Institut für Mikrobiologie und Weinforschung

Understanding of stuck fermentations: investigations on the relationship between the microbial diversity and chemical composition of must

Dissertation
Zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie
Der Johannes Gutenberg-Universität Mainz

vorgelegt von
Eva Christ
geboren am 21.12.1981 in Frankfurt am Main

Mainz, im Januar 2015

Erklärung

Die Experimente dieser Arbeit wurden von Mai 2010 bis September 2014 am Institut für Mikrobiologie und Weinforschung der Johannes Gutenberg-Universität Mainz durchgeführt.

Ich erkläre, dass ich die Arbeit selbstständig angefertigt und nur die angegebenen Hilfsmittel benutzt habe. Alle Stellen, die dem Wortlaut oder dem Sinn nach anderen Werken, gegebenenfalls auch elektronischen Medien, entnommen sind, sind von mir durch Angabe der Quelle als Entlehnung kenntlich gemacht.

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Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung: 19.02.2015

Vineyard `Winninger Uhlen`of a winery from the upper Moselle in Germany

Abbreviations

%	percent
°C	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
Ala	alanine
Arg	arginine
AS	amino acid
Asp	aspartic acid
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAT8	zinc cluster-encoding gene
CBS	Centraalbureau voor Schimmelcultures, Utrecht
cf.	compare
CFU	colony forming units
cm	centimeter
COX2	gene for the subunit II of the cytochrome c oxidase
CRP	Centre de Recherche Public Gabriel Lippmann
CYR1	gene for the adenylate cyclase
d	days
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphate
d.o.f.	day of fermentation
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig/Germany (German Collection of Microorgansims and Cell Cultures)
eH_2O	deionized water
et al.	and others
Fig.	figure
FMOC-Cl	fluorenylmethyloxycarbonyl chloride
g	gravity
g	gram
Glu	glutamic acid
GPYA	glucose - peptone - yeast extract - agar

GSY1	gene for the glycogen synthase
GYP	glucose - yeast extract - peptone
h	hour
H.	*Hanseniaspora*
His	histidine
HPLC	high performance liquid chromatography
IMW	Institute for Microbiology and Wine Research, Mainz/Germany
ITS	internal transcribed spacer
kg	kilogram
L	liter
Lb.	*Lactobacillus*
Lc.	*Leuconostoc*
M	molar
MALDI-TOF-MS	matrix assisted laser desorption/ionization – time of flight – mass spectrometry
Met	methionine
MET6	gene for the cobalamin-independent methionine synthase
mg	milligram
min	minute
ml	milliliter
mM	millimolar
NCBI	National Center of Biotechnology Information
n.d.	not detected
nm	nanometer
O.	*Oenococcus*
OD	optical density
OPY1	overproduction-induced pheromone-resistant yeast gene
P.	*Pediococcus*
PDA	potato – dextrose – agar
PCR	polymerase chain reaction
rDNA	ribosomal desoxyribonucleic acid
resp.	respectively
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
S.	*Saccharomyces*
SAPD	specifically amplified polymorphic DNA

SCAR	sequence characterized amplified region
Ser	serine
T	type strain
Tab.	table
Taq	*Thermophilus aquaticus*
TBE	TRIS – Borate – EDTA
Thr	threonine
TJ	tomato juice medium
Trp	tryptophan
TSA	tryptone – soja – agar
UV	ultra violet
V	volt
var.	variety
YAN	yeast assimilable nitrogen
YPM	yeast extract – peptone – mannitol

Contents

1 Introduction

1.1 Spontaneous fermentation versus other fermentation procedures

Spontaneous fermentation is the oldest form of producing alcoholic beverages and is still applied, especially by wine growers of the upper class segment, to produce wines with a characteristic sensory profile and high quality. The sensory profile of wines produced by spontaneous fermentation depends on the grape variety, the terroir (soil, climate etc.), the conditions in the cellar and the used materials as well as the microbiota which are responsible for the fermentation (Belitz et al., 2008; Fleet, 2003). In natural must, the dominating flora consists of yeasts, due to the low pH, high sugar content and mostly anaerobic conditions (Bisson and Joseph, 2009). In general, at the beginning of spontaneous fermentation, non-*Saccharomyces* yeasts like the genera *Hanseniaspora*, *Candida* and *Metschnikowia* are most present in must (Dittrich and Großmann, 2005). Although most of the non-*Saccharomyces* wine-related yeast species are known to have a low fermentation power and rate (Ciani et al., 2010), these yeast species are important for the development of the unique aroma profile and the complexity of a wine. They are replaced by the more ethanol-tolerant *Saccharomyces* species during the fermentation. Spontaneous fermentations rely on the indigenous microbiota of the grapes and of the winery. This means, that yeast and bacteria may already be present in the vineyard and on the grapes or might get in must at the different stages of fermentation. The apiculate yeasts *Hanseniaspora*/*Kloeckera* are the predominant species on the surface of grape berries (Pretorius, 2000). The composition of the yeast flora in the vineyard is dependent on climatic conditions (Parrish and Carroll, 1985) and the grape variety (Schütz and Gafner, 1994). It is also influenced by the vinification technology (Charoenchai et al., 1998). For example, yeasts are able to survive in the equipment of the winery like wooden casks. In that way, the composition of microbiota in a winery may vary from one vintage to another and also between the different casks in the cellar. This variety of microorganisms leads to a unique and extraordinary sensory profile. With the benefits of spontaneous fermentation, the wine growers have the opportunity to create their own brand with its characteristic taste.

In contrast, many winemakers make use of commercial starter cultures as this is the most secure way to produce wines in short time with a minimum of fermentation problems. In the beginning of the fermentation, sulfite needs to be added to the must because most of the non-*Saccharomyces* wine-related yeast species have a low SO_2 resistance (Ciani et al., 2010). Nevertheless, some microorganisms can survive and grow in sulfite-added must (Takahashi et al., 2014). After inhibiting the wild yeasts of the must, special starter cultures can be added. The commercial starter cultures are selected to be tolerant against low temperatures of 12-15°C in the cellar or high amounts of more than 200 g/L of sugar, for example. The winemaker is able to select a yeast strain which is most suitable for the conditions in his winery and has knowledge

about the sensory profile this yeast strain will most likely produce. Today, there is a huge variety of industrial produced starter cultures available for a huge number of conditions. These yeast strains are supposed to guarantee a high fermentation activity with a minimum of negative attributes. Although *Saccharomyces* yeast strains, especially *S. cerevisiae* and *S. bayanus*, are the main fermenting wine yeasts in spontaneous fermentation as well as starter cultures, there is a tendency for using wild yeasts as commercial cultures or as mixed cultures combined with *Saccharomyces* yeasts. These selected non-*Saccharomyces* yeast strains are able to enrich the sensory profile of the wine in addition to the *Saccharomyces* species. For that reason, the use of *Saccharomyces* and non-*Saccharomyces* yeast strains as mixed starter cultures is of increasing interest for quality enhancement and improved complexity of wines (Comitini et al., 2011). The wild yeasts are able to enhance wine complexity by modulating the concentration of volatile compounds, such as ethyl acetate and 2-methyl 1-butanol (Domizio et al., 2011).
When selecting a yeast strain as a starter culture, it is necessary to have knowledge of the conditions in the cellar and the chemical composition of the must. Since there is a huge range of starter cultures on the market they can be selected for a wide range of temperature, alcohol tolerance and demand for yeast assimilable nitrogen (YAN). However, besides the positive aspects, commercial starter cultures do not offer such a complex and extraordinary sensory profile which is typical for the vineyard and can only be produced by spontaneous fermentation.

There is a third possible procedure for winemaking, the managed fermentation. This is a combination of the benefits of both, spontaneous fermentation and the use of selected yeast strains. In this case, the natural diversity of wild yeasts in the beginning of the fermentation is used to improve the sensory profile of the wine and is complemented by the use of starter cultures at a certain concentration of ethanol to avoid sluggish or stuck fermentations. Fig. 1.1 shows an overview of the different mentioned fermentation procedures in which different microbiota are involved.
However, managed fermentation can only be successful if there are no spoilage yeasts present and if the *Saccharomyces* wine yeast is able to exert its dominance in time (Jolly et al., 2014). A successful way to shape the characteristic and extraordinary wine profile of a vineyard without the risk of stuck fermentation is to combine the controlled fermentation with the use of yeast strains which have been previously isolated from wines of the vineyard and have been selected in the laboratory for certain characteristic features and aroma production. These yeast strains can be added to wines in high cell counts later on and can therefore be helpful to avoid sluggish fermentations combined with an individual taste.

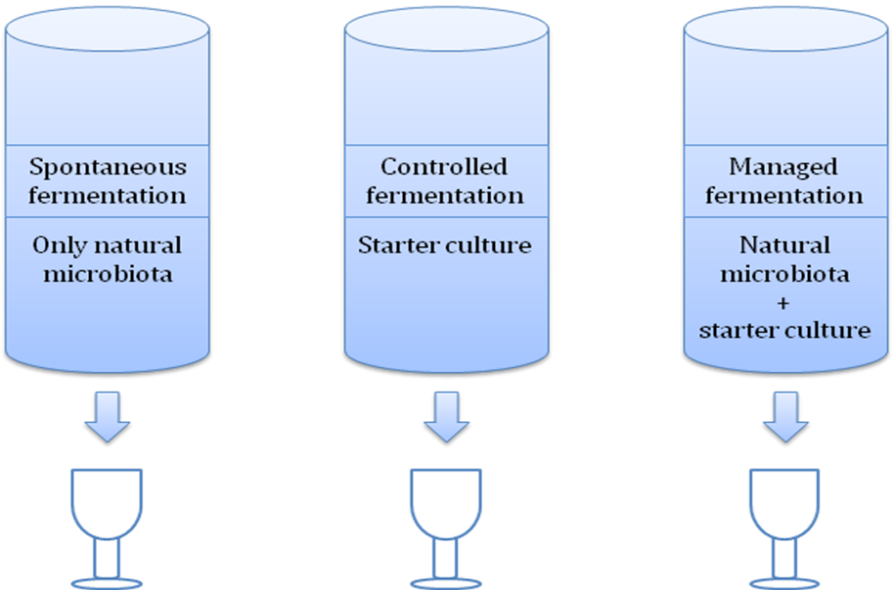


Fig. 1.1: Scheme of different fermentation procedures and the microbiota involved

1.1.1 Succession of yeasts during spontaneous fermentation

The microbiota in must consists of yeasts and bacteria from the vineyard, the terroir, the grapes and the equipment of the winery. All these factors can change from one year to another and are dependent on the soil, the treatment of the vineyard, the climate and other parameters which cannot be influenced. At the beginning of the fermentation, wild yeasts of the species *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* and *Candida stellata* (Bisson and Joseph, 2009; Lopandic and Prillinger, 2007; Mills et al., 2002) are most frequently because they exist on the grapes in higher cell counts. *Hanseniaspora sp*. may reach cell counts of up to 10^6 to 10^7 cells/mL, depending on the nature of the grapes (Phister et al., 2007). These apiculate yeasts are important for the chemical composition and quality of wine (Gil et al., 1996). They produce substances like esters and acids which contribute to the diverse aroma of wines from spontaneous fermentation. For that reason, wild yeasts are also added to mixed population starter cultures. In general, non-*Saccharomyces* yeasts found in grape must can be divided in three groups after their physiological behaviour: (1) yeasts that are largely aerobic like *Pichia sp*. and *Candida sp*. for example, (2) apiculate yeasts with low fermentative activity like *Hanseniaspora uvarum* and (3) yeasts with fermentative activity like *Zygosaccharomyces bailii* (Jolly et al., 2014). Above all, *Hanseniaspora uvarum* is regarded as the dominating species during this first part of the fermentation. With increasing concentrations of alcohol, the ethanol-tolerant yeast species of the genus *Saccharomyces* replace the wild yeasts (Lopandic and Prillinger, 2007) and continue fermentation. *Saccharomyces cerevisiae* is considered as the most common wine yeast, but also other species of the genus *Saccharomyces* and their interspecific hybrids may dominate the later stages of fermentation.

1.1.2 Succession of bacteria during spontaneous fermentation

Like yeasts, bacteria can get into the must from grape skins or from the equipment of the winery and the cellar. Phytosanitary treatments like the use of copper in the vineyard can have a negative impact on bacterial communities on grapes (Martins et al., 2012). Regarding bacteria, lactic acid and acetic acid bacteria are most common in wine. Lactic acid bacteria of the genera *Oenococcus, Lactobacillus, Pediococcus, Weisella* and *Leuconostoc* may grow in the anaerobic milieu of fermenation (Ribereau-Gayon et al., 2006). They are able to transform malate to lactate. This process is known as malolactic fermentation and can be beneficial in winemaking. The most important organism for malolactic fermentation is *Oenococcus oeni* and this species is added to wine by the winegrowers which are dependent on the reduction of acid because they have wines with high acid contents. Nevertheless, lactic acid bacteria may also cause wine faults like mousy smell. Species of the genera *Pediococcus* and *Leuconostoc* can be involved in the ropiness fault and *lactobacilli* have been isolated as being responsible for lactic spoilage from sluggish fermentations (Blasco et al., 2003). Members of the genera *Pediococcus*, *Weisella*, *Leuconostoc* and *Lactobacillus* are producers of exopolysaccharide slimes, acetic acid and other off-flavors (Blättel et al., 2009). In addition, many lactic acid bacteria are able to form biogenic amines, like histamine, which can cause allergic reactions (König et al., 2013). For these reasons, it is important to be able to control the succession of lactic acid bacteria timely during fermentation. This can be achieved by the use of multiplex PCR (Petri et al., 2013, Pfannebecker and Fröhlich, 2008). With multiplex PCR, different wine-relevant lactic acid bacteria can be detected during fermentation in a single detection method which is not time-consuming. With this knowledge, favored bacteria like *Oenococcus oeni* may be added if they are not present in must with higher cell counts. This would be a necessary treatment if acid in wine is too high and malolactic fermentation is needed to reduce the acid concentration.

Another group of bacteria which may occur in wine are the aerobic acetic acid bacteria. Acetic acid bacteria are able to metabolize ethanol by producing acetate and ethyl acetate (Mills et al., 2008). This is the reason why they are considered as wine spoilers. The most common acetic acid bacteria in wine belong to the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* (Dicks et al., 2009). High temperatures during winemaking and high pH favor the development and metabolism of acetic acid bacteria (Joyeux et al., 1984). As the growth of acetic acid bacteria is not desired in wine, attention should be paid to ensure that casks are as full as possible to avoid the accumulation of oxygen which is needed by the acetic acid bacteria for growth.

Besides these two important groups of bacteria, several different genera may occur in wine, depending on the terroir and the equipment of the cellar. Therefore, it is very important to pay attention to hygiene in the cellar to avoid the growth of bacteria in higher cell counts. In addition, the growth of bacteria is more likely in red wine, as this is produced by the process of mac-

eration when the grape as a whole with skin is fermented because a lot of bacteria are present on the grape skin.

1.2 Chemical composition of must and wine

Grape juice and must is characterized by a high amount of sugars, especially glucose and fructose. The sugar content depends on the soil, the climate and the maturity of grapes. A total concentration of about 200 g/L of these sugars is common and they usually are present in a 1:1 ratio (Dittrich and Großmann, 2005). Glucose and fructose are degraded to alcohol and carbon dioxide during the process of alcoholic fermentation. Wine yeasts are able to consume glucose as well as fructose, although most of these yeasts have a preference for glucose which may lead to residual fructose amounts in wine and stuck fermentation. Besides glucose and fructose, other hexoses like mannose may occur in wine as well as pentoses like arabinose but they are not fermented by the wine yeasts. For the enrichment of wines with low sugar content, sucrose is used. This disaccharide can be split by the yeast to glucose and fructose in a ratio of 1:1.

Beside the main products of fermentation, there are several by-products which may occur in wine in varying concentrations. The production of microbial by-products is dependent on factors like the fermenting yeast strain, the sugar content of must and the fermentation conditions. One of the most important primary by-product in quantity terms is glycerol. The formation of this trivalent alcohol is correlated to the production of ethanol and depending on the sugar content. *S. cerevisiae* usually transforms 4 % of sugar to glycerol (Dittrich and Großmann, 2005). The glycerol concentration in wine accounts for roughly 10% of the ethanol concentration. Glycerol is supposed to protect the yeasts from osmotic stress and to contribute to the body of the wine.

Lactic acid can be formed by the hydrogenation of pyruvate during fermentation by yeasts or result from malolactic fermentation of lactic acid bacteria. Acetic acid can also be produced by yeasts or originate from spoilage acetic acid bacteria. The legal limit for acetic acid in German wine is 1.08 g/L in white wine and 1.20 g/L in red wine (Dittrich and Großmann, 2005). Further organic acids like succinate, pyruvate and citric acid can also be present in wine as a by-product of fermentation.

Secondary by-products are generally formed from sugar degradation products. But higher alcohols are by-products of yeast cell growth (Dittrich and Großmann, 2005). Esters, aldehydes and higher ethanols contribute to the sensory profile of the wine in a very high level.

1.3 Reasons for stuck fermentation

Two general types of problematic fermentations can be distinguished: (1) stuck fermentations have a higher than desired residual sugar content at the end of the fermentation and (2) sluggish fermentations are characterized by a low rate of sugar consumption by the yeast (Bisson, 1999).

Both stuck and sluggish fermentations are characterized by very low yeast viability at the end of the fermentation (Blateyron and Sablayrolles, 2001) and can result in high residual sugar contents and low alcohol which may cause high financial losses for the winemaker if the wine can not be sold. The risk of stuck fermentation is increasing with spontaneous fermentations because the natural yeasts are not selected for high fermentation rates. Malherbe et al. (2007) differentiate between the following general factors which may cause fermentation arrest: vineyard and viticultural factors, harvest conditions and cellar management. Within these factors, some reasons for sluggish or stuck fermentations are low temperatures, nutrient deficiencies, the presence of toxic substances and a too low glucose/fructose ratio (Malherbe et al., 2007). Yeast nutrition consists of several factors like nitrogen, phosphate, oxygen, vitamins and minerals. Nitrogen deficiency slows down yeast growth and can result in stuck fermentation. Nitrogen limitation is one of the most common causes for stuck or sluggish fermentation in winemaking, and it is usually dealt with by supplementing grape juice with either ammonium salts or organic nutrients (Martínez-Moreno et al., 2014). However, this supplementation may influence the sensory profile of the wine. Oxygen and certain lipids are survival factors which decrease the inhibitory effects of alcohol. Lower fermentation temperatures also lead to a greater tolerance for ethanol (Henschke, 1997). In addition, some minerals such as magnesium and zinc can affect the fermentative activity of yeasts (Malherbe et al., 2007). Besides a lack of nutrition, several substances may have an inhibitory effect on yeasts, especially depending on the concentration of the substance. The product of the alcoholic fermentation, ethanol can also be toxic for yeasts at higher concentrations. The toxicity of ethanol is influenced by different factors like temperature. Acetic acid produced during bacterial metabolism can not only lead to sensory wine faults but can also cause sluggish or stuck fermentation (Rasmussen et al., 1995). Toxic substances can also be produced by the yeasts themselves, the so called killer yeast strains. These yeasts secrete a proteinaceous extracellular toxin that kills other sensitive yeast strains (Carrau et al., 1993; Malherbe et al., 2007). In this way, the microbial flora of the must can be affected and it can come to sluggish or stuck fermentation. If killer yeast strains occur in the natural microbiota of the wine it can be helpful to add a resistant starter culture in high cell counts to replace the natural yeasts. Many commercial starter culture yeast strains have killer toxin activity to dominate the original microbiota of the must. For example, most of the Oenoferm® yeast strains (Erbslöh, Geisenheim/Germany) feature killer activity.

As stuck fermentations cause high financial losses for winegrowers each year because they cannot sell the wines with higher than desired residual sugar content, the wine industry is continuously providing new products to the market as solutions for avoiding or resolving stuck fermentations. One possibility is to add a combination of nutrient salts and yeast cell walls to the must which is supposed to supply the wine yeasts with amino acids, vitamins and surviving factors.

Yeast cell walls are also used to protect the wine yeasts against their own toxic substances, especially in the final stage of fermentation.

Recently, there is a tendency to use special mixed populations of yeasts or selected hybrids as starter cultures in order to avoid stuck fermentation and increase the aroma profile. One example is the commercial yeast Exotics SPH (Schliessmann Kellerei-Chemie, Schwäbisch Hall/Germany) which is a hybrid of *S. cereviaise* and *S. paradoxus* and is supposed to combine the positive aspects of spontaneous fermentation with the reliability of a commercial yeast culture. Another commercial hybrid yeast is Oenoferm® X-treme (Erbslöh, Geisenheim/Germany) which was produced by fusion of the protoplasts of two different strains of *S. cerevisiae.* This hybrid strain is able to grow at low temperatures and needs a low concentration of nutrients.

Despite these known reasons, fermentation problems can still not be solved in all cases. The composition of must and wine is very complex and many ingredients are needed for several metabolic processes. Physical parameters like temperature and pH also influence the fermentation activity of yeasts. Therefore, the process of alcoholic fermentation is influenced by numerous factors which can also interact with each other. For that reason it is not recommendable to add substances in excess as they can also have inhibitory effects depending on the concentration or change flavour and taste of the wine. Especially those winegrowers who are settled in the upper-class price segment are very reliant on their unique and characteristic sensory profile of wine. Therefore, it would be advantageous to gain more information about unusual causes of stuck fermentations in order to avoid them without influencing or changing the aromatic profile of the wine.

1.4 Identification of *Saccharomyces* species in winemaking

Not only in case of sluggish or stuck fermentation, but although to control a normal fermentation, it can be necessary to isolate and clearly identify the fermenting yeast species from wine. The well-known and most important wine yeast species *Saccharomyces cerevisiae* as well as *Saccharomyces bayanus* belong to the so called *Saccharomyces sensu stricto* complex. Besides *S. bayanus* and *S. cerevisiae*, this complex includes *S. paradoxus*, *S. mikatae*, *S. arboricolus*, *S. kudriavzevii* and *S. pastorianus* (Naumov et al., 2000b; Scannell et al., 2011; Wang and Bai, 2008), which is a hybrid of *S. cerevisiae* and *S. bayanus* (Nakao et al., 2009). Members of this group are closely related species and show a high degree of similarity concerning morphological, physiological and genetical properties. Therefore, a simple identification and differentiation of strains is difficult. Nevertheless, the identification of these species and also their interspecific hybrid can be important to control fermentations and avoid stuck fermentations. Although the *Saccharomyces* species are closely related they have different characteristics and therefore are suitable for diverse fermentation conditions. It can also be necessary to control the growth of an applied *Saccharomyces* starter culture. Two different methods to discriminate the different *sensu*

stricto species are: a DNA fingerprint method, the specifically amplified polymorphic DNA (SAPD-) PCR and fingerprinting with MALDI Biotyper (MALDI-ToF MS) (Blättel et al. 2013).
Interspecific hybrids of *Saccharomyces* yeasts can also occur in natural must and they can be added as commercial starter cultures because these yeasts are supposed to combine the characteristics of both parental species. For example, *S. bayanus* is supposed to be more tolerant at low fermentation temperatures, whereas *S. cerevisiae* seems to be more suitable for higher temperatures and high ethanol concentrations (Belloch et al., 2008). Of course, those characteristics may vary from one strain of a certain species to another. The interspecific hybrids are sterile, having non-viable ascospores (Naumov et al., 2000b). For the identification of *Saccharomyces* hybrids, the methods mentioned before can be insufficient. In these cases, a further restriction enzyme digestion can be used (Gonzàlez et al., 2006) in order to identify the parental species of the hybrids. This information can give a hint to the characteristics of the isolated hybrids and is necessary if the hybrid yeast strains can possibly be used as commercial cultures.
In addition, identification of natural occurring hybrids a vineyard can show if these specific hybrids change during several years depending on different conditions in the cellar and in must. The interspecific hybrids are able to adapt to fermentative stress by inheriting competitive traits from their parental species (Belloch et al., 2008). With this knowledge, the isolated and identified hybrids can be helpful later on in the winery to avoid or resolve stuck fermentation.

1.5 Goals of the investigations

Despite the fact that many reasons for stuck fermentations are known, fermentation problems still occur in many wineries every year and can not always be solved. Most of the winegrowers of the upper class segment do not want to use commercial starter cultures or any other treatment that might change the characteristic sensory profile of their wines. For that reason, unconventional solutions for their specific fermentation problems are very important for those wineries and high financial losses could be avoided. The aim of this work was to study the succession of microorganisms during fermentation in detail and to collect information about the chemical composition of must and young wine in different stages of vinification in order to detect unusual factors that may induce sluggish or stuck fermentations. With this knowledge, new ways for avoiding or solving fermentation problems should be developed.

2 Material and methods

2.1 Organisms

The organisms used in this thesis are shown in table 2.1.

Table 2.1: *Saccharomyces* strains

Species/Hybrids	Strain	Origin
Saccharomyces bayanus	DSM 70412^T	DSMZ
Saccharomyces cerevisiae	DSM 70449^T	DSMZ
Saccharomyces cerevisiae	Fermivin®	DSM Food Specialties Delft/The Netherlands
Saccharomyces kudriavzevii	CBS 8840^T	CBS
Saccharomyces paradoxus	CBS 432^T	CBS
S. bayanus	HL 77	IMW
S. cerevisiae x S. kudriavzevii x S. bayanus	HL 78	IMW
S. cerevisiae x S. kudriavzevii x S. bayanus	82.14	IMW
S. cerevisiae x S. kudriavzevii x S. bayanus	96.1	IMW

CBS: Centraalbureau voor Schimmelcultures, Utrecht/The Netherlands
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig/Germany
IMW: Institute for Microbiology and Wine Research, Johannes Gutenberg University Mainz/Germany
^{T}: type strain

2.2 Biochemicals, kits and enzymes

The biochemicals used in this thesis are compiled in table 2.2.

Table 2.2: Biochemicals

Name	Manufacturer
Agarose peqGold Standard-Agarose	Peqlab (Erlangen/Germany)
Cycloheximid	Sigma-Aldrich (Steinheim/Germany)
DNA-Standard Generuler DNA Ladder Mix	Thermo Fisher Scientific (Waltham/USA)
dNTP-Mix[1]	Peqlab (Erlangen/Germany)
Enhancer Solution P for PCR	Peqlab (Erlangen/Germany)
$MgCl_2$ solution for PCR	Peqlab (Erlangen/Germany)
PCR reaction buffer S (10x)[2] / Y (10x)[3]	Peqlab (Erlangen/Germany)
InstaGene™-Matrix	Bio-Rad (München/Germany)

[1]: 40 mM, 10 mM for each nucleotide (dATP, dCTP, dGTP, dTTP)
[2]: 100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.1 % Tween 20, 15 mM $MgCl_2$
[3]: 200 mM Tris-HCl (pH 8.55), 160 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 20 mM $MgCl_2$

Enzymes used in this thesis are presented in table 2.3.

Table 2.3: Enzymes

Enzyme	**Manufacturer**
Restriction enzymes: *Hae*III, *Hha*I, *Hinf*I, *Msp*I, *ScrF*I, HpaII, *BsuR*I	Thermo Fisher Scientific (Waltham/USA)
Lysozyme from chicken protein	Sigma-Aldrich (Steinheim/Germany)
Lyticase from *Arthrobacter luteus*	Sigma-Aldrich (Steinheim/Germany)
Taq-Polymerase from *Thermus aquaticus*	Peqlab (Erlangen/Germany)

Table 2.4 shows the kits used in this thesis.

Table 2.4: Kits

Kit	**Manufacturer**
DNeasy® Blood & Tissue Kit	Qiagen (Hilden/Germany)
Multiplex PCR Kit	Qiagen (Hilden/Germany)
Purification Kit	Qiagen (Hilden/Germany)

2.3 Equipment and accessories

Equipment and accessories used in this thesis are represented in table 2.5.

Table 2.5: Equipment and accessories

Name	**Manufacturer**
Autoclaves: Certoclav	CertoClav Sterilizer GmbH (Traun/Austria)
Systec	Systec GmbH (Wettenberg/Germany)
Gel documentation unit Biovision CN3000	Vilber-Lourmat (Eberhardzell/Germany)
Thermocycler: Mastercycler gradient	Eppendorf (Hamburg/Germany)
Micromanipulator: Mikromanipulateur de Fonbrune	Etablissements Beaudoin (Paris/France)
pH-Meter CG840	Schott (Mainz/Germany)
Ultrapure water systems: Milli-Q Plus 185	Millipore (Eschborn/Germany)
Millipore RiOs 30	Millipore (Eschborn/Germany)
UV-Transilluminator FLX-20M	Vilber-Lourmat (Eberhardzell/Germany)
Balances	Sartorius (Göttingen/Germany)
Centrifuges: Centrifuge 5403	Eppendorf (Hamburg/Germany)
Micro Star 17	VWR (Radnor, PA/USA)

2.4 Sampling

The sampling took place in regular intervals (generally every two weeks) in the two years 2011 and 2012 at the vinery in Winningen, Germany. Wine barrels of 2500 L were sampled. Additionally, the staff of the winery took samples every 2-3 days which were stored at –20 °C. During the vintage 2011/2012 5 tanks were sampled altogether. The stainless steel cask 3007 was sampled from the first day after the filling of the cask (27.09.2011). This day will be referred to as day 1. The last day of sampling (06.12.11) was shortly before the sulphuring (09.12.11). Four wooden casks were sampled when their fermentation was stuck. For these casks with the numbers 2607 (day of filling 17.10.2011), 2608 (day of filling 14.10.2011), 2609 (day of filling 14.10.2011) and 2612 (day of filling 18.10.2011), the sampling began shortly after the fermentations were sluggish and lasted until the fermentation was finished. The day of their filling will be referred to as day 1. One of these wooden casks (2608) was sampled for the investigation of microbial interactions, the others were tested for their chemical composition only. During the vintage 2012/2013 four tanks were sampled. The steel cask S 101 was sampled for microbial interactions (day of filling 18.10.2012) and was stuck after 41 days of fermentation (27.11.2012), another steel cask (Rö 104, day of filling 18.10.2012) and two wooden casks (STO 101, d.o.f. 29.10.2012; UB 101, d.o.f. 5.11.2012) were tested for their chemical composition only. The samples were taken by opening the tap of the casks and afterwards transported in a cooling box to the CRP-Gabriel Lippmann as well as to the IMW and frozen at -20 °C. Table 2.6 shows an overview of the sampled casks in this thesis.

Table 2.6: Overview of the sampled casks from a German winery from the upper Moselle

Cask	Vintage	Grape variety	Fermentation	Parameters tested
3007	2011	Riesling	normal	microbial and chemical
2608	2011	Riesling	stuck	microbial and chemical
2607	2011	Riesling	stuck	chemical
2609	2011	Riesling	stuck	chemical
2612	2011	Riesling	stuck	chemical
S 101	2012	Riesling	stuck	microbial and chemical

2.5 Cultivation of microorganisms

The microorganisms were isolated by plating. Hundred µL of the must sample or the appropriate dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}; dilution in 0.9% solution of NaCl) per plate were spread with a Drigalski-spatula. The plates were incubated two to seven days at room temperature. In order to

get pure cultures, three visual similar colonies were subsequently plated several times. Afterwards, the plates were stored at 4 °C.
Lactic acid bacteria were enriched in a tomato juice medium (TJ). Therefore, 1 mL of the must sample was mixed with 9 mL of the liquid tomato juice medium and cultivated for 14 days at room temperature. To inhibit the growth of yeasts cycloheximide was added.

2.6 Culture media

In order to isolate a broad spectrum of microorganisms seven different culture media were used. Cycloheximide (20 mg/L) was added to the media for cultivation of bacteria after sterilization. Therefore, the media were cooled down to 60 °C first.

2.6.1 Media for cultivation of yeasts

- *GPYA (glucose - peptone - yeast extract - agar; CBS 2008)*
- *PDA (potato - dextrose - agar; CBS 2008)*
- *GYP (glucose - yeast extract - peptone)*

10.00 g	yeast extract
20.00 g	peptone
20.00 g	glucose
15.00 g	agar
ad 1000 mL	eH_2O

2.6.2 Media for cultivation of bacteria

- *YPM (yeast extract - peptone - mannitol; DSMZ 2008) for acetic acid bacteria*
- *MRS (de Man et al., 1960) for lactic acid bacteria*
- *Tomato juice medium (TJ) for lactic acid bacteria*

basic medium:

5.00 g	peptone
5.00 g	yeast extract
20.00 g	tryptone
5.00 g	glucose
5.00 g	fructose
3.00 g	citric acid
1.00 g	tween 80
0.50 g	$MgSO_4$ x 7 H_2O
0.67 g	potassium sorbate
ad 1000 mL	eH_2O

The pH-value was adjusted to 6.0. Seven hundred and fifty mL of the basis medium were filled up with 250 mL of centrifuged tomato juice and 12.00 g agar were added.
In addition, for the enrichment of lactic acid bacteria a liquid tomato juice medium without agar was used (TJ-liquid).

- *TSA (tryptone - soja - agar)*

  15.00 g tryptone
  5.00 g peptone from soja
  5.00 g sodium chloride
  12.00 g agar
  0.67 g potassium sorbate
  ad 1000 mL eH_2O

2.6.3 Synthetic medium for growth experiments

For growth experiments modified B-medium (Heerde and Radler, 1978) was used:

25.00 g glucose
25.00 g fructose
20.00 g malate
1.50 g $(NH_4)_2SO_4$
1.00 g $MgSO_4$ x 7 H_2O
0.50 g sodium citrate
0.04 g inositol
100 ml amino acid solution,
10 ml vitamin solution,
10 ml trace mineral solution,
ad 1000 mL eH_2O, pH 3.3.

In order to investigate the influence of different amino acid concentrations on the growth and metabolism of isolated yeasts, synthetic B-medium with 100%, 50% and 25% of the amino acid solution was used. In addition, experiments were carried out with one amino acid of the amino acid solution missing in each parallel of synthetic B-medium.

- Amino acid solution

  0.75 g L-alanine
  3.50 g L-arginine
  0.20 g L-histidine
  0.40 g L-methionine
  0.50 g L-serine
  2.00 g L-threonine
  0.40 g L-tryptophane
  0.50 g L-aspartic acid
  3.00 g L-glutamic acid
  ad 1000 mL eH_2O.

- Trace mineral solution

  300.00 mg potassium hydrogen tartrate
  30.00 mg H_3BO_3
  30.00 mg $FeCl_3$ x 6 H_2O
  30.00 mg $ZnSO_4$ x 7 H_2O
  30.00 mg $MnSO_4$ x H_2O
  30.00 mg $AlCl_3$ x 6 H_2O
  15.00 mg KJ
  15.00 mg $CnSO_4$ x 5 H_2O
  15.00 mg $NaMoO_4$ x 2 H_2O
  15.00 mg $CoCl_2$ x 6 H_2O
  15.00 mg $LiSO_4$ x H_2O
  ad 150 mL eH_2O.

- Vitamin solution

  40.00 mg 4-aminobenzoic acid
  40.00 mg nicotinic acid
  40.00 mg Ca^{++}-D-pantothenate
  20.00 mg pyridoxolim chloride
  40.00 mg thiaminium dichloride
  40.00 ml biotin 2 mg / 100 ml dH_2O [containing 5 ml saline solution (1.5 g KH_2PO_4 + 1.5 g K_2HPO_4 / 10 ml)]
  40.00 ml folic acid 2 mg / 100 ml H_2O (NaOH)
  20.00 ml riboflavin 20 mg / 20 ml H_2O (NaOH)
  ad 400 mL eH_2O.

Infusion bottles were filled with B-medium and inoculated with 10^4 cells/mL. The experiments were performed in four parallels for each condition.

2.7 Micromanipulation

Micromanipulation was performed for single cell separation of yeast cells in order to ensure purity of the isolated hybrid strains. The used micromanipulation system is based on a development of Pierre de Fonbrune (US-Patent 1987733 - 1987734) and was manufactured by Etablissements Beaudoin (Paris/Frankreich). Fig. 2.1 shows the micromanipulation system used in this thesis.

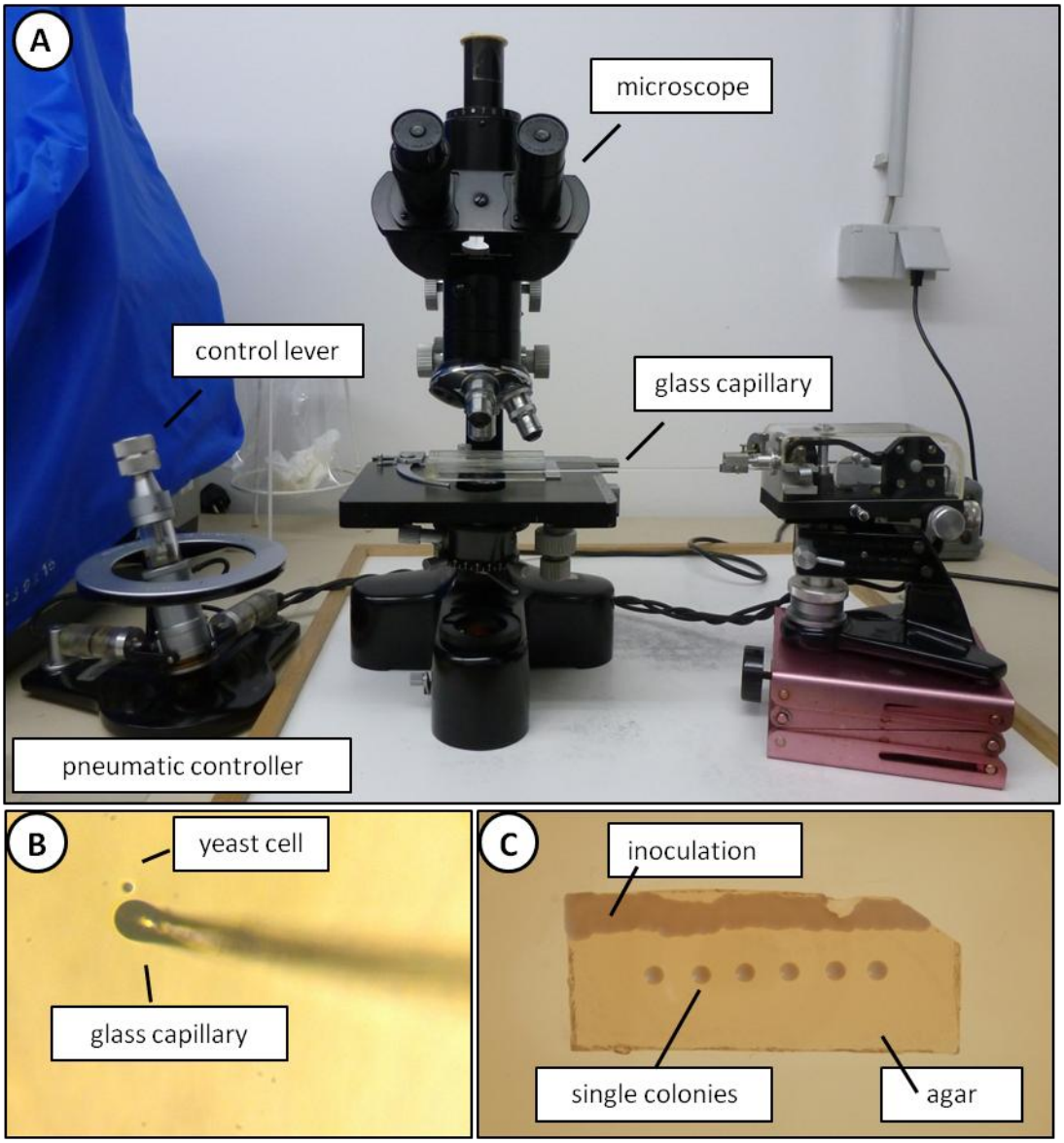


Fig. 2.1: Micromanipulator system
A: arrangement of the micromanipulation system; B: microscopic image of a glass capillary with a separated single yeast cell; C: agar layer with colonies from separated single yeast cells

2.8 DNA isolation

2.8.1 DNA isolation of yeasts

The pure cultures were picked from the petri dishes with an inoculation loop and suspended in 100 µL InstaGene™-Matrix (Bio-Rad, München/Germany). Ten µL of lyticase (10000 U/mL) were added to the suspensions which were shaken afterwards for 60 min at 37 °C. After that the suspensions were shaken for 56 min at 37 °C to disrupt cell aggregations. In the following step the suspensions were incubated for 8 min at 99 °C in order to destroy remaining cell structures. After centrifugation for 4 min at 10000 rpm the DNA remained in the supernatant and was separated from the other cell material. The supernatants were stored at -20 °C.

For some applications high-quality DNA extracts without contaminants and enzyme inhibitors are required. Therefore, DNA that was used for SAPD-PCR was purified using the DNeasy blood & tissue kit (Qiagen, Hilden/Germany), according to the manufacturer's protocol for purification of total DNA from yeasts.

2.8.2 DNA isolation of bacteria

The genomic DNA of bacteria was isolated using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden/Germany). The extraction was performed as recommended by the manufacturer for gram-positive bacteria. The pure cultures were picked from the plates with an inoculation loop.

2.9 Polymerase chain reaction (PCR)

2.9.1 Amplification

For amplification of the 16S rDNA genes of the bacteria universal oligonucleotide primers (Eubak5 and C1392R) were used, which bind to the 5'- or to the 3'-end of the bacterial 16S rDNA genes (cf. Tab. 2.7), respectively. For identification of the isolated yeasts the internal transcribed spacer (ITS 1-5.8S-ITS 2) regions were sequenced. Amplification of the ITS regions was performed with oligonucleotide primers ITS 4 and ITS 5 (cf. Tab. 2.7). All primers were from Eurofins MWG Operon (Ebersberg/Germany). All other used PCR components were purchased from peqlab (Erlangen/Germany). For PCR, ultrapure water was filtrated through a 0.2 µm cellulose acetate filter, irradiated with UV-light (Transilluminator, Biometra, Göttingen/Germany) and steam autoclaved for the highest possible level of sterility. The PCR products were checked by agarose gel electrophoresis (1.5%) and stained by incubating the gel for 30 min in an ethidium bromide staining solution. After short washing of the gel in eH_2O the bands were visualised under UV light. The stained gels were photographed (BioVision CN 3000, Vilber-Lourmat, Eberhardzell/Germany).

Tab. 2.7: Primers for amplification of DNA from bacteria and yeasts

Primer	Direction	Sequence (5´ --- 3´)	Length[a] (bp)	T_m[b]	Final sequence	Position[c]
Eubak5	forward	AGA GTT TGA TCM TGG CT	17	50	SSU rRNA Eubacteria	Eco 8-25
C1392R	reverse	CCA CGG GCG GTG TGT AC	17	58	SSU rRNA Bacteria	Eco 1406-1392
ITS 4[d]	reverse	TCC TCC GCT TAT TGA TAT GC	20	55,3	LSU rRNA Eucaryotes	
ITS 5[d]	forward	GGA AGT AAA AGT CGT AAC AAG G	22	56,5	SSU rRNA Eucaryotes	

[a] nucleotides in the part of the oligonucleotides complementary to the SSU-rDNA-Sequenz.
[b] melting temperature of the oligonucleotides in °C.
[c] the position data refer to the SSU rRNA-Sequence of *Escherichia coli* (Brosius et al., 1981).
[d] primer according to White et al. (1990)

2.9.2 Multiplex PCR

The multiplex PCR was performed in order to identify the microorganisms in the enrichment cultures (Petri et al., 2012). Therefore, the QIAgen® Multiplex PCR kit (Qiagen, Hilden/Germany) was used. This kit contained a Mastermix, including a „Hot Start" *Taq*-DNA-Polymerase, dNTPs, $MgCl_2$ and reaction buffer. For the primer mixes (cf. Tab 2.8), specific primers were used. These sequence characterized amplified region (SCAR) primers were developed according to Paran (Paran and Michelmore, 1993).

For checking purposes the PCR products were separated by agarose gel electrophoresis (1.5%) and stained by incubating the gel for 30 min in an ethidium bromide staining solution. After short washing of the gel in eH_2O the bands were visualized under UV light. The stained gels were photographed.

2.9.3 Specifically amplified polymorphic DNA (SAPD)-PCR

For the differentiation of species belonging to the *Saccharomyces sensu stricto* complex the SAPD-PCR (Blättel et al., 2013; Fröhlich and Pfannebecker, 2007), a DNA fingerprint method was applied. PCR was performed as described by Pfannebecker and Fröhlich (2008) using the primer A-Not (5'-AGCGGCCGCA-3'), C-Not (5'-AGCGGCCGCC-3') and T-Not (5'-AGCGGCCGCT-3'). Resulting PCR products were analysed by gel electrophoresis at 55 V for 3-4 h. The used gels contained 1.5% agarose and 0.035% sodium silicate (Na_2SiO_3). Gels were stained and documented as described before. Obtained fingerprint patterns were converted into a binary matrix using the Bio-1D software (Vilber Lourmat, Eberhardzell/Germany).

Tab. 2.8: Set of Primer-Mix A and Primer-Mix B

Primer[a] Mix A	Sequence (5´ --- 3´)	Specific for	Length (bp)	Tm[b]	Product (bp)
SCAR-OENI-F	GGT AGA TTA ACC CGC GAC G	*Oenococcus oeni*	19	58.8	1588
SCAR-OENI-R	GGA ATC GGT AGC ATC CTG	*Oenococcus oeni*	18	56.0	1588
SCAR-LBR-F	GGA AGA TCA AGA ATA TCG GTG	*Lactobacillus brevis*	21	55.9	1361
SCAR-LBR-R	GCG TCT CTA ATT CAC TGA GC	*Lactobacillus brevis*	20	57.3	1361
SCAR-LPL-F	GAA GAT TTG CCC ATC GGT G	*Lactobacillus plantarum*	19	56.7	1113
SCAR-LPL-R	CGT TTG ATG GTA GCG TTG C	*Lactobacillus plantarum*	19	56.7	1113
SCAR-LEU-F	GTG GTC ATG GGT CTT AGC	*Leuconostoc mesenteroides*	18	59.9	886
SCAR-LEU-R	GGA TCA AGA CTA GCC AAT GG	*Leuconostoc mesenteroides*	20	60.4	886
SCAR-WPA-F	GCT GAT GAA CCC ATA CCT C	*Weissella paramesenteroides*	19	56.7	641
SCAR-WPA-R	GAC CTG ATT CGC TCG TTG	*Weissella paramesenteroides*	18	56.0	641
SCAR-PDA-F	GTC TAA ACT GGT GGT TAA ACG	*Pediococcus damnosus*	21	58.6	470
SCAR-PDA-R	ATC GCA CCT GGT TCA ATG C	*Pediococcus damnosus*	19	60,1	470
SCAR-PPA-F	GCA TGA ATC ACT TTT CGC TC	*Pediococcus parvulus*	20	58.3	331
SCAR-PPA-R	CAA AGA TTG TGA CCC AGT TG	*Pediococcus parvulus*	58,3	58.3	331
Primer[a] Mix B	**Sequence (5´ --- 3´)**	**Specific for**	**Length (bp)**	**Tm[b]**	**Product (bp)**
SCAR-LBU-F	CTA TCT TTA ACC GCA TTG CCG	*Lactobacillus buchneri*	21	57,9	1007
SCAR-LBU-R	GAC ACG CTT CTC ATG ATT GTC	*Lactobacillus buchneri*	21	57,9	1007
SCAR-PAC-F	ATG ATG GAC AGA CTC CCT G	*Pediococcus acidilactici*	19	60,4	776
SCAR-PAC-R	CGA GCT GCG TAG ATA TGT C	*Pediococcus acidilactici*	19	60,4	776
SCAR-LBH-F	TTC CTT GGT AAT GTG CTT GC	*Lactobacillus hilgardii*	20	58,3	684
SCAR-LBH-R	AAT GGC AAT CGC AAT GGA CG	*Lactobacillus hilgardii*	20	60,4	684
SCAR-PIN-F	CTA TCC TTA CAA TGT GCA TCG	*Pediococcus inopinatus*	21	58,6	567
SCAR-PIN-R	TGG TGC GTC AGT AAA TGT AAG	*Pediococcus inopinatus*	21	58,6	567
SCAR-LCU-F	CCA GAT CCA TCA GAA GAT ACG	*Lactobacillus curvatus*	21	57,9	480
SCAR-LCU-R	GCT AAC TTA CCA CTA ACG ACC	*Lactobacillus curvatus*	21	59,7	480
SCAR-PPE-F	GGG AAC GGT TTT AGT TTT ATA CG	*Pediococcus pentosaceus*	23	59,9	396
SCAR-PPE-R	CTA AGA GCG GTG ATG ATA AG	*Pediococcus pentosaceus*	20	58,3	396

[a] primer name: F: forward; R: reverse; [b] melting temperature of the oligonucleotides in °C.

2.10 Restriction enzyme digestion

After the PCR a restriction enzyme digestion of the amplified regions was performed in order to identify the isolated organisms. The bacterial 16S PCR products were restricted with enzymes *Bsu*RI (interface 5´-GG^CC-3´) and *Hpa*II (interface 5´-C^CGG-3´). For the ITS amplification product of yeasts, the following FastDigest® restriction enzymes were used: *Hha*I (interface 5´-GC^GC-3´), *Hae*III (interface 5´-GG^CC-3´) and *Hinf*I (interface 5´-G^ANTC-3´, N stands for a random base) (Divol and Lonvaud-Funel, 2005). The mixtures were incubated at 30 °C for 2 h. Afterwards the enzyme was inactivated at 65 °C for 20 min. For the identification of hybrids, a further restriction enzyme digestion was carried out according to González et al. (2006).

The resulting DNA fragments were analysed with an electrophoresis (Bio-Rad, München/Germany) using a 2 % agarose gel. For length estimation of the DNA fragments, 5 µl of the GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, Waltham MA/USA) were applied. The products of the restriction were stained by incubating the gel for 30 min in an ethidium bromide staining solution. After short washing of the gel in eH_2O the bands were visualised under UV light. The stained gels were photographed.

The resulting restriction patterns were compared with the data of Wacker (2010). Those patterns which could not be assigned to the patterns described by Wacker (2010) were divided into groups. One amplificate of each group was sequenced by LGC Genomics (Berlin/Germany) in order to identify the organism. The bacterial 16S rDNA fragments were sequenced with primer C1392R and the ITS region fragments of the yeasts were sequenced with primer ITS 5 (cf. Tab. 2.7). The resulting sequences were identified via NCBI BLAST.

2.11 Yeast assimilable nitrogen

Yeast assimilable nitrogen was determined by the CRP (Belvaux/Luxembourg). The analysis was performed according to a protocol available at www.vignevin-sudouest.com, 17.03.2010. The yeast assimilable nitrogen was determined by the Soerensen titration. Twenty mL of a sample were adjusted to pH 7 with NaOH (0.1 M), 20 mL formaldehyde (previously adjusted to pH 7 with NaOH; 1 M) were added and the solution was titrated to pH 7 with NaOH (0.1 M). The volume of consumed NaOH (0.1 M) was used to calculate the yeast assimilable nitrogen.

Yeast ass. nitrogen [mg/L] = volume(NaOH)•c(NaOH)•14•1000 /volume(sample)

2.12 Sugar, alcohol and organic acid analysis

Sugar, alcohol and organic acids in the samples were analyzed with high performance liquid chromatography (HPLC; autoinjector SIL-10AD, pump LC-10AD, integrator C-R8A, Shimadzu, Kyoto/Japan) on an Aminex®HPX-87H ion exclusion column (300 mm x 7.8 mm, Biorad, Munich, Germany), according to Pfeiffer and Radler (1985), using an external standard. Each sample was

analyzed three times. Malate was analyzed in an enzymatic process (D-Malate/L-Malate-Kit, Roche, Grenzach-Whylen/Germny) because the HPLC analysis did not offer a sufficient differentiation of malate and fructose due to similar retention times. The enzymatically determined concentration of malate was substracted from the detected concentration of fructose using the values of response factor and area. The chemicals for the enzymatic determination of malate were purchased from Roche (Grenzach-Wyhlen/ Germany). Most of the components were analyzed in diluted must (dilution 1:3). Succinate, lactate, citrate and acetate were analyzed in undiluted must and young wine. Amino acids in must samples were also determined with HPLC (autoinjector SIL – 10Ai, pump LC – 10Ai, UV-detector RF – 535, Shimadzu, Kyoto/Japan) on a ProntoSIL Spheribond ODS 2 column (250 mm x 4 mm, Bischoff, Leonberg/Germany) using an external standard. Derivatization was carried out with FMOC-Cl. Each sample was analyzed three times. Fig. 2.2 shows the scheme of an HPLC system.

The glucose and fructose concentration in synthetic media were analyzed enzymatically. The chemicals for the enzymatic determinations (D-Glucose/D-Fructose-Kit) were purchased from Roche (Grenzach-Wyhlen/Germany).

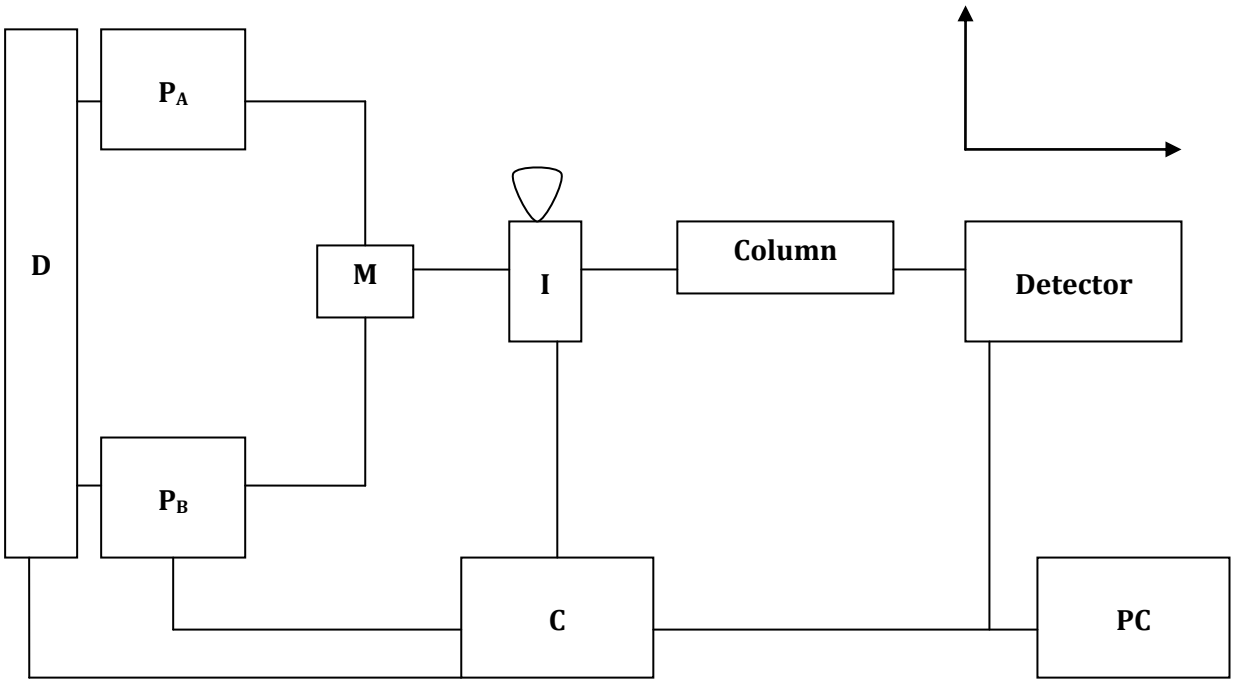


Fig. 2.2: Scheme of an HPLC system
D: Degaser; P_A: Pump A; P_B: Pump B; M: Mixing-cell; I: Injector; C: Controller

3 Results

3.1 Succession of yeasts

The succession of yeast strains present in must and young wine was investigated at intervals of two weeks during fermentation in two following years. The yeast strains were identified based on restriction patterns of the ITS region using the restriction enzymes *Hae*III, *Hha*I and *Hinf*I.

3.1.1 Differentiation of Hanseniaspora uvarum and Saccharomyces bayanus.

Since *Hanseniaspora uvarum* and *Saccharomyces bayanus* were the main yeast strains during fermentation, a reliable differentiation of the wild yeast *Hanseniaspora uvarum* and the wine yeast *Saccharomaces bayanus* is very important for the study of the succession. The isolate *Hanseniaspora uvarum* could be cultivated on the media containing cycloheximide in contrast to *Saccharomyces bayanus.* On GPYA agar *Hanseniaspora uvarum* showed slightly brownish colonies, while *Saccharomyces bayanus* formed colonies of a lighter colour (cf. Fig. 3.1). Thereby, these two yeast species could be differentiated on the four media with certainty.

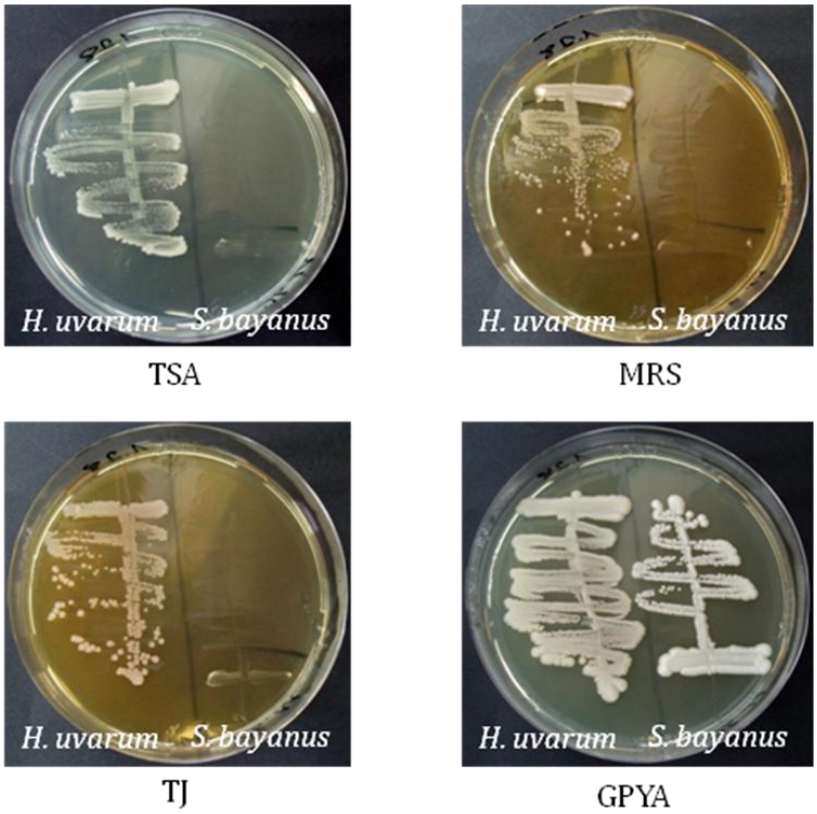


Fig. 3.1: Yeast isolates from the vineyard Heymann/Löwenstein on different culture media
H. uvarum: Hanseniaspora uvarum*; *S. bayanus: Saccharomyces bayanus

3.1.2 Succession of yeasts in the first vintage 2011/2012

Right after the filling of the cask and start of the fermentation, yeasts of the genera *Hanseniaspora*, *Candida*, *Cryptococcus*, *Pichia, Metschnikowia* and *Aureobasidium* were found (cf. Tab. 3.5) in steel cask 3007. The species *Candida zemplinina* was the most frequently found yeast species at day 1 with a cell count of 10^6 cells/mL, followed by *Hansensiaspora uvarum, Pichia kluyveri* and *Cryptococcus flavescens* with about 10^5 cells/mL. The cell titre was determined via colony forming units (CPU). No yeast species of the genus *Saccharomyces* was found on day 1, but from day 15 the species *Saccharomyces bayanus* dominated the fermentation with a cell count of up to 10^7 cells/mL. Identification of *Saccharomyces* yeast species was performed via SAPD-PCR (cf. Tab. 3.1). Patterns were compared to those of *Saccharomyces* type strains.

Wild yeasts of the genera *Hanseniaspora, Pichia* and *Metschnikowia* could not be found after day 15. Species of the genus *Saccharomycopsis* were only found on day 15 with a cell count of about 10^2/mL. The species *Candida boidinii* was detected from day 15 until the end of the fermentation on day 57 with a cell count of 10^2 or 10^3 cells/mL, respectively. Towards the end of the fermentation on day 43 and 57, yeasts of the genera *Saccharomycetes* or *Pichia,* were found with a cell count of 10^2 cells/mL additionally. Table 3.5 shows a summary of the isolated yeast strains from steel cask 3007 at the different dates of sampling during fermentation.

Table 3.1: SAPD-PCR fragment patterns from *Saccharomyces* species used in this study

Species	Strain	Amplification products using the primer A-Not in bp rounded to 10
S. bayanus	DSM 70412^T	2,630 2,300 1,960 1,730 1,570 1,400 1,100 1,020 890 730 610 550 310
S. bayanus	HL 77	2,630 2,300 1,960 1,730 1,570 1,400 1,100 1,020 890 730 610 550 310

^{T}: type strain

The sampling of wooden cask 2608 started on day 26 of the fermentation, because at that time the fermentation was sluggish in this cask. From this first sampling only yeasts of the species *Saccharomyces bayanus* could be isolated (10^6 cells/mL). On day 54 the fermentation continued and at that time besides *Saccharomyces bayanus* (10^4 cells/mL) *Saccharomyces cerevisiae* (10^4 cells/mL)*, Saccharomycetes sp.* or *Pichia membranifaciens*, respectively (10^0 cells/ mL), and *Candida bituminiphila* (10^1 cells/mL) were found as well. Table 3.6 shows a summary of the isolated yeasts from wooden cask 2608 at the different times of sampling during fermentation.

In addition, an organism was isolated from that sampling with a remarkable restriction pattern using enzyme *Hae*III. This pattern was a mixture of the patterns type I and type II of the *sensu stricto*-strains belonging to the genera *Saccharomyces* (cf. Tab. 3.2). Single cell separation was performed in order to ensure purity of these cultures. Afterwards, this organism was identified via further restriction analysis according to Gonzàlez et al. (2006) as a hybrid of *S. cerevisiae* x

S. kudriavzevii x *S. bayanus* (cf. Tab. 3.4). From day 54 on this hybrid was the most represented yeast strain in the wooden cask 2608.

Table 3.2: Restriction fragment patterns of the ITS region with enzyme *Hae*III from wine-relevant *Saccharomyces* species

Restriction pattern	Fragments using *Hae*III in bp rounded to 10	*Saccharomyces* species
I	860 740 650 500 240 170	*S. cerevisiae*, *S. paradoxus*
II	580 320 240 170	*S. bayanus, S. kudriavzevii*

3.1.3 Succession of yeasts in the second vintage 2012/2013

Right after the filling of the cask yeasts of the genera *Hanseniaspora*, *Cryptococcus*, *Pichia, Metschnikowia, Rhodotorula, Saccharomycopsis* and *Aureobasidium* were found (cf. Tab. 3.7) in steel cask S 101. The species *Hanseniaspora uvarum* was the most frequently found yeast at day 1 with a cell count of 10^4 cells/mL, followed by *Cryptococcus macerans and Rhodotorula glutinis* with about 10^3 cells/mL. The cell titre was determined via colony forming units. No yeast species of the genus *Saccharomyces* was found on day 1, but from day 13 the species *Saccharomyces bayanus* dominated the fermentation with a cell count of up to 10^5 cells/mL. From day 40 there was a stuck of fermentation and it did not continue until the cask was stopped on day 78 by sulphuring. The hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* was not found in this cask before or during the stuck. Table 3.5 shows a summary of the isolated yeasts from steel cask S 101 at the different dates of sampling during fermentation. Fig. 3.3 shows the characteristic restriction patterns of the yeasts isolated during the second vintage in 2012/2013 from cask S 101.

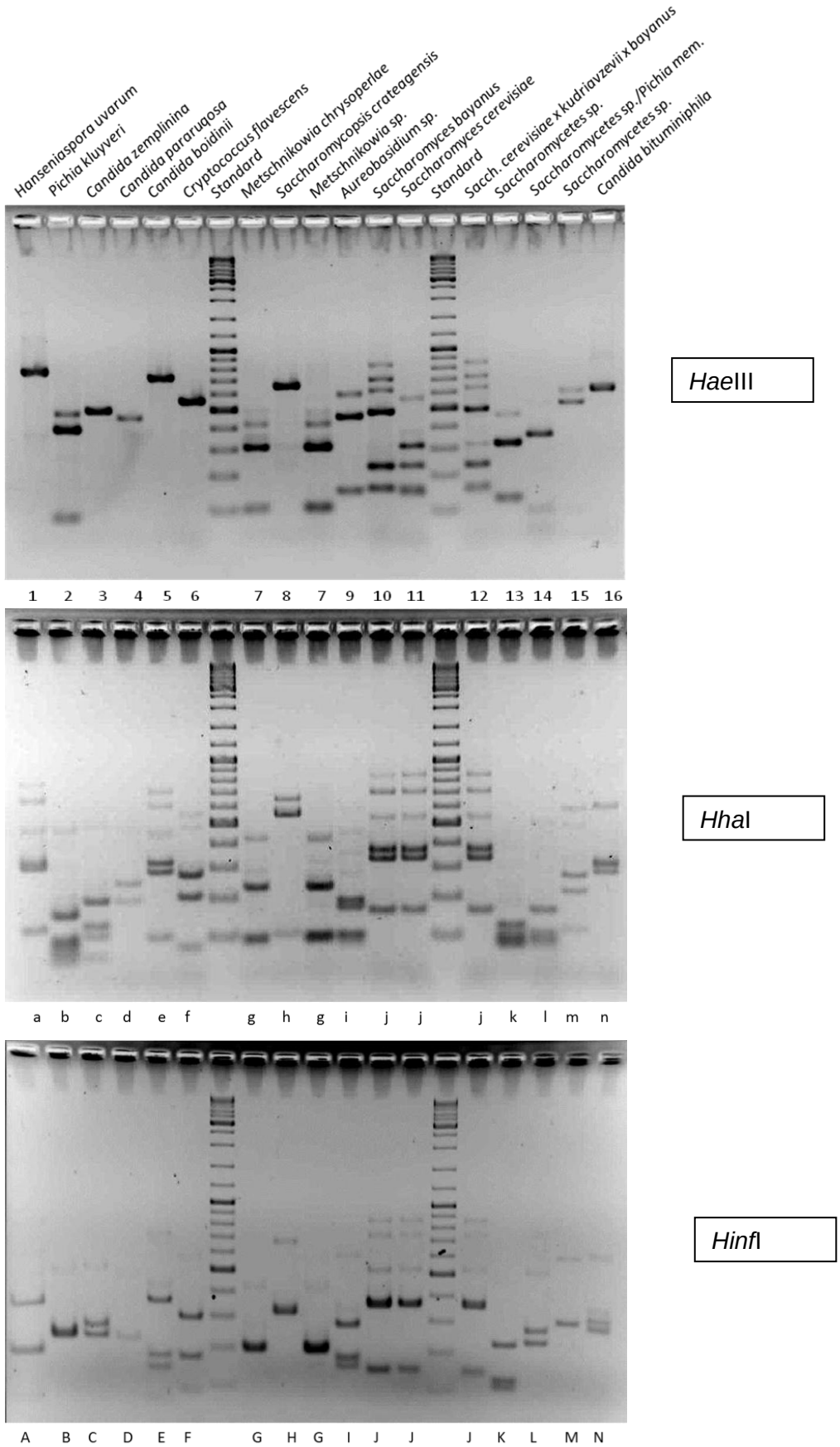
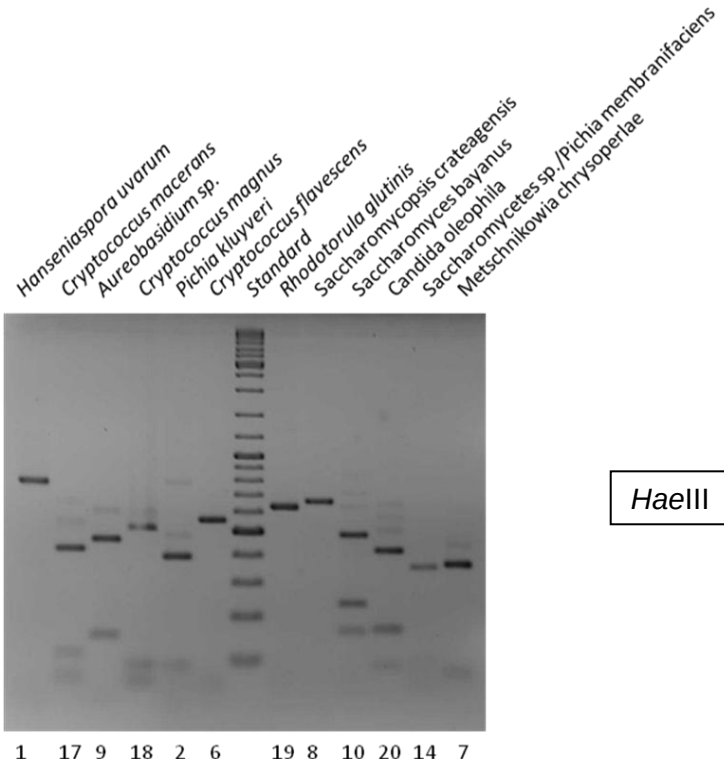


Fig. 3.2: Restriction patterns of isolated yeast strains from the first vintage 2011/2012
Total isolates from two casks; restriction patterns of the ITS region using restriction enzymes *Hae*III, *Hha*I and *Hinf*I; the characteristic combination of patterns for each yeast strain is given in Tab. 3.5 and 3.6



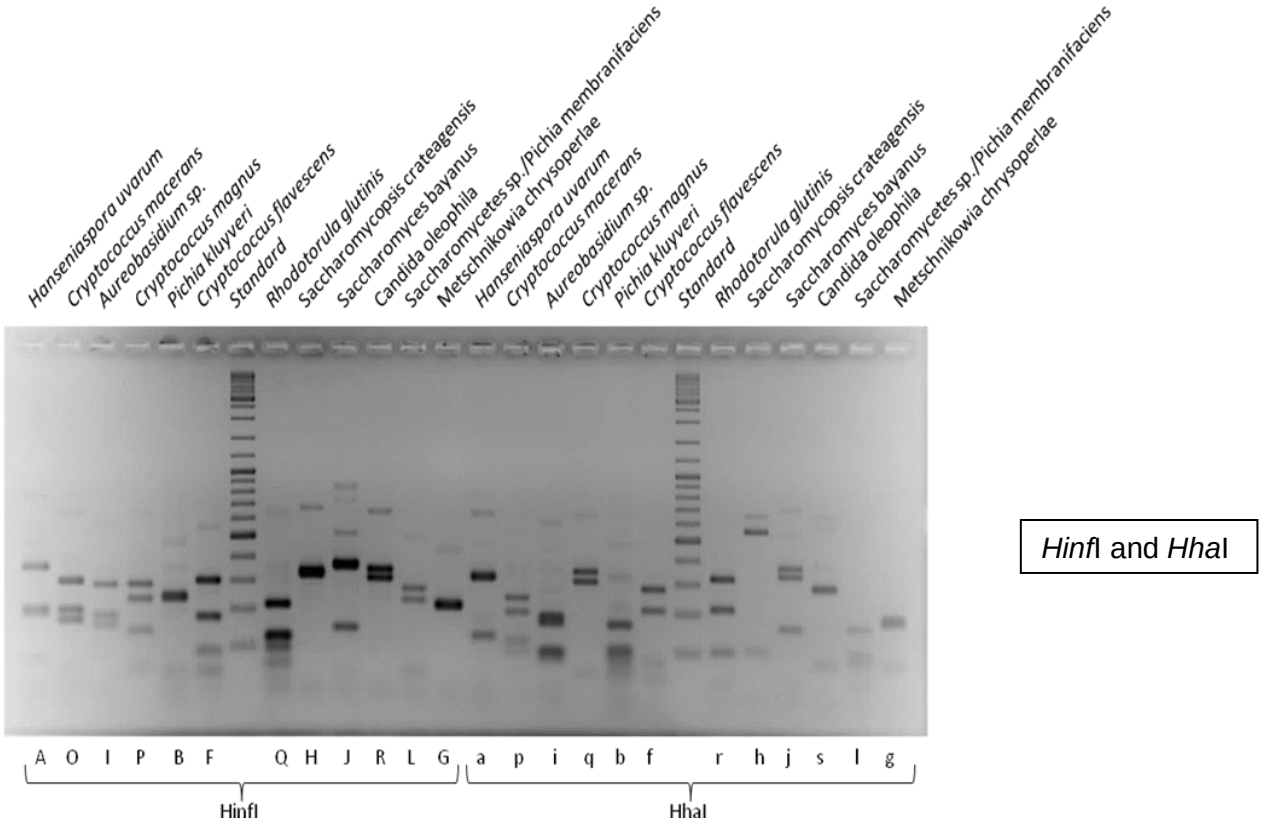


Fig. 3.3: Restriction patterns of isolated yeast strains from the second vintage in 2012/2013
Total isolates from one cask; restriction patterns of the ITS region using restriction enzymes *Hae*III, *Hha*I and *Hinf*I; the characteristic combination of patterns for each yeast strain is given in Tab. 3.7.

Tab. 3.3: Restriction patterns of the isolated yeast strains from vintage 2011/2012 and vintage 2012/2013.
Based on restriction patterns of the ITS region using the restriction enzymes *Hae*III, *Hha*I and *Hinf*I (the characteristic combination of patterns for each yeast strain is given in Tab. 3.5 – 3.7 and shown in Fig. 3.2 and Fig 3.3)

Enzyme *Hae*III		Enzyme *Hha*I		Enyzme *Hinf*I	
Restriction pattern	**Restriction fragment length [bp]**	**Restriction pattern**	**Restriction fragment length [bp]**	**Restriction pattern**	**Restriction fragment length [bp]**
1	780	a	780 630 460 310 110	A	350 200
2	480 390 80	b	460 150 80	B	500 240
3	500	c	190 120 100 40	C	520 270 240
4	460	d	240 190	D	230
5	730	e	710 600 450 320 290 100	E	730 420 360 180 150
6	550	f	560 480 270 200 70	F	570 300 180 100
7	420 310 110	g	420 230 90	G	410 200
8	660 320	h	650 550 100	H	680 320
9	600 470 160	i	460 370 190 170 100	I	570 270 180 150
10	860 740 650 500 240 170	j	860 710 540 380 340 170	J	820 710 500 340 140
11	580 320 240 170	k	120 90	K	210 120 100
12	860 740 650 500 320 240 170	l	520 170 90	L	480 250 210
13	480 340 140	m	580 500 270 220 110	M	550 270
14	380 100	n	600 320 290	N	570 270 250
15	640 560	o	300 200 180	O	300 200 180
16	650	p	250 210 140 100	P	300 250 150
17	700 550 450 120 80	q	650 350 300	Q	120 100 50
18	600 540 100 50	r	330 210 100	R	650 360 320
19	640	s	650 550 280 80		
20	620 550 490 420 160 100				

Tab. 3.4: RFLP analysis of different gene regions of the DNA from yeasts of the genus *Saccharomyces* and isolated hybrids.
HL78, 82.14, 96.1: isolates from three different times of sampling after stuck fermentation; *GSY1*, *OPY1*, *CYR1*, *CAT8*, *MET6*: nuclear genes, located in different chromosomes; A/B/C/D: restriction fragment length patterns of different gene regions and restriction enzymes; AB/AC/ABC: combinations of restriction patterns

Gene and restriction enzyme / Species/ Strain	*GSY1*	*OPY1*		*CYR1*		*CAT8*			*MET6*	
	*Msp*I	*Scr*FI	*Hae*III	*Msp*I	*Hae*III	*Hha*I	*Msp*I	*Hinf*I	*Hae*III	*Scr*FI
S. kudriavzevii CBS 8840^T	350 300 200	750	510 250	(570) 460 (150)	290 200 170 100	590 200	490 240	620	680	680
	A	A	A	A	A	A	A	A	A	A
S. bayanus DSM 70412^T	400 350	560 310 260 220	760 400 350	570	570	750	320 280 240	620	480 200	680 610
	B	B	B	B	B	B	B	A	B	B
S. cerevisiae DSM 70449^T	600 200	460 310	760	400 200	570	750	690	450 200	680	680
	C	C	C	C	B	B	C	B	A	A
S. paradoxus CBS 432^T	450 350	460 310	510 250	400 200	390 220	750	750	620	680	680
	D	C	A	C	D	B	D	A	A	A
Isolate Strain HL 78	600 350 300	750 460 310	760 400 250	570 460 400 150	570 290 200 170	750 590 200	690 490 240	620	680	680
	AC	AC	ABC	ABC	AB	AB	AC	A	A	A
Isolate Strain 82.14	600 350 300	750 460 310	760 400 250	570 460 400 150	570 290 200 170	750 590 200	690 490 240	620	680	680
	AC	AC	ABC	ABC	AB	AB	AC	A	A	A
Isolate Strain 96.1	600 350 300	750 460 310	760 510 250	570 460 400 150	570 290 200 170	750 590 200	690 490 240	620	680	680
	AC	AC	AC	ABC	AB	AB	AC	A	A	A

Table 3.5: Succession of the yeast isolates in steel cask 3007 during vintage 2011/2012 (table 3.5 shows an approximation of the cell titer determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; - : not found)

Cell titer (CFU)											
Day of fermentation						Organism	Medium[d]	Length[a] (bp)	Restriction patterns[b]		
1	15	29	43	57					*Hae*III	*Hha*I	*Hinf*I
10^5	10^5	-	-	-		*Hanseniaspora uvarum*	GYP, TJ, TSA, YPM, PDA, GPYA,MRS	800	1	a	A
10^5	10^4	-	-	-		*Pichia kluyveri*	GPYA, PDA, GYP, YPM	500	2	b	B
10^6	-	-	-	-		*Candida zemplinina*	GPYA, PDA, GYP	500	3	c	C
10^5	-	-	-	-		*Cryptococcus flavescens*	GYP	550	6	f	F
10^4	10^3	-	-	-		*Metschnikowia chrysoperlae*	GYP	450	7	g	G
10^4	-	-	-	-		*Metschnikowia sp.*	GPYA, PDA, GYP	450	7	g	G
10^4	-	-	-	-		*Aureobasidium sp.*	GYP	550	9	i	I
-	10^7	10^7	10^7	10^5		*Saccharomyces bayanus*	GPYA, PDA, GYP	900	10	j	J
-	10^4					*Saccharomycetes sp.*	GYP	450	13	k	K
-	10^2	10^3	10^2	10^3		*Candida boidinii*	YPM, TJ, MRS, GYP, TSA	750	5	e	E
-	10^2					*Saccharomycopsis crateagensis*	GPYA, YPM, TJ	700	8	h	H
-	-	-	10^2	10^2		*Saccharomycetes sp. / Pichia membranifaciens* (resp.)[c]	GYP, TSA	500	14	l	L
-	-	-	-	10^2		*Candida pararugosa*	TSA	450	4	d	D

[a]length of the PCR product from the amplified ITS region

[b]restriction patterns according to table 3.3

[c]distinction not possible

[d] cf. 2.6 Culture media

Table 3.6: Succession of the yeast species in wooden cask No. 2608 during vintage 2011/2012 (table 3.6 shows an approximation of the cell titer determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; - : not found)

Cell titer (CFU)	Day of fermentation					Organism	Medium[d]	Length[a] (bp)	Restriction patterns[b]		
	26	40	54	82	96				*Hae*III	*Hha*I	*Hinf*I
	10^6	10^5	10^4	-	10^5	*Saccharomyces bayanus*	PDA, GYP, GPYA	900	10	j	J
	-	-	10^0	10^1	-	*Saccharomycetes sp. / Pichia membranifaciens* (resp.)[c]	GYP	500	14	l	L
	-	-	10^4	-	10^5	*Saccharomyces cerevisiae*	PDA	900	11	j	J
	-	-	10^5	10^6	10^6	*S. cerevisiae* x S. *kudriavzevii* x *S. bayanus*	GYP, GPYA, PDA	900	12	j	J
	-	-	10^1	-	-	*Saccharomycetes sp.*	YPM	650	15	m	M
	-	-	10^1	-	-	*Candida bituminiphila*	TSA	650	16	n	N

[a]length of the PCR product from the amplified ITS region

[b]Restriction patterns according to table 3.3

[c]distinction not possible

[d] cf. 2.6 Culture media

Table 3.7: Succession of the yeast species in steel cask No. S 101 during vintage 2012/2013 (table 3.7 shows an approximation of the cell titer determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; - : not found)

Cell titer (CFU)	Day of fermentation							Organism	Medium[d]	Length[a] (bp)	Restriction patterns[b]		
	1	13	27	40	51	62	78				*Hae*III	*Hha*I	*Hinf*I
	10^4	10^5	-	-	-	-	-	*Hanseniaspora uvarum*	GYP, PDA, TJ, GPYA, YPM	800	1	a	A
	10^3	-	-	-	-	-	-	*Cryptococcus macerans*	GYP, PDA	700	17	p	O
	10^2	-	-	-	-	-	-	*Aureobasidium sp.*	GYP, PDA, GPYA	550	9	i	I
	10^1	10^4	-	-	-	-	-	*Pichia kluyveri*	GYP, GPYA, PDA	500	2	b	B
	10^2	-	-	-	-	-	-	*Cryptococcus flavescens*	PDA	550	6	f	F
	10^2	-	-	-	-	-	-	*Cryptococcus magnus*	GYP, PDA	690	18	q	P
	10^1	-	-	-	-	-	-	*Saccharomycopsis crateagensis*	GPYA	700	8	h	H
	10^3	-	-	-	-	-	-	*Rhodotorula glutinis*	GPYA	700	19	r	Q
	-	10^5	10^5	10^5	10^4	10^3	10^3	*Saccharomyces bayanus*	GYP, PDA, GPYA	900	10	j	J
	-	10^2	-	-	-	10^3	-	*Saccharomycetes sp./Pichia membranifaciens* (resp.)[b]	GYP	500	14	l	L
	-	10^4	-	-	-	-	-	*Candida oleophila*	GYP, GPYA	800	20	s	R
	-	-	-	-	-	10^3	-	*Metschnikowia chrysoperlae*	GYP, PDA, GPYA	450	7	g	G

[a]length of the PCR product from the amplified ITS region

[b]restriction patterns according to table 3.3

[c]distinction not possible

[d] cf. 2.6 Culture media

3.2 Succession of bacteria

Bacteria were isolated from must in order to find out if stuck fermentation was influenced by bacterial succession. The bacteria strains were identified based on restriction patterns of the 16s rDNA using the restriction enzymes *BsuR*I and *Hpa*I (cf. Fig. 3.4 and 3.5, Tab. 3.8 - 3.11).

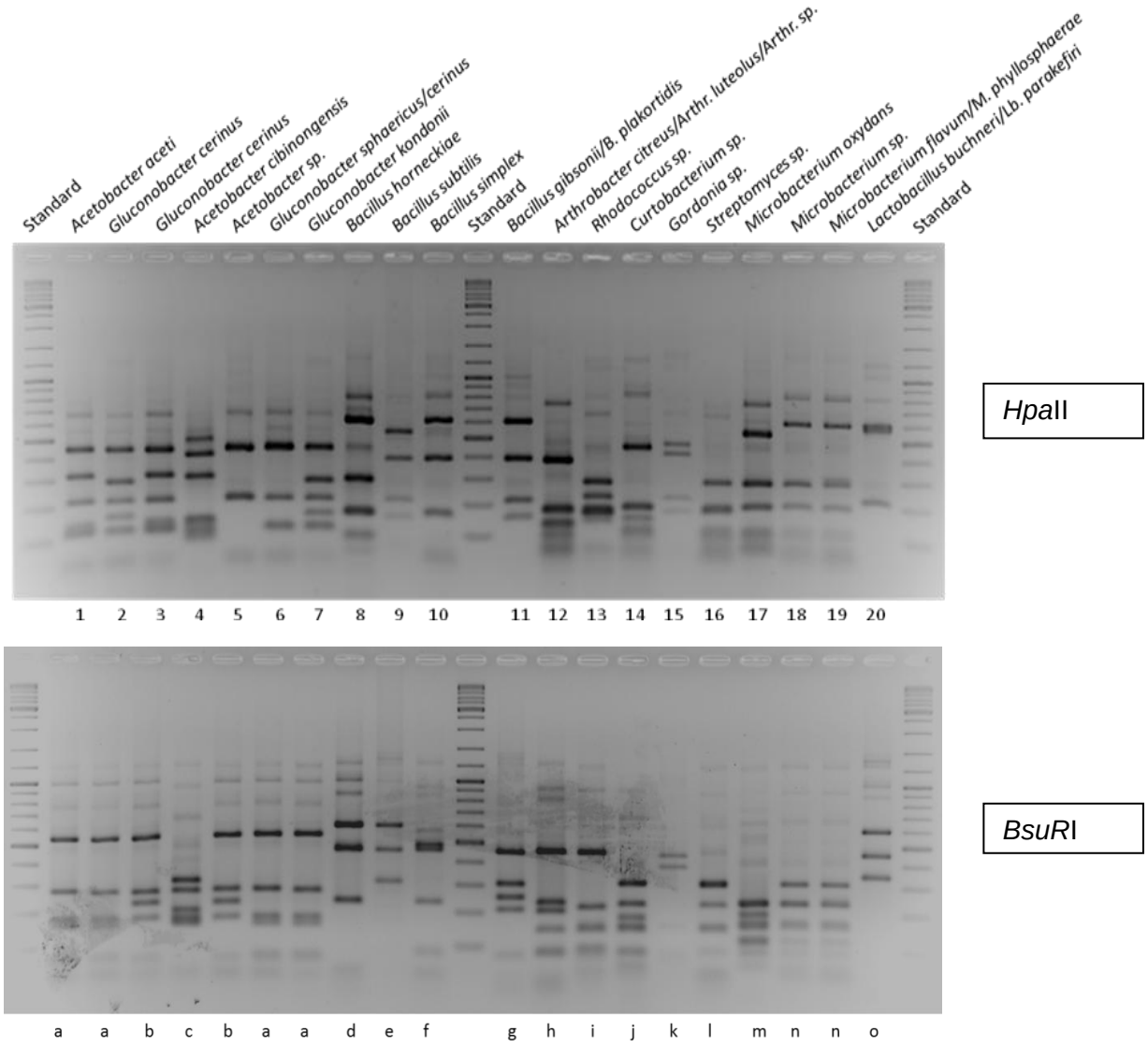


Fig. 3.4: Restriction patterns of isolated bacteria strains from the first vintage 2011/2012. Total isolates from two casks; restriction patterns of 16S rDNA using restriction enzymes *Hpa*II and *BsuR*I; the characteristic combination for each strain is given in Tab. 3.9 and 3.10.

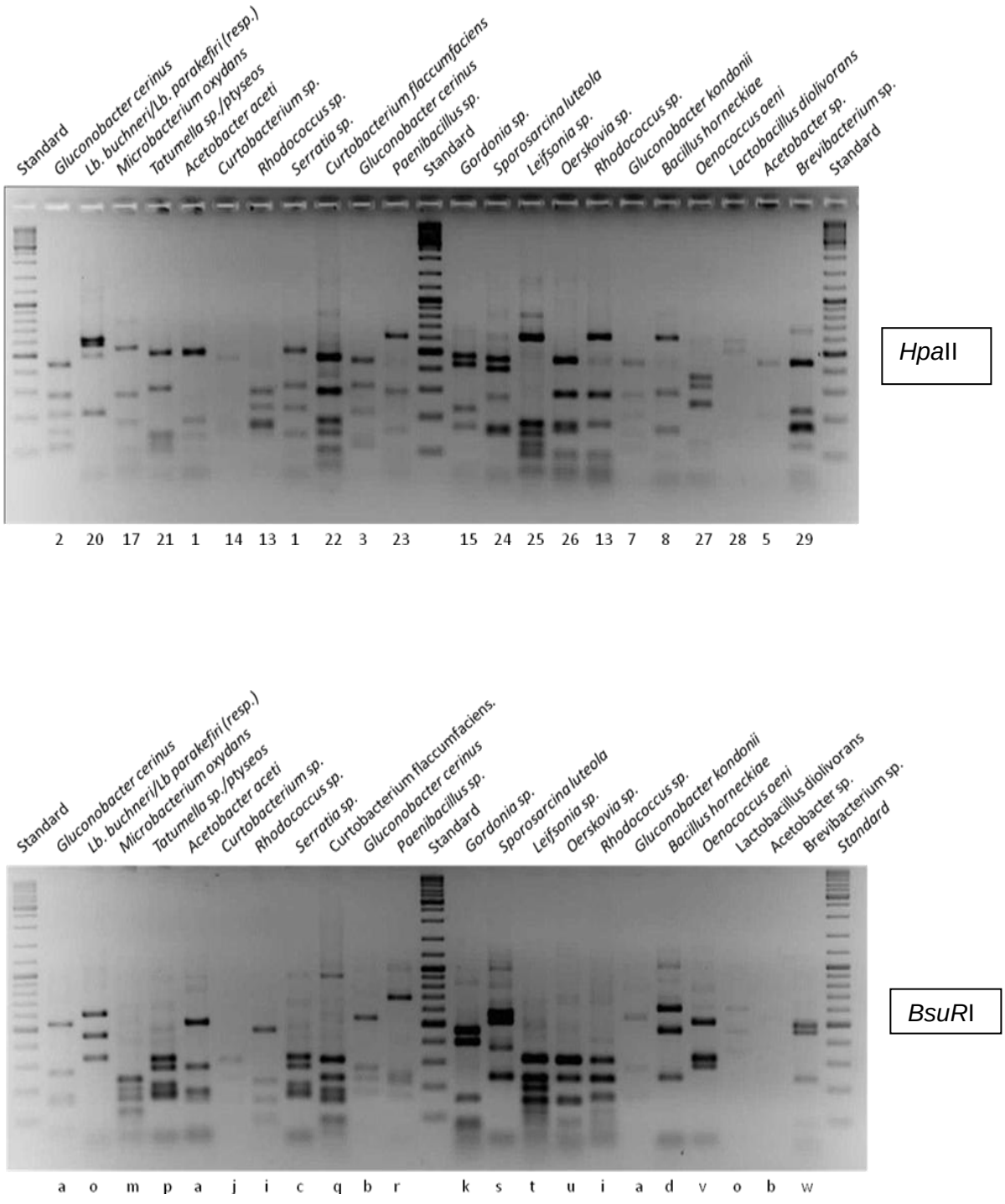


Fig. 3.5: Restriction patterns of isolated bacteria strains from the second vintage 2012/2013. Total isolates from one cask; restriction patterns from 16*S* rDNA using restriction enzymes *Hpa*II and *BsuR*I; the characteristic combination for each strain is given in Tab. 3.11.

Tab. 3.8: Restriction patterns for bacteria strains isolated in two vintages.
Based on restriction patterns of the 16S rDNA region using the restriction enzymes *Hpa*II and *BsuR*I (the characteristic combination of patterns for each bacteria species is given in Tab. 3.9 - 3.11 and shown in Fig. 3.4 and 3.5)

Enzyme *Hpa*II		**Enzyme *Bsur*I**	
Restriction pattern	**Restriction fragment length [bp]**	**Restriction pattern**	**Restriction fragment length [bp]**
1	670 450 310 240 140 120	a	1200 1040 780 540 280 180 160
2	670 450 290 240 160 120	b	1200 1040 780 540 280 230 180
3	670 450 310 240 140 120	c	1200 950 670 490 410 320 280 210 180
4	500 420 320 160 110	d	1250 1050 870 610 470 240
5	670 450 240	e	1310 1020 610 470 320
6	670 560 450 240 140	f	1250 950 850 570 490 460 240
7	670 560 450 300 240 180 140	g	450 310 250 210 80
8	800 700 600 450 310 190 110	h	1220 920 810 450 240 210 150 90
9	540 400 230 160	i	910 450 220 160 90
10	1000 800 620 540 400 180	j	910 670 310 230 190 150 90
11	1000 800 620 400 220 170	k	430 380
12	740 380 190 150 100 60	l	1190 840 590 440 300 230 160
13	780 660 450 290 240 190	m	1190 700 610 530 400 230 190 160 110
14	1240 840 450 200 160 120	n	1190 880 630 300 230 160
15	470 420 230 180	o	1270 1190 960 820 560 430 330
16	720 630 290 190 110	p	320 300 200 160
17	740 600 530 440 280 200 100 60	q	950 300 250 200 160 100
18	790 580 280 200 110	r	700 240 180
19	790 580 290 280 200 110	s	1000 800 580 520 360 250
20	1200 1000 760 560 540 210	t	320 250 200 160
21	500 300 150 100	u	300 240 160 80
22	480 300 200 160 100 50	v	800 510 320 290
23	620 300 150	w	500 460 240
24	450 400 280 160		
25	800 600 180 160 120 100 50		
26	450 290 180 100 50		
27	350 300 250		
28	600 500		
29	680 450 230 180 140		

3.2.1 Succession of bacteria in the first vintage 2011/2012

On day 1 acetic acid bacteria of the species *Acetobacter aceti* and *Gluconobacter cerinus* were isolated from the must with cell counts of about 10^5 cells/mL in steel cask 3007. During the fermentation period, *Gluconobacter cerinus* was found with cell counts of 10^2 cells/mL to 10^4 cells/mL. Additionally bacteria of the species *Bacillus horneckiae* (up to 10^2 cells/mL), *Bacillus simplex* (10^1 cells/mL), *Bacillus subtilis* (10^4 cells/mL) and *Bacillus gibsonii* or *Bacillus plakortidis* (10^0 cells/mL), respectively, could be isolated. It has to be assumed that those bacilli occurred most likely as spores in must and young wine, because the isolates were not able to grow in must later on. *Acetobacter cibinongensis* was only found in the sample from day 15 with a cell count of 10^2 cells/mL. Strains of the genera *Microbacterium* and *Gordonia* were detected in lower cell counts of 10^1/mL to 10^2/mL, as well as *Streptomyces* with 10^1cells/mL. Enrichment cultures with liquid tomato juice medium were used in order to prove the growth of lactic acid bacteria with low cell counts. In those enrichment cultures of steel cask 3007 bacteria of the species *Oenococcus oeni* could be identified via multiplex PCR from day 15 on. Table 3.9 shows a summary of the isolated bacteria from steel cask 3007 at the different times of sampling during fermentation.

The cask 2608 was sampled from day 26 on because at that time sluggish fermentation was observed. At this first time of sampling acetic acid bacteria of the species *Gluconobacter cerinus* and *Acetobacter sp.* were isolated with cell counts of 10^5 cells/mL. *Gluconobacter cerinus* was found continuously until day 82, cell counts decreased from 10^4 cells/mL to 10^2 cells/mL. The *Acetobacter sp.* strain was only found on day 40 again with a cell count of 10^3 cells/mL. Additionally isolates of *Gluconobacter sphaericus* or *cerinus,* respectively, and *Acetobacter aceti* were found at that time with cell counts of 10^2 cells/mL each. Concerning lactic acid bacteria*, only Lactobacillus buchneri* or *Lb*. *parakefiri* could be isolated on day 96 (10^2 cells/mL). But in the enrichment cultures of wooden cask 2608 *Oenococcus oeni* and *Pediococcus damnosus* could be identified via multiplex PCR. Strains of the genus *Microbacterium* were found on day 40. Table 3.10 shows a summary of the isolated bacteria from wooden cask 2608 at the different times of sampling during fermentation.

3.2.2 Succession of bacteria in the second vintage 2012/2013

On day 1 acetic acid bacteria of the species *Acetobacter aceti* and *Gluconobacter cerinus* were isolated from the must of steel cask S 101 as well as strains of the genera *Microbacterium*, *Tatumella*, *Curtobacterium*, *Rhodococcus*, *Lactobacillus* and *Serratia*. Later on during fermentation, strains of the genera *Paenibacillus*, *Gordonia*, *Sporosarcina*, *Leifsonia*, *Oerskovia* and *Brevibacterium* were isolated, as well as the lactic acid bacteria species *Oenococcus oeni* and *Lac-*

tobacillus diolivorans. Compared to steel cask 3007 from vintage 2011/2012, a higher variety of species was observed in this cask S 101 (cf. Tab. 3.11).

3.3 Identification of lactic acid bacteria via multiplex PCR

Using the multiplex PCR developed by Petri et al. (2012) lactic acid bacteria could be identified in the enrichment cultures from the different samples of steel cask 3007 and wooden cask 2608 from vintage 2011/2012 as well as from cask S 101 from vintage 2012/2013, although those isolates did not show sufficient cell titres in must to be observed on the culture media. For each sample, one mixture with primer-mix A and one mixture with primer-mix B was prepared and amplified. The products of the PCR were checked with gel electrophoresis. DNA-mix A and DNA-mix B, containing DNA of the lactic acid bacteria most common in wine, served for comparison. Figure 3.6 presents an agarose gel electrophoresis with the products of the multiplex PCR from vintage 2011/2012.

In the enrichment cultures of steel cask 3007 from the first vintage, lactic acid bacteria of the species *Oenococcus oeni* were found in all samples from day 15 on (cf. Tab.3.9). *Oenococcus oeni* was found in all enrichment cultures from wooden cask 2608 as well. In addition, bacteria of the species *Pediococcus damnosus* were found in the enrichment cultures of this wooden cask from day 40 until the end of the fermentation (cf. Tab. 3.10).

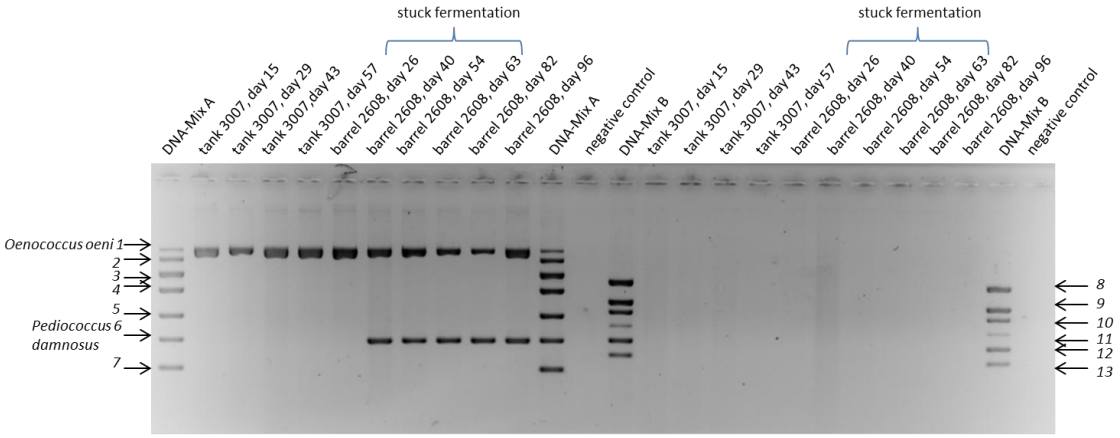


Fig.3.6: Identification of lactic acid bacteria from vintage 2011/2012 via multiplex PCR
The position of the characteristic band for each organism is presented in the DNA primer mixtures A and B; the brackets mark the beginning and the end of the stuck fermentation; 1: *Oenococcus oeni*, 2: *Lactobacillus brevis*, 3: *Lactobacillus plantarum*, 4: *Leuconostoc mesenteroides*, 5: *Weissella parameseteroides*, 6: *Pediococcus damnosus*, 7: *Pediococcus parvulus*, 8: *Lactobacillus buchneri*, 9: *Pediococcus acidilactici*, 10: *Lactobacillus hilgardii*, 11: *Pediococcus inopinatus*, 12: *Lactobacillus curbatus*, 13: *Pediococcus pentosaceus*

In the enrichment cultures of steel cask S 101 from vintage 2012/2013, isolates of the species *Oenococcus oeni* and *Lactobacillus buchneri* could be identified via multiplex PCR. Table 3.11 compiles a summary of the isolated bacteria from steel cask S 101 at the different times of sampling during fermentation.

Table 3.9: Succession of the bacteria in steel cask 3007 during vintage 2011/2012 (table 3.9 shows an approximation of the cell titre determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; * identification via Multiplex-PCR after enrichment culture; - : not found)

Cell titer (CFU)	Day of fermentation					Organism	Medium[d]	Length[a] (bp)	Restriction patterns[b]	
	1	15	29	43	57				*Hpa*II	*BsuR*I
	10^5	-	-	-	10^2	*Acetobacter aceti*	MRS, YPM	1400	1	a
	10^5	10^2	10^2	10^4	10^3	*Gluconobacter cerinus*	YEP, YPM, TSA, MRS, TJ	650	2/3	a/b
	-	10^1	10^2	10^1	10^1	*Bacillus horneckiae*	TSA	650	8	d
	-	10^2	-	-	-	*Acetobacter cibinongensis*	YPM	650	4	c
	-	10^1	-	-	-	*Microbacterium oxydans*	TSA, YPM	650	17	m
	-	10^1	-	-	-	*Arthrobacter citreus/A. luteolus/A. sp.* (resp.)[c]	TSA	650	12	h
	-	10^1	-	-	-	*Rhodococcus sp.*	TSA, YPM	1400	13	i
	-	10^1	10^1	10^1	10^2	*Microbacterium sp.*	YPM, TSA	1400	18	n
	-	10^1	-	-	-	*Curtobacterium sp.*	YPM	650	14	j
	-	10^2	-	-	-	*Gluconobacter kondonii*	YPM	650	7	a
	-	-	10^4	-	-	*Bacillus subtilis*	TSA	650	9	e
	-	-	10^1	-	-	*Bacillus simplex*	TSA	650	10	f
	-	-	10^0	-	-	*Bacillus gibsonii/Bacillus plakortidis* (resp.)[c]	TSA	650	11	g
	-	-	10^1	-	10^1	*Microbacterium flavum/M. phyllosphaerae* (resp.)[c]	TSA	650	19	n
	-	10^2	-	-	-	*Gordonia sp.*	YPM	650	15	k
	-	10^1	-	-	-	*Streptomyces sp.*	YPM	1400	16	l
	-	*	*	*	*	*Oenococcus oeni*	Enrichment TJ-liquid			

[a]length of the PCR product from the amplified 16S rDNA

[b]restriction patterns according to table 3.8

[c]distinction not possible

[d] cf. 2.6 Culture media

Table 3.10: Succession of the bacteria in wooden cask 2608 during vintage 2011/2012. (table 3.10 shows an approximation of the cell titre determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; * identification via Multiplex-PCR after enrichment culture; - : not found)

Cell titer (CFU)	Day of fermentation					Organism	Medium[d]	Length[a] (bp)	Restriction patterns[b]	
	26	40	54	82	96				*Hpa*II	*Bsu*RI
	10^5	10^4	10^4	10^2	-	*Gluconobacter cerinus*	MRS, YEP, TSA, YPM, GPYA, TJ	1400	2/3	a/b
	10^5	10^3	-	-	-	*Acetobacter sp.*	MRS, TSA, GPYA, TJ, PDA	1400	5	b
	-	10^2	-	-	-	*Gluconobacter sphaericus/cerinus* (resp.)[c]	TJ	1400	6	a
	-	10^2	-	-	-	*Acetobacter aceti*	MRS	1400	1	a
	-	10^4	-	-	-	*Microbacterium sp.*	YEP, YPM, TSA	1400	18	n
	-	-	-	-	10^2	*Lactobacillus buchneri/parakefiri* (resp.)[c]	TJ	1400	20	o
	*	*	*	*	*	*Oenococcus oeni*	Enrichment TJ-liquid			
		*	*	*	*	*Pediococcus damnosus*	Enrichment TJ-liquid			

[a]length of the PCR product from the amplified 16S rDNA

[b]restriction patterns according to table 3.8

[c]distinction not possible

[d] cf. 2.6 Culture media

Table 3.11: Succession of the bacteria in steel cask S 101 during vintage 2012/2013 (table 3.11 shows an approximation of the cell titer determined via colony forming units, correlating to the day of fermentation; for each organism the media it was isolated from are shown; * identification via Multiplex-PCR after enrichment culture; - : not found)

Cell titer (CFU)												
Day of fermentation							**Organism**	**Medium[d]**	**Length[a] (bp)**	**Restriction patterns[b]**	
1	**13**	**27**	**40**	**51**	**62**	**78**				***Hpa*II**	***Bsur*I**
10^2	*	-	-	-	-	-	*Lactobacillus buchneri/parakefiri*	MRS, TJ, GYP	1400	20	o
10^3	-	-	10^3	-	-	-	*Acetobacter aceti*	TSA, TJ	1400	1	a
-	-	-	-	10^2	10^2	-	*Bacillus horneckiae*	TSA	650	8	d
10^4	-	-	-	-	10^3	10^3	*Gluconobacter cerinus*	GYP, PDA, GPYA, TSA, YPM, MRS, TJ	650	3/2	b/a
-	-	-	-	-	10^3	10^3	*Acetobacter sp.*	YPM, GYP, PDA	1400	5	b
10^2	-	-	-	-	-	-	*Microbacterium oxydans*	YPM, TSA	650	17	m
10^4	-	10^3	-	-	-	-	*Microbacterium sp.*	YPM, TSA	1400	18	n
10^2	-	-	-	-	-	-	*Tatumella sp./ptyseos*	YPM	1300	21	p
10^2	-	-	-	-	-	-	*Curtobacterium sp.*	TSA	650	14	j
10^1	-	10^2	-	-	-	-	*Rhodococcus sp.*	TSA	1400	13	i
10^1	-	-	-	-	-	-	*Serratia sp.*	TSA	1300	1	c
10^1	-	-	-	-	-	-	*Curtobacterium flaccumfaciens*	TSA	1400	22	q
-	10^2	-	-	-	-	-	*Paenibacillus sp.*	YPM	1300	23	r
-	10^2	10^2	-	-	-	-	*Gordonia sp.*	YPM, TSA	650	15	k
-	10^2	-	-	-	-	-	*Sporosarcina luteola*	TSA	1300	24	s
-	-	10^3	-	-	-	-	*Leifsonia sp.*	YPM, TSA	1400	25	t
-	-	10^2	-	-	-	-	*Oerskovia sp.*	TSA	1400	26	u
-	-	-	-	10^4	-	-	*Gluconobacter kondonii*	GYP	650	7	a
*	*	-	*	10^2	*	*	*Oenococcus oeni*	TJ	1400	27	v
-	-	-	-	-	10^2	-	*Lactobacillus diolivorans*	TJ	1400	28	o
-	-	-	-	-	-	10^5	*Brevibacterium sp.*	GYP	1400	29	w

[a]length of the PCR product from the amplified 16S rDNA

[b]restriction patterns according to table 3.8

[c]distinction not possible

[d] cf. 2.6 Culture media

3.4 Characterization of the isolated yeasts

For further characterization of the isolated wine yeast species *S. bayanus* (strain HL 77) and the triple hybrid *S. cerevisiae* x S. *kudriavzevii* x S. *bayanus* (strain HL 78) were cultivated in synthetic B-medium with different concentrations of sugars, amino acids and ammonium sulfate.

3.4.1 Sugar consumption and growth curves at a glucosee:fructose ratio of 1:1

S. bayanus (strain HL 77) reached an OD of 0.5 after 7 days of growth (cf. Fig. 3.7), whereas the triple hybrid *S. cerevisiae* x S. *kudriavzevii* x S. *bayanus* strain HL 78 needed 10 days for the same OD (cf. Fig. 3.8). The hybrid reacheed a stationary phase after 14 days which lasted 4 days until OD increased again. The commercial starter culture *S. cerevisiae* (Fermivin®, DSM Food Specialties, Delft/The Netherlands) which was used as a control reached an OD of 0.46 after 15 days (cf. Fig. 3.9).

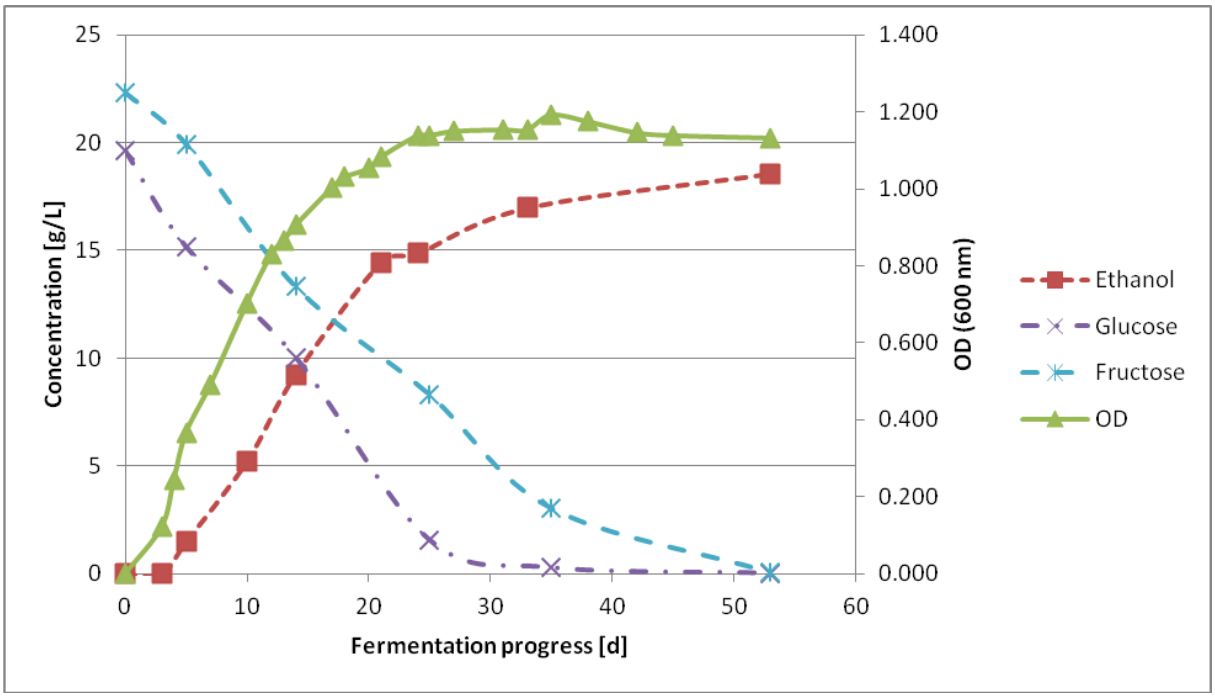


Fig. 3.7: Sugar consumption and ethanol production of *S. bayanus* strain HL 77

All three tested yeast strains used glucose better than fructose, but *S. bayanus* and the triple hybrid degraded both sugars from the beginning and *S. cerevisiae* showed delayed sugar consumption for 10 days. Although the triple hybrid showed delayed growth compared to *S. bayanus*, this strain was able to use fructose significantly faster, especially in the second half of the fermentation (cf. Tab. 3.14). At the end of the fermentation, the highest ethanol concentration of 21.2 g/L (2.65 vol%) was produced by the *S. cerevisiae* strain (cf. Fig.3 .9) and no residual sugar was left in any of the samples (cf. Tab. 3.12, 3.14, 3.16).

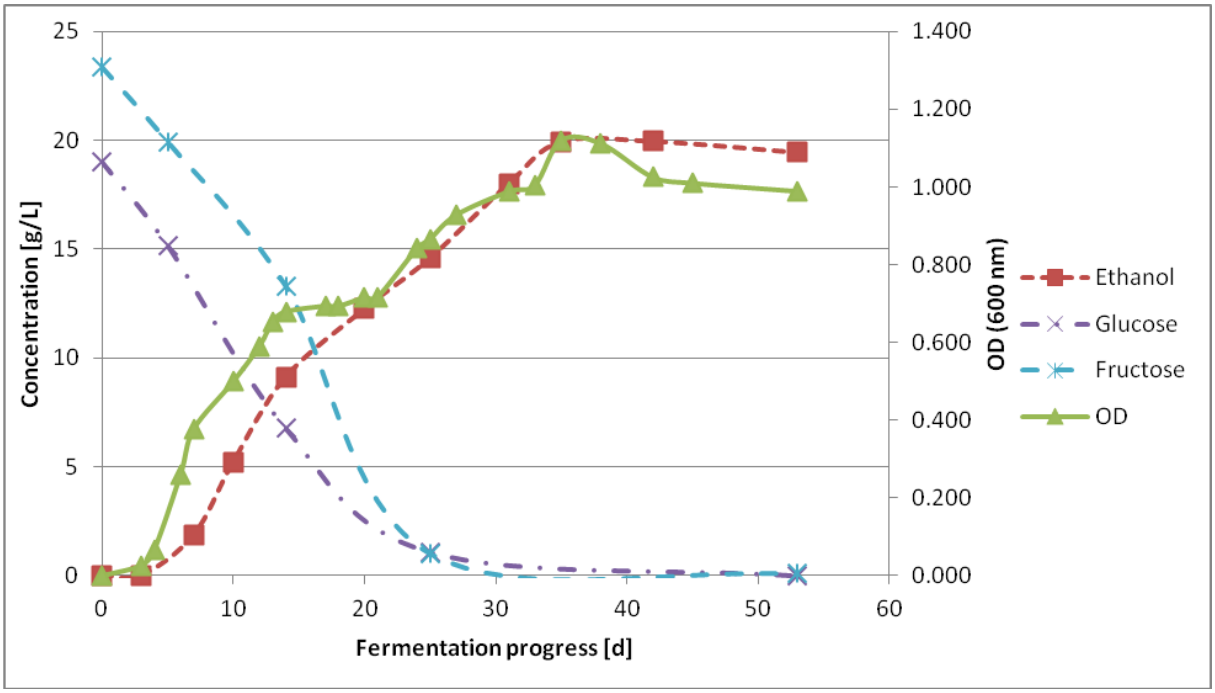


Fig. 3.8: Sugar consumption and ethanol production of the triple hybrid *S. cerevisiae* x S. *kudriavzevii* x S. *bayanus* strain HL 78

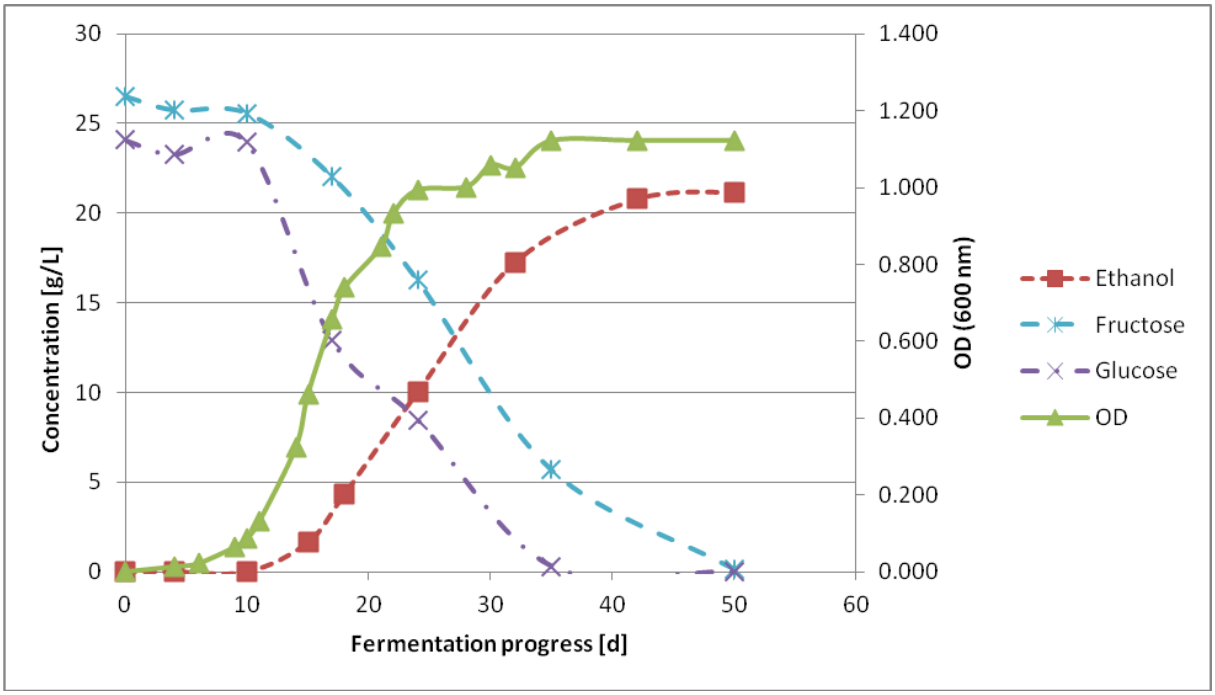


Fig. 3.9: Sugar consumption and ethanol production of *S. cerevisiae* (Fermivin®)

Tab. 3.12: Glucose and fructose concentration [g/L] in synthetic B-medium (cf. 2.6.3) inoculated with *S. bayanus* strain HL 77 during fermentation (cf. Fig. 3.7)

Days of fermentation	0	5	14	25	35	53
Glucose	19.63	15.17	10.04	1.56	0.27	0.01
Fructose	22.31	19.93	13.32	8.32	3.06	0.09

Tab. 3.13: Ethanol production [g/L] of *S. bayanus* strain HL 77 during fermentation

Days of fermentation	0	3	5	10	14	21	24	33	53
Ethanol	0.00	0.00	1.49	5.22	9.25	14.43	14.87	16.98	18.57

Tab. 3.14: Glucose and fructose concentration [g/L] in synthetic B-medium (cf. 2.6.3) inoculated with *S. cerevisiae x S. kudriavzevii x S. bayanus* strain HL 78 during fermentation (cf. Fig. 3.8)

Days of fermentation	0	5	14	25	53
Glucose	19.00	15.17	6.79	1.09	0.01
Fructose	23.36	19.93	13.32	1.04	0.09

Tab. 3.15: Ethanol production [g/L] of *S. cerevisiae x S. kudriavzevii x S. bayanus* strain HL 78 during fermentation

Days of fermentation	0	3	7	10	14	20	25	31	35
Ethanol	0.00	0.00	1.86	5.20	9.11	12.25	14.59	18.01	19.94

Tab. 3.16: Glucose and fructose concentration [g/L] in synthetic B-medium (cf. 2.6.3) inoculated with *S. cerevisiae* (Fermivin®) during fermentation (cf. Fig. 3.9)

Days of fermentation	0	4	10	17	24	35	50
Glucose	24.09	23.29	23.94	12.90	8.48	0.32	0.01
Fructose	26.51	25.75	25.58	22.03	16.25	5.68	0.16

Tab. 3.17: Ethanol production [g/L] of *S. cerevisiae* (Fermivin®) during fermentation

Days of fermentation	0	4	10	15	18	24	32	42	50
Ethanol	0.00	0.00	0.00	1.64	4.34	10.03	17.27	20.79	21.17

3.4.2 Growth and sugar consumption in relation to the ammonium concentration

In order to test how different levels of ammonium influence the sugar consumption of the isolated yeasts, they were cultivated in synthetic B-medium with varied concentrations of $(NH_4)_2SO_4$ between 0.0 g/L and 2.5 g/L. From the tested yeasts, the growth of *S. bayanus* strain HL 77 was

the least affected by varying concentrations of ammonium (cf. Fig. 3.10). Only with the highest tested concentration of 2.5 g/L $(NH_4)_2SO_4$ *S. bayanus* showed slightly delayed growth compared to the lower concentrations.

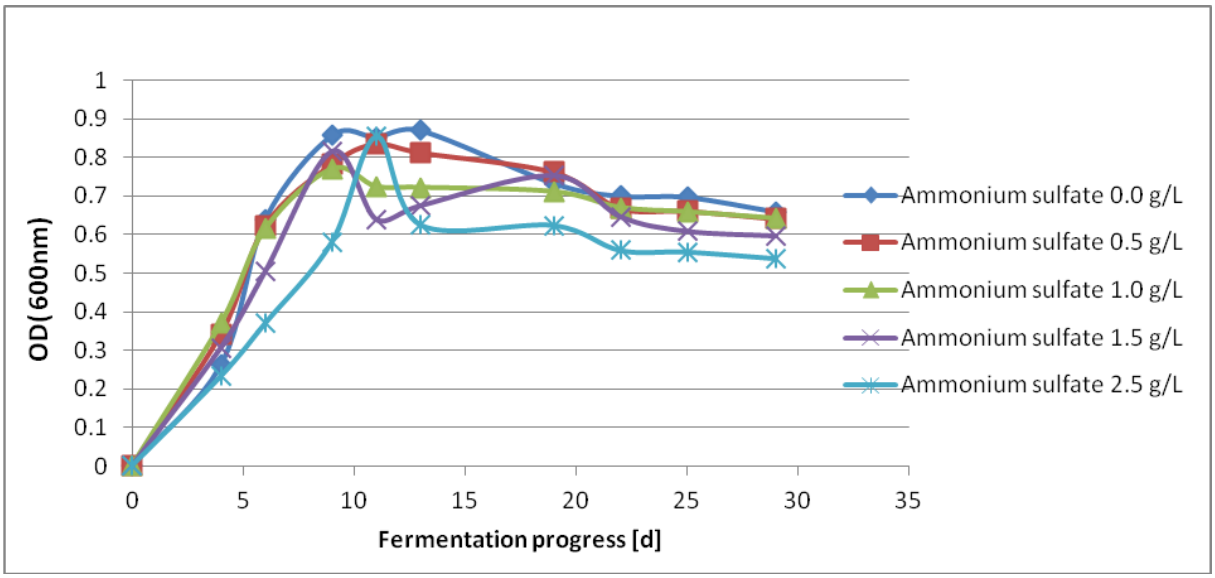


Fig.3.10: Growth of the *S. bayanus* strain HL 77 in relation to the concentration of ammonium sulfate in synthetic B-medium

The triple hybrid *S. cerevisiae* x S. *kudriavzevii* x S. *bayanus* strain HL 78 showed delayed growth of about ten days at 0.0 g/L $(NH_4)_2SO_4$ compared to the other concentrations of ammonium sulfate. With increasing levels of $(NH_4)_2SO_4$ the triple hybrid grew faster, but the maximum OD at the highest tested concentration of 2.5 g/L $(NH_4)_2SO_4$ was below the growth curves of the lower nitrogen levels (cf. Fig. 3.11). In general, the maximum OD of the triple hybrid stayed below the OD of *S. bayanus*.

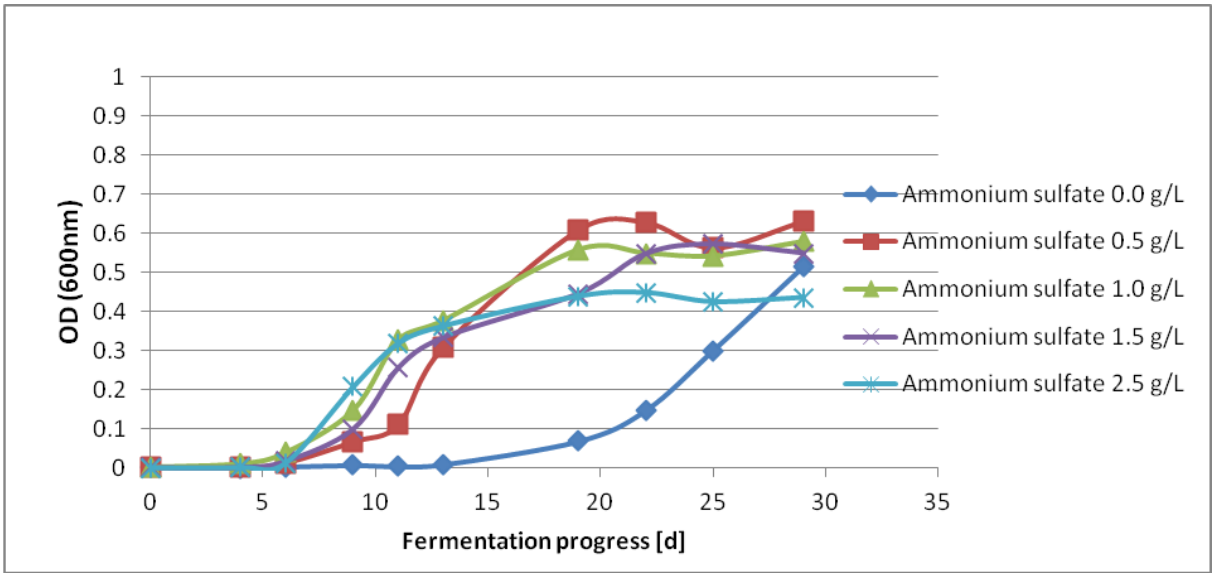


Fig.3.11: Growth of the triple hybrid *S. cerevisiae x S. kudriavzevii x S. bayanus* (strain HL 78) in relation to the concentration of ammonium sulfate in synthetic B-medium

The yeast strain used as control, *S. cerevisiae* (FermiVin®), showed no growth at all at 0 g/L $(NH_4)_2SO_4$. Growth was delayed with the two highest levels of ammonium sulfate and the fastest and highest growth was reached with moderate $(NH_4)_2SO_4$ concentrations of 0.5 and 1.0 g/L (cf. Fig. 3.12). The maximum OD was similar to *S. bayanus* and higher compared to the triple hybrid.

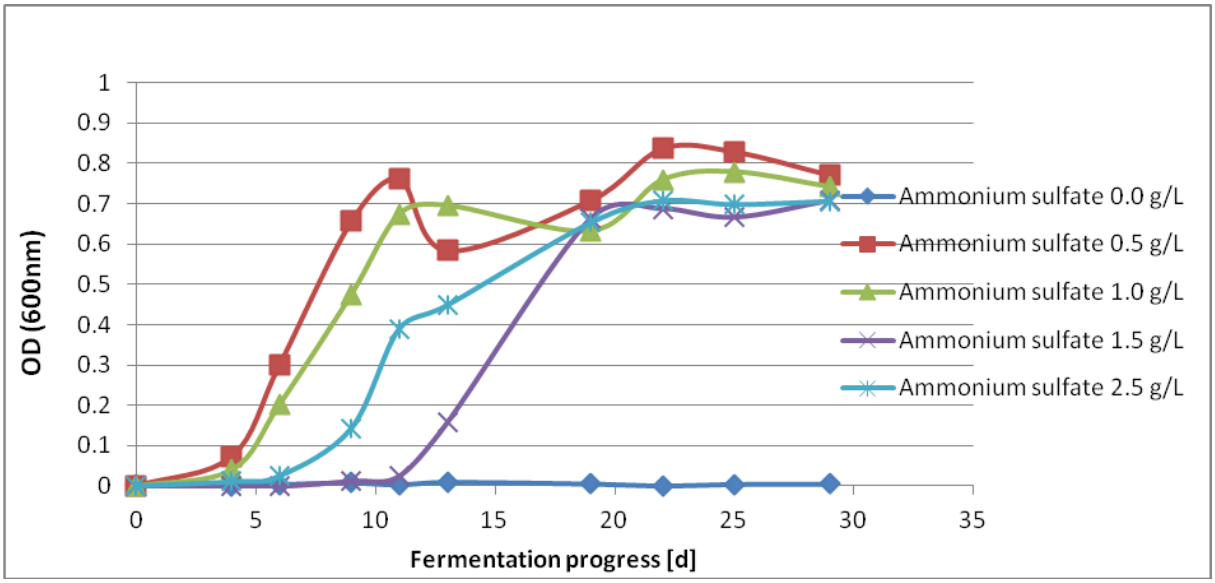


Fig.3.12: Growth of *S. cerevisiae* (Fermivin®) in relation to the concentration of ammonium sulfate in synthetic B-medium

Consumption of both sugars by *S. bayanus* strain HL 77 was not affected by varying concentrations from 0.0 to 2.0 g/L ammonium sulfate, but at the highest level of 2.5 g/L $(NH_4)_2SO_4$, glucose and fructose uptake was decreased. In general, *S. bayanus* strain HL 77 degraded glucose better than fructose (cf. Fig. 3.13), but no residual sugar was left at the end of the fermentation (cf. Tab. 3.17).

The triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 showed delayed growth and also delayed consumption of both sugars glucose and fructose with 0.0 g/L $(NH_4)_2SO_4$ in the medium, sugar degradation started on day 25 of the fermentation (cf. Fig. 3.14) at this level of ammonium sulfate. It could be seen that fructose consumption increased in the second part of the fermentation and the triple hybrid showed the fastest usage of both sugars at the highest concentration of 2.5 g/L $(NH_4)_2SO_4$ (cf. Tab. 3.18).

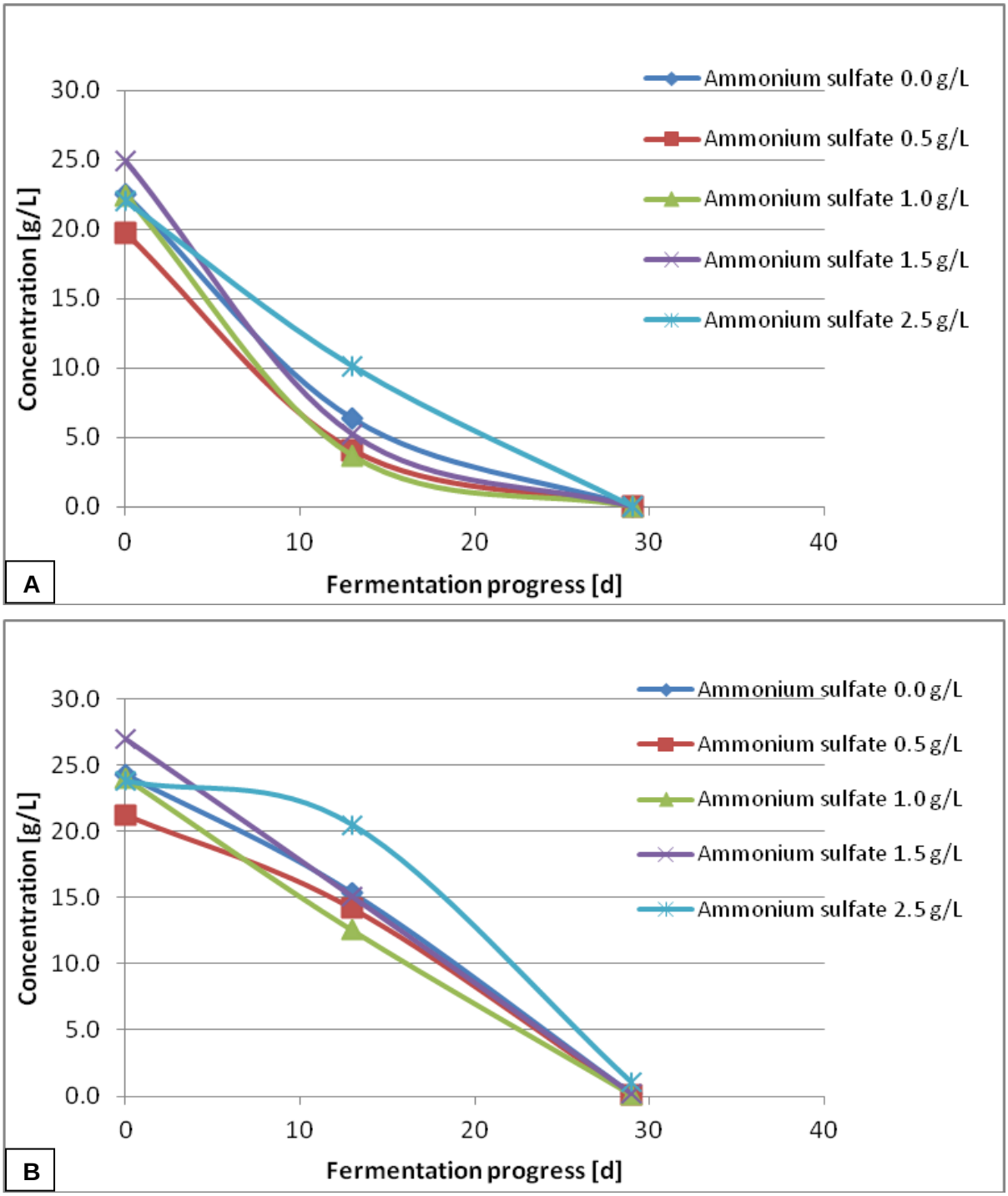


Fig. 3.13: Glucose (A) and fructose (B) consumption of *S. bayanus* strain HL 77 in relation to the concentration of ammonium sulfate in synthetic B-medium (cf. 2.6.3)

Tab.3.17: Glucose and fructose concentration [g/L] in synthetic B-medium (cf. 2.6.3) inoculated with *S. bayanus* strain HL 77 in relation to ammonium sulfate

Glucose					
$(NH_4)_2SO_4$ / days	0.0 g/L	0.5 g/L	1.0 g/L	1.5 g/L	2.5 g/L
0	22.51	19.74	22.42	24.93	22.08
13	6.32	4.07	3.64	5.19	10.13
29	0.00	0.00	0.00	0.00	0.01
Fructose					
$(NH_4)_2SO_4$ / days	0.0 g/L	0.5 g/L	1.0 g/L	1.5 g/L	2.5 g/L
0	24.31	21.26	24.05	27.02	23.79
13	15.34	14.21	12.55	15.08	20.48
29	0.16	0.12	0.13	0.17	1.02

Tab.3.18: Glucose and fructose concentration [g/L] in synthetic B-medium (cf. 2.6.3) inoculated with the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 in relation to ammonium sulfate (n.d.: not determined)

Glucose					
$(NH_4)_2SO_4$ / days	0.0 g/L	0.5 g/L	1.0 g/L	1.5 g/L	2.5 g/L
0	20.78	19.13	19.74	17.66	21.82
13	n.d.	16.62	11.26	13.33	6.93
25	19.48	n.d.	n.d.	n.d.	n.d.
29	8.22	0.10	0.00	0.00	0.00
Fructose					
$(NH_4)_2SO_4$ / days	0.0 g/L	0.5 g/L	1.0 g/L	1.5 g/L	2.5 g/L
0	23.27	20.92	21.79	19.52	23.44
13	n.d.	21.44	20.92	19.87	14.21
25	23.27	n.d.	n.d.	n.d.	n.d.
29	14.99	0.21	0.01	0.01	0.10

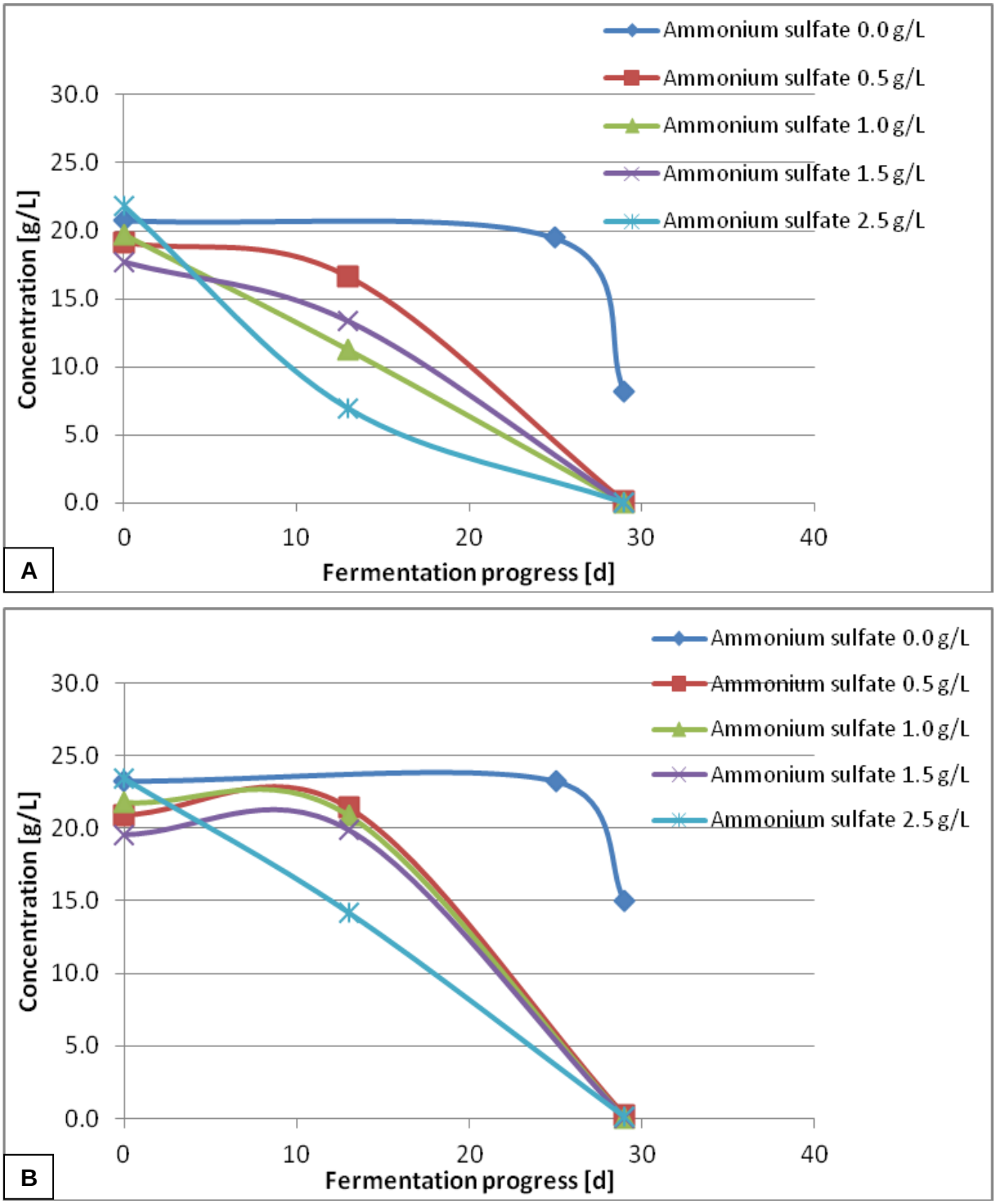


Fig. 3.14: Glucose (A) and fructose (B) consumption of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x S. *bayanus* strain HL 78 in relation to the concentration of ammonium sulfate in synthetic B-medium (cf. 2.6.3)

3.4.3 Growth and sugar consumption in relation to amino acid concentrations

In order to test the influence of fermentation temperature on the growth of the isolated yeasts, the yeast strains *S. bayanus* HL 77 and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* HL 78 were cultivated in synthetic B-medium (cf. 2.6.3) with different concentrations of amino

acids and at 15 °C and 20 °C, respectively. The temperature of the cellar in the investigated winery is usually 12-15 °C.

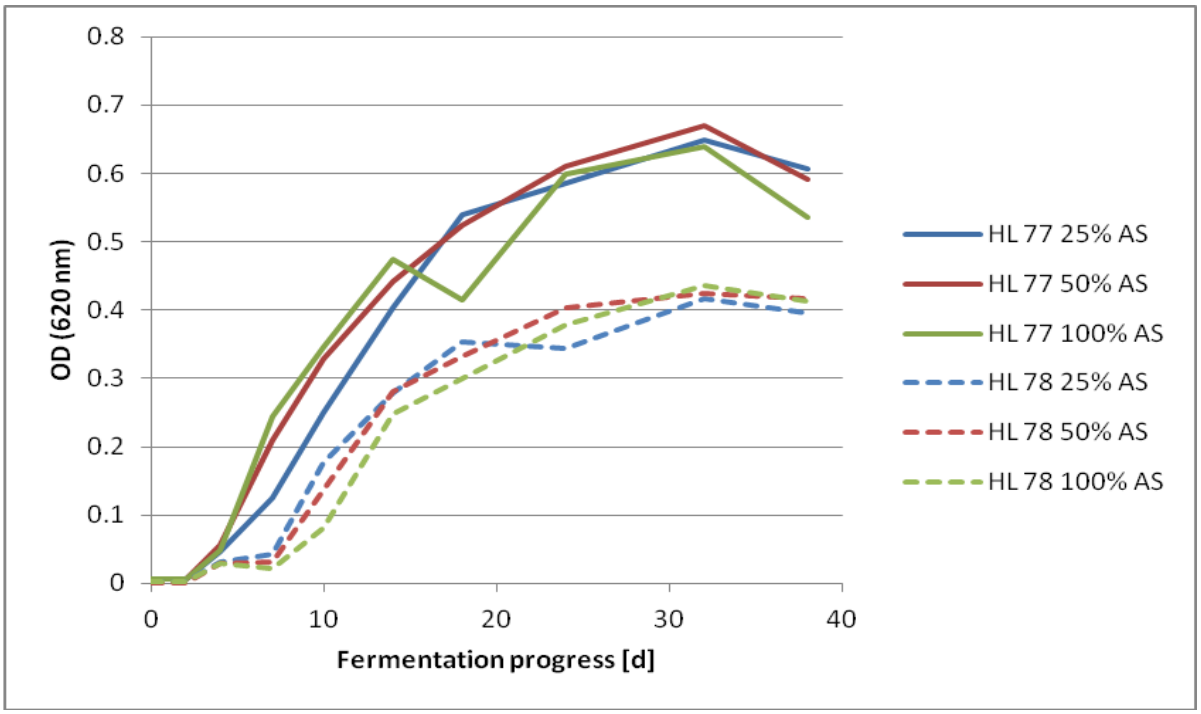


Fig. 3.15: Growth of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 and *S. bayanus* strain HL 77 at different amino acid concentrations (cf. 2.6.3) in synthetic B-medium at 15 °C

The triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 showed delayed growth at 15 °C compared to *S. bayanus* strain HL 77 and did only reach a maximum OD of 0.4, whereas *S. bayanus* reached a maximum OD of 0.67. At a temperature of 15 °C, the triple hybrid showed a slightly lower growth with 100 % of the amino acid solution in the medium and reached an OD of 0.08 after 10 days of fermentation compared to 0.14 with 50 % of amino acids and 0.18 with 100 %. *S. bayanus* showed delayed growth at reduced amino acid levels with an OD of 0.13 at 25 %, 0.21 at 50 % and 0.24 at 100 % of amino acids after 7 days of fermentation (cf. Fig. 3.15).
At 20 °C, the triple hybrid had reached a little bit higher maximum OD of 0.4 and variation of amino acid levels did not influence the growth curves, whereas *S. bayanus* showed different growth curves at this temperature when amino acid concentration was reduced. At 25 % of amino acids, the maximum OD was 0.37, compared to 0.42 at 100 % and 0.49 at 50 % (cf. Fig. 3.16).

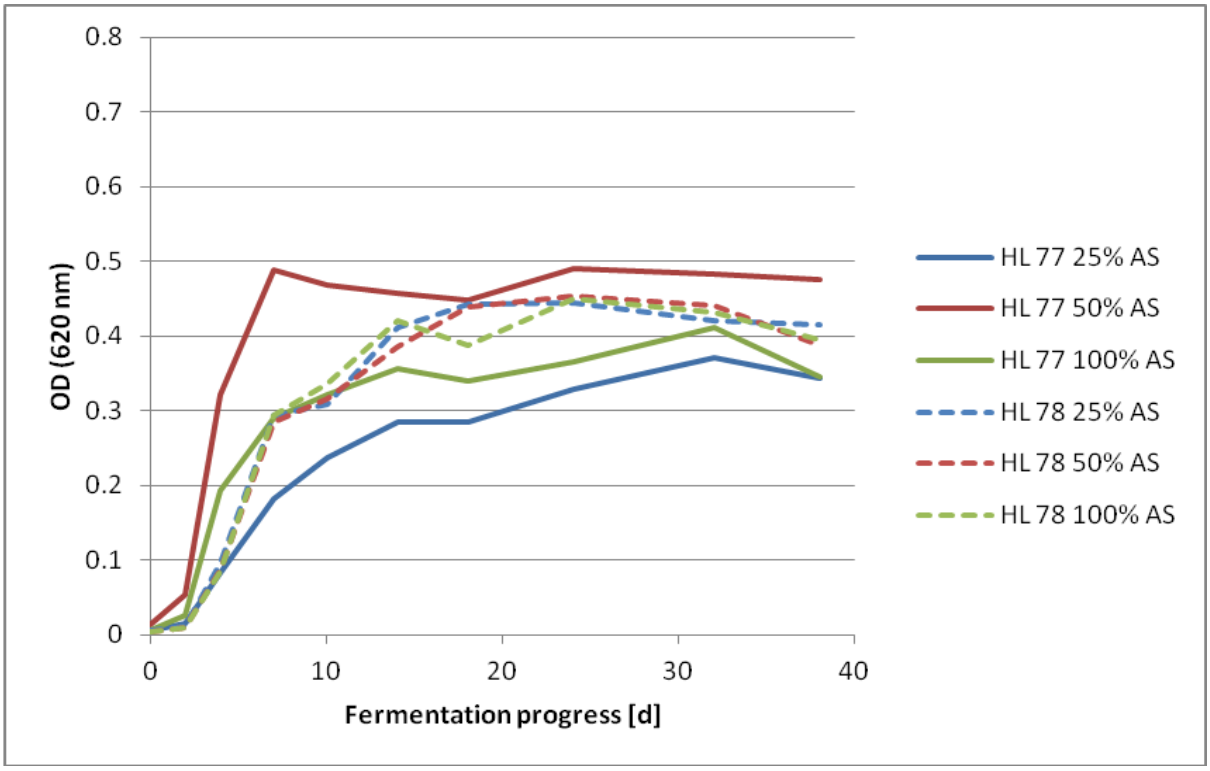


Fig. 3.16: Growth of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 and *S. bayanus* strain HL 77 at different amino acid concentrations (see 2.6.3) in synthetic B-medium at 20 °C

Sugar consumption of the isolated yeast strains was tested at 15 °C, as this is close to the temperature in the winery. Amino acid concentration of the medium was varied to test whether this parameter effects sugar consumption.

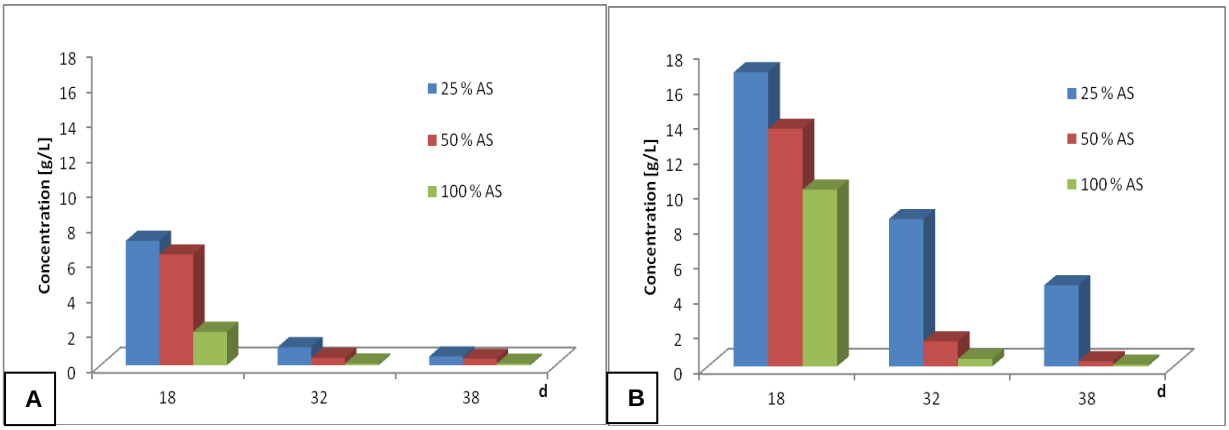


Fig. 3.17: Residual glucose (A) and fructose (B) concentration in a culture supernatant of *S. bayanus* strain HL 77 in relation to the amino acid concentration of synthetic B-medium (cf. 2.6.3) after 18, 32 and 38 days of fermentation

At reduced amino acid concentrations of 50% and 25%, *S. bayanus* strain HL 77 utilized both glucose and fructose slower than with 100 % of amino acids (cf. 2.6.3). Although this yeast strain

has a higher affinity for glucose, no residual fructose was left as well after 38 days of fermentation at full supply of amino acids (cf. Fig. 3.17, Tab. 3.19).

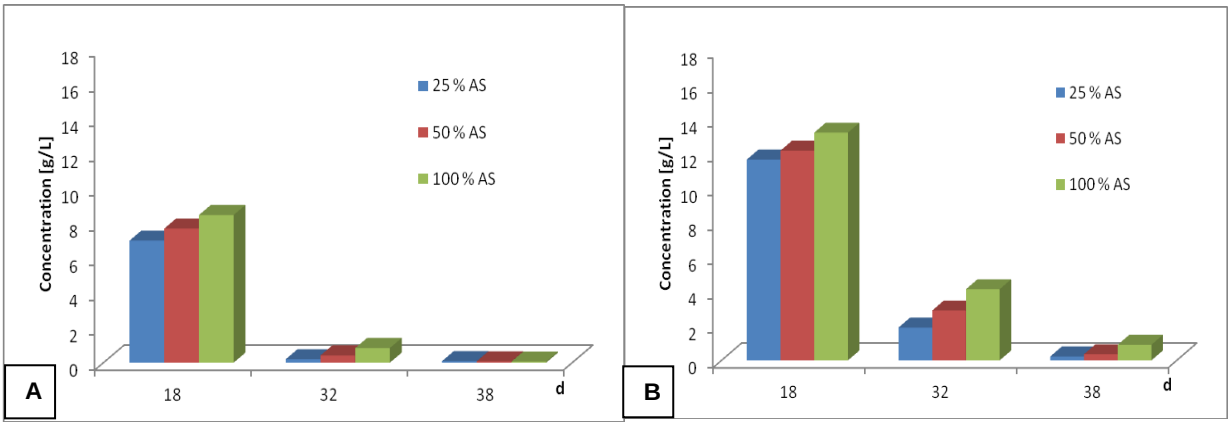


Fig. 3.18: Residual glucose (A) and fructose (B) concentration in a culture supernatant of the triple hybrid *S. cerevisiae x S. kudriavzevii x S. bayanus* strain HL 78 in relation to the amino acid concentration of synthetic B-medium (cf. 2.6.3) after 18, 32 and 38 days of fermentation

In the medium inoculated with *S. bayanus*, after 38 days of fermentation 4.64 g/L fructose was left with 25 % amino acid concentration in the medium and 0.11 g/L fructose with 100 % amino acids (cf. Tab. 3.19).

Tab. 3.19: Residual glucose and fructose concentration [g/L] in a culture supernatant of *S. bayanus* strain HL 77 in relation to the amino acid concentration of synthetic B-medium (cf. 2.6.3)

Residual glucose			
Amino acids / Days of fermentation	25 %	50 %	100 %
18	7.10	6.32	1.91
32	1.03	0.42	0.10
38	0.50	0.37	0.09
Residual fructose			
Amino acids / Days of fermentation	25 %	50 %	100 %
18	16.82	13.60	10.11
32	8.43	1.43	0.43
38	4.64	0.30	0.11

The triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 had a lower residual glucose and fructose concentration compared to *S. bayanus* at reduced amino acid concentrations of 50% and 25% (cf. *2.6.3 Synthetic medium for growth experiments*) in the synthetic B-medium (cf. Fig. 3.18, Tab. 3.20). The hybrid showed the lowest residual concentrations of both sugars at the lowest amino acid concentration: 0.23 g/L fructose were left after 38 days of fermentation with 25 % amino acids compared to 0.91 g/L with 100 % amino acid concentration (cf. Tab. 3.20).

Tab. 3.20: Residual glucose and fructose concentration [g/L] in a culture supernatant of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x S. *bayanus* strain HL 78 in relation to the amino acid concentration of synthetic B-medium (cf. 2.6.3)

Residual glucose			
Amino acids / Days of fermentation	25 %	50 %	100 %
18	7.01	7.71	8.48
32	0.20	0.42	0.84
38	0.09	0.08	0.06
Residual fructose			
Amino acids / Days of fermentation	25 %	50 %	100 %
18	11.68	12.20	13.25
32	1.91	2.90	4.14
38	0.23	0.36	0.91

3.4.4 Growth and sugar consumption in relation to lacking amino acids

In order to test whether any of the amino acids in the amino acid solution of the synthetic B-medium (cf. *2.6.3 Synthetic medium for growth experiments*) is essential for growth of the isolated yeasts, *S. bayanus* strain HL 77 and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 were cultivated at 15 °C in several parallels with one amino acid of the solution lacking in each of the nine parallels.

S. bayanus grew delayed for five days and sugar consumption was reduced when methionine was missing in the synthetic medium but reached a similar OD to the other parallels with 0.74 (cf. Fig. 3.19, Tab. 3.21a).

The triple hybrid *S. cerevisiae x S. kudriavzevii x S. bayanus* strain HL 78 showed delayed growth and sugar consumption without histidine, tryptophan or alanine (cf. Tab. 3.21b). Especially, the lack of tryptophan seemed to slow down growth for about five days compared to the parallels including trytophan. The parallel with full amino acid supply (control) grew slower than the parallels with one amino acid missing but a little bit faster than the parallel without tryptophan (cf. Fig. 3.20). Consumption of glucose and fructose was correlated to the results of the growth curves (cf. Fig. 3.21 – 3.24, Tab. 3.21).

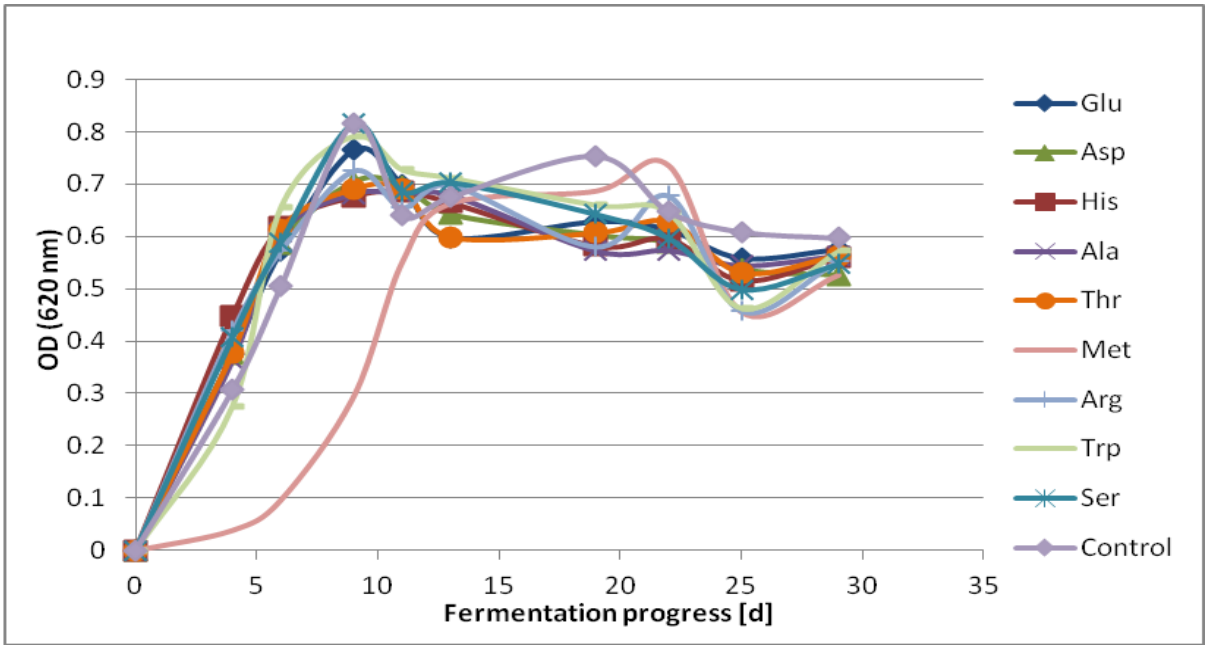


Fig. 3.19: Growth of *S. bayanus* strain HL 77 in synthetic B-medium with one amino acid lacking in each parallel (cf. 2.6.3)

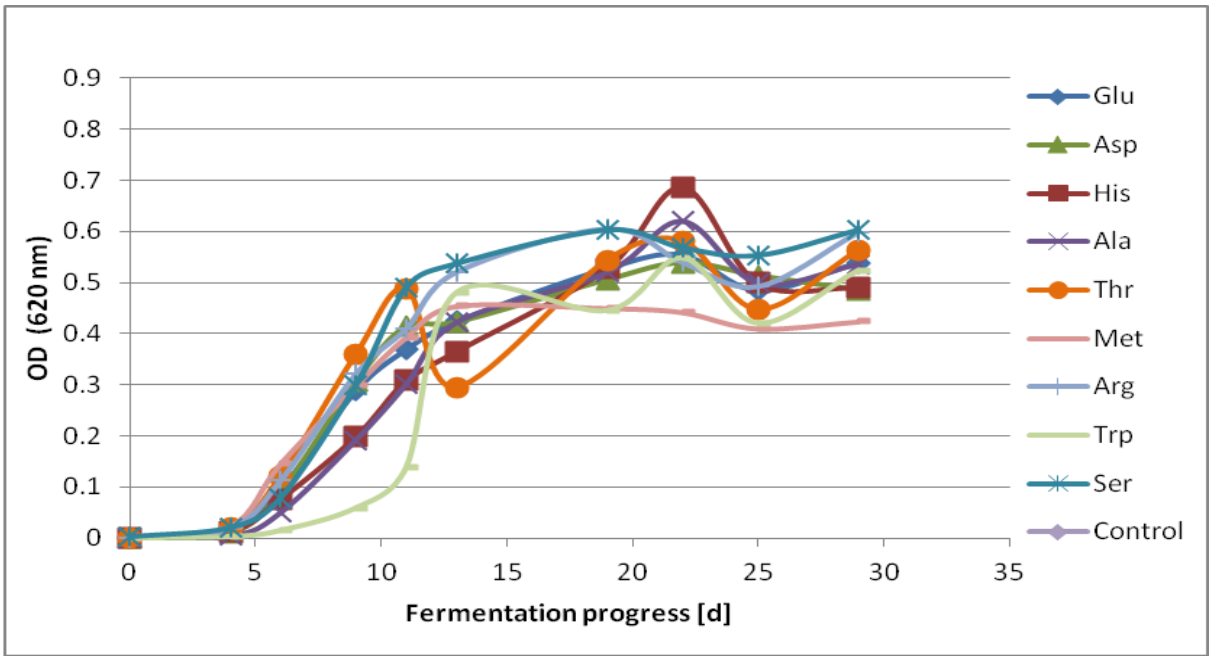


Fig. 3.20: Growth of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x S. *bayanus* strain HL 78 with one amino acid lacking in each parallel (cf. 2.6.3)

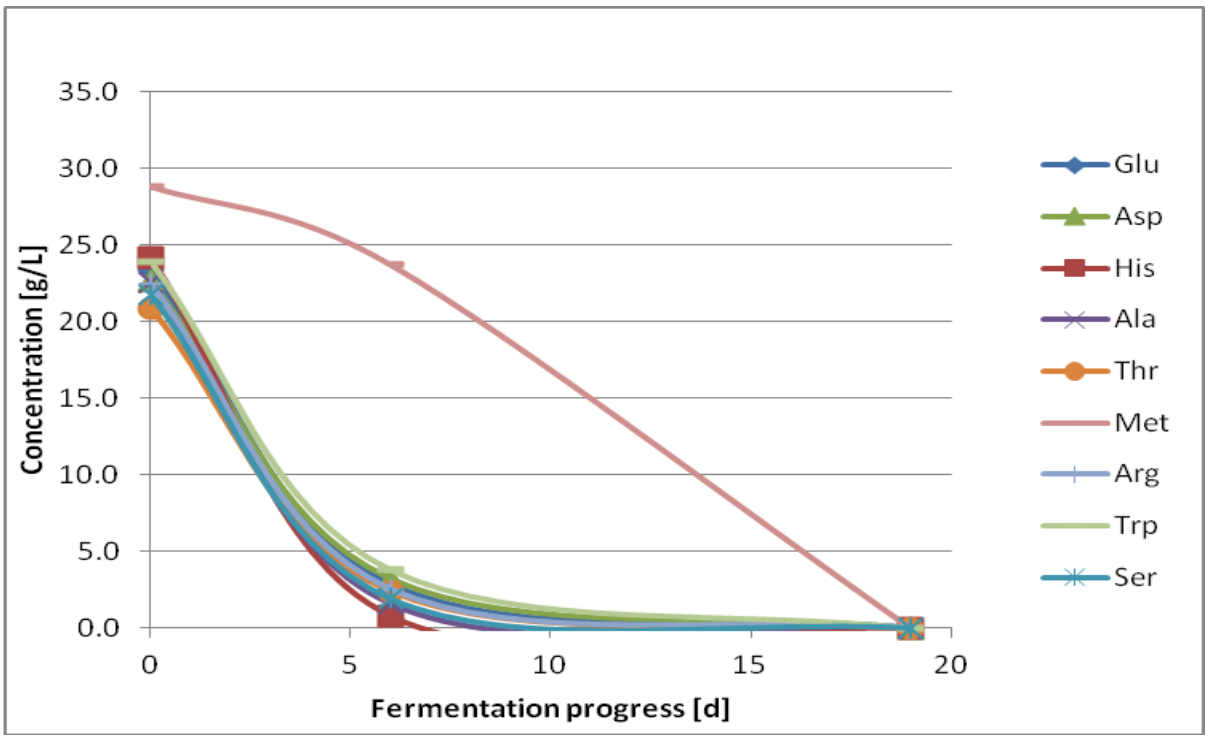


Fig.3.21: Glucose consumption [g/L] of *S. bayanus* strain HL 77 in relation to the absence of one amino acid in synthetic B-medium (cf. 2.6.3)

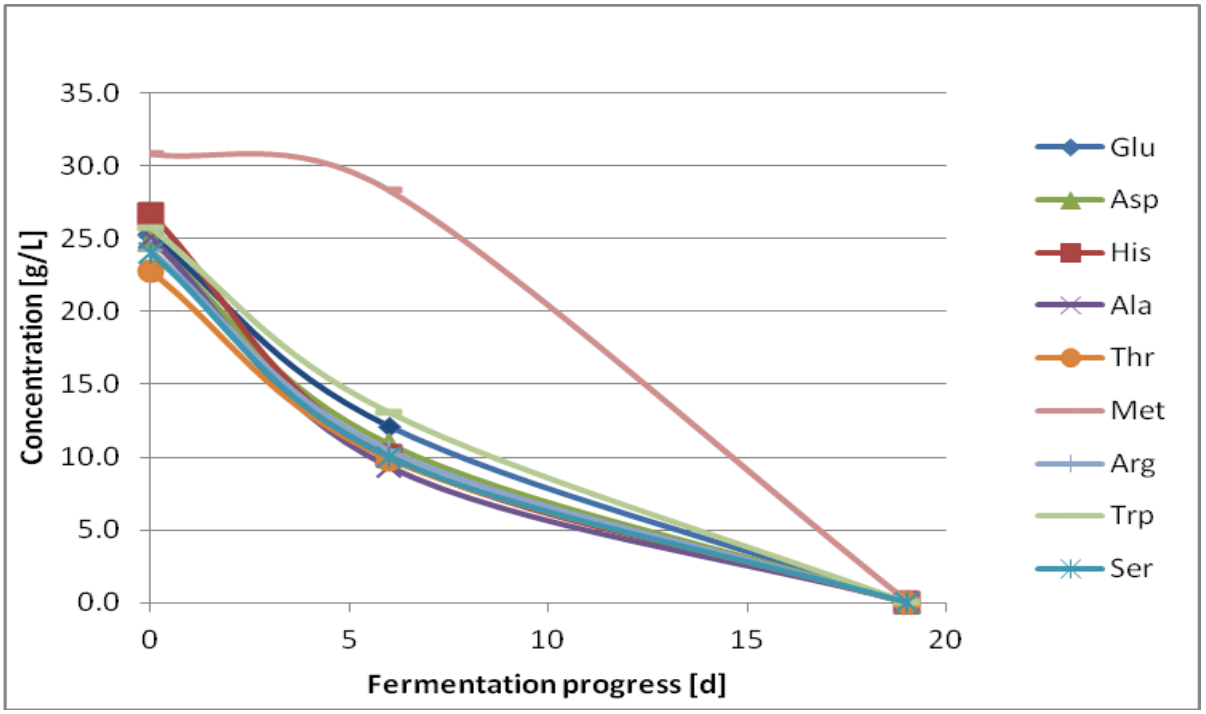


Fig.3.22: Fructose consumption [g/L] of *S. bayanus* strain HL 77 in relation to the lack of one amino acid in synthetic B-medium (cf. 2.6.3)

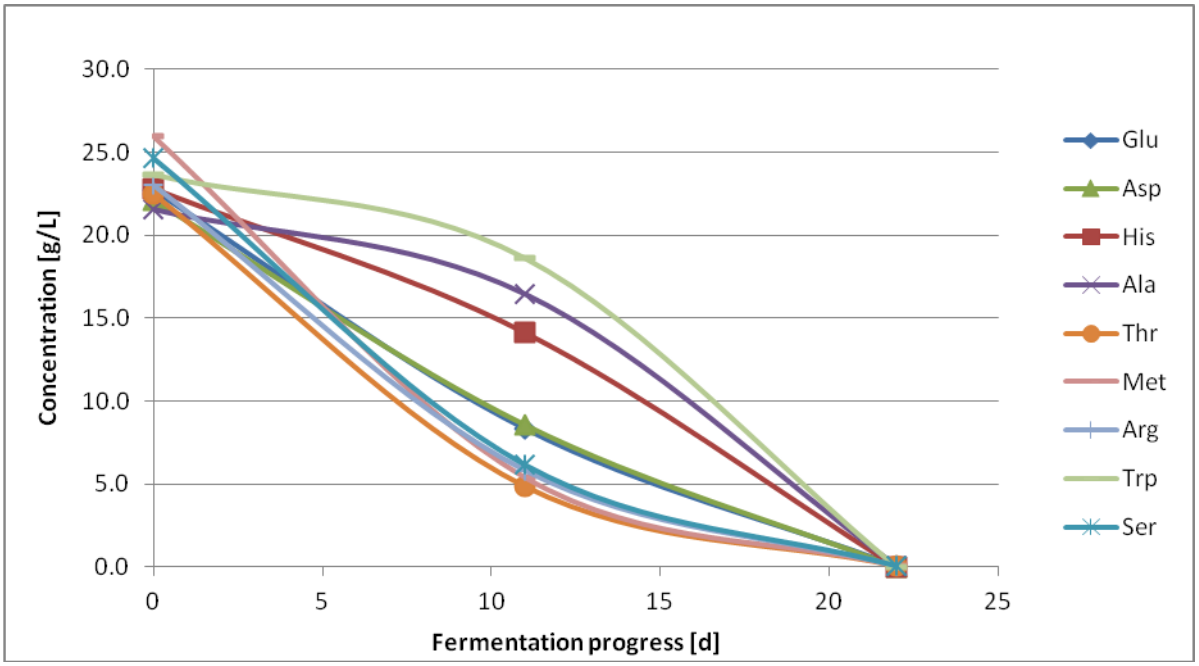


Fig. 3.23: Glucose consumption [g/L] of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 in relation to the missing of single amino acids in synthetic B-medium (cf. 2.6.3)

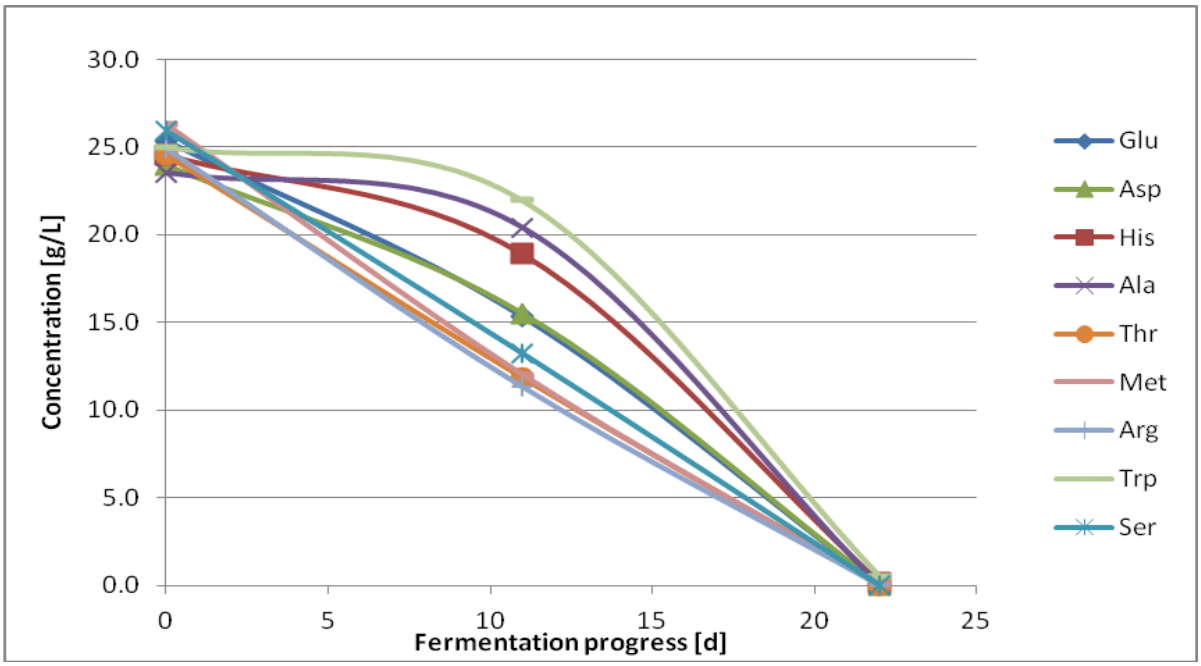


Fig. 3.24: Fructose consumption [g/L] of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 in relation to the lack of single amino acids in synthetic B-medium (cf. 2.6.3)

Tab. 3.21a: Glucose and fructose consumption [g/L] of *S. bayanus* strain HL 77 in relation to the lack of single amino acids in synthetic B-medium (cf. 2.6.3)

Residual glucose			
Day of fermentation / Lacking amino acid	0	6	19
Aspartic acid	22.60	3.20	0.00
Glutamic acid	23.38	2.86	0.01
Histidine	24.15	0.78	0.00
Alanine	22.51	1.56	0.00
Threonine	20.86	2.42	0.00
Methionine	28.83	23.72	0.00
Arginine	22.42	2.51	0.00
Tryptophan	23.89	3.81	0.00
Serine	21.73	1.91	0.00
Residual fructose			
Day of fermentation / Lacking amino acid	0	6	19
Aspartic acid	24.92	10.89	0.02
Glutamic acid	25.27	12.11	0.02
Histidine	25.97	18.21	0.01
Alanine	25.10	9.33	0.00
Threonine	22.75	9.85	0.01
Methionine	30.85	28.32	0.10
Arginine	24.23	10.46	0.01
Tryptophan	25.80	13.07	0.00
Serine	24.05	10.02	0.01

Tab. 3.21b: Glucose and fructose consumption [g/L] of the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 in relation to the lack of single amino acids in synthetic B-medium (cf. 2.6.3)

Residual glucose			
Day of fermentation / Lacking amino acid	0	11	22
Aspartic acid	22.60	3.20	0.00
Glutamic acid	23.38	2.86	0.01
Histidine	24.15	0.78	0.00
Alanine	22.51	1.56	0.00
Threonine	20.86	2.42	0.00
Methionine	28.83	23.72	0.00
Arginine	22.42	2.51	0.00
Tryptophan	23.89	3.81	0.00
Serine	21.73	1.91	0.00
Residual fructose			
Day of fermentation / Lacking amino acid	0	11	22
Aspartic acid	24.92	10.89	0.02
Glutamic acid	25.27	12.11	0.02
Histidine	25.97	18.21	0.01
Alanine	25.10	9.33	0.00
Threonine	22.75	9.85	0.01
Methionine	30.85	28.32	0.10
Arginine	24.23	10.46	0.01
Tryptophan	25.80	13.07	0.00
Serine	24.05	10.02	0.01

3.5 Chemical composition of must and young wine

3.5.1 Concentration of yeast assimilable nitrogen

Nitrogen is an important nutrient for wine yeasts and a lack of nitrogen can lead to stuck fermentation. Yeast assimilable nitrogen was measured by the CRP (Belvaux/Luxembourg). The concentration of yeast assimilable nitrogen in steel cask 3007 was 160.0 mg/L at the beginning of the fermentation in fresh must and decreased to 140.0 mg/L after two weeks of fermentation and remained on an approximately constant level until the end of fermentation. Yeast assimilable nitrogen was only measured in the steel cask 3007 which was sampled since the start of the fermentation (cf. Fig. 3.25, Tab. 3.22).

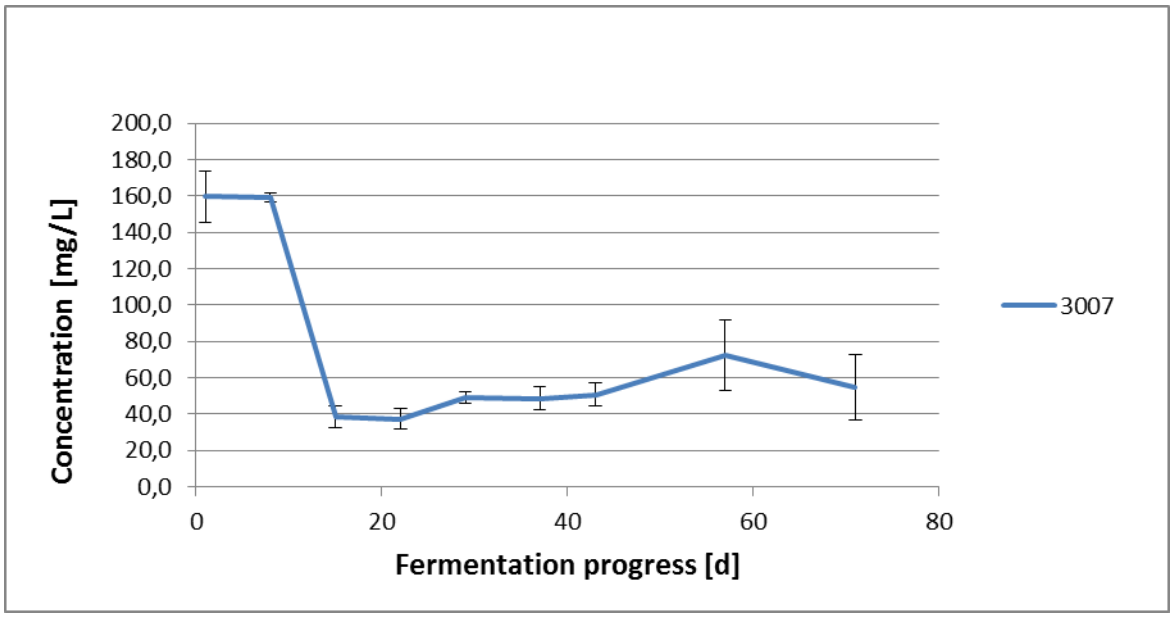


Fig. 3.25: Development of yeast assimilable nitrogen during the fermentation progress in steel cask 3007 (normal fermentation during vintage 2011/2012)

Tab. 3.22: Concentration of YAN [mg/L] in cask 3007 during vintage 2011/2012

Day of fermentation	Yeast assimilable nitrogen
1	160.00
8	159.00
15	39.00
22	37.00
29	49.00
37	49.00
43	51.00
57	72.00

3.5.2 Concentration of sugars during fermentation

The glucose : fructose ratio in must can cause fermentation problems because many of the wine yeasts are not able to use both sugars equally. Glucose and fructose were analyzed in diluted must and young wine (dilution 1:3). Each sample was analyzed three times.

On day one when the steel cask 3007 was filled (27.9.2011) 119.1 g/L glucose and ca. 102.4 g/L fructose were measured in the must. Both sugars continuously decreased during the fermentation. Glucose was not detected from day 33 (cf. Tab. 3.23). Fructose was measured to be 2.8 g/L at the time of sulphuring.

On day 26 when the first sampling took place in the wooden casks 2607, 2608, 2609 and 2612, sluggish fermentation was observed. Analysis with HPLC showed a fructose : glucose ratio of approximately 11:1 for wooden cask 2607 (40.0 g/L fructose and 3.8 g/L glucose; cf. Tab. 3.24), 22:1 for wooden cask 2608 (33.0 g/L fructose and 1.5 g/L glucose; cf. Tab. 3.25), 9:1 for wooden cask 2609 (43.0 g/L fructose and 4.9 g/L glucose; cf. Tab. 3.26) and 15:1 for wooden cask 2612 (40.0 g/L fructose and 2.7 g/L glucose; cf. Tab. 3.27). In wooden cask No. 2607, both glucose and fructose decreased continuously until no glucose was found on day 61 while 1.9 g/L fructose was left at the end of the fermentation on day 91. Sugar was added to wooden cask 2608 on day 40 according to the information of the staff, so the fructose : glucose ratio was decreased to 3:1 in the sample of day 40 (41.2 g/L fructose and 12.0 g/L glucose; cf. Tab. 3.25). From this day on both sugars decreased continuously in wooden cask 2608 during fermentation so that no glucose could be measured in the sample from day 57 while 0.8 g/L fructose were left at the end of the fermentation. The fermentation in wooden cask 2609 was stuck for 37 more days until day 63. From that point on both glucose and fructose decreased so that no glucose was found on day 92 and 2.7 g/L fructose were left on day 96. In wooden cask 2612 the fermentation was sluggish for 14 more days until day 40. From that point on the sugars decreased slowly. No glucose was left on day 81 and 5.0 g/L of fructose were left at the end of the fermentation.

In the second vintage, the sampled cask S 101 had an amount of 135.3 g/L glucose and 103.5 g/L fructose on day 1. Both sugars decreased until day 40 when it came to stuck fermentation and 20.1 g/L glucose and 48.4 g/L fructose were left (cf. Tab. 3.28). Figures 3.26 and 3.27 show the development of glucose and fructose in all six tested casks during two years of fermentation.

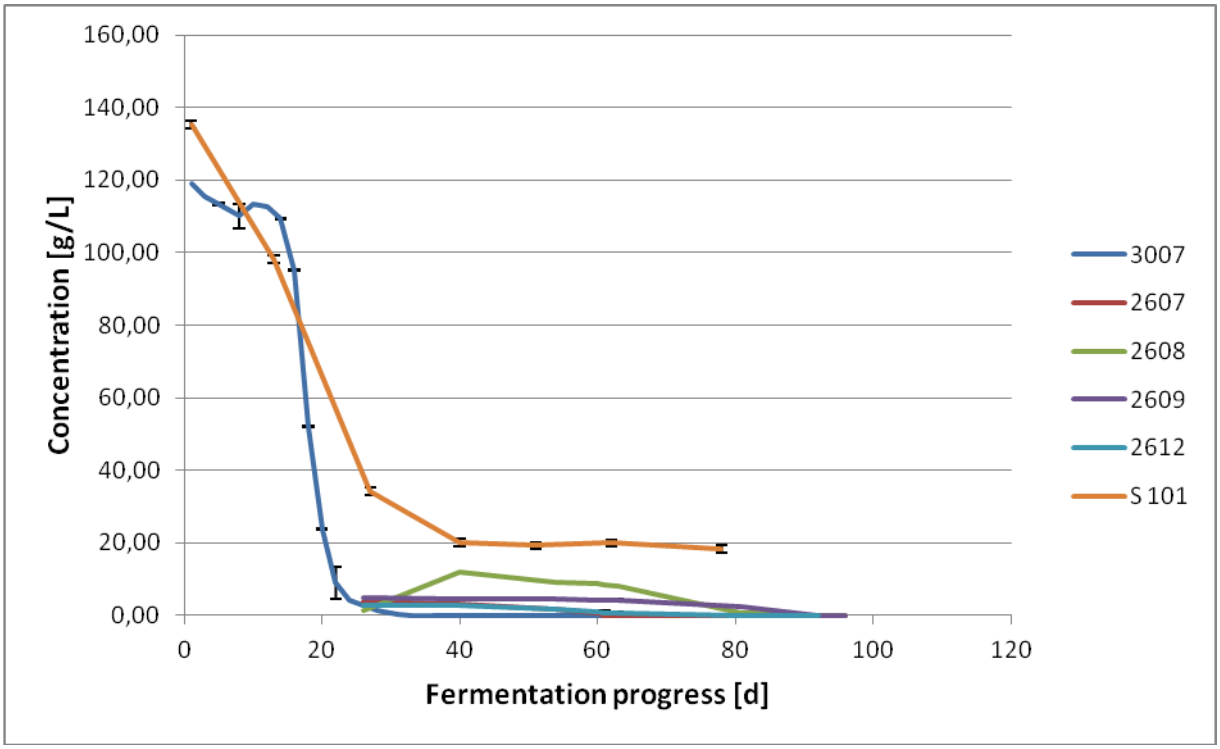


Fig. 3.26: Consumption of glucose during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

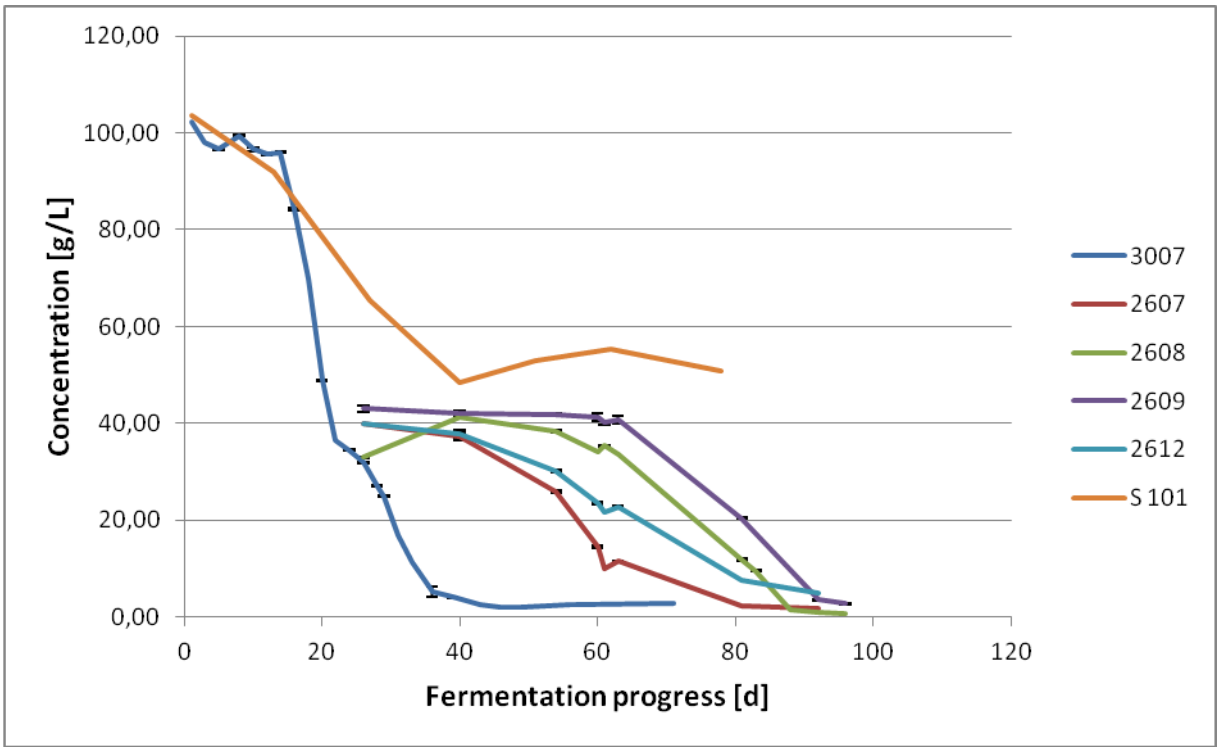


Fig. 3.27: Consumption of fructose during fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

Tab. 3.23: Alteration of glucose and fructose during fermentation in steel cask 3007. Determined in vintage 2011/2012

Day of fermentation	Glucose [g/L]	Fructose [g/L]
1	119.10	107.88
3	115.50	103.86
5	113.43	101.93
8	110.04	104.38
10	113.40	101.58
12	112.65	100.66
14	109.32	100.46
16	95.07	94.48
18	52.03	73.49
20	23.74	52.14
22	8.95	39.80
24	4.20	37.68
26	2.60	35.29
28	1.39	30.46
29	1.00	28.39
31	0.35	20.30
33	0.00	14.78
36	0.00	8.36
39	0.00	2.53
43	0.00	2.34
46	0.00	2.29
49	0.00	2.31
56	0.00	2.21
71	0.00	2.29

Tab. 3.24: Alteration of glucose and fructose during fermentation in wooden cask 2607. Determined in vintage 2011/2012

Day of fermentation	Glucose [g/L]	Fructose g/L]
23	3.77	40.05
37	3.14	37.29
51	1.52	25.88
57	0.47	14.46
58	0.00	9.93
60	0.00	11.51
78	0.00	2.25
89	0.00	1.90

Tab. 3.25: Alteration of glucose and fructose during fermentation in wooden cask 2608. Determined in vintage 2011/2012

Day of fermentation	Glucose [g/L]	Fructose [g/L]
26	1.46	32.95
40	11.98	41.23
54	9.16	38.30
60	8.65	34.09
61	8.39	35.33
63	8.06	33.61
81	0.77	11.84
83	0.53	9.59
88	0.00	1.47
92	0.00	1.00
96	0.00	0.77

Tab. 3.26: Alteration of glucose and fructose during fermentation in wooden cask 2609. Determined in vintage 2011/2012

Day of fermentation	Glucose [g/L]	Fructose [g/L]
26	4.87	43.02
40	4.64	42.17
54	4.59	41.85
60	4.27	41.19
61	4.11	40.13
63	4.26	40.74
81	2.27	20.43
92	0.00	3.52
96	0.00	2.72

Tab. 3.27: Alteration of glucose and fructose during fermentation in wooden cask 2612. Determined in vintage 2011/2012

Day of fermentation	Glucose [g/L]	Fructose [g/L]
22	2.67	40.00
36	2.62	37.89
50	1.76	30.13
56	1.00	23.57
57	0.73	21.71
59	0.78	22.78
77	0.00	7.54
88	0.00	4.97

Tab. 3.28: Alteration of glucose and fructose during fermentation in steel cask S 101. Determined in vintage 2012/2013

Day of fermentation	Glucose [g/L]	Fructose [g/L]
1	135.25	103.50
13	98.14	92.04
27	34.23	65.36
40	20.14	48.40
51	19.19	53.05
62	19.94	55.47
78	18.24	50.84

3.5.3 Concentration of organic acids during fermentation

Organic acids can influence the growth and viability of microorganisms and therefore may potentially cause stuck fermentation. Succinate, lactate, citrate and acetate were analyzed in undiluted must, tartrate was analyzed in diluted must (dilution 1:3). Each sample was analyzed three times. Malate was analyzed enzymatically.

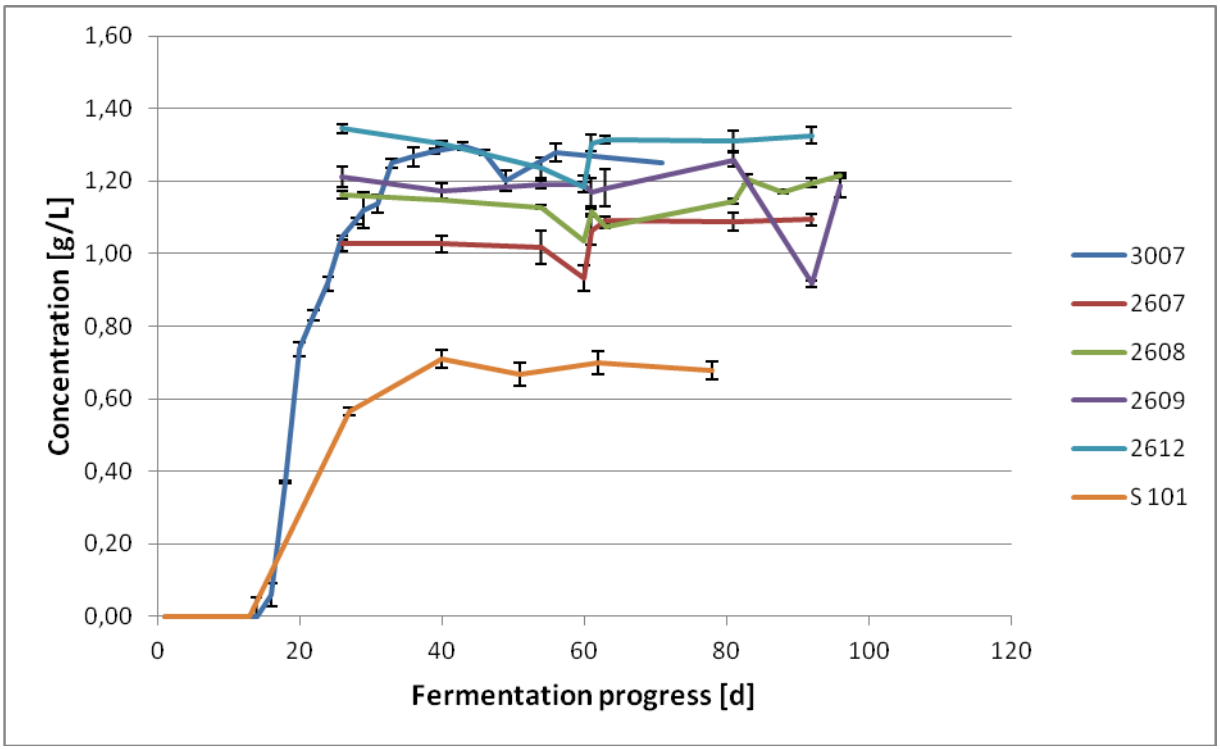


Fig. 3.28: Development of succinate during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

During fermentation 1.25 g/L of succinate were formed in steel cask 3007 (cf. Tab. 3.29). The concentration of succinate in the wooden casks with sluggish fermentation was quite similar to

that in the steel cask with normal fermentation (cf. Tab. 3.30 - 3.33). In the steel cask S 101 from the second vintage 2012/2013 it came to stuck fermentation on day 40. Up to that point, 0.68 g/L of succinate were produced (cf. Tab. 3.34). Figure 3.28 shows the development of succinate in all six tested casks during two years of fermentation.

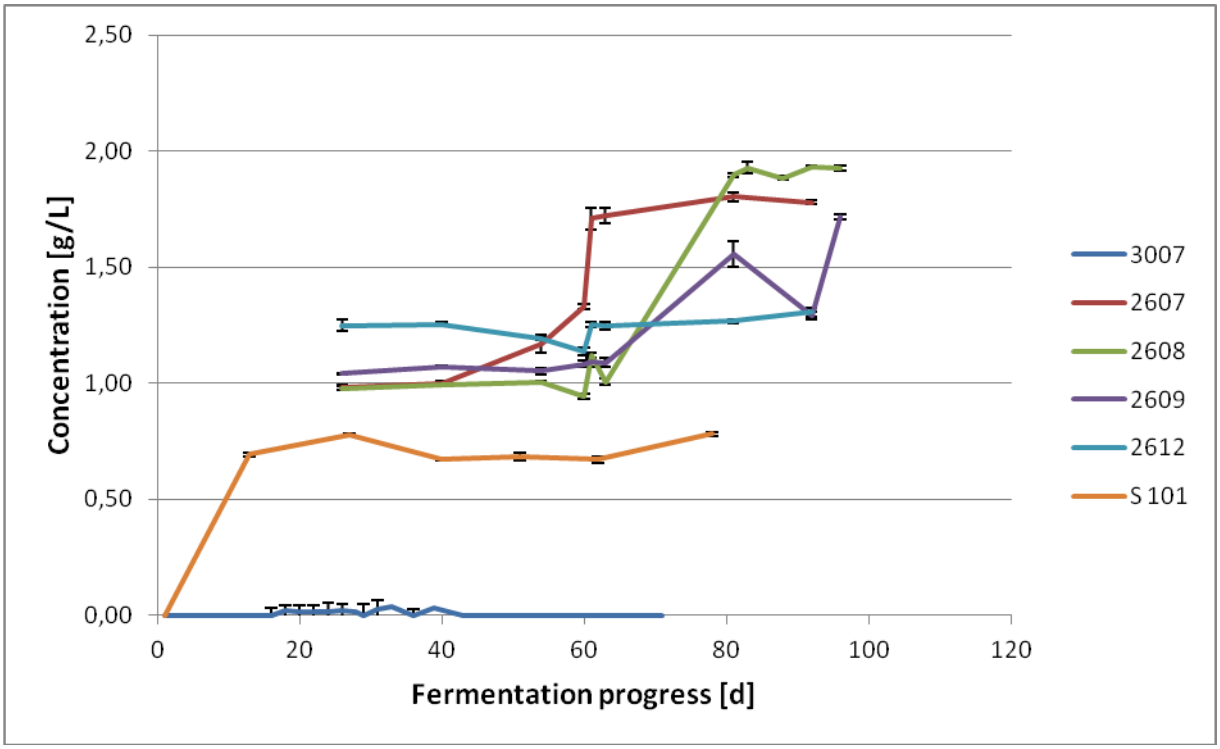


Fig. 3.29: Development of lactate during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

Lactate was found in steel cask 3007 only on few days and in concentrations less than 0.1 g/L (cf. Tab. 3.29), which is below the detection limit of this method. In the wooden casks with sluggish fermentation, up to 1.9 g/L lactate was detected (cf. Fig. 3.29, Tables 3.30 - 3.33). In the vintage 2012/2013, 0.78 g/L of lactate were found in cask S 101 (cf. Tab. 3.34).

Malate was found in a concentration of 4.5 g/L in steel cask 3007 on day 1 and decreased to 2.3 g/L during fermentation (cf. Tab. 3.29). In the wooden casks with sluggish fermentation lower concentrations of malate were found than in the steel cask with normal fermentation (cf. Fig. 3.30) and except for wooden cask 2612 no malate was found in any of the other tested wooden casks at the end of fermentation (cf. Tab. 3.30 - 3.33). In the vintage 2012/2013, 3.05 g/L of malate were found at the beginning of the fermentation and it decreased to 1.85 g/L during fermentation (cf. Tab. 3.34).

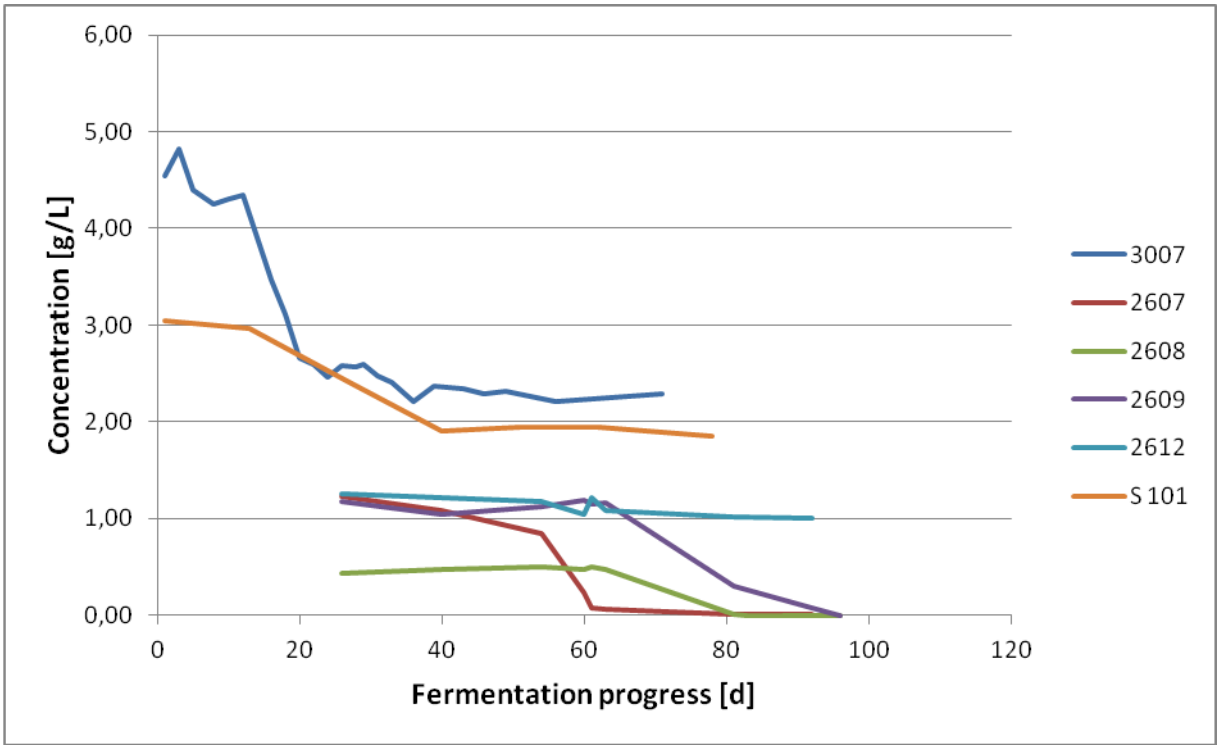


Fig. 3.30: Development of malate during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

Tartrate was found in varying concentrations which might be caused by the freezing of the samples. It was found in lower concentrations in the steel cask 3007 than in the wooden casks with sluggish fermentation (cf. Tab. 3.29 – 3.33). In the vintage in 2012/2013, tartrate concentrations varied from 4.43 to 3.12 g/L in steel cask S 101 (cf. Tab. 3.34).

Tables 3.29 - 3.34 show the concentrations of succinate, lactate, malate and tartrate in the tested casks. Acetate was detected in all casks with less than 0.2 g/L citrate was detected with less than 0.3 g/L.

Tab. 3.29: Development of organic acids during fermentation in cask 3007. Determined during vintage 2011/2012

Day of fermentation	Succinate [g/L]	Lactate[g/L]	Malate [g/L]	Tartrate [g/L]
1	0.00	0.00	4.54	2.97
3	0.00	0.00	4.82	2.61
5	0.00	0.00	4.40	2.97
8	0.00	0.00	4.25	3.09
10	0.00	0.00	4.30	2.70
12	0.00	0.00	4.35	3.00
14	0.00	0.00	3.91	2.73
16	0.06	0.00	3.47	2.23
18	0.37	0.02	3.11	3.22
20	0.74	0.01	2.66	3.56
22	0.83	0.02	2.59	2.79
24	0.92	0.02	2.46	3.34
26	1.04	0.02	2.58	3.22
28	1.09	0.02	2.57	3.49
29	1.12	0.00	2.60	2.73
31	1.14	0.03	2.47	3.39
33	1.25	0.04	2.41	2.46
36	1.27	0.00	2.21	2.53
39	1.28	0.03	2.37	2.57
43	1.30	0.00	2.34	2.51
46	1.28	0.00	2.29	2.33
49	1.20	0.00	2.31	2.46
56	1.28	0.00	2.21	2.44
71	1.25	0.00	2.29	1.73

Tab. 3.30: Development of organic acids during fermentation in cask 2607. Determined during vintage 2011/2012

Day of fermentation	Succinate [g/L]	Lactate [g/L]	Malate [g/L]	Tartrate [g/L]
23	1.03	0.98	1.23	4.41
37	1.03	1.00	1.09	3.62
51	1.02	1.17	0.84	4.20
57	0.93	1.33	0.24	4.18
58	1.06	1.71	0.07	4.15
60	1.09	1.72	0.06	4.65
78	1.09	1.80	0.01	3.72
89	1.09	1.78	0.01	4.05

Tab. 3.31: Development of organic acids during fermentation in cask 2608. Determined during vintage 2011/2012

Day of fermentation	Succinate [g/L]	Lactate [g/L]	Malate [g/L]	Tartrate [g/L]
26	1.16	0.98	0.43	3.48
40	1.15	0.99	0.47	3.25
54	1.13	1.00	0.50	3.55
60	1.03	0.94	0.47	3.54
61	1.12	1.12	0.50	3.42
63	1.07	1.01	0.47	3.71
81	1.14	1.90	0.01	3.33
83	1.20	1.93	0.00	3.34
88	1.17	1.88	0.00	3.01
92	1.20	1.93	0.00	3.03
96	1.22	1.93	0.00	2.86

Tab. 3.32: Development of organic acids during fermentation in cask 2609. Determined during vintage 2011/2012

Day of fermentation	Succinate [g/L]	Lactate [g/L]	Malate [g/L]	Tartrate [g/L]
26	1.21	1.04	1.18	4.08
40	1.17	1.07	1.04	3.36
54	1.19	1.05	1.12	3.75
60	1.19	1.08	1.19	3.51
61	1.17	1.09	1.15	3.84
63	1.18	1.09	1.16	4.00
81	1.26	1.56	0.30	4.98
92	0.92	1.29	0.08	4.00
96	1.19	1.72	0.00	3.51

Tab. 3.33: Development of organic acids during fermentation in cask 2612. Determined during vintage 2011/2012

Day of fermentation	Succinate [g/L]	Lactate [g/L]	Malate [g/L]	Tartrate [g/L]
22	1.34	1.25	1.26	5.01
36	1.30	1.25	1.21	3.81
50	1.24	1.19	1.18	4.65
56	1.18	1.14	1.05	4.61
57	1.31	1.25	1.22	5.09
59	1.31	1.25	1.08	4.75
77	1.31	1.27	1.02	4.34
88	1.33	1.31	1.01	4.33

Tab. 3.34: Development of organic acids during fermentation in cask S 101. Determined during vintage 2012/2013

Day of fermentation	Succinate [g/L]	Lactate [g/L]	Malate [g/L]	Tartrate [g/L]
1	0.00	0.00	3.05	3.62
13	0.00	0.69	2.96	4.19
27	0.57	0.78	2.41	4.43
40	0.71	0.67	1.91	3.19
51	0.67	0.68	1.94	3.38
62	0.70	0.67	1.94	3.31
78	0.68	0.78	1.85	3.12

3.5.4 Concentration of alcohols and glycerol during fermentation

Alcohols and glycerol are known to effect the growth of yeasts and bacteria. For that reason, these chemical components were also studied during fermentation. Ethanol and glycerol were analyzed in diluted must and young wine (dilution 1:3), threo-butandiol and meso-butandiol were analyzed in undiluted samples. Each sample was analyzed three times.

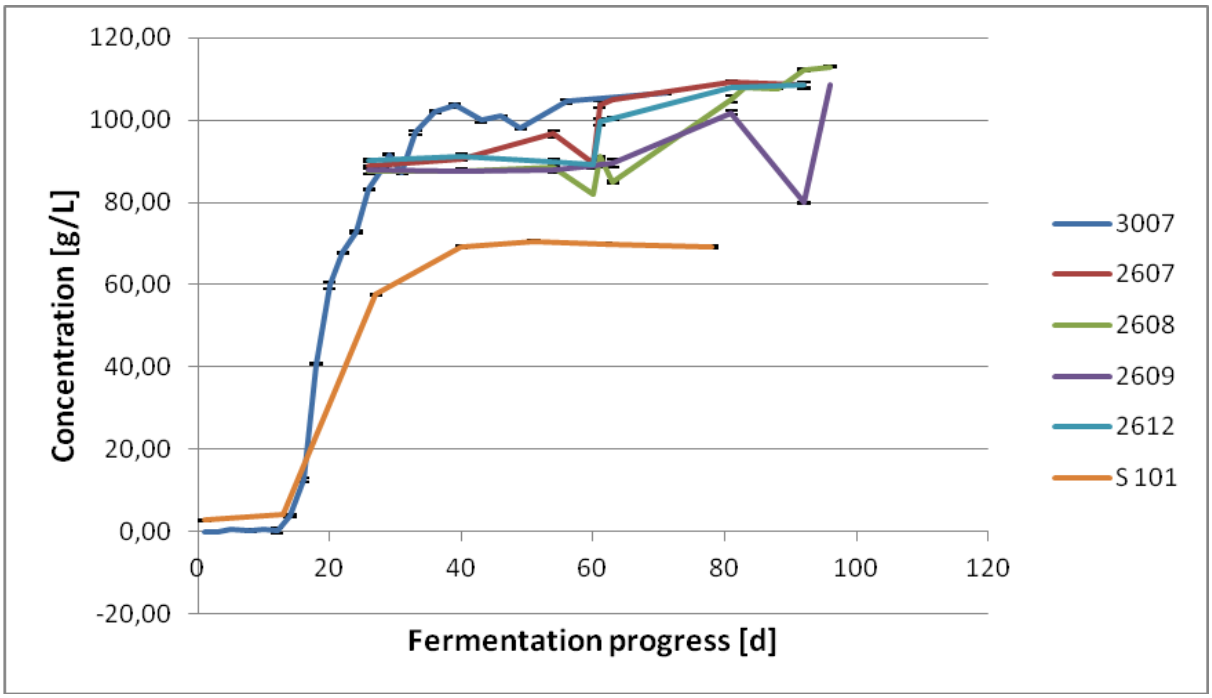


Fig. 3.31: Development of ethanol during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

In steel cask 3007 of vintage 2011/2012, 106.0 g/L of ethanol were formed during fermentation (cf. Tab. 3.36). The ethanol concentration of the four wooden casks with sluggish fermentation was similar to that in the control cask (cf. Fig. 3.31). In steel cask S 101 from the vintage

2012/2013, 69.1 g/L of ethanol were produced until it came to stuck fermentation (cf. Tab. 3.40, Fig. 3.31).

At the end of fermentation, 10.5 g/L of glycerol were found in the wine of steel cask 3007 (cf. Tab. 3.36). The concentration of glycerol in the wooden casks was slightly lower, except for wooden cask 2612, which contained 12.2 g/L at the end of the fermentation. In the cask S 101, 6.8 g/L of glycerol were found (cf. Fig. 3.32).

Threo-Butandiol was detected in similar concentrations in the steel cask 3007 and in the wooden casks 2607 and 2608. In wooden casks 2609 and 2612 the content of threo-butandiol was slightly lower (cf. Fig. 3.33, Tab. 3.35 – 3.39). In the second vintage in 2012/2013, 1.0 g/L threo-butandiol was measured in cask S 101 with stuck fermentation (cf. Tab. 3.40).

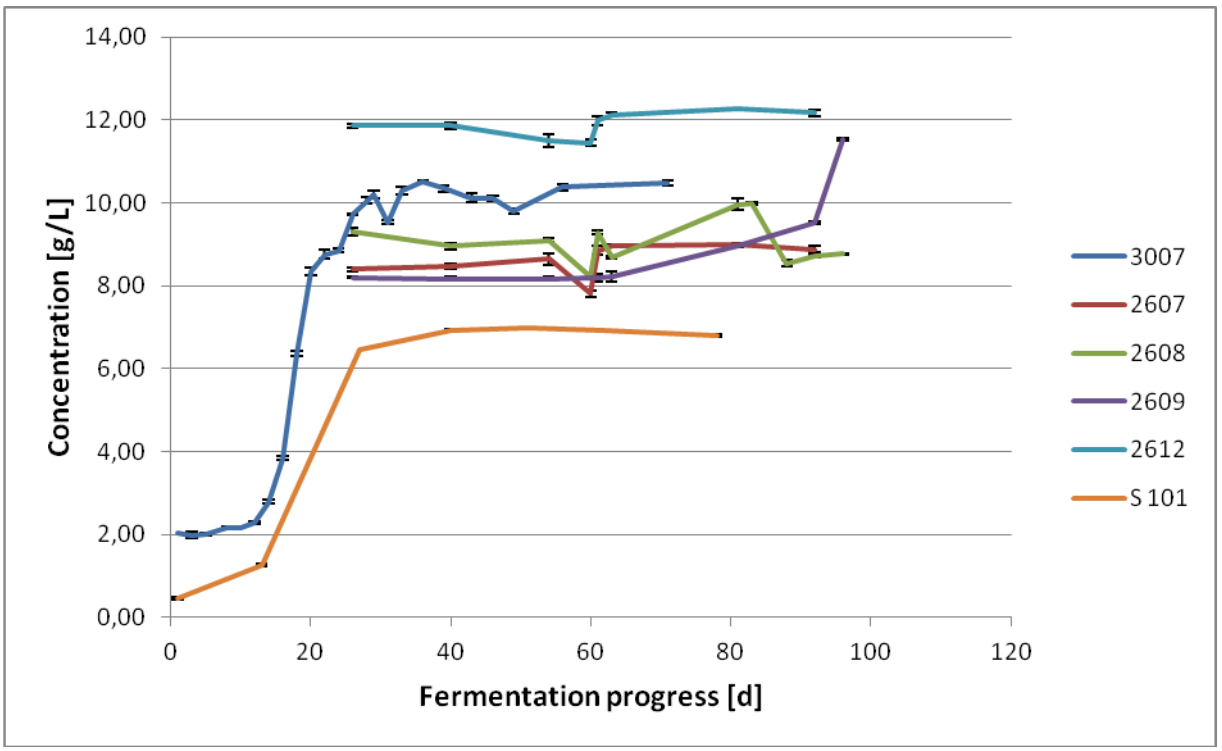


Fig. 3.32: Development of glycerol during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

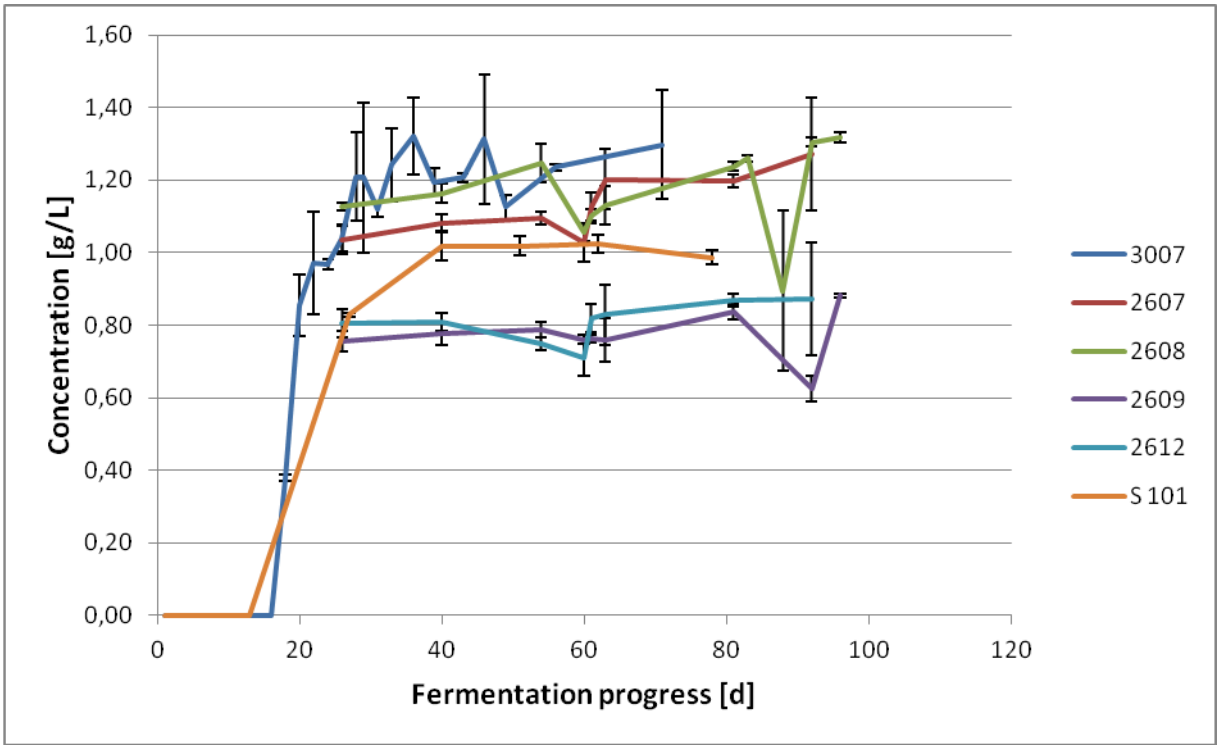


Fig. 3.33: Development of threo-butandiol during the fermentation progress
Control cask: steel cask 3007; casks with sluggish fermentation: wooden casks 2607, 2608, 2609 and 2612; steel cask S 101 from the second vintage 2012/2013 with stuck fermentation

Tables 3.35 - 3.40 show the concentrations of alcohols and glycerol in the six tested casks.

Tab. 3.35: Development of alcohol and glycerol [g/l] during fermentation in cask 2608. Determined in vintage 2011/2012

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
26	87.53	9.30	1.13	0.14
40	87.61	8.95	1.16	0.09
54	88.61	9.10	1.25	0.18
60	81.91	8.23	1.06	0.11
61	91.08	9.29	1.10	0.12
63	84.87	8.68	1.13	0.15
81	105.02	9.96	1.24	0.09
83	108.04	9.98	1.26	0.13
88	107.71	8.53	0.89	0.15
92	112.07	8.72	1.30	0.13
96	112.90	8.77	1.32	0.14

Tab. 3.36: Development of alcohols and glycerol [g/L] during fermentation in cask 3007. Determined in vintage 2011/2012

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
1	0.00	2.04	0.00	0.00
3	0.00	1.98	0.00	0.00
5	0.42	2.01	0.00	0.00
8	0.38	2.16	0.00	0.00
10	0.57	2.16	0.00	0.00
12	0.39	2.28	0.00	0.00
14	3.84	2.79	0.00	0.00
16	12.65	3.84	0.00	0.00
18	40.77	6.36	0.38	0.03
20	59.82	8.35	0.85	0.17
22	67.78	8.76	0.97	0.13
24	72.64	8,85	0.97	0.09
26	83.19	9.72	1.04	0.16
28	88.06	10.05	1.21	0.21
29	91.62	10.20	1.21	0.21
31	87.22	9.53	1.12	0.18
33	96.95	10.29	1.24	0.21
36	102.01	10.51	1.32	0.26
39	103.47	10.35	1.19	0.11
43	99.95	10.12	1.21	0.17
46	100.87	10.12	1.31	0.18
49	98.00	9.80	1.13	0.13
56	104.59	10.38	1.24	0.16
71	106.49	10.48	1.30	0.19

Tab. 3.37: Development of alcohol and glycerol [g/L] during fermentation in cask 2607. Determined in vintage 2011/2012

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
23	88.75	8.40	1.04	0.06
37	90.50	8.46	1.08	0.14
51	96.60	8.64	1.09	0.17
57	89.49	7.81	1.03	0.15
58	103.71	8.84	1.13	0.06
60	105.03	8.97	1.20	0.19
78	109.29	9.00	1.20	0.18
89	108.44	8.88	1.27	0.15

Tab. 3.38: Development of alcohol and glycerol [g/L] during fermentation in cask 2609. Determined in vintage 2011/2012

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
26	87.82	8.20	0.76	0.04
40	87.67	8.16	0.78	0.11
54	87.91	8.17	0.79	0.06
60	88.86	8.20	0.76	0.09
61	89.31	8.19	0.76	0.10
63	89.61	8.21	0.76	0.10
81	101.78	8.96	0.84	0.11
92	79.96	9.53	0.63	0.02
96	108.49	11.53	0.88	0.10

Tab. 3.39: Development of alcohol and glycerol [g/L] during fermentation in cask 2612. Determined in vintage 2011/2012

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
22	90.21	11.87	0.81	0.05
36	91.31	11.86	0.81	0.04
50	89.74	11.50	0.75	0.03
56	89.19	11.44	0.71	0.02
57	99.59	11.98	0.82	0.03
59	100.37	12.12	0.83	0.07
77	107.87	12.26	0.87	0.02
88	108.61	12.17	0.87	0.03

Tab. 3.40: Development of alcohol and glycerol [g/L] during fermentation in cask S 101. Determined in vintage 2012/2013

Day of fermentation	Ethanol	Glycerol	Threo-Butandiol	Meso-Butandiol
1	2.84	0.46	0.00	0.00
13	4.27	1.25	0.00	0.00
27	57.56	6.45	0.83	0.00
40	69.03	6.93	1.02	0.04
51	70.47	6.98	1.02	0.00
62	69.66	6.92	1.02	0.00
78	69.09	6.80	0.99	0.03

4 Discussion

4.1 Succession and diversity of yeasts during fermentation

The microbial succession was studied in two following years in order to find out if stuck fermentations in the cooperative winery were caused by microorganisms. In the vintage 2011/2012, one cask with normal fermentation (cask 3007) and one cask with stuck fermentation (cask 2608) were studied. In the second vintage 2012/2013, one cask with stuck fermentation was studied (cask S 101). Right after the filling of the cask 3007 and start of the fermentation, yeasts of the genera *Hanseniaspora*, *Candida*, *Cryptococcus*, *Pichia, Metschnikowia,* and *Aureobasidium* were found in vintage 2011/2012. The presence of non-*Saccharomyces* yeasts in spontaneous fermentations is well known (Fleet, 1997; Herrmann, et al., 2008; Lopandic and Prillinger, 2007) and the genera *Hanseniaspora* and *Candida* are most present. *Pichia* and *Metschnikowia* occur in lower cell counts (Fleet et al., 1984; Mills et al., 2002). These wild yeasts metabolize glucose and fructose almost equally (Dittrich and Großmann, 2005). Maqueda Gil et al. (2006) analyzed population and biodiversity of yeasts in 53 spontaneous fermentations and found that in must from deteriorated grapes the amount of apiculate yeasts was significantly higher. They also described that fermentation speed increased with high initial cell titers of *Saccharomyces* yeasts.

No yeast species of the genus *Saccharomyces* was found on day 1 in cask 3007, but from day 15 the species *Saccharomyces bayanus* dominated the fermentation with a cell count of up to 10^7 cells/mL. Tofalo et al. (2009) investigated yeast populations involved in a spontaneous fermentation of a high sugar must in central Italy. They found that only a restricted number of osmotolerant non-*Saccharomyces* yeast species were present throughout the fermentation process, while *Saccharomyces cerevisiae* prevailed after 15 days of fermentation.

The species *Saccharomyces bayanus* is also called *Saccharomyces bayanus* var. *uvarum* (Bartowsky and Pretorius, 2009; Naumov et al., 2000a; Tamai et al., 2007). Ultee et al. (2013) also found *S. bayanus*, which was referred to as *S. uvarum* by that time, as the main wine yeast species instead of *S. cerevisiae* in this winery. The absence of the classical wine yeast species *S. cerevisiae* might be due to the low temperatures in the cellar of about 12 °C – 15 °C as *S. cerevisiae* is known as a more mesophilic wine yeast whereas *S. bayanus* is a more cryophilic wine yeast species which is useful for fermentations at low temperatures (Kishimoto, 1994). The sampling of the cask 2608 started on day 26 of the fermentation, because at that time stuck fermentation was observed in this cask. In wooden cask 2608 which was also sampled in the first vintage 2011/2012 only yeasts of the species *S. bayanus* were found exclusively while the fermentation was stuck and the concentration of ethanol was already 88 g/l in the wine. By that time the glucose : fructose ratio was 1:22 (1.5 g/L glucose and 33 g/L fructose). Fermentation may stuck when the glucose : fructose ratio is lower than 1:10 (Pulver and Gafner, 2003). Sugar was added to the wine at that point according to the staff of the winery so that the glucose : fruc-

tose ratio increased to 1:3. Nevertheless, fermentation was stuck for more than four weeks. At the end of the stuck a triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* was found and finished fermentation. This hybrid is supposed to be useful for winemaking in the middle of Europe due to the combined characteristics of its parents, e.g. tolerance for alcohol and sugar of *S. cerevisiae* and tolerance for low temperature of *S. kudriavzevii* and *S. bayanus* (Gonzàlez et al., 2006). Several *Saccharomyces* species and their hybrids were tested by Tronchoni et al. (2009) for sugar consumption and the *S. kudriavzevii* type strain was the least affected by variations of temperature during fermentation. When the fermentation continued the hybrid *S. cerevisiae* x *S*. *kudriavzevii* x *S. bayanus* was the dominating yeast species, although *S. cerevisiae* and *S. bayanus* were found sporadically on the last day of sampling as well. Several authors have isolated this type of hybrids in Europe (Bradbury et al., 2006; González et al., 2008, Lopes et al., 2010), although the strains of *S. kudriavzevii* known so far have been isolated from decaying leaves in Japan (Naumov et al., 2000b) and from oak bark samples in Portugal (Sampaio and Gonçalves, 2008), but not yet from grapes or must. They isolated hybrid species might have come from the grapes but it is more likely that they have persisted on the surfaces and equipment of the winery as they have also been found in single samples from previous years (Blättel, 2012). *Saccharomyces* hybrids have already been described to be involved in the winemaking process (Arroyo-López et al., 2009; González et al., 2006). However, their role in overcoming stuck fermentation occurring during spontaneous fermentation has not yet been described.

In the second vintage 2012/2013, right after the filling of the cask yeast species of the genera *Hanseniaspora*, *Cryptococcus*, *Pichia, Metschnikowia, Rhodotorula, Saccharomycopsis* and *Aureobasidium* were found in the investigated cask S 101. As in the year before, no yeast species of the genus *Saccharomyces* was found at the beginning of the fermentation, but later on from day 13 the species *Saccharomyces bayanus* dominated the fermentation again at ethanol concentrations of 7 %vol and above with a cell count of up to 10^5 cells/mL. Figure 4.1 shows the percentage of total yeast isolates from cask 3007 (vintage 2011/2012) and cask S 101 (vintage 2012/2013). The variety of yeast species in vintage 2011/2012 was similar to the variety in vintage 2012/2013 although *Hanseniaspora uvarum* was not that predominant in the beginning of the fermentation in the first year. From day 40 there was a stuck of fermentation and fermentation did not continue until the cask was stopped by the winemaker on day 78 by sulphuring because he wanted to use this wine for coupage. The triple hybrid *S. cerevisiae* x *S*. *kudriavzevii* x *S. bayanus* was not found in this cask before or during the stuck. But before sulphuring, ten litres of this cask were taken to the IMW. Five litres were inoculated with the triple hybrid and five litres were just cultivated without special inoculation. The inoculated triple hybrid was immediately able to grow in the young wine and finish fermentation. The other five litres sample also restarted fermentation after four weeks and at that stage of restart, the triple hybrid was isolat-

ed from this sample as well. This means that the triple hybrid was also present at low cell titer in this stuck must as well alike to the observations of the previous year but first grows up during stuck at a stage of fermentation when nutrients and sugars are already reduced and *S. bayanus* decreased in cell counts. It is also possible, that this triple hybrid has become a resident of this winery and does not come from the grapes but from the equipment of the winery. Santamaría et al. (2005) found such a resident *S. cerevisiae* strain which took over spontaneous fermentation in all vintages studied in this winery.

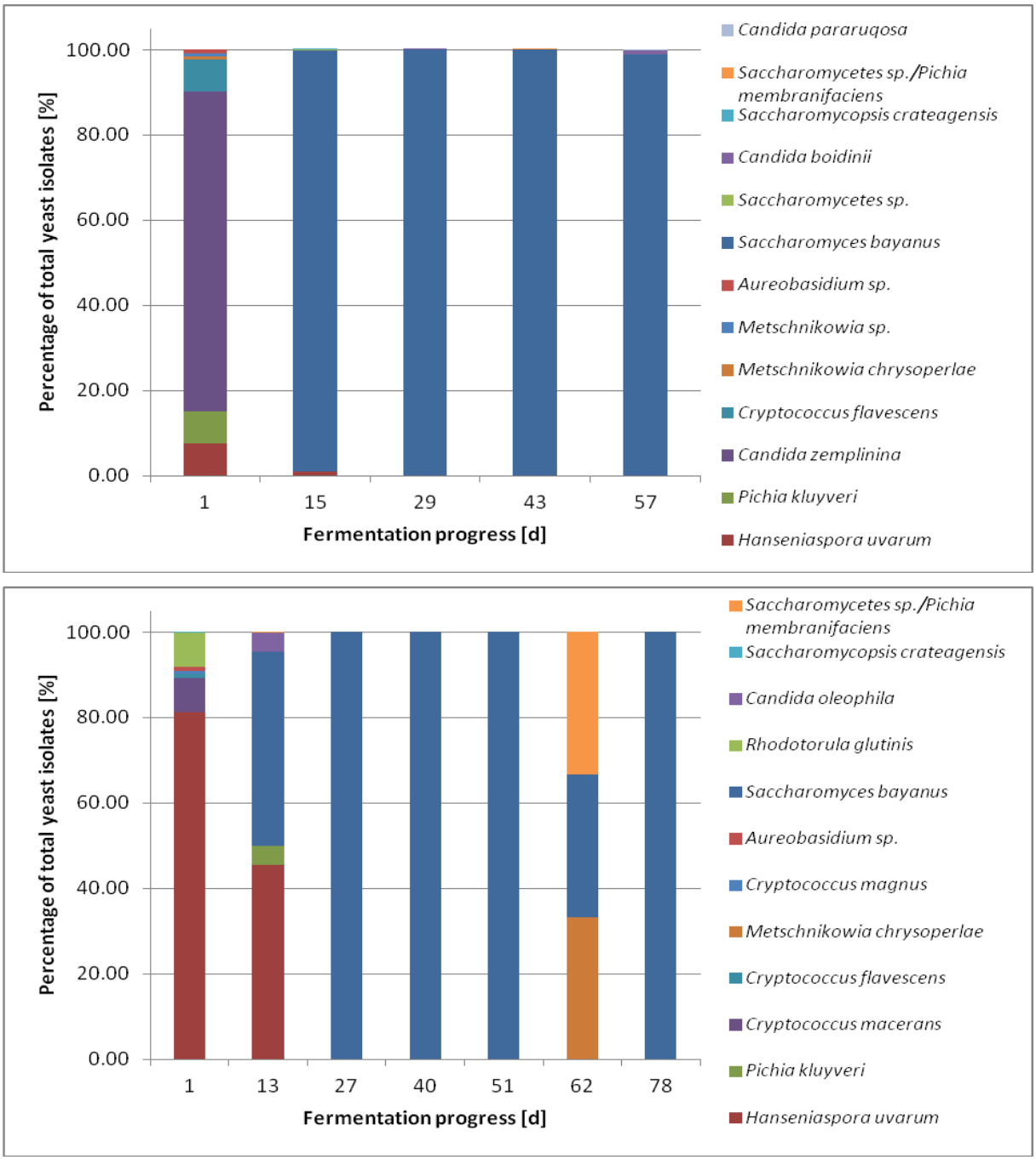


Fig. 4.1: Percentage of total yeast isolates during fermentation
A: Isolates from cask 3007 from vintage 2011/2012; B: Isolates from cask S101 from vintage 2012/3013; total cell titers varied from 10^3/mL to 10^7/mL

Spontaneous fermentations have a higher risk of stuck fermentations, especially because of the fermentation start by non-*Saccharomyces* yeasts. If the natural occurring *Saccharomyces* yeasts are not able to dominate the wild yeasts in the later stage, fermentation is going to stuck. Ciani et al. (2006) studied multistarter fermentations of *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* together with *Saccharomyces cerevisiae.* They found out that the mixed trials showed a comparable or even better fermentation behavior than *S. cerevisiae* single culture trials at high sugar contents in must. But they also proved that multistarter fermentations with non-*Saccharomyces* yeasts may cause stuck fermentations under stress conditions like high ethanol content and/or low temperature.

4.2 Bacterial succession and diversity during fermentation

In winemaking, bacteria can get in the must in different ways. They already exist on grapes in the vineyard and can be transferred to must by the pressing of grapes. Martins et al. (2012) analyzed the grape berry bacterial microbiota during the whole process of ripening and found over 44 species from 21 genera. In addition, they can survive on the equipment of the winery.

The bacteria most common in wine are lactic acid bacteria and acetic acid bacteria. Some species are able to grow during fermentation despite of the high concentrations of ethanol caused by yeasts (Fleet, 1997). Spores of bacilli and clostridia may also occur in low cell counts (Dicks et al., 2009; Mills et al., 2008). In fresh must from day 1 of the fermentation, acetic acid bacteria of the species *Acetobacter aceti* and *Gluconobacter cerinus* were found in steel cask 3007 from vintage 2011/2012 with cell counts of 10^5/mL. *Gluconobacter cerinus* was isolated continuously during fermentation. In addition, bacteria of the species *Acetobacter cibinongensis* were found on day 15. Although acetic acid bacteria are aerobic and need oxygen for their metabolism, they may occur in must and wine under certain conditions and may even proliferate (Mills et al., 2008). But the general assumption is that they don`t grow during alcoholic fermentation due to the anaerobic conditions (Du Toit et al., 2005). At the first sampling of wooden cask 2608 with stuck fermentation on day 26 of vintage 2011/2012, acetic acid bacteria of the species *Gluconobacter cerinus* and *Acetobacter sp.* were found with cell counts of 10^5/mL during the stuck fermentation. *Gluconobacter cerinus* was isolated continuously in the further course of the fermentation until day 82. Furthermore, on day 40 bacteria of the species *Acetobacter aceti* and *Gluconobacter sphaericus/*Gluconobacter *cerinus* resp. were found with cell counts of 10^2/mL. Despite the occurrence of acetic acid bacteria throughout the fermentation they did not produce significant amounts of acetic acid and therefore do not seem relevant for stuck fermentation. In vintage 2012/2013, on day one acetic acid bacteria of the species *Acetobacter aceti* and *Gluconobacter cerinus* were also isolated from the must of steel cask S 101 as well as strains of the genera *Microbacterium*, *Tatumella*, *Curtobacterium*, *Rhodococcus*, *Lactobacillus* and *Serratia*. Bacteria of the genus *Microbacterium* have not been associated with wine so far and especially

not with the causes of stuck fermentations. In contrast, *Tatumella ptyseos* has already been found in must (Nisiotou et al., 2011). Later on during fermentation, strains of the genera *Paenibacillus*, *Gordonia*, *Sporosarcina*, *Leifsonia*, *Oerskovia* and *Brevibacterium* were isolated, as well as the lactic acid bacteria species *Oenococcus oeni* and *Lactobacillus diolivorans*. Compared to steel cask 3007 from vintage 2011/2012, a higher variety of bacterial species was observed in the cask S 101 from the second vintage (cf. Fig. 4.2).

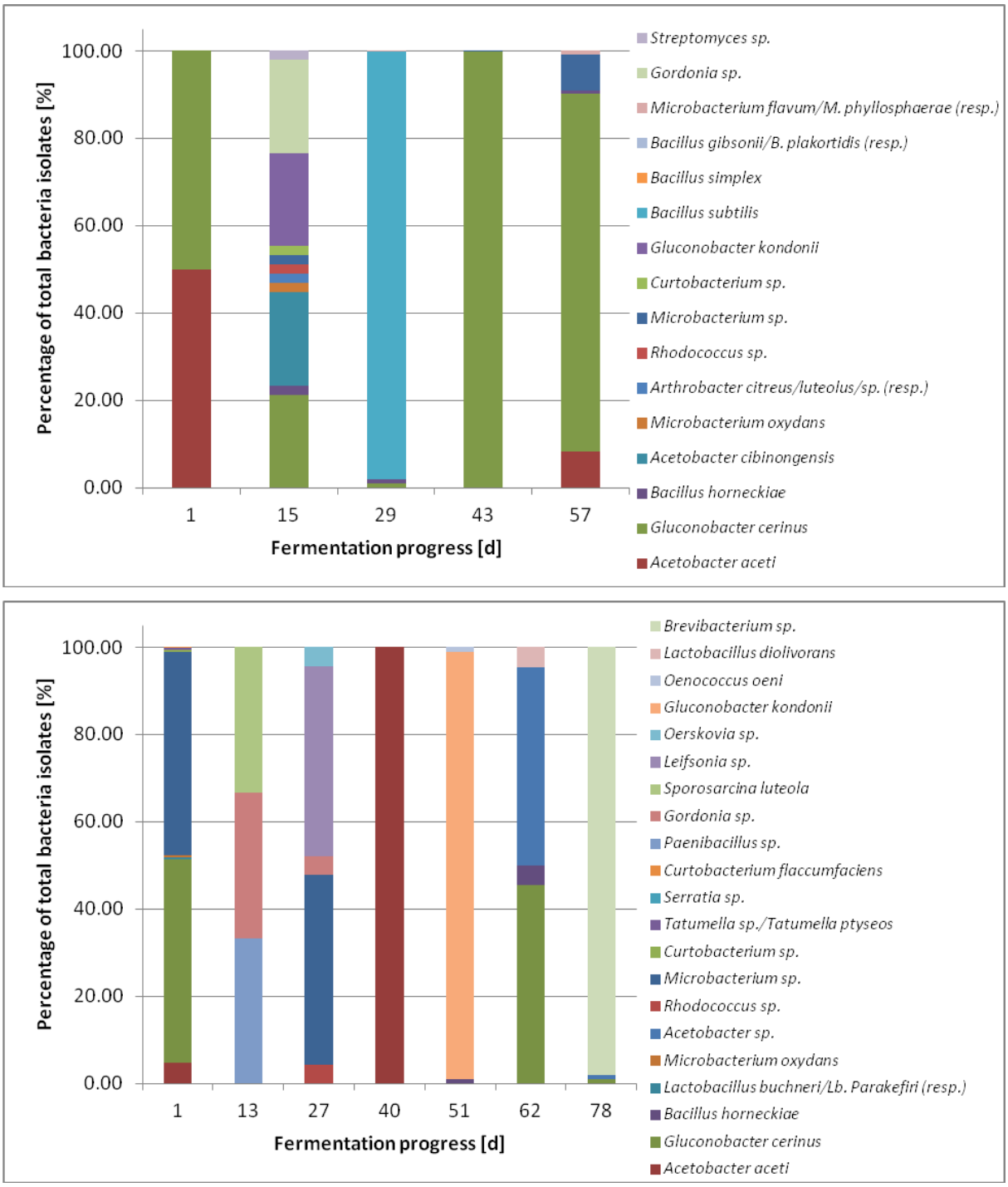


Fig. 4.1: Percentage of total bacteria isolates during fermentation
A: Isolates from cask 3007 from vintage 2011/2012; B: Isolates from cask S101 from vintage 2012/3013; total cell titers varied from 10^3/mL to 10^5/mL

Besides the acetic acid bacteria, some bacteria of the genus *Bacillus* were found in steel cask 3007 from vintage 2011/2012 without stuck fermentation. *Bacillus horneckiae* was found, as well as the species *B. subtilis, B. simplex* and *B. gibsonii*/*B. plakortidis* (resp.). Several authors have reported about the isolation of bacilli from wine and must in the past (Gini and Vaughn, 1962; Mills et al., 2008). As the isolated bacilli strains were not able to grow in must, it is assumed that they have only been in the samples as spores but not as living cells and do not play an important role for stuck fermentation.

Very few lactic acid bacteria were isolated from the solid media. Only bacteria of the species *Lactobacillus buchneri* or *Lb*. *parakefiri,* respectively, were isolated on day 96 with cell counts of 10^2/mL in wooden cask 2608 of vintage 2011/2012. Therefore, enrichment cultures for the lactic acid bacteria were cultivated as well. In the enrichment cultures of steel cask 3007 without stuck fermentation of vintage 2011/2012 lactic acid bacteria of the species *Oenococcus oeni* could be identified via multiplex PCR. These bacteria are desirable especially in wines with a high content of acid because they are responsible for malolactic fermentation (Dittrich and Großmann, 2005). During malolactic fermentation L-malate is metabolized to L-lactate, which leads to a more harmonious acid profile of the wine (Dittrich and Großmann, 2005). In steel cask 3007 in vintage 2011/2012 ca. 2.2 g/L malate were decreased, but no detectable amounts of lactate were built due to the low cell titres of lactic acid bacteria. In the enrichment cultures of wooden cask 2608 bacteria of the species *Pediococcus damnosus* were found besides *Oenococcus oeni*. At the time of the first sampling on day 26 during the stuck, only 0.4 g/L of malate were left in this barrel. Until the end of the fermentation 1.9 g/L lactate were built. In the second vintage in 2012/2013, 3.05 g/L of malate were found in fresh must and it decreased to 1.85 g/L during fermentation. The lactic acid bacteria species *Oenococcus oeni* and *Lactobacillus diolivorans* and *Lactobacillus buchneri*/*Lb*. *parakefiri* (resp.) were isolated via plating with cell counts of 10^2/mL and multiplex PCR. From these results, there are no indications that the succession of bacteria leads to stuck fermentation in this winery.

4.3 Contents of must and young wine

At the beginning of the fermentation in the first vintage in 2011/2012, a total amount of 221.5 g/L sugar in a glucose : fructose ratio of 1.2:1 was determined in must from cask 3007 which showed no stuck during fermentation. In the second vintage in 2012/2013, the total sugar amount was 238.8 g/L with a glucose : fructose ratio of 1.3:1 in fresh must of the cask S 101 which came to stuck later on. In general, about a total of 200.0 g/L glucose and fructose can be found in must and the ratio is 1:1 (Dittrich and Großmann, 2005). Charoenchai et al. (1998) investigated the influence of the sugar concentration in juice on the growth of yeasts. An increase from 200 g/L to 300 g/L led to decreased growth rates for some yeasts and decreased final cell

biomass for all of the tested yeasts. The initial glucose : fructose ratio changes from season to season as a result of climate and ripeness level (Malherbe et al., 2007).
The wooden casks 2607, 2608, 2609 and 2612 from the first vintage in 2011/2012 all had a sluggish or stuck fermentation when the first sampling took place on day 26. The four wooden casks showed fructose : glucose ratios of approximately 11:1 (wooden cask 2607), 22:1 (wooden cask 2608), 9:1 (wooden cask 2609) and 15:1 (wooden cask 2612). A fructose : glucose ratio of 10:1 may lead to stuck fermentation according to Pulver and Gafner (2003). Sugar was added to wooden cask 2608 with the highest fructose : glucose ratio which increased the ratio to 3:1 and from that point on both sugars decreased continuously. No sugar was added to the other wooden casks but both glucose and fructose decreased, which means that despite of the high fructose : glucose ratios the fermentation continued. In the second vintage in 2012/2013, the investigated cask S 101 showed a fructose : glucose ratio 2.4:1 when it came to stuck fermentation after 40 days. In the winery, it could be observed that in the beginning of the fermentation, both sugars were decreased almost equally. But in the second half of the fermentation, glucose seemed to be utilized faster than fructose before it comes to stuck fermentation. Tronchoni et al. (2009) observed that fructose fermentation was more inhibited by high ethanol levels than glucose fermentation. From these results it was assumed that the glucose : fructose ratio represents one part of the complex problem of stuck fermentation. For this reason, investigations on the sugar utilization of the isolated yeasts were performed later on.

Up to 1.25 g/L succinate were built during fermentation in the investigated casks of both years. In wine, 0.0 - 2.0 g/L succinate can be found usually (Radler, 1992; Riberau-Gayon et al., 1972). Succinate is regarded as favorable for wine quality because of its salt-bitter acidic taste (Remize et al., 1999). Up to 1.9 g/L lactate were found during both years of sampling and the concentration was higher in the casks with stuck fermentation compared to normal fermentation. Postel et al. (1973) tested 99 wines for lactic acid and found total concentrations from 0.10 - 5.61 g/L. There was a tendency for a higher decrease of malate in the casks with stuck fermentation. Malate might be converted to succinate via a reductive pathway of yeasts (Remize et al., 1999) but it is also transformed to lactate during malolactic fermentation. Acetate was found in concentrations of not more than 0.20 g/l in all casks from both vintages which is clearly below the limiting value of 1.08 g/l for acetate in white wine. In general, 0.2 - 0.8 g/L acetate can be found in wine (Fleet and Heard, 1992; Ribereau-Gayon et al. 1972). So despite the existence of acetic acid bacteria, the production of acetic acid is down to the lowest limit in the investigated wines. There is no hint that these levels of acetic acid or any other of the acetic acids may have caused or influenced stuck fermentation.

4.4 Physiological properties of the isolated yeast strains

From the results of the first two years of sampling concerning succession of microorganisms and chemical composition of the must it was assumed that the two isolated main fermenting yeasts play an important role for recurring stuck fermentation observed in this winery. Therefore, several experiments were performed in order to characterize the isolated yeast species. Under controlled laboratory conditions in synthetic B-medium close to grape must, the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 showed delayed growth compared to *S. bayanus* strain HL 77 just like it could be observed in the winery. These results verify the hypothesis that *S. bayanus* dominates fermentation after the wild yeasts in this winery because of faster growth compared to the triple hybrid. Concerning sugar utilization, the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* HL 78 was able to use fructose significantly better than *S. bayanus* strain HL 77, especially in the second half of the fermentation. In all casks with stuck fermentation, low glucose : fructose ratios were measured. These observations are a strong hint for the ability of the hybrid to grow up when sugar is already decreased or low glucose : fructose ratios are reached and *S. bayanus* is already struggling with stuck fermentation. Many of the wine yeasts have a higher affinity for glucose, which may lead to undesirable residual amounts of fructose in wine and sluggish fermentation (Berthels et al., 2004; Gafner and Schütz, 1993). D`Amore et al (1988) tested a *S. cerevisiae* strain for sugar uptake and they found out that glucose inhibited fructose uptake by 60 %, and likewise fructose inhibited glucose uptake by 40 %. Therefore, they suspected that both sugars share the same membrane transport components. Several hexose transporter (*HXT*) genes were studied by Guillaume et al. (2007) of a *S. cerevisiae* wine yeast strain with an enhanced fructose utilization capacity. They found a mutated *HXT3* allele which was sufficient for producing an increase in fructose utilization during fermentation. Besides the sugar transport across the membrane, fructose utilization can also be influenced by hexose phosphorylation. Glucose and fructose are phosphorylated by the hexokinases Hxk1 and Hxk2, whereas the glucokinase Glk1 is only specific for glucose (Entian, 1997). To avoid sluggish or stuck fermentations, the use of fructophilic yeast strains like the triple hybrid is advantageous in the production of dry wines.

The amount of available nitrogen is considered to be one of the main limiting factors for yeast growth in musts (Pretorius, 2000). Blateyron and Sablayrolles (2001) investigated slow, sluggish and stuck fermentations and they found that slow fermentations with low fermentation rate throughout the whole process were always due to low concentrations of yeast assimilable nitrogen in must. On the other hand, they came to the conclusion that stuck and sluggish fermentations with low yeast viability at the end of the fermentation could not be predicted from the analytical data. Stuck fermentations because of nitrogen deficiency might be due to the inhibition of the synthesis of proteins transporting the sugar through the cell membrane

(Malherbe et al., 2007). The demand for YAN varies between 140 mg/L and 880 mg/L (Dittrich and Großmann, 2005). Both isolated yeast strains, *S. bayanus* strain HL 77 and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* HL 78 were able to grow in the absence of $(NH_4)_2SO_4$ to the synthetic medium when amino acids were present. The *S. cerevisiae* strain used as control (Fermivin®) could not grow without ammonium. It has been reported that concentrations of assimilable nitrogen below 140 mg/L impair fermentation by *S. cerevisiae* at normal sugar concentrations, and a concentration of ammonium ions below 25 mg/L is generally considered to be undesirable (Bely et al., 1990). Kinetics of alcoholic fermentation of *Saccharomyces cerevisiae* wine strains in a synthetic medium with high sugar content were investigated by Taillandier et al. (2007) with initial nitrogen amounts from 120 to 290 mg N/L assimilable nitrogen. They found out that in general, the influence of yeast assimilable nitrogen was greater on sugar consumption rates than on growth of the yeasts. Mendes-Ferreira et al. (2004) described that fermentation rate and the time required for completion of the alcoholic fermentation by *S. cerevisiae* were strongly dependent on nitrogen availability. Their tested *S. cerevisiae* strain required a minimum of 267 mg N/L to attain complete dryness of media and lower levels were enough to support growth, but lead to sluggish or stuck fermentation. This higher demand for ammonium of *S. cerevisiae* seems to be one of the main reasons why *S. bayanus* and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* were found as dominating wine yeasts in this winery and not *S. cerevisiae*, as only 160 mg N/L were found in the beginning of the fermentation. This already low nitrogen concentration decreased down to 40 mg N/L within the first two weeks. These results show that all three tested yeast strains react in different ways on ammonium concentration. Therefore, it is not useful to apply ammonium supplementation on a routine basis without having information about the requirements of the yeast strain in use. Excess of assimilable nitrogen can lead to inhibition of sugar consumption for some wine yeast strains (Taillandier et al., 2007). Over-supplementation may also have negative effects on quality related parameters, notably on volatile acidity and aroma complexity (Martínez-Moreno et al., 2014). In addition, the fact that the isolated yeast strains are less dependent on nitrogen supplementation makes them useful as commercial starter cultures.

Low levels of YAN were measured in must from the investigated winery, but the isolated yeast strains *S. bayanus* strain HL 77 and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 must have found a way to use nitrogen from other sources like amino acids. Therefore, the isolated yeast strains were cultivated in synthetic B-medium with different concentrations of amino acids and at 15 °C. The temperature of the cellar in the investigated winery is usually 12-15 °C. The triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* strain HL 78 showed delayed growth at 15 °C compared to *S. bayanus* strain HL 77 and reached a lower OD (600 nm). This could be one of the main reasons why *S. bayanus* is the dominating wine yeast

species until it comes to stuck fermentation in the winery. At this temperature of 15 °C, the triple hybrid strain HL 78 showed a slightly lower growth at high amino acid concentrations whereas *S. bayanus* strain HL 77 showed delayed growth at reduced amino acid levels. Sugar consumption of the isolated yeast strains in relation to amino acid concentration of the medium was also tested at 15 °C, as this is close to the temperature in the winery. Amino acid concentration of the medium was varied to test whether this parameter effects sugar consumption. At reduced amino acid concentrations, *S. bayanus* strain HL 77 utilized both glucose and fructose slower than with 100 % of amino acids. The triple hybrid *S. cerevisiae* x *S*. *kudriavzevii* x *S*. *bayanus* strain HL 78 used both sugars glucose and fructose faster at reduced amino acid concentrations. These results show that the triple hybrid *S. cerevisiae* x *S*. *kudriavzevii* x *S*. *bayanus* is less dependent on the amino acid concentration of the medium and is able to utilize both glucose and fructose with reduced amino acid supply which might be one of the main reasons why this yeast strain replaces *S. bayanus* at a later stage of fermentation, when sugars and nutrients are already reduced and fermentation became stuck. In case of amino acid starvation, the protein synthesis in yeasts has to be controlled. Rolfes and Hinnebusch (1993) found increased transcription of more than 30 genes encoding amino acid biosynthetic enzymes in numerous pathways in response to starvation of a single amino acid. This regulatory mechanism is known as general amino acid control and involves binding of the transcriptional activator protein GCN4 to the promoter region of each gene subject to the general control (de Aldana, 1995). One of the most important mechanisms for translation control is the eukaryotic initiation factor (eIF)-2. When it comes to amino acid starvation, high levels of uncharged tRNA accumulate and this leads to a stimulation of the GCN2 kinase (Wek, 1994). This protein kinase is present in yeasts and phosphorylates the α-subunit of the eIF-2 (de Aldana, 1995) and therefore regulates translation initiation (Wek, 1994). The triple hybrid might be adapted better to limitations or changes of the concentration of amino acids in must.

In order to test if any of the amino acids in the amino acid solution of the synthetic B-medium is essential for growth of the isolated yeasts, *S. bayanus* strain HL 77 and the triple hybrid *S. cerevisiae* x *S*. *kudriavzevii* x *S*. *bayanus* strain HL 78 were cultivated at 15 °C in several parallels with one amino acid of the solution missing in each parallel. *S. bayanus* showed delayed growth of five days when methionine was missing in the synthetic B-medium but reached a similar OD (600 nm) to the other parallels. The triple hybrid *S. cerevisiae x S. kudriavzevii x S. bayanus* showed delayed growth without histidine, tryptophan or alanine. Especially, the lack of tryptophan seems to slow down growth for about five days compared to the parallels including trytophan. The parallel with full amino acid supply (control) grows slower than the parallels with one amino acid missing but a little bit faster than the parallel without tryptophan.

HPLC analysis of the must samples from casks with normal and with stuck fermentation showed that during the stuck, amino acid concentrations were lower than in the control cask and especially methionine was reduced. Malherbe et al. (2007) described that the rate of fermentation by yeast and bacteria is considerably influenced by the amino acid composition of the must. Jiranek et al. (1995) showed that amino acid utilization can vary from one strain to another even between different wine yeasts of *S. cerevisiae*.

The characterization of the isolated yeast strains *S. bayanus* HL 77 and the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* HL 78 verify the thesis that the triple hybrid is able to grow in a stage of fermentation when nutrients are already reduced and the glucose : fructose ratio is low. The observations lead to the conclusion that the hybrid could be useful for fermentations because this yeast strain seems to be able to adapt rapidly to changing conditions in must. Kishimoto (1994) used *S. bayanus* for hybridization with *S. cerevisiae* and found improved low temperature fermentation ability as well as higher production of malic acid and flavor compounds like higher ethanols and lower amounts of acetic acid compared to the single *S. cerevisiae* strain. Nevertheless, *Saccharomyces* hybrids have not been known yet to be able to overcome stuck or sluggish fermentations. This knowledge offers new ways to avoid stuck fermentations in winerys by using their own *Saccharomyces* yeast strains at different times of fermentation without the risk of changing the characteristic and desired sensory profile of the wines. For winegrowers of the upper class segment, it is of highest importance not to change their unique aroma profile by any treatment of the wine or the use of starter cultures.

5 Outlook

The role of *Saccharomyces* hybrids in overcoming stuck fermentation has not been mentioned before and it is not yet fully understood why the triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. bayanus* is able to use fructose more efficiently than *S. bayanus* in the second half of the fermentation. More experiments concerning sugar uptake and utilization are currently carried out within a doctoral thesis (pers. communication). In addition, it would be interesting to find explanations for the ability of the isolated yeast strains to grow in the absence of ammonium as long as amino acids are present. Possible differences of the enzyme composition of these yeasts would reveal important information for the wine industry and possible commercial culture strains could be selected for a low demand of yeast assimilable nitrogen.

6 Summary

The goal of this thesis was to gain more information about unconventional reasons for stuck fermentations and find new ways to overcome this problem. Microbial succession and chemical composition during fermentation were studied in two following years in a winery from the upper Moselle in Germany. From the results, there was no hint that the isolated bacterial species or chemical components of must and young wine were involved in sluggish or stuck fermentation. Furthermore, during this work it could be shown that *Saccharomyces bayanus* was the dominating wine yeast in this winery instead of the classical and well-known wine yeast *Saccharomyces cerevisiae*. During stuck fermentation, a triple hybrid *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* x *Saccharomyces bayanus* grew up, replaced *Saccharomyces bayanus* and finished fermentation. Both isolated yeast strains, *Saccharomyces bayanus* strain HL 77 and the triple hybrid *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* x *Saccharomyces bayanus* strain HL 78 were able to use glucose and fructose from the beginning and were able to grow at low temperatures of 15 °C and in the absence of yeast assimilable nitrogen in form of ammonium as long as amino acids were present in the medium in contrast to a commercial *Saccharomyces cerevisiae* yeast strain. Chemical investigations revealed that yeast assimilable nitrogen was very limited in the must from the cooperative winery with a maximum of 160 mg/L at the beginning of the fermentation which decreased to 40 mg/L after two weeks. For that reason, both isolated yeast strains are interesting as starter cultures in this winery and it may also explain why *Saccharomyces cerevisiae* is not the main fermenting yeast in this case besides the low temperature in the cellar. The triple hybrid *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* x *Saccharomyces bayanus* strain HL 78 is able to use fructose even more efficiently than *Saccharomyces bayanus* strain HL 77 and is less dependent on the amino acid concentration. This strain has already successfully been used during this project to restart fermentation in the cooperative winery it was isolated from. *Saccharomyces* hybrids have already been described to be involved in wine making, but their role in overcoming stuck fermentations has not been mentioned yet. These results are useful to avoid or overcome stuck fermentation with the selective use of these yeast strains at different stages of fermentation. The cooperative winery which is placed in the upper quality segment had problems with stuck fermentation every year. Therefore, the application of the triple hybrid *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* x *Saccharomyces bayanus* strain HL 78 is a great opportunity to avoid stuck fermentations and financial losses without using commercial starter cultures or other common practices which can lead to a change in the aroma profile. The described investigations could be taken as a model procedure to overcome fermentation problems also in other wineries applying spontaneous fermentation.

7 References

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8 Danksagung

Diese Arbeit wurde durch die DFG (KO 785/17-1) finanziell unterstützt.