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Biochemistry of Beer Fermentation

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Chapter 1
An Overview of the Brewing Process

Abstract The first chapter of this book has an introductory character, which discusses the basics of brewing. This includes not only the essential ingredients of beer, but also the steps in the process that transforms the raw materials (grains, hops) into fermented and maturated beer. Special attention is given to the processes involving an organized action of enzymes, which convert the polymeric macromolecules present in malt (such as proteins and polysaccharides) into simple sugars and amino acids; making them available/assimilable for the yeast during fermentation.

A Brief History of Brewing

Beer has a strong bond with human society. This fermented beverage was most likely created by accident thousands of years ago. Despite the massive technological growth that separates ancient brewing from today's high-tech breweries, the process in its traditional version remains entirely unchanged. However, even though our ancestors could make primitive beers from doughs and cereals, they did not know the biochemical steps involved in the process.

Some historians suggest that beer-like beverages were brewed in China as early as 7000 BC (Bai et al. 2012), but the first written records involving beer consumption only date from 2800 BC in Mesopotamia. However, there is strong evidence that "beer" was born as early as 9000 BC during the Neolithic Revolution (Hornsey 2004), when mankind left nomadism for a more settled life. With this new lifestyle, came the need for growing crops and for the storage of grains. Thus, it is likely that natural granaries produced the first "unintentional" batches of beer.

From Mesopotamia, the beer culture spreads through Egypt around 3000 BC. Until shortly before the years of Christ (30 BC), beer was the beverage of choice among Egyptian people (Geller 1992). Thereafter, Egypt fell under Roman domain, introducing a wine culture into the region. However, even with wine as a choice, beer endured as the sovereign beverage among the Egyptian general population (Meussdoerffer 2009). Through the Roman dominion, wine was a drink for the nobles. At that time, beer was regarded as the drink of "barbarians" because

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wine was the conqueror's beverage (Nelson 2003). In fact, before the expansion of the Roman Empire, beer was the queen beverage of all Celtic peoples in France, Spain, Portugal, Belgium, Germany, and Britain. Then, together with the expansion of the Roman Empire, came the development of the wine culture (Nelson 2003). When Romans lost control, mainly by Germanic conquering of Western Europe in the fifth century AD, beer took back the place as the sovereign drink.

The first evidence of commercial brewing is in the old drawings of a brewery, found in the monastery of Saint Gall, and date from 820 AD (Horn and Born 1979). Before the twelfth century, only monasteries produced beer in amounts considered as "commercial scale" (Hornsey 2004). Monks started to make more beer than they could drink or give to pilgrims, the poor, or guests. They were allowed to sell beer in the monastery "pubs" (Rabin and Forget 1998). The basis of the brewing industry, however, was born in the growing urban centers where large markets began to emerge. Brewers began to provide good profits for the pubs, and the independent inns became tied public houses. Thus, most of the fundamentals for manufacturing and selling of beer in our time were established in London by 1850 (Mathias 1959).

The Ingredients

Beer holds one of the oldest acts in the history of food regulation—the *Reinheitsgebot* (1487). Most known as the "German Beer Purity Law" or as the "Bavarian Purity Law", it was originally designed to avoid the use of wheat or rye in beer making. This act ensured the availability of primary grains for the bakers, thus keeping bread's prices low. From that time forth, the law restricted the ingredients for making beer to barley, water, and hops. Naturally, this purity law has been adapted over time. For example, yeast was not present in the original text as it was unknown by that time. The current law (*Vorläufiges Biergesetz*) is at stake since 1993 and comprises a slightly expanded version of the *Reinheitsgebot*. It limits water, malted barley, hops, and yeast for making bottom-fermented beers, while to make top-fermented beers, different kinds of malt and sugars adjuncts are allowed. However, it is well known that breweries around the world often use starchy and sugars adjuncts also for the production of bottom-fermented beers.

The basic beer ingredient will be described in the following chapters as well as the main technological steps with focus on bottom-fermented lager beer, the most widespread beer type in the world.

Water

Water is the primary raw material used not only as a component of beer, but also in the brewing process for cleaning, rinsing, and other purposes. Thus, the quality of the "liquor," which is how brewers call the water as an ingredient, will also determine the quality of the beer. Thereafter, the brewing liquor is often controlled

by legislation. It has to be potable, free of pathogens as well as fine controlled by chemical and microbial analyses. In addition, different beer styles require different compositions of brewing liquor.

Water has to be often adjusted previously to be ready as brewing liquor. Adjustments involve removal of suspended solids, reduction of unwanted mineral content, and removal of microbial contamination. Thus, different mineral ions will affect the brewing process or the final beer's taste differently. For example, sulfates increase beer's hardness and dryness, but also favor the hop bouquet. High iron and manganese contents may change beer's color and taste.

Calcium is perhaps the most important ion in the brewing liquor. It protects α-amylase from the early inactivation by lowering the pH toward the optimum for enzymatic activity. Throughout boiling, it not only supports the precipitation of the excess of nitrogen compounds, but also acts in the prevention in over-extraction of hops components (Comrie 1967). Furthermore, calcium also plays a crucial role through fermentation, since it is mandatory for yeast flocculation (Stratford 1989), as discussed in the next chapter. Yeast growth and fermentation are favored by zinc ions, but hindered by nitrites (Heyse 2000; Narziss 1992; Wunderlich and Back 2009).

Malted Barley and Adjuncts

The barley plant is, in fact, a grass. The product of interest for the brewers is the reproductive parts (seeds) of the plant known as grains or kernels displayed on the ears of the plants. Depending on the species of the barley, the plant will expose one or more kernel per node of the ear. Mainly, two species of barley are used in brewing: the two-row barley (with one grain per node) and the six-row barley (with three grains per node). To put it simple, the fewer are the kernels per node, the bigger and richer in starch they are. Conversely, the six-row barley has less starch but higher protein content. Therefore, if the brewer wants to increase the extract content, the two-row barley is the best option, whereas if enzymatic strength is the aim, the six-row will be the best choice (Wunderlich and Back 2009).

Worldwide, most breweries use alternative starch sources (adjuncts) in addition to malted barley. Adjuncts are used to reduce the final cost of the recipe and/or improve beer's color and flavor/aroma. The most common adjuncts are unmalted barley, wheat, rice, or corn, but other sugar sources such as starch, sucrose, glucose, and corresponding sirup are also used. The use of adjuncts is only feasible because light malts (i.e., Pilsener malt) have enough enzymes to breakdown up to twice their weight of starch granules. However, each country regulates the maximum allowed amount of adjuncts for making beer. Until the current days, the Bavarian Purity Law regulates the use of adjuncts in Germany, whereas "outlaw" countries such as USA and Brazil often exaggerate the use of adjuncts. In the USA, commercial breweries can use up to 34 % (w/w) of unmalted cereals of the total weight of grist. In Brazil, unmalted grains such as corn and rice are allowed in amounts as high as 45 % of the total recipe content. Poreda et al. (2014) assessed the impact of corn grist adjuncts

on the brewing process and beer quality under full-scale conditions. The use of corn in up to 20 % of the formula affected some of the technological aspects of wort production and quality, but caused no significant effect in the physicochemical properties of the final beer. Nonetheless, the impact on beer's flavor profile was not considered. The abuse of maize and/or rice is known to impair the beer with a predominant aroma of cooked corn or "popcorn aroma" (Taylor et al. 2013).

Malting

It is important to emphasize that unmalted grains are the dormant seeds of grass plants, i.e., *Hordeum* spp. (barley) and *Triticum* spp. (wheat). Through the malting process, the grains are germinated controllably to produce the corresponding malt. However, the correct extent of germination is the key for producing good malt.

During germination, the embryo grows at the expense of reserve material stored in the kernel. As soon as the grain makes contact with suitable conditions during steeping (moist and adequate temperature), all enzymatic apparatus is gradually activated to break the reserves of starch and proteins to form a new plant. Here lie the crucial roles of malting, which are enriching the malt with enzymes (amylolytic, proteolytic, etc.), modification of kernel endosperm, and formation of flavor and aroma compounds. Starch-degrading enzymes (such as α-amylase, β-amylase, α-glucosidase, and limit dextrinase) produced during germination are better characterized than the proteolytic counterparts (Schmitt et al. 2013).

It is easy to understand that the optimum stage for interrupting the germination is when the malt is rich in enzymes, achieved sufficient endosperm modification and have consumed as little reserve materials (starch, proteins) as possible during embryo development. At this point, germination is arrested by kilning (drying). After complete kilning, the pale-malted barley is known as Pilsener malt. All other varieties of malt derive from this point by kilning or roasting at different temperatures. However, the more the malt is heat treated, the greater is the damage to the enzymes. So, while Pilsener malts are the richest in enzymes, chocolate malt (thoroughly roasted) have no enzymatic activity at all.

Hops

Compared to water and malts, hops are lesser of the ingredients used in brewing, but no lesser is the contribution it makes to the final beer. Hops influence to a large extent the final character of beer. Brewers use the flowers (cones) from the female plants of *Humulus lupulus*. As there are numerous varieties of this plant spread worldwide, it is predictable that the quality and characteristics of the flowers also vary. Thus, some hops are known as "aroma/flavor hops" while others as "bitter hops." The α-acids are responsible for the bitterness of a given hop, whereas aroma

is tied to essential oils from hop cones. Thus, aroma hops are usually weaker in α-acids but rich in essential oils. Conversely, bitter hops have higher contents of α-acid but may lack on essential oils.

Nowadays, breweries rarely use cones, but pellets and hop extracts instead. Pellets are made from raw hops by drying, grinding, screening, mixing, and pelletizing. Extracts result from extraction with ethanol or carbon dioxide. The resulting product is a concentrated, resin-like sticky substance. The extracts and pallets are easier to be stored and have higher shelf life but also different chemical compositions than hop cones.

Yeast

Genus of *Saccharomyces* has always been involved in brewing since ancient times, but through the vast majority of the brewing history our ancestors had no idea that living cells were the responsible entities for fermentation.

Although Antonie van Leeuwenhoek was the first to see yeast cells through a microscope in 1680, it was not before the studies by Louis Pasteur that conversion of wort into beer was awarded to living cells. Pasteur made careful microscopic examination of beer fermentations and published the results in *Études sur la bière* (1876), which means "Studies about beer." Pasteur observed the growth of brewing yeast cells and demonstrated that these were responsible for fermentation. Given the importance of the brewing yeasts to beer characteristics, the next chapter of this book is entirely dedicated to them.

Wort Production

Milling

Before mashing, the malt and other grains must be milled in order to increase the contact surfaces between the brewing liquor and malt. The ground malt (with or without other unmalted grains) is called grist. Some traditional breweries still use lauter tuns for wort filtration and, in these cases, the grain's husks should not be too damaged because it functions as a filter material. However, other breweries use mash filters as an alternative and thus no husks or coarse grits are necessary. The appropriate milling is usually attained either by roller or hammer mills.

The finer are the particles the better is usually the breakdown of the malt material into fermentable sugars and assimilable nitrogen compounds. However, the particle size directly interferes with the rate of wort separation. Unmalted grains also hamper the rate of wort recovery by increasing the proportion of insoluble aggregates of protein, hemicellulose, starch granules, and lipids (Barrett et al. 1975).

Although the vast majority of breweries perform a dry milling, Lenz (1967) suggested several decades ago an alternative wet milling and Szwajgier (2011) has recently discussed the advantages of the process. The author compared wet and dry millings, proving that the former improves the extraction rate of fermentable sugars from the filtration bed into the wort, thus reducing lautering time. Moreover, the author observed that the wet method can also reduce the amount of phenolic compounds extracted during mashing, which could enhance the colloidal stability of beer produced (Delvaux et al. 2001). However, the wet milling also increases protein extraction, which should be monitored to prevent haze formation (Szwajgier 2011).

Mashing

To initiate mashing, the grist is mixed with water (mashing-in) at a prespecified temperature to produce a slurry known as mash. Subsequently, the mash is heated to optimum temperatures of the technologically most important enzymes and allowed to rest.

There are two main mashing strategies. Either the entire mash is heated up according to a predefined pathway (infusion mashing), or the temperature of the mash is increased by removing, boiling, and pumping back parts of the mash (decoction mashing). A considerable breakdown of starch is only attained after the temperature is high enough to cause gelatinization, which broadly exposes the binding sites to the enzymes. As the temperature rises, enzyme activity accelerates, but also does the rate of enzyme denaturation. In addition to temperature, enzyme activity and stability is also influenced by pH and wort composition (Rajesh et al. 2013).

The breakdown of starch into fermentable sugars is quantitatively the most important task occurring during mashing. Although barley malts have four starch-degrading enzymes (α-amylase, β-amylase, α-glucosidase, and limit dextrinase), the heavy work of breaking starch to fermentable sugars throughout mashing depends on α-amylase and β-amylase. The degradation of starch starts by action of α-amylases (optimum temperature 72–75 °C, optimum pH 5.6–5.8), which have much broader work option than β-amylases (optimum temperature 60–65 °C, optimum pH 5.4–5.5). That is because β-amylases can only "attack" the non-reducing ends of starch and dextrin chains. Despite β-amylases have a higher affinity with long chains of starch molecules (Ma et al. 2000), the fast action of α-amylases makes dextrin more accessible increasing the availability of binding sites for β-amylases. Therefore, the smallest product of action of β-amylases is maltose, while α-amylases can virtually break an entire starch chain into glucose. Thus, the final wort consists of fermentable sugars (glucose, maltose, and maltotriose) and non-fermentable small (limit) dextrins. Simultaneously with enzymatic starch degradation, other processes such as protein breakdown, β-glucan degradation, changes in lipids and polyphenols, and acidification reactions take place.

At the end of the mashing, it is necessary to separate the aqueous solution of the extract (wort) from the insoluble fraction called spent grains. For this purpose, lautering (filtration) is carried out either in lauter tuns or in mash filters of different constructions. In lauter tuns, the complete separation of extract is achieved through sparging of the spent grains with water. In mash filter, the extract adsorbed in spent grains is recovered with the use of filter cloths.

The amount of solid malt (grist) transferred into soluble extract enables to calculate the brewhouse yield (efficiency of operations) and determines the "strength" of the wort. The wort concentration is usually expressed as the mass of extract (kg) per hl wort in % w/v.

Wort Boiling

After separation from the residual solids (brewer's spent grains), the hot sugary liquid (wort) is boiled with hops. Additionally, some special recipes also use all kinds of "seasoning" to the wort on this step such as coriander seeds, orange peel, cinnamon, and cloves. Furthermore, it is also in this stage that sugar adjuncts as sucrose, malt sirup, and sugarcane may be added as "wort extenders" to increase extract.

The whole process takes from 90 to 120 min and according to Miedaner (1986), the crucial processes taking place during wort boiling are: inactivation of enzymes; sterilization; precipitation of proteins (hot break); evaporation of water and unwanted volatiles such as dimethyl sulfide (DMS); isomerization of hop α-acids; and the formation of flavor compounds through Maillard reaction. After separation of hot break and cooling, the wort is aerated and it is ready for pitching.

Fermentation and Maturation

After pitched into chilled and aerated wort, brewing yeast will initiate assimilating fermentable sugars, amino acids, minerals, and other nutrients. From this time forth, the yeast starts excreting a wide range of compounds such as ethanol, CO_2, higher alcohols, and esters, as a result of cellular metabolism. Whereas the large cut of these metabolic by-products are toxic for the yeast cells at higher concentrations, they are the wanted products of beer fermentation at reasonable amounts.

After cooling and aeration, the wort must be pitched (inoculated with suspended yeast cells) as fast as possible to avoid contaminations. Common pitching rates are about 15–20 × 10^6 cells mL^{-1}. However, higher dosages are often used in high gravity brewing (HBG). While small to medium size breweries still may use open fermenters, large breweries mostly replaced them by closed stainless steel cylindroconical vessels (CCVs). These closed fermenters not only offer larger productivity and good hygienic standards, but also provide operating advantages through temperature and pressure control (Landaud et al. 2001).

The amount of fermented extract determines the attenuation of wort, which is the main parameter indicating the course of fermentation. Regular worts contain about 80 % of fermentable extract. At the stage of beer transfer, movement of the green beer from fermentation cellar to lager cellar, the green beer should contain approximately 10 % of unfermented fermentable extract in order to obtain sufficient formation of dissolved CO_2 during maturation. However, some breweries allow all extract to be utilized during primary fermentation and then add more of the original wort (or sugar adjuncts) for carbonation. A proper primary fermentation can be achieved usually in about 5–7 days, but the exact duration will strongly depend on the original wort extract, fermentation temperature (7–15 °C for lager beers), and yeast physiology.

Maturation further exhausts the residual extract to form CO_2, which in turn helps at removing some unwanted volatile substances as aldehydes and sulfur compounds ("CO_2 wash"). During maturation, also other processes take place such as beer clarification (precipitation and sedimentation of cold break particles), yeast sedimentation, and flavor formation. The main parameter determining the state of maturation is the removal of diacetyl formed during primary fermentation. Although this process can take several weeks, modern breweries may use specific yeast strains, high pitching rates, and elevated temperatures to accelerate diacetyl removal. After diacetyl concentration falls below perception threshold (0.1 mg L^{-1}), the temperature of the lager tanks or CCVs is decreased (−2 to 3 °C for lager beers) to clarify and stabilize the beer. Thereafter, beer is ready to proceed into final processing stages, which may include all or just some of the following operations: filtration, colloidal stabilization, packaging, and pasteurization.

The next chapter of this book thoroughly discusses yeast metabolism and fermentation.

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Chapter 2
The Brewing Yeast

Abstract The concept of brewing science is very recent when compared with the history of beer. It began with the microscopic observations of Louis Pasteur and evolved through the last century with improvements in engineering, microbiology, and instrumental analysis. However, the most profound insight into brewing processes only emerged in the past decades through the advances in molecular biology and genetic engineering. These techniques allowed scientists to not only affirm their experiences and past findings, but also to clarify a vast number of links between cellular structures and their role within the metabolic pathways in yeast. This chapter is therefore dedicated to the behavior of the brewing yeast during fermentation. The discussion puts together the recent findings in the core carbon and nitrogen metabolism of the model yeast Saccharomyces cerevisiae and their fermentation performance.

Introduction

Brewing yeasts are eukaryotic, unicellular, heterotrophic, and facultative anaerobic microorganisms. During beer fermentation, they reproduce exclusively asexually by budding. A single yeast cell can bud approximately 10–30 times (Powell et al. 2000) and each cell division will leave on the mother cell a scar (bud scar), the counting of which indicates the cell's age. A fully grown yeast cell has an ovoid shape and measures around 5–10 μm in diameter.

The word "*Saccharomyces*" means "sugar fungus" (from the Greek Saccharo = sugar and myces = fungus). The species "*cerevisiae*" comes from the Latin and means "of beer." As the name clearly suggests, in nature, yeasts from the genus *Saccharomyces* are commonly found in sugary environments as in the surface of ripe fruits. Throughout evolution, strains of *Saccharomyces* spp. have developed very sophisticated ways to survive and move around the globe. One example is the ability to travel great distances in the guts of migratory birds (Francesca et al. 2012). Moreover, yeast can also disseminate within crops in the body and

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digestive tracts of flying insects (Stefanini et al. 2012; Asahina et al. 2008, 2009; Fogleman et al. 1981). To an evolutionary point of view, this mobility allows different strains to mate and even endure all over the winter (Stefanini et al. 2012). It is also believed that esters are produced on purpose by the yeast aiming at luring fruit flies such as *Drosophila* spp. (Asahina et al. 2008, 2009). In this case, esters would be serving as flight tickets, allowing yeast to disseminate effectively.

There are two groups of brewing yeasts that present very distinctive, genomic, physiological, and fermentation characteristics: ale and lager strains. Therefore, many features may significantly vary between these groups such as flocculation behavior (Holle et al. 2012; Soares 2011); fermentation time; stress tolerance and trehalose storage capacity (Bleoanca et al. 2013; Ekberg et al. 2013); and organoleptic impression added to beer. The most distinguishing feature used to differentiate individuals of these groups is the inability of ale yeasts to ferment melibiose (a disaccharide of galactose–glucose). Conversely, lager yeasts can hydrolyze 5-bromo-4-chloro-3-indolyl-α-D-galactoside, growing as blue colonies in Petri dishes with media containing this indicator, whereas ale yeast colonies will remain uncolored (Tubb and Liljeström 1986).

Saccharomyces cerevisiae strains are associated with the brewing process since ancient times. They are called "top-fermenting" and produce ale-type beers. The term top-fermenting is related to the fact that they often accumulate in the foam during fermentation. However, with the hydrostatic pressure applied in modern large-scale cylindroconical vessels (CCVs), even ale yeasts are harvested from the bottom cone of the CCVs. *S. cerevisiae* works properly in temperatures ranging from 18 to 25 °C, resulting in fast fermentations, and beers strongly marked by fruity aromas. The vast majority of the knowledge built so far about yeast (including the pathways of nutrient sensing, signaling, formation of products cell aging and chronological life span) regards to *S. cerevisiae*, because it is a widely accepted eukaryotic cell model.

Lager yeasts are "bottom-fermenting," on account of their tendency to sink in open fermenters. Formerly referred as *S. carlsbergensis* or *S. uvarum*, lager yeasts strains have a current accepted nomenclature of *S. pastorianus*. They are natural, aneuploid hybrids of *S. cerevisiae* and a non-*cerevisiae Saccharomyces* species (Bolat et al. 2013). Nakao et al. (2009) performed the first complete genome sequence of a lager brewing strain attributing the non-*cerevisiae* part of the genome to *S. bayanus var. bayanus*. Two years later, a closer look in the genome of *S. eubayanus* revealed that this cryotolerant yeast was, in fact, responsible for the non-*cerevisiae* genome of *S. pastorianus* (Libkind et al. 2011).

Irrespective of the species, the yeast used for brewing purposes lives a considerable different life than it would have in the natural environment. Throughout successive fermentations, yeast cells are regularly exposed to fluctuating conditions, forcing the cells equally to modify the transcriptome in order to keep homeostasis. Thus, in the course of a given fermentation, a single yeast cell will exhaustively express, repress, and derepress genes, and build and destroy (autophagy) cellular components according to the immediate needs. Thus, yeast cells are continuously monitoring the intracellular and extracellular environments to assess nutrient availability and potential harsh conditions, and respond by induction or repression of specific genes, while the modulation of metabolic pathways is mediated through stimulatory or inhibitory effects of metabolites.

Yeast Flocculation

Flocculation is the reversible, asexual process by which yeast cells stick to each other to form large cell aggregates known as flocs. Yeast uses this feature as a defense mechanism that allows it to flee quickly from the harsh environment developed throughout fermentation. To the industry, on the other hand, flocculation provides a free of charge method to separate yeast from the freshly made beer. If flocculation fails, unwanted high residual yeast counts may remain suspended in the green beer. If this happens, the remaining yeast is recovered by other mechanisms (e.g., centrifugation), consequently increasing production costs. Conversely, if yeast flocculates prematurely, insufficient cells will remain suspended to finish the fermentation. In other words, yeast must flocculate properly at the end of the primary fermentation, leaving an adequate amount ($10–15 \times 10^6$ cells mL^{-1}) of cells for maturation, and therefore, the ideal brewing yeast must exhibit constant flocculation capacity throughout successive rounds of fermenting, cropping, washing, storing, and repitching.

The lectin-like proteins (sugar-binding proteins, also called flocculins) mediate the best known mechanism of yeast flocculation. Eddy and Rudin (1958) took the first step toward the elucidation of the lectin hypothesis by identifying ionizable entities in the cell wall of *S. carlsbergensis* with fluctuating changes through starvation. However, the role of proteins encoded by FLO genes in flocculation was only modeled in the work of Miki et al. (1982). Flocculins from one cell bind to mannose residues in the cell wall of surrounding cells and this chain reaction results in large clusters of cells. The presence of calcium is mandatory for lectin-mediated flocculation (Stratford 1989; Miki et al. 1982; Veelders et al. 2010). Miki et al. (1982) first suggested that Ca^{++} would change the structural conformation of flocculins. However, not long ago Veelders et al. (2010) shown that calcium is directly involved in flocculin to carbohydrate binding.

S. cerevisiae have five flocculin-encoding genes (FLO1, FLO5, FLO9, FLO10, and FLO11) (Caro et al. 1997). The genes FLO1, FLO5, FLO9, and FLO10 encrypt proteins related to cell–cell adhesion and flocculation. FLO11 is encoding a protein responsible for cellular adhesion to substrates (such as plastics and agar), diploid pseudohyphae formation, and haploid invasive growth (Guo et al. 2000; Lambrechts et al. 1996; Lo and Dranginis 1998). Other important FLO genes are FLO2 and FLO4, which are alleles of FLO1, as well as FLO8, which is encoding a transcriptional activator of FLO1 and FLO9.

There are two dominant phenotypes expressed by the brewing yeast: the Flo1 and the NewFlo. In the former, flocculation can only be inhibited by mannose. In the NewFlo, flocculation is disrupted by a broader range of sugars including mannose and glucose (Stratford and Assinder 1991; Kobayashi et al. 1998; Sim et al. 2013). In this manner, free mannose (for Flo1 phenotype) and other sugars (for NewFlo phenotype) competitively displace cell wall mannose residues from flocculin binding sites, separating them in consequence (Fig. 2.1). Stratford and Assinder (1991) were the first to describe the NewFlo phenotype in lager strains. Kobayashi et al. (1998) have further shown that flocculent strains of *S. pastorianus* had a gene homologous to FLO1 called Lg-FLO1, which was responsible for the NewFlo phenotype. Indeed,

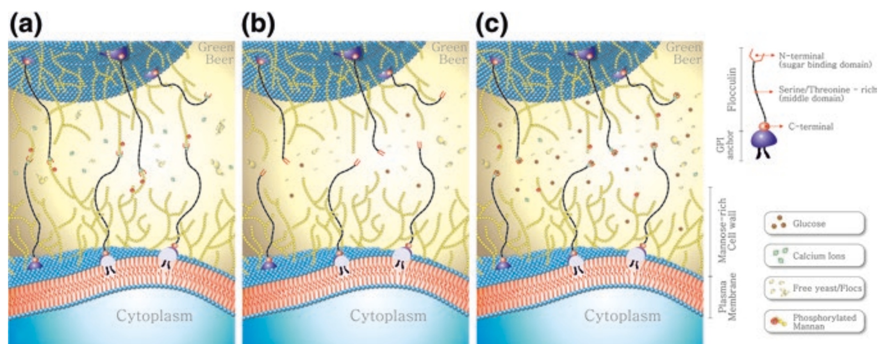

Fig. 2.1 Schematic view of the NewFlo yeast phenotype under different situations of beer fermentation, where **a** flocculation is established because free sugars (e.g., glucose) have been exhausted, calcium ions are present and associated with the N-terminals of flocculins, and mannan residues in cell wall are phosphorylated; **b** flocculation cannot occur because there are neither calcium ions nor phosphorylated mannans; and **c** flocculation is prone to occur, but the sugar-binding domains of flocculins are occupied with free sugars of the unfinished beer fermentation

Ogata et al. (2008) further confirmed that Lg-FLO1 was a *S. pastorianus*-specific gene located on *S. cerevisiae*-type chromosome VIII. However, Lg-FLO1 was also found in some *S. cerevisiae* (ale) strains proving the flocculation gene variability in industrial brewing yeast strains (Van Mulders et al. 2010). More recently, Sim et al. (2013) demonstrated that Lg-Flo1 flocculins would bind to phosphorylated mannans rather than non-phosphorylated mannans in the yeast's cell wall.

Both environmental (e.g., pH, metal ions, and nutrients) and genetic factors affect flocculation. However, these factors should never be considered separately as the environment may influence the expression of FLO genes (Verstrepen and Klis 2006). Because flocculation is mainly a defense mechanism, nutrient starvation and stress conditions will trigger the expression of flocculins (Stratford 1992). Nothing represents this better than the competitive attachment of simple sugars to the flocculin binding sites, working as a signaling mechanism of nutrient availability. Indeed, Ogata (2012) has suggested that yeast expresses Lg-FLO1 in response to nutritional starvation, and it is regulated by a nitrogen catabolite repression-like mechanism. In fact, FLO genes are under tight transcriptional control of several interacting regulatory pathways such as Ras/cAMP/PKA, MAPK, and main glucose repression (Verstrepen and Klis 2006; Gagiano et al. 2002).

Ethanol has a positive effect on flocculation as it reduces the negative electrostatic repulsion between cells (Dengis et al. 1995) and increases cell-surface hydrophobicity (Jin et al. 2001). Moreover, it has also been suggested that ethanol acts directly on the expression of FLO genes (Soares et al. 2004; Soares and Vroman 2003).

Hydrodynamic conditions may also have an impact on flocculation as liquid agitation increases the chance of cell collision; however, vigorous movement may also break up cell clusters (Klein et al. 2005). Additionally, concentration of yeast cells in suspension must be sufficient to cause the number of collisions necessary

to form flocs (van Hamersveld et al. 1997). Moreover, factors that increase cell-surface hydrophobicity and that decrease the repulsive negative electrostatic charges on the cell wall cause stronger flocculation as they increase the probability of cell–cell contact (Jin and Speers 2000).

Most yeast strains flocculate in a wide range of pH (2.5–9.0), but brewing strains expressing NewFlo phenotype can only flocculate in a significantly narrower pH range of 2.5–5.5 (Miki et al. 1982; Sim et al. 2013; Stratford 1996). In fact, Sim et al. (2013) have recently shown that Lg-FLO1 expressing strains flocculate optimally at pH 5.0, with cell–cell binding strength decreasing rapidly at lower pH. Lower fermentation temperatures decrease yeast metabolism and hence CO_2 production. The agitation caused by CO_2 bubbles determines to a large extent the number of cells in suspension during active fermentation (Speers et al. 2006).

Apart from flocculation, individual yeast cells may slowly sediment if size and density overcome the Brownian motion that would keep cells suspended (Stratford 1992). The sedimentation rate is also dependent on particle size: Smaller particles settle more slowly than larger particles of the same density, because they are relatively more retarded by friction (viscosity). Therefore, older yeast cells sediment faster than younger, smaller cells (Powell et al. 2003). However, the sedimentation of individual cells is too slow to be relevant in beer fermentations. Instead, there is a continuous exchange between cells entrapped in flocs and free cells. Therefore, single cells are continually leaving the flocs, while others become attached.

Carbohydrate Transport and Metabolism

The brewing wort is a complex solution of sugars, amino acids, peptides, vitamins, minerals, and a long list of other dissolved substances. When it comes to carbohydrate metabolism associated to the brewing process, the first thing that comes in mind is the conversion of fermentable sugars to ethanol. However, this would be an oversimplification for such an organized and sophisticated process.

The brewing yeast (either *S. cerevisiae* or *S. pastorianus*) can only assimilate and metabolize small sugar units as sucrose, glucose, fructose, maltose, and maltotriose. Invertases hydrolyze sucrose into glucose and fructose outside the yeast cell, whereas all the other sugars are transported into the cytoplasm for further processing. Both maltose and maltotriose are hydrolyzed into glucose within the cell by α-glucosidase. However, the intake of sugars occurs in a very orderly manner, being glucose and fructose absorbed first than maltose and maltotriose. Glucose and fructose compete for the same permease in the plasma membrane. However, glucose has a higher affinity for the permeases, which hinders the passage of fructose (Berthels et al. 2004, 2008).

Throughout fermentation, the brewing yeast lives in a fluctuating environment, going through moments of plenty and starvation. For that reason, yeast cells developed an efficient mechanism of sensing the nutritional availability, which enable cellular adaption through adversities. There are two well-known pathways triggered by

the presence of glucose: the main glucose repression pathway (or catabolite repression pathway), and the Ras/cAMP/protein kinase A (PKA) pathway. The first pathway inhibits the expression of several genes involved in the transport of maltose and maltotriose if preferable sugars such as sucrose and glucose are present. It also represses genes involved in gluconeogenesis and respiration (Carling et al. 2011; Garcia-Salcedo et al. 2014; Hardie et al. 2012). The Ras/cAMP/PKA regulates genes involved in metabolism, proliferation, and stress resistance. Thus, in times of plenty (i.e., after wort pitching), both the main glucose repression pathway and the Ras/cAMP/PKA pathway are activated because levels of glucose are high. In short, simultaneous activation of these pathways leads mainly to the arresting of both respiration and intake of less preferable carbohydrates, as well as to temporary loss of cell's stress resistance.

Main Glucose Repression Pathway

After fructose, glucose is the lesser of the fermentable sugars in all-malt worts. Nonetheless, when yeast is pitched in a new batch, glucose blocks the uptake and utilization of the main fermentable sugars in the brewing wort: maltose and maltotriose.

The Snf1 protein kinase is a major player in the main glucose repression pathway. This protein is the catalytic subunit of the SNF1 complex that also contains a regulatory subunit (Snf4) and one of the three alternative subunits (Gal83, Sip1, or Sip2) (Garcia-Salcedo et al. 2014). When glucose is present, unphosphorylated transcriptional regulator Mig1 is translocated from the cytoplasm to the nucleus where it recruits two general repressors (Tup1 and Ssn6) (Papamichos-Chronakis et al. 2004). Within the nucleus, this complex binds to promoters and downregulates genes involved in gluconeogenesis, respiration, and utilization of alternative carbon sources. When glucose is depleted extracellularly, the kinases Sak1, Tos3, and Elm1 phosphorylate the SNF1 complex, which in turn phosphorylates the transcriptional regulator Mig1 (Ghillebert et al. 2011; Treitel et al. 1998; Garcia-Salcedo et al. 2014; Papamichos-Chronakis et al. 2004). The phosphorylation of Mig1 abolishes the interaction with the corepressors Ssn6 and Tup1 and stimulates Mig1 export from the nucleus (Treitel et al. 1998; Smith et al. 1999; Papamichos-Chronakis et al. 2004).

Garcia-Salcedo et al. (2014) have recently added new perspectives about Snf1 phosphorylation. The authors over-expressed the Snf1-phosphorylating kinase Sak1 and observed that this genetically modified strain could phosphorylate and activate Snf1 even in the presence of high concentration of glucose. Conversely, the over-expressing Sak1 strain and the control cells showed an identical Mig1 mobility between nucleus and cytoplasm. Therefore, the enhanced Snf1 activity at high glucose levels did not result in increased Mig1 phosphorylation. To unravel this inconsistency, the authors co-over-expressed the regulatory subunit Reg1 of the Glc7–Reg1 phosphatase, partially restoring the regulation of Snf1 phosphorylation in cells with increased Sak1 activity. Additionally, when compared to

the control strains, cells over-expressing Reg1 had identical Snf1 activity, which indicates that increased Reg1 level does not disrupt the glucose regulation of Snf1 phosphorylation. Moreover, the enhanced dephosphorylating activity promoted by Reg1 over-expression alters the utilization of alternative carbon sources and regulation of Mig1 phosphorylation (Garcia-Salcedo et al. 2014). Thus, considering that Mig1 activity was not affected by the enhanced phosphorylation of Snf1 at high levels of glucose, Garcia-Salcedo et al. (2014) concluded that Glc7–Reg1 dephosphorylates both Snf1 and Mig1 forming a feed-forward loop on glucose repression/derepression (Fig. 2.2).

The major negative aspect of the main glucose repression pathway over brewing fermentations is the sequential uptake of sugars. Maltose (60 %) and

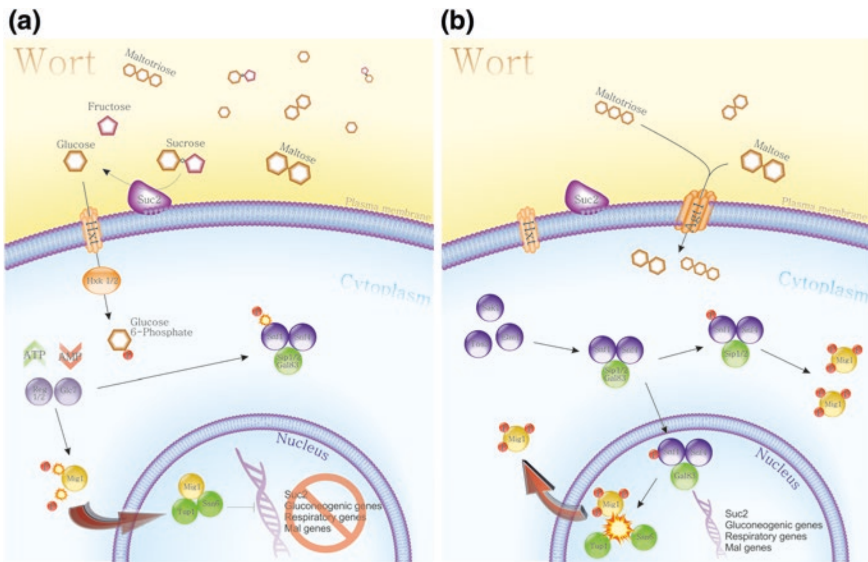

Fig. 2.2 The main glucose repression pathway in the brewing yeast. **a** When glucose is available in the wort, it is taken up by a hexose transporter (Hxt) and immediately phosphorylated by one of the yeast's hexokinases (Hxk1 or Hxk2). The phosphorylation of glucose and/or the depletion of AMP due to increased production of ATP inactivates the central protein kinase Snf1 by action of the Glc7–Reg1/2 phosphatase that dephosphorylates Snf1. Inactive Snf1 is unable to phosphorylate Mig1 and together with the parallel dephosphorylating activity of Glc7–Reg1/2 over Mig1, results in increased pool of dephosphorylated Mig1. In this state, Mig1 migrate to the nucleus where it recruits the general repressors Tup1 and Ssn6 and binds to the promoters of several genes, including those involved in gluconeogenesis, respiration, and the uptake and breakdown of alternative carbon sources, such as maltose or maltotriose. **b** When glucose is depleted from the brewing wort, the upstream kinases Sak1, Elm1, and Tos3 phosphorylate and activate Snf1. If the active complex Snf1 and Snf4 are associated with the β-subunits Sip1 or Sip2, the complex will be acting in the cytoplasm in the phosphorylation of Mig1, arresting it in the cytoplasmic region. When the active complex Snf1–Snf4 is linked with Gal83, it migrates to the nucleus and phosphorylates Mig1 forcing its exclusion from the nucleus. Without Mig1, Tup1, and Ssn6 yeast can no longer repress the expression of glucose-repressed genes

maltotriose (25 %) represent the largest part of energy in the form of assimilable carbohydrates present in the brewing wort. Therefore, the processing of these sugars into ethanol is the most time-consuming step in alcoholic fermentation. However, for the reasons above mentioned, as long as sucrose or glucose is present, all the machinery involved in the transport and hydrolysis of maltose and maltotriose is downregulated. All this turns out hindering fermentation rates. In fact, beer fermentations would be faster if yeast could assimilate and process all fermentable sugars simultaneously (Shimizu et al. 2002).

Glucose-Sensing System—Ras/cAMP/PKA Pathway

The Ras/cAMP/PKA pathway mediates the responses to levels of glucose through a dual glucose-sensing mechanism. Firstly, glucose from the extracellular environment is detected by a G-protein-coupled receptor (GPCR) system composed by a transmembrane protein (Gpr1), which is associated with Gα protein (Gpa2). However, there is evidence that Gpa2 and Gpr1 are not inseparable (Broggi et al. 2013; Zaman et al. 2009). In addition to the external stimuli, intracellular phosphorylation of glucose triggers the activation of Ras proteins (Colombo et al. 2004) through a yet-unknown pathway (Conrad et al. 2014). Thus, the cAMP-producing adenylate cyclase collects signals from two G-proteins (Ras and Gpa2), each mediating an independent branch of a glucose-sensing pathway (Fig. 2.3). However, GPCR system alone is unable to induce adenylate cyclase to produce cAMP (Rolland et al. 2000). This evidence undermines the existence of an extracellular glucose-sensing system, a subject yet to be unraveled by science. Whereas glucose and sucrose activate both intracellular and extracellular cascades, other sugars such as fructose, maltose, and maltotriose cannot trigger a strong cAMP/PKA activity (Rolland et al. 2001).

The forward/reverse switch of GDP↔GTP controls the operation of the monomeric GTPase Ras (Broach and Deschenes 1990). Thus, Ras is active when bounded to GTP, whereas it is inactive if linked to GDP. Although Ras possesses intrinsic GTPase activity, it depends on the help of other proteins to work properly. Thus, the guanine nucleotide-exchange factors (GEFs; Cdc25 and Sdc25) aid in the activation of Ras (Broek et al. 1987; Boy-Marcotte et al. 1996). Conversely, GTPase-activating proteins (GAPs: Ira1 and Ira2) stimulate the hydrolysis of bound GTP to GDP, hampering Ras activity (Tanaka et al. 1990).

The brewing yeast encodes two Ras (Ras1 and Ras2) proteins, sharing more than 70 % amino acid similarity (Powers et al. 1984; Kataoka et al. 1984). Ras binds to yeast's membranes through the C-terminal domain (Kato et al. 1992). Recent studies revealed that Ras (plus associated regulating GTPases) and adenylate cyclase are not only present in the plasma membrane, but also in the membranes of internal organelles such as mitochondria and nucleus (Belotti et al. 2011, 2012; Broggi et al. 2013). Broggi et al. (2013) further observed that nutritional availability of glucose determines the subcellular location of Ras proteins.

If the glucose is present, Ras is preferentially located in the plasma and nuclear membranes. On the other hand, under glucose starvation, Ras accumulates in the mitochondria and the original location is reestablished upon addition of glucose (Broggi et al. 2013). This evidence takes the investigations in the regulation of the Ras signaling system to a whole new ground.

PKA is a tetrameric protein that consists of two catalytic and two regulatory subunits. TPK (1, 2, and 3) genes encrypt the catalytic units, whereas BCY1 gene encodes the regulatory parts (Toda et al. 1987a, b). The binding of cAMP to the regulatory subunits governs the activation of PKA, which in turn dissociate from the catalytic part (Fig. 2.3). Conversely, PKA is deactivated by the hydrolysis of cAMP performed by a low- and high-affinity phosphodiesterases, Pde1 and Pde2, respectively (Nikawa et al. 1987; Sass et al. 1986). Moreover, PKA regulates the expression of Pde1 and Pde2, thereby performing an autoregulation (Hu et al.

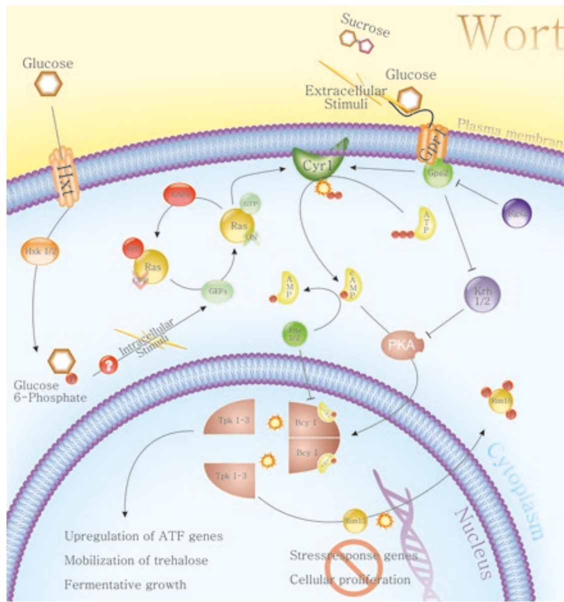

Fig. 2.3 The Ras/cAMP/PKA pathway governing a dual-glucose-sensing mechanism through beer fermentation. Intracellular phosphorylation of glucose activates Ras proteins by switching its bound GDP to GTP. This switch is carried out by guanine nucleotide-exchange factors (GEFs; Cdc25 and Sdc25), whereas inactivation (hydrolysis of GTP) is helped by GTPase-activating proteins (GAPs; Ira1 and Ira2). Active Ras stimulates adenylate cyclase (Cyr1) to produce cAMP from ATP. Further, cAMP binds to the regulatory subunits of PKA (Bcy1), thereby dissociating it from the catalytic subunits (Tpk 1–Tpk 3). Simultaneously, extracellular glucose or sucrose is sensed by a transmembrane G-protein-coupled receptor (GPCR) system, consisting of the receptor Gpr1 and the Gα subunit Gpa2. Gpa2 has intrinsic GTPase activity and is directly inhibited by Rgs2. *A*ctive Gpa2 enhances Cyr1 activity generating a transitory cAMP peak immediately after yeast is exposed to glucose or sucrose, i.e., after pitching in fresh beer wort. The kelch-repeat proteins (Krh 1/2) are inhibited by Gpa2, mediating an alternative route (cAMP-independent) of activating PKA by lowering the affinity between Bcy1 and Tpk 1–Tpk 3

2010; Ma et al. 1999). The catalytic subunits mediate a broad range of cellular processes such as metabolic pathways (glycolysis and gluconeogenesis); cellular growth, proliferation, and aging; accumulation of reserve carbohydrates; and pseudohyphae differentiation, invasive growth, and sporulation.

Harashima et al. (2006) observed that Ras GAPs (Ira1, Ira2) were also stimulated by two components of the GPCR-Gα signaling module: Gpb1 and Gpb2 (also known as kelch-repeat proteins, Krh1 and Krh2). Peeters and colleagues (2006) suggested that kelch-repeat proteins reestablish the link between PKA's regulatory and catalytic subunits, therefore, lowering PKA activity. In short, activated Gpa2 inhibits the activity of the kelch-repeat proteins allowing direct activation of PKA, representing an alternative route of activating PKA (Peeters et al. 2006; Lu and Hirsch 2005). Furthermore, kelch-repeat proteins were found to avoid the degradation of PKA's regulatory subunits (Bcy1), granting their availability under glucose starvation (Budhwar et al. 2010, 2011).

The Impact of the Glucose-Sensing System on Fermentation

Throughout beer fermentation, yeast cells are exposed to fluctuations in dissolved oxygen, pH, osmolarity, ethanol and dissolved CO_2 concentrations, nutrient supply status, pressure, and temperature (Gibson et al. 2007). Despite the brewing yeast is well prepared to respond to these changes, the presence of glucose triggers the Ras/cAMP/PKA pathway, which inactivates most of the cellular responses to environmental stress. Therefore, stress-responsive genes are all downregulated when cells are pitched into fresh wort, whereas nutritional and ethanol stress in the late stages of wort fermentation causes cellular cycle arrest and entrance into stationary phase thereby upregulating all PKA targets.

Among the several downregulated genes mediated by PKA activity are the genes encoding heat-shock proteins (HSPs) such as Hsp12 and Hsp104 (Brosnan et al. 2000; Varela et al. 1995). HSPs are specialized nursing proteins capable of remodeling cellular structures to protect the yeast against thermal damage, or other environmental stresses (see Verghese et al. (2012) for a review). Varela et al. (1995) have shown that the Hsp12 (which protects the yeast against high-osmolarity/glycerol, HOG pathway) is under negative control of the Ras/cAMP/PKA pathway. Under stress conditions, Hsp12 stabilizes membranes by modulating fluidity (Welker et al. 2010). Brosnan et al. (2000) observed an active downregulation of Hsp104 during both brewery fermentation and glucose-rich medium. HSP104 is required for thermotolerance, and deletion of this gene reduces cell survival (Sanchez et al. 1992).

High-gravity brewing (HGB) and very high-gravity brewing (VHGB) have become a common practice in modern breweries owing to the enhancement in productivity with few/none extra investment in equipment. However, in such conditions, the yeast faces more challenging environments where the hindered stress response (caused by Ras/cAMP/PKA pathway) often leads to sluggish or

stuck fermentations, even autolysis (Ivorra et al. 1999; Blieck et al. 2007). Yeast autolysis during fermentation strongly impairs beer aroma by leakage of intracellular components such as fatty acids and esterases. The small branched fatty acid 4-ethyloctanoic acid impairs the beer an intense, unpleasant goat-like aroma with very low flavor threshold (Carballo 2012). While this fatty acid directly damages beer aroma, the released esterases diminish the pleasant fruity notes of the beer by hydrolyzing the esters (Neven et al. 1997). Moreover, the extended exposition to glucose in HGB and VHGB may reduce yeast replicative lifespan (Maskell et al. 2001) and affect the structural stability of short chromosomes (Sato et al. 2002b). Ras/cAMP/PKA pathway is responsible for the induction of alcohol acetyltransferase (ATF) genes in response to glucose (Verstrepen et al. 2003). The expression of ATF genes determine to a large extent the amount of esters produced during fermentation (see Chap. 3 of this book for more details). Whereas an adequate amount of esters is beneficial for an overall impression of beer's bouquet, in excess they may be detrimental.

Trehalose is a non-reducing disaccharide comprised by two glucose units linked by a α-1-1-glycosidic bond. This sugar was formerly believed to be a reserve carbohydrate, but there is increasing evidence that its role is rather stress protectant (Trevisol et al. 2014; Wang et al. 2014; Jain and Roy 2010). The protective character of trehalose is attributed to the physical and chemical properties of this sugar (i.e., low reactivity, non-reducing, hydrophilic character, and polymorphism). These characteristics make trehalose suitable for stabilizing unfolded proteins and inhibiting protein aggregation (Jain and Roy 2010). However, through PKA activation, intracellular trehalose is immediately degraded when starved yeast is pitched into sugary-rich wort (Blieck et al. 2007; Wang et al. 2014).

Transport of α-Glucosides

Successful beer fermentations depend on the ability of the brewing yeast to transport the fermentable sugars from the brewing wort efficiently into the cytoplasm. Whereas glucose and fructose are passively diffused into yeast cells through hexose transporters (Hxt), α-glucosides as maltose and maltotriose are transported at the expense of energy by proton symporters (Palma et al. 2007). Fermentation of maltose requires that the strain possesses at least one of the five independent multi-gene MAL loci (in chromosome): MAL1 (VII), MAL2 (III), MAL3 (II), MAL4 (XI), and MAL6 (VIII) (Naumov et al. 1994). Each loci is a group of three genes involved in maltose utilization: one encoding a maltose permease; second encrypting a maltase (α-glucosidase); and third gene that encodes a regulator/activator factor that mediates the expression of the former two genes (Chow et al. 1989). Maltose permeases determine to a large extent the course of fermentation rate (Rautio and Londesborough 2003; Vidgren et al. 2009, 2014). Brewing strains often have two or more MAL loci, which have been long suggested to be a result of yeast adaptation to the high maltose environment of wort

(Ernandes et al. 1993). Indeed, Kuthan et al. (2003) have shown that yeast exposed to a long-term cultivation in glucose-rich medium lose the ability to derepress genes encoding maltose permeases and maltases when inoculated in maltose containing medium. More recently, Huuskonen et al. (2010) looked for robust yeast variants selected after a batch of VHGB beer fermentation. After isolation, the authors assessed viable cells that could grow in maltose or maltotriose under the harsh conditions such as high ethanol concentrations, low nutrient availability, and complete lack of oxygen. The selected variants showed improved performance in HGB and VHGB fermentations.

Maltotriose is the second most abundant (approximately 25 %) fermentable sugar in the brewing wort and shares with maltose the same MAL-encoded permeases to reach the cytoplasm (Vidgren et al. 2009). Since maltotriose is the last carbohydrate used throughout fermentation, it is commonly found as a residual sugar in beers produced over HGB and VHGB. Several permeases can transport maltose: Agt1 (alpha-glucoside transporter), Mphx, Mtt1 (also known as Mty1), and several versions of Malx (Jespersen et al. 1999; Vidgren et al. 2005; Salema-Oom et al. 2005). Among these, only Agt1 and Mtt1 can carry maltotriose (Alves et al. 2008; Salema-Oom et al. 2005; Cousseau et al. 2013). There is evidence that Agt1 is the most frequently present maltose transporter in the brewing yeast (Vidgren et al. 2005). Additionally, Agt1 is the only known permease to transport maltotriose in ale strains since Mtt1 is exclusive of lager strains (Salema-Oom et al. 2005).

Vidgren et al. (2014) have recently raised an interesting discussion about the temperature-dependent activity of Agt1. The authors were intrigued with the capabilities of ale and lager strains in absorbing maltose under different temperature conditions. It is believed that the most efficient fermentation performance of lager strains at lower temperatures has been inherited from the ancestor *S. eubayanus* (Sato et al. 2002a). With that in mind, Vidgren et al. (2014) compared the activity of three homologues of Agt1 under different fermentation temperatures. The authors proved that the activity of Agt1 was not only dependent on the temperature, but also on the genotype of the host yeast (mainly on the nature of plasma membrane) and on yeast-handling procedures (Vidgren et al. 2014).

Nitrogen Metabolism

The brewing yeast can assimilate and use a vast variety of nitrogen sources, ranging from simple ammonia, urea, and amino acids to complex nucleic acids and small peptides. In response to this array of options, yeast has evolved equally extensive degradative enzyme systems and sophisticated strategies of enzymatic regulation. A clear example of this is the ability of yeast in assimilating preferably those nitrogen-containing compounds able to be readily converted into the primary amino acid precursors. When the preferred amino acids are completely consumed,

yeast will express the machinery necessary for using alternative/less preferred ones. The nitrogen catabolite repression (NCR) is the pathway coordinating this mechanism.

Throughout the fermentation and maturation processes, the availability of nutrients continually drops, while the impact of some stress factors increases (ethanol stress, cold shock). In order to deal with this fluctuation, the brewing yeast unceasingly modifies gene expression to adapt both metabolism and nutrient uptake. Several pathways are in charge of continuously coping with recognition of nutritional deficiencies and with remodeling of transcriptome. For example, when amino acids are available, intracellularly a central serine/threonine protein kinase called target of rapamycin (Tor) commands a cascade of signals that activate the synthesis of proteins and consequently cellular growth. During this time, Tor is also inhibiting unnecessary degradation of proteins through autophagy. Conversely, under starvation conditions, Tor is inactive, which ceases cell growth and triggers the recycling of cellular components to maintain homeostasis. Moreover, under normal conditions the brewing yeast keep high basal expression of amino acid biosynthetic enzymes. However, under starvation of any amino acid, the transcription of these enzymes is significantly increased. This response has been designated as the general amino acid control (GAAC) pathway because derepression is not specific for the lacking amino acid.

Although often discussed separately, metabolic pathways work together to keep cellular functions throughout fluctuating growth conditions. This, in fact, is also a target of recent research (Staschke et al. 2010).

Target of Rapamycin (Tor) Pathway

Heitman et al. (1991) performed genetic modifications that equipped yeast with resistance to rapamycin (an immunosuppressant that inhibit cell growth). The authors were the first to recognize Tor as the primary protein affected by rapamycin. Thereafter, Tor has been described as central protein that integrates a wide range of intracellular and extracellular signals to modulate cellular growth. The Tor pathway is ubiquitous to all eukaryotes, which shares conserved function in the regulation of metabolism, translation, autophagy, and cellular growth (Kim and Guan 2011). Barbet et al. (1996) suggested that the Tor pathway could be triggered by extracellular nutrient signaling. However, there is growing evidence that TOR pathway would be rather involved in mobilization of nitrogen reserves from the vacuole in response to intracellular nitrogen availability (Conrad et al. 2014).

Differently from other eukaryotes that only have one Tor-encoding gene, *S. cerevisiae* has two similar (67 %) TOR genes (TOR1 and TOR2), encrypting homologous proteins with common biological functions (Helliwell et al. 1994). These core proteins work in cooperation with other protein subsets, forming complexes with distinctive functional versatilities (Wullschleger et al. 2006; Helliwell et al. 1994). Tor complex 1 (TorC1) has either Tor1 or Tor2 proteins in close

association with Kog1, Lst8, and Tco89 subunits (Loewith et al. 2002). Tor complex 2 (TorC2) has exclusively Tor2 in association with the proteins Avo1-3, Bit61, and Lst8 (Loewith et al. 2002; Wedaman et al. 2003; Reinke et al. 2004). Besides the regulatory role in the cellular growth, TorC1 is also involved in transcription, cell cycle, meiosis, and autophagy (Conrad et al. 2014; Laor et al. 2014). The role of TorC2 to cellular functions is not as well understood as those of TorC1. It is known, however, that rapamycin cannot inhibit TorC2 and that this complex is in charge of cytoskeleton organization, endocytosis, lipid synthesis, and cell survival (Conrad et al. 2014; Laor et al. 2014).

Such wide range of biological processes under control of the TorC1 drew attention to the subcellular location of the complex. Sturgill et al. (2008) inserted DNA cassettes encoding green fluorescent proteins in both the TOR1 and TOR2 genes in living cells of *S. cerevisiae*. The authors observed that Tor1 concentrated in the vacuolar membrane, but it also appeared spread through the cytoplasm. Tor2 was also present in the cytoplasm, but it was found mostly in the plasma membrane. The distinct pattern of subcellular location of the two proteins is consistent with the regulation of cellular processes controlled by the two independent complexes (Sturgill et al. 2008). In fact, not only the whole TorC1, but also the activator (EGO complex) and downstream effectors (such as Tap42–Sit4 phosphatases and Sch9 kinase) are confined in the vacuolar membrane (Fig. 2.4a) (Binda et al. 2009; Kim et al. 2008; Dubouloz et al. 2005; Urban et al. 2007; Yan et al. 2006; Zhang et al. 2012).

The EGO complex activates TorC1 when the intracellular environment is rich in amino acids and favorable to proceed with the translation of proteins and cellular growth (Dubouloz et al. 2005). As just mentioned, this complex is located in close association with TorC1 in the vacuolar membrane and consists of four proteins: Ego1, Ego3, Gtr1, and Gtr2 (De Virgilio and Loewith 2006; Dubouloz et al. 2005). Zhang et al. (2012) demonstrated that the structural conformation of Ego3 is essential in the anchoring of the entire EGO complex to the vacuolar membrane. The authors have shown that Ego3 is required for both recruiting Ego1 to the vacuolar membrane and also for the docking of the heterodimer Gtr1–Gtr2 to the vacuolar anchor Ego1. Amino acids are sensed intracellularly by Gtr1–Gtr2 (Ras-related GTPases), which is activated by the simultaneous binding of GTP and GDP, respectively (Kim et al. 2008; Binda et al. 2009; Sekiguchi et al. 2014).

Dokudovskaya et al. (2011) described the SEA complex (SEAC, also associated to the vacuolar membrane) in *S. cerevisiae* that contains the following: the nucleoporin Seh1 and Sec13; the upstream regulators of TorC1 kinase, Npr2 and Npr3 proteins; and four previously uncharacterized proteins (Sea1–Sea4). More recently, Panchaud et al. (2013a) identified a new protein (Iml1) working in a complex with Npr2 and Npr3 as a GTPase-activating protein for Gtr1. The authors observed that upon amino acid starvation, Iml1 transiently interact with Gtr1 at the vacuolar membrane to stimulate Gtr1's intrinsic GTPase activity, consequently interrupting the positive stimuli over TorC1. For this reason, the subcomplex Iml1–Npr2–Npr3 has been named SEACIT, referring to SEAC subcomplex inhibiting TorC1 signaling (Panchaud et al. 2013a, b). Conversely, SEAC has been

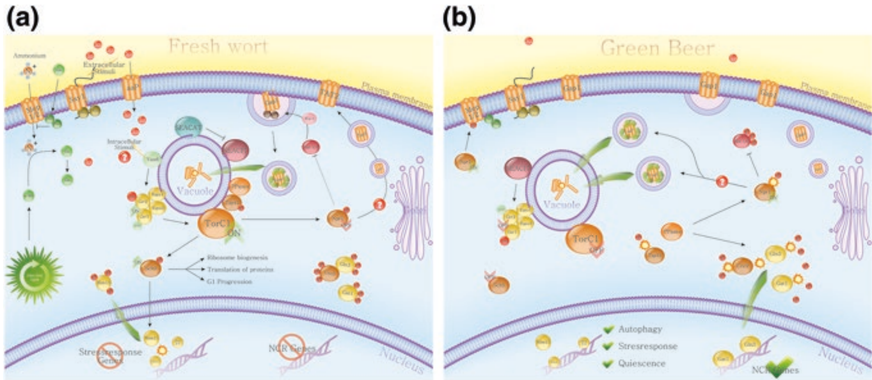

Fig. 2.4 Some interactions between the TorC1 and the NCR in the management of nitrogen sources through beer fermentation. **a** If good nitrogen sources, such as glutamine (Gln), are available for uptake, the ammonium permease Mep2 (ammonium is incorporated into the carbon skeleton of α-ketoglutarate leading to glutamate and glutamine) is inhibited via plasma membrane Psr1- and Psr2-redundant phosphatases. Specific amino acid permeases (aaP) are synthetized and send to the plasma membrane according to their specific availability in the wort. This recognition and further signaling is carried out by the SPS (Ssy1–Ptr3–Ssy5) system. The global increase in the intracellular levels of glutamate and glutamine is the main driver in the repression of genes involved in the absorption and metabolism of less preferred nitrogen sources (NCR genes). Under such condition, Ure2, Gln3, and Gat1 are hyperphosphorylated because the phosphatase complex (PPases—Pph21/Pph22 and Sit4) is arrested in the vacuolar surroundings by Tap42 owing to its phosphorylation commanded by active TorC1. In these circumstances, the transcription factors Gln3 and Gat1 are kept outside the nucleus and cannot activate NCR genes. Increasing intracellular levels of glutamine and other amino acids encourages the activity of guanine nucleotide-exchange factors (GEFs, through a yet-unknown mechanism) as Vam6 in the switching of GDP to GTP in the GTPases (Gtr 1–Gtr 2) of EGO complex, activating it. The active EGO complex activates the TorC1, which in turn phosphorylates Sch9, Tap42, and Npr1. Most of TorC1 control is hence performed by the effector Sch9. Together with the glucose inhibition over Rim15 through the Ras/cAMP/PKA pathway, active Sch9 also phosphorylates Rim15, arresting it in the cytoplasm where it is unable to activate the transcription factors Gis1 and Msn 2/Msn 4; thus inhibiting stress-responsive genes. On the other hand, phosphorylation inactivates Npr1 that stabilize aaPs such as Tat2 through a yet-unrevealed mechanism. Moreover, the inability of Npr1 to phosphorylate arrestin-like proteins, such as Bul 1–Bul 2, allows these proteins to assemble Rsp5 ubiquitin (Ub) ligase, which in turn target (by ubiquitylation) unnecessary Gap1 for endocytosis and destruction in the vacuole. **b** After the primary fermentation, the green beer is poor in nutrients, including assimilable nitrogen sources. In this situation, the intracellular levels of glutamate and glutamine drop triggering the activity of SEACIT over Gtr1 in the EGO complex, thus activating its intrinsic GTPase activity. This increases the GDP-bound state of Gtr1, inactivating the EGO complex. The inactive EGO complex can no longer activate TorC1, thus dissociating Tap42 and related PPases. Increased phosphatase activity causes massive dephosphorylation of Ure2, Gln3, and Gat1. The unphosphorylated transcription factors (Gln3 and Gat1) may not migrate to the nucleus and activate NCR genes including GAP1 in order to harvest the remaining amino acids from the green beer. The PPases also dephosphorylate and activate Npr1 kinase, which in turn phosphorylate Bul proteins. This protects Gap1 by preventing the recruitment of Rsp5 and subsequent targeting for destruction. Active Npr1 is also responsible for the vacuolar sorting of specific aaPs such as Tat2 through a yet-unknown mechanism. Still, active Npr1 has been recently shown to phosphorylate Mep2 permease, triggering its activity.

shown to reestablish TorC1 activity by abolishing SEACIT inhibition (Fig. 2.4a) (Panchaud et al. 2013a, b). Therefore, SEAC has been recently renamed as SEACAT (SEAC Subcomplex Activating TorC1 signaling) (Panchaud et al. 2013a, b). Binda et al. (2009) have also shown that TorC1 is reversibly inactivated in response to leucine starvation (and less pronouncedly in response to the lack of lysine or histidine). Besides, the authors have also shown that the conserved GEF Vam6 regulates the GTP/GDP status of Gtr1. Vam6 (a subunit of a large hexameric protein complex responsible for mediating the link and fusion of vacuoles) controls TorC1 signaling in response to amino acids, yet through an unknown mechanism (Ostrowicz et al. 2008). Later, Bonfils et al. (2012) have shown that leucine activates TorC1 through the interaction of leucyl-tRNA synthetase Cdc60 with Gtr1.

After receiving the signals that amino acids are available within the cell, TorC1 will command cellular growth not only by positively regulating ribosome biogenesis and translation, but also by inhibiting stress responses that would be incompatible with these processes (De Virgilio 2012). Two major effector branches execute TorC1 commands: the Sch9 kinase and the Tap42–phosphatase complex (Loewith and Hall 2011; Broach 2012; Urban et al. 2007).

Urban et al. (2007) have shown that TorC1 directly phosphorylate Sch9 at multiple C-terminal sites. However, this phosphorylation is abolished under either nitrogen or carbon starvation and transiently reduced when cells are subjected to stress conditions. One of the primary functions of phosphorylated Sch9 is to control the synthesis of proteins and cellular size before division (Jorgensen et al. 2004). Additionally, both phosphorylated Sch9 and PKA signals converge at Rim15 to inhibit/reduce stress responses, stationary phase, viability in stationary phase, and autophagy (Conrad et al. 2014).

Therefore, under nutrient abundance (such as in the early stages of beer fermentation), Rim15 is phosphorylated by either Sch9 or PKA, which sequesters Rim15 in the cytosol where it can no longer stimulate transcription factors such as Gis1 and Msn2/4 (Wanke et al. 2008). Indeed, Wei et al. (2008) have shown that Rim15 was mandatory for the cellular chronological life span extension caused by deletions in SCH9, TOR1, RAS2, and calorie restriction. These authors further noted a 10-fold increase in chronological life span in a double-knockout (*sch9Δ* and *ras2Δ*) strain growing under calorie restriction. More recently, Nagarajan et al. (2014) found divergent expressions of RIM15 in yeast cells immobilized in alginate beads from freely suspended cells growing under nutrient-sufficient conditions. RIM15 gene was highly expressed in encapsulated but not in planktonic yeast. Moreover, encapsulated wild-type but not *rim15Δ* cells cease to reproduce and show extended chronological life span. Therefore, the authors concluded that Rim15 induces cell cycle arrest and increases stress resistance in alginate-immobilized yeast. Though immobilized, well-fed yeast ceases to divide, it retains high fermentative capacity (Nagarajan et al. 2014). In fact, a misfunction in the Rim15p is responsible for the defective entry into the quiescent state and high fermentation rates observed in sake yeast strains (Watanabe et al. 2012; Inai et al. 2013).

Tap42–phosphatase complex executes the other branch of actions of TorC1. Active TorC1 phosphorylates Tap42, which consequently recruits and inhibits the phosphatases Pph21/22 and Sit4 (Jiang and Broach 1999). PPH21 and PPH22 redundantly encrypt the major protein phosphatase 2A (Pp2A) catalytic protein in yeast (Sneddon et al. 1990). When linked to phosphatases, Tap42 is localized in the internal membranes of yeast cells in close association to TorC1 complex (Aronova et al. 2007). Inactivation of TorC1 by either rapamycin treatment or nitrogen starvation releases Tap42–phosphatase complex in the cytosol, where it slowly dissociates owing to dephosphorylation of Tap42 (Yan et al. 2006). Cdc55 and Tpd3 regulate the activity of Tap42–Pp2A both by direct competition to the binding with Pp2A and dephosphorylation of Tap42 (Jiang and Broach 1999). This dephosphorylation activates Pp2A and Sit4 phosphatases that will mediate the expression of nitrogen catabolite repressed genes and genes involved in stress response (Duvel et al. 2003).

Nitrogen Catabolite Repression (NCR)

As they do for fermentable sugars, brewers yeast also orderly absorb and use nitrogen-containing compounds. Therefore, when yeast are exposed to nitrogen-rich environment, they repress the machinery involved in the use of less preferred nitrogen sources. Such repressive effect is widely known as NCR. The expression of genes affected by NCR is coordinated by Ure2 protein and four DNA-binding GATA transcription factors: two activators (Gln3 and Gat1) and two repressors (Dal80 and Gzf3) (Cooper 2002; Magasanik 2005; Conrad et al. 2014). When preferred nitrogen sources are broadly available, Ure2 arrests virtually all Gln3 and Gat1 in the cytoplasm where these activators cannot trigger the expression of NCR-sensitive genes (Blinder et al. 1996). Conversely, when the preferred nitrogen sources run out, the phosphatases Sit4 and Pp2A dephosphorilate Ure2, Gln3, and Gat1. Thereafter, the transcription activators Gln3 and Gat1 quickly relocate to the nucleus where they activate the transcription of the machinery necessary for using alternative nitrogen sources (Fig. 2.4b) (Rai et al. 2013; Broach 2012; Conrad et al. 2014). Gln3 is constitutively expressed and responsible for derepression of NCR-sensitive genes (including expression of other transcription factors) when preferred nitrogen sources are depleted (Mitchell and Magasanik 1984).

The exclusion of Gln3 from the nucleus is determined by the phosphorylation state of the 146 phosphorylation sites it possesses (Rai et al. 2013). Much attention has been given to Gln3 as the primary activator of NCR-sensitive gene expression, but Georis et al. (2009) highlighted several characteristics of Gat1 worthy of mentioning. The authors found that Gat1 was a limiting factor for derepression of NCR-sensitive genes. Moreover, both negative regulators Dal80 and Gzf3 interfered with Gat1 binding to DNA. Eventually, Gat1 was necessary for Gln3 binding to some promoters (Georis et al. 2009).

TorC1 involvement in NCR was first shown by Beck and Hall (1999). These authors evidenced that upon the addition of rapamycin to cells growing in nitrogen-rich environment, they behaved as if growing under nitrogen limitation. The observation was supported by nuclear localization of Gln3 and Gat1 activating the transcription of NCR-sensitive genes (Beck and Hall 1999). However, more recent works show that nutrient starvation and rapamycin relocate GATA factors to the nucleus through different pathways (Tate et al. 2010; Georis et al. 2011; Rai et al. 2013). Rai et al. (2013) showed that a structural modification in Gln3 diminishes its ability to remain sequestered in the cytoplasm under nitrogen-rich growth and that the same modification entirely abolished the response of Gln3 to rapamycin, but left NCR response to limiting nitrogen untouched. The authors were intrigued in whether TorC1-mediated activity represented sequential steps of a single regulatory pathway or two independent regulatory mechanisms were working in concert to control the traffic and function of Gln3. The authors concluded that Tor1 association-dependent (rapamycin-elicited) Gln3 regulation is a distinct and genetically separable pathway from nitrogen source-responsive, NCR-sensitive Gln3 regulation. Cooper et al. (2014a) have later demonstrated that rapamycin interacts with Gln3 through a separate site than that used by Gln3 to interact with Tor1. Thus, events triggered by rapamycin inhibition over TorC1 occur outside of the Gln3's site interacting with Tor1 or responding to nitrogen availability (Cooper et al. 2014a).

Because the interaction between Tor1 and Gln3 is required for the cytoplasmic sequestration of Gln3 under nitrogen-rich growth, Cooper et al. (2014b) raised the possibility of TorC1-activator EGO complex and Vam6 being also involved in the cytoplasmic allocation of Gln3 when preferred nitrogen sources are available. Both EGOC/Vam6-knockout and wild-type strains presented Gln3 sequestered in the cytoplasm when growing in nitrogen-rich medium. The first hypothesis raised by the authors was that Gln3 sequestration would occur in response to a TorC1-independent regulatory pathway. Otherwise, TorC1 activation can occur via both EGOC/Vam6-dependent and EGOC/Vam6-independent regulatory pathways (Cooper et al. 2014b).

Fayyadkazan et al. (2014) have recently shown that vacuolar protein sorting (Vps—responsible to Golgi-to-vacuole protein transport) components are required for Gln3 activity in response to rapamycin under poor nitrogen conditions. These authors have also speculated that Vps proteins in *S. cerevisiae* could be involved in amino acid sensing from the extracellular environment, similar to what happens in mammalian cells where Vps34 sense and triggers Tor pathway in response to external amino acids (Backer 2008).

Ogata (2012) has recently demonstrated that expression of Lg-FLO1 and flocculation in bottom-fermenting strains are under control of an NCR-like mechanism. Moreover, the author proved that transcription of Lg-FLO1 gene depended on the binding of Gln3 to the promoter region in the DNA in either nitrogen-starved cells or cells growing in medium containing only non-preferred nitrogen source (proline). The same author has also recently correlated the increased production of hydrogen sulfide and thiol off-flavor compounds with the induction

of NCR-sensitive genes during beer fermentations of worts containing reduced nitrogen content (Ogata 2013). The author used both strains with disrupted expression of GLN3 and GAT1 and over-expressing DAL80, GZF3, and URE2. While on the one hand, strains over-producing negative transcriptional factors were not conclusive with respect to reduced production of hydrogen sulfide, on the other hand, deletion of GLN3 and GAT1 successfully reduced the off-flavor formation (Ogata 2013).

General Amino Acid Control (GAAC)

The GAAC in yeast is responsible for certifying that all amino acids remain available inside the cell in response to deprivation of one or more of these building blocks. Accordingly, when lacking in amino acids, the yeast cell stop with the indiscriminate translation of proteins and focus their cellular machinery on preservation of energy and protection from stress. The Gcn4 is the central protein activator capable of inducing the manifestation of almost one-tenth of the total yeast genome in response to amino acid starvation (Hinnebusch 1993, 2005). The majority of genes induced by Gnc4 are directly involved in the increase of the intracellular pool of amino acids as genes encoding: amino acid biosynthetic enzymes, peroxisomal components, mitochondrial carrier proteins, amino acid transporters, and autophagy proteins (Staschke et al. 2010). The gene GCN4 has three positive regulatory genes (GCN1, GCN2, and GCN3) and five negative regulators (GCD1, GCD2, GCD6, GCD7, and GCD11) (Hinnebusch 1988, 2005).

Gcn4 has a short lifetime, being continually phosphorylated and tagged by ubiquitylation for proteasome degradation (Zhang et al. 2008). This permits a continued translation of GCN4 mRNA in non-starved cells, thus keeping a low level of redundant Gcn4. Intense degradation also allows rapid restoration of the basal level of Gcn4 when amino acids are replenished in starved cells. Recently, Rawal et al. (2014) have shown that accumulation of the β-aspartate semialdehyde (ASA—an intermediate in the synthesis of threonine) attenuates the GAAC transcriptional response by hastening degradation of Gcn4 in cells starved for isoleucine and valine.

Godard et al. (2007) noted that the expression of Gcn4 depends on the nitrogen source supplied, and it is subject to NCR, suggesting the interconnection between NCR and GAAC. The authors observed a pronounced activation of GAAC in yeast cells growing in the presence of non-preferred nitrogen sources. In addition, these authors have also found a reduced growth behavior of a knockdown Gcn4-activator (*gcn2Δ*) strain under poor nitrogen conditions. Previously, Sosa et al. (2003) had already raised the hypothesis of a physiological role of Gcn4 in the nitrogen discrimination pathway. These authors showed that when growing in nitrogen-rich conditions, a double-deleted (*ure2Δ gcn4Δ*) strain had the highest expression of DAL5 when exposed to rapamycin. These results suggest that Tor pathway, Ure2, and Gcn4 are acting through independent routes preventing the

expression of NCR-sensitive genes by Gln3 transcriptional activity and also that Gcn4 and Ure2 act in synergy in NCR control.

Transport and Control of Nitrogen Sources

Throughout beer fermentation, yeast cells are concomitantly controlling the catabolic routs of extracellular nitrogen sources and anabolic routes of amino acids and nucleotides. A perfect coordination of these complex processes can only be attained through constant monitoring of the nutrient availability in both intracellular and extracellular environments. Immediately after pitching in fresh wort, the brewing yeast "checks" the environment for the presence of amino acids through specialized sensors located in the plasma membrane, which are made of three proteins—Ssy1, Ptr3, and Ssy5 (SPS) (Fig. 2.4). Ssy1 is a permease-like protein devoid of transport activity (Forsberg and Ljungdahl 2001). Ssy5 is a protease responsible for the endoproteolytic activation of the transcription factors Stp1 and Stp2 (Andreasson and Ljungdahl 2002). Omnus and Ljungdahl (2013) recently showed that Ptr3 facilitates the activating signal carried out by Ssy5. Thus, in the early stages of fermentation, Ssy1 senses external amino acids, which triggers the proteolytic activity of Ssy5 and results in the activation of Stp1/2. These transcription factors induce the expression of a broad array of genes encoding amino acid-specific permeases as well as transporters for small peptides. Among the carriers are the TAT2, AGP1, BAP2, and BAP3 genes (for amino acids) and PTR2 gene (for di and tripeptides) (Fig. 2.5) (Ljungdahl and Daignan-Fornier 2012).

Once located intracellularly, amino acids or any other nitrogen-containing compounds are directly used in biosynthetic processes, deaminated to generate ammonium, or used as substrate for transaminases that catalyzes the transfer of amino groups to α-ketoglutarate to form glutamate. In this last case, what remains from the amino acid after transamination (i.e., α-keto-acid) is converted to higher alcohols as discussed in the next chapter of this book. Glutamine can be further synthetized from glutamate and ammonium, which is catalyzed by glutamine synthase encoded by GLN1. Ultimately, all incorporated cellular nitrogen originates from the amino nitrogen donated by glutamate and glutamine.

The brewing yeast can encode 24 different amino acid permeases (Nelissen et al. 1997), which are expressed according to yeast's need and quality of nitrogen sources available in the environment. However, it is important to emphasize that whereas some permeases are constitutive, others are only expressed when required, and still, unnecessary permeases are often targeted for recycling by autophagy. The NCR governs the expression of the general amino acid permease Gap1, and therefore, it is broadly present in the plasma membrane of yeast exposed to limited nitrogen conditions such as at the end of the primary beer fermentation. The intracellular trafficking of Gap1 is carried out in endosomes leaving the Golgi complex to the plasma membrane (in case of its translation in nitrogen-starved cells—Fig. 2.4b) and from the plasma membrane to the vacuole

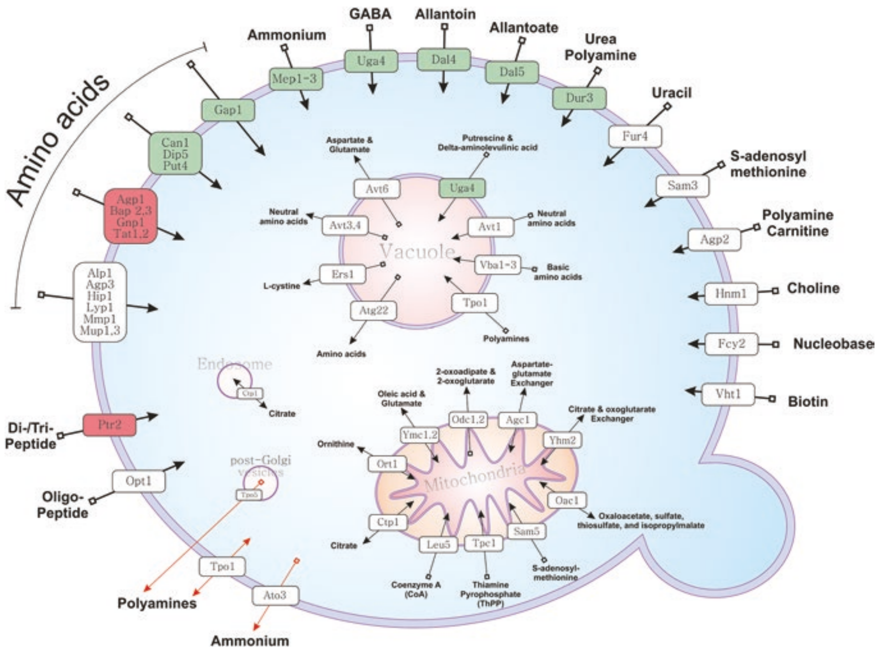

Fig. 2.5 The complex membrane transport system of nitrogen-containing compounds in the brewing yeast. The permeases/transporters are displayed with the corresponding substrate. The *arrows* signalize the direction through which the permease can transport the respective substrates. The transporters displayed within *green boxes* are under NCR control, whereas *red boxes* represent the permeases encoded through the stimuli of SPS system. Top1 catalyzes intake of polyamines at alkaline pH and excretion at acidic pH. It also mediates the export of polyamines during oxidative stress, which controls timing of expression of stress-responsive genes. Ato3 eliminates the excess ammonia that arises because of a potential defect in ammonia assimilation

for recycling (autophagy) when nutritional conditions are reestablished (Fig. 2.4a). As early discussed, the activation of TorC1 will recruit Tap42 to the vacuolar membrane, arresting the phosphatases Sti4 and PP2A. Thus, when starved yeast is pitched in fresh wort, the recycling of Gap1 starts with the TorC1-dependent phosphorylation (inhibition) of the Npr1 kinase. The inactive Npr1 can no longer phosphorylate the arrestin-like Bul1 and Bul2 adaptors, which recruits the Rsp5 ubiquitin ligase to Gap1 (Helliwell et al. 2001). Gap1 ubiquitylation is then carried out by Rsp5, which catalyzes the addition of ubiquitin moieties to lysine residues in Gap1, condemning it to internalization and further destruction in vacuole (Fig. 2.4b) (Springael and Andre 1998). Conversely, in the late stages of fermentation, inactive TorC1 releases Tap42–phosphatase complex in the cytosol that dephosphorylates and activates Npr1 kinase, which in turn phosphorylates Bul proteins (Merhi and Andre 2012; MacGurn et al. 2011). Thus, the Npr1-dependent phosphorylation of arrestin-like proteins prevents the recruitment of Rsp5 ubiquitin ligase to its plasma-membrane targets (e.g., Gap1) protecting them from ubiquitylation, endocytosis, and degradation in the vacuole (Fig. 2.4b) (MacGurn

et al. 2011). Therefore, Npr1 is responsible for both stabilizing Gap1 in the plasma membrane and for the endocytosis of specific amino acid permeases (AAPs) through a yet-unknown mechanism (Conrad et al. 2014).

Very recently, Crapeau et al. (2014) have shown that besides nutrient-replenishment-dependent targeting and dismantling of Gap1, this permease would be also ubiquitylated under stress conditions. This stress-induced pathway would allow yeast to retrieve amino acids from permease degradation improving the chances of survival when exposed to harsh conditions. Still recently, Van Zeebroeck et al. (2014) have elucidated alternative mechanisms of permease sorting acting in parallel to TorC1/Npr1-mediated signaling. The authors observed that the addition of various amino acids to starved cells (expressing Gap1) triggered different responses in regard to oligoubiquitylation and endocytosis of Gap1. Moreover, the authors have also demonstrated that the targeting of Gap1 for endocytosis does not necessarily require amino acids transport through Gap1 and also that some amino acids weakly induce Gap1's destruction.

Long ago, Jones and Pierce (1964) classified the amino acids present in wort into four separate groups, based on their uptake rate by yeast throughout beer fermentation: (A) absorption with complete uptake within the first 20 h after pitching; (B) gradually absorbed through the entire fermentation; (C) slowly absorbed, normally presenting an extended lag phase; and (D) proline as poorly absorbed (Table 2.1). Despite a minor change in the regrouping of methionine to the group of fast absorption, the original classification is still current (Gibson et al. 2009; Krogerus and Gibson 2013).

The brewing yeast possesses a family of three highly similar transporters responsible for the intake of ammonium ions from the wort. These permeases are encoded by MEP 1–MEP 3 genes, which are under NCR control. Although ammonium is already a good nitrogen source, the presence of "better" (preferred) ones such as glutamate and glutamine inhibits the expression of MEP genes (Marini et al. 1997). Very recently, this controversy has been clarified by Boeckstaens et al. (2014), who demonstrated that unlike other permeases that are targets for destruction by ubiquitylation, ammonium transporters would be rather "deactivated" by phosphorylation (Fig. 2.4b). The authors reported that active Npr1 kinase modulates Mep2's

Table 2.1 Classification of amino acids by speed of absorption during beer fermentation according to Jones and Pierce (1964)

Fast absorption (A)	Gradual absorption (B)	Slow absorption (C)	Poor absorption (D)
Glutamate	Valine	Glycine	Proline
Aspartate	Methionine	Phenylalanine	
Asparagine	Leucine	Tyrosine	
Glutamine	Isoleucine	Tryptophan	
Serine	Histidine	Alanine	
Threonine		Ammonia	
Lysine			
Arginine			

activity by phospho-silencing the carboxy-terminal autoinhibitory domain S457. Supplementation of glutamine stimulates the activity of the plasma membrane-redundant phosphatases Psr1 and Psr2 (Fig. 2.4a) immediately dephosphorylating the carboxy-terminal S457 and inactivating Mep2 (Boeckstaens et al. 2014).

Eukaryotic cells such as the brewing yeast have a complex intracellular system of membranes (forming organelles and other cellular structures), which makes the discussion about nitrogen transport even more complex. Besides the transporters mentioned so far (that mediate the intake of nitrogen compounds through the plasma membrane), there are also specific permeases in the membranes of organelles such as in the vacuole and mitochondria managing with the cytoplasmic availability of nitrogen compounds. In the end, all these transporters will work together to maintain the cytoplasmic environment rich in the necessary amino acids for essential proteosynthesis and cellular homeostasis. This complex array of transporters can be better understood if demonstrated graphically (Fig. 2.5).

Alcoholic Fermentation

At first sight, it seems unwise from the brewing yeast to opt for fermentation in the presence of glucose and oxygen. However, as mentioned earlier, the main glucose repression pathway will divert yeast into fermentative state. Thus, despite the brewing yeasts have the means to carry out aerobic respiration, they will choose to produce ethanol and this event is known as “Crabtree effect.” The great advantage of fermentation is the suppression of microorganisms competing for the food source by producing ethanol. It is good to remember that not all microorganisms feel as comfortable as *Saccharomyces* spp. in an alcoholic environment. Moreover, while other microorganisms spend energy producing antimicrobial molecules, ethanol after providing the competitive advantage can be used by yeast as a source of energy and carbon (diauxic shift). The reason why yeast has evolved aerobic fermentation has been recently reviewed by Dashko et al. (2014).

The alcoholic fermentation starts with the breakdown of glucose in the cytoplasm in a series of reactions that ultimately results in two molecules of a core metabolite—pyruvate. This metabolic pathway is known as glycolysis. The next step toward ethanol formation is the decarboxylation of pyruvate to form acetaldehyde and CO_2 catalyzed by pyruvate decarboxylase (Pdc). The activity of Pdc depends on the help of the coenzymes thiamine pyrophosphate (TPP) and magnesium (Kutter et al. 2009). The ethanol is further formed through the reduction of acetaldehyde performed by alcohol dehydrogenases (Fig. 2.6).

The predominant isoform of Pdc is encoded by PDC1 gene, and it is strongly expressed in the brewing yeast during fermentation (Seeboth et al. 1990). Besides Pdc1, *Saccharomyces* spp. also encodes two other Pdcs (Pdc5 and Pdc6). From these two, only Pdc5p is involved in glucose fermentation. However, Pdc5 seems to be rather a backup isoenzyme because it is hardly detectable under normal fermentation conditions. Moreover, the expression of Pdc5 is greatly enhanced by

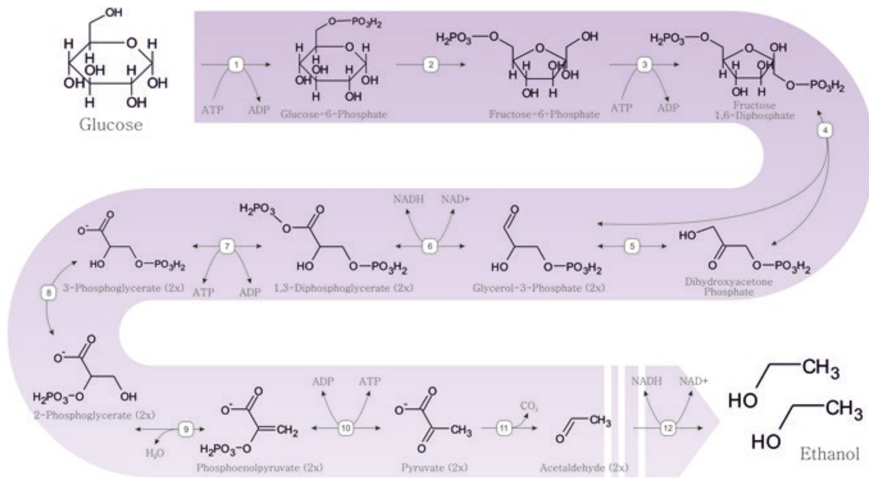

Fig. 2.6 Diagram of alcoholic fermentation performed by yeast through the Embden–Meyerhof–Parnas pathway (most common type of glycolysis). Within the yeast cell, glucose is phosphorylated by (*1*) hexokinase, which uses the phosphate from ATP. Glucose-6-phosphate enters the glycolytic chain that will ultimately convert it into two molecules of pyruvate, through the action of (*2*) glucose-6-phosphate isomerase; (*3*) 6-phosphofructokinase; (*4*) fructose diphosphate aldolase; (*5*) triose-phosphate isomerase (converts the intermediate dihydroxyacetone phosphate into glycerol-3-phosphate); (*6*) glyceraldehyde-3-phosphate dehydrogenase; (*7*) phosphoglycerate kinase; (*8*) phosphoglycerate mutase; (*9*) phosphopyruvate hydratase; (*10*) pyruvate kinase. Pyruvate is further decarboxylated by (*11*) pyruvate decarboxylase, releasing CO_2 and forming acetaldehyde, which is then reduced by (*12*) alcohol dehydrogenase to ethanol. The net product of the alcoholic fermentation from 1 mol of glucose is then 2 mol of CO_2; 2 mol of ATP; and 2 mol of ethanol

PDC1 deletion (Schaaff et al. 1989). The expression of both PDC1 and PDC5 genes is subject to autoregulation, and therefore, their promoters are activated in the absence of Pdc1 (Eberhardt et al. 1999). Moreover, the transcription of PDC1 requires the transcription factor Pdc2, which is broadly available intracellularly during fermentation (Velmurugan et al. 1997). A *pdc2Δ* strain is unable to grow in glucose because it fails to express both PDC1 and PDC2 (Velmurugan et al. 1997).

As the glucose induces a fermentative state in the brewing yeast, it was first thought that this hexose would trigger the expression of PDC1 (Boles and Zimmermann 1993). However, few years later, Liesen et al. (1996) have shown that the transcription of PDC1 would be controlled by ethanol repression rather than by glucose induction. This feedback inhibition would be mediated by a cis-acting element (named as “ERA”), which has also been suggested by the authors to be involved in the autoregulatory process, mediating the increase in the transcription of PDC gene promoters when PDC1 is deleted.

Until recently, much attention had been given to the regulation in the expression of PDC genes, and little was known about the direct regulation of enzymatic activity. Long ago, Eberhardt et al. (1999) have demonstrated the crucial role of an intact conformation in the binding site for the coenzyme TPP to Pdc’s activity.

Throughout fermentation, the peak activity of Pdc in yeast is reached in the exponential growth phase and decreases when glucose is exhausted (Weusthuis et al. 1994; Assis et al. 2013). Recently, Assis et al. (2013) have shown that Pdc1 is activated by phosphorylation when yeast is exposed to high levels of glucose. As discussed above, glucose concentration does not interfere with the genetic expression of PDC1, which has been also observed by the authors. Therefore, Assis et al. (2013) focused on the post-translational activation of Pdc. The authors have shown that Sit4 is required for a proper Pdc1 phosphorylation during exponential growth. However, as Sit4 has phosphatase activity, the authors concluded that Pdc1 would clearly not be a direct target. In addition, the authors have also shown that knockout of the SIT4 gene decreases the affinity of Pdc1 for TPP, thus reducing activity of the Pdc1. Pdcs are also involved in the catabolism of amino acids as discussed in the next chapter of this book.

The brewing yeast is capable of both producing and degrading ethanol through the action of alcohol dehydrogenases Adh1 and Adh2, respectively. The former is constitutively encoded by ADH1 gene, whereas ADH2 is only derepressed when the sugar levels drops, e.g., at the end of the primary beer fermentation (Wills 1976). Both these enzymes have a common ancestor called Adh_A that has been cloned by Thomson et al. (2005). The authors proved that the ancestral counterpart was optimized only to produce, never to consume ethanol. This is consistent with the hypothesis that Adh_A was originally prepared to recycle NADH generated in the glycolytic pathway. The need to evolve two homologues with diverging functions is believed to coincide with the appearance of juicy fruits in the Cretaceous age (Thomson et al. 2005). These observations only strengthen the early evolutionary discussion of producing ethanol to get rid of competing microorganisms.

For industrial purposes, the Adh1 is clearly the enzyme of interest, and therefore, it is the most studied one. The yeast Adh1 is a tetrameric protein containing four identical subunits with 347 amino acids (Bennetzen and Hall 1982). Each of these subunits has been shown by Raj et al. (2014) to possess two zinc entities: one is catalytic, and the other is structural. The authors concluded that the coordination between catalytic zincs may be essential to displace the zinc-bound water to give place to alcohol or aldehyde substrates.

In beer fermentation, the expression level of ADH1 has been directly correlated with the initial sugar concentration and fermentation temperature. Among several genes tested, ADH1 had the highest gene expression under fermentation conditions tested (Saerens et al. 2008). Recently, Wang et al. (2013) induced mutation in strains of industrial brewing yeast and isolated mutants with defective ADH2 expression. The beer produced by the selected mutants had nearly 82 % less acetaldehyde and 1 % more ethanol when compared to fermentations performed by parental strains. These mutants could have real practical use because the reduced acetaldehyde would reflect in better flavor, whereas avoiding ethanol oxidation by Adh2 results in better ethanol yield hence improved fermentation performance. Different mutants were selected in the work of Yu et al. (2012) who isolated *S. pastorianus* strains with improved sugar transport performance and enhanced ADH activity. These mutants were successfully used in the production of flavor-balanced beer fermented under

very high gravity conditions. A genetically modified strain of *S. cerevisiae* was patented long ago for the production of alcohol-free beers (Dziondziak 1989). This strain had the advantage of improving beer's body due to increased glycerol content. However, the excessive acetaldehyde produced (owing to the lack of ADH) had to be washed out from the beer by CO_2 injection, which could lead to detrimental cowashing of positive aroma constituents (Brányik et al. 2012).

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Chapter 3
By-products of Beer Fermentation

Abstract Among the most important factors influencing beer quality is the presence of well-adjusted amounts of higher alcohols and esters; as well as the successful reduction of undesirable by-products such as diacetyl. While higher alcohols and esters contribute rather positively to the beer aroma, diacetyl is mostly unwelcome for beer types with lighter taste. Thus, the complex metabolic pathways in yeast responsible for the synthesis of both pleasant and unpleasant by-products of fermentation were given special attention in this last chapter.

Introduction

Beer is one of the most pleasant beverages in the world, the taste/aroma of which is formed by several hundreds of compounds, with a different flavor activity, produced in the course of every step of brewing. A significant part of these substances are produced during the fermentation phase and consist of metabolic intermediates or by-products of yeast. Higher alcohols, esters, and vicinal diketones (VDKs) are compounds produced by yeast, which cocreate the final quality of the beer. While higher alcohols and esters are to a certain extent desirable volatile constituents, VDKs are often considered as off-flavors. In addition, yeast metabolism contributes to formation and conversion of another three groups of chemical compounds: organic acids, sulfur compounds, and aldehydes.

All flavor-active components in beer must be kept within certain limits. Otherwise, a single compound or group of compounds may predominate and impair the flavor balance. Furthermore, aroma compounds such as esters may act in synergy with other components affecting beer flavor/aroma in concentrations well below their threshold values (Meilgaard 1975a). However, each type of beer has its own aromatic character codetermined by the yeast strain chosen (Ramos-Jeunehomme et al. 1991; Peddie 1990; Nykanen and Nykanen 1977; Rossouw et al. 2008) and parameters used during fermentation (Berner and Arneborg 2012; Blasco et al. 2011; Bravi et al. 2009; Hiralal et al. 2013; Lodolo et al. 2008; Verbelen et al. 2009a; Saerens et al. 2008a; Dekoninck et al. 2012). For example, while there are only the isoamyl acetate concentrations above the

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Table 3.1 Threshold values of most important esters and higher alcohols present in lager beer (Meilgaard 1975b; Engan 1974, 1981)

Compound	Threshold (mg L^{-1})	Concentration range (mg L^{-1})	Aroma impression
Acetate esters			
Ethyl acetate	25–30	8–32	Fruity, solvent
Isoamyl acetate	1.2–2	0.3–3.8	Banana
Phenylethyl acetate	0.2–3.8	0.1–0.73	Roses, honey
MCFA ethyl esters			
Ethyl hexanoate	0.2–0.23	0.05–0.21	Apple, fruity
Ethyl octanoate	0.9–1.0	0.04–0.53	Apple, aniseed
Higher alcohols			
n-Propanol	600	4–17	Alcohol, sweet
Isobutanol	100	4–57	Solvent
Isoamyl alcohol	50–65	25–123	Alcoholic, banana
Amyl alcohol	50–70	7–34	Alcoholic, solvent
2-phenylethanol	40	5–102	Roses
VDKs			
2,3-Butanedione (diacetyl)	0.1–0.15	0.02–0.07	Sweet, buttery
2,3-Pentanedione	0.9–1.0	0.01–0.02	Buttery, toffee-like

threshold levels in lager beers, ales typically contain also ethyl acetate and ethyl hexanoate in significant amounts (Meilgaard 1975b; Alvarez et al. 1994). Similarly, other flavor-active compounds such as diacetyl (VDK) are kept below the threshold values in lager beers (buttery off-flavor), but their presence in ales or specialty beers is less detrimental or it can be even desirable. Table 3.1 shows the threshold values of the principal esters, higher alcohols, and VDKs and typical concentrations in lager beers.

Pleasant By-products

Higher Alcohols

Also known as fusel alcohols, higher alcohols are the most abundant organoleptic compounds present in beer. The brewing yeast absorbs amino acids present in wort, from which they remove the amino group, so it can be incorporated into newly synthesized structures. What is left from the amino acid (α-keto acid) enters in an irreversible chain reaction that will ultimately create a by-product—higher alcohols. This pathway was suggested long ago by Ehrlich (1907), who was intrigued with the structural molecular similarities between the active amyl alcohol with isoleucine and isoamyl alcohol with leucine. This observation has led Ehrlich to investigate whether these amino acids were involved in higher alcohol synthesis or not. When

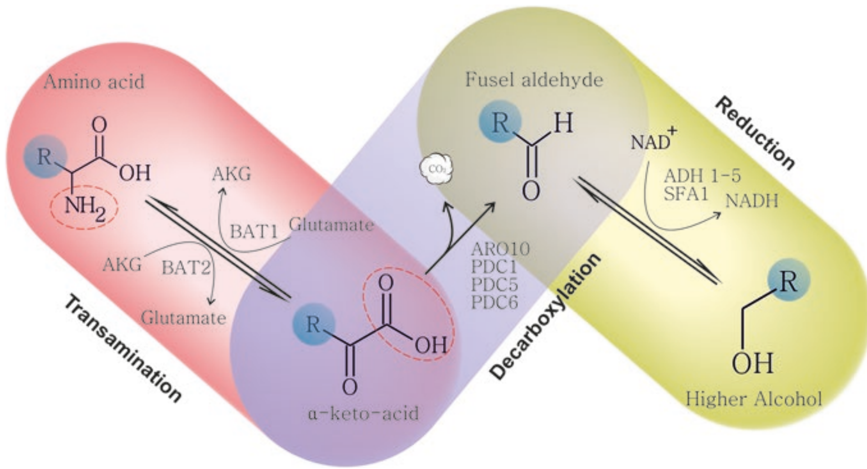

Fig. 3.1 The Ehrlich pathway and the main genes involved in the synthesis of enzymes catalyzing each reaction. The reversible transamination reaction uses different BAT-encrypted enzymes—while Bat2 catalyzes the transfer of the amino group from the amino acid to α-ketoglutarate (AKG), Bat1 is usually required on the reverse transamination for amino acid biosynthesis

supplementing the fermenting medium with those amino acids, Ehrlich evidenced an increased production of fusel alcohols. This observation led Ehrlich to state that amino acids were enzymatically hydrolyzed to form the corresponding fusel alcohols, along with ammonia and carbon dioxide. As the ammonia was not detected in the medium, it was assumed to be incorporated into yeast proteins. Few years later, Neubauer and Fromherz (1911) proposed a few intermediate steps to the Ehrlich pathway, completing the metabolic scheme as it is known until today. However, a detailed enzymatic chain reaction was only demonstrated several decades later (Sentheshanuganathan 1960; Sentheshanmuganathan and Elsden 1958). The currently accepted elementary enzymatic sequence for the Ehrlich pathway involves transaminase, decarboxylase, and alcohol dehydrogenase (Fig. 3.1). Although this pathway is the most studied and discussed, higher alcohols are also formed during upstream (anabolic pathway) biosynthesis of amino acids (Chen 1978; Oshita et al. 1995; Dickinson and Norte 1993). The most important is the de novo synthesis of branched-chain amino acids (BCAA) through the isoleucine–leucine–valine (ILV) pathway (Dickinson and Norte 1993).

Transamination

The first step in Ehrlich pathway involves four enzymes encoded by the genes BAT1 (TWT1 or ECA39), BAT2 (TWT2 or ECA40), ARO8, and ARO9. These enzymes are transaminases that catalyze the transfer of amines between amino acids and respective α-keto acid, using glutamate/α-ketoglutarate as a donor/acceptor.

While Bat1- and Bat2-encrypted enzymes are involved in the BCAA transamination (Kispal et al. 1996; Eden et al. 1996), Aro8 and Aro9 were first described as being aromatic amino acid aminotransferases I and II, respectively (Iraqui et al. 1999). Further studies carried out by Urrestarazu et al. (1998) demonstrated that Aro8- and Aro9-encoded enzymes had broad-substrate specificity than just for aromatic amino acids. This was confirmed in the work performed by Boer et al. (2007), who cultivated *Saccharomyces cerevisiae* using six independent nitrogen sources followed by transcriptome analysis. All phenylalanine, methionine, or leucine activated the transcription of ARO9 and BAT2 genes.

A recent study mapped almost entirely (97 %) the proteome of *S. cerevisiae* (Picotti et al. 2013). The authors organized the proteome into a network of functionally related proteins, which they called as "modules." Within these modules, they highlighted the one comprising of Bat1p, Bat2p, Rpn11p, Hsp60p, and Ilv2p, which they termed B1B2 module. The core of this module is composed by Bat1p and Bat2p—two paralogous enzymes involved in the metabolism of the BCAA. While Bat1p is mainly involved in the anabolism of BCAA (amination of α-keto acids), Bat2p is almost exclusively involved in the catabolism of BCAA (deamination of BCAA). Thus, BAT1- and BAT2-encoded proteins catalyze the same metabolic reaction in opposite directions. Strictly related to these two proteins is the Ilv2-encrypted enzyme, which catalyzes an early step in the synthesis of BCAA from pyruvate (Picotti et al. 2013).

The subcellular location of enzymes catalyzing the synthesis of fusel alcohols has been studied in the past (Schoondermark-Stolk et al. 2005; Kispal et al. 1996) and recently reaffirmed (Avalos et al. 2013). Isobutanol is produced by yeast originally in the cytoplasm via Ehrlich pathway or by anabolic synthesis inside the mitochondria (Kohlhaw 2003). Avalos et al. (2013) redirected the entire enzymatic biosynthetic pathway of that fusel alcohol to the mitochondrial matrix. Compartmentalization of the Ehrlich pathway within the mitochondria increased isobutanol production by 260 %, whereas overexpression of the same pathway in the cytoplasm only improved yields by 10 %. These results are justified by the most favorable environmental conditions found in the mitochondria matrix, which enhanced enzymatic activity.

Decarboxylation

After transamination, the remaining α-keto acids can be decarboxylated to form the respective aldehyde, and this is the point of no return in the Ehrlich pathway (Dickinson et al. 1997). There are five genes encoding decarboxylases in *S. cerevisiae*: three encoding pyruvate decarboxylases (PDC1, PDC5, and PDC6), ARO10, and THI3 (Romagnoli et al. 2012; Dickinson et al. 1997; Bolat et al. 2013). All PDCs depend on the cofactor thiamine diphosphate (TPP) to work properly. Among those genes, only PDC5 and ARO10 were described to encode decarboxylases with a broad-substrate specificity (Vuralhan et al. 2003, 2005; Romagnoli et al. 2012). Dickinson et al. (1998)

have shown that the valine is decarboxylated by any of the enzymes encrypted by PDC1, PDC5, or PDC6. In the case of isoleucine, all five decarboxylases of the family can produce active amyl alcohol (Dickinson et al. 2000). THI3-encoded enzyme cannot catalyze the decarboxylation of the aromatic amino acids phenylalanine and tyrosine, while all other four can (Dickinson et al. 2003). The single expression of THI3 in a quadruple gene-deleted (*pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ) *S. cerevisiae* strain had no α-keto acid decarboxylase activity (Vuralhan et al. 2003, 2005). Further studies involving Thi3 suggest that the role of this enzyme in the Ehrlich pathway is rather regulatory than catalytic (Mojzita and Hohmann 2006).

Although the lager brewing yeast *S. pastorianus* is long known to be a natural aneuploid hybrid of *S. cerevisiae* with another *Saccharomyces* spp. (Vaughan and Kurtzman 1985), only recently the missing link was proven to be *S. eubayanus* (Libkind et al. 2011). This fact has called the attention of Bolat et al. (2013) upon the contribution of ARO10 gene expression from each of the subgenomes on the production of higher alcohols. The authors amplified by PCR both *S. eubayanus*-like and *S. cerevisiae*-like alleles of ARO10 (*LgSeubARO10* and *LgScARO10*, respectively) from genomic DNA of *S. pastorianus*. The alleles showed a sequence identity of 80 % at the DNA level and 84 % at the protein level. The results have also shown that *S. cerevisiae* alleles of ARO10 are present in a ratio of 3:1 to those present in *S. eubayanus* subgenome. These authors have equally demonstrated that both *S. eubayanus*-like and *S. cerevisiae*-like ARO10-encoded isoenzymes had similar activity for most of the substrates tested with preferred decarboxylation action against phenylpyruvate. However, the activity of *LgSeubARO10*-encrypted enzyme toward ketoisovalerate (precursor of isobutanol) was twofold higher than that encoded by *LgScARO10*. Moreover, they also suggest that *S. eubayanus*-like and *S. cerevisiae*-like ARO10-derived α-oxo acid decarboxylases exert different roles during beer fermentation by *S. pastorianus*. Fusel alcohols produced by Ehrlich pathway would involve the *S. cerevisiae*-like ARO10 decarboxylase preferentially. Conversely, higher alcohols formed by de novo synthesis would rely almost exclusively on the *LgSeubARO10*-encrypted isoenzyme.

Reduction to Higher Alcohols

After decarboxylation, the fusel aldehydes enter the last step of the Ehrlich pathway, in which they are converted into their respective alcohols by action of alcohol dehydrogenases. Any one of the *S. cerevisiae* alcohol dehydrogenases or the formaldehyde dehydrogenase encrypted by SFA1 can catalyze the conversion of fusel aldehydes into higher alcohols (Dickinson et al. 2003). Thus, studies related to these genes often discuss ethanol production rather than fusel alcohols. A detailed discussion about alcohol dehydrogenases is presented in the last chapter of this book.

Regulation of Higher Alcohols

Iraqui et al. (1999) were the first to identify the ARO80 gene as a pathway-specific regulator of the Aro9 transaminase and Aro10 decarboxylase in the presence of the aromatic amino acids tryptophan, phenylalanine, and tyrosine. Recent findings have shown that ARO9 and ARO10 transcription also requires the NCR-related GATA activators Gln3 and Gat1 (Lee and Hahn 2013). Therefore, not only ARO80 induces the transcription of ARO9 and ARO10 by directly binding to their promoter in the presence of aromatic amino acids, but it is also required for the recruitment of Gat1 and Gln3 activators. Lee et al. (2013) assessed whether environmental conditions would also affect ARO9 and ARO10 expression. Among the environments tested, only heat shock could activate ARO9 and ARO10 transcription. Thereafter, the authors examined a knocked-down *aro80Δ* strain upon the same stress conditions, and no ARO9 or ARO10 expression was observed during the heat-shock growth. These data strongly suggest that the transcription of ARO9 and ARO10 is activated by ARO80 under heat-shock stress in *S. cerevisiae*. Back in the studies of Bolat et al. (2013) with *S. pastorianus*, a deletion of ARO80 from *S. eubayanus*-like allele did not eliminate phenylalanine induction of LgSeubARO10. This finding suggests that LgScARO80 can also cross-activate LgSeubARO10 compensating the loss of *S. cerevisiae*-type activator.

The Anabolic Pathway

The brewing wort normally has all proteinogenic amino acids required by the fermenting yeast for growth. However, α-keto acids (intermediates in the Ehrlich pathway) are also formed via de novo biosynthesis of amino acids through carbohydrate metabolism (Fig. 3.2) (Chen 1978). Thus, in order to evaluate the contribution of anabolic pathway in the synthesis of higher alcohols, Eden et al. (2001) have blocked the transamination of amino acids from the growth medium by using a knockout strain (*eca39Δ and eca40Δ*). In addition to these deletions, *ilv2Δ* was also investigated, and thus, the activity of acetolactate synthase encoded by ILV2 could be assessed. Without ILV2, the synthesis of isoleucine is hindered, causing an increase of the primary precursor (after pyruvate)—α-ketobutyrate. As this α-keto acid is a precursor of propanol, the authors evidenced a significant increase in this fusel alcohol produced by *eca39Δ eca40Δ ilv2Δ* strain (Eden et al. 2001). This strain was also unable to produce isobutanol as α-acetolactate could not be synthesized from pyruvate due to lack of ILV2. Thus, as no external amino acid could be used in the Ehrlich pathway due to *eca39Δ eca40Δ,* the role of ILV2 gene was confirmed in the anabolic pathway of isobutanol. On the other hand, active amyl alcohol and isoamyl alcohol synthesis was reduced, but still unexpectedly present (Eden et al. 2001). ILV2 was recently addressed to be integrated to a protein network module of functional similar proteins involved in BCAA and

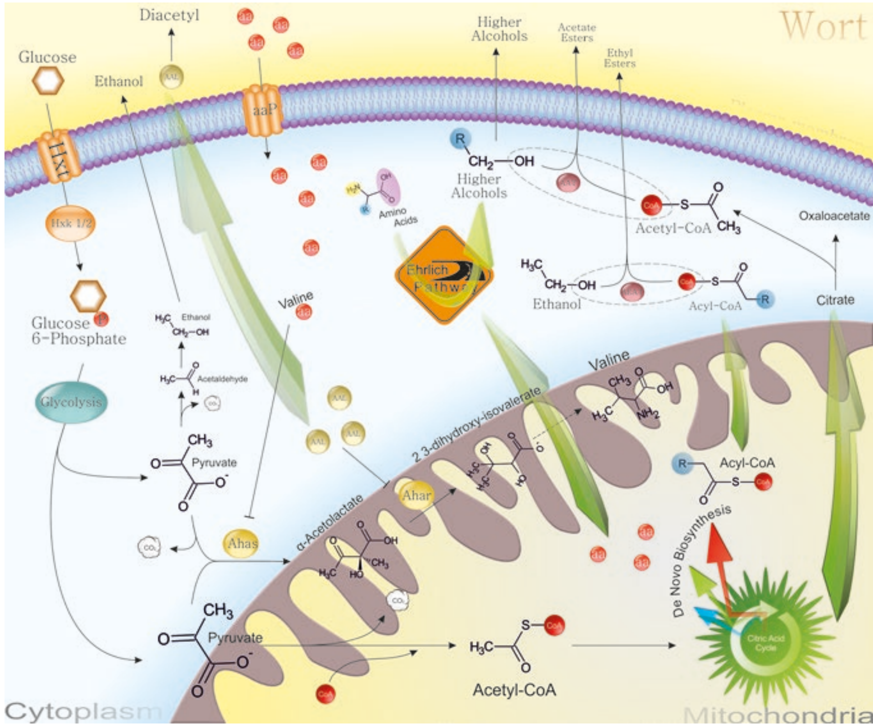

Fig. 3.2 A schematic overview of the central metabolic routes to the formation of higher alcohols, esters, and diacetyl when yeast is inserted in the fermenting wort. When glucose enters the yeast cell, it is phosphorylated by hexokinases (Hxk 1/2). Glucose-6-phosphate then enters the glycolytic pathway that breaks it into two molecules of pyruvate. Thereafter, pyruvate enters the mitochondria where it is oxidized in the pyruvate dehydrogenase complex to form acetyl coenzyme A (Acetyl-CoA). Still in the mitochondria, acetyl-CoA either directly or indirectly through intermediates of the citric acid cycle will originate the majority of amino acids via synthesis de novo. Another amino acid biosynthetic pathway (isoleucine, leucine, valine—ILV pathway) occurs in parallel through the condensation of two molecules of pyruvate to form α-acetolactate. This first reaction is catalyzed by ILV2-encoded enzyme—acetohydroxyacid synthase (Ahas). The second reaction in the ILV pathway is catalyzed by the acetohydroxyacid reductoisomerase (Ahar) encoded by ILV5. The accumulation of α-acetolactate (AAL) within the mitochondria hampers the activity of the ILV5-encoded enzyme, and therefore, the yeast excretes it. Outside the cell, AAL is spontaneously decarboxylated to form diacetyl—a potent buttery odorant in beer. Higher alcohols are formed through the Ehrlich pathway either from absorbed amino acids (through specific amino acid permeases—aaPs) or from those arising from de novo biosynthesis. Cytosolic acetyl-CoA is originated from the excessive citrate formed within the mitochondria. Therefore, outside the organelle, citrate is converted into acetyl-CoA and oxaloacetate. Then, in the cytosol, acetyl-CoA can be enzymatically (by alcohol acetyltransferases—AAT) condensed with a higher alcohol to form acetate esters. Ethyl esters are formed through a condensation reaction between an acyl-CoA unity and ethanol, catalyzed by two acyl-CoA:ethanol O-acyltransferases (AEAT)

physically connected to the mitochondria (Picotti et al. 2013). The activity of acetolactate synthase is also crucial in the formation of the VDKs as further discussed in this chapter.

Esters

Compared to other yeast metabolites, esters are only trace elements. Nevertheless, despite being "a drop in the ocean" of beer's constituents, esters are the most important aroma components produced by yeast. That is because esters have a very low odor threshold in beer (Meilgaard 1975b; Saison et al. 2009) and yet to a large extent may define its final aroma (Engan 1974; Hiralal et al. 2013; Meilgaard 1991; Nykanen and Suomalainen 1983; Saerens et al. 2008a; Saison et al. 2009; Verbelen et al. 2009a; Peddie 1990; Suomalainen 1981). However, if overproduced, they can negatively affect the beer with fruity taste. Thus, it is crucial for the brewer to keep the optimum conditions to obtain a balanced beer in terms of ester profile.

Esters are mainly formed during the vigorous phase of primary fermentation by enzymatic condensation of organic acids and alcohols. Volatile esters in beer can be divided into two major groups: the acetate esters and the medium-chain fatty acid (MCFA) ethyl esters. The former group comprises esters synthesized from acetic acid (acetate) with ethanol or higher alcohol. In ethyl esters' family, ethanol will form the alcohol radical and the acid side is an MCFA. Although dozens of different esters can be found in any beer (Meilgaard 1975b; Engan 1974), six of them are of major importance as aromatic constituents: ethyl acetate (solvent-like aroma); isoamyl acetate (banana aroma); isobutyl acetate (fruity aroma); phenyl ethyl acetate (roses and honey aroma); ethyl hexanoate (sweet apple aroma); and ethyl octanoate (sour apple aroma).

Esters are synthesized in the cytoplasm of the brewing yeast, but readily leave the cell as they are lipophilic. However, while small-chain acetate esters quickly diffuse through the plasmatic membrane, the passage of MCFA is hindered (Nykanen and Nykanen 1977; Dufour 1994; Nykiinen et al. 1977).

To be synthesized into esters, organic acids must be linked to a coenzyme A to form an acyl-CoA molecule. Acyl-CoAs are highly energetic entities, which in the presence of oxygen can be β-oxidized ("cut") into smaller units (acetyl-CoA) in the mitochondria. This will happen unless the organic acid involved is already the acetic acid, which in this case will be turned into acetyl-CoA. However, the vast majority of acetyl-CoA produced by the yeast cells comes from the oxidative decarboxylation of pyruvate. During respiration, acetyl-CoA migrates to the mitochondria to enter in the Krebs cycle and produce high levels of ATP. Throughout fermentation, acetyl-CoA is enzymatically esterified with an alcohol to form the acetate esters. Additionally, longer chains of acyl-CoA are enzymatically condensed with ethanol to form MCFA ethyl esters. Figure 3.2 drafts the main metabolic routes in the formation of flavoring compounds during beer fermentation.

Biosynthesis of Acetate Esters

Acetate esters are the primary flavoring components, in the ester family, because they are present in much higher concentrations in beer if compared to the MCFA ethyl ester counterparts. The involvement of enzymes in the production of esters dates from the 1960s (Nordström 1962). However, the enzyme in charge was only purified and named as alcohol acetyltransferase (AAT) back in 1981 by Yoshioka and Hashimoto (1981). The most studied and best characterized enzymes responsible for ester synthesis are the AATases I and II, encoded by the genes ATF1 and ATF2 (Yoshioka and Hashimoto 1981; Verstrepen et al. 2003b; Malcorps and Dufour 1992; Fujii et al. 1994; Zhang et al. 2013; Nagasawa et al. 1998; Yoshimoto et al. 1998; Dekoninck et al. 2012; Molina et al. 2007). It was also found that bottom-fermenting lager yeasts have an extra ATF1 homologous gene (Lg-ATF1) (Fujii et al. 1994) that encodes an AAT very similar to that encoded from the original ATF1 gene (Fujii et al. 1996). This additional gene expression in lager yeast enhances acetate ester production and ultimately the beer's aroma profile. Figure 3.3a schematizes the chemical reaction for the production of the chief acetate esters and genes involved in these reactions.

The best way to understand the role of a gene's expression is by either overexpressing or deleting it. A substantial body of literature focuses on these genetic modifications to better understand the role of ATF1, ATF2, and Lg-ATF1 gene

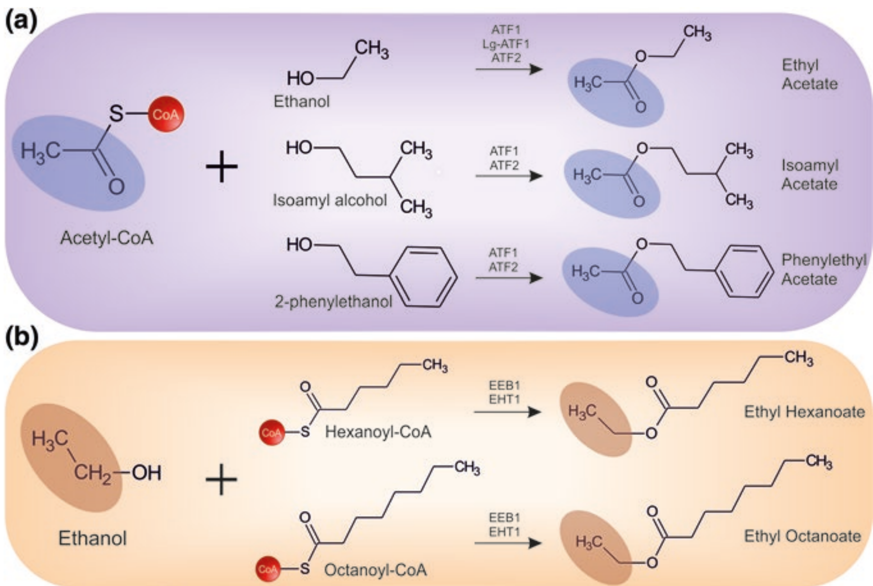

Fig. 3.3 A scheme of the chemical reactions involving acetate esters (**a**) and medium-chain fatty acid (MCFA) ethyl ester (**b**) biosynthesis. The genes encoding the primary enzymes involved in each reaction are indicated

expression on the total acetate ester production (Yoshimoto et al. 1998; Verstrepen et al. 2003b; Nagasawa et al. 1998; Fujii et al. 1994, 1996; Zhang et al. 2013). Very recently, a brewing yeast strain was designed to increase the ester/higher alcohol ratio by overexpressing ATF1 and knocking down a gene related to higher alcohol synthesis (Zhang et al. 2013). Ester production by the genetically modified strains was considerably higher than that of parental cells. Verstrepen et al. (2003b) have earlier carried out a more detailed work concerning deletion and overexpression of not only the AFT1 and ATF2, but also its homologous Lg-ATF1. As others in the past (Nagasawa et al. 1998; Fujii et al. 1994, 1996), those authors clearly demonstrated the substantial impact exerted by the expression levels of ATF genes on acetate ester production. For example, they have shown that overexpressing ATF1 strains may have up to 180-fold increased the isoamyl acetate production and 30-fold increased the ethyl acetate production, when compared to wild-type cells. In fact, their analysis also revealed that ATF1-encrypted ATTases seem to be responsible for the vast majority of acetate ester production. Through specific deletion of ATF1 and ATF2, no acetate esters originated from alcohols with more than five carbon atoms (such as isoamyl acetate and phenyl ethyl acetate) were formed. This means that the banana aroma (isoamyl acetate) in beer depends exclusively on ATF1- and ATF2-encoded enzymes. Later in 2008, Saerens et al. (2008b) confirmed that the maximum expression levels of ATF1 and ATF2 are directly correlated with the final concentration of acetate esters. However, the knockdown (*atf1*Δ*atf*2Δ) executed by Verstrepen et al. (2003b) could only reduce the production of smaller esters such as ethyl acetate by 50 %. Together with other pieces of evidence (Malcorps and Dufour 1992; Malcorps et al. 1991), this result makes clear that there might be more ATTases involved in acetate ester production, but this goes beyond the knowledge in currently published data. Given the importance of acetate esters to Chinese rice wine, Zhang et al. (2014) cloned the Lg-ATF1 from a lager brewing strain and inserted it into a Chinese rice wine yeast (which does not have such homologue). The genetically modified variant, expressing Lg-ATF1, greatly enhanced the production of both ethyl acetate and isoamyl acetate with values reaching 70.91 and 8.66 mg L^{-1}, respectively.

The presence of acetate esters in alcohol-free beers (AFBs) is imperative. AFBs can be produced either from physical removal of ethanol from the finished beer or by controlling the biological process involved in beer fermentation (Branyik et al. 2012). AFBs produced by membrane processes have usually less body and a low aromatic profile, thermally dealcoholized AFBs may suffer heat damages, while beers obtained by biological methods have often a sweet and worty off-flavor (Montanari et al. 2009). The lack of ethanol itself significantly affects the retention of volatile flavor-/aroma-active compounds (Perpete and Collin 2000). Very recently, Strejc et al. (2013) isolated a brewing yeast mutant capable of overproducing isoamyl acetate and isoamyl alcohol. The sweet banana odor from isoamyl acetate could then be a solution to overcome the undesirable worty off-flavor of AFB. Sensory analyses showed that the increased level of isoamyl acetate ester had a positive effect on the fruity (banana) palate fullness and aroma intensity of the AFB produced.

Biosynthesis of Ethyl Esters

From a historical perspective, it is clear that MCFA ethyl esters were devoted less research attention. The reason for this is their lower concentration in beer, when compared to their acetate counterparts. Nonetheless, works focused on ethyl esters in brewing fermentations have become much more common in the past decade, most of them carried out by Saerens et al. (2006, 2008a). Based on evidences published long ago (Malcorps and Dufour 1992), Mason and Dufour (2000) suggested that apart from ATF1- and ATF2-encoded enzymes, there should be a different enzyme involved in ethyl ester synthesis. The authors called it ethanol hexanoyl transferase, responsible for mediating the esterification between ethanol and hexanoyl-CoA to form ethyl hexanoate (Mason and Dufour 2000). Saerens et al. (2006) further proved that MCFA ethyl esters are formed through a condensation reaction between an acyl-CoA and ethanol (Fig. 3.3b), catalyzed by two acyl-CoA:ethanol O-acyltransferases (AEATases) encoded by EeB1 and EHT1 genes. Moreover, these authors further attested the role of each of these genes on the final MCFA ethyl ester content. A single deletion on EeB1 reduced the formation of ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate by 36, 88, 45, and 40 %, respectively. EHT1 knocked out strain and, on the other hand, only had ethyl hexanoate and ethyl octanoate productions affected. Additionally, a double deletion (*eeb1Δ and eht1Δ*) strain produced an ethyl ester profile similar to the *eeb1Δ* single deletion strain. This means that EeB1 is the most relevant gene in MCFA ethyl ester synthesis (Saerens et al. 2006). However, even though double deletion caused a pronounced drop in detected ethyl esters, only ethyl hexanoate production was virtually extinguished. Thus, there must be another, yet unknown, AEATases involved in the MCFA ethyl ester synthesis. Also, overexpression of those genes did not increase MCFA ethyl ester production even when more precursors of these esters were added to the fermenting medium. This fact was explained as a consequence of extra esterase (breakdown) activity exerted by EeB1- and EHT1-encoded proteins, which was also demonstrated in vitro by the same authors (Saerens et al. 2006).

Ester Regulation

The net rate of ester production depends not only on the availability of the substrates (Saerens et al. 2006; Hiralal et al. 2013), but to a significant extent on the enzymatic balance of synthesis (Saerens et al. 2006; Verstrepen et al. 2003b; Zhang et al. 2013; Mason and Dufour 2000; Yoshimoto et al. 1998) and breakdown (by esterases) of esters (Fukuda et al. 1998a, 1996; Lilly et al. 2006). Esterases are a group of hydrolyzing enzymes that catalyze the cleavage and/or prevent the formation of ester bonds.

Fukuda et al. (1998b) have chosen another strategy to raise the final net production of isoamyl acetate by a sake strain of *S. cerevisiae*. Instead of enhancing the activity of AATases, they avoided isoamyl acetate cleavage by deleting the acetate-hydrolyzing esterase gene (IAH1, previous known as EST2) encoding a carboxylesterase (Fukuda et al. 1996). The IAH1-deficient strain produced approximately 19 times higher amounts of isoamyl acetate when compared with the parental strain. Fukuda et al. (1998a) have further proven the essential activity balance between AATases and esterases for the net rate of ester formation by *S. cerevisiae*. More evidence of the IAH1-encoded esterase influence on the breakdown of esters was presented by Lilly et al. (2006). In addition to isoamyl acetate, the authors also reported a decreased production of ethyl acetate, phenyl ethyl acetate, and hexyl acetate by the overexpressing IAH1 mutant strain. These findings are in agreement with recently published data by Ma et al. (2011) whose work determined the crystalline structure of the enzyme encrypted by IAH1 gene. They have shown that an additional *C*-terminus was involved in the substrate-binding region. Furthermore, it was also demonstrated that this *C*-terminus restricts the access to the active site of the enzyme, playing a vital role in determining substrate specificity. Non-modified IAH1-encoded esterase had the highest hydrolytic activity against shorter acetate esters. Moreover, this activity was significantly reduced against ethyl hexanoate and almost null for ethyl decanoate, which suggests that IAH1-encrypted enzyme preferentially breaks shorter-chain esters. This was confirmed by truncating the other *C*-terminus present in the enzyme. The modified variant with a truncated *C*-terminus was now able to hydrolyze longer ethyl ester chains such as decanoate. The authors concluded that the deletion of the *C*-terminus provides better access to the active site of the enzyme, which allows accommodating longer acyl chains (Ma et al. 2011).

Esters in Beer Aging

The ester profile of a given beer may change drastically during storage either by action of yeast (bottle refermentation) (Vanderhaegen et al. 2003) or by spontaneous chemical condensation of organic acids with ethanol (Saison et al. 2009; Rodrigues et al. 2011; Vanderhaegen et al. 2006). With time, hop-derived components are oxidized to form 3-methyl butyric and 2-methyl butyric acid, which are spontaneously esterified to their respective ethyl esters (3-methyl butyrate and 2-methyl butyrate) (Williams and Wagner 1979). The formation of these esters imparts the aged beer a winy aroma (Williams and Wagner 1978). In addition, some esters such as isoamyl acetate are hydrolyzed during the storage of beer (Neven et al. 1997). Chemical hydrolysis and esterification are acid-catalyzed (Vanderhaegen et al. 2006), but the esterases from yeast autolysis can also play their role in unpasteurized beers (Neven et al. 1997). Other ethyl esters such as ethyl nicotinate (medicinal, solvent, anislike aromas), ethyl pyruvate (peas, freshly cut grass), and ethyl lactate (fruity, buttery) are also formed during beer aging

(Saison et al. 2009). For all the above-mentioned reasons, beers during aging tend to lose their fresh fruity aroma, often being replaced by sweeter odors.

Unpleasant By-products

Vicinal Diketones (VDKs)

The two relevant VDKs in beer fermentation are the 2,3-butanedione (diacetyl) and the 2,3-pentanedione. They are formed as by-products of cellular biosynthesis of amino acids, i.e., valine and isoleucine, respectively. When in concentrations above the flavor threshold, these VDKs impair the beer with a sweetish buttery flavor/aroma. However, diacetyl has a ten times lower flavor threshold than 2,3-pentanedione, being therefore sensorially more important. This is why the reduction of diacetyl below the flavor threshold defines for many brewers the end of beer maturation. Nonetheless, it is important to emphasize that whereas VDKs are particularly detrimental for lager beers, they do no harm to stronger beers or they are even desired in some beer styles. Given the importance of diacetyl for the brewing process, a discussion will be henceforth focused on this by-product.

As for many other amino acids, the biosynthesis of valine takes place within the mitochondria (Ryan and Kohlhaw 1974). It is a four-step pathway that starts with the enzymatic condensation of two molecules of pyruvate to form α-acetolactate (AAL). This reaction is catalyzed by acetohydroxyacid synthase (Ahas), which is encoded by the ILV2 gene (Falco et al. 1985). This gene is under GAAC (Xiao and Rank 1988), which means that it will be upregulated if the brewing yeast starves either for valine or for any other amino acid as discussed in the previous chapter. The second step in the pathway is the conversion of α-acetolactate into 2,3-dihydroxy isovalerate, catalyzed by the ILV5-encoded acetohydroxyacid reductoisomerase (Ahar). The accumulation of α-acetolactate (AAL) within the mitochondria is rate-limiting for the action of Ahar, and therefore, it is excreted to the fermenting beer (Krogerus and Gibson 2013). Diacetyl is further formed outside yeast cells through the spontaneous (non-enzymatic) oxidative decarboxylation of α-acetolactate. The exact mechanism and why yeast excretes α-acetolactate to the beer is not known. Probably, the most acceptable hypothesis has been raised by Dasari and Kolling (2011) who attributed the excretion of α-acetolactate to its easier access to the extracellular environment when formed in the cytoplasm owing to deficient internalization of Ahas by the mitochondria. These authors demonstrated that petite yeast mutants (which lacks in capacity to generate ATP by oxidative phosphorylation) produce more diacetyl than wild strains owing to the compromised potential across the inner mitochondrial membrane that hampers the internalization of mitochondrial targeted proteins such as Ahas. If α-acetolactate is formed in the cytosol, it would only have to transpose the plasma membrane to reach the fermenting wort/beer, whereas if synthesized within the mitochondria, three membranes (besides plasma membrane, mitochondrial inner and outer

membrane) would be separating α-acetolactate from the extracellular environment. The authors also suggested that other enzymes relevant for AHAS activity such as Ilv5 and Ilv6 may not be present in the cytosol (Dasari and Kolling 2011).

Diacetyl is formed during cellular growth and division, which means that it is also a by-product of primary beer fermentation. Throughout maturation, the yeasts reabsorb diacetyl and reduce it to 2,3-butanediol by action of acetoin reductase and several other ketone reductases (Bamforth and Kanauchi 2004). Diols have much higher flavor threshold than VDKs; therefore, they do not represent any flavor risk to the finished beer. However, the reduction of VDKs to diols through maturation can take weeks, being in turn the most time-consuming step of beer fermentation. Nonetheless, it is important to emphasize that the delay in diacetyl reduction has nothing to do with the ability of yeast in assimilating and reducing this VDK. Instead, it has been shown that the real rate-limiting step in diacetyl removal is the spontaneous decarboxylation of α-acetolactate to diacetyl (Boulton and Box 2008). Therefore, most of the efforts in brewing science have been focused on avoiding diacetyl formation (i.e., reducing valine biosynthesis) and/or enhancing the decarboxylation step, rather than favoring its reduction.

An important clue in reducing valine biosynthesis is that this amino acid acts in the feedback inhibition of Ahas activity (Magee and Robichon-Szulmajster 1968). This inhibition has been recently found by Gibson et al. (2014) to be mediated by a regulatory subunit encoded by ILV6. Additionally, it has been also demonstrated that the Ilv6 (encoded by the *S. cerevisiae* branch of genome—Sc-ILV6 gene) enhances Ahas activity and works as a perfect marker for measuring diacetyl productivity (Gibson et al. 2014; Duong et al. 2011). Duong et al. (2011) exploited the natural diversity of *S. pastorianus* strains to track strains with low diacetyl production. These authors evidenced that lower expressions of the homologue Sc-ILV6 gene correlated well with lower diacetyl production. The authors further confirmed this observation by double-deleting Sc-ILV6 in commercial lager strains, which in response produced 65 % less diacetyl during fermentation.

Not surprisingly, much attention has been given to the valine uptake rate in the attempt of increasing the intracellular levels of this amino acid, which in turn would reduce the activity of Ahas and hence α-acetolactate formation. Valine enters the yeast cell mainly through specific (branched-chain amino acid permeases—Bap 2/3) and non-specific (Gap1) membrane transporters. As discussed in the previous chapter, Gap1 is under NCR control and therefore targeted for destruction in nitrogen-rich conditions and derepressed for the uptake of amino acids in poor nitrogen conditions. However, Gap1 has little affinity for BCAA and unfortunately notably lower for valine (Stanbrough and Magasanik 1995). Furthermore, the transcription of BAP2 depends on the previous external stimuli of valine or other BCAA through the SPS complex (discussed in Chap. 2) causing expression delays in BAP2. Didion et al. (1996) have shown that valine has a weak induction power over the expression of BAP2. Thus, while the majority of preferred amino acids is absorbed by yeast through the first 12 h of fermentation, most of valine is still to be absorbed (Perpète et al. 2005; Gibson et al. 2009). Romkes and Lewis (1971) observed that lager yeast taken from stationary phase

had deficient valine uptake, which was among the amino acids with the longest lag period for assimilation. This observation is in accordance with the studies of Kodama et al. (2001), who have shown that the transcription of the homologous gene Lg-BAP2 (inherited from the *S. eubayanus* ancestor) in lager yeast is repressed in the early stages of fermentation and it is only transcribed when the majority of amino acids have been taken up from the wort. Industrial lager strains presented the same behavior in the work of Gibson et al. (2009) as BAP2 was only expressed in the late stages of primary fermentation causing delays in valine uptake.

Strain upgrades through genetic modifications have become a common practice for scientists who want to achieve the best results possible in fermentation performance. Given the importance in time savings that reduced diacetyl formation would bring to commercial breweries, genetic constructions often involve strategies to reduce the formation of this VDK. The most logical approach is by disrupting the Ahas-encoding ILV2 gene. Wang et al. (2008) reported an average reduction of 60 % in diacetyl formation by disrupted ILV2 strains when compared to parental strains under the same fermentation conditions. Accordingly, the *ilv2Δ*-constructed strain tested by Liu et al. (2007) could reduce diacetyl formation by 66 %, and maturation time was reduced from 7 to 4 days. Another option in decreasing diacetyl formation is by pushing forward the chain reaction of valine biosynthesis, i.e., by increasing Ilv5 activity. Overexpression of acetohydroxyacid reductoisomerase will ultimately use the available α-acetolactate, avoiding its accumulation and further excretion. Therefore, genetically modified strains overexpressing ILV5 have been designed by several authors (Qin and Park 2012; Dillemans et al. 1987; Kusunoki and Ogata 2012; Gjermansen et al. 1988) and all of them observed reduced diacetyl formation when compared to fermentations performed by the parental strains. Slightly different strategy has been carried out by Omura (2008) who redirected the expression of ILV5 (originally present in the mitochondria) to the cytosol. The author overexpressed a modified ILV5 with deleted *N*-terminal that resulted in the arrest of acetohydroxyacid reductoisomerase in the cytosol. This was useful in lowering diacetyl production without any significant change in beer quality. However, methods involving genetic modifications have limited application in commercial breweries due to uncertain consumer acceptance and legal regulations.

Yeast Response to Fermentation Parameters

Yeast Strain

The production of many flavor-/aroma-active compounds depends on the yeast strain chosen for the fermentation. The genome-associated phenotypic character of each strain is unique and will strongly impact the final flavor/aroma profile of the product (Ramos-Jeunehomme et al. 1991; Rossouw et al. 2008). This makes

the selection of the right strain an extremely important task to make good beer. However, it is crucial that the brewer keeps his strain safe not only from contamination, but also from genetic (mutation) or metabolic (physiological) drifts that may occur in the course of serial repitching (Jenkins et al. 2003; Powell and Diacetis 2007; Sato et al. 1994). Whereas the serial repitching of yeast will not cause loss of prominent physiological characteristics of the brewing yeast (Buhligen et al. 2013; Powell and Diacetis 2007; Vieira et al. 2013), the accumulation of variant with a different stress response may eventually cause certain features to linger on subsequent generations. Indeed, it is now clear that the phenotypic heterogeneity regularly emerges from within microbial population, leading to the appearance of deleterious phenotypes among cellular fractions of individuals during industrial bioprocesses (Delvigne and Goffin 2014). This phenotypic heterogeneity occurs due to random alterations in gene expression levels that can be amplified by specific genetic circuits such as positive feedback loops. This stochasticity needs a specific tool to be analyzed, such as a combination of fluorescent reporter gene with real-time flow cytometry (Brognaux et al. 2013). More recently, another source of heterogeneity has been pointed out and relies on post-transcriptional regulations such as the plasticity of the metabolism (de Lorenzo 2014; van Heerden et al. 2014). For all these reasons, brewers must keep frozen stocks of original yeast strains for periodical restart of fresh pitching cultures.

A clear example of how different yeast strains can behave during beer fermentations can be found in a recent work performed by Gibson et al. (2014). The authors screened 14 different brewing strains of *S. pastorianus,* and variances as great as ninefold in the production of diacetyl at equivalent stages of beer fermentation (using the same conditions) were observed. In an attempt to obtain better results in highly pitched fermentations, Verbelen et al. (2008) assessed the performance of 11 lager yeast strains. Despite the fact that cell density had an apparent impact on the flavor profile (increased higher alcohol and residual diacetyl), this effect was strain dependent. Therefore, advantage could be taken by finding the correct strain to be used in highly pitched beer fermentations.

Recently, He et al. (2014) assessed the contribution of each of the ancestry subgenomes of *S. pastorianus* (*S. cerevisiae and S. eubayanus*) to the final concentration of higher alcohols and esters in beer. The authors noted a significantly higher transcription of *S. eubayanus* genes (BAP2, BAT2, ATF1, ATF2, EHT1, and IAH1) when compared to the same orthologous genes encoded by the *S. cerevisiae* genome. This differential expression of orthologous genes was also observed during fermentation, suggesting that Sc-type and Sb-type genes may have different functionalities during beer fermentation (He et al. 2014).

Temperature

A precise control of temperature is another critical parameter for successful beer fermentation. Landaud et al. (2001) have shown that temperature increases

fermentation rate, productivity, and final concentration of higher alcohols, independently of the top pressure applied (1.05–1.8 bar). Increased fermentation temperatures trigger a higher formation of diacetyl in the early stages of fermentation due to increased cellular growth. However, it does not change the final concentration of diacetyl as there will also be more yeast to reduce it (Krogerus and Gibson 2013; Saerens et al. 2008b). Moreover, increased temperatures also hasten the oxidative decarboxylation of α-acetolactate into diacetyl, which is rate-limiting for diacetyl reduction (García et al. 1994).

It has been reported that rising fermentation temperatures increase BAP2 expression in the brewing yeast *S. cerevisiae* (Yukiko et al. 2001). This gene is encoding a broad-substrate specificity permease that promotes the transport of the BCAAs (valine, leucine, and isoleucine) into the yeast cell (Didion et al. 1996). The greater availability of amino acids within the cell favors the catalytic Ehrlich pathway, increasing thus the higher alcohol formation (Yukiko et al. 2001). Saerens et al. (2008b) obtained increasing levels of propanol, isobutanol, isoamyl alcohol, and phenyl ethanol by rising the fermentation temperature using two different brewing yeast strains. Conversely, these authors have shown that despite the fact that increasing temperatures promote the expression of BAT1, BAT2, or BAP2, only BAT1 could be strongly correlated with the final concentration of higher alcohols, in particular propanol (Saerens et al. 2008b).

As formation of higher alcohols is temperature dependent (Landaud et al. 2001), changes in temperature may cause changes in the availability of fusel alcohols, which are necessary for ester formation (Calderbank and Hammond 1994). Indeed, a slight change in temperature from 10 to 12 °C can increase ester production by up to 75 % (Engan and Aubert 1977). Saerens et al. (2008b) have shown that the AATases-encrypting genes ATF1 and ATF2 are upregulated with increasing temperatures during beer fermentation. Furthermore, the maximum expression of these genes clearly correlated with the final concentration of ethyl acetate, isoamyl acetate, and phenyl ethyl acetate. Fermentation temperature is mainly essential for ethyl ester formation such as ethyl octanoate and decanoate because (as opposed to acetate ester production) the precursor availability has a significant role in ethyl ester production (Saerens et al. 2008a). More recently, Hiralal et al. (2014) have shown that an increase in the fermentation temperature from 18 to 22 °C increased the acetate ester and total ethyl ester concentration in beer by 14.42 and 62.82 %, respectively. This is also consistent with the findings of Saerens et al. (2006, 2008a).

Hydrostatic Pressure

With increasing market demands, breweries are continuously increasing the reactor sizes for beer production. The incredibly large fermenters (up to 12,000 hl) naturally generate a massive hydrostatic pressure that increases the concentration of carbon dioxide dissolved in beer. Increasing concentrations of dissolved CO_2

suppress yeast growth by unbalancing decarboxylation reactions (Rice et al. 1977; Knatchbull and Slaughter 1987; Renger et al. 1992; Shanta Kumara et al. 1995; Landaud et al. 2001). As said before, decarboxylation is a fundamental step in either higher alcohol or acetyl-CoA synthesis. As acetyl-CoA is the primary precursor of acetate esters, hydrostatic pressure unbalances beer flavor most probably by limiting the substrate availability for ester formation (Landaud et al. 2001). In a previous work carried out by Renger et al. (1992), both higher alcohols and esters decreased with increasing pressure, but ester formation was more affected. Again, these authors attributed this reduced production of flavor-active compounds (by 70 % less at 2 bar) to the decrease in biomass growth. Conversely, the reduced yeast proliferation and decreased formation of by-products is very useful in high-gravity brewing (HGB), as high-gravity worts also increase the formation of higher alcohols and esters. In this manner, pressure can counterbalance the over production of by-products.

Wort Composition

It is not hard to understand that wort composition will significantly influence the final beer flavor/aroma. After all, the fermenting wort is the growth medium, from which the brewing yeasts absorb nutrients for living and to where they excrete the metabolic by-products. Thus, changes in the amount and composition of nutrients will trigger different yeast responses through the pathways discussed earlier in Chap. 2.

Sugars

HGB or even very high-gravity brewing (VHG) became a standard practice in many breweries as it can bring significant economic benefits (Yu et al. 2012; Lei et al. 2013b). The use of HGB can not only increase the brewery capacity by up to 20–30 % without any significant investment in equipment, but it was also claimed to improve the haze and smoothness of the beer (Stewart 2007). However, HGB often brings an unbalanced flavor profile to the finished beer, the most common perturbation being the overproduction of acetate esters, impairing the beer with fruity and solvent-like aromas (Anderson and Kirsop 1974; Peddie 1990; Saerens et al. 2008b). Anderson and Kirsop (1974) observed up to eightfold increase in acetate ester production when the specific gravity of the wort was doubled. Saerens et al. (2008b) have tested ale and lager strains upon increasing specific wort gravity. Although all higher alcohols showed an increased accumulation, after dilution to reach the standard ethanol content (5.1 % v/v), only the fermentations conducted by the ale strain remained with unbalanced high levels of fusel alcohols. Simultaneously, all acetate esters were overproduced by both lager and ale strains (Saerens et al. 2008b).

However, not only the amount, but also the type of sugars may influence the changes in the aromatic profile of the final beer. Quickly assimilable glucose- and fructose-rich worts typically generate beers with higher contents of esters than those rich in maltose (Younis and Stewart 1998, 1999, 2000; Piddocke et al. 2009). Fermentations of both 21 and 24 °P worts enriched with maltose syrup, performed by Piddocke et al. (2009), produced fewer acetate esters compared to fermentations carried out with glucose syrup-enriched worts. The reason why an individual assimilable sugar has a different effect on ester production has not been fully elucidated. Younis and Stewart (1998) suggested that higher levels of glucose increase acetyl-CoA formation, which is the primary substrate for acetate ester synthesis. In the same way, maltose-rich worts may only weakly induce acetyl-CoA formation acetate ester production (Shindo et al. 1992). Moreover, while glucose rapidly enhances ester synthase activity in carbon-starved cells by directly inducing ATF1 transcription through Ras/cAMP/PKA nutrient pathway, maltose is only absorbed and metabolized later (Verstrepen et al. 2003a). Increasing levels of maltose as sole carbon source in synthetic medium showed an increasing tendency to accumulate acetate esters (Saerens et al. 2008a). Conversely, Dekoninck et al. (2012) have shown that although sucrose had greater impact on ATF1 expression when compared to maltose, a remarkable decrease in acetate esters was observed during HGB. The high amount of sucrose-stimulated yeast growth and metabolism, which ultimately increased the uptake of amino acids. This leads to another important feature of HGB altering aroma profile of the beer, namely the carbon-to-nitrogen (C|N) ratio. The addition of sugary syrups is a common practice to increase the specific gravity of the wort in HGB. However, these syrups lack nitrogen, which typically reduces the total free amino nitrogen (FAN) content of the wort. Therefore, adjuncts usually increase the C|N ratio, which in turn may lead nitrogen to be a growth-limiting factor (Lei et al. 2012, 2013a; Saerens et al. 2008a; Verstrepen et al. 2003a). Any alteration in sugar or FAN levels affects the formation of acetate esters, but not ethyl esters (Saerens et al. 2008a). Additionally, diluted FAN content found in HGB leads to abnormal yeast physiology and unbalanced beer flavor (Lei et al. 2012).

Adaptive evolution can be used to obtain robust industrial strains, namely for HGB. With this in mind, Ekberg et al. (2013) isolated an osmotolerant *S. pastorianus* variant with improved fermentation capacity. The enhanced capacity could be attributed to the reduced transcription of hexose permeases and increased transcription of the MAL1 and MAL2 genes. Therefore, the variant strain showed significantly shorter fermentation time than the parental strain, producing a beer with similar organoleptic properties. However, VDKs and acetate esters were higher by up to 75 and 50 % in the beer produced by the osmotolerant strain.

Free Amino Nitrogen (FANs)

Although a wide range of nitrogen-containing compounds are dissolved in the wort, the brewing yeast can only assimilate the smaller molecules, called FANs. The discussion of FANs interfering with beer aroma will inevitably lead to the

absorption of amino acids to form higher alcohols through the Ehrlich pathway. The type and amount of amino acids under assimilation will also lead the yeast to different responses and ultimately to final beer aromatic profile (Lei et al. 2013a; Äyräptää 1971). In fact, treating the wort with proteases increases the final FAN and ultimately increases the production of higher alcohols and esters by the brewing yeast in either HGB or normal gravity brewing (Lei et al. 2013c). The addition of BCAAs such as valine, leucine, and isoleucine to the fermenting wort increases the formation of their respective fusel alcohols—isobutanol, isoamyl alcohol, and amyl alcohol (Äyräptää 1971; Engan 1970; Procopio et al. 2013). Recently, Procopio et al. (2013) have shown that not only the addition of valine, leucine, and isoleucine increased the formation of fusel alcohols, but also did proline. Since proline cannot be converted into a higher alcohol via Ehrlich pathway, its role on fusel alcohol formation induction was attributed to the synthesis of glutamate from this amino acid. A recent study showed that the supplementation of wort with lysine and histidine improved the performance of a lager brewing yeast in HGB (Lei et al. 2013a). Compared to lysine, histidine significantly affected the aromatic profile by increasing the formation of higher alcohols and esters. Moreover, recent reports confirmed that FAN content of wort can affect the transcription of both ATF1 and BAT1 genes (Lei et al. 2012; Saerens et al. 2008b).

As discussed in the first chapter of this book, commercial breweries are incessantly looking for alternative methods to decrease the production costs, and using unmalted grains as adjuncts is one of the most widespread strategies. However, unmalted cereals are poor in FANs and do not contribute to the enzymatic activity during mashing. Therefore, the higher the ratio of unmalted grains used in the recipe is, the poorer in FAN the wort will be. Yeast will try to compensate this lack of FAN through the anabolic pathway of amino acids from carbohydrates, leading inevitably to increased formation of higher alcohols. Liu et al. (2014) executed a double deletion in LEU2 genes aiming at decreasing the production of higher alcohols in high adjunct beer (60 % of malt substituted by rice). The LEU2 gene encodes the enzyme b-isopropylmalate dehydrogenase, which mediates the third step in the biosynthesis of leucine (Hsu and Kohlhaw 1980). The disruption of LEU2 reduced the formation of total higher alcohols by nearly 26 % if compared to parental strains. Conversely, overexpression of LEU2 can increase higher alcohol production 3–4-fold (Park et al. 2014).

Increased production of higher alcohols is also a common issue in continuous beer fermentation (Willaert and Nedovic 2006). Pires et al. (2014) recently suggested that increased production of fusel alcohols through continuous fermentation is a result of both intense catabolic and anabolic pathways. On the one hand, the incessant injection of amino acids into continuous fermenter inevitably raises the higher alcohol formation by the Ehrlich pathway. On the other hand, the increased availability of preferred amino acids impairs the intake of the less preferred ones consequently triggering the anabolic route because of the GAAC pathway (Chap. 2).

There is an increasing evidence that the FAN content and composition are the primary factors influencing diacetyl formation in beer fermentation (Pires et al. 2014; Lei et al. 2013c; Gibson et al. 2009). Gibson et al. (2009) demonstrated

that worts with less FAN produced less diacetyl during fermentation. Although Pugh et al. (1997) have evidenced the same correlation, FAN levels lower than 122 mg L^{-1} began to increase diacetyl production. It was clear that the depletion of FAN below critical levels stimulated the de novo synthesis of valine increasing the pool of α-acetolactate. Recently, Lei et al. (2013c) noted that the uptake of valine decreased with increasing FAN content. More recently, Pires et al. (2014) performed a long-term continuous beer fermentation and saw very interesting patterns linking diacetyl productivity over time with the FAN consumption rate. All these pieces of evidence are in accordance with the moderate speed of absorption of valine when compared to that of preferred amino acids with faster absorption. The lesser the FANs (consequently less amino acids) are, the quicker the preferred amino acids are consumed, which gives better chances for valine to enter the cell. Conversely, the more the amino acids are available to enter the yeast cell, the greater the challenge for valine to have access to the permeases is.

Oxygen and Unsaturated Fatty Acids (UFAs)

Dissolved oxygen and UFAs in wort are remarkably known as negative regulators of ester synthesis by brewing yeast (Fujii et al. 1997; Anderson and Kirsop 1974; Thurston et al. 1982; Taylor et al. 1979; Malcorps et al. 1991; Fujiwara et al. 1998; Anderson and Kirsop 1975a, b). Oxygen was originally believed to reduce ester formation by decreasing acetyl-CoA availability (Anderson and Kirsop 1974). However, when genetic studies came into fashion, oxygen and UFAs were proven directly to inhibit the expression of ATF1 and ATF2 (Fujii et al. 1997). Fujiwara et al. (1998) have further complemented that oxygen and UFAs repress the expression of ATF1 by different regulatory pathways. Oxygen represses ATF1 through the Rox1–Tup1–Ssn6 hypoxic repressor complex (Fujiwara et al. 1999), whereas UFAs inhibit ATF1 through the low-oxygen response element (Vasconcelles et al. 2001). In addition to acetate esters, it has been also shown that increasing levels of UFAs in the fermenting medium reduce the production of ethyl esters by the brewing yeast (Saerens et al. 2008a).

Considering what is written above, Moonjai et al. (2002) assessed the potential of UFA-rich lipid supplements to decrease the need of wort aeration. The results have shown that the yeast treated with UFAs can be pitched into poor-oxygenated worts without losing fermentation potency or influencing the organoleptic quality of the product. A reduced amount of oxygen supplied to the wort may increase flavor stability of the final beer and will limit potential oxidative stress upon the brewing yeast (Gibson et al. 2008). Inspired by this potential, Hull (2008) assessed the replacement of wort oxygenation by treatment of the pitching yeast with olive oil rich in UFAs. The industrial scale test succeeded without major effects on the acceptability of the produced beer. Therefore, UFA-treated yeast may be of particular help in HGB, once worts with specific high gravity have limited oxygen solubility (Baker and Morton 1977).

Verbelen et al. (2009b) evaluated the use of different oxygen conditions (such as wort aeration/oxygenation and yeast preoxygenation) over the performance of high-cell-density beer fermentations. Expectedly, wort oxygenation exerted a substantial negative impact on ester formation owing to decreased expression of ATF1. BAP2, ILV2, and ILV5 were screened in parallel under the same conditions. The authors observed that BAP2 was highly expressed only 1 h after pitching in the fermentations using non-preoxygenated yeast with both oxygenated and aerated worts. However, 4.5 h later, the expression of BAP2 was significantly reduced in all fermentations. Whereas either wort oxygenation (51.8 ppm oxygen in wort) or aeration (7.8 ppm oxygen in wort) had no effect on the expression of both ILV2 and ILV5, the total diacetyl measured in the experiments using increased pitching rates (80×10^6 cells mL^{-1}) was considerably higher (~10 times) than in the control fermentation (20×10^6 cells mL^{-1}). The authors hypothesized that other factors such as yeast physiology and wort composition might have influenced diacetyl overproduction (Verbelen et al. 2009b).

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