An integrative approach to identify novel target genes for reduction of diacetyl production in lager yeast

vorgelegt von
B.Sc-Cam Thuy Duong
aus Hanoi

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Abbreviations

2D	Two-dimensional
AHAS	acetohydroxyacid synthase
BCAA	Branched-chain amino acid
bp	base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CGH	comparative genomic hybridisation
DEPC	Diethyl Pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetic acid
EtBr	Ethidium bromide
FAD	Flavin adenin dinucleotide
Fig.	Figure
G418	Geneticine
GC-ECD	Gas Chromatography - Electron Capture Detector
GCOS	GeneChip Operating System
GMOs	Genetically modified organisms
h	hour
hl	hectorlitre
IAA	Idoacetamid
IVT	In vitro transcription
kb	kilobase
Km^R	Kanamycin resistance
LB	Luria-Bertani
M, mM	Molar, Millimolar
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
MEBAK	Mitteleuropäische Brautechnische Analysenkommission
min	minute
nm	nanometre

nt	nucleotide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl flourid
RNA	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. monacensis	*Saccharomyces monacensis*
S. pastorianus	*Saccharomyces pastorianus*
s	second
SGD	*Saccharomyces* Genome Database
SDS	Sodium dodecyl sulphate
Tab.	Table
TEMED	Tetramethylethylenediamine
ThDP	Thiamin pyrophosphate
Tris	Tris (hydroxymethyl) aminomethane
V	Voltage
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

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I Literature review

Brewers' yeast: Targets and strategies for strain improvement

1 Introduction

1.1 Overview about brewers' yeast: history, taxonomy and genetic features

1.1.1 History

Beer brewing is one of the oldest technologies in the world and its history can be traced for several millenniums. For most of the time, beer brewing was considered as a spontaneous event. It was originally performed based on the experience that cereal grains used for brewing would potentially result in alcohol production when they had been stored under wet condition (Corran, 1975). Although beer brewing has an ancient history, the role of yeast in beer fermentation has been only known from 19th century. In 1680, Antonie Van Leeuwenhook observed "yeast flocs" in fermenting wort through a microscope. Nevertheless, no comment about the role of yeast in fermentation was stated (Briggs *et al*., 2004). The presence of microorganisms in fermentation was only recognised between 1836 to 1838 as the result of independent works of Theodore Schwann, Friedrich Traugott Kuetzig and Charles Cagniard-Latour (Briggs *et al*., 2004). Based on observation of "yeast" cells under miscroscopes, Kuetzig and Cagniard assumed they were living organisms and were necessary in the brewing process. Schwann also observed the growth of yeast cells through a microscope and designated them as 'Zuckerpilz''. The theory of living organism being responsible for the alcoholic fermentation process encountered a strong opposition by some eminent chemists for a long time (Barnett, 2004). Only in 1861s was the importance of yeast in fermentation generally accepted, thank to the work of Louis Pasteur. Another milestone in the history of brewing was the work of Emile Hansen (1883). By developing the technique of generating pure cultures in solid media invented by Robert Koch (1881), Hansen isolated the first pure culture

brewing yeast named “Carlsberg Yeast Number 1”. From that time, the use of pure cultured yeast became popular in beer brewing.

In general, there are two main kinds of beer i.e. ale and lager beer, these are dependent on the yeast and conditions used for fermentation. For ale beer brewing, the fermentation is carried out at room temperature using ale yeast strains (from about 20 to 25^{o}C). The fermentation of lager beer is performed under lower temperatures (8-14^{o}C) using the lager brewers’ yeast strains. After the main fermentation, ale beer production is subjected to a short period of aging whilst the lager beer production has to undergo a long maturation period lasting from one to three weeks at low temperature (around 0^{o}C). Ale beer has a fruity aroma whilst lager beer is paler, drier and usually has a lower alcohol content (Polaina, 2002). At the end of fermentation, ale yeast rises to the top of the fermentation vessels whilst lager yeast settles down to the bottom. They are therefore called top-fermenting and bottom-fermenting yeast, respectively. Whilst ale beer is believed to be produced in 3000 BC in Mesopotamia (Corran, 1975), history of lager beer is much shorter, only being recorded from the 19th century. Bottom-fermenting yeast was secretly used by Bavarian brewers’ until the 1840s when it was illegally transported to Czechoslovakia and Denmark (Boulton and Quain, 2001). The lager yeasts were then spread throughout other parts of Europe and North America. Currently, lager beers are brewed worldwide and comprise of 90% beer production of the world while ale beers are mostly produced on the British Isles (Kodama *et al*., 2006).

1.1.2 Taxonomy

Recent descriptions about brewers’ yeast taxonomy were given by Boulton and Quain (2001) and by Briggs and colleagues (2004). Whilst ale yeast is classified as *Saccharomyces cerevisiae*, lager brewers’ yeast is taxonomically much more complicated and has been renamed several times. Barnett (2004), in his review about yeast taxonomy study pointed out factors for the instability in yeast

nomenclature including: i) criteria used for classification, ii) development of lab techniques, iii) discovery of new kinds of yeast and iv) nomenclature correction of one taxon which is unintentionally named several times. The change of lager yeast's nomenclature is predominantly consequence of the first two factors listed.

The aforementioned first pure brewers' yeast strain in the world, propagated by Emile Hansen, was a lager brewers' yeast strain. In 1908, Hansen named this bottom-fermenting yeast as *S. carlbergensis* in recognition of its difference from ale yeast which has been used in the traditional beer production of Belgium, Germany and Britain. This strain is suggested to be closely related to most current lager brewers' yeast strains (Hansen and Kielland-Brandt, 2003). Lager brewers' yeast was then consolidated in *S. uvarum* since it was recognised to be almost undistinguishable from this kind of wine yeast (Campbell, 2000). Later, based on the criteria of nurtrient consumption, cell morphology and mode of reproduction, Yarrow (1984) assigned lager brewers' yeast assigned to the species *S. cerevisiae*.

From the beginning to Yarrow's classification, taxonomy of brewers' yeast was mostly based on its ability to assimilate certain substrates, its colony and cell morphology, mode of reproduction and based on microscopic experience of scientists. With the development of recombinant DNA technology, from 1985, DNA characteristic criteria have been applied and provided a more precise classification of brewers' yeast. By using DNA re-association, Vaughan-Martini and Kurztman (1985) demonstrated that the DNA of the original *S. carlbergensis* showed high homology to both *S. cerevisiae* (53%) and *S. bayanus* (72%). Since the genomes of *S. bayanus* and *S. cerevisiae* showed little similarity (20%), Vaughan-Martini (1985) suggested that lager brewers' yeast was the hybrid of *S. cerevisiae* and *S. bayanus*. Later, lager brewers' yeast was grouped into *S. pastorianus* based on the fact that they were 93% homologous in genome constitution (Vaughan-Martini and Martini, 1987). Until recently, it has been generally accepted that ale yeast is *S. cerevisiae* and lager yeast is *S. pastorianus*. Compared to Yarrow's classification, this taxonomy seems to

be more “comfort” to brewers in its clear reflection of the physiological differences between ale and lager brewers’ yeast.

1.1.3 Genetic features

According to their genetic constution, ale and lager yeast are different. In addition, ale yeast strains are much more diverse than lager yeast strains. A chromosomal fingerprinting study proved that most lager yeast in the brewing world have one or two basic fingerprints namely “Turborg” or “Carlsberg” while ale yeasts failed to show any common fingerprint (Casey, 1996). Ale yeast strains revealed to be polyploid and closely related to laboratory strains of *S. cerevisiae.* In contrast, lager yeast strains are allopolyploid hybrids of *S. cerevisiae* and another *Saccharomyces* yeast (see I.1.1.2). In comparison to laboratory yeast strains, the amplified fragment length polymorphism (AFLP) pattern of ale yeasts showed 93.7% commonality while it was only about 74.6 % in the case of lager yeasts (Azumi and Goto-Yamamoto, 2001). Two-dimensional gel electrophoresis of the proteomes also showed that ale yeast strains were much closely related to lab yeast S288c than the lager yeast strains (Kobi *et al*., 2004).

Using DNA re-association experiment, Vaughan Martini (1985) was the first author to verify the hybrid nature of lager brewers’ yeast (see I.1.1.2). Since then, the existence of diverged genomes in lager yeast was confirmed in a series of studies using different methods such as Southern analysis of several genes, *kar*-mediated single chromosome transfer and hybridisation of radioactive probes to chromosome-sized DNA separated pulse-field electrophoresis (review by Kodama *et al*., 2006). Several attempts have been aimed in elucidating the origins of lager brewers’ yeast. Even though most studies agree that *S. cerevisiae* is the first parent of lager brewers’ yeast, there were different ideas about its second ancestor. By comparing DNA homology, Vaughan Martini *et al*. (1985) recognized the second parent of lager brewing yeast as a *S. bayanus* type strain (CBS 380). This hypothesis was supported

by the investigation of homology in the the non-*S. cerevisiae* sequences between lager brewers' yeast and *S. bayanus* (Tamai *et al*., 1998; Yamagishi and Ogata, 1999; Casaregola *et al*., 2001; Kodama *et al*., 2001a). In contrast, several studies based on Southern analysis and molecular cloning suggested that an *S. monacensis* type strain (CBS 1503) could be the other contributor of the lager brewing genome (Pedersen, 1986a; Pedersen, 1986b; Hansen and Kielland-Brandt, 1994). However, it was revealed that *S. bayanus* CBS 380 (Hansen and Kielland-Brandt, 1994; Pedersen, 1986a) and *S. monacensis* CBS 1503 (Andersen *et al*., 2000; Casaregola *et al*., 2001) themselves are hybrids containing divergent versions of many genes.

The species *S. bayanus* contains two varieties: *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum*. It was proved that between these two varieties, only *S. bayanus* var. *bayanus* contained strains which contributed to lager brewers' yeast genomes (Naumova *et al*., 2005). This variety contains a collection of hybrid strains which are similar to *S. bayanus* CBS380. Lager brewers' yeast therefore seems to represent one among many hybridisation events occurred between *S. cerevisiae and S. bayanus.* In a laborious work using sequencing and restriction analysis of 48 gene fragments chosen randomly within the *S. bayanus* genome, Rainieri and colleagues (2006) identified the third group of *S. bayanus* which only contains two pure genetic lines: IF0539 and IFO1948 without sequence from *S. cerevisiae*. These two isolates are supposed to represent the pure non-*S. cerevisiae* genomic content of lager brewers' yeast (Kodama *et al*., 2006; Rainieri *et al*., 2006).

1.2 Brewing process and role of yeast in beer production

The aim of the brewing process is the conversion of grain starch and proteins to fermentable sugars and amino acids, subsequently to extract these nutrients with water and to ferment them with yeast to produce beer, an alcoholic, carbonated and aromatic beverage. The brewing process involves five main stages: i) malting,

ii) milling, mashing and wort production, iii) wort boiling, iv) fermentation and v) post fermentation treatment.

In the malting step, barley is germinated by being steeped into water. The germination process is about two weeks long and results in the biosynthesis and activation of amylotic and proteolytic enzymes to convert barley starches and proteins into fermentable sugars and free amino nitrogen, respectively. This germination is stopped by heating. In the second step, the dry malt is milled and mixed with water. The temperature of this mixture is then increased in several steps to provide optimal conditions for the activity of amylotic and proteolytic enzymes which facilitate sugar and protein degradation. After that, sweet wort is produced by separating the aqueous phase from the residual grains. Sweet wort is then boiled with hops to extract aroma and bitter hop compounds. The product is called brewers' (hopped) wort ready for use in fermentation. Following this, freshly propagated yeast is inoculated into wort and the fermentation begins. In this process, yeast utilises fermentable sugars and nutrients in the wort for growth and maintenance and in turn releases ethanol, carbon dioxide and various by-products to form "green beer". The green beer becomes "drinkable beer" after the maturation, filtration and pasteurisation. The maturation course is also called "secondary fermentation" and is needed for the improvement of beer flavour and aroma.

In ale brewing, the fermentation lasts about two or three days at room temperature, whilst lager brewing fermentation takes from 5 to 10 days at lower temperatures of between 8-15^{o}C. At the end of fermentation, yeast is harvested and often used in subsequent fermentations. Depending on the type of fermentation, yeast cells are collected either from the surface (ale yeast) or from the bottom (lager yeast) of the fermentation vessels. During the maturation period, many undesirable organoleptic compounds are reduced to the acceptable levels. Among these undesirable substances, diacetyl is of the most concern to brewers, especially in lager beer brewing. Even present at low concentrations, it has a strong impact by causing a butter-like flavour in beer (Virkajarvi, 2006). Diacetyl may be a part of the

flavour in some ale beers; however, it is an off-flavour in lager beers. Removing diacetyl in lager beer production requires a long maturation process which may last from one to three weeks.

Brewers' yeast only participates in two phases, i.e the fermentation and maturation of beer brewing process. The role of yeast in the fermentation, however, is active and decisive by the fact that most of beer components such as alcohol, carbon dioxide and flavours compounds are brewing yeast's metabolites and this metabolite pattern depends strongly on brewing yeast genotypes. Improvement of brewers' yeast strain has therefore received a great attention in the optimisation of the fermentation process. In the following part, I will give an overview of targets and strategies for the genetic improvement of brewers' yeast strain. The "omics" studies of brewers' yeast are also mentioned. The application of "omics" technologies in brewers' yeast studies has led to an increased knowledge about cellular activities of brewers' yeast during the main fermentation. The lager brewers' yeast genome sequence is emphasized in its perspective in global studies of brewers' yeast. The accessibility of brewing yeast genome database can provide useful tools for global studies; thus endowing an insight into the nature of brewers' yeast. The enlargement of knowledge about yeast nature will be a valuable basis for the strain improvement of brewers' yeast.

2 Targets and strategies for optimisation of brewers' yeast strains

2.1 Improvement of carbohydrate consumption

2.1.1 Dextrin

During the malting and mashing process, barley starch is degraded to simple sugars which are fermentable by brewers' yeast. These fermentable sugars are comprised of 75% of wort carbohydrates, including glucose, fructose, maltose, galactose and maltotriose. Another product of barley starch degradation is polysaccharides (dextrins) of varying length. Dextrins constitute at least 20% of brewers' wort carbohydrates; however, they are not utilisable by brewers' yeast. The creation of brewers' yeast capable of fermenting dextrin has become a target of industrial brewing in the production of low calorie beer and the production of higher alcoholic amounts from the same amount of malt (Campbell, 2000).

Dextrins are mixtures of D-glucose polymers which have linear glycosidic α-1,4 and branched glycosidic α-1,6 linkages. Among *Saccharomyces* yeasts, *S. diastaticus* is known to have the capability of hydrolysing and fermenting dextrin by producing extracellular glucoamylases. These enzymes are encoded by three unlinked polymeric genes i.e. *STA1, STA2* and *STA3.* Several attemptes were made to confer this feature of *S. diastaticus* to brewers' yeast. The first approach was the hybridisation of brewers' yeast strain with the wild yeast *S. diastaticus*. The resulting progeny was able to utilise dextrin, however, it also governs other genetic make-up from *S. diastaticus*, notably the inheritance of the *POF1* gene. The presence of this gene in the hybrid genome confers the ability of ferulic acid decarboxylation, resulting in a phenolic off-flavour in beer (Hansen and Kielland-Brandt, 1997).

Other attempts to improve dextrin utilisation in brewers' yeast involved the direct transfer of either *S. diastaticus STA1* or *STA2* gene of to brewers' yeast using plasmid expression or integration approaches (Meaden and Tubb, 1985; Perry and Meaden, 1988; Sakai *et al*., 1989; Vakeria and Hinchliffe, 1989; Park *et al*., 1990). These studies more or less gained certain success especially those which involved

the integrated approaches and thus conferred a better genetic stability to the transformants. Nonetheless, a drawback of glucoamylases derived from *S. diastaticus* is the lack of α-1,6 debranching activity leading to a high amount of unfermented dextrin. To gain higher dextrin-fermenting efficiency, genes endowing both α-1,6 and α-1,4 glucoamylase activity from other fungi were introduced into brewers' yeast (Yocum, 1986a; Cole *et al*., 1988; Gopal and Hammond, 1992). Integration of the glucoseamylase gene from *Aspergillus niger* to brewers' yeast genome was highly successful. This was demonstrated by the fact that 50% of wort dextrin was utilised resulting in a 20% increase in ethanol concentration (Gopal and Hammond, 1992). This enzyme from *Aspergillus niger*, however, is heat stable and therefore not being denatured after pasteurisation of beer. The presence of active glucoamylase made the beer became sweet during the storage. To overcome this problem, the *GAM1* gene from *Swanniomyces occidentalis* was introduced into brewers' yeast (Lancashire *et al*., 1989). The resulting glucoamylase is both heat-labil and possesses debranching activity and the transformant can ferment dextrin efficiently.

2.1.2 Maltose and maltotriose

Maltose is the most abundant sugar in brewing wort accounting for ca. 60% of total fermentable sugars (Vidgren *et al*., 2005). The fermentation of maltose only starts when 50% of wort glucose is consumed (Stewart *et al*., 1983). This phenomenon results from the glucose repression of genes which are responsible for the uptake and hydrolysis of these sugars in the cell. The improvement of maltose utilisation of brewers' yeast is important in brewing fermentation, especially in the high gravity fermentation in which glucose is present at high amounts and in accelerating the rate of fermentation.

Maltose assimilation in yeast requires the presence of at least one among five unlinked *MAL* loci namely *MAL1-4* and *MAL6*. Each *MAL* locus consists of three

genes *MALxT*, *MALxR* and *MALxS* (with x referring to the number of the locus) encoding for a maltose permease, a positive regulator and a maltase, respectively. These three genes are repressed by Mig1p in the presence of glucose (Hu *et al*., 1995). In addition, *MALT* and *MALS* expression is induced by maltose. The repression involves *MIG1* while induction involves *MALR* (Klein *et al*., 1996). Apart from transcriptional regulation, maltose assimilation also involves post-transcriptional regulation and post-translational control in which the presence of glucose leads to the increase in the lability of the *MALS* transcript and to the inactivation of the maltose permease (Go¨rts, 1969; Siro and Lo¨vgren, 1979; Federoff *et al*., 1983; Peinado and Loureiro-Dias, 1986; Hu *et al*., 1995; Lucero *et al*., 1993). Besides maltose permease, maltose is also being taken up via *AGT1*-encoded transporter which is a broad specificity α-glucosidase transporter (Han *et al*., 1995). *AGT1* is allelic but is only 57% identical to *MAL1T*.

To improve maltose fermentation efficiency, Kodama and colleagues (1994) overexpressed *MAL* genes by using a constitutive promoter which is not repressed by glucose in one brewers' yeast strain. In high gravity fermentation, the constitutive expression of *MALT* gene was effective to improve maltose fermentation efficiency, whilst the expression of *MALS* or *MALR* had no impact on maltose consumption. Another attempt to accelerate maltose fermentation in yeast was based on the removal of the repression factor which regulated the transcription of *MAL* genes (Klein *et al*., 1996). Disruption of the *MIG1* gene resulted in a decrease in maltose repression only in a haploid laboratory strain while it led to a stricter glucose control on maltose metabolism in an industrial yeast strain. That effect on the industrial strain is supposed to be caused by the increased glucose control on the maltose permease resulting in the alteration in the uptake of maltose (Klein *et al*., 1998).

Some recent studies have focused on the clarification of *MALT* gene combination and on functionality of maltose transporters in brewers' yeast. In an examination involving 25 lager and 5 ale yeast strains, Jesperson and colleagues (1999), by using hybridisation genes probes to seperate chromosomes, showed that different brewers'

yeast strains had diverse combinations of *MAL* genes. In fact, all 30 studied brewers' strains contained *MAL1T, MAL3T* and *AGT1* and only one of those strains lacked the *MAL4* gene*. MAL2T* was not detected in 12 lager yeast strains, nor in any of the tested ale strains. *MAL6T* was not found in any of all 30 tested brewers' yeast strains*.* Through the use chromosome blot and hybridisation, another study on different maltose transporters showed that maltose was mostly taken up via the *MALxT* transporters in lager strains while in ale strains it was predominantly carried out by *AGT1*-encoded transporter (Vidgren *et al*., 2005). This study also indicated that some apparent multiple maltose transporter genes in several brewers' yeast strains did not encode functional transporters.

Besides maltose, the fermentation of maltotriose is also of concern in brewing fermentation. Maltotriose is the second most abundant fermentable sugar in brewing wort (comprising 15-20 %); however, it is least preferred to be taken up by yeast cells compared to glucose and maltose (Sergio L. Alves *et al*., 2008). The consumption of maltotriose in ale yeast is significantly slower than in lager yeast and is therefore more problematic in the ale brewing fermentation. Hydrolysis of maltose and maltotriose requires the same maltase, however, it was unclear whether there exists a specific transporter for maltotriose or if maltotriose is co-transported with maltose via maltose transporters (Salema-Oom *et al*., 2005). Recently, a novel gene *MTY1* encoding an α-glucoside transporter was identified in lager brewers' yeast (Salema-Oom *et al*., 2005). This new gene is 90% and 45% identical to *MAL3T* and *AGT1* genes respectively. Overexpression of *MTY1* conferred the capability of fermenting maltose and maltotriose in an *S. cerevisiae* Mal^- strain. Interestingly, the Mytp is distinct from other α-glucoside transporters as it has higher affinity for maltotriose than maltose.

2.2 Improvement of by-product profile

2.2.1 Reduction of diacetyl production

Vicinal diketones (diacetyl and 2,3-pentanedione) impart undesirable butter-like flavour to beer. Among these two substances, diacetyl is of more concern to brewers since it has a much lower taste threshold than 2,3-pentanedione. Diacetyl is a by-product of the valine biosynthetic pathway which is formed from the non-enzymatic oxidative decarboxylation of α-acetolactate. The latter compound leaks out from the cells during the main fermentation (Fig. 1). Diacetyl was then reabsorbed into yeast cells and there it was reduced to acetoin and subsequently to 2,3-butanediol, a compound which has much higher taste threshold in beer. Diacetyl is reduced to acceptable levels during the maturation. The main purpose of the maturation process in lager beer brewing is indeed the diacetyl removal and the completion of this process may last from one to three weeks. Prevention of diacetyl production would therefore help to shorten the maturation process thus accelerating lager beer production.

In general, diacetyl production can be reduced by different strategies: i) elimination of diacetyl formation from its precursor α-acletolactate, ii) reduction of α-acetolactate production and iii) increase of the conversion of α-acetolactate towards the valine biosynthetic pathway.

In the first approach, to prevent the formation of diacetyl from its precursor α-acetolactate, heterogeneous α-acetolactate decarboxylase was either introduced into green beer or expressed in brewers' yeast. This enzyme catalyzes the direct conversion of α-acetolactate to acetoin, thereby eliminating diacetyl formation (Fig. 1). The addition of α-acetolactate decarboxylase isolated from *Enterobacter aerogenes* to green beer led to a decrease in vicinal diaketones levels under the taste-threshold after 24 h at 10^{o}C (Godtfredsen *et al*., 1987). The use of acetolactate decarboxylases in brewing was approved in 2001 by US Food and Drug Administration in USA (Hannemann, 2002); however, the addition of this enzyme is

incompatible with the German beer purity law (Donalies *et al*., 2008). Alternatively, *ALDC* genes encoding α-acetolactate decarboxylase from different bacteria i.e. *Enterobacter aerogenes*, *Klebsiella terrigena*, *Lactococcus lactis* and *Acetobacter aceti* were expressed in yeast using either episomal plasmids, genomic or rDNA integration (Sone *et al*., 1987; Goelling and Stahl, 1988; Sone *et al*., 1988; Fujii *et al*., 1990; Blomqvist *et al*., 1991; Yamano *et al*., 1994). By using this strategy, diacetyl formation was reduced efficiently and in some cases, the maturation period could be ignored. The *ALDC* genes from *Lactococcus lactis* and *Acetobacter aceti* are considered to be more acceptable for food application since these organisms have been already used in food production (Hammond, 1995). In the current opinion, the "self-cloned" yeast strains which do not contain any additional heterogeneous DNA are assumed to be more accepted in food and beverage approval (Akada, 2002).

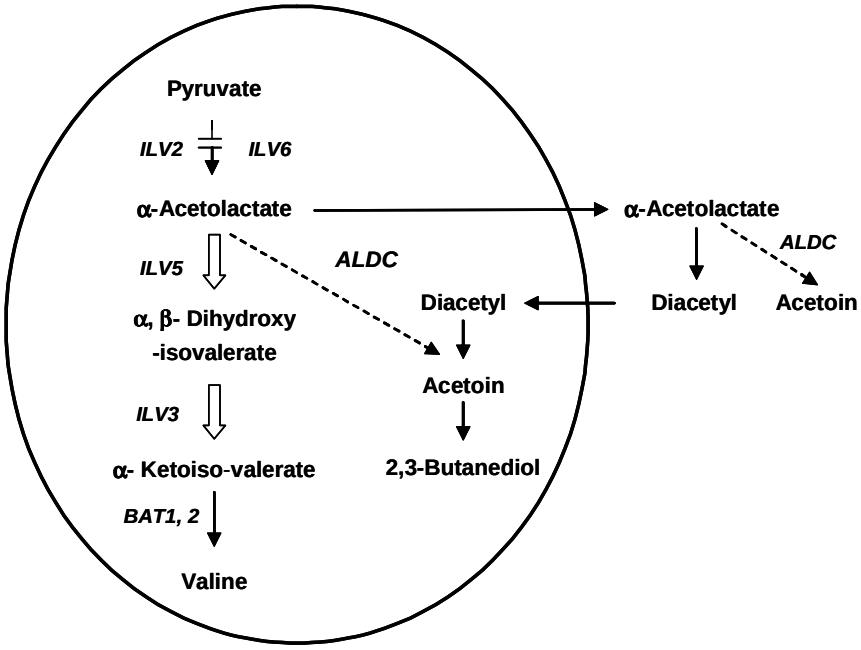

Fig. 1 Previous modifications of the valine biosynthetic pathway to reduce diacetyl production in brewers' yeast. The big and disrupted arrows indicate overexpression and prevention of corresponding enzyme activity respectively. The dash arrow indicates the introduction of heterogenouos enzyme. *ILV2, ILV6*: acetohydroxyacid synthase, *ILV5*: reductoisomerase, *ILV3*: dihydroxyacid dehydratase; *BAT, BAT2*: branched-chain amino acid transaminase.

The second genetic strategy to reduce diacetyl has been based on blocking the formation of its precursor α-acetolactate. As acetohydroxyacid synthase (also called acetolactate synthase) is the enzyme responsible for the formation of α-acetolactate, there have been different attempts in eliminating the activity of this enzyme. Gjermansen *et al*. (1988) completely remove acetolactate synthase activity in one lager brewers' yeast strain by introduction of *in vitro* constructed *ilv2* deletion. The resulting deletion strain no longer produced α-acetolactate but encountered nutrient deficiency since valine uptake from extracellular medium was not sufficient for growth (Kiellandt-Brandt *et al*., 1995). It was discovered that sulfometuron methyl (SM) is an inhibitor of acetolactate synthase and mutation of the *ILV2* gene leads to the insensitivity of this enzyme to SM (Falco and Dumas, 1985). Based on this fact, a partial block of acetolactate synthase was obtained. In this approach, at first spontaneous primary allodiploid mutants which were resistant to SM and prototrophic for valine and isoleucine were isolated (Kiellandt-Brandt *et al*., 1989). These mutants were then treated with UV radiation and the secondary mutants reversely sensitive for SM were screened. In *S. cerevisiae*, this procedure would result in the strain carrying two copies of wildtype *ILV2* gene dues to the high frequency of mitotic recombination. In brewers' yeast, since these mutants were alloploid, the frequency of mitotic recombination was low. Thus, these secondary mutants were expected to carry one copy of wild-type *ILV2* gene while the SM resistant gene was inactivated. Secondary mutants were selected by screening for slow growing colonies on medium lacking of valine and isoleucine. Mating of these secondary mutants resulted in allotetraploid brewing yeast strains which had a lower diacetyl production and acceptable brewing characteristics (Kiellandt-Brandt *et al*., 1995). Another method to reduce acetolactate synthase activity involved the exploitation of *ILV2* anti-sense mRNA (Vakeria *et al*., 1991). In this case, lower diacetyl production was obtained but the resulting transformant was not able to ferment wort well. Recently, some authors combined the disruption of the *ILV2* gene with integration of either *AMY, LSD1, FLONS* genes into the *ILV2* locus (Liu *et al*., 2004; Wang *et al*., 2008; Zhang *et al*.,

2008). Apart from producing less diacetyl, the resulting brewing yeast mutants conferred other beneficial brewing phenotypes: capability to utilise starch (*AMY*), capability to utilise dextran T-70 (*LSD1*) and controllable NewFlo flocculation property (*FLONS*), respectively.

Diacetyl reduction can also be achieved by increasing the flux towards the formation of valine. To this end, overexpression of *ILV3* encoding dihydroxyacid reductase and *ILV5* encoding reductoisomerase was performed (Goossens *et al*., 1987; Villanueba *et al*., 1990; Goossens *et al*., 1993; Mithieux and Weiss, 1995). Both approacheas led to the enhancement in activity of their corresponding enzymes, however, only an increase in activity of *ILV5* encoding enzyme resulted in a reduction of diacetyl level. A decrease in vicinal diketone concentration of up to 60% was obtained in brewers' yeast strains with overexpression of *ILV5* encoding enzyme (Villanueba *et al*., 1990; Goossens *et al*., 1993; Mithieux and Weiss, 1995).

It is reported that Ilv5p has a high turnover in mitochondria. As Ilv5p is responsible for the valine biosynthesis and mitochondrial DNA maintenance, the overexpression of Ilv5p might therefore cause abnormal situation relating to stoichiometry of mitochondrial DNA and nucleoid which is might be undesirable from a view point of the quality of brewers' yeast strain (MacAlpine *et al*., 2000). Besides that, manipulation of a certain metabolic pathway can result in unwanted change in the organoleptic properties of beer. To avoid this problem, Omura (2008) aimed to overexpress a functional cytosolic Ilv5p enzyme. For this purpose, Ilv5p mutants with different N-terminal truncations were generated. Among those, the mutant which had 46 residues deleted (Ilv5p-Δ46), was found to stably function solely in the cytosol but was not present in the mitochondria. Overexpression of the Ilv5p-Δ46 in a lager yeast strain resulted in the same reduction of VDK production as the overexpression of wild-type Ilv5p using a constitutive promoter. Moreover, cytosolic Ilv5p-Δ46 overexpression did not alter the production of aromatic compounds and organic acids important for organoleptic properties of beer. In contrast, there existed an alteration of production of some organic acids (pyruvate, acetate), fusel alcohols (amyl

alcohols, isobutyl alcohol) and acetate ester (isoamyl acetate) in the case of wild-type Ilv5p overexpression.

2.2.2 Increased production of acetate esters

Besides ethanol and carbon dioxide, during the fermentation, brewers' yeast produces other organoleptic compounds which define beer flavour and aroma. The largest group of those compounds is fusel alcohols and their acetate esters. They are intermediates of branched-chain amino acid pathways i.e. valine, isoleucine and leucine. Among those, isoamyl acetate is the most important component which imparts distinct banana and pear flavour to beer. It is a by-product of leucine biosynthesis and is formed from the esterification of acetyl-coA and isoamyl alcohol by catalysis of the *ATF1* and *ATF2* encoded alcohol acetyltransferases. Isoamyl alcohol is formed from a-ketoisocaproate in two enzymatic steps. Alpha-ketoisocaproate is the intermediate of the leucine biosynthetic pathway which is formed from a-ketoisovalerate in three enzymatic steps. The enzyme responsible for the first of the three steps is α-isopropylmalate synthase which catalyses the conversion of a-ketoisovalerate to α-isopropylmalate. Thus, one strategy to increase isoamyl acetate production was based on the alteration of the activity of α-isopropylmalate synthase. This enzyme is encoded by the *LEU4* gene and is feedback-inhibited by leucine. Overexpression of the *LEU4* gene in a sake *S. cerevisiae* yeast resulted in a slight increase in the amount of isoamyl alcohol and its acetate esters (Hirata *et al*., 1992). In addition, it was revealed that the *LEU4* encoding enzyme was strikingly insensitive to leucine inhibition in the mutant which is resistant to one toxic analogue of leucine (Santyanarayana *et al*., 1968). This strategy was applied to achieve a bottom fermenting strain which produced a higher amount of isoamyl alcohol and its corresponding acetate ester (Lee *et al*., 1995). In addition, α-ketoisocaproate is formed by the degradation of leucine via Ehrlich pathway. Thus, the increase in leucin uptake could result in an increase in α-ketoisocaproate level and isoamyl acetate levels. It was shown that the constitutive

expression of *BAP2*, a gene encoding branched-chain amino acid permease in brewers' yeast showed an increase in α-ketoisocaproate and in isoamyl alcohol levels (Kodama *et al*., 2001b).

Overexpression of either *ATF1* or *ATF2* genes in brewers' yeast resulted in a great enhancement of isoamyl acetate production (Fujii *et al*., 1994; Nagasawa *et al*., 1998; Verstrepen *et al*., 2003a; Verstrepen *et al*., 2003b). In addition, increases of other acetate esters such as ethyl acetate, isobutyl acetate, pentyl acetate, hexyl acetate and octyl acetate were also obsevered. As lager brewers' yeast is the hybrid of *S. cerevisiae* and another non-*Saccharomyces* yeast, it contains two diverged versions of many genes in its genome, namely *S. cerevisiae* type gene (Sc-type) and non-*Saccharomyces* type gene (non-Sc-type). It has been demonstrated that overexpression of different alleles of *ATF* genes e.g. *Sc-ATF1, Sc-ATF2,* non-Sc-*ATF1* led to different impacts on rate of ester production (Fujii *et al*., 1994; Verstrepen *et al*., 2003b). Based on this knowledge, Verstrepen (2003b) suggested that different aroma patterns produced by different brewers' yeast strains might result from various mutations of their *ATF* genes.

2.2.3 Increase of sulphite production

Sulphite plays an important role in beer flavour stabilization. As an antioxidant, sulphite prevents oxidative reactions that may occur during post-fermentation processes, thereby helping to increase beer's shelf life. In addition, it stabilizes beer flavour by trapping undesirable carbonyl compounds. These complexes of carbonyl-sulphite have much higher taste threshold compared to free carbonyls. Sulphite is an intermediate of the reductive sulphate assimilation which is significant for the biosynthesis of the sulfur-containing amino acids i.e. methionine and cystein (Fig. 2). Sulphite, however, is usually produced in yeast at low levels. For the improvement of beer flavour stability, several efforts have concentrated on increasing sulphite production in brewers' yeast.

One approach was based on prevention of sulphite ($S0_2$) reduction to hydrogen sulphide (H_2S). In yeast, sulphite is reduced to sulphide by the activity of sulphite reductase. This enzyme is a heterogeneous tetramer which is composed of two subunits α and β, encoded by the genes *MET10* and *MET5* respectively (Fig. 2). Hansen and Kielland-Brandt (1996a) eliminated sulphite reductase activity in a lager brewers' yeast strain by disrupting all *MET10* alleles. The resulting mutant showed a striking enhancement of sulphite production. Moreover, this study also succeeded in eliminating hydrogen sulphide, an unwanted by-product causing a rotten-egg flavour to beer.

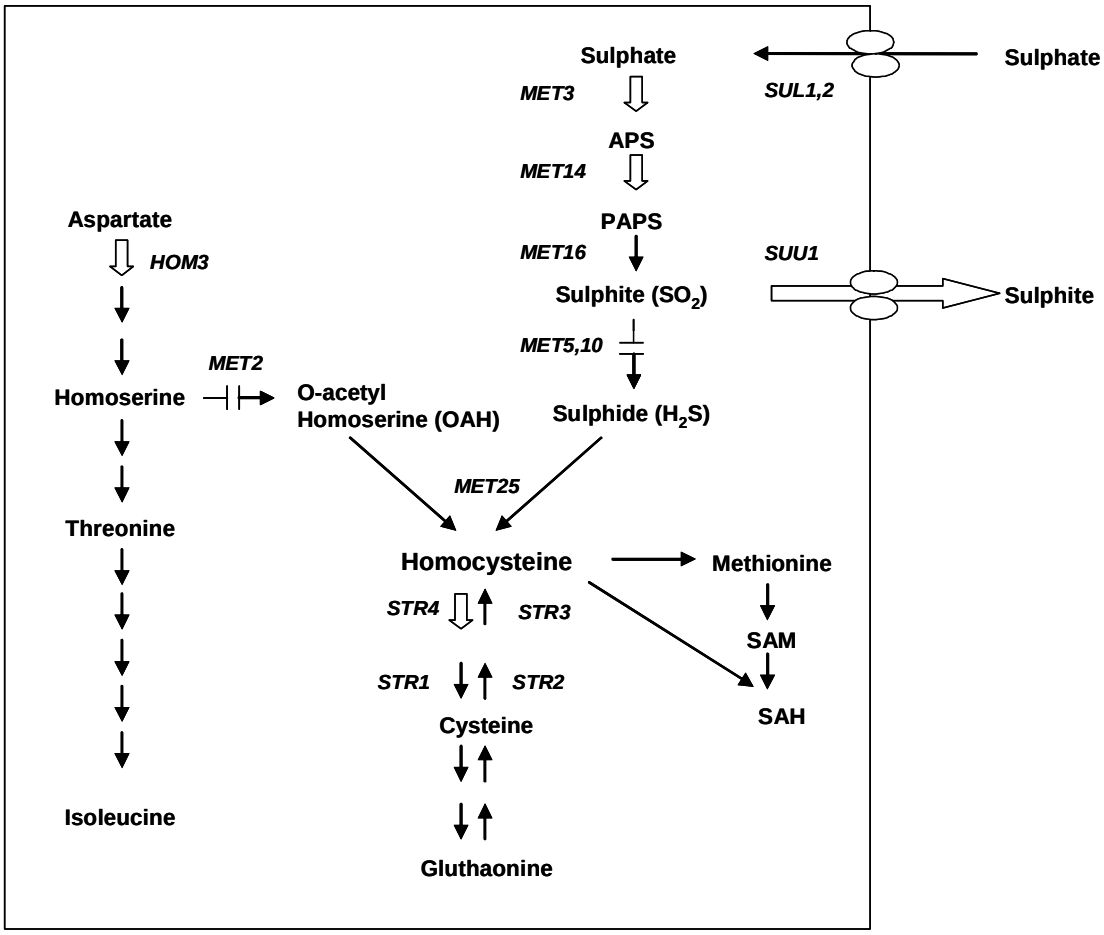

Fig. 2 Modifications of sulphate assimilation and sulphur-containing amino acid biosynthetic pathway to control sulphite and sulphide production. APS: adenosyl phosphosulphate, PAPS: phosphoadenosyl phosphosulphate. SAM: S-adenosyl homomethionine, SAH: S-adenosyl homocystein. Big arrows indicate overexpression of correspondent enzymes. Interrupted arrows signify decrease of enzyme activity.

Another approach to increase sulphite production in brewers' yeast was based on enhancing the flux towards from sulphate to sulphite (Fig. 2). To this end, *MET3 and MET14* encoding ATP sufurylase and APS kinase respectively were overexpressed in brewers' yeast from multi-copy plasmids. It was reported that overexpression of *MET14* had the highest impact on sulphite production and it even led to an increase in sulphite production in a met5 mutant (Korch *et al*., 1991). Expression of *MET14* under the control of the strong promoter *TIP1* in one sulphite reductase deficient *S. cerevisiae* strain also resulted in an increase in sulphite production. Moreover, Donalies *et al.* increased the production of sulphite 10-fold in a *S. cerevisiae* strain by combining the enhancement *MET14* encoded enzyme activity with the overexpression of *SSU1,* the gene encoding a sulphite efflux pump (Donalies and Stahl, 2002).

Repression of the transcription of *MET* genes is mediated by cysteine. *MET2* encodes L-homoserine-O-acetyltransferase which catalyzes the conversion of homoserine to O-acetyl homoserine (OAH) (Fig. 2). Disruption of *MET2* in brewers' yeast led to the shortage of OAH and consequently to the prevention of cysteine formation. In this way, genes participating in sulphate assimilation were depressed, leading to an increase in sulphite production. An enhancement in hydrogen sulphide production, however, was also observed (Hansen and Kielland-Brandt, 1996b).

It has been reported that sometimes a brewers' yeast strain with low sulphite production is desirable for beer brewing (Hansen and Kielland-Brandt, 2003). It derives from the fact that due to being early accumulated; sulphite will form complexes with carbonyl compounds and therefore prevent them from being reduced to their corresponding alcohols. Consequently, the flavour will be negatively affected (Dufour, 1991). By inactivating 4 copies of *MET14* gene in one brewers' yeast strain, Johanesen *et al.* proved that sulphite production during main fermentation resulted in an increase of acetaldehyde in beer (Johannesen *et al*., 1999). A brewers' yeast strain with a late formation of sulphite is therefore necessary for beer brewing in preventing the accumulation of acetaldehyde. This demand can be afforded by the

utilisation of *HSP26* and *HPS30* promoters which allowed gene expression at the end of the exponential phase or stationary phase, respectively. It was shown that overexpression of the *MET14* gene under control of *HPS26* promoter led to a delayed increase in sulphite production (Donalies and Stahl, 2002).

Compared to baker's yeast, lager brewers' yeast produces higher amounts of sulphite (SO_2) and hydrogen sulphide (H_2S). In a recent study, Yoshida *et al*. (2008), via using integrated metabolome and transcriptome analyses, found out the genetic basis for these differences. The higher amounts of SO_2 and H_2S produced by lager brewers' yeast than baker's yeast were due to the limiting amount of OAH. The study also revealed that the flux from aspartate to OAH had a greater effect on production of H_2S than sulphite (SO_2) (Fig. 2). In contrast, the flux from sulphate to SO_2 had a greater effect on SO_2 production than H_2S production. With the aim of increasing sulphite production, Yoshida created a prototype brewers' yeast strain by simultaneously increasing the flux from aspartate to OAH (Sc-*HOM3* overexpression) and the flux from sulphate to sulphite (*MET14* overexpression). The resulting mutant showed a higher level of sulphite and lower level of hydrogen sulphide than the parental strain.

2.2.4 Elimination of sulphide compounds

Hydrogen sulphide is an unwanted by-product causing a rotten-egg flavour to beer. It is generated via sulphate assimilation or degradation of sulphur containing amino acids when nitrogen is depleted. As its taste threshold flavour is low, small amounts of hydrogen sulphide cause an organoleptic problem in beer. Different strategies to reduce hydrogen sulphide formation in brewers' yeast were developed. As previously mentioned, the deletion of all *MET10* genes in brewers' yeast led to the inactivation of sulphite reductase. This resulted in a strong abolishment of H_2S formation and an accumulation of sulphite in the mutant strain (see I.2.2.3) (Hansen and Kielland-Brandt, 1996a). Moreover, hydrogen sulphide can also be reduced by

the overexpression of both *Sc-HOM3* and *MET14* (see I.2.2.3) (Yoshida *et al*., 2008). Besides the reduction in H_2S production, the resulting mutant also showed a higher sulphite production than the wild-type strain.

Other approaches to eliminate H_2S involved the orientation of H_2S into the flux of sulphur containing amino acid biosynthesis. The expression of *MET25* under the control of a constitutive promoter in brewers' yeast gave rise to a several fold enhancement of homocystein synthase (Met25p) activity. In the pilot-scale fermentation, the resulting mutant showed approximately 10-fold decrease in sulphide production (Omura and Shibano, 1995). In another study, overexpression of *STR4* encoding cysthaonine β-synthase in bottom fermenting yeasts resulted in the suppression of sulphide formation (Tezuka *et al*., 1992). The suppression of H_2S production was partly due to an increased requirement for homocysteine when *STR4* was overexpressed. In addition, it was also explained by the authors that *STR4* overexpression led to an increased amount of intracellular cysteine, thus causing to an increased repression of sulphur assimilation genes, comprising those responsible for H_2S formation (Hansen and Kielland-Brandt, 1994). Moreover, hydrogen sulphide can also be reduced by the overexpression of both *Sc-HOM3* and *MET14* as indicated in section I.2.2.3 (Yoshida *et al*., 2008). Besides the reduction in H_2S, the resulting mutant also showed a higher sulphite production than the wildtype strain.

Dimethyl sulphide (DMS) is also a compound affecting organoleptic beer characteristics, especially in the case of lager beer. The presence of DMS causes an unwanted corn-like smell and flavour in beer. It is formed both in the wort boiling stage by thermal degradation of S-methyl methionine and during fermentation by reduction of dimethyl sulfoxide (DMSO). Hansen *et al.* (1999) revealed that disruption of *MXR1*, a gene encoding methionine sulfoxide reductase led to the incapability of DMSO reduction in a laboratory yeast strain. Based on that fact, a brewers' yeast strain producing lower level of DMS was obtained by disrupting the *MXR1* gene (Hansen *et al*., 2002).

2.3 Alteration of flocculation behaviour

Yeast flocculation is a common phenomenon in beer brewing which involves the spontaneous asexual aggregation of yeast cells into flocs and their subsequent removal from fermentation medium by sedimentation (lager yeast) or floating to the surface (ale yeast) of the fermentation tank. Flocculation is beneficial to beer brewing since it provides an easy and effective way to separate yeast cells from green beer. Yeast strains with good flocculation behaviour are of great concern to the brewers. Besides being strongly aggregated, an ideal yeast strain must flocculate at the proper time of fermentation (Dequin, 2001). An early flocculation leads to unfinished fermentation and subsequently to abnormal flavour and aroma of beer. On the other hand, late flocculation results in cloudy beer due to incomplete separation of yeast cells. In general, yeast strain with strong flocculation behaviour towards the end of the main fermentation is necessary for the production of aromatic sufficient and apparent beer.

Even though the exact mechanism of flocculation is still unclear, it is generally accepted that flocculation results from the interaction between lectin-like proteins (flocculins) of a cell with the sugar residues of adjacent cells (Stratford, 1989; Stratford, 1992). Flocculation is inhibited by the presence of free saccharide molecules in the medium, seemingly because these free sugars competitively interact with flocculins, thereby preventing them from interacting with the sugar residuals on cell surface. Depending on the types of sugar affecting inhibition, different flocculation phenotypes have been described i.e.: i) the Flo1 phenotype where flocculation is inhibited by mannose but not by other sugars like glucose, maltose, sucrose, galactose (Stratford and Assinder, 1991), ii) the NewFlo phenotype in which flocculation is inhibited by mannose and other sugars like glucose or maltose (Stratford and Assinder, 1991) and iii) the third flocculation phenotype which is not inhibited by mannose (Masy *et al*., 1992; Dengis and Rouxhet, 1997).

It is also reported that flocculation is controlled by genetic factors. Flocculins are encoded by flocculation genes. Sequence analysis revealed that flocculation genes

belonged to a multi-gene family which localized to the telomeric regions (Teunissen and Steensma, 1995). Among those, two dominant flocculation genes conferring the Flo1 phenotype in yeast are *FLO1* and *FLO5* (Johnston and Reader, 1982; Johnston and H. P, 1983). Besides that, the flocculation gene family includes the genes *FLO9* and *FLO10* which are 94% and 58% homologous to *FLO1*, respectively (Teunissen and Steensma, 1995; Sieiro *et al*., 1997). In lager brewers' yeast, another homologue of *FLO1* gene named Lg-*FLO1* was identified. This gene is not present in ale yeast strains and is responsible for the NewFlo phenotype of most lager brewers' yeast strains (Kobayashi *et al*., 1995; Kobayashi *et al*., 1998; Sato *et al*., 2002). In addition to these structural genes, flocculation gene family also includes *FLO8*, which encodes a transcription activator required for regulation of flocculation as well as other phenotypes such as diploid filamentous growth, and haploid invasive growth (Liu *et al*., 1996; Kobayashi *et al*., 1996; Kobayashi *et al*., 1999; Pan and Heitman, 1999). Another dominant flocculation gene is *FLO11* which encodes a Flo1-type flocculin (Lo and Dranginis, 1996; Bayly *et al*., 2005). *FLO11* is distinct from other flocculation genes in that it locates near the centromere instead of the telomere (Lo and Dranginis, 1996). Besides being regulated by Flo8p, the expression of *FLO11* is also controlled by other factors such as mating type.

Several attempts have been made in altering the flocculation behaviour of brewers' yeast. The protoplast fusion of one non-flocculent brewers' yeast strain with a flocculent *S. cerevisiae* strain resulted in the formation of a flocculent yeast strain which could be used for beer brewing (Urano *et al*., 1993). Other efforts to control the flocculation in brewers' yeast involved the manipulation of flocculation genes. The constitutive expression of the *FLO1* gene using the *ADH1* promoter resulted in a strong flocculation phenotype in one non-flocculent brewers' yeast strain (Watari *et al*., 1994). Nevertheless, this strain was not suitable for beer brewing since the onset of flocculation occurred too early, thus leading to the incomplete fermentation. Identification of promoters which can precisely control the gene expression under

industrial fermentation conditions is therefore necessary for the improvement of the flocculation behaviour in yeast.

The promoter *HSP30* is induced during the entry of yeast cells into stationary phase of the fermentation (Riou *et al*., 1997). In the *S. cerevisiae* FY32 strain, a mutation of *FLO8* gene led to the inactivation of transcription of the *FLO* genes and thus to the non-flocculent phenotype in this strain (Winston *et al*., 1995; Liu *et al*., 1996; Verstrepen *et al*., 2005). For the alteration of flocculation phenotype in this strain, the wild-type *FLO1* promoter was replaced by *HSP30* promoter (Verstrepen *et al*., 2001). The resulting transformant showed a strong flocculation toward the end of the laboratory fermentation. Besides that, *ADH2* promoter, which is repressed during the growth on glucose (Price *et al*., 1990; Gancedo, 1998) and is derepressed with transition on the growth on ethanol (Noronha *et al*., 1998), is a possible system to alter the flocculation in yeast. Govender *et al.* (2008) placed the dominant flocculation genes *FLO1, FLO5* and *FLO11* of the *S. cerevisiae* FY32 strain under the transcriptional control of either *ADH2* or *HSP30* promoters. It was shown that the six gene-promoter combinations resulted in specific flocculation phenotypes in terms of timing and intensity. The results suggested that the flocculation behaviour of brewers' yeast could be improved by fine-tuning the expression of dominant flocculation genes.

3 “Omics” technologies in studies regarding brewers’ yeast

The progress in DNA recombinant technology has enabled the improvement of yeast strains via metabolic engineering. In metabolic engineering, one crucial factor for the determination of targets and approaches for strain improvement is the understanding of how a phenotype is determined by a genotype (Attfield and Bell, 2003). This knowledge has been accumulated mostly via reductionistic approaches in which the linkage between the genotype and the phenotype has been identified via the modification of a particular gene (Bro and Nielsen, 2004). However, the fact that a phenotype can be defined by multiple genes or the modification of a single gene may lead to pleiotrophic effects can make it difficult to discover this genotype-phenotype relationship.

The accessibility of complete genome sequences of several organisms has facilitated the development of the so-called “omics” technologies mainly genomics, transcriptomic and proteomics. The development of “omics” technologies in turn has allowed the studies of cellular activities on a global scale and thus has provided an insight into the cellular responses to genetic alterations or environmental changes. The availability of the complete genome sequence of *S. cerevisiae* has laid the foundation for the utilisation of “omics” technologies in yeast studies i.e. the construction of DNA microarrays for global genome and transcriptome analyses and the protein database for proteome studies and thus has led to an accumulation of the huge knowledge about cellular activities. The accumulation of the knowledge is highly advantegous for strain improvement.

As previously mentioned, lager brewers’ yeast strains are aneuploid hybrids between *S. cerevisiae* and another *Saccharomyces* yeast. As the genome sequence of brewers’ yeast has not been publicly accessible, the application of “omics” technologies in the brewers’ yeast studies has been performed mostly by exploiting the current knowledge of *S. cerevisiae* genome sequence. In this section, I review

some recent applications of genomics, transcriptomics and proteomics technologies in studies regarding brewers' yeast.

3.1 Genomics

Microarray-based comparative genomic hybridisation (CGH) is a useful tool for global scale genome analysis. Microarray-based CGH has been used successfully in detecting gene deletions, quantification of gene copy numbers and giving information on the chromosomal aneuploidies as well as the translocations at genomic scale (Winzeler *et al*., 1999; Daran-Lapujade *et al*., 2003). Identification of genomic differences between strains showing different degrees of a certain phenotype can directly reveal relevant target genes for strain improvement as long as they can linke to this phenotype. In brewers' yeast, DNA microarray-based hybridisation was used to study the complexity of lager brewers' yeast genome. By hybridising total genomic DNA of two lager brewers' yeast strains to the *S. cerevsiae* array, Bond *et al.* (2004) detected conserved and discrete translocation regions in the genomes of the studied lager brewers' yeast strains. In addition, large regions of *S. cerevisiae* genome were found to be absent in lager brewers' yeast. The study provided more evidence about the aneuploid nature of lager brewers' yeast as well as the diversity of genome composition between different lager brewers' yeast strains.

Pope *et al*. (2007) used different genomic fingerprinting approaches to discriminate different lager, ale and *S. cerevisiae* strains. Among the genomic fingerprinting methods, amplified fragment length polymorphism providing a snapshot of DNA sequences across the whole genome resulted in relatively good discrimination of the studied strains. In contrast, the array-based GCH by means of *S. cerevisiae* microarray failed in providing meaningful differentiation of studied strains. The unsatifying result obtained by using microarray-based CGH had supposedly been caused by the lack of the non-*S. cerevisiae* component in the analysis.

Recently, a two-species microarray has been developed based on the genome sequence of the strain *S. cerevisiae* S.288C and contig sequences of one *S. bayanus* var. *uvarum* strain (CBS 7001 type strain) (Dunn and Sherlock, 2008). The analysis of 17 different lager brewers' yeast strains using this two-species microarray revealed the presence of two genomically distinct groups of lager brewers' yeast strains which correlated to specific breweries and geographical regions. The first identified group lack a significant portion of the *S. cerevisiae* genome but retains all of the S*. bayanus* genome. The second group retains nearly all of the genomic content of the both genomes. The 1st group represents Saarz type beer and Carlsberg brewery strains while the 2nd group contains strains from the Netherlands, non-Carlsberg Danish breweries and two North American breweries. The analysis also revealed some consistent break points or regions of amplifications or deletions in the studied strains and thus presumably included genes of selective importance in brewing conditions

3.2 Transcriptomics

DNA microarray is also a powerful tool for the global-scale transcriptome analysis. Microarray-based transcriptome analyis enables the examination of abundance of all transcripts in the cell at a given state or condition and thus allows the identification of genes which are co-regulated as well as the analysis of global responses to genomic mutations. Furthermore, by comparing the transcriptional profiles of one strain in different conditions or between various strains showing different phenotypes, genetic basis relevant to these differences can be revealed (Pandey *et al*., 2007). Based on that, target genes for strain improvement can be identified.

DNA microaray has been applied to study transcriptional profiles of brewers' yeast during fermentation. So far, these studies have been performed by the means of the *S. cerevisiae* array. Olesen *et al*. (2002) studied the dynamics of one lager brewers' yeast transcriptome at different points of time during a pilot-scale brewery

fermentation. The analysis revealed that the average gene expression increased rapidly and reached a maximum value after two days of the main fermentation. The average expression was then declined as sugar was consumed. Those genes with high average expression value were mostly genes involved in protein synthesis, glycolysis and lipid synthesis while a large number of genes with unknown biological functions showed a low average expression. Another study using the *S. cerevisiae* microarray to study the transcriptomes of two lager brewers' yeast strains at different points of time during a small-scale brewery fermentation revealed a high level of expression of ORFs involved in fatty acid and ergosterol biosynthesis early in fermentation (James *et al*., 2003). Genes involved in respiration and mitochondrial protein synthesis also showed a high level of expression early in the fermentation. Furthermore, a near complete repression of many stress response genes and gene involved in protein biosynthesis was observed at the end of fermentation compared to that at the start of fermentation.

3.3 Proteomics

Like transcriptomics, proteomics reveals the global response of gene expression to environmental and genetic changes. Nevertheless, compared to transcriptome analysis, proteomics brings us one level closer to the phenotype (Bro and Nielsen, 2004) by disclosing the questions related to gene functions such as the mRNA translation efficiency, protein translation modification or protein stability. The standard method for quantitative protein analysis involves the protein separation by two-dimensional gel electrophoresis (2D gel electrophoresis) and mass spectrometry (MS) or tandem MS (MS/MS) identification of protein spots (Gygi *et al*., 2000). Despite its potential in study of gene function, the disadvantage in proteome analysis is caused by the standard method used for proteomic study. 2D gel electrophoresis is considered more laborious, less sensitive and less reproducible than DNA microarray, the common method used in global-scale transcriptome study. However,

the optimisation of the 2D gel electrophoresis and the development of new methods for global quantification of proteins based on mass spectrometry will bring about more perspectives for global-scale proteome studies (Aebersold and Mann, 2003).

Proteome analysis using 2D gel electrophoresis has been used to define the relatedness between different kinds of brewers' yeast as well as between brewers' yeast and other *Saccharomyces* yeasts (Joubert *et al*., 2000; Kobi *et al*., 2004). The first proteome maps of lager brewers' yeast and ale brewers' yeast were respectively presented by Joubert *et al*. in 2000 and Kobi et al. in 2004. Comparison of the proteomes of one ale yeast strain, one lager yeast strain and the *S. cerevisiae* strain S288C confirmed that the ale yeast strain is much more closely related to *S. cerevisiae* than the lager yeast strain at proteomic level (Kobi *et al*., 2004). In agreement with the hypothesis that lager brewers' yeast is a hybrid of at least two different *Saccharomyces* yeasts, the proteome of lager brewers' yeast appeared to be the superimposition of two elementary patterns, one corresponded to *S. cerevisiae* proteins and the other was best represented by one *S. pastorianus* strain (Joubert *et al*., 2000).

In addition, through the use of 2D gel electrophoresis and differential gel exposure in which the proteomes of *S. cerevisiae* strain S288C and one lager brewers' yeast strain were labelled with different isotopes and then being separated in one 2D gel, Joubert *et al.* (2000) discovered that a large percentage of *S. cerevisiae* proteins (83%) was co-migrated with lager brewers' yeast proteins. In contrast, the co-migration of proteins of either *S. bayanus* or *S. uvarum* type strains with this lager yeast strain was markedly lower, only 35% and 37%, respectively. Furthermore, the proteome analysis using 2D gel electrophoresis, MS, MS/MS and *S. cerevisiae* database searching allowed the identification of many novel non-*S. cerevisiae* proteins of lager brewers' yeast (Joubert *et al*., 2001). These newly identified proteins of lager brewers' yeast corresponded to the protein spots that did not co-migrate with known proteins of *S. cerevisiae* separated on the 2D gels.

In addition to the study of strain relatedness, proteomics was applied in studying brewers' yeast gene expression at different stages of fermentation and as well as at different generations of successive fermentations (Kobi *et al*., 2004). The proteome of one ale yeast strain during production-scale fermentation was studied at the beginning and at the end of the first and the third usage of the yeast (the 1st and 3rd generation of successive fermentations). It was shown that the most pronounced changes in protein expression occurred in the 1st generation, during the switch from aerobic propagation to anaerobic fermentation. Even though yeast propagation was performed in saccharose medium before inoculated in brewing wort, no drastic protein change directly related to the change in sugar source (from saccharose medium to wort) was observed. The variation in protein expression in the 3rd generation was much lower in comparison to the 1st generation. Unsurprisingly, no difference in protein expression related to the switch from aerobic to anaerobic condition in the 3rd generation was observed. However, certain stress response proteins i.e. Hsp26p, Ssa4p, Pnc1p were induced during first generation and constitutively expressed in the subsequent generations. These are stress-response proteins induced by variety of treatments. The induction of these stress-response proteins during the first fermentation suggested that the switch from oxidative to fermentative condition was an environmental stress to yeast cells (Kobi *et al*., 2004). In addition, the authors explained that the constituve expression of these stress response proteins in subsequent fermentations was probably important for the maintance of viability of the yeast cells which encountered stressful fermentative conditions.

There has not yet been any study regarding the dynamics of lager brewers' yeast proteome during fermentation. However, proteome analysis was used to identified the proteins which are induced during the lag and early exponential phase (early-induced proteins) in glucose-containing medium (Brejning *et al*., 2005). After 5 h of inoculation, several proteins were identified as early-induced including Ade17p, Eno2p, Ilv5gp, Sam1p, Rsp21 and Ssa2p. The induction of most of these proteins did

not match the transcriptional expression of genes in the glucose-containing medium. Nevertheless, under brewing conditions, the transcriptional expression of these genes, except for *ENO2* and *SSA2*, were strongly induced early in the lag phase (Brejning *et al*., 2005). The monitor of these early-induced genes and proteins could be useful in creating physiological markers for optimisation and control of growth initiation during brewing fermentation.

4 Lager brewers' yeast genome sequence and its perspective in brewers' yeast global studies

The application of "omics" technologies based on current knowledge of *S. cerevisie* genome sequence in brewers' yeast studies has certain limitations, especially regarding the lager brewers' yeast studies. As aforementioned, the microarray-based global genome analysis failed to discriminate different lager brewers' yeast strains due to the lack of non-*S. cerevisiae* sequences in the microarray (Pope *et al*., 2007). The usage of the two-species array composed of *S. cerevisiae* and *S. bayanus* var. *uvarum* sequences in global genome analysis provided a better opportunity to precisely differentiate lager brewers' yeast strains. However, it was estimated that the *S. bayanus* sequence which contributed to the lager brewers' yeast genome was about 10% divergent to the sequence of the *S. bayanus* var. *uvarum* strain (Dunn and Sherlock, 2008), thus the exploitation of this two-species microarray could not fully evaluate the genotype of lager brewers' yeast. In addition, the transcriptome analysis of lager brewers' yeast using *S. cerevisiae* arrays only revealed the expression pattern of half of the genome while the other half is uncovered. The proteome studies have also been obstructed due to the lack of the non-*S. cerevisiae* sequences. Non-*S. cerevisiae* proteins cannot be unambiguously identified by using the common method of peptide fingerpringting (MALDI-TOF MS) and database searching with *S. cerevisiae* sequence. The identification of non-*S. cerevisiae* proteins thus requires more time-consuming

methods such as tandem MS or nano-electrospray tandem MS/MS (Joubert *et al*., 2001; Brejning *et al*., 2005) and has to be based on sequence homologies.

Recently, the genome of one lager brewers' yeast strain i.e WH 34/70 was sequenced (Kodama *et al*., 2006). The total size of the lager brewers' yeast genome was 23.2 million bp, approximately twice the size of the *S. cerevisiae* laboratory yeast genome. The contig sequences of the lager brewers' yeast genome are divided in two groups: i) Sc-type with more than 98% to homomology to *S. cerevisiae* sequences and ii) non-Sc-type with identity around 85% identical to *S. cerevisiae* sequences. The sequencing project also confirmed the hybrid nature of lager brewers' yeast with the presence of three kinds of chromosomes: Sc-type, non-Sc-type and various chimeral types.

Although the sequence of the lager brewers' yeast genome has not been publicly available, production of bottom fermenting yeast microarray based on this sequence has been announced (Nakao *et al*., 2008). In general, the bottom fermenting yeast DNA microarray contains 22,977 probesets representing 22,483 regions from the whole genome sequence information of the lager brewing yeast strain 34/70, 403 *S. cerevisiae* ORFs which are not identified in the WH 34/70 strain, 64 control genes and 27 *S. pastorianus* ORFs submitted in Genbank (http://www.ncbi.nlm.nih.gov/Genbank/). The 22,483 regions from strain WH 34/70 composed of 7640 Sc-type ORFs, 6307 non-Sc-type ORFs, 28 mitochondrial ORFs, 7955 intergenic regions and 553 other sequences which showed a similarity to *S. cerevisiae* proteins by NCBIblastX homology searching (Nakao *et al*., 2008). The availability of the bottom fermenting yeast DNA microarray will allow more reliable global transcriptome and genome analyses of lager brewers' yeast strains while the accessibility of the bottom fermenting yeast DNA sequence will bring about more convenience for proteomic studies. These advancements will give rise to an outburst knowlege about brewers' yeast physiology in the near future and is thus highly advantageous for the improvement of brewers' yeast strain.

5 Conclusions

The progress of genetic engineering has led to the creation of numerous novel brewers' yeast strains highly beneficial for brewing industry. Many of these strains were constructed according to requirements for commercial production, e.g the generation of "self-cloned" strains which contains no heterologous gene and no addition of selectable marker. However, the use of recombinant strains in beer production has not been worldwide approved. So far, there is only one brewers' yeast strain received official approval for commercial use from British government. Even so, it has not yet been used commercially. The limited public acceptance for genetically modified organisms (GMOs) is clearly the obstacle for the development of novel brewers' yeast strains. This phenomenon is derived from the concern of consumers about the danger of genetically modified (GM) foods and beverages. In the future, a better communication between scientists and consumers as well as between scientists and legislators should be established to improve the community's knowledge about the low risk and high benefit that certain GMOs can bring about. This will be the important requirement for application of biotechnology in food and beverage industry in general and in brewing industry in particular.

The application of global molecular methods i.e. genomic, transcriptomic and proteomics has already enabled the accumulation of some knowledge about cellular activities of brewers' yeast during fermentation. So far, these global analyses have been mostly performed based on current knowledge about *S. cerevisiae* genome sequence and thus have certain limitations in studying lager brewers' yeast, the hybrid between *S. cerevisiae* and another *Saccharomyces* yeast. Nevertheless, recent achievements regarding the complete sequence of bottom fermenting yeast sequence and the bottom fermenting yeast DNA microarray will strongly facilitate the enlargement of the basic knowledge and the improvement of brewers' yeast strain.

II Experimental part

An integrative approach to identify novel target genes for reduction of diacetyl production in lager yeast

1 Introduction

1.1 The need to optimise brewers' yeast

Although beer brewing is a well-established traditional process, the current brewers' yeast strains are far from optimised for beer production as stated by Hammond (1995). For example, beer has to undergo a secondary fermentation process which lasts about two weeks to remove diacetyl, an off-flavour in beer. As this process requires a lot of time and capacity, it would be a great benefit to have a yeast strain with a low diacetyl production. For beer brewing, an "ideal" yeast strain should comprise a number of good features including the ability to consume a wide range of substrates, the fast fermentation of wort sugars under low temperature, a good flocculation towards the end of main fermentation, a balanced pattern of by-products and a low level of diacetyl production. There are brewers' strains with ideal features; however, these features are not included in one single strain. Therefore, a complete understanding of the relationship between phenotype and genotype is needed to allow the transfer of a good trait from one strain to another strain.

1.2 Former attempts to improve brewers' yeast

Due to the demand for brewers' yeast strains with improved properties, there has been much research focusing on engineering brewers' yeast. As lager beer constitutes 90% of world beer production (Kodama *et al*., 2006), the object for most of this research has been the bottom fermenting yeast. In general, genetic

improvement of brewers' yeast strains has been achieved through traditional genetic manipulations (breeding, traditional mutagenesis and cytocyduction) and rational metabolic engineering. Application of these approaches in brewers' yeast improvement has advantages and drawbacks which will be discussed as follows.

1.2.1 Classical genetic manipulations

The first approach for genetic improvement of yeast strains was mating (also denoted as breeding or cross hybridisation) of parents owning favourable traits and selection for progenies with combined desirable phenotypes. The approach produces high genetic diversity and can be used to combine optimal genotypes and traits in one strain (Attfield and Bell, 2003). Moreover, it does not relate to the matter of "Genetically modified organism" (GMO). In general, this approach is applicable to any yeast strain which can produce a number of viable spores (Attfield and Bell, 2003).

Among industrial yeasts, lager brewers' yeast is striking in its genetic constitution of being an aneuploid hybrid between *S. cerevisiae* and another *Saccharomyces* yeast, probably *S. bayanus*. Besides this, lager brewers' yeast sporulates poorly and even if it sporulates, a low number of spores can survive (Hammond, 1995). Due to this limitation, early studies encountered problem when breeding brewers' yeasts (Andersen *et al*., 2000). Nevertheless, matable spores from brewers' yeast were isolated and some novel brewers' yeast strains with optimised features were created by cross hybridisation. Gjemansen isolated segregants of one lager brewers' yeast strain and obtained viable spores of the two mating types (Gjermansen and Sigsgaard, 1981). Pairwise cross was performed between spores of opposite mating types resulting in a number of hybrids. Investigation of these hybrids under brewery conditions revealed one strain as good as the parental strain. Besides that, the hybridisation of maters from brewers' yeast with maters from other yeasts such as *S. cerevisiae* led to the formation brewers' yeast strains which harboured good traits from the non-brewing parent (Bilinski *et al*., 1987; Bilinski and Casey, 1989).

Still, breeding of brewers' yeast, in particular lager brewers' yeast is troublesome since it is quite laborious and the results cannot be anticipated.

Classical genetic approaches also involve mutagenesis by inducing UV radiation or alkylating reagents. The approach itself is indirect and the frequency of obtaining a desirable phenotype is very low. In addition, it always has a high frequency of generating harmful gene alterations. As it was previously mentioned, bottom fermenting yeast strains are polyploid or aneuploid. Therefore, if the mutation was not dominant, it must occur in all alleles of a gene for the alteration of the phenotype (Attfield and Bell, 2003). Thus, even though this method is possible for creation of desirable haploid laboratory yeast strains, it is almost not accessible for polyploid organisms such as brewers' yeast.

Another strategy of traditional genetic manipulations of brewers' yeast is protoplast fusion. This approach allows the hybridisation of individuals without considering the mating types (Hansen and Kielland-Brandt, 2003). Early studies using this method led to the creation of hybrid brewers' yeast strains producing off-flavours in beer (Attfield and Bell, 2003). Nonetheless, protoplast fusion was successfully used for improving the flocculation behaviour of one brewers' yeast strain. The fusion of protoplast of one flocculent *S. cerevisiae* strain with a non flocculent brewers' yeast led to the formation of a flocculent yeast strain which could be used for beer brewing (Urano *et al*., 1993). However, this approach is unpredictable since the characteristics of the two parents are not averaged. The protoplast fusion tends to lead to chromosomal dominance of one nucleus from one parent while the other nucleus is the subordinate which containes the loss of most nuclear genome (Attfield and Bell, 2003). The desirable trait from parents thus can be absent in the offspring.

To sum up, application of traditional genetic approaches has gained several successes for strain improvement of brewers' yeast. However, they are not direct and the probability of having the correct combination of desirable traits is low (Attfield and Bell, 2003).

1.2.2 Rational metabolic engineering

The development of recombinant DNA technology has allowed the improvement of yeast strains by rational metabolic engineering. The approach was defined as "*the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions with the use of recombinant DNA technology*" (Bailey, 1991). In general, rational metabolic engineering consists of two important parts: i) identification of target genes for genetic manipulation and ii) genetic engineering of the cell for construction of recombinant strain (Ostergaard *et al*., 2000). The rational metabolic engineering is distinguished from other classical genetic approaches as it allows the direct transfer of genetic information via genetic manipulation of the target genes.

In rational metabolic engineering, the identification of target genes is based on knowledge about enzymes, pathways and regulatory factors relevant to the phenotype. From that, a rate-controlling step is identified or a model system is constructed. The problem is then solved by genetic manipulation of the identified target genes. Application of this approach has led to a number of successes in strain improvement of *Saccharomyces* yeast in general and brewers' yeast in particular.

Rational metabolic engineering was effectively employed to improve numerous phenotypes of brewers' yeast. Concretely, it has been used in the alteration of by-product formation i.e. the prevention of unwanted substances diacetyl (Sone *et al*., 1987; Villanueba *et al*., 1990) and sulphide compounds (Omura and Shibano, 1995) as well as the enhancement of production of desirable by-products like acetate esters (Lee *et al*., 1995) and sulphite (Korch *et al*., 1991; Hansen and Kielland-Brandt, 1996a). In addition, rational metabolic engineering has been used to improve brewers' yeast utilisation of carbohydrates such as dextrin (Yocum, 1986b; Park *et al*., 1990) and maltose (Kodama *et al*., 1994). Optimisation of flocculation phenotype in brewers' yeast was also achieved by rational metabolic engineering (Watari *et al*., 1991). Thus, the application of rational metabolic genetic engineering has actually covered most targets for brewers' yeast strain improvement.

Even though numerous achievements have been made via employing rational metabolic engineering, many studies failed or were not as successful as expected. In some cases, the genetically modified strains governed other unfavourable phenotypes in addition to the desirable traits. For example, the reduction of diacetyl formation in brewers' yeast by the complete elimination of acetolactate synthase activity led to the inability of the mutant strain to produce valine. This led to a nutrient deficiency in the mutant strain since valine uptake from the extracellular medium was not sufficient for cellular activities (Gjermansen *et al*., 1988; Kiellandt-Brandt *et al*., 1995). The reason for these failures is likely due to an incomplete understanding of the complex global metabolic network and its response to genetic alterations (Bailey *et al*., 2002; Nevoigt, 2008).

Identification of the target for rational metabolic engineering is based on the available knowledge regarding the relationship between phenotype and genotype. Therefore, the more knowledge about the targets and the relevant factors for genetic manipulation is accumulated, the more successful is the approach altogether. Regarding this aspect, rational metabolic engineering is less accessible to brewers' yeast compared to laboratory yeast. As the genome sequence of lager brewers' yeast has not yet been published, the knowledge used for genetic engineering of brewers' yeast so far has been mostly based on studies of *S. cerevisiae*. However, the genome of lager brewers' yeast is strikingly different from the laboratory yeast. As previously mentioned, the genome of lager brewers' yeast is comprised of sequences from both *S. cerevisiae* and another *Saccharomyces* yeast, probably *S. bayanus* (Vaughan-Martini and Kurztman, 1985; Naumova *et al*., 2005; Kodama *et al*., 2006; Rainieri *et al*., 2006). In fact, there have been several examples that genetic engineering of certain target genes led to different results in laboratory and brewers' yeast backgrounds (Klein *et al*., 1996). Rational metabolic engineering to improve brewers' yeast is therefore limited since the effects of gene homologs from the non *S. cerevisiae* origin on the phenotype of lager brewers' yeast cannot be correctly predicted.

1.3 Inverse metabolic engineering as an alternative approach for improving brewers' yeast

To overcome the limitations of rational metabolic engineering, inverse metabolic engineering can be used an alternative for the improvement of brewers' yeast. The concept of inverse metabolic engineering was codified by Bailey *et al*. in 2002 and was described as "*The elucidation of a metabolic engineering strategy by first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or particular environmental process factors conferring the phenotypes; and third, endowing that phenotype on another strains or organism by directed engineering environmental manipulation*".

The selection of the desired phenotype is the first step of inverse metabolic engineering. This desired phenotype can arise naturally or can be obtained via appropriate evolutionary engineering (Sauer and Schlattner, 2004). The second step is the identification of the target genes for genetic modification. It has been carried out by analysing the molecular basis for the differences between the strains with desirable phenotypes and the host/production strain, i.e the strain to be modified. This identification of target genes is considered the most challenging step in inverse metabolic engineering. However, the availability of methods for genome-wide and global functional analyses has enabled the screening of differences at various molecular levels (i.e. genome, transcriptome, proteome, metabolome) and thus has facilitated the identification of crucial genetic information relevant for a certain phenotypic trait (Nevoigt, 2008). In the last step, the desirable trait is conferred to the host strain by genetic engineering.

In contrast to rational metabolic engineering which tries to genetically engineer the cellular activities based on available knowledge or on a "human-deduced model system", inverse metabolic engineering is more advantageous as it is based on a "living model system" i.e. a desirable phenotype that exists in nature (Sauer and Schlattner, 2004). Besides that, inverse metabolic engineering comprises many other benefits compared to the rational metabolic engineering (Nevoigt, 2008). Firstly, it

requires no preliminary knowledge about cellular subsystems e.g. enzymes, pathways and regulation factors relevant to the favourable trait. This is actually helpful for the improvement of brewers' yeast strain since the accumulated knowledge about the cellular activities of brewers' yeast is still limited (see above). Another advantage of inverse metabolic engineering is that one can directly use the industrial strains and industrially relevant conditions to identify the target genes for genetic modification. Moreover, inverse metabolic engineering can be applied to combine various valuable traits in one strain. In this model of analysis, one strain with the 1st desirable trait will play the role of the host while the other strain with the 2nd favourable trait plays the role of the model strain. By comparison the genotypes and gene expression patterns of the two studied strains, the genetic basis for the 2nd valuable trait will be identified and genetic manipulation of the explored target genes in the host strain will lead to the creation of a yeast strain with combined favourable traits. In an approach where the host and the model organism are taxonomically closely related, e.g. they both belong to one species, genetic modification will then be based on homologous genes. The modified strains therefore can be considered as "self-cloned" and can be better accepted in food and beverage industry. Lastly, by studying naturally diverse strains, inverse metabolic engineering approach provides good chances to explore novel target genes for strain improvement i.e. those which have never been found in the rational approach.

Inverse metabolic engineering was effectively employed for yeast strain optimisation in an increasing number of studies (Nevoigt, 2008). For example, it was applied to improve the galactose utilisation in S. cerevisiae (Bro *et al*., 2005). Another application was to construct a yeast strain with the improvement in xylose uptake and ethanol production (Jin *et al*., 2005). Recently, inverse metabolic engineering was successfully employed to increase sulphite production in a bottom fermenting yeast strain (Yoshida *et al*., 2008). It can be said that inverse metabolic engineering, with its outstanding advantages, is clearly a powerful approach for yeast strain improvement.

2 Aim of work

Diacetyl is a by-product of the valine biosynthetic pathway which causes an unwanted butter flavour in beer. The reduction of diacetyl production is one of the most relevant targets for brewer' yeast strain improvement since it helps to shorten the maturation process and thereby enabling an acceleration of lager beer production (see also section I.2.2.1).

The aim of this thesis is to reduce diacetyl production in lager brewers' yeast by means of inverse metabolic engineering. In inverse metabolic engineering, the most important step is the identification of target genes for genetic modification. This task herein is performed by means of an integrative approach using global analyses at different genetic molecular levels.

A step by step scenario reflected by the following key questions was designed to find out the most suitable solution:

1) What is the strategy for brewers' yeast improvement using inverse metabolic engineering?

Firstly, lager brewers' yeast strains producing various levels of diacetyl will be selected. Secondly, the genetic basis responsible for the phenotypic differences in the selected strains will be identified. The last step will be the improvement of a production brewers' yeast strain via genetic engineering of the target genes.

2) How novel target genes for reducing diacetyl production in lager brewers' yeast are identified?

To identify the genetic basis for the strain's phenotypic differences relevant to brewing, an integrative approach is chosen using global analyses at different molecular levels of genome (microarray-based comparative genomic hybridisation), transcriptome (microarray-based transcriptome analysis) and proteome (two-dimensional gel electrophoresis, MALDI-TOF MS). For the microarray-based genome and transcriptome analyses, bottom fermenting yeast DNA microarrays will

be used. Incorporating data of these analyses will lead to the identification of novel target genes for strain improvement.

3) How the roles of the relevant target genes for diacetyl production are verified?

Genetic manipulations of the relevant target genes will be performed in one production lager yeast strain. Fermentations of the resulting recombinant strains will be carried out under laboratory and industrially relevant brewery conditions. The roles of target genes will be verified by measuring diacetyl productions of the wildtype and the recombinant strains during the main fermentation.

4) *How do the genetic modifications affect the fermentation performance of the lager brewer' strain?*

Fermentation performances of the recombinant strains will be investigated under industrially relevant brewery conditions. During the fermentation, the time courses of apparent extract and time couses of pH will be recorded. In addition, the concentrations of non-sedimented cells in the wort medium will be measured. At the end of the main fermentations, the green beers produced by the recombinant strains will be taken for by-product analyses.

3 Materials and methods

3.1 Materials

3.1.1 Equipment

Autoclave	Varioklav 500 EV (H+P Labortechnik, Oberschleissheim)
Array	Bottom fermenting yeast DNA microarray (Affymetrix customer array)
Array washing system	Fluidics Station 450
Balances	Type 1907 (Sartorius, Göttingen)
Centrifuges	Sorvall RC-5B (for SS34- and GSA-Rotors) (Sorvall DuPont, Bad Homburg); Microrapid/K (Hettich, Tuttlingen); DW41 (Qualitron, Korea)
Clean bench	Uniflow UVUB1200 (UniEquip, Martinsried)
Electrophoresis chambers	Mini-Sub and Wide Mini-Sub DNA-Cell (Biorad); Ettan™ IPGphor™ Isoelectric Focusing System; Ettan™ Dalt *six* Electrophoresis Unit (Amersham Pharmacia Biotech)
Homogenizer	Micro-Dismembrator; Braun, Melsungen
Hybridization oven	Compact Line OV4 (Biometra, Göttingen)
Incubators	Typ B6420 & FunctionLine Typ B20 (Heraeus Instruments, Hanau); MultiTemp III (Amersham Pharmacia Biotech)
Microscope	Labophot (Nikon, Japan)
PCR equipment	GeneAmp 9600 (PerkinElmer, Norwalk, USA); MyCycler™ thermal cycler (Biorad)
pH-electrode	IntLab 412 (Mettler-Toledo, Urdorf, Schweiz)
pH meter	Digital-pH-Meter (Knick)
Pipetting equipment	Gilson Pipetman P10, P20, P200, and P1000 (Abimed, Langenfeld)
Shaker	Certomat U (Braun Biotech, Melsungen); Polymax 1040T (Heidolph, Kelheim)
Scanner	Affymetrix Array Scanner 3000, Image Scanner (Amersham)

Spectrophotometer	UV-160A (Shimadzu, Japan)
Vacuum equipment	Laborota 4000 (Heidolph, Kelheim)
Water bath	Lauda A100 (Wobser, Lauda-Königshofen)
Other materials	Immobiline^{TM} DryStrip Gels pH 3-7

3.1.2 Enzymes, chemicals and kits

Agarose	Seakem LE, GTG & Gold, Incert^{R} Agarose (FMC Bioproducts, Denmark)
Biochemicals/chemicals	Acetylated BSA, Control Oligo B2, DMSO, Eukaryotic Hybridisation Buffer (Affymetrix); Pharmalyte pH 3-7, PlusOne APS, PlusOne CHAPS, PlusOne Dithiothreitol, PlusOne Drytrip Cover Fluid, PhastGel^{TM} Blue R, PlusOne Glycin, PlusOne SDS, PlusOne Urea, (Amersham Pharmacia Biotech, Uppsala Sweden); Ampiciline (BioMol, Hamburg); PAA 29:1, UltraPure Urea (ICN Biomedicals, US); BSA, Geneticin, UltraPure™ Tris, (Invitrogen, Karlsruhe); Phleomycin (Invivogen, Toulouse, France); Agar-Agar, Yeast extract, Peptone, Triptone, Glucose, Maltose, Isoamyl Alcohol (Merck, Darmstadt; Serva, Heidelberg; Difco, MI, USA); DEPC, Sodium Acetate (Fluka, Switzland); BPB, Chloroform, Isoamyl Alcohol, Hydrochloric acid (MERK, Darmstadt); Biotin-N6-ddATP (NEL); 6x SSPE Buffer (NIPPON GENE); One-Phor-All Buffer (Pharmacia); Herring Sperm (Promega); Acetic acid, Ethanol, Glycerol, Isopropanol, (Roth, Karlsruhe); EDTA, IAA, Phenol, PMSF, TEMED, Acetoin, Thiamin pyrophosphate, FAD, Creatine, Alpha-napthol, Pyruvate, Triton-X (Sigma, St. Louis, USA);
Enzymes	Restriction enzymes (New England Biolabs, Schwalbach); DNAseI (Amersham); Zymolase, Protease, RNAse (Gibco BLR)
Kits	BioRad Protein-Assay (BioRad, München); AccuPre® PCR

	Purification Kit (Bioneer, Seoul), Genomic extraction Kit (Quiagen); GeneChip IVT Labelling Kits (Affymetrix)
Nucleic acids	λ-DNA (Roche Diagnostic, Mannheim); GeneRuler TM DNA Ladder Mix (MBI Fermentas, Litauen), 100bp Plus DNA ladder (Bioneer, Seoul)
Oligonucleotides	Metabion, Berlin
PCR-reagents	Deoxynicleoside-Triosephosphate Set, Top-polymerase, Pfu-polymerase (Roche Diagnostics, Mannheim; Bioneer, Seoul)

All chemicals not listed above were obtained from the following companies: Fluka, Merck, Roche Diagnostic, Sigma, Serva, Pharmacia and were of analytical grade or better quality.

3.1.3 Strains

Table 1. Strains used in this study

Strain	Lab name	Genotype	Source or reference
S. cerevisiae			
BY4741		*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
Brewers' yeast			
Sc-06136	A	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin
Sc-06168 or H06	B	Bottom fermenting yeast	Institut für Gaerungsgewerbe Berlin
Sc-06165	C	Bottom fermenting yeast, arose from strain A via single cell isolation	Institut für Gaerungsgewerbe Berlin
Sc-06165-*ilv6Δ*	*Sc-ilv6Δ*	Sc-*ILV6*/ Sc-ilv6Δ::loxP-kanMX-loxP	This study
Sc-06165- *ilv6ΔΔ*	*Sc-ilv6Δ/ Sc-ilv6Δ*	Sc-ilv6Δ::loxP-kanMX-loxP/ Sc-ilv6Δ::loxP-ble^{r}-loxP	This study
E. coli		*SupE44, ΔlacU169(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Melson M. and Yuan R. (1968)

3.1.4 Media and culture conditions

E. coli

E. coli strains were cultivated in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) at 37^{o}C. Recombinant *E. coli* strains were selected in LB medium with 150 μg/ml of Ampicilline. Solid LB medium suppelementing with 150 μg/ml of Ampicilline was obtained by adding 1.5% agar. For stock culture storage, 1 ml of liquid culture was mixed with 0.3 ml glycerol 100% and then being kept at -70^{o}C.

Yeasts

In general, *S. cerevisiae* and wild-type brewers' yeast strains were grown in Erlenmeyer flasks on a rotary shaker at 170 rpm in YEPD medium (2% peptone, 1% yeast extract, and 2% glucose) or on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) at 30^{o}C.

The mutant *Sc-ilv6Δ* where one copy of Sc-*ILV6* was replaced by the loxP-kanMX-loxP cassette was first propagated at 30^{o}C in YEPD-containing shaking flask. Cells were then grown on selective YED plates (1% yeast extract, 2% glucose; pH 6.3) supplemented with 17.5 μg/ml G418. YEPD plate was not being used since the presence of peptone increased the threshold of selective concentration of this antibiotic to yeast.

The mutant *Sc-ilv6Δ/Sc-ilv6Δ* which carries deletions in both copies of Sc-*ILV6* ORFs and thus habouring the kanMX and ble^{r} markers was first generated on YEPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30^{o}C. After that, cells were replica-selected on YEPD plates supplemented with 17.5 μg/ml phleomycin and afterwards on YED plates plus 50 μg/ml G418.

For storage, 1 ml stock of yeast culture was mixed with 0.3 ml glycerol and kept at -70^{o}C. For preparation of any stock cultures, yeast strains were grown in selective media.

3.1.5 Plasmids

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pUG6	*E. coli/ S. cerevisiae* shuttle vector, containing Amp^+, loxP-kanMX-loxP disruption cassette	Güldner *et al*., 1996 ; Güldner *et al*., 2002
pUG66	*E. coli/ S. cerevisiae* shuttle vector, containing Amp^+, loxP-ble^r-loxP disruption cassette	Güldner *et al*., 2002

3.1.6 Oligonucleotides

The oligonucleotides used in this work were synthesized by Metabion (Berlin)

1) Primers used for disruption of Sc-*ILV6* gene in lager brewers' yeast

P1 (Forward primer):

5'- TACAGAATCTTTAGAACATCTGAGCTCACTAACCCAGTCTTTCTAccgccagctgaagcttcg - 3´

P2 (Reverse primer):

5' - ATTTCGGCGACCAATTCTTGGGAGTCAGCGGCGCCAGCATTGGTGgcataggccactagtggatc - 3´

2) Primers used to verify the *Sc-ILV6* deletion:

P3 (Forward primer): 5´- ATATGGAAGTACATAGTTCG - 3´

P4 (Reverse primer): 5´- TTCGGCGACCAATTCTTG - 3´

3) Primers used to verify the existence of non-Sc-*ILV6*

P5 (Forward primer): 5´- TAAGTCACATACGTAGTTTG - 3´

P6 (Reverse primer): 5´- TCGGCAACTAACTCGTTG - 3´

3.2 Methods

3.2.1 DNA methods

3.2.1.1 Isolation of yeast genomic DNA

Genomic DNA was isolated from *S. cerevisiae* and brewers' yeast following the method of Hoffman and Winston (1987). Yeast cells were grown overnight in 5 ml YEPD medium. Next, 1.5 ml of the culture was harvested by centrifuging for 10 minutes at 12000 rpm at 4^{o}C and washed with 500 µl sterile distilled water. After centrifugation, cell pellet was resuspended in residual water. For the cell disruption, 200 µl of lysis buffer (2% Triton X-100; 1% SDS; 0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA), 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 300 mg of glass beads (0.45-0.5 mm) were added to the cell suspension. The tube was vortexed vigorously for 4 minutes and then centrifuged for 5 minutes at 12000 rpm at 4^{o}C and the supernatant was collected. For the removal of protein residuals, one volume of chroloform was added to the supernatant. The mixture was vortexed for 30 s and centrifuged again for 5 minutes at 12000 rpm at 4^{o}C. The upper phase was collected and 0.7 volume of isopropanol was added for DNA precipitation. The precipitated DNA was obtained by centrifuging for 15 minutes at 12000 rpm at 4^{o}C. DNA pellet was washed with 70% ethanol and then dissolved in 50 µl of sterile distilled water.

3.2.1.2 Isolation of plasmid DNA from *E. coli* (minipreparation)

For analysis of *E. coli* transformants, the plasmid DNA was isolated from 1.5 ml culture using the alkaline method of Birnboim and Doly (1979). Lager amounts of plasmid DNA were isolated using GeneJET^{TM} Plasmid Miniprep Kit (Fermentas).

3.2.1.3 Agarose DNA gel electrophoresis

Agarose gels of 0.8% (w/v) were used for DNA electrophoresis. The gel was prepared by boiling agarose in 1x TBE buffer until agarose was totally dissolved.

Gels were casted at about 60^{o}C. DNA samples were mixed with 1/4 volume of loading buffer (20 mM EDTA, pH 8.0, 0.025% bromophenol blue, 60% saccharose) and loaded into the lanes of agarose gel. The electrophoresis was run in 1x TBE buffer (89 mM Tris; 89 mM Boric acid; 2 mM EDTA, pH 8.0) at about 80 mA. For size determination of DNA fragments, either the DNA size marker "GeneRuler^{TM}" (Fermentas) or "100bp Plus DNA leader" (Bioneer) was used. The gels were stained in 0.5 mg/ml EtBr solution for 20 minutes and the DNA fragments were visualized under UV light (λ = 254 nm).

3.2.1.4 PCR

PCR were performed using either GeneAmp9600 (Perkin Elmer) or Mycycler (BioRad). The reactions were performed in 25 µl or 50 µl volume in 0.2 ml reaction tubes (GeneAmp9600). The reaction mix contained 0.25 mM of each dNTP; 1 µM of each primer; 1 ng/µl of template DNA; 1x reaction buffer, 1-2.5 units of DNA polymerase and H_2O up to the final volume. For generation of disruption cassettes, *Pfu* DNA polymerase (Bioneer) was used. For PCR diagnostic of *Sc-ILV6* disruption, Top DNA polymerase (Bioneer) was used.

In general, PCR programe set up included following steps: i) pre-heat treatment at 95^{o}C for 2 minutes; ii) 25 cycles as followed: denaturation for 45s at 94^{o}C, 45s at annealing temperature and elongation at 72^{o}C and iii) end-elongation for 10 min at 72^{o}C.

The annealing temperature was calculated based on melting temperatures of the forward and reverse primers. The melting temperature of the primers was calculated using pDRAW32 software. The annealing temperature was adjusted to two degree lower than melting temperatures of the primers. The elongation time was calculated depending on the length of the expected PCR. On average, 60 s elongation times was used for the amplification of DNA fragment of 1 kb length.

3.2.1.5 Transformation of lager brewers' yeast

Transformation of disruption cassettes into brewers' yeast was performed using the Lithiumacetate/PEG method. Yeast strains were precultured overnight in 20 ml YEPD medium at 30^{o}C. For main culture preparation, 200 ml YEPD was inoculated with the preculture by adjusting to an OD of 0.2. The main cultures were grown at 30^{o}C till an OD of 0.7 and cells were harvested by centrifugation for 2 minutes at 6000 rpm. The supernatant was decanted and cells were washed with 25 ml distilled water. The cells were then centrifuged for 2 minutes at 6000 rpm and resuspended in 1 ml distilled water. After that, 400 µl of the cell suspension was washed and resuspended again in 400 µl water. Next, this cell suspension was dispatched into aliquots of 100 µl. The aliquots were kept on ice until being added with transformation mixture.

The transformation mixture (without the DNA cassette) contained 240 µl PEG 4000 (50 % w/v), 36 µl 1 M lithium acetate, 50 µl single stranded carrier DNA (herring sperm DNA 2 mg/ml). The carrier DNA was boiled and cooled on ice for generation of single stranded DNA before being added to mixture.

Up to 1 µg of PCR product (dissolved in 34 µl water) was added to the yeast cell suspension (100µl). The tube was vortexed vigorously to allow good contact between cells and DNA. Next, the previously prepared transformation mix was added to the tube. The complete mixture was vortexed vigorously and incubated for 40 minutes at 42^{o}C. Afterwards, the mixture was transferred into an Erlenmeyer flask containing 5 ml YEPD. The flask was shaked overnight at 170 rpm and 30^{o}C for the expression of enzymes which conferred antibiotic resistance to the transformants. Cells were harvested by centrifugation (13000 rpm, 30 sec) and then washed with 5 ml of 0.85% NaCl. After that, cell pellet was resuspended in 1ml of 0.85% NaCl. Cell suspension was spread onto selective agar plates. The plates were incubated for 2-4 days at 30^{o}C for the appearance of colonies.

3.2.1.6 Transformation of *E. coli*

Transformation of *E. coli* was carried out using the heat shock method according to Inoue *et al*. (1990). *E. coli* cells were made competent by $CaCl_2$ treatment. For competent cells preparation, *E. coli* cells were precultured in 20 ml LB-medium from a frozen stock culture (-70^{o}C) for 16 hours at 37^{o}C. Next, 1 ml of preculture was transferred into an Erlenmeyer flask containing 100 ml of LB-medium. Cells were grown until an OD of 0.4 and the main culture was kept on ice for 1 hour. Cells were then collected by centrifuging for 5 minutes at 4^{o}C at 4000 rpm. Cells were washed with 100 ml of ice-cold solution I (0.1 M $MgCl_2$; 0.01 M Tris-HCl, pH 7.6). After that, cells were dissolved in 50 ml of ice-cold solution II (0.1 M $CaCl_2$; 0.01 M Tris-HCl, pH 7.6) by gently mixing. The cell suspension was then centrifuged again as in the previous step and the supernatant was decanted. Cell pellet was then resuspended in 4 ml of ice-cold solution II and the suspension was kept on ice for 30 minutes. The competent cells were either directly used for transformation or stored at -70^{o}C after the addition of glycerol (15% final concentration).

For transformation, approximately 100 ng of plasmid DNA were mixed gently with 200 µl of competent cells. The mixture was kept on ice for 30 minutes and then subjected to a heat shock at 42^{o}C for 30 sec. The cells were then put immediately on ice for 1 minute and then mixed with 1 ml of LB medium. The tube was incubated with agitation at 37^{o}C for 1 h. After that, various dilutions of the sample were spread onto LB plates containing ampiciline (150 µg/ml).

3.2.1.7 Microarray-based comparative genomic hybridisation

Sample preparation and array hybridisation

Yeast cells were grown in YEPD medium and genomic DNA was extracted from 50 ml of yeast culture at an OD of 1.5 using genomic extraction kit from QIAGEN. Protein and RNA were removed using zymolase, protease K and RNAse treatment for 45 minutes. Sample preparation was carried out as following method described by

Winzeler (2003) with slight modifications. Genomic DNA (10 µg) was fragmented with 0.15 units DNAseI (Gibco BLR, PCR grade) in 1x One-Phor-All buffer (Pharmacia) supplemented with 1.5 mM $CoCl_2$ for 3 minutes at 37^{o}C. DNAse I reaction was inactivated by heating the sample at 95^{o}C for 15 minutes. Fragmented DNA was labelled with 1 nmol Biotin-N6-ddATP (NEL) using 25 units termininal transferase (Roche) at 37^{o}C for 1 hr. Labelled DNA fragments were dissolved in 200 µl hybridisation solution containing 50 pM Control Oligonucleotide B2 (Affymetrix), 1x Eukaryotic Hybridisation Controls (Affymetrix), 20 µg herring sperm (Promega), 6x SSPE (0.9 M NaCl, 60 mM NaH_2P0_4, 6 mM EDTA) (NIPPON-GENE) and 0.005% Triton-X (SIGMA). After 10 minutes incubation at 100^{o}C, the hybridisation solution was transferred on ice for a few minutes and afterwards hybridised to DNA microarray.

Hybridisation was carried out for 16 h at 42^{o}C in hybridisation oven with permanent mixing at 60 rpm. Genomic DNA of each lager yeast strain was hybridized to one single array. Washing, staining and scanning of arrayss were carried out as described in Affymetrix Technical manual (Affymetrix, 2004).

Data acquisition and analysis

Data analyses were performed in collobration with of Yoshihiro Nakao (Suntory Ltd). Detection of signal intensities of microarrays was carried out using Affymetrix Gene Chip Analysis Basic System and Affymetrix GeneChip® Operating Software (GCOS) v 1.0. A probeset was detected as absent “A” or present “P” based on detection p-value calculated by detection algorithm with default parameter in GCOS. In each pairwise comparison, signal $\log_2$ ratio and change p-value of every probe set was calculated. A probeset had a change call of decrease “D”, increase “I”, medium increase “MI”, medium decrease “MD” or not change “NC” based on the change p-value calculated by Change Algorithm with default parameter in GCOS.

3.2.2 RNA method: Microarray-based comparative transcriptome analysis

3.2.2.1 Isolation of brewers' yeast total RNA

Brewers' yeast strains were grown in 30 litre fermenters under relevant brewing conditions (11.5^{o}P brewers' wort, 11^{o}C). For total RNA isolation, brewers' yeast cells were collected at apparent extract of 8%. Cell sampling was performed as described by Piper (2002) with slight changes. Roughly 20 ml of culture corresponding to 240 mg yeast wet weight was harvested in triplicate for each strain from the 30 liter scale fermentations. The broth was frozen instantly by pouring it directly into a beaker containing liquid nitrogen. The frozen sample was then broken into small fragments and transferred to two 50 ml centrifuge tubes. The sample was then thawed at 0^{o}C by subsequent vigorous vortexing and keeping on ice. Next, the samples were centrifuged at 5000 rpm at 0^{o}C for 4 minutes and re-suspended in 1.8 ml AE-cold buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). The content of the two tubes was pooled and afterwards aliquoted into 10 eppendorf tubes, 400 µl each. RNA extraction was carried out using the hot phenol method (Schmitt *et al*., 1990). The resulted RNA was treated with DNAseI (Amersham) (approximately 0.1 units per 1 µg RNA) to remove DNA. RNA sample was then purified again with phenol:chloroform:isoamyl alcohol (25:24:1) for DNAseI removal. After that, RNA sample was precipitated by adding 0.7 volumes of isopropanol and 0.1 volumes of 3 M sodium acetate, pH 5.2. The RNA pellet was washed with 80% ethanol and resuspended in DEPC-treated water. The RNA sample was then kept at -80^{o}C until being used for hybridization.

3.2.2.2 Sample preparation and array hybridization

cDNA synthesis, biotin-labeled cRNA synthesis and fragmentation, hybridization and washing, scanning of the array were performed following Affymetrix user's manual (Affymetrix, 2004). In short, single strand cDNAs was synthesed from total RNA (15 µg) by incorporating T7 RNA-polymerase promoter. Subsequently, double

strand cDNAs was synthesized and was then used as the template for the synthesis of biotin-labeled cRNA using GeneChip IVT Labeling Kit (Affymetrix). Biotin-labelled cRNA was then fragmented. Hybridization cocktail (300 µl) containing 15 µg fragmented Biotin-Labeled cRNA, 50 pM Control Oligonucleotide B2, 1x Eykaryotic Hybridization Controls, 0.1 mg/ml Herring Sperm DNA, 0.5 mg/ml Acetylated BSA, 1x hybridisation buffer and 10% DMSO (Affymetrix) was applied to bottom fermenting yeast DNA microarray. The hybridization was carried out for 16 hour at 45^{o}C and at 60 rpm rotation. Array hybridizations were carried out in technical triplicate, i.e. the three independent RNA isolations for each strain, respectively. The arrays were washed and stained as described in Affymetrix user's manual (Affymetrix, 2004) using Fluidics Station 450. Arrays were then scanned with Affymetrix GeneChip Scanner 3000.

3.2.2.3 Data acquisition and analysis

Data analyses were performed in collaboration with Dr. Matthias E. Fuschik (Humbolt University) and Yoshihiro Nakao (Suntory Ltd.). Detection of signal intensities of micoarrays was carried out using Affymetrix Gene Chip Analysis Basic System and Affymetrix GeneChip® Operating Software (GCOS) v 1.0. Every transcript was flagged as absent "A", present "P" or marginal present "M" based on the detection p-value calculated by Detection Algorithm with default parameter in GCOS. Subsequently, the data was adjusted by quantile normalization (Bolstad *et al*., 2003). For every transcript in each pairwise comparison, we calculated the logged average fold-change. The significance of differential expression was assessed using the CyperT approach, which is based on a Bayesian t-test (Baldi and Long, 2001). As significant threshold for the pairwise comparisons, a false discovery rate of 0.001 was chosen. Pathway analysis of significant differences was performed using SGD database and Microsoft Access program.

3.2.3 Protein methods

3.2.3.1 Comparative proteome analysis

3.2.3.1.1 Isolation of total protein from bottom fermenting yeast

Brewers' yeast strains were grown in 3 litre fermenters under relevant brewing conditions (11.38^{o}P brewers' wort, 12^{o}C). For protein isolation, brewers' yeast cells were collected at an apparent extract of 8% by centrifugation at 6000 rpm for 5 minutes. Subsequently, 1 g wet weight of yeast cells was washed twice with 100 ml and then with 50 ml of ice-cold 10 mM Tris-HCl, pH 8.0 buffer and centrifuged at 4000 rpm for 3 minutes in each step. Cells were then resuspended in the same buffer and dropped into liquid nitrogen in the form of small bits. Frozen cells were ground into powder using Braun Dismembrator at 2500 rpm for 2 minutes. Frozen cell powder was transferred into an eppendorf tube and thawed to 4^{o}C. Next, 10 µl of 100mM PMSF was added to the cell samples and the mixtures were centrifuged for 8 minutes at 8000 g at 4^{o}C. Supernatant was then collected and centrifuged again at 16000 rpm for 30 minutes at 4^{o}C. Protein concentration of the supernatant was determined using Bradford assay. Protein sample was dispatched into eppendorf tubes in small aliquots of 70 µl and stored at -80^{o}C until being used for electrophoresis.

3.2.3.1.2 Two-dimensional gel electrophoresis

Protein sample (800 µg) was solublized in 600 µl reswelling solution (8 M urea, 1% CHAPS, 0.4% DTT, 0.5 v/v Pharmalyte 3-10, 0.002% bromophenol blue) by shaking for 30 minutes at 600 rpm. The samples were then centrifuged for 5 minutes at 12000 rpm and the supernatants were applied to a 24 cm, nonlinear Immobiline Drystrip, pH 3-7 (Amersham Biosciences). The strips were covered with silicon oil. Protein focusing was performed on IPGphor at 20^{o}C as follow: 30 V for 15 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, gradient 8000 V for 30 minutes, and 4 h at 8000 V. The strips were equilibrated twice for 15 minutes firstly in 10 ml buffer

(6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.0, 30% glycerol) supplemented with 1% (w/v) DTT and then in 10 ml of the same buffer supplemented with 2.5% (w/v) idoacetamid.

After equilibration, IPG strips were load onto 12.5% acrylamide/bis-acrylamide (29:1) gels. The two gels were sealed with 0.5% (w/v) agarose solution containing 0.002% BPB. The second dimension was perfomed using Ettan^{TM} Dalt six Electrophoresis Unit (Amersham Biosciences). The gels were run at 20^{o}C at 3W per gel for 30 minutes and then at 20 W per gel for 4.5 h in the electrophoresis buffer containing 25 mM Tris-base, 192 mM glycine, 1% (w/v) SDS. Gels were stained overnight with Comassie Brillant Blue and washed several times with destaining solution (25% Ethanol, 5% acetic acid). After that, gels were washed again with sterile distilled water for 30 minutes and scanned with the Image Scanner (Amersham).

Protein samples of the three strains were isolated in triplicate from two independent fermentations. Concretely, protein samples of three strains were isolated twice from the first fermentation and once from the second fermentation. For each strain, 2D gel of protein extract was run in triplicate. Protein samples of the three selected strains were always run concurrently.

Scanned images of analytical gels were analysed using Delta 2D Software v 4.0 (Decodon, Greifswald, GmbH). A master gel for each strain was created from all replicates using all-to-one warping strategy. The average volume (in percentage to the total volume) and standard deviation of each spot in replicates were calculated. The master gels were used for pairwise comparisons between selected lager yeast strains. Differentially expressed proteins were selected with a fold change of 2 of average volumes. Statistical analysis was performed allowing a standard deviation ≤ 30% for each spot from the three replicates and a *p*-value of 0.05 or below in the Student's t-test at pairwise comparison.

3.2.3.1.3 MALDI-TOF mass spectrometry

Protein spots were excised from 2D gels; trypsin digested and identified using MALDI-TOF mass spectrometry (Kohler *et al*., 2005). The analysis was performed using the service of Greifswald University, Institut für Marine Biotechnologie (Prof. Thomas Schweder group). MS data were investigated using Mascot search engine (Matrix Science Ltd) against the protein database of *S. cerevisiae*. Peptides that yield a protein score of at least 100 and a sequence coverage ≥ 30% for duplicate identifications were regarded as positively detected.

3.2.3.2 Determination of protein concentration

The protein concentration in cell extracts was determined using Bradford assay (1976). The dye reagent (Bio-Rad) was diluted 1:4 with distilled water. Cell protein extract (200 µl) was mix with 800 µl of the diluted dye solution and incubated for 5-10 minutes at room temperature. The extinction was measured at a wavelength of 595 nm against a control made up of 800 µl of the prepared dye solution and 200 µl of the buffer used to dissolve cell protein extract. For protein determination, a calibration curve of BSA with concentrations of 20-70 µg/ml was prepared.

3.2.3.3 Determination of enzyme activity

3.2.3.3.1 Preparation of permeabilized cell proteins

Yeast strains were pre-cultivated in 20ml wort by shaking at 24^{o}C for one day. Yeast strains were then inoculated at the concentration of 1 x 10^{7} cells/ml in 90 ml brewers' wort (11.38^{o}P). Fermentations were carried out in 100 ml bottles closed with airlocks at 12^{o}C until the apparent extract was reduced to a value varying from 8.3 to 8.7. Cells were harvested by centrifuging for 5 minutes at 5000 rpm at 4^{o}C and washed twice with sterile distilled water. Next, cells were resuspended in 1 ml ice-cold buffer (0.1 M Tris-HCl pH7.5, 0.1 M NaCl and 0.1 M EDTA) supplemented with 2 mM PMSF. Cells were permeabilized by adding 100 µl chloroform and

vortexing for 30 s. Cell samples were then centrifuged at 6000 rpm for 5 minutes 4^{o}C and the supernatant was removed. Permeated cell were resuspended in the same buffer and were placed at 4^{o}C for being used for AHAS assay within 2 h.

3.2.3.3.2 *In vitro* acetohydroxyacid synthase (AHAS) assay

AHAS was assayed based on its activity to convert pyruvate into α-acetolactate. The enzyme activity was assayed using the method described by Byrne and Meacock (2001) with some modifications. The assay was performed in a volume of 100 µl. For each sample, two tubes, the control and sample itself were set up. A 90 µl mixture containing 65 µl cell suspension, 5 µl ThDP 20 mM, 5 µl 0.2 M $MgCl_2$, 5 µl of 4 mM FAD and 10 µl of 1 M K_3PO_4 (pH7.5) was added to each tube and incubated for 10 minutes at 30^{o}C. After that, 10 µl 1 M pyruvate was added to the mixture and the reactions were carried out for 20 minutes at 30^{o}C. The reactions were then stopped by the addition of 11.3 µl 9.9 M H_2SO_4 to the sample and 150 µl 6M NaOH to the control tube. The sample tubes were then incubated at 60^{o}C for 30 minutes to allow the efficient conversion of α-acetolactate to acetoin. After that, 140 µl 8 M NaOH was added to each sample tube to stop the reaction. At this point, the sample and control had the same volume (250 µl) and the same pH. The yield of α-acetolactate was determined by the amount of acetoin produced in the decarboxylation reaction which was taken place in the acidic condition at high temperature. In the control tubes, α-acetolactate was not decarboxylated to acetoin, therefore the amount of acetoin produced in the decarboxylation reaction was measured by subtracting the amount acetoin in the background (control tube) from the total amount of acetoin (sample tube).

The concentration of acetoin in the control and sample tubes was determined using colorimetric method (Westerfeld, 1945). Each tube was filled with 750 µl water, 200 µl 0.5% creatine and 200 µl 5% α-napthol freshly prepared in 2.5 M NaOH. The tubes were vortexed for 2 s and kept for 1 h at RT to allow colour development. The

reaction mixtures were centrifuged for 2 minutes for clarification. Absorbances of the supernatants at 525 nm were measured against the blank made up of 1 ml water, 200 µl 0.5% creatine and 200 µl 5% α-napthol. AHAS activity was calculated as acetoin produced per mg of permeabilized cell protein per h.

3.2.4 Fermentations

Yeast cells and green beers used for different experiments (e.g. protein isolation, RNA isolation and green beer analysis) were harvested from different fermentations carried out under sightly different conditions.

For determination of characteristics of green beers produced by brewers' yeast strains and the fermentation performance of brewers' yeast strains, yeast strains were cultivated and fermented using brewers' wort with original gravity of 11.38^{o}P kindly provided by a German brewery. Yeast strains were cultivated in 200 ml wort by shaking at room temperature. After three days, 600 ml wort was added and the cultures was cultivated under the same condition for another two days. The cultures were then filled with 1000 ml wort and incubated without shaking at 12^{o}C for 24 h. The cells were harvested by fermentation and inoculated into fresh wort at the density of 1 x 10^{7} cells/ml for primary fermentation. The fermentations were carried out in 3 litre glass fermenters with stirring at 50 rmp at 10^{o}C. The fermenters were closed with airlocks for elimination of oxygen but allowing the release of gases. During the main fermentation, cell density, decrease of wort apparent extract and the diacetyl concentration were recorded. The fermentations were finished when wort apparent extract decreased to a value between 2.8% to 3%. After the main fermentation, green beers produced by yeast strains were taken for analysis. Besides ethanol and glycerol, other by-products were analysed included higher alcohol, organic acids, vicinal diketones, esters and fatty acids.

For protein isolation, yeast cells were cultivated and fermented in 3 litre-scale galss fermenters as described above. Yeast cells were harvested at an apparent extract of 8% for total protein isolation.

For isolation of brewers' yeast total RNA**,** yeast strains were cultivated and fermented using brewers' wort of 11.5^{o}P produced by our collaboration partner from VLB (Prof. Frank Jürgen Methner group). Yeast strains were pre-cultivated in 30ml wort by shaking at 24^{o}C for one day and then transferred into a bottle containing 500 ml wort. The secondary pre-cultures were cultivated under the same condition until they reached a cell density of 1 x 10^{8} cells/ml. Next, 5 litre wort was inoculated with each secondary preculture and yeast strains were cultivated under the same condition. Main fermentations were carried out in 30 litre scale-tanks at 11^{o}C. Wort was inoculated at a density of approximately 1.6 x 10^{7} cells/ml for the main fermentation. For RNA isolation, cells were harvested at an apparent extract of 8%.

For determination of fermentation performance and vicinal diketone production by the engineered strain *Sc-ilv6Δ/Sc-ilv6Δ,* yeast strains (the reference strain C and strain *Sc-ilv6*Δ/*Sc-ilv6*Δ) were cultivated and fermented under relevant industrial brewery conditions using brewers' wort with original gravity of 11.38^{o}P. Yeast strains were innoculated in 5 litre bottles at the cell density of about 1.1 x 10^{7} cells/ml. The cultures were incubated at RT (20-25^{o}C) until they reached the cell density of ca. 1.3 x 10^{8} cells/ml. Wort was inoculated with the precultures at the cell density of approximately 1 x 10^{7} cells/ml for the main fermentation. Main fermentations were carried out in 30-litre fementation tank filled with 24 litre brewers' wort at 10.5^{o}C. During the fermentation, cell density, decrease of wort apparent extract and the diacetyl concentration in wort medium were recorded. The fermentations were finished until wort apparent extract was decreased to a value from 2.8% to 3%. After the main fermentation, green beers produced by yeast strains were taken for analysis. The measured compounds included ethanol, glycerol and other flavour-relevant products such as vicinal diketones, fusel alcohols, esters and fatty acids.

For *in vitro* AHAS assay and determination of diacetyl production levels of strains B, C and Sc-ilv6 mutants, yeast strains were cultivated and fermented using brewers' wort with original gravity of 11.38^{o}P kindly provided by a German brewery. Yeast strains were precultured in 20 ml wort by shaking at room temperature. The second preculture was performed in 100 ml wort until it reached to a cell density varying from 5×10^7 to 1×10^8 cells/ml. Cells were harvested by centrifugation. For fementation, wort was inoculated with the preculture at concentration of 1×10^7 cells/ml. The fermentations were carried out in 100 ml bottles containing 90 ml wort. The strains were fermented in 100 ml bottles closed with airlocks at 12^{o}C until an apparent extract varying from 8.3 to 8.8 was reached. Cells were then harvested for preparation of permeabilized cell proteins and the culture supernatant was collected for diacetyl measurement.

3.2.5 Analytical methods

Vicinal diketone concentration of the wort medium harvested from the laboratory scale fermentations was determined using GC-ECD method derived from MEBAK (Band II, 1.2.1, 1996). Vicinal diketone measurement was carried out by our collobration partner at "Institut für Versuchs- und Lehranstalt für Brauerei in Berlin" (VLB) (Prof. Frank-JürgenMethner group).

Determination of apparent extract of wort medium harvested from the laboratory scale fermentations was performed using the method according to MEBAK (Band III, 1.1.1, 1.1.4, 1996).

Determination of apparent extract of wort medium and components of the green beer derived from the relevan industrial brewery fermentations were performed according to international standardized methods edited by European Brewery Convention (1998). These analyses were carried out by our collobration partner in a German beer factory.

4 Results

4.1 Phenotypes of the three selected lager brewers' yeast strains producing different levels of diacetyl

This work focuses on applying the strategy of inverse metabolic engineering to reduce diacetyl production in lager brewers' yeast. To that aim, we at first selected lager brewers' yeast strains which showed different levels of diacetyl production. These strains are derived from the collection of "Institut für Versuchs- und Lehranstalt für Brauerei in Berlin" (VLB) with the lab names A, B and C (see materials and methods). Strain A produces the highest level of diacetyl. Strain B was previously selected for a very low level of diacetyl production. Strain C arose from strain A from single cell isolation and produces a slightly higher level of diacetyl than strain A. Strain C is a common production strain for lager beer brewing.

The performances of these brewers' yeast strains were investigated under industrially relevant brewing conditions (11.38^{o}P wort, 12^{o}C). For each strain, the time courses of apparent extract were recorded. The time courses of apparent extract are the readouts for the consumption of wort sugar during the fermentation of brewers' yeast strains. At the end of the main fermentation, green beer produced by each strain was taken for product analysis. The measured compounds included ethanol, glycerol and other flavour-relevant products such as vicinal diketones, fusel alcohols, esters and fatty acids.

4.1.1 Wort sugar consumption during the main fermentation of the three selected lager brewers' yeast strains

The decreases of wort gravity in strains A and C were almost similar. However, a difference in fermentation rate between strain B and the other strains was observed after day 3 of the main fermentation. Thus, compared to strains A and C, strain B showed a slower rate of wort sugar consumption. Strain B needed more time,

i.e. 60hrs and 40 hours, respectively than strains A and C to reach the wort attenuation (apparent extract of 3%) (Fig. 3).

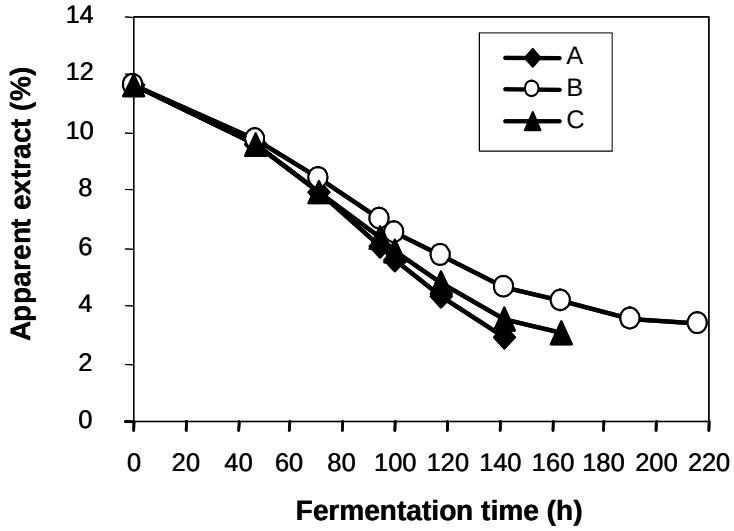

Fig. 3 Time courses of apparent extract during the fermentation by the three selected lager brewers' yeast strains. Fermentation was carried out in 3 litre glass fermenters under conditions relevant to industrial brewery fermentations (11.38^{o}P brewers' wort, 11^{o}C).

4.1.2 Diacetyl production

The relative difference in diacetyl production of the three selected lager brewers' yeast strains was investigated under industrially relevant brewing conditions (11.38^{o}P wort, 12^{o}C) in 3 litre glass fermenters. The fermentations were carried out in duplicate. For each strain, diacetyl was investigated at apparent extract of 8%, 6% and 3% corresponding to the beginning, the middle and the end stages of the main fermentation, respectively (Fig. 4). It is obvious from Fig. 4 that from all investigated points of apparent extract; strain A produced the highest level of diacetyl while the diacetyl production of strain B was the lowest. By the end of fermentation (ca. apparent extract of 3%), the diacetyl concentration of beer produced by strain B was of about 17% and 34% compared to those of strains A and C, respectively. Interestingly, the diacetyl production of strain B by the end the main fermentation was below the beer diacetyl taste-threshold of 0.1 mg/ml (Fig. 4).

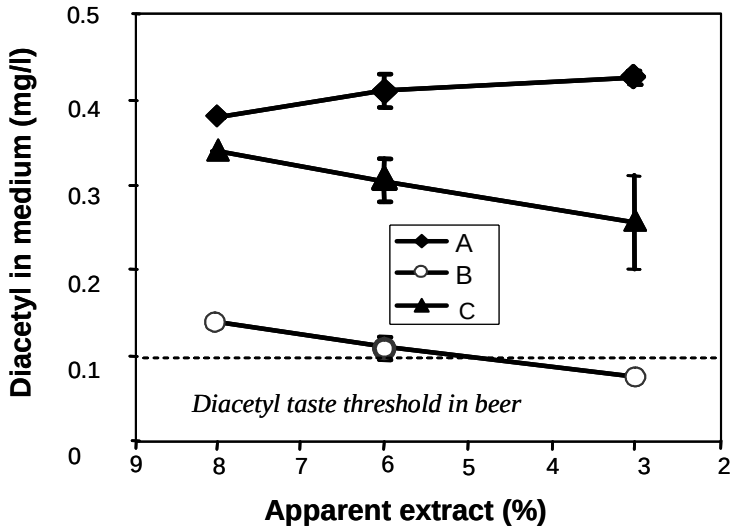

Fig. 4 Different diacetyl productions of the three studied lager brewers' yeast strains. Fermentation was carried out in 3 litre glass fermenters under conditions relevant to industrial brewery fermentations (11.38°P brewers' wort, 11°C). Values at apparent extract of 6% and 3% are the average from two independent experiments, including standard deviations. Values at apparent extract of 8% were measured from a single fermentation.

4.1.3 Flocculation behaviour

Fig. 5 Difference in flocculation behaviour between the studied lager brewers' yeast strains. Fermentation was carried out in *Lietz* device using 11.38°P brewers' wort at 12°C. The picture was taken after 7 days fermentation. Strain B flocculated much earlier than strains A and B.

Despite the very low level of diacetyl production (Fig. 4), strain B is not useful for brewing industry since it flocculates very early. The flocculation behaviour of three selected lager brewers' yeast strains was easily detectable using *Lietz* fermentation devices. It can be seen from Fig. 5 that the medium of strain B was much clearer than that of strains A or C after 7 days of the main fermentation. The low number of non-sedimented cells left in the medium explaines the fact that strain B needed more time than strains A and C to reach the wort attenuation (II.4.1.1).

4.1.4 Other fermentation by-products

Green beer analysis showed that the patterns of by-products of the three selected lager brewers' yeast strains were almost similar except for some slight differences in isobutyl acetate, acetaldehyde and 2,3-pentanedione concentrations (Table 3). The difference in 2,3-pentanedione production in the studied strains corresponds to their differences in diacetyl productions. This result matches the fact that the precursors of these two by-products are both formed by the activity of acetohydroxyacid synthase.

Table 3. **Analysis of green beers produced by the three studied brewers' lager yeast strains (harvested at apparent extract of 3%)**. Fermentation was carried out as indicated in Fig. 3. Except as noted, the results shown are mean values of two independent experiments including standard deviations. The bold lines indicate difference of by-product production in the studied strains.

Strains	**A**	**B**	**C**
Ethanol (g l^{-1})	37.65 ± 0.25	36.30 ± 0.80	37.30 ± 0.07
Glycerol (g l^{-1}) *	1.5	1.5	1.5
pH	4.155 ± 0.015	4.325 ± 0.065	4.185 ± 0.020
Acetaldehyde (mg l^{-1})*	1.76	4.70	2.65
Fusel alcohols			
n-propyl alcohol (mg l^{-1})*	19.3	16.3	20.9
Isoamyl alcohol (mg l^{-1})*	39.2	38.1	47.1
Iso-butyl alcohol (mg l^{-1})*	8.9	6.5	10.6
2-Phenylethyl alcohol (mg l^{-1})	12.10 ± 0.00	13.60 ± 1.60	14.50 ± 1.55
Vicinal diketones			
2,3-pentandione (mg l^{-1})	0.355 ± 0.005	0.090 ± 0.000	0.260 ± 0.060
Diacetyl (mg l^{-1})	0.425 ± 0.005	0.075 ± 0.005	0.255 ± 0.110
VDK (mg l^{-1})	0.780 ± 0.010	0.165 ± 0.005	0.515 ± 0.180
Esters			
Ethyl acetate (mg l-1) *	11.62	11.52	10.6
Butyl acetate (mg l^{-1})	0 ± 0	0 ± 0	0 ± 0
Isoamyl acetate (mg l^{-1})	0.495 ± 0.035	0.408 ± 0.120	0.475 ± 0.030
Isobutyl acetate (mg l^{-1})	0.017 ± 0.008	0.040 ± 0.012	0.030 ± 0.014
2-Phenylethyl acetate (mg l^{-1})	0.150 ± 0.000	0.175 ± 0.025	0.170 ± 0.010
Ethyl butyrate (mg l^{-1})	0.040 ± 0.000	0.045 ± 0.005	0.045 ± 0.000
Ethyl caproate (mg l^{-1})	0.110 ± 0.010	0.085 ± 0.015	0.090 ± 0.010
Ethyl caprate (mg l^{-1})	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Ethyl caprylate (mg l^{-1})	0.170 ± 0.010	0.135 ± 0.015	0.150 ± 0.000
Fatty acids			
Isovalerate (mg l^{-1})	1.58 ± 0.06	1.36 ± 0.11	1.42 ± 0.12
Caproate (mg l^{-1})	1.385 ± 0.005	1.245 ± 0.015	1.265 ± 0.030
2-Ethyl caproate (mg l^{-1})	0.120 ± 0.051	0.150 ± 0.093	0.110 ± 0.065
Caprate (mg l^{-1})	0.23± 0.00	0.26 ± 0.00	0.235 ± 0.02
Caprylate (mg l^{-1})	3.145 ± 0.035	3.035 ± 0.075	2.920 ± 0.010

*) Values were recorded from a single experiment

4.2 Global molecular analyses of the three selected brewers' yeast strains possessing variations in diacetyl production

To understand the genetic basis leading to the difference in diacetyl production in the selected lager brewers' yeast strains, molecular global analyses of the strains were carried out at the level of genome, transcriptome and proteome. The cell samples used for transcriptome and proteome analyses were harvested at apparent extract of 8% of the main fermentation. At this point of time, all strains were in the logarithmic growth and had the same cell densities (Fig. 3).

4.2.1 Genome level: Microarray-based comparative genome hybridization using bottom fermenting yeast DNA microarray

It is well known that lager brewers' yeast is an aneuploid hybrid between *S. cerevisiae* and another *Sacchromyces* yeast, probably *S. bayanus* (Kodama *et al*., 2006; Rainieri *et al*., 2006). The DNA component from *S. cerevisiae* of lager brewers' yeast is often referred to Sc-like type (Sc-, cer-, -CE) whilst its other DNA component is differently denoted as *S. pastorianus* (-Sp), lager (-Lg), non-*S. cerevisiae* (non-Sc) or *S. carlsbergensis* (-CA) (Kodama *et al*., 2006). Here the terms Sc-type and non-Sc-type are consistently used.

Before having a closer look at the results obtained at genome level, it is important to review some recent knowledge regarding the chromosomal structure of lager brewers' yeast. Different studies have shown that lager brewers' yeast genome contains three kinds of chromosomes: Sc-type, non-Sc-type and various chimeral types (Fig. 6) (De Barros Lopes *et al*., 2002; Kodama *et al*., 2006). By applying comparative genomic hybridisation (CGH) with the use of *S. cerevisiae* array, Kodama *et al*. (2006) revealed that different lager brewers' yeast strains could have variable compositions of chromosomes. Concretely, they can contain different types and copy number of some certain chromosomes in their genetic set-up. For example,

most lager yeast strains possess chromosome XVI in two different Sc/non-Sc hybrid types while only few strains contain Sc-type and non-Sc-type ones.

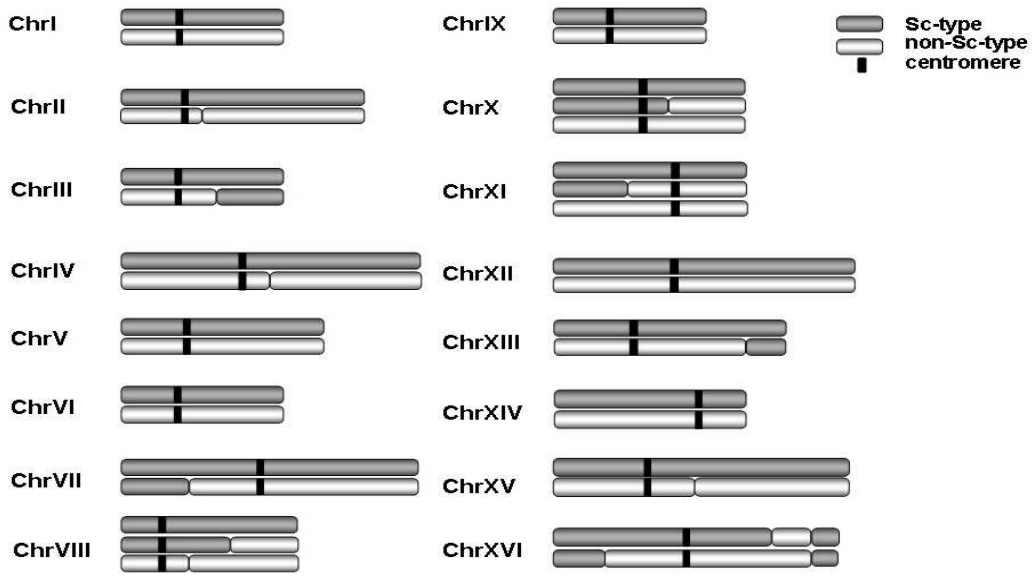

Fig. 6 Putative chromosomal structure of lager brewers' yeast strain Weihenstephan Nr.4 (34/70) (Kodama *et al*., 2006). The break points between Sc-type and non-Sc-type DNA in chromosomes are shown as constrictions.

Furthermore, one notable point about chromosomal structure of lager brewers' yeast is that the pure non-Sc-type of chromosomes III, VII and XVI are mostly not observed. Instead of that, these chromosomes are mostly found in Sc-type and non-Sc/Sc hybrid type (Fig. 6) (Y. Nakao, pers.comm.). In addition, lager brewers' yeast genome is remarkable by the translocations between non-Sc-types of chromosomes II and IV; chromosomes VIII and XV which resulted in the presence of hybrid non-Sc-type between these chromosomes (Fig. 7) (Kodama *et al*., 2006), (Y. Nakao, pers.comm.).

To investigate the genetic basis for the differences in diacetyl production of the threeselected lager brewers' yeast strains at genomic level, we employed microarray-based CGH by means of bottom fermenting yeast DNA microarray to study our strains. In the pairwise comparison, a sequence was designated as "increased" if the

calculated change p-value was ≤ 0.002 and as “decreased” if the change p-value was ≥ 0.998. In general, the genome analysis revealed thousands of significant changes in the studies strains i.e. A vs B (5730); B vs C (6003) and A vs C (2094). These include differences regarding the coding and intergenic regions. This result is consistent to the fact that strain A and C are genetically related and different from strain B.

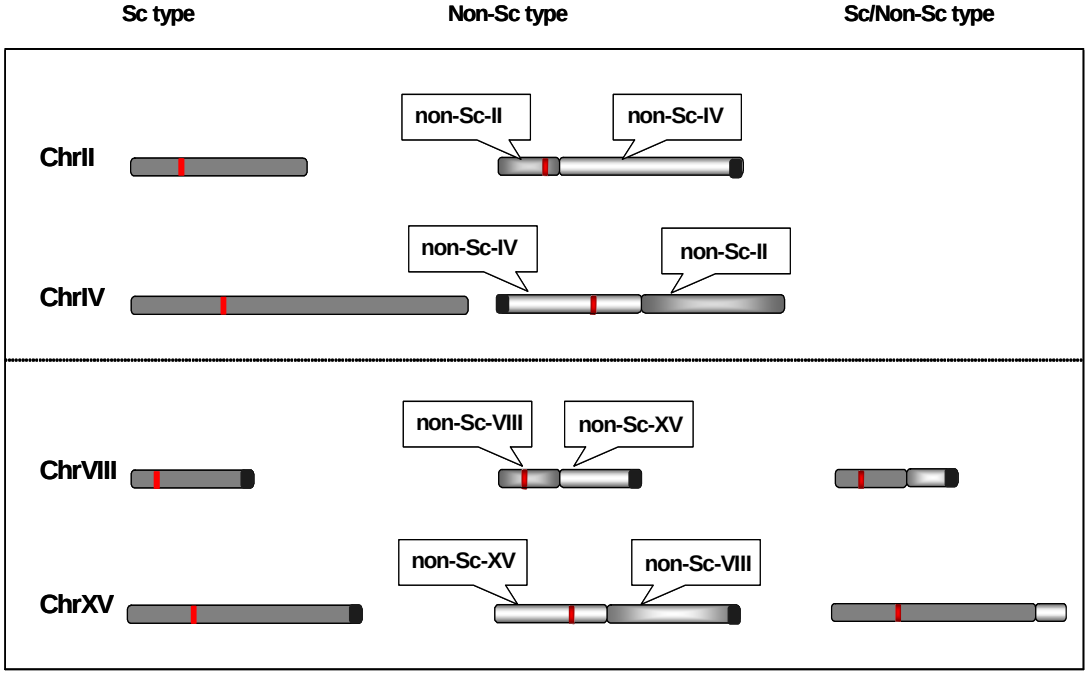

Fig. 7 Translocation between the non-Sc-type chromosomes II and IV and between the non-Sc-type chromosomes VIII and XV in lager brewers' yeast (*Y.Nakao, Genomic analysis of lager brewing yeast and its application to brewing*). The break points between Sc-type DNA and non-Sc-type DNA in chromosomes are shown as constrictions.

In each pairwise strain comparison, $\log_2$ hybridisation ratio for every single gene was calculated. If the DNA components of two strains were identical, a $\log_2$ hybridisation ratio of 0 was expected while deviations of this value indicated variations between the two samples. To estimate the differences in the chromosome constitutions between the compared strains, the $\log_2$ hybridisation ratio of each single ORF was plotted versus its position on the chromosomes, using the gene order of *S. cerevisiae*. Since strains C and A are genetically closely related, we expected that

the genome patterns of strains A and C would display high similarity and be different from that of strain B. The chromosomal comparison, therefore, was focused on two pairs: strain A vs C and strain B vs C.

4.2.1.1 Differences of the studied strains in their chromosome patterns

4.2.1.1.1 Strain A vs strain C

In accordance to the fact that strains A and C are genetically related, microarray-based CGH revealed that the signal intensities of genes on most of chromosomes of these two strains were in fact very similar (Fig. 8B). Comparison of these two strains only showed changes in relative $\log_2$ hybridisation ratios for part of chromosomes VIII and XV (Fig. 8B). The relative $\log_2$ hybridisation ratios of Sc-type and non-Sc-type ORFs signals on other chromosomes were equal to 0 indicating that the two compared strains possess a similar constitution of these chromosomes in their genomes.

In the case of chromosome VIII, $\log_2$ hybridisation ratios of Sc-type ORFs between strains A and C were about 0.45 (Fig. 8B). These $\log_2$ hybridisation ratios corresponded to a hybridisation ratio of 1.36, suggesting that strain A contains more copy numbers of Sc-type chromosome VIII than strain C. For example, strain A may contain 4 copies while strain C may possess 3 copies of this chromosome.

Log_2 hybridisation analysis showed one region of no difference in Sc-type chromosome XV while in the other parts, the relative $\log_2$ hybridisation ratio was equal to 0.42 corresponding to the relative hybridisation of 1.34 (Fig. 8B). The region of no signal difference spread from the gene y0r343w to yor065c, similar to the previously described “jump region” or the translocation points on chromosome XV in brewers’ yeast (Bond *et al*., 2004). As lager brewer’ yeast has three types of chromosome XV: Sc-type, non-Sc and hybrid Sc/non-Sc types (Fig. 7) I deduced that genome of strain A might have more copies of the hybrid type of chromosome XV than strain C.

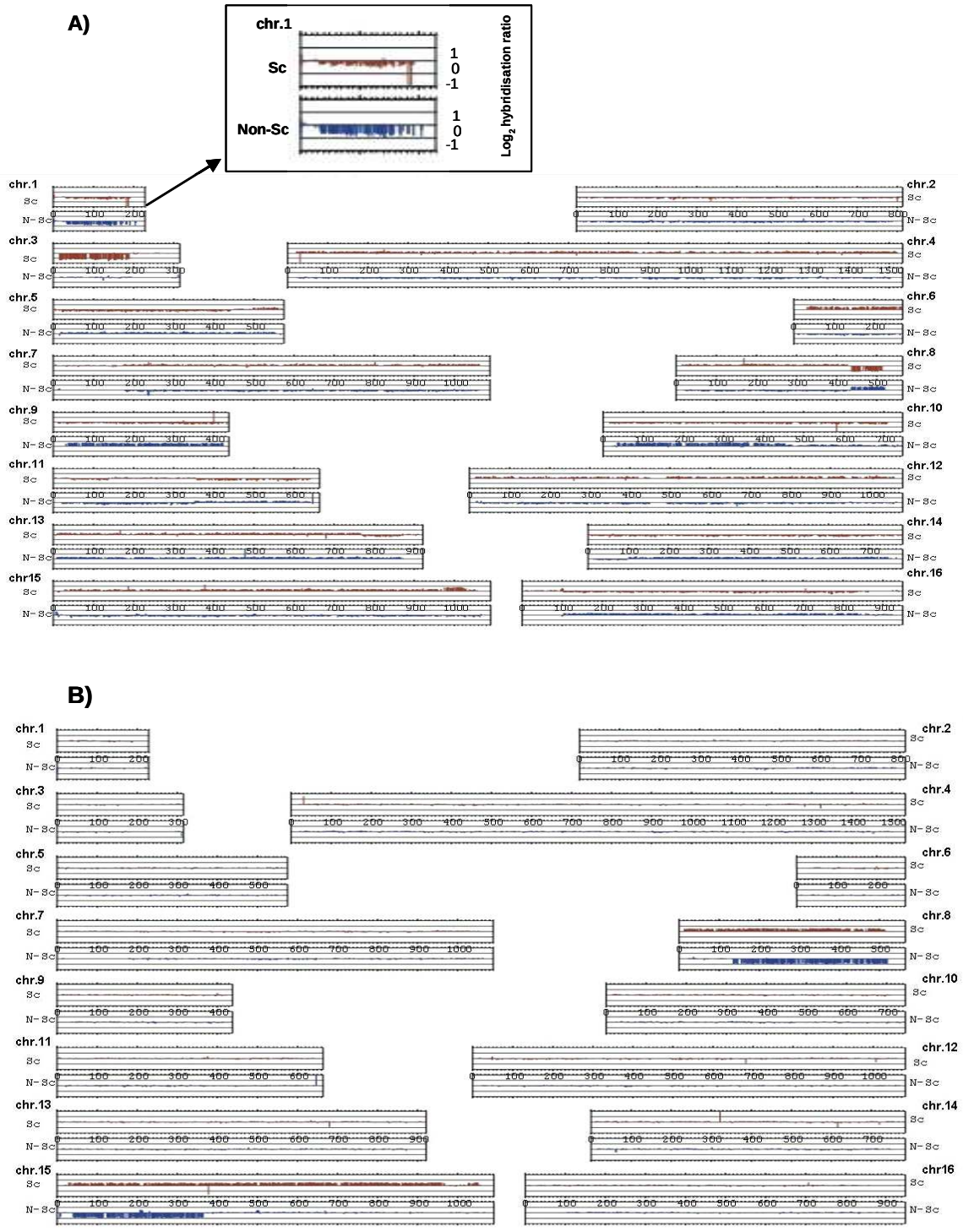

Fig. 8 Array-based genomic comparison of the studied lager brewers' yeast strains by means of bottom fermenting yeast DNA microarray. A) Strain B vs C. B) Strain A vs C. In each pairwise comparison, the log_2 hybridisation ratio of each ORF was plotted versus its position on chromosome**.** The red and blue colours indicate the log_2 hybridisation ratios of Sc-type and non-Sc-type ORFs, respectively. The points where signal show abrupt changes are considered sites of recombination which gave rise to chimeral chromosomes and are simply denoted as translocation points.

Non-Sc type chromosome VIII and non-Sc-type XV in A vs C comparison showed regions of relative $\log_2$ hybridisation of -1 respectively on the left side and right side (Fig. 8B). That means the hybridisation signals of the ORFs located on these regions in this chromosome of strain C were two times higher compared to strain A. As aforementioned, the non-Sc-type chromosomes VIII and XV lager brewers' yeast are present as the heterogenic hybrid types in comparison to *S. cerevisiae* chromosomes (Fig. 7). Based on this knowledge, I deduce that strain C contains a higher copy number of non-Sc type chromosomes XV compared to strain A.

4.2.1.1.2 Strain B vs strain C

A number of slight differences in relative hybridisation ratios were observed in chromosomal comparison between strains B and C. These included differences in chromosomes II, IV, V, VII, XI, XII, XIII, XIV and XVI (Fig. 8A). As a high percentage of ORFs on these chromosomes have been detected as "not changed" in the comparison of the two strains, we concluded that these differences were not due to the difference in chromosomes copy numbers. It is more likely that these differences resulted from the varying specificities of the probesets of the bottom fermenting yeast DNA microarray to the genomes sequences of the two compared lager brewers' strains.

The most striking differences in strains B and C comparison were found on chromosomes I, III, VI, VIII, IX, X and XV (Fig. 8A). Based on the relative hybridisation signals and the chromosome structure of lager brewers' yeast, we suppose some differences in chromosomes constitutions between strains B and C (Fig. 9).

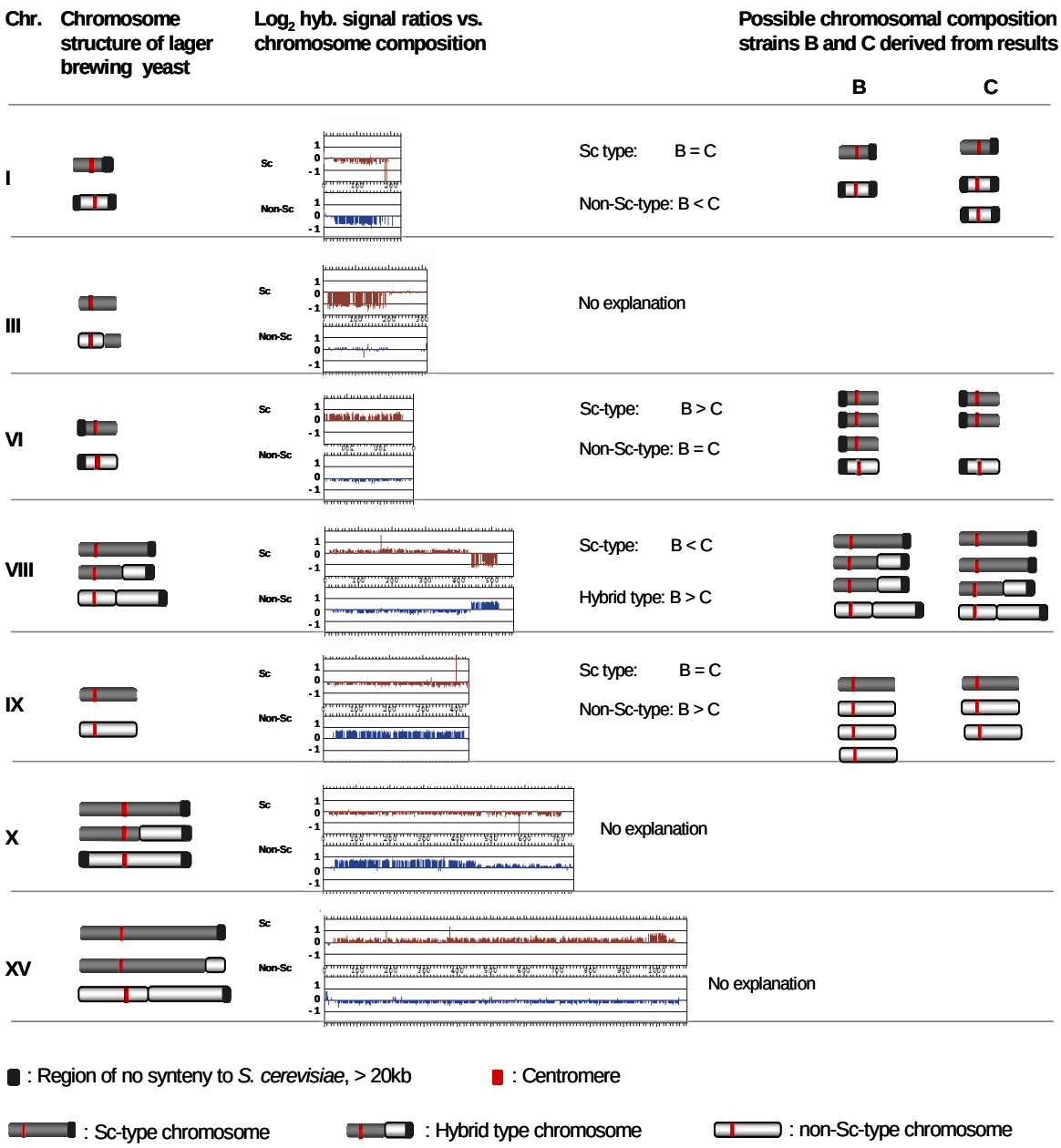

Fig. 9 Chromosomal differences and possible compositions of some chromosomes in strains B and C. The red and blue colours indicate the $\log_2$ hybridisation signal ratios of Sc-type ORFs and non-Sc-type ORFs, respectively. The points where signal show abrupt changes are considered sites of recombination which gave rise to chimeral choromosomes and are simply denoted as translocation points.

Regarding chromosome VIII, both Sc-type and non-Sc-type showed regions of no change in its left side in B vs C comparison (Fig. 9). In contrast, there was one region on the right side of Sc-type chromosome VIII which exhibit a relative hybridisation ratio of 2 times lower in strain B compared to strain C. In addition, another region on

the right side of non-Sc-type chromosome VIII with the relative ratio of about 1.5 times higher in strain B compared to strain C was observed (Fig. 9). Based on the fact that the chromosome VIII of lager brewers' existed in three types: Sc, non-Sc, Sc/non-Sc hybrid types (Fig. 6) (Rainieri *et al*., 2006), we assumed that strains B and C contained the same copy numbers of non-Sc-type chromosome VIII. In addition, strain B contained more copies of hybrid type while strain C possessed a higher copy number of Sc-type chromosome VIII (Fig. 9).

Comparison of chromosomes I, VI, IX of two strains B and C showed that the relative hybridisation signal ratios of one type of these chromosomes (either Sc-type or non-Sc-type) was the same while the other type was different. Since there was no hybrid type in these chromosomes, we assumed that the two compared strains possessed the same copy numbers in either Sc-type or non-Sc type but different copy numbers in the other type of these chromosomes. For example, relative Sc-type hybridisation ratio on chromosomes IX was equal to 0 while of relative non-Sc-type hybridisation signal ratio was higher in strain B. Thus, we presumed that regarding chromosome IX, strain B had the same copy numbers of Sc-type and more copies of non-Sc-type compared to strain C. As the relative non-Sc-type hybridisation signal ratio was about 1.5 times higher in strain B, it was supposed strain B contained 3 copies while strain C possessed 2 copies of the non-Sc-type chromosome IX (Fig. 9).

Chromosomes III, X, XV displayed the same relative hybridisation ratio patterns. Relative hybridisation signal of either Sc-type or non-Sc type were the same in both strains while that of other type (either Sc or non-Sc) was only similar in one region of these chromosomes (Fig. 9). So far, we do not have a reasonable explanation for these differences on chromosomal level.

4.2.1.2 Identification of differences in copy number of known genes relevant to diacetyl formation and flocculation

The genes identified as different in the studied strains at genomic level were incorporated to yeast pathways listed by *Saccharomyces* Genome Database (SGD) using the Microsoft Access software. As diacetyl is the by-product of the valine biosynthetic pathway, we at first focused on comparing the hybridisation ratios of genes encoding enzymes participated in this pathway in the selected strains (Fig. 10). Based on the results obtained in microarray-based CGH, differences related to the valine biosynthetic pathway were detected including Sc-*IVL6,* Sc-*BAT1* and non-Sc-*BAT1* (Fig. 10). All of these ORFs were found to be different in both B vs C and B vs A pairwise comparisons (Fig. 10). This result is consistent to the fact that the diacetyl production of strain B strikingly differences from those of strains A and C (Fig. 4).

Among the studied strains, strain A produced the slightly higher of level of diacetyl than strain C while diacetyl production of strain B was much lower than those of strains A and C. In accordance to this fact, the level of Sc-*BAT1* hybridisation signal was highest in strain A and lowest in strain B (Fig. 10). Since the difference in hybridisation signal of each ORF is directly related to the difference in gene copy numbers, from the hybridisation ratios, I deduced that strains A, C and B might contain three, two and one copies of Sc-*BAT1* ORF, respectively. The pairwise comparison of hybridisation signals of non-Sc-*BAT1* and Sc-*ILV6* genes also suggested that the three studied strains possessed different copy numbers of these genes. Concretely, strain B might contain one copy whilst strains A and C might contain two or three copies of *Sc-ILV6* gene*.* These differences in gene copy number might be the reason for the different expression levels of these genes and thus for difference in diacetyl phenotype of the studied strains. However, this assumption has to be confirmed by incorporating with results obtained from other analyses at transcriptome and proteome levels.

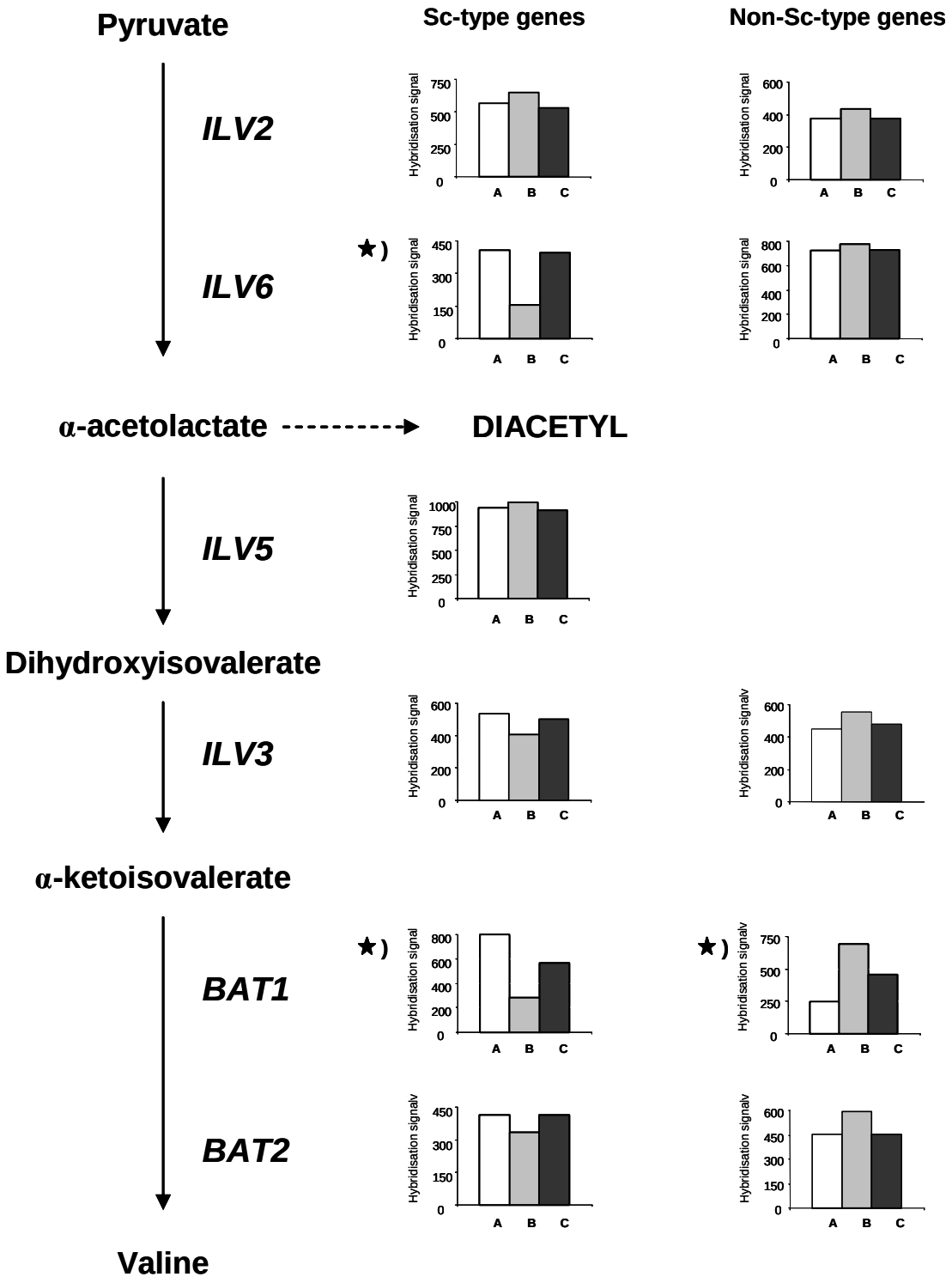

Fig. 10 Microarray DNA hybridisation signals of genes encoding enzymes of the valine biosynthetic pathway in the studied lager brewers' yeast strains. The microoarray-based CGH among the studied strains was performed using bottom fermenting yeast DNA microarray. The stars indicate significant differences in the studied strains. Signinicant differences were selected with change p-value ≤ 0.002 or ≥ 0.998. *ILV2, ILV6*: acetohydroxyacid synthase, *ILV5*: reductoisomerase, *ILV3*: dihydroxyacid dehydratase; *BAT1, BAT2*: branched-chain amino acid transaminase.

Besides the differences directly related to diacetyl formation, global analysis at genome level also revealed differences in copy numbers of flocculation genes in the studied strains (Table 4). The comparison between the highly flocculent strain B and other strains revealed many differences including Sc-*FLO1*, non-Sc-*FLO1*, Sc-*FLO8,* non-Sc-*FLO9*, non-Sc-*FLO10*, *Sc-FLO11*, non-Sc-*FLO11* (Table 4). Among those, the sequence of non-Sc-*FLO10* (id-fix Lg_4227_1) was found to be present only in strain B. In addition, except for Sc-*FLO1* (id-fix Sc_2439_1) other genes showed higher hybridisation ratios in strain B than in both strains C and A, suggesting that strain B contained more copies number of flocculation genes than the two other strains. No difference was detected in the comparison between strains A and C. This result was reasonable since flocculation phenotype of the strains A and C was similar.

Table 4. **Differences of genes belonged to the flocculation gene family identified at genome level in the studied lager yeast strains.** The DNA analysis was performed using microarray-based CGH by means of bottom fermenting yeast DNA microarray. Black sheets indicate changes in the pairwise strain comparison. Changes were selected based on a change p-value ≤ 0.002 or ≥ 0.998. Some sequences, e.g. *FLO1* appeared several times as the results being divided in two halves or more because of frame shift and stop codon mutation. Gene was detected as present based on a detection p-value of 0.05.

				Gene detection			**Hybridisation ratios**		
Gene name	**Gene type**	**id_fix**	**sys.gene**	**A**	**B**	**C**	**A/B**	**A/C**	**C/B**
FLO1	Sc	Sc_2439_1	YAR050W	P	P	P	-1.5	1.1	-1.7
FLO1	Sc	Lg_6958_1	YAR050W	P	P	P	1.9	-1.1	1.9
FLO1	non-Sc	Lg_1617_1	YAR050W	P	P	P	1.6	1.1	1.6
FLO1	non-Sc	Lg_3309_1	YAR050W	P	P	P	1.2	1.0	1.2
FLO1	non-Sc	Lg_3309_1	YAR050W	P	P	P	1.3	-1.1	1.3
FLO1	non-Sc	Lg_4229_1	YAR050W	P	P	P	1.3	1.0	1.4
FLO8	Sc	Sc_4622_1	YER109C	P	P	P	1.1	1.0	1.2
FLO9	Sc	Lg_427_1	YAL063C	P	P	P	-1.4	1.1	-1.4
FLO9	non-Sc	Lg_934_2	YAL063C	P	P	P	1.2	1.0	1.2
FLO10	non-Sc	Lg_4227_1	YKR102W	A	P	A			
FLO10	non-Sc	Lg_4227_2	YKR102W	P	P	P	1.2	1.0	1.2
FLO11	Sc	Sc_1180_1	YIR019C	P	P	P	1.3	1.0	1.3
FLO11	Sc	Sc_1180_2	YIR019C	P	P	P	1.3	1.0	1.2
FLO11	Sc	Lg_2876_1	YIR019C	P	P	P	-1.4	1.1	-1.5
FLO11	non-Sc	Lg_2876_2	YIR019C	P	P	P	-1.3	1.0	-1.4

*) Gene detection: A means absent, P means present

To conclude, by using microarray-based CGH, we identified differences regarding the type and copy number of some chromosomes between the studied strains. Differences directly related to diacetyl and flocculation phenotypes were determined. The differences relating to diacetyl phenotype will be integrated with the results obtained at the level of transcriptome and proteome for identifying target genes for reduction of diacetyl production in lager yeast.

4.2.2 Transcriptome level: Microarray-based comparative transcriptome analysis

4.2.2.1 Transcriptome analysis using bottom fermenting yeast DNA microarray

To identify the genetic basis for the phenotypic differences at transcriptome level, we carried out comparative microarray-based transcriptome analysis on the selected lager brewers' yeast strains. The transcriptome profiles of lager brewers' yeast strains were examined using yeast cells harvested at apparent extract of 8% of the main fermentation. Customized bottom fermenting yeast DNA microarrays were used in order to differentiate the expressional levels of Sc-type and non-Sc-type genes.

Transcriptome of each strain was studied in technical triplicates, i.e. total RNA of each strain was isolated in three replicates from cells harvested from a single fermentation. Mean values and statistical analysis for triplicate microarray hybridisations were calculated for each strain. In each pairwise comparison, the logged average fold-changes were calculated and statistical analysis was performed using Cyper-T approach with a false discovery rate of 0.001.

Using this criterion, 1851 significant differences were identified at transcriptome level among the three studied strains. The number and categories of significant differences in each pairwise comparison are shown in Table 5. In general, the comparison of strain B to either strain A or C revealed more than 1000 significant differences while the number of significant differences in the A vs C comparison was much lower (rougly 300). More than 600 common significant differences were found

in both A vs B and B vs C comparison (data not shown). The results were consistent to the fact that strains A and C are genetically related. Transcriptional profiling also revealed differences regarding SGD-type genes i.e. Sc-type ORFs from SGD database that are not present in WH34/70, in the studied strains (Table 5). Furthermore, differences reagrding several *S. patorianus* sequences published in the Genbank, intergenic regions and other sequences, which showed similarity to S. *cerevisiae* proteins by NCBIblastX homology searching, were also detected at transcriptomic level. There were no differences regarding mitochondrial genes among the studied strains.

Table 5. Number of significant differences identified at transcriptome level among the studied lager yeast strains via analysis using bottom fermenting yeast DNA microaray. Significant differences were chosen with a false discovery rate of 0.001. Sc: *S. cerevisiae* type; non-Sc: non-*S. cerevisiae* type; SGD-type: Sc-type sequences from SGD database which are not detected in the genome sequence of the lager brewers' yeast strain WH34/70. Others: sequences from *S. pastorianus* published in Genbank, intergenic sequences, sequences which showed similarity to *S. cerevisiae* proteins by NCBIblastX homology searching

Pairs	Type genes	No of significant differences	
A vs B	Sc-type	488	1176
	Non-Sc-type	471	
	SGD-type	20	
	Others	197	
A vs C	Sc-type	86	338
	Non-Sc-type	181	
	SGD-type	3	
	Others	68	
C vs B	Sc-type	560	1236
	Non-Sc-type	436	
	SGD-type	16	
	Others	224	

4.2.2.2 Identification of differences at transcriptional level of genes relevant to diacetyl and flocculation

The identified differentially expressed ORFs between the studied strains have been further analysed using the Microsoft Access software and *Saccharomyces* Genome Database (SGD). The differentially expressed ORFs in the studied strains were incorporated into 90 of the total 156 biological pathways listed in SGDs. Valine biosynthetic pathway (from which diacetyl is formed as a by-product) was one of the pathways which showed many differences in the studied strains. These included Sc-*ILV6,* Sc-*BAT1,* non-Sc-*BAT1* and non-Sc-*BAT2* (Fig. 11). For data evaluation, the result of genomic analysis of genes participated in valine biosynthesis were incorporated with result of transcriptome level (Fig. 11).

It is shown from (Fig. 11) that Sc-*BAT1* gene and Sc-*ILV6* transcript were both less abundant in strain B in comparison to strains A and C. In addition, the abundance of non-Sc-*BAT1* ORF and transcript were both lowest A and highest strain B. This positive correlation between genome and transcriptome analyses suggested that the differences regarding abundance of Sc-*ILV6*, Sc-*BAT1* and non-Sc-*BAT1* transcripts among the studied strains might result from the differences in the gene copy numbers.

Besides that, the result at transcriptional level regarding non-Sc-*BAT2* ORF did not match that of genome analysis. In the genome analysis, non-Sc-*BAT2* gene was identified as “not change” in the studied strains. However, at mRNA level it was about two-fold higher in strain B compared to strains A and C. Thus, the difference of non-Sc-*BAT2* gene cannot be due to the difference in gene copy numbers but it rather resulted from other factors such as differences in transcriptional regulation, promoter strength or mRNA stability.

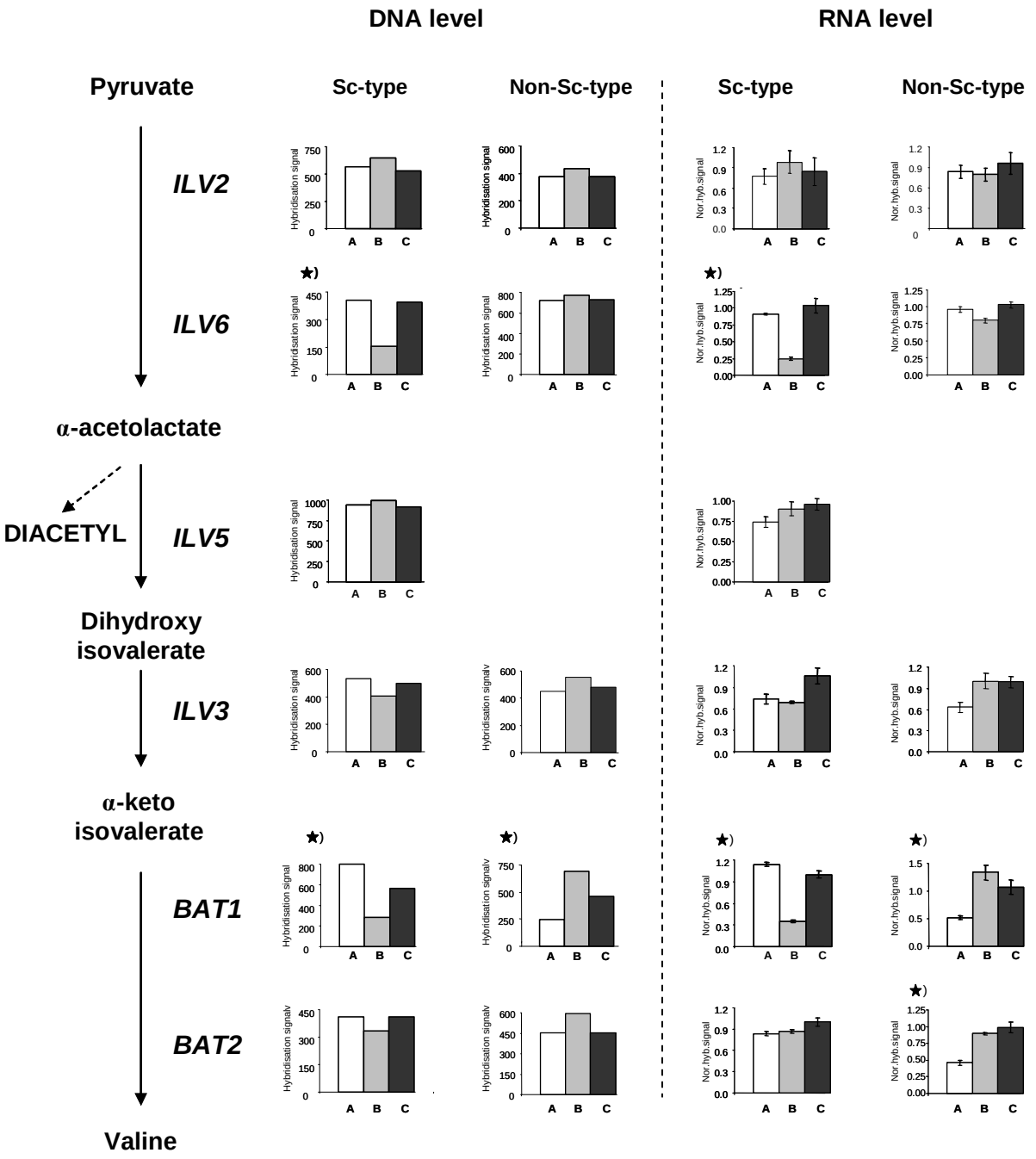

Fig. 11 Microarray-based DNA and microarray-based normalised RNA hybridisation signals of genes encoding enzymes involved in the valine biosynthetic pathway in the studied strains. The experiments were performed using bottom fermenting yeast DNA microarrays. In each strain analysis, the hybridisation signals at DNA level were obtained from a single array hybridisation whilst the normalized hybridisation signals at RNA level were the average of normalized signals from triplicate array hybridisations with standard deviations. The stars indicate significant differences in the studied strains. Significant differences at DNA level were chosen with change p-value ≤ 0.002 or ≥ 0.998. Significant differences at RNA level were selected based on a false discovery rate of 0.001.

Nor.hyb.signal: normalised hybridisation signal. For each ORF, the Sc-type and non-Sc-type signals were generated. *ILV5* non-Sc-type ORF and transcript are absent since the bottom fermenting yeast DNA microarray does not contain probeset for this ORF. *ILV2, ILV6*: acetohydroxyacid synthase, *ILV5*: reductoisomerase, *ILV3*: dihydroxyacid dehydratase; *BAT1, BAT2*: branched-chain amino acid transaminase

Table 6. Differences in flocculation genes identified at level of genome and transcriptome in the studies strains. The genome and transcriptome analyses were performed using comparative microarray analysis by means of bottom fermenting yeast DNA microarray**.** Bold sheets indicate significant changes or differences in a pairwise comparison. Changes at DNA level were chosen with a change p-value ≤ 0.002 or ≥ 0.998. Significant differences at RNA level were selected based on a false discovery rate of 0.001. Some sequences appeared more than once as the result of being divided in two halves or more because of frame shift or stop codon mutation. For gene detection and transcript flag, P means present; A means absent; A, P: ambiguously detected (either present or absent).

			DNA level						**RNA level**					
			Gene detection			**Hybridisation ratios**			**Transcript flags**			**Hybridisation ratios**		
Gene name	**Gene type**	**id_fix**	**A**	**B**	**C**	**A/B**	**A/C**	**C/B**	**A**	**B**	**C**	**A/B**	**A/C**	**C/B**
FLO1	Sc	Sc_2439_1	P	P	P	-1.5	1.1	-1.7	P	P	P	-6.3	-1.8	-3.5
FLO1	Sc	Lg_6958_1	P	P	P	1.9	-1.1	1.9	P	P	P	1.6	1.3	1.2
FLO1	non-Sc	Lg_1617_1	P	P	P	1.6	1.1	1.6	P,A	P, A	P	1.1	-1.2	1.4
FLO1	non-Sc	Lg_3309_1	P	P	P	1.2	1.0	1.2	P	P	P	1.2	1.2	1.0
FLO1	non-Sc	Lg_3309_1	P	P	P	1.3	-1.1	1.3	P	P	P	1.0	1.2	-1.3
FLO1	non-Sc	Lg_4229_1	P	P	P	1.3	1.0	1.4	P	P	P	1.4	1.3	1.1
FLO8	Sc	Sc_4622_1	P	P	P	1.1	1.0	1.2	P	P	P	1.2	1.0	1.2
FLO9	Sc	Sc_2692_1	P	P	P	1.0	1.0	-1.1	P	P	P	-91.3	1.2	-111.0
FLO9	Sc	Lg_427_1	P	P	P	-1.4	1.1	-1.4	P	P	P	-37.3	1.8	-68.5
FLO9	non-Sc	Lg_934_2	P	P	P	1.2	1.0	1.2	P	P	P	-1.2	1.3	-1.6
FLO10	non-Sc	Lg_4227_1	A	P	A				P,A	P	P,A			
FLO10	non-Sc	Lg_4227_2	P	P	P	1.2	1.0	1.2	P	P	P	1.4	1.3	1.1
FLO11	Sc	Sc_1180_1	P	P	P	1.3	1.0	1.3	P	P	P	-2.4	1.4	-3.3
FLO11	Sc	Sc_1180_1	P	P	P	1.1	-1.1	1.1	P	P	P	-2.5	1.3	-3.3
FLO11	Sc	Sc_1180_2	P	P	P	1.3	1.0	1.2	P,A	P	P,A	-2.8	1.8	-4.9
FLO11	Sc	Lg_2876_1	P	P	P	-1.4	1.1	-1.5	P	P	P	-2.1	1.0	-2.2
FLO11	non-Sc	Lg_2876_2	P	P	P	-1.3	1.0	-1.4	A	P, A	A	-2.2	1.2	-2.6

Regarding the flocculation phenotype, no difference was observed at transcription level in the comparison between strains A and C. However, many flocculation genes were found to be up-regulated in strain B compared to strains A and C (Table 6). These included Sc-*FLO1,* Sc-*FLO9,* non-Sc-*FLO9,* non-Sc-*FLO10*, Sc-*FLO11,* non-Sc-*FLO11*. Among those, Sc-*FLO9* (id-fix Sc_2692_1) was more than 90-fold up regulated in strain B in compared to strains A and C. The result was consistent with the fact that strain B flocculates much earlier than strains A and C during the main fermentation (see II.4.1.3). In this data set, the detected difference of non-Sc-*FLO10* (id-fix Lg_4227_1) in the studied strains was due to the absence of this sequence in

genome of strains A and C. Interestingly, in A vs B and B vs C comparisons, some genes which were not more than 2-fold changed at genomic level showed big differences at transcriptional level, i.e. *Sc-FLO1*, id_fix Sc_2349_1 (6.3-fold lower in A vs B, 4-fold lower in C vs B); Sc-*FLO9*, id-fix Sc_2692_1 (91-fold lower in A vs B, 111-fold lower in C vs B), non-Sc-*FLO9*, id-fix Lg_427_1 (37-fold lower in A vs B, 69-fold lower in C vs B comparisons). This result suggested that the differences in the expression levels of these genes should result from factors such as differences in transcriptional regulation, promoter strength or mRNA stability.

To sum up, the microarray-based comparative transcriptome analysis revealed many significant differences directly relevant to diacetyl and flocculation behavious in the studied strain. The identified differences directly related diacetyl formation included Sc-*ILV6,* Sc-*BAT1,* non-Sc-*BAT1* and non-Sc-*BAT1*. The differences regarding the abundance of these transcripts might be responsible for the difference in diacetyl production of the studied strains. Nevertheles, integration of the result at transcriptome level with the result obtained at proteome levels is needed for the identification of potential target genes for reducing diacetyl production in lager brewers' yeast.

4.2.3 Proteome level: Comparative proteome analysis using two-dimensional gel electrophoresis

4.2.3.1 Identification of protein spots which showed significant different intensities among the three studied lager brewers' yeast strains

To identify genetic basis for differences in diacetyl production of the studied lager brewers' yeast strains at proteome level, two-dimensional (2D) gel analysis was performed. Protein samples were isolated from the cells harvested at apparent extract of 8% from two independent fermentations. The 2D gel electrophoresis was carried out in triplicate as described in the materials and methods section. The 2D gels of brewers' yeast strains were scanned and analysed using the Delta 2D

software version 4.0. Separation of total protein by 2D gel electrophoresis resulted in the detection of 520 spots in total in the 2D gel of each studied strain (Fig. 12). Statistical analysis was performed allowing a standard deviation ≤ 30% for each spot from the three replicates and a *p*-value of 0.05 or below in the Student's t-test at pairwise comparison. Using these criteria, 9 spots were identified as more than 2-fold significantly different among the three studied lager yeast strains. Among these 9 spots, 4 spots were identified in A vs B comparison, 4 spots were identified as significantly different in B vs C comparison and 1 spot was identified as significantly different in the comparison between B vs A/C (Fig. 13).

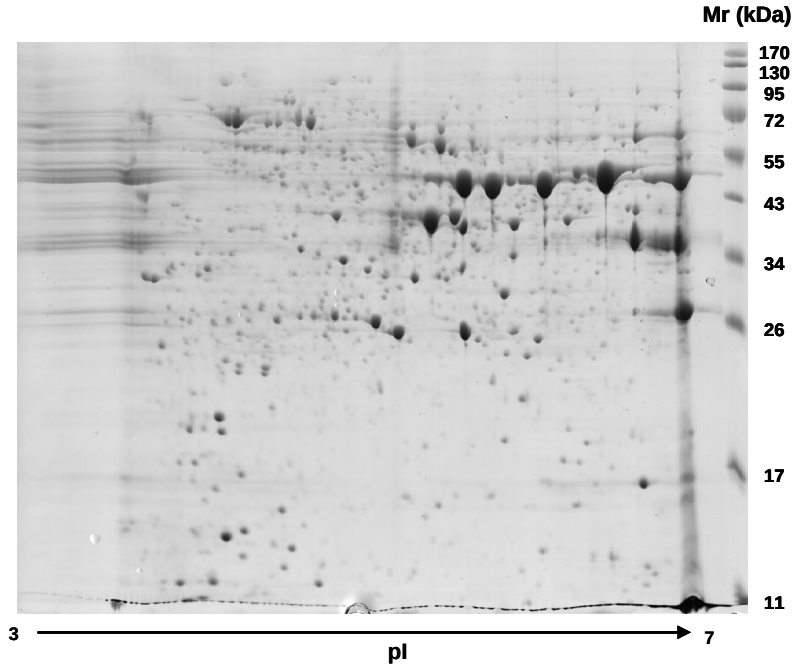

Fig. 12 Two-dimensional gel image of a lager brewers' yeast strain (strain C) at apparent extract of 8%. Gel was stained with Comassie Brillant Blue.

4.2.3.2 Mass spectrometry identification of differentially expressed proteins

Protein spots identified as different in studied strains were excised from the gels and characterised using MALDI-TOF MS. Among the 9 spots analysed by MALDI-TOF MS, spots 1-8 were positively identified with a protein score of at least 100 and a sequence coverage ≥ 30% for duplicate identification (Table 7). Spot 9

was identified as Eno2p with protein sequence coverage of about 25%. As this sequence coverage was quite low, it was considered as being ambiguously identified. In addition, it is well known that lager brewers' yeast is a hybrid of two *Saccharomyces* yeast, thus it proteome may contain two version of certain proteins, Sc-type and non-Sc-type. As a protein database of lager brewers' yeast has not been available, Mascot search engine of peptides generated in the MALDI-TOF experiment was only performed with the protein database of *S. cerevisiae*. Due to that fact, a protein of non-Sc may not be detectable as in the case of spot 9.

Table 7. MALDI-TOF mass spectrometry identification of significantly different protein spots detected in the proteome comparisons of the three studied lager yeast strains

Spots	Accession No	Name	pI	MW (kD)	Mowse Score	Protein coverage (%)	Identification
1	gi|6321968	*ENO2*	5.67	46.89	576	61.09	Eno2p [*S. cerevisiae*]
2	gi|6322790	*FBA1*	5.51	39.60	942	68.8	Fructose 1,6-bisphosphate aldolase
3	gi|10383781	*PGK1*	7.11	44.71	514	66.35	3-phosphoglycerate kinase
4	gi|151942494	*HSP31*	5.26	25.62	500	82.7	heat-shock protein
5	gi|10383781	*PGK1*	7.11	44.71	266	52.59	3-phosphoglycerate kinase
6	gi|151944335	*SSB2*	5.32	66.55	546	53.51	stress-seventy subfamily B protein
7	gi|151941387	*SSA1*	5	69.60	506	46.88	stress-seventy subfamily A protein
8	gi|6321968	*ENO2*	5.67	46.89	422	38.92	Eno2p [*S. cerevisiae*]
9	gi|157830958	*ENO2*	6.04	46.60	447	25%	Ambiguously identified

The eight spots identified by MALDI-TOF included two stress-seventy subfamily proteins (Ssa1p, Ssb2p), one heat shock protein (Hsp31p) and three proteins participating in the glycolytic pathway (Fba1p, Eno2p, Pgk1p). Some spots at different positions on the 2D gels were identified as one protein, i.e. spots 1 and 8 were identified as Eno2p, spots 3 and 5 were detected as Pgk1p. Spot 5 and spot 8 appeared respectively to be fragments of Pgk1p and Eno2p since their molecular weights were smaller than those of corresponding proteins.

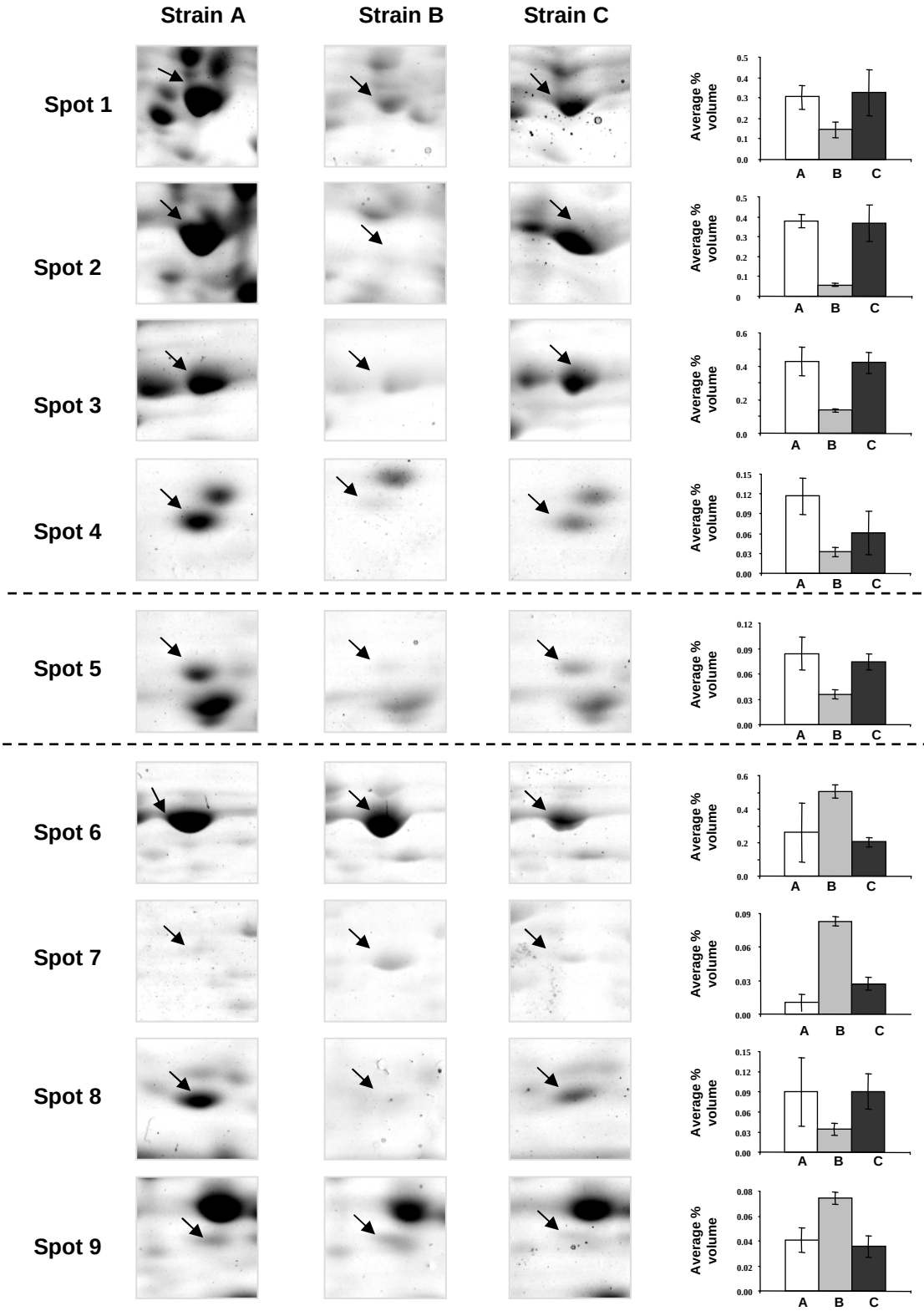

Fig. 13 Significant differentially expressed protein spots among the three studied lager brewers' yeast strains at apparent extract of 8% detected in 2D gels. Significant differences were identified with *p*-value of 0.05, a spot standard deviation ≥ 30% and 2 fold-change regulated. Spot 1-4: significant differences between strain A and B. Spot 6-9: Significant differences between strain C and B. Spot 5 was the significant difference identified between strain B vs strains A and C.

All of these 9 spots were shown to be different in the comparison of strain B versus strains A and C. Spots 1, 2, 3, 4, 5 and 8 were shown to be at the highest level while spots 6, 7, 9 were least abundant in strain B. No significant difference was observed in proteome comparison between strains A and C. In addition to the global genetic analyses at transcriptomic and genomic levels, the result obtained at proteome level once again fitted well to the fact that strains A and C are closely related and are phenotypically different from strain B.

To conclude, global analysis at proteome level only revealed differences regarding glycolytic and stress proteins. None of these differences directly related to the diacetyl and flocculation phenotypes.

4.3 Sc-*ILV6*, a potential novel target gene for reducing diacetyl production in brewers' yeast

Global molecular analyses only revealed differences directly relevant to diacetyl and flocculation phenotypes at level of genome and transcriptome. Among those, Sc-*ILV6* is one of the most promising targets for the reduction of diacetyl production. Significant differences regarding this ORF were identified at both genome and transcriptome levels in the studied strains. Sc-*ILV6* is proposed to encode a regulatory subunit of acetohydroxyacid synthase (AHAS, Ilv2p). AHAS catalyzes the conversion of pyruvate to α-acetolactate which is the precursor of diacetyl. Compared to strains A and C, strain B contains a lower copy number of *Sc-ILV6* ORF and a lower level of Sc-*ILV6* transcript. The lower concentration of Sc-*ILV6* mRNA in strain B might be responsible for a lower activity of AHAS and thus for the lower levels of α-acetolactate and diacetyl in this strain.

4.4 *In vitro* acetohydroxyacid synthase activity in the studied strains

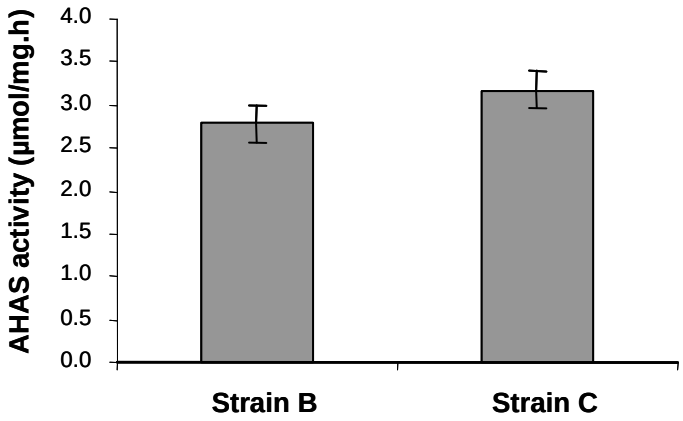

Fig. 14 *In vitro* activity of acetohydroxyacid synthase (AHAS or Ilv2p) in strains B and C. AHAS activity was measured using permeabilized cell proteins. For this experiment, fermentation was carried out in 100 ml bottle using 11.38°P brewers' wort at 12°C. Permeabilized cell proteins were prepared from cells harvested at apparent extract of 8% of the fermentation. Results shown are mean values of two independent experiments including standard deviations.

To demonstrate the hypothesis that the lower level of Sc-*ILV6* transcript in strain B compared to strain A and C could lead to the lower AHAS activity in this strain (section II.4.3), *in vitro* AHAS activity in two strains B and C was measured. *In vitro* AHAS activity in strain B was only slightly different from that in strain C (about 87%) (Fig. 14). In spite of this result, we decided to delete the Sc-*ILV6* in strain C to verify its role in the formation of diacetyl in brewers' yeast.

4.5 Disruption of Sc-*ILV6* in strain C for reduced diacetyl production

4.5.1 Deletion of the first Sc-*ILV6* gene copy in strain C: generation of a *Sc-ilv6Δ* single deletion strain

Disruption of Sc-*ILV6* in strain C was mediated via homologous recombination. The first copy of Sc-*ILV6* in strain C was deleted by using a loxP-kanMX-loxP disruption cassette amplified from the plasmid pUG6 (Gueldener *et al*., 2002).

The disruption cassette consisted of the kanMX module which was flanked by two direct repeated 34 bp loxP sequence. The kanMX module itself consisted of *kan*^{r} gene flanked by the *TEF* promoter and terminator originated from filamentous fungus *Ashybya gossypii*. The *kan*^{r} gene of the kanMX module originated from *E.coli* transposon Tn 903. Insertion of the loxP-kanMX-loxP module into the genome of brewers' yeast would confer the resistance to the antibiotic Geneticin 418 (G418) to this strain. Once inserted to the yeast genome, the kanMX marker can be rescued by transformation of a plasmid expressing Cre recombinase under control of *GAL* promoter. Upon growth on galactose, Cre recombinase action at the repeated loxP sites would excise the kanMX marker, leaving behind one loxP sequence at the site of the Sc-*ILV6* disruption cassette.

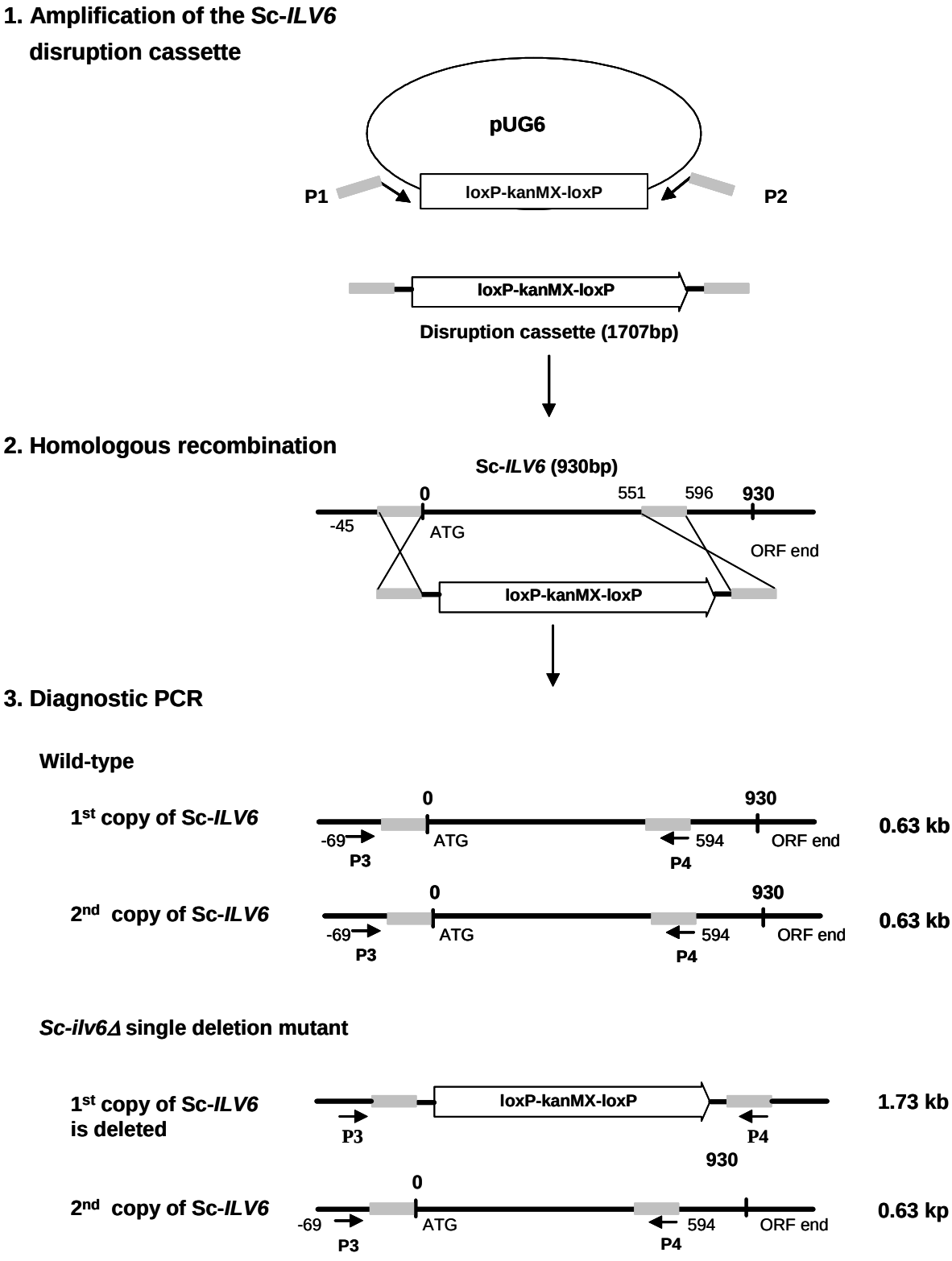

Fig. 15 Disruption of the first copy of Sc-*ILV6* in strain C. Gray bars indicate 45 bp homologous regions needed for mediating the homologous recombination. The integration was verified by diagnostic PCR using primers P3 and P4.

The 1.7 kb loxP-kanMX-loxP cassette was generated via PCR using recombinogenic primers P1 and P2 (see materials and methods). The forward P1 and reverse P2 primers respectively contained 18 bp and 20 bp homologous to plasmid pUG6 at 3' end which were necessary for PCR amplification of the disruption cassette. At the 5' ends, the two primers contained 45 bp homologous to brewers' yeast sequences. The 45 bp at the 5'end of P1 primer was designed as the sequence flanking the left side of Sc-*ILV6* gene while the 45 bp at 5'end of P2 primer was designed as the sequence at position -551 till -596 of Sc-*ILV6* gene (Fig. 15). These homologous sequences were selected as specific for Sc-*ILV6* replacement to prevent the disruption of non-Sc-*ILV6* ORF. The sequence of non-Sc-*ILV6* ORF as well as its flanking sequences was kindly provided by Dr. Kodama from Suntory Ltd. Homologous recombination would result in the replacement of 596 bp of the Sc-*ILV6* coding region, starting from the ATG start codon, by the disruption cassette.

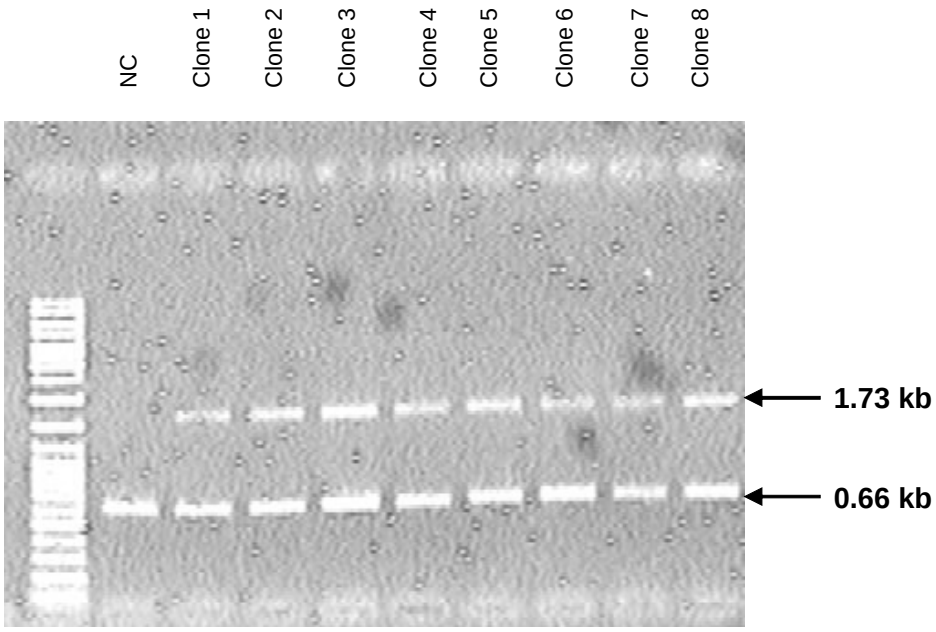

Fig. 16 Diagnostic PCR to check the correct single deletion mutant *Sc-ilv6Δ* in the 8 selected clones. NC: negative control, strain C, which contains two copies of Sc-*ILV6*

The PCR product was introduced into strain C using the PEG/Lithium acetate transformation method. The transformants carrying loxP-kanMX-loxP cassette were selected on YED plate supplemented with 17.5 µg/ml of G418. After that, the transformants were transferred to a new YED plate with a higher concentration of G418 (50 µg/ml). Eight randomly chosen transformants which were able to grow on the latter medium were then selected for further investigation. The disruption of Sc-*ILV6* in these transformants was confirmed by diagnostic PCR using primers P3 and P4 (see materials and methods). The primer P3 located 49 bp upstream and the primer P4 spread from position 551 to 596 of the Sc-*ILV6* (Fig. 15). Microarray CGH revealed that strain C contained two copies of Sc-*ILV6*. Thus, diagnostic PCR using these two primers in the mutant strain where one copy of Sc-*ILV6* was deleted would result in two fragments of 1.7 kb (loxP-kanMX-loxP band) and 660 bp (the remaining Sc-*ILV6* band) (Fig. 15). In contrast, the untransformed strain C led to the amplification of only one fragment of 660 bp (control band). The results showed that all of the eight selected transformants carried the correct single deletion of Sc-*ILV6* (Fig. 16).

4.5.2 Deletion of the second Sc-*ILV6* gene copy: generation of a *Sc-ilv6Δ/Sc-ilv6Δ* double deletion strain

One of the *Sc-ilv6*Δ single deletion mutants was used for the generation of the *Sc-ilv6*Δ/*Sc-ilv6*Δ double deletion strain. The disruption of the second copy of Sc-*ILV6* in strain C was also mediated via homologous recombination. The plasmid pUG66 was used as the template for PCR amplification of the second disruption cassette loxP-ble^r-loxP (Gueldener *et al*., 2002). The reaction was carried out with the same primers P1 and P2 previously used to amplify the loxP-kanMX-loxP cassette. The *ble^r* module of the second disruption cassette was composed of the *ble^r* gene which was flanked by the *TEF* promoter and terminator of *Ashybya gossypii*. The *ble^r* gene of the plasmid pUG66 originated from transposon Tn5. The transformant harbouring the *ble^r* module in the genome would render resistance to the antibiotic phleomycine.

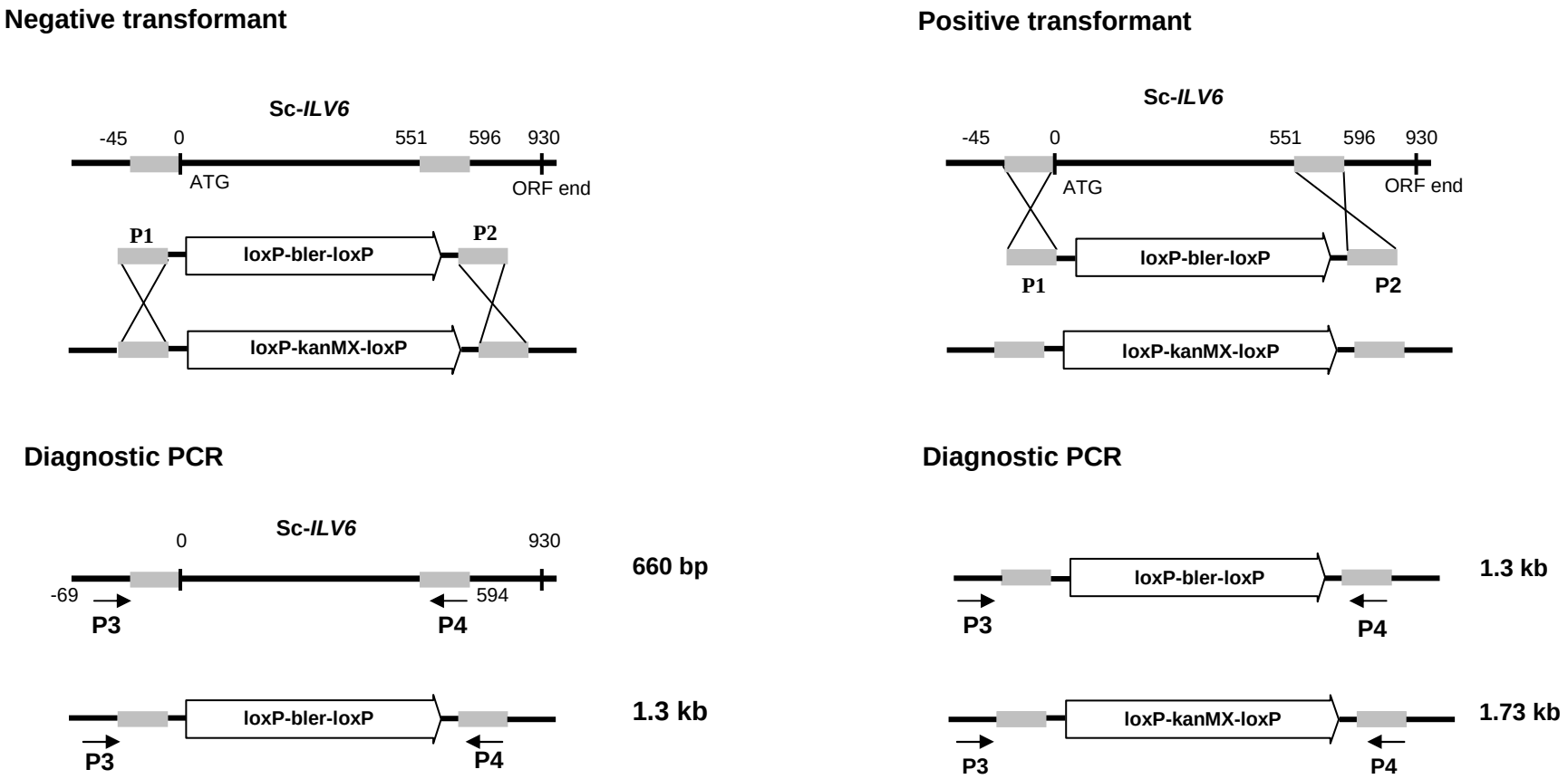

Fig. 17 Disruption of the second copy of Sc-*ILV6* in the *Sc-ilv6Δ* single deletion strain. Gray bars indicate 45 bp of homolougous regions for mediating homologous recombination. The integration of disruption cassette was diagnosed by PCR using primers P3 and P4. In the positive transformant, the loxP-ble^{r}-loxP cassette replaced the second copy Sc-*ILV6*, diagnostic PCR resulted in two bands of 1.3 kb (loxP-bler-loxP containing band) and 1.73 kb (loxP-kanMX-loxP containing band). In the negative transformant, loxP-bler-loxP cassette replaced loxP-kanMX-loxP cassette, diagnostic PCR resulted in two bands of 660 bp (Sc-*ILV6* containing band) and 1.3 kb (loxP-ble^{r}-loxP containing band)

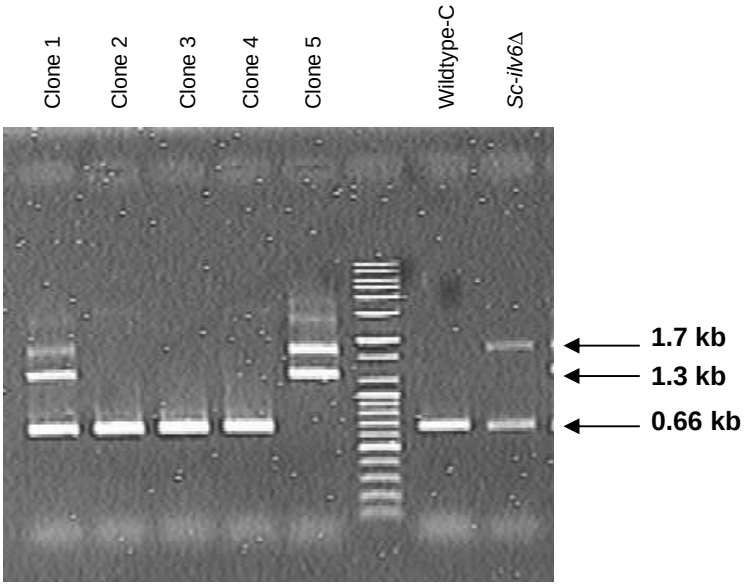

Fig. 18 Diagnostic PCR to confirm the correct *Sc-ilv6Δ/Sc-ilv6Δ* double deletion in the selected clones

The second disruption cassette loxP-ble^{r}-loxP was transformed into the strain *Sc-ilv6*Δ using the PEG/Lithium acetate method. As the two disruption cassettes loxP-kanMX-loxP and loxP-ble^{r}-loxP were amplified by using the same primers, integration of the second disruption cassette loxP-ble^{r}-loxP into the genome of the strain *Sc-ilv6*Δ would appear in two possibilities: i) loxP-ble^{r}-loxP replaced loxP-kanMX-loxP cassette and the 2nd copy of Sc-*ILV6* remained (negative transformant) and ii) the loxP-kanMX-loxP cassette substituted the 2nd copy of Sc-*ILV6*, generating the double *Sc-ilv6*Δ/*Sc-ilv6*Δ strain (positive transformant) (Fig. 17). The *Sc-ilv6*Δ/*Sc-ilv6*Δ double deletion mutants harbouring both loxP-ble^{r}-loxP and loxP-kanMX-loxP cassettes rendered resistance to both G418 and phleomycine. Thus, for the selection of the double deletion mutants, transformants were first selected on a YEPD plate containing 17.5 µg/ml phleomycine and then replica selected on a YED plate containing 50 µg/ml of G418. On the first selective plates (YEPD plus 17.5 µg/ml phleomycine), 130 transformants were obtained. The transfer of 80 of these transformants onto the second selective plates (YED plus

50µg/ml of G418) led to the growth of 9 transformants. Five of these transformants were used for diagnostic PCR using primers P3 and P4 (Fig. 17).

In the original *Sc-ilv6*Δ single deletion mutant containing one copy of Sc-*ILV6* and the loxP-kanMX-loxP cassette, diagnostic PCR would result in two bands of 1.7 kb and 660 bp (Fig. 17). In the negative transformant which the loxP-kanMX-loxP cassette was replaced by loxP-ble^{r}-loxP cassette, it resulted in two bands of 1.3 kb and 660 bp. The desired double deletion transformant carried no copy of Sc-*ILV6* and thus, led to an amplification of two fragments of 1.3 kb and 1.7 kb (Fig. 17)

Diagnostic PCR revealed that only one of these five tested transformants was the correct *Sc-ilv6Δ*/*Sc-ilv6Δ* double deletion mutant (Fig. 18) (clone 5). In addition, PCR of one transformant (clone 1) resulted in three bands of 1.7 kb, 1.3 kb and 660 bp. Thus, this clone appeared to be the mixture of the correct double deletion strain either with the single deletion strain or with the negative transformant. Diagnostic PCR of the other three transformants only resulted in one band of 660 bp. The appearance of only one band of 660 bp indicates that these three clones contained no disruption cassette. However, why these clones were able to grow on G418 and phleomycin selective media remains questionable to us.

As aforementioned, brewers' yeast containes two versions of many genes (Sc-type and non-Sc-type). The microarray-based CGH analysis revealed that all the studied brewers' yeast strains in this work contained both Sc-*ILV6* and non-Sc-*ILV6* ORFs. Sequence analysis showed that these two ORFs had the same length and were about 86% identical (Dr. Yukiko Kodama, personal information). To verify that the disruptions were specific for Sc-*ILV6* ORF, diagnostic PCR using non-Sc primers P5 and P6 (see material and methods) was set up for the generated *Sc-ilv6*Δ single and *Sc-ilv6*Δ/*Sc-ilv6*Δ double deletion strains (Fig. 19). The primer P5 located 46 bp upstream while primer P6 was designed as the sequence located from position 575 to 593 of the non-Sc-*ILV6* ORF. PCR amplification using non-Sc primers P5 and P6 gave no product in *S.cereviae* strain BY4741. In contrast, it resulted in one band of 660 bp (non-Sc-type *ILV6* band) in the wild-type strain C, the single deletion mutant

*Sc-ilv6*Δ and the double deletion mutant *Sc-ilv6*Δ/*Sc-ilv6*Δ. The result confirmed the correct disruption of Sc-*ILV6* ORF in the mutant strains.

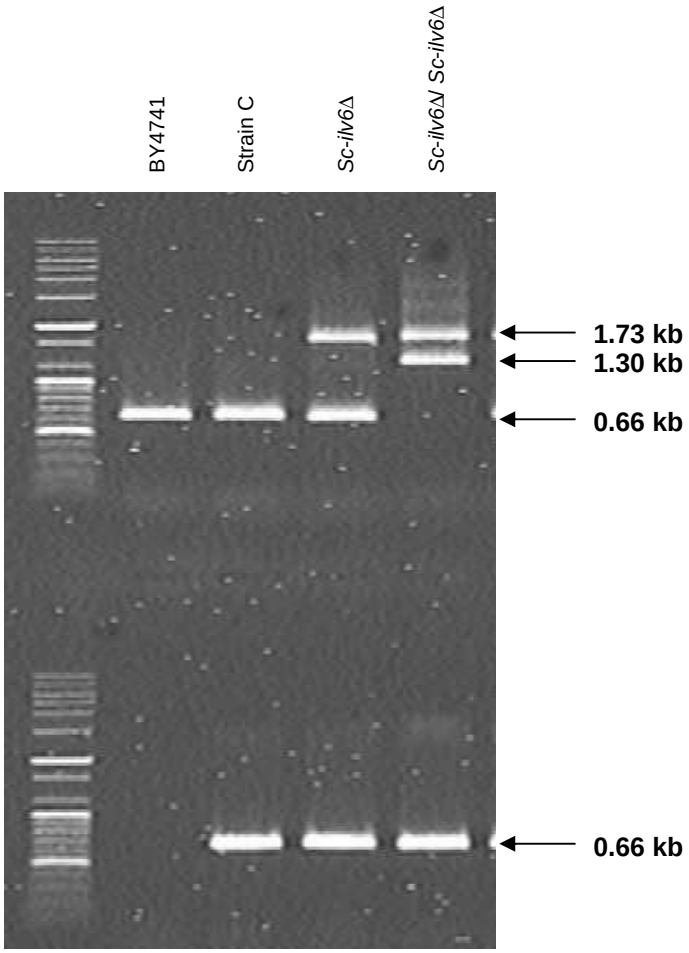

Fig. 19 Diagnostic PCR to check the correct disruption of Sc-*ILV6* instead of the wrong disruption of non-Sc-*ILV6* ORF. Upper gel: Diagnostic PCR using the Sc-type primers P3 and P4. Lower gel: Diagnostic PCR using non-Sc-type primers P5 and P6.

4.5.3 *In vitro* acetohydroxyacid synthase activity in strains *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ*

As previsouly mentioned, Ilv6p has been proposed as an enhancer of acetohydroxyacid synthase (Ilvp2, AHAS). Thus, the activity of AHAS in the Sc-ilv6 single (*Sc-ilv6Δ*) and in the double deletion strain (*Sc-ilv6Δ/Sc-ilv6Δ*) was measured (Fig. 20). It was showed that the AHAS activity in the two mutants was slightly different in comparison to that in the reference strain C. Concretely, the AHAS activity in the Sc-ilv6 single and double deletion strains was about 97% and 90% compared to that in the reference strain C. The AHAS activity of strain B which contains one copy of *Sc-ILV6* ORF was about 82% compared to strain C.

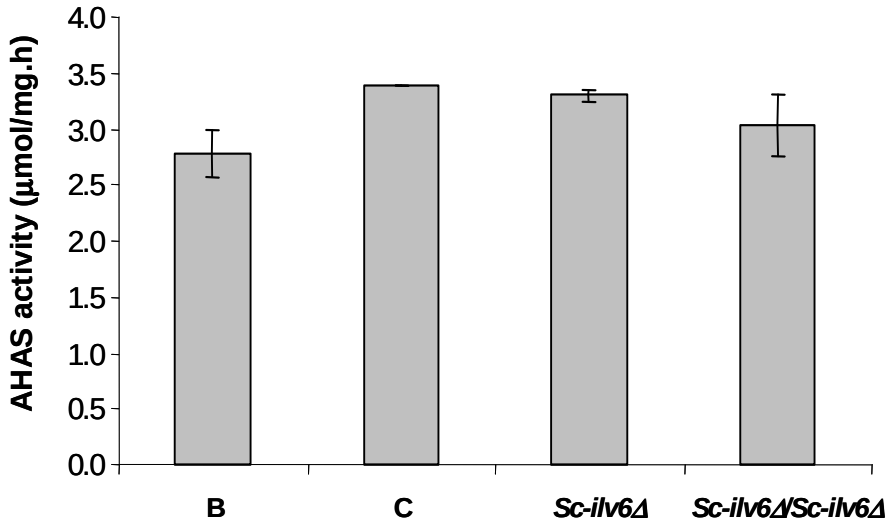

Fig. 20 *In vitro* activity of acetohydroxyacid synthase (AHAS or Ilv2p) in strains B, C, *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ*. AHAS activity was measured using permeabilized cell proteins. For this experiment, fermentation was carried out in 100 ml bottle using 11.38°P brewers' wort at 12°C. Permeabilized cell proteins were prepared from cells harvested at apparent extract of 8% of the fermentation. Results shown are mean values of two independent experiments including standard deviations.

4.5.4 Fermentation characteristics and vicinal diketone production of strains *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ* under the laboratory scale fermentation

The diacetyl productions of Sc-ilv6 deletion mutant strains were investigated under laboratory scale fermentation (see materials and methods). For each strain, the fermentations were done in duplicate. Diacetyl was always measured when the apparent extract varying from 8.3 to 8.8% was reached. The results showed that the production of 2,3-pentanedione of the two mutant strains was similar to that of the reference strain C. In contrast, a strong decrease in diacetyl formation was observed in the two mutant strains (Fig. 21). The diacetyl production of strains *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ* were reduced to about 87% and 60% compared to that of the wildtype strain C. Nevertheless, vicinal diketone production of strain B which contains one copy of Sc-*ILV6* was still much lower than those of strains *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ*. Diacetyl and 2,3-pentanedione productions of strain B at apparent extract of 8% was about 9% and 25% compared to those of strain C, respectively. The diacetyl concentration of these strains had a correlation to the AHAS activity even though the difference in AHAS activity was low.

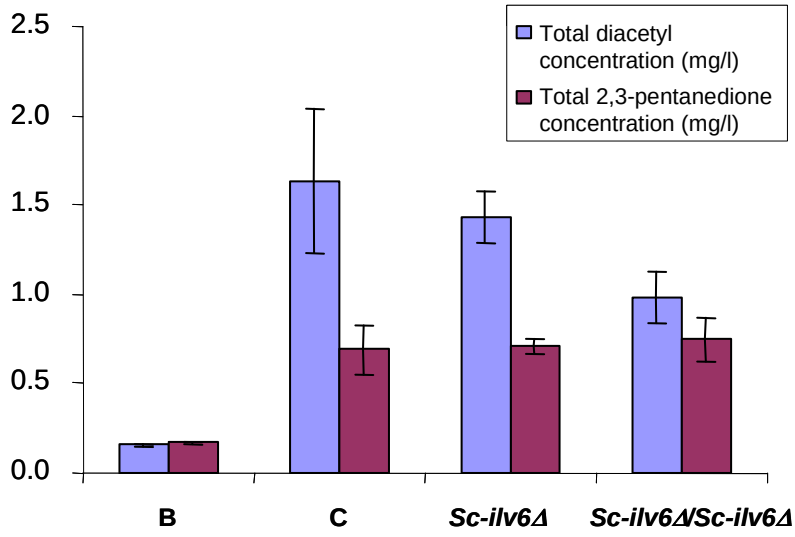

Fig. 21 Vicinal diketone production by strains B, C and *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ*. Diacetyl and 2,3-pentanedione were measured at apparent extract varying from 8.3 to 8.8%. Yeast strains were fermented in 100 ml bottles using 11.38°P brewers' wort at 12°C. Total diacetyl: the sum diacetyl and its precursor α-acetolacte in the wort medium; total 2,3-pentanedione: the sum of 2,3-pentanedione and its precursor α-aceto-α-hydroxybutyrate in the medium.

4.5.5 Fermentation characteristics and vicinal diketone production of strain *Sc-ilv6Δ/Sc-ilv6Δ* under industrially relevant brewery fermentation

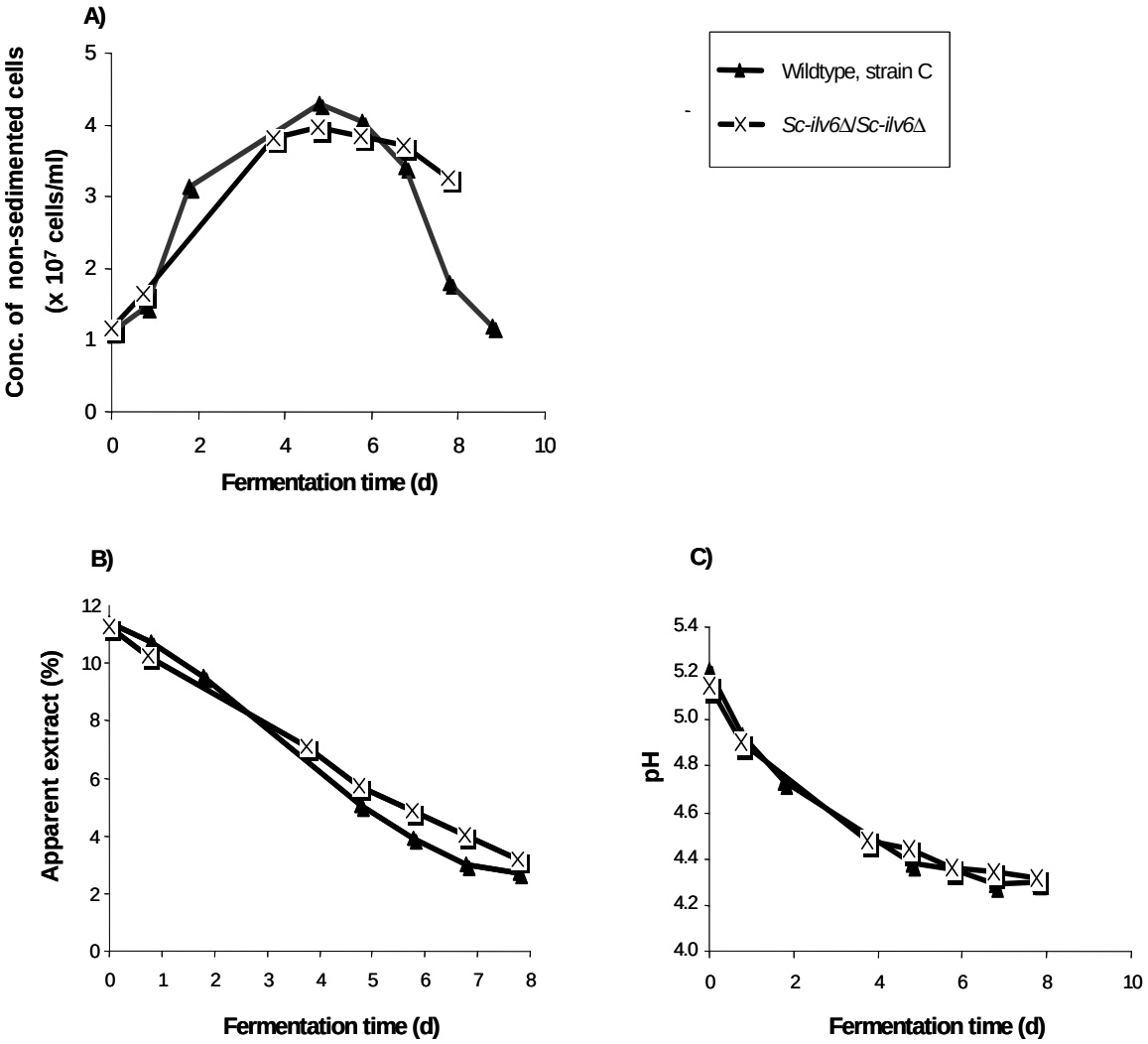

Fig. 22 Fermentation performance of strain *Sc-ilv6Δ/Sc-ilv6Δ* in comparison to the wild type strain C. Fermentation was carried out in 30-litre tank under industrially relevant brewing conditions (10.5°C, 11.38°P brewers' wort). A) Concentration of non-sedimented cells, B) Time courses of apparent extract, C) Time courses of pH

The growth of the strain *Sc-ilv6*Δ/*Sc-ilv6*Δ was virtually similar to that of the reference strain C during the main fermentation (Fig. 22A). At the end of fermentation, the strain *Sc-ilv6*Δ/*Sc-ilv6*Δ showed a slower sedimentation compared to the reference strain C (Fig. 22A).

By the end of the main fermentation, the consumption of wort sugars in the strain *Sc-ilv6*Δ/*Sc-ilv6*Δ was slightly slower in comparison to the wildtype. The reference strain C needed seven days while it took the strain *Sc-ilv6*Δ/*Sc-ilv6*Δ eight days to reach wort attenuation (Fig. 22B).

The time courses of the pH values were not influenced by the disruption of Sc-*ILV6* ORFs (Fig. 22C).

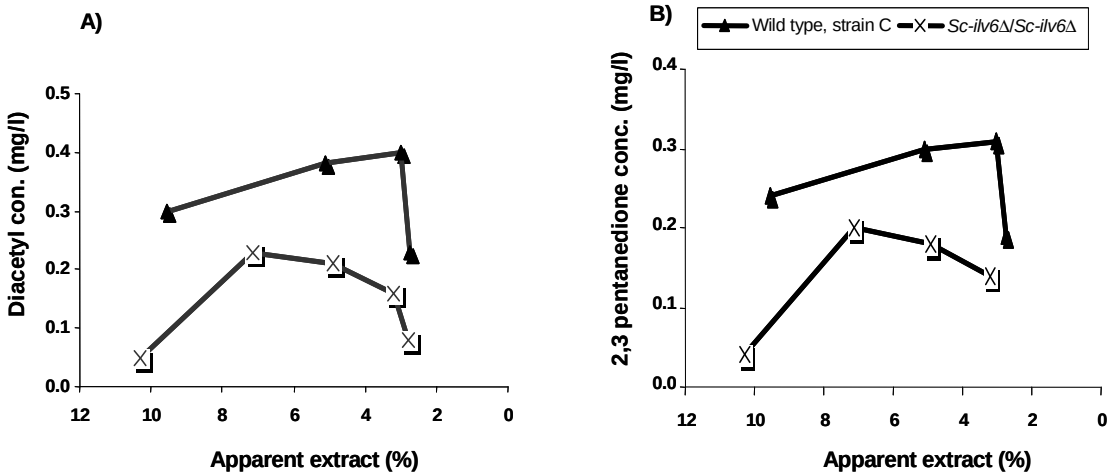

Fig. 23 Vicinal diketone production by strain *Sc-ilv6Δ/Sc-ilv6Δ* in comparison to the wild type strain C. Fermentation was carried out in 30-litre tank under industrially relevant brewing conditions (10.5°C, 11.38°P brewers' wort). A) Diacetyl concentration during fermentation. B) 2,3-pentanedione concentration during fermentation

The diacetyl content of the *Sc-ilv6Δ/Sc-ilv6Δ* double deletion strain was investigated under brewing condition. Diacetyl contents were measured at different apparent extract during the main fermentation. By the end of fermentation (at apparent extract of 2.8%), the diacetyl production of strain *Sc-ilv6*Δ/*Sc-ilv6*Δ was reduced by 65% in comparison to that of the wild type.

However, the complete disruption of Sc-*ILV6* gene in strain C only resulted in a slighter change in the final production of 2,3-pentanedione. Compared to the wild type, the strain *Sc-ilv6*Δ/*Sc-ilv6*Δ showed a 26% reduction in 2,3-pentanedione concentration by the end of the fermentation (Fig. 22).

4.5.6 Determination of flavour-relevant products in green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ*

Compared to the reference beer, the green beer produced by the mutant *Sc-ilv6*Δ/*Sc-ilv6*Δ showed a 25% reduction in acetaldehyde concentration (Table 8). Moreover, an increase in production of some acetate esters (ethyl acetate, 2-phenylethyl acetate, isoamyl acetate) and ethyl esters (ethyl caprate, ethyl formiate) was observed. A decrease in production of some fusel alcohols (iso-butyl alcohol, isoamyl alcohol, 2-phenylethyl alcohol) and fatty acid (capric acid) was also observed. However, the concentrations of these by-products were in the beer normal range.

Table 8. Analysis of green beers produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* and the reference strain C (harvested at apparent extract of 3%). Fermentation was carried out in 3 litre glass fermenters under conditions relevant to industrial brewing fermentation (11.38°P, 12°C). The results obtained for the strain *Sc-ilv6Δ/Sc-ilv6Δ* are mean values of two dependent experiments including standard deviations. Bold lines indicate alteration in production of some by-products

Strains	**C**	***Sc-ilv6Δ/Sc-ilv6Δ***
Ethanol (g l^{-1})	38.4	36.95 ± 0.35
pH	4.29	4.27 ± 0.06
Acetaldehyde (mg l^{-1})	5.25	3.93 ± 0.04
Organic acids		
Acetic acid (mg l^{-1})	187.11	176.12 ± 49.12
Butyric acid (mg l^{-1})	1.47	1.77 ± 0.11
Fusel alcohols		
1-propanol (mg l^{-1})	21.2	23.19 ± 0.49
Active amyl alcohol (mg l^{-1})	15.6	9.9 ± 0.4
Isoamyl alcohol (mg l^{-1})	49.3	31.45 ± 0.95
Iso-butyl alcohol (mg l^{-1})	13.4	8.2 ± 1.2
2-phenylethyl alcohol (mg l^{-1})	48.1	36.1 ± 6.8
Esters		
Ethyl acetate (mg l^{-1})	3.34	7.91 ± 2.09
Isoamyl acetate (mg l^{-1})	0.35	0.46 ± 0.09
Isobutyl acetate (mg l^{-1})	0	0 ± 0
2-Phenylethyl acetate (mg l^{-1})	1.05	1.47 ± 0.09
Ethyl formiate (mg l^{-1})	0.82	0.65 ± 0.03
Ethyl butyrate (mg l^{-1})	0.04	0.050 ± 0.005
Ethyl caproate (mg l^{-1})	0.07	0.080 ± 0.005
Ethyl caprate (mg l^{-1})	1.45	2.81 ± 0.12
Fatty acids		
Isovalerate (mg l^{-1})	6.48	6.59 ± 0.56
Caproate (mg l^{-1})	5.17	4.75 ± 0.79
2-Ethyl capronate (mg l^{-1})	4.7	4.26 ± 0.17
Caprate (mg l^{-1})	4.61	3.47± 0.02
Caprylate (mg l^{-1})	9.28	7.86 ± 0.31
Phenylacetic acid (mg.l^{-1})	0.86	0.95 ± 0.14

5 Discussion

So far, the improvement of brewers' yeast has been mostly attempted by classical genetic manipulation or rational metabolic engineering. In this study, we used the inverse metabolic engineering approach to identify novel target genes for optimisation of lager brewers' yeast strains. The predominant target addressed in the current work is the reduction of diacetyl production. Diacetyl causes an unwanted butter-like flavour in beer. The reduction of diacetyl to an acceptable level in beer during maturation requires a lot of time. A lager brewers' strain producing low level of dicetyl would be of great advantage for industry since it helps to accelerate the beer brewing process, i.e. it would save alot of time and storage capacity.

Comparative global molecular analyses of different lager brewers' yeast strains producing various levels of diacetyl revealed that Sc-*ILV6* was one of the potential novel target genes for reduction of diacetyl production. Sc-Ilv6p is proposed to be the regulatory subunit of Ilv2p. The latter enzyme is directly involved in diacetyl formation since it catalyses the reaction to convert pyruvate into α-acetolacte which is the precursor of diacetyl. The significant differences regarding Sc-*ILV6* were found at both level of genome (gene copy number) and transcriptome (mRNA concentration) in the studied strains. The resulting difference regarding Sc-*ILV6* expression level in the studied strains might be the reason for the difference in activity of Ilv2p (AHAS) and thus might have led to the difference in diacetyl production in the studies strains. The *in vitro* activity of Ilv2p was shown to be slightly different in the studied strains. Despite of this fact, I subsequently deleted two copies of Sc-*ILV6* in one production industrial brewers' yeast strain to verify its role in diacetyl formation.

The disruption of the Sc-*ILV6* in brewers' yeast only led to a slight reduction in AHAS activity and 2,3-pentanedione concentration. However, a significant decrease in diacetyl production was achieved. Growth and wort sugar consumption of strain *Sc-ilv6Δ/Sc-ilv6Δ* were slightly slower than those of the reference strain. Analysis of green beer produced by the *Sc-ilv6Δ/Sc-ilv6Δ* mutant revealed a slight change in concentrations of acetaldehyde, some fusel alcohols, esters and fatty acids.

5.1 Global genetic analyses of the three lager brewers' yeast strains producing different levels of diacetyl

To identify the crucial differences determining the various levels of diacetyl in the three selected lager yeast strains, comparative global analyses were performed at the level of genome, transcriptome and proteome. The genome and transcriptome analyses were carried out by means of comparative microarray analysis using customized bottom fermenting yeast DNA microarrays. The proteomes of the selected strains were studied using 2D gel electrophoresis and mass spectrometry (MALDI-TOF MS). To obtain an insight into the transcriptome and proteome at the same point in time, mRNA and protein samples were always isolated from cells harvested at apparent extract of 8% which corresponds to the early phase of the main fermentation.

Global analysis at genomic level using bottom fermenting yeast DNA probes (22,977 probesets including coding and intergenic regions) revealed thousands of sequences which showed to be significantly differently abundant in the there studied strains. In each pairwise comparison at the genome level, a sequence was designated as "increased" if the calculated change p-value was ≤ 0.002 and as "decreased" if the change p-value was ≥ 0.998. Global genome analysis revealed that the chromosome patterns of the two strains A and C were in fact quite similar and were different from that of strain B (Fig. 8). Expression analysis at transcriptome level using the same bottom fermenting yeast DNA microarray resulted in the identification of total 1851 transcripts whose levels were significant different in the studied strains (false discovery rate of 0.001). Among those, 338 transcripts were found to be differentially abundant between strains A and C (Table 5). In contrast, the number of significant differences identified in comparison between strain B and the two other strains are quite high i.e 1176 (B vs A) and 1236 (B vs C). In addition, the transcriptome analysis releaved many common differences (more than 600) in the B vs A and B vs C comparisons. The results obtained at genome and transcriptome

levels were consistent to the fact that strains A and C are genetically closely related and phenotypic different from strain B.

The differences identified at both genome and transcriptome levels included those which have an obvious link to the diacetyl and flocculation phenotypes of the investigated strains. In addition to Sc-*ILV6*, non-Sc-*BAT1* which can be linked to the diacetyl production pathway, many differences directly related to flocculation were obtained (e.g. those regarding *FLO1*, *FLO8* and *FLO9)*. In a number of cases, the identified genes showed significantly different abundances at both levels, i.e. gene copy number and mRNA level. The results indicate that genome and transcriptome analyses with the use of bottom fermenting yeast DNA microarray is a useful tool to study the genetic basis of brewing relevant phenotypic differences of lager brewers' yeast strains.

Microarray-based genome and transcriptome analyses of lager brewers' yeast in previous studies were solely performed with *S. cerevisiae* arrays (Olesen *et al*., 2002; James *et al*., 2003; Bond *et al*., 2004; Pope *et al*., 2007). Thus, these analyses only allowed the identification of the differences regarding Sc-type genes and transcripts while the non-Sc-type ones were not accessible. The use of bottom fermenting yeast DNA microarrays (kindly provided by Suntory Ltd.) in the current study allowed a more profound study of significant lager brewer's yeast strain's differences and revealed whether such a difference was based on Sc-type or non-Sc-type gene or transcript. Therefore, it led to more reliable transcriptome and genome data which are important for the identification of real target genes for strain improvement.

Furthermore, the integration of results obtained for a certain gene at both genome and transcriptome levels allowed us to draw a hypothesis about the factors affecting its differential expression in the studied strains. For example, both Sc-*ILV6* copy number and Sc-*ILV6* transcript (mRNA) showed a low abundance in strain B (low diacetyl producer) compared to the other two strains. This positive correlation between gene copy number and transcript abundant level suggested that the higher Sc-*ILV6* transcript level in the strain B might result from a higher gene copy number.

In contrast, non-Sc-*BAT2* showed the same gene copy number among all strains but a higher mRNA level in strain B (Fig. 11). One can conclude that the higher Sc-*BAT2* transcript level in strain B must result from other factors than copy number such as transcriptional activity and regulation or mRNA stability. The combination of global analyses at different genetic molecular levels is therefore crucial for a better understanding of the differences in gene expression and for deducing rational strategies for the strain improvement.

The expression patterns of genes directly related to diacetyl and flocculation behaviour at transcriptome level did not match at all the result at proteome level. Indeed, no differences directly related to diacetyl and flocculation phenotype was identified when comparing the proteomes of three studied strains. In contrast to genome and transcriptome analyses, the number of differences identified in proteome comparisons of the studied strains was very low. It only revealed 6 proteins which were at least 2-fold differentially expressed (up- or down-regulated) in the studied strains with a t-test p-value ≤ 0.05 and spot standard deviation of 30% (Table 7). These differentially abundant proteins included 3 glycolytic enzymes (Fba1p, Eno2p and Pgk1p) and 3 stress proteins (Ssa1p, Ssb2p and Hsp31p) (Table 7, Fig. 13). Among those, 4 proteins were found to be differentially expressed in A vs B comparison including Eno2p, Fba1p, Pgk1p and Hsp31p. In addition, 4 proteins were identified as differentially expressed in B vs C comparison including Ssb2p, Ssa1p, Eno2p and Pgk1p. No difference was identified when proteome of strain A was compared to that of strain C. Similar to the analyses at genome and transcriptome levels, the comparison at peoteome level fitted well to the fact that strains A and C are closely related and phenotypically different from strain B. As diacetyl and flocculation phenotypes of strain B was considerably different from those of strains A and C, the significant differences identified in both A vs B and B vs C comparison were considered to be most important for the phenotype, i.e. Eno2p and Pgk1p. Interestingly, no differences regarding *ENO2* and *PGK1* mRNA abundances were found in transcriptome analysis. The result suggested that the differences in

protein concentration must have resulted from differences in translational efficiency or protein stability.

Different levels of the two glycolytic enzymes Eno2p and Pgk1p might even be related to the differences in diacetyl production in the studied lager yeast strains. It was found that strain B has a lower amount of two glycolytic enzyme Eno2p and Pgk1p in comparison to strains C and A. The lower abundance of these enzymes in strain B (compared to strains A and C) could have led to a lower level of pyruvate intermediate concentration in strain B. As pyruvate is the substrate for the reaction catalysed by AHAS to form the precursors for vicinal diketones (diacetyl and 2,3-pentanedione), the lower level of pyruvate could result in a lower level of vicinal diketone production in strain B.

The negative correlation between the identified differentially expressed mRNA species and proteins can be due to a number of reasons e.g. the sensitivity and reproducibility of the used methods, post-translational modifications of protein or time incompatible between levels of expression of a transcript and a protein. In this study, the relative low number of differences identified at proteome level might result from the common limitations of the protein analysis method used in this study. The detection limit of a protein in a 2D gel is estimated to be at least 1000 protein copies per cell (Mijalski *et al*., 2005). In contrast, the DNA microarray allows detecting less than one copy of mRNA per 20 yeast cells (Lockhart *et al*., 1996; Wodicka *et al*., 1997). Thus, low abundant proteins might not have been detected in our 2D gels. Since the 2D gel electrophoresis requires many stages of manual work, its reproducibility is lower than that of the microarray method. In this study, protein analysis was carried out in biological triplicates (cell samples for protein isolation were taken in triplicate from two independent fermentations) while the transcriptome analysis was performed in technical triplicates (total RNA samples were isolated in triplicate from cells harvested from one single fermentation). The difference in the triplicate models used in the transcriptome and proteome analyses could account for the lower reproducibility of our proteome analysis compared to that of transcriptome

analysis. Due to these facts, only a low number of significant differences (regarding high abundant proteins) were identified at proteom level. In addition, flocculation proteins are integral cell wall proteins and thus might have been lost during the protein extraction. Due to these reasons, the strain specific differences directly related to diacetyl production and flocculation (found at transcriptome level) might not be detectable at proteome level.

5.2 Identification of potential novel target genes for reduction of diacetyl production: Sc-*ILV6*, Sc-*BAT1,* non-Sc-*BAT1*, non-Sc-*BAT2*

As aforementioned, diacetyl is a by-product of the valine biosynthetic pathway, which is formed from the non-enzymatic decarboxylation of α-acetolactate. When transcriptomes of the three studied lager brewers' yeast strains were compared, several significant differences were identified for genes whose products are involved in the valine biosynthesis pathway (Sc-*ILV6*, Sc-*BAT1*, non-Sc-*BAT1* and non-Sc-*BAT2)* and differences in their expression could thus be directly relevant for diacetyl formation (Fig. 11). Among those, the levels of Sc-*BAT1* and Sc-*ILV6* transcripts were respectively 3-fold and 4-fold lower in strain B compared to strains A and C. Strain B produced the lowest level of diacetyl while strain A has a slightly higher diacetyl production than strain C (Fig. 4). The lower levels of Sc-*ILV6* and Sc-*BAT1* mRNA in strain B might therefore be responsible for the lower level of diacetyl production in strain B compared to A and C. In addition, the level of non-Sc-*BAT1* and non-Sc-*BAT2* transcripts were about 2-fold lower in strain A compared to strains B and C. These differences might thus be related to the highest diacetyl production in strain A.

ILV6 which is one of the differentially expressed genes in the studied strains, is assumed to encode a regulatory subunit of Ilv2p. This protein confers acetohydroxyacid synthase (AHAS) activity and is responsible for the formation of α-acetolactate from pyruvate. The different studies that have resulted in the

assumption that Ilv6p is a regulatory subunit of Ilv2p are as follows. First, three active AHAS isozymes (AHASI, AHASII, and AHASIII) exist in most bacteria, e.g. *E. coli* and *Samonella typhimurium*. All three isozymes have a tetrameric structure ($\alpha 2\beta 2$) and each consists of two subunits of different molecular weights (small subunits: ≈ 10-17 kD, large subunits ≈ 60 kD) (Pang and Duggleby, 1999). It was demonstrated that AHAS activity was conferred by the large subunits while the small subunits act as a regulator by enhancing the activity of the catalytic subunits and conferring sensitivity to feedback inhibition by valine of the enzyme. In *S. cerevisiae*, only one isozyme of AHAS has been characterized which is also composed of a catalytic subunit (Ilv2p) and a regulatory subunit (Ilv6p) (McCourt and Duggleby, 2006). The disruption of *ILV6* in *S. cerevisiae* led to the insensitivity of AHAS to feedback inhibition by valine (Cullin *et al*., 1996). Although the role of yeast Ilv6p in feedback regulation of the enzyme is obvious, its role as an enhancer for catalytic subunit Ilv2p is still unclear. It was showed that an ilv6 null mutant did not show any change in AHAS *in vitro* activity (Cullin *et al*., 1996). Nevertheless, Pang *et al*. (1999) overexpressed yeast Ilv2p and Ilv6p in *E. coli* and carried out *in vitro* reconstitution of these two proteins. In fact, the activity of the reconstituted enzyme in high salt concentration buffer was 7-fold higher than that of the catalytic subunit (Ilv2p) alone.

Global genetic analysis at mRNA level revealed that Sc-*ILV6* transcript was about 4-fold less abundant in strain B compared to strains A and C. As Ilv6p probably acts as an enhancer for Ilv2p in yeast, the lower expression level of *Sc-ILV6* in strain B may lead to lower the AHAS activity and accordingly to the lower level of diacetyl production in this strain. Regarding this fact, Sc-*ILV6* is a potential target gene for the reduction of diacetyl production in lager brewers' yeast. The influence of genetic modification of Sc-*ILV6* on diacetyl production and fermentation performances of lager brewers' yeast will be discussed in details in the latter parts.

Apart from the detected difference regarding Sc-*ILV6* transcript abundance, strain B also contained a lower level of Sc-*BAT1* transcript (approximately 2-fold) compared to strains A and C (Fig. 11). *BAT1* encodes a mitochondrial branched-chain amino

acid (BCAA) transaminase which is required both for the BCAA biosynthetic pathway and the BCAA degradation via the Ehrlich pathway (Fig. 24). In yeast, this enzyme is highly expressed during logarithmic phase and repressed during stationary phase. In the first period of the fermentation which can be considered as the “logarithmic phase”, brewers’ yeast cells do not need to produce BCAAs since they can be taken up from the medium. Thus, the higher level of Sc-*BAT1* expression in strains A and C could lead to a higher valine consumption and to a higher level of α-keto-isovalerate formed from the valine degradation in these strains. The higher level of α-keto-isovalerate could result in a lower metabolic flux through the valine biosynthetic pathway. This could lead to a higher level of intermediate α-acetolactate and accordingly a higher level of diacetyl in strains A and C compared to strain B.

Strain A produced the highest level of diacetyl among the studied strains. It was shown that non-Sc-*BAT1* and non-Sc-*BAT2* transcripts were least abundant in strain A compared to strains B and C (Fig. 11). The difference regarding non-Sc-*BAT2* expression level might be responsible for difference in diacetyl production between strain A and C or in other words, for the highest diacetyl production in strain A compared to strains B and C. *BAT2* encodes a cytosolic BCAA transaminase which is, like *BAT1*, involves in the BCAA biosynthetic pathway and the BCAA degradation via the Ehrlich pathway (Fig. 24). In contrast to *BAT1*, this enzyme is repressed during logarithmic phase and highly expressed during stationary phase. Starting from the middle of the fermentation which corresponds to the “balance phase”, brewers’ yeast has to synthesize BCAAs needed for the cellular activities via the BCAA biosynthetic pathway as the BAACs in wort medium is depleted. Thus, the lower level of non-Sc-*BAT2* expression in this period in strain A could lead to the lower level of valine formation in this strain. As AHAS is feedback inhibited by valine, the lower level of valine formation might result in the lesser extent of valine inhibition to AHAS and thus in the higher activity of AHAS in strain A. The higher activity of this enzyme in turn might lead to a higher level of diacetyl production in strains A and C. In this study, the transcriptomes of the studied strains were analysed during the early stage

of fermentation, which corresponded to the late logarithmic growth of the brewers' yeast. Thus, the lower level of non-Sc-*BAT2* in strain A during the early stage might not be necessarily reflect the lower level of this transcript in strain A at the middle stage (the balance phase) of the fermentation. To confirm this hypothesis, the investigation of the expression levels of non-Sc-*BAT2* in the studied strains during the middle stage of fermentation is required.

In addition to the low level of non-Sc-*BAT2* transcript, strain A also showed a lower level of non-Sc-*BAT1* transcript compared to strain C. As discussed above, the lower level of *BAT1* expression may lead to the higher level of diacetyl production. Although strain A has the lower level of non-Sc-*BAT1* transcript, it still produced higher level of diacetyl than strain C. One possible explanation for this result is the low level of non-Sc-*BAT1*, which might result in the lower level of diacetyl production, could not compensate the higher expression level of non-Sc-*BAT2,* which might result in the higher level of diacetyl production in strain A. Thus, in total strain A produced higher level of diacetyl than strain C. It was demonstrated that Sc-type and non-Sc-type ORFs are about 85% homologous (Kodama *et al*., 2006). Therefore, the negative correlation between non-Sc-*BAT1* expression level and diacetyl production in strain A could be also explained by the possible difference in the function of Sc-Bat1p and non-Sc-Bat1p. Due to that reason, the lower level of non-Sc-*BAT1* might not be related to higher level of diactyl production in strain A.

In brewers' yeast, so far there has not been any study of *ILV6*, *BAT1* and *BAT2* expression level in relation to diacetyl formation. Regarding these above analyses, these Sc-*ILV6*, Sc-*BAT1*, and non-Sc-*BAT2* ORFs can be considered as the novel potential target genes for reducing diacetyl production in yeast. The roles of these genes in diacetyl formation can be verified via the deletion of Sc-*BAT1* and Sc-*ILV6* in strains A or C or via overexpression of non-Sc-*BAT2* genes in strain A. In addition, the role of non-Sc-*BAT1* in diacetyl formation can also be verified via the overexpression of this gene in any selected lager yeast strain. Besides the genetic modification of every single gene, the simultaneous manipulation of both Sc-*BAT1*

and Sc-*ILV6* or both non-Sc-*BAT1* and non-Sc-*BAT2* or of even all of these genes might be crucial to verify their role in diacetyl production.

In this thesis, I performed the genetic modification of one of these promising target genes, Sc-*ILV6* to reduce diacetyl production in lager yeast. For that purpose, Sc-*ILV6* gene was disrupted in strain C which is the production strain of an industrial German brewery. The success in reducing diacetyl production in this strain may lead to the creation of a brewers' strain producing low level of diacetyl which is useful for beer brewing. In addition, the *BAT1* and *BAT2* genes were addressed in a parallel work carried out by Lysann Strack.

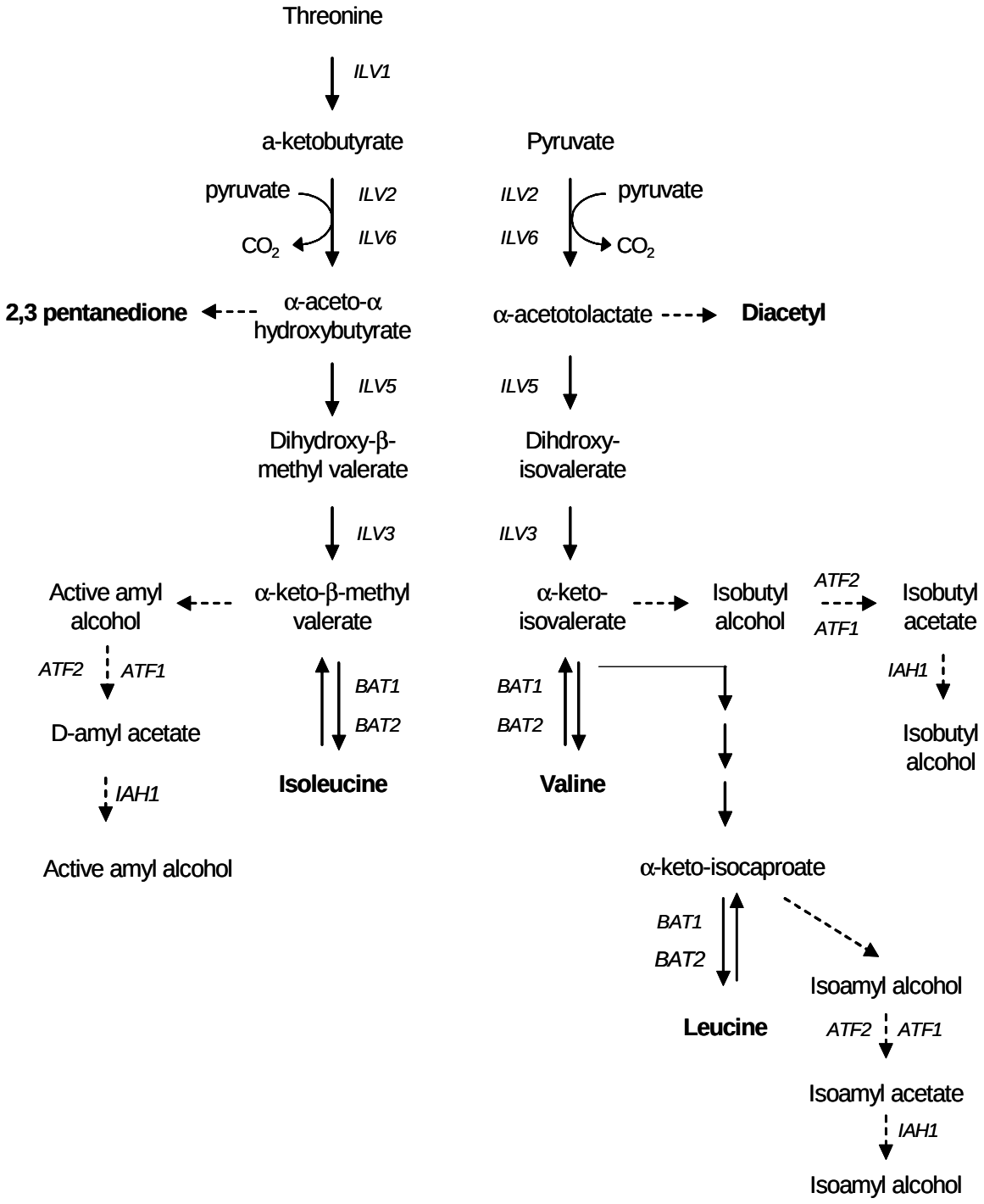

Fig. 24 Branched-chain amino acid biosynthesis and degradation in yeast and some related metabolites. *ILV1*: threonin deaminase; *ILV2, ILV6*: acetohydroxyacid synthase, *ILV3*: dihydroxyacid dehydratase; *ILV5*: reductoisomerase; *BAT1, BAT2*: branched-chain amino acids transaminase; *ATF1, ATF2*: alcohol acetyltransferese; *IAH1*: isoamyl acetate-hydrolyzing esterase

5.3 Impact of Sc-*ILV6* disruption on *in vitro* AHAS activity and vicinal diketone reduction

The disruption of one and two copies of Sc-*ILV6* gene in the reference strain C only led to insignificant changes in *in vitro* AHAS activity (Fig. 20). The AHAS activity of the Sc-ilv6 single deletion mutant was about 97% compared to that of the reference strain C (Fig. 20). Here, only the mean values were taken into account. Roughly, 10% reduction in AHAS activity was obtained in the Sc-*ILV6* double deletion strain. These results are consistent with the work of Cullin (1996) in which the disruption of *ILV6* ORF also did not result in the alteration of AHAS activity in a laboratory *S. cerevisiae* strain. In contrast to the insignificant differences regarding AHAS activity, a remarkable reduction in diacetyl production in Sc-ilv6 mutants was observed (Fig. 21, Fig. 23). The analysis of wort at apparent extract of 8% in laboratory scale fermentations showed that the diacetyl production of strains *Sc-ilv6Δ* and *Sc-ilv6Δ/S-ilv6Δ* was reduced by 13% and 40%, respectively compared to that of the wildtype. In addition, under conditions relevant to industrial brewery fermentations**,** the diacetyl production of strain *Sc-ilv6Δ/Sc-ilv6Δ* showed a decrease of about 65% compared to the reference strain C at the end of the primary fermentation (Fig. 23).

As previously mentioned, diacetyl is formed from the non-enzymatic decarboxylation of α-acetolactate. As AHAS catalyses the reaction to convert pyruvate into α-acetolactate, the reduction of dicetyl production in the Sc-ilv6 mutants can be considered as the readout for the reduction of *in vivo* AHAS activity in the Sc-ilv6 mutant strains. It is a common fact that the *in vitro* activtity does not necessarily reflect the *in vivo* activity of an enzyme due to the lack of allosteric regulation or of some unknown crucial regulation factors or simply due to the fact that the *in vitro* conditions are not optimal for the stability and activity of the enzyme. Compared to previous studies, our study was the first study which showed a reduction of diacetyl production by disrupting *ILV6* in yeast, and thus was successful in providing data which supports the role of Ilv6p as the enhancer of AHAS *in vivo*.

Comparative genome analysis revealed that strain B contained one copy of Sc-*ILV6* while strain C contained two copies of Sc-*ILV6*. The strains *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ* generated from strain C contains one and zero copy of Sc-*ILV6*, respectively. It was shown that the *Sc-ilv6Δ* and *Sc-ilv6Δ/Sc-ilv6Δ* respectively showed a diacetyl reduction of 13% and 40% compared to the reference strain C. Nevertheless, these two Sc-ilv6 mutants produced higher levels of diacetyl compared to strain B (Fig. 21). The results implied that the difference in diacetyl production between strain B and C not solely resulted from the difference in Sc-*ILV6* copy numbers. Other possible factors causing the different levels of diacetyl production in strains B and C could involve the differential expression levels of Sc-*BAT1* or glycolytic enzymes as previously discussed in section II.5.2 and II.5.1, respectively.

It was shown that the disruption of Sc-*ILV6* gene in the reference strain C resulted in the stronger impact on the reduction of diacetyl than of 2,3-pentanedione (Fig. 21, Fig. 23). Diacetyl and 2,3-pentanedione are formed from the non-enzymatic oxidative decarboxylation of their precursors α-acetolactate and α-aceto-α-hydroxybutyrate, respectively (Fig. 24). Alpha-acetolactate and α-aceto-α-hydroxybutyrate are formed by the condensation of one pyruvate molecule respectively with another pyruvate molecule and one molecule α-ketobutyrate, respectively (Fig. 24). These two reactions are both catalyzed by AHAS. One possible elucidation for the different impacts of Sc-*ILV6* deletion on diacetyl and 2,3-pentanedione reduction could be that the deletion had a much stronger influence on reducing the activity of AHAS in the reaction to form α-acetolactate than in the reaction to form α-aceto-α-hydroxybutyrate. For example, the reaction to form α-aceto-α-hydroxybutyrate might be catalyzed by only AHAS large subunit (Ilv2p) while the reaction to form α-acetolactate might be catalysed by both catalytic subunit (Ilv2p) and the holoenzyme (Ilv2p+Ilv6p). The deletion of Sc-*ILV6* could lead to the absence of holoenzyme and thus to the stronger reduction in diacetyl than 2,3-pentanedione in strain *Sc-ilv6Δ/Sc-ilv6Δ*.

5.4 Fermentation performance of the Sc-ilv6 double deletion mutant

Investigation of fermentation performance of strain *Sc-ilv6Δ/Sc-ilv6Δ* under industrially relevant brewery conditions showed a slightly slower decrease in wort gravity in comparison to that of the reference strain C (Fig. 22B). It seemed that the decrease in wort sugar consumption started from middle of the main fermentation (approximately from day 3 or day 4). Due to the slower wort consumption, it took strain *Sc-ilv6Δ/Sc-ilv6Δ* about 20 hrs more than the reference strain to reach wort attenuation. Growth of strain *Sc-ilv6Δ/Sc-ilv6Δ* was virtually similar to that of the reference strain C during the fermentation. Nonetheless, by the end of the main fermentation, the strain *Sc-ilv6Δ/Sc-ilv6Δ* showed a slower sedimentation rate than the reference strain C (Fig. 22A). Integration of these results suggested that the slower sedimentation in the mutant strain might result from the slower consumption of wort sugars compared to the reference strain.

In the beginning of the main fermentation, brewers' yeast takes up valine as well as other branched-chain amino acids (BCAAs) from the extracellular wort medium for growth and maintenance (Petersen *et al*., 2004). As the internal valine concentration increases during the uptake of valine, AHAS is inhibited (Inoue and Kashihara, 1995). When the valine in wort is depleted, valine and other BCAAs are produced via the BCAA biosynthetic pathway. Based on this knowledge, we supposed that the slower wort sugar consumption starting from the middle of the main fermentation in strain *Sc-ilv6/Sc-ilv6Δ* could result from the lower level of BCAAs formed during this period. In the beginning of the fermentation, growth of the reference strain C and strain *Sc-ilv6Δ/Sc-ilv6Δ* were similar as there was no difference in the level of valine taken up from the extracellular wort medium. As valine from the wort was consumed, the yeast cells had to synthesize BCAAs necessary for the cellular activities. In the strain *Sc-ilv6Δ/Sc-ilv6Δ*, AHAS activity was lower than that in the reference strain C due to the absence of Sc-Ilv6p. The lower level in AHAS activity in strain *Sc-ilv6Δ/Sc-ilv6Δ* could result in lower level of BCAAs and thus to the slower consumption of pyruvate substrate. Due to that reason, strain *Sc-ilv6Δ/Sc-ilv6Δ*

needed more time to consume wort sugars and thus sedimented slower than the reference strain C.

5.5 Slight change of by-product profile in the green beer produced by strain *Sc-ilv6Δ/ Sc-ilv6Δ*

The green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* showed differences in concentrations of some acetate esters (isoamyl acetate, 2-phenylethyl acetate, ethyl acetate) and ethyl esters (ethyl formiate, ethyl butyrate, ethyl caprate) (Table 8). Besides that, some fusel alcohols (isoamyl alcohol, iso-butyl alcohol, active amyl alcohol, 2-phenylethyl alcohol) and some fatty acids (capric acid, caprylic acid) were reduced. A decrease in acetaldehyde level in the green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* was also observed. However, the concentrations of these by-products were in the normal range for lager beer and no significant difference in the taste of beer produced by the strain *Sc-ilv6Δ/Sc-ilv6Δ* was detected in comparison to that of the reference beer.

Isoamyl alcohol, active amyl alcohol and iso-butyl alcohol are intermediates of the BCAA biosynthetic pathway (Fig. 24). The concentrations of these fusel alcohols were lower in green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* in comparison to those of the reference beer (Table 8). In addition to the decreaded isoamyl alcohol, an increase in its corresponding acetate ester (isoamyl acetate) was observerd. Besides that, a decrease in the production of 2-phenylethyl alcohol and an increase in the production of its acetate ester (2-phenylethyl acetate) were also observed (Table 8). Therefore, the lower level of these fusel alcohols might be caused by the increase of their corresponding acetate esters.

In addition to the enhancement in concentrations of acetate esters, green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* also showed an increased level of some ethyl esters (ethyl formiate, ethyl butyrate and ethyl caprate). Ethyl esters are formed from the esterification of ethanol with the fatty acids under activity of esterase. In addition

to the increase of ethyl caprate, a decrease in its corresponding fatty acid (capric acid) concentration was observed.

The decrease of fusel alcohols in correlation to the increase of their acetate esters and the decrease of fatty acids in correlation to the increase of the corresponding ethyl esters could be explained by the alteration of esterases that are responsible for the esterification reactions between acetyl-coA and fusel alcohols and the esterification reations between ethanol and fatty acids. The disruption of Sc-*ILV6* might have unknown impact resuting in an increase in activity of these esterases. Due to that reason, the reactions between acetyl-coA and fusel alcohols as well as reactions between ethanol and fatty acids could be accelerated, thereby resulting in the decreased fusel alcohols and fatty acids as well as the increase of the corresponding acetate esters and ethyl ester production. The question raised here is why the production of some esters was altered when the concentrations of other esters were unchanged. One possible explanation could be that the disruption of Sc-*ILV6* could have resulted in the increase of activity of some certain esterases, which catalyzed specific esterification reactions between ethanol and some certain fatty acids as well as between acetyl-coA and some certain fusel alcohols.

5.6 Concluding remarks and outlook

The global analyses are powerful tools for the identification of potential novel target genes for diacetyl reduction i.e. Sc-*ILV6*, Sc-*BAT1*, non-Sc-*BAT1* and non-Sc-*BAT2* in lager brewers' yeast in particular when the results of different molecular level were integrated. A striking decrease in diacetyl production was obtained by the disruption of one and two copies Sc-*ILV6* in an industrial lager brewers' yeast strain. The strain *Sc-ilv6Δ/Sc-ilv6Δ* which containes no copy of Sc-*ILV6* ORF showed a reduction in diacetyl production by 65% under the relevant industrial brewery fermentation. The result supports the role Sc-ilv6p as an enhancer

of Ilv2p. In addition, the results confirm that inverse metabolic engineering is a useful tool for identifying novel target genes for the improvement of brewers' strain.

Besides the reduction of diacetyl production, small changes in concentrations of alcetaldehyde, esters, fusel alcohols, fatty acids in green beer produced by the strain *Sc-ilv6Δ/Sc-ilv6Δ* were observed. The concentrations of these by-products, however, are in the normal range for lager beer. The taste of the beer produced by the strain *Sc-ilv6Δ/Sc-ilv6Δ* showed no significant difference compared to that of the reference beer. The characterisation of the beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* suggested that such a modification strain would be useful for the beer brewing with a shortening lagering period.

In the genome of strain *Sc-ilv6Δ/Sc-ilv6Δ*, the two copies of Sc-*ILV6* ORF were replaced by the loxP-kanMX-loxP and loxP-ble^r-loxP disruption cassettes. One of the aims of the future work will be the removal of these disruption cassettes for conferring a better acceptance in commercial use to this strain. This task can be carried out by introducing a plasmid expressing Cre recombinase under the control of *GAL* promoter. In the presence of galactose, Cre recombinase action at the repeated loxP sites will excise the kanMX and ble^r markers, leaving behind one loxP sequence at the site of the each disruption cassette. After that, the Cre recombinase plasmid can be removed by subcultivations under non-selective condition. To this end, apart from the two loxP sequences, strain *Sc-ilv6Δ/Sc-ilv6Δ* will contain no other hetorologous DNA. In this case, the engineered strain can be considered as "self-cloned" and can be better accepted in food and beverage industry.

It is very likely that diacetyl can be reduced even more. The expression levels of Sc-*BAT1*, non-Sc-*BAT2* and non-Sc-*BAT*1 as well as the abundant level of Eno2p and Pgk1p could influence the level of diacetyl production of lager brewers' yeast. Thus, the outlook of this thesis involves the additional genetic manipulation of other target genes directly relevant to diacetyl formation i.e Sc-*BAT1*, non-Sc-*BAT2* and non-Sc-*BAT1* or even of target genes which may be relate to diacetyl formation i.e. *ENO2* and *PGK1*. The verification of the hypothesis that the expression levels of

these genes could have influenced the diacetyl production can provide more knowledge about the genotype-phenotype relationship in brewers' yeast and is crucial for the construction of new brewers' yeast strain with desired diacetyl phenotype.

6 Summary

This thesis aimed at improving lager brewers' yeast by means of inverse metabolic engineering. The primary target is the reduction of diacetyl production. To that aim, three lager brewers' yeast strains producing different levels of diacetyl were selected: i) strain A which produces the highest level of diacetyl, ii) strain B which shows a very low level of diacetyl production and iii) strain C, a currently used industrial production strain, whose diacetyl level is slightly lower than that produced by strain A but much higher than strain B. Although strain B seems to be an ideal strain due to its low diacetyl production, it is not useful for beer brewing because of a very strong and early flocculation which results in uncomplete wort attenuation.

In order to identify the genetic basis for the strain's phenotypic differences relevant to brewing, an integrated approach was chosen using global analyses at different molecular levels which influence protein expression, i.e. gene copy numbers (analysed by microarray-based comparative genome hybridisation), mRNA concentrations (via microarray-based comparative transcriptome analysis) and protein abundance (two-dimensional gel electrophoresis plus mass spectrometry). Based on the result, an industrial production strain was modified and its diacetyl production could be significantly reduced without negatively affecting any important property of the strain relevant in brewing.

The main results obtained were as follows:

1) Genome and transcriptome analyses revealed numerous significant differences regarding the abundance of gene copies and transcripts in the studied strains. Among those, several differences obviously related to flocculation and diacetyl phenotype were identified. In contrast, the number of significant differences at proteome level was very low and none of these few was directly related to the difference in diacetyl and flocculation phenotypes.

2) The comparative transcriptome analysis of the three studied strains revealed several differences in mRNA concentrations of genes, i.e Sc-*ILV6*, Sc-*BAT1*, non-Sc-*BAT1* and non-Sc-*BAT2,* whose products are directly involved in the valine biosynthesis pathway and could thus have affected the production of diacetyl, a by-product of valine biosynthesis, simply by strain-dependent differences in their protein activities. Thus, these genes were considered promising targets for the reduction of diacetyl production in brewers' yeast.

3) Among the potential target genes, Sc-*ILV6* was chosen for further investigations. In fact, Ilv6p has previously been proposed to be a regulatory subunit of Ilv2p (AHAS), the enzyme which is responsible for the formation of α-acetolactate, the precursor of diacetyl. To verify the role of Sc-*ILV*6 gene in diacetyl formation, two copies of Sc-*ILV6* were subsequently disrupted in the industrial production strain C, leading to the generation of a single mutant (*Sc-ilv6Δ*) and a double mutant (*Sc-ilv6Δ/Sc-ilv6Δ)*, respectively.

4) The disruption of two copies of Sc-*ILV6* in the production strain only led to an insignificant decrease in *in vitro* AHAS activity. However, an industrially relevant brewery fermentation revealed that the diacetyl production of strain *Sc-ilv6Δ/Sc-ilv6Δ* was reduced by 65% at the end of the main fermentation and this concentration is 0.08 mg/ml and below the taste-threshold of 0.1 mg/ml for diacetyl in beer. The concentration of 2,3-pentanedione was only slightly reduced by Sc-*ILV6* deletion

5) Comparative genome analysis revealed that strain B contained one copy of Sc-*ILV6* while strain C contained two copies of Sc-*ILV6* ORF. However, compared to strain B, the diacetyl production of the Sc-ilv6 double deletion mutant (derived from strain C) was still much higher. The results imply that the lower level of diacetyl production in strain B compared to strain C could not be solely caused by the higher copy number of Sc-*ILV6* in strain C.

6) Examination of the Sc-ilv6 double deletion strain under conditions relevant in industrial brewery fermentations revealed that the green beer produced by strain *Sc-ilv6Δ/Sc-ilv6Δ* showed only small alterations in the concentrations of some fusel alcohols, esters, fatty acids and acetaldehyde. Nevertheless, the levels of these by-products are in the normal range for lager beer. Sensory investigation revealed no significant differences in the taste of the beer produced by the *Sc-ilv6Δ/Sc-ilv6Δ* compared to that of the reference beer.

7 Zusammenfassung

Die vorliegende Arbeit beschäftigt sich mit der Optimierung von untergärigen Brauhefen mittels *Inversem Metabolic Engineering*. Das vorrangige Ziel war die Reduzierung der Diacetylproduktion. Zu diesem Zweck wurden drei untergärige Brauhefen mit unterschiedlicher Diacetylproduktion selektiert: i) Stamm A, der am meisten Diacetyl bildet, ii) Stamm B, der das niedrigste Diacetyl-Niveau zeigt und iii) Stamm C, ein zur Zeit eingesetzter industrieller Produktionsstamm, der etwas weniger Diacetyl bildet als Stamm A, jedoch wesentlich mehr als Stamm B. Trotz seiner geringen Diacetylbildung ist Stamm B nicht für Brauereien geeignet, da dieser Stamm stark und sehr früh flockuliert, was zu einer unvollständigen Würzevergärung führt.

Um die genetische Basis für die brauerei-relevanten stammspezifischen phenotypischen Unterschiede zu identifizieren wurde ein integrierter Ansatz gewählt, der globale Analysenmethoden beeinhaltete um unterschiedlichen molekulare Ebenen zu analysieren, die einen Einfluß auf die Proteinexpression haben, d.h. Genkopiezahl (*Microarray*-basierte vergleichende Genomhybridisierung), mRNA Konzentrationen (genomweite Transkriptom-Analyse mittels Hefe-*Microarrays*) und Proteinkonzentrationen (zweidimensionale Gelelektrophorese und Massen-spektrometrie). Basierend auf den Ergebnissen wurde der Produktionsstamm C so verändert, dass die Diacetylproduktion signifikant reduziert und keine der anderen brauerei-relevante Eigenschaften negativ beeinflußt war.

Im folgenden sind die wesentlichen Ergebnisse der Arbeit zusammengefaßt:

1) Die Genom- und Transkriptomanalyse ergab eine Vielzahl von signifikanten stammspezifischen Unterschieden. Unter den identifizierten Genen waren einige, die offensichtlich einen direkten Bezug zum Diacetyl- bzw. Flockulationsphenotyp hatten. Dagegen war die Anzahl der stammspezifischen Unterschiede auf der Proteomebene nur sehr gering und die wenigen detektierten Gene hatten keinen direkten Bezug zum Diacetyl- bzw. Flockulationsphenotyp.

2) Die vergleichende Transkriptomanalyse der drei Stämme ergab Unterschiede in den Konzentrationen von verschiedenen mRNAs, d. h. Sc-*ILV6*, Sc-*BAT1*, non-Sc-*BAT1*, non-Sc-*BAT2,* dessen Genprodukte direkt in die Valinbiosynthese involviert sind. Stammspezifische Unterschiede bezüglich der entsprechenden

Proteinaktivitäten könnten die Produktion von Diacetyl beeinflußt haben, welches ein Nebenprodukt der Valinbiosynthese ist. Daher wurden diese Gene als potentielle Targets für eine Reduzierung der Diacetylproduktion in Brauhefen betrachtet.

3) Aus den potentiellen Targetgenen, wurde Sc-*ILV6* für weitere Untersuchungen ausgewählt. Arbeiten anderer Autoren ließen vermuten, dass Ilv6p eine regulatorische Untereinheit von Ilv2p (AHAS) ist, welches für die Bildung von α-Acetolaktat verantwortlich ist, dem Precursor von Diacetyl. Um die Rolle von Sc-*ILV6* in der Diacetylbildung zu verifizieren, wurden beide Kopien dieses Gens im industriellen Produktionsstamm C nacheinander deletiert; d. h. es wurde sowohl eine Einfachmutante (*Sc-ilv6Δ*) und eine Doppelmutante (*Sc-ilv6Δ/Sc-ilv6Δ)* generiert.

4) Die Deletion der beiden Sc-*ILV6* Kopien in Produktionsstamm C führte zu einer nicht-signifikanten Verminderung der *in vitro* AHAS-Aktivität. Allerdings zeigten Fermentationen unter industriell relevanten Bedingungen, dass die Diacetylkonzentration der Doppelmutante *Sc-ilv6Δ/Sc-ilv6Δ* am Ende der Fermentation im Vergleich zur Kontrolle um 65% reduziert war. Die erreichte Konzentration von 0.08 mg/ml lag sogar unter dem Geschmacksschwellenwert von Diacetyl im Bier (0.1 mg/l)

5) Die vergleichende Genomanalyse zeigte, dass Stamm B eine Kopie von Sc-*ILV6* enthielt, während Stamm C zwei Kopien aufwies. Allerdings produzierte die *Sc-ilv6Δ/Sc-ilv6Δ* Doppelmutante (die von Stamm C abgeleitet wurde) immer noch wesentlich mehr Diacetyl als Stamm B. Diese Ergebnisse lassen folgern, dass das niedrige Diacetyl-Niveau in Stamm B nicht ausschließlich auf der niedrigeren Kopiezahl von Sc-*ILV6* in diesem Stamm beruhen kann.

6) Untersuchungen der *Sc-ilv6Δ/Sc-ilv6Δ* Doppelmutante unter brauerei-relevanten Fermentationsbedingungen ergaben, dass das produzierte Jungbier nur geringfüge Veränderungen in Bezug auf die Konzentrationen einiger höherer Alkohole, Ester, Fettsäuren und Acetaldehyd aufwies. Insgesamt lagen die Konznetrationen jedoch alle in einem Bereich, der für untergäriges Bier als normal angesehen wird. Sensorische Tests ergaben keine erkennbaren Unterschiede im Geschmack im Vergleich zum Referenzbier.

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