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Ethyl acetate in ethanol fermented beverages

Elucidation of pathways
and parameters affecting production

Bachelor's thesis in Chemistry
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Acknowledgments

Abstract

In this bachelor thesis, endeavors to elucidate the pathways of ethyl acetate have been conducted and described in ethanol fermented beverages. Results from reviewed studies implies ethyl acetate production during fermentation through biosynthesized pathways in the yeast cells of *Saccharomyces cerevisiae* via enzyme-catalyst esterification by *alcohol acetyl transferases* (AAT) enzymes such as *ethanol acetyl-CoA transferase 1* (Eat1), *acetyl transferase 1* (Atf1) and *acetyl transferase 2* (Atf2), along with indication of *hemiacetal alcohol dehydrogenases* (HADH) activity. Even though the pathways are consistent with previous findings, several emphasize that the enzyme activity and the interaction between the enzymes is still not fully understood.

During the storage process after ended fermentation, oxidation sequences are deemed as the pathway for ethyl acetate production as well as chemical esterification with an acid-catalyst. However, is chemical esterification not determined as significance for the ethyl acetate production during fermentation. In addition are the reactions taking place during the storage process neither fully understood to this date.

This review also describes the importance of parameters' affect on the production of ethyl acetate in order to achieve a beverage with preferred concentration. These parameters include: unsaturated fatty acids, pH, carbon and nitrogen content, temperature, ethanol concentration, storage time, carbon dioxide pressure, interaction of microorganisms and genetic modification.

Sammendrag

Bacheloroppgaven omhandler et forsøkt på å kartlegge reaksjonsveier til etylacetat i etanolfermenterte næringsmidler. Resultatene fra gjennomgåtte studier antyder at etylacetat blir dannet under fermentering ved biosyntetiserte reaksjoner i gjærcellene til *Saccharomyces cerevisiae* via *alkoholacetyltransferase* (AAT) enzymer som katalysatorer, deriblant; *etanolacetyl-CoA-transferase 1* (Eat1), *acetyltransferase 1* (Atf1) og *acetyltransferase 2* (Atf2), sammen med indikasjon på *hemiacetal alkohol dehydrogenase* (HADH) aktivitet. Selv om flere studier viser til funn og støtter nevnte reaksjonsveier, presiserer flere at enzymaktiviteten og interaksjonen mellom enzymer, fortsatt ikke er godt nok forstått.

Under lagringsprosessen etter endt fermentering pekes det mot dannelse av etylacetat fra oksidasjonsreaksjoner så vel som kjemisk esterifisering med en syrekatalysator til stedet. Derimot ansees det at kjemisk esterifisering, ikke er av signifikans for formasjonen av etylacetat under fermenteringsprosessen i tillegg til at reaksjonsmekanismene i sin helhet under lagringsprosessen fortsatt ikke er fullstendig forstått.

Denne bacheloroppgaven beskriver også betydningen av parameternes innvirkning på produksjonen av etylacetat for å oppnå et næringsmiddel med foretrukket konsentrasjon. Disse parameterne inkluderer: umettede fettsyrer, pH, karbon- og nitrogeninnhold i fermenteringsmediet, temperatur, etanolkonsentrasjon, lagringstid, karbondioksidtrykk, interaksjoner fra andre mikroorganismer og genetisk modifikasjon.

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Abbreviations

AAT *Alcohol acetyl transferases*

ABV *Alcohol by volume*

Acs *Acetyl-CoA synthetase*

Adh *Alcohol dehydrogenase*

ADP *Adenosine diphosphate*

Ald *Acetaldehyde dehydrogenase*

AMP *Adenosine monophosphate*

Atf1 *Acetyl transferase 1*

Atf2 *Acetyl transferase 2*

ATP *Adenosine triphosphate*

C:N ratio *Carbon-to-nitrogen ratio*

Eat1 *Ethanol acetyl-CoA transferase 1*

FAN *Free amino nitrogen*

HADH *Hemiacetal alcohol dehydrogenases*

MO *Microorganisms*

NAD$^+$ *Nicotinamide adenine dinucleotide*

NADH *Nicotinamide adenine dinucleotide hydride*

Pdh *Pyruvate dehydrogenase*

PIX *Positional isotope exchange*

TCA-cycle *Tricarboxylic acid cycle*

UFA *Unsaturated fatty acid*

1　Introduction

In nature, esters occur naturally in a wide range, they are often formed during fermentation from fruits and grains.[1] During fermentation, the yeast *Saccharomyces cerevisiae* produces a broad range of volatile compounds where the majority consists of volatile esters, after higher alcohols (fusel alcohols), composition overview in wine shown in Figure 2.1.[2] Even though volatile compounds only are found in trace amounts, they are crucial and conjointly form the complex flavor in alcoholic beverages.[3] Thus, only minor changes in the concentrations of the volatile compounds can make a large impact on the final product. In industry, volatile esters are particular interesting, since they determine the preponderance of fruity odors in beverages.[4,5]

Ester are generally volatile compounds due to their lack of hydrogen bonds, since the oxygen atom does not have any hydrogen bonded to it.[6] As a result, esters have weaker intramolecular bonds compared to their counterparts of acids and alcohols of similar molecular weight. This in turn results in a lower boiling point, i.e., higher vapour pressure.[7] Although, since esters are polar molecules, they can form hydrogen bonds in a solution with water. This makes esters slightly soluble in water, but less soluble than carboxylic acids.[6]

The formation of esters during fermentation is dependent on which strain of yeast is used and parameters affecting the yeast, such as dissolved oxygen, fatty acids, temperature and ratio of assimilable nitrogen- and carbon contents in the medium (the solution in a fermentation tank).[2,4] While these parameters have a vital effect on the ester production, they only inflict minor adjustments to the ester concentrations of the final product.[8] Nevertheless, to not be aware of the effects from the parameters and yeast strain can easily lead to a suboptimal ester balance after the fermentation.

Ethyl acetate is commonly known as the major ester produced during ethanol fermentation and storage in beverages, with its fruity to nail polish odor which is dependent on its concentration in the beverage.[3,9] Ethyl acetate also has multiple industrial applications and can be applied for the synthesis of paints, adhesives, biodiesels, herbicides, and resins, where the latter can further be used with aramid (kevlar), carbon fibers and fiber glass.[10,11] Additionally, the ester is seen as an environmentally friendly solvent with properties like biodegradability, non-toxicity and even medicinal with anti-inflammatory and vasodilative functions.[12,13] Fischer-Speier esterification was the conventional method for industrial production of ethyl acetate through acid-catalyzed esterification, where ethanol and acetic acid reacted under temperature reaching 200–250 °C with concentrated sulfuric acid as the catalyst.[12,14] Due to the process being highly energy consuming and produces hazardous byproducts, biosynthesis of the ester through enzyme-catalyzed esterification have been shown as a more environmentally sustainable and promising alternative.[10,15]

Alcoholic beverages are an intricate mixtures of hundreds of compounds, many of which contribute substantially to a beverage's flavor profile.[16] Alcoholic beverages are produced by ethanol fermentation, where the yeast produces ethanol by fermentation of the available carbon content in the medium.[17] For wine the source of the carbon content is natural sugars in grapes, beer uses grains containing starches that have been converted to sugars by an enzyme called *amylase*,

present in grain kernels. Sake on the other hand, which is a Japanese beverage, uses rice starches that have been converted to sugar by the mold *Aspergillus oryzae* instead of amylase. Generalized, distilled wine is known as brandy, beer as whisky and sake as shōchū. A lesser-known beverage to the western world is baijiu, a traditional Chinese distillate with a over two thousand year old history.[13] The production of baijiu is closely related to shōchū with a higher alcohol by volume (ABV) content of typical 40-60 %. Instead of rice as the main source of carbon content, baijiu uses a combination of rice and a grain cereal called sorghum. Baijiu has an unique flavor where the most important flavor compound in the beverage is ethyl acetate with its characteristic of solvent to a pear-cherry fruity odor.[18,19] In Chinese national standard, ethyl acetate is used as an indicator to judge the baijiu's style and also as a clear quality trait of the product.[20] In addition to the total annual yield of over 12 billion dollar in 2016, it is crucial for the baijiu industry to improve and strengthen the overall quality by improving and controlling the levels of ethyl acetate in the products.[13,20,21] This is due to the baijiu's reputation for often being related to quantity in production over quality in the products. In contrast to baijiu, ethyl acetate levels in wine and beer is preferred to be much lower, often below the recognition threshold.[22–24] Therefore, it is of significance to control the levels of ethyl acetate accordingly to the type of product and consumers which it is aimed at.

In this bachelor thesis it will be reviewed how ethyl acetate is produced during ethanol fermentation, storage and method to control the levels of the ester. In consequence of the time and length that have been assigned to the thesis, limitation must be implemented. Firstly the thesis will mainly focus on the ester ethyl acetate, which is just one of the hundreds of volatile compounds contributing to the flavors in alcoholic beverages. Secondly, the ethanol fermentation of the yeast *Saccharomyces cerevisiae* in batch culture, will be simplified to its species and not focused on the yeast's strains as well as fusel alcohols, analytic methods and finally only briefly focused on other yeast species and genetic modification.

2 Theory

2.1 Sensation of flavors, thresholds and beverage composition

When food or beverages are consumed, the sense of taste (gustatory) and smell (olfactory) with nerves in the mouth and nose receives simultaneously stimuli from receptor cells.[25] Flavors are defined as a complex combination of the olfactory, gustatory and nerve sensations perceived during tasting,[26] i.e., flavor is a multiple sensory sensation that combines individual stimuli to give a synergistic perception of a compound. An example of this is how vanillin (molecule with vanilla flavor) with other sweeteners added, will intensify the smell of vanillin. As well as how the addition of vanillin to sweeteners can enhance the perception of sweetness.[27,28]

A threshold of an odor is divided in a stimulus threshold and a recognition threshold, which it varies depending on the composition of the solution, where pure ethyl acetate in air has a stimulus threshold at 3.6 ppm (volume by volume).[3,26,29] The recognition threshold is the minimum concentration required of a compound to be correctly recognized, whereas stimulus threshold is the minimum concentration required to give rise to a olfactory sensation. Ethyl acetate has a odor characterized as pear drops, cherry or fruity below the recognition threshold (20-100 mg/L), enhancing the beverages collective odor and thus it is quality trait (in wine often referred to as *bouquet*).[9,17,30] On the contrary when ethyl acetate is above the recognition threshold (>160 mg/L) it usually give off an off-odor of solvent or nail polish, which is deemed as spoilage of the odor's floral complexity.[31–33] While high concentrations of ethyl acetate is considered as a spoilage in wine and beer, it is deemed as a quality trait

in baijiu with concentrations comparison an order of magnitude higher (>2000 mg/L).[13,34] An overview of reported threshold values and ethyl acetate concentration from previous studies in different beverages is shown in Table 2.1, 2.2 and 2.3.

Table 2.1: Reported values of stimulus thresholds for certain alcoholic beverages.

Stimulus threshold	
Beverage	Ethyl acetate [mg/L]
Beer	5-10[35]
Wine	12-14[36–38]
Ethanol 20 %	7.5[9]
Spirit 34 %	17[9]

Table 2.2: Reported values of recognition thresholds for certain alcoholic beverages.

Recognition threshold	
Beverage	Ethyl acetate [mg/L]
Beer	25-50[35]
Wine	60-160[16,31,39]
Ice wine	198[32]

Table 2.3: Reported values of ethyl acetate concentration in certain alcoholic beverages.

Concentration	
Beverage	Ethyl acetate [mg/L]
Beer	8-32[3]
Wine	30-60[16]
Whisky	112[9]
Baijiu	>2000[13]

As mention, deviations in thresholds values varies depending on the composition of the beverage, but also as a consequence of human factors and subjectivity.[40] Subsequently are there no successful universal classification of odors that ascribe words corresponding to e.g., sweet and bitter.[41] The best alternatives are words like flowery, fruity or foul, which are largely subjective.

For a more visual example of a beverage's composition, Figure 2.1 shows the composition of the major components in an average wine.[16]
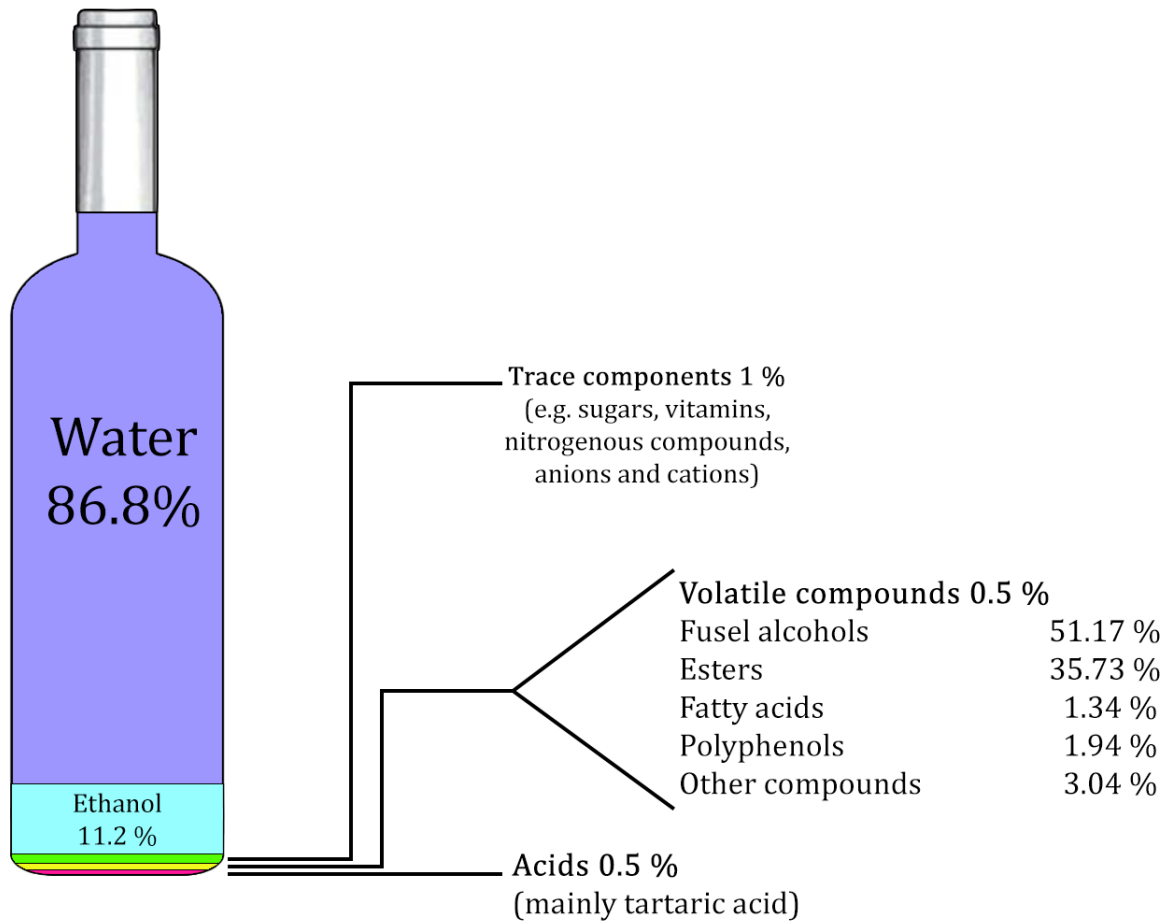

**Figure 2.1:** The average composition of a wine's major components, figure adopted.[16]

2.2 Ethanol fermentation

For millennia, alcoholic beverages have been an integrated part of the human culture.[42,43] Although alcoholic beverages have been a close companion to humans since early Neolithic period (7000–5000 BCE), it would not be until the seventeenth century that its protagonist would be discovered by Leeuwenhoek.[44,45] That being the microorganisms (MO), more precisely a yeast, an eukaryotic single-celled fungus. A century after Leeuwenhoek, the reaction between sugar, ethanol and carbon dioxide (CO_2) within the fermentation process was described by Lavoisier.[46,47] Much like how Lavoisier described the fermentation process in the eighteenth century, is still how

the metabolic process is simplified and explained today; with the yeast turning fermentable sugar in form of glucose into ethanol and CO_2, shown in Scheme 1. The most well-known yeast species used for ethanol fermentation is *Saccharomyces cerevisiae*, which is also known by it is more common name, brewer's yeast.[48] Even though *S. cerevisiae* is the most commonly used, there are thousands of varieties and strains of the yeast, some carefully cultivated others genetic modified to give a selection of desirable flavors to beers, wines and spirits.[17,21]
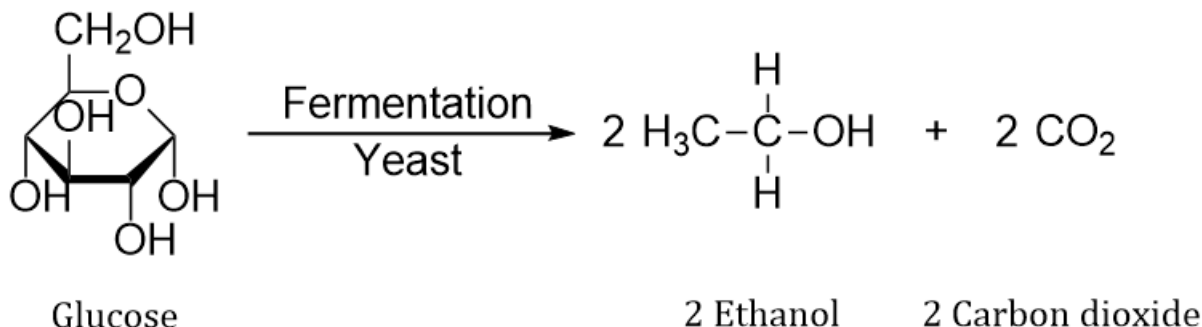

Scheme 1: Simplified scheme of the ethanol fermentation process, one molecule of glucose converted though fermentation to two ethanol and two CO_2 molecules, scheme adopted.[17]

S. cerevisiae is a facultative anaerobic MO, meaning it can produce energy in form of adenosine triphosphate (ATP) in both aerobic- (presence of oxygen) and anaerobic environments (absent of oxygen).[17] In aerobic environments the yeast produce ATP through aerobic respiration, while in anaerobic conditions it will turn to fermentation. Fermentation is a metabolic process where fermentable sugar is converted into metabolites such as esters, acids, gases and alcohols, along with other compounds, shown in Scheme 2.

The first step of the metabolic process regardless of whether it is anaerobic or aerobic, is glycolysis.[49] Glycolysis is a catabolic process that breaks down a molecule of glucose through ten enzymatic steps into two molecules of pyruvate, 2 ATP and 2 nicotinamide adenine dinucleotide hydride (NADH) molecules. For glycolysis to continue, NADH needs to be oxidized by an electron acceptor to be regenerate as nicotinamide adenine dinucleotide (NAD^+). In aerobic respiration, this would take place in the electron transport chain, however in ethanol fermentation, NADH reduces the derivative of pyruvate, acetaldehyde, which in turns regenerate NAD^+ and produces ethanol from acetaldehyde catalyzed by the enzyme *alcohol dehydrogenase* (Adh) .[49]
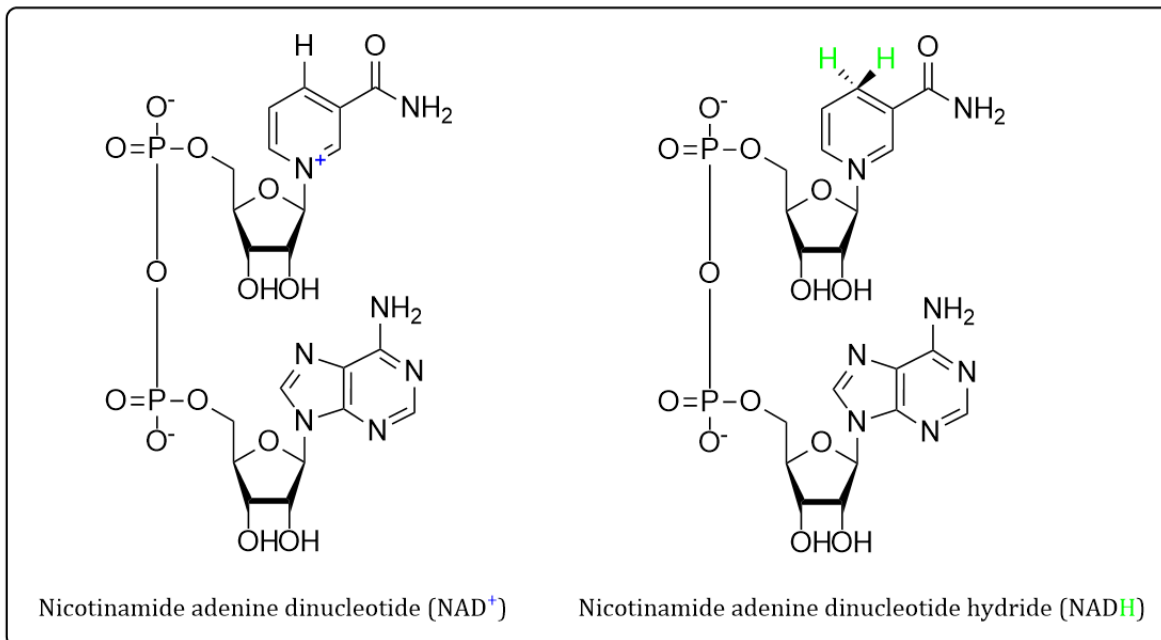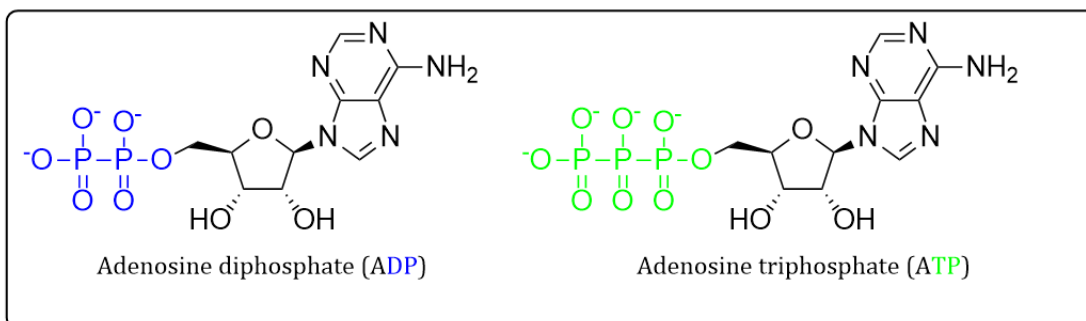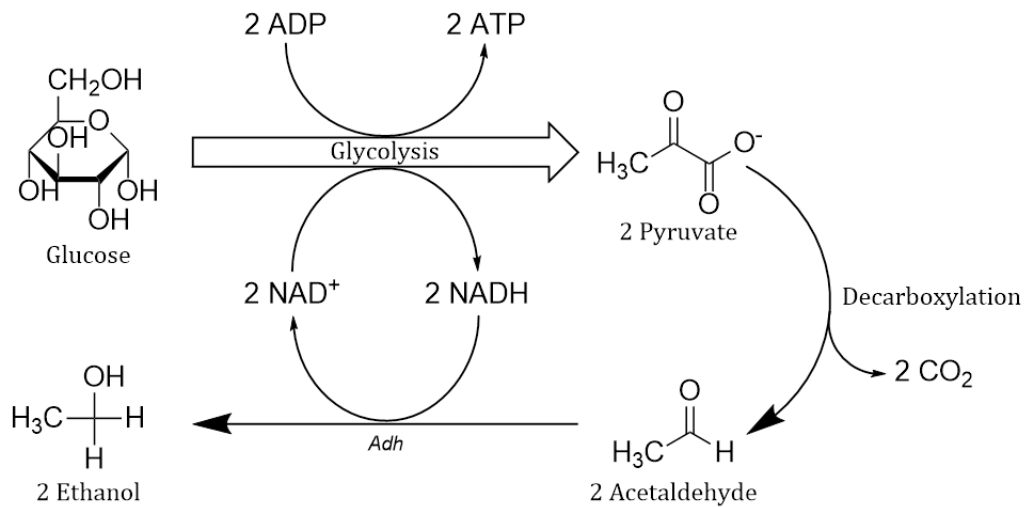
Scheme 2: The main steps in the ethanol fermentation process, scheme modified and adopted.[17,49]

2.3 Biosynthesized esterification

Ethyl acetate is the most prevalent ester within ethanol fermentation due to the large quantities of ethanol that is present, formed by the yeast.[31] Not only because ethanol is a substrate for the esterification of ethyl acetate, but also since primary alcohols are more reactive than secondary (R_2-CH-OH) and tertiary alcohols (R_3-C-OH). In addition to ethanol, a substantial amount of other different alcohols and acids are also formed. In turn, this lays the foundation for a widespread diversity of ester formations.

Ethyl acetate can be biosynthesized by fermenting yeast cells in an enzyme-catalyzed intracellular reaction.[50] Scheme 3 shows potential pathways of the ethyl acetate formation in a yeast cell's cytosol and mitochondrion. The last step in the formation of ethyl acetate from Scheme 3 is shown more in details in Scheme 4. During an anaerobic environment following the blue pathway, ethanol is formed, as earlier illustrated in Scheme 2. In the step before ethanol formation, acetaldehyde can through the pathway to the right rather be oxidized by NAD^+ to acetate, catalyzed by *acetaldehyde dehydrogenase* (Ald), than being reduced by NADH to ethanol.[18,49–52] In addition, acetate forms acetyl-coenzyme A (acetyl-CoA) via hydrolysis of ATP to adenosine monophosphate (AMP), catalyzed by *acetyl-CoA synthetase* (Acs). The last step in anaerobic formation of ethyl acetate is with the substrates ethanol and the coenzyme acetyl-CoA, where the latter is required by an *acetyl transferase* (Atf) enzyme to function as a catalyst.[8] Atf enzymes belongs to a group called *alcohol acetyl transferases* (AAT), that are the main ethyl acetate producing enzymes within yeast cells.[51] Furthermore, it is specifically the enzymes *acetyl transferase 1* (Atf1) and *acetyl transferase 2* (Atf2) that are responsible for 50 % of the formation of ethyl acetate, where Atf2 plays a minor role compared to Atf1.[8] The enzyme responsible for the remaining 50 % of ethyl acetate was unknown until 2017 and is putatively the mitochondrial enzyme *ethanol acetyl-CoA transferase 1* (Eat1) also belonging to the AAT group.[53] In an aerobic environment (pathway to the left, Scheme 3), pyruvate is transported to the mitochondrion, Acetyl-CoA is then formed from pyruvate through decarbonization catalyzed by *pyruvate dehydrogenase* (Pdh), which is then oxidized in the tricarboxylic acid cycle (TCA-cycle) to CO_2.[54] By contrast, if acetyl-CoA should not enter the TCA-cycle and accumulate in the mitochondrion, acetyl-CoA would react with ethanol transported from the cytosol, catalyzed by Eat1 and form ethyl acetate to reduce the amount of acetyl-CoA in the mitochondrion and regenerate free CoA.[51,55]
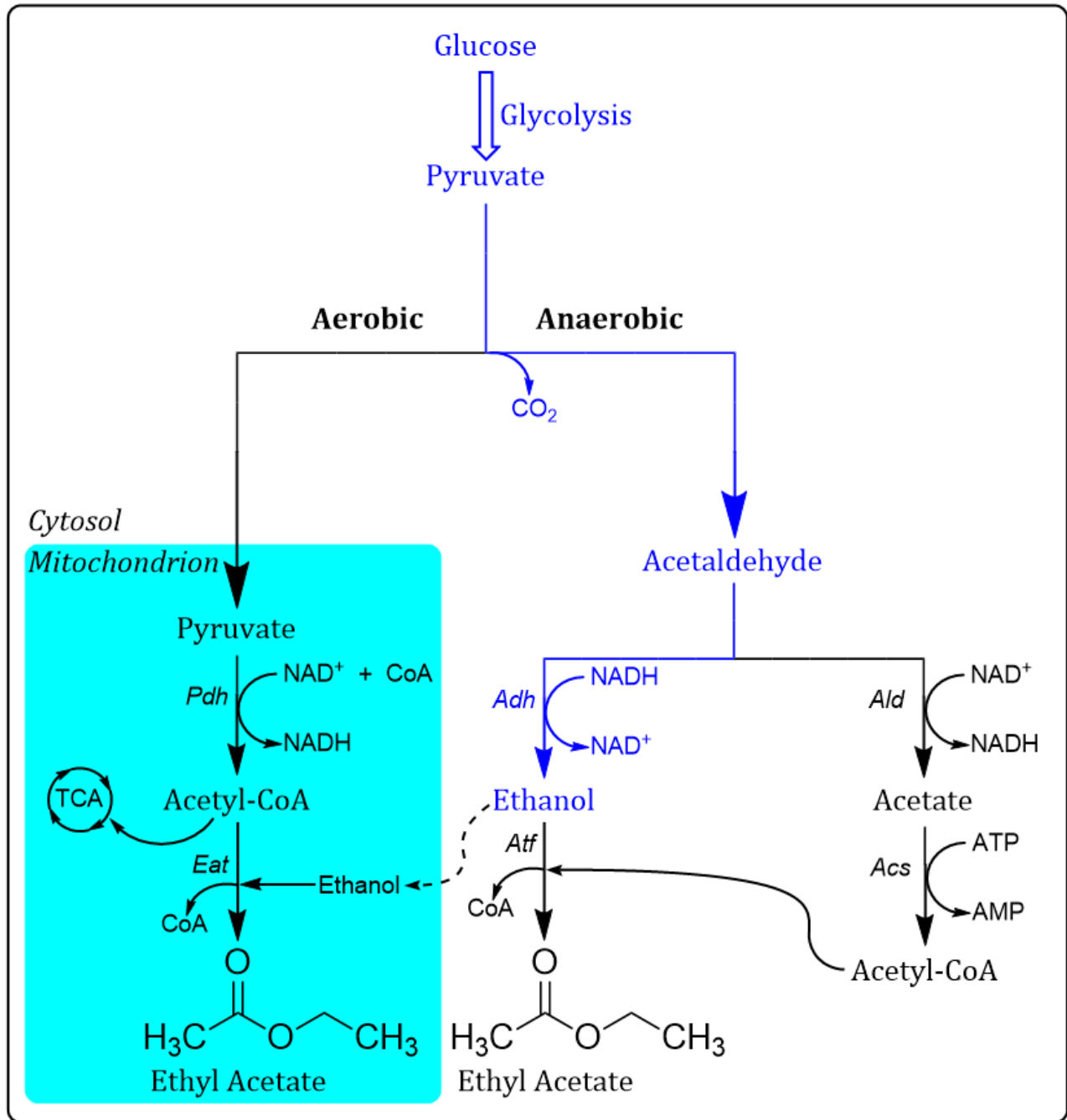
Scheme 3: Potential pathways of ethyl acetate formations within the yeast cell, pathway in blue are the steps shown from Scheme 2. The cell's cytosol is represented as a squircle[56] and the mitochondrion are colored within the cytosol as turquoise, scheme modified and adopted.[1,12,51,53]
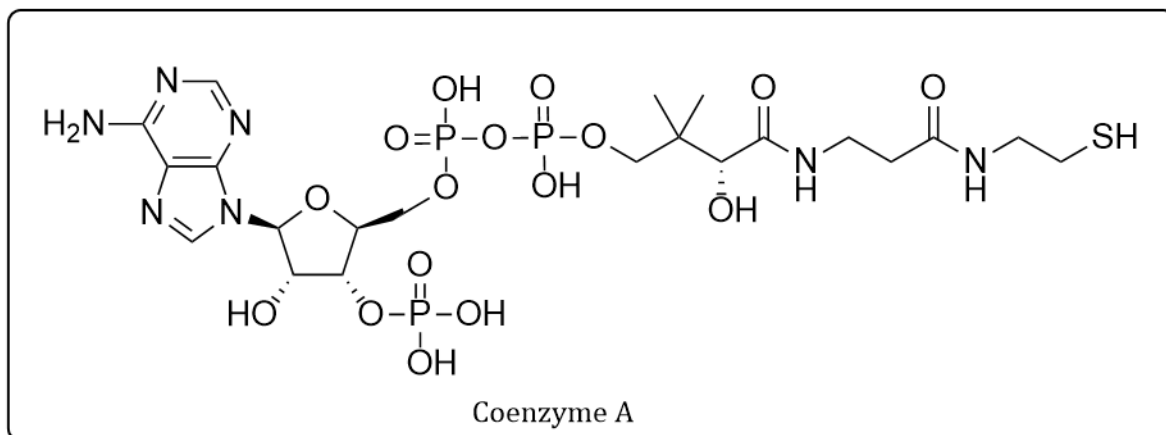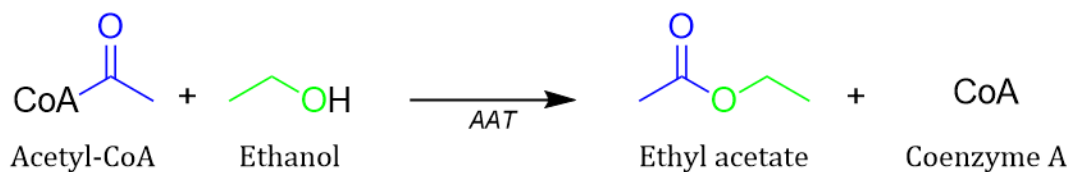
Scheme 4: Formation of ethyl acetate through enzyme-catalyst esterification by AAT, scheme modified and adopted.[53,57]

Esterase is an enzyme that can catalyse hydrolysis reaction and form an alcohol and an acid from an ester in aqueous conditions.[58] As a consequence of their abundance in all domains of life, reverse esterase have been suggested as a pathway of ethyl acetate formation as well as *hemiacetal alcohol dehydrogenases* (HADH). Hemiacetals are formed by an addition reaction from ethanol and acetaldehyde, where HADH can oxidize hemiacetals to form ethyl acetate. The enzymatic reactions' change in free energy (ΔG_r) is listed in Table 2.4.

Table 2.4: The presumably enzymatic reactions forming ethyl acetate in the yeast cells. The reported values for change in free energy (ΔG_r) of the reactions were calculated by Kruis et al.[53]

Enzymatic reactions	ΔG_r [kJ/mol]
Reverse esterase	28.8
HADH	-18.2
AAT	-18.3

2.4 Chemical esterification

Esters are chemical compounds that contains an ester functional group (R^1-COO-R^2).[59] Industrially produced esters have usually been derived by reacting a carboxylic acid and alcohol with a strong acid as a catalyst.[60] Due to the formation of an intermediate during the esterification reaction an isotopic labeling or oxygen-isotope scrambling method,[61] called *positional isotope exchange*

(PIX), has been used for the elucidation of the mechanism.[62,63] Alcohol containing ^{18}O-isotope have been used and further analyzed with mass spectrometry to determine the reaction mechanism. It reveals that it is the hydroxyl group from the alcohol and not from carboxylic acid that's in the ester product. Acid-catalyzed esterification is shown in Scheme 5, whereas the acid's hydroxyl group (R_1-OH) is marked in blue and the alcohol's oxygen-atom (R_2-O) is marked in green.
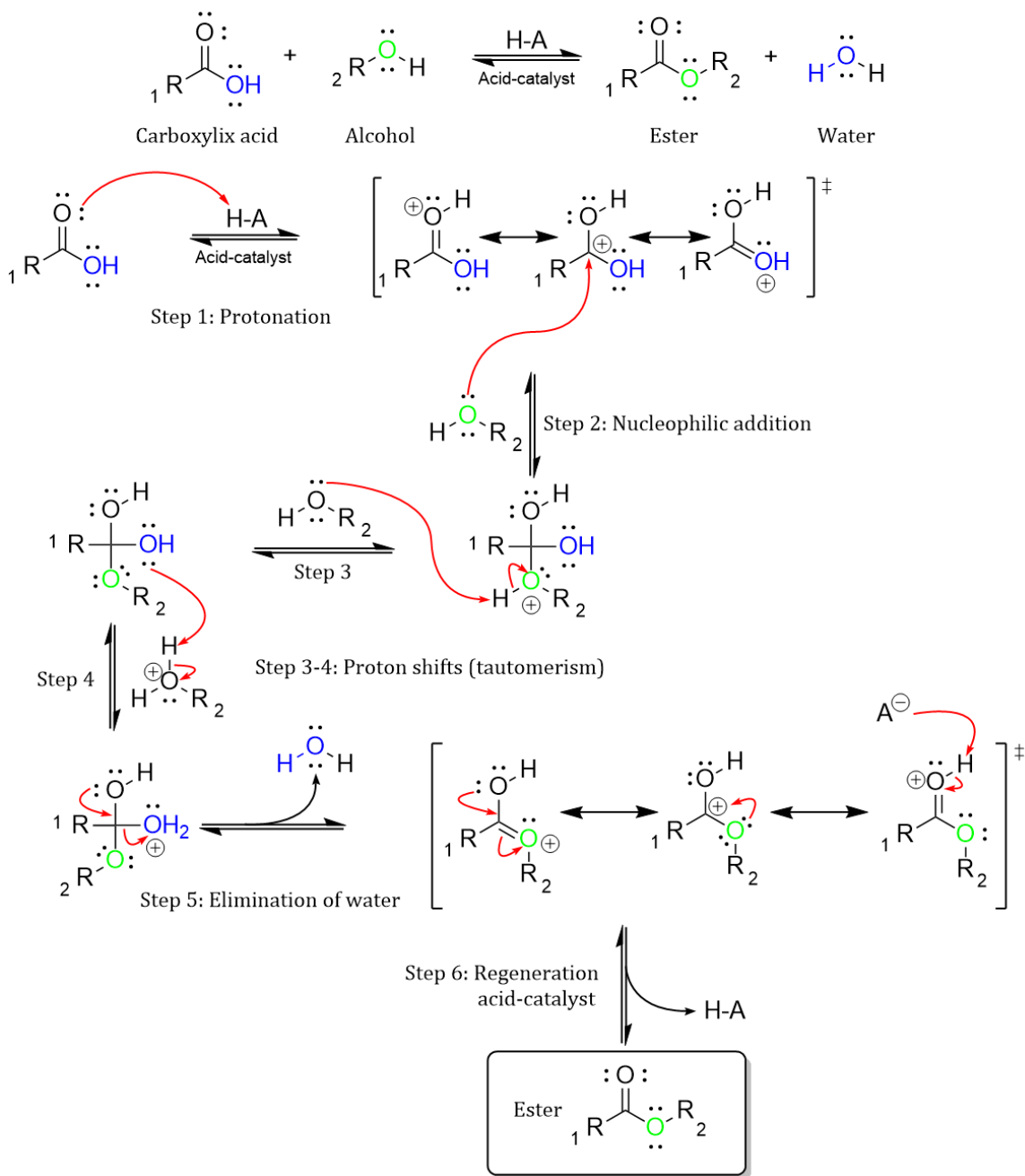
**Scheme 5:** Acid-catalyzed esterification reaction between carboxylic acids and alcohols, scheme modified and adopted.[12,17,34]

Scheme 5 shows an acid-catalyzed esterification reaction which often is called Fischer- or *Fischer-Speier esterification*[64] and is an example of a nucleophilic addition-elimination.[65] In step 1, the acid's carbonyl group is protonated by the acid-catalyst (H-A) making it to a more reactive electrophilic group that favors the nucleophilic attack of the alcohol (R_2-OH) in step 2.[66,67] Step 2 yields a tetrahedral intermediate that undergoes a proton shift with another alcohol through step 3 and 4, followed by step 5 elimination of water as a leaving group containing the acid's hydroxyl group. In step 6 the acid-catalyst (H-A) is regenerated by deprotonation of the intermediate and yielding the ester product.

The reaction is an equilibrium reaction with reversible pathways, where the product tends to spontaneously hydrolyze to regenerate the reactants, called ester hydrolysis.[66] Hence, the difference in the activation energy in either directions is presumably insignificant. Consequently, the accumulation of water from the reaction reduces the yield of the esters over time due to the Le Châtelier's principle.[67] Water and alcohol, in this case ethanol, have a minor difference in pKa values at 15.7 and 16, respectively.[68,69] Additionally the difference in the molecules size is also a factor and makes water a slightly stronger nucleophile than alcohol.[12,70] As a consequence, water will compete with the alcohol, reducing the concentration of acid-alcohol intermediates and thus stagnating the esterification reaction.[71] This resulting in an equilibrium between the alcohol and water, as followed:

$$R\text{-}OH_2^+ + H_2O \rightleftharpoons H_3O^+ + R\text{-}OH \quad (2.1)$$
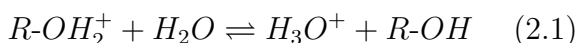
To favor the esterification reaction, the equilibrium may be influenced by either removing one product from the reaction solution or by employing an excess of one reactant. Often the latter is used by adding an excess of alcohol.[67] Removal of water is also possible with a Dean-Stark trap, which collects water through an azeotropic distillation.[72]

In the past decades the industry have seen an increasing transition from chemical to biosynthesized esters, as a results of a more environment sustainable alternative.[10,15]

2.5 Storage

Storage, aging or often called maturation of the beverage is a mandatory step for many alcoholic beverages during production.[9] This is essential for whisky, cognac, most types of red wines and baijiu.[73] The process of storage typical considered the most fundamental step for evolving the beverages flavor profile, however long storage of white wine and some red wine can cause the bouquet's fruitiness to decrease.[31] The development of new flavors and diminishing of others is due to interaction of chemical reactions, such as esterification, oxidation and hydrolysis.[73] Per date the chemical reaction mechanisms occurring during storage have yet to be determined. Concentration of ethyl acetate during storage over a period of 6 years can be seen listed in Table 2.5.

Table 2.5: Concentration of ethyl acetate in whisky in once-used American bourbon barrels and baijiu in unknown container during storage over 6 years.

Concentration during storage [mg/L]			
Beverage	0 year	3 years	6 years
Whisky[9]	148	411	523
Baijiu[73]	1900	750	2300

2.6 Genetic modification and other microorganisms

Genetic modification of MO has seen a rapid rise the last decades and modifications on yeasts have been no exception.[3,16] The contribution of genetic modification on *S. cerevisiae* have helped to elucidate which enzymes is responsible for the production of ethyl acetate, thereby deleting or overexpressing certain genes encoding for AAT enzymes.[74] Modification has also been done to enhance MO ability to either produce metabolites or resistance to certain environments.[12] As a case in point, a strain of *S. cerevisiae* was modified for improved ethyl acetate production by overexpressing certain genes to accumulate a high amount of acetyl-CoA in the cell and ending with a yield over 60 times higher than the original strain at 610.26 ($\pm$14.28) mg/L.[21] Other relevant yeasts spices used for ethyl acetate production either alone or in a simultaneous fermentation with *S. cerevisiae* are among others, *Kloeckera apiculata*, *Candida krusei* and *Hansenula anomala*.[36–38] Reported yields from the yeasts is gathered and shown in Table 2.6.

Table 2.6: Reported levels of ethyl acetate produced by different yeast species during ethanol fermentation and genetic modified stain of *S. cervisiae* for improved ethyl acetate production.

Species	Ethyl acetate produced [mg/L]
Saccharomyces cerivisiae	10-100[36–38]
Genetic modified *S. cervisiae*	610.26 ($\pm$14.28)[21]
Kloeckera apiculata	25-375[36–38]
Candida krusei	220-730[36–38]
Hansenula anomala	137-2150[36–38]

2.7 Parameters affecting ethyl acetate production

Production of ethyl acetate can be affected in multiple ways and the most intuitive is by the amount of substrates (ethanol and acetyl-CoA) in biosynthesized- or reactants (ethanol and acetic acid) in chemical esterification, as well as the reactions equilibrium. During fermentation, aeration of the medium is important for growth, but can as well lead to more O_2 and CO_2 in the medium than desirable. Where O_2 can induce unsaturated fatty acid (UFA) growth and CO_2 can inhibit the enzymatic activity of AAT.[1]

The level of pH is crucial for the yeast's metabolism, e.g., correct growth and for amino acids to function properly.[9,17] The right temperature is also profound as a parameter for the yeast's metabolism. The rate of metabolism increases by temperature, but so does the amount of different byproducts as well.[75] At temperature above 40 °C the yeast stop producing ethanol. How pH and temperature affect the production of ethyl acetate is shown respectively, in Table 2.7 and 2.8.

Table 2.7: The effect of pH on the formation of ethyl acetate, showing initial values from the start of fermentation and final values in parentheses.[9,76]

	pH, Initial (final)					
	4 (3.1)	4.5 (3.5)	5 (3.8)	5.5 (4.2)	6 (4.4)	7 (5.1)
Ethyl Acetate [mg/L]	31	39	48	48.5	48	42

Table 2.8: The effect of temperature on the formation of ethyl acetate with open and closed fermentation tank.[9,76]

	Temperature, open [°C]				Temperature, closed [°C]			
	20	25	30	35	20	25	30	35
Ethyl Acetate [mg/L]	30.5	50.1	59.7	40.9	29.4	44.9	57	42.5

Parameters that have been evidently shown to affect ethyl acetate production are among others; the concentration of UFA, the carbon-to-nitrogen (C:N) ratio in the fermentation medium and fermentation temperature, shown respectively in Figure 2.2, 2.3, 2.4 and 2.5.[2]

By adding UFAs to ethanol fermentation Figure 2.2 shows a decrease in ethyl acetate production of 37 % compared to a standard batch as blank sample.[2]
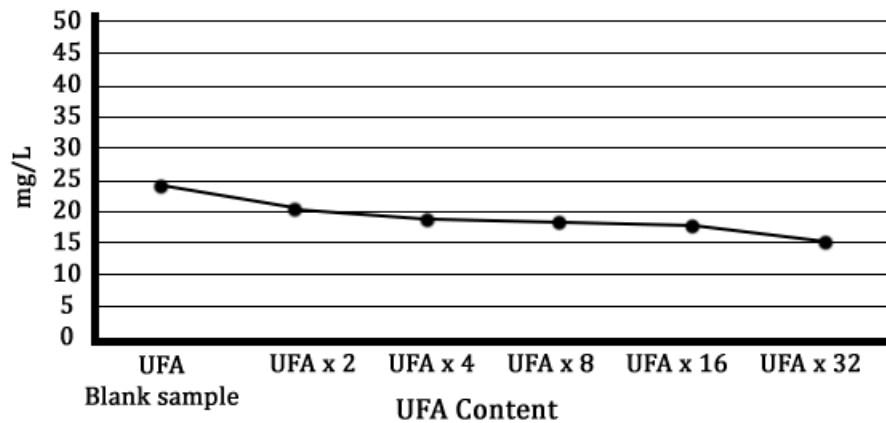
Figure 2.2: Effect on ethyl acetate production from the amount of UFA in the medium. The blank sample of UFA consisted of 0.34 mg/L oleic acid, 1.82 mg/L linoleic acid, and 0.50 mg/L linolenic acid, figure adopted.[2]

The carbon content is the total content of fermentable sugar in the medium, i.e., the amount of sugar which can be assimilated by the yeast. By increasing the carbon content ethyl acetate had an increase of 50 %.[2]
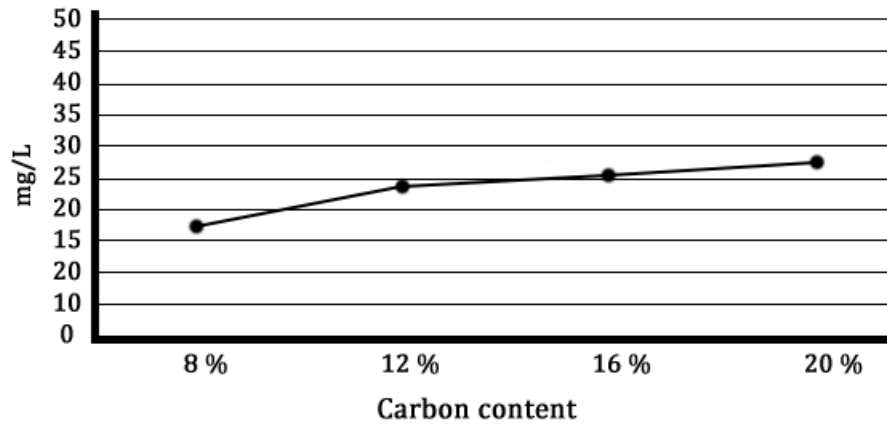
Figure 2.3: Effect on ethyl acetate production from the amount of carbon content in the medium, figure adopted.[2]

The nitrogen content assimilated by the yeast is called *free amino nitrogen* (FAN) and consists of short peptides and individual amino acids.[77] In beer fermentation, FAN is produced by the hydrolysis of the malt protein by proteolytic enzymes.[78] An increase in available nitrogen resulted in an increase of about 30 % ethyl acetate.[2]
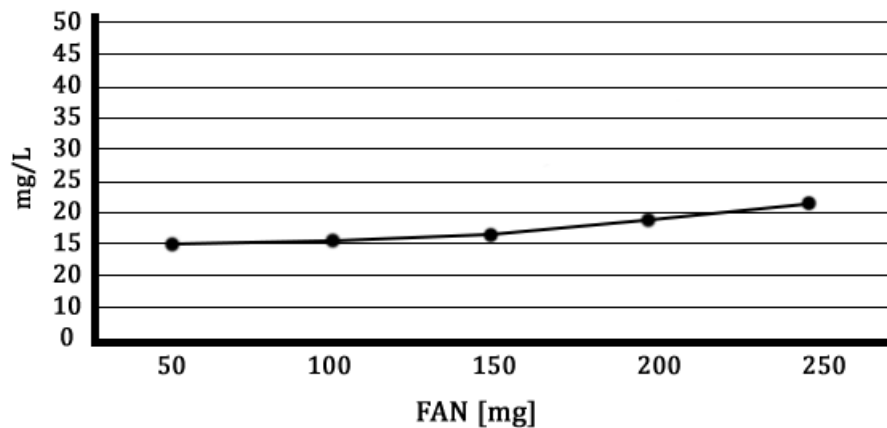
Figure 2.4: Effect on ethyl acetate production from the amount of FAN in the medium, figure adopted.[2]

Additionally production gradually increased with increasing fermentation temperature.[2]
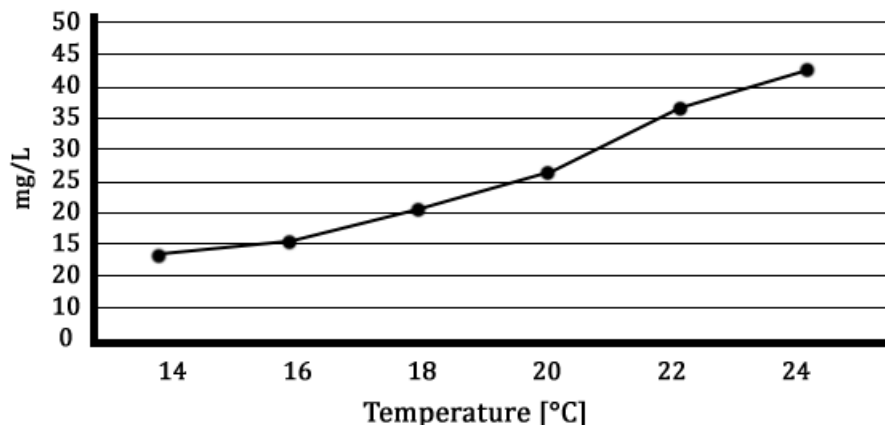
Figure 2.5: Effect on ethyl acetate production from the fermentation temperature, figure adopted.[2]

An overview of parameters from previous studies as well as their optimal value for highest ethyl acetate production is shown in Table 2.9.[2,9,21,36–38,73,79–81]

Table 2.9: Gathered data over optimal values for ethyl acetate production in alcoholic beverages.[2,9,21,36–38,73,79–81] Each parameter has been measured individually, thereby may effect each other if adjusted. The value of UFA is summarized from initial values of oleic acid, linoleic acid and linolenic acid. [W] values for whisky and [B] values for baijiu.

Parameters	Optimal value
UFA	$\leq$7.16 mg/L[2]
pH, Initial (final)	$\leq$5.5, ($\leq$4.2)[9]
Excess of carbon content	20 %[2]
FAN	250 mg/L[2]
CO_2 pressure	<1 bar[2]
Fermentation temperature	$\leq$26 °C[2]
Ethanol, fermentation	<330 mg/L[79,80]
Ethanol, storage	No data
Storage time	$\leq$1 year[B73], $\leq$6 years[W9]
Malolactic fermentation	Yes[81]
Simultaneous fermentation	Yes[36–38]
Overexpress AAT genes	Yes[21]

3 Discussion

The pathways of ethyl acetate have been a discussed topic over several decades of research. While there is good supported evidence over certain pathways, there are still fundamental features of the ester's formation and the yeast's metabolism that are still not fully understood, which leads to some inconsistency.

In results gathered from previous studies of enzyme-catalyzed esterification, some show conflicting results. The enzymes Atf and Eat reportedly stand for 50 % of the ethyl acetate production each, i.e., AAT stands for the total, 100 %, production of ethyl acetate.[8,53] However, it is reported that some ethyl acetate is formed due to a spontaneous hemiacetal reaction.[82] This rises the question if AAT are responsible for the total ethyl acetate production alone. Since ethanol and acetaldehyde are toxic for most organisms in high concentrations, this pathway may act as detoxifying mechanism for the yeast.[83] Where hemiacetal is formed from ethanol and acetaldehyde and then is converted to ethyl acetate by an Adh enzyme, often referred to as HADH.[84] HADH is strictly not considered as an enzyme by itself, but rather as a side activity of Adh enzymes.[58] The matter is debated, whereas HADH has shown other metabolic significance as it is required for the yeast to reach an optimal growth, due to the ability to process oleic acid,[85] a common mono-unsaturated omega-9 fatty acid.[86]

An observation in certain red wines from Bordeaux can be viewed as supporting evidence that Atf and Eat are not responsible for the sole production of ethyl acetate.[81] Here it has been observed high levels of the ester, but only after the occurrence of malolactic fermentation, a bacterial fermentation by lactic acid bacteria commonly performed by *Oenococcus oeni* or in some cases by strains of *Lactobacillus* or *Pediococcus*.[87] Malolactic fermentation converts the tart malic acid to the more mellow lactic acid leading to lower levels of acidity and also the production of multiple flavor compounds.[88] Nevertheless, malolactic fermentation is often prevented in white wine by the addition of sulfur dioxide (SO_2) to kill off the bacteria, filtration or by malolactic-inhibiting enzyme to maintain a fresh acidic flavor and bouquet.[17,89]

Esterase activity has been a matter of discussion coupled to ethyl acetate, if the enzyme could catalyze a reverse esterase reaction during fermentation.[58] This is rather unlikely due to the role of the thermodynamic equilibrium, where the reaction has a reportedly positive ΔG value of 28.8 kJ/mol, Table 2.4.[53] Therefore, the reaction is thermodynamically unfavourable, which instead strongly favors ethyl acetate hydrolysis under aqueous conditions. The reverse esterase reaction will yield ethyl acetate in high concentration only if high concentration of acetic acid and ethanol are present or if water concentrations are low, where the latter is seen as less feasible in a fermentation medium.[90]

In the case of an chemical esterification as Fischer-Speier esterification, there are multiple obstacles that are lowering the probability in comparison to the biosynthesized esterification of AAT during ethanol fermentation. Firstly the catalyst of an Fischer-Speier esterification is a strong acid, typical sulfuric acid, which can lower the pH to a dangerous level for the yeast.[91] The usage of sulfuric acid during ethanol fermentation is mainly as a pretreatment to control bacterial contamination and remove any other MO before adding the yeast, hence removing any other competitor for

the resources for growth.[92] Secondly the temperature for Fischer-Speier esterification would optimally be above 48 °C,[93] which would break the interactions in many proteins and denature them,[94] thus inhibiting any enzyme activity and likely kill the yeast itself. Even though *S. cerevisiae* has a relative high resistance to temperature stress compared to other yeast species, it will not ferment over 37 °C and start dying above 40 °C.[25,95] Thirdly the presence of high water concentration in a fermentation medium will favor the reaction equilibrium to go backwards, from products to reactants, due to Le Châtelier's principle.[67] Additionally, as mention in Section 2.4, water will compete with ethanol as a nucleophile (shown in Scheme 5, Step 2) and it will reduce the amount of acid-alcohol intermediates and consequently lower the yields of ethyl acetate.[71] Finally should sulfuric acid or other strong acids be used as catalysts, the final beverage product may needed to be filtered for byproducts that could cause an allergic reaction or in other ways be harmful for the consumer.[96–99]

Despite every obstacle, there are reports of chemical esterification during ethanol fermentation.[34,50,52] However, it is also mentioned that the reaction rate is too slow to account for any significance part of esters present from the fermentation. Due to the slow reaction rate it is assumed that the reaction is occurring without a catalyst, without any evidence to support this. On the other hand, both chemical formation of ethyl acetate is well reported in storage of alcoholic beverages.[9,16,31,100] During storage in oak barrels, oxygen continuously diffuses through the pores of the wood and transfers both tannins (polyphenols) and flavor compounds from the wood into the beverage.[100] While the flavor compounds from the fermentation decrease, new compounds are form through multiple reactions and creates a more complex flavor. In result, the formation of esters continues throughout the storage process due to large quantities of available ethanol and the formation of acids.[31] Without a storage process, synthesis of esters would have ceased when fermentation had ended due to the yeast runs out of nutrition.[79]

Isotopic labeling of radioactive ethanol-1-^{14}C ($CH_3{}^{14}CH_2OH$) was conducted to determine which compounds are derived from ethanol during storage.[101] It was shown that ethanol through a slow sequences of oxidation reactions formed acetic acid and finally by an esterification reacted with ethanol formed ethyl acetate, as shown in Scheme 5.[9] Analysis of alcoholic beverages have generally indicated that the total ester concentration increases slowly throughout storage, but with the most significant increase close after fermentation.[9,73,81] The ester responsible for the majority of the concentration is ethyl acetate.[102] In Table 2.5 it is listed how the concentration fluctuate over 6 years for baijiu and is steadily rising for whisky. An explanation for baijiu's fluctuation, could be when in the storage process the measurements were taken, whereas baijiu after one year of storage has a concentration of about 2200 mg/L (results not shown in table) before rapidly decreasing to 750 mg/L.[73] The sudden drop of ester values has been reported as a combination of vaporization and hydrolysis of the esters adjusting for the esterification equilibrium.[9,31,73] Furthermore the slowly increase after 2-3 years have been associated with the dissolved oxygen that gradually oxidize compounds.[73] As a result it have been suggested that this shows how the chemical reactions occurring (esterification, hydrolysis, oxidation etc.) are intertwined during storage.

Comparatively to how the formation of ethyl acetate occurs, it is also undoubtedly

important to understand which parameters that affect the formation itself. It is possible to generalise the rate of ester formation during fermentation and narrow it down to two important factors; the concentrations of the substrates (acetyl-CoA and ethanol) and the enzyme activity of the involved enzymes.[2] The parameters that have been shown results to affect ethyl acetate production is the concentration of UFA, the C:N ratio in the fermentation medium, aeration of the medium, fermentation temperature, and hydrostatic pressure in the fermentation tank. The latter parameter and the aeration of the wort medium attribute to the increase of dissolved CO_2 in the medium as well as dissolved O_2.[103] The increase of dissolved O_2 will promote the synthesis of fatty acids, which uses acetyl-CoA as substrate and results in less available acetyl-CoA for ester production.[104] On the other hand, it has been shown difficulty to perform hydrostatic pressure experiments in laboratory analysis, hence there have been no studies on how hydrostatic pressure influences the ester production in details, but studies have reported that the formation of esters by AAT is negatively correlated with the depth of the fermentation tank.[1,2] Additionally, it is suggested that one of the causes of a high hydrostatic pressure is closely related to the dissolved CO_2 in the medium at larger depths.[105,106] High CO_2 pressure (1 bar CO_2) over time will inhibit the cell's uptake of nutrition through the cell membrane along with inhibition of enzymes, thus lowering the intracellular pH. In turn this results in a increase of cell size, lower viability and production of esters and fusel alcohol, but is a minor impact on the ethanol production.[1,107] The effect of CO_2 exposure can eventually result in yeast cells having a lower ATP level, however the factors resulting in AAT inhibition and growth reduction are not fully understood.[1,108]

The graph in Figure 2.2 indicates a decrease in ethyl acetate production when UFA was added to the fermentation medium, a decrease of 37 % from a standardized batch as a blank sample.[2] The decreasing effect from UFA is consistent with previous findings,[109] where there is evidence upon UFA's repressing the ATF1 gene in yeast that encodes for the enzyme Atf1 and in turn as described earlier is one of the enzymes responsible for the major production of ethyl acetate.[110,111]

In the fermentation medium, the C:N ratio is crucial for the yeast to reach a optimal growth and to procure the desired products.[112] Where an increase in the carbon- or nitrogen contents in the fermentation medium is shown to have a positive correlation with the increase of ethyl acetate.[2,112] From the graph in Figure 2.4, ethyl acetate levels is shown to increase with about 30 % when FAN was added and Figure 2.3 shows a increase of 50 % when the carbon content of the fermentation medium was enhanced.[2] The latter is consistent with result indicating that the ATF1 gene expression is induced by the addition of fermentable sugar to carbon-starved yeast cells.[113] One peculiar note from the results displayed in the graph,[2] even though more carbon content was added, the amount of fermentable sugar consumed by the yeast was the same in every fermentation. Only the residual sugar concentration was higher when higher carbon content concentrations were used. There is no good explanation for this incident, however there could be an influence from the *Crabtree effect* without any evidence to directly support it.[114] Where the Crabtree effect is the occurrence of alcoholic fermentation in an aerobic environment in the response from the yeast to provision of excess levels of fermentable sugar.[115] Therefore the Crabtree effect could be a possibility if the rise in ester

formation occurred in the starting phase of fermentation, but since it is not stated when during the fermentation process, it is hard to predict any correlations.[116]

It is well-known that the growth and metabolism of MO are profoundly affected by the temperature, which also applies to *S. cerevisiae*.[117,118] During fermentation higher temperatures result in a higher metabolism rate for the yeast and thereby higher enzyme activity. Table 2.5 show results of a steady increase of ethyl acetate concentration along with a increase of temperature from 14 to 26 °C.[2] Although the graph shows positive correlation, a temperature over 28 °C have shown a negatively effect on the levels of esters and seems to be consistent with results in Table 2.8.[49] Since enzymes are still function at temperature of 28 to 30 °C, it is explained by the rapid release of CO_2 that entrains compounds from the medium.

Ethanol's effect on the ethyl acetate production during fermentation is as the Swiss physician and chemist Paracelsus ostensibly expressed "the dose makes the poison".[119] The concentration of ethanol strongly correlates to the amount of ethyl acetate being produced up to a certain point of 330 mg/L.[79,80] Above 330 mg/L, ethanol shifts from being an addition of substrate to inhibit the yeast's growth. The positive effect of ethanol is suggested to be from the AAT enzyme creating a hydrophobic area at the catalytic site, resulting in shifting the equilibrium towards ester formation in the absent of water.[53]

In conclusion, in how the parameters affect ethyl acetate production, can it be implemented in beverage production to acquired the preferred levels of ethyl acetate. As an example, a winemaker can lower the amount in white wine to achive a fruitier bouquet by e.g., increasing the amount of UFA. While a spirit producer can increase the levels in an already existing product or to make a beverage more lucrative for a new marked. The latter can be illustrated in how a western spirit producer can aim to make a beverage appeal more to a Chinese marked accustomed with baijiu by increasing the amount of ethyl acetate. In comparison can also a Chinese producer enhance the quality trait in baijiu by higher ethyl acetate levels. The optimal values for increasing ethyl acetate production for each parameter are listed in Table 2.9. On the contrary, the listed parameters are examined individually and therefore will potentially affect each other upon adjustment. Consequently, this implies more research is needed on how the parameters in combination affect production of ethyl acetate.

4 Conclusion

The pathways of ethyl acetate are still not completely elucidated and there are yet areas with only limited understanding to the yeast's metabolism. Especially the interaction and possible intertwining between enzymes is not fully understood. During fermentation, the production of ethyl acetate is mainly pointed towards biosynthesis by AAT enzymes, however there is evidence that HADH also may be playing a role in the production.

While chemical esterification is deemed to have no significance during fermentation, it is evidently the major contributor during storage along with naturally occurring oxidation. Parameters affecting ethyl acetate production have been well-studied and are paramount for controlling the final levels in a beverage. Which can lead to decisive improvement in terms of quality. However, there are more studies on the individual parameter and few on their conjoined effect on the ester and the final flavor. In conclusion, more research is needed to further elucidate the pathways of ethyl acetate, as well as the combined effect from parameters.

Bibliography

[1]   S. Holt, M. H. Miks, B. T. de Carvalho, M. R. Foulquia-Moreno, J. M. Thevelein, *FEMS Microbiology Reviews* **2019**, *43*, 193–222.

[2]   S. M. G. Saerens, F. Delvaux, K. J. Verstrepen, P. Van Dijck, J. M. Thevelein, F. R. Delvaux, *Applied and Environmental Microbiology* **2008**, *74*, 454–461.

[3]   K. J. Verstrepen, G. Derdelinckx, J.-P. Dufour, J. Winderickx, J. M. Thevelein, I. S. Pretorius, F. R. Delvaux, *Journal of Bioscience and Bioengineering* **2003**, *96*, 110–118.

[4]   H. A. B. Peddie, *Journal of the Institute of Brewing* **1990**, *96*, 327–331.

[5]   G. Stewart, *Fermentation* **2017**, *3*, 63.

[6]   R. J. Ouellette, J. D. Rawn in *Organic Chemistry*, (Eds.: R. J. Ouellette, J. D. Rawn), Elsevier, Boston, **2014**, pp. 699–745.

[7]   P. W. Atkins, J. De Paula, J. Keeler, *Atkins' Physical chemistry*, Eleventh edition, Oxford University Press, Oxford, United Kingdom ; New York, NY, **2018**, 908 pp.

[8]   K. J. Verstrepen, S. D. M. Van Laere, B. M. P. Vanderhaegen, G. Derdelinckx, J.-P. Dufour, I. S. Pretorius, J. Winderickx, J. M. Thevelein, F. R. Delvaux, *Applied and Environmental Microbiology* **2003**, *69*, 5228–5237.

[9]   G. H. Miller, *Whisky Science: A Condensed Distillation*, Springer International Publishing, Cham, **2019**.

[10] R. S. Alavijeh, F. Tabandeh, O. Tavakoli, A. Karkhane, P. Shariati, *Journal of Oleo Science* **2015**, *64*, 69–74.

[11] M. K. Modi, J. Reddy, B. Rao, R. Prasad, *Bioresource Technology* **2007**, *98*, 1260–1264.

[12] S. Zhang, F. Guo, W. Yan, W. Dong, J. Zhou, W. Zhang, F. Xin, M. Jiang, *Applied Microbiology and Biotechnology* **2020**, *104*, 7239–7245.

[13] J. Hong, D. Zhao, B. Sun, *Food Reviews International* **2021**, 1–27.

[14] C. Löser, T. Urit, P. Keil, T. Bley, *Applied Microbiology and Biotechnology* **2015**, *99*, 1131–1144.

[15] P. Patra, M. Das, P. Kundu, A. Ghosh, *Biotechnology Advances* **2021**, *47*, 107695.

[16] K. M. Sumby, P. R. Grbin, V. Jiranek, *Food Chemistry* **2010**, *121*, 1–16.

[17] J. M. deMan, J. W. Finley, W. J. Hurst, C. Y. Lee, *Principles of Food Chemistry*, Springer International Publishing, Cham, **2018**.

[18] W. Shi, J. Li, Y. Chen, X. Liu, Y. Chen, X. Guo, D. Xiao, *ACS Synthetic Biology* **2021**, *10*, 495–504.

[19] X.-W. Zheng, B.-Z. Han, *Journal of Ethnic Foods* **2016**, *3*, 19–25.

[20] G. Fan, B. Sun, D. Xu, C. Teng, Z. Fu, Y. Du, X. Li, *Journal of the American Society of Brewing Chemists* **2018**, *76*, 117–124.

[21] J. Dong, P. Wang, X. Fu, S. Dong, X. Li, D. Xiao, *Journal of Industrial Microbiology and Biotechnology* **2019**, *46*, 801–808.

[22] A. Miranda, V. Pereira, M. Pontes, F. Albuquerque, J. C. Marques, *Ciência e Técnica Vitivinícola* **2017**, *32*, 1–11.

[23] K. Grainger, *Wine Faults and Flaws: A Practical Guide*, 1st ed., Wiley, **2021**.

[24] Wine flavours, faults and taints, The Australian Wine Research Institute, https://www.awri.com.au/industry_support/winemaking_resources/sensory_

`assessment/recognition-of-wine-faults-and-taints/wine_faults/` (visited on 04/22/2022).

[25]   *Molecular Mechanisms in Yeast Carbon Metabolism*, (Eds.: J. Piškur, C. Compagno), Springer Berlin Heidelberg, Berlin, Heidelberg, **2014**.

[26]   ISO:5492:2008(en), Sensory Analysis' Vocabulary, **2008**, `https://www.iso.org/obp/ui#iso:std:iso:5492:ed-2:v1:en` (visited on 03/04/2022).

[27]   B. G. Green, D. Nachtigal, S. Hammond, J. Lim, *Chemical Senses* **2012**, *37*, 77–86.

[28]   N. Sakai, T. Kobayakawa, N. Gotow, S. Saito, S. Imada, *Perceptual and Motor Skills* **2001**, *92*, 1002–1008.

[29]   PubChem, Ethyl acetate, `https://pubchem.ncbi.nlm.nih.gov/compound/8857` (visited on 04/11/2022).

[30]   D. D. l. C. García, S. Magnaghi, M. Reichenbächer, K. Danzer, *Journal of High Resolution Chromatography* **1996**, *19*, 257–262.

[31]   P. Ribéreau-Gayon, D. Dubourdieu, B. Donèche, *Handbook of enology Volume 2 The Chemistry of Wine Stabilization and Treatments*, 2nd ed, John Wiley, Chichester, West Sussex, England ; Hoboken, NJ, **2006**.

[32]   M. A. Cliff, G. J. Pickering, *Journal of Wine Research* **2006**, *17*, 45–52.

[33]   S. Holt, M. H. Miks, B. T. de Carvalho, M. R. Foulquié-Moreno, J. M. Thevelein, *FEMS Microbiology Reviews* **2019**, *43*, 193–222.

[34]   H. Shi, Y. Wang, R. Hua, *Physical Chemistry Chemical Physics* **2015**, *17*, 30279–30291.

[35]   B. V. Humia, K. S. Santos, A. M. Barbosa, M. Sawata, M. d. C. Mendonça, F. F. Padilha, *Molecules* **2019**, *24*, 1568.

[36]   K. C. Fugelsang, *Wine microbiology*, Chapman & Hall, New York, **1997**, 245 pp.

[37]   Y. Margalit, *Concepts in wine chemistry*, 2. ed., new ed, Wine Appreciation Guild, San Francisco, Calif, **2011**, 476 pp.

[38]   *Wine analysis and production*, Nachdr. 2010, (Ed.: B. W. Zoecklein), Kluwer Academic, New York, **2010**, 621 pp.

[39]   C. Corison, C. Ough, H. Berg, K. Nelson, *American Journal of Enology and Viticulture* **1979**, *30*, 130–134.

[40]   M. Ocvirk, N. K. Mlinarič, I. J. Košir, *Journal of the Science of Food and Agriculture* **2018**, *98*, 3627–3635.

[41]   R. Moncrieff, *The Chemical Senses*, 2nd ed., L. Hill, Cornell University, London, **1951**.

[42]   P. E. McGovern, *Uncorking the past: the quest for wine, beer, and other alcoholic beverages*, University of California Press, Berkeley, **2009**, 330 pp.

[43]   *Nature* **2008**, *455*, 699–699.

[44]   N. Lane, *Philosophical Transactions of the Royal Society B: Biological Sciences* **2015**, *370*, 20140344.

[45]   A. van Leewenhoeck, *Philosophical Transactions (1665-1678)* **1677**, *12*, 821–831.

[46]   D. Duveen, *Isis* **1950**, *41*, 168–171.

[47]   J. A. Barnett, *Microbiology* **2003**, *149*, 557–567.

[48]   M. A. Moyad, *Urologic Nursing* **2008**, *28*, 4.

[49] P. Ribéreau-Gayon, D. Dubourdieu, B. Donèche, A. Lonvaud, *Handbook of enology Volume 1 The Microbiology of Wine and Vinifications*, 2nd ed, John Wiley, Chichester, West Sussex, England ; Hoboken, NJ, **2006**.

[50] K. Nordstrøm, *Journal of the Institute of Brewing* **1964**, *70*, 226–233.

[51] A. J. Kruis, A. E. Mars, S. W. M. Kengen, J. W. Borst, J. van der Oost, R. A. Weusthuis, *Applied and Environmental Microbiology* **2018**, *84*, (Ed.: C. Vieille), e01640–18.

[52] A. B. Mason, J.-P. Dufour, *Yeast* **2000**, *16*, 1287–1298.

[53] A. J. Kruis, M. Levisson, A. E. Mars, M. van der Ploeg, F. Garcés Daza, V. Ellena, S. W. Kengen, J. van der Oost, R. A. Weusthuis, *Metabolic Engineering* **2017**, *41*, 92–101.

[54] T. Pfeiffer, A. Morley, *Frontiers in Molecular Biosciences* **2014**, *1*.

[55] C. Löser, T. Urit, T. Bley, *Applied Microbiology and Biotechnology* **2014**, *98*, 5397–5415.

[56] E. W. Weisstein, Squircle, Publisher: Wolfram Research, Inc., `https://mathworld.wolfram.com/Squircle.html` (visited on 04/14/2022).

[57] MetaCyc EC 2.3.1.268, `https://www.biocyc.org/META/NEW-IMAGE?type=REACTION&object=RXN-12363` (visited on 04/28/2022).

[58] A. J. Kruis, A. C. Bohnenkamp, C. Patinios, Y. M. van Nuland, M. Levisson, A. E. Mars, C. van den Berg, S. W. Kengen, R. A. Weusthuis, *Biotechnology Advances* **2019**, *37*, 107407.

[59] *The IUPAC Compendium of Chemical Terminology: The Gold Book*, 4th ed., (Ed.: V. Gold), International Union of Pure and Applied Chemistry (IUPAC), Research Triangle Park, NC, **2019**.

[60] O. D. Sparkman, Z. E. Penton, F. G. Kitson in *Gas Chromatography and Mass Spectrometry: A Practical Guide*, Elsevier, **2011**, pp. 285–292.

[61] I. Roberts, H. C. Urey, *Journal of the American Chemical Society* **1938**, *60*, 2391–2393.

[62] C. F. Midelfort, I. A. Rose, *Journal of Biological Chemistry* **1976**, *251*, 5881–5887.

[63] M. R. Webb, *Biochemistry* **1980**, *19*, 4744–4748.

[64] E. Fischer, A. Speier, *Berichte der deutschen chemischen Gesellschaft* **1895**, *28*, 3252–3258.

[65] R. V. Cash, *Journal of Chemical Education* **1964**, *41*, 108.

[66] M. Vafaeezadeh, A. Fattahi, *Computational and Theoretical Chemistry* **2015**, *1071*, 27–32.

[67] R. Vitiello, C. Li, V. Russo, R. Tesser, R. Turco, M. Di Serio, *Rendiconti Lincei* **2017**, *28*, 117–123.

[68] T. P. Silverstein, S. T. Heller, *Journal of Chemical Education* **2017**, *94*, 690–695.

[69] P. Ballinger, F. A. Long, *Journal of the American Chemical Society* **1960**, *82*, 795–798.

[70] K. A. Savin in *Writing Reaction Mechanisms in Organic Chemistry*, Elsevier, **2014**, pp. 1–53.

[71] H. A. Smith, *Journal of the American Chemical Society* **1939**, *61*, 254–260.

[72] W. H. Puterbaugh, C. H. Vanselow, K. Nelson, E. J. Shrawder, *Journal of Chemical Education* **1963**, *40*, 349.

[73] Y. Deng, A. Xiong, K. Zhao, Y. Hu, B. Kuang, X. Xiong, Z. Yang, Y. Yu, Q. Zheng, *Scientific Reports* **2020**, *10*, 17169.

[74] S. Holt, B. Trindade de Carvalho, M. R. Foulquié-Moreno, J. M. Thevelein, *mBio* **2018**, *9*, (Ed.: F. M. Winston), e01279–18.

[75] M. Zakhartsev, X. Yang, M. Reuss, H. O. Pörtner, *Journal of Thermal Biology* **2015**, *52*, 117–129.

[76] C. Ramsay, D. Berry, *Food Microbiology* **1984**, *1*, 117–121.

[77] K. G. Duodu, F. E. Dowell in *Sorghum and Millets (Second Edition)*, (Eds.: J. R. N. Taylor, K. G. Duodu), AACC International Press, **2019**, pp. 421–442.

[78] J. R. N. Taylor, K. G. Duodu in *Cereal Grains*, (Eds.: C. W. Wrigley, I. L. Batey), Woodhead Publishing Series in Food Science, Technology and Nutrition, Woodhead Publishing, **2010**, pp. 237–263.

[79] K. Nordstrøm, *Journal of the Institute of Brewing* **1961**, *67*, 173–181.

[80] K. Nordstrøm, *Journal of the Institute of Brewing* **1964**, *70*, 328–336.

[81] A. D. Webb, *Biotechnology and Bioengineering* **1967**, *9*, 305–319.

[82] M. Kusano, Y. Sakai, N. Kato, H. Yoshimoto, Y. Tamai, *Journal of Bioscience and Bioengineering* **1999**, *87*, 690–692.

[83] H. Yurimoto, B. Lee, F. Yasuda, Y. Sakai, N. Kato, *Yeast* **2004**, *21*, 341–350.

[84] M. Kusano, Y. Sakai, N. Kato, H. Yoshimoto, H. Sone, Y. Tamai, *Bioscience Biotechnology and Biochemistry* **1998**, *62*, 1956–1961.

[85] Y.-M. Qin, M. S. Marttila, A. M. Haapalainen, K. M. Siivari, T. Glumoff, J. K. Hiltunen, *Journal of Biological Chemistry* **1999**, *274*, 28619–28625.

[86] N. H. Choulis in *Side Effects of Drugs Annual*, (Ed.: J. K. Aronson), A worldwide yearly survey of new data in adverse drug reactions, Elsevier, **2011**, pp. 1009–1029.

[87] I. Gil-Sánchez, B. Bartolomé Suáldea, M. Victoria Moreno-Arribas in *Red Wine Technology*, Elsevier, **2019**, pp. 85–98.

[88] G. Scollary in *Encyclopedia of Food and Health*, Elsevier, **2016**, pp. 571–576.

[89] F. Matei, M. Kosseva in *Science and Technology of Fruit Wine Production*, Elsevier, **2017**, pp. 73–103.

[90] P.-Y. Stergiou, A. Foukis, M. Filippou, M. Koukouritaki, M. Parapouli, L. G. Theodorou, E. Hatziloukas, A. Afendra, A. Pandey, E. M. Papamichael, *Biotechnology Advances* **2013**, *31*, 1846–1859.

[91] N. V. Narendranath, R. Power, *Applied and Environmental Microbiology* **2005**, *71*, 2239–2243.

[92] Y.-Q. Tang, M.-Z. An, Y.-L. Zhong, M. Shigeru, X.-L. Wu, K. Kida, *Journal of Bioscience and Bioengineering* **2010**, *109*, 41–46.

[93] F. S. Atalay, *Developments in Chemical Engineering and Mineral Processing* **2008**, *2*, 181–184.

[94] B. K. Shoichet, W. A. Baase, R. Kuroki, B. W. Matthews, *Proceedings of the National Academy of Sciences* **1995**, *92*, 452–456.

[95] C. Belloch, *International Journal of Food Microbiology* **2008**, *122*, 188–195.

[96] K. Gupta, D. Roy, V. Subramanian, P. Chattaraj, *Journal of Molecular Structure: THEOCHEM* **2007**, *812*, 13–24.

[97] R. Koster, B. van der Linden, E. Poels, A. Bliek, *Journal of Catalysis* **2001**, *204*, 333–338.

[98] The Preservatives in Food Regulations 1989, `https://www.legislation.gov.uk/uksi/1989/533/made` (visited on 04/18/2022).

[99] Veileder om tilsetningsstoffer i næringsmidler, `https://www.mattilsynet.no/om_mattilsynet/gjeldende_regelverk/veiledere/veileder_til_forskrift_om_tilsetningsstoffer.4594/binary/Veileder%20til%20forskrift%20om%20tilsetningsstoffer` (visited on 04/18/2022).

[100] J. Câmara, M. Alves, J. Marques, *Analytica Chimica Acta* **2006**, *563*, 188–197.

[101] G. H. Reazin, S. Baldwin, H. S. Scales, H. W. Washington, A. A. Andreasen, *Journal of AOAC INTERNATIONAL* **1976**, *59*, 770–776.

[102] M. Onishi, J. F. Guymon, E. A. Crowell, *American Journal of Enology and Viticulture* **1977**, *28*, 152–158.

[103] S. Landaud, E. Latrille, G. Corrieu, *Journal of the Institute of Brewing* **2001**, *107*.

[104] *Brewing: science and practice*, (Ed.: D. E. Briggs), CRC Press ; Woodhead Pub. Ltd, Boca Raton : Cambridge, England, **2004**, 881 pp.

[105] M. Meilgaard, *Journal of the Institute of Brewing* **2001**, *107*, 271–286.

[106] A. Vrieling, *Eur Brew Conv Monograph* **1978**, *5*, 135–45.

[107] F. B. Knatchbull, J. C. Slaughter, *Journal of the Institute of Brewing* **1987**, *93*, 420–424.

[108] L. Richard, S. E. Guillouet, J.-L. Uribelarrea, *Process Biochemistry* **2014**, *49*, 1808–1818.

[109] T. Fujii, O. Kobayashi, H. Yoshimoto, S. Furukawa, Y. Tamai, *Applied and Environmental Microbiology* **1997**, *63*, 910–915.

[110] D. Fujiwara, H. Yoshimoto, H. Sone, S. Harashima, Y. Tamai, *Yeast (Chichester England)* **1998**, *14*, 711–721.

[111] D. Fujiwara, O. Kobayashi, H. Yoshimoto, S. Harashima, Y. Tamai, *Yeast (Chichester England)* **1999**, *15*, 1183–1197.

[112] E. D. G. Danesi, Â. S. M. Miguel, C. d. O. Rangel-Yagui, J. C. M. de Carvalho, A. Pessoa, *Journal of Food Engineering* **2006**, *75*, 96–103.

[113] K. Verstrepen, G. Derdelinckx, J. Dufour, J. Winderickx, I. Pretorius, J. Thevelein, F. Delvaux, *FEMS Yeast Research* **2003**, *4*, 285–296.

[114] R. H. De Deken, *Journal of General Microbiology* **1966**, *44*, 149–156.

[115] A. Hagman, T. Säll, J. Piškur, *The FEBS journal* **2014**, *281*, 4805–4814.

[116] P. M. Doran, *Bioprocess engineering principles*, Reprint, Elsevier/Acad. Press, Amsterdam, **2004**, 439 pp.

[117] H. T. BROWN, *Annals of Botany* **1914**, *28*, Publisher: Oxford University Press, 197–226.

[118] R. C. Jones, J. S. 1. Hough, *Microbiology Society* **1969**, *60*, 107–116.

[119] U. L. Gantenbein in *Toxicology in the Middle Ages and Renaissance*, Elsevier, **2017**, pp. 1–10.