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Brewer's Yeast Modification: Effects Both on Diacetyl Production and Alcohol Formation*

New industrial brewing yeasts*1 have been generated by introduction of two genes involved in valine biosynthetic pathway in order to clarify their roles for the reduction of diacetyl content in beer. The *Saccharomyces cerevisiae* genes as well as lager*2 genes both *BAT1* (encoding branched-chain amino acid aminotransferase) and *BAT2* (encoding branched-chain amino acid aminotransaminase) have been transferred into brewer's yeast. Within this approach it was possible to decrease diacetyl levels at the end of main fermentation without affecting the by-product profile of the beer negatively. Furthermore, our results pointed to the regulation of these genes concerning diacetyl formation.

Descriptors: brewer's yeast, metabolic engineering, diacetyl, maturation

1 Introduction

The presence of diacetyl in green beer is one of the main reasons for the expensive, time-consuming flavour maturation period after primary fermentation is complete. Diacetyl is a major off-flavour (*butterscotch*) in beer with a very low threshold of 0.1 ppm and is formed spontanously during the growth phase of yeast in an oxidative decarboxylation reaction from α-acetolactate. During maturation process diacetyl is converted to acetoine and further to 2,3-butanediol with both metabolites having a higher threshold and therefore not having any influence on the taste of the beverage.

The diacetyl precursor α-acetolactate is an intermediate of the valine biosynthetic pathway which diffuses from yeast cells into the fermenting wort (Fig. 1). The synthesis of α-acetolactate from pyruvate is catalyzed by acetolactate synthase (*ILV2*). The appropriate protein has a regulatory subunit (ilv6p) which enhances the activity of the enzyme during the initial step of amino acid biosynthesis. As acetolactate reductoisomerase encoded by *ILV5* reduces α-acetolactate to dihydroxy-isovalerate, this is converted to keto-isovalerate by water elimination (*ILV3* coding for dihydroxyacid-dehydratase). Keto acids are biosynthetic precursors of higher alcohols which have a bearing on beer flavour [3]. The amino group transfer from α-keto-acid to amino acid is encoded by the genes *BAT1* and *BAT2*. The branched chain amino acid valine – like leucine and isoleucine – is synthesized in yeast mitochondria.

Brewing yeast strains show considerable differences concerning diacetyl production levels. Certain yeasts produce high diacetyl concentrations whereas others produce levels of diacetyl even under the taste threshold. But these yeasts often have disadvantages such as incomplete sugar consumption or an unbalanced by-product profile so that these yeasts cannot ensure an ideal fermentation process. The differing diacetyl concentrations imply that the genes involved in valine biosynthesis in yeast are regulated diversely or show different enzyme activities. For that reason there is a huge demand on the optimization of brewer's yeasts in order to shorten maturation time and to safe storage capacities.

With the aim of accelerating beer production, various genetic approaches to control the level of diacetyl in beer by manipulating the genes of amino acid biosynthetic pathway leading to the formation of valine in yeasts have been evaluated [6–9]. For example the bacterial gene for acetolactate decarboxylase (ALDC) has been isolated from several species and introduced into yeast [10–13]. This enzyme catalyses the direct conversion of α-acetolactate to acetoin and thus prevents its oxidation. These genetic modifications lead to significant decreases in diacetyl production levels during main fermentations [14]. By the introduction of bacterial genes which additionally underly specific promoter control e.g. alcohol dehydrogenase (ADH1) the reduction of the unwanted by-product could be achieved as well so that maturation became redundant [15]. However, cloning of foreign to species genes into organisms which are used for food production are not accepted by consumers so that these yeasts are not used in breweries.

As a result attempts have been made for the reduction of diacetyl contents in beer without the introduction of yeast external genes. This has been achieved by adapting yeasts with the *ILV3* and *ILV5* genes or by deletion of *ILV2* gene (see Fig. 2) in order to increase the fluxes in valine biosynthetic pathway [4, 16–18]. Although diacetyl could be reduced within these approaches e.g. valine and leucine auxotrophic strains had been generated [19]. Consequently, these strains are not qualified for beer production. Therefore, the aim should be the reduction of diacetyl concentrations at the end of the main fermentation without any changes concerning physiological properties of the yeast strain.

To date, only *ILV* genes had been modified in order to gain knowledge concerning their influence on the diacetyl production in yeast. But as the genes *BAT1* and *BAT2* encode a branched-chain amino

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Tables and figures see Appendix

acid transferase and a branched-chain amino acid transaminase, respectively, and therefore undertake terminal enzyme functions to form valine these genes may also have an effect on diacetyl levels in beer (Fig. 1). These genes have merely been described regarding their influence on alcohol and ester formation [3, 20–23]. Here, we present a study in which the genes *BAT1* and *BAT2* have been characterized regarding diacetyl production and have been transferred into brewer's yeast without having any negative impact on beer quality and taste, respectively.

2 Materials and Methods

2.1 Strains and media

S. carlsbergensis Sa-06165 (bottom-fermenting yeast) was fermented as described below.

Escherichia coli DH5α was used for transfer experiments and for extracting plasmid DNA used in yeast transformation experiments. *E. coli* was cultivated in Luria-Bertani (LB) medium at 37 °C, as previously described [24].

2.2 Construction of plasmids

The construction of plasmids for the overexpression of the *S. cerevisiae* genome's branched-chain amino acid transaminase/ transferase genes (*BAT1*/ *BAT2*) was carried out using PCR techniques. Oligonucleotides used are listed in table 1.

The *BAT1*/ *BAT2* coding region were prepared by PCR with genomic DNA of yeast Sa-06165 as template using the oligonucleotides 1 and 3 or 2 and 4 as primers. Amplified PCR products were purified (Accu Prep® PCR Purification Kit, Bioneer) and then subcloned into pGEM®-T Easy Vector System (Promega, Madison) according to the supplier's instructions. The integrity of PCR fragments was verified by agarose gel electrophoresis. After digestion with *SacI* and *NotI*, the Sc*BAT1* or Sc*BAT2**3 open reading frame (ORF) was inserted into the *SacI*-*NotI* gap of pYCGPY2 [25] to yield ScBAT1pYCGPY2 and ScBAT2pYCGPY2. Plasmids holding *lager BAT* genes were provided by Suntory© Ltd./Osaka, Japan (original isolation from lager brewing yeast BH-225 according to [25]). Insertion of plasmids was checked using PCR techniques and oligonucleotides 3–8 (Table 1). After transformation of the plasmids in brewer's yeast primer 7 was used in order to get PCR products with higher base pair numbers (580 bp for G418 without gene, Sc*BAT1*/ Sc*BAT2*: ca. 3500 bp; Lg*BAT1*/ Lg*BAT2*: ca. 3000 bp) for better differentiation after gel electrophoresis (for plasmid structure see [25]).

2.3 DNA methods

DNA for yeast transformation was isolated from *E. coli* using a plasmid purification kit (Accu Prep® Plasmid Extraction Kit, Bioneer) as recommended by the manufacturer.

E. coli transformation was done according to Himeno *et al.*, 1984 [26] and yeast transformation was performed using the lithium acetate method [27] but both slightly modified. The selections for positive clones were carried out on either LB plates containing 150 µg/ ml Ampicillin or YPD (1 % yeast extract, 2 % peptone, 2 % glucose) plates supplemented with 100 µg of G418 ml.

2.4 Brewery-scale fermentations

Strain Sa-06165 and the generated yeast strains were fermented under brewery conditions. Hopped wort was used with an apparent extract of 11.5 % which was fermented at 11 °C in 30 l scale-tanks. Yeast strains were precultivated in 30ml wort while shaking at 24 °C and transferred into 500 ml vessels. When propagation reached 1*10^8 cells/ml (cell counting/Thoma chamber) the cells were inoculated in next size flasks (5 l wort) at 24 °C. Main fermentations were inoculated with $1.6^{\wedge}10^7$ cells ml. The number of non-sedimented cells, the apparent extract (Beer Analyzer, Paar Austria) and pH were recorded within 24 hour interval. Moreover, the diacetyl-production was measured every morning within a 2 hour time frame. The fermentation time was constituted up to 8 days. When the apparent extract fell to 2.8–3.0 % the beer was filled into steel kegs. After maturation for two weeks at 0 °C the beer was bottled. The beverage was analyzed concerning by-products such as higher alcohols and esters. Furthermore, sensors were investigated by a trained panel (VLB Berlin e.V.) to control the taste of the beer.

2.5 Analysis during and after fermentations

The method for diacetyl-measuring via GC-ECD was derived from MEBAK (Band III, 1.2.1, 1996). For one sample two vials were prepared. The beer samples were diluted according to the expected diacetyl content (1:2, 1:5) to a volume of 10 ml. The concentrations of diacetyl in the headspace was increased by addition of NaCl (4 g per vial). An internal standard (IS, hexane-2,3-dione, stock solution about 250mg/ 50ml according to MEBAK, Band III, 1.2.2, 1996) was used which had been solved until 0,1 ppm were present in the vial (0,5/ 50// 0,02/ 10). Also, a calibration was created over 0,005 to 0,2 ppm (stock solution about 250 mg/ 50 ml each diacetyl, pentane-2,3-dione and IS hexane-2,3-dion according to MEBAK, Band III, 1.2.2, 1996). After convertion of α-acetolactate to diacetyl the solutions were analyzed by GC-ECD. Results were related to the definite weighted sample for evaluation of the real concentration of diacetyl in beer.

Additionally, by-products such as esters and higher alcohols were detected after beer maturation in steel kegs for three weeks at 0 °C. Analysis methods originate from MEBAK (Band III, 1.1.1, 1996).

Diacetyl:

HP GC system, µECD Ni63

Column: DBWax 60.0 m, Ø 0.32 mm, film size 0.5 µm

Gerstel MPS2 Multi Purpose Sampler

Esters and higher alcohols:

HP 5890/ II

Column: DBWax 30.0 m, Ø 0.25 mm, film size 0.25 µm

Gerstel MPS2L Multi purpose Sampler

3 Results and discussion

3.1 Plasmid constructions and transformations

After yeast DNA isolation of Sa-06165 and amplification of *S. cerevisiae* genes both *BAT1* and *BAT2* using PCR these genes have been cloned into the linearized pGEMT®Easy vector system in order to gain overhangs for specific cloning. The gene-containing, cut plasmid solutions were ligated to linearized pYCGPY2 vector. Plasmid transformations were carried out in *E. coli*. A yield of transformants ranging between 20 and 100 transformants per µg plasmid DNA was obtained while no transformants were observed in the controls lacking the plasmid. After control of transformants desired plasmids for overexpression of *BAT* genes in yeast were available. Both the pYCGPY2 plasmids which contained the appropriate *BAT*-gene and the gene lacking reference plasmid were transferred into mentioned brewing yeast. The transformants were also propagated after replica-plating on medium containing Geneticin (G418). Five of the generated transformants per gene were checked by PCR after yeast DNA isolation. Agarose gel electrophoresis of plasmid DNA of these yeast clones indicated that all yeast transformants tested contained the specific plasmid vector. Therefore, the *S. cerevisiae* and *lager* BAT genes are present additionally to the genome in brewer's yeast which shall result in their higher protein expression levels. The strain names are referred to as LSa (Wildtype containing reference plasmid pYCGPY2), LSb (LSpScBAT1), LSc (LSpScBAT2), LSd (LSpLgBAT1) and LSe (LSpLgBAT2) containing either *S. cerevisiae* or *lager BAT* gene, respectively.

3.2 Brewery fermentations and recorded analyses

New, plasmid-carrying brewer's yeasts were fermented with hopped wort. During main fermentation, parameters such as pH, cell concentration and apparent extract were recorded (data not shown). Carbon sources were consumed nearly completely (apparent extract ranged from Es [%] = 3,7–2,5) so that fermentations could be finished after 8 days as usual. Values of pH ranged from 5 to 4.39 and 4.48. Cell numbers increased up to $4.4*10^7$ cells/ml in the middle of the fermentation. It can be concluded that there were no significant differences between generated strains and the wildtype strain.

3.3 Diacetyl

Since the role of genes *BAT1* and *BAT2* on diacetyl formation in yeast has not yet been clarified these genes were overexpressed using a plasmid vector. According to the genetic modifications a reduction of diacetyl concentration could be achieved up to 20 % with the strain LSb holding an extra copy of S. cerevisiae BAT1 gene (day 4, Fig. 2). As a result of *S. cerevisiae BAT2* overexpression the diacetyl concentration was highest (+ 66 %) at the end of the main fermentation when directly compared to the wildtype strain. The yeast strains which contained an additional *BAT2* copy (either *S. cerevisiae* or *lager*) showed severe increased diacetyl levels compared to those strains comprising the specific *BAT1* copies (either *S. cerevisiae* or *lager*). But it is striking that only one modified strain (LSb) could reduce the diacetyl content in beer compared to the wildtype strain.

The gene *BAT1* seems to be involved in diacetyl production more likely than *BAT2* due to the fact that *BAT1* is expressed during logarithmical phase of yeast growth and *BAT2* is induced during stationary phase [28]. Therefore, the continuous increase of diacetyl values measured after fermentation with yeast strains in which *BAT2* had been overexpressed can be explained by late expression of *BAT2*. Moreover, results indicate that *S. cerevisiae* genes *BAT1* and *BAT2* act stronger on the diacetyl production than corresponding genes of the *lager* genome part and that *lager* genes are regulated in a different way than *S. cerevisiae* genes. This assumption could be confirmed by observations regarding *BAP2* genes (branched-chain amino acid permeases for valine, leucine and isoleucine) as *S. cerevisiae BAP2* is induced by supplementary leucine whereas *lager BAP2* is not [25].

As could be shown (Fig. 2) overexpression of *S. cerevisiae BAT1* leads to higher valine concentrations which results in diacetyl reduction. The reason for this is the enhancement of valine concentration hampering functional acetolactate synthase as the gene products of *ILV2* and *ILV6* underly a feedback inhibition caused by valine [29, 30]. Hence, sufficient valine is available for protein biosynthesis whereby ilv2p is inhibited resulting in a decrease of α-acetolactate. The formation of valine is counteracted and less diacetyl is produced consequently.

In contrast to these findings *BAT2* gene product probably catalyzes the reversible reaction to form the α-keto-acid (opposed to bat1p) since increased diacetyl concentrations were obtained after enhancing *BAT2* activity (Fig. 2) [20]. Thus, the precursor of valine (keto-isovalerate) accumulates which leads to enhanced *ILV2* gene activities and further to a strengthened diacetyl production especially during the stationary phase of yeast growth.

3.4 Alcohol/ Esters

The higher alcohol and ester contents of generated strains LSa-LSe measured after beer maturation differed from each other (see Fig. 3). However, the values correspond to usual ranges for bottom-fermented beers and the differences did not have any negative impact on the taste of the beer after sensory tests [31]. However, it was observed that engineered strains LSc (containing *S. cerevisiae BAT2*) and LSe (containing *lager BAT2*) showed the largest variations for isobutanol and for isoamyl acetate compared to the wildtype strain bearing the reference plasmid and strain Sa-06165. As with diacetyl also enhanced amounts of higher alcohols and esters were detected with aforementioned yeast strains. So strain LSe produced 2.1-fold amounts of isoamyl alcohol and strain LSc even produced 2.8-fold concentrations of isoamyl alcohol (Fig. 4, 5).

These alcohols are formed after desamination and decarboxylation reactions of amino acids valine, leucine and isoleucine via Ehrlich-Pathway [32]. So from valine isobutanol and from leucine isoamyl alcohol is formed and the corresponding alcohol of isoleucine is active amyl alcohol [21]. Higher aliphatic alcohols convey a sweetish taste and have a wide influence on the beverage flavour [33]. Furthermore, higher contents of alcohol occur when amino acid concentration in wort decreases. Therefore, higher isoamyl alcohol contents resulting from lacking leucine and increased

isobutanol due to valine deficiency were detected [31]. Derrick and Large (1993) published equivalent results regarding valine lack [34]. Thus unsurprisingly, changes in yeast *BAT* gene activity affected higher alcohol formation. The single deletion of *BAT2* caused a huge decrease in isoamyl alcohol production [22]. Eden *et al.* (2001) found out the deletion of *BAT2* leading to extremely reduced isobutanol contents [20]. As aforementioned, we achieved higher isobutanol concentrations after *BAT2* overexpression which correlates with previous results. When indeed *BAT1* was single deleted or a double deletion of both genes *BAT1* and *BAT2* was present other transaminases undertook their enzymatic functions. However, it is striking that no increasing regulation is noticed when just *BAT2* was deactivated. Thus, it can be outlined that the *BAT1* gene product is not that important for alcohol production in yeast as is bat2p [3]. These findings and our presented approach allow the conclusion that overexpression of *BAT2* gene achieves the accumulation of α-keto-acid and therefore can lead to increased alcohol contents.

The formation of esters is catalyzed by acetyl transferases (ATF) which transfer acetyl-CoA to alcohols [35]. The corresponding esters of amino acids are isobutyl acetate originated from valine, isoamyl acetate from leucine and isoleucine is converted into 2-methyl butyrate [21]. Consequence of higher *BAT2* gene expression level is the enhancement of isoamyl acetate values [23] which conforms to our results presented in this study. In contrast, with yeast bearing an additional *BAT1* gene copy converse effects have been registered. These findings again suggest higher amounts of alcohols and resultant esters being the result of α-keto-acid accumulation in the cell due to raised *BAT2* gene activity. Overexpression of the gene *BAT1* leads to lower contents of esters (see Fig. 5). Thus, it can be concluded that bat1p catalyzes the formation of the amino acid whereas the gene product of *BAT2* promotes the convertion of the corresponding α-keto-acid.

Although the generated yeasts bear marker sequences and thus cannot be applied for industrial beer production the outcome of this work is the clarification of *BAT* gene regulation and its role on diacetyl production in yeast. The knowledge concerning metabolic fluxes can lead to new approaches in order to select brewer's yeast strains in a natural way. In our opinion it should be possible to apply natural engineering techniques for gaining yeast mutants with the "right" genes (increased or decreased expression levels) so that yeast strains can be created which produce less diacetyl without any changes in by-product profile.

Footnotes

* This study is the second part of a two-section publication. The first part with the title "Brewer's yeast and "omics" technologies" came in BrewingScience, **62** (2009), no. 11/12, pp 187-190.

[*1] Brewing yeast have formerly been named *Saccharomyces carlsbergensis* but these multiple genetic lines which contain the *S. cerevisiae* genome and *S. bayanus* genome parts shall now be named *S. pastorianus* [1].

[*2] The genes which belong to the *S. bayanus* genome part of yeast hybrids [2] are herein referred to as *lager* genes.

[*3] Denotations for genes of each *Saccharomyces cerevisiae* and *lager* genome part are abbreviated as Sc and Lg in this paper.

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Appendix

Table 1 Primer designation and oligonucleotide sequences. GCG (primers 1–4) are protection nucleotides, inserted restriction sites are set between hyphens (*NotI*: 5'-G C^GGCCGC-3'; *SacI*: 5'-GAGCT^C-3'), other regions are relevant for annealing

Primer designation	Sequence (5' → 3')
1, Fw-Sc-*BAT1*	GCG-GAGCTC-AAAAA TGTCTTTGCAGAGACATTCCT
2, Fw-Sc-*BAT2*	GCG-GAGCTC-AAAAATGTCTACCTTGGCACCCCTA
3, Rv-Sc-*BAT1*	GCG-GCGGCCGC-TTAGTTCAAGTCGGCAACAG
4, Rv-Sc-*BAT2*	GCG-GCGGCCGC-TCAGTTCAAATCAGTAACAACC
5, Rev.-Lager *BAT1*	GACGAACGGCCTTGAAACCGG
6, Rev.-Lager *BAT2*	GCAAGGAGCATAGTTGGCGCCC
7, Fw-Kanamycin-resistence2	GGGAAGCCCGATGCGCCAGAGTTG
8, Rev-Kanamycin-resistence2	CACCGAGGCAGTTCCATAGGATGGC

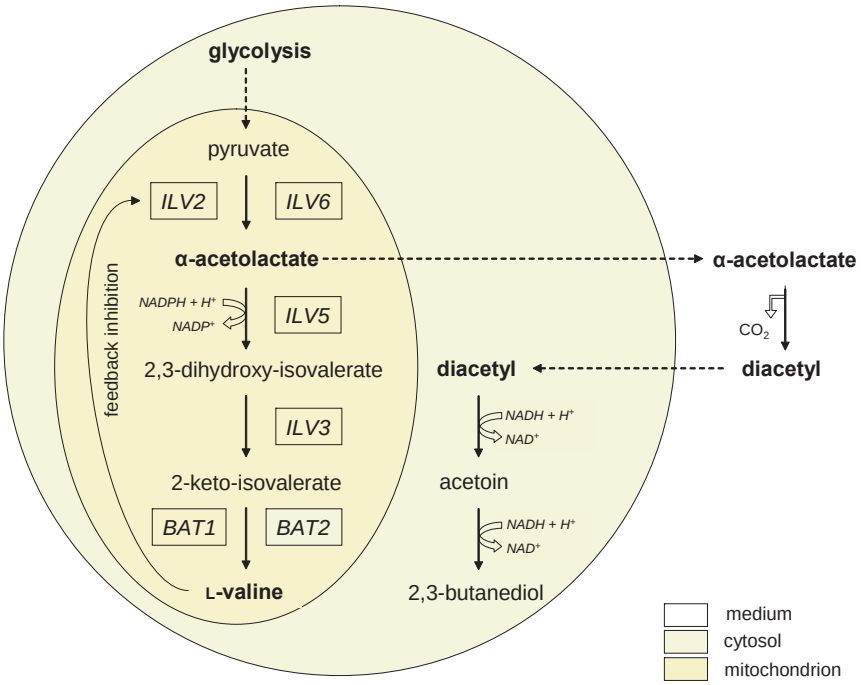

Fig. 1 Diacetyl formation in yeast (according to [4, 5]). *ILV2*: acetolactate synthase, *ILV3*: dihydroxyacid dehydratase, *ILV5*: acetolactate reductoisomerase, *ILV6*: regulatory subunit of *ILV2*, *BAT1*: branched-chain amino acid aminotransferase, *BAT2*: branched-chain amino acid aminotransaminase

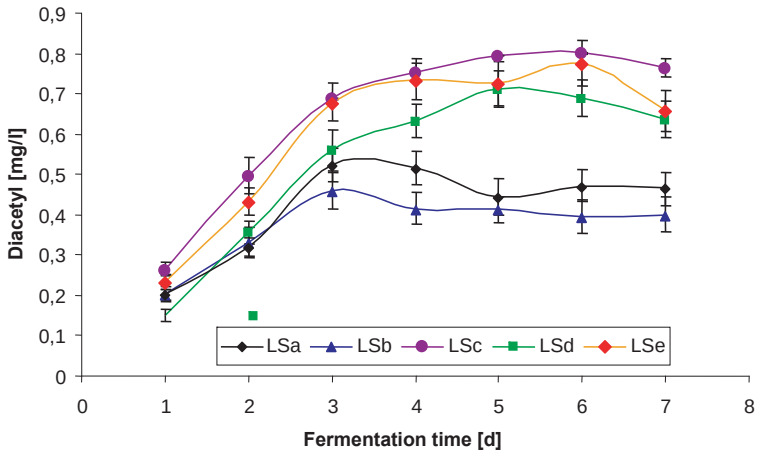

Fig. 2 **Fermentations with four genetic engineered brewing yeast strains (containing an additional copy of either *S. cerevisiae* or *lager BAT1/ BAT2*) and the wildtype strain containing reference plasmid. Strain designation is referred to as LSa (Wildtype containing reference plasmid pYCGPY2), LSb (LSpScBAT1), LSc (LSpScBAT2), LSd (LSpLgBAT1) and LSe (LSpLgBAT2). Here the concentrations of extracellular diacetyl* during main fermentation in 30 l-tanks with hopped wort (T = 11 °C, Original gravity of wort: 11,5 %) are shown for one fermentation (mean values and standard deviations from two samples each measured twice)**

*** Here the total diacetyl concentration is demonstrated which means the sum of free diacetyl and its precursor α-acetolactate**

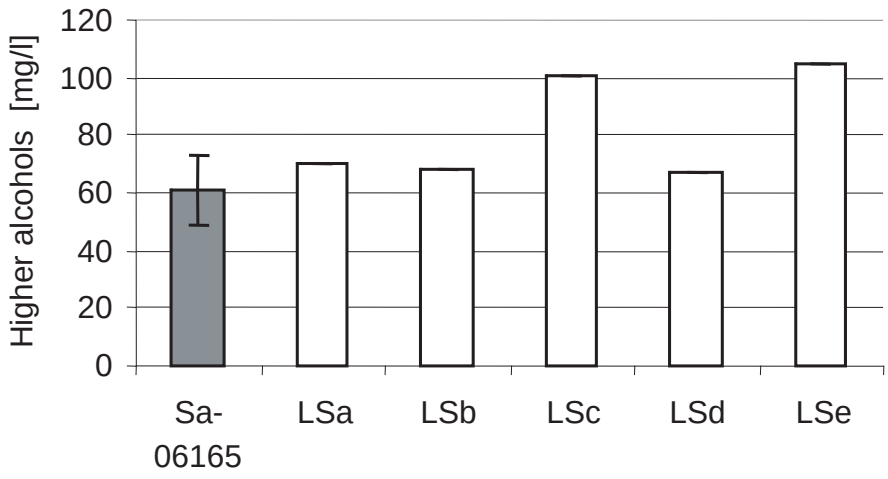

Fig. 3 **Fermentations with four genetic engineered brewing yeast strains (containing an additional copy of either *S. cerevisiae* or *lager BAT1/ BAT2*), the wildtype strain containing reference plasmid and strain Sa-06165. Strain designation is referred to as LSa (Wildtype containing reference plasmid pYCGPY2), LSb (LSpScBAT1), LSc (LSpScBAT2), LSd (LSpLgBAT1) and LSe (LSpLgBAT2). Here the concentrations of higher alcohols (C3-C5) are shown for one fermentation in maturated beer (4 weeks, T = 0 °C) after main fermentation in 30 l-tanks with hopped wort (T = 11 °C, Original gravity of wort: 11,5 %). As concentrations for higher alcohols and esters showed the same levels and standard deviation ranges for LSa and Sa-06165 the modified strains had been fermented in singlicate.**

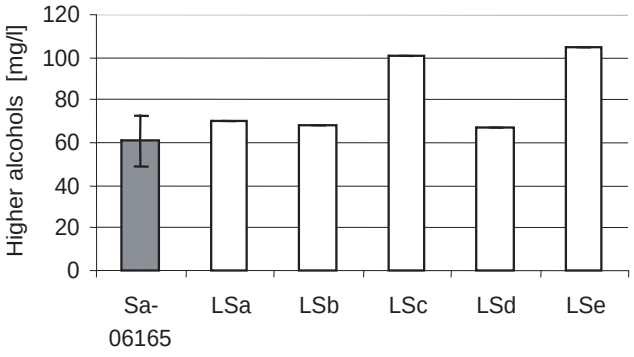

Fig. 4 **Content of isobutanol in beer.**

Strains and brewing conditions are the same as in figure 3

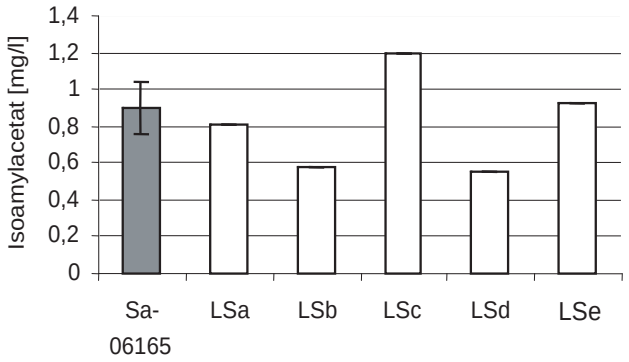

Fig. 5 **Content of isoamyl acetate in beer.**

Strains and brewing conditions are the same as in figure 3