1 activity differences, Strain F had a three-fold higher activity than Strain B. This decrease in the protein activity of Strain B despite protein abundance shows that a large portion of the synthesized Gap1p is in their inactive form. Previous works on Gap1p have reported that mutations that occur in Gap1 proteins reaching the plasma membrane results in unstable and totally inactive forms of the protein (Merhi, 2011).

Based on the quality of nitrogen supply, yeast enforce new programs of gene expression. Gap1p expression in the cell which are highly regulated based on the available nitrogen supply, is also known to positively coregulate other genes involved in the synthesis of preferred nitrogen sources like glutamate (*GDH1*) and glutamine (*GLN1*) from low-quality nitrogen sources internalized by Gap1p (Kaufmann, 2010). When brewers' yeast strains have different Gap1p levels, these strains could also show differences in the coexpression of related genes, which could indirectly affect diacetyl levels.

The variation in the diacetyl production with varying Gap1p levels could be due to changes in the assimilation and metabolism of extracellular valine. The uptake rate of valine is highly significant for the study of diacetyl production during beer fermentation (Krogerus, 2013). Increased uptake of valine into yeast results in a negative feedback loop in the valine biosynthesis pathway, leading to decreased levels of diacetyl. The increased diacetyl production in strains with higher Gap1p amounts could be due to downregulation of the expression of genes involved in valine uptake leading to decreased assimilation of extracellular valine in these strains (Figure 40).

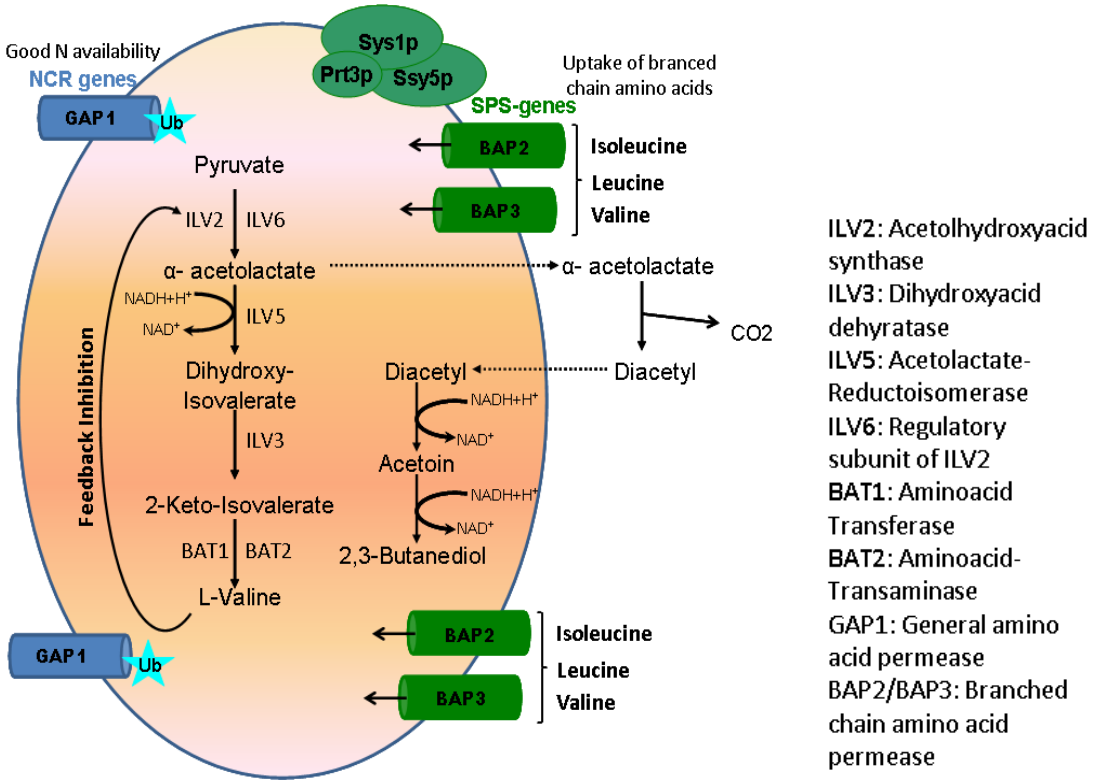


Figure 40: Proposed model showing role of *GAP1* on influencing diacetyl levels in yeast during beer fermentation

Amino acid import is a highly regulated mechanism in yeast as they prefer amino acid uptake via Ssy1-Ptr3-Ssy5 (SPS) sensor regulated specific permeases over nitrogen catabolite repression (NCR) regulated broad-range permeases. *GAP1* is regulated by the available nitrogen source through NCR and therefore is repressed at the start of the fermentation, due to high levels of amino acid nutrients in the wort. Under nitrogen-rich conditions, Gap1p at the plasma membrane is endocytosed and newly synthesized Gap1p is degraded at the vacuole via Gap1p ubiquitination (Ub). Presence of branched chain amino acids in the medium induces the transcription and activity of their respective transporters (*BAP2*/*BAP3*) via SPS sensor thereby enabling their import into yeast. *GAP1* which acts as a nutrient sensor in yeast, could play an important role in upregulation of specific permeases required for amino acid import into the cell. Higher Gap1p amounts could lead to downregulation of the expression of the SPS-regulated genes like *BAP2* and *BAP3* which in turn impedes the uptake of branched chain amino acids into the cell. Lower uptake of extracellular valine results in the activation of valine biosynthesis pathway leading to higher diacetyl levels.

5.2 Hypothesis testing I: Influence of Gap1p on diacetyl production verified in Saccharomyces cerevisiae model strain

Now the major question that needed to be addressed was if these differences in Gap1p seen at the protein level either directly or indirectly influence diacetyl production in brewers' yeast strains. How does the permease which functions both as a transporter and as a receptor for activation of important pathways in response to depletion of amino acids (Rubio-Texeira, 2012), play a role in altering diacetyl production in yeast? The *GAP1* transcriptome and proteome studies in the wild-type brewers' yeast strains

revealed that increase in transcript amounts accounts for the increase in protein amounts and the subsequent increase in permease activity. The question that arose as a result of this observation was, whether higher *GAP1* copy number achieved using a self-replicating plasmid and the resultant increase in the permease activity would lead to an increase in diacetyl production in the respective yeast strain.

Before attempting to answer these questions in industrial brewing strains, in view of its higher-ploidy nature, the first part of the functional studies was carried out in haploid laboratory BY4741 strain. This strain carries a couple of auxotrophies and is unable to synthesize certain essential and non-essential amino acids namely histidine, leucine, metheonine and uracil, thereby rendering it unsuitable for industrial applications.

Since one of the best ways to understand the function of a gene is by means of gene disruption, BY4741 *Δgap1* strain was also included in the study. The consequences of *GAP1* overexpression and deletion on diacetyl production were investigated under nitrogen repressed conditions. The growth of the strains in WMIX-Proline medium enables highest difference in Gap1p levels between strains.

For overexpression of *GAP1*, either a low- or multi-copy plasmid could be used. While the use of 2 µ-based multi-copy plasmids results in a strong gene overexpression, fine-tuning of gene expression cannot be carried out (Nevoigt, 2008). Moreover the maintenance of high-copy plasmids could be a metabolic burden to the cells and there could be variations in the gene expression due to copy number variance between individual cells in a culture (Camps, 2010). Due to these reasons the use of a CEN-based low copy plasmid was preferred initially. However with the expression of *GAP1* under a strong constitutive *TEF6* promoter, the variant of *TEF1* promoter of *S. cerevisiae,* a 1.5-fold increase in the gene expression was achieved in the *GAP1* OE strain as compared to the wild-type (Figure 17).

The growth of the BY4741 strains in WMIX-Proline medium supplemented with amino acids that cannot be otherwise synthesized by the strains due to their auxotrophies, allows maximum Gap1p expression in them. The increase in Gap1 protein amounts in the BY4741 *GAP1* overexpression strain could have resulted in a corresponding increase in its permease activity as compared to the wild-type (not measured). As for the *Δgap1* strain, in which no Gap1p activity is expected, no significant difference in its growth behaviour compared to the wild-type even in the medium containing Proline as its sole nitrogen source (Figure 18). This is due to the fact that in *S. cerevisiae*, proline is taken up both by Gap1 and by the proline-specific permease, Put4p. So proline transport in a *Δgap1* strain can occur via one of the two permeases (Kaufmann, 2010). On the contrary, previous studies showed that when Gap1p is the sole transporter of a particular amino acid as in the case of citrulline, growth of *Δgap1* will be affected on a medium with

citrulline as the sole nitrogen source (Marco De Boer, 1998). Similarly Gap1p is the main transporter of D-amino acids and deletion of the *GAP1* gene is expected to affect the uptake of these amino acids. However in general, the lack of Gap1p activity in a strain can be compensated by other amino acid permeases (Didion, 1998).

Similar to *Δgap1* strain, the *GAP1* overexpression strain shows growth behaviour which is comparable to the wild-type strain (Figure 18). This shows that the strain's growth was not therefore affected by the biosynthetic burden due to plasmid maintenance or increase in gene copy number.

To have a better understanding of the *GAP1* expression in yeast during beer fermentation, an analogy between different Gap1p levels in these BY4741 model strains and lager yeast strains involved in beer brewing can be considered. During fermentation, the cells evolve from a nitrogen-repressed situation at the beginning of the process to a nitrogen derepressed situation as the nitrogen is consumed (Beltran, 2004). In other words, under good nitrogen conditions, *GAP1* expression is considered to be almost negligible as in the case of BY4741 *Δgap1*. But as soon as nitrogen depletion occurs, *GAP1* expression is set in motion and escalates to the maximum. This is comparable to the *GAP1* expression in the wild-type and in the *GAP1* overexpression strains respectively, growing in WMIX-Proline medium.

Differences were observed in the diacetyl content of these strains after changing their respective Gap1p level (Figure 19). Upon increasing the *GAP1* gene copy number in the cell (1 or 2 copies per cell) using the self-replicating CEN-based plasmid, the overall diacetyl production in the strain increased by 30 % as compared with wild-type. Conversely a decrease of 20 % was achieved upon deletion of *GAP1* in the *Δgap1* strain (Figure 19). These differences in the diacetyl production were found to be significant with a p-value of 0.0101, when the significance level is set to $p<0.05$. Principally these results support the research hypothesis (H_1) which says that strains with higher levels of Gap1p produced more diacetyl than strains with lower Gap1p levels.

The consumption of valine plays a significant role in diacetyl metabolism. 0.1 % of casamino acids used in the WMIX-Proline medium contains 55.7 mg valine/100 ml of 1 % of casamino acid solution (Johnson A.H., 1965) which is taken up by the yeast through specific permeases. Considering the diacetyl synthesis phase (up to 24 h), the *GAP1* OE strain seems to have a slower valine uptake rate leading to increased diacetyl levels compared to wild-type and *Δgap1* strains.

The role of *GAP1* in influencing diacetyl levels in the cell could be understood from the point of view of function of the permease not only as a transporter, but also as a receptor involved in signalling process in yeast upon nitrogen depletion (Kriel, 2011). In general the availability of amino acids to the cells is known to play an important role in diacetyl

production (Eßlinger, 2009). The repressed state of *GAP1* in response to preferred nitrogen sources could invoke responses from the branched-chain amino acid permeases such as Bap2p, Bap3p, and Tat1p which are known to be upregulated during conditions of *GAP1* repression in the presence of the respective amino acids they transport (Kodama, 2001).

Thinking along these lines, it can be said that decrease in *GAP1* levels in the cell could be a signal for cells to upregulate the transcription of the branched-chain amino acid permeases, which in turn results in an increased uptake of branched-chain amino acids (Didion, 1998). Diacetyl production is then reduced with an increased uptake of valine into the cells due to feed back inhibition in the intracellular valine biosynthesis pathway. The converse is then true for increased *GAP1* levels in the cell. A more vivid description on the theory behind the influencing of diacetyl production using *GAP1* is provided in section 5.6.

Despite the complete inactivation of *GAP1* in the *Δgap1* strain, its impact on diacetyl reduction is seemingly low compared to the wild-type strain having higher levels of *GAP1*. This however could be explained as the effect seen during growth of the cells in WMIX medium with relatively low levels of amino acids (casamino acids 1 g/L). Hence, even though the transcription of *GAP1* is completely absent, the cells require sufficient supply of amino acids in the medium for better uptake and a resultant decrease in diacetyl production.

From these results we can draw a good picture of how the diacetyl production of brewing yeast could vary under conditions of *GAP1* overexpression and deletion (Figure 40).

5.3 Hypothesis testing II: Overexpression of *GAP1* in brewers' yeast Strain C leads to increase in diacetyl production

Diacetyl production in brewers' yeast is a complex process influenced by various environmental and genetic factors (section 2.3). The findings using the *S. cerevisiae* model strain have certainly thrown in some light on the subject (sections 4.3 and 5.2). However it is also known that results obtained with laboratory yeasts cannot necessarily be transferred to brewers' yeast since they are genetically and physiologically different. Although the regulatory machinery in brewers' yeast are quite complex compared with haploid laboratory yeast, gaining a clearer understanding of the regulations inside brewing yeast that directly or indirectly affect their diacetyl production was considered imperative.

Along these lines, a lager brewing yeast Strain C was chosen to analyze the influence of varying expression rates of *GAP1* on diacetyl production. To vary *GAP1* expression levels in the cell by increasing its gene copy number, both low-copy (CEN/ARS-based

p416TEF6*GAP1*) and multicopy (2 µ−based YEpTKm*TEF6GAP1*CycT) yeast expression plasmids were used. The use of the strong *TEF6* promoter was meant to increase the transcription rates.

Both plasmids contained the *GAP1* gene cloned from the genomic library derived from *S. cerevisiae* (BY4741) strain. Accordingly the amino acid sequence homology between *S. cerevisiae* Gap1p and Lg-Gap1p was 89 % (Figure 8). Since the structural composition of the chromosome (Sc-type, non-Sc-type and Sc/non-Sc-type chimera chromosomes) are known to vary among different lager brewing yeast strains, any further increase in the copy number of Sc-*GAP1* could lead to major alterations in the gene's transcriptional and translational activity.

Using the p416 plasmid, the *GAP1* gene copy number was further increased in the order of 1-2 copies per cell. On the other hand, the copy number of most YEp plasmids ranges from 10-40 copies per cell (Schneiter, 2004) and therefore turns out to be a convenient way to monitor the effects of overproduction of the gene product.

Both protein levels and activity measurements were carried out under conditions of maximum *GAP1* expression (WMIX-Proline medium). A two-fold and a five-fold increase in Gap1p amounts was obtained in the overexpression strains compared with the wild-type using the centromeric and the 2 µ plasmids respectively (Figure 25). Consequently, a maximizing of the permease activity by three-fold was obtained using the 2 µ vector (Figure 26). This increase of activity in the overexpression Strain C YEpT*GAP1*can be attributed to the increase in protein expression level achieved using the 2µ plasmid. However the lack of perfect correlation between the Gap1p level and permease activity could be due to strong post-translational control. A more detailed explanation for the same is given below in this section.

A similar increase in the permease activity, proportionate to the *GAP1* overexpression using the low-copy plasmid is supposedly achieved in Strain C p416*GAP1* (not measured). Thus using these plasmids, a crude adjustment of Gap1 protein expression was achievable in the brewing yeast as in the case of lab strain.

The diacetyl production in the green beer during the primary fermentation was measured in the two *GAP1* overexpression strains and in the wild-type strain carrying the empty plasmid (as a control). Distinct increases in the total diacetyl production (free diacetyl + α-acetolactate) were obtained with increase in *GAP1* levels in the respective strains (Figures 29 and 31).

Compared with the wild-type strain, the production of diacetyl was increased by 14 % in StrainC-p416*GAP1* (low copy plasmid) and by 40 % in Strain C YEpT*GAP1* (multicopy plamid). Broadly speaking, the increase in the diacetyl production in the *GAP1* overexpression strains compared with the wild-type is statistically significant, with a p-

value of 0.009 (with the significance level set to $p < 0.05$). Through the use of plasmids with different copy numbers, it is possible to show that a step wise increase in the *GAP1* gene copy number will produce an equivalent increase in the strain's diacetyl production.

When these results are seen from the point of view of the transcriptome data analysis of the six brewing yeast strains differing in their diacetyl production, it can be seen that the level of *GAP1* in the strain definitely speaks for its diacetyl production. In other words, among the other genetic differences seen within brewing strains, the differences in their *GAP1* levels can now be taken into account. All in all, the hypothesis (H_1) was successfully proved in the industrial lager brewing Strain C.

Now there are a few questions that need to be addressed to have a better understanding of the various regulations that occur inside the brewers' yeast strain upon *GAP1* overexpression.

1. Why is the increase in the diacetyl production of the *GAP1* overexpression strains, in particular in Strain C-YEpT*GAP1* not proportional to the protein overexpression achieved using the respective plasmids?
2. Are there significant differences in *GAP1* activity between wild-type and overexpression strains during the beer fermentation process?
3. Why is the overall diacetyl production in Strain C (a medium diacetyl producer), seemingly higher in both wild-type and overexpression strains.

To understand why there was only a moderate increase in the diacetyl production after *GAP1* overexpression in Strain C, the first factor that should be taken into account is that protein expression and activity measurements were initially carried out in WMIX-Proline medium in which *GAP1* is highly expressed. However when the strains are transferred into a nitrogen- rich medium like wort for beer fermentation, Gap1p is subjected to ubiquitination, internalization and degradation in order to substitute them with a range of other specific amino acid transporters. This preference of the yeast to use specific transporters over general permeases under nitrogen-rich conditions could allow for better fine-tuning of the amino acid uptake according to the requirements of various metabolic processes in the cell (Springael & André, 1998).

During the fermentation process, although strains with a strong constitutive expression of *GAP1* were used, the plethora of synthesized Gap1 proteins could nevertheless be subjected to a strong post-translational regulation, thereby allowing only a limited expression of *GAP1*. Since brewers' yeast differ in their Gap1p levels, their protein regulation could also be different. A previous study on the branched- chain amino acid permease *BAP2* showed that during beer fermentation, the transcription profiles of the Sc-*BAP2* and Lg-*BAP2* are different from one another (Kodama, 2001). Likewise the

overexpression of Sc-*GAP1* in Strain C could follow a different transcriptional profiling or other post-transcriptional regulations than Lg-*GAP1*. The mechanism by which these regulations take place in the yeast is complicated and is not well understood in most cases.

Another possible explanation is the instability of the multicopy plasmid (YEpTKmTEF6*GAP1*) used for *GAP1* overexpression. Most YEp plasmids are relatively unstable and is said that only 60 % to 95 % of the cells retain the plasmid even when the cells grow under selection pressure (Sherman, 1998). So during the long fermentation runs, it is likely that some of the cells do not retain the plasmid necessary for *GAP1* overexpression.

To answer the question about the behavior of *GAP1* both in wild-type and overexpression strains, it is necessary to measure the permease activity under brewing conditions. This will give a better picture of the expression of the Gap1 protein and their relation to diacetyl production (Section 5.5).

To answer the final question, diacetyl production in strains could be greatly influenced by fermentation conditions. Although Strain C is a medium diacetyl producer, the production of diacetyl even in the wild-type was up to 0.9 ppm. Diacetyl production may have increased due to various factors like higher fermentation temperature used (20 °C instead of the preferred 6-14 °C), plasmid maintenance and other stress factors that occur during fermentation that might have produced greater impact on diacetyl levels in Strain C. Ultimately, the critical part is to determine differences in the diacetyl production between strains which are all treated in the same manner and fermented under same conditions.

5.4 Hypothesis testing III: *GAP1* overexpression in brewers' yeast Strain F significantly increases diacetyl production

As a next and final example to show that overexpression of *GAP1* in industrial brewing strains might bring about an increase in its diacetyl production, Strain F (Weihenstephan 34/70 lager yeast) known for its low diacetyl production, was used. Lager brewing yeasts are divided into two groups based on their genomic contents. One group has lost a significant portion of *S. cerevisiae* genome and the other group with contents from both *S. bayanus* and *S. cerevisiae* parental strains. Strain F, which has been completely sequenced and considered a representative of the lager brewing production strains, falls into the latter category. (Nakao, 2009).

Since wild-type Strain F was found to have lower levels of Gap1p (Figure 10 B), the 2µ-based YEpTkmTF6*GAP1* plasmid was used to obtain a maximum expression of *GAP1* in order to study the influence on its diacetyl production. As YEp is a multicopy vector

leading to variations in the plasmid copy number among positive clones, an immunoblot analysis was made. Among the four clones used, all of them showed a considerable increase in Gap1p, in the order of two to four-fold higher protein amounts compared with the wild-type strain (Figure 33). These differences can also be reflective of the cell to cell variation in the plasmid-copy number when using a multicopy plasmid (discussed in the previous section). The activity measurement using the clone which showed the highest Gap1p expression in the immunoblot showed a 2.5-fold increase compared with the wild-type strain (Figure 34). As in the previous cases, both the immunoblot and activity measurements were carried out after growing the strains in WMIX-Proline medium and therefore the increases measured represent maximum *GAP1* expression obtained under nitrogen-repressed conditions.

Now the diacetyl measurements were carried out under brewing conditions to check for the influence produced by *GAP1* overexpression. The diacetyl produced by the Strain F-YepT*GAP1* clone which showed the highest protein expression among other strains, was compared with that of the wild-type strain carrying empty plasmid. The result showed that the overexpression strain, which otherwise showed no significant differences in the fermentation behaviour compared to the wild-type strain, produced a three-fold higher diacetyl than the parental strain (Figure 36). These differences have proved beyond doubt that the level of *GAP1* in a strain plays a crucial role in changing its diacetyl production. The differences in the diacetyl production of the wild-type and *GAP1* overexpression strains were found to be statistically highly significant having a p-value =0.0005 (with the significance level set at $p \leq 0.05$), thereby proving the hypothesis (H_1).

Now comparing the *GAP1* overexpression in Strain C, we can see that the differences in the diacetyl production with their respective wild-type strains are higher in Strain F than in Strain C. Although these bottom-fermenting yeast strains share several common fermentation properties, several attempts made in the past to characterize the genomes of industrial yeast strains reveal that there are inter-species differences such as single nucleotide polymorphisms (SNPs), strain specific ORFs and variations in gene copy numbers among them (Borneman, 2011). Since it has been previously checked in this work that the Gap1p expression and activity are different in both wild-type strains (Strain C>Strain F), it can be said that the phenotypic responses to Gap1p overexpression could also vary. The reasons behind these differences could be manifold.

1) Under brewing conditions, Strain C might have stricter regulations on Gap1p (post-transcriptional and post-translational) than Strain F.
2) In addition to *GAP1*, the copy number variations in other amino acid permeases between strains must also be taken into account.

For example, if in Strain C both *GAP1* and *BAP* (branched-chain amino acid permease) have same copy number, as long as there is good Nitrogen source available, *GAP1* expression is limited. So the increase in the *GAP1* copy number in this strain using a self-replicating plasmid may produce limited overexpression of this permease as long as there is a strong expression of BAP under conditions of good nitrogen availability. On the contrary if in Strain F both *BAP* and *GAP1* have low expression, and then upon plasmid overexpression, a stronger expression of *GAP1* is facilitated in this strain under brewing conditions.

3) Strain-specific responses to stress conditions.

Each strain is specifically programmed to meet its nutrient requirements. Cells respond to changes in the environment by regulating the transcription of appropriate genes to meet its needs (Yoshida, 2007). It is also known that various cellular processes such as mRNA or protein degradation can confer beneficial phenotype to the cell (von der Haar, 2007). In general, each strain utilizes *GAP1* only as required. In the attempt to prevent excess levels of Gap1 when not required, expression of the permease is repressed and newly synthesized *GAP1* are degraded and these responses could greatly influence *GAP1* overexpression in these strains.

Nevertheless, when the diacetyl production in the *GAP1* overexpression strains (Strain F YEpT*GAP1*, Strain C p416*GAP1* and Strain C YEpT*GAP1*) are considered, the overexpression strains always have higher diacetyl levels than their respective wild-type strains. Thus there is a good indication that inter-species variations in the *GAP1* gene copy number causes changes in their diacetyl.

Although both Gap1 protein expression and activity were measured under Nitrogen repressed conditions, the differences observed between different strains are still representative of the differences expected to be seen during beer fermentation when the strains transit from nitrogen-rich to poor conditions. However, to have a better understanding of the behaviour of *GAP1* during beer brewing, further comparison of the inter-strain permease differences was carried out under brewing conditions.

5.5 *GAP1* expression under brewing conditions

Wort used for beer brewing has a rich supply of Nitrogen in the form of amino acids and low-molecular-weight peptides required for the growth and maintenance of the yeast which are in turn indispensable for the fermentation quality (Eßlinger, 2009). Through a mechanism known as Nitrogen Catabolite Repression (NCR), yeast selects the best nitrogen sources in a rich medium. A previous study on wine yeast showed that *GAP1* is repressed by the nitrogen catabolite repression (NCR) mechanism in the beginning of the fermentation when there is a good supply of nitrogen and is derepressed when good

nitrogen sources like ammonium were consumed (Beltran, 2004). These nitrogen transport systems are said to be highly conserved in *S. cerevisiae* species (Crépin L, 2012) and therefore we can draw a good parallel between regulation of *GAP1* in wine and beer fermenting yeast strains.

Four wild-type brewing yeast strains that varied in their diacetyl production (C, D, B and F) and two *GAP1* overexpression strains (C-YEpT*GAP1* and F-YEpT*GAP1*) were fermented using strandard wort with 12 °P. The activity of the general amino acid permease was analyzed when the apparent extract concentration reached 6 %, a time point when there is slow down in fermentation rate as the yeasts are in their late log/early stationary phase. Diacetyl production of the respective strains corresponding to this time point (AE = 6 %) were also measured (Figure 38).

Given the fact that during the stationary phase yeast carries out flavour adjustments which includes reabsorption of the VDKs, the measured diacetyl levels of all the strains during that time point (AE = 6 %) will be low. Although the permease activity was expected to be almost negligible at the start of the fermentation when there is high nitrogen content, during late log and stationary phase it was expected that the rapid depletion of the good nitrogen sources results in derepression of NCR-regulated *GAP1* gene.

Gap1p activity of the six brewing yeast strains (4 wild-type and 2 *GAP1* overexpression strains) which showed differences in their Gap1p expression levels was previously measured when the cells were grown under poor-nitrogen conditions (Sections 5.1, 5.2 and 5.3). As mentioned earlier, the Gap1p activity under these conditions is at its maximum level and is not entirely representative of Gap1p expression in a nitrogen-rich medium like wort. Therefore, it was necessary to verify these differences in the permease activity between the strains during beer fermentation. The aim of this section of work was to show that the differences in diacetyl production between strains can be correlated with corresponding differences in the Gap1p activity of the respective strains at any determined time point.

As seen in the case of Gap1p activity measurements under nitrogen repressed conditions, all six brewing strains differed from one another in their permease activity under brewing conditions. From this we can understand that these Gap1p activity differences occur due to strain specific gene expression machinery such as gene copy number or transcription rate. Looking firstly at the activity differences between the wild-type brewers' yeast strains, Strain D showed the highest activity followed by Strains C and F. Strain B showed the lowest level of Gap1p activity among all strains. Similarly in the case of the overexpression strains, the activity differences due to plasmid overexpression were significant compared to their respective wild-type strains. However the difference in the

activity between Strains F and F-YEpT*GAP1* was more pronounced than that between Strains C and C-YEpT*GAP1*.

Now comparing the activities of these strains between nitrogen rich wort and nitrogen repressed WMIX-Proline medium, we can see the influence that the nitrogen based repression brings on Gap1p activity. The order of differences in the permease activity measured in wort, agree with the activity values of the same strains measured under nitrogen repressed conditions. However in wort, the activity measurements of Strains C and F were quite similar, contrary to the measured differences in WMIX-Proline medium (Strain C>Strain F). The reason for this could be that in a nitrogen rich medium, the onset of *GAP1* expression in Strain C was slower owing to stronger transcriptional regulations in this strain than in Strain F, causing their permease activities to be similar at that time point.

Likewise the permease activities of the wild-type and their respective *GAP1* overexpression strains also showed a different behavior in wort and in WMIX-Proline media. The differences in the permease activities of Strains C and C-YEpT*GAP1* dropped radically in wort compared to WMIX-Proline resulting in a 93 % decrease. This shows that in Strain C growing in the nitrogen rich wort, *GAP1* is subjected to a strong post-transcriptional/translational regulation leading to a controlled expression of the permease despite overexpression using a constitutive promoter. Such a strict regulation of the permease expression in Strain C clearly explains the lower differences in diacetyl measured between its wild-type and Gap1 overexpression strains (Section 5.3.).

On the other hand, strains F and F-YEpT*GAP1* still showed sufficiently high differences in their activity in wort as in WMIX-Proline. This indicates that the regulations in Strain F toward *GAP1* could be less stringent than in Strain C which in turn leads to higher differences in the diacetyl production between wild-type and overexpression strains (Section 5.4).

Now the major question that needs to be addressed is if these differences in Gap1p activities measured during beer fermentation are reflective of the differences in diacetyl production of the respective strains at that time point (AE = 6 %).

The diacetyl values measured from each strain at the time point when the apparent extract concentration was 6 % showed that given the same conditions, different strains produce different levels of diacetyl. Among the wild-type strains, Strain D showed the highest diacetyl production followed by Strains C and F and Strain B was the lowest diacetyl producer. Diacetyl produced in Strains C and F was highly similar at this time point. Also comparing the wild-type and the *GAP1* overexpression strains of C and F, the diacetyl production in the overexpression strains was clearly higher than their respective

wild-type strains. As seen in the previous cases these differences in the diacetyl values was more pronounced for Strains F and F-YEpT*GAP1* than for strains C and C-YEpT*GAP1*.

Interestingly, a tight correlation can be seen between diacetyl production and Gap1p activity in all six strains under brewing conditions. Starting with the high diacetyl producers, Strain D with the highest Gap1p activity showed highest diacetyl production among all strains. This was followed by Strain F-YEpT*GAP1* and then by Strain C-YEpT*GAP1*, the *GAP1* overexpression strains in which the permease levels were substantially increased using a multicopy plasmid. Since Strain C-YEpT*GAP1* was subjected to a stronger regulation in the permease activity than Strain F-YEpT*GAP1*, the diacetyl production of the latter was higher than the former. The next in line was the diacetyl production in the wild-type Strains C and F whose diacetyl productions were close together as was their Gap1p activities. Finally Strain B which measured a very low Gap1p activity also had a very low diacetyl production.

We can therefore conclude that the production of diacetyl in a strain is greatly influenced by its Gap1p activity at the given time point. Altogether the above results help gain insight into the complex regulations involved in *GAP1* and diacetyl levels in yeast.

5.6 The theory behind *GAP1* regulation and its influence on diacetyl production in brewers' yeast

Production of diacetyl is known to be greatly influenced both by valine content in wort and its uptake efficiency by yeast during beer fermentation. The transport of valine into the yeast, along with other branched-chain amino acids like leucine and isoleucine, is mediated by at least four transport systems namely, the general amino acid transporter, Gap1p; the branched-chain amino acid permease, Bap2p; the chief tyrosine transporter, Tat1p; and closest Bap2p homologue amino acid permease, Bap3p (Didion, 1998). In media rich in ammonium ions, most of the branched-chain amino acid uptake is mediated by Bap2p, Tat1p and Bap3p, whereas Gap1p transports amino acids during poor nitrogen conditions.

To understand the role of *GAP1* in diacetyl production, it is necessary to understand the function of the permease both as a transporter and as an amino acid sensor. During alcoholic fermentation, yeasts utilize good nitrogen sources through amino acid permeases controlled by the SPS (Ssy1-Ptr3-Ssy5)-sensing mechanism while the *GAP1* gene under the regulation of NCR-mechanism is repressed. However as the good nitrogen sources in the medium are depleted, *GAP1* is derepressed which in turn causes the repression of SPS- mechanism regulated genes (Crépin, 2012). A study on the amino acid transporters in wine yeast showed that the depletion of ammonium ions in the must (pressed grapes) activated *GAP1* expression even when there was sufficient concentration of yeast assimilable nitrogen compounds left (Beltran, 2004). This shows

that the onset of *GAP1* expression in yeast begins halfway through the alcoholic fermentation.

GAP1 gene repression is considered to be a good NCR marker in yeast through which they utilize good nitrogen sources available in the growth environment (Beltran, 2004). Under conditions of good nitrogen availability, the lower levels of Gap1p in the cell could therefore be an indicator for the upregulation of other specific amino acid permeases (e.g. branched chain amino acid permeases) that are under the control of SPS (Ssy1-Ptr3-Ssy5)-sensing mechanism (Section 1.3). This theory is further confirmed by the study performed during wine fermentation, where the uptake of the branched-chain amino acids was found to be better in the *Δgap1* strain than in the respective wild-type *S. cerevisiae* strain (Chiva R., 2009). The repression of *GAP1* gene by the NCR mechanism seems to modify the gene expression of other amino acid permeases and in particular the branched chain amino acid permeases. Additionally, a study on branched chain amino acid permeases (*BAP2, BAP3* and *GAP1*) in brewing yeasts showed that these permeases with the same or overlapping substrate ranges could compensate for the loss of each other (Didion T., 1998). Together these findings show that the permeases involved in BCAA transport and *GAP1* work in close association with one another.

Looking at the *GAP1* expression profile during wine fermentation (Appendix: Figure D), *GAP1* was repressed in the first hours after the inoculation into the must-like media (Beltran, 2004). However the expression of the Gap1p sets in from 12 to 18 h after inoculation and increases with depletion of nitrogen in the media. In principle, the expression of *GAP1* is low in the beginning of the fermentation (up to 18 h after inoculation), when the pitched in yeast begins to acclimatize to the environment (lag phase). During this stage yeast begins to take up minerals and amino acids (through specific amino acid permeases) from wort. The next is the exponential growth phase (1-4 days) in which cells grow rapidly and consume the available sugars and nitrogen in the wort. It is also during this stage that the *GAP1* expression increases to the maximum indicating that the cells after exhausting the preferred good nitrogen sources in the wort is driven to utilize the available non-preferred nitrogen sources in the medium. The full-fledged activity of Gap1p mediates the signaling to the protein kinase A pathway thereby preparing the cells to enter into the stationary phase. As the yeast reach their stationary phase of growth (3-10 days) whose primary purpose is flavour balancing, further consumption of nitrogen is unrequired and therefore the expression of *GAP1* continuously diminishes.

This *GAP1* transcription profile can be plainly transferred to brewing yeast during beer fermentation. The Gap1p activity measured during the late log/early stationary phase (AE = 6 %) showed that the permease was active in all strains at that time point, although

the extent of their activity varied among strains (Figure 38). When the results from Beltran concerning the *GAP1* expression are extrapolated to beer fermentation, it could be seen that the Gap1p activity starts as the cells enter into their exponential growth phase and continues its expression through the stationary phase until the expression decreases as the cells flocculate and the fermentation is complete.

Since yeasts require sufficient nitrogen for its metabolic activities, its availability is highly monitored throughout the course of fermentation. Upon starvation of amino acids (a major source of nitrogen), various genes involved in the amino acid metabolism are induced by the general amino acid control (GAAC) pathway (Wek, 2004). Since valine biosynthesis in yeast is the principal pathway for diacetyl production, we can have a closer look at the same. Upon insufficient availability of valine, the cells produce intracellular valine by activation of *ILV2* gene (encoding acetolactate synthase) which is under the control of GAAC pathway (Xiao, 1988; Kingsbury, 2004).

On the other hand, the uptake of valine results in feedback inhibition of acetohydroxy acid synthase (AHAS), an enzyme which catalyses the conversion of pyruvate to α-acetolactate (diacetyl precursor). Works have been previously performed in supplementing the wort with branched-chain amino acids to check its influence on the production of vicinal diketones (Krogerus, 2013). According to their results, supplementation of wort with valine not only increased its uptake into the yeast but also resulted in lower levels of diacetyl production. In addition to valine, concentrations of other amino acids in the wort, in particular BCAAs may also have an indirect effect on diacetyl production as they could affect valine uptake into the cell (Barton, 1992). Increased uptake of leucine and isoleucine also resulted in reduced diacetyl production. Leucine in particular had an inhibiting effect on the AHAS mediated conversion of pyruvate to α-acetolactate (Magee, 1968, Barton, 1992). These results clearly show that there is a negative correlation between the uptake rate of branched-chain amino acids and diacetyl production.

In order to have a better uptake of BCAAs into the yeast, highly active branched chain amino acid transporting permeases are required. The transcriptional regulation of these permeases (Bap2p, Bap3p and Tat1p) is complex involving several transcription factors that control their amino acid induced transcription (Nielsen, 2001). As seen previously, one of the important factors that increase BCAA consumption through higher activity of the *BAP* genes is repression of *GAP1* via NCR. Like Ssy1p, Gap1p that also functions as an amino acid sensor could signal the induction of *BAP* transcription. As long as good nitrogen sources are available for the cells to utilize, Gap1p activity is preferentially repressed in order to prevent accumulation of unneeded amino acids which could prove toxic to the cells. The increased *BAP* activity through *GAP1* repression causes improved

uptake of branched-chain amino acids which in turn affects the intracellular valine biosynthesis and thereby reduces diacetyl.

Furthermore, since elevated levels of *GAP1* in the cells indicate insufficient nitrogen availability in the medium, the general amino acid pathway could be activated leading to induction of the valine biosynthesis pathway through activation of *ILV2* by the transcriptional activator Gcn4p (Holmberg, 1988).

So putting all these results together, it is possible to understand how *GAP1*, an amino acid permease and a sensor, plays an important role in influencing diacetyl levels in beer.

5.7 Conclusion and Outlook

Lager brewers' yeast strains that naturally show variation in their *GAP1* transcription levels are good examples for strain improvement via genome translocations. These translocations that shaped its genome could have taken place as a result of stressful environment such as occurring during alcoholic fermentation (Perez-Ortin, 2002). In order for the strains to adapt to various changes and requirements in its environment, deletions of gene or chromosomal region take place.

Results from TDA verification of four brewers' yeast strains using immunoblot and Gap1p activity measurements showed that brewers' yeast with different diacetyl production showed differences in their *GAP1* activity. Furthermore, fermentations carried out with *GAP1* overexpression strains and their wild-type showed that diacetyl production of *GAP1* overexpression strains (*S. cerevisiae* BY4741, Strain C and Strain F) is always higher than that of their respective wild-type strains. Since the extent of influence resulting from Gap1p overexpression on diacetyl production varies among these strains, we can propose that the regulation of Gap1p is strain specific.

Gap1p is a highly regulated amino acid transporter and sensor whose activity levels in yeast produce an inducing effect leading to increased transcription levels of branched chain amino acid permeases. Higher activity of BAP permits increased uptake of BCAAs which in turn results in reduced diacetyl production. Differences seen in the permease activity as a result of changes in its transcription rate could be either the result of variations in their promoter strength (due to point mutations) or due to variations in the gene copy numbers. These differences in *GAP1* expression seen could also be part of the cellular mechanism to reduce increased competition among amino acids for permease interactions, which could affect their uptake into yeast.

Supplementing the wort with BCAA's is said to have a positive effect on its uptake rate as it induces higher levels of BAP activity. However altering the ratio of free amino nitrogen (FAN) content to fermentable sugar in an initial wort composition could affect the flavour profile of the beer (Forster, 2003). Similarly maintaining a *GAP1* repressed condition

throughout the fermentation by use of excess nitrogen could affect the production of various metabolites which gives the beer its characteristic taste and flavour. Furthermore, such a nitrogen repressed condition could have adverse effects on the microbial stability during lagering of beer (Beltran, 2004).

The diacetyl production can indeed be reduced by reducing the activity of Gap1p in a high diacetyl producing strain. This could be achieved by blocking the transcription of *GAP1* using genetic approaches or using classical mutagenesis techniques and screening for variants with reduced *GAP1* activity. The effect of *GAP1* mutation on diacetyl production could be further improved by overexpressing the branched chain amino acid transporters at the genome level or by supplementation of wort with appropriate levels of BCAAs. The transcriptome data analysis of the six brewers' yeast can be further checked for other amino acid transporters or permeases which might, together with *GAP1*, have a combinatory effect in reducing diacetyl production in brewers' yeast.

6. Summary

The thesis aimed at using an inverse metabolic engineering approach to influence diacetyl levels in brewers' yeast strains to reduce time needed for beer maturation. Gene targets for reducing diacetyl production were identified using microarray-based transcriptome analysis of six lager brewing yeast strains with differences in their diacetyl levels. *GAP1* encoding for General Amino acid Permease was selected as a potential candidate in several genes that showed differential expression during beer fermentation process. Gap1p was also selected based on its function as a transporter and receptor (transceptors) for amino acids and its expression is highly regulated depending on the availability of amino acids in the medium.

Based on the TDA results, a hypothesis was proposed that there is a positive correlation between amounts of Gap1p and diacetyl levels. The correlation seen in the Gap1p expression and activity among four lager yeast strains agreed with the transcriptome data results.

For verification of the hypothesis diacetyl production in yeast strains were analysed under varying Gap1p levels. Diacetyl levels were examined in *Δgap1* and in *GAP1* overexpression strains of *S. cerevisiae* (BY4741) and compared to the wild type. Results indicate that increasing Gap1p levels in yeast indeed leads to significant increase in their diacetyl levels. Similarly, *GAP1* overexpression in bottom fermenting yeast strains (Strain C, a medium diacetyl producer and Strain F, a low diacetyl producer) resulted in increased diacetyl levels compared to their respective wild-type strains. The correlation between Gap1p activity and diacetyl production in the respective brewing strains (4 wildtype and 2 transformants) were studied under brewing conditions and the results showed that higher levels of *GAP1* in the strains gave rise to a corresponding increase their diacetyl production in all strains.

Previous studies have shown that the uptake of branched-chain amino acids (BCAAs) like valine, leucine and isoleucine were better under *GAP1* repressed conditions indicating that the branched chain amino acid permeases like Bap2p, Bap3p (transporters of BCAAs) are more active under lower levels of Gap1p. Such a condition enables better uptake of preferred nitrogen sources. Increase in valine uptake results in reduced diacetyl production due to feedback inhibition in the valine biosynthesis pathway. Among several genetic factors that cause variation in the diacetyl production in different brewing yeast strains, the variation in the rate of Gap1p expression is identified.

7. Zusammenfassung

Die vorliegende Arbeit hatte zum Ziel, durch gezielte genetische Modifikationen (metabolic engineering) die Diacetylwerte in Brauhefestämmen zu reduzieren und dadurch die Lagerungsdauer signifikant zu verkürzen. Die Gentargets (GOI) für eine verringerte Diacetylproduktion wurden mittels Microarray-basierter Transkriptomanalyse von sechs unterschiedlichen Brauhefestämmen (Lagerbier) mit jeweils abweichenden Diacetylwerten ermittelt.

Aus den umfangreichen Daten der Transkriptionsanalyse konnte *GAP1* (General Amino acid Permease) aufgrund unterschiedlicher Expressionsmuster während des Brauprozesses als potentieller Kandidat identifiziert werden. Ein weiterer Grund für die Auswahl war die Funktion von Gap1p als Transporter und Rezeptor (Transceptor) für Aminosäuren. Ausgehend von den Transkriptomdaten wurde die Hypothese aufgestellt, dass eine positive Korrelation zwischen der Menge von Gap1p, deren Aktivität sowie der Diacetylkonzentration besteht.

Die Überprüfung der Hypothese der Diacetylproduktion wurde in Hefestämmen mit veränderten Gap1p-Expression analysiert. Hierfür wurden *Δgap1* Deletionsmutanten als auch Mutanten mit einer erhöhten *GAP1* Expression von *S. cerevisiae* (BY4741) erzeugt und mit dem Wildtyp verglichen. Die Ergebnisse zeigen, dass die erhöhte Expression von *GAP1* in Hefen tatsächlich zu signifikant gesteigerten Diacetylkonzentrationen führen.

Darüber hinaus wurden unter Produktionsbedingungen Brauhefestämme (Transformant) mit erhöhter *GAP1* Expression als auch deren Wildtyp untersucht (Stamm C, ein mittlerer Diacetylproduzent und Stamm F, ein niedriger Diacetylproduzent). Die Überexpression von *GAP1* in den Brauhefestämmen C und F führt zum Anstieg der Diacetylproduktion. Weiter konnte in vier Wildtypstämmen (Stamm B, C, D, F) und zwei Transformanten (von Stamm C und F) mit verschiedenen Gap1p-Expressionsraten gezeigt werden, dass eine direkte Korrelation zwischen der Gap1p-Aktivität und der Produktion von Diacetyl existiert.

Die Expression von Gap1p ist stark reduziert in der Anfangsphase des Brauprozesses, wenn hochwertige Stickstoffquellen im Überschuss verfügbar sind. Unter diesen Bedingungen ist die Aktivität von verzweigtkettigen Aminosäurepermeasen (z.B. Bap2p und Bap3p) erhöht. Wenn die hochwertigen Stickstoffverbindungen verbraucht sind, werden die spezifischen Aminosäurepermeasen reprimiert und Gap1p hochreguliert. Die erhöhte Aktivität von BAP resultiert in einer verstärkten Aufnahme von verzweigtkettigen Aminosäuren, was wiederum zu einer verminderten Diacetylproduktion führt. Es exsitieren viele verschiedene genetische Faktoren die Diaycetylproduktion zu beeinflussen. In dieser Arbeit konnte nachgewiesen werden, dass einer dieser genetischen Faktoren *GAP1* ist.

7. References

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Appendix

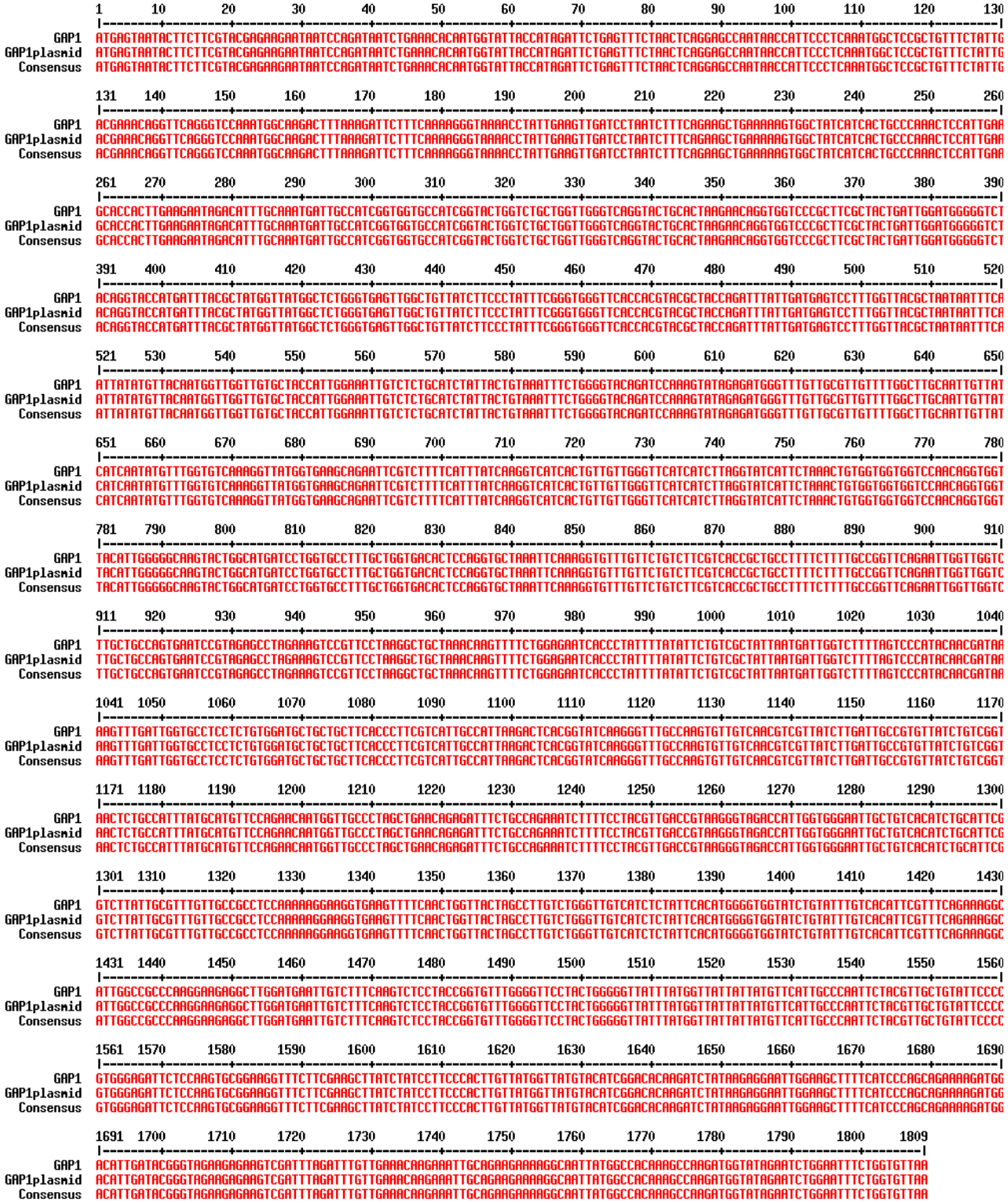

Figure A: Sequence alignment of *GAP1*-CDS with sequence of *GAP1* cloned into p416TEF6*GAP1* plasmid.

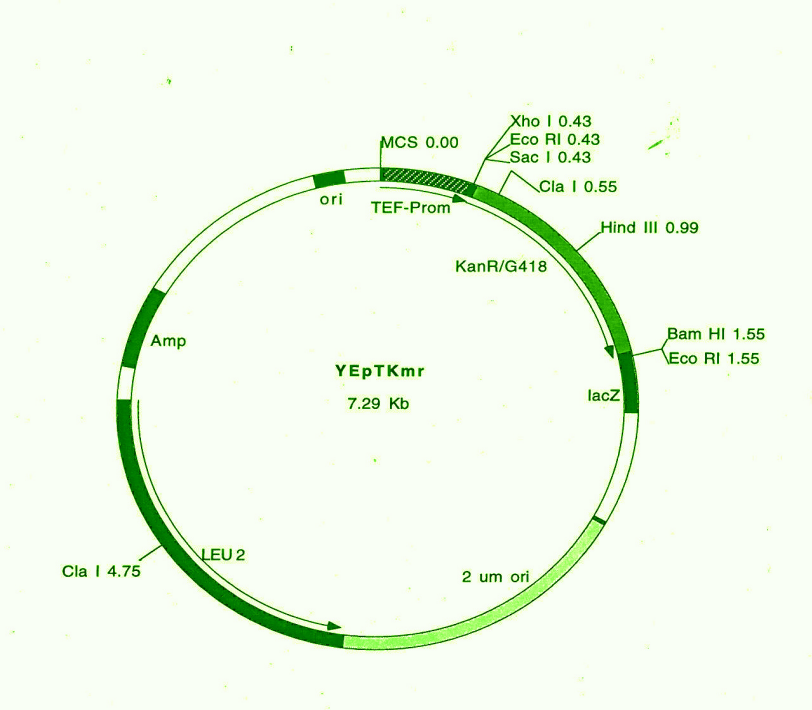

Figure B: Plasmid map of YEpTKmr

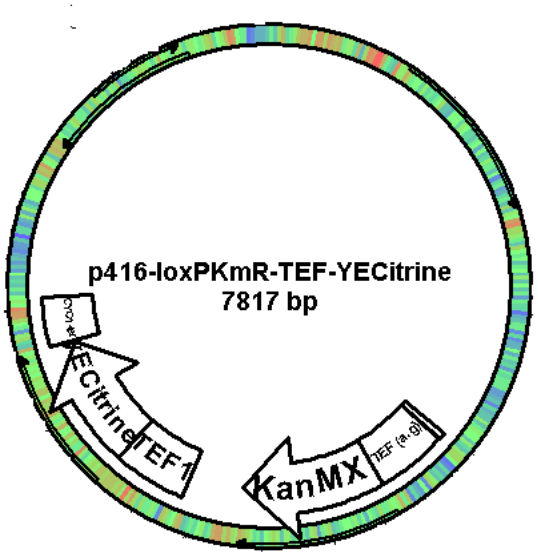

Figure C: Plasmid map of p416TEF6*YECitrine*

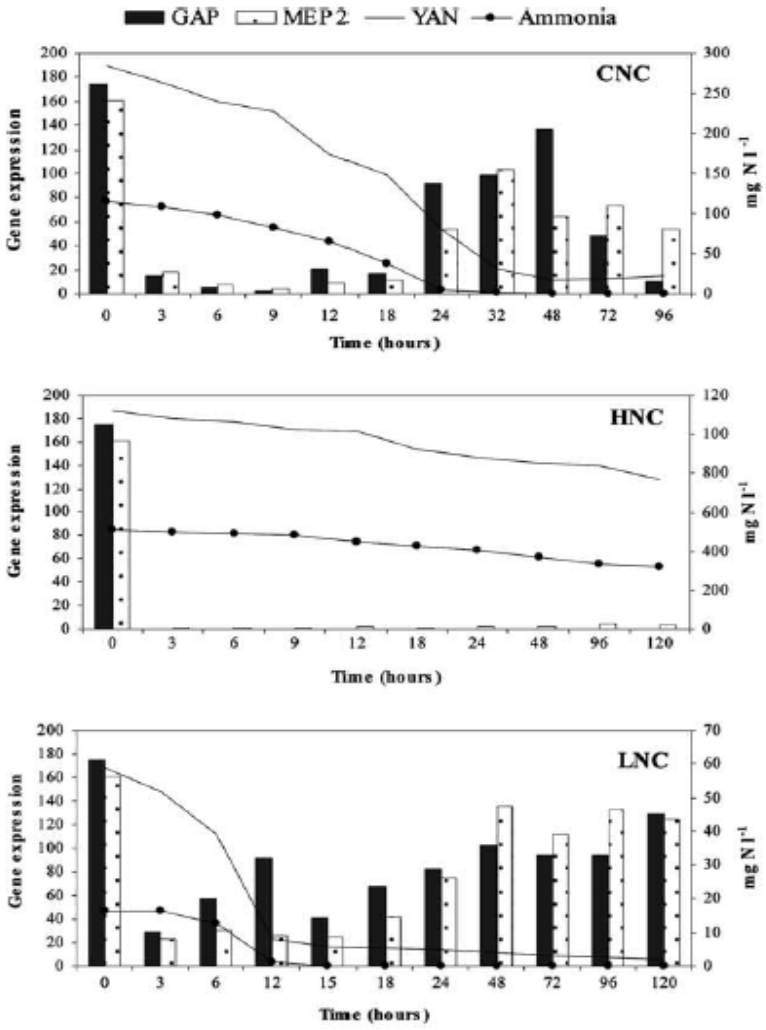

Figure D: *GAP1* expression profile during wine fermentation. Refer Section 5.6.

HNC: High Nitrogen Content Fermentation with yeast-assimilable nitrogen (YAN) content of 1200 mg N L^{-1}, CNC: Control Fermentation with YAN content of 300 mg N L^{-1}, LNC: Low Nitrogen Content Fermentation with YAN content of 60 mg N L^{-1}). Above data represents the relative gene expression of ammonia permease (*MEP2*) and general amino acid permease (*GAP1*) at several fermentation points. Time point zero (before inoculation), Exponential phase up to 24 h and Stationary phase up to 96 h (Beltran, 2004).

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Selbstständigkeitserklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind. Diese Arbeit wurde in der jetzigen oder einer ähnlichen Form keiner anderen Prüfungsbehörde vorgelegt. Ich habe mich weder anderwärts um einen Doktorgrad beworben, noch besitze ich einen entsprechenden Doktorgrad. Die dem Verfahren zugrunde liegende Promotionsordnung ist mir begannt.

Berlin, den 05 Dezember 2013 James, Nisha