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Yeasts as postfermentation agents in beer

***Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts are used since a long time in Belgian speciality beers for postfermentation improvements of maturating and conditioned beer. The use of *S. cerevisiae* strains is justified by carbonation effects and some oxygen removal. In contrast, the effects of non-*Saccharomyces sp*., which are present in sour beers mainly, are effective regarding flavour evolution predominantly. This study reviews successively the physiology (energy) aspects of the several yeasts species encountered in Belgian speciality beer and the potential of *Saccharomyces cerevisiae* strains by flavour modification after beer conditioning. The more practical aspects regarding pitching and yeast preparation are considered too. Further, several interesting features of *Brettanomyces sp., Dekkera sp., Kloeckera sp., Candida sp., Cryptococcus sp.* and *Torulopsis sp.* are studied. The influence of some enzymatic activity as alcohol dehydrogenase, aldehyde dehydrogenase, β-glucosidase and alcohol oxidase under peculiar conditions can allow the development of some typical flavours. The use of herbs, spices or fruit together with these yeasts will accelerate, modify, improve and probably, sometimes, alter the "final touch" of the beer. Scientific research and projects are going on to understand and control further the production of speciality beers.**

BC 40 General (brewery biology)

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Saccharomyces sp.

For beer refermentation most often a pure culture of a *Saccharomyces cerevisiae* strain is used. Yeast and fermentable sugars are added before the bottle filling [10, 31, 40]. The fermentable sugars added to the beer are generally glucose and sucrose, which are commercially available as granulated sugar or as syrups. The glucose, fructose and sucrose are rapidly utilized. Sucrose is hydrolysed at the outer surface of the cell to glucose and fructose by a periplasmatic invertase. The monosaccharides are transported into the cell by a transporter enzyme.

Energetic, redox and biomass balance of the postfermentation

Balance of the cell budding phase

When a microbial population is inoculated into a fresh medium, growth usually does not begin immediately. Changes in the physical nature of the environment induce a lag phase. Once adaptation to the new conditions commences and sufficient reserves are built up, the rate of growth increases until the cells enter the exponential phase. During this phase of growth the population increases at a constant rate.

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Aerobic part

Oxygen dissolved in beer or molecular oxygen in the bottleneck determines the fermentation rate since it is essential for the synthesis of unsaturated fatty acids and sterols, which are incorporated in the cell membrane. Dissolved oxygen removal is quite fast (a few hours for the dissolved oxygen) by the yeast mainly while the oxygen of the bottleneck is only metabolised at a rate of about 30%, the other 70% are removed by oxidation in beer [10]. The fate of the dissolved oxygen in refermented beer is still not completely clear. In fact, one could expect that the oxygen should be taken up by the yeast but it is established that the dissolved oxygen present at the beginning of the refermentation reacts also with compounds involved in beer ageing. Indeed, it was demonstrated by *Derdelinckx* et al. [10] by tastings and by measurements of the colour evolution that ageing occurs more quickly in beer aerated before bottling; such a beer is characterized by less flavour stability although the carbonation efficiency (CO_2 production/time) by the yeast is better. Therefore yeast preoxygenation could be advised. In the yeast cell itself, the oxygen must be further assimilated both in the lipidic pathways involved in the unsaturated fatty acids and sterols necessary for the membrane and by respiration. Indeed, even under fermentative conditions for *S. cerevisiae* (see paragraph on: Regulation of carbohydrate metabolism), we calculate that by addition of 10 000 mg/L of fermentable sugar in order to produce 5 g of carbonic acid pro litre beer, 200 mg of these sugar can be metabolised by the Krebs cycle of the respiratory pathway. The theoretical oxygen demand to carry out the full respiration reactions is 214 mg O_2 pro liter of beer; this value is much higher than the dissolved oxygen available at maximum in flat beer (at maximum 10.0 mg pro liter). The limiting factor for sugar respiration during beer refermentation is thus the oxygen available; in consequence, less than 5% of the sugar added can be metabolised by the respiration pathway.

Only low amounts of glucose and fructose taken up, are used in anabolic pathways. Most of the sugar is metabolised in the cytosol through the Embden-Meyerhof-Parnas route (also termed EMP, glycolytic pathway or glycolysis). The monosaccharides are metabolised to two molecules of the three-carbon compound pyruvate

yielding two molecules of ATP for each molecule of hexose converted to pyruvate. At the same time, two molecules of NAD^+ are reduced to NADH.

Under aerobic conditions and in the absence of repressing levels of glucose, acetyl coenzyme A is obtained from the oxidative decarboxylation of pyruvate available from the glycolysis. The decarboxylation of pyruvate is catalysed by a cluster of three enzymes called the pyruvate dehydrogenase complex and is located in the mitochondria. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle by the transfer of its acetyl group to the four-carbon acceptor oxaloacetate generating citrate. Through the subsequent steps of the cycle, two molecules of CO_2, 3 NADH, 1 $FADH_2$ and 1 GTP are produced.

The final stage of glucose catabolism by respiring yeast also occurs within the mitochondria where the reduced cofactors NADH and $FADH_2$, formed by the complete oxidation from glucose, are themselves oxidized to regenerate NAD^+ and FAD respectively. The released electrons are shuttled along the respiratory chain (including ubiquinone, iron-sulphur-containing carriers and cytochromes) to molecular oxygen. The oxidation by electron transport of each molecule of NADH produced by the citric acid cycle may generate up to 2.5 molecules of ATP, whereas oxidation of a molecule of $FADH_2$ yields 1.5 ATP molecules. A summary of the major routes for the aerobic glucose catabolism is presented in figure 1[19].

The NADH generated by glycolysis in the cytosol cannot be oxidized directly within the mitochondria since these organelles do not contain a transport system for these reduced coenzymes.

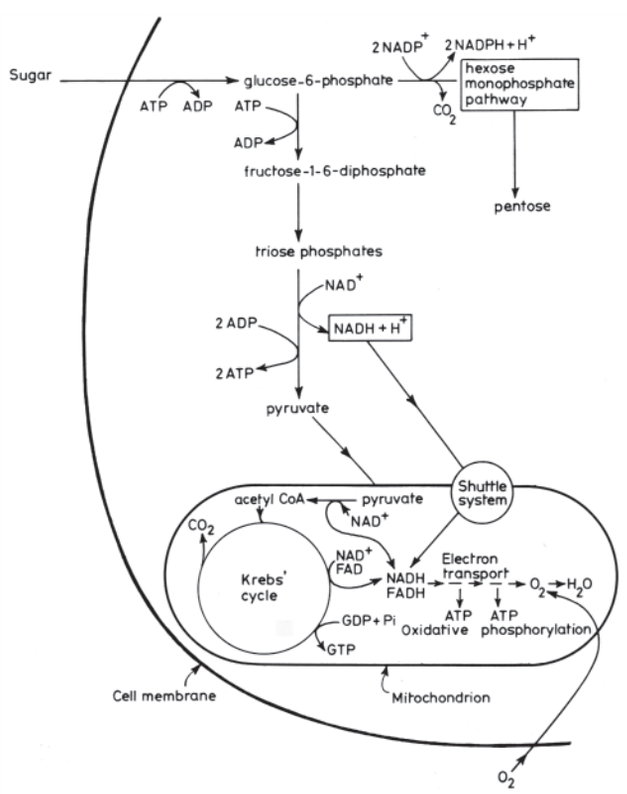

Figure 1 Aerobic metabolism of sugar [19]: effective for less than 5.0% of the fermentable sugar added to the beer for refermentation

Two shuttle systems, the glycerophosphate shuttle and the malate shuttle, may be used to import electrons from the cytosolic NADH to the respiratory chain. In both systems, a cytosolic dehydrogenase utilizes NADH to reduce organic molecules, which may enter the mitochondria whereupon a mitochondrial dehydrogenase oxidizes them to yield reduced cofactors. In the glycerophosphate shuttle system, dihydroxyacetone phosphate is reduced to glycerol-3-phosphate, which enters the mitochondria where it is oxidized by an FAD-dependent dehydrogenase. The $FADH_2$ produced reacts with the respiratory chain. In the second shuttle, cytosolic NADH reduces oxaloacetate to malate, which is carried across the inner membrane by a specific transporter. Inside the mitochondria, the malate is re-oxidized to oxaloacetate, reducing NAD^+ to NADH.

Anaerobic part

Under anaerobic conditions, the pyruvate produced by the EMP pathway is decarboxylated by the enzyme pyruvate decarboxylase with the formation of acetaldehyde and CO_2. The formed acetaldehyde acts, in the absence of molecular oxygen, as an electron acceptor and is used to oxidize NADH with the formation of ethanol. This reaction is carried out by an alcohol dehydrogenase. During the cell budding phase, removal of pyruvate for biosynthesis may lead to a build up of NADH and thus to a halt in sugar catabolism. To prevent this, cells reduce dihydroxyacetone phosphate to glycerol phosphate. This, in turn, is dephosphorylated to produce glycerol, which is excreted [19].

Under anaerobic conditions, the levels of the TCA cycle enzymes in yeast are greatly lowered. Two mechanisms, an oxidative pathway and a reductive pathway, have been proposed by which the yeast cell can synthesize organic acids essential for biosynthetic reactions and cell growth under these circumstances. In both mechanisms pyruvate is converted either to acetyl-CoA by pyruvate dehydrogenase or to oxaloacetate by pyruvate carboxylase. In the oxidative pathway, there is a limited operation of the Krebs cycle with the formation of succinic acid. The reductive pathway, oxaloacetate to malate to succinate, leads to the formation of succinic acid involving the synthesis of additional enzymes [19]. The major routes for glucose catabolism in anaerobic conditions are shown in figure 2.

The regulation of the carbohydrate metabolism is described by the Pasteur effect and the Crabtree effect.

Aerobic respiration leads to a greater release of energy per mole glucose (± 300 kcal/mole glucose) than fermentation (22 kcal/mole glucose). Less substrate is required for the release of a given amount of energy and less substrate is metabolised for energy production. This phenomenon is referred to as the "Pasteur effect". This effect arises from the feedback mechanisms associated with hexose transport, phosphofructokinase and isocitrate dehydrogenase as a result of which the fermentative catabolism of glucose to ethanol, CO_2 and energy is blocked.

Glucose in excess is, even in the presence of molecular oxygen, metabolised via the glycolytic pathway with the production of ethanol and CO_2 i.e. alcoholic fermentation. Glucose in excess causes changes in the enzymatic composition of the yeast cell and influences the cell structure e.g. the structure of the mitochondria as a result of the loss of cytochromes. This process is known as the Crabtree or glucose effect. Practically, even in the presence of molecular dissolved oxygen, glucose is partly metabolised by the fermentative way once that its concentration is higher than 0.8%. In fact, the ratio glucose fermented / glucose respired is 0.49 in

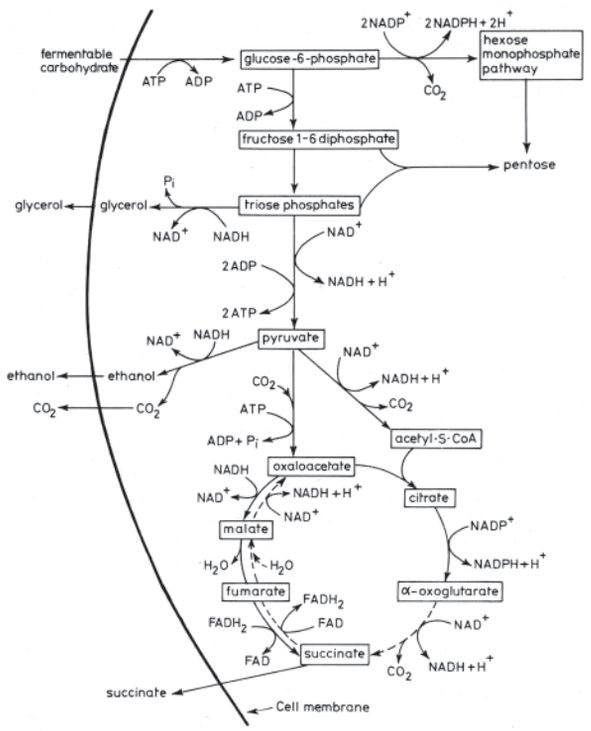

Figure 2 Anaerobic metabolism of sugar [19]: effective for more than 95.0% of the fermentable sugar added to the beer for refermentation

case of *S. cerevisiae* [9]; but we demonstrated earlier that the limiting factor regarding the respiratory metabolism by bottle refermentation and even by regular wort fermentation is the concentration of oxygen available in the liquid.

Balance of the stationary phase

Due to the lack of some growth conditions, accumulation of waste products from the microbiological metabolism the budding will stop and the fermentation goes further until all the fermentable carbohydrates are metabolised, growth rate declines and the yeast enters the stationary phase. In the stationary phase, there is no net increase or decrease in cell number. In unagitated cultures, the biomass concentration in the medium may decline as cells flocculate and, even in shaken cultures, the concentration of biomass will decline after a period of stationary phase, not because of the change in cell number but because of the depletion of the stored carbohydrate reserve [7].

CO_2-production phase

The decarboxylation reaction of pyruvate with the formation of acetaldehyde by pyruvate decarboxylase is the main reaction by which CO_2 is formed. There is also some direct CO_2 production through the glycolysis and the Krebs cycle.

Although glycolysis represents the major pathway for glucose catabolism, some glucose is metabolised by an alternative route, the hexose monophosphate (HMP) pathway. The major functions of this pathway are the generation of NADPH for anabolic reduction reactions and the supply of pentose sugars for biosynthesis. This pathway, which only takes place in the stationary phase, can consume up to 20% of the amount of glucose and is also responsible for some direct CO_2 production.

Generally, the CO_2-content changes from about 1.5 g/L till 7.5 g/L. The CO_2-production speed is quite proportional to the quantity of added yeast considering a same yeast quality and a same beer. Values as high as 9.0 g/L can be reached but practically the best results are obtained with CO_2 concentrations between 7.0 g/L and 7.5 g/L [10].

Phase of inactivity

Phase of resting cells

The phase of resting cells gives no important changes in concentrations of metabolites. During this phase, the exchanges between the yeast cells and the environment are rather limited presumably and the survival of the yeast cells must be dependent on some parameters of the yeast itself like glycogen content, trehalose, and the environment like alcohol content and eventual growth inhibitors from the raw material like some Maillard compounds [28, 35]. It is clearly established that the source of the yeast used by refermentation influences the further evolution of the cell during the beer-ageing phase [2, 7, 24, 28]

Autolysis

After a prolonged period in the stationary phase cells die and autolyse. Because of the absence of oxygen for membrane synthesis yeast cells fail to grow and loss of membrane integrity results in cell death. When all the storage carbohydrates are metabolised rather completely, metabolic activity stops and the permeability of the cell membrane alters. Cells burst open with the release of the internal cellular pool and liberation of the hydrolytic enzymes of the vacuole content [39].

Flavouring effects of the postfermentation

Beer postfermentation is a complex biochemical and chemical event concerning much more than some oxygen removal and carbonation by gas production alone. During refermentation, there is a clear evolution in the flavour profile due to exchange of compounds between the yeast and the beer. These exchanges can vary from the uptake of amino acids by the active yeast to the final release of intracellular material, such as enzymes, during autolysis [18].

Cell budding phase

During the growth phase yeast takes up all the oxygen present in beer. Later on budding, the absorption of amino acids and fermentable sugars starts. Amino acids are essential for the synthesis of proteins and other biomolecules. The concentration of amino acids in beer is only 1/3 to 1/5 of the concentration in wort. During the fermentation amino acids are taken up sequentially. Considering the four classes of amino acids, a decrease varying between 12% (first group), 18% (second group) and 6% (third group) is observed [30]. Transamination of surplus amino acids followed by decarboxylation leads to the formation of aldehydes. These can be reduced by a NADH-dependent alcohol dehydrogenase to the corresponding higher alcohols with one carbon atom less than the original amino acid.

Table 1 Important higher alcohols in beer derived from the corresponding amino acids through desamination and oxidative decarboxylation [19]

amino acids	oxo acids	aldehydes	alcohols
alanine	pyruvic acid	acetaldehyde	ethanol
α-aminobutyric acid	α-ketobutyric acid	propionaldehyde	propanol
valine	α-ketovaleric acid	isobutyraldehyde	isobutanol
leucine	α-ketoisocaproic acid	isovaleraldehyde	isoamyl alcohol
isoleucine	α-keto-β-methyl valeric acid	2-methylbutanal	2-methyl-butanol
phenylalanine	phenylpyruvic acid	phenylpyruvaldehyde	phenylethyl alcohol

Higher alcohols can also be derived from the anabolic or Genevois pathways along which amino acids are synthesized. In these pathways, pyruvate and acetyl-CoA act as precursors for amino acid synthesis. Both pathways are active in brewer's yeast and can account for the presence of higher alcohols in beer. The most important higher alcohols, derived from their respective amino acids through desamination and oxidative decarboxylation are presented in table 1.

Esters constitute one of the largest and most important groups of compounds importing flavour to beer. Esters can be formed through a strictly chemical condensation between an alcohol and an organic acid. Since the rate of this uncatalysed reaction is too slow to account for the ester concentration present in beer, most of the esters are formed biochemically. In these reactions involved compounds are: ethanol or higher alcohols, fatty acids, coenzyme A and ester synthases. Activated acyl-CoA compounds acts as acyl-CoA donors (figure 3).

Compared to higher alcohols, threshold values of esters are very low which implies that small variations in concentrations can have a profound effect on the overall flavour and aroma of beer. When expressed in concentration only little changes occur, but due to the low threshold values of ethyl caproate, ethyl caprylate and isoamyl acetate the flavour potential of beer can increase by about one flavour unit [30]. (*Flavour unit : absolute concentration of the compound / threshold*). Two very interesting reviews covering the scope of this topic in case of wine were published by *Lambrechts* and *Pretorius* [23] and by *Pretorius* [34].

Stationary phase

Results for beer

Refermentation in the bottle is often regarded as a process, which causes a favourable evolution of the beer's flavour. However, once the beer is saturated with carbon dioxide, yeast cells rapidly loose viability and inactive cells remain present in the bottle. This presence has a negative impact on the evolution of the fruity flavour and aroma during storage. The importance of yeast autolysis should not be underestimated. The enzymatic autodegradation of the cells and the concomitant increase of free amino nitrogen possibly influences the formation of specific lactones and esters during storage [5].

During beer ageing, three zones can be considered: a zone of unmaturity, a maturity phase where beer quality reaches its maximum and finally a phase of ageing where beers presents flavour deviations characterized as "Madere wine" or "old Port wine" [5]. The loss of viability is accompanied by yeast autolysis, which influences the flavour profile. The major consequence of yeast autolysis is the liberation of hydrolytic enzymes of the vacuole content in the beer. This influences the beer characteristics as foam stability by hydrolysis of hydrophobic proteins and flavour. Aroma is strongly affected by an increase of volatile fatty acids with a low threshold value. Due to cell autolysis the concentration of isovaleric acid, caproic acid, caprylic acid and capric acid increase enough to develop a negative effect on the flavour [31].

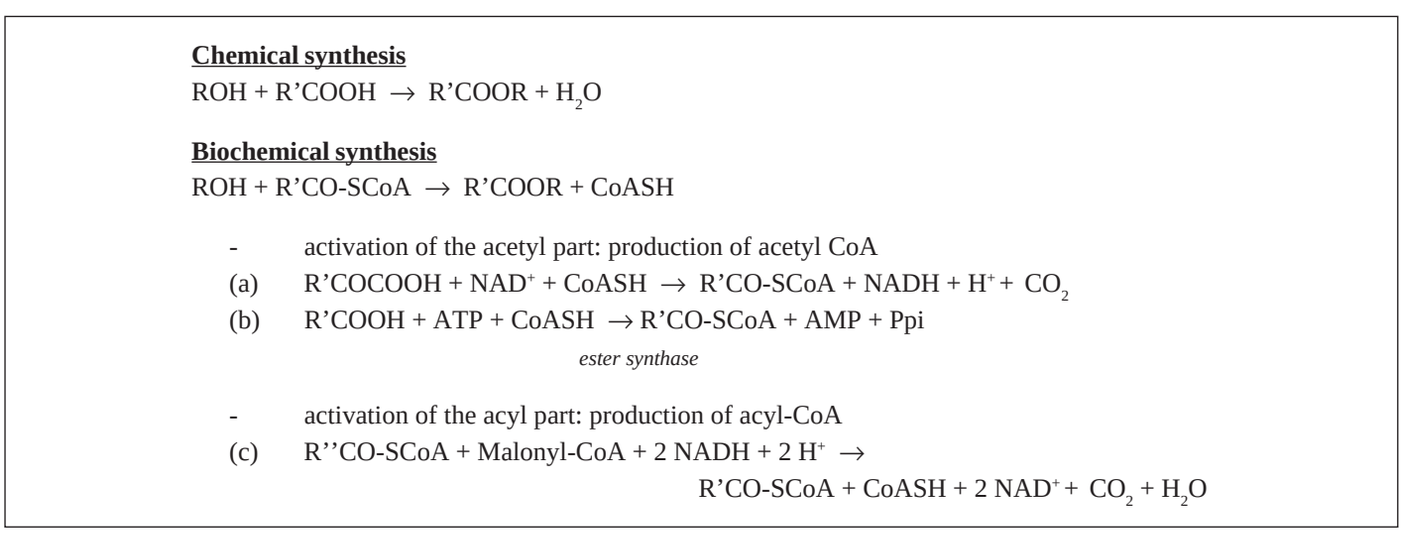

Figure 3 Chemical and biochemical formation of esters [19]

Table 2 Evolution of the concentration of higher alcohols (n-propanol, isobutanol, iso-amyl alcohol) and esters (ethyl acetate, iso-amyl acetate, ethyl hexanoate, ethyl octanoate) (ppm) during bottle refermentation and subsequent storage at 24°C of a non-pasteurized beer. Results are expressed as the average of triplicate analyses. A change in flavor units is expressed as ΔFU (*Neven*, 1997).

Active compound (Threshold; flavor)	After maturation	After bottle conditioning	After 6 months	ΔFU
n-Propanol (800 ppm, alcohol)	24	24	24	0
Isobutanol (200 ppm, alcohol)	22	22	22	0
Iso-amyl Alcohols (70 ppm; banana)	122	123	125	0
Ethyl Acetate (30 ppm; fruity, solvent)	54	53	52	0
Iso-amyl Acetate (1.6 ppm; pear, banana)	**5.9**	**5.9**	**4.4**	**-1**
Ethyl Hexanoate (0.23 ppm; fruity)	0.3	0.2	0.2	0
Ethyl Octanoate (0.9 ppm; fruity, apple)	0.4	0.2	0.2	0

Besides a slow chemical hydrolysis, a biochemical hydrolysis plays an important role in the evolution of esters throughout storage. Enzymes that hydrolyze esters are synthesized during fermentation and maturation by *Saccharomyces cerevisiae*. An important esterase activity is observed towards iso-amyl acetate, ethyl hexanoate (caproate) and ethyl octanoate (caprylate). The optimal pH for this activity is situated near 7.5. However at common beer pH conditions this enzymatic activity is still present. As a consequence the consumer will experience a diminution of the fruity flavour when tasting aged beer (table 2).

The production of sulphur dioxide during refermentation is another factor which will influence the flavour profile. It is an intermediate in the metabolism of the amino acids cysteine and methionine and is excreted when yeast growth is limited and excess sulphite is present intracellular. Since refermentation takes place in a closed container it cannot escape and combines with aldehydes. Since aldehydes have positive or negative flavour properties, reaction with sulphite can thus be considered positive or negative [30, 31].

Results for wine

The term "*méthode champenoise*" refers to a second fermentation of white wine in the bottle. During bottling the "*tirage de liqueur*" (a mixture of wine, sugar and yeast cells) is added to primary fermented white wine. To insure secondary fermentation, a minimum of 1 million cells per milliliter should be added to each bottle [49]. Following sealing, sparkling wines are stored for the "*prise de mousse*". The secondary fermentation, during which the yeast cells convert the added sugars to ethanol, takes one to two months. During the subsequent storage of the bottles in cool cellars, the Champagne, still in contact with the yeast cells, continues to age during 9 months to 5 years.

During this ageing process, which is responsible for the development of the typical "*méthode champenoise bouquet*", excretion and autolysis of yeast cells occur. Cytoplasmatic compounds are degraded due to the release of enzymes, such as proteases and hydrolases, from the vacuole content. The result of this "*storage sur lie*" is an enrichment with amino acids [5]. This difference in amino acid constituents between the cuvée and the final wine contribute to the character and complexity of "*méthode champenoise*" wines and explains the sensory difference between "*méthode champenoise*" and charmat-produced sparkling wines. Amides, fatty acids and terpenoids are all shown to increase during autolysis [26, 39].

Furthermore, aged wines are rich in esters and certain compounds which can be considered very specific e.g. methyl-2-ethoxy-furan. This compound originates from the dehydratation reaction between ethanol and alcohols produced in the Maillard reaction pathway [50]. Not only the flavour of sparkling wines is affected by refermentation, also bubble size and foam properties change. Fermentation conditions, indigenous yeast populations [14], concentration of nitrogen compounds in the original wine and yeast autolysis (figure 4) are important for these effects.

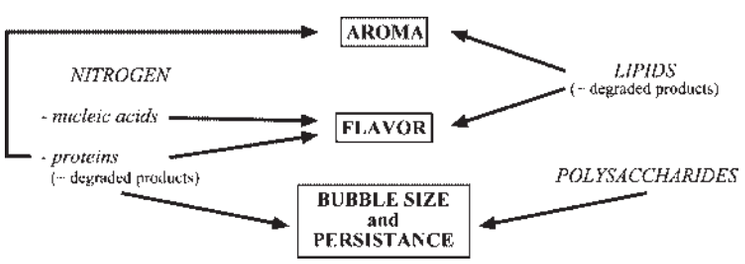

Figure 4 Reported impact of yeast autolysis on various attributes of sparkling wine quality [39]

Practical Aspects

The easiest way to perform refermentation is by using the same yeast strain as the strain used for the main fermentation. Practically, normal production wort after two days fermentation is used as pitching solution. It is also possible to use the yeast of a previous main fermentation. The collected yeast slurry has to be stored at 4 °C and stirred for 5 minutes twice a day to prevent temperature increase and loss of cell viability [10]. But this manner of working can lead to colloidal haze in the conditioned beer since the wort used isn't stabilized and that the cell concentration in fermenting wort is rather low versus the quantities obtainable using other ways.

Since circumstances during the secondary fermentation are very different from the primary fermentation (low pH, high alcohol content, low oxygen content, presence of CO_2, small amounts of nutrients), some breweries use strains of *Saccharomyces cerevisiae var. cerevisiae* selected especially for refermentation. In this case, a separate propagation in sterilized hopped wort is necessary. The propagation medium has to be oxygenated just before pitching to allow an optimal fatty acid synthesis and a maximum yield. The yeast propagation system in the brewery must be designed to a high standard to limit the possibility of the culture becoming contaminated with wild yeasts or bacteria. Especially attention should be paid during the transfer from the laboratory culture to the propagator [9, 11].

The third possibility is the use of a commercially available bakers' yeast strain *Saccharomyces cerevisiae var. pastorianus*. It's an easy technology but let us keep in mind that the flavour obtained using bakers' yeast can't be the same as the one obtained using an aromatic brewers' yeast since the activity regarding fermentable sugars can vary amongst them.

Another manner to perform the refermentation is the use of freeze-dried or spray-dried-yeast. There are several advantages in using dried brewing yeast. Dried yeast has less bulk, it may be stored at ambient temperatures for several months and is readily reconstituted by dispersion in water. The quality of the dried brewing yeast available on the market has improved considerably in recent years. Also the long term capital investment and labour costs associated with the propagation plant are replaced by the short term costs for purchasing dried yeast [40]. The process of drying yeast cells makes them more resistant against mutations since non-growing cells are less prone to copying errors during the replication from its genetic material than dividing cells. A disadvantage of using dried yeast is high mortality of some yeast strains during the drying process. Different strains are affected differently and higher viability is generally obtained if the process is carefully controlled; further, research to isolate a novel yeast factor that allows an increasing of the yeast brewing performances must be helpful too [13, 40].

Table 3 Non-*Saccharomyces* yeast isolated from acid Belgian ales (*Van Oevelen* et al. [42] , *Martens* et al. [27], *Derdelinckx* et al. [11], *Shanta Kumara* [37] and *Verachtert* [44])

	Respiration	Fermentation	Anaerobic growth
Brettanomyces clausenii *Brettanomyces custersianus* *Brettanomyces intermedius* *Brettanomyces lambicus*	yes	yes	yes
Candida sp *Candida guillermondii* *Candida datilla* *Torulopsis sp.* *Cryptococcus sp.*	yes	yes	no
Dekkera bruxellensis	yes	no	no
Kloeckera apiculata	yes	yes	yes
Pichia sp.	yes	anaerobic in pregrown cells	no
Rhodotorula sp.	yes	no	no

Table 4 Effectiveness of the Crabtree effect in some yeasts [9]

Yeast	Ratio glucose fermented /glucose respired	Crabtree effect
S. cerevisiae	49.0	+
Brettanomyces lambicus	23.0	+
Candida utilis	0	–
Cryptococcus sp.	nd	nd
Dekkera bruxellensis	23.0	+
Hansenula anomala	0	–
Kloeckera apiculata	nd	nd
Pichia fermentans	0.16	–
Rhodotorula sp.	0	–
Torulopsis sake	0	–

Non-*Saccharomyces sp*.

Non-*Saccharomyces sp*. can play an important role on beer flavour since some peculiar metabolisms distinguish these yeasts from *S. cerevisiae sp*. The carbon and energy metabolism as well as the nitrogen metabolism, the phosphorus and phenol metabolism and some specialized metabolisms can be considered. Their influence depends on the physiological characteristics of the considered yeast strains and on the environmental conditions developed by the production process.

The typical non-*Saccharomyces* micro-organisms isolated by Van *Oevelen e*t al. [42] , *Shanta Kumara* [37], *Martens* et al. [27] are listed below (table 3); they are listed according to their growth characteristics and their ability to survive without oxygen. The major difference regarding the carbohydrates metabolism concerns the use of sugars and the regulatory phenomena. The yeasts isolated from bottle conditioned Belgian acid ales can be listed according to their sugar catabolism pathway (table 4).

Carbohydrate metabolism and regulatory phenomena

The regulatory phenomena in the sugar metabolism of yeasts are also a distinguishing feature in some cases.

In the case of *Brettanomyces sp., Dekkera sp*. and *Candida sp*. the Custers effect occurs, while *S. cerevisiae* and other *Saccharomyces sp.* are characterised by a more or less effectiveness of both Pasteur effect and Crabtree effect (also termed catabolite repression).

Table 5 Biopolymer degradation/utilisation by yeasts [46]

Biopolymer	Micro-organisms	Enzymes involved
Starch	*Candida sp.* *Pichia sp.* *(Saccharomyces diastaticus)* *Brettanomyces naardenensis*	Extracellular α-amylases and sometimes gluco-amylase and cyclodextrinase activity
Hemicellulose	*Cryptococcus sp.*	Xylanase activity
Pectin	*Candida sp.*	Polygalacturonase activit

The Custers effect is defined as the transient inhibition of fermentation by anaerobiosis and is observed when small levels of oxygen or organic hydrogen-acceptors (e.g. acetoin) abolish this anaerobic inhibition of fermentation. Thus, oxygen stimulates ethanol production. The Custers effect is effective in *Brettanomyces sp*. and *Dekkera sp*. and is due to a lack of NAD^+ caused by the secretion of acetate by the cells, provoking a "crucial deficiency" on the potential hydrogen-acceptor acetaldehyde, which isn't formed sufficiently. The NAD^+/NADH ratio is altered and this results in an unfavourable redox balance in the cell. NADH re-oxydation can occur only very slowly under anaerobic conditions in *Brettanomyces sp.* and *Dekkera sp.*

$$C_6H_{12}O_6 + 4\,NAD^+ + 2\,H_2O \rightarrow 2\,CH_3COOH + 2\,CO_2 + 4\,NADH + 4H^+$$

(as comparison: anaerobic catabolism in *S. cerevisiae*:
$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2$)

The Kluyver effect explains the fact that some yeasts like *Candida sp*. which can ferment glucose anaerobically are able to aerobically assimilate but not ferment other sugars like certain disaccharides. It was demonstrated by *Weusthuis* et al. [48] that ethanol causes a suppression of the maltose utilisation by *Candida utilis*.

Since some living yeasts are isolated after 24 months of bottling, the biopolymer metabolism can be considered as important too. Some of the yeasts possess a peculiar biopolymer activity (Table 5).

The metabolism of lower aliphatic alcohols in some strains like these studied by V*an den Bremt* et al. [41] showed attractive results too. These authors demonstrated that the alcohol oxidase, which allows the oxidation of methanol to formaldehyde in *Candida methanolovescens*, is also suitable to transform even cyclic alcohols like salicyl alcohol into salicyl aldehyde. This molecule is typed by a pleasant almond note of which the threshold is 10^3 lower than the one of the corresponding alcohol [20].

Table 6 Yeasts and bacteria isolated after 6 months in acid ales

Yeasts	Bacteria
Brettanomyces lambicus	*Pediococcus cerevisiae (= damnosus)*
Brettanomyces custersianus	
Candida sp.	
Cryptococcus sp.	
Dekkera bruxellensis	
Pichia sp.	
Rhodotorula sp.	
Torulopsis sp.	

Therefore, the study of the metabolisms of yeasts leading to aldehydes, esters, fatty acids, and some phenols and lactones and a few higher alcohols is of major importance since they are mainly responsible for the bioflavour found in these beers.

In contrast to *S. cerevisiae*, some yeasts isolated from the microflora present during the secondary alcoholic fermentation (after 6 – 8 months) have an oxidative metabolisms. Although strains as *Candida utilis anomala, Pichia fermentans* and *Torulopsis sake* are strictly aerobic yeasts, it needs to be mentioned that some species of these genera were isolated from acid Belgian ales after 6 to 24 months of storage (table 6).

Methylotrophic yeasts play a very important role regarding the flavour that they import. Indeed, their alcohol oxidase, necessary to metabolise methanol, is also able to convert other alcohols into aldehydes [32].

Even some simple unbranched aldehydes have a fresh, fruity flavour (table 7). They can be grouped according to their chain length. Aldehydes with a chain length of 2 to 5 carbon atoms have generally a green flavour while aldehydes with a chain length of 6 to 11 carbon atoms are often characterised by a bitter, orange peel flavour. The threshold of these compounds decreases with increasing chain length, for example, 1 ppm for n-butanal (flavour: melon, green leaves, varnish) to 6 ppb for n-decanal (flavour: bitter, aldehyde, orange peel). Additionally, when these unbranched aldehydes have a mono-unsaturated function, an extra pleasant fruity flavour is obtained [36]. Their influence on the beer flavour can not be neglected.

Furthermore, studies by *Shennan* and *Levi* [38] and by *Cartledge* [6] concerning the hydrocarbon metabolism of *Candida sp., Pichia sp., Rhodotorula sp.* and *Torulopsis sp.*, indicated that these micro-organisms have the enzymatic capability of transforming linear long chain alcohols (C10-C20) to their corresponding aldehyde by an NAD^+-linked long chain alcohol dehydrogenase and further by the corresponding NAD^+-linked long chain aldehyde dehydrogenase.

Van den Bremt et al. [41] mentioned also a very interesting approach using a methylotrophic yeast, *Candida methanolovescens* characterised by a β-glucosidase activity. These yeasts are able to hydrolyse the link between the sugar structure and the aglycon part. This aglycon part undergoes a biological oxidation producing an aldehyde characterised by a low threshold.

Since some *S. cerevisiae* strains, some species of *Torulopsis molinschiana* and *Hansenula wickerhamii* possess these enzymatic features, these phenomena will play an important role for the flavour evolution of speciality beers. This is especially true for beers characterised by the use of hop cones, dry hopping, herbs, spices or fruits due to the liberation of aglycons. In some cases,

Table 7 Role played by oxidative yeast metabolisms on beer flavour: redox steps from alcoholic till acidic stage

	Concentration range in beer (ppm)	Threshold (ppm)	Flavour of the aldehydic form
C5			
Isoamyl alcohol (3-methyl butanol)	30-70	70	
Isovaleraldehyde	0.01-0.3	0.6	*Unripe banana, apple, cherry, cheese*
Isovaleric acid	0.1-2	1.5	
C6			
n-Hexanol	0.05-0.33	4.0	
Hexanal	0.003-0.007	0.35	*Vinous, bitter*
Caproic acid (hexanoic acid)	1-5	8	
C8			
Octanol	0,02	0.9	
Octanal	0.001-0.02	0.04	*Orange peel, vinous, bitter*
Caprylic acid (octanoic acid)	2-12	13-15	
Phenylethanol	8-35	125	
Phenylacetaldehyde	0.005	1.6	*Hyacinth, lilac, flowery*
Phenylacetic acid	0.93	2.5	
C10			
n-Decanol	0.01	0.18	
Decanal	0-0.003	0.006	*Orange peel, bitter*
Capric acid (decanoic acid)	0.1-4	10	

Table 8 Small length acids and corresponding ethyl esters synthesis by non-*Saccharomyces* yeast [42]. Concentrations are in ppm

	Brettanomyces lambicus	*Dekkera bruxellensis*	*Kloeckera apiculata*	*S. cerevisiae*
Acetic acid	922	366	360	190
Isobutyric acid	16.8	31.1	2.4	2.7
Lactic acid	297	508	8.1	14.0
Ethyl acetate	62.5	15.5	< 0.5	< 0.5
Ethyl isobutyrate	—	—	—	—
Ethyl lactate	< 0.5	< 0.5	< 0.5	< 0.5

these aglycons (i.e. (+)-catechin) have reducing properties [47]. Their importance in the production of carbohydrate-flavour conjugates in wine by the microflora present in the mouth was revealed by *Hemingway* et al. [17].

The results of *Van den Bremt* et al. associated to those described by *Shennan* and *Levi* [38] and by *Cartledge* [6] makes clear that research is necessary out to check out the cascade flavour synthesis presented hereafter (see table 7). Some of the molecules are already described and their role in flavour generation is of major importance.

Studies of *Licker* et al. [25] in wine showed the role of *Brettanomyces sp*. in giving a peculiar flavour known as "Brett" flavour. The analysis by gas chromatography olfactometry revealed two predominant odour active compounds: isovaleric acid and a second unknown compound; but other molecules are odour active too (see ester metabolism and phenol metabolism).

Ester synthesis

Due to their metabolism one can expect that *Brettanomyces lambicus* and *Dekkera bruxellensis* produce in pure cultures a high quantity of acetic acid, lactic acid and isobutyric acid, resulting in the synthesis of their corresponding ethyl esters (see table 8). The results reveal that chemical synthesis of the esters hardly occurs and that biochemical synthesis mainly occurs when the corresponding coenzyme A derivative of the acid is produced by the yeast.

Taken in account the acid fractions produced by the metabolisms of *Candida sp., Cryptocuccus sp., Pichia sp., Rhodotorula sp*. and *Torulopsis sp*. one can expect a significant presence of their corresponding ester. The esters play a major role in speciality beer flavour due to their low threshold (see table 9).

Moreover, the conjugate presence of some yeasts under coculture conditions will influence the fatty acid pattern and the flavour profile due to ester synthesis. *Fugelsang* et al. [15] confirmed a

Table 9 Concentration range of some important higher alcohols, organic acids and their corresponding ester found in Belgian refermented speciality ales, their respective threshold and flavour

	Concentration Range in beer (ppm)	Threshold (ppm)	Flavour
C7			
ethanol	4500	14000	
isovaleric acid	0.02-0.06	1,5	
Ethyl isovalerate	0.02	0.018-0.2	*Fruity, grapes, sweat*
C8			
caproic acid	1-5	8	
ethanol	4500	1400	
Ethylcaproate	0.2	0.17-0.21	*Apple, fruity, anise*
C10			
isovaleric acid	1.5	1.5	
isoamyl alcohol	50-55	70	
Isoamyl isovalerate	0.1	0.019	*Fat, fruity, solvent, perfume*
acetic acid	1350	175	
2-phenylethanol	40	125	
2-phenylethylacetate	3-4	3.8	*Roses, honey, apple, sweetish*
C12			
capric acid	10	13	
ethanol	4500	1400	
Ethyl caprate	1.5	0.11-0.21	*Apple, fruity, sweetish, aniseed*
C13			
caproic acid	3	8	
isoamylalcohol	50-55	70	
Isoamylcaproate	0.5-1	0.9	*Fatty acids, fruity, solvent, perfume*

Table 10 Comparison of fatty acid levels in pure and coculture fermentations

Yeast	Acetic acid (ppm)	Caprylic acid (ppm)	Capric acid (ppm)
Saccharomyces	82	18	7
Sacch. + Brett.	249	93	16
Sacch. + Dekk.	672	93	11

Table 11 Physico-chemical character of the beers studied

	Strong pale ale	Belgian wheat beer	Kriek
Orig. extract (°P)	18	12	11.5
Alcohol (v/v)	7.5	4.9	4.2
Att. Rate (%)	85	82	89
CO_2 (g/l)	8	5.2	4.5
Bitterness (BU)	25	17	15
pH	4.1	4.0	3.85
Total acidity	20	20	33
Total polyphenols	180	180	120

strong production of fatty acids in cocultures of *S. cerevisiae* together with *Brettanomyces sp*. and *Dekkera sp*.. They assumed that the large amounts of acetic acid produced, results from the oxidation of ethanol rather than coming from pyruvate. In the same way, the influence of the metabolites produced, as lactic acid and acetic acid, on the yeast physiology cannot be neglected too [29] (see table 10).

Most of the flavours arising in Belgian speciality beers are described and one needs to mention that the combination of esters together with volatile acids and aldehydes deliver some typical flavours to well known Belgian speciality beers [33].

Phenol metabolism

The phenol metabolism of some non-*Saccharomyces* yeasts plays also an important role regarding the flavour pattern of certain speciality ales in a similar way as in wines [8].

Due to their resistance to severe stress constraints and to their ability to metabolise some typical molecules as phenols, some yeasts, like *Brettanomyces sp*. and *Dekkera sp.*, develop a characteristic aroma [16, 25].

Fugelsang et al. [15] proposed a pathway for the formation of volatile phenols by *Brettanomyces sp.* and by *Dekkera sp*. involving two enzymatic steps: first a cinnamate decarboxylase followed by the action of a vinyl-phenol reductase and by transforming p-coumaric and ferulic acid in 4-ethyl-phenol and 4-ethyl-guaiacol. The role played by some yeasts regarding the typical phenolic flavour of most German Weissbeers is described by *Wackerbauer* and *Methner* [45].

Amino acid metabolism

Beside the production of higher alcohols as described by *Van Oevelen* [42] and *Martens* [43], *Heresztyn* [18] mentions the production of "mousey" flavour developed in some wines infected by *Brettanomyces sp.* The offensive compounds are reported to be amino acid derivatives, as the heterocyclic bases 2-acetyl-1,4,5,6 tetrahydropyridine and 2-acetyl-3,4,5,6 tetrahydropyridine. Their synthesis by *Brettanomyces intermedius* and *B. lambicus* requires lysine and ethanol. Another aspect regarding health, the eventual production of some polyamines and biogene amines, is known but this feature is linked to some peculiar species. Let us only mention that *Saccharomyces* strains and non-*Saccharomyces* strains can be considered exceptionally. Some environmental conditions like co-cultures can be critical. The values stated can eventually exceed legal limits but the process adaptations needed to prevent this problem are known.

Case studies of refermented speciality beers

Several speciality beers have in their process the use of herbs, spices and even fruits. The alcohol dehydrogenase activity, the aldehyde dehydrogenase activity, the glucosidase activity and the alcohol oxidase activity of some yeasts influence the flavour pattern of those beer types. To explain the potential of non-*Saccharomyces* yeasts as flavouring agents in practice, we will illustrate the flavour evolution of three different speciality beers: a strong speciality beer, a Belgian wheat beer containing spices and a sour fruity beer named Kriek (table 11).

Table 12 Flavour modification of the ester profile of a traditional strong ale by maturation in casks in the presence of *Brettanomyces lambicus.*

	Concentration variation (ppm)	FU (Flavour Unit)
Ethyl acetate	+ 15	+ 0.5
Isoamyl acetate	- 7	- 4.4
n-propyl acetate	+ 0.010	+ 0.00033
i-butyl acetate	- 0.035	- 0.047
Ethyl isobutyrate	+ 0.020	+ 0.004

The evolution of higher alcohols and esters present in the strong pale ale follows a flavour cascade as previously described (tables 8 and 9). The kinetics is dependent on the environmental conditions (temperature, pH) and on the micro-organisms involved. The influence of oxidative yeast on the esters pattern is already well described [42, 44] (table 12).

Concerning white beer, one needs to mention the major constituents of the spices used i.e. coriander and bitter orange peels. A compound found in both spices is linalool (fig. 5), typed by a floral, bergamot, lavender flavour and by a threshold of 6 ppb [1].

Due to its chemical structure, linalool (formerly named coriandrol) cannot be oxidised to the aldehyde and acid form. It was demonstrated that linalool reacts with acetate to form linalyl acetate; the presence of this compound is mentioned, but the threshold is not available [21].

Geraniol (citral) is a major compound of bitter orange peels and the reactivity of its structure regarding alcohol dehydrogenase and ester synthesis (the description of the oxidation of geraniol into its acid form, geranic acid or citronellic acid was not found by our literature study) is not described.

Starting from geranial, geraniol and geranyl propionate, geranyl acetate and geranyl isobutyrate are produced potentially. Analysis are achieved actually to elucidate the sequence (table 13)

An analogous scheme can be shown in case of neral (citral B) which is an isomer of citral with a sweeter lemon odour and a higher threshold.

In the sour speciality beer of Brussels called Kriek and produced with mature fresh cherries containing the kernel (if processed in the traditional way), β-glucosidase and alcohol oxidase can play

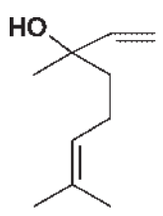

Figure 5 Structure of linalool, a terpene molecule that represents about 80% of the essential oil of coriander but also appears in bitter orange peel and hops

Table 13 Putative evolution of geranial (citral A) under the influence of biochemical and chemical reactions arising in Belgian white beer and sour beer

	Threshold	Flavour
Geranial	30 ppb	Refreshing, intensive lemon, bitter
Geraniol	40 – 75 ppb	Floral
Geranyl acetate	9 ppb	Floral,fruity
Geranyl lactate	unknown	
Geranyl propionate	10 ppb	
Geranyl isobutyrate	13 ppb	

an important role due to the presence of prunasin. Prunasin is a glucoside consisting of D(-)-mandelonitril-D-glucoside (Pharmacopeial drugs). We compared Kriek beers processed by two different ways, one by addition of whole cherries and maturation of 4 months in oak casks, the other one used pasteurised cherry juice. The chromatogram of the beer containing the whole cherry fruit doesn't show the typical aldehyde arising from hydrolysis of the glucoside i.e. benzaldehyde while this molecule is present in the beer produced with the juice. The conclusion of this experiment let us presume that there is no glucosidase activity from the yeasts present in the sour beer (lambic) during maturation (probably due to the peculiar environment conditions in sour beer). The presence of benzaldehyde in beer with juice is presumably due to thermal stabilisation of the juice and consecutive hydrolysis of the molecule to benzaldehyde, hydrocyanic acid and glucose. The absence of benzaldehyde could be a method to eventually distinguish both production types (see figure 6).

General conclusion

Since maturation in bottle or in casks is applied for the production of speciality beers, one needs to consider the different aspects of this process. Initially, refermentation was often used to carbonate beer essentially and, mostly erroneous, to improve beer flavour stability. Due to research performed in Belgian brewing institutes essentially, the major step of microbiology and of technical knowledge of bottle refermentation is now known and the essential difficulties of production methods involving the use of *S. cerevisiae* are described scientifically. Further research is now carried out to study the influence of *S. cerevisiae* physiology on beer flavour stability distinguishing the three important phases linked to the yeast activity in the bottle (budding, fermentation, resting cells and autolysis).

In a second step, regarding flavours, it was demonstrated that non-*Saccharomyces* yeasts can be used eventually and that in some cases, the results obtained are surprising. For example, in the case of fruity beers where it was showed that the sour beer environment didn't allow β-glucosidase activity and in consequence liberation of benzaldehyde while the latter molecule is present in beer produced with pasteurised cherry juice. In conclusion the potential of *S. cerevisiae* and non-*Saccharomyces* yeasts regarding their use as postfermentation agents are far from understood and exploited fully. A lot of beer improvements and flavours will be obtained if serious research is carried out further in this field.

Zusammenfassung

Vanderhaegen, B., Coghe, S., Vanbeneden, N., Van Landschoot, A., Vanderhasselt, B., und Derdelinckx, G.: Hefen als Hilfsmittel zur Nachgärung von Bier — Monatsschrift für Brauwissenschaft 55, Nr. 11/12, 218 – 232, 2002

BC 40 Allgemeines (Brauereibiologie)

Saccharomyces cerevisiae und Nicht-Saccharomyceshefen werden in Belgien schon seit Langem in Spezialbieren zur Verbesserung der Nachgärung bei Lagerkeller- und konditioniertem Bier (Flaschen- bzw. Fassgärung) verwendet. Der Einsatz von *S. Cerevisiae* Stämmen ist durch die Aufkarbonisierung und eine gewisse Sauerstoffreduzierung gerechtfertigt. Im Gegenteil dazu gelten die Auswirkungen von Nicht- Saccharomyces Spezies, welche hauptsächlich in mit Säure bildenden Mikroorganismen vergorenen Bieren vorkommen, überwiegend der Geschmacksbildung. Diese Studie gibt einen Überblick über die physiologischen Aspekte mehrerer in belgischen Spezialbieren vorkommender Hefespezies, sowie über das Potential von *Saccharomyces cerevisiae* Stämmen bei der Modifikation des Geschmacks nach der Bierkonditionierung. Auch werden die eher praktischen Gesichtspunkte bezüglich des Anstellens und der Hefevorbereitung besprochen. Darüber hinaus werden einige interessante Eigenschaften von *Brettanomyces sp., Dekkera sp., Kloeckera sp., Candida sp., Cryptococcus sp.* und *Torulopsis sp.* untersucht. Der Einfluss der enzymatischen Aktivität von Alkoholdehydrogenase, Aldehyddehydrogenase, ß-Glucosidase und Alkoholoxidase unter ungewöhnlichen Bedingungen kann die Entwicklung typischer Geschmacksrichtungen ermöglichen. Die Verwendung von Kräutern, Gewürzen oder Früchten wird zusammen mit diesen Hefen eingesetzt, um die endgültige Geschmacksrichtung des Bieres zu verstärken, zu modifizieren, zu verbessern und manchmal vielleicht sogar zu verändern. Wissenschaftliche Forschung und Projekte werden durchgeführt, um die Produktion von Spezialbieren tiefgreifender zu verstehen und gezielt zu beeinflussen.

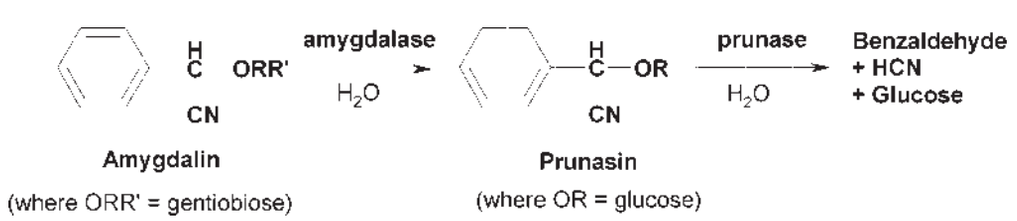

Figure 6 Enzymic hydrolysis of amygdalin (glycoside found in cherries (*Prunus sp.*)) to benzaldehyde. This hydrolysis can occur chemically by thermal treatments as pasteurising but was not detected under the cold acid/ethanol/water extraction of the cherries as by sour fruit beer

Vanderhaegen, B., Coghe, S., Vanbeneden, N., Van Landschoot, A., Vanderhasselt, B., et Derdelinckx, G.: *Saccharomyces* et non-*Saccharomyces sp*.: agents de maturation et de conditionnement de la bière — Monatsschrift für Brauwissenschaft 55, No 11/12, 218 – 232, 2002

BC 40 Généralités (Biologie brassicole)

Plusieurs bières spéciales belges se caractérisent par la présence de souches de levures *Saccharomyces* et non-*Saccharomyces*; le but recherché est l'amélioration du produit après la phase de fermentation c'est-à-dire au courant de la garde et après soutirage. L'utilisation de *S. cerevisiae* se justifie essentiellement pour des raisons de saturation (production de CO_2) et d'élimination de l'oxygène dissous. Par contre, les souches sauvages (non-*Saccharomyces cerevisiae* et non-*Saccharomyces sp.*) essentiellement présentes dans les bières de type acide sont surtout utilisées pour leurs caractéristiques aromatiques. Ce travail passe en revue les aspects métaboliques (energétiques) caractérisant les différentes souches de levures rencontrées dans les bières spéciales belges ainsi que les effets des souches de *S. cerevisiae* en ce qui concernent la modification de la saveur de la bière après conditionnement. Différents aspects pratiques relatifs au conditionnement de la levure et à l'innoculation sont mentionnés. D'intéressantes propriétés de *Brettanomyces sp., Dekkera sp., Kloeckera sp., Candida sp., Cryptococcus sp.* et *Torulopsis sp.* sont décrites. L'activité d'enzymes typiques telles les alcool déshydrogénases, aldéhyde déhydrogénases, alpha-glucosidases et alcool oxydases permet sous certaines conditions le développement des aromes typiques. L'utilisation d'épices, d'herbes ou de fruit en combinaison avec ces levures modifiera voire améliorera et même parfois altérera la saveur finale de la bière. C'est pourquoi, ces phénomènes jusqu'à présent incompris font l'objet de la poursuite de notre recherche scientifique.

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