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Microbial biochemistry

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1. Introduction

The process of making wine from grapes can be seen from a technological point of view (which encompasses the handling, treatment, transport and storage of the grapes and the liquids[87]) or also, for example, from a biochemical point of view. Only this latter aspect will be considered in this review. The final product, wine, is the result of a vast series of biochemical reactions that have been gradually recognized over the last century. These reactions are catalyzed by enzymes that are produced either by the grape itself or by microorganisms (i.e. yeasts, filamentous molds, bacteria). Of course, the main reactions that eventually turn grape must into wine are those of the well-known alcoholic fermentation of the hexoses by the enzymes of yeasts. Even this rather simple alcoholic fermentation requires the growth of the yeast cells and thus a plethora of biochemical reactions is involved in the overall process. As it is impossible to cover even the main aspects of the microbial biochemistry involved in wine-making this review article will be devoted to some catabolic reactions, mainly the metabolism of carboxylic acids by yeasts and lactic acid bacteria, the biochemistry of killer-proteins from yeasts, and some metabolic activities of the mold *Botrytis cinerea*. For more detailed information on this and on other topics such as the metabolism of nitrogen, sulfur, aromatic or flavor compounds recent textbooks or reviews should be consulted[1, 3, 19, 42, 43, 53, 72].

2. Fermentative metabolism of carboxylic acids

Grape must and wine contain a variety of carboxylic acids. Their concentration varies greatly and depends on the condition and maturity of the grapes. The main acids and some of the minor acids are listed in table 1. The main acids of must are malate and tartrate that are produced by the grape. Many of the acids of wine are of microbial origin.

Malate

The metabolism of malate has been studied intensively. The usual wine yeasts of the genus *Saccharomyces* are capable of metabolizing malate during the fermentation, but generally only about one fifth of the acid that is present in the must is fermented[28, 69, 90]. Therefore the change in total acidity is small. The biochemical mechanism for the decomposition of malate is the same for the yeasts of the genera *Saccharomyces*[29] and *Schizosaccharomyces*[18, 83]. Malate is first oxidatively decarboxylated to pyruvate by an NAD-dependent malic enzyme. Pyruvate is decarboxylated to acetaldehyde which is reduced to ethanol. These reactions are catalyzed by two enzymes that are involved in the alcoholic fermentation. The overall result is that one molecule of malate yields two molecules of carbon dioxide and one molecule of ethanol. In addition, part of the malate is probably transformed to succinate via fumarate[40]. So far the attempts to employ

Table 1. The carboxylic acids of wine, their origin and microbial metabolism

Carboxylic acid	Content in wine (g/l)	Grape	Yeasts	Lactic acid bacteria	*Botrytis*	Acetic acid bacteria
Main acids						
Malate	0–8	F	F M	M		
L-Lactate	0.1–6	–	F[a]	F		
Tartrate	1–4	F	–	M[a]		
Succinate	0.5–2	–	F	F[a]		
Gluconate	0–3	–	–	M	F	F
Acetate	0–3	–	F	F	F	F
Amino acids	1–6	F	F M	M	M	
Minor acids						
Citrate	0–0.5	F	–	M		
D-Lactate	0.1–0.5		F	F		
Pyruvate	0–0.5		F M	M		
2-Oxoglutarate	0–0.2		F	M		
Galactarate	0–1					F

F, formed; M, metabolized; –, metabolism not known or insignificant; [a] formed or metabolized by certain strains or under special conditions only.

yeasts with an ability to ferment malate in wine-making have not been satisfactory.

Yeasts of the genus *Schizosaccharomyces* that ferment malate completely have a requirement for elevated temperatures and are therefore easily outgrown by the less exacting wine yeasts of the genus *Saccharomyces*. In addition, a tendency to produce undesirable off-flavors has been observed with *Schizosaccharomyes*[5]. *Saccharomyces bailii* (synonym *Zygosaccharomyces bailii*) is also able to ferment malate completely[2]. This yeast is no usual wine yeast, although it is occasionally found in wine. Due to its high resistance against preservative chemicals (sulphur dioxide and sorbic acid, among others) and high sugar concentrations, this yeast is a well-known spoilage organism, particularly of concentrates and beverages. The malic enzymes of *Saccharomyces cerevisiae* and *S. bailii* are somewhat different, in particular the substrate affinities (expressed as K_m-values). However, this difference is insufficient to explain why *S. bailii*, and of course *Schizosaccharomyces*, can metabolize malate completely whereas the wine yeast (*S. cerevisiae*) cannot[40]. Malate enters the cells of *S. cerevisiae* obviously by simple diffusion. In *S. bailii* the transport of malate is mediated by a carrier protein that is specific for the L(−)-isomer. As experiments have shown, this protein is not always present in cells. Surprisingly, it is only synthesized when the yeast cells are grown in glucose. Cells grown on other carbon sources like fructose or glycerol (aerobically) are unable to transport malate rapidly into the cells, whereas glucose-grown cells transport malate by facilitated diffusion. Thus the effective metabolism of malate by *S. bailii* depends on the presence of a special transport system (a carrier protein for L-malate) and a malic enzyme with a higher affinity for malate than in *S. cerevisiae*[2].

Much better known than the inconspicuous transformation of malate by yeasts is the fermentation of malate by lactic acid bacteria[41, 55, 62]. These organisms, of which members of the genera *Leuconostoc*, *Lactobacillus* and *Pediococcus* may be found in wine, are able to convert L-malate almost quantitatively to L(+)-lactate and carbon dioxide. Recent investigations have shown that the fermentation of malate to lactate is caused by one enzyme (one single protein) that has been tentatively called malolactic enzyme[12, 49]. Originally it was assumed that this 'malolactic fermentation' of wine is catalyzed by several enzymes. One possible sequence of reactions could consist of the following series of enzymes: malate dehydrogenase, oxalacetate decarboxylase and L-lactate dehydrogenase with the intermediates oxalacetate and pyruvate. Since the discovery of NAD- or NADP- dependent malic enzymes the malolactic fermentation was assumed to be catalyzed by a malic enzyme that oxidatively decarboxylates malate to pyruvate, and an L-lactate dehydrogenase that reduces pyruvate to L-lactate.

The malolactic enzyme was purified to homogeneity from *Lactobacillus plantarum* by common biochemical procedures[12]. As the enzyme is present in the bacteria in high concentration already, a 50-fold purification leads to a homogeneous protein. Malolactic enzyme of *L. plantarum* is a single protein with a molecular weight of about 140,000. The purified enzyme has a very high specific activity of more than 300 U/mg protein. L-Malate is decarboxylated to L-lactate in the presence of NAD and manganese. Oxalacetate is decarboxylated to pyruvate. In the presence of $NADH_2$ neither oxalacetate nor pyruvate is reduced at a rate of more than 0.5% of the decarboxylation of malate. Thus the purified malolactic enzyme does not show significant activities of lactate dehydrogenase or malate dehydrogenase. If malolactic enzyme is treated with sodium dodecyl sulphate it is easily split into subunits. These subunits have a molecular weight of about 70,000. Thus it can be assumed that malolactic enzyme of *L. plantarum* consists of two protein subunits of the same molecular weight, which are probably identical.

The malolactic enzyme of *Leuconostoc mesenteroides* has been partially purified[49]. The enzyme preparation showed a specific activity of about 42 U/mg. The molecular weight was determined as approximately 235,000; the existence of subunits was not shown but was assumed from the kinetic behavior of the enzyme. Recently the malolactic enzyme from another strain of *L. mesenteroides* was purified to homogeneity. Gel electrophoresis showed that the protein of the most active enzyme preparation had a molecular weight of about 70,000, but the protein tends to form aggregates and by ultracentrifugation a molecular weight of 140,000 was determined (Radler and Battermann, unpublished results).

The malolactic enzyme of *Leuconostoc* is certainly different from this enzyme of *Lactobacillus plantarum*. Rabbit antibody prepared against the purified malolactic enzyme of *Lactobacillus plantarum* did cross-react with the *Leuconostoc* enzyme, but whereas malolactic enzymes from other *Lactobacillus* species or from *Pediococcus* showed a continuous band with the Ouchterlony agar diffusion technique, the band of the *Leuconostoc* enzyme showed spurs, indicating the presence of additional determinants[11].

So far the malolactic enzyme has been found in lactic acid bacteria only[12]. Most strains contain it, and the activity can be conveniently determined with a CO_2-electrode[48]. The reaction does not yield energy that can be used by the bacteria, which need at least small amounts of sugars for growth[38, 61]. Apparently the malolactic reaction is a mechanism to remove protons that inhibit bacterial growth. A few lactic acid bacteria (i.e. *Lactobacillus casei, Strepto-*

coccus faecalis) possess a true malic enzyme[44-47,77]. Therefore these strains, which can grow on pyruvate as energy source, can also grow on malate in the absence of a fermentable sugar. A further mechanism operates in some strains of *Lactobacillus fermentum* which are able to metabolize malate but contain neither malolactic enzyme nor malic enzyme. Cells of this organism form L- and D-lactate, acetate, succinate and carbon dioxide from malate. The fermentation products depend on the hydrogen concentration. At low pH-values lactic acid is the main product, at pH-values of about pH 5 mainly succinic and acetic acids are formed from malate[11]. The formation of succinate from malate by lactic acid bacteria has been observed by Carr and Whiting[9,91]. At low pH-values, malate is probably converted to D, L-lactate via oxalacetate and pyruvate. The final result resembles the reaction of the malolactic enzyme, but due to the presence of the respective lactate dehydrogenases, both L- and D-lactate are produced. At a pH-value of about pH 5, part of the pyruvate is oxidized to acetate. The resulting hydrogen equivalents are obviously transferred to fumarate which is reduced to succinate. The formation of fumarate is catalyzed by fumarase, which is present in lactic acid bacteria at a high level of activity. This unusual reaction of malate, which may be called a dismutation[11] because of the partial reduction and oxidation, is probably of little importance in wines that usually have pH-values of less than 4 and, of course, most lactic acid bacteria possess the malolactic enzyme anyhow. However, in cider with a somewhat higher pH-value, this reaction may occur occasionally. The various reactions by which malate may be metabolized by yeasts or lactic acid bacteria are summarized in figure 1.

In wines produced from ripe grapes with a low content of malic acid, there is no need to transform this acid by microorganisms. On the other hand it may be of interest to remove an excess of malate in wines from less mature grapes with high acidity. The use of malate-decomposing yeasts is possible but even selected strains of *Schizosaccharomyces* or *Saccharomyces bailii* have not been completely satisfactory. Also, attempts to hybridize suitable wine yeasts with malate-decomposing strains of *S. bailii* by conjugation or cell fusion have not been successful (Radler, unpublished). An interesting new approach is the transfer of the gene for malolactic enzyme from a lactic acid bacterium (*Lactobacillus delbrueckii*) into a yeast by genetic engineering with a shuttle plasmid that is replicated in a bacterial cell or in a yeast cell[94]. The genetic manipulation has been successful and the resulting yeast strains synthesized the bacterial malolactic enzyme. However, only small amounts of malate were decarboxylated to lactate. I assume that the transfer of the malolactic enzyme into a yeast cell is not sufficient for a rapid metabolism of malate, for it is likely that an additional specific transport system for malate is required, as has been demonstrated in *S. bailii*[2].

Although in wine the reactions causing a decomposition of malate predominate, it has been observed that strains of yeast exist that are able to produce malate[92]. Such strains have been isolated from cider but they are found among wine yeasts too. This malate formation is greatly influenced by the culture conditions and is favored by high sugar concentrations (20–30%), at pH-values of about pH 5 and at limiting concentrations of nitrogen compounds (100–250 mg N/l). The presence of carbon dioxide is required[65]. Radioactive carbon dioxide is incorporated in about equal amounts into malate and succinate (Schwartz and Radler, unpublished). The mechanism of malate formation is not exactly known. A backward reaction of malic enzyme is possible but not very likely. In wine the usually high concentration of amino acids and the low pH may prevent the formation of malate, and therefore its decomposition prevails, but depending on the conditions, some part of the malate found after fermentation might have been synthesized by yeasts.

Succinate

Succinate is the main carboxylic acid produced by yeasts during fermentation, in amounts up to 2 g/l[84]. Its formation depends on the yeast strain and it is particularly favored by glutamate[33]. The formation of succinate is correlated with the formation of 2-oxoglutarate. Obviously the oxidative pathway (via succinyl-CoA) is operative for the synthesis of succinate by yeasts when growing on glutamate. Accordingly, the important citric acid cycle enzyme 2-oxoglutarate dehydrogenase has been found in fermenting yeast cells; this enables them to produce succinate during fermentation by the oxidative reac-

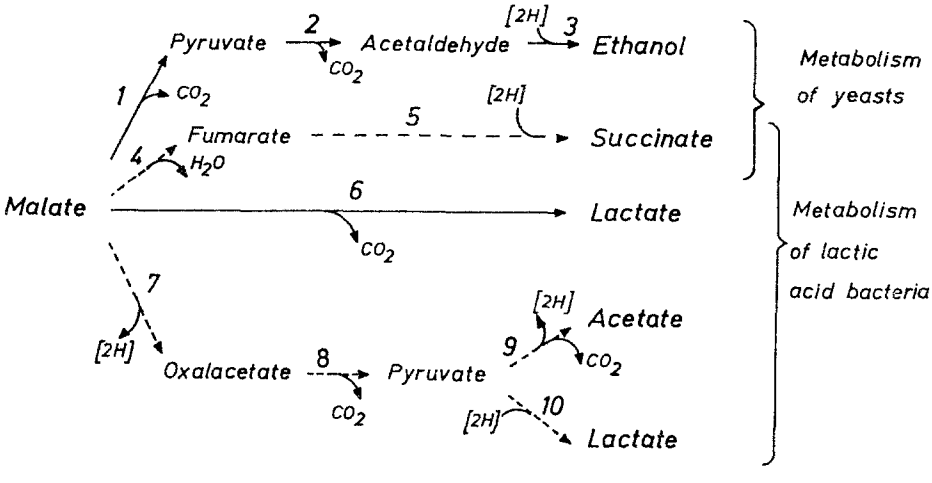

Figure 1. Metabolism of malate by yeasts and lactic acid bacteria. Full lines = main reactions. Dotted lines = minor reactions. 1, malic enzyme; 2, pyruvate decarboxylase; 3, alcohol dehydrogenase; 4, fumarase; 5, fumarate reductase; 6, malolactic enzyme; 7, malate dehydrogenase; 8, oxalacetate decarboxylase; 9, pyruvate dehydrogenase; 10, lactate dehydrogenase.

tions. In accordance with this assumption is the fact that fermenting yeasts contain succinyl-CoA synthetase. This enzyme has been partially purified and characterized[78]. Malate and aspartate may be converted to small amounts of succinate by the enzymes of the reductive pathway (via oxalacetate, malate, and fumarate). If fumarate is added to must before fermentation it is metabolized by fumarase to malate which is further metabolized by malic enzyme. Thus fumarate that is known to inhibit the bacterial malolactic fermentation[14] cannot be used as an acidulant for grape must before the alcoholic fermentation is completed[59, 89].

Pyruvate and 2-oxoglutarate

The two oxo acids pyruvate and 2-oxoglutarate are present in grape must in low concentrations. During fermentation their amount increases, and pyruvate is later partially metabolized by the yeasts. Both acids may be formed from the corresponding amino acids alanine and glutamate but they are also excreted by the yeast cells at low concentrations of nitrogen compounds in the medium[60]. The oxo acids are of importance in wine fermentation, for they are able to bind to SO_2, thus lowering the content of free SO_2 in wine which is regarded as the biologically most active part and is necessary for the safe preservation of wine. Lactic acid bacteria contain lactate dehydrogenase and the pyruvate dehydrogenase complex. Thus pyruvate may be reduced to lactate or oxidized to carbon dioxide, acetate and C_4-compounds like acetoin, diacetyl or butanediol-2.3. Some lactic acid bacteria contain alcohol dehydrogenase, therefore some ethanol may be formed.

Only a few strains of lactic acid bacteria are unable to attack 2-oxoglutarate. Most species reduce it to 2-hydroxyglutarate. An exception is *Leuconostoc oenos,* the most important organism of the malolactic fermentation. *L. oenos* decarboxylates 2-oxoglutarate to succinic semialdehyde, and that is reduced to 4-hydroxybutyrate from which the corresponding γ-butyrolactone is formed. Cells of *L. oenos* oxidize part of the succinic semialdehyde to succinate[64]. The metabolism of 2-oxoglutarate is shown in figure 2. Although pyruvate and 2-oxoglutarate are readily metabolized by most lactic acid bacteria, it is so far unknown to what extent the low concentrations of these acids that are present in wine are subject to bacterial metabolism[20].

Tartrate and citrate

No yeasts are known that are capable of degrading tartrate anaerobically. Even among the lactic acid bacteria only a few strains ferment tartrate[34, 55]. However, as soon as tartrate is being fermented by lactic acid bacteria, a wine can certainly be regarded as spoiled. This wine disease has been known since the beginning of the century. The main biochemical reaction is the conversion of tartrate to oxalacetate by a tartrate dehydratase; so far among lactic acid bacteria this reaction has only been observed in some strains of *Lactobacillus*[68]. The further reactions proceed along two different pathways. If the bacterium possesses malolactic enzyme, then the intermediate oxalacetate is decarboxylated to pyruvate, which is in part reduced to lactate and in part oxidized to acetate and carbon dioxide. Thus the end products of the fermentation of tartrate are carbon dioxide, acetate and lactate. If the organism does not contain malolactic enzyme, then most of the tartrate is converted to succinate with the probable intermediates oxalacetate, malate and fumarate. Obviously part of the oxalacetate is decarboxylated and the resulting pyruvate is oxidized to acetate and carbon dioxide. The hydrogen equivalents are probably used to reduce fumarate but the enzyme and its cofactor requirements are still unknown.

Citrate is present in grape must in quantities usually much less than 0.5 g/l. This acid is also metabolized by lactic acid bacteria only. It is split by a citrate lyase to oxalacetate and acetate. The reaction sequence was originally proposed by Deffner[17]. Oxalacetate is then probably metabolized to acetate and carbon dioxide. Important by-products may be C_4-compounds as acetoin and diacetyl, which occur in wine, but their significance has been more extensively studied in dairy products[51, 82].

Lactate and acetate

Lactic acid bacteria produce lactate from almost all fermentable sugars and from carboxylic acids. Lactate occurs as two stereoisomers which are produced by the reduction of pyruvate by the corresponding L- or D-lactate dehydrogenases. One exception is the malolactic en-

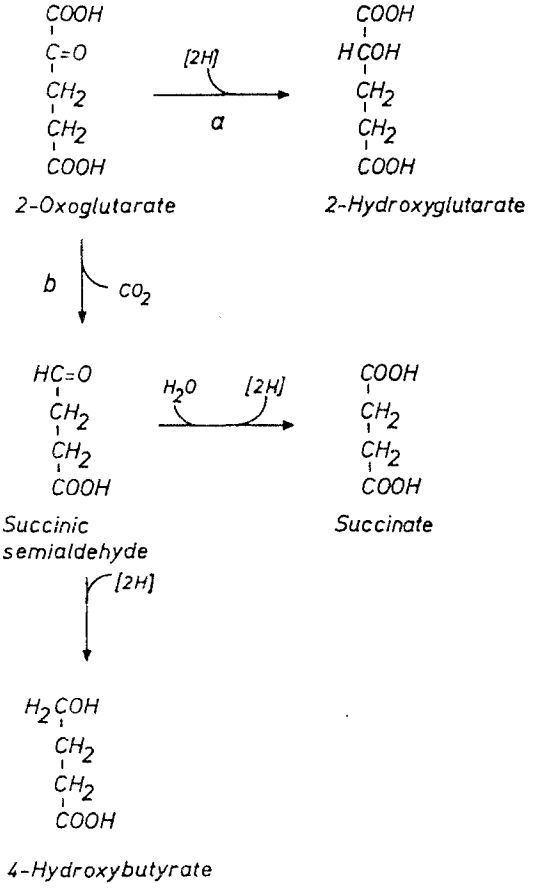

Figure 2. The metabolism of 2-oxoglutarate by lactic acid bacteria. *a* Reduction of 2-oxoglutarate to 2-hydroxyglutarate by most lactic acid bacteria. *b* Decarboxylation of 2-oxoglutarate and formation of 4-hydroxybutyrate and succinate by *Leuconostoc oenos*.

zyme that quantitatively yields L-lactate from L-malate in the absence of a lactate dehydrogenase[12,76].

Usually yeasts produce only small amounts of free carboxylic acids during fermentation (about 20–25 meq/l); of these acids only a small fraction is lactate. It has been observed that *Saccharomyces veronae* produces L-lactate[56]. Among several hundred yeast strains investigated, a few strains were observed that produced comparatively large quantities of L-lactate (up to 100 meq or 9 g/l)[63]. These yeasts belong to the species *Saccharomyces pretoriensis* which closely resembles *S. cerevisiae*. The acid is obviously synthesized from sugar by reduction of pyruvate by an L-lactate dehydrogenase which has been partially purified[30].

Acetate is the main product of the oxidation of ethanol by acetic acid bacteria. But acetate can also be formed anaerobically by lactic acid bacteria from sugars, particularly pentoses, and from various carboxylic acids. Wine is legally regarded as spoiled when its acetate content is higher than about 20 meq/l. (The exact figure is different for various types of wines and varies in different countries. Usually acetic acid is determined as 'volatile acid'). Acetate is also a normal regular by-product of the alcoholic fermentation of yeasts. Various yeast strains show great differences in the anaerobic production of acetate. Strains producing high amounts of acetate contain an unusually high activity of acetaldehyde dehydrogenase (Hannemann and Radler, unpublished). Such strains are not suitable for wine fermentation.

Gluconate

Gluconate is not present in musts prepared from sound grapes. But grapes infected with the mold *Botrytis cinerea* may contain several grams of gluconate per liter[17]. Gluconate is probably produced by the mold but acetic acid bacteria may be involved in its formation. Some but not all strains of lactic acid bacteria from wine, especially the species *Leuconostoc oenos* and *Lactobacillus brevis* ferment gluconic acid. The bacteria possess gluconokinase, an enzyme that can be inducible. The final products of the fermentation of gluconate are lactate, acetate and carbon dioxide[66]. The fermentative mechanism is not quite certain but it can be assumed to resemble the hexosemonophosphate pathway. Yeasts are apparently unable to metabolize gluconate fermentatively[4].

Sorbate

Sorbate is not a natural constituent of grape must or wine. However, it is a widely-employed preservative and its addition to wine, usually as potassium sorbate, is legal in many countries. Sorbate inhibits yeast growth, it is not effective against lactic acid bacteria. These bacteria are not inhibited, but some strains apparently reduce the acid to the corresponding alcohol (2,4-hexadien-1-ol)[16]. At the acid pH of wine this substance is chemically esterified by ethanol and rearranged to 2-ethoxy-hexa-3,5-diene. This compound produces a distinct off-flavor that spoils a wine completely[98]. Therefore, if sorbate is added to wine, this should be done just before bottling, when the development of lactic acid bacteria can be prevented by the addition of sulphur dioxide.

Glycerol and butanediol-2,3

Glycerol is by far the most important secondary product of fermentation. The amount of glycerol formed is generally assumed to be in the range of one tenth to one fifteenth of the ethanol formed. The formation of glycerol is not constant but depends on various factors, such as oxygen, fermentation temperature, pH, sugar concentration, amino acids and in particular thiamine. The yeast strain is of great importance too, as different strains form very varying amounts of glycerol. The enzymes leading directly to ethanol and glycerol-3-phosphate (the direct precursor of glycerol) are alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase respectively. Apparently the amount of glycerol-formed in the result of the competition between these two enzymes for the reduced coenzyme $NADH_2$. High and low glycerol-forming yeast strains were found to show large differences in the activity of glycerol-3-phosphate dehydrogenase and only small variations in the activity of alcohol dehydrogenase[67]. Must prepared from sound grapes contains insignificant amounts of glycerol. However, grapes infected with the mold *Botrytis cinerea* may contain considerable quantities of glycerol; thus in some wines the glycerol content may be of two different origins[36].

A few rare strains of lactic acid bacteria are able to ferment glycerol, and the bacterial spoilage of wine due to glycerol fermentation has been known since the turn of the century. Today, with modern technology, this disease of wine is almost unknown. The formation of acrolein and its reaction with tannin was regarded as important for this spoilage, but it was observed that acrolein is formed during distillation of mashes in which acrolein is not formed by lactic acid bacteria, but by thermal dehydration of 3-hydroxypropanal at acid pH during distillation[79]. The fermentation of glycerol by a heterofermentative lactic acid bacterium was found to yield propanediol-1,2[80]. This ability to carry out glycerol fermentation is rare among lactic acid bacteria. Among 54 strains belonging to nine species of *Lactobacillus* and *Leuconostoc* only three strains of *Lactobacillus brevis* and one strain of *Lactobacillus buchneri* metabolized glycerol, which was dehydrated to 3-hydroxypropanal and subsequently reduced to propanediol-1,3[74]. Glycerol was not used as the only substrate but was metabolized jointly with glucose. The joint fermentation of glycerol and glucose by *L. brevis* (fig. 3) results in the formation of D(−)- and L(+)-lactate, acetate, carbon dioxide, propanediol-1,2 and no or very little alcohol. The essential enzyme for this fermentation of glycerol is the cobamide-requiring propanediol-1,2 dehydratase. The enzyme has been purified[75]. It reacts with the substrates propanediol-1,2, glycerol and ethanediol-1,2 with the relative activities of about 3:2:1. The apparent molecular weight was determined as $M_r = 180{,}000$.

Besides glycerol the diol butanediol-2,3 is an important by-product of fermentation; it is observed in wine in amounts of up to 3 g/l[31,39]. Usually its concentration is about 1 g/l and its contribution to the taste of wine is probably negligible. Between one third and about half of the butanediol-2,3 belong to the meso-isomer, the remainder is optically active. Butanediol-2,3 is formed by yeast via acetoin that is reduced by an acetoin reductase.

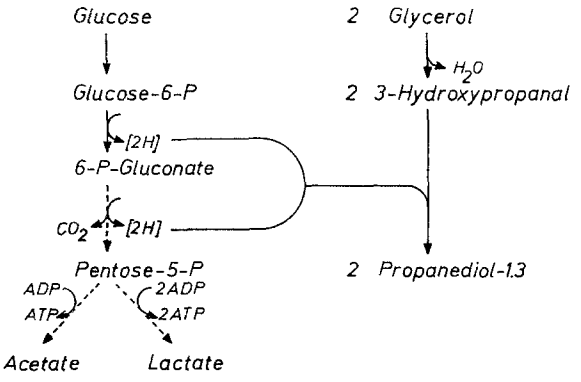

Figure 3. A simplified scheme of the joint metabolism of glycerol and glucose in *Lactobacillus brevis*.

This enzyme has been partially purified, and its molecular weight determined as 95,000[86].

In spoiled wines, the presence of butanol-2 has been observed, and it was demonstrated[88], that lactic acid bacteria produce this alcohol from meso-butanediol-2,3[35]. The enzyme responsible for this reaction was purified from *Lactobacillus brevis*. It is identical with the diol-dehydratase of glycerol metabolism. The substrate affinity for meso-butanediol-2,3 is much lower than for glycerol (Radler and Zorg, unpublished). As with glycerol, the diol meso-butanediol-2,3 does not serve as the sole growth substrate for the bacteria; it is only metabolized when a fermentable substrate like glucose is present. In some distillates butanol-2 is regarded as a normal constituent[70].

Although the metabolism of both yeasts and lactic acid bacteria contributes to the composition of the wine, it appears that the degradative reactions of the bacteria are more diverse than the reactions of the yeasts. In table 2 the various substrates (acids, sugars, polyols and also amino acids) and the products formed from them are compiled. In addition a number of alcohols and aldehydes are formed by lactic acid bacteria from unknown substrates, probably from sugars. Many of these alcohols and aldehydes are also fermentation products of yeasts.

Table 2. Substrates and fermentation products of lactic acid bacteria

	Substrates	Products
Acids	L-Malate	L-Lactate, CO_2 (succinate, acetate)
	Citrate; pyruvate	Lactate, acetate, CO_2, acetoin
	Gluconate	Lactate, acetate, CO_2
	2-Oxoglutarate	4-Hydroxybutyrate, succinate, CO_2
	Tartrate	Lactate, acetate, CO_2, succinate
	Sorbinate	2,4-Hexadien-1-ol
	Chlorogenate	Ethylcatechol, dihydroshikimate
Sugars	Glucose	Lactate, ethanol, acetate, CO_2
	Fructose	Lactate, ethanol, acetate, CO_2, mannitol
	Arabinose, xylose or ribose	Lactate, acetate
Polyols	Mannitol	(Probably as from glucose)
	Butanediol-2.3	Butanol-2
	Glycerol	Propanediol-1.3
Amino acids	Arginine	Ornithine, CO_2, NH_3
	Histidine	Histamine, CO_2
	Phenylalanine	2-Phenylethylamine, CO_2
	Tyrosine	Tyramine, CO_2
	Ornithine	Prutescine, CO_2
	Lysine	Cadaverine, CO_2
	Serine	Ethanolamine, CO_2
	Glutamate	γ-Aminobutyrate, CO_2
Unknown substrates (probably sugars)		Propanol, isopropanol, isobutanol, methyl-2-butanol-1, methyl-3-butanol-1, ethylacetate, acetaldehyde, n-hexanol, n-octanol, glycerol, butanediol-2.3, erythrol, arabitol, dextrane, diacetyl

3. Biochemistry of killer proteins of yeasts

Since Makower and Bevan[50] observed yeast strains that produce substances by which sensitive yeasts are killed, this phenomenon has received widespread attention. The available information has been compiled in recent reviews[5, 85, 93]. The active substance is called killer toxin and was regarded as a protein[95]. The purified killer toxin has been identified as protein[54]. On the other hand, it has been reported that the killer toxin is glycoprotein in nature in *Saccharomyces cerevisiae*[6, 57], *Torulopsis glabrata*[8] and *Pichia kluyveri*[52]. So far the carbohydrates of the glycoproteins have not been identified. The protein consists of the usual amino acids. Table 3 shows the molecular weights and the overall composition of the amino acids of three different killer proteins, the toxins K_1, K_2 and the toxin of strain 28. Of course, the overall composition is not so important as the sequence of the amino acids. Recent work on the sequence of the K_1-toxin indicates that it is obviously composed of two parts, the α-toxin and the β-toxin[85]. This may lead to a different calculation of the molecular weight and a different amino acid composition.

In wine, killer yeasts of type K_2 may be found. The strain 28 of *Saccharomyces cerevisiae*, which produces a different type of toxin, was originally isolated from a grape. It was found with strain 28 that the toxin is primarily produced at low pH-values[57]. At pH-values above pH 6 this toxin is rapidly inactivated, at pH 5 and below it is stable. The optimum activities of other killer proteins are different, usually in the range of pH 4.2 or pH 4.7[58]. The toxin of strain 28 has an optimum above pH 5. Strain 28 produces the toxin, when grown in grape must at about pH

Table 3. Composition of amino acids and molecular weights calculated from the amino acid residues of the killer toxins of several strains of *Saccharomyces cerevisiae*

Amino acid	Number of residues per molecule		
	K_1 (strain T158C)	K_2 (strain 399)	Strain 28
Alanine	10	9	12
Arginine	0	4	3
Aspartic acid	13	8	9
Cysteine	8	1	5
Glutamic acid	10	9	11
Glycine	13	7	6
Histidine	2	2	2
Isoleucine	5	6	5
Leucine	7	8	7
Lysine	5	3	3
Methionine	3	2	2
Phenylalanine	4	4	3
Proline	0	8	6
Serine	7	6	12
Threonine	6	8	10
Trytophan	5	3	2
Tyrosine	5	6	6
Valine	6	9	7
Number of residues	109	103	111
Mol. wt	11,470	13,207	14,045

3.5. This means that killer protein may be produced during wine fermentation. However, at the lower pH, the killer activity is very weak. Therefore this toxin, although stable in wine, may have only a limited effect on sensitive yeasts, if they are present during fermentation. Of course, this may be different with different types of toxins.
The killer factor, i.e. the genetic information for the formation of the toxin, has been introduced into wine yeasts[32], and wild type killer strains are available as dried yeast for use as starter cultures. In theory, a killer yeast should be able to prevent the growth of other yeasts. Of course, this can be expected for sensitive yeasts only. Many but not all strains of *Saccharomyces* are sensitive; many other yeasts are not affected by killer yeasts. On the other hand, if an active starter culture is used to inoculate about 10^6 cells per ml, then the risk is small that some other yeast than the starter culture will develop. So far it is difficult to assess whether the use of killer yeasts is advantageous for wine-making.

4. Reactions and enzymes of Botrytis cinerea

The importance of the development of the grey mold *Botrytis cinerea* on the grape berry for enology has been recently summarized by Ribéreau-Gayon et al.[73]. *B. cinerea* causes both a very destructive grey mold rot and, in certain conditions, the so-called 'noble rot', which yields special wines like Sauternes or Traubenbeerenauslese. Between the two types of mold, there are all sorts of intermediate types, depending to a large extent on the climatic conditions. The situation is in fact very complicated as further organisms are involved like acetic acid bacteria, yeasts and other molds.

Formation of acids and polyalcohols

When *B. cinerea* parasitizes on grape berries and causes noble rot important chemical changes take place, that are usually studied by comparing the composition of must and wine prepared from healthy berries or noble rot berries[13,21,22]. The perforated cuticle of the berry leads to a rapid water loss and thus the must contains a high concentration of solutes. *B. cinerea* degrades sugar but acids are proportionately more metabolized, therefore the rich wines prepared from noble rot grapes can be smooth and not too acid[73].
Some acids are formed by *B. cinerea*. Gluconic acid is an oxidation product of glucose. This acid is absent from healthy berries but is regularly present in wines produced from infected berries[22]. As the amount of gluconic acid is not greatly changed by fermenting yeasts, its presence indicates that the grapes were infected by *B. cinerea*. (However, some lactic acid bacteria are able to ferment gluconic acid[66]). Sometimes a sediment of the calcium salt of mucic acid (galactaric acid) is observed in bottles of wines from *Botrytis*-infected grapes[96]. Mucic acid is probably formed by the enzymatic oxidation of galacturonic acid by *Botrytis cinerea*. This latter acid is a component of the pectic substances of the grape berry. Very significant amounts of several polyols may be formed by *B. cinerea*. Glycerol is by far the most important. It may be found in musts from infected grapes in concentrations up to 14 g/l[22]. Several other polyols are formed (erythrol, arabitol, mannitol, meso-inositol, and the disaccharide trehalose), but their concentration attains only about several hundred mg/l at the most[73]. No or only small differences were observed in the concentration of several pentoses and hexoses between must and wines from normal or *Botrytis* infected grapes[23].
Wines prepared from *Botrytis* infected grapes have a high combining ability with sulphur dioxide. In order to obtain the necessary level of free sulphur dioxide, the addition of higher amounts of sulphur dioxide is required. The main sulphur dioxide binding compounds are acetaldehyde, ketoacids like pyruvic and 2-oxoglutaric aids, and furthermore, amongst other compounds, 2-ketogalactonic acid, 5-ketogluconic acid and xylosone[73,97]. Some of these compounds are mainly observed in wines from *Botrytis* infected grapes or they are found in such wines in higher concentration than in wines from healthy grapes. However, the situation is complicated by the fact that besides *B. cinerea* other microorganisms might take part in their formation. The comparatively high concentration of galacturonic, glucuronic, 2- and 5-oxo-gluconic acids in some German wines of high quality indicates that these compounds are likely to be formed in *Botrytis*-infected grapes[81].

Formation of polysaccharides

In addition to the polysaccharides of the grape, in berries infected with *B. cinerea* further saccharides have been found. These are of great technological importance because they tend to clog the filters by forming a glutinous layer on them if they are present in wine in quantity[73]. This occurs particularly if there has been excessive mechanical handling of the grape berries, because in the infected grape berry, the fungal polysaccharide is located between the epidermal cells and the pulp. Two different polysaccharides have been shown to be produced from infected berries. One polysaccharide has a high molecular weight of $M_r = 10^5$–10^6, and consists entirely of glucose subunits. It is a (1→3:1→6)-β-D-glucan which is synthesized from hexoses of the must[26]. Its structure was studied by degradation[27]. The second polysaccharide has a lower molecular weight ($M_r = 20$–$50 \cdot 10^3$) and is composed of mannose (60–70%), galactose (ca 20%) and traces of glucose and rhamnose[73]. This latter heteropolysaccharide is particularly interesting, not only because of its toxicity to plant cells[37] but because of its effect on yeast fermentation. It causes an inhibition of the fermentation, which is accompanied by an increase in the formation of acetic acid and glycerol[71].

Excreted enzymes

In addition to the o-diphenol oxidase (tyrosinase) of the grape, musts prepared from *Botrytis*-infected grapes contain a second oxidase, the p-diphenol oxidase (laccase) which is excreted by the fungus[73]. The fungal laccase is characterized by its action on p-diphenols and can be distinguished from the tyrosinase of the grape by its activity on p-phenylenediamine, ascorbic acid and certain m-diphenols[24]. Both oxidases act on o-diphenols and on monophenols. The fungal laccase is stable at the pH of must and wine and it oxidizes many phenolic compounds

in wine, especially the important anthocyanins and tannins, the coloring matter of red wines, which are scarcely affected by the tyrosinase of the grape. Thus, a good red wine can never be made from grapes that are even slightly moldy. White wines contain much lower amounts of phenolic compounds, and they can be treated with sulphur dioxide to inhibit the oxidase activity without risk of bleaching the wine. Compared with tyrosinase the fungal laccase is much more stable against the action of sulphur dioxide. Therefore this enzyme is of considerable importance in wine-making[73].

Proteases are enzymes that hydrolyze polypeptides into their low molecular weight subunits, that is into amino acids or small peptides. In healthy berries of grapes the proteases are associated with solid particles and specifically with the cell membranes. In berries infected with *Botrytis* the protease activity is found in the juice[15, 73]. Recently an extracellular esterase has been detected in culture filtrates of *Botrytis cinerea* and in must or wine from infected grapes. This enzyme hydrolyzes esters of fatty acids and it is assumed that this may be of importance for the flavor development of wine[25].

5. Conclusion

The important biochemical reactions of fermenting yeasts are well understood. Besides the main catabolic processes a great number of enzymatic reactions are involved in the process of wine-making. These reactions lead to the many by-products that may contribute in very varying degrees to the development of taste, the stability of wine, and the ability to bind sulfur dioxide. Besides the enzymes of the grape, the enzymes of yeasts, and often of lactic acid bacteria, and of the mold *Botrytis cinerea* contribute to the great complexity of fermentation. Since the availability of new and specific methods of analysis it has become possible to study many aspects of microbial biochemistry. These investigations lead to an understanding of the various processes, explain the origin of minor compounds and may eventually be used to control the enzymatic reactions of microorganisms. In spite of many investigations it is still difficult to predict all aspects of the biochemical reactions of fermenting organisms. So far, few attempts have been made to alter the genetic setup to improve their biochemical abilities, but this possibility will certainly be exploited in the future.

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Analytical chemistry

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During the last twenty years, analytical chemistry has benefited considerably from the evolution of techniques and methods. This evolution – one could even call it a revolution – allows the detection and determination of some substances at ever lower concentrations. At the same time, our knowledge has been increased by the discovery of new substances in natural products and in our environment.

The grape, coming from a great variety of vines growing in different soils and climates, and the wine, marked by varied conditions of processing, preservation, and in some cases aging (and commercialization to varying extents), have benefited from this development. A better understanding or the final products and of the factors which affect them, beneficial and harmful, has favored the obtaining of quality products.

We shall briefly summarize the well-known origins of this important development, which has benefited every branch of enology.

In 1951, in his work 'Analyse des vins', P. Jaulmes[87] pointed out that 150 different components were present in wine; ten years later, J. Ribéreau-Gayon and E. Peynaud[139] took into account more than 200. The methods worked out since then have made it possible to go even farther; more than 1000 components are now considered to be present.

The last decades have also been marked by the development of research into food quality; the consumer has become harder to please, with quality playing a vital part in the international market. In order to define this quality, some precisely defined regulations were established and their analytical control organized.

The same applies to wine, and both chemical and sensory analysis are used more and more to define the quality of wine with greater precision. We shall not discuss sensory analysis here, but we want to emphasize its supreme importance in the appraisal of wine quality and the efforts made to connect its results with those of chemical analysis.

The greater part of this report will be directed towards chemical analysis of wines during their processing in cellars and their aging in wine stores, with particular attention placed on official methods for the analysis of wines involved in commercial transactions. Our laboratory has participated actively in the work carried out in this field by the Office International de la Vigne et du Vin (OIV), under the auspices of the Sub-Committee on Methods for Wine Analysis and Evaluation, since its creation in 1951. Mme S. Brun has been a member of this committee since 1961.

1. Analytical methods and wine composition

Improvements in analysis are undeniably bound up with the development of chromatography.

Paper chromatography contributed to our early knowledge about the composition of coloring matter in grapes and wines[142]; it is still used routinely to reveal diglucoside anthocyanins, which are characteristic of American vines and of wines produced from hybrids, and it is used to provide evidence in disputes. For this reason paper chromatography is incluced in many official procedures; see for example 'Receuil des Méthodes Internationales des Vins' published by the OIV[127].