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Development of a genetically defined diploid yeast strain for the application in spirit production

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„Dosis facit venenum“

Paracelsus, 1493 - 1541

CONTENTS

CHAPTER I

SCOPE AND OUTLINE OF THE Ph.D. THESIS

Scope

The diversity and the composition of the yeast micropopulation during fruit fermentations contributes significantly to the sensory characteristics of the spirits. The growth of each yeast species is characterized by specific metabolic activities, which determine concentrations of flavour compounds in the final product (Walker, 1998). However, it should be pointed out that, within each yeast species, significant strain variability has been recorded (Younis and Steward, 1998). The wide use of starter cultures of *Saccharomyces cerevisiae*, mainly applied to reduce the risk of spoilage and unpredictable changes of flavour, ensures a balanced quality. On the other hand it may also cause a loss of characteristic aroma and flavour determinants.

Therefore it could be of great benefit to select and combine certain characteristics of different yeast strains. These could be adjusted according to need not only in spirit production, but also in wine and beer making, to optimize and ensure a reproducible quality. Currently there is a large number of different yeast strains for spirit and wine production on the market. These have been isolated, selected and cultivated from spontaneous fermentations, are readily available and are all claimed to have perfect fermentation skills. In general, little genetic research has been devoted to yeast strains used in fermentation and baking industries. If any, this has concentrated on the winery busyness (Pretorius, 2000). Since financial resources are very scarce for scientific investigations in spirit productions, little attention has been paid to biological improvements.

Accordingly, the yeast strains commonly employed for alcohol production are genetically largely undefined and highly heterogeneous (Benitez *et al.*, 1996). Thus, little is known about their chromosomal constitution and aneuploidy is frequently observed (Bidenne *et al.*, 1992, Cardinali and Martini, 1994, Vezinhet, 1981). This prevents the use of standard genetic manipulations such as crossings and tetrad analysis for strain improvement. Furthermore, it complicates the application of the majority of modern methods developed in yeast molecular biology (Pretorius, 2000). The application of laboratory yeast strains for industrial purposes offers the potential of a genetic and physiological design, since the complete genome

sequence of *S. cerevisiae* is available (Goffeau *et al.*, 1996; Zagulski *et al.*, 1998). Recently, laboratory strains have been developed with improved metabolic features (van Dijken *et al.*, 2000). The efficiency of fermentation could further be improved e. g. by a better sugar utilization, an increased ethanol tolerance, resistance to zymocins and heavy metals, reduced formation of foam, induced flocculance at the end of fermentation, the production of extracellular (or liberated) enzymes or the reduced formation of undesired metabolites. For example, ethyl carbamate (EC) which is mainly found in fermented foods and beverages, has been listed as a carcinogenic agent. Especially in stone fruit brandies EC can additionally origin from the fruit itself. EC forms in fermented food by the reaction of urea and ethanol (Ough et al., 1988a, Pretorius, 2000). It has been assumed that yeast contributes substantially to EC formation since urea is formed during arginine degradation (Ough et al., 1988b, Kitamoto et al., 1991).

Regarding the performance in alcoholic fermentation, it has been claimed that laboratory strains show worse ethanol production kinetics. Furthermore, it is generally believed, that such strains lead to the appearance of undesired aromatic compounds in fermented fruit. Based on the prospect of strain improvement in this work, a genetically well defined prototrophic diploid laboratory yeast strain should be constructed and tested for its fermentative and sensory performances in spirit production. Such a strain offers the potential for further genetic modification by classical breeding and modern molecular genetic techniques, to adjust yeast physiology to special production schemes.

Outline

Chapter II provides an introduction to (i) the fundamentals of the distillation process, (ii) yeast metabolism with regard to the degradation of carbohydrates and nitrogen compounds as well as the formation of secondary fermentation products and flavours, and (iii) the relevance of ethyl carbamate in spirits with a special focus on its origins.

Chapter III describes the construction of a laboratory yeast strain and its suitability for fermentation of fruit mashes in spirit production. The fermentation skills of the laboratory strain are compared to industrial yeast strains. Finally, the influence of the different yeast strains employed on the sensory quality of the spirits has been determined. An outline for future applied research is given, involving genetic possibilities for improvements in spirit

production. This chapter has been published in: Schehl, B., C. Müller, T. Senn, and J. J. Heinisch: A laboratory strain suitable for spirit production. *Yeast* 21:1375-89, 2004.

Chapter IV comprises experiments evaluating the influence of the stone content on the quality and flavour of plum and cherry spirits combined with analytical assessments of the spirits using the laboratory strain and some industrial yeast strains. This chapter has been published in: Schehl B., T. Senn, and J. J. Heinisch. Effect of the stone content on the quality of plum and cherry spirits produced from mash fermentations with commercial and laboratory yeast strains. *J. Agric. Food Chem.* 53:8230-38, 2005.

Chapter V describes the characteristics of spirit production using the established laboratory strain HHD1 compared with its genetically modified mutant HHD1*delCAR1* in laboratory scale experiments. Furthermore the dependence of the EC content on the yeast strain employed has been investigated. Finally, the data are related to the technological procedure used for spirit production. This chapter has been submitted for publication: Schehl, B., D. Lachenmeier, T. Senn, and J. J. Heinisch: Reduction of ethyl carbamate in stone fruit spirits by manipulation of the fermenting yeast strain. *Appl. Environ. Microbiol.*, submitted.

In chapter VI a statistical analysis of a database with regard to ethyl carbamate in stone fruit spirits in the Southern part of Germany over the last 15 years is reported. A discussion on acceptable methods of spirit production based on “state of the art technology” is supported by these data. This chapter has been published in: Lachenmeier, D. W., B. Schehl, T. Kuballa, W. Frank, and T. Senn: Retrospective trends and current status of ethyl carbamate in German stone-fruit spirits. *Food Additives and Contaminants* 22:397-405, 2005.

CONTRIBUTIONS OF CO-AUTHORS

The co-authors **Prof. Dr. Jürgen J. Heinisch** and **Priv. Doz. Dr. Thomas Senn** (Chapters 3 to 5) contributed by their supervision, financial support and many fruitful suggestions to the publications and the studies which have been carried out at the University of Hohenheim, Institute of Food Technology, Section Fermentation Technology. Some of the genetic work was done during a short-term stay at the Department of Biology at the University of Osnabrück, Germany.

Dr. Dirk W. Lachenmeier also contributed by intensive discussions and carried out the ethyl carbamate analyses at the *Chemisches und Veterinäruntersuchungsamt (CVUA)*, Karlsruhe, Germany.

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CHAPTER II

GENERAL INTRODUCTION

OVERVIEW OF THE DISTILLATION PROCESS

The manufacturing of fruit brandies is divided into several steps: mashing process (with cleaning and comminution of the fruit), fermentation, distillation, dilution, filtration and storage of the spirits. These will be discussed separately in the following.

Raw material and mashing

Any saccharine (sweet) or amylaceous (starchy) substance can be used as raw material for alcoholic fermentation (Rideal, 1920, Kreipe 1981). Raw materials like pip fruit, stone fruit, berries and starch rich corn cultivars owe their use in spirit production to the fact that a high sugar content and distinctive characteristic aromas are essential for a good quantity and quality of a distilled spirit. Fruit to be mashed should be ripe and clean. Putrid fruits deteriorate the mash and may cause faults in fermentation and consequently result in a worse distillate. The fruits should be crushed mechanically. Depending on the type of fruit, roller mills, masher or fruit mixer and pumps with a feed screw, are used. In order to reach an optimal fermentation, yeast and an appropriate acid to adjust the pH-value to 3.0 should be added to reduce the risk of bacterial contaminations (Pieper et al., 1993, Bernath et al., 1999).

Fermentation

Yeast is a living organism and therefore is generally not regarded as a “raw material”. It is largely responsible for fermentation and is thus more than a mere constituent. The strains used invariably belong to the species *Saccharomyces cerevisiae*, in addition, the so called “wild yeasts” which belong to other species such as *Kloeckera apiculata*, *Debaryomyces Kluyveromyces, Pichia, Brettanomyces, Schizosaccharomyces* and *Torulaspora* are usually introduced with the raw material (Pieper et al., 1993, Pretorius, 2000). The fermentation takes place in the absence of oxygen in closed tanks and is normally finished after 10 - 20 days. The fermentation process can be further controlled if the tanks are equipped with a stirrer and a temperature controller. It should be noted, that even in the presence of oxygen, yeast would predominantly ferment the available sugars (Lagunas, 1979, Lagunas, 1986).

Distillation

Basically distillation is a process used to separate a composite mixture into its constituting substances. It involves a change of state, usually from liquid to gas, and a subsequent condensation into the liquid phase. Thus, distillation has been defined as "*the separation of the constituents of a liquid mixture by partial vaporisation and separate recovery of the vapour and residue*" (Roempp, 2005). It takes advantage of the fact, that alcohol has a higher vapour pressure and lower boiling point than water. If a mixture of alcohol and water is heated to its boiling point the vapour will be richer in alcohol than the liquid. Therefore, when the vapour is condensed, the liquid collected will have a higher alcohol content than the original mixture. It should be realised that the products of fermentation are not simply alcohol and carbon dioxide, but include many other compounds, such as aldehydes, esters and higher alcohols. These are present at low levels, but need to be taken into account in the distillation regime. Since different constituents, such as water, alcohol, and fusel oils, have different boiling points, the elements separate. The distilled material is then collected in a vessel. Heads and tails (undesirable elements like acetaldehyde and fusel oils) are excluded from drinkable alcohol (Pieper et al., 1993).

Types of pot stills used in the spirit industry are the alembic pot still and the reflux or column pot still. The alembic pot still is the oldest and commonly known still design. The flask or kettle is typically made from copper and resembles a huge onion shape, which liberates the alcohol from the mixture. The vapours rise and pass through a narrow pipe and then through a serpentine coil. A cold-water bath condenses the vapours in the coils. The modern reflux or column pot still is technologically more advanced, usually more efficient, mainly steam-heated and requires only a single distillation done in one continuous operation (Figure 1). This type of still allows more exactly tuned separations. Also, changing the reflux rate (defined as the ratio of the amount of condensate being refluxed to the amount being withdrawn as product) provides great flexibility in the style and quality of the spirit produced. Distillations can be carried out either as a batch or a continuous process. This in turn has produced significant changes in practice (Alcohol Textbook, 1999). The size of the distillation unit depends on the quantity of the raw material to be processed. Nonetheless, the basic operations are all shared.

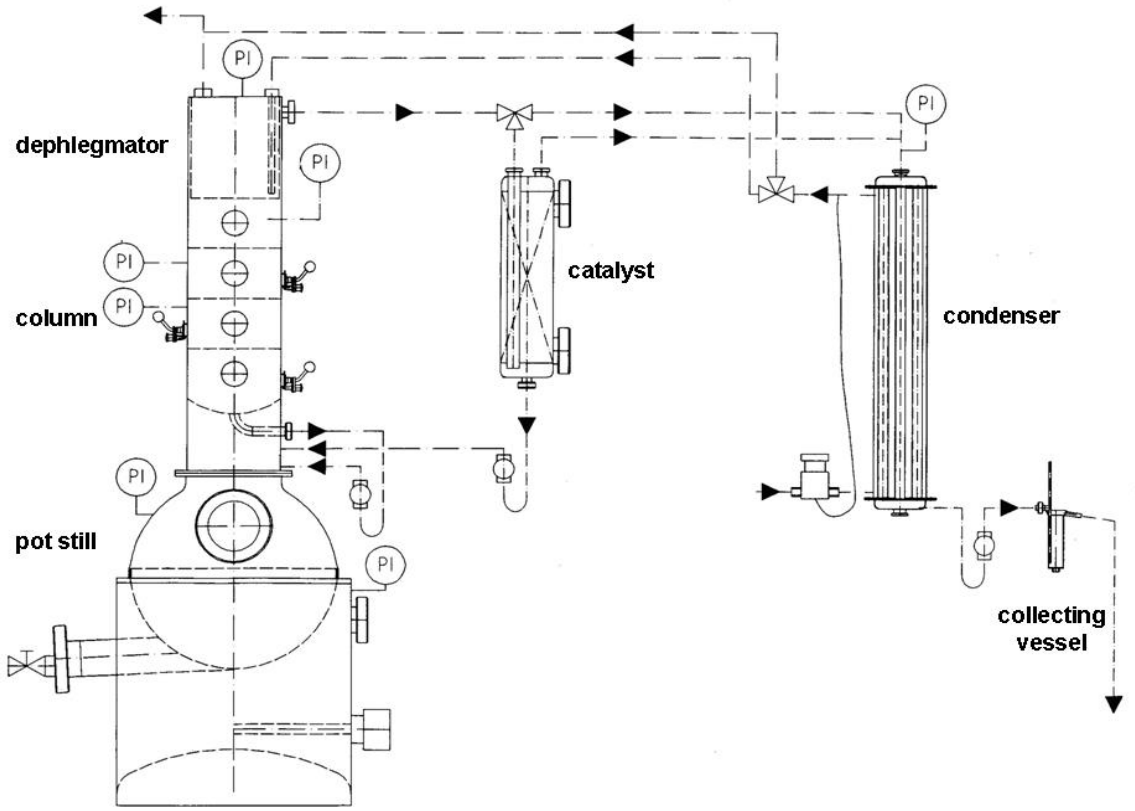


Fig. 1: Schematic overview over the distillation process in fruit spirit production (J. Carl, Göppingen); see text for detailed descriptions

Dilution, Filtration, Storage and Product Quality

After distillation the collected product fractions are diluted with deionised water to an alcohol content of 40% (v/v) and cold filtered at 4°C. Heads and tailings can be discarded. The spirits diluted and filtered are stored up to several months to improve their aroma profile by the formation of acetales and esters. In general, qualities of fruit brandies are judged by the quality and quantity of the flavour of the original fruit which they convey. Some varieties of fruits have stronger flavours when distilled than others. For instance, Williams-pears, though not necessarily the most flavoursome variety of pear when eaten fresh, produce a much richer flavour when distilled, than does any other pear variety. Cherry spirits on the other hand do not taste much like cherries. Rather, the characteristic, almost almond flavour is determined by the fact that the stones are also fermented and distilled together with the flesh of the cherries. In general during distillation, the flavour of the fruit is transferred in the form of oily esters dissolved in the hot alcohol vapour.

Sensory aspects

Regarding the features just explained, clearly the ethanol content is not the major determinant for the production of a high quality spirit. Fruit spirits are considered as a premium product, if the typical flavour of the fruit is entirely retained in the distillate, though tasting is smooth and clean. A panel of trained and experienced probationers is necessary to reach such a conclusion on the quality of a fruit spirit and to provide an objective, reproducible and statistically significant result.

BASIC PRINCIPLES OF THE DISTILLATION PROCESS

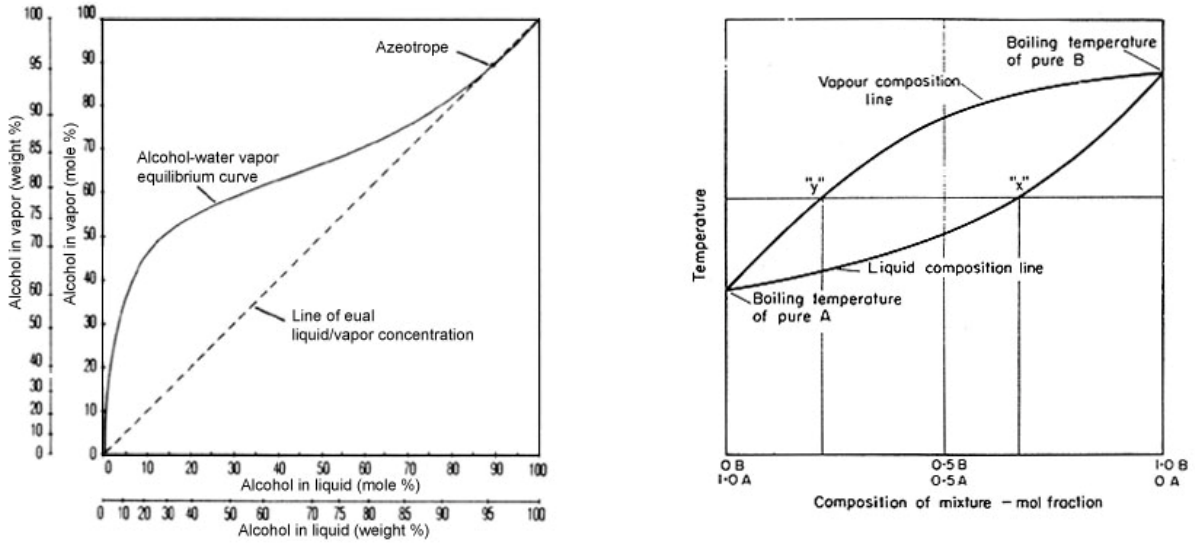
Distillation of alcohol-water-mixtures

Distillation is a separation process, which is used to separate mixtures of liquids with different boiling points (i.e. of volatile from non-volatile materials), such as ethyl alcohol and water. Ethyl alcohol boils at 78.3°C, while water boils at 100°C. A mixture of these two liquids will boil at any temperature between 78.3° and 100°C, depending on the ratio of alcohol to water.

When vapours are produced from a mixture, they contain the components of the original mixture, but in proportions which are determined by the relative volatilities of these components. The vapour is richer in the more volatile components leading to separation. In fractional distillation, the vapour is condensed and then again evaporated for further separation.

The amount of water vapour and that of alcohol vapour contained in the gaseous mixture above the liquid will reach a constant value, which is dependent on the temperature and pressure. The liquid and vapour mixtures reach an "equilibrium", a condition under which there is no change in the liquid/vapour ratio or in the alcohol/water ratio within either the liquid or vapour mixture. However, the ratio of alcohol to water in the vapour phase is generally greater than the ratio in the liquid phase, because alcohol is more volatile than water (Figure 2a and 2b). The equilibrium curves for two-component vapour-liquid mixtures can be presented in two forms: as boiling temperature/concentration curves or as vapour/liquid concentration distribution curves. Both forms are related as derive from the same data and the concentration distribution curves, which are much the same as the equilibrium curves used in extraction, can readily be obtained from the boiling temperature/

concentration curves. The vapour-versus-liquid composition in an alcohol-water mixture under atmospheric pressure is shown in Figure 2a.



a) b)

Figure 2: a) Equilibrium relationship between gaseous and liquid alcohol-water mixtures under atmospheric pressure b) Boiling temperature/concentration diagram (both modified from Pieper et al., 1993 and The Alcohol Textbook, 1999)
The dotted line in Figure 2a represents an equal concentration of alcohol in both the liquid and the vapour state. Note that the alcohol concentration is consistently higher in the vapour phase than in the liquid phase for most of the graph. Figure 2b is further explained in the text.

A boiling temperature/concentration diagram is shown in Figure 2b. If a horizontal (constant temperature) line is drawn across the diagram within the limit temperatures of the two curves, it will cut both curves. This horizontal line corresponds to a particular boiling temperature, the point at which it cuts the lower line gives the concentration of the liquid boiling at this temperature, the point at which it cuts the upper line gives the concentration of the vapour condensing at this same temperature. Thus the two points give the two concentrations which are in equilibrium. They give in fact two corresponding values on the concentration distribution curves, the point on the liquid line corresponding to an *x* point (that is to the concentration in the heavier phase) and the point on the vapour line to a *y* point (concentration in the lighter phase). The diagram shows that the *y* value is richer in the more volatile component of the mixture than *x*, and this is the basis for separation by distillation.

It is difficult and sometimes impossible to prepare pure components in this way, but a reasonably high degree of separation can easily be obtained if the volatilities are sufficiently different. Where higher purity is required, successive distillations may be used. By the

separation of a controlled series of successive sequences of evaporation and condensation in each step condensation, from the previous vapour state achieves a higher alcohol concentration. Thereby alcohol-water mixtures of up to 95.6%mas alcohol can be achieved. At this concentration, the two substances cannot further be separated. A mixture of this composition is called an "azeotropic mixture". For fruit spirit production, successive rounds of distillation are halted much below this point, at approximately 80%vol alcohol.

Pot-Type Distillation Process

The main parts of a conventional distillation setup for the continuous fractionation of liquids consist of a boiler (in which the necessary heat to vaporize the liquid is supplied), a column (in which the actual contact stages for the distillation separation are provided), and a condenser (for condensation of the final top product).

In the pot distillation process, the entire batch of fermented mash is heated to boiling in a copper pot still, the alcohol-water vapours are condensed and channelled into the distillation column. At the end of the distillation, the liquid remaining in the still is withdrawn as the residue (so called “Schlempe”). Such a process will always be a batch procedure and involves only the use of a rectifying column, since the stripping is done as the alcohol vapours are boiled off from the pot. A modern pot still is illustrated in Figure 3.

The condenser and the steam heated boiler are straightforward. The fractionation column (Figure 4a) is more complicated, as it has to provide a series of contact stages for contacting the liquid and the vapour. The conventional arrangement is in the form of "bubble-plates”, whose design is shown in Figure 4b. The column can be constructed from copper, iron, or steel pipe and fittings. Aluminium is not suitable because it can react chemically with the alcohol.

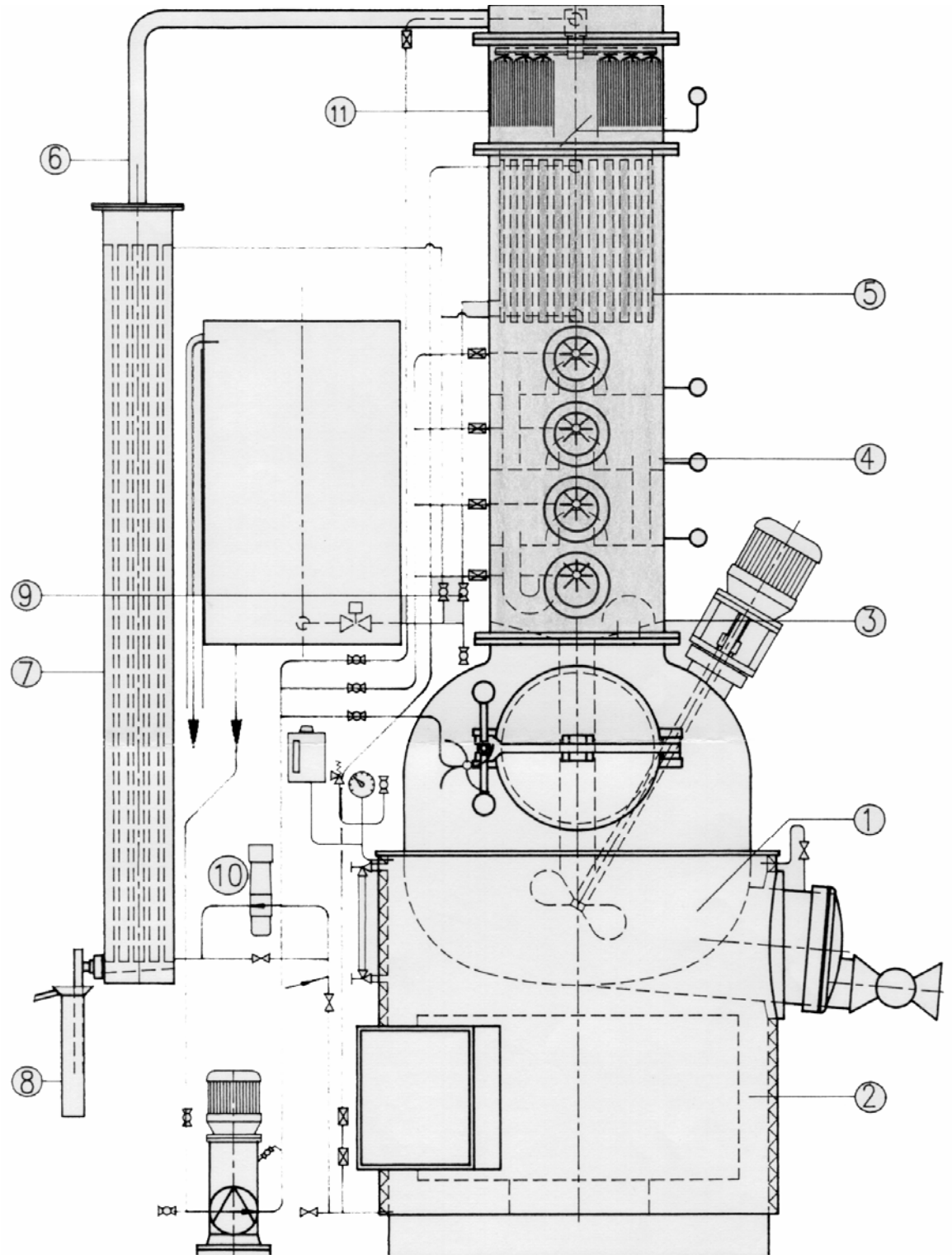
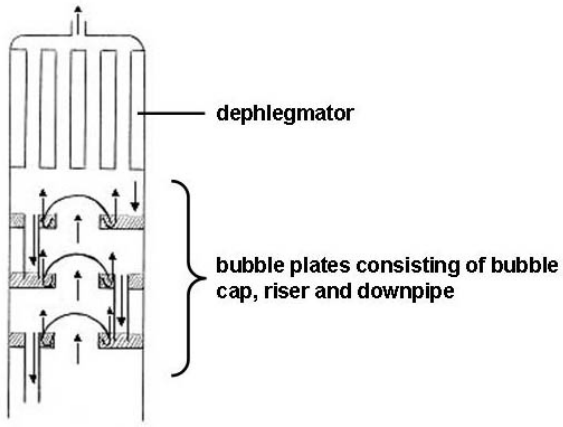


Fig. 3: Assembly of a 200 liter copper pot still (J. Carl, Göppingen)
Boiling vessel (1), water bath (2), overboil mechanism (3), intensifier (4), dephlegmator (5), condenser (6), water-jacketed cooler (7), collection pot (8), switch (9), water cooling controller (10), catalyst (11)

a)



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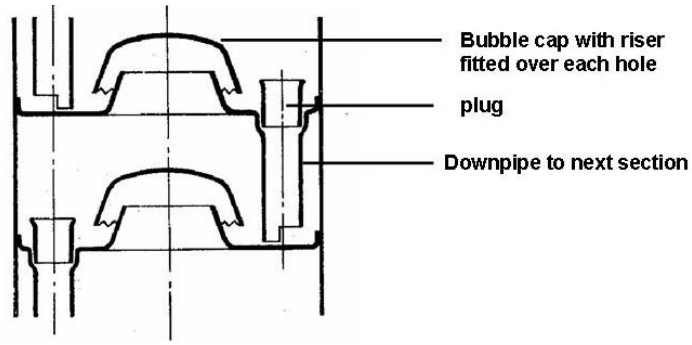

Fig. 4: a) Typical column with bubble-plates and dephlegmator b) Assembly and mode of operation of "bubble-plates" (Kolb, 2002)
A bubble cap tray has riser or chimney fitted over each hole, and a cap that covers it. The cap is mounted so that there is a space between riser and cap to allow the passage of vapour. Vapour rises through the chimney and is directed downward by the cap, finally discharging through slots in the cap, and finally bubbling through the liquid on the tray.

As the liquid begins to boil, vapours rise in the column. After a while, the column will achieve a constant temperature, and an equilibrium will be established. As the vapours from the still pot ascend through the column, they condense on the bubble caps and drip downward. The liquid flows across the trays past the bubble plate where it contacts the vapour and then over a weir and down to the next tray. The descending liquid flows downward from plate to plate through down-pipes. Not much of the liquid does flow through the down-pipe, because of the pressure exerted by the ascending vapour. Thus, a certain amount of liquid is "trapped" on each plate and, as the vapours bubble through it, alcohol is removed from the descending liquid. The result is equivalent to a separate "distillation" being performed at each plate.

Each tray represents a contact stage and a sufficient number of stages must be provided to reach the desired separation of the components (note that German legislation allows for the use of a maximum of only three trays in spirit production). Additional ascending vapours contact the descending liquid (called "reflux") and revaporize it. Thus, as the vapours slowly work their way up the column, they increase in alcohol content. In this process higher reflux ratios usually result in higher proof. Meanwhile, the descending liquid is stripped of its alcohol. The overall effect is that the distillation is performed in one continuous operation and the liquid in the pot still is stripped of its alcohol.

The top of the column is connected to a condenser. There the concentrated alcohol-water vapour of 80-95% alcohol is condensed to liquid by cooling. The condenser can consist of several coils of stainless steel inside a suitable container as illustrated in Figure 5. Normally water is used as the heat exchange medium. Cold water is circulated through the coil to condense a portion of the ascending vapours and, thus, increase the amount of reflux. Adjustment of cooling water in the reflux coils must be very precise. On small stills, air cooled condensers have also been used. Most importantly the condenser has to be large enough to cool all of the vapours from the still to temperatures below 20°C.

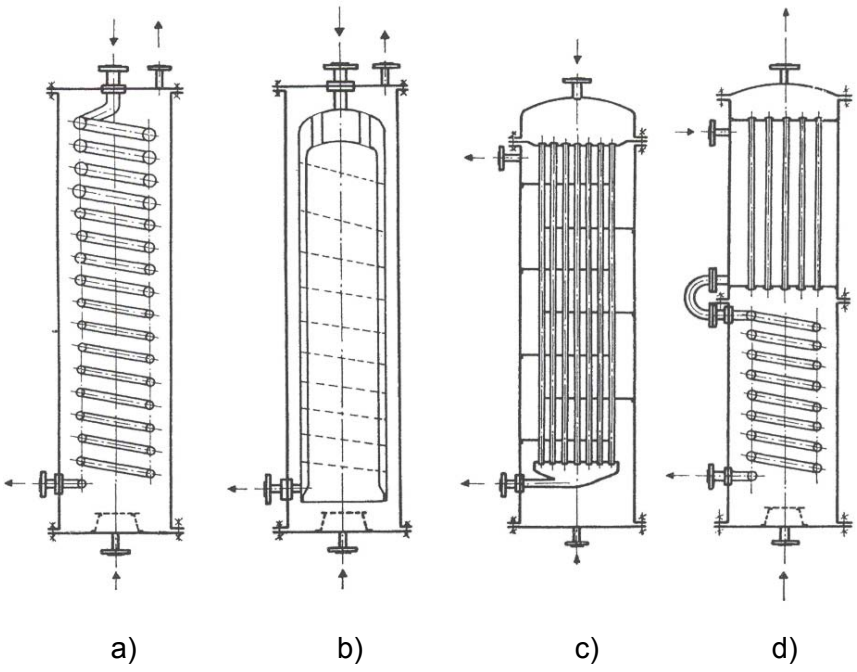

Fig. 5: Different types of condenser (Kolb, 2002)
Condenser with spiral cooling tubes (a), Condenser with cylindrical cooling section (b), Condenser with straight-lined tubes (c), Condenser with combined straight-lined tubes and spiral section (d)

It should be noted that during the early stages of distillation, certain low boiling vapours may already reach the condenser. A small amount of liquid is thus collected, that is not ethanol. This liquid is composed of substances in the mash that have a lower boiling point than ethanol. Also, the distillation progresses, the vapours in the still pot will contain an increasing percentage of water and a correspondingly lesser proportion of alcohol. The still pot vapour temperature will then rise. Eventually, a point will be reached, where there is too little alcohol in the vapour for the column to achieve effective separation. The temperature at the still head will rise slightly and the proof of the product will be lower. At this point, the product coming from the condenser should be collected in the low proof container mentioned above. The distillation should be continued until the temperature at the still head equals the temperature of the vapours in the boiler, which will be near 100 °C, depending on altitude, atmospheric pressure, and the amount of dissolved material in the mash. When the ethanol concentration in the distilled fractions comes under 5%(v/v), all the alcohol has been removed from the mash and the distillation is normally complete.

FERMENTATION BIOCHEMISTRY AND YEAST METABOLISM

'Yeast' in every day language is synonymous with *Saccharomyces cerevisiae* (the name was created for a yeast strain observed in malt in 1837; FEBS, 2000). This species is probably one of the oldest domesticated organisms used since thousands of years to make bread, wine and beer. Scientifically, yeast fermentation and its ability to ferment sugar were first investigated by Louis Pasteur in 1857. In terms of application, *S. cerevisiae* is the most important of approximately 700 known yeast species (Barnett et al., 2000). They all belong to the kingdom of fungi which are estimated to contain 700.000 different species. Moreover, *S. cerevisiae* and other yeasts offer a variety of industrial and medical applications beneficial to human life. Potable and industrial alcohol worldwide is almost exclusively produced by yeast fermentation employing *S. cerevisiae*. Ethanol is, quantitatively and economically the world's premier biotechnological commodity, and is produced at 24 billion liter per year (Walker, 1998, Alcohol Textbook, 1999). The potable alcohol industry produces brewing, winery and distillery products destined for human consumption, while the non-potable alcohol industry manufactures ethanol for fuel and industrial purposes (Figure 6).

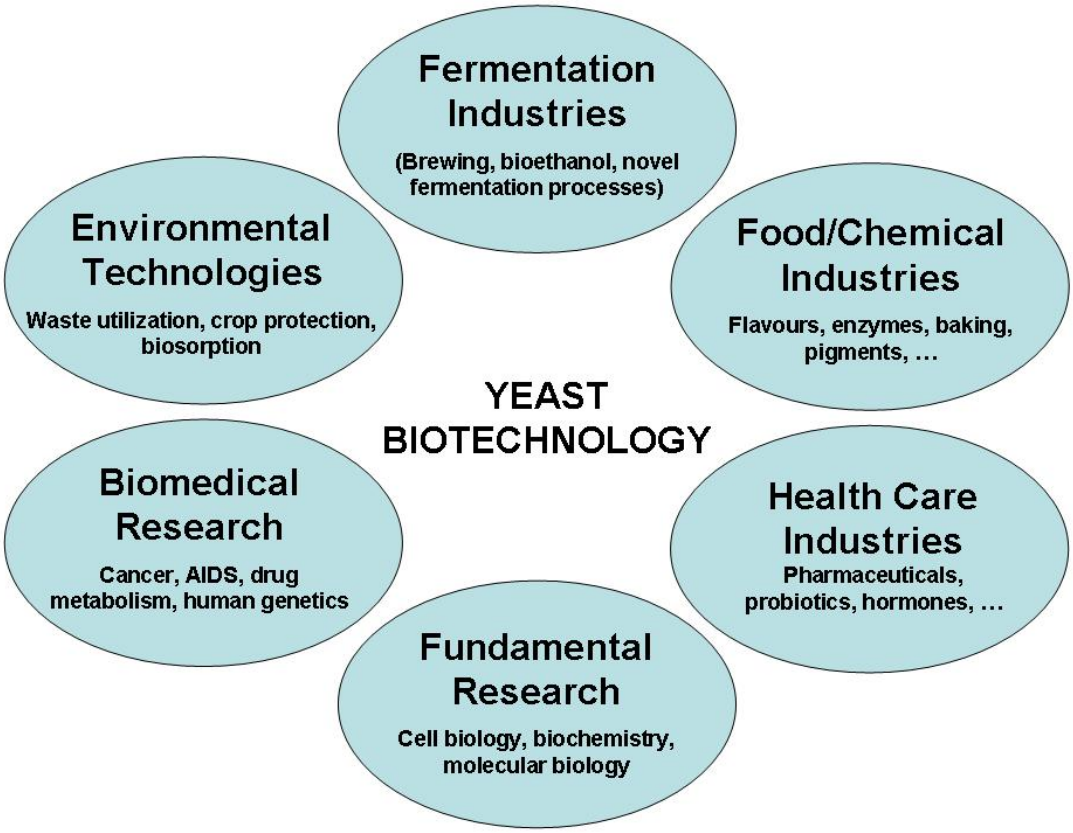

Fig. 6: Diversity of outlets involving yeast biotechnology (Walker, 1998)

Yeast has been introduced as an experimental organism in the mid-thirties of the 20th century (Roman, 1981) and has since received increasing attention. The elegance of yeast genetics and the ease of manipulation of yeast, and finally the technical breakthrough of yeast transformation to be used in reverse genetics, have substantially contributed to the enormous advances in yeast molecular biology (Strathern et al., 1981; Broach et al., 1991; Guthrie and Fink, 1991). This success is also due to the fact, that the extent to which basic biological structures and processes have been conserved throughout eukaryotic life is remarkable.

The following paragraphs focus on physiological and metabolic aspects of *S. cerevisiae* related to fermentation in spirit production.

Yeast physiology

S. cerevisiae is a eukaryote and defined as an ascomycetous fungus that reproduces vegetatively by budding or fission with sexual states which are not enclosed in a fruiting body. It is a single-cell organism that, as it grows and ferments, produces alcohol and carbon dioxide. An idealized yeast cell is schematically shown in Figure 7. Yeast cells share most of the structural and functional features of higher eukaryotes. In contrast to mammalian cells, yeast cells are surrounded by a rigid cell wall and develop scars during cell division. The vacuole corresponds to lysomes in higher cells. The subcellular organisation of yeast cells has been extensively studied in Walker, 1998.

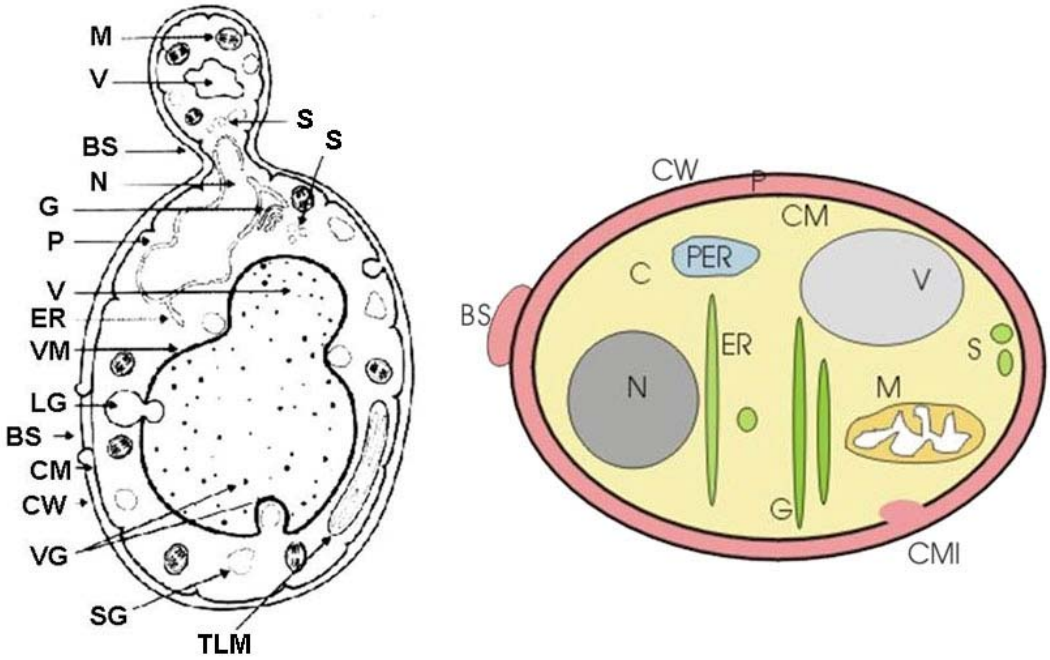

Fig. 7: Idealized schematic structure of a yeast cell (Walker, 1998, Pretorius, 2000)
mitochondrion (M), vacuole (V), bud scar (BS), nucleus (N), Golgi apparatus (G), periplasm (P), endoplasmic reticulum (ER), vacuolar membrane (VM), lipid granule (LG), cell membrane (CM), cell wall (CW), vacuolar granules (VG), storage granule (SG), thread-like mitochondrion (TLM), secretory vesicles (S), cytosole (C), peroxisome (PER), invagination (CMI)

Life cycle

The life cycle of *S. cerevisiae* (Figure 8) can alternate between a diplophase and a haplophase. Both phases are stable and propagate by budding. In heterothallic strains, haploid cells are either of two mating types, **a** or α. Mating of **a** and α cells yields in **a**/α diploids, that are unable to mate but can undergo meiosis. The four haploid products resulting from meiosis of a diploid cell are contained within the wall of the mother cell (the ascus). Digestion of the ascus and separation of the spores by micromanipulation yields clones of the four haploid meiotic products. Analysis of the segregation patterns of different heterozygous markers among the four spores by tetrad analysis has been widely used to determine the linkage between genes (Mortimer and Schild, 1991).

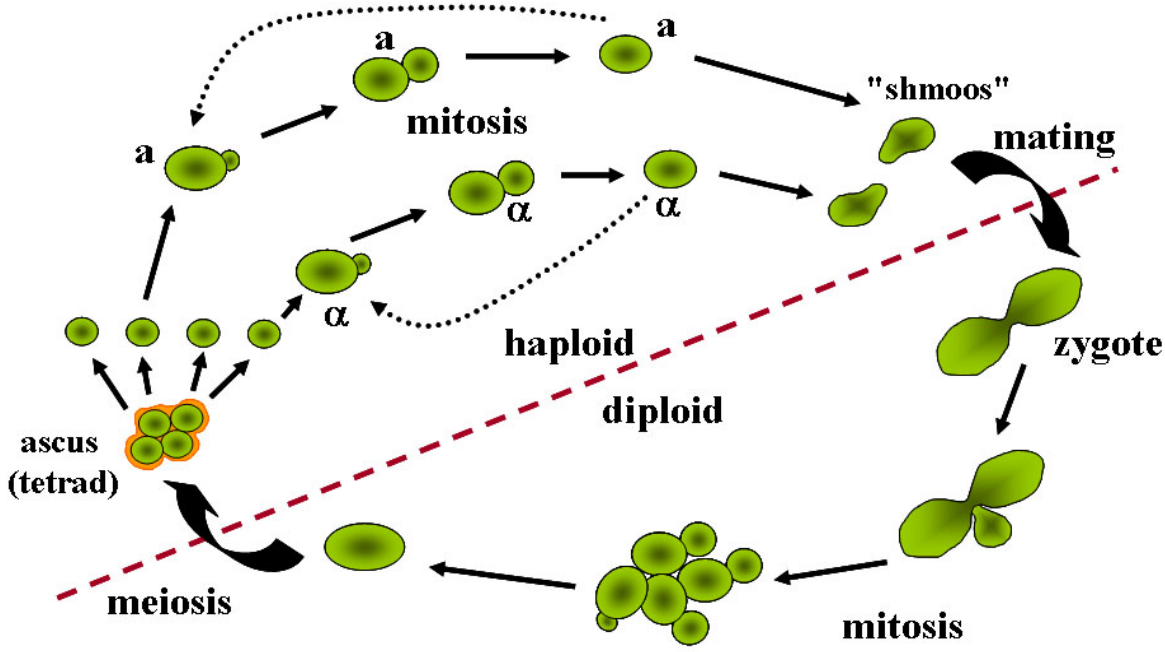

Fig. 8: Life cycle of *S. cerevisiae* (Heinisch, 2002)

Metabolism

Yeast can grow both aerobically and anaerobically. The two metabolic modes are depicted in Figure 9. It is often wrongfully claimed, that yeast switches between these two modes depending on the availability of oxygen (the so called “Pasteur-effect”). Yet, while the respiratory pathway exists in yeast, most of the available sugar is usually fermented, even in the presence of oxygen (Lagunas, 1979; Heinisch, 2002).

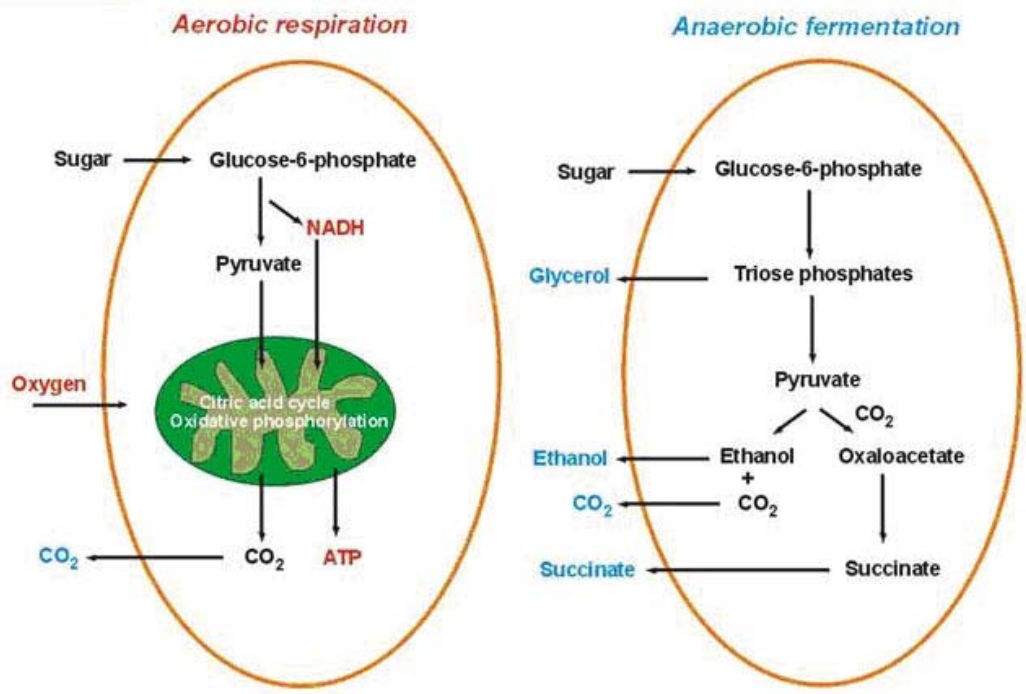

Fig. 9: Overview of the aerobic and anaerobic metabolism of *S. cerevisiae* (Walker, 1998)

The majority of organisms, including micro organisms like yeasts and bacteria can utilize glucose as a carbon- and energy source by channelling it through glycolysis (Heinisch and Hollenberg, 1993). This process is a series of consecutive chemical conversions that require the participation of different enzymes, which have been thoroughly studied. Glycolysis begins with a single molecule of glucose and concludes with the production of two molecules of pyruvate. The pathway is mainly catabolic, and occurs in two major stages:

The first is the conversion of the various sugars to a common intermediate, glucose-6-phosphate and in the second stage this is converted to pyruvate. Some of the energy that is liberated upon degradation of glucose is conserved by the simultaneous formation of the so-called high-energy molecule adenosin triphosphate (ATP). Two reactions of the glycolytic sequence proceed with the concomitant production of ATP, thus ATP synthesis is said to be coupled to glycolysis. Hundreds of enzymatic reactions, particularly those involved in the synthesis of cellular components and those that allow the cell to perform mechanical work, require the participation of ATP as a source of chemical energy. Glycolysis yields two pyruvate molecules, and a net gain of 2 ATP and two NADH per glucose (Figure 10).

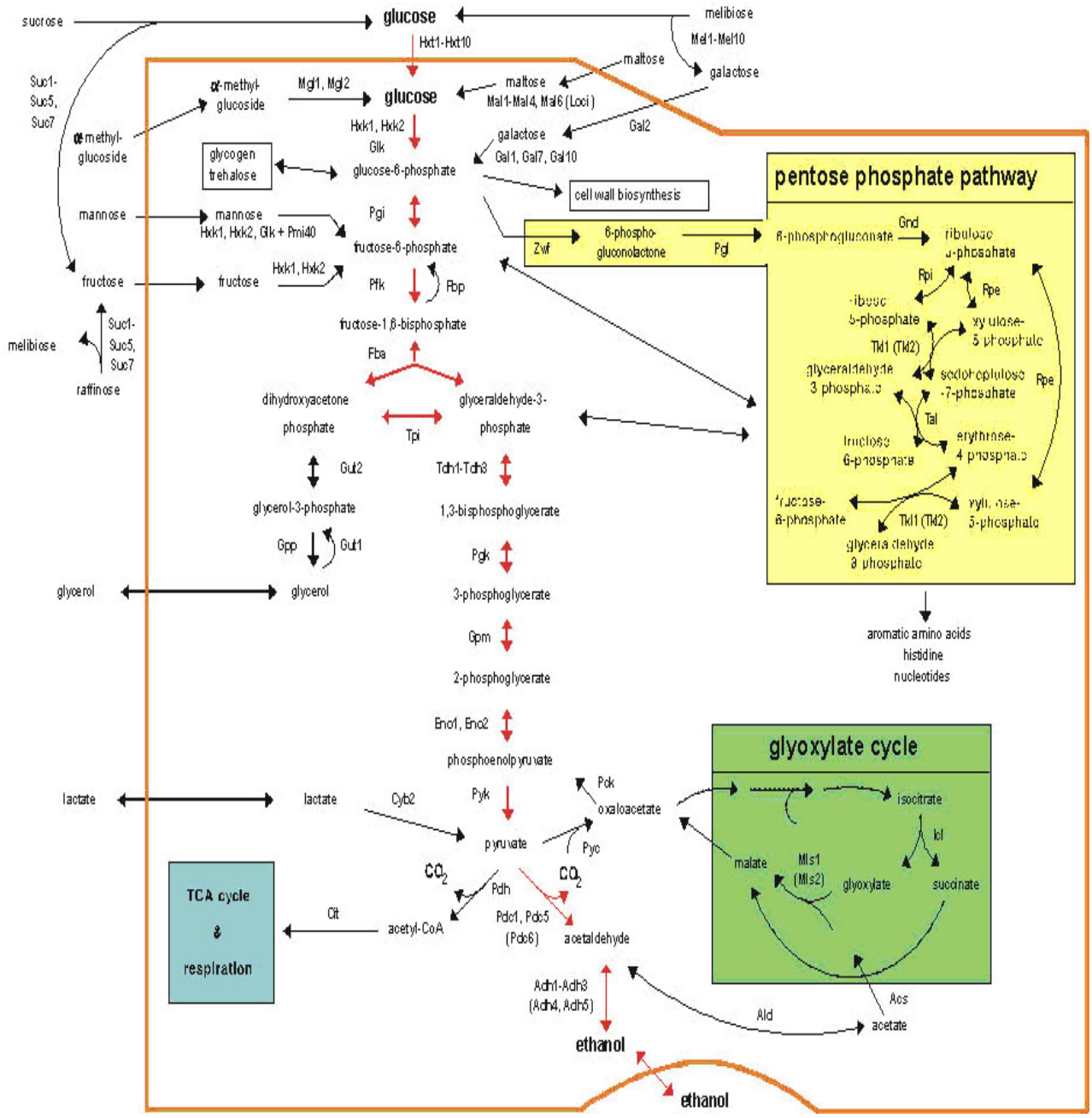

Fig. 10 Schematic overview of carbohydrate metabolism in *S. cerevisiae* (Heinisch and Hollenberg, 1993)
Enzymes important in the context of this work are: glycolytic pathway enzymes (hexokinase *HXK*, glucokinase *GLK*, phosphoglucose isomerase *PGI*, phosphofructokinase *PFK*, aldolase FBA, triosephosphate isomerase *TPI*, glyceraldehydes-3-phosphate dehydrogenase *TPH*, phosphorglycerate kinase *PGK*, phosphoglycerate mutase *GPM*, enolase *ENO*, pyruvate kinase *PYK*, pyruvate decarboxylase *PDC*, alcohol dehydrogenase *ADH*) The boxed reactions belong to the pentose phosphate pathway (6-phosphogluconate dehydrogenase, ribose phosphate isomerase, ribose phosphate epimerase, transketolase, transaldolase, glucose-6-phosphate dehydrogenase). Furthermore: Glycerol-3-phosphate dehydrogenase, glycerol-1-phosphatase, glycerolkinase, phosphoenolpyruvate carboxylase, lactate dehydrogenase, pyruvate dehydrogenase, pyruvate carboxylase and citrate synthase (for further details see Heinisch and Hollenberg, 1993).

An alternative mode of glucose oxidation is the pentose phosphate cycle, which provides the cell with pentose sugars and cytosolic NADPH. The latter is necessary for biosynthetic reactions, such as the production of fatty acids, amino acids and sugar alcohols. Since the pentose phosphate pathway accounts for a maximum of 2.5% of the glucose degraded by yeast (Gancedo, 1998) and has not been directly related to alcoholic fermentation, it will not be discussed in detail here.

Further downstream, different routes of fermentation have been developed by different organisms to dispose of the pyruvate under anaerobic conditions. These include lactic acid fermentation, anaerobic respiration and alcoholic fermentation (Schlegel, 1992). Yeast even under aerobic conditions in the presence of high sugar concentrations (> 0.05%) uses an enzymatic two-step process to yield ethanol and CO_2 (Figure 11).

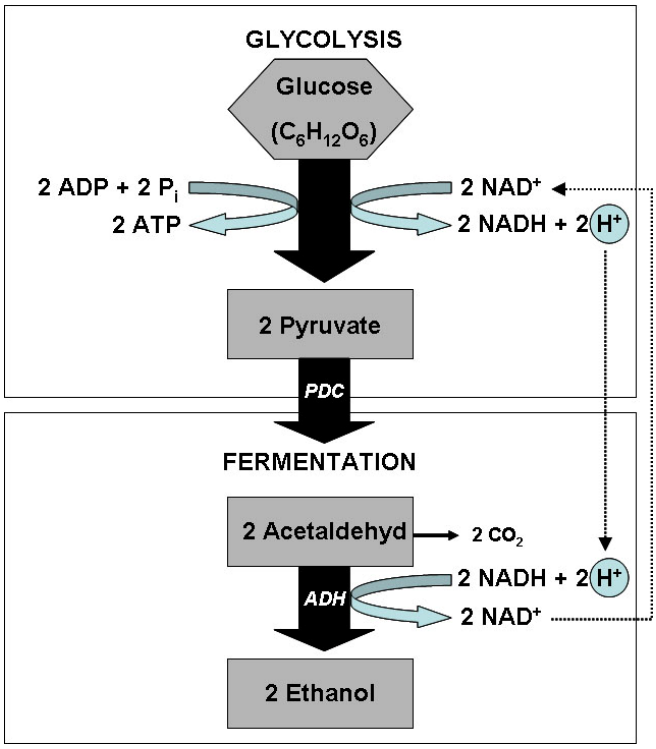

Fig. 11: Formation of ethanol from sugar – the alcoholic fermentation (Pyruvate decarboxylase (PDC), Alcohol dehydrogenase (ADH); modified from Schlegel, 1992)

Under aerobic conditions (aerobic respiration), most organisms will use the citric acid cycle and the electron transport chain to produce their ATP. In eukaryotes, these processes occur in the mitochondria, while in prokaryotes they occur at the plasma membrane. In yeast, it has been estimated that only approximately 5% of the pyruvate produced in glycolysis is further metabolized through the citric acid cycle (Figure 12) and respiration to yield ATP.

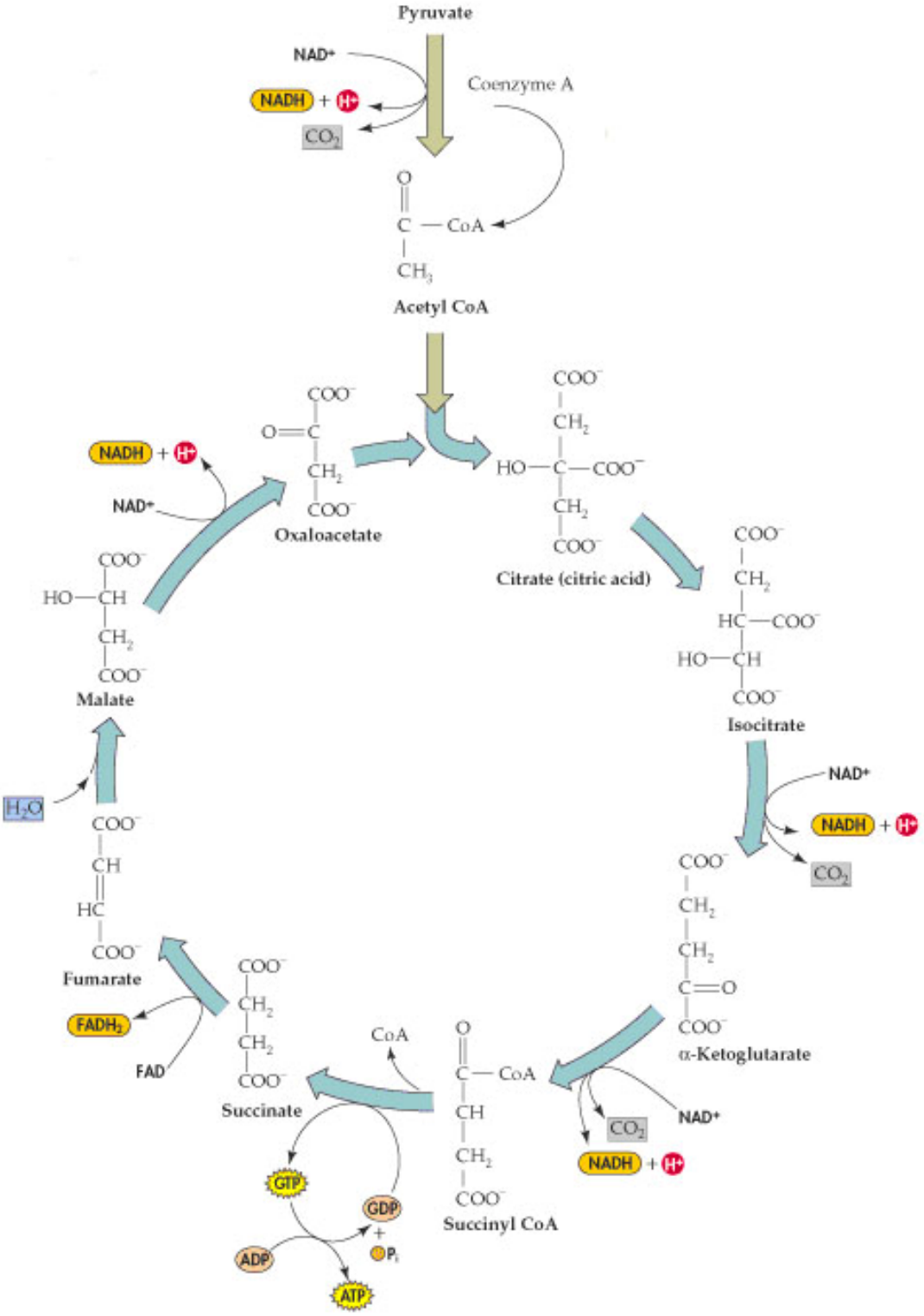

Fig. 12: The citric acid cycle (also known as Krebs or TCA Cycle). Pyruvate oxidation and the citric acid cycle take place in the mitochondrial matrix (Roempp, 2005)
The reduction equivalents produced in this cycle are used in respiratory chain to generate a proton motive source leading to the generation of ATP.

A significant part of the aromatic compounds (e. g. organic acids, glycerol, higher alcohols) found in spirits is derived from yeast metabolism. Note that some of the intermediates of the citric acid cycle may be liberated by yeast during fermentation to contribute to the aromatic composition of potable alcohols. While the citric acid cycle *per se* is not essential for energy production during anaerobic fermentation, the organic acids produced by some of the enzymatic reactions of the cycle are still needed for biosynthetic purposes and are not of much consequence as flavour components. When mash is inoculated with aerobically grown *S. cerevisiae*, ethanol is not immediately produced. In respiring cells, pyruvate decarboxylase and alcohol dehydrogenase activities initially are low and have to be induced by the presence of glucose. This leads to the production of compounds other than ethanol in the early stages of fermentation. Glycerol, pyruvate and succinate are formed at this time as are other organic acids (Boulton et al., 1996). Glycerol may serve as an alternative route for regeneration of NAD^+ from NADH. Since no net ATP can be produced if all triose units are shunted to glycerol, this pathway is down-regulated in the course of fermentation.

It has been observed, that during all yeast fermentations, small amounts of higher alcohols (mainly 2-methyl butanol, 3-methyl butanol, 2-methyl propanol, 1-propanol and many others) are formed. These can be of major importance for the sensory properties of distillates. Indeed, they build an oily layer on the surface of the product with a bad (foul) smell and were thus named *fusel oils*. The formation of higher alcohols seems to be a common characteristic of all yeast species, including non-fermenting yeasts such as *Pichia*, but amounts formed are genus-, species- and strain-dependent (Webb and Ingraham, 1963). The biochemical pathways for the formation of these alcohols except for the very last steps, are identical with those for the formation of the similarly structured amino acids, leucine, isoleucin, valine, and threonine. The higher alcohols are formed either anabolically from sugars, employing these pathways, or as the transamination products of these amino acids (Figure 13). The physiological properties and the formation of higher alcohols from the respective amino acid have been reviewed by Webb and Ingraham (1963) and are further discussed below in the section of nitrogen metabolism.

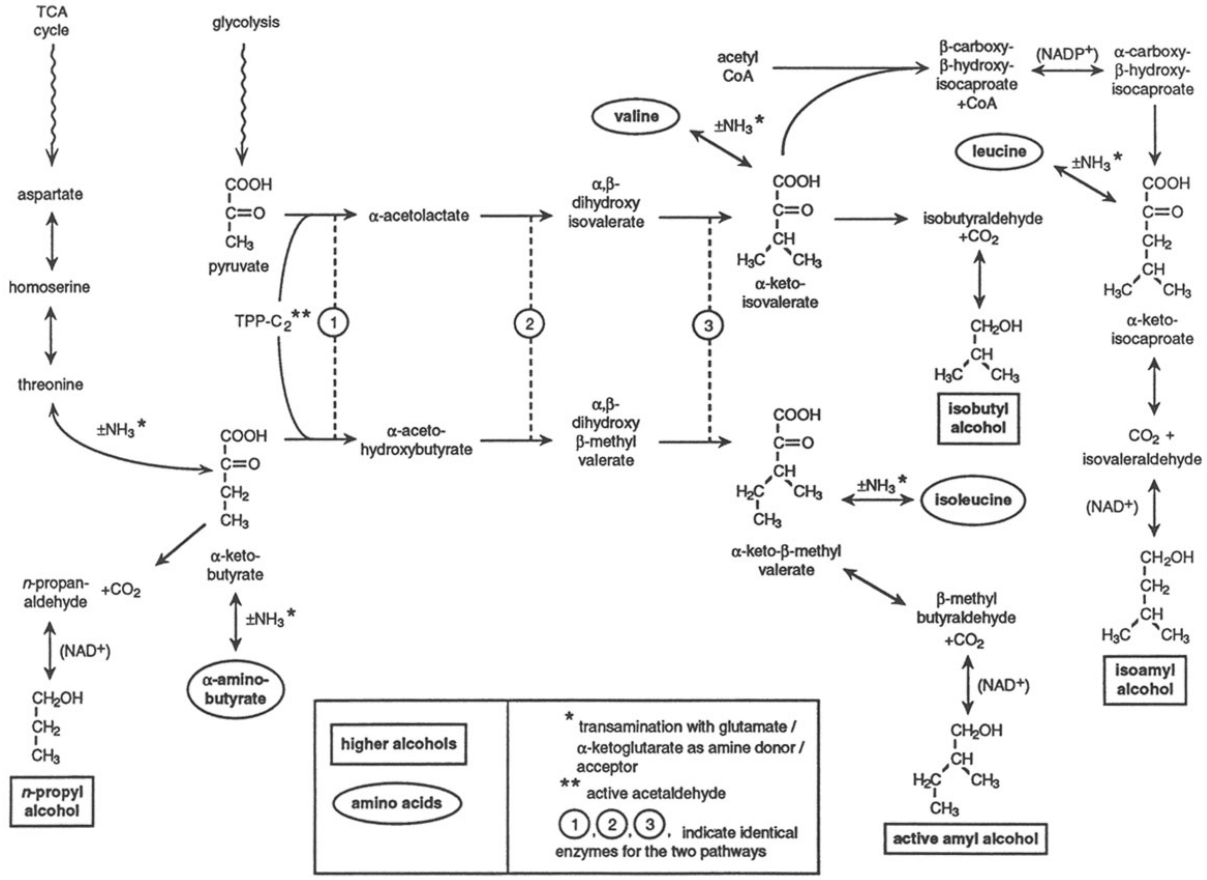

Fig. 13: Pathway for formation of higher alcohols from glucose (Boulton et al., 1996)

In addition to hexose sugars other yeast species can utilize a number of 'non-conventional' carbon sources, such as biopolymers, pentoses, alcohols, polyols, hydrocarbons, fatty acids and organic acids (Middelhoven et al., 2004). For example, many species can easily use disaccharides as nutrients by their hydrolysis into the constituent monosaccharides. In contrast pentoses can only be fermented to ethanol by very few yeast species. Many yeasts (e. g. *Hansenula polymorpha, Pichia pastoris* and several *Candida* species) have the capability of metabolizing ethanol or methanol, an approach used in biomass production of yeasts of biotechnological interest. Even *S. cerevisiae* can use ethanol as a sole carbon source (Bonnet et al., 1980).

Overall, the spectrum of end products of carbon metabolism found in the spirit depends on a variety of factors. The growth conditions of the inoculum dictate the initial enzymatic composition of the cell. Availability of and need to regenerate cofactors also affects the cell's ability to conduct certain types of reactions. The presence of other microorganisms compli-

cates the situation and may have an impact on the metabolic activity of the yeast and thus affect the end product (Drysdale and Fleet, 1989).

With regard to nitrogen metabolism, most yeasts are capable of assimilating simple nitrogen sources to biosynthesize amino acids (and consequently peptides and proteins), polyamines, nucleic acids and vitamins as shown in Figure 14. Nitrogen-containing compounds are either utilized directly in biosynthesis, or converted to a related compound, or degraded releasing nitrogen either as free ammonium ion or as bound nitrogen. Note that nitrate cannot be used by *S. cerevisiae* (Barnett et al., 2000).

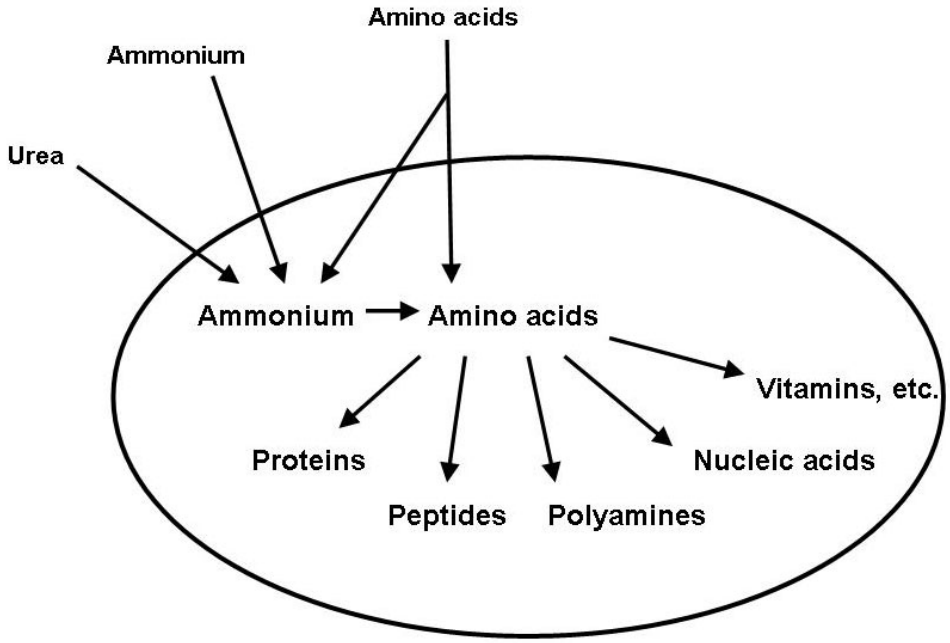

Fig. 14: Schematic overview of nitrogen assimilation by yeast (Walker, 1998)

Protein degradation and synthesis are seldom complementary - either no additional protein is needed or the amino acid composition of the synthesized proteins is not identical to the protein being hydrolyzed. Ammonium is the preferred nitrogen source. It is consumed, the amino acids from the medium are taken up in a pattern determined by their concentration relative to yeast's requirements for biosynthesis and to total nitrogen availability (Salmon 1988). In contrast to bacteria, yeasts are able to take up nitrogen-containing compounds very fast in typical fermentation processes prior to the start of growth. Biosynthetic pools (e. g. in the vacuoles) of amino acids are filled first, before degradation of compounds as nitrogen

sources occurs. Once pools have been filled and growth commences, nitrogen compounds will be taken up and degraded in a specific order of preference.

Nitrogen can be channelled into metabolism from a variety of forms. Ammonium ion and glutamate are generally the two most preferred nitrogen sources. Glutamine since it can generate ammonium ion and glutamate is also a preferred nitrogen source. In general, most yeast species will deplete the medium of these three nitrogen compounds first, before attacking other sources of nitrogen. The order of utilization of nitrogen-containing compounds may chance depending upon environmental, physiological and strain-specific factors (Boulton et al., 1996). Note that glycine, lysine, histidine, thymine and thymidine cannot be used by most strains of *Saccharomyces* as sources for nitrogen, but they can be readily employed as biosynthetic precursors.

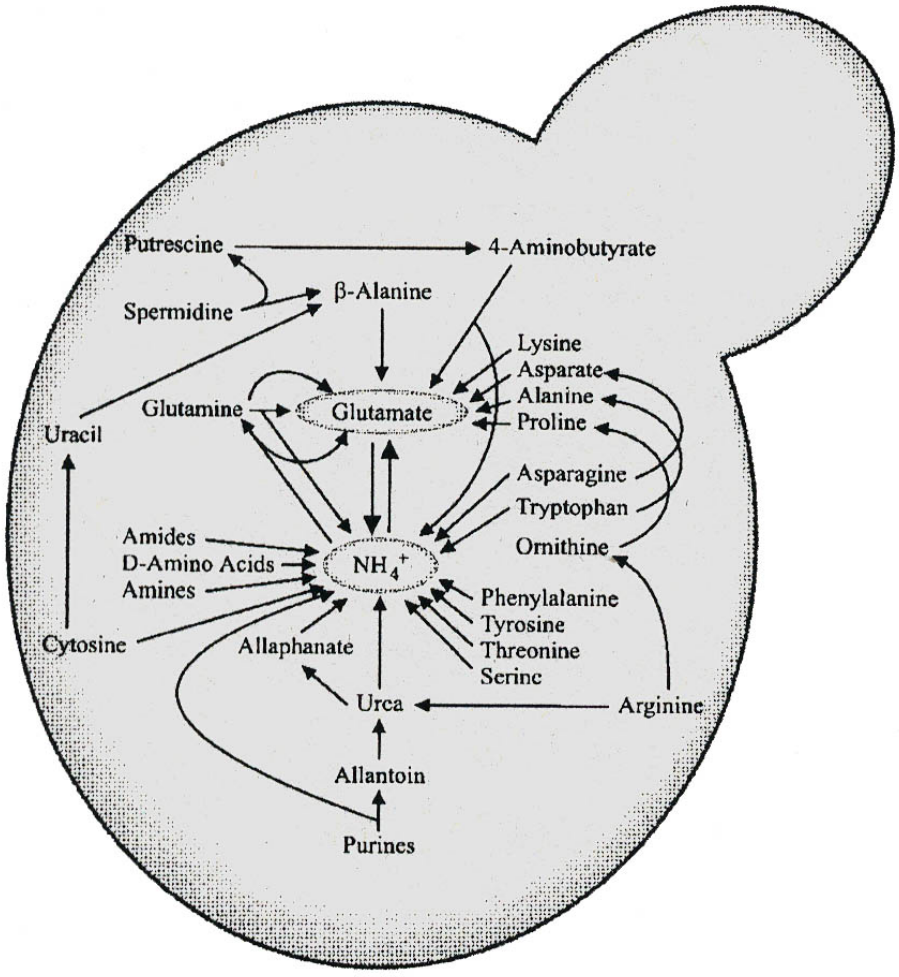

Fig. 15: Schematic representation of the degradation of nitrogenous compounds by yeast (Henschke and Jiranek, 1993)

Ammonium ions, either supplied as nutrient or derived from the catabolism of other nitrogenous compounds, are provided for metabolism as glutamate or glutamine, which can then serve as donors of the amino group for other amino acids. The major route for assimilation of ammonium is the reaction of the NADPH-dependent glutamate dehydrogenase (GDH) which forms glutamate from α-ketoglutarate and ammonium. Whenever ammonium ion concentrations are low, but also as a prerequisite for the synthesis of many nitrogenous compounds, glutamine synthase is activated, which forms glutamine from α–ketoglutarate and ammonium in an ATP-dependent reaction. Glutamine is absolutely required as a prominent precursor in several important pathways, such as the synthesis of asparagine, tryptophan, histidine, arginine, carbamyl phosphate, CTP, AMP, GMP, glucosamine, and NAD (Figure 15, Pretorius, 2000).

The metabolism of nitrogen-containing compounds yields end products of lesser sensory importance for instance in wine, but deamination of amino acids can result in the formation of α–keto acids or of higher (fusel) alcohols. In addition to being produced by deamination, decarboxylation and reduction of nitrogen-source amino acids, higher alcohols can also be produced during biosynthesis of amino acids from the excess of their corresponding keto acids (Nykanen, 1986). A known paradoxon is that the formation of higher alcohols also occurs late in fermentation, i. e. after the period of rapid consumption of amino acids (Webb and Ingraham, 1963). Studies of Nykanen (1986) demonstrated that higher alcohols could also be formed from carbon substrates. The major higher alcohols produced from yeast metabolism and their precursors are summarized in Table 1.

Tab. 1: Derivatives of amino acid metabolism (Boulton et al., 1996)

Amino Acid	α-keto acid	Higher alcohol
Leucine	α -Isocaproate	3-methylbutanol
Isoleucine	α –Keto-β-methyl-valerate	2-methylbutanol
Valine	α -Ketoisovalerate	Isobutanol
Threonine	α -Ketobutyrate	Propanol
Tyrosine	3-(4-Hydroxyphenyl)-2-ketopropionate	Tyrosol
Phenylalanine	3-Phenyl-2-ketoproprionate	Phenylethyl alcohol
Tryptophan	--	Tryptophol

The exact function of higher alcohol production is not known. Three possible explanations have been given to account for the production of higher alcohols: the detoxification of some of the higher aldehydes produced during amino acid catabolism, the regulatory role in amino acid synthesis and it has also been speculated, that the reduction of the aldehydes could serve as a means of regeneration of NADH. This seems rather unlikely, since there appears to be ample acetaldehyde and alcohol dehydrogenase to serve this purpose (Boulton et al., 1996).

The formation of metabolic by-products and flavour compounds during fermentation

As mentioned above, after ethanol and carbon dioxide glycerol is produced in significant amounts by yeast fermentation. It is an important compound in that it helps to maintain the cell's redox balance. When a yeast cell is growing, removal of pyruvate for biosynthetic processes can lead to a build up of NADH, which can halt catabolism (Pretorius, 2000). In response, the cell reduces dihydroxyacetone phosphate to glycerol phosphate, which is then dephosphorylated to glycerol and excreted into the growth medium (Figure 16). Thus, redox balance and energy metabolism are highly coordinated and the biosynthesis of new cellular material results in the production of a surplus of reducing equivalents of NADH. This is especially a problem under anaerobic conditions where respiration is not operating *S. cerevisiae* then relies on glycerol production to re-oxidize the NADH formed during anaerobic conditions. However, during aerobic conditions, the surplus of NADH in the cytoplasm is delivered to the respiratory chain in the mitochondria. The second function of glycerol is its role as an osmoprotectant. When medium osmolarity is high, the cell would be drained of water. To counteract this effect, glycerol can be accumulated intracellularly. Consequently, the stress of high osmotic pressure and heat shock both enhance glycerol production (Hohmann, 2002).

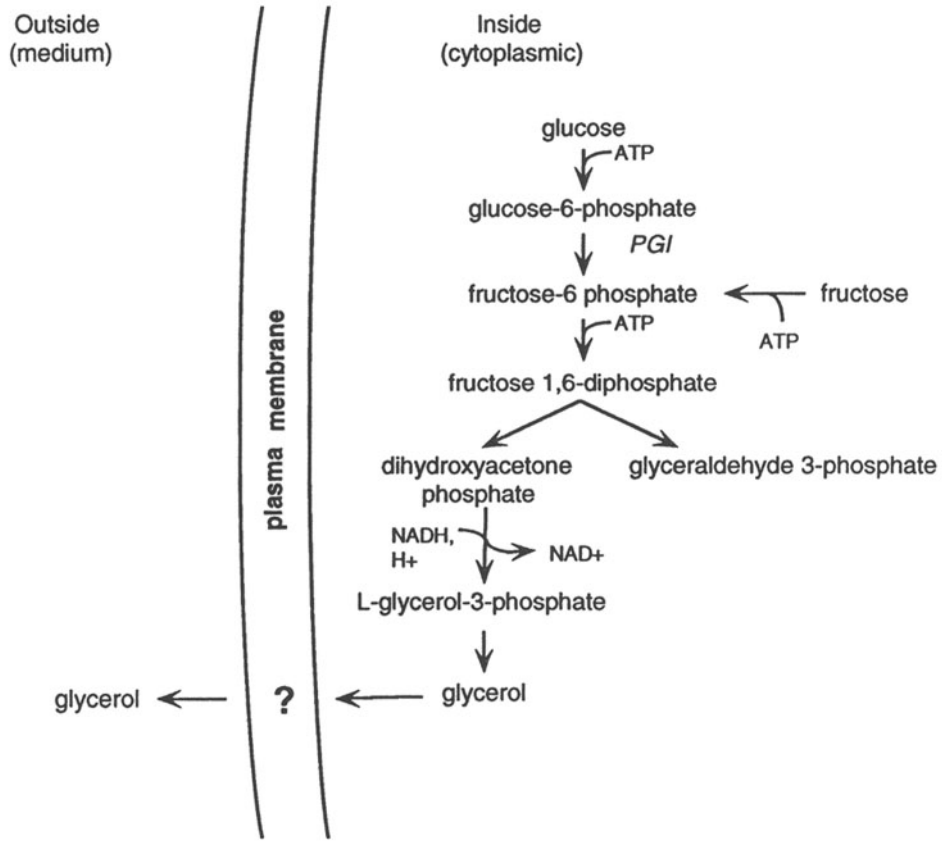

Fig. 16: Enzymatic steps leading to glycerol formation (Boulton et al., 1996)

Regarding wine production glycerol is generally supposed to improve the product quality by leading to a better mouth feeling, an enhanced viscosity and taste (Boulton et al., 1996). On the other hand glycerol can be degraded *via* hydroxypropion aldehyde by certain contaminating lactic acid bacteria and turned based on the high temperatures during the distillation process (60-100°C) to undesired acrolein in the final spirit. Acrolein is carcinogenic, has an intensely bitter taste and degrades product quality very much.

Similarly, organic acids add different flavours and aromas to beverages ranging from acidic to rancid or cheesy. Succinic acid is a main secondary by-product of alcoholic fermentation. It is believed to be synthesized and secreted by yeasts either following limited operation of the citric acid cycle or by reductive pathways involving some citric acid cycle enzymes. Low concentrations of pyruvic, malic, fumaric, oxaloacetic, citric, α-ketoglutaric, glutamic, propionic, lactic and acetic acids are also produced during fermentation, mostly as intermediates of the citric acid cycle.

Another intermediate of yeast carbohydrate metabolism is acetaldehyde, which is the carbonyl compound that occurs in the highest concentration. It is generated from pyruvate in a decarboxylation step. Usually, it is further reduced to ethanol, by the alcohol dehydrogenase. To a minor extent (which varies in different yeast species), it can also be oxidized to acetic acid. The latter then poses a problem in many fruit fermentations. It has been observed, that the level of acetaldehyde increases in the course of fermentation and then decreases again in the later stages (Stanley and Pamment, 1993).

Second carbonyl compound formed is diacetyl, which causes a characteristic aroma and taste described as 'buttery'. Two modes of generation have been described: bacterial formation of diacetyl originates mainly from catabolism of citric acid and has been extensively studied in lactobacilli (Hugenholtz and Starrenburg, 1992). In yeasts, diacetyl is generated from an oxidative decarboxylation of α-acetolactic acid (Figure 17). It is generally assumed, that in spirit and wine production the formation of diacetyl by bacteria predominates over the small amount produced by yeast. Two other end products, acetoin (2-hydroxy-2-butanone) and butylene glycol (2,3-butan diol) are closely related to diacetyl (2,3-butane dione). Neither of them is thought to have a significant impact on the sensory characteristics of the wine or spirit.

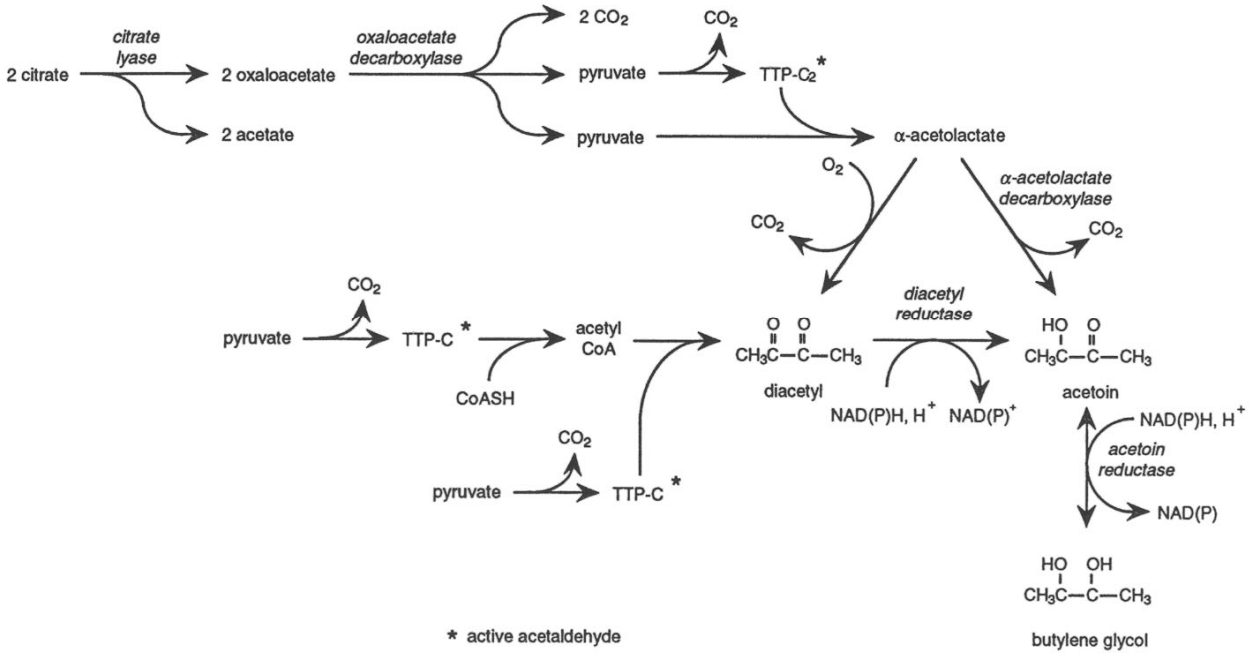

Fig. 17: Pathways of diacetyl formation (Boulton et al., 1996)

From a quantitative point of view, the most important higher alcohols (fusel oils) produced are n-propanol and amyl alcohols (3-methylbutanol and 2-methylbutanol). In addition, many more alcohols can be identified by gas chromatography. Higher alcohols are produced as by-products of amino acid catabolism or *via* pyruvate derived from carbohydrate metabolism as described in detail above (page 20ff). The catabolic route involves a pathway in which the keto acid produced from an amino acid transamination is decarboxylated to the corresponding aldehyde and then reduced to the alcohol *via* an NADH-linked dehydrogenase. Thus, isobutanol may be produced from valine, 3-methyl-1-butanol from leucine and 2-methyl-1-butanol from isoleucine (Table 1).

As depicted in Figure 13, higher alcohols are either formed from intermediates in the synthesis or in the degradation of amino acids. Therefore, the available free nitrogen in the medium determines the composition of the higher alcohols produced. A low free nitrogen concentration results in a growth inhibition and a concomitant increase in the production of higher alcohols. Furthermore, a different subset of these alcohols is produced when ammonium or urea serve as nitrogen sources (e. g. isobutanol, isoamyl alcohols; Boulton et al., 1996).

Finally, esters are a product of yeast metabolism and there are over 100 distinct esters identified in fermentation beverages. The most abundant ester is ethyl acetate. Other esters produced include isoamyl acetate, isobutyl acetate, ethyl caproate and 2-phenylethylacetate. Esters are minor components of spirits. The metabolic role for ester formation is still not well understood but it may provide a route for reducing the toxic effects of fatty acids by their esterification and removal. Nutrients that promote yeast growth tend to decrease ester levels in alcoholic fermentation. Likewise an increased oxygen supply tends to reduce ester levels. On the other hand, higher temperatures lead to increased ester formation and increased levels of higher alcohols (Boulton et al., 1996). These physiological considerations are of great relevance in the application for the production of wine, beer and spirits. Thus knowing how off-flavours are formed and how to limit their production helps to improve product quality (Boulton et al., 1996).

ETHYL CARBAMATE (URETHANE, CARBAMIC ACID ETHYL ESTER)

In the past decade much attention has been directed to ethyl carbamate (EC), a compound suspected to be carcinogenic. It occurs in fermented foods and has at least in part been attributed to the metabolic activity of fermenting microorganisms such as yeast (Ough, 1976). EC, the ethyl ester of carbamic acid (Figure 18), is an odourless, white crystalline powder that is produced commercially for use in the preparation and modification of amino resins and as a co-solvent during the manufacture of pesticides, fumigants, and cosmetics.

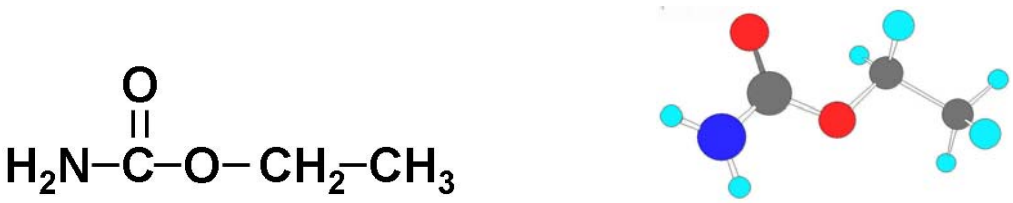

Fig. 18: Chemical structure of ethyl carbamate (also known as urethane, carbamic acid ethyl ester; Roempp, 2005)

It has also been used as a chemical intermediate in the textile industry, as a cosolvent with drugs, and, for a brief period, as an agent for the treatment of chronic leukemia (IARC, 1974). As stated above, EC has also been identified in food as a by-product of fermentation (Ough, 1976). Fermented food and beverages are the major source of human exposure to EC. EC in combination with ethanol was nominated by different Food and Drug Administrations for in-depth toxicological evaluation because of the widespread exposure to EC in alcoholic beverages and a lack of adequate dose-response carcinogenicity data to allow for risk assessments. Known precursors of EC (with respect to yeast metabolism) are urea, citrulline, carbamyl phosphate and n-carbamyl amino acids (Monteiro et al. 1989; Ough et al., 1988).

If urea is not present in the mash, the amino acid arginine is thought to be the main precursor. Generally, arginine is one of the most abundant amino acids available to yeast in fruit fermentations. Degradation of this amino acid by yeast arginase results in the formation of urea (Figure 19).

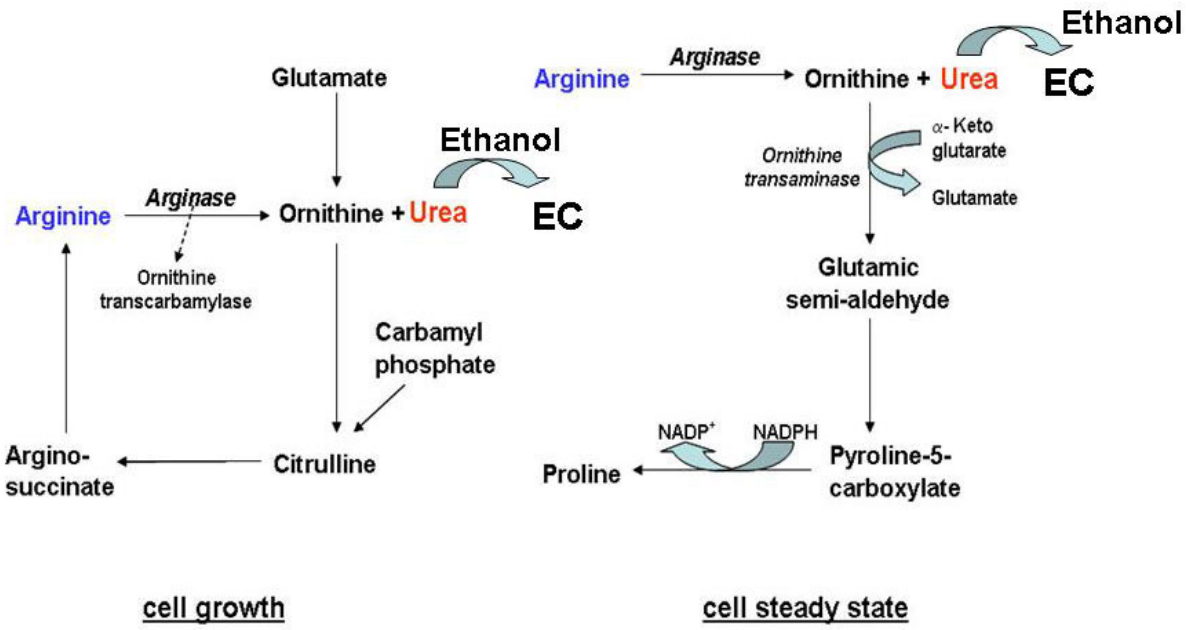

Fig. 19: Reactions of *S. cerevisiae* during cell growth and steady state which produce urea and other possible ethyl carbamate precursors (modified according to Ough et al., 1988)

If the latter can not be further metabolized and starts to accumulate, it is secreted into the medium. Moreover, it is also released at the end of fermentation, presumably by yeast cells undergoing autolysis. Urea is often formed during the early or middle stages of fermentation, with subsequent yeast generations utilizing it as a nitrogen source during the later stages. Different yeast strains differ in their urea secretion and uptake kinetics during fermentation (Ough et al., 1989). Urea can spontaneously react with the alcohol in the medium to form EC (Ough et al., 1988). The chemical reaction between urea and ethanol is exponentially accelerated by the concentrations of either precursor. EC concentrations also increase with time and temperature. Because urea is the principal precursor of ethyl carbamate, controlling the urea concentration may thus be crucial in limiting EC levels. Factors influencing urea concentrations in fruit fermentations include the arginine content of the fruit, the yeast strain employed, fortification, the timing of fortification, the temperature and the duration of storage of the end product (Stevens and Ough, 1993, Ough et al., 1988). Therefore, yeast selection provides one means to minimize the potential for EC formation.

To a lesser extent citrulline, an amino acid which is not incorporated into proteins, and is formed during arginine biosynthesis, can also serve as an EC precursor. In addition to the yeast metabolism, it can also be formed by contaminating lactic acid bacteria in fruit fermentations.

Ethyl carbamate deriving from the fruit

Yeast metabolism is not the only way to form EC. As a matter of fact, EC is also formed from precursors available in the raw material depending on the specific food/beverage considered,. Especially in stone-fruit distillates, hydrogen cyanide together with photochemically active substances can lead to the formation of EC. In this case the main part of EC is formed after distillation involving photochemical reactions. As already mentioned above, the formation of EC is correlated to the concentration of urea and its precursors, ethanol and temperature. In wine (and probably fruit mashes) significant EC formation seems to follow a heat treatment. While in distillates hydrogen cyanide is the most important single precursor, in wine different carbamyl compounds, mainly urea, seem to be involved in EC formation. Despite this apparent difference a common EC formation pathway is discussed for all alcoholic beverages by assuming cyanic-/isocyanic acid as an important ultimate reactant with ethanol (Wucherpfennig *et al.* 1987, Battaglia *et al.* 1990, MacKenzie *et al.* 1990, Taki et al., 1992, Aresta *et al.* 2001).

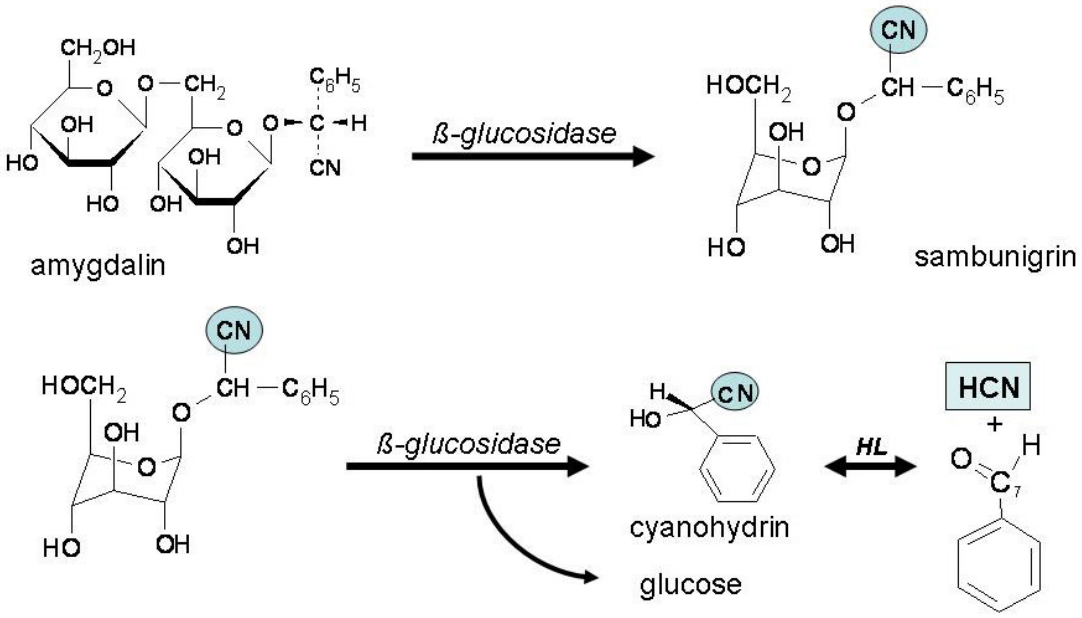

Fig. 20: Detachment of cyanide by enzymatic action and thermal cleavage of amygdaline in stone fruit *Prunaceae* (Hydrolase (HL); Wucherpfennig et al., 1987)

From what is said above, the formation of EC in spirits can occur before, during, and after the distillation process (Mackenzie et al., 1990). It has been claimed that the EC formed in the processes before distillation does not contribute significantly to the final content in the spirits (Lachenmeier et al., 2005). Rather, it is removed in the distillation process to the final amount

not significant, because EC has a boiling point of 185°C (Cook et al., 1990). Therefore, cyanide ion has been proposed to be the most important EC precursor in spirits. As shown in Figure 20 it is formed by enzymatic action and thermal cleavage of cyanogenic glycosides such as amygdaline in stone fruits (Battaglia et al., 1990).

Two chemical pathways have been proposed to most likely occur in the formation of EC from cyanide. The first is based on the complexation of cyanide with Cu^{2+} followed by its oxidation to cyanogen, with a subsequent disproportionation to cyanate and cyanide (Beattie, 1995). Cyanate may react with ethanol to form EC. Copper can be released from the distillation apparatus upon corrosion. Different copper(I) cyanide species were detected in the pot still apparatus, supporting the idea that the formation of EC could start during the distillation process (Bourton, 1992). The second pathway is based on self-oxidation of unsaturated compounds present in alcoholic beverages under UV light (Guerrain and Leblond, 1992), which produce free radicals (organic peroxides or hydro-peroxides), which catalyze the oxidation of cyanide to cyanate, again followed by the reaction with ethanol to generate EC.
According to more recent works the factors influencing EC formation from cyanide are pH, light, ethanol content, temperature, vicinity of carbonyl groups in organic molecules, and concentration of Cu^{2+} ions in the beverage (Battaglia, 1990, Riffkin et al., 1989a). Some authors have proposed pathways other than cyanide for the formation of EC in spirits, e. g. the reaction of proteins with ethanol catalyzed by Cu^{2+} ions (Riffkin et al., 1989b). Another proposal considers cyanic acid released directly from thermal decomposition of urea present in the mash as described above. However, for the decomposition of isocyanate kinetics for the formation of EC, indicating an intermediate reaction for converting cyanate into EC was reported (Bourton, 1992). However, all EC in the distillate is formed at ~24-48 h after the distillation (Riffkin, 1989a).

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CHAPTER III

A LABORATORY YEAST STRAIN SUITABLE FOR SPIRIT PRODUCTION

Abstract

Yeast strains of the species *Saccharomyces cerevisiae* currently in use for the production of consumable alcohols such as beer, wine and spirits are genetically largely undefined. This prevents the use of standard genetic manipulations such as crossings and tetrad analysis for strain improvement. Furthermore, it complicates the application of the majority of modern methods developed in yeast molecular biology. Here we used two haploid laboratory strains with suitable auxotrophic markers for the construction of a genetically well defined, prototrophic diploid production strain. This strain was tested for its fermentative and sensory performances in comparison to commercially available yeasts. Three different fruit mashes (cherries, plums and pears) were fermented in a 90 kg scale. These were then subjected to distillation and used for the production of spirits with a final ethanol content of 40% (v/v). Fermentation parameters assayed included growth, sugar utilization, ethanol production and generation of volatile compounds, higher alcohols and glycerol. The spirits were also tested for their sensory performances and the data obtained statistically consolidated. Our results clearly demonstrate that this laboratory strain does not display any disadvantage over commercial yeasts in spirit production for any of the parameters tested. Yet, it offers the potential to apply both classical breeding and modern molecular genetic techniques for adjusting yeast physiology to special production schemes.

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INTRODUCTION

Long before Pasteur´s demonstration that yeasts are the agents which cause alcoholic fermentation, they had been in practical use for the production of beer, wine, and spirits (reviewed in Barnett, 1997 and Huxley, 1873). Whereas traditionally these yeasts were derived from the raw material employed (i.e. yeasts colonize the fruit to a minor extent and constantly increase in number after the onset of fermentation), modern industrial standards recommend the addition of cultured *Saccharomyces cerevisiae* strains to speed up fermentation and to avoid production of deleterious metabolites by biological contaminants (see Grossmann *et al.*, 2000, and references therein).

The yeast strains commonly employed for alcohol production are genetically largely undefined and highly heterogenous (Benitez *et al.*, 1996). Thus, little is known about their chromosomal constitution and aneuploidy is frequently observed (Bidenne *et al.*, 1992, Cardinali and Martini, 1994, Vezinhet, 1981). Moreover, sporulation is extremely poor (if observed at all) and is accompanied by poor spore viabilities (Maraz, 2002). Therefore, such strains cannot be manipulated by most techniques developed for classical yeast genetics (i.e. sporulation, crossing, tetrad analysis), which would be analogous to breeding in plant and animal genetics. The methods usually employed to manipulate yeast in modern molecular genetics are limited to the use of dominant selectable markers due to the uncertainties in genomic composition and the lack of auxotrophic markers (Pretorius, 2000).

These features of industrial yeast strains explain the huge gap between the vast amount of knowledge gathered on the genetics and physiology of *S. cerevisiae* (summarized e.g. in Sherman, 2002 and in Walker, 1998) and its application in fermentation industries. The genome of *S. cerevisiae* was the first of a eukaryotic organism to be completely sequenced (Goffeau *et al.*, 1996; Zagulski *et al.*, 1998). Functional analysis resulted in the availability of single deletion mutants in more than 5000 chromosomal gene copies (e.g. available from EUROSCARF, Frankfurt, Germany; http://www.srd-biotec.de). In particular, the genetics and physiology of glycolysis and sugar transport, as a basis for alcoholic fermentation, have been extensively studied (reviewed in Heinisch and Hollenberg, 1993 and Boles and Hollenberg, 1997). This basic knowledge has resulted in comparatively few attempts to engineer industrial yeast strains. These include the production of amylases for brewing purposes (Jansen and Pretorius, 1995), of peptide antibiotics in wine strains (Pretorius, 2000), and the reduction of the presumed cancerogenic compound urethane, by mani-pulating arginine metabolism in sake yeasts (Kitamoto *et al.*, 1991). Certain strains have also been genetically

engineered to produce esterases in order to enhance the generation of volatile compounds (Hirata *et al.*, 1992, Lee *et al.*, 1995).

The use of laboratory yeast strains for industrial purposes has so far been prevented by the general belief that they are not as competitive as natural isolates. With microbiological contaminants inherent in the raw materials employed, they are thought to have lower fermentative capacities and to produce undesirable flavour compounds due to differences in their secondary metabolism. Although it has been frequently claimed, that laboratory yeast strains display these characteristics, comparatively few sound scientific studies have been published in this respect (Tuite, 1992, Bothast *et al.*, 1999, Gimren-Alcaniz and Matallana, 2001, Romano *et al.*, 2003). Recently, laboratory strains have been developed with improved genetic and physiological performances (van Dijken *et al.*, 2000). The latter work showed that one of these strains, CEN.PK122, was amenable to genetic, physiological and biochemical studies under controlled laboratory conditions. It performed sufficiently well in batch- as well as in steady-state chemostat cultures in defined mineral media and displayed growth rates, sugar utilization capabilities and biomass yields similar to the other strains tested.

Spirit production on a commercial basis differs from controlled laboratory conditions in various aspects: Due to the differences in fruit composition, yeast strains used for fermentation have to adapt to different environments (e.g. sugar compositions and concentrations, presence of organic acids etc.). In addition, depending on the fruit of choice and varying climatic conditions, the yeast employed has to compete for sugar utilization with other microorganisms present in the mashes (e.g. other yeast species such as *Kloeckera apiculata* {= *Hanseniaspora uvarum*}, and with bacteria such as various lactic acid bacteria (Pieper *et al.*, 1993, Narendranath *et al.*, 1997). Furthermore, a major quality of spirits lies in their flavour compounds, rather than merely the speed and amount of ethanol production. The most abundant esters and higher alcohols in fermented beverages are ethylacetate, isoamylacetate, amylalcohols and isobutanol (Renger *et al.*, 1992). Interestingly, different yeast strains will usually produce individual ester- and alcohol profiles when fermenting similar media (Younis and Steward, 1998). On the other hand, the type of sugar being fermented also affects volatile compound production (Engan, 1972, Gil *et al.*, 1996, Pollock and Weir, 1976, Pretorius, 2000). Owing to the distillation following the fermentation process, secondary fermentation compounds produced by the yeast may be concentrated in spirits and preclude product consumption (Piggott, 1983, Postel and Adam, 1989, Meinl, 1995).

Such profound differences between applied and laboratory growth conditions have to be taken into account when adjusting a laboratory yeast strain for production purposes.

We used here a derivative of the *S. cerevisiae* CEN.PK122 strain (van Dijken *et al.*, 2000) to test its performance on the fermentation of different fruit mashes under medium-scale production conditions. In contrast to the general opinion, our results demonstrate that the laboratory strain HHD1 performs in a similar manner to the industrial breeds for all parameters tested.

MATERIALS AND METHODS

Yeast strains employed

Apart from the laboratory strain (HHD1) constructed here (*MAT**a**/*α *ura3-52/URA3 leu2-3,112/LEU2 MAL2-8^C/MAL2-8^C SUC2/SUC2*; obtained from a cross of CEN.PK113-5D with CEN.PK113-16B; Entian and Kötter, 1998), the haploid parents and two representative spirit production strains of *S. cerevisiae* were employed: *Siha Aktiv6* (manufacturers trade name, further abbreviated herein as "Siha") and *Uvaferm CGC62* (manufacturers trade name, further abbreviated herein as "Uvaferm"; both purchased from Begerow GmbH & Co., Langenlonsheim, Germany). The latter two strains were packaged as dried yeast in 500 g aliquots. First, experiments with these strains were performed in pilot scales (1.5 kg) to observe their general fermentation behaviour (data not shown). Based on these results, fermentations on a technical scale (90 kg) which are reported here were initiated.

Media, culture conditions and growth determinations

Rich media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with 2% glucose (YEPD). Haploid strains were also grown on YEPD and tested for markers on minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids, adenine and uracil (Sherman *et al.*, 1986), with the omission of uracil or leucine as required. For growth on plates 1.5% agar was added to the described media. All strains were incubated at 30°C.

For small scale experiments, strains were grown in YEPD and inoculated to an OD_{578} of 0.1 in 1.5 kg apple mashes, cultivar *Rubinette* (*Cox-Orange* x *Golden Delicious*). Growth rates were determined in cherry mashes on an even smaller scale of 0.8 kg. For this purpose, the

fruit was stoned and passed through a filter to obtain a homogenous medium. The mashes were inoculated with an OD_{578} of 0.05 and samples regularly taken over a period of 120 hours. Cells were plated from appropriate dilutions onto YEPD and after two days of incubation at 30°C the number of colony forming units (cfu) was determined. Experiments for each strain were performed in duplicate.

For spirit production scales, strains were grown in 5 ml YEPD overnight on a rotor shaker (30°C, 140 rpm), transferred into 500 ml shake-flask cultures with fresh YEPD, incubated for 12 h and harvested by centrifugation (3500 x g for 5 min at room temperature). Cell pellets were washed twice with 25 ml NaCl/peptone (0.85% NaCl, 0.05% peptone), resuspended in 25 ml of the same medium and transferred to 1.5 liter YEPD in 3 liter shake flasks. After 20 hours of incubation at 30°C at 140 rpm, yeasts from each culture were again harvested by centrifugation (3500 x g for 10 min at room temperature), washed twice as described above and resuspended in 100 ml NaCl/peptone solution. The cell density was calculated by optical measurements at 578 nm in appropriate dilutions, assuming that 1 OD_{578} equals 10^7 cells/ml. From this, the yeasts were added to the mashes at a final density of 10^6 cells/ml, each.

Sugar utilization

All strains were tested for their ability to utilize different sugars. The utilization of glucose, fructose, galactose, maltose, sucrose and raffinose was tested on agar-plates. The media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with the particular sugar (at 1% concentration). The strains were also incubated in the respective media in liquid at 30°C. After 12 hours at 140 rpm the cells were harvested, washed and resuspended in NaCl/peptone solution to an OD_{578} of 1. Serial dilutions of 10^{-1} – 10^{-5} in NaCl/peptone solution were prepared and 5µl of each dropped onto rich medium containing the respective sugar and 3 ppm antimycin A (Sigma-Aldrich, Germany) to block respiration. Growth was assessed after 3 days of incubation at 30°C by visual inspection. Each experiment was performed in triplicate.

Raw materials and mashing process

For pilot scale experiments (0.8 kg or 1.5 kg, as indicated) apple mashes, cultivar *Rubinette* (*Cox-Orange* x *Golden Delicious*) inoculated with the yeast strains listed above were used. Technical scale studies (90 kg) were performed on three different fruit mashes: cherries (cv. *Dollenseppler*), plums (cv. *Ersinger Frühzwetschge*) and pears (cv. *Bartlett*).

Mashes were prepared according to standard procedures. The pipfruits were washed and crushed by passing them through a rasp mill (Wahler, Stuttgart, Germany). The stone fruits (exempted from peduncles) were washed and subsequently chopped using a drill machine attached to a beater, so that the stones remained undamaged. Immediately after this, the mashes were adjusted topH values between pH 3.0 and pH 4.0 with sulphuric acid (technical grade). After 24 hours of fermentation, the pH was again adjusted to 3.0. To increase the decomposition of pectin, pectinolytic enzyme was added to the pipfruit mashes (Pectinex Ultra SP-L; Novoenzymes, Denmark at 8 ml/hl mash). No pectinolytic enzyme was added to the stone fruit mashes.

Fruit and mash qualities

The cherries were in an excellent condition, like fresh dessert fruit. No bruised or decayed fruit were present. This minimizes the risk of a bacterial contamination. The pears were generally in a faultless condition. Foul fruit were removed. They were stored at 15°C for a few days to achieve a doughy consistence which facilitates the mashing process. The plums were windfallen and, therefore, microbiologically in a more critical condition. To reduce the amount of by-products formed by spontaneous fermentation (which had already begun in the plum mashes), and to cope with the presumed higher load of bacterial contamination, plums were processed immediately.

The mashes were divided into approximately 90 kg lots and transferred into 120 liter vessels. The vessels were then sealed with a fermentation bung and incubated with the various yeast strains. All experiments were performed at least in triplicate and different parameters such as ethanol yields, extract, sugar utilization, sugar content, yeast metabolites and pH-values were determined at regular intervals during the fermentation period.

Fermentation

The mashes (prepared as described above) were inoculated with the selected commercial yeast strains or the laboratory strain HHD1 (all standardized to be in the same physiological state and cell density as described above) and fermented to completion at 15-17°C. During fermentation, mashes were agitated and samples were collected and analysed with regard to the different parameters as indicated in the results section.

Analytical methods

During fermentation, changes in the pH were monitored using a pH-meter 521 (WTW, Weilheim, Germany). As a preliminary indication, the decrease in fermentable carbohydrates (% sugar) was determined with a hand refractometer (Carl Zeiss, Jena, Germany) and the synthesis of ethanol was determined using steam-distillation (Vapodest 20, Gerhardt, Bonn, Germany) and a density-meter DMA48 (Paar Physica, Graz/Straßburg) according to the standard procedures described in Chemisch-Technische Bestimmungen (Chemisch-Technische Bestimmungen, 1980). The total (titratable) acidity was measured by titration with NaOH and calculated in tartaric acid equivalents according to Adam *et al.* (1995).

For the determination of various yeast metabolites and the compounds ethylacetate and 3-methyl-butylacetate, 2-methyl-1-propanol, 1-propanol and the isoamylalcohols (3-methyl-1-butanol and 2-methyl-1-butanol), a headspace gas chromatograph (Perkin Elmer HS40, GC 8420) equipped with a packed crossbond phenylmethylpolysiloxane column (Rtx-volatiles; 60 m by 0.32 mm, film thickness 1.5 µm; Resteck GmbH, Bad Homburg, Germany), a flame ionization detector and a CLASS VP 4.2 integrator (Shimadzu, Duisburg) were employed. As an internal standard, n-Butanol (200 mg/l; purchased from Merck, Darmstadt, Germany) was used. For mashes, the method described in Brautechnische Analysenmethoden (Brautechnische Analysemethoden, 1996) and according to Boettger and Pieper (1994) was used. The following temperature profile was employed: 50°C for 7 min, increase from 50 to 120°C at 15°C/min, a hold at 120°C for 2 min and an increase from 120 to 250°C at 20°C/min. The profile for the spirit analyses was: 60°C for 2 min, an increase from 60 to 70°C at 2°C/min, a further increase from 70 to 160°C at 8°C/min, a hold at 160°C for 2 min, and further increases from 160 to 200°C at 4°C/min and from 200 to 250°C at 15°C/min, with a final hold at 250°C for 10 min. Nitrogen was applied at a flow rate of 5 ml/min, hydrogen/synthetic air at 30 ml/min and helium as the carrier gas at 1.7 ml/min. All gases were supplied by Sauerstoffwerk GmbH (Friedrichshafen, Germany).

The exact decrease of the fermentable sugars (glucose and fructose) and the formation of the volatile compounds acetic acid, propionic acid and lactic acid, as well as the exact ethanol content were determined by HPLC (Bischoff Modell 2200 HPLC using a Bischoff Modell 728 Autosampler; Bischoff, Leonberg, Germany), using an Aminex HPX-87 H column (Biorad, Munich, Germany), a RI detector ERC7510 (ERC, Altegolfsheim, Germany) and a McDAcq15 Integrator (Bischoff, Leonberg, Germany). 0.1N sulphuric acid (technical grade) was used for elution.

Distillation

After 6-8 weeks of fermentation, the mashes were distilled using a 200 liter copper pot (Jacob-Carl, Göppingen, Germany; see Chapter II, Figure 13 for further details) fitted with an enrichment section consisting of three bubble plates, a dephlegmator and a cyan catalyst (Holstein, Markdorf, Germany). This modern plant allows for distillation under technical and standard conditions. The dephlegmator was run with a flow rate of 120 liter/h. The catalyst was used for cherries and plums, but not for pears. Fermented cherry mashes were distilled with one plate in operation, pears and plums with two plates. The distillates were collected in fractions with a volume of 250-300 ml, each. In the vicinity of the switching points (heads to product fractions and product fractions to tailings) smaller volumes of 150 ml were collected. The heads were identified with the detaching test determining acet-aldehyde according to Pieper and Rau (Pieper *et al.*, 1987). The tailings were screened by organoleptic assessment.

Spirit fractions

The product fractions were stored for at least one week at 17°C, then diluted with deionised water to an alcohol content of 40% (v/v), cold filtered at 4°C (Macherey Nagel, Düren, Germany) and kept for another four weeks at 17°C prior to further analysis and sensory assessment. Heads and tailings were discarded.

Sensoric evaluations

The spirits produced with the three different yeast strains were tested and assessed for their characteristic flavour quality using order-of-precedence and triangle-tests (Jellinek, 1981). Sensoric evaluation of the spirits was conducted with a panel of at least ten judges, previously trained for their ability to correctly match spirits. To enhance statistical significance, larger panels (>20 persons) with a short introductory training were also employed.

Statistical analyses

Data were analysed by the statistical software SigmaStat (Jandel Scientific) using "One-Way-ANOVA" on ranks. This non-parametric test compares several different experimental groups which received different treatments (for purposes of this study the only parameter difference being the three yeast strains employed). To isolate the group or groups that differed, all pairwise multiple comparison procedures (according to the Student-Newman-Keuls method) were performed at 5% significance level (Fox *et al.*, 1995). Means of mash samples were not compared because of an inferior replication extent (n=2).

RESULTS

Growth characteristics of commercial and laboratory yeast strains

The aim of this work was to obtain a strain of *S. cerevisiae* suitable for the production of various spirits that is amenable to both classical and modern genetic manipulations. For this purpose we chose two haploid segregants from an isogenic series previously shown to perform well in chemostat cultures (van Dijken *et al.*, 2000). The diploid strain (HHD1) is prototrophic for all amino acid and base requirements but heterozygous for the *ura3-52* and the *leu2-3,112* markers, which are commonly used for genetic manipulations. First, we assessed the ability of this strain to ferment different carbon sources (glucose, fructose, maltose, galactose, sucrose and raffinose) in a serial dilution test on rich media containing antimycin A to block respiration. Simultaneously, we also tested the two industrial strains (Siha and Uvaferm) on the same media (Fig. 1a). No significant difference was observed for any of the strains, indicating that the commonly available fruit sugars serve equally well as carbon sources (note that the Uvaferm strain seems to be more suitable for fermentation of raffinose than the other strains tested).

In order to assess the performance of the different strains under applied fermentation conditions, we inoculated cherry mashes at lower cell densities of 0.05 OD_{578} (in a small scale of 0.8 kg) and determined growth at 30°C by plate counts. Again, no significant differences in viabilities were observed between the commercial preparations and the diploid laboratory strain (Fig. 1b). Although the latter showed an elongated lag in the initial growth phase, it reached the same cell density as the commercial strains after approximately 90 hours of fermentation. It is generally assumed that yeast strains for industrial purposes need at least a diploid set of chromosomes to cope with the requirements of good fermentation capacity (Oda and Ouchi, 1989). This assumption was confirmed for our laboratory strain when we tested the haploid parental strains. Neither could compete for growth or for fermentation capacity with their diploid derivative or the industrial yeasts (Fig. 1b).

a)

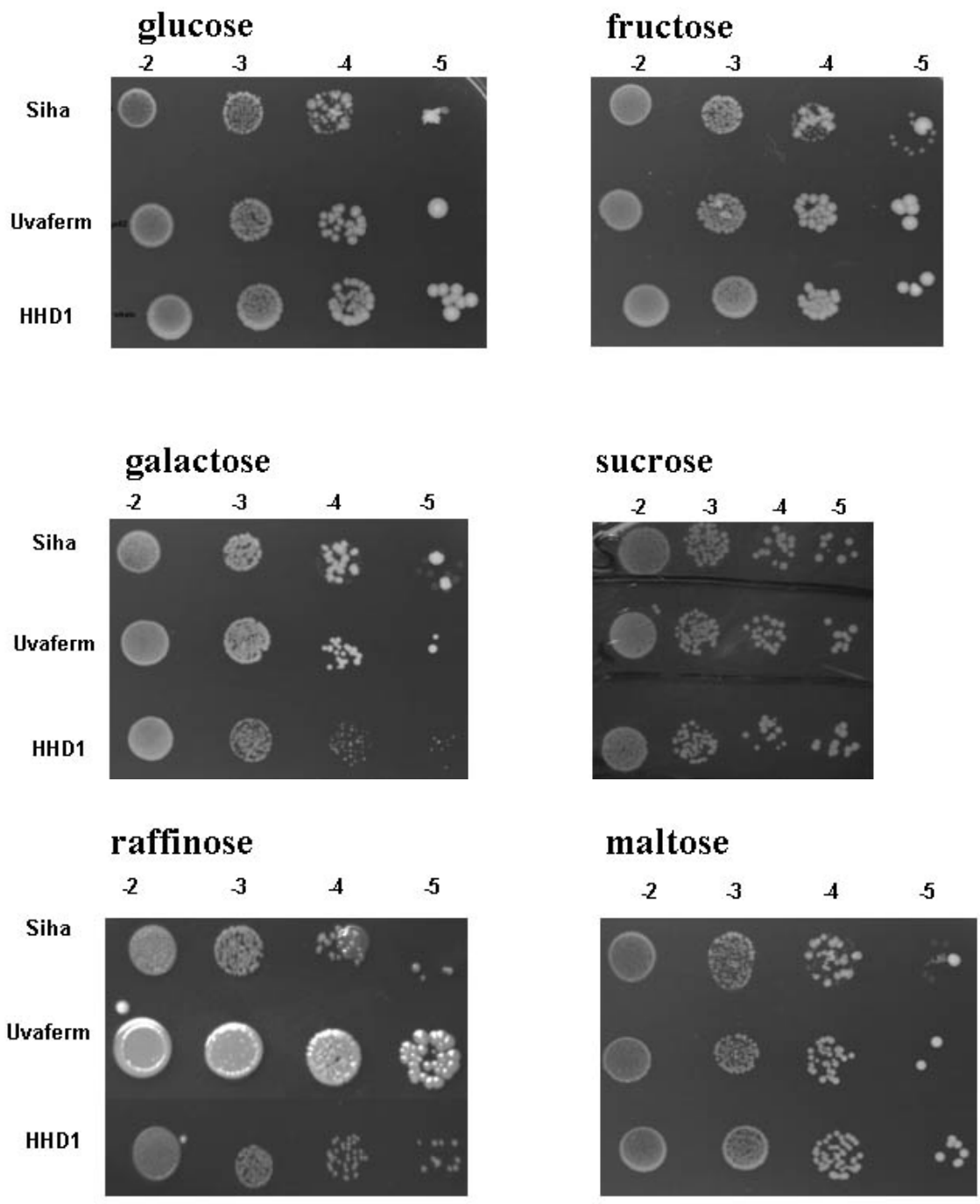

Fig. 1a: Sugar utilization and growth characteristics of commercial and laboratory yeast strains.

Strains were pregrown in liquid rich media containing the indicated sugars at 1% concentrations. Serial dilutions of 10^{-2} to 10^{-5} were prepared inNaCl/peptone solution and 5µl of each was dropped onto rich media containing 1% of the sugars as indicated. Respiration was blocked by the addition of 3ppm antimycin A to the plates. Growth was assessed after 3 days of incubation at 30°C.

b)

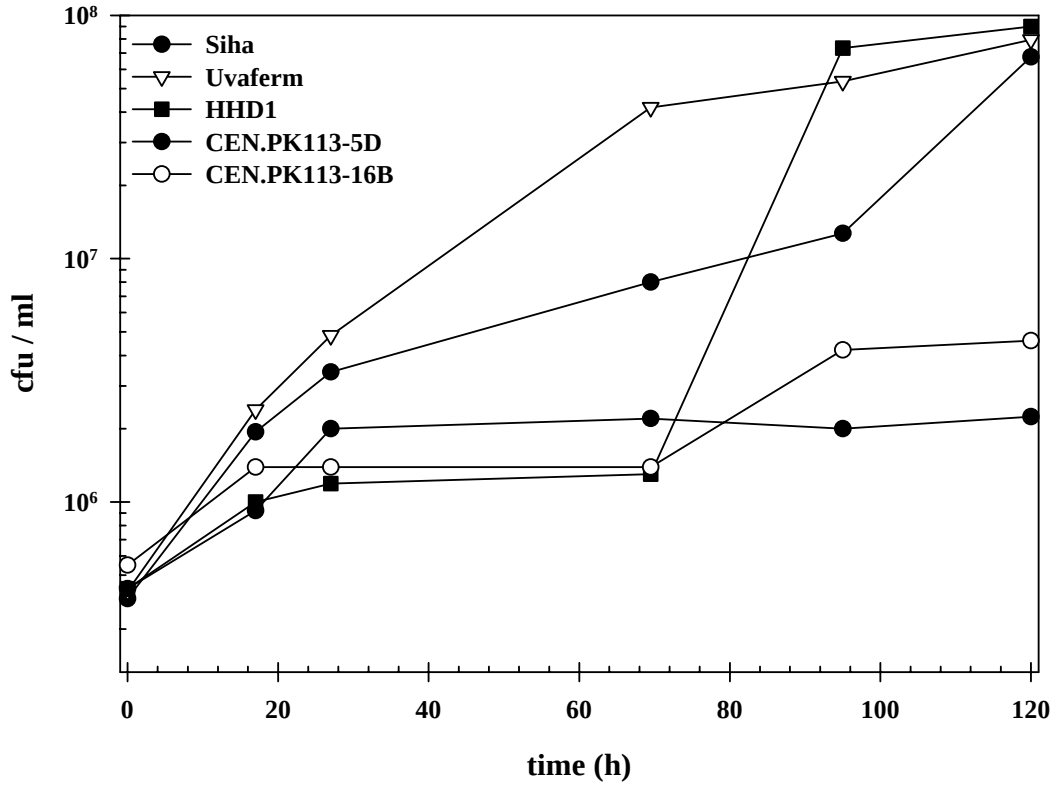

Fig. 1b: Sugar utilization and growth characteristics of commercial and laboratory yeast strains.

Cherry mashes, prepared and filtered as described in materials and methods, were inoculated with the yeast strains indicated and incubated at 17 °C. Samples were taken at the indicated times, diluted and plated onto YEPD to give rise to approximately 100 colonies/plate (cfu = colony forming units). Viable cell densities in the mashes were calculated from these counts. CEN.PK113-5D and CEN.PK113-16B are the haploid parental laboratory strains from which the diploid strain HHD1 has been derived.

Analyses of fermentation parameters in mashes

To assess the fermentation capacity of the laboratory yeast strain (HHD1) under real production conditions, different fruit mashes (cherries, plums and pears; 90 kg each) were inoculated in triplicate with 10^6 yeast cells/ml and allowed to ferment under semi-anaerobic, non-sterile conditions at low temperatures (see Materials and Methods). As controls, both the Siha and the Uvaferm yeasts were tested under the same conditions. Samples were taken weekly for microscopic examination and it was confirmed that no significant bacterial contaminations were present within the mashes. However, growth of a layer of wild yeast contaminants on the surface, due to exposure to oxygen during sampling, was observed to a similar extent in all cases.

Tab. 1: Sugar content in mashes and alcohol yields after fermentation

Mash	% Plato [a]						Theor. alcohol yield [b]	Observed alcohol yield in mashes [c]		
	Siha		Uvaferm		HHD1			Siha	Uva-ferm	HHD1
	initial	final	initial	final	initial	final				
Cherries	20.4	4.1	20.4	4.2	20.4	4.3	7.33	7.45	6.69	7.84
Plums	12.5	1.8	12.5	2.0	12.5	1.8	4.26	4.62	4.92	5.28
Pears	11.6	2.2	11.6	2.4	11.6	2.2	4.13	4.67	4.70	4.47

a) % Plato equals %mas sucrose per 100 g mash liquid

b) The theoretical alcohol yield was calculated as liter alcohol per 100 liter of mash [(%Plato – non-fermentable substances) × 0.56 × TF], with TF values: cherries=0.85, plums=0.885, pears=0.91 and non-fermentable substances: cherries=5.0%, plums=4.0%, pears= 3.5%

c) Calculated as total alcohol content after distillation (v/v) × liter distillate/liter mash

To assess the amount of fermentable carbohydrates present in the mashes, we determined their refraction index with the assumption that 1% Plato corresponds to approximately 1 g sucrose per 100 g mash liquid (Schobinger *et al.*, 2001). The approximate alcohol content in different samples was determined by steam distillation. Table 1 summarizes the data obtained for the different fruit mashes, including the theoretical and practical alcohol yields. As expected from the higher initial sucrose content, the highest yields for all strains tested were obtained in the cherry mashes, as opposed to those of plums and pears. Comparison

of the three yeast strains within each of the mashes did not reveal significant differences in the final alcohol concentrations.

As a more accurate measure of yeast metabolic activity, we determined the kinetics of glucose degradation and the increase in ethanol concentrations over a period of 48-55 days by HPLC (Fig. 2; the kinetics of fructose degradation was also determined and behaved similar to the ones of glucose after a longer lag-phase; data not shown). A steady state was reached after a maximum of 10 days of fermentation in all cases. Supporting the data summarized in Table 1, no significant differences were observed between the performances of Siha, Uvaferm and HHD1 in this respect. Only in the case of the cherry mashes, HHD1 displayed a longer lag-phase before the onset of fermentation, but then degraded the carbohydrates and produced ethanol to the same final levels as observed for the two industrial strains (Fig. 2). Note that the total sugar content as deduced from the % Plato given in Table 1 was similar for plum and pear mashes, but the content of free glucose is considerably lower in pear mashes.

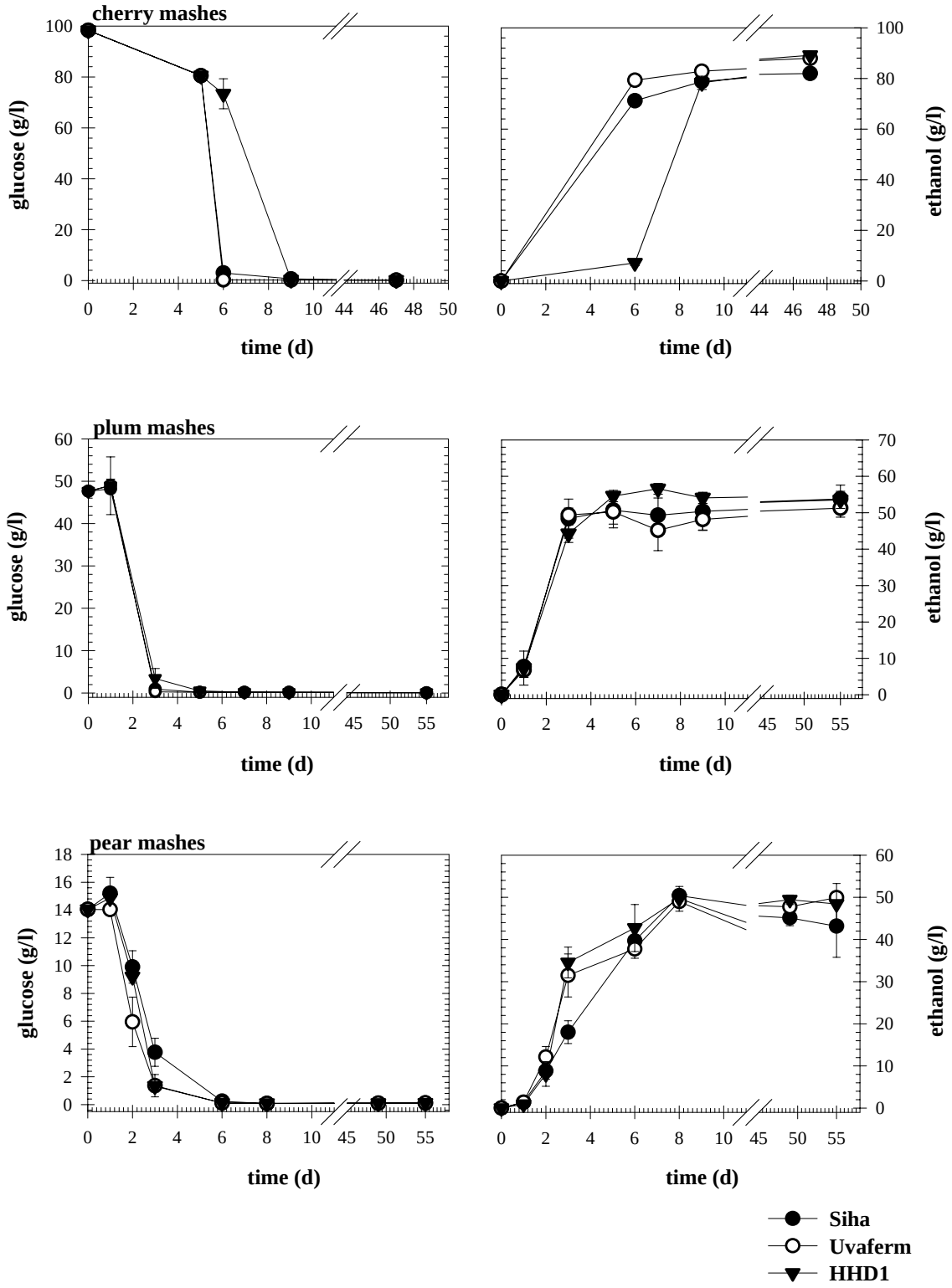

Fig. 2: Glucose consumption and ethanol production in different fruit mashes by commercial and laboratory yeast strains.

Mashes were prepared and inoculated with approximately 10^6 cells/ml of precultured yeasts as described in materials and methods. Fermentation at 15 to 17 °C was followed for a minimum of 48 days. All experiments were performed in triplicate, with bars indicating the standard deviation.

Organic acids and glycerol production during fermentation

Other important parameters of mash and product quality are the acidity of the mashes and the compounds determining their viscosities. We therefore proceeded by determining the concentrations of some key organic acids and of glycerol in the mashes. Our examinations concentrated at first on the changes of the mash pH during fermentation. After an initial decrease within the first two days of fermentation, values then reached a slightly higher constant pH, after approximately 5 days (data not shown). Whilst these did not change significantly over the elongated period of 48-55 days, an increase in the total titratable acidity (calculated as equivalents of tartaric acid) was observed for the pear mashes. This was most pronounced with the Uvaferm yeast, but observed in all fermentations. On the other hand, acidities decreased during fermentation of plum and cherry mashes (Table 2).

Tab. 2: Acidities of mashes before and after fermentation

Mash	Acidity [a]						Final pH [b]		
	Siha		Uvaferm		HHD1		Siha	Uva-ferm	HHD1
	initial	final	initial	final	initial	final			
Cherries	8.60	6.50	8.60	7.50	8.60	7.90	3.90	3.76	3.80
Plums	10.30	8.03	10.30	7.03	10.30	7.43	3.56	3.50	3.50
Pears	2.60	5.43	2.60	6.56	2.60	5.10	2.96	2.90	3.00

a) The total acidity was calculated as tartaric acid equivalents according to Adam *et al.* (1995).
b) The final pH values are the mean of all values measured between 10-50 days and did not vary by more than 0.2 within this period.

Accurate determinations of specific organic acids and of glycerol where obtained by HPLC measurements (Table 3). Although HHD1 seems to produce a little less glycerol (approximately 14% lower) than the industrial strains in the pear and plum mashes, this is not observed for the cherry mashes. Likewise, the higher content of acetic acid detected for the laboratory strain in the pear mashes is not consistent with the other fruit mashes. Similarily, differences in concentrations of lactic and propionic acids did not generally correlate with any of the yeast strains employed for fermentation.

Tab. 3: Concentration of organic acids and glycerol (g/l) in fruit mashes after complete fermentation

Fruit	Compound	Yeast strain employed		
		Siha	Uvaferm	HHD1
Cherries	acetic acid	0.26 ± 0.11	0.16 ± 0.09	0.11 ± 0.05
	propionic acid	0.26 ± 0.08	0.20 ± 0.22	0.52 ± 0.20
	lactic acid	2.25 ± 0.19	2.51 ± 0.08	2.40 ± 0.37
	glycerol	6.54 ± 0.15	6.01 ± 0.17	6.58 ± 0.33
Plums	acetic acid	1.66 ± 0.19	0.80 ± 0.22	1.07 ± 0.24
	propionic acid	0.47 ± 0.21	0.57 ± 0.13	0.61 ± 0.29
	lactic acid	1.73 ± 0.83	2.74 ± 0.69	1.91 ± 1.03
	glycerol	4.57 ± 0.15	4.85 ± 0.32	3.94 ± 0.75
Pears	acetic acid	1.02 ± 0.17	0.92 ± 0.11	1.45 ± 0.23
	propionic acid	0.34 ± 0.17	0.06 ± 0.00	0.23 ± 0.04
	lactic acid	0.22 ± 0.02	0.35 ± 0.01	0.21 ± 0.00
	glycerol	4.28 ± 0.19	4.46 ± 0.12	3.71 ± 0.17

Secondary fermentation products

Other volatile compounds such as esters, aldehydes and higher alcohols present in the mashes after fermentation, are of crucial importance for the quality of the final spirits. Therefore, we also quantified some of these key compounds in the mashes by headspace gas chromatography (Fig. 3a). Siha yeast consistantly produced the lowest amounts of acetaldehyde concentrations in the mashes, whilst Uvaferm and HHD1 produced approximately double (although at very low overall concentrations of 30-60 mg/l, i.e. below the threshholds for sensoric detection). In the case of 1-propanol concentrations, no consistency between the different mashes and the three yeast strains employed could be observed. However, HHD1 invariably correlated with the highest concentrations (again, all below sensoric threshholds), although the Uvaferm strain produced similar amounts in the case of the cherry mashes, whereas Siha led to the lowest 1-propanol amounts in cherry and plum fermentations (Fig. 3a). 2-methyl-1-propanol and isoamylalcohol (3-methyl-1-butanol and 2-methyl-1-butanol) concentrations did not differ signifycantly between the yeast strains in the different mashes (with the exception of the cherry mashes, where the Siha strain produced approximately 30% more 3-methyl-1-butanol than Uvaferm and HHD1).

a)

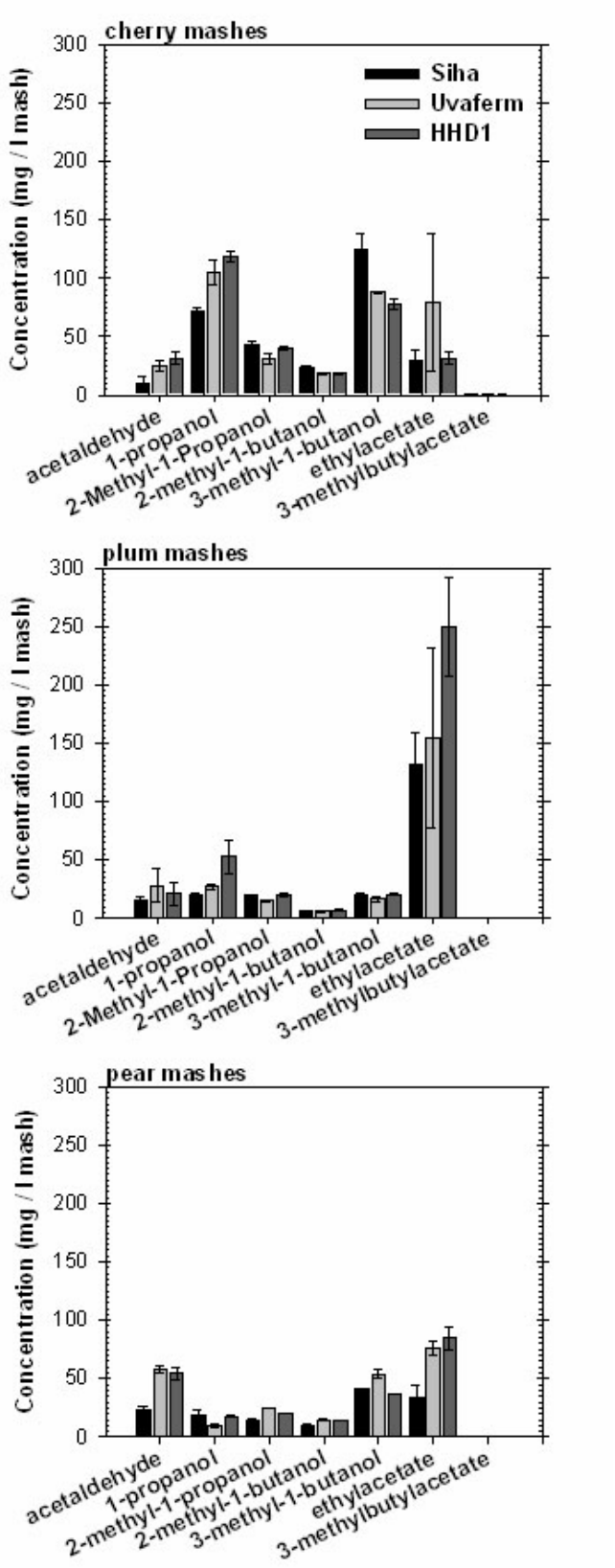

Fig. 3a: Concentration of volatile substances in mashes produced by fermentation with commercial and laboratory yeast strains (determined after complete fermentation, see materials and methods for details)

b)

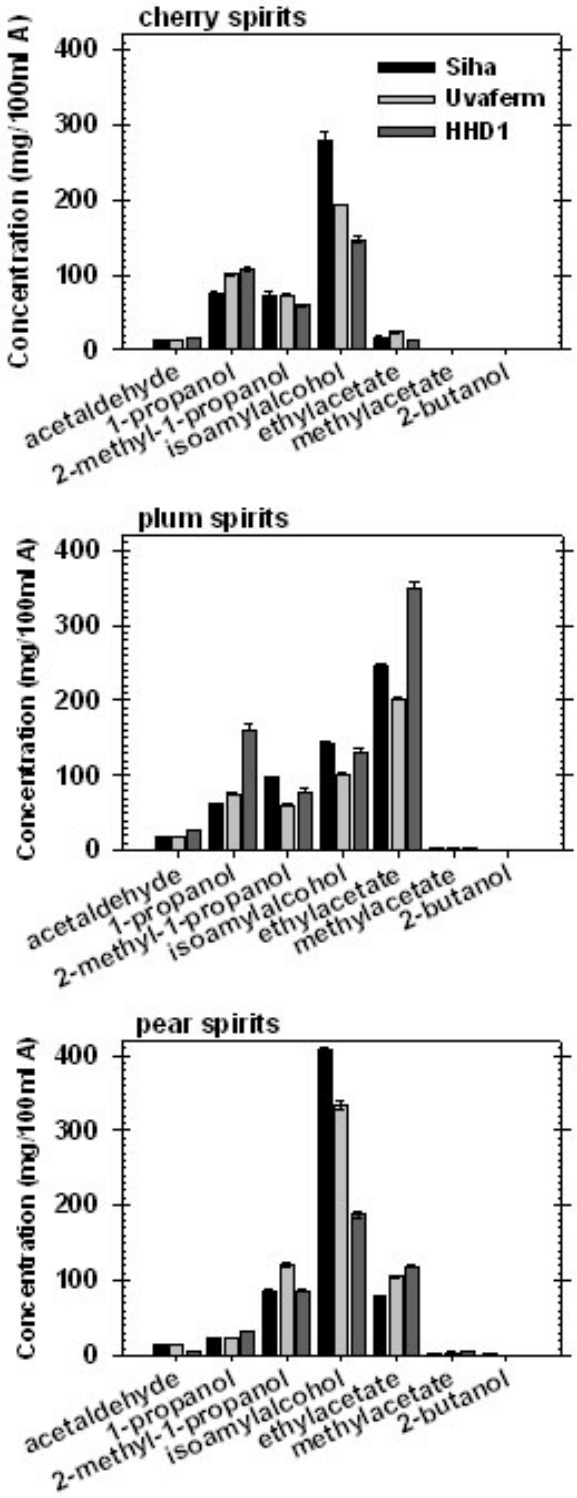

Fig. 3b: Concentration of volatile substances in spirits produced by fermentation with commercial and laboratory yeast strains

Concentrations of key volatile compounds (in mg per 100 ml of alcohol = A) observed in the spirits produced from the mashes tested above. All experiments were performed in triplicate, with bars indicating the standard deviation. Note that concentrations in mashes and spirits are not directly comparable, since their references (liter of mash and 100 ml of alcohol, respectively) are different.

Ethylacetate concentrations were considerably higher in the plum mashes than in the pear and cherry mashes, presumably due to the lower quality of the fruit employed (see Materials and Methods). For pear and plum mashes, fermentation with either Uvaferm or the laboratory strain yielded higher values than with the Siha yeast. This correlation was not observed for the cherry mashes, where HHD1 led to similar low values as the Siha preparation. 3-methyl-butylacetate concentrations scarcely exceeded detectable levels. Finally, the metha-nol content (introduced by the pectinases from the fruit) of the pear and plum mashes (Table 4), although slightly higher for the laboratory strain than for the two industrial yeasts, was within acceptable limits (i.e. below 400 mg/liter). In the cherry mashes, variations between the three yeast strains with respect to the final methanol concentrations were much less pro-nounced (Table 4).

Distillation and spirit analyses

Although the composition of mashes has a crucial influence on the final quality of the spirits produced, distillation leads to the elimination of a variety of volatile compounds and to thermal changes within others. Therefore, we examined all distillates with headspace gas chromatography for their aromatic compounds (partly repeating those already tested in the fermented mashes). The concentrations were calculated in mg/100 ml of pure alcohol and are shown in Fig. 3B. As expected for a successful distillation process, acetaldehyde levels were extremely low in the spirits. Generally, 1-propanol concentrations were higher in the spirits produced with the laboratory strain, especially in plum spirits (correlating with the higher values already observed in the mashes). The spirits produced from plums showed significantly higher levels of ethylacetate when fermented with HHD1. This was not observed for pear and cherry spirits, for which ethylacetate concentrations were generally lower than those of plums. 3-Methyl-1-butanol concentrations were considerably lower in pear and cherry spirits produced with the laboratory strain. All other compounds exhibited approximately the same concentrations for all strains and distillates tested. Moreover, differences in methanol concentrations observed in the mashes for the different yeast strains were generally leveled out by the distillation process (Table 4).

Tab. 4: Methanol concentrations in different fruit mashes after complete fermentation and in drinkable spirits produced from these mashes

Fruit	Siha		Uvaferm		HHD1	
	mash[a)]	spirit[b)]	mash[a)]	spirit[b)]	mash[a)]	spirit[b)]
Cherries	327 ± 21	565 ± 13	307 ± 7	548 ± 3	355 ± 10	717 ± 8
Plums	241 ± 18	256 ± 4	269 ± 25	210 ± 20	319 ± 2	252 ± 72
Pears	252 ± 41	766 ± 39	339 ± 18	741 ± 22	380 ± 22	742 ± 12

a) The methanol concentration in the mashes was calculated in mg per liter mash

b) The concentration of methanol in the spirits was calculated in mg per 100 ml alcohol.

Sensory evaluation

Despite the highly sensitive detection equipment employed above, it is not yet possible to predict the quality of spirits merely by their known chemical composition (Busch-Stockfisch, 2002, Jellinek, 1981, Koch, 1986, Neuman and Molnár, 1991). We used, therefore, two different approaches to determine the sensory properties of the products:

In a first series of sensory evaluations we performed an "order-of-precedence test", in which the three different spirits, fermented with the different yeast strains, were placed in order of decreasing quality (fruity character, smell and taste). With a panel consisting of 25 probationers, no statistical preference for any of the yeast strains could be established (data not shown). Secondly, the so-called "triangle test" was employed to determine whether the yeast strains had any detectable influence on the flavour or taste of the spirits produced. Up to 65 test persons participated in evaluating the laboratory strain against either Uvaferm or Siha products. Probationers were trained to detect the principle taste qualities (sweet, sour, salty, bitter) and then presented with three spirit samples, two of which were identical for each spirit. By simply asking each tester to identify the different sample, even small differences in taste or flavour of a spirit could be detected. The test persons were also asked to judge which of the samples was of better quality. For statistical reasons, only the answers of those able to identify the differing sample, were used in our calculations for the latter (Koch, 1986). Table 5 shows the results and statistical analysis of this test.

Tab. 5: Sensory analyses ("triangle test") of spirits produced from different fruit mashes fermented with commercial and laboratory yeast strains

Fruit	Spirits	Number of test persons	Differences detected [a]				Preference [b]		
			number	$\chi^2_{theoret.}$	$\chi^2_{calcul.}$	significic. (α=5%)	$\chi^2_{theor.}$	$\chi^2_{calcul.}$	Prefer. yeast
Cherries	HHD1 *vs* Siha	65	41	2.71	24.56	yes	3.84	0.39	none
	HHD1 *vs* Uvaferm	56	25	2.71	2.73	yes	3.84	0.64	none
	Siha vs Uvaferm	18	10	2.71	3.06	yes	3.84	0.40	none
Pears	HHD1 *vs* Siha	55	32	2.71	14.18	yes	3.84	6.13	HHD1
	HHD1 *vs* Uvaferm	56	34	2.71	17.68	yes	3.84	7.53	HHD1
	Siha *vs* Uvaferm	19	8	2.71	0.32	no	3.84	3.13	none
Plums	HHD1 *vs* Siha	55	22	2.71	0.82	no	3.84	0.05	none
	HHD1 *vs* Uvaferm	55	21	2.71	0.38	no	3.84	0.00	none
	Siha *vs* Uvaferm	13	3	2.71	0.24	no	3.84	0.30	none

Spirits were subjected to triangle tests (see materials and methods for details) to detect differences introduced by the yeast strain used for fermentation of the mashes (e.g. HHD1 *versus* Siha = HHD1 *vs* Siha).

[a] The numbers of test persons detecting a difference were subjected to χ^2 analyses and differences are given (yes = significantly different; no = not significantly different).

[b] If differences were detected, the test persons were asked to judge their preference. Again, these data were subjected to statistical analyses (using the forced choice technique) and evaluated for the preference of the yeast strain employed in the fermentation of the mashes.

In the case of pear and cherry spirits, a significant statistical difference was observed between the spirits produced with the laboratory strain and those produced with the Siha or the Uvaferm yeast. In the case where the pear mashes were fermented and distilled, HHD1 was judged to produce a spirit of significantly higher quality. No differential quality judgement could be made for the cherry spirits. Spirits produced from plums using the three different yeast strains could not be distinguished at all by the panel.

DISCUSSION

In the fruit fermentation industry, whether for the production of wines or spirits, the addition of a selected strain of *S. cerevisiae* to avoid bacterial contamination and to ensure a reproducible performance and product quality is common practice (Pretorius, 2000). Commercially available yeast preparations used for this purpose have not been sufficiently characterised regarding their life cycle and genetic composition (Heinisch and Hollenberg, 1993). Therefore, reproducible performance is threatened by strain evolution caused by sporulation and mating, mutations, gene conversions and genetic transpositions. Moreover, targeted genetic manipulations for strain improvement are quite laborious. Thus, protoplast fusions between different *S. cerevisiae* strains, as well as with other yeast species, have been employed to circumvent the problems caused by the non-sporulating phenotype of most commercial strains (Spencer and Spencer, 1996). Genetic instability is an obvious by-product of the latter procedure. On the other hand, transformation with plasmids or DNA carrying heterologous genes (e.g. for the production of enzymes, vaccines etc.) relies on the introduction of dominant genetic markers and does not find public acceptance where food production processes are concerned (Danner, 1997, Drewnoski and Rock, 1995, Nishiura *et al.*, 2002). These problems could be avoided by the use of a genetically well-defined yeast strain that can be easily sporulated and thus crossed to combine the desired properties. Furthermore, the availability of haploid segregants would ease phenotypic selection procedures.

In the study presented here we confirmed that haploid laboratory yeast strains carrying auxotrophic markers are indeed not suitable for practical applications. Growth, sugar consumption and ethanol production were decreased in comparison to the commercial yeast preparations when tested in cherry mashes. However, this was not observed for the diploid derivative constructed from the two laboratory strains by simple mating. HHD1 showed similar fermentation rates and survived just as well as the commonly used Siha- and

Uvaferm yeasts. This observation substantiates its ability to ferment all sugars usually found in fruit mashes, i.e. glucose, fructose, sucrose and raffinose. Although the start of fermentation was delayed by 2-3 days in some cases when employing the laboratory strain, it caught up with the commercial yeasts within the first 10 days. Since mash fermentations usually last over a period of at least one month in spirit production (Pieper *et al.*, 1993), this does not present an obstacle. It has been suggested that the longer alcohol production is delayed the greater the risk of bacterial contamination (Bayrock and Ingledew, 2001). Yet, this was not observed (neither by microscopic examination nor by the distribution of fermentation by-products) even in the case of the pear mashes, where we employed low quality fruit in order to test the prevalence of the yeast preparations over bacteria being introduced by the raw material. It seems noteworthy that interactions between lactic acid bacteria and various yeast species are frequently inherent to fruit fermenting processes (Addis *et al.*, 2001, Corsetti *et al.*, 2001, Eliseeva *et al.*, 2001, van Beek and Priest, 2002). Metabolic activity of bacteria is usually indicated by increased amounts of acetic acid and lactic acid. These may in part explain the growth inhibitory effect of bacterial contaminations on the yeast population (Boidron, 1969, Thomas *et al.*, 2001). *Vice versa*, bacterial growth is enhanced by the presence of yeasts as they serve as a nutrient source to provide essential compounds. Massive bacterial growth leads to competition for the sugars present in the mashes and may result in a considerable reduction in the alcohol yield (Thomas *et al.*, 2001). None of these deleterious effects was observed in the mashes fermented in this work, indicating that the competitive fitness of the laboratory strain equals that of the industrial yeasts employed. This is also true for the competition with "wild yeasts" commonly found on fruit, such as *Kloeckera apiculata* (= *Hanseniaspora uvarum*; Meyer *et al.*, 1978).

A major by-product of yeast carbohydrate metabolism which also reduces the alcohol yield is glycerol. Therefore, this compound is of significant interest to wine-, beer- and ethanol-production industries (Cronwright *et al.*, 2002). Moreover, overproduction of glycerol in an engineered *S. cerevisiae* strain leads to substantial changes in the formation of other by-products and to a stimulation of the fermentation rate in stationary phase cells (Remize *et al.*, 1999). Again, the laboratory strain produced similar glycerol concentrations as compared to the two industrial strains (4-5g/l) in all fruit mashes tested in this work. In this respect, no negative effect could be observed.

For spirits and other alcoholic beverages a major factor for their application is the sensory performance. This in turn is mainly influenced by a combination of higher alcohols and volatile compounds, such as organic esters (Goranov, 1983). Although some of the key

substances may escape detection, we here employed both HPLC- and GC-methods to determine the exact concentrations of some of the major constituents, both in the mashes and the final products. In general, concentrations of higher alcohols and esters varied depending more on the raw material used than on the yeast strain employed for the fermentation. Yet, a fairly consistent higher production of 1-propanol, that was carried over into the distillates, could be observed for the laboratory strain as opposed to the two commercial preparations tested. However, this did not result in an altered sensory performance. Likewise, methanol levels in the final spirit were largely independent of the yeast strain employed for fermentation and remained below the legal limits in all cases. It is most interesting to note that a difference between the spirits produced with the commercial strains and the laboratory strain could be detected, with statistical significance for two of the three fruit spirits tested (i.e. pear and cherry). The preference for the use of the laboratory strain ("triangle test") in the preparation of the pear spirits, although statistically significant, should be taken with care. Since tailings have to be excluded by organoleptic assessment in the distillation process, slight differences observed in the products may result from a relatively small amount of tailing included in one case and not the other (Postel and Adam, 1989). This result would have to be verified by the large-scale fermentation of higher numbers of pear mashes, which would exceed our possibilities with the equipment available to us. On the other hand, since none of the commercial strains were preferred, it seems safe to conclude that the laboratory strain at least does not have a negative influence on the sensory properties of the final spirits. This conclusion is further supported by the order of preference test, where none of the yeast strains employed was consistently associated with a preferred spirit.

CONCLUSIONS AND OUTLOOK

The data presented here indicate that the diploid laboratory strain tested for the fermentation of various fruit mashes and spirit production is as suitable as commercially available yeast preparations with respect to both fermentation capacity and sensoric performance. However, albeit these preliminary experiments are very promising, a number of questions remain to be addressed. Thus, the laboratory strain should be adjusted for improved fermentation at lower temperatures. This may resolve the problem of delayed fermentation start observed in some cases. Furthermore, to be of commercial value, large scale strain production and preparation of dry yeast would be desirable. Thus, growth and viability of the laboratory strain under

industrial production conditions need to be tested. Given that these problems can be solved, the strain would be of great value for the production of spirits and may also be tested in other beverages and for bioethanol production. Its known genetic constitution will greatly simplify the adjustment to such different purposes.

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CHAPTER IV

EFFECT OF THE STONE CONTENT ON THE QUALITY OF PLUM AND CHERRY SPIRITS PRODUCED FROM MASH FERMENTATIONS WITH COMMERCIAL AND LABORATORY YEAST STRAINS

Abstract

In order to evaluate the influence of stone content on spirit quality from stone fruit, cherry and plum mashes were prepared and fermented with a commercial and a diploid laboratory yeast strain. Fermentation parameters such as sugar content and ethanol production were followed. Despite an initial lag-phase in cherry spirits, both yeast strains performed similarily, as substantiated by the determination of specific flavour compounds and methanol in the mashes and after distillation. The spirits produced were subjected to sensory analyses by trained panels of at least 25 judges. Although mashes retaining the stones could be clearly distinguished from those where the stones had been removed, no significant preference could be attributed to either spirit, indicating that qualities added by the presence of stones during fermentation are largely a matter of personal taste. Interestingly, the yeast strain used for fermentation seemed to have little influence on the spirit quality.

Schehl, B., T. Senn, and J. J. Heinisch. 2005. Effect of the stone content on the quality of plum and cherry spirits produced from mash fermentations with commercial and laboratory yeast strains. *J. Agric. Food. Chem.* 53:8230-8238.

INTRODUCTION

Since time immemorial alcoholic beverages and spirits have been produced from a variety of fruit by yeast-based fermentations. Fermentation and distillation technologies have been especially improved in the course of the last century and refined methods are continuously developed. Like with other spirits, using stone and pip fruit as approved raw materials, these developments are aimed at the production of high quality distillates. As a result of the increasing competition in the spirit production busyness, consumer's interests shifted from "low cost" commodities to high-quality beverages. Although the definition of high quality is somewhat prone to personal preferences, there are certain legal requirements to be fulfilled and also some rules in production to be followed to ensure a widely excepted spirit quality. Nevertheless, obeying all these rules does not necessarily guarantee a high quality and commercially successful product. In addition, attributes such as social acceptability, healthiness and enjoyment in the consumption are values which can be at least partially influenced by the producer (e.g. by reducing the concentrations of potentially hazardous compounds). Moreover, the judgement of sensory attributes by expert panels is necessary for the development of production schemes that will result in beverages with reproducible quality and good consumer acceptance.

Sensory performance is dependent on the concentration of flavour compounds. These have their origin in the fruit employed as raw material, in the fermentation process itself with substances coming from yeast metabolism or from the degradation of fruit ingredients, and from chemical reactions between these compounds during fermentation, distillation and storage (Dürr, 1997).

Besides the aspects concerning the raw material employed, market-orientated yeast strains are currently being developed for the cost-competitive production of alcoholic beverages with minimized resource inputs, improved quality and low environmental impact (Pretorius and Bauer, 2002). Thus, *Saccharomyces cerevisiae* strains are developed, showing improved fermentation, processing and biopreservation abilities, as well as improved sensory qualities of the beverages. Different yeast strains will usually produce individual quality profiles (Younis and Steward, 1998). Therefore, genetically well-defined or even modified yeast strains are more and more constructed for the alcoholic beverage industry (Pretorius and Bauer, 2002, Schehl et al., 2004).

Regarding stone fruit as raw materials, consumers often desire the typical "bitter-almond" character in the final spirits. However, such positive flavour compounds introduced from the stones may be accompanied by detrimental influences and even health risks. Thus, fermentation of stone fruit and subsequent spirit production has been claimed to frequently result in the formation of the carcinogenic compound ethyl carbamate (also referred to as urethane; Melzoch et al., 1996, Ough, 1976, Pretorius, 2000). It was proposed that this compound can form when amygdaline from the stones is degraded to cyanide and exposed to light (Baumann and Zimmerli, 1986, Arresta et al., 2001, Mildau et al., 1987). Another possible source of ethyl carbamate may be yeast metabolism and secretion of urea into the medium, as an intermediate of arginine metabolism (Kitamoto et al. 1991, An and Ough, 1993).

Thus, removal of stones remains an option for the production of spirits with different flavour and a "healthier" spirit. In this work, we tested the effect of such a removal prior to fermentation on the sensory quality and the concentration of several flavour compounds within the spirits produced.

MATERIALS AND METHODS

Yeast strains employed

In this work the commercially available yeast strains named *Uvaferm CGC62, Freddo, Forte* (manufacturers trade names; all purchased from Begerow GmbH & Co., Langenlonsheim, Germany) and the laboratory strain HHD1 (*MAT**a**/α ura3-52/URA3 leu2-3*,*112/LEU2 MAL2-8^c/MAL2-8^c SUC2/SUC2*; *4*) which is closely related to the *S. cerevisiae* CEN.PK122 strain (Van Dijken et al., 2000), were used. The commercial strains were packaged as dried yeast in 500 g aliquots.

Media and culture conditions

Rich media were based on 1% yeast extract and 2% bacto peptone and supplemented with 2% glucose (YEPD). All strains were incubated at 30°C. For standardized conditions all strains were grown in 5 ml YEPD overnight on a rotor shaker (30°C, 140 rpm), transferred into 500 ml shake-flask cultures with fresh YEPD, incubated for 12 h and harvested by centrifugation (3500 x g for 5 min at room temperature). Cell pellets were washed twice with 25 ml NaCl/peptone (0.85% NaCl, 0.05% peptone), resuspended in 25 ml of the same medium and transferred to 1.5 liter YEPD in 3 liter shake flasks. After 24 hours of incubation

at 30°C at 140 rpm yeasts from each culture were again harvested by centrifugation (3500 x g for 10 min at room temperature), washed twice as described above and re-suspended in 100 ml NaCl/peptone solution. The cell density was calculated from optical measurements at 578 nm in appropriate dilutions, assuming that 1 OD_{578} equals 10^7 cells/ml. From this, the yeasts were added to the mashes at a final density of 10^6 cells/ml each.

Raw material and mashing process

Based on former results fermentations on a technical scale (90 kg) were initiated (Schehl et al., 2004). The studies were performed with two different stone fruit mashes: cherries (cv. *Dollenseppler*) and plums (cv. *Ersinger Frühzwetschge*). Cherry mashes were inoculated with the yeast strains listed above. The plum mashes and the remaining stones were only fermented with the Uvaferm strain and HHD1. The cherries were in an excellent condition like fresh dessert fruit, no bruised or decayed fruit were present. The plums were in a faultless but rather more critical condition, so that foul fruit had to be sorted out prior to mashing.

Mashes were prepared according to standard procedures. Thus the fruit (exempted from peduncles) were washed and chopped using a drill machine attached to a beater so that the stones remained undamaged, and then divided into equal lots. One fraction was not treated any further, the other portion was passed through a pulping machine and destoner (filter-width 4 mm, capacity 50-250 kg/h; Bockmeyer, Nürtingen, Germany) for the total removal of the stones. Immediately after comminution or pitting the fruit, the pH-value was adjusted to 3.0 with technical sulphuric acid (technical grade). The remaining stones were collected and fermented separately without addition of sulphuric acid.

The mash was divided in 90 kg-lots each and separated in 120 liter vessels. For fermentation the vessels were sealed with a fermentation bung and incubated with the two different yeast strains. All experiments were performed in triplicate and different parameters like ethanol yields, extract, sugar content, yeast metabolites and pH were determined over the fermentation period. The stones were only fermented without checking any of these parameters, distilled and finally used for sensory assessment.

Fermentation

The fruit mashes (90 kg each) were fermented in 120 liter plastic barrels. The mashes were inoculated with the selected commercial yeast strains Uvaferm, Freddo, Forte, the laboratory strain HHD1 (all standardized to be in the same physiological state and cell density as described above) and fermented to completion at 15-17°C. During fermentation, mashes were agitated at times and samples were collected and analysed at the same time for the different parameters indicated.

Distillation

After 8 weeks of fermentation, the mashes were distilled using a 200 liter copper pot (Jacob-Carl, Göppingen, Germany) fitted with an enrichment section consisting of three bubble plates, a dephlegmator and a cyan catalyst (Holstein, Markdorf, Germany). This modern plant facilitates distillation under technical and standardized conditions. The dephlegmator was run with a flow rate of 120 liter/h and the catalyst was used. The fermented mashes were distilled with two plates in operation. The distillates were collected in fractions with a volume of 250-300 ml, each. In the vicinity of the switching points (heads to product fractions and product fractions to tailings) smaller volumes of 100-150 ml were collected. The heads were identified with the detaching test determining acetaldehyde according to Pieper et al., 1987. The tailings were screened by detachment at 72%vol and partly by organoleptic assessment. The stones were distilled on a 19 liter plant with three plates, a dephlegmator and without a catalyst. Fractions of 100 ml each were collected and the heads and tailings discarded as described above.

Spirit fractions

The product fractions were stored for at least one week at 17°C, then diluted with deionised water to an alcohol content of 40% (v/v), cold filtered at 4°C (Macherey Nagel, Düren, Germany) and kept for another four weeks at 17°C prior to further analysis and sensory assessment.

Analytical methods

As a preliminary indication to observe the fermentation process, the pH was followed using a pH-meter (WTW521, Weilheim, Germany), the decrease of fermentable carbohydrates (%sugar) was determined with a hand refractometer (Carl Zeiss, Jena, Germany).

The exact decrease of the fermentable sugars (glucose and fructose), the ethanol content as well as the formation of the volatile compounds acetic acid, propionic acid and lactic acid were determined by HPLC (Bischoff Modell 2200 HPLC using a Bischoff Modell 728 Autosampler; Bischoff, Leonberg, Germany), using an Aminex HPX-87H column (Biorad, Munich, Germany), a RI detector ERC7510 (ERC, Altegolfsheim, Germany) and a Mc-DAcq15 Integrator (Bischoff, Leonberg, Germany). Sulphuric acid (0.1N, technical grade) was used for elution.

Quantitative GC-FID analyses were performed to determine methanol and the major various yeast metabolites and aroma components like acetaldehyde, methyl- and ethylacetate, 3-methyl-butylacetate, 2-methyl-1-propanol, 1-propanol and the isoamylalcohols (3-methyl-1-butanol and 2-methyl-1-butanol). Therefore a headspace gas chromatograph from Perkin Elmer (Modell HS40, GC 8420) equipped with a packed crossbond phenylmethyl-polysiloxane column (Rtx-volatiles; 60 m by 0.32 mm, film thickness 1.5 µm; Resteck GmbH, Bad Homburg, Germany), a flame ionization detector and a CLASS VP 4.2 integrator (Shimadzu, Duisburg). As an internal standard n-Butanol (200 mg/l; Merck, Darmstadt, Germany) was used. For mashes, the method described in Brautechnische Analysen-methoden (MEBAK, 1996) according to Boettger and Pieper (1994) was used. All gases were supplied by Sauerstoffwerk GmbH (Friedrichshafen, Germany).

Sensory analyses

The fruit spirits produced in different technological ways and fermented with different yeast strains, were analyzed by both sensory and physical methods. They were assessed for their characteristic flavour quality using order-of-precedence and triangle-tests (Jellinek, 1981, Roth et al., 1977).

Before sessions, a panellist training (staff and graduate students from the University Hohenheim, Department of Food Technology) was accomplished. The participants were trained in evaluation of the basic flavours (salty, bitter, sweet and sour) and in detecting differences between typical ingredients of heads and tailings in spirits. To enhance statistical significance larger panels of at least 20 judges were employed.

Statistical analyses

Data were analysed by the statistical software SigmaStat (Jandel Scientific) using "One-Way-ANOVA" on ranks. This non-parametric test compares several different experimental groups which received different treatments. To isolate the group or groups that differed, all pairwise

multiple comparison procedures (according to the Student-Newman-Keuls method) were performed at 5% significance level (Fox et al., 1995).

RESULTS

Analyses of fermentation parameters in mashes with and without stones

In order to investigate how the presence of stones affects spirit quality, cherry and plum mashes were fermented with different yeast strains and a set of fermentation parameters was followed. For this purpose, 90 kg each of the fruit mashes with and without stones were inoculated in triplicate with 10^6 cells/ml either of a commercial Uvaferm yeast or the laboratory diploid yeast strain HHD1. Fermentation was accomplished under semi-anaerobic, non-sterile conditions at low temperatures. Samples were taken weekly for microscopic examination and it was confirmed that no excessive bacterial contaminations were present in the mashes. However, complete mashes (with stones) developed a layer of wild yeast contaminants on the surface, due to exposure to oxygen during sampling. Interestingly, this layer of wild yeasts did not occur on the mashes where stones had been removed (i.e. stoneless mashes).

Table 1 summarizes the general fermentation parameters, including the theoretical and practical alcohol yields. As expected from the higher initial sucrose content, higher alcohol yields were obtained in the cherry mashes than from those of plums. Regarding the use of different yeast strains for fermentation, the presence or absence of stones did not affect the final alcohol yield in the mashes fermented with the Uvaferm strain. The laboratory strain produced slightly more ethanol when stones were removed from the cherry mashes. In contrast, slightly less ethanol was produced from the stoneless plum mashes than from those of the complete mashes.

Tab. 1: Sugar content and alcohol yield during mash fermentations

Mash	% Plato[a)]				Theoretical alcohol yield[b)]	Observed alcohol yield in mashes [c)]	
	Uvaferm		HHD1			Uvaferm	HHD1
	Initial	Final	Initial	Final			
Cherries with stones	26.0	14.3	25.4	14.5	9.85	14.00	12.73
Cherries w/o stones	23.9	11.6	24.1	12.4	9.04	14.42	15.12
Plums with stones	17.2	9.5	16.7	10.6	6.42	11.64	10.30
Plums w/o stones	17.1	10.9	16.2	9.5	6.28	11.64	8.83

a) % Plato = g sucrose per 100 g mash liquid

b) The theoretical alcohol yield was calculated as follows: liter alcohol / 100 liter mash = (%Plato - non-fermentable matters) x 0.56 x TF (with non-fermentable matters for cherries = 5% and for plums = 4% and TF for cherries = 0.850 and for plums = 0.885)

c) Observed alcohol yield = alcohol content of the spirit (v/v) x liter spirit per liter mash; for the mashes 20% for cherries and 24% for plums was assumed.

w/o = without

As a more accurate measure of yeast metabolic activity, we determined the kinetics of glucose and fructose degradation as well as the production of ethanol over a period of 50-60 days by HPLC (Fig. 1). In the initial phase of fermentation, we noted a difference in the performance of the Uvaferm strain in comparison to the laboratory strain within the cherry mashes: HHD1 displayed a longer lag phase in the onset of fermentation as judged from all three parameters measured. Nevertheless, after a maximum of 10 days of fermentation, all mashes, regardless of their stone contents, were equally well fermented by both the commercial and the laboratory yeast strains. In these determinations, the laboratory strain initially produced higher amounts of ethanol from the stoneless plum mashes than the Uvaferm strain. However, this difference diminished later-on during fermentation.

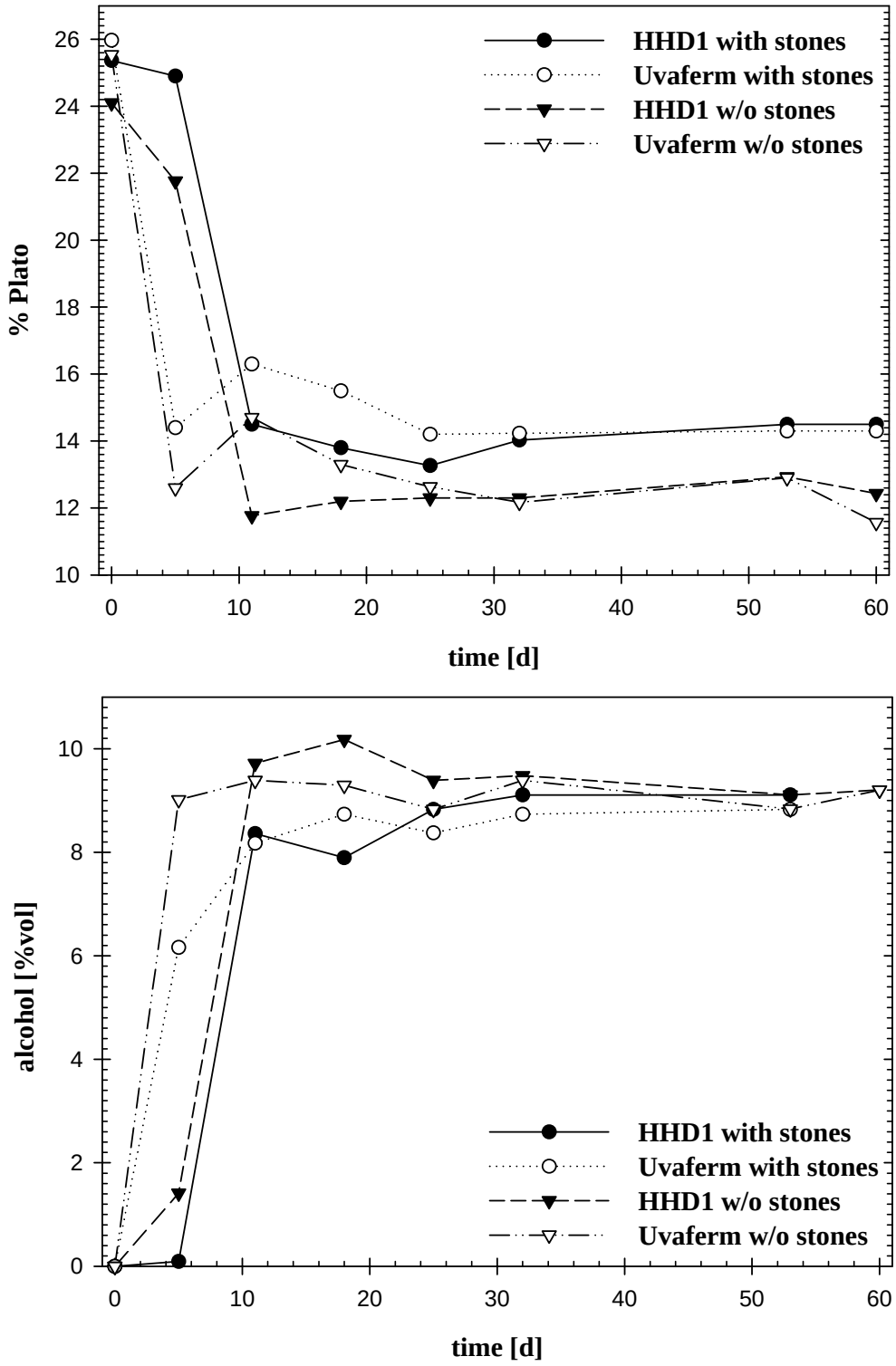


Fig. 1a: % Plato and alcohol (v/v) content in the cherry mashes during fermentation

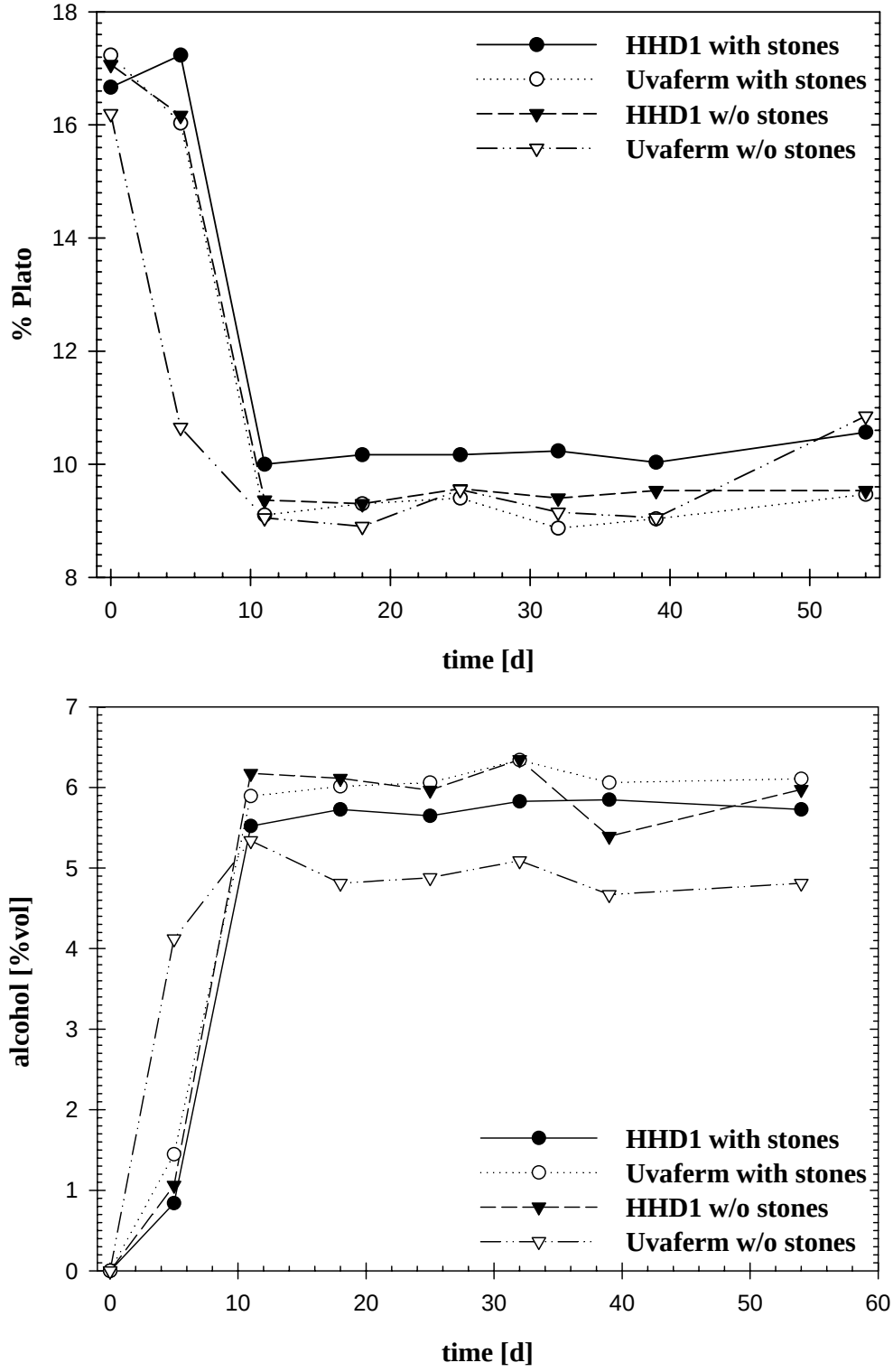


Fig. 1b: % Plato and alcohol (v/v) content in the plum mashes during fermentation

The commercial Uvaferm strain and the laboratory yeast strain HHD1 were employed for fermentations. Mashes were prepared and inoculated with approximately 10^6 cells/ml of precultured yeasts as described in materials and methods. Fermentation at 17 °C was followed for up to 60 days. All experiments were performed in triplicate.

Organic acids and glycerol

Some organic acids and glycerol play an important role for the quality of the mashes and the spirits produced from them (Pieper et al., 1993). We therefore proceeded by determining the concentrations of acetic, propionic and lactic acid and of glycerol in the mashes by HPLC (Table 2).

Tab. 2: Organic acids and glycerol contents in mashes after fermentation (50-60 days).

Mash	Compound	Yeast strain and stone content			
		Uvaferm with stones	Uvaferm w/o stones	HHD1 with stones	HHD1 w/o stones
Cherries	**acetic acid**	0.41 ± 0.28	0.35 ± 0.23	0.45 ± 0.28	0.42 ± 0.34
	propionic acid	1.31 ± 0.26	0.86 ± 0.22	1.20 ± 0.33	0.81 ± 0.14
	lactic acid	1.24 ± 0.57	4.95 ± 1.26	2.86 ± 0.44	5.49 ± 0.99
	glycerol	8.07 ± 0.37	8.33 ± 0.49	9.58 ± 0.68	9.10 ± 0.37
Plums	**acetic acid**	< 0.05	< 0.05	< 0.05	< 0.05
	propionic acid	< 0.05	< 0.05	< 0.05	< 0.05
	lactic acid	0.47 ± 0.26	0.53 ± 0.12	0.62 ± 0.44	0.58 ± 0.22
	glycerol	6.29 ± 0.87	5.37 ± 0.21	6.77 ± 0.75	6.13 ± 0.66

Concentrations of all compounds are given in g per liter mash.

Acetic and propionic acid concentration ranged below detectable levels in the plum mashes. For the cherries, slightly lower values were found in the stoneless fermentations than in complete mashes. For the two yeast strains employed, no significant differences were detected. Glycerol production did not vary significantly either under all conditions tested. Only the lactic acid concentrations were increased in the cherry mashes as compared to the plum mashes. Furthermore, the stoneless cherry mashes showed a 2-3fold increase in the amount of lactic acid compared to the complete mashes. The latter observation indicates a higher load of bacterial contamination.

Secondary fermentation products and methanol

Other volatile compounds such as esters, aldehydes, methanol and higher alcohols present in the mashes after fermentation, are of crucial importance for the quality of the final spirits. Therefore, we also quantified some of these key compounds in the mashes by headspace gas chromatography (Table 3a).

Tab. 3a: Metabolites in mashes after 50-60 days of fermentation.

Mash	Compound	Yeast strain and stone content			
		Uvaferm with stones	Uvaferm w/o stones	HHD1 with stones	HHD1 w/o stones
Cherries	**methanol**	398.58 ± 23.4	320.83 ± 20.89	440.08 ± 10.97	342.00 ± 22.86
	acetaldehyde	27.73 ± 1.7	107.20 ± 35.24	17.52 ± 1.48	88.68 ±37.93
	1-propanol	2.90 ± 0.6	3.45 ± 0.20	5.39 ± 1.46	11.25 ± 1.11
	2-methyl-1-propanol	21.07 ± 1.5	18.08 ± 1.35	19.42 ± 1.23	15.04 ± 1.47
	3-methyl-1-butanol	98.63 ± 5.5	86.47 ± 11.96	91.16 ± 3.27	89.32 ± 17.37
	ethylacetate	137.65 ± 3.1	147.18 ± 13.13	157.30 ± 0.77	225.95 ± 24.25
	3-methyl-butylacetate	0.21 ± 0.05	0.32± 0.02	0.23 ± 0.05	0.27 ± 0
Plums	**methanol**	530.78 ± 49.27	539.37 ± 80.60	413.48 ± 67.59	442.87 ± 85.72
	acetaldehyde	22.83 ± 5.32	27.85 ± 2.17	6.92 ± 5.74	45.22 ± 3.92
	1-propanol	67.95 ± 32.01	66.28 ± 30.25	8.18 ± 5.99	124.25 ± 17.89
	2-methyl-1-propanol	22.57 ± 4.76	16.35 ± 16.07	11.14 ± 7.07	13.83 ± 1.46
	3-methyl-1-butanol	76.99 ± 14.11	66.53 ± 5.35	37.13 ± 21.27	43.10 ± 5.34
	ethylacetate	53.58 ± 3.00	28.81 ± 6.09	144.52 ± 62.18	29.91 ± 0.96
	3-methyl-butylacetate	0.30 ± 0.04	0.33 ± 0.31	0.15 ± 0.09	0.30 ± 0.04

w/o = without

The methanol content of the cherry mashes containing stones was higher than in the stoneless mashes regardless of the yeast strain employed. From the plum mashes fermented with the Uvaferm yeast slightly more methanol could be detected than from those fermented with the laboratory strain. Invariably, the concentrations remained below critical thresholds (i.e. 1000 mg/l).

Acetaldehyde concentrations were higher in the mashes fermented without stones than in those with stones. Likewise, the concentrations of 1-propanol were generally higher in the stoneless mashes, with the exception of the plums fermented with the Uvaferm strain. *Vice versa*, the ethylacetate content was higher when plums were fermented with stones than in the stoneless mashes. This difference was not observed for the cherry mashes. The other compounds tested did not differ significantly between the different fermentation sets, although a high variability was found within the plum mashes.

Distillation and spirit analyses

Although the quality and treatment of the mashes play a key role, distillation conditions still have an influence on the performance of the final spirits (Ande, 2004). Thus, through the process of distillation many volatile compounds can be either removed or concentrated and thermal reactions will produce further compounds. We therefore first also examined the

distillates for some aromatic compounds (Table 3b). As expected for a successful distillation process, acetaldehyde levels were all generally low in the spirits (note that concentrations in this case are given per 100 ml of total alcohol).

Tab. 3b: Metabolites in spirits after distillation.

Spirits	Compound	Yeast strain and stone content			
		Uvaferm with stones	Uvaferm w/o stones	HHD1 with stones	HHD1 w/o stones
Cherries	**methanol**	562.27 ± 2.76	568.05 ± 176.75	590.31 ± 6.68	582.32 ± 94.04
	acetaldehyde	57.46 ± 0.16	40.55 ± 32.84	55.20 ± 0.39	86.32 ± 17.02
	1-propanol	195.86 ± 1.07	204.92 ± 68.49	192.70 ± 1.36	170.35 ± 41.86
	2-methyl-1-butanol	307.82 ± 2.11	284.94 ± 97.28	276.43 ± 1.26	296.01 ± 36.27
	3-methyl-1-butanol	417.57 ± 2.16	450.27 ± 134.98	377.02 ± 2.05	416.82 ± 74.69
	ethylacetate	119.47 ± 0.64	117.13 ± 42.79	115.88 ± 0.75	142.30 ± 19.53
	methylacetate	0.85 ± 0.12	2.46 ± 0.36	0.92 ± 0.02	2.57 ± 0.13
	3-methyl-butylacetate	1.19 ± 0.75	2.41 ± 1.48	1.39 ± 0.30	2.63 ± 5.19
Plums	**methanol**	891.24 ± 79.31	1010.63 ± 69.68	732.05 ± 50.41	876.90 ± 3.25
	acetaldehyde	13.48 ± 1.29	31.39 ± 1.93	18.81 ± 1.11	31.26 ± 0.15
	1-propanol	185.85 ± 13.72	181.93 ± 1.42	198.76 ± 15.96	274.29 ± 11.74
	2-methyl-1-butanol	449.48 ± 20.11	341.08 ± 2.07	306.25 ± 41.39	216.77 ± 16.90
	3-methyl-1-butanol	477.49 ± 16.55	376.64 ± 2.30	252.93 ± 43.77	197.04 ± 27.15
	ethylacetate	251.67 ± 10.52	138.41 ± 0.59	151.41 ± 22.19	110.18 ± 9.50
	methylacetate	0.22 ± 0.23	1.88 ± 0.02	3.78 ± 0.04	2.88 ± 0.01
	3-methyl-butylacetate	2.75 ± 0.13	0.73 ± 0.11	2.30 ± 0.69	2.58 ± 0.07

Concentrations are given in mg per 100 ml alcohol.

The differences in methanol concentrations discussed above for the mashes were abolished by the distillation process. It should be noted however, that for the plum spirits produced from mashes fermented with Uvaferm methanol concentrations approached the critical limits of 1000 mg/100 ml alcohol. The spirits produced from mashes fermented with the laboratory strain stayed clearly below that concentration.

For the amounts of the other compounds tested, substantial variabilities were observed. However, the concentrations did not differ significantly comparing stone content or the employed yeast strains.

Sensory evaluation

Despite the highly sensitive detection equipment employed above, it is not yet possible to predict the quality of spirits merely by their known chemical composition (Jellinek, 1981, Busch-Stockfisch, 2002, Koch, 1986, Neumann and Molnar, 1991). Therefore, two different evaluation methods were employed to determine the sensory properties of the spirits.

In the first series of sensory evaluations we performed "triangle tests" to determine the influence of stone content on flavour and taste of the spirits. By simply asking each tester to identify the different sample, even small differences in taste or flavour of a spirit can be detected by this method. Up to 70 test persons participated in evaluating the effect of the two yeast strains employed and the different production schemes, i.e. fermentation with or without stones. The test persons were also asked to judge which of the samples was of better quality. For statistical reasons, only the answers of those able to identify the differing sample were used in the latter calculations (Koch, 1986). Table 4 shows the results and statistical analyses of these tests. Spirits produced from mashes with stones could always be distinguished from those of the stoneless mashes. Yet, neither was preferred. Spirits produced from stoneless mashes with the laboratory yeast strain and with the Uvaferm strain could only be distinguished in the case of plums, but not in the cherry spirits. Again, no preference was given in this test.

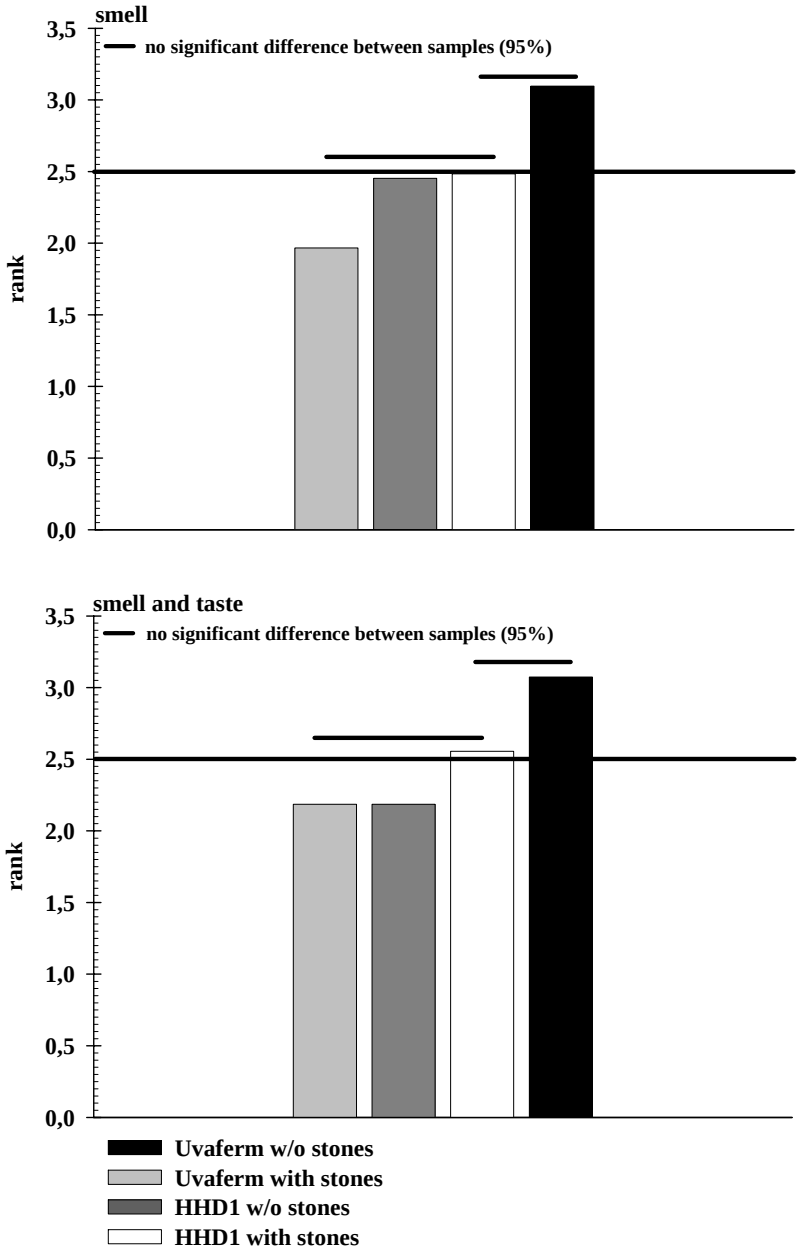
Tab. 4: Sensory analyses ("triangle test") of spirits produced from mashes with and without stones by different yeasts.

Fruit	Comparison	Number of test persons	Differences detected				Preference		
			Recognized	$\chi^2_{theoret}$	χ^2_{calc}	Significance [a] (α = 5%)	$\chi^2_{theoret}$	χ^2_{calc}	Preferred spirit [b]
Cherries	Uvaferm with vs. w/o stones	64	37	3.84	16.17	**yes**	3.84	0.02	**none**
	HHD1 with vs. w/o stones	44	24	3.84	7.23	**yes**	3.84	0.46	**none**
	Uvaferm vs. HHD1 w/o stones	70	26	3.84	0.30	**no**	3.84	0.35	**none**
Plums	Uvaferm with vs. w/o stones	64	31	3.84	5.91	**yes**	3.84	1.16	**none**
	HHD1 with vs. w/o stones	44	22	3.84	4.78	**yes**	3.84	0.05	**none**
	Uvaferm vs. HHD1 w/o stones	70	35	3.84	8.02	**yes**	3.84	0.03	**none**

[a] The number of test persons detecting a difference were subjected to statistical analysis and differences are given (yes = significantly different; no = not significantly different).
[b] If differences were detected the test persons were asked to judge their preference.
w/o = without; vs. = versus

Secondly, we made “order-of-precedence tests" in different combinations. At least 25 trained test persons were asked to place the spirits in an order of decreasing quality. As shown in Fig. 2a no significant difference for Uvaferm or HHD1 in cherry spirits produced without stones could be shown (also not with other commercial strains, data not shown). In the same test, spirits produced with HHD1 from mashes with stones were given a significantly worse ranking than those produced with the Uvaferm strain. In case of the plums, for the spirits from mashes with stones, no differences between Uvaferm and HHD1 were found in the overall quality, i.e. combining the ranking of smell and taste (Fig. 2b). Where stones had been removed, the laboratory strain performed better than the Uvaferm strain.

a)



b)

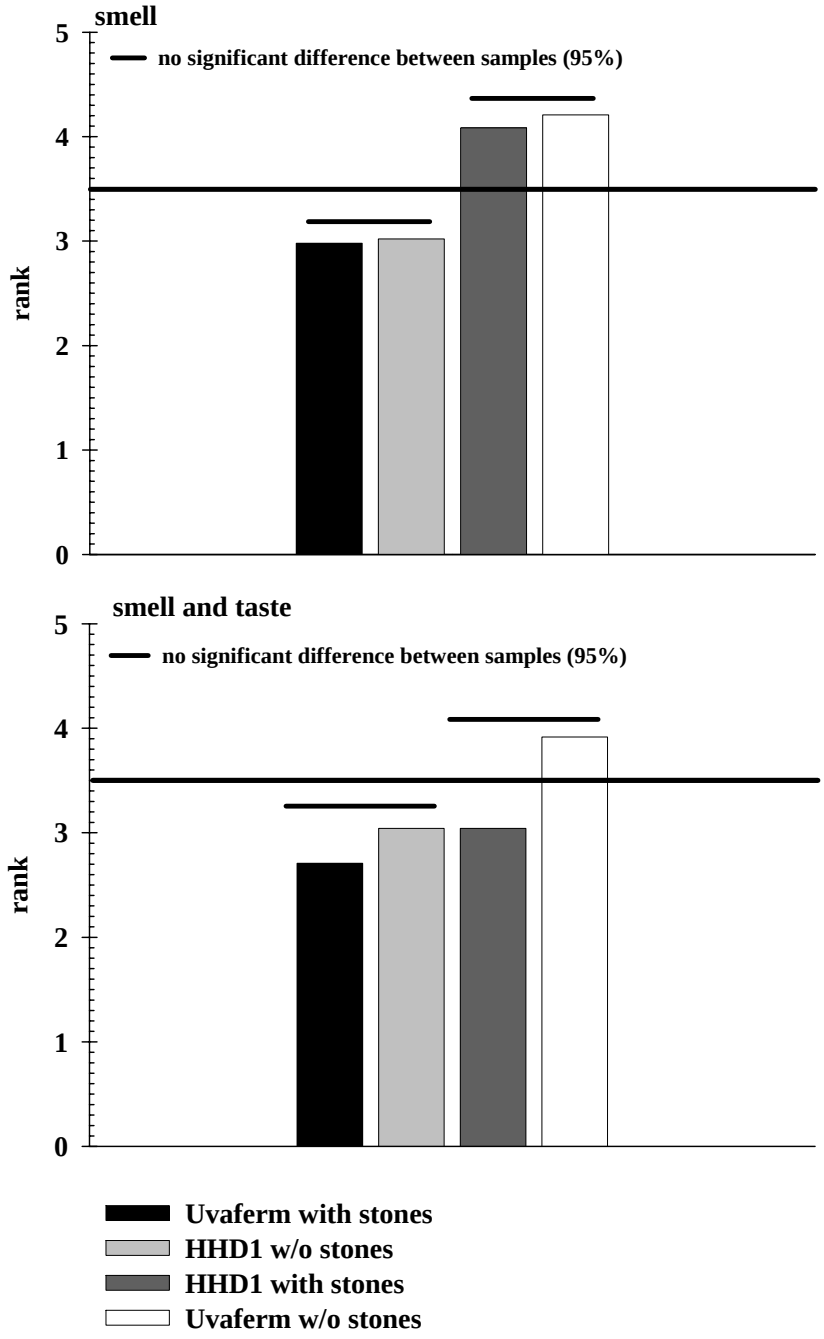


Fig. 2: Order of precedence tests (a = cherries; b = plums)

Spirits produced from the mashes as indicated (a = cherry spirits; b = plum spirits) were judged by a panel of 25 trained test persons. They were asked to give rankings from 1 to 5 to each spirit judging either smell alone, or smell and taste in conjunction. The data obtained were statistically analysed as described in materials and methods. In general, the lower the final ranking number, the better the quality of the spirit. The average rank is indicated by a continuous line. Short bars above the columns indicate those spirits, that did not show a statistically significant difference.

Finally, in order to evaluate the effect of stone contents in an independent experiment, we also produced spirits from the stone fractions themselves. For plums, such pure stone distillates earned the worst rank sums in the order of precedence test, as might have been expected (with an average rank of 4.1). Surprisingly, mixing cherry spirits from mashes without stones (obtained with the Uvaferm strain) gradually with up to 40% of the respective pure stone distillate did not alter preferences consistently (data not shown).

DISCUSSION

This work was aimed to determine the influence of stone content on the fruit spirits produced from cherries and plums as raw materials. In addition, the performance of a genetically defined, diploid laboratory yeast strain (HHD1) as a fermentation agent opposed to commercially available yeasts was further characterized.

As observed previously, neither the speed of fermentation nor the general quality of spirits produced from mashes without stones was significantly different when we used the laboratory strain in comparison to a set of different commercially available yeast strains (with special attention paid to the Uvaferm strain). However, a distinct lag-phase in the onset of fermentation in cherry mashes can be reproducibly observed for the laboratory strain, which is even more pronounced in mashes where stones have not been removed. Yet, this difference to the use of commercial yeasts was not found in fermentations of plum or pear mashes (this work; *4*). It can be concluded that cherry mashes contain some growth-inhibitory compound(s) like sulphur compounds (forming because of acidifying with sulphuric acid) which show antifungal activity to which the laboratory strains is more sensitive (Kyung and Fleming, 1997, Egilson et al., 1986). In this context an inadequate supply of nitrogen can crucial influence the growth of the yeast and initiate malolactic fermentation (Rauhut, 2004). It should be noted that despite this initial disadvantage, the laboratory strain adapts within the first 5 days of fermentation and then rapidly reaches the performance of the commercial yeasts. Since mash fermentations are usually carried out for a period of 50-60 days, in terms of sugar consumption and alcohol production, the initial lag phase thus has no practical consequences (Schehl et al., 2004).

Regarding the microbial environment, we found that mashes without stones were largely devoid of wild yeasts growth on the surface, in contrast to the mashes retaining the stones. This could possibly be a result of the treatment of the mashes: The removal of stones produces a kind of mechanical sieve composed of the stones themselves and residual fruit material such as skin fragments. Since wild yeasts found on the fruit surface frequently form hyphae, they may be retained more readily than single-cell yeast species that stay in suspension during the fermentation process.

Vice versa, we found that lactic acid concentrations, which indicate a higher load of bacterial contamination, were generally increased in the mashes fermented without stones compared to those retaining the stones. In fruit fermentations, lactic acid bacteria constitute the prevalent prokaryotic genera (Carr et al., 2002, van Beek, 2002). A special feature of these bacteria is their ample need for amino acid and vitamin supplies. One can assume that such compounds are usually scarce in mashes due to the rapid depletion by the yeasts added for fermentation. A further shortage produced from the wild yeasts growing at the surface of the mashes retaining the stones may therefore explain a certain level of protection against such bacteria.

Corresponding to the longer lag-phase of the laboratory strain in cherry mashes, which is enhanced by the presence of stones, the overall alcohol yield using this yeast strain was higher in the stoneless cherry mashes. For the plum mashes, where the lag-phase was absent, the opposite behaviour was found. Since no significant differences were found for the Uvaferm strain with regard to alcohol yields from all mashes, one can conclude that the stone content does not affect sugar degradation or final alcohol yields. Interestingly, no significant differences in the concentrations of organic acids and glycerol were found between the different mashes fermented with different yeasts (with the exception of lactic acid, which is due to a higher bacterial load as discussed above). This indicates that i) yeast carbohydrate metabolism (of which acetic acid and glycerol may form as by-products) is not influenced dramatically by the stone-content of the mashes, and ii) that both yeasts perform equally well in this respect. The same holds true for secondary fermentation products such as esters, aldehydes and higher alcohols. The concentration of these compounds was generally within the normal limits, although the amount of acetaldehyde was higher in stoneless mashes of both fruits tested. Although not detectable in the long-run fermentation kinetics, this may reflect a certain inhibition in yeast stationary phase metabolism, which contributes to the degradation of acetaldehyde, e.g. in beer production (Nevoigt et al., 2002).

On the other hand, methanol is produced from fruit-specific enzymes and not from yeast metabolism (Kolb, 2002, Bindler et al., 1988). Accordingly, a fruit-dependence is prevalent in that more methanol is produced from cherry mashes than from plum mashes, regardless of the stone content and yeast strain employed. The observation that fermentation by the Uvaferm strain produced slightly more methanol from plum mashes than those fermented with the laboratory strain may thus be due to minor variabilities in the fermentation conditions rather than the yeasts themselves. It should be noted that the experimental setup with "real" fermentations does not allow a large sample number and a judgement on statistical variations in this respect. Nevertheless, methanol contents remained within acceptable limits in all experiments performed in this work.

The minor differences in measurable quality-determining compounds within the mashes as discussed above were further diminished during the distillation process, as expected. We would like to emphasize that no catalyst (such as a copper surface) was included in the distillation to allow for the detection of even minor differences within the spirits produced. These can only be judged by sensory evaluations as the final and most important test. Despite personal preferences, the experimental setup employed in this work and the number of probationers involved, allows for a statistically significant assessment of spirit quality. Some general conclusions can be drawn from these data: i) Invariably, spirits produced from stoneless mashes could always be distinguished from those produced from complete mashes, regardless of the yeast strain used for fermentation. ii) For the plum mashes without stones, spirits produced from fermentations with the Uvaferm strain were recognized as different from those of the laboratory strain. iii) Even if differences as discussed in i) and ii) were detected, no preference could be assigned to either spirit. This indicates that the quality of the spirits is similar in all cases and preferences for either are a matter of personal taste. iv) As expected, spirits distilled from pure stone fermentations were always judged to be worse in the order of precedence test. Surprisingly, however, mixing up to 40% of these spirits with those from stoneless cherry mashes did not result in a change of preferences. Thus, whatever ingredients render the pure stone spirits less acceptable in taste and/or smell are near the sensory threshold so that the components of the traditionally produced spirit prevail in the mixture. v) For the cherry spirits produced from complete mashes, the laboratory strain performs worse than the Uvaferm strain. This may again be attributed to the enhanced lag-phase in the onset of fermentation, allowing other microbial contaminants to produce a certain amount of deleterious compounds, before being inhibited by yeast growth and metabolism. This lack of performance of the laboratory strain is not observed in fermentations of stoneless cherry mashes, presumably due to the less pronounced lag-

phase in the onset of fermentation. Supporting this view is the absence of strain-dependent quality differences in plum spirits, were fermentation kinetics in the mashes are similar between the two yeasts employed.

CONCLUSION AND OUTLOOK

In summary, this is the first experimental work comparing simultaneously the influence of stone content in fruit mashes and the employment of different yeast strains on the quality of spirits that can be produced from such mashes. We find that in contrast to the general believe, the presence or absence of stones in the mashes cannot be used as a general quality criterion. Rather, our data provide strong evidence that the preference for one or the other spirit will remain a matter of personal taste. Nevertheless, although the differences cannot be assigned to a specific organic acid or volatile compound, sensory analyses can clearly distinguish between these two kinds of spirits. Moreover, with little differences in fermentation performance our results offer the possibility to apply metabolic design techniques to a genetically defined yeast to be employed in large-scale fermentations. One may for instance reduce the health risk of spirit consumption implemented by substances such as the cancerogen ethyl carbamate. We are in the process of testing this hypothesis for fruit mash fermentations.

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CHAPTER V

REDUCTION OF ETHYL CARBAMATE IN STONE FRUIT SPIRITS BY MANIPULATION OF THE FERMENTING YEAST STRAIN

Abstract

Fermented fruit and beverages frequently contain ethyl carbamate (EC), a carcinogenic compound which can be formed by the reaction of urea with ethanol. Urea is produced as a by-product in arginine metabolism by the yeast *Saccharomyces cerevisiae*. EC can also derive in spirit production from cyanide introduced by stone fruit. In order to determine the relative contribution of yeast metabolism to EC production, we genetically engineered a diploid laboratory strain to reduce the arginase activity leading to urea production. For this purpose, strains with either a heterozygous *CAR1/car1* deletion or a homozygous defect (*car1/car1*) were constructed. The heterozygous strain was compared in mash fermentations and spirit production to the wild-type parental and to an industrial yeast strain. Whereas the EC content in the fermented mashes of cherries and plums generally was below detectable levels, the engineered strains showed a significant reduction of this compound in the final spirits from cherry mashes as compared to the non-engineered controls.

Schehl, B., D. W. Lachenmeier, T. Senn and J. J. Heinisch. Reduction of ethyl carbamate in stone fruit spirits by manipulation of the fermenting yeast strain. *Appl. Environ. Microbiol.,* submitted.

Saccharomyces cerevisiae is the microorganism responsible for most of the world's ethanol production (either as bioethanol for industrial purposes or as drink alcohol in beer, wine and spirits). The past decades have seen a tremendous increase in knowledge on the physiology and genetics of this yeast, comparatively little of which has made its way into industrial applications (see Pretorius 2000, and references therein). This is in part explained by the notion, that laboratory yeast strains would not be suitable for industrial production purposes, either because of a worse performance in ethanol production (for bioethanol) or the generation of unwanted metabolic by-products (for drink alcohol). On the other hand, the yeast strains currently employed by the fermentation and baking industries are genetically largely undefined, limiting the application of modern genetic engineering techniques in strain improvement (Tuite, 1992, Benitez *et al.*, 1996, Bothast *et al.*, 1999, Pretorius, 2000).

We have recently shown that a diploid laboratory yeast strain (HHD1) can be used in fruit mash fermentations for spirit production without any apparent loss of fermentation time or product quality (Schehl et al., 2004). Since the parental haploid strains are basically isogenic, but carry different auxotrophic markers (*ura3-52* and *leu2-3,112*, respectively), this offers the opportunity of metabolic design, e.g. by blocking certain enzymatic reactions. Gene deletions can be introduced in the heterozygous state into the diploid laboratory strain. If necessary, the strain can be sporulated, subjected to tetrad analysis and the segregants may be crossed back to generate a diploid strain homozygous for the respective deletion.

Here we used this approach to study the contribution of the fermenting yeast strain to the generation of ethyl carbamate (EC), also known as urethane. Regarding stone fruit as raw materials, consumers often desire the typical "bitter-almond" character in the final spirits. However, such positive flavour compounds introduced from the stones may be accompanied by detrimental influences and even health risks. Thus, fermentation of stone fruit and subsequent spirit production has been claimed to frequently result in the formation of EC as a carcinogen (Ough, 1976, Melzoch et al., 1996, Pretorius, 2000). It was proposed that this compound can form when amygdalin from the stones is degraded to cyanide and exposed to light (Baumann and Zimmerli, 1988; Arresta et al., 2001; Mildau et al., 1987). Another established source of EC is urea formed during the degradation of arginine by yeast. Arginase, encoded by the *CAR1* gene, catalyzes the hydrolysis of L-arginine to L-ornithine and urea (Whitney and Magasanik, 1973). Urea is then secreted into the medium, where it reacts with ethanol to form EC (Monteiro et al., 1988; Kitamoto et al., 1991; An and Ough, 1993). *CAR1* gene expression is regulated in response to a variety of environmental signals (Smart et al., 1996). Deletions of the gene resulted in yeast strains which did not produce

urea, anymore (Kitamoto et al., 1991). Moreover, it has been shown that a homozygous *car1*/*car1* deletion in a commercial Baker's yeast enhanced its freeze-tolerance, presumably due to the intracellular accumulation of arginine or glutamate (Shima et al., 2003).

Deletion of the *CAR1* gene in a diploid laboratory strain suitable for spirit production

The observations described above and the success in eliminating EC production by genetic engineering of a Sake yeast strain (Kitamoto et al., 1991) prompted us to investigate the effects of a yeast *car1* deletion on the production of EC in stone fruit spirits. For this purpose, the diploid strain HHD1 (*MAT**a**/MAT*α *URA3/ura3-53 LEU2/leu2-3,112 MAL2-8^c/MAL2-8^c SUC2/SUC2*) was transformed with a fragment containing the *kanMX* expression cassette from pUG6 (Güldener *et al*., 1996), which was amplified by PCR using an oligonucleotide pair with flanking sequences of the *CAR1* gene (delCAR1-5': 5'-ATGGAAACAGGACCTCAT TACAACTACTACAAAAATCGCGAATTGTCCCTTCGTACGCTGCAGGTCGAC-3', and delCAR1-3': 5'-CTACAATAAGGTTTCACCCAATGCACACCTTGCAATGGCGCAACCTGGC ATAGGCCACTAGTGGATCTG-3'; sequences underlined designate the complementary strands to pUG6, non-underlined bases correspond to the 5'- and 3'-end of the *CAR1* coding sequence). After homologous recombination and selection for G418 (Sigma-Aldrich, Germany) resistance, the resulting strain (HHD1/*car1hez*) contained a heterozygous *car1* deletion (Fig. 1a). In order to generate a homozygous deletion strain the diploid was sporulated, subjected to tetrad analysis and two segregants with complementary *ura3* and *leu2* markers, but being G418 resistant, were crossed to yield a prototrophic diploid strain (HHD1/*car1hoz*). Correct deletions were confirmed by PCR with flanking oligonucleotides (CAR1-5': 5'-GAGGATTCAGTATGCGACTCG-3' and CAR1-3': 5'-GTGTCCACTCGTGTTAT AGG-3') for both strains (Fig. 1b).

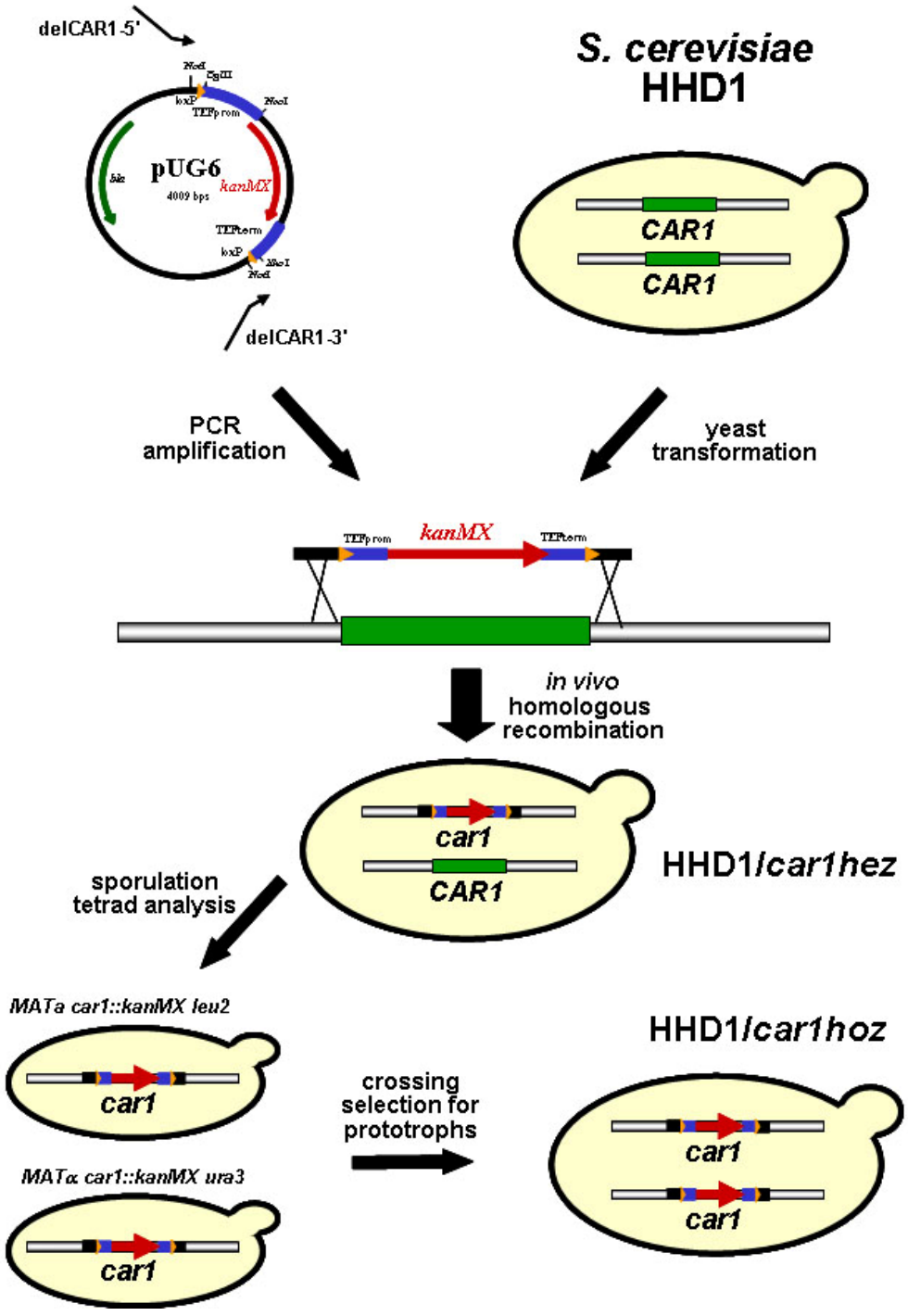

Fig. 1a: Deletion of the Arginase-encoding *CAR1* gene in HHD1

The diploid strain HHD1 was transformed with a fragment containing the *kanMX* expression cassette from pUG6 (amplified by Herculase™ enhanced Polyymerase, Stratagene, USA). After homologous recombination and selection for G418 resistance, the resulting strain (HHD1/*car1hez*) contained a heterozygous *car1* deletion

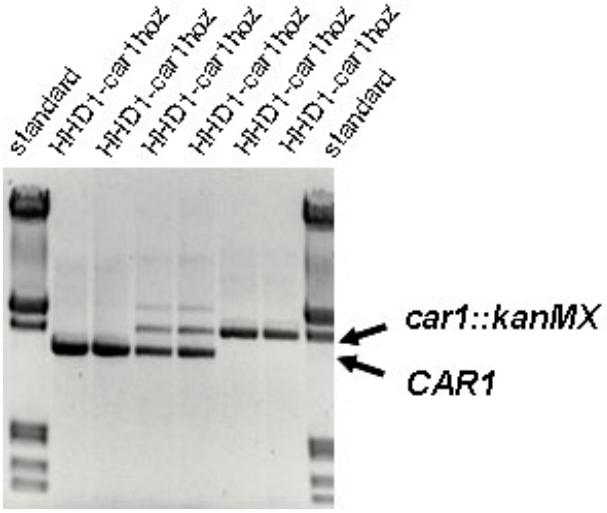

Fig. 1b: PCR of the *car1* deletion mutant

HHD1/*car1hez* was sporulated and subjected to tetrad analysis. The two segregants with complementary *ura3* and *leu2* markers, but being G418 resistant, were crossed to yield a prototrophic diploid strain (HHD1/*car1hoz*). Deletions were confirmed by PCR with flanking oligonucleotides (see text for details)

Effect of a heterozygous *car1* deletion on the aromatic composition of cherry spirits

It is to be expected that the heterozygous *car1* deletion would generate a yeast strain with half the specific arginase activity than the corresponding wild-type diploid. We first aimed to confirm that this reduction in enzymatic activity does not have a negative effect on the quality of the spirits produced. The heterozygous *car1* deletion strain (HHD1/*car1hez*) and the wild-type parental strain HHD1 were therefore used in cherry mash fermentations, either with complete cherry mashes retaining the stones, or with cherry mashes were the stones had been removed prior to fermentation (stoneless mashes). After standard distillation and storage, spirits were produced and tested for some key aromatic compounds. Growth of the yeast strains, fermentation and distillation conditions were employed as described in Chapter III and IV. As shown in Table 1, the concentrations of key aromatic compounds did not vary significantly between the different spirits produced with either strain. As expected, the concentrations of benzaldehyde are about five-fold increased in the spirits produced from the complete cherry mashes as compared to those from the stoneless mashes. Nevertheless, the *car1* deletion did not have an influence on the benzaldehyde concentrations. The only exception is provided by the acetaldehyde concentrations in the spirits produced from complete cherry mashes. There, the heterozygous deletion strain displayed a much higher

value. Since this is not observed in spirits from the stoneless mashes, it may not be a characteristic feature of the yeast strain. Rather, it could be attributed to a poor separation of the fractions during distillation. Further experiments would have to be performed to confirm a statistical significance.

Tab. 1: Concentrations of the key compounds found in the cherry spirits produced with the laboratory yeast strain HHD1 and heterozygous *car1* deletion strain (HHD1/*car1hez*)

Compound (mg/100 ml alcohol)	Complete cherries		Stoneless cherries	
	Employed yeast strain		Employed yeast strain	
	HHD1	HHD1/*car1hez*	HHD1	HHD1/*car1hez*
methanol	175.2	264.7	153.3	159.7
acetaldehyde	0.9	16.8	5.4	6.6
1-propanol	160.2	135.7	109.3	114.9
1-butanol	1	1.1	0.8	0.8
iso-butanol	26.2	21	21.5	23
amylalcohol	197.7	144.9	173.1	165.1
1-hexanol	0.5	< 0.01	0.5	< 0.01
methylacetate	< 0.01	< 0.01	< 0.01	< 0.01
ethylacetate	5.6	7.8	4.3	5.5
ethyllactate	< 0.01	0.6	0.3	0.4
benzaldehyde	7.7	10.1	1.5	2.2

Compounds are calculated in mg per 100 ml alcohol

Effect of a heterozygous *car1* deletion on the production of ethyl carbamate

The strains described above and a commercial Uvaferm yeast strain were further employed for the fermentation of cherry and plum mashes, with and without stones, and in one case the fermentation of the isolated stone fraction from cherry mashes. Interestingly, after completion of fermentation but prior to distillation, EC contents in all but one of the mashes ranged below detectable levels (Table 2). The higher EC values in the fermented cherry mashes with stones of the vintage 2003 were not reproduced in the vintage of 2004, indicating that small variations in fermentation conditions may be responsible, rather than being a contribution of the yeast strain employed. As expected, cyanide concentrations were generally much higher in mashes containing stones than in those were the stones had been removed (Table 2).

Tab. 2: *Ethyl carbamate* (EC) and *hydrocyanic acid* (HCN) concentrations of standardised produced stone fruit mashes

Fruit	Mash Treatment	Employed Yeast strain	EC OS [mg l^{-1}]	EC UV [mg l^{-1}]	HCN [mg $100ml^{-1}$]
Vintage 2003					
Cherries	complete	*Uva*	< 0.01	< 0.01	0.57
		HHD1	0.1	0.09	0.47
	w/o stones	*Uva*	< 0.01	< 0.01	0.14
		HHD1	< 0.01	< 0.01	< 0.01
Plums	complete	*Uva*	< 0.01	< 0.01	0.13
		HHD1	< 0.01	< 0.01	< 0.01
	w/o stones	*Uva*	< 0.01	< 0.01	< 0.01
		HHD1	< 0.01	< 0.01	< 0.01
Vintage 2004					
Cherries	complete	*Uva*	< 0.01	< 0.01	0.42
		HHD1	< 0.01	< 0.01	0.43
		HHD1/*car1hez*	< 0.01	< 0.01	0.36
	w/o stones	*Uva*	< 0.01	< 0.01	0.11
		HHD1	< 0.01	< 0.01	0.08
		HHD1/*car1hez*	< 0.01	< 0.01	0.08
	pure stones	*Uva*	< 0.01	< 0.01	1.87
		HHD1	< 0.01	< 0.01	1.87

The fruit derived always from the same cultivation and region. The mashes were completely fermented and all treated standardised (see Chapter IV for details). Mashes were fermented with and without stones, employing different yeast strains (see text for details); OS: original samples, UV: 4h irradiated samples, <0.01: not detected

More significantly, we also determined the EC contents in the final spirits after storage and additionally after UV irradiation to induce EC formation from cyanide (Table 3). Whereas a maximum of a two-fold variation in the final EC contents was observed between the commercial Uvaferm and the wild-type laboratory strain (which may well be attributed to statistical variations), the heterozygous *car1* deletion derivative produced drastically lower amounts of EC.

Tab. 3: Ethyl carbamate (EC) and hydrocyanic acid (HCN) concentrations of spirits produced by state-of-the-art technology with and without stones

Fruit	Mash Treatment	Employed Yeast strain	EC OS [mg l^{-1}]	EC UV [mg l^{-1}]	HCN [mg 100ml A^{-1}]
Vintage 2004					
Cherries	complete	*Uva*	0.29	1.2	< 0.01
		HHD1	0.14	0.78	< 0.01
		HHD1/*car1hez*	< 0.01	< 0.01	< 0.01
	w/o stones	*Uva*	0.06	0.34	0.04
		HHD1	0.12	0.62	0.03
		HHD1/*car1hez*	< 0.01	0.06	0.03

The fruit were collected during seasons over the years 2002-2004. The mashes were treated as described in materials and methods (Chapter IV). The spirits were produced under controlled and standardised conditions; OS: original samples, UV: 4h irradiated samples, <0.01: not detected

This observation stands in contrast to the data reported on an engineered Sake yeast (Kitamoto et al., 1991). In that work, the authors found, that the heterozygous *car1* deletion only led to slight reductions in the final EC contents, especially after long-term storage for 150 days. The decrease below detectable amounts of EC was only observed with strains carrying a homozygous *car1* deletion (*car1/car1*). Clearly, our data need further confirmation by additional fermentation experiments to provide statistical significance. Furthermore, we are in the process of also investigating the performance of the homozygous *car1* deletion derivative of the laboratory strain. Should these experiments substantiate the data provided in Table 3, one could draw some important conclusions:

i) A reduction in arginase activity is sufficient in spirit production to drastically reduce the risk of EC formation in the final product. This raises the question of the contribution of cyanide to EC formation. Our data indicate that this contribution may be comparatively low, since the genetic composition of the yeast strain used for fermentation should then have a minor influence.

ii) Provided that the fermentation performance of the homozygous *car1* deletion strain is not worse than for the strains tested here, one could further reduce the risk of EC formation by completely abolishing arginase activity and urea formation. This assumption is supported by the data on the Sake yeast cited above (Kitamoto et al., 1991), where neither a loss of fermentation capacity nor a sensory influence on the final product was observed by using the *car1* deletion strains.

iii) Given the fact, that a homozygous *car1* deletion leads to an increased freeze-tolerance in a Baker's yeast strain, one might expect that such strains would also be more viable after the drying process. The latter is a prerequisite for the industrial application of such genetically engineered yeast strains.

In summary, we here provided a first example of the application of genetic engineering techniques in a genetically defined, diploid laboratory yeast strain suitable for spirit production. This work aimed at the reduction of EC as a carcinogenic compound probably constituting a health risk to consumers. Clearly, similar approaches can now be established for other metabolic manipulations, e.g. the reduction of diacetyl in beer production.

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CHAPTER VI

RETROSPECTIVE TRENDS AND CURRENT STATUS OF ETHYL CAR-BAMATE IN GERMAN STONE-FRUIT SPIRITS

Abstract

Ethyl carbamate (EC) is a known genotoxic carcinogen of wide-spread occurrence in fermented food and beverages with highest concentrations found in stone-fruit sprits. Between 1986 and 2004, 631 cherry, plum or mirabelle (yellow plum) spirits were analysed for EC using gas chromatography in combination with mass spectrometry after extrelut extraction. The EC concentration of the samples ranged between 0.01 mg l^{-1} and 18 mg l^{-1} (mean 1.4 mg l^{-1}). After exposition of the samples to UV light, significantly higher concentrations between 0.01 mg l^{-1} and 26 mg l^{-1} (mean 2.3 mg l^{-1}) were determined. The EC concentration increased in average by 1.3 mg l^{-1}. If a linear correlation is done between year of sampling and EC concentration, a statistically significant but very slight decrease was found. However, if only officially complained samples are considered exceeding the upper limit of 0.4 mg l^{-1} more than twice, a significant reduction of the quota could be proven. This documents that measures to reduce EC were successively introduced in many distilleries. However, nearly 20 years after the first warnings about EC in spirit drinks, the problem persists especially in products derived from small distilleries. During experimental production of stone-fruit spirits using state-of-the-art technologies, it was proven that the occurrence of EC in stone fruit spirits can be prevented. Even for small distilleries, simple possibilities like destoning exist to minimize the EC content

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INTRODUCTION

Ethyl carbamate (EC) is a known genotoxic carcinogen of widespread occurrence in fermented food and beverages (Dennis *et al.,* 1989, Battaglia *et al.,* 1990, Schlatter and Lutz, 1990, Zimmerli and Schlatter, 1991, Sen *et al.,* 1992, Sen *et al.,* 1993, Benson and Beland, 1997, Kim *et al.,* 2000). Public health concern of EC in alcoholic beverages has begun in 1985 when relatively high levels were detected by Canadian authorities including spirit drinks imported from Germany (Conacher and Page, 1986). The highest EC concentrations were found in spirits derived from stone fruit of the species *Prunus* L. (Rosaceae; like cherries, plums, mirabelles (yellow plums), or apricots; Battaglia *et al.,* 1990, Zimmerli and Schlatter, 1991). Subsequently, Canada established an upper limit of 0.4 mg l^{-1} EC for fruit spirits (Conacher and Page, 1986), which was adopted by Germany and many other countries.

The disposal of cyanogenic glycosides such as amygdalin in stone fruit by enzymatic action (mainly β-glucosidase) leads to the formation of cyanide, which is the most important precursor of EC in spirits. Cyanide is oxidised to cyanate, which reacts with ethanol to form EC (Wucherpfennig *et al.,* 1987, Battaglia *et al.,* 1990, MacKenzie *et al.,* 1990, Taki *et al.,* 1992, Aresta *et al.,* 2001). The wide range of EC concentrations in stone-fruit spirits reflects its light-induced and time-dependent formation after distillation and storage (Andrey, 1987, Mildau *et al.,* 1987, Baumann and Zimmerli, 1988, Zimmerli and Schlatter, 1991, Suzuki *et al.,* 2001).

Many preventive actions to avoid EC formation in alcoholic beverages have been proposed. Besides, self-evident measures of good manufacturing practice like the use of high-quality, non spoiled raw material, and high standards of hygiene during fermentation and storage of the fruit mashes (Dürr, 1992, Lafuente and Fabre, 2000), the mashing and distillation conditions must be optimised. To avoid the release of cyanide, it is essential to avoid breaking the stones, to minimize light irradiation, and to shorten storage time (Christoph and Bauer-Christoph, 1998). Some authors proposed the addition of enzymes to decompose cyanide or a complete destoning of the fruit prior to mashing. The mashes have to be distilled slowly with an early switch at 65% (v/v) to the tailing-fraction (Dürr, 1992). Further preventive actions are the addition of patented copper salts to precipitate cyanide in the mash (Christoph and Bauer-Christoph, 1998, Christoph and Bauer-Christoph, 1999), the distillation using copper

catalysts (Pieper *et al.,* 1992a, Kaufmann *et al.,* 1993) or the application of steam washers (Nusser *et al.,* 2001). However, the use of copper can generate environmental problems due to hazardous waste.

MATERIALS AND METHODS

Sample collective

Between 1986 and 2004, 631 stone fruit spirits submitted to the CVUA Karlsruhe were analysed for EC. The institute covers as a part in official food control in Baden-Württemberg the district of Karlsruhe in North Baden (Germany), which has a population of approximately 2.7 million and includes the northern part of the Black Forest, a territory with around 14 000 approved distilleries (including South Baden) producing well-known specialties like Black Forest Kirsch (cherry spirit). The sampling was conducted by local authorities directly at the distilleries or from retail trade. Generally, spirits already diluted to drinking strength as offered to the end-consumer were taken. Since 2001, an interview protocol at sampling has been made including questions about preventive actions, age of the distillery, cleaning of the distillery, fermentation conditions, storage of the fruit mashes, and distillation conditions in general. To eliminate the possibility of EC formation during transport and sample storage, the bottles were wrapped in aluminium foil directly after sampling.

Experimental production of stone-fruit spirits

To show the state-of-the-art in the production of stone-fruit spirits in comparison to commercial samples, cherry and plum spirits of different vintages were produced under completely standardised conditions as described in Chapter III and IV at the Institute of Fermentation Technology Hohenheim. Thereby appropriate and commonly employed commercial available yeast strains were used. All strains were purchased from Begerow GmbH & Co. (Langenlonsheim, Germany). Media, culture conditions and incubation of the yeast strains were standardised and carried out according to Schehl *et al.* (2004).

Raw material and mashing process

The studies were performed with two different stone fruit mashes: cherries (cv. *Dollenseppler*) and plums (cv. *Ersinger Frühzwetschge*). The cherries were in an excellent condition like fresh dessert fruit, no bruised or decayed fruit were present. The plums were in faultless but in a bit more critical condition, so that single foul fruit were sorted out.

Mashes were prepared according to standard procedures. Indeed the fruit (exempted from peduncles) were washed and chopped using a stirrer attached to a drill machine, so that the stones remained undamaged (see Hagmann, 2002) and then divided into equal lots. One fraction was not treated any further (further named as *complete mashes*), the other portion was passed through a pulping machine and destoner (filter-width 4 mm, capacity 50-250 kg h^{-1}; Bockmeyer, Nürtingen, Germany) for the total removal of the stones (further named as *stoneless mashes*). Immediately after comminution respectively pitting the fruit, the pH-value was adjusted to 3.0 with sulphuric acid (technical grade). The remaining stones were collected and fermented separately without addition of sulphuric acid.

Fermentation

The mash was divided in 90 kg-lots each and separated in 120 litre vessels. For fermentation, the vessels were sealed with a fermentation bung and inoculated with the selected yeast strains (all standardised to be in the same physiological state and cell density) and fermented to completion at 15-17°C. All experiments were performed in triplicate and the classical fermentation parameters were observed over the whole fermentation period (for details see Chapter III, Schehl *et al.,* 2004). The remaining stones were separately fermented and distilled.

Distillation

The distillation was accomplished under technical and standardised conditions using a 200 litre copper pot still (Jacob-Carl, Göppingen, Germany) fitted with an enrichment section consisting of three bubble plates, a dephlegmator and a copper catalyst (Holstein, Markdorf, Germany). The dephlegmator was run with a flow rate of 120 l h^{-1} and the copper catalyst was used. The fermented mashes were distilled with two plates in operation. The distillates were collected in fractions with a volume of 250-300 ml, each. In the vicinity of the switching points (heads to product fractions and product fractions to tailings) smaller volumes of 150 ml were collected. The heads were identified with

the detaching test determining acetaldehyde according to Pieper *et al.* (1987). The tailings were screened by detachment at 72% (v/v) and partly by organoleptic assessment. The stones were distilled on a 19-litre plant with three plates, a dephlegmator and without a catalyst. Fractions were collected and the heads and tailings discarded.

Spirit fractions

The product fractions were stored for at least one week at 17°C, then diluted with deionised water to an alcohol content of 40% (v/v), cold filtered at 4°C (Macherey Nagel, Düren, Germany) and stored in darkness for another four weeks at 17°C prior to further analysis.

Quantitative determination of ethyl carbamate and cyanide

The analysis of EC was done using previously published procedures combining the extrelut extraction procedure of Baumann and Zimmerli (1986) with gas chromatography and mass spectrometry (GC/MS) according to Mildau *et al.* (1987) (analyses 1986-2003) or tandem mass spectrometry (GC/MS/MS) according to Lachenmeier *et al.* (2004) (analyses in 2004). For sample preparation, 20 ml of stone-fruit spirit or 20 ml of filtrated mash were spiked with 50 µl of ethyl carbamate-d_5 (1 µg ml^{-1}), that was synthesised according to Funch and Lisbjerg (1988), and directly applied to the extraction column. The extrelut column was wrapped in aluminium foil to eliminate the possibility of EC formation during extraction. After 15 min of equilibration, the column was washed with 2 x 20 ml of n-pentane. Next, the analytes were extracted using 3 x 30 ml of dichloromethane. The eluates were combined in a brown flask and reduced to 2-3 ml in a rotary evaporator (30°C, 300 mbar). After that, the solution was adjusted to 10 ml with ethanol in a measuring flask and directly injected into the GC/MS or GC/MS/MS system. In addition, to evaluate the light-induced EC formation capability of the products, the samples were exposed to UV light for 4 hours using a 360 W high-pressure mercury lamp Psorilux 3060 (Heraeus, Hanau, Germany) and extracted as described above. The recovery of EC was 100.4±9.4%. The limit of detection was 0.01 mg l^{-1} of EC. The precision never exceeded 7.8% (intraday) and 10.1% (interday) as well as the trueness never exceeded 11.3% (intraday) and 12.2% (interday), indicating good assay accuracy (Lachenmeier *et al.* 2004).

The total hydrocyanic acid (HCN) in the stone fruit spirits was photometrically determined after hydrolysis with potassium hydroxide and reaction with chloramine-T

and pyridine/barbituric acid reagent using the method of Wurzinger and Bandion (1985). For the determination of mashes, hydrocyanic acid was separated from the matrix by distillation before the photometric analysis (Wurzinger and Bandion 1993). The limit of detection was 0.15 mg l^{-1} of hydrocyanic acid.

Statistics

All data were evaluated using standard statistical packages for Windows. Statistical significance was assumed at below the 0.05 probability level. Groups of two cases were compared using t-tests. One-way analysis of variance (ANOVA) was used to test whether three or more cases have the same mean including the Bonferroni post hoc means comparison. Pearson's test was used to evaluate the significance of linear relations.

RESULTS

The results of 631 analysed stone-fruit spirit samples from commercial trade are given in Table 1.

Tab. 1: EC concentrations of 631 stone-fruit spirits.

	All samples (Total amount)		**Cherries**		**Plums**		**Mirabelles**	
	OS	**UV**	**OS**	**UV**	**OS**	**UV**	**OS**	**UV**
n	631	538	312	256	212	187	107	95
positive	89%	88%	93%	93%	83%	81%	86%	87%
Mean ± SD [mg l^{-1}]	1.4±1.7	2.3±3.2	1.5±1.9	2.7±3.5	1.2±1.5	1.8±2.6	1.2±1.6	2.3±3.0
Range [mg l^{-1}]	0.01-18	0.01-26	0.01-18	0.06-26	0.01-8.8	0.01-16.5	0.06-9.2	0.07-11.8
Median [mg l^{-1}]	0.74	1.05	1.0	1.5	0.6	0.5	0.6	0.8

The samples were collected and measured over a period of 19 years (OS: original samples, UV: 4 h irradiated samples, SD: standard deviation).

The EC concentration of the samples ranged between 0.01 mg l^{-1} and 18 mg l^{-1} (mean 1.4 mg l^{-1}). After exposition of the samples to UV light, significantly (p=0.001) higher

concentrations between 0.01 mg l^{-1} and 26 mg l^{-1} (mean 2.3 mg l^{-1}) were determined. Using ANOVA, no significant difference between the three fruit groups in the EC content could be determined for the dark-stored samples (p=0.07). However, after irradiation with UV light, a significant difference of the mean could be proven between cherry and plum spirit, but not between the cherry and mirabelle or plum and mirabelle (ANOVA p=0.03). The EC concentration increased in average by 1.3 mg l^{-1} (Table 2), with the highest formation capability usually found in cherry spirits.

Tab. 2: Light-induced formation of EC after exposition to UV light (4 h).

	All samples (Total)	Cherries	Plums	Mirabelles
n	538	256	187	95
Samples with formation	69%	77%	55%	72%
Increase mean ± SD [mg l^{-1}]	1.3±2.4	1.5±2.7	1.0±1.7	1.4±2.2
Increase range [mg l^{-1}]	0.01-21	0.01-21	0.01-11	0.01-9
Increase median [mg l^{-1}]	0.4	0.5	0.3	0.4

However, on average the formation capability of all fruit groups is the same (ANOVA p=0.20). Figure 1 and Table 3 show the distribution of the EC concentrations between different concentration categories. More than 50% of the samples had EC concentrations above the Canadian upper limit.

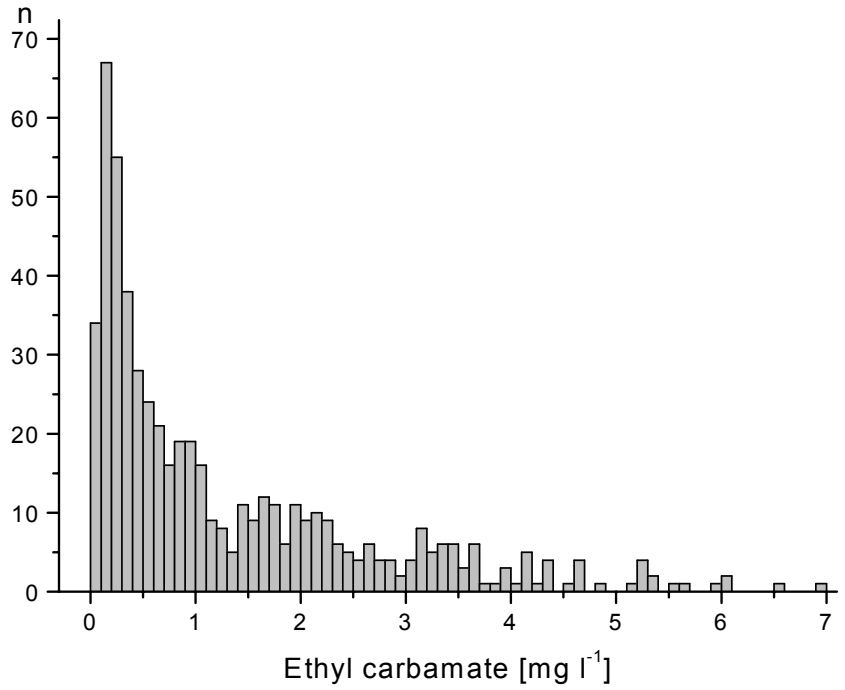

Figure 1: Statistical distribution of EC concentrations in 631 stone-fruit spirits analysed between 1986 and 2004

Tab. 3: Distribution of EC concentrations

	All samples		Cherries		Plums		Mirabelles	
	OS	UV	OS	UV	OS	UV	OS	UV
n	631	538	312	256	212	187	107	95
nd	11%	12%	7%	7%	17%	18%	14%	13%
<0.4 mg l^{-1}	31%	27%	29%	26%	32%	34%	32%	19%
0.4-0.8 mg l^{-1}	14%	13%	13%	11%	13%	9%	21%	24%
>0.8 mg l^{-1}	44%	48%	51%	56%	38%	39%	33%	44%

OS: original samples, UV: 4 h irradiated samples, nd: not detected

Figure 2 visualizes the retrospective trend of EC in German stone fruit spirits analysed since 1986. Using ANOVA, a significant difference between the means could be determined (p=0.002).

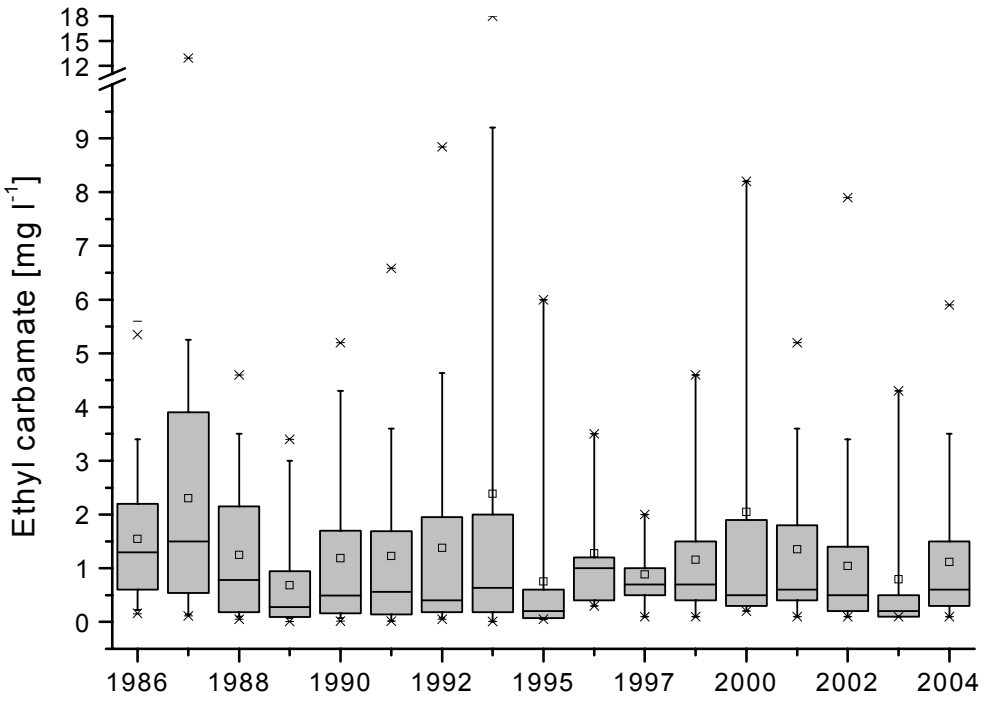

Fig. 2: Box-plots for the EC concentrations in 631 stone-fruit spirits analysed between 1986 and 2004 (for 1994 and 1998 no data was available)

Only a minor reduction (R=-0.096) could be proven over this period of time.

However in the post hoc means comparison, there were no significant differences between any of the sub groups. Therefore, no consistent trend could be seen. If a linear correlation is done between the year of sampling and the EC concentration, a statistically significant but only very slight decrease (R=-0.10) was found (Table 4). All in all, our data state that the average EC content of stone-fruit spirits remains nearly constant over the years. However, if only officially complained samples are considered exceeding the upper limit of 0.4 mg l^{-1} more than twice, a significant reduction of the quota could be proven (Figure 3). In 1986, more than 65% of the analysed samples had to be rejected. Nowadays, the rejection quota varies between 25% and 40%.

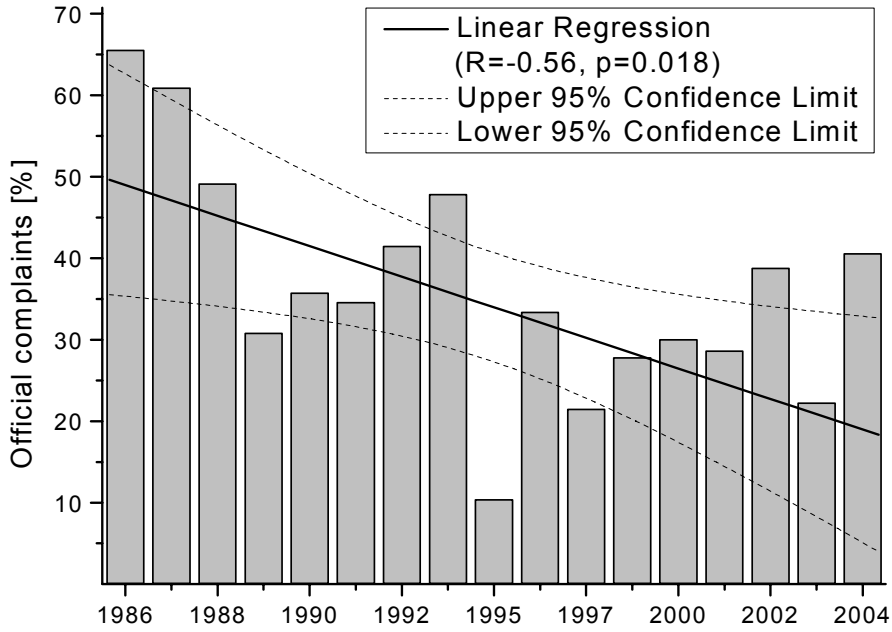

Fig. 3: Percentage of samples with EC concentrations higher than 0.8 mg l^{-1}, which led to official complaints

A significant reduction (R=-0.56) of the quota could be proven between 1986 and 2004.

The HCN concentration of the samples ranged between 0.15 and 22 mg l^{-1} (mean 1.96 ± 2.52 mg l^{-1}). No correlation could be found between EC and its main precursor cyanide, neither for the dark-stored samples nor for the UV-irradiated samples (Table 4).

Tab. 4. Results of linear correlation between EC concentrations of original or UV irradiated samples and year of sampling (1986-2004), concentration of total hydrocyanic acid (HCN) as well as the age of the used distillery

Correlation of EC with		Original sample		UV irradiated sample	
	n	R	p	R	p
Year of sampling	559	-0.096	0.024	-0.146	0.001
HCN	132	0.118	0.180	0.141	0.107
Age of distillery	39	-0.259	0.116	-0.418	0.008

There was also no correlation between hydrocyanic acid and the light induced increase of EC (R=-0.06, p=0.51). However if the EC concentrations of HCN-negative and HCN-positive samples are compared, the positive ones showed a significantly higher EC concentration and, of course, a higher formation capability (Table 5).

Tab. 5: EC concentrations of hydrocyanic acid (HCN) negative and positive cases

		EC [mg l^{-1}]	
	n	**Original sample**	**UV irradiated sample**
HCN negative	142	0.42 ± 0.75 (0.01-4.64)	0.48 ± 0.97 (0.01-6.65)
HCN positive	138	1.92 ± 2.40 (0.06-18)	3.61 ± 4.23 (0.07-26)
p		<0.0001	<0.0001

If the interview protocols are considered, a significant negative correlation was provable between the age of distillery and the EC content after irradiation (Table 4), attributed to the fact that new distilleries are usually equipped with copper catalysts or other preventive measures. The comparison between EC concentrations of spirits produced using copper catalysts and spirits produced without preventive actions confirms this relation. The samples distilled over copper catalysts (apart from a single distillate with 1 mg l^{-1}) had a significantly lower EC concentration below the upper limit (Table 6). No correlation between the other information of the interview protocol like mash storage time or state of cleaning of the distillery and EC or hydrocyanic acid content could be made. The results of the experimental and standardised production of stone-fruit spirits are shown in Table 7 and 8.

Tab. 6: EC concentrations of cases with and without the use of preventive actions to avoid the contaminant.

		EC [mg l^{-1}]	
	n	**Original sample**	**UV irradiated sample**
Copper catalyst	12	0.28 ± 0.29 (0.08-1)	0.32 ± 0.35 (0.07-1.2)
No preventive actions	40	1.32 ± 1.44 (0.06-7.88)	1.86 ± 1.84 (0.09-8.7)
p		0.0079	0.0073

Tab. 7: EC and hydrocyanic acid (HCN) concentrations of standardised produced stone fruit mashes intended to produce spirit drinks

Fruit	Mash Treatment	Status	EC OS [mg l^{-1}]	EC UV [mg l^{-1}]	HCN [mg l^{-1}]
Vintage 2003					
Cherry	complete	unfermented	<0.01	<0.01	0.7
		fermented	0.1	0.1	4.7
	stoneless	unfermented	<0.01	<0.01	1.3
		fermented	<0.01	<0.01	1.4
Plum	complete	unfermented	<0.01	<0.01	<0.01
		fermented	<0.01	<0.01	1.3
	stoneless	unfermented	<0.01	<0.01	<0.01
		fermented	<0.01	<0.01	<0.01
Vintage 2004					
Cherry	complete	unfermented	<0.01	<0.01	<0.01
		fermented	<0.01	<0.01	4.0
	stoneless	unfermented	<0.01	<0.01	<0.01
		fermented	<0.01	<0.01	0.9
	Stones	unfermented	<0.01	<0.01	<0.01
		fermented	<0.01	<0.01	18.7

The fruit derived always from the same cultivation and region. The mashes were treated standardised but in different technological ways with and without stones; OS: original samples, UV: 4 h irradiated samples, <0.01: not detected.

Apart from one sample with a very low concentration, EC was not detected in any of the mashes. Hydrocyanic acid was found in concentrations between 0.7 and 4.7 mg l^{-1} with lower or not detectable contents in the stoneless mashes than in the complete mashes. In the spirits of the years 2002-2003 (distilled from the complete and stoneless mashes), no EC was detected. In contrast, the stones had a very high concentration of hydrocyanic acid after fermentation, and the EC concentration in the distillate exceeded the upper limit. Two cherry spirits from the year 2004 showed low values of EC (0.2 mg l^{-1} in the complete mash and 0.1 mg l^{-1} in the stoneless mash). In these positive samples, the EC concentrations were below the upper limit; only the 'complete mash' sample had the capacity for EC formation up to 1 mg l^{-1}. Therefore, the results from 2004 show that removing the stones reduced the hydrocyanic acid

concentration in the mash and hence the EC content in the distillate as well as the formation capability (based on good technological manufacturing).

Tab. 8: EC and hydrocyanic acid (HCN) concentrations of spirits produced by state-of-the-art technology with and without stones

Fruit	Mash Treatment	EC OS [mg l^{-1}]	EC UV [mg l^{-1}]	HCN [mg l^{-1}]
Vintage 2002				
Cherry	complete	<0.01	<0.01	<0.01
Plum	complete	<0.01	<0.01	<0.01
Vintage 2003				
Cherry	complete	<0.01	<0.01	<0.01
	stoneless	<0.01	<0.01	<0.01
Plum	complete	<0.01	<0.01	<0.01
	stoneless	<0.01	<0.01	<0.01
	stones	1.9	4.0	4.8
Vintage 2004				
Cherry	complete	0.2	1.0	<0.01
	stoneless	0.1	0.3	<0.01

The fruit were collected during seasons over the years 2002-2004. The mashes were treated as described in materials and methods. The spirits were produced under controlled and standardised conditions; OS: original samples, UV: 4 h irradiated samples, <0.01: not detected.

DISCUSSION

Food regulatory viewpoints

In our study, an enormously wide range of EC concentrations was found in stone-fruit spirits, varying in more than three orders of magnitude, which corresponds well to the results of previous studies (Zimmerli and Schlatter, 1991, Adam and Postel, 1992). The statistical distribution of our samples corresponds also to that of a study of Andrey (1987), who analysed 135 Swiss cherry spirits, resembling a normal distribution. However, in our sample collective more samples with a higher EC content were found. These samples were officially objected, because they were produced contrary to European law. According to Council Regulation (EEC) No 315/93 laying down

Community procedures for contaminants in food (Council of the European Communities, 1993), no food containing a contaminant in an amount unacceptable from the public health viewpoint and in particular at a toxicological level shall be placed on the market. Furthermore, contaminant levels shall be kept as low as reasonably can be achieved by following good practices. In our opinion, an offence against good practices can be assumed, if the upper limit is exceeded more than twice. In consideration of lot-to-lot differences and inhomogeneities, the manufacturers were advised of their duty to exercise diligence and to use the state-of-the-art measures needed to reduce the content of EC. In 1999, the German health authorities stated that measures taken so far by manufacturers to reduce EC levels have led to a drop in contamination particularly in products from large distilleries (BgVV, 1999). In principle, this statement is in full accordance to our results. The decrease in the rejection quota since 1986 impressively documents that the measures were successively introduced in the distilleries.

However, as the relatively stable mean EC concentrations document, this process is very slow. And from our experience, the problem encompasses particularly small distilleries, which have not introduced improved technologies. In this context it must be stated, that our sampling was biased towards those small distilleries, which are often one-man businesses. In the context of a risk assessment, the authorities included more of those types of distilleries and products for sampling that were likely of posing a hazard to the consumer. The few large distilleries, producing for the mass market, have all introduced the described good manufacturing practices and produce stone-fruit distillates with only traces of EC.

Light-induced formation as risk for the consumer

In spite of the efforts of official food control to prevent EC formation after sampling, this concentration reflecting the status after bottling or in trade is not entirely of interest to the consumer. Only the EC concentration at consumption would be relevant. In many cases this is the maximum content because spirit drinks are usually not stored light protected either in trade or at the consumers'. Therefore, to achieve a better consumer protection, the EC formation capability of stone-fruit spirits should be evaluated in food control. As the results show, the EC concentration regularly increased over the upper limit after irradiation with UV light. Regrettably, the results published in 1987 (Mildau *et al.,* 1987), which showed significant delay of EC formation in brown glass bottles, did

not start a process of rethinking the use of the traditional white glass bottles. The use of UV filters in the white glass nowadays proposed by some breweries to prolong the shelf-life of beer could be a novel alternative to reduce the formation of EC.

Cyanide as precursor of ethyl carbamate

The findings of several authors that besides cyanide one or several further factors are additionally needed to form EC in stone-fruit distillates are verified by our results. Besides light, the factors influencing EC formation from cyanide are pH, ethanol content, temperature, vicinity of carbonyl groups in organic molecules and concentration of copper or iron-ions in the beverage (Baumann and Zimmerli, 1988, Battaglia *et al.,* 1990, Aresta *et al.,* 2001). But EC is also found in a variety of fermented beverages and foods (Ough 1976). It is proposed that EC derives from different yeast metabolites such as urea (Pretorius, 2000). Nevertheless, urea causes only negligible low values of EC in this context; the main influencing factor for the formation of EC is cyanide, deriving from the stones of the fruit.

In contrast to the study of Aresta et al. (2001), who found a relatively high correlation (R=0.597) between cyanide and EC in Brazilian sugar cane spirits, we only found a very low correlation between these parameters. However, as it is shown in Table 5, the determination of cyanide can be used as a simple screening for EC. If cyanide is negative, the EC concentration can be assumed to be below the upper limit. This is in accordance to previous research that no EC is formed in appreciable amount under light exposure when the distillates are free of cyanide (Baumann and Zimmerli, 1988). The advantage is that simple test-kits for cyanide are available, which can be used directly at the distilleries for product control, whereas EC analysis is only possible in specialised laboratories.

Reduction of ethyl carbamate

Because of its carcinogenic and mutagenic properties, no limit value below which health risks could be reliably excluded can be formulated for EC. Therefore, the goal must be to consistently reduce the contents by means of technological measures (BgVV, 1999). The first priority has to be the quality of the raw material and hygiene during fermentation, distillation and storage. The content of cyanide in the mash depends on the condition of the fruit. Damaged and microbiologically spoiled fruit contain more free cyanide (Hesford, 1998). This is confirmed by the observation that

samples with an EC content above the upper limit often also contain high levels of propanol-1 or butanol-2. These alcoholic congeners indicate an unwanted fermentation by spoilage microorganisms (Frank, 1983). Pieper *et al.* (1992b) stated that the formation of EC can be avoided by a defined and careful procedure in the production of stone-fruit spirits.

To reduce the EC levels as low as technologically possible, the use of further measures like copper catalysts is advisable, which cause a significant reduction during distillation. However, it should be noted that the catalysts have to be regularly cleaned and maintained (Hesford, 1998). Otherwise, EC concentrations above the upper limit are nevertheless possible.

Destoning to eliminate the precursor cyanide

Copper catalysts or other techniques to reduce EC were primarily established by large distilleries, whereas small distilleries could not afford the investment or had problems with correct maintenance in the daily routine. Therefore, simpler possibilities to avoid EC are required that must be both economical and adaptable by small distilleries. Since the discovery of cyanide as the main EC precursor, the simplest alternative would be to remove the stones prior to mashing, and therefore remove the precursor cyanide, which is bound as glucoside inside of the stones. Such destoned mashes do not have the potential to form EC during distillation, so that no further measures would be required. However, for a long time, this method was restricted because the possibility to distil high-quality spirits from destoned mashes was questioned (Pieper *et al.,* 1992b). The distillates were described as not typical of the fruit (Dürr, 1992) or the sensory quality as not satisfactory (Kaufmann *et al.,* 1993). Nowadays, a process of rethinking has begun. Of course, the destoned distillates do not have the typical and often appreciated ‘stone flavour’, which is induced by the bitter almond aroma of benzaldehyde. However, this has the advantage that the typical flavour of the fruit itself can now clearly emerge. In addition, the consumer can significantly better perceive the kind of fruit mashed, because the strong stone aroma does not cover the delicate, fruit typical components. Sieving and destoning machines are available allowing a simple removal of the stones (Jung, 2003). In this work, the use of the so-called ‘complete cherry mash’ was demonstrated towards the stoneless mashes. On a small scale this low cost machine allows to separate the fruit flesh from the stones and simultaneously makes a homogeneous mash. Dependent on the time of the separation, distillates with

a subtle bitter almond aroma but with distinct fruit flavour emerge (Hagmann, 2002). Worth mentioning is the fact, that the stones stay undamaged during the process (Senn and Jung, 1999). The results of our experimental production of stone-fruit spirits demonstrate in striking difference to the commercial samples, that the production of EC -free spirits is possible even for small distilleries.

CONCLUSION AND OUTLOOK

The results show that nearly 20 years after the first warnings about EC in spirit drinks, the problem especially persists in products from small distilleries. Even if the intake cannot be completely avoided because of its natural occurrence in all kinds of fermented foods and beverages, we showed that using state-of-the-art technologies, the occurrence of EC in stone fruit spirits can be prevented. Even for small distilleries, simple possibilities like destoning or process control using cyanide test-kits exist to minimize the EC content.

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Chapter VII

CONCLUDING REMARKS

Since Louis Pasteur revealed in 1863 the microbial activity of *Saccharomyces cerevisiae* during fermentations and proved that yeast is responsible for the biotransformation of sugars into alcohol and carbon dioxide, the process of spirit and wine production was continuously advanced. The aim of this work was to further contribute to an improvement of spirit quality by a better understanding of the underlying biology of fermentation and a directed manipulation of the major fermentation organism, i.e. the yeast *S. cerevisiae*.

Spirits have long been produced from a variety of fruits, especially in the Southern parts of Germany. During the past decade, consumer's demands have turned to higher spirit quality, prompting some research into how a reproducible high standard in spirit production can be achieved. In this context the quality of fruit spirits is determined by three major parameters: 1) Clearly, the most important factor is the quality of the fruit itself, which is used as raw material. 2) The biology of the fermentation process also contributes significantly, since the population of microorganisms (with yeast as the major determinant, but also including lactic or acetic acid bacteria), influences the product quality by the production of primary and secondary metabolites. 3) The application of optimized distillation conditions (i.e. "state-of-the-art-technology") ensures reproducible handling of the fermented mashes and avoids loss of important aromatic compounds. Before going further into a detailed discussion of the contributions provided by this work, these three parameters will be briefly discussed in the following:

1) Fruit quality is mostly dependent on seasonal and meteorological conditions, with a limited human influence (such as selection of only acceptable fruit by laborious manual screening and reducing the bacterial load by harvesting in time and a sorrow cleaning of the fruit prior to mashing).

2) Microorganisms, with mainly lactic acid and acetic acid bacteria from the prokaryotic side, also have a significant influence on fruit and spirit quality. Bacterial contaminations can hardly be avoided, since sterilization of the raw material would be cost-intensive and, depending on the methods employed, could lead to the loss of essential aromatic compounds. Therefore, one usually tries to diminish the bacterial load by taking advantage of

their biological properties. Thus, acetic acid bacteria are mainly controlled by the anaerobic conditions developing during the fermentation process, since they are strictly oxygen-dependent. However, especially when appearing in the early phases of fermentation, these bacteria often lead to a lower final alcohol yield and a negative aroma profile.

Lactic acid bacteria are partly controlled by their limited metabolic capacity. "Wild yeasts" present on the fruit and *S. cerevisiae* added for fermentation share the ability to drain amino acids from the medium and store them intracellular in their vacuoles (Boulton et al., 1996). Once this occurs, lactic acid bacteria will lack essential nutrients and will not pose a problem until the end of fermentation, when some yeast lysis occurs, again setting free some of the bacterial nutrients. Bacterial contaminations other then lactic and acetic acid bacteria are usually reduced by the adjustment of mashes to a low pH. Since most bacteria cannot grow in more acidic solutions, this provides a growth advantage for different yeast species.

In addition to the desired fermentation organism, *S. cerevisiae*, which is usually added in access from starter cultures, “wild yeasts” are also found on fruit as eukaryotic organisms. These belong predominantly to yeasts of the *Kloeckera* variety, but also *Brettanomyces, Torulaspora* etc. can be found on the fruit and in the resulting mashes. Even if left alone in the fermentation process (i.e. without the addition of starter cultures), the different “wild yeasts" and bacteria are usually outnumbered by *S. cerevisiae* at the end of fermentation (Fleet and Heard, 1993, Versavaud et al., 1995). Initially yeasts and bacteria grow rapidly until oxygen and nutrients other than carbohydrates are depleted. Yeast growth is accompanied by an increasing alcohol content, which rapidly becomes inhibitory for growth of bacteria and "wild yeasts".

The consequence of the above is a co-existence of bacteria and yeast on the fruit and in the mashes, whose interdependence in fruit fermentations is not understood at present (for further reading see Jakob et al., 1979, Trost, 1980, Dittrich, 1987, Boulton et al., 1996). Although the physiology of the "industrial" yeast strains of *S. cerevisiae* used as starter cultures has been well studied regarding suitability, their genetics largely remains a mystery (Benitez et al., 1996). How research in this direction can be applied for an improvement of spirit quality was the main subject of this work and is discussed in more detail, below. This is accompanied by the fact that changes in production technology to improve the reliability of fermentation, quality and economics of production have placed new demands on the performance of the yeast strains employed.

3) Distillation conditions and distillation apparatus have been optimized from the technical point of view over the past decades, with various contributions from the group in Hohenheim (Luz, 1990, Pieper et al., 1993, Guan, 1997, Glaub et al., 1998, Heil, 2001, Ande, 2004).

Although still a matter of investigation, further major breakthroughs by an optimization of the apparatus are not to be expected. On the other hand, thorough investigations of the distillation conditions may well provide further improvements. In Germany, technical advances are further limited by legislation. Thus, whereas a refinement of aromatic composition in the final spirits can be achieved by the use of various distillation schemes in other countries (e.g. the use of more than ten "bubble plates" in the distillation apparatus, whereas in Germany spirit production is limited to the use of no more than three plates; Brennereiordnung BGBI, 1998).

From the points made above, it seems logical that a further improvement in spirit production and spirit quality has to come from a sound knowledge of the biology of the fermentation process itself. This includes an application-driven design of the yeast strains employed. In the long range, a detailed understanding of the interdependences of the entire population of microorganisms present in the mashes will further aid to this purpose.

As a first measure of yeast improvement, two haploid laboratory strains with suitable auxotrophic markers were used in this work for the construction of a genetically well defined, prototrophic diploid production strain (Note that all data provided in this work has been extensively discussed in the respective chapter; **Chapter III**). Until now, it has been generally believed that laboratory yeast strains are not suitable for spirit production either because of a much lower rate of alcohol production and/or the production of negative aroma compounds in the fermentation process (Walker, 1998, Pretorius, 2000). In order to validate this assumption, the diploid laboratory strain was tested for its fermentative and sensory performances in comparison to commercially available yeasts, sold for the purpose of mash fermentations. Fermentation parameters assayed included growth, sugar utilization, ethanol production and generation of volatile compounds, higher alcohols and glycerol. The spirits were produced based on modern distillation conditions and finally tested for their sensory performances by a trained panel of judges. Overall, the laboratory strain did not show any disadvantage towards the commercial yeasts for the parameters tested. If anything, spirits produced with the laboratory strain were preferred in the sensory analyses towards those from the industrial yeast strains. As a slight disadvantage, the laboratory strain showed a delayed onset of fermentation, but only for cherry mashes, which was levelled out within the first few days of fermentation. Such a lag-phase was neither observed in pear nor in plum mashes. Overall, the data obtained confirmed the suitability of a laboratory yeast strain for spirit production. This offers the opportunity for further genetic improvements, since such a strain can be crossed and breeded as practised with plants and animals since centuries.

Although the data gathered so far seem to demonstrate that the laboratory strain tested here may be suitable for spirit production, there are several points to be answered before its industrial application: 1) A minor concern is the genetic stability of the strain. Diploid yeast strains go through meiosis when subjected to nitrogen limitation in a medium with a poor carbon source. This results in the production of haploid segregants, which theoretically can propagate in the haploid state. However, in mashes this is of minor importance. As demonstrated here (**Chapter III**), haploid yeast strains perform much worse in mash fermentations and are likely to be outgrown by their diploid counterparts. In addition, if left to germinate from the ascus, the haploid segregants will immediately mate and produce a diploid progeny. 2) To be of commercial value, the laboratory strain would have to be produced in large scales and prepared for shipment. This necessitates the preparation of dry yeast with reasonable viability. This question has not yet been addressed for our laboratory strain. However, if solved, it would not only pave the way for the application in fruit spirit production. Given the similar performance to commercially available yeasts in alcohol production rates, the strain could be used for other large scale fermentation processes such as bio-ethanol production.

Stone fruit spirits are characterized by a specific almond flavour, if stones are not removed from the mashes prior to fermentation. Despite determining the typical character, the benefits of the stone content for the quality of the resulting spirits remains controversial. To provide a statistical basis for what appears like personal preferences, a series of experiments was performed in this work a cherry and plum mashes with different stone contents (**Chapter IV**). In order to further substantiate the suitability of the laboratory yeast strain in spirit production, mashes were prepared and fermented both with the commercial Uvaferm strain and with the diploid laboratory yeast strain HHD1. The spirits produced were again tested by a panel of trained judges for their sensory qualities. Not surprisingly, the mashes retaining the stones could always clearly be distinguished from those where the stones had been removed. The yeast strain used for fermentation did not to have a significant influence on the spirit quality in these tests. It seems that the characteristic flavour introduced by the handling of the fruit has too strong an influence to allow the differentiation of minor metabolic contributions of the fermenting organism. As observed in the previous chapter, both yeast strains showed little variation regarding the measured fermentation parameters. The data obtained from these experiments indicate that indeed the personal taste is the decisive quality criterion, rather than the presence or absence of stones in the mashes (**Chapter IV**). Interestingly, neither yeast carbohydrate metabolism nor the production of secondary metabolites with influence on smell or taste (e.g. esters, aldehydes and higher alcohols), seemed to depend on the

stone-content of the mashes. Any disadvantages of the laboratory strain that may appear in the future as compared to commercial yeast strains may be outweigh by the application of metabolic design techniques, for instance to reduce health risks.

As stated above, using stone fruit as raw materials, consumers often desire the typical "bitter-almond" character in the final spirits. However, such positive flavour compounds introduced from the stones may be accompanied by detrimental influences and even health risks. Thus, fermentation of stone fruit and subsequent spirit production has been claimed to frequently result in the formation of ethyl carbamate (also referred to as urethane; Ough, 1976, Pretorius, 2000). It was proposed that this compound can form when amygdalin from the stones is degraded to cyanide and exposed to light (Mildau et al., 1987, Baumann and Zimmerli, 1988, Arresta et al., 2001). In summary, removal of stones remains an option for the production of spirits with different flavour and a "healthier" spirit.

Ethyl carbamate (EC) is a known genotoxic carcinogen of widespread occurrence in fermented food and beverages with highest concentrations found in stone-fruit spirits. Besides the "chemical" formation of EC from the stones, it has been proposed that some EC can also be formed as a consequence of yeast metabolism. There, potential precursors are N-carbamyl compounds such as urea, citrulline, allantoin and carbamyl phosphate (Ough et al., 1988a). It is believed that EC forms by the reaction of urea and ethanol (Ough et al., 1988b). It has been strongly suggested that urea contained in the spirits is produced exclusively by the arginase reaction of the yeast and that EC is formed during long term storage. To lower the urea content in mashes, addition of an acid urease has been employed (Ough et al., 1988b). Since this is not very cost-effective, a non-producing laboratory yeast strain has been constructed in this work that should be unable to produce urea, by deletion of the arginase encoding gene (*car1::kanMX/car1::kanMX*). This strain was again used for the fermentation of cherry and plum mashes and compared to its parental diploid laboratory yeast strain HHD1 (*CAR1/CAR1*) and a commercial yeast strain (**Chapter V**). In order to determine the contribution of the stones on the EC content, the mashes were produced with and without stones. To exclude a negative influence of the deletion on the overall performance of the newly constructed yeast strain, general fermentation parameters such as sugar content and ethanol production were followed. Importantly, the modified laboratory strain did not differ significantly from its parental strain HHD1 in glucose consumption and alcohol production. The concentrations of specific flavour compounds and methanol, determined by using Fourier Transform Infrared (FTIR) spectroscopy and GC/MS (**Chapter VI**), all were in a similar range, too. Likewise, the concentration of secondary fermentation

products such as esters, aldehydes and higher alcohols were generally within the normal limits. Regarding the EC content, the results suggest that the contribution of the yeast strain in these cases can be neglected. Rather, the main factor for the formation of EC seems to derive from the stones and seems to be cyanide. However, since the EC concentrations in these experiments ranged near the detection limits, a general conclusion cannot be drawn, yet. Unless fermentation conditions are developed where significant amounts of EC are produced from both urea and from the stones in the mashes, the contribution of yeast metabolism remains to be determined.

In order to further establish the relationship between distillation conditions and EC content, a few hundred fruit stone spirits produced in the years between 1986 and 2004 were analysed for EC in the final part of this work (**Chapter VI**). For this purpose, EC contents were analysed using gas chromatography in combination with mass spectrometry after extrelut extraction. As expected from the data available in the literature, exposure of the spirits to UV light generally led to higher EC concentrations. In the course of experimental production of stone-fruit spirits, it was shown that the EC content could be controlled using state-of-the-art distillation technologies. It has been previously observed, that damaged and microbiologically spoiled fruit contain more free cyanide (Hesford, 1998). This was also indicated in this work by the observation that samples with an EC content above the upper limit also contained high levels of propanol-1 or butanol-2 (i.e. compounds typically observed upon the occurrence of bacterial contaminations). As a rule derived from these observations, the determination of cyanide can be used as a simple prediction for the occurrence of EC. If no cyanide is present in the spirits, EC can be assumed to stay well below the upper limit. In summary, the quality of the raw material, a fast and complete fermentation, a modern distillation and a proper storage constitute the most important factors in avoiding EC formation in stone fruit spirits (**Chapter VI**).

The second point can be directly related to the yeast strain employed for fermentation. In this context, the diploid laboratory strain HHD1 established in this work, offers the potential to adjust yeast physiology to a variety of production schemes. With respect to spirit production, it could further be optimized for specific tasks. Thus, once negative aromatic compounds are identified, the vast knowledge on yeast physiology, the complete genome sequence, and the possibility of genetic engineering can be applied to prevent its formation. The application in urea metabolism discussed above just serves as one example. Moreover, classical genetic methods like crossing and breeding cannot be applied to the commercial yeast strains employed in breweries, wineries and bakeries, today. The HHD1 strain offers the opportunity

to use such techniques, avoiding the controversial use of modern genetic methods. Regarding commercial yeast strains, clone selection, mutagenesis, hybridization, rare-mating and spheroplast fusion have proved to be valuable tools in strain development programmes. However, these methods lack the specificity required to modify yeasts in a well-controlled manner (Pretorius, 2000). It may not be possible, for example, to precisely define the change required, and a new strain may bring an improvement in some aspects, while compromising other desired characteristics. Yeast geneticists must be able to alter the characteristics of yeasts in specific ways: an existing property must be modified, or a new one introduced without adversely affecting other desirable features.

Molecular-genetic techniques like gene cloning and recombinant DNA technology offer exciting prospects for improving yeasts. The strain HHD1 introduced in this work is also extremely useful in this respect. It can be subjected to tetrad analysis, yielding haploid progeny with *ura3* and *leu2* selectable markers. These allow the introduction both of extra-chromosomally replicating plasmids, as well the use of the wild-type alleles for genomic (and thus more stable) modifications. By using such procedures it is possible to construct new yeast strains differing from the original parental strain only in a single specific characteristic. Laborious protoplast fusions between different *S. cerevisiae* strains, as well as with other yeast species, have been employed to circumvent the problems caused by the non-sporulating phenotype of most commercial strains (Spencer and Spencer, 1996). However, since whole genomes are combined by these methods, genetic instability is an unavoidable by-product. On the other hand, transformation with plasmids or DNA carrying heterologous genes (e.g. for the production of enzymes, vaccines etc.) still relies on the introduction of dominant genetic markers and does not find public acceptance where food production processes are concerned (Danner, 1997, Drewnoski and Rock, 1995, Nishiura *et al.*, 2002). Homologous yeast markers, such as the *URA3* and *LEU2* genes applicable in our system, could avoid these problems.

Amongst the features that would be desirable if the laboratory strain was to be used for commercial purposes are the following:

The efficiency of fermentation could be markedly improved by a better sugar utilization (e.g. broadening the spectrum of different carbohydrates to be metabolised). Furthermore, an increased tolerance to ethanol, resistance to zymocins and heavy metals in some cases would also improve the fermentation capacity on special substrates. Moreover, a reduced formation of foam and an induction of flocculance at the end of fermentation could be useful in certain production schemes. And finally, the production of extracellular enzymes like

pectinases, glucanases, xylanases and proteases may aid in the degradation of certain biopolymers. For instance, secretion of glucanases and glucosidases may enhance the flavour by hydrolysis of flavour precursor glycosides (Canal-Llauberes, 1993, Walker, 1998, Pretorius, 2000). Over-expression of the yeast's own alcohol acetyltransferase has been shown to be the first step towards enhanced ester production, thereby adjusting the aroma profile considerably (Herraiz and Ough, 1993). Another possibility is the development of yeast strains with antimicrobial activity as a bio-inhibitor against bacteria. Especially in fruit fermentations bacterial contaminations could thus be effectively controlled, resulting in improved spirit qualities, as exemplified above. In addition, external preservatives could be significantly reduced if yeast strains secreted natural antimicrobial peptides (such as bacteriocins) during fermentation, thereby playing an auto-sterilizing role (Du Toit and Pretorius, 2000). Preliminary results indicate that it is indeed possible to develop bactericidal yeast strains that could be useful in fermentation processes with reduced levels of potentially harmful chemical preservatives or ingrediences. Since such peptides are natural bacterial products e.g. of lactic acid bacteria found in milk products, the appearance of clinical resistances would not pose a problem, in contrast to classical antibiotics.

Undoubtedly, future work will thus see the improvement of existing yeast strains through traditional strain adaptation and genetic engineering. In predicting the improvements in future strains, it is important to consider what parameters would be most advantageous for industrial fermentations. The critical traits in the context of this work are for instance high ethanol yield and productivity, genetic stability, inhibitor and ethanol tolerance. It should be noted that genetic stability is essential even for non-contiguous fermentations because of the large number of generations that pass between the original colony and the final seed culture for large-scale fermentations. Other desirable traits in the alcohol industries include the ability to simultaneously use multiple sugars, to grow at lower pHs and/or higher temperatures, and to produce e. g. oligosaccharide-hydrolyzing enzymes (such as α-glucosidases) for simultaneous saccharification and fermentation.

Especially in Europe, genetic engineering is controversially discussed in the general public. One thus has to take into account the fears of consumers when it comes to the presence of genetically engineered microorganims in food. In this context, it should be relatively easy to convey that genetically engineered yeasts in spirit production pose the least risk of all. One of the final steps in spirit production is the distillation process. Since yeasts survive a maximum of 60°C only for a few minutes, one can be sure that no living genetically engineered organism can appear in the final product.

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Chapter VIII

ZUSAMMENFASSUNG

Hefen sind einzellige Pilze und in der Natur mit vielen Arten weit verbreitet. Seit Urzeiten werden Hefen zur Herstellung von Brot, Bier, Essig, Wein und anderer Lebensmittel eingesetzt. Der Gärorganismus *Saccharomyces cerevisiae* spielt besonders bei der Herstellung alkoholischer Getränke, insbesondere der Obstbrennerei, eine entscheidende Rolle. Ohne geeignete Konservierungsmaßnahmen beginnen Fruchtmaischen auch ohne einen Zusatz von heute erhältlichen Reinzuchthefen zu gären. Dieser als Spontangärung bezeichnete Vorgang führt durch sogenannte „wilde Hefen“ zur Bildung erwünschter wie unerwünschter Gärungsnebenprodukte (wie Glycerin, organische Säuren und höhere Alkohole). Diese Nebenprodukte können in den Branntweinerzeugnissen Geruchs- und Geschmacksfehler erzeugen.

Im Laufe der Jahrzehnte ist durch gezielte Selektion und Kreuzung bestimmter Stämme eine Spezialisierung in Form der heute erhältlichen Hefe-Reinzuchtstämme erfolgt. Durch deren Einsatz sollen Nachteile wie Fehlgärungen und mangelnde Ausbeuten vermieden werden. Reinzuchthefen, die in der Brennerei zum Einsatz kommen, zeichnen sich in der Regel durch gute Alkoholverträglichkeit (bis 16%vol), hohe Belastbarkeit, gutes Durchgären und damit hohe Alkoholausbeuten aus. Geschmacksstoff- und Aromabildung werden zudem positiv beeinflusst.

Betrachtet man den heutigen nationalen wie internationalen Markt, so fällt es dennoch schwer, die erhältlichen Hefepräparate seinen Bedürfnissen entsprechend einzuordnen. Ein gezielter und optimaler Hefeeinsatz im Bereich der Obstbrennerei kann im Allgemeinen nur nach einem überlegten Zusammenspiel von anbaulichen, verarbeitungs- und gärungstechnischen Voraussetzungen im Betrieb erfolgen. Dennoch sind die auf dem Markt erhältlichen Reinzuchthefen auf Grund hoher Mutationsraten stark heterogen, so dass die „angezüchteten“ Eigenschaften weitestgehend verloren gehen können. Eine gezielte genetische Modifikation zur Verbesserung bestimmter Gäreigenschaften der Hefe wird dadurch unmöglich gemacht.

Seit der Sequenzierung des gesamten Hefegenoms 1996 werden weltweit Anstrengungen unternommen die Funktionen der über 6000 Gene zu verstehen, um gezielte genetische Modifikationen bzw. Verbesserungen in den verschiedensten Einsatzbereichen von Hefen zu ermöglichen. Die Anwendung genetisch definierter und modifizierter Laborstämme konnte sich bisher in der Praxis insbesondere bei der Spirituosenherstellung nicht durchsetzen. Laborstämmen wird im Allgemeinen die Unbeständigkeit sich gegenüber der Konkurenzflora (Bakterien, wilde Hefen) in Obstmaischen durchzusetzen und somit die gewünschte Aromenbildung und Alkohol-Ausbeute zu erbringen, nachgesagt.

Ziel dieser Arbeit war es, einen genetisch definierten Hefestamm zu konstruieren und diesen speziell im Bereich der Obstbrennerei auf seine Eignung im Vergleich zu handelsüblichen Präparaten zu etablieren.

Kapitel III: Die Eignung eines Laborstammes in der Obstbrand-Herstellung

Die Konstruktion eines diploiden Laborstammes und dessen Anwendung im halbtechnischen Maßstab bei der Obstbrand-Herstellung wird beschrieben. Der durch Kreuzung zweier haploider Elternstämme konstruierte diploide, prototrophe Stamm HHD1 wurde auf seine Gäreigenschaften, ebenso wie auf sensorische Eigenschaften getestet. Der Laborstamm HHD1 konnte sich in seiner Anwendung gegenüber den handelsüblichen Hefepräparaten behaupten und wies keinerlei Nachteile hinsichtlich der getesteten Parameter wie Zuckerverwertung, Alkoholausbildung und Bildung sekundärer Gärungsnebenprodukte auf. Des Weiteren wurden die mit HHD1 vergorenen Maischen und die daraus gewonnen Destillate bei sensorischen Tests nicht abgelehnt, im Falle von Kirschwassern sogar bevorzugt.

Kapitel IV: Einfluss des Steingehaltes fermentierter Maischen mit kommerziellen Hefepräparaten und dem Laborstamm HHD1 auf die Qualität von Pflaumen und Kirschdestillaten

Der Einfluss des Steingehaltes auf die sensorischen Eigenschaften von Steinobstdestillaten wurde untersucht. Ein Ansatz der Maischen wurde entsteint, ein anderer Teil herkömmlich eingemaischt und vergoren. Zur Fermentation der Pflaumen- und Kirschmaischen wurde das Handelspräparat Uvaferm und der konstruierte Laborstamm HHD1 eingesetzt. Zucker-verwertung, Alkoholausbeute und Gärungsnebenprodukte wurden analysiert und gegen-übergestellt. Es konnten keine Unterschiede im Bezug auf den eingesetzten Labor-Hefestamm gezeigt werden.

Kapitel V: Deletion des Arginase-kodierenden Gens *CAR1* zur Reduktion von Ethylcarbamat in Fermentationsprozessen

Das Arginase-kodierende Gen *CAR1* wurde mittels eines dominanten Markers, homologer Rekombination und Tetradenanalyse in dem Labortsamm HHD1 deletiert. Die heterozygote diploide Deletionsmutante wurde gegenüber dem Elternstamm HHD1 und dem Handelspräparat Uvaferm auf seine Eignung zur Vergärung von Kirschmaischen getestet. Neben Fermentationsparametern wurde mittels modernster GC/MS/MS-Methode die Bildung von Ethylcarbamat analysiert. Es konnte gezeigt werden, dass die Deletionsmutante keine Nachteile gegenüber HHD1 oder Uvaferm bei der Fermentation aufweist und in Kirschwassern zu deutlich niedrigeren EC-Gehalten führt.

Kapitel VI: Status und Erhebung von Ethylcarbamatgehalten in Deutschen Steinfrucht Destillaten der vergangenen 20 Jahre

Einige hundert Steinobstdestillate wurden auf ihren Ethylcarbamatgehalt analysiert. Die Probendaten wurden über einen Zeitraum von 1989 bis 2004 statistisch ausgewertet. Die Ergebnisse wurden anhand modernster Destillationstechniken interpretiert und statistisch ausgewertet. Es konnte gezeigt werden, dass sich bei sachgemäßen Fermentations- und Destillationsbedingungen die immer wieder diskutierte Ethylcarbamatproblematik in Steinobstdestillaten lösen lässt. Zudem wurde die Eignung eines modifizierten Hefestammes (in Bezug zum EC-Precursor-Bildungspotential seitens des Gärorganismus´) weiter untersucht. Der modifizierte Stamm zeigte gegenüber dem Elternstamm HHD1 und einem Handelspräparat seine fermentative Eignung. Des Weiteren bildete dieser Stamm etwaige Vorstufen für EC (aus dem Hefestoffwechsel resultierend) nicht mehr. Durch Kombination eines solchen Stammes und der Anwendung moderner und sachgemäßer Destillationstechniken lässt sich EC innerhalb der gesetzlichen Grenzwerte problemlos vermeiden.

Abschließend kann gesagt werden, dass der in dieser Arbeit konstruierte Laborstamm HHD1 durchaus für die Vergärung von Obstmaischen geeignet ist. Er bietet somit eine Basis für weitere genetische Modifikationen, angepasst an die jeweiligen Belange (wie z. B. eine verbesserte Zuckerverwertung durch die Steigerung der Glykolyserate, eine höhere Widerstandsfähigkeit gegenüber kontaminierenden Bakterien, hohe Alkoholausbeuten oder ein hohes Bildungspotential an Aromastoffen). Nicht nur im Bereich der Obstbrand-Herstellung, sondern vielmehr auch in aktuell diskutierten Nachernte-Technologien (z. B. alternative Energiegewinnung durch die Vergärung nachwachsender Rohstoffe) kann ein solcher genetisch modifizierter Stamm durchaus viele Verbesserungsmöglichkeiten bieten.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig gefertigt, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche kenntlich gemacht habe (§9 Abs. 2 Ziffer 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat. vom 04.05.1993).

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(Beatus Schehl)

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