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Review Article

Review of Aroma Formation through Metabolic Pathways of *Saccharomyces Cerevisiae* in Beverage Fermentations

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Abstract: Fermentation has historically played an important role in the production of several commodities, such as bread and alcoholic beverages. Today fermentation can be used to produce specific flavor compounds in multiple industries. Flavor compounds are secondary metabolites produced during fermentation in addition to primary metabolites such as ethanol. Secondary metabolism is influenced by fermentable carbon, nitrogen makeup and the fermentation environment. A better understanding of how these variables effect the physiology of yeast strains to produce flavor compound can improve a number of industrial commodities. Systems biology is an attractive method for studying the complex dynamics of secondary metabolism. While applying a systems biology approach to winemaking or brewing is not a new concept, making direct linkages between -omics data and the production of flavor compounds is a novel approach to improving flavor production in fermentation. Thus far, the bulk of the work in which systems biology methods have been applied to fermentation relies heavily on laboratory strains of *S. cerevisiae* that lack metabolism-relevant genes present in industrial yeast strains. Therefore,

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investigation of industrial strains using systems biology will provide a deeper understanding of secondary metabolism in the industrial setting. Ultimately, integrating multiple -omics approaches will lay the foundation for predictive models of *S. cerevisiae* fermentation and optimal flavor production.

Key words: *Saccharomyces cerevisiae*, fermentation, systems biology, beer/wine/cider

Introduction

In the earliest fermentations, bread and alcohol were produced mainly by spontaneous inoculation of wild yeast from the environment. Starting in the late 19th century, industrial fermentations were purposefully inoculated with commercially produced yeast strains. These strains were propagated and sold based on the efficiency of production of the desired commodity, resulting in the specialization of yeast strains to specific industries (Richter and Pugh 2012). Today fermentation is used to produce specific metabolites utilized in the beverage, food, cosmetic, medical and biofuel industries (Vandamme and Soetaert 2002). Therefore, research aimed at maximizing the quality and efficiency of metabolite production will provide significant technical and economic benefit to fermentation industries. This review will describe the metabolic pathways involved in the formation of aroma through fermentation.

In 2013 the worldwide flavor and fragrance market was estimated to be worth US $23.9 billion (Venkataraman et al. 2014). Over 100 flavor molecules are produced via the conversion of natural precursors using microbial enzymes or by microbial fermentation using native yeast strains or strains with gene modifications (Vandamme and Soetaert 2002). Compounds produced by yeast fermentation include glycerol, aldehydes, alcohols, propanediol, organic acids, isoprenoids, esters and steroids.

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Generation of aroma through yeast metabolism

Although *Saccharomyces* is the most commonly used fermentation organism many other genera of yeast can also be used including: *Saccharomycodes, Schizosaccharomyces, Candida, Torulaspora, Cryptococcus, Debaryomyces, Issatchenkia, Pichia, Kluyveromyces, Metschnikowia, Hanseniaspora (Kloeckera), Rhodotorula, Brettanomyces (Dekkera)* and *Zygosaccharomyces* (Vandamme and Soetaert 2002). Strains or species of yeast are selected based on the aesthetic/flavor components they produce in commercial food and beverage products. "Flavor" will be defined here as the volatile and non-volatile components related to aroma and mouthfeel, respectively. Non-volatile compounds include glycerol, mono- and poly-saccharides, phenolics and organic acids; whereas, volatile compounds include alcohols, aldehydes, esters, dicarbonyls, short-chain fatty acids, medium chain fatty acids, methyl ketones, lactones, phenolic compounds, terpenes and sulfur compounds (Moreno-Arribas and Polo 2009). The many flavor metabolites that yeast produce during fermentation (Figure 1) are generated de novo or by the transformation and volatilization of precursor compounds in the starting material (fruit juice, hops, grains, etc.) (Lambrechts and Pretorius 2000).

The metabolism of fermenting *S. cerevisiae* can be divided into two classes: primary and secondary. Primary metabolism is essential for growth, cell division and survival, producing metabolites such as ethanol, glycerol, acetaldehyde and acetic acid (Styger et al. 2011). Secondary metabolism is non-essential for growth and produces small molecules that include the fusel alcohols, esters, carbonyls, sulfur compounds, thiols and terpenoids (Figure 1) (Styger et al. 2011). Secondary metabolites can contribute to the organoleptic properties of food and beverage products and are important commodities for several industries. Secondary metabolism is greatly

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influenced by fermentable carbon, nitrogen makeup and the fermentation environment (Henschke and Jiranek 1993, Verstrepen et al. 2004). A better understanding of how these variables influence yeast aroma compound production can be used to improve beverage, food, perfume and cosmetic products.

Regulation of Secondary Metabolism

Fusel alcohols

Fusel alcohols are the most abundant volatile components produced during fermentation and contribute to essential aroma and flavors in fermented beverages and food (Hazelwood et al. 2008). Fusel alcohols include propanol, isoamyl alcohol, isobutanol, active amyl alcohol, 2-phenylethanol and tyrosol (Swiegers and Pretorius 2005). Fusel alcohol formation occurs through the Ehrlich pathway or central carbon metabolism (Figure 2A). The first step in the Ehrlich pathway is a transamination reaction between an amino acid and 2-oxoglutarate (Sentheshanmuganathan 1960). The transamination step converts the amino acids to α-keto acids which may also come from central carbon metabolism (Sentheshanmuganathan 1960). Subsequently, multiple pyruvate decarboxylases catalyze the conversion of the α-keto acid into a branched chain aldehyde (Sentheshanmuganathan 1960). Lastly, an alcohol dehydrogenase catalyzes the NADH-dependent final step that reduces the aldehyde to a fusel alcohol (Figure 2A) (Hazelwood et al. 2008). Not all enzymes involved in the catalysis of this pathway are known. One of the difficulties in elucidating specific proteins necessary for fusel alcohol production is the large degree of redundancy in the genome. Multiple aminotransferases, decarboxylases and alcohol dehydrogenases make traditional genetic approaches to understanding fusel production difficult.

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Fusel alcohols can have positive or negative sensory impacts depending on the concentration. Fusel alcohol concentrations of 400 mg/L or greater can result in a pungent, solvent-like aroma in wine; whereas, concentrations of less than 300 mg/L are often described as a desirable fruity characteristic (Swiegers and Pretorius 2005). In ciders high concentrations of fusels, particularly 2-phenylethanol, are important contributors to the typical flavor (Beech 1972). Propanol, butanol and isobutanol are described as having an alcoholic odor; whereas, active amyl alcohol and isoamyl alcohol are described as having a marzipan-like or banana aroma (Lambrechts and Pretorius 2000). Tyrosol and 2-phenylethanol impart honey-like and floral aromas, respectively (Lambrechts and Pretorius 2000). In the appropriate concentrations fusel alcohols can impart a beneficial complexity and also serve as precursors for the formation of acetate esters.

Acetate & Ethyl Esters

Esters contribute to the floral and fruity characteristics associated with wines and beer and are generated by the esterification of alcohol and acids at low pH (Saerens et al. 2010). The reaction requires an alcohol molecule, acetyl-CoA, an ester synthesizing enzyme and ATP (Figure 2B, 2C) (Saerens et al. 2010). Both the production and degradation of esters are tightly regulated.

The two types of esters produced during fermentation are acetate esters and ethyl esters. Acetate esters are the result of esterification of acetyl-CoA and an alcohol (Figure 2B). These include ethyl acetate, isoamyl acetate, 2-methylbutyl acetate and phenylethyl acetate and are described as banana, apple, fruit and aromatic sweetness (Saerens et al. 2010). Ethyl acetate is

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the most common acetate ester, primarily because of the large quantities of ethanol and the more reactive nature of primary alcohols (Saerens et al. 2010).

The regulation of acetate ester production is primarily controlled by the expression of two alcohol acetyl transferases (AATase), Atf1p, 2p (Figure 2B) (Yoshimoto et al. 1998). Atf1p has the greatest AATase activity; introducing multiple copies of *ATF1* into laboratory strains resulted in increased production of acetate esters (Verstrepen et al. 2003). In contrast, deletion of Atf1p resulted in a significant decrease in acetate ester concentration (Verstrepen et al. 2003). The production of acetate esters is also substrate dependent, relying on the availability of fusel alcohols (Lilly et al. 2006).

The second group, the ethyl esters, are composed of ethanol and a medium chain fatty acid (MCFA) (Figure 2C) (Saerens et al. 2010). Ethyl esters include: ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate (Saerens et al. 2010). These ethyl esters vary in their sensorial attributes but descriptions include apple, fruit, strawberry, pear and aniseed (Saerens et al. 2010). During late exponential growth phase MCFA intermediates are released prematurely from the cytoplasmic fatty acid synthase (FAS) complex which triggers ester synthesis (Taylor and Kirsop 1977). The MCFAs are activated by coenzyme A, and in conjunction with ATP, ethanol and enzymes, are esterified (Figure 2C) (Saerens et al. 2010).

Evidence points to three regulatory pathways responsible for the release of MCFAs and the subsequent production of ethyl esters: 1) decreased acetyl-CoA carboxylase activity, 2) the upregulation of fatty acid biosynthesis genes *FAS1, FAS2, EEB1, EHT1* and 3) the concentration of MCFAs (Saerens et al. 2006, Dufour et al. 2008). Inhibition of acetyl-CoA carboxylase initiates the release of MCFAs from the FAS complex (Dufour et al. 2008). Deletion of *EEB1*

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and *EHT1* decreases the production of ethyl esters; however, overexpression of both enzymes does not result in an increase (Saerens et al. 2006). Several genes have been implicated in the production and degradation of esters; however, direct linkages between precursors, enzyme activity, substrate concentration and ester production have not yet been found, particularly for ethyl esters.

Carbonyls: Diacetyl

Diacetyl is described as having a toasty, butterscotch, nutty aroma but at high concentrations can smell like rancid butter (Swiegers and Pretorius 2005). Yeast can synthesize diacetyl during fermentation; however, diacetyl is predominantly produced in wine by lactic acid bacteria (Laurent et al. 1994). Diacetyl is formed extracellularly by chemically-driven decarboxylation of α-acetolactate. α-Acetolactate is synthesized as an intermediate in the 2-ketoisovalerate pathway or produced from acetaldehyde by Ilv2p (Figure 1) (Suomalainen and Ronkainen 1968). Diacetyl is subsequently converted to acetoin followed by 2,3-butanediol, both of which have a higher sensory threshold and, thus, less sensory impact (Swiegers and Pretorius 2005).

Sulfur compounds

Sulfur-containing compounds have a very low sensory detection threshold and are often described as having cabbage, rotten eggs or onion aroma (Rauhut 1993). There are five categories of sulfur compounds: sulfides, polysulfides, thiols, thioesters, and heterocyclic compounds (Rauhut 1993). Aromatic sulfur compounds originate from sulfur-containing fungicides or by the degradation of sulfur-containing amino acids during fermentation; however, the pathways responsible for these processes have not been fully elucidated (Rauhut 1993).

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Hydrogen sulfide (H_2S) is one of the primary sulfur off-notes; it is produced during fermentation by the sulfate reduction sequence (SRS) pathway. In this pathway, sulfate is taken up from the external medium via a sulfate permease (Sul1p, 2p) and reduced to sulfite then sulfide via Met5p and Met10p, respectively (Rauhut 1993, Spiropoulos and Bisson 2000). In the presence of non-limiting cysteine or methionine, sulfide combines with O-acetylserine or O-acetylhomoserine to form homocysteine (Swiegers and Pretorius 2005). Under methionine and cysteine limited conditions, O-acetylserine and O-acetylhomoserine are also limited resulting in excess sulfide that is converted to H_2S (Thomas and Surdin-Kerjan 1997).

Agricultural use of elemental sulfur, pantothenate deficiency and high concentrations of threonine can also affect H_2S production during fermentation (Spiropoulos and Bisson 2000). Hydrogen sulfide is very reactive, often resulting in additional off-notes in wine. One of the more common off-notes produced from H_2S reactivity is ethanethiol, which is formed when H_2S reacts with ethanol (Spiropoulos and Bisson 2000). Hydrogen sulfide can be removed from wine by copper stripping or nitrogen aeration but these treatments can remove positive aromatics as well (Swiegers and Pretorius 2005).

In contrast to other sulfur compounds volatile thiols can have beneficial aromatic characteristics. These are described as boxwood, passion fruit, black currant or grapefruit (Tominaga et al. 1998). These compounds are often S-cysteine-bound or glutathione-bound in grape or hops and released during fermentation (Tominaga et al. 1998). Irc7p and Str3p cleave the carbon-sulfur bond between cysteine and the thiol to release the aroma (Figure 1) (Tominaga et al. 1998).

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The three most impactful volatile thiols are 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). Harsch *et al.* (2013) identified (E)-2-hexenal and its corresponding alcohol, (E)-2-hexen-1-ol, as precursors to the formation of 3MH (Harsch et al. 2013). This work established the potential for enhancing 3MH and 3MHA in the presence of a sulfur donor and (E)-2-hexenal and (E)-2-hexen-1-ol, opening the door to the possibility of using yeast to enhance positive aromatic thiols (Figure 3) (Harsch et al. 2013).

Terpenoids

Terpenoids are a class of aromatics that define varietal characteristics in fruit and hops, producing aromas described as rose, geranium and floral (Swiegers and Pretorius 2005). Terpenoids are essential flavor components in a variety of commercial products including wine, beer, food additives, perfumes and cosmetics. Optimizing the production of volatile terpenes during yeast fermentation is a major goal in industrial fermentation.

Monoterpenoids are produced by the precursor geraniol pyrophosphate (GPP) in plants and some fungi (King and Richard Dickinson 2000). *Vitis vinifera* (grapevines) and *Humulus lupulus* (hops) synthesize monoterpenoids such as geraniol, linalool, nerol and citronellol (Swiegers and Pretorius 2005). Terpenoids can be found in both free and bound forms, with the bound form more prevalent (Swiegers and Pretorius 2005). Yeast glycosidase enzymes release and volatilize these aromatic compounds during fermentation (Figure 1) (Gunata et al. 1988).

The enzymatic release of monoterpenes is a one- or two-step process depending on the sugar bound to the precursor glucoside (a mono- or di-saccharide) (Gunata et al. 1988). For disaccharide glycosides, the first step involves the release of the terminal sugar via an

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arabinofuranosidase, rhamnopyransosidase, or apiofuranosidase followed by β-glucosidase cleavage releasing the terpenoid (Gunata et al. 1988). Wine strains overexpressing cell wall protein Exg1p produced elevated levels of terpenes in both synthetic media and grape must (Gil et al. 2005).

Terpenoid production has primarily been attributed to hydrolysis of glycosidic linkages; however, *S. cerevisiae* is capable of producing monoterpenes through the mevalonic acid (MVA) pathway (Figure 2D). Carrau *et al.* (2005) showed that *S. cerevisiae* and *H. uvarum* were able to produce terpenes in chemically defined medium lacking grape juice, terpenes and glycoconjugates (Carrau et al. 2005). This work revealed that linalool and α-terpineol were produced by *S. cerevisiae* under microaerobic and high assimilable nitrogen conditions in synthetic media. Carrau *et al.* (2005) have predicted an alternative pathway for monoterpene synthesis (Figure 2D); the monoterpenes are formed de-novo in the mitochondria, linked to leucine catabolism and a novel GPP synthase (Carrau et al. 2005). Leucine catabolism and isoprenoid metabolism have been linked and studied in the fungi *Aspergillus nidulans*, providing precedence for this alternative pathway (Rodríguez et al. 2004).

Impact of starting materials on production of flavor components

Fermentation requires a carbon source (sugar), nitrogen (ammonia and amino acids), water and yeast. The aromatic by-products produced during fermentation can vary drastically depending on the starting material (fruit, grain or extract), the concentration of carbon and nitrogen and the yeast strain (Henschke and Jiranek 1993, Verstrepen et al. 2004, Richter and Pugh 2012). Because of this variability, industrial fermentation has been optimized depending on the commodity. For example, fermentations for alcoholic beverage products seek to enhance specific

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flavor compounds. Fruity and flowery esters are desirable in wine (Moreno-Arribas and Polo 2009) but may be less desirable in certain beer styles. Fusel alcohol levels exceeding 300 mg/L are sought after in hard ciders (Beech 1972) but can produce off-notes in wine.

The starting material plays a crucial role in affecting the final flavor profile of the wine, beer, cider or sake. For example, free terpenoid compounds in grapes impart specific aromatic characteristics and vary depending on the grape varietal (Conde et al. 2007). Muscat grapes have the highest concentration of free monoterpenes, providing a distinct floral aroma (Conde et al. 2007). In contrast, the monoterpenes in Cabernet Sauvignon, Sauvignon blanc, Merlot, Shiraz and Chardonnay are typically below sensory threshold (Conde et al. 2007).

Apples impart unique flavor components to cider due to high concentrations of phenolics and acidity (Barker and Burroughs 1953, Beech 1972). The aromatic profile produced from apple juice concentrate (apple aroma essence) primarily comprises six compounds including: ethyl-2-methyl butyrate, 1-hexanal, trans-2-hexanal, ethyl acetate, 1-butanol and cis-3-hexenol (Beech 1972). The sensory profile varies when freshly pressed apples are used rather than concentrate. Fresh apple juice has a lower overall concentration of the apple aroma essence, which is created through evaporation, condensation and multiple rounds of concentration of fresh juice (Beech 1972). Another important class of compounds crucial for cider flavor is the fusel alcohols (Beech 1972). Yeast produce fusel alcohols during fermentation, which is influenced by apple variety, juice pre-treatment prior to fermentation, yeast strain, storage condition and carbon and nitrogen content of the juice or concentrate (Beech 1972).

Beer requires four essential ingredients: water, hops, yeast and grain, all of which can impact the flavor profile. The cereal grain used to generate malt for brewing is typically barley

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246 but can also include wheat, rye, oats, sorghum or millet (Briggs et al. 2004). The use of non-
247 barley malt imparts distinct flavor characteristics to beer and fermented beverages. For example,
248 beers produced from 50% malt and 50% polished sorghum have lower concentrations of
249 isobutanol (alcoholic aroma), 2-methylbutanol (marzipan) and dimethylsulphide (cabbage and
250 gasoline) but have higher levels of n-propanol (stupefying aroma) and diacetyl (butterscotch,
251 butter) compared to beers brewed with 100% malt (Dale et al. 1990). Additionally, batches of
252 malted oats used in place of barley malt produced lower alcohol, higher pH beers with lower
253 overall concentrations of fusel alcohols and the esters: ethyl and isoamyl acetate (Klose et al.
254 2011). The concentration of vicinal diketones such as 2,3-pentanedione and diacetyl were above
255 threshold in oat malt beers and below threshold for barley malt beers imparting an overall yogurt
256 and berry-like aroma to the beer made exclusively with oat malt (Klose et al. 2011). In contrast,
257 beers brewed with 100% barley malt had an apple-like flavor (Klose et al. 2011). These studies
258 reveal that altering the grain source drastically changes the flavor components of the final beer,
259 which can be exploited to target different flavor profiles during fermentation.

260 *Carbon source and its impact on fermentation*

261 In industrial fermentation sugars provide essential carbon. The concentration of usable
262 sugars will vary depending on the starting material (fruit or grain) (Figure 4A). Wine, cider and
263 beer will vary in their sensorial characteristics in part due to the different sugars and relative
264 ratios of those sugars in the starting material (Figure 4A). Although many sugars may be
265 available, *S. cerevisiae* preferentially utilizes glucose and sucrose by down-regulating genes
266 involved in the uptake of alternative carbon sources (Figure 4B) (De Deken 1966). Glucose also
267 limits the uptake of fructose by *S. cerevisiae* because they utilize the same sugar transporters in

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the cell (Reifenberger et al. 1997). Fermentations with high residual fructose will affect the overall sweetness and flavor of the final product, as fructose is sweeter than glucose.

High concentrations of glucose and sucrose also downregulate the stress signaling pathway in *S. cerevisiae* by triggering the activation of the Ras/cyclic-adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, which decreases stress resistance of yeast cells (Figure 4B) (Rolland et al. 2000). Downregulation of the stress pathways can result in sluggish fermentations and cell autolysis. Cell autolysis increases "goat-like" flavors by releasing fatty acids into the fermentation media and decreases fruity or floral flavors through ester hydrolysis (Anderson and Kirsop 1974).

Once glucose and sucrose have been depleted by *S. cerevisiae*, catabolite de-repression occurs, allowing the uptake of alternative sugars (Figure 4C). Depending on the starting concentration of glucose in the must or malt, the transition time to catabolite de-repression will vary. For example, de-repression occurs more rapidly in wort than fruit must because of the lower concentration of glucose and sucrose (Figure 4A). Carbon source has a huge impact on beer aroma; specifically, worts with high levels of glucose and fructose have higher levels of acetate esters (Anderson and Kirsop 1974). This can be problematic for high-gravity (high sugar) brewing as the beers are overly fruity and chemical. For example, ethyl acetate and isoamyl acetate concentrations were four-fold higher in high gravity beers than in low gravity beers (Anderson and Kirsop 1974). Worts supplemented with maltose syrups; however, had a reduction of ethyl acetate (10%) and isoamyl acetate concentrations (40%) (Younis and Stewart 1999), suggesting that high-gravity beers can be brewed with higher concentrations of maltose to reduce the overall concentration of acetate esters (Younis and Stewart 2000). These studies also

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highlight the linkage between carbon source and flavor profiles. These processes are likely transcriptionally and translationally controlled through yeast metabolism (Figure 4B and 4C).

Nitrogen's impact on fermentation

In addition to carbon, nitrogen is important for growth, cell division and secondary metabolism. The two forms of yeast-assimilable nitrogen (YAN) are primary amino acids and ammonium (Henschke and Jiranek 1993). In nitrogen rich conditions, *S. cerevisiae* undergoes nitrogen catabolite repression (NCR) controlled by the Tor complex (Figure 4B) (Hardwick et al. 1999). NCR downregulates genes encoding permeases and enzymes needed for the uptake and transport of poor nitrogen sources (Figure 4B). Tor1p is also responsible for regulating glucose activation, glycolysis and the TCA cycle, creating a tight link between carbon and nitrogen uptake in the cell (Hardwick et al. 1999).

NCR is reversed as nitrogen conditions shift from rich to poor, forcing *S. cerevisiae* to utilize alternative sources of nitrogen (Figure 4C). Depending on the starting material (fruit or grain) and the yeast strain, amino acids will be taken up by order of preference. Typically, glutamic acid, aspartic acid, asparagine, glutamine, serine, threonine, lysine and arginine are preferred amino acid sources for *S. cerevisiae* (Jones and Pierce 1964).

The production of secondary metabolites is tightly linked to nitrogen source. Fusel alcohols can be formed through the catabolic Ehrlich pathway (Sentheshanmuganathan 1960; Hazelwood et al. 2008), which directly utilizes the amino acids: leucine, valine, isoleucine, phenylalanine, tyrosine and tryptophan. Thus, the nitrogen concentration and relative ratio of amino acids directly affects the formation of fusel alcohols (Hazelwood et al. 2008). Nitrogen concentration will also affect ester formation, as fusel alcohols are precursors for acetate esters

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(Saerens et al. 2010). The majority of studies exploring the link between nitrogen supplementation and the production of aromatics during fermentation have been conducted in synthetic media or model grape juice (Hernandez-Orte et al. 2006, Carrau et al. 2008), which may not reflect true conditions in a complex starting matrix. While there is extensive knowledge of the genes regulating fusel alcohol and ester production in laboratory strains and synthetic media little is known about nitrogen regulation using commercially relevant strains under production conditions.

Using –omics biology to understand flavor production

Flavor profiles can be optimized during industrial fermentations using a more integrative approach. Systems biology often refers to -omic approaches including: genomics, transcriptomics, proteomics and metabolomics. The data from multiple -omics approaches can be integrated, graphically viewed and modeled to predict how a particular system functions under a given set of conditions (Ideker et al. 2001). Systems biology methods have been applied to winemaking and brewing to understand how specific genes link to specific variations in phenotype and aroma production (Table 1). Making direct linkages between -omics data and the production of secondary aromatic metabolites is a novel approach to improving flavor production in industrial fermentation.

Systems biology approaches have been used to explore the link between yeast gene expression and metabolomics but the majority of these utilized the lab strain S288c, which lacks a number of genes found in wine, ale and lager yeast strains of *S. cerevisiae* (Dunn et al. 2005). Genetic dissimilarity between laboratory and industrial strains are also due to hybridization, introgression and copy number variation (Dunn et al. 2012). Differences in secondary metabolite

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334 composition is observed when industrial strains are used in fermentation, suggesting that genetic
335 differences between lab and industrial strains account for the differences in secondary metabolite
336 production (Howell et al. 2005, Rossouw et al. 2008, Richter et al. 2013). The majority of
337 transcriptomic studies that analyzed the impact of wine-relevant environmental alterations to
338 yeast fermentation are limited by the use of only a small number of industrially-relevant strains
339 (Rossouw et al. 2008, Steyer et al. 2012, García-Ríos et al. 2014). Thus, a broader assessment of
340 commercially relevant *S. cerevisiae* strains is required to fully elucidate the regulation of flavor
341 compounds produced during fermentation.

342 Transcriptomics studies of *S. cerevisiae* have yielded some success in understanding the
343 linkage between gene expression and the production of specific aromatic compounds. Many
344 studies have explored genome-wide changes in gene expression by using microarray, serial
345 analysis of gene expression (SAGE) and direct RNA sequencing (RNAseq) (James et al. 2003,
346 Rossouw et al. 2008, Rossouw and Bauer 2009, Rossouw et al. 2010) (Table 1).

347 Rossouw *et al.* 2008 analyzed the transcriptome and metabolome profiles of five
348 industrially relevant wine strains to predict the impact of individual gene expression on aroma
349 production (Rossouw et al. 2008). Five genes were selected for overexpression in one yeast
350 strain based on a model built from transcriptomic data and the predicted contribution to
351 production of higher alcohols and esters. Four of the five overexpressed genes showed
352 substantial changes in aroma profiles that had statistically significant alignment with the
353 aromatic changes predicted by the models. This study illustrates how a systems biology approach
354 can guide genetic manipulation of wine sensory characteristics (Rossouw et al. 2008).

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Several -omics approaches have also been applied to brewing to analyze the two types of yeast used for beer: top fermenting *S. cerevisiae* (ale/weiss) or bottom fermenting *S. pastorianus* (lager) (James et al. 2003, Pham et al. 2006). These studies have been limited by the use of *S. cerevisiae* arrays for both ale and lager yeast. Due to the hybrid nature of lager strains (Smart 2007) lager-specific microarray chips are required for a more rigorous transcriptomic analysis of lager yeast.

In the brewing studies, global transcription, amino acid uptake and production of nine volatile compounds were monitored during wort fermentation using *S. cerevisiae* strain S81 or *S. pastorianus* strain S23 (Procopio et al. 2014). Both the sequential uptake of amino acids and the aroma profiles differed between the two strains indicating that these two facets may be linked. The authors suggested that differences in gene transcription, specifically amino acid permeases, affected the uptake of amino acids and the subsequent production of higher alcohols (Procopio et al. 2014). Global transcription patterns coupled with aroma production may help identify genes and metabolites involved in aroma production; however, the hypotheses generated must be tested to confirm a true linkage. For example, juice manipulation experiments were conducted in Sauvignon blanc must to validate hypotheses generated from a metabolomics study (Pinu et al. 2014). The juice manipulation studies confirmed the predicted hypotheses and drew causal relationships between specific metabolites and the production of 3-MH, 3-MHA and 4-MMP (Pinu et al. 2014). Confirmation of the linkage between specific genes and aromatics can then be used to build predictive models for altering aroma production during fermentation.

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Conclusions

Here we describe how secondary metabolism, starting material, carbon and nitrogen profiles and the fermentation environment affect the production of secondary metabolites produced by *S. cerevisiae* during industrial fermentation. These variables can all be manipulated to produce higher yields of desirable flavor compounds. The power of systems biology can be paired with synthetic biology to improve the yield and quality of targeted secondary metabolites in the cosmetic, perfume and biofuels industries. In the food and beverage industries systems biology can be used to map biochemical pathways but innate genetic variability must be exploited to enhance flavor production. The sheer amount of genomic and experimental data in conjunction with *S. cerevisiae*'s long history in industrial fermentation make it an ideal target for improving and enhancing its application in biotechnology, particularly for the production of small molecules.

Systems biology approaches applied to *S. cerevisiae* fermentation have focused on differential gene expression (Rossouw and Bauer 2009), correlation of gene to protein expression (Rossouw et al. 2010), differentiating phenotype of industrial strains (Rossouw et al. 2008, Rossouw and Bauer 2009) and linking gene and protein expression to the production of secondary aromatic compounds (Rossouw et al. 2008, Rossouw et al. 2010). Despite this volume of work we are only beginning to understand how altering the fermentation environment results in an altered secondary metabolism of *S. cerevisiae*.

The bulk of literature in which systems biology methods are applied to fermentation rely heavily on laboratory strains of *S. cerevisiae*; however, many industrial yeast strains are hybrids of *S. cerevisiae* and other *Saccharomyces* species or contain genes absent in lab strains.

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Continued investigation of industrial strains, and non-*S. cerevisiae* yeast, will uncover genes and pathways responsible for regulating the production of secondary aromatic compounds. Ultimately, integrating multiple -omics approaches will lay the foundation for predicting the types and concentrations of flavor-relevant metabolites produced when fermentation conditions are altered. This approach will provide the framework for tailoring industrial fermentation to produce the highest quality and yield of metabolites using *S. cerevisiae* across a range of industries.

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Table 1 Omics approaches applied to *Saccharomyces cerevisiae* fermentation and flavor production

Reference	Genomic	Transcriptomic	Metabolomic
Howell KS, Cozzolino D, Bartowsky EJ et al. Metabolomic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. FEMS Yeast Res 2006; 6: 91-101.			√
Marullo P, Aigle M, Bely M, et al. Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine *Saccharomyces cerevisiae* strains. FEMS Yeast Res 2007; 7: 941-952.	√		
Rossouw D, Næs T and Bauer FF Linking gene regulation and the exo-metabolome: a comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. BMC Genomics 2008; 9: 530.		√	√
Yoshida S, Imoto J, Minato T, et al. Development of bottom-fermenting *Saccharomyces* strains that produce high SO2 levels, using integrated metabolome and transcriptome analysis. Appl Environ Microbiol 2008; 74: 2787-2796.		√	√
Rossouw D and Bauer FF Comparing the transcriptomes of wine yeast strains: toward understanding the interaction between environment and transcriptome during fermentation. Appl Microbiol Biotechnol 2009; 84: 937-954.		√	
Rossouw D, Olivares-Hernandes R, Nielsen J, et al. Comparative transcriptomic approach to investigate differences in wine yeast physiology and metabolism during fermentation. Appl Environ Microbiol 2009; 75: 6600-6612.		√	√
Rossouw D, Jacobson D and Bauer FF Transcriptional regulation and the diversification of metabolism in wine yeast strains. Genetics 2012; 190: 251-261.		√	
Celton M, Sanchez I, Goelzer A, et al. A comparative transcriptomic, fluxomic and metabolomic analysis of the response of *Saccharomyces cerevisiae* to increases in NADPH oxidation. BMC Genomics 2012; 13: 317.		√	√
Procopio S, Brunner M and Becker T Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation. Eur Food Res Technol 2014; 1-19.		√	
Silva Ferreira AnCs, Monforte AR, Teixeira CS, et al. Monitoring alcoholic fermentation: an untargeted approach. J Agric Food Chem 2014; 62: 6784-6793.			√
Pinu FR, Edwards PJ, Jouanneau S, et al. Sauvignon blanc metabolomics: grape juice metabolites affecting the development of varietal thiols and other aroma compounds in wines. Metabolomics 2014; 10: 556-573.			√

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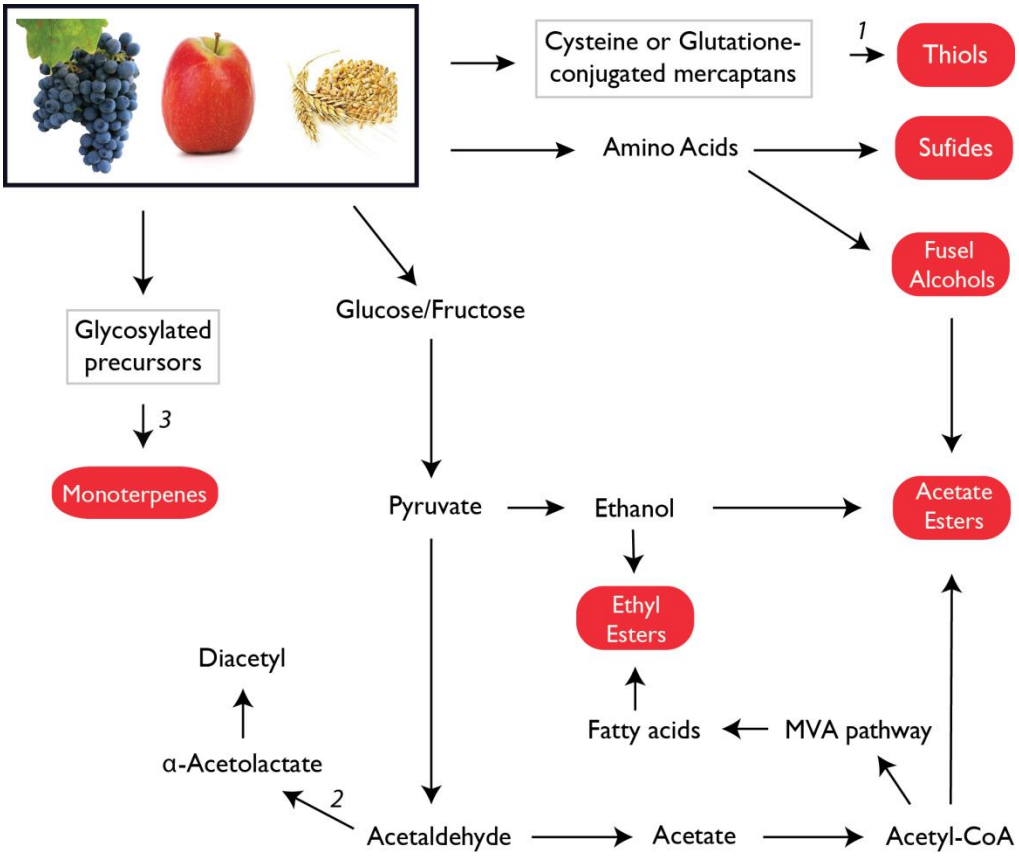

Figure 1 Aromatic metabolites produced by *S. cerevisiae* during fermentation (red boxes). Non-volatile precursor metabolites released during fermentation (gray boxes). Cysteine or glutathione-conjugated mercaptans are found in grapes and hops; glycosylated precursors are found in fruit, but not grain. Italicized numbers represent enzymes catalyzing those reactions. *1*:Irc7 and Str3, *2*: Ilv2 and *3:* Exg1.

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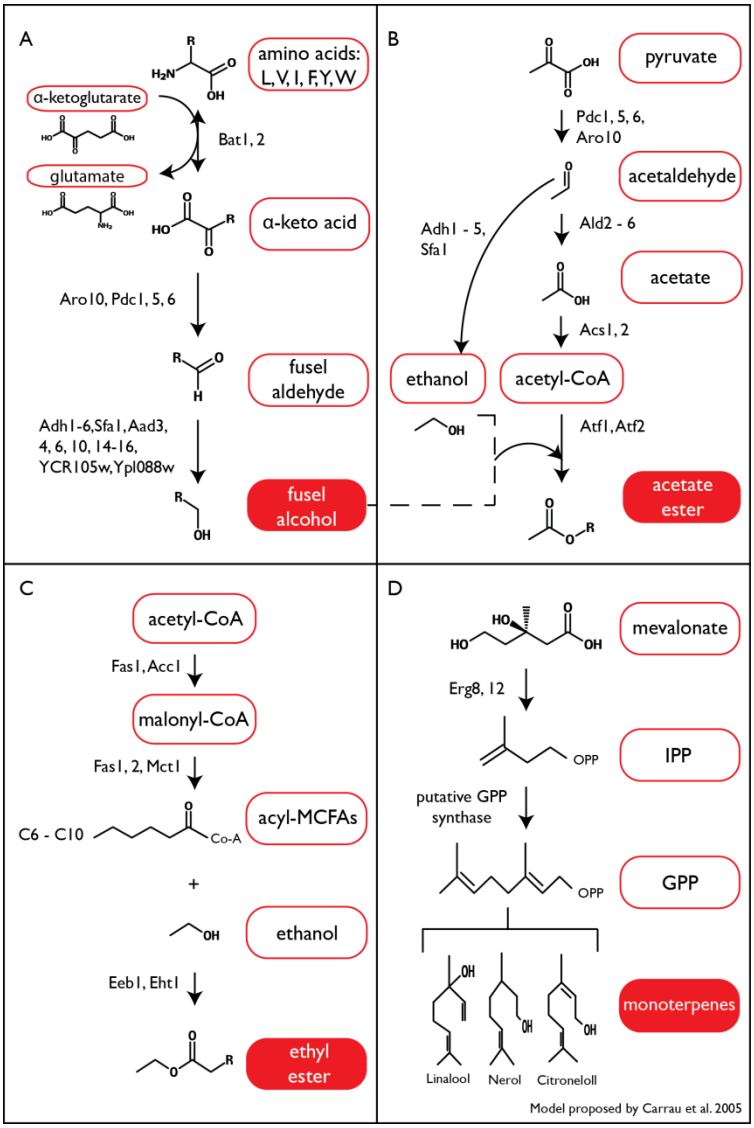

Figure 2 Detailed metabolic pathways depicting the reactions leading to the formation of aromatic compounds (Carrau et al. 2005) produced by *S. cerevisiae* during fermentation. (A) The fusel alcohol pathway (Ehrlich Pathway) is the transformation of an amino acid to an alcohol through multiple steps. Amino acids include: leucine (L), valine (V), isoleucine (I), phenylalanine (F), tyrosine (Y) and tryptophan (W). (B, C) The formation of esters requires an alcohol. The alcohol can be either ethanol or a fusel alcohol. (B) The acetate ester pathway. (C) The ethyl ester synthesis pathway. (D) *In vivo* synthesis of monoterpenes through the mevalonate pathway. Red boxes: volatile aroma compounds.

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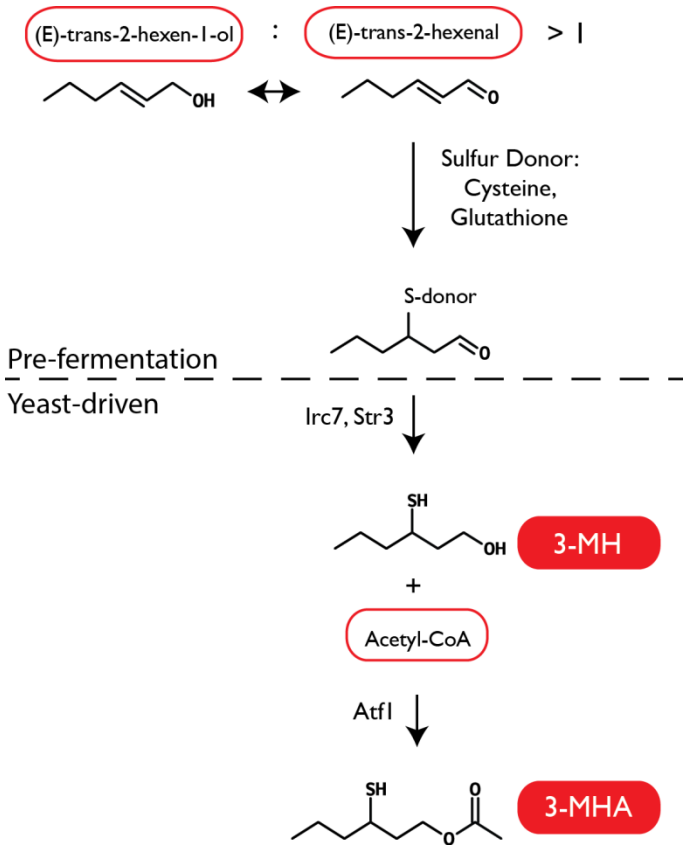

Figure 3 The predicted pathway leading to the production of the aromatic thiols 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA) (adapted from Harsch *et al.* 2013). When the six-carbon (C6) precursors, (E)-trans-2-hexen-1-ol and (E)-trans-2-hexanal have a relative ratio greater than one and a sulfur donor is present, the reaction is driven to produce 3-MH and 3-MHA. When fermentation begins yeast release the aromatic thiol 3-MH. 3-MH can be acetylated to form 3-MHA.

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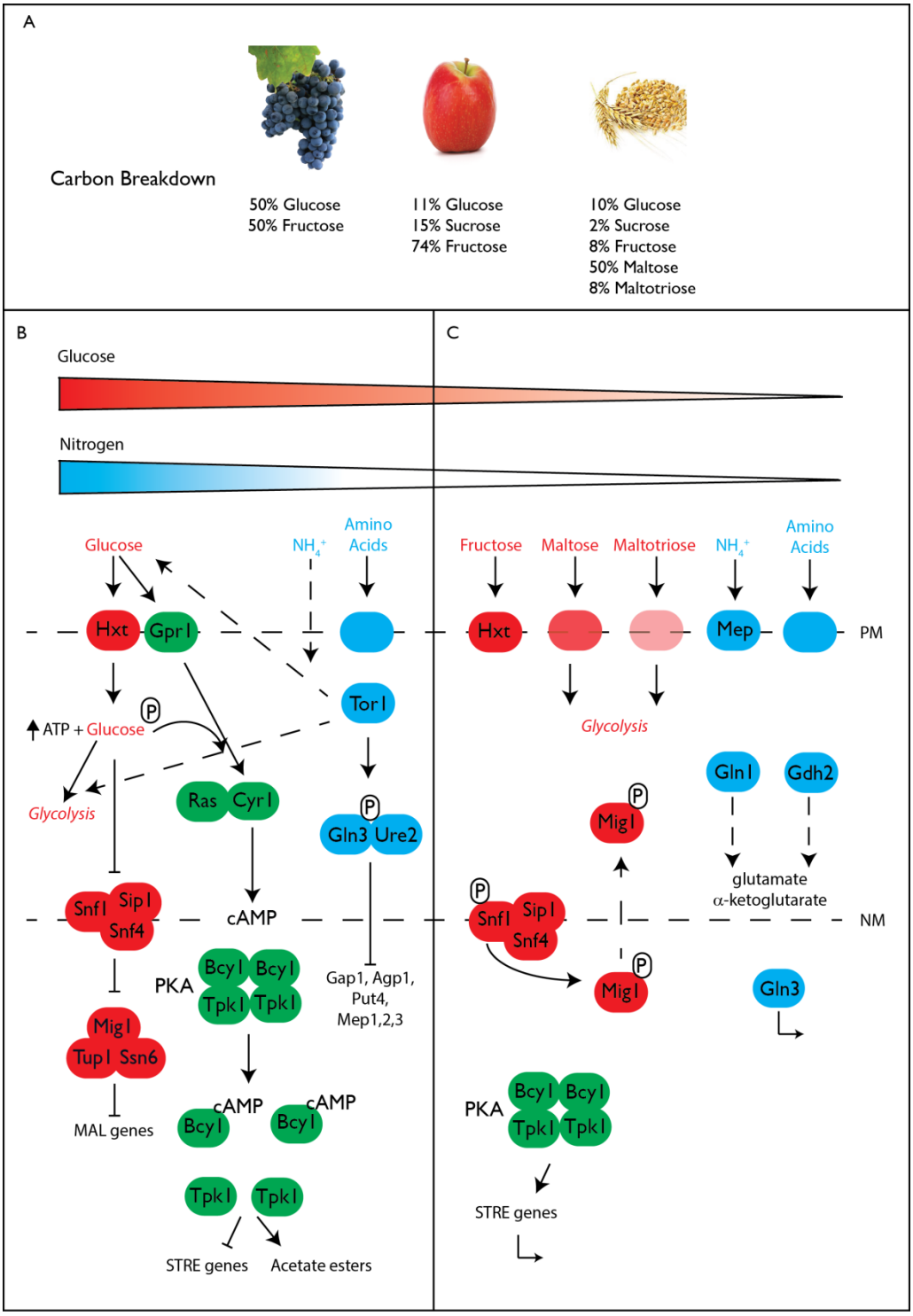

Figure 4 The starting material (A) used in fermentation will affect glucose and nitrogen catabolite repression (B) and de-repression (C). Sugar concentration, the types of sugar and their

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relative ratios will impact the length of catabolite repression and the flavor compounds produced during fermentation. (B) In high sugar environments, glucose is preferentially taken up by the hexose transporters (Hxt) and phosphorylated to glucose-6-phosphate in glycolysis (De Deken 1966). The rise in glucose-6-phosphate and rise of ATP in the cell inactivates the Snf1 complex (Wilson et al. 1996). When Snf1 is inactive, Mip1 moves to the nucleus, recruits the repressors Tup1 and Ssn6 and the MAL genes involved in the uptake of alternative carbon sources are blocked (red proteins and metabolites) (De Vit et al. 1997). Extracellular glucose is sensed by Gpr1 (G-protein-coupled receptor) and Gpa2 (not shown) and a rise in glucose-6-phosphate increases Cyr1 activity (Rolland et al. 2000). The signal creates a rise in cAMP that binds to Bcy1 (of the PKA complex), releasing the Tpk 1 catalytic subunits (Toda et al. 1987). Tpk1 phosphorylates several proteins outside of the nucleus which block the transcription of the stress responsive-element (STRE) genes (ex. Hsp12 and Hsp104) and enhances acetate ester synthesis (green proteins, metabolites) (Thevelein 1994). In the presence of good nitrogen sources, ammonium is taken up in the cell and specific amino acids (glutamatine, glutamate) are taken up preferentially by amino acid permeases. The Tor complex is activated, phosphorylating Gln3, which then recruits the repressor Ure2 effectively blocking amino acid uptake of poorer nitrogen sources (blue proteins and metabolites) (Bertram et al. 2000). Tor1 is linked to carbon metabolism, as it is involved in regulating glucose activation and glycolysis during fermentation (Hardwick et al. 1999) (dashed arrows). (C) In low glucose environments Snf1 and Mig1 are phosphorylated initiating translocation to the cytoplasm (De Vit et al. 1997). The proteins involved in the uptake of alternative carbon sources (maltose and maltotriose) are translated (red proteins and enzymes) (De Vit et al. 1997). Additionally, the PKA complex is not activated, allowing the STRE genes to be expressed (green proteins and enzymes). Low nitrogen conditions do not trigger activation of the Tor complex, allowing Gln3 to activate multiple nitrogen permeases for nitrogen uptake including Mep1,2,3 (ammonium permeases), Gap1, Agp1 (general amino acid permeases) and Put4 (proline specific permease) (Beck and Hall 1999). Gln1p and Gdh2p are translated and involved in glutamate and α-ketoglutarate synthesis, respectively (blue proteins and enzymes) (Miller and Magasanik 1990; Filetici et al. 1996). PM = plasma membrane, NM = nuclear membrane. The sugar transporters are represented by shades of red, darker red represents higher sugar uptake preference.