CHAPTER 1

INTRODUCTION AND LITERATURE REVIEWS

1.1 Introduction

Mineral nitrogen in the form of ammonia salts, is most prevalent in grape flesh cells during the vegetative growth phase. Ammonia nitrogen represents 80 per cent of total nitrogen in grapes originating, of course, from nitrates extracted from soil by the roots. At colour change, ammonia nitrogen is still largly predominant. Concentrations decrease rapidly, however, due to transamination, producing more elaborate forms of organic nitrogen. A transamination reaction with α-keto acids, via the Kreb's cycle and the respiration of the sugars, initially converts ammonia nitrogen mainly into free amino acids and then into bonded forms of peptides, polypeptides and proteins. When grapes are fully ripe, mineral nitrogen represents less than 10 per cent of total nitrogen, or a few tens of mg/L expressed in ammonia. Ammonia, or more exactly the NH_4^+, ammonium ion, is the form most directly assimilable by yeasts. Its concentration affects the rapidity with which must start to ferment as well as its potential ferment ability. Nitrogenous compounds are important components of grape juice for wine production. Low level of yeast assimilable nitrogenous compounds (YAN: it includes α-amino acids and ammonia) have been related to lower fermentation rates and longer fermentations. Limiting YAN is thought to affect yeast by reducing yeast cell multiplication and by decreasing indirectly the rate of glycolysis (Bely, Sablayrolles and Barre, 1990a).

Yeast utilizes nitrogen sources by rapidly uptaking and storing the nitrogen available in order to use it when needs. It is supposed to be an adaptive response to its natural habitat (Bisson, 1991), because the ethanol accumulated in the medium throughout the fermentation is a strong inhibitor of amino acid transport.

1.2 Objectives

1. To study the suitable time for the addition of ammonium phosphate and ammonium sulfate in the must as nitrogen source for *Saccharomyces cerevisiae*.
2. To study the suitable concentrations of ammonium salts for *Saccharomyces cerevisiae*.

1.3 Wine Yeasts

The origin of wine making is lose in antiquity. Assyrin and Egyptian documents going back more 3 millenia mentioned grapes and wines. Yeast fermentation of grapes and berries occurs spontaneously in late fall, when the fruit is ripe and its surface is injured. Fresh berries start to ferment and assume a vinous odour if left at room temperature. These fermentations are induced by yeasts on the surface of the fruit. As soon as the fruit is cut or crushed, or the yeast gains entrance along the stem, fermentation of the fruit sugar begins. This in contrast to the fermentation of cereal (bread and beer) in which the sugars have to be formed by enzymatic action before the fermentation can begin. (Adams, 1954)

The production of wine had become highly sophisticated as an art when Pasture's studies on the nature of fermentations opened the door to scientific investigation of the process. Today, more than a century after Pasture's pioneering work, the role of art in wine making is still considerable.

1.3.1 Natural Fermentation

Spontaneous or natural fermentation has been practiced for several thousand years. It is still practiced in the majority of less industrialized countries. It is also practiced in many wineries in highly industrialized countries, mainly in wineries in which wine making by traditional methods is highly prized. There is a succession of species of yeasts beginning with the genera *Kloekera*, *Hansenula*, *Hanseniaspora*, *Cantida* and *Pichia*. *Torulaspora delbrueckelii* is often encountered. In the most active stages of fermentation and toward the end, these yeasts are replaced by

Saccharomyces cerevisiae. There has been some controversy over the relative merits spontaneous fermentations of the flora of the wineries and fermentations carried out with inoculations with selected yeast strains. The latter can be carried out by growing pure culture of a selected yeast in the winery or with an active dry wine yeast. Traditional wine makers have strongly suggested that spontaneous fermentations affect the quality, particularly the organoleptic of the wine. Specifically, it is claimed that such wines lead to a more complex-that is, better-aroma. Some of the extensive literatures on this question have been reviewed by Kraus, Reed and Villettaz (1984). It was found that spontaneous fermentation produced a better rounded and more complex aromatic quality (Benda, 1982). It was also found that a significant preference for wine produced with selected yeasts (Schmitt, Cursmann and Koehler, 1979). Still, others found significant differences in the aroma or flavour of wines made by spontaneous fermentations versus wines made with selected yeasts, but can not find significant preferences for wines made by either method (Bidan and Maugenet, 1981; Rossini, Bertoluccioli and Pasquale, 1981).

Spontaneous fermentations by their very nature showed a great deal of variability and hence the reproducibility when compared with selected yeast fermentation had to be poor. There is an additional complicating factor. It seems to have been tacitly assumed that inoculation with a selected *Saccharomyces cerevisiae* leads to the immediate dominance of that strain and to the rapid disappearance of the nature flora. However, figure 1.1 showed prolong survival of *Kloeckara apiculata* and *Candida stellata* in a must inoculated with 10^5 to 10^7 cells of *Saccharomyces cerevisiae* per milliliter (Heard and Fleet, 1985).

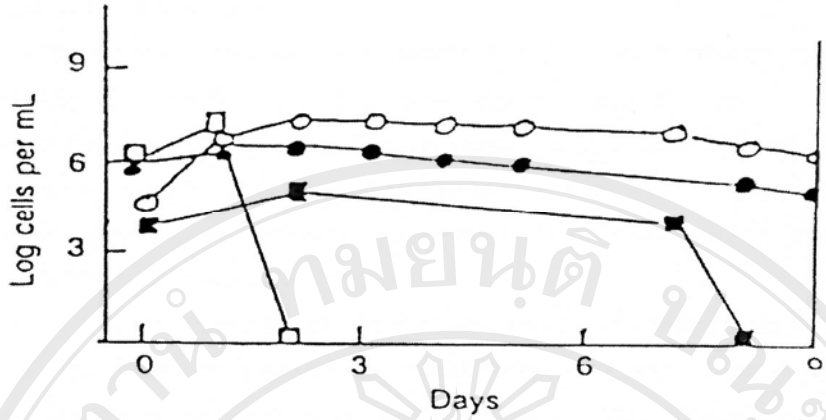


Figure1.1 Growth of Yeast During the Fermentation of White Wine Inoculated with *Saccharomyces cerevisiae*: O = *Saccharomyces cerevisiae*; □ = *Candida stellata*; ● = *Kloeckara apiculata*; ■ = *Candida pulcherrima.*

Source : Heard and Fleet, 1985.

1.3.2 Description of Species

The following genera and species of yeast occur most frequently in fermenting juices and musts (Ferreira, 1959).

Saccharomyces.-The yeast of this genus is the strongest fermenters. The cells are usually spheroid, ovoid, ellipsoidal. They propagate vegetatively by multilateral budding. Sporulation medium can be acetate agar. Ascus carry from 1 to 4 spores.

The most frequently encountered species is a *S. cerevisiae*. A varity, *S. cerevisiae var. ellipsoideus* has a cell size of (3-7) x (4-12.5) micron. Under suitable conditions, these yeasts produce 18 to 20 per cent ethanol (by volume). Other species of *Saccharomyces* which produce high concentrations of ethanol are: *S.chevalieri* (round to oval cells); *S. bayanus* (oval to elongate cells) is often used for the secondary Champagne fermentation, and *S. becicus* (round to short oval cells) for sherry fermentation. Both produce high concentrations of ethanol. *S. rosei* occurs frequently in musts. It produces lesser concentrations of ethanol. *S. carbergensis* (now classified as *S. uvarum*) some strains of which are bottom-fermenting brewer'yeasts, also occur in musts. *S. rouxii* (round to oval cells) is osmophilic yeast which occurs occasionally as spoilage organisms in sweet wines.

Pichia.- the cells have short ellipsoid to cylindrical shape and propagate by multilateral budding. On sporation, 1 to 4 spores are found in the ascus. Some of the species from true hypae. *Pichia membranefaciens* with a cell size of (2.5-4.5) x (5-14) micron occurs almost in all musts. It does not produce more than 1 to 2 per cent ethanol, but it readily survives higher alcohol concentrations. It grows readily on the surface of young wines, it forms a continuous film.

Hansenula.- these yeasts are spherical, ovoid or cylindrical with a cell size of (2-5.5) x (5.5-20) micron. They propagate by multilateral budding; 1 to 4 spores are found per ascus. A pseudo-mycelium and sometimes a true mycelium can be found. *Hansenula anomala* is the species of particular interest in wine making. It may produce up to 10 per cent ethanol by volume. *H. anomala* produces large concentrations of ethyl acetate and other esters.

Saccharomycodes.- *S. ludwigii* has rather large cells, (5-8) x (10-30) micron. The cells have cylindrical, sausage and sometimes lemon shapes. It is highly resistant to SO_2 and can ferment juices with up to 500 ppm SO_2. Consequently, it may occur as a spoilage organism in highly sulfited wines. However, this has been denied. It is probably sensitive to free SO_2. *S. ludwigi* produces up to 8 to 11 per cent ethanol by volume.

Schizosaccharomyces.- the fission yeasts reproduce vegetatively by forming a wall across the middle of the elongated cells. Ascus formation follows conjugation of two vegetative cells and 4 to 8 spores are formed per ascus. The yeasts do not produce high concentration of ethanol and do not occur too frequently in grape musts. *Schizosaccharomyces pombe* (cell size (3-5) x (6-16) micron) ferments malic acid to ethanol and CO_2, and has been used commercially in rum fermentations.

Kloeckera.- the small cells have point, oval or lemon shapes. *Kloeckera apiculata* occurs abundantly in musts and often dominate the early phase of fermentation. Fermentation can be carried to 5 to 6 per cent ethanol by volume, but the yeast survives at higher alcohol concentrations. They form larger amounts of volatile acids and esters. The cells are small, (2-4.5) x (5-8) micron.

Candida.- the non-sporeforming yeasts are round to cylindrical in shape and reproduce by multilateral budding. They form pseudomycelia or true mycelia. *Candida mycoderme* occurs in the majority of young musts and wines. The cell size is

(2-4) x (4-18) micron. The fermentation capacity of this yeast is weak, and not more than 1 per cent ethanol is produced. At alcohol concentrations of 6 to 8 per cent, the yeast readily forms a cohesive skin over the surface of the young wine.

Torulopsis.- *Torulopsis bacillaris*, a common species of this genus, is oval to long oval, and propagates by multilateral budding. No pseudomycelium is formed, and fermentation capacity is poor.

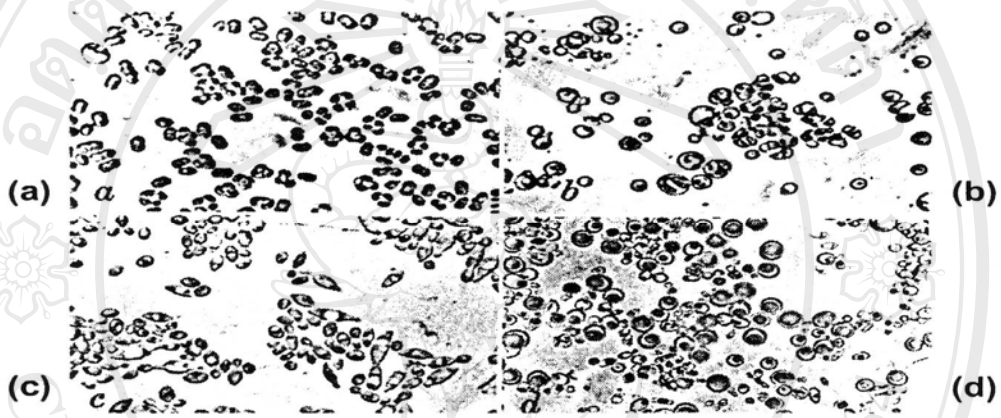


Figure 1.2 Varieties of Yeast Species, *Saccharomyces* sp. (a), *Hansenula* sp. (b), *Kloeckera* sp. (c) and *Canadida* sp.(d).

Source : Gerald and Henry, 1973.

1.4 Alcoholic Fermentation

1.4.1 Fermentation

Wine is usually batch-fermented. Thus, nutrient availability is maximal at the beginning of fermentation and declines progressively thereafter. By the end of fermentation, most sugars have been metabolized, leaving the wine dry. Batch fermentation generally shows a growth pattern consisting of four phase lag, log, stationary and decline. Immediately following inoculation, cells need to adjust to the new environment, because some cells do not acclimate successfully, there is the initial period in which the number that die. This is called the lag phase. Once adaptation is complete, most cells begin to multiply at a steady rate until conditions become

unfavourable. Most microbes are unicellular, the growth curve is termed the exponential or log (logarithmic) phase. During this period, the population of the viable cells rapidly increases to its maximum value. Under batch conditions, the nutrient content falls and toxic metabolic by-products accumulate. Thus, after a period of rapid growth, the rate of cell division (growth) declines and approaches the rate at which cells die. The culture is now said to have entered the stationary phase. As nutrient conditions continue to deteriorate and the concentration of toxic metabolites increase, more cells die than divide. At this point, the culture enters a decline phase. Most viable cells are not replaced, the colony eventually perishes, or become dormant (Ribereau- Gayon, 1985).

Although similar, the population growth pattern displayed by yeast growth in grape juice shows several variations from the norm (Figure. 1.3). Typically the lag phase is short or undetectable; the exponential growth phase is relatively short (seldom amounting to more than eight 8 divisions); the stationary phase may be short and commence long before nutrients become limiting; and the decline phase is atypically long and the viable cell population remains high for several months. As much as 40 per cent of the sugar metabolized to alcohol occurs during the decline phase (Ribereau-Gayon, 1985).

The brevity or apparent absence of a lag phase in yeast growth may result from the preadaped state of the cells initiating fermentation. Active dry yeast, commonly used for juice or must inoculation, comes from cultures grown exponentially in aerated media. Although, these cells have functional mitocondria, capable of respiration, they also have a full complement of fermentative enzymes. Thus, little time is required for the conversion from respiratory to fermentatory metabolism in grape juice or must. Semilary, the indigenous yeast population on grape requires little enzymatic adaptation to commence rapid cell growth. The absence of a prolonged lag period with spontaneous fermentation also may be an artifact. Indigenous yeast cells are commonly bathed in the juice released from broken grapes during harvested and may pass through the lag phase before fermentation “officially” begins in the winery. In addition, yeasts growing on berry skins may exist under limited, but concentrated nutrient conditions. (Ribereau-Gayon, 1985).

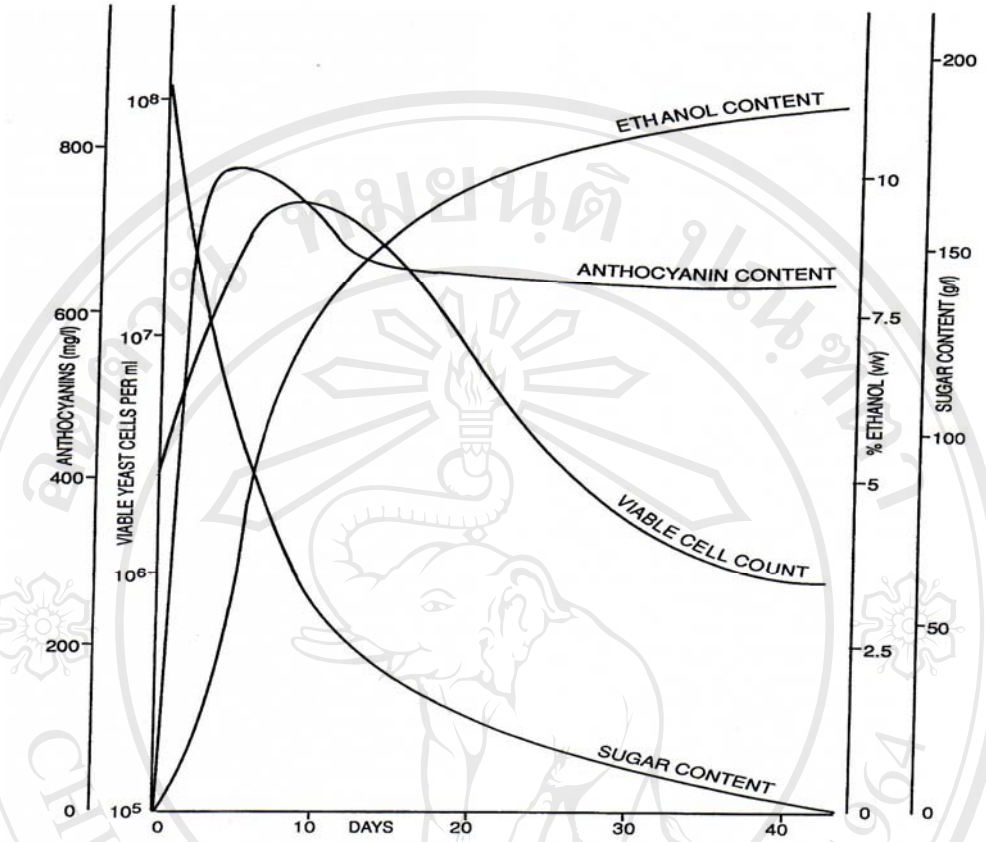


Figure 1.3 Growth Cycle of Yeasts and Fermentation Kinetics in Grape Must with a High Sugar Content.

Source : Ribereau-Gayon, 1985

Although, physiological adjustment to growth in juice appears to be minimal, a lag phase may be observed when conditions are less than optimal. Conditions such as low temperature (≤10 ^{o}C) and excessive protection of the juice from oxygen exposure during crushing may be disadvantage yeast cells. Active dry yeast cells are often leaky, and initially may lose vital nutrients (Kraus, Scoop and Chen, 1981). In addition, nitrogen deficiency and low juice pH can prolong the lag period. The latter probably results from the enhancement of the antimicrobial effects by added sulfur dioxide (Ough, 1996a). High ^{o}Brix values or ethanol contents (second fermentation in sparkling wine production) also suppress yeast growth and fermentation rate (Ough, 1996a,b).

During the exponential phase, cells grow and reproduce at the maximal rate permitted by the prevailing condition. The presence or absence of oxygen does not appear to affect the rate (Schulze *et. al*., 1996). The protein content of the cytoplasm approaches 60 per cent (w/v) and the RNA content about 15 per cent (w/v). Little storage carbohydrate (glycerol and trehalose) accumulates.

The early termination of exponential growth may be partially explained by the large inoculum supplied from grapes or added during inoculation. Juice or must often contians about 10^6 cells/mL at the onset of fermentation, which rises to little more than 10^8 viable cells/mL. Considerably more cell divisions may occur when the cell concentration is initially low or reduced during clarification. Ethanol accumulation and sensitivity may partially explain why the viable cell count seldom reaches more than 10^8 cells/mL. However, other factors appear to be more important because population reaching 10^6 to 10^8 cells/mL can develop in juice initially fortified to 8 per cent ethanol. These factors probably include the inability of yeasts to synthesize essential stereols and long-chain unsaturated fatty acids, and the accumulation of toxic, mid-size, carboxylic acid, by-products of yeasts metabolism that can affect the function of fermentation of yeast cell membranes. One factor not involved is a lack of fermentable nutrients (the stationary phase commences with approximately one-half the sugar content unused). Nevertheless, a reduction in the supply of nitrogen may accentuate catabolic repression (Bely *et. al*., 1994) and ethanol accumulation may disrupt glucose transport into the cell. The remaining and decline phases-constituting up to 80 per cent of the total fermentation period (Ribereau-Gayon, 1985).

As the cells enter the stationary phase, there is a change in the enzyme complement, the production of several heat-shock proteins (Hsp) (Riou *et. al*., 1997), and the accumulation of trehalose and glycerol. Trehalose stabilizes membrane fluidity (Iwahashi *et. al*., 1995), and limits protein denaturation (Hottiger *et. al*., 1994). Heat-shock proteins, produced by many organisms under stress conditions, also limit protein denaturation (Parsel *et. al*., 1994). These may play important roles in prolonging cell viability during the subsequent decline phase. It is at this time that protection against oxidation is required the blanketing of the ferment with carbon dioxide produced during intense fermentation dissipates.

The initiation of the decline phase probably results from the increasing membrane dysfunction becoming progressively lethal to cellular function. Membrane disruption results from the combined effects of ethanol (Hallsworth, 1998) and mid-chain fatty (carboxylic) acid toxicity (Viegas, Sa-Correia and Novais, 1998), plus a shortage in sterol precursors. The absence of oxygen may be an additional factor. Molecular oxygen is required for the synthesis of nicotinic acid, a vital component of the electron carriers NAD^+ and $NADP^+$. However, why the decline initially stabilizes at approximately 10^5 to 10^6 cells/mL is unknown. Cell viability is improved by maceration (red wines). The cells die slowly over the next few months.

Another distinction from most industrial fermentation is the non pure status of wine fermentation is to sterilize the nutrient medium before inoculation. Except for continuous fermentation, grape juice or must is not sterilized. In Europe, the traditional procedure has been to allow indigenous yeasts to conduct the fermentation. However, this is changing, with a shift toward induced fermentation with selected yeast strains (Barre and Vezinhet, 1984). Induced fermentation is standard throughout much of the world. Although sulfur dioxide often is added to inhibit the growth of indigenous (wild) yeasts, it may be ineffectual in this regard (Martinez, Millan and Ortega, 1989).

At one time, there was no adequate means of determining whether indigenous strains of *S. cerevisiae* were controlled by sulfur dioxide. With techniques such as mitochondrial DNA sequencing (Dubourdieu *et. al.,* 1987) and gene marker analysis (Petering, Henschke and Langride, 1991), it is now possible to identify the strain(s) conducting fermentation. Although species occurring on grapes or winery equipment occasionally may dominate the fermentation of inoculated juice (Bouix, Leveau and Cuineir, 1981), inoculated yeasts appear to be the primary if not the yeast detectable at the end of most fermentation (Querol *et. al*., 1992).

The vinification of red must routinely occurs in the presence of high concentrations of indigenous yeasts, regardless of yeast inoculation. White grapes, which are pressed shortly after crushing, are cold settled, and quickly population. Nevertheless, white juice may still possess a significant indigenous yeasts may be present and viable, whether they are metabolically active during inoculated fermentation is unclear.

1.4.2 Biochemistry of Alcoholic Fermentation

Glucose and fructose are metabolized to ethanol primarily via glycolysis (Embden-Meyerhof pathway) (Figure.1.4). Although, the primary by-product is ethanol, additional yeast metabolites generate the most common aromatic compounds

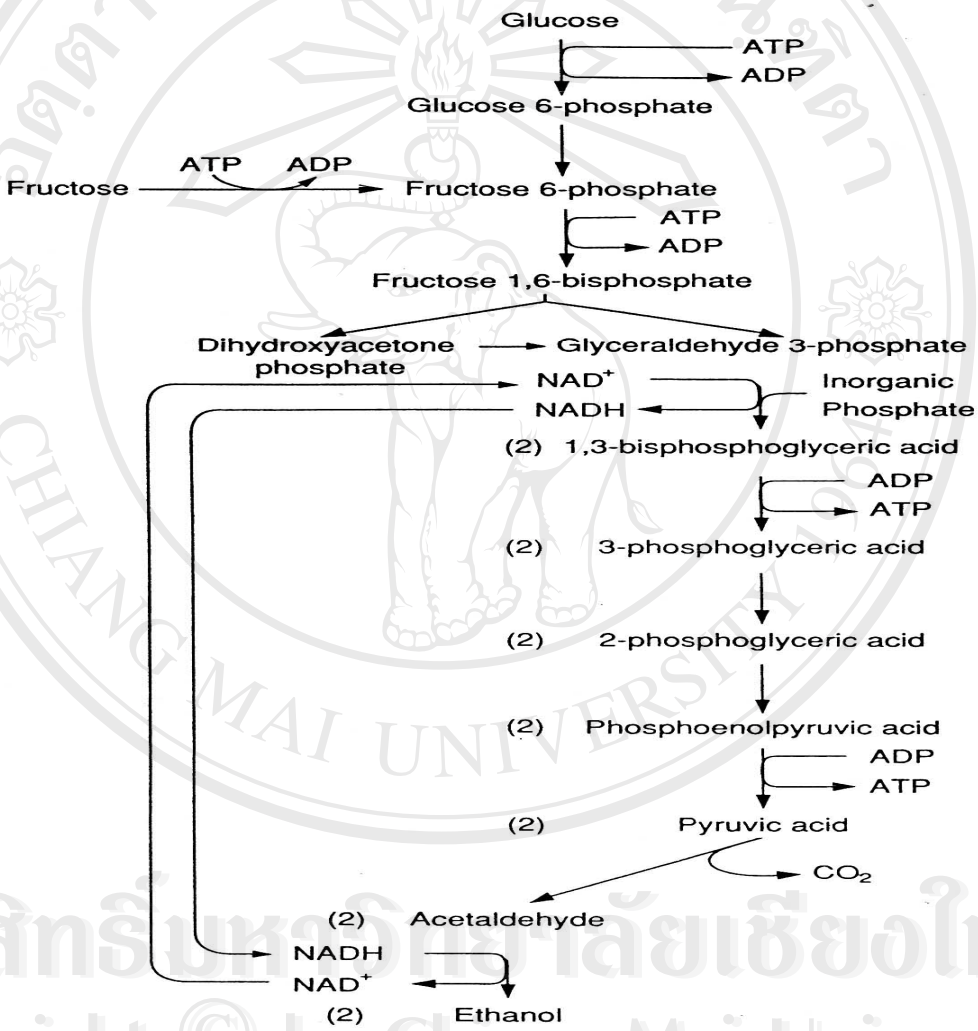


Figure 1.4 Alcoholic Fermentation via the Glycolytic Pathway.
Source : Laffort, Romat and Darriet, 1989.

found in wine. Yeast action also may influence the development of the varietal aroma by hydrolyzing nonvolatile aroma precursors, thus potentially liberating aromatic terpenes, phenols, and norisoprenoids (Laffort, Romat and Darriet, 1989). In addition,

the changing physicochemical conditions produced during fermentation progressively modify yeast metabolism. This is reflected in the various phases of colony growth noted previously, related adjustments in the substances released and absorbed throughout fermentation. Thus, much of the fragrance of wine can be interpreted in terms of the modifications of primary and intermediary yeast cell metabolism.

1.4.3 Energy Balance and the Synthesis of Metabolic Intermediates

During the changing phases of colony growth, yeasts have differing requirements for ATP and reducing power (in the forms of NADH or NADPH). These energy carrying chemicals are required to activate cellular functions and maintain an acceptable ionic and redox balance in the cell. “Redox balance” refers primarily to the equilibrium between the oxidized and reduced forms of the two major pyridine nucleotides (NAD^+/NADH and $NADP^+$/NADPH) (Aristidou *et. al*., 1999).

As shown in Figure. 1.4, glucose and fructose are oxidized to pyruvate, primarily via glycolysis. During the process, electrons are transferred to NAD^+, reducing it to NADH. Pyruvate subsequently may be decarboxylated to acetaldehyde from NADH. In the process, NADH is reoxidized to NAD^+.

The release of energy from glucose and fructose and its storage in ATP and NADH are much less efficient via fermentation than by respiration. Most of the chemical energy initially associated with glucose and fructose remains bound in the end product, ethanol. Furthermore, the energy trapped in NADH is used to reduce acetaldehyde to ethanol. This process is necessary to maintain an acceptable redox balance. As do most cells, yeasts contain only a limited supply of NAD^+. In the absence of oxygen, yeast cells are unable to transfer the energy stored in NADH to ADP (which is more abundant), generating ATP (adenosine triphosphate). Under the anaerobic conditions of fermentation, the regeneration of oxidized NAD^+ requires the reduction of an organic molecule. In the most case, this is acetaldehyde, and the by-product is ethanol. Without the regeneration of NAD^+, the fermentation of glucose would cease. The consequence is that alcoholic fermentation generates only about two molecules of ATP per sugar molecule, in contrast to the potential the potential 24 to 34 ATPs produced via respiration. Most of the ethanol during fermentation escape

from the cell and accumulates in the surrounding juice (Boles, Lehnert and Zimmermann, 1993).

The low respiratory capacity of *S. cerevisiae* reflects its limited ability to produce the requisite enzymes. The high proportion of glycolytic enzymes in yeast cytoplasm (about 50 per cent of the soluble protein content) clearly demonstrates the importance of fermentation to wine yeasts (Hess, Boiteuxand and Kruger, 1969). Yeasts show high rates of glycolysis, usually about 200 to 300 micromol glucose/min/g cell weight (de Deken, 1966). It is estimated that about 85 per cent of the sugar incorporated by *S. cerevisiae* are used in energy production, whereas only about 15 per cent is involved in biosynthetic reactions. Specific values vary depending on the prevailing conditions during the fermentation.

Although, most fermentable sugars in juice are metabolized via glycolysis, some are channeled through the pentose phosphate pathway (PPP) (Figure. 1.5, upper right. This diversion is important to the production of pentose sugars needed for nucleic acid synthesis. The PPP also generates the NADPH required to activate various cellular functions. Amino acids availability in the juice can decrease the activity of the PPP; NADPH is primary required for amino acids biosynthesis (Gancelos and Serrano, 1989). Intermediates of the PPP not required for biosynthesis are normally directed through phosphoglycerate to pyruvate.

During alcoholic sugar fermentation, the redox balance is maintained, but no NADH accumulates. However, yeasts need reducing power for growth and reproduction during the early stage of juice fermentation. Some of the required reducing power comes from the operation of the pentose phosphate pathway (noteably NADPH) and the oxidation of pyruvic acid to acetic acid (yielding NADPH). Additional supplies come from NADH generated in glycolysis. The division of NADPH to biosynthetic functions means that acetaldehyde is not reduced to ethanol and released into the fermenting juice (Bruinenberg *et. al.,*1985).

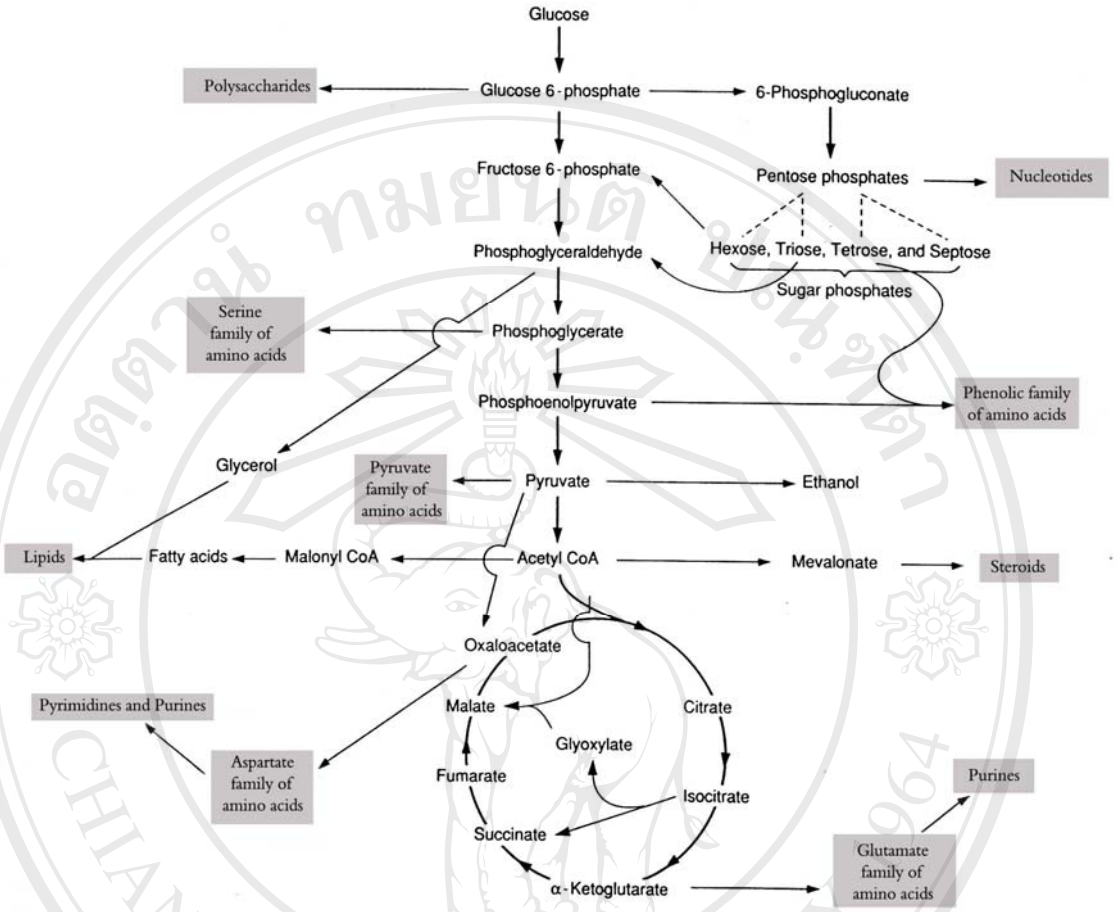


Figure 1.5 Core Reactions of Metabolism Showing the Main Energy-Yielding Pathway (bold arrows) and the Major Biosynthetic Products Derived from Central Metabolism (boxes).

Source : Gancelos and Serrano, 1989.

The central pathway is the Embden-Meyerhof pathway of glycolysis, the top right shows a highly schematic pentose phosphate pathway (PPP), and the bottom is the TCA (tricarboxylic acid) cycle. Each pathway has been simplified for clarity by the omission of several intermediates. The directions of the reactions are shown as being unidirectional, although several are reversible. Energy transformations and the loss or addition of carbon dioxide are not shown. Under the anaerobic conditions of vinification, the TCA-cycle does not function. However, except for the enzyme involved in the conversion of succinate to fumarate, those TCA enzymes present

appear to be active only in the cytoplasm. In addition, decarboxylation of pyruvate to acetyl CoA is inactive and the glyoxylic acid pathway is suppressed (by glucose).

The changing needs of yeasts for reducing power during fermentation probably explains why compounds, such as acetaldehyde and acetic acid, are initially released into juice during fermentation, but are subsequently reincorporated (Figure. 1.6 and 1.7). Early in fermentation, growth and cell division require considerable quantities of reducing power. In contrast, in the decline phase, NADH and NADPH have a tendency to accumulate. The latter suppresses sugar fermentation by diminishing the supply of the requisite NAD^+ (Figure. 1.4). The incorporation and reduction of compounds such as acetaldehyde and acetic acid help to balance the redox potential and permit continued fermentation (Amerine and Joslyn, 1970).

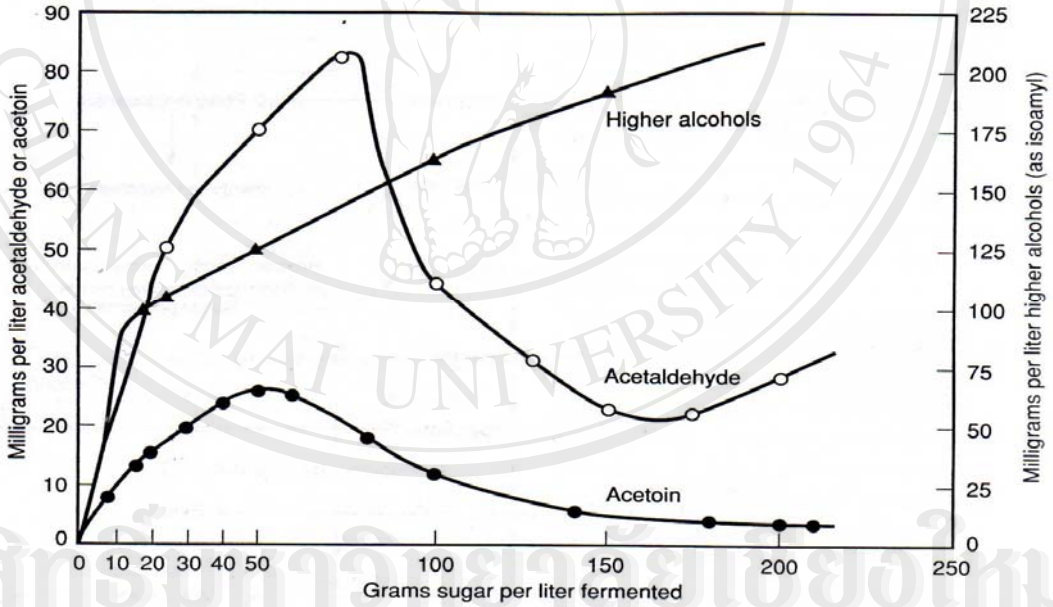


Figure 1.6 Formation of Acetaldehyde, Acetoin and Higher Alcohols During Alcoholic Fermentation. the Dynamics of the Production of These Compounds Varies Considerably with the Yeast Strain**.**

Source : Amerine and Joslyn, 1970**.**

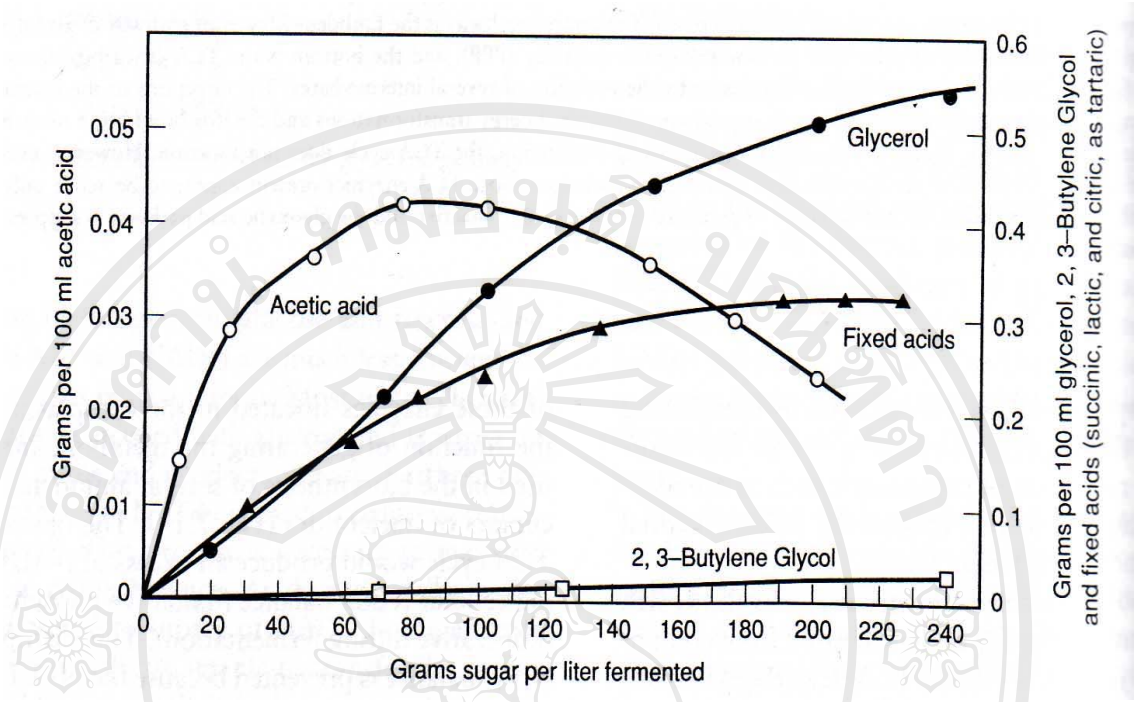


Figure 1.7 Formation of Acetic Acid, Glycerol, 2,3-Butylene Glycol and Fixed Acid During Alcoholic Fermentation. the Dynamics of the Production of These Compounds Varies with the Yeast Strain.

Source : Amerine and Joslyn, 1970.

The metabolic intermediates needed for cell growth and maintenance generally are synthesized from components of the glycolytic, PPP, and TCA (tricarboxylic acid) cycles of central metabolism (Figure.1.5). However, during vinification, most of the TCA-cycle enzymes in the mitochondrion are inactive. Isoenzymic versions of most of the enzyme (located in the cytoplasm) take over the function of generating the metabolic intermediates used in the biosynthesis of several amino acids and precursors of nucleotides (Figure.1.5). The operation of the TCA cycle would produce an excess of NADPH, disrupting cellular redox balance (oxidative phosphorylation is inoperative during fermentation). this disruption of the redox balance is prevented because the NADH produced during the oxidation of citrate to succinate (the right-hand side of the TCA-cycle) can be oxidized back to NAD^+ by reducing oxaloacetate to succinate (the left-hand side of the TCA-cycle). The result is no net change in the redox balance associated with the synthesis of

needed metabolic intermediates. In addition, NADH generated in glycolysis may be oxidized in the reduction of oxaloacetate to succinate, rather than in the reduction of acetaldehyde to ethanol. In both situations, an excess of succinate is generated. This probably explains why succinate is not of the major by-products of yeast fermentation (Gancelos and Serrano, 1989).

The replacement of TCA-cycle intermediates lost to biosynthesis probably comes from pyruvate. Pyruvate may be directly channeled through acetate, carboxylated to oxaloacetate, or indirectly routed via the glyoxylate pathway. The last pathway, if active, is probably active only near the end of fermentation. the glyoxylate pathway is suppressed by glucose. The involvement of biotin in the carboxylation of pyruvate to oxaloacetate probably accounts for its requirement by yeast cells.

The accumulation of another major by-product of fermentation, glycerol, also has its origin in the need to maintain a favourable redox balance. The reduction of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate can oxidize the NADH generated in the oxidation of glyceraldehyde 3-phosphate in glycolysis (Figure.1.8). However, the couplings of these two reactions dose not generate ATP. This is in contrast to the net production of two ATP molecules during the fermentation of glucose to ethanol (Pines *et. al.,* 1997).

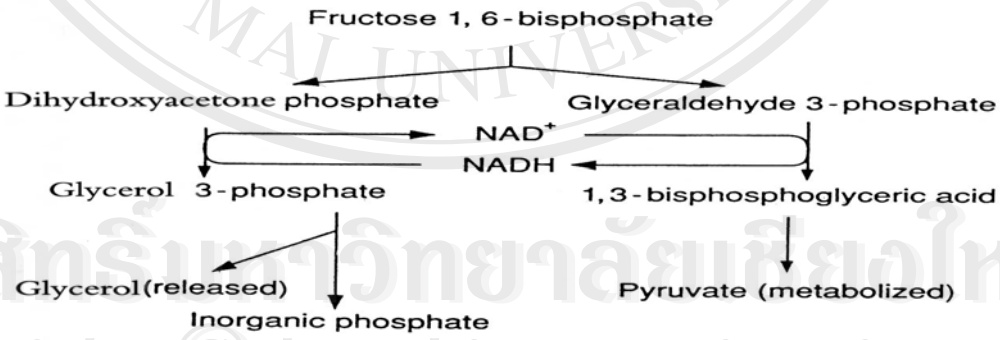


Figure 1.8 Simplified Pathway Showing How NADH Derived from the Oxidation of Glyceraldehyde 3-Phosphate to 1,3-Bisphosphoglyceric Acid is Used in the Reduction of Dihydroxyacetone Phosphate to Glycerol. As a Consequence, NADH is Unavailable to Reduce Acetaldehyde to Ethanol.

Source : Pines *et. al.,* 1997.

Increased glycerol production in the presence of sulfur dioxide probably comes from the need to regenerate NAD^+. The binding of sulfur dioxide to acetaldehyde inhibits its reduction to ethanol, the usual means by which NAD^+ is regenerated during alcoholic fermentation. Throughout fermentation, yeast cells adjust physiologically to the changing conditions in the juice to produce adequate levels of ATP, maintain favourable redox and ionic balances, and synthesize the necessary metabolic intermediates. Consequently, the concentration of yeast by-products in the juice changes continually during fermentation (Figure.1.6 and 1.7). Several of the products are aromatics, for example, acetic acid, acetoin (primarily by conversion from diacetyl), and succinic acid, their presence can affect bouquet development. Adjustments to the diminishing availability of nitrogen sources can also affect the relative production and release of organic acids, fatty acids, and reduced sulfur compounds (Amerine and Joslyn, 1970).

Although, different strains of *S. cerevisiae* possess similar enzymes, their relative proportions and catalytic activities may vary. These differences probably depend on the precise functioning of the regulatory systems of the cells and the number of copies of each gene in the cells. Thus, no two yeast strains are likely to respond identically to the same set of environmental conditions. This variability in response undoubtedly accounts for much of the subtle, and not so subtle, different yeast strains. For example, over expression of the enzyme, glycerol 3-phosphate dehydrogenase, not only results in a marked increase in glycerol production, but also augments the accumulation of acetaldehyde, pyruvate, acetate, 2,3-butanediol, succinate, and especially acetoin (Michnick *et. al.,* 1997). In addition, over expression of cytoplasmic malate dehydrogenase increases not only the accumulation of malic acid, but also fumaric acid and citric acid (Pines *et. al.,* 1997).

1.5 Utilization of Nitrogen Sources

Yeasts find the nitrogen supply necessary for their growth in grape must. The ammonium ion is easily assimilated and can satisfy yeast nitrogen needs, in particular, for the synthesis of amino acids. Polypeptides and proteins do not participate in

S. cerevisiae growth, since this yeast can not hydrolyze these substances. *S. cerevisiae* does not need amino acids as part of its nitrogen supply, since it is capable of synthesizing them individually, but their addition stimulates yeasts more than ammonical nitrogen. A mixure of amino acids and ammonical nitrogen is an even more effective stimulant. Yeasts use amino acids according to three mechanisms (Henschke and Jiranek, 1992):

1. Direct integration without transformation into proteins.

2. Decomposition of the amino group, which is used for the biosynthesis of different amino constituents. The corresponding carbon molecule is excreted.

Such a reaction is one of the pathways of higher alcohol formation present in wine:

$$R\text{-}CHNH_2\text{-}COOH \longrightarrow R\text{-}CH_2OH + CO_2 + NH_3$$

Yeasts are probably capable of obtaining ammonical nitrogen from amino acids through other pathway.

3. The amino acids molecule can be used as a source of carbon in metabolic reaction. The yeast simultaneously recuperates the corresponding ammonical nitrogen.

The assimilation of different amino acids depends on the functioning of transport systems and the regulation of metabolic systems.

The must sugar concentration also affects the impact of the nitrogen supply on fermentation kinetics, especially the successful completion of fermentation. For moderate concentrations of sugar (less than 20 g/L), the addition of nitrogen increases biomass of yeast formed and in consequence the fermentation speed; the fermentation is completed a few days in advance. For high concentrations of sugar, the fermentation is accelerated at the beginning with respect to the control sample, but as the fermentation continues, the gap between the control sample and the supplemented sample decreases. Finally, their fermentations spontaneously stop with similar quantities of reducing sugar remaining. Curve II in Figure 1.9 depicts the effect of supplemental nitrogen (or other activator effect) on a must with a high sugar concentration, having a normal nitrogen concentration. On the other hand, if fermentation sluggishness is due to a nitrogen deficiency, the addition of ammonium

salts manifestly stimulates it (Curve III, Figure 1.9). Stuck fermentations can sometimes be avoided in this manner.

Other factors affect the assimilation of nitrogen during fermentation. Yeasts have strain-specific capabilities. Henschke and Jiranek (1992) reported that different *S. cerevisiae* strains fermenting grape must assimilated quantities of nitrogen varying from 329 to 451 mg/L at 15 ^{o}C and from 392 to 473 mg/L at 20 ^{o}C. These last figures also show, among other things, that temperature increases nitrogen assimilation.

Oxygen, however, has the most effect on the assimilation of nitrogen. Yeasts have long been know to use considerably more nitrogen in the presence of oxygen (Ribereau-Gayon *et. al.,* 1975). It has been observed that yeasts fermenting in the complete absence of oxygen assimilate 200 mg of nitrogen per liter. When they develop in the presence of oxygen, their assimilation increases to 300 mg/L. in aerobiosis, they can assimilate up to 735 mg/L without a proportional increase in cellular multiplication.

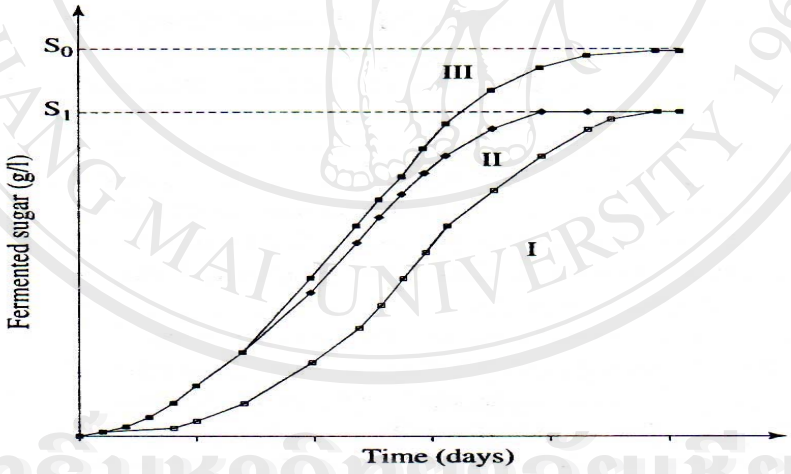


Figure 1.9 Example of a Theoretical Activation of Grape Must Fermentation (S_0 = initial sugar concentration). (I) Control: fermentation stops, leaving unfermented sugar ($S_0 - S_1$). (II) Activation at the initiation of fermentation in musts having high sugar concentrations is not improved. (III) Activation acting on yeast population growth and survival; fermentation is complete

Source : Henschke and Jiranek, 1992.

Several conditions can be reduced must nitrogen content. Nitrogen deficiency in the vineyard and the clarification can limit or diminish, respectively, the assimilable nitrogen content of the juice. If sufficiently marked, inadequate nitrogen level slow fermentation and can cause it to become stuck. This may result from the irreversible inactivation of sugar transport by ammonia starvation (Lagunas, 1986). The half-life of the main glucose transport system is approximately 12 h, with complete inactivation occurring within approximately 50 h (Schulze *et. al.,* 1996). This results from the cessation of protein synthesis and enzyme degradation. The lack of ammonia can also negate the allosteric activation of crucial glycolytic enzymes, such as phosphofructokinase and pyruvic kinase. This is turn, further inhibits the uptake of glucose (Bely, Sablayrolles and Barre, 1994).

Juice nitrogen content may also be reduced by 33 to 80 per cent in grapes infected by *Botrytis cinerea* (Rapp and Reuther, 1971). In sparkling wine production, nitrogen deficiency caused by the initial fermentation and clarification of the cuvee wines is usually counteracted by the addition of ammonium salts such as ammonium phosphate. A rapid technique for the assessment of assimilable nitrogen (Dukes and Butzke, 1998) may facilitate assessment of actual nitrogen need.

The juice of some grape varities is more likely to show nitrogen limitation than other, for example, Chardonney and Colombard. This is especially true when the juice has been given undue centrifugation or filtration.

Nitrogen is incorporated most rapidly during the exponential growth phase during fermentation. This correlates with the period of cell growth and division. Subsequently, there is a slow release of nitrogen-containing compounds back into the fermenting juice (Figure. 1.10).

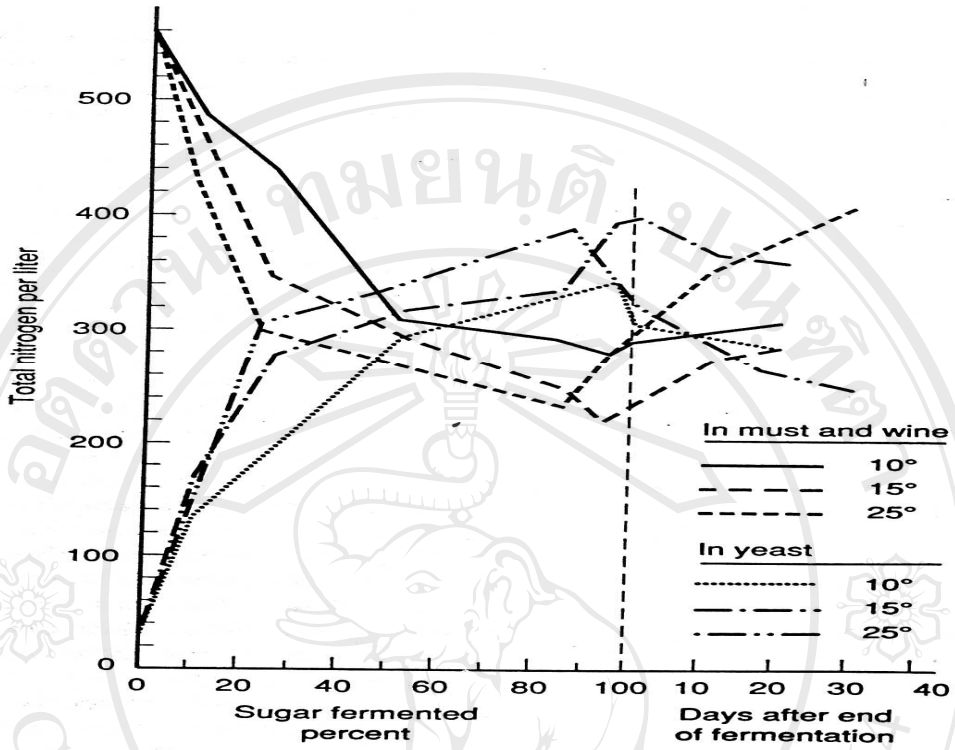


Figure 1.10 Changes in Total Nitrogen During and after Fermentation in Musts and in Yeasts Fermenting Them.

Source : Nilov and Valuiko, 1958**.**

Of the inorganic nitrogen sources, ammonia is incorporated preferentially. The oxidized state of ammonia permits its direct incorporation into organic compounds. Although, ammonia is potentially capable of repressing the uptake of amino acids, its normal concentration in grape juice is insufficient to have this effect *Saccharomyces cerevisiae* has several amino acids transport systems (Cartwright *et. al.,* 1989). One is nonspecific and directs the uptake of all amino acids except proline. The other systems are more selective, transporting only particular groups of amino acids. Certain amino acids are preferentially incorporated, such as phenylalanine, leucine, isoleucine and tryptophan; whereas others are poorly assimilated, for example, alanine, arginine and proline (Ough *et. al*., 1991).

The primary amino acids available in grape must are proline and arginine. Proline is not used as a nitrogen source; it requires the presence of molecular oxygen for its metabolism (Tomenchok and Brandriss, 1987). Thus, arginine is the principal

amino acid used as a nitrogen source. Regrettably, arginine may be degraded to carbon dioxide and ammonia (the latter assimilated in amino acid synthesis), it may be excreted. This is undesirable, because urea has been implicated in the production of the carcinogen ethyl carbamate (Ough *et. al*., 1990).

Amines and peptides also may be incorporated as nitrogen sources, but protein nitrogen is unavailable. Wine yeasts are capable of neither transporting protein across the cell membrane nor enzymatically degrading them to amino acids outside the cell.

Nitrogen contents can influence the synthesis of aromatic compounds during fermentation. Most noticeable is the reduction in fusel alcohol content in the presence of ammonia and urea. This effect can be reversed by the assimilation of certain amino acids from the juice or must. These opposing effects appear to result from the use of fusel alcohols in the biosynthesis of amino acids from ammonia and urea, and their release by deamination of amino acids assimilated from the must. The sensory impact of this equilibrium, if any, has not been established. Under nitrogen starvasion, there is a dramatic increase in the production of glycerol and trehalose (Schulze *et. al.,* 1996). Cell division also ceases in G1.

1.6 Metabolism of Nitrogen Compounds

1.6.1 Amino Acid Synthesis Pathways

The ammonium ion and amino acids found in grape must supply the yeast with nitrogen. The yeasts can also synthesize most of amino acids necessary for constructing its proteins. It fixes an ammonium ion on a carbon skeleton derived from the metabolism of sugar. The yeast use the same reactional pathways as all organisms. Glutamate and glutamine play an important role in this process (Cooper, 1982; Magasanik, 1992).

The $NADP^+$ glutamate dehydrogenase ($NADP^+$- GDH), product of the GDH1 gene, produces glutamate (Figure1.11) from an ammonium ion and an α-ketoglutarate molecule. The latter is an intermediary product of citric acid cycle.

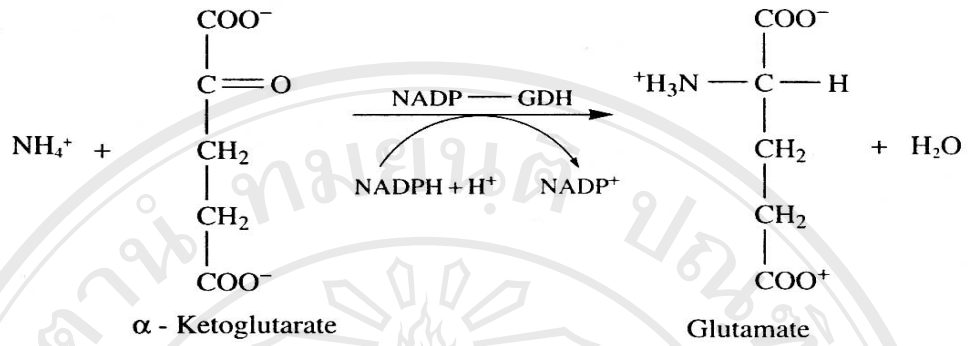


Figure 1.11 Incorporation of the Ammonium Ion in α-ketoglutarate Catalyzed by NADH Glutamate Dehydrogenase (NADP - GDP).

Source : Cooper, 1982; Magasanik, 1992.

The yeast also processes an NAD^+ glutamate dehydrogenase (NAD^+- GDH), product of the GDH2 gene. This dehydrogenase is involved in the oxidative catabolism of glutamate. It produces the inverse reaction of the precedent, liberating the ammonium ion used in the synthesis of glutamine. NAD^+- GDH activity is at its maximum when the yeast is cultivated on a medium exclusively ammonium ion as its source of nitrogen. The NAD^+- GDH activity, however, is at its highest level when the principal source of nitrogen is glutamate. Glutamine synthetase (GS) produces glutamine from glutamate and ammonium ion. This animation requires the hydrolysis of an ATP molecule (Figure 1.12).

Through, tranamination reactions, glutamate then serves as an amino acid. Pyridoxal phosphate is the transaminase cofactor (Figure1.13); it is derived from pyridoxine (Vitamin B_6).

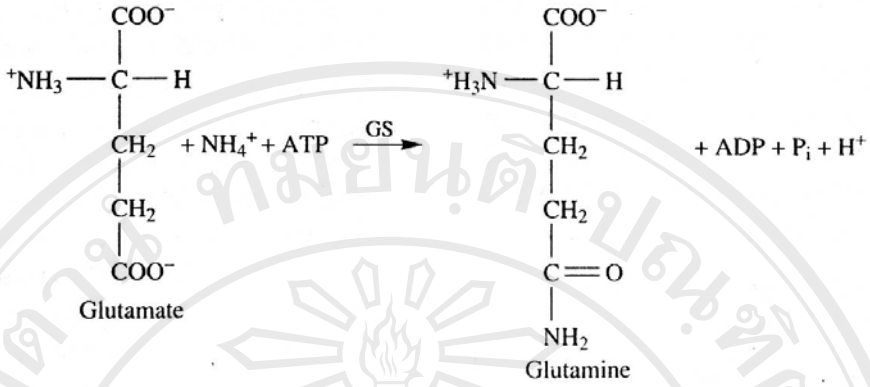


Figure 1.12 Amidation of Glutamate into Glutamine by Glutamine Synthetase (GS).
Source : Cooper, 1982; Magasanik, 1992.

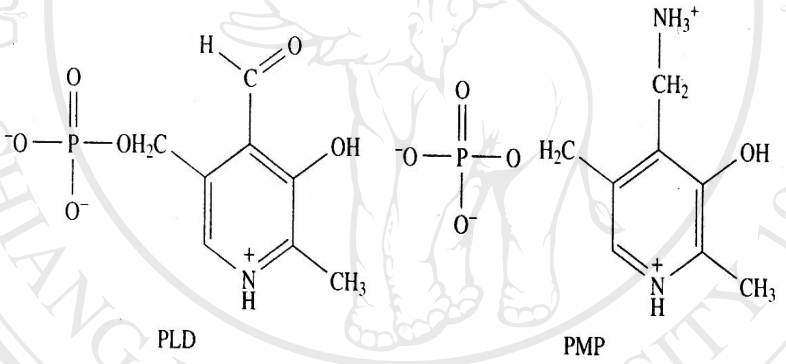


Figure 1.13 Pyridoxal Phosphate (PLP) and Pyridoxamine Phosphate (PMP).
Source : Cooper, 1982; Magasanik, 1992.

The carbon skeleton of amino acids originates from glycolysis intermediary products (pyruvate, 3-phosphoglycerate, phosphoenolpyruvate), the citric acid cycle (α-ketoglutarate, oxaloacetate) or the pentose phosphate cycle (ribose 5-phosphate, erythrose 4-phosphate). Some of these reactions are very simple, such as the formation of aspartate or alanine by transamination of glutamate into oxaloacetate or pyruvate:

- oxaloacetate + glutamate ⟶ aspartate + α-ketoglutarate
- pyruvate + glutamate ⟶ alanine + α-ketoglutarate

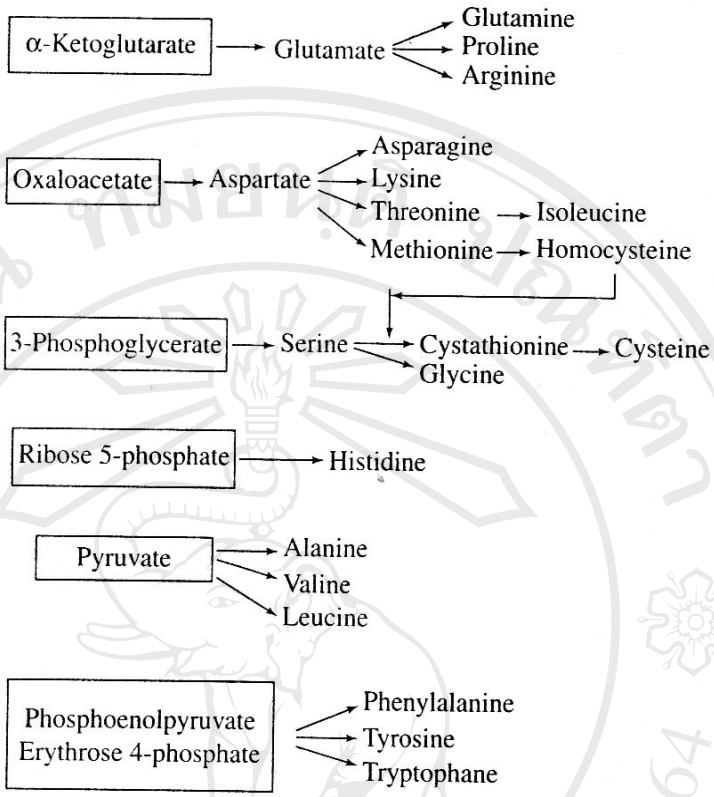


Figure 1.14 General Biosynthesis Pathway of Amino Acids.
Source : Cooper, 1982; Magasanik, 1992.

Other biosynthetic pathways are more complex, but still occur in yeasts as in the rest of the living world. The amino acids can be classified into six biosynthetic families depending on their nature and their carbon precursor (Figure 1.14):

1. In addition to glutamate and glutamine, proline and arginine are formed α-ketoglutarate.

2. Asparagine, methionine , lysine, threonine and isolucine are derived from aspartate, which is issued from oxaloacetate. ATP can activate methionine to form *S*-adenosylmethionine, which can be demethylated to form adenosylhomocysteine, the hydrolysis of which liberates adenine to produce homocysteine.

3. Pyruvate is the starting point for the synthesis of alanine, valine and leucine.

4. 3-Phosphoglycerate leads to the formation of serine and glycine. The condensation of homocysteine and serine produce cystathionine, a precursor of cysteine.

5. The imidazole cycle of histidine is formed ribose 5-phosphate and adenine of ATP.

6. The amino acids possessing an aromatic cycle (tyrosine, phenylalanine, trytophan) are derived from erythrose 4-phosphate and phosphoenolpyruvate.These two compounds are intermediaries of the pentose cycle and glycolysis, respectively. Their condensation forms shikimate. The condensation of this compound with another molecule of this compound with another molecule of phosphoenolpyruvate produces chorismate, a precursor of aromatic amino acids.

1.6.2 Assimilation Mechanisms of Ammonium Ion and Amino Acids

The penetration of ammonium ion and amino acids into the yeast cell activates numerous membrane proteinic transporters or permeases. *Saccharomyces cerevisiae* has at least two specific ammonium ion transporters (Dubois and Grenson,1979). Their activity is inhibited by several amino acids, in a non-competitive manner.

Two distinct categories of transporters ensure amino acid transport:

1. A general amino acid permease (GAP) transports all of the amino acids. The ammonium ion inhibits and represses the GAP. The GAP, therefore only appears to be active during the second half of fermentation, when the must no longer contains ammonium. It acts as a 'nitrogen scavenger' towards amino acids (Cartwright *et. al.*,1989).

2. *Saccharomyces cerevisiae* also has many specific amino acid permeases (at least 11). Each one ensures the transport of one or more amino acids. In Contrast to GAP, the ammonium ion dose not limits their activity. From the beginning of fermentation, these transporters ensure the rapid assimilation of must amino acids.

Glutamate and glutamine, crossroads of amino acid synthesis, are not the only amino acids rapidly assimilated. Most of the amino acids are practically depleted from the must by the time the first 30 g of sugar have been fermented. Alanine and arginine are the principal amino acids found in must. Yeasts slightly after the depletion of

other amino acids. Furthermore, yeasts massively assimilate arginine only after the disappearance of ammonium from the medium. Sometimes, yeasts do not completely consume γ-aminobutyric acid. Yeasts utilize proline during fermentation although, it is one of the principal amino acids found in must (Dubois and Grenson,1979).

During fermentation, yeasts assimilate between 1 and 2 g/L of amino acids. Towards the end of fermentation, yeasts excrete significant, but variable amounts of different amino acids. Finally, at the end of alcoholic fermentation, a few hundred milligrams of amino acids per liter remain; proline generally represent half (Dubois and Grenson,1979).

Contrary to must hexoses that penetrate the cell by facilitated diffusion, ammonium and amino acids require active transport. Their concentration in the cell is generally higher than in the external medium. The permease involved couples the transport of an amino acid molecules (or ammonium ion) with the transport of a hydrogen ion. The hydrogen ion moves in the direction of the concentration gradient: the concentration of hydrogen ions in the must is higher than in the cytoplasm. The amino acids and the hydrogen ions are linked to the same transport protein and penetrate the cell simultaneously. This concerted transport of two substances in the same direction is called symport (Figure 1.15). Obviously, the hydrogen ion that penetrates the cell must then be exported to avoid acidification of the concentration gradient and requires energy. The membrane ATPase ensures the excretion of the hydrogen ion across the plasmic membrane, acting as a hydrogen ion pump (Courchesne and Magasanik, 1983).

Ethanol strongly limits amino acid transport. It modifies the composition and the properties of the phospholipids of the plasmic membrane. The membrane becomes more permeable. The hydrogen ions of the medium massively penetrate the interior of the cell by simple diffusion. The membrane ATPase must increases its operation to control the intracellular pH. As soon as this task monopolizes the ATPase, the symport of the amino acids no longer functions. In other words, at the beginning of fermentation, and of as long as the ethanol concentration in the must is low, yeasts can rapidly assimilate amino acids and concentrate them in the vacuoles for later use, according to their biosynthesis needs (Courchesne and Magasanik, 1983).

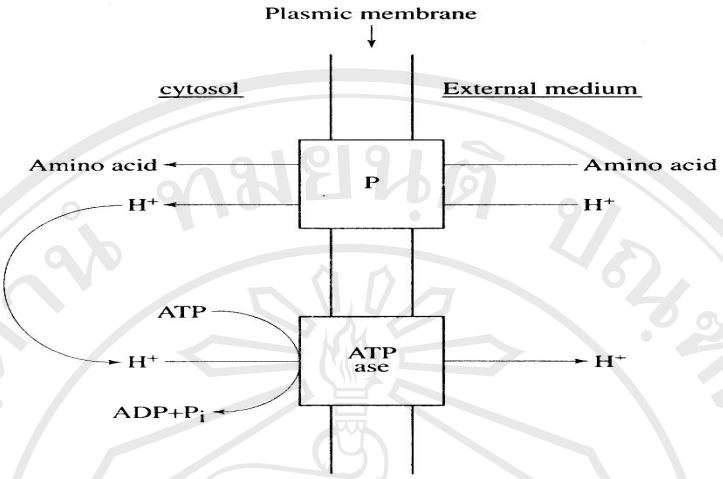


Figure 1.15 Active Amino Acid Transfer Mechanisms in the Yeast Plasma Membrane. P = protein playing the role of an amino acid 'symporter'.
Source : Courchesne and Magasanik, 1983.

1.6.3 Catabolism of Amino Acids

The ammonium ion is essential for the synthesis of amino acids necessary for building proteins, but yeasts cannot always find sufficient quantities in their environment. Fortunately, they can obtain ammonium ion from available amino acids through various reactions (Boulton, Singleton and Kunkee, 1996).

The most common pathway is the transfer of an α-amino group, originating form one of many different amino acids, onto α-ketoglutaric acid to form glutamate. Aminotransferase or transaminase catalyze this reaction, whose prosthetic group is pyridoxal phosphate (PLP). Glutamate is then determinated by oxidative pathway to form NH_4^+ (Figure1.16). These two reactions can be summarized as follow:

$$\alpha\text{-amino acid} + NAD^+ + H_2O \longrightarrow \alpha\text{-ketonic acid} + NH_4^+ + NADH+H^+$$

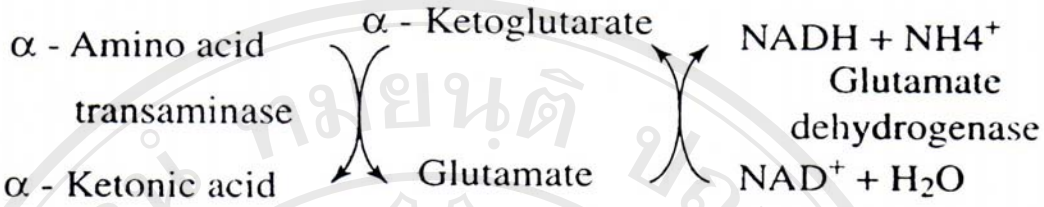


Figure 1.16 Oxidative Deamination of an Amino Acid, Catalyzed by a Transaminase and Glutamate Dehydrogenase.

Source : Boulton, Singleton and Kunkee, 1996.

During transamination, pyridoxal phosphate is temporarily transformed into pyridoxamine phosphate (PMP). The PLP aldehydic group is linked to a lysine residue ε-amino group on the active site of the aminotransferase to form an intermediary product (E-PLP) (Figure1.17). The α-amino group of amino acid substrance of the transamination displaces the lysine residue ε-amino group linked to PLP. The cleavage of this intermediary product liberates PMP and ketonic acid, corresponding to the amino acid substrate. PMP can in turn react with another ketonic acid to furnish a second amino acid and regenerate pyridoxal phosphate. The partial reactions can be written in the following manner:

- amino acid 1 + E-PLP ⟶ ketonic acid 1 + E-PLP
- ketonic acid 2 + E-PLP ⟶ amino acid 2 + E-PLP

the balance sheet for which is:

amino acid 1 + ketonic acid 2 ⟶ ketonic acid 1 + amino acid 2

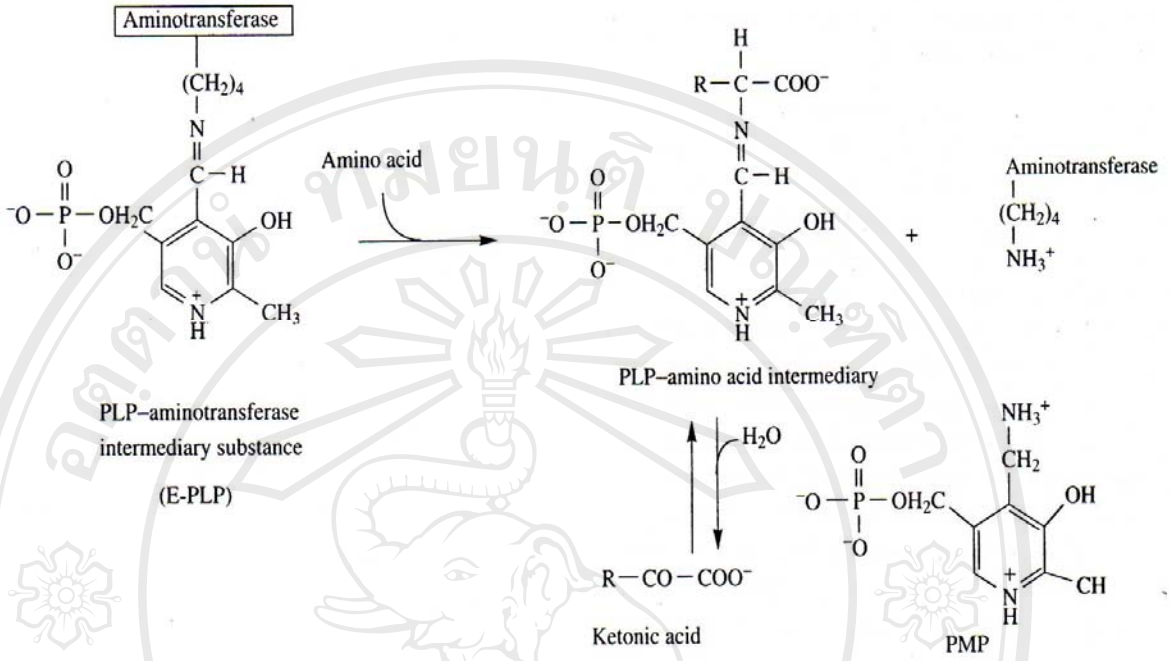


Figure 1.17 Mode of Action of Pyridoxal Phosphate (PLP) in Transamination Reactions. Formation of intermediary products between PLP and aminotransferase or the amino acid substrate.

Source : Boulton, Singleton and Kunkee, 1996.

Some amino acids, such as serine and threonine, possess a hydroxyl group on their β carbon. They can be directly deaminated by dehydration. A dehydratase catalyses this reaction, producing the corresponding ketonic acid and ammonium ion (Figure1.18)

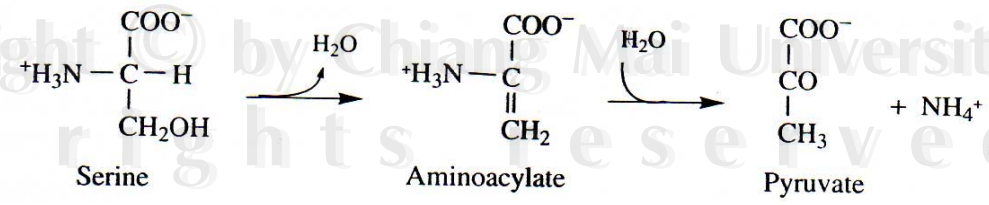


Figure 1.18 Deamination of Serine by a Dehydratase**.**

Source : Cooper, 1982

1.6.4 Formation of Higher Alcohols and Esters

Yeasts can excrete ketonic acids originating form the deamination of amino acids only after their decarboxylation into aldehyde and reduction into alcohol (Figure 1.19). This mechanism, known as the Ehrich reaction, explains in part the formation of higher alcohols in wines. Table 1 lists the principal higher alcohols and their corresponding amino acids, possible precursors of these alcohols (Cooper, 1982).

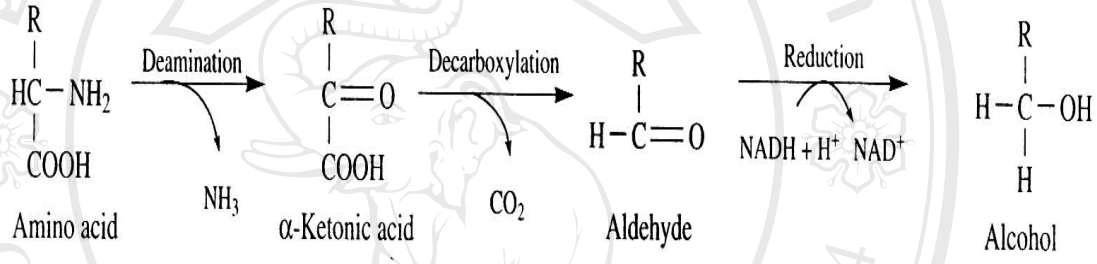
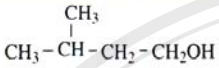
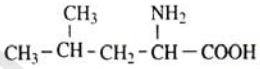
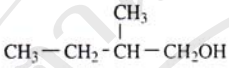
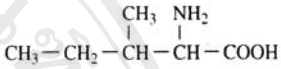
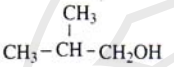
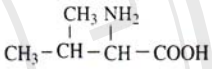
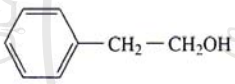
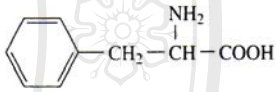
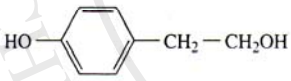
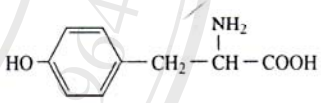
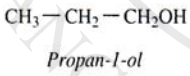
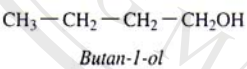
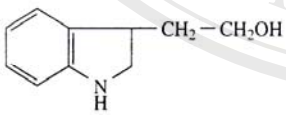
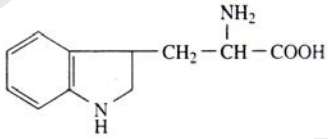
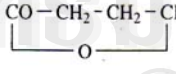
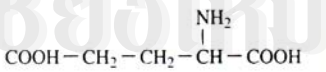
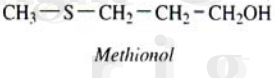
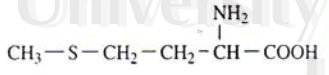


Figure 1.19 Formation of Higher Alcohols Form Amino Acids (Ehrlich reactions).
Source : Cooper, 1982.

Table 1.1 The Principal Alcohols Found in Wines and Their Amino Acid Precursors

Higher alcohol	Concentration in wine ($mg.l^{-1}$)	Amino acid precursor
 *3-methyln-buta-1-ol or isoamyl alcohol*	80-300	 *Leucine*
 *2-methyl-butan-2-ol or active amyl alcohol*	30-100	 *Isoleucine*
 *2-methyl-propan-1-ol or isobutyl alcohol*	50-150	 *Valine*
 *Phenylethanol*	10-100	 *Phenylalanine*
 *Tyrosol*	20-50	 *Tyrosine*
 *Propan-1-ol*	10-50	?
 *Butan-1-ol*	1-10	?
 *Thyptophol*	0-1	 *Tryptophane*
 *γ-Butyrolatone*	0-5	 *Glutamic acid*
 *Methionol*	0-5	 *Methionine*

Source : Rapp and Mandery, 1986.

Several experiments clearly indicate, however, that the degradation of amino acids is not the only pathway for forming higher alcohols in wine. In fact, certain ones, such as 1-propanol and 1-butanol, 1-propanol do not have amino acids precursors. Moreover, certain mutants deficient in the synthesis of specific amino acid do not produce the corresponding higher alcohol, even if the amino acids are present in the culture medium. There is no relationship between the amount of amino acids in must and the amount of corresponding higher alcohols in wines (Sponholz, 1988).

Higher alcohols production by yeasts appears to be linked not only to the catabolism of amino acids, but also to their synthesis via the corresponding ketonic acids. These acids are derived from the metabolism sugar. For example, 1-propanol has no corresponding amino acid. It is derived from α-ketobutyrate which can be formed pyruvate and acetyl coenzyme A. α-Ketoisocaporate is a precursor of isoamyl alcohol and the intermediary product in the synthesis of lucine. It too can be produced from α-acetolactate, which is derived from pyruvate. Most higher alcohols in wines can also be formed by the metabolism of amino acids (Chen, 1978).

The physiological function of higher alcohols production by yeasts is not clear. It many be a simple waste of sugars, a detoxification process of the intracellular medium, or a means of regulating the metabolism of amino acids.

With the exception of phenylethanol, which has a rose-like fragrance, higher alcohols smell bad. Most, such as isomylic alcohol, have heavy solvent-like odours. Methanol is a peculiar alcohol, because it contains a sulfur atom. Its cooked cabbage odour has the lowest perception threshold (1.2 mg/L). It can be responsible for the most persistent and disagreeable olfactory flaws of reduction, especially in white wines. In general, the winemaker should avoid excessive higher alcohol odours. Fortunately, their organoleptical impact is limited at their usual concentrations in wine, but it depends on the overall aromatic intensity of the wine. Excessive yields and rain at the end of maturation can dilute the must, in which case the wines will have a low aromatic intensity and the heavy, common character of higher alcohols can be pronounced (Rapp and Guntert, 1986.)

The winemaking parameters that increase higher alcohol production by yeasts are well known: high pH, elevated fermentation temperature, and aeration. In red winemaking, the extraction of pomace constituents and the concern for rapid and complete fermentations impose aeration and elevated temperatures, and in this case higher alcohol production by yeasts cannot be limited. In white winemaking, a fermentation temperature between 20 and 22 ^{o}C limits the formation of higher alcohols (Rapp and Guntert, 1986.)

Ammonium ion and amino acids deficiencies in must lead to an increased formation of higher alcohols. In these conditions, the yeast appears to recuperate all of the animated nitrogen available by transamination. It abandons the unused carbon skeleton in form of higher alcohols. Racking white must also limits the production of higher alcohols (Sponholz, 1988).

The nature of the yeasts (species, strains) responsible for fermentation also affects the production of higher alcohols. Certain species, such as *Hansenula anomala*, have long been known to produce a lot, especially in aerobiosis (Guymon, Ingraham and Crowell, 1961). Yet, production by wine yeasts is limited, even in spontaneous fermentation. More recently, various researchers have shown that most *S. cerevisiae* depends on the strain. A limited higher alcohol production (with the exception of phenylethanol) should be among selection criteria for wine yeasts. Due to their esterase activities, yeasts form various esters (a few milligrams per liter). The most important acetates of higher alcohols are isoamyl acetate (banana aroma) and phenylethyl acetate (rose aroma). Although, they are not linked to nitrogen metabolism, ethyl esters of medium chain fatty acids are also involved. They are formed by the condensation of acetyl coenzyme A. These esters have more interesting aromas than the others. Hexanoate has a flowery and fruity aroma reminiscent of green apples. Ethyl decanoate has a soap-like odour. In white winemaking, the production of these esters can be increased by lowering the fermentation temperature and increasing must clarification. Certain yeast strains (71B) produce large quantities of these compounds, which contributes to the fermentation aroma of young wines. They are rapidly hydrolyzed during their first year in bottle and have no long-term influence on the aromatic character of white wines (Nykanen, 1986).