IMPACT OF THIAMINE AND PYRIDOXINE ON ALCOHOLIC FERMENTATIONS OF SYNTHETIC GRAPE JUICE

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of HUAJING XING find it satisfactory and recommend that it be accepted.

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Chair

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IMPACT OF THIAMINE AND PYRIDOXINE ON ALCOHOLIC FERMENTATIONS OF SYNTHETIC GRAPE JUICE

Abstract

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Sluggish fermentation and H_2S production are serious problems found in the wine industry since they are directly related to wine quality and economic issues. There are several factors that can cause these problems, such as nitrogen and vitamin deficiencies.

In this study, the effects of thiamine (vitamin B_1) and pyridoxine (vitamin B_6) on alcoholic fermentation rates and hydrogen sulfide (H_2S) production were studied. Using a synthetic grape juice base, three fermentations were conducted: (1) a 2×3 factorial design with nitrogen (60 and 250 mg/L) and thiamine (0, 0.2, and 0.5 mg/L) as variables with a sufficient concentration of pyridoxine (2 mg/L), (2) a 2×3 factorial design with nitrogen (60 and 250 mg/L) and pyridoxine (0, 0.25, and 0.5 mg/L) as variables with a sufficient concentration of thiamine (0.5 mg/L), and (3) a 3×3 factorial design for comparing the effects of thiamine (0, 0.2, and 0.5 mg/L) and pyridoxine (0, 0.25, and 0.5 mg/L) at the low concentration of nitrogen (60 mg/L). Fermentations were conducted with *Saccharomyces cerevisiae* UCD 522 at 22°C.

Thiamine, pyridoxine, nitrogen and their interactions were found to affect fermentation rate and H_2S production. At low levels of thiamine and pyridoxine, yeast

exhibited slower fermentation rate regardless of nitrogen concentration, which indicated that thiamine and pyridoxine deficiencies may cause sluggish fermentations. Hydrogen sulfide production was significantly different ($p \leq 0.001$) at different concentrations of thiamine and pyridoxine. Furthermore, interactions between thiamine, pyridoxine and nitrogen also highly affected hydrogen sulfide production. Sensory evaluation methods were applied to the synthetic wine samples fermented at different thiamine, pyridoxine and nitrogen concentration combinations and hydrogen sulfide was found to be significant different in aroma attribute rating.

By adjusting thiamine, pyridoxine and nitrogen concentrations in grape juice, sluggish fermentation and excessive hydrogen sulfide production can be reduced.

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DEDICATION

This thesis is dedicated to my loving and supportive husband, parents, and friends.

INTRODUCTION

Wine fermentation is the process of converting grape sugars, mainly glucose and fructose, into ethanol and carbon dioxide. In 1815, Gay-Lussac proposed a chemical equation for alcoholic fermentation (Rose 1970):

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

In alcoholic fermentation, one sugar molecule can be converted into two molecules of ethanol and two molecules of carbon dioxide by yeast. Besides ethanol and carbon dioxide, other volatile compounds are produced that determine the sensory characteristics of wine. These volatile compounds include glycerol, pyruvate, acetaldehyde, lactic acid, diacetyl, succinate, acetic acid and hydrogen sulfide (Boulton et al. 1999, Farkaš 1988, Jackson 2000, Ribéreau-Gayon et al. 2000).

Both sluggish/stuck fermentation and excessive hydrogen sulfide production are serious problems in the wine industry since they are directly related to wine quality and economic issues (Bisson 1999, Kunkee 1991). These problems can also cause undesirable sweetness for finished wines and sometimes the optimal alcohol content is not achieved. Furthermore, hydrogen sulfide, characterized by a rotten egg odor, is one of the most undesirable metabolites of alcoholic fermentation (Eschenbruch 1974, Jackson 2000).

During alcoholic fermentation, nutrients including nitrogen play important roles in yeast metabolism. In fact, nitrogen deficiency is one of the most important factors that lead to sluggish/stuck fermentation and excessive H_2S production (Bisson 1999, Giudici and Kunkee 1994, Sablayrolles 1996). In addition to nitrogen, vitamins also play important roles during wine fermentation. Some yeast strains have an absolute

requirement for certain vitamins, such as biotin and inositol (Dixon and Rose 1964, 1966, Ough et al. 1989). Vitamin deficiency is also regarded as one of the factors that cause sluggish/stuck fermentations (Alexandre and Charpentier 1998, Ough et al. 1989, Wang et al. 2003).

Thiamine (vitamin B_1) and pyridoxine (vitamin B_6) are two B vitamins that serve as coenzymes in yeast metabolism during fermentation (Trevelyan and Harrison 1954). Thiamine and pyridoxine can assist in the synthesis of one and the other (Leonian and Lilly 1942, Chiao and Peterson 1956), which means yeast may still reach normal growth in thiamine or pyridoxine deficient media. On the other hand, interaction effects between these two vitamins have been found in yeast metabolism. For instance, in pyridoxine-free medium, yeast growth inhibition was caused by addition of thiamine. However, it could be solved by the addition of pyridoxine (Schultz 1947, Chiao and Peterson 1956). The effects of thiamine and pyridoxine, together with nitrogen, on fermentation rate and hydrogen sulfide production during fermentation have not been demonstrated.

The objective of this study was to determine how thiamine, pyridoxine, and nitrogen affect yeast growth, fermentation rate, and hydrogen sulfide production under fermentation conditions.

LITERATURE REVIEW

Wine Yeast—*Saccharomyces*

The genera *Saccharomyces* is undoubtedly the most important yeast in the wine industry and conducts the majority of alcoholic fermentations (Alfenore et al. 2002, Jackson 2000, Kunkee and Bisson 1993, Taherzadeh et al. 1996). The yeast strains used will affect the fermentation speed, color extraction, alcohol content, nature and quantity of secondary products formed, and the aromatic characters of the wine (Ribéreau-Gayon et al. 2000). A good wine yeast strain should be able to conduct a vigorous fermentation, possess good alcohol tolerance and not produce excessive off-odor compounds (Boulton et al. 1999).

Taxonomy and general ecology

The initial classification of *Saccharomyces* was based principally on morphological and physiological characteristics of the genera (Boulton et al. 1999). The taxonomy of *Saccharomyces* has changed in the past 30 years and resulted in the *Saccharomyces sensu stricto* complex, which is composed of four species: *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, and *S. pastorianus* (synonym *S. carlsbergensis*) (Naumov et al. 2000, 2003, Torriani et al. 1999, Vaughan-Martini 1989, Vaughan-Martini and Kurtzman 1985, Vaughan-Martini and Martini 1987, 1998, Viti et al. 2000). The predominant yeast involved in wine fermentation is *S. cerevisiae* and it represents most of the yeasts selected for wine fermentation (Boulton et al. 1999, Frezier and Dubourdieu 1992, Martini et al. 1996, Ribéreau-Gayon et al. 2000).

S. cerevisiae may appear microscopically as round, oval or oblong cells. Some of the cells form spores, and the spores can be smooth, round, globular or hat-shaped (Cook 1958, Farkaš 1988, Tuite and Oliver 1991). *S. cerevisiae* can exist either as a single celled organism or as pseudomycelia, and reproduce by multilateral budding (Dickinson and Schweizer 2004, Fleet et al. 2002).

Saccharomyces species are present in the vineyard or around the winery, including grapes leaves, soil, hands, and equipment surfaces (Jackson 2000). They are rarely present on grapes and seldom occur in significant numbers on grape leaves or stems (Fleet and Heard 1993, Jackson 2000). Although the surfaces of wine equipment in the winery may be the major sources of *S. cerevisiae*, commercial yeast inoculation is widely used in the wine industry (Fleet and Heard 1993, Schuller et al. 2005).

S. cerevisiae catabolize sugars present in the grape juice, primarily glucose and fructose, to produce energy for growth and metabolism (Farkaš 1988). Different strains show various fermentation capabilities and are influenced by environmental conditions, such as presence of sulfur dioxide, sugar concentration, and sensitivity to ethanol. *S. cerevisiae* produce large amounts of ethanol and have high resistance to sulfur dioxide (Ribéreau-Gayon et al. 2000).

<u>Commercial use</u>

Starter cultures are utilized to obtain desirable yeast characteristics (Mateo et al. 1999). Starter cultures can be either active dry yeast (ADY) or a single strain from a yeast collection. Since the commercial use of ADY began in the 1960s (Kunkee and Bisson 1993, Reed and Nagodawithana 1988), it has been widely accepted and used in new wine

making regions, especially in the United States. It can conduct good fermentations with desirable characteristics (Boulton et al. 1999, Schuller et al. 2005). Selected strains of *S. cerevisiae* can grow to more than 10^7 cells/mL to dominate the fermentation within several days. The production of ADY does not always result in a pure strain and may contain some bacteria or other yeasts (Boulton et al. 1999).

Among *S. cerevisiae* strains, UCD 522 (Montrachet) is popular for producing both red and white wines and has been widely applied in enology research (Ciani 2002, Davenport 1985, Liu and Gallander 1982, Ough et al. 1988, 1989, Porter and Ough 1982, Spiropoulos et al. 2000). The Montrachet strain conducts vigorous fermentation, has good ethanol tolerance, and can ferment grape musts and fruit juices to dryness, defined as less than 2 g/L reducing sugars. Montrachet is noted for producing low volatile acidity, good flavor complexity, and intensely colored wines (Eisenman 1999). It is also known to have the highest nitrogen demand during fermentation and produces a higher level of H_2S compared to other *S. cerevisiae* strains (Henschke and Jiranek 1993). It is not recommended to use Montrachet with grapes that have been recently dusted with sulfur because of its tendency to produce H_2S in the presence of the high concentrations of sulfur compounds (Acree et al. 1972, Park et al. 2000, Rupela and Tauro 1984).

Biochemistry of Alcoholic Fermentation

Although ethanol and carbon dioxide are the main products of fermentation, many volatile compounds are also produced. Normally, more than 95% of the sugar is converted into ethanol and carbon dioxide, with 1 to 4% converted into cellular materials and other end products (Boulton et al. 1999).

Glycolysis

Glycolysis, or the Embden-Meyerhof-Parnas pathway (EMP), is the major pathway by which *Saccharomyces* utilizes glucose and fructose in the cell cytoplasm (Fig. 1). The first step of glycolysis is the transport of glucose, fructose, and mannose into the cell by facilitated diffusion. Sucrose, the most prevalent disaccharide in grape must, is cleaved to glucose and fructose by a secreted invertase before transport (Boulton et al. 1999).

The second step of glycolysis is the phosphorylation of monosaccharides by kinase enzymes, which include hexokinase PI (A), hexokinase PII (B) and glucokinase (Boulton et al. 1999). All three enzymes can utilize glucose and mannose as substrates, but only the hexokinases efficiently phosphorylate fructose. Hexokinase PII is dominant during the log phase of fermentation at high sugar concentration while Hexokinase PI is present during the stationary phase of growth and fermentation (Bisson 1991).

The following steps result in the conversion of the phorsphorylated sugars to pyruvate (Fig. 1). These procedures involve phosphoglucose isomerase, phosphofructokinase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase. The last step in glycolysis is that pyruvate is decarboxylated into acetaldehyde by pyruvate decarboxylase and then converted into ethanol by alcohol dehydrogenase. In the later reaction, NADH is oxidized to NAD^+ and requires thiamine pyrophosphate (TPP) as a cofactor under anaerobic conditions.

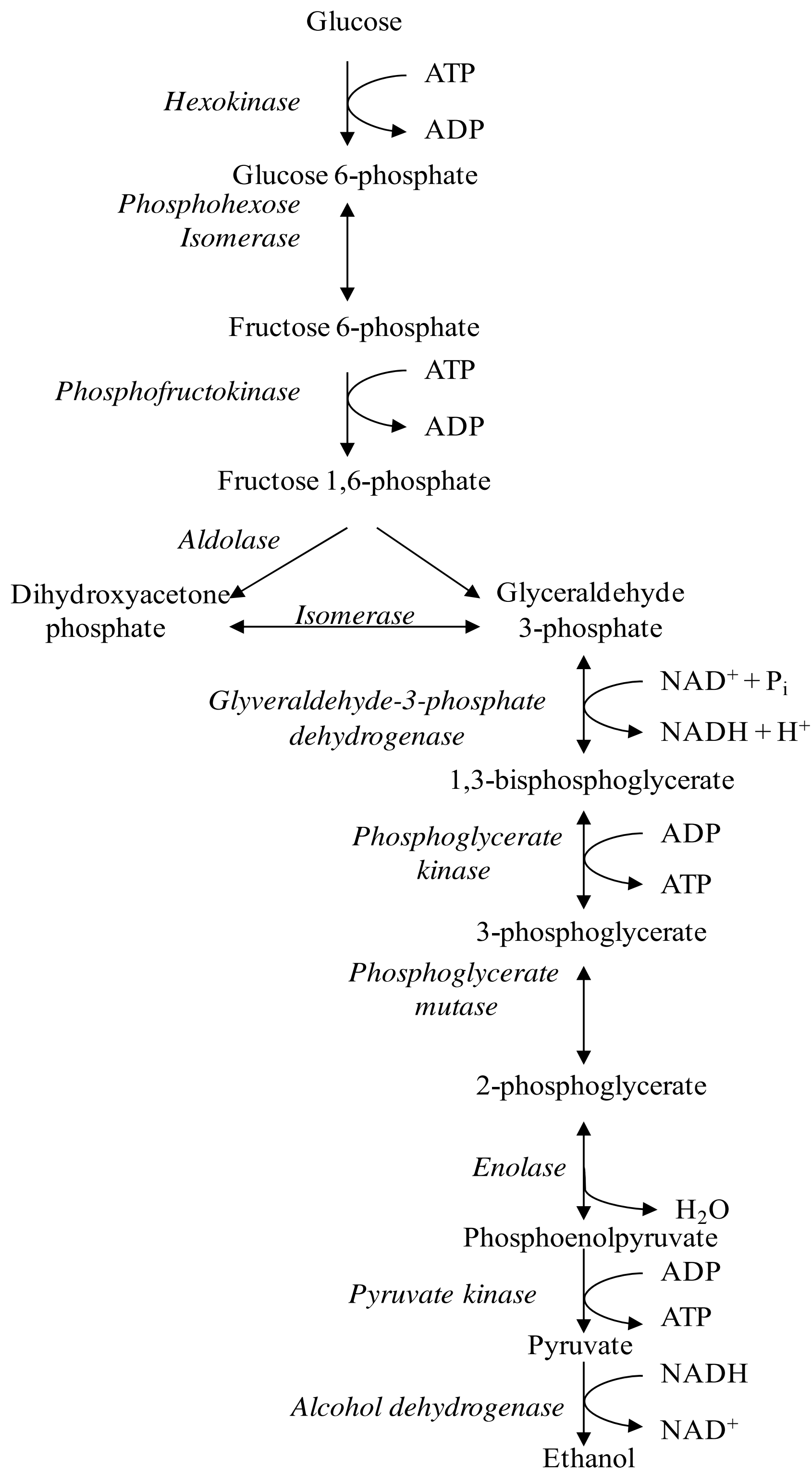


Figure 1. Glycolysis pathway (Embden-Meyerhof-Parnas pathway)

Crabtree effect

Under aerobic conditions, yeast metabolism is through respiration enzyme systems, which include glycolysis, tricarboxylic acid cycle (TCA cycle) (also named citric acid or Krebs cycle), and oxidative phosphorylation (electron transfer). However, at high concentration of glucose, respiration is inhibited even when oxygen is present. Thus, fermentative pathway is used to metabolize sugars. This phenomenon was discovered by Crabtree in 1929 and has been named the catabolic repression, Crabtree effects, or Pasteur contrary effects (Gancedo 1998). The minimum amount of glucose required for Crabtree effect to take place is 9 g/L glucose (Ribéreau-Gayon et al. 2000). Under winemaking conditions, yeast undergo fermentative metabolism with oxygen present during the fermentation since grape musts contain high concentrations of glucose.

Nutrients Important for Alcoholic Fermentation: Nitrogen

Besides sugars, nitrogen containing compounds are also essential to yeast growth and metabolism. The nitrogen content in grape must varies greatly depending on grape cultivar, climate, vineyard, year, and fertilization treatments (Bell et al. 1979, Bisson 1991, Kliewer 1970, Kluba et al. 1978, Sponholz 1991).

Grape musts normally contain 60 to 2400 mg N/L of nitrogen-containing compounds, many of which are utilized by yeast (Amerine et al. 1980, Ough and Amerine 1988). The components of nitrogen in grape juice include different compounds, such as free amino acids, ammonium ions, peptides and proteins. While polypeptides and proteins cannot be utilized by *S. cerevisiae*, ammonium ions and most free amino acids can be utilized by *S. cerevisiae* (Farkaš 1988, Ribéreau-Gayon et al. 2000). The major

amino acids in grape juice are proline, arginine, alanine, glutamate, glutamine, serine, and threonine (Butzke 1998, Spayd and Andersen-Bagge 1996, Kunkee and Bisson 1993). Ammonia and amino acids, except proline, are referred as yeast assimilable nitrogen (YAN), which are nitrogen sources that can be utilized by yeast during fermentation (Henschke and Jiranek 1991, Sablayrolles et al. 1996). YAN is calculated as mg N/L based on the concentration of amino acids and ammonia and it is used as a measurement of nitrogen concentration in grape juice.

It is difficult to determine the amount of nitrogen yeast need for fermentation. Nitrogen requirements depend on fermentation purposes, such as whether or not non-*Saccharomyces* yeast are encouraged to be involved in fermentation (Fleet 1993). Other factors include yeast strains, fermentable sugar concentration, oxygen supply and temperature of fermentation (Jiranek et al. 1990, 1991, 1995a, Julien et al. 2000, Malherbe et al. 2003, Vos et al. 1980). Although it is estimated that at least 140 mg/L assimilable nitrogen is needed to complete the fermentation to dryness (Agenbach 1977), most grape musts are deficient in nitrogen for yeast utilization (Butzke 1998, Spayd and Andersen-Bagge 1996, Varela et al. 2004). Henschke and Jiranek (1993) suggested that optimum levels for assimilable nitrogen are 400 to 500 mg/L.

Nitrogen uptake and metabolism during fermentation

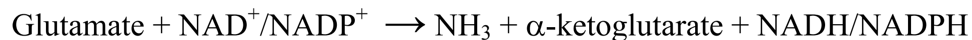
Ammonia and amino acids are taken up by yeast during the early stage of fermentation through permeases. The uptake rate is dependent on the activity and number of permeases in the membrane available for different compounds (Bisson 1991, Boulton et al. 1999, Darte and Grenson 1975).

Ammonium ions, glutamate and glutamine are generally the most preferred nitrogen sources because they can be directly utilized for biosynthesis (Cantarelli 1957, Ribéreau-Gayon et al. 2000). The pathways for ammonium ion and glutamate metabolism are the glutamate dehydrogenase system and the glutamine synthetase-glutamate synthetase system (Cooper 1982, Henschke and Jiranek 1991). In the glutamate dehydrogenase system, ammonia and α-ketoglutarate are catalyzed by NADP-dependent glutamate dehydrogenase (NADP-GDH) to form glutamate with NADH or NADPH transferred to NAD^+ or $NADP^+$. NAD-dependent glutamate dehydrogenase (NAD-GDH) catalyzes the reverse reaction (Fleet 1993, Henschke and Jiranek 1991):

NADP-GDH

$$NH_3 + \alpha\text{-ketoglutarate} + NADH/NADPH \longrightarrow \text{glutamate} + NAD^+/NADP^+$$

NAD-GDH



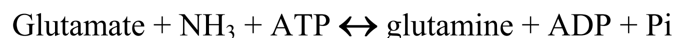
$$\text{Glutamate} + NAD^+/NADP^+ \longrightarrow NH_3 + \alpha\text{-ketoglutarate} + NADH/NADPH$$

The glutamine synthetase-glutamate synthetase system includes two reversible reactions, which can generate glutamate and ammonium (Magasanik and Kaiser 2002). These two reactions are catalyzed by glutamate synthetase and glutamine synthetase:

Glutamate synthetase

$$\text{Glutamine} + \alpha\text{-ketoglutarate} + NADH \leftrightarrow 2\ \text{glutamate} + NAD^+$$

Glutamine synthetase



$$\text{Glutamate} + NH_3 + ATP \leftrightarrow \text{glutamine} + ADP + Pi$$

Most amino acids can be utilized as direct nitrogen sources by *Saccharomyces* through transamination reactions (Fig. 2). These reactions exchange the amino group from one amino acid to a different keto acid with no overall loss or gain of nitrogen from the system. α-Ketoglutarate is an important component for glutamate and ammonium ion metabolism, which is also involved in transamination reactions. Pyridoxal phosphate (PLP), the biologically active form of pyridoxine, is required as a cofactor to complete these transamination reactions.

<u>Nitrogen effect on fermentation</u>

Nitrogen metabolism can affect the availability of amino acid precursors for the biosynthesis of proteins, yeast cell biomass, and glycolysis pathway, and therefore influence the fermentation rate (Boulton et al. 1999, Schulze et al. 1996, Varela et al. 2004). An insufficient nitrogen concentration can cause a slow fermentation rate. Nitrogen limitation accelerates turnover of glucose permeases and decreases fermentation capacity (Cramer et al. 2002, Jackson 2000, Jiranek et al. 1990, Lagunas 1979, Salmon 1989, Varela et al. 2004) .

Nitrogen, especially amino acids, highly affects H_2S production (Butzke 1998, Jiranek et al. 1990, Moreira et al. 2002). Sulfur containing amino acids, such as methionine and cysteine, are involved in sulfur metabolism, and therefore influence H_2S production (Moreira et al. 2002, Park et al. 2000).

During fermentation, some nitrogen-containing compounds in the grape musts can be synthesized to aroma compounds, such as esters and higher alcohols (Cabrera et al. 1988, Giudici et al. 1993, Herraiz and Ough 1993, Inoue 1975). Higher alcohols are those

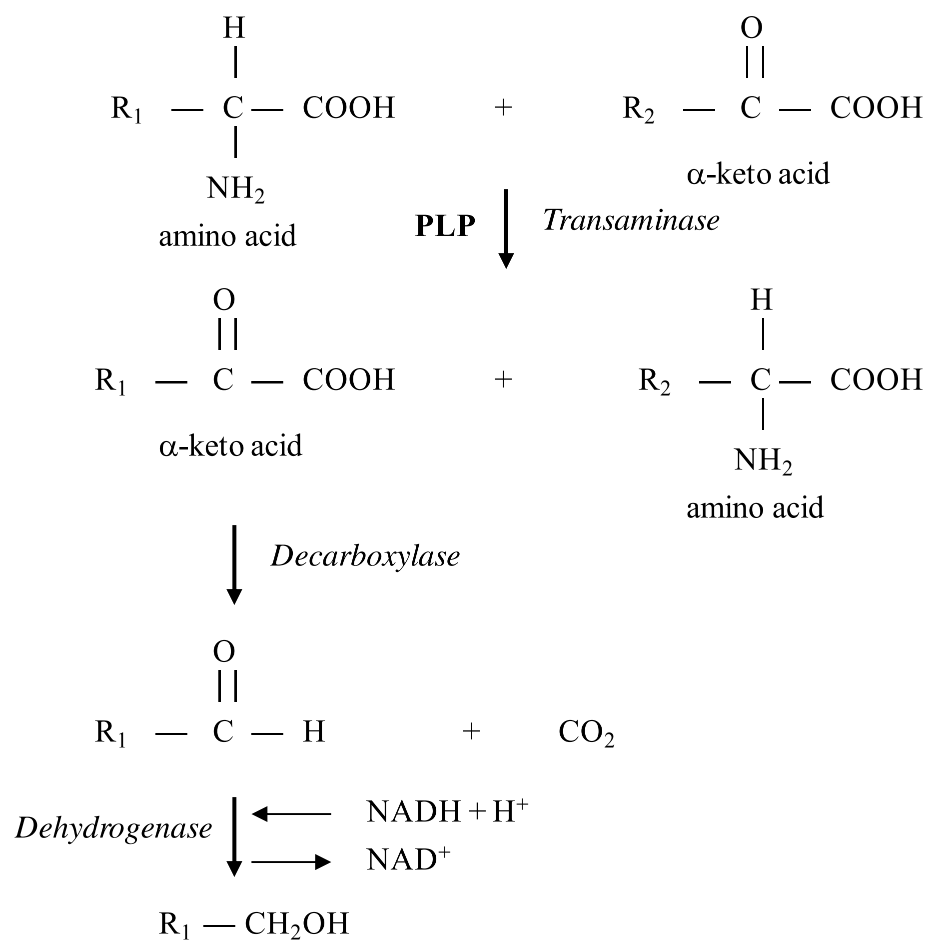


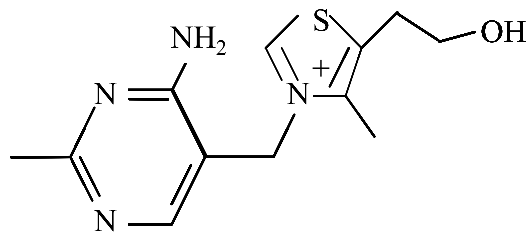
Figure 2. Transamination reaction between amino acid and α-keto acid. R_1 and R_2 are different amino side chain groups.

with three or more carbons, normally including *n*-propyl alcohol, isobutyl alcohol, isoamyl alcohol and amyl alcohol (Farkaš 1988). Higher alcohol formation involves transamination reactions utilizing available amino acids and α- keto acids (Fig 2). Although higher alcohols are normally present in low concentrations in finished wines, these influence the bouquet and taste of wine (Bisson 1991, Boulton et al. 1999, Farkaš 1988, Jiranek et al. 1990, Rankine 1967).

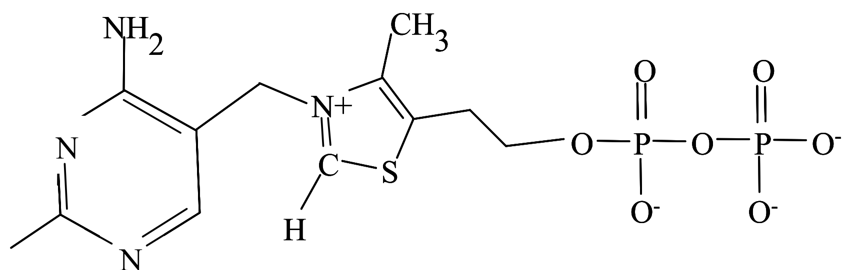
Nutrients Important for Alcoholic Fermentation: Thiamine

Vitamin B_1 is most often known as thiamine or thiamin, but the term "aneurin" is also used (Tanphaichitr 2001). Thiamine, 3-(4-amino-2-methylpyrimidin-5-pyrimidinyl)-5-(2-hydroxyethyl)-4-methylthiazolium, is a water soluble vitamin within the B complex (Fig. 3 A). Thiamine consists of a pyrimidine ring and a thiazole ring, connected by a carbon link. Thiazolium salts, such as thiamine hydrochloride and thiamine mononitrate, are common commercial sources of thiamine. Thiamine is regarded as an important nutrient for yeast metabolism during wine fermentation (Koser 1968, Laser 1941, Muller et al. 1999, Schenk et al. 1998, Trevelyan and Harrison 1954, Williams et al. 1941, Williams and Roehm 1930).

The most biologically active form of thiamine is thiamine pyrophosphate (TPP), sometimes called thiamine diphosphate (TDP) (Fig. 3 B). This form is also known as cocarboxylase, which was first recognized as the coenzyme of carboxylase, involved in converting pyruvic acid to carbon dioxide and acetaldehyde (Trevelyan and Harrison 1954). Yeast can synthesize TPP from free thiamine either inside or outside the cell by



(A)



(B)

Figure 3. Chemical structure of thiamine (A) and thiamine pyrophosphate (TPP) (B).

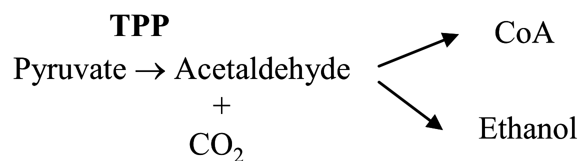
thiamine pyrophosphokinase (Bataillon et al. 1996, Hohmann and Meacock 1998, Tanphaichitr 2001).

Content in grape musts

Ribéreau-Gayon et al. (1975) reported that the concentration for thiamine in grape musts ranges from 160 to 450 μg/L. However, yeast can utilize more thiamine (600-800 μg/L) than grape must contains (Ribéreau-Gayon et al. 2000). There are no data available on thiamine content in U. S. grape musts to date.

Biochemical roles

TPP plays an important role in yeast metabolism, where it is a coenzyme for α-ketoacid dehydrogenase. This enzyme cleaves a C-C bond adjacent to a carbonyl group, releasing carbon dioxide and an acetaldehyde (Laser 1941, Muller et al. 1999, O'Fallon 1975, Park et al. 2003, Schenk et al. 1998, Williams et al. 1941, Zeidler et al. 2002). The resulting product is then transferred to coenzyme A (CoA) (Cooper and Benedict 1966, Muller et al. 1999, Park et al. 2003, Schenk et al. 1998, Schneider and Lindqvist 1993, Singleton 1997). This reaction is a non-oxidative decarboxylation and is regarded as direct decarboxylation:



After acetaldehyde is formed, it can also be directly reduced to ethanol by alcohol dehydrogenase (Hohmann and Meacock 1998).

TPP is also the coenzyme for oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase complex (PDHC). In this process, the ketoacid is decarboxylated to carbon dioxide with electron transfer systems involved (Cooper and Benedict 1966, Koser 1968):

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{TPP}} \text{acetyl CoA} + CO_2 + \text{NADH} + H^+$$

Acetyl CoA and CoA are both involved in amino acids and sulfur metabolism during fermentation, therefore TPP can also indirectly affect these metabolisms.

Impact on fermentation and wine quality

Thiamine serves as an activator for fermentation and was found to improve yeast cell growth and fermentation rate (Bataillon et al. 1996, Laser 1941, Ournac 1969, Schultz et al. 1937a, Trevelyan and Harrison 1954). The observation that thiamine could accelerate alcoholic fermentation was first found by Schultz et al. (1937). Later work by Trevelyan and Harrison (1954) found that thiamine addition decreased pyruvate accumulation, which indicated the increase in converting pyruvate into ethanol, and implied the increase of fermentation rate. Ough et al. (1989) found that the elimination of thiamine from fermentation showed a decrease in fermentation rate. Bataillon et al. (1996) obtained similar results that fermentation rate of the synthetic grape must by *S. cerevisiae* dramatically decreased at thiamine depletion condition, while the addition of 0.25 mg/L thiamine to the must exhibited a normal fermentation rate. The author also found that the influence of thiamine on fermentation kinetics was dependent on the existing nitrogen

concentration. Thiamine was found to not influence the fermentation rate at high nitrogen level, whereas, little effect was shown at low nitrogen concentration.

On the other hand, thiamine was found to have an inhibiting effect on yeast growth and alcoholic fermentation rate in pyridoxine-free medium (Haskell and Snell 1970, Kotarska et al. 2006, Nakamura et al. 1981, Rabinowitz and Snell 1951,1953, Schultz et al. 1940). Schultz et al. (1940) showed that the addition of 0.33 mg/L thiamine to pyridoxine-free basal medium caused a decrease in yeast growth for strains of both *S. cerevisiae* and *S. carlsbergensis*. Thiamine can be split into thiazole and pyrimidine. The former contains an inhibitor that exhibits an inhibitory effect on yeast growth (Rabinowitz and Snell 1951, 1953). Nakamura et al. (1981) summarized their work on thiamine effect on *S. carlsbergensis* to say that with thiamine present in the medium, yeast exhibit lower respiratory activity compared with the cells grown in the absence of thiamine.

Besides yeast growth and fermentation rate, thiamine can also affect the synthesis of other compounds during fermentation. For instance, adequate concentrations of thiamine can reduce the synthesis of carbonyl compounds that bind to sulfur dioxide, thereby more SO_2 is available to control spoilage organisms (Tuite and Oliver 1991). In addition to limiting carbonyl synthesis, thiamine also reduces the concentration and relative proportions of higher alcohols produced during fermentation (Jackson 2000). Thiamine also serves as a coenzyme in sulfite and sulfate reductions, which affects hydrogen sulfide formation (Eschenbruch 1974, Wainwright 1971).

Nutrients Important for Alcoholic Fermentation: Pyridoxine

Vitamin B_6 is one of the eight B complexes. Naturally occurring vitamin B_6 was first isolated in 1938 by György and he proposed "pyridoxine" as a synonym for "vitamin B_6" (Fig. 4 A) (Moss 1973). Two other natural compounds possessing vitamin B_6 activity were detected in 1944 and recognized as the aldehyde and amine forms of pyridoxine, which were named as "pyridoxal" and "pyridoxamine", respectively (Fig. 4 B and C) (Leklem 2001, Moss 1973). These three vitamin B_6 forms are highly active and can be converted from one form to the other (Koser 1968). Pyridoxal phosphate (PLP) (Fig. 4 D) is the biologically active form and is involved in several important reactions of amino acid metabolism (Davenport 1985, Gunsalus et al. 1944, John 1995, Kondo et al. 2004).

Vitamin B_6 is regarded as an important nutrient for yeast metabolism during wine fermentation (Holden et al. 1949, Holden and Snell 1949, Kondo et al. 2004). It is also important in microbial metabolism of some *lactobacilli*, yeast, and some fungi (Koser 1968).

<u>Content in grape musts</u>

Ribéreau-Gayon et al. (1975) reported that the concentration for pyridoxine in musts ranges from 160 to 500 μg/L. A small decrease in pyridoxine concentration was found during fermentation, showing that not all the pyridoxine contained in musts was utilized by yeast (Castor 1953). The U. S. Department of Agriculture (USDA) Handbook No. 8 Series shows that 1.6 mg/L pyridoxine is contained in per 100 g foods; however it

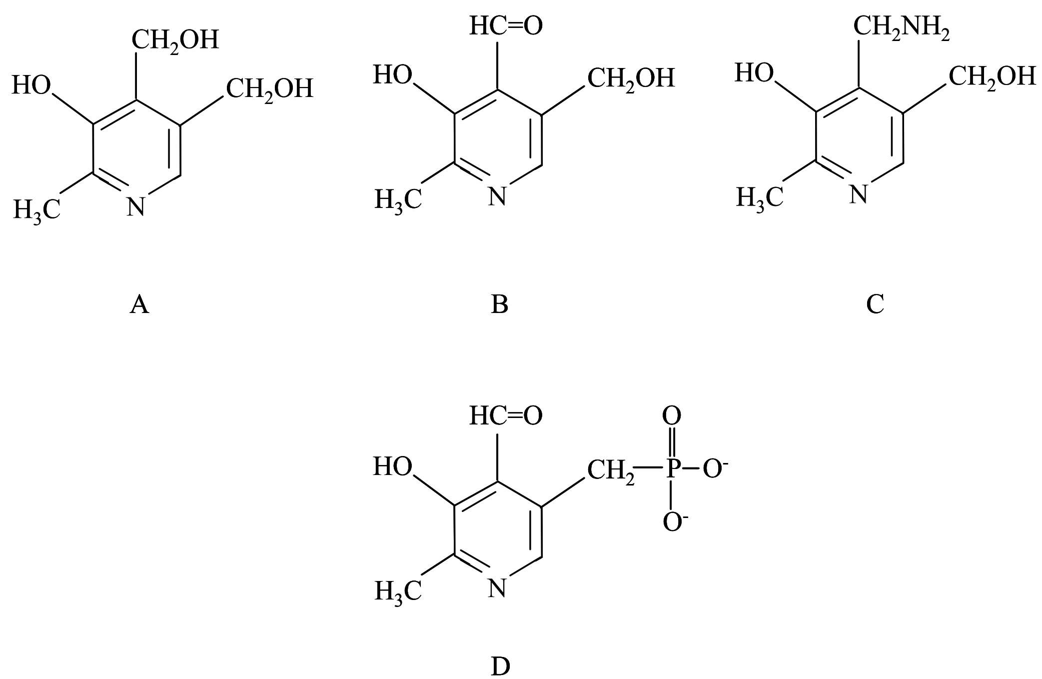
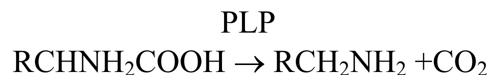


Figure 4. Pyridoxine (A), pyridoxal (B), pyridoxamine (C) and pyridoxal phosphate (D)

did not specify the food sources where the nutrients were obtained. There are no data available on pyridoxine content in U. S. grape musts to date.

<u>Biochemical roles</u>

Pyridoxine and pyridoxamine are converted to PLP prior to metabolic use. PLP serves as coenzyme for transamination, racemization and decarbolylation reactions. In transaminations, an amino group is moved to α-keto compounds with the formation of different amino compounds. Racemization is the convertible change of the L- and D-forms of amino acids, such as L-alanine and D-alanine. Both racemization and transamination result from the release of the α-hydrogen of amino acids. Decarboxylation is the liberation of a carbon dioxide molecule from an amino acid with the formation of an amine compound (Koser 1968):



$$RCHNH_2COOH \xrightarrow{PLP} RCH_2NH_2 + CO_2$$

The mechanism of PLP serving as a coenzyme in the completion of these reactions has been studied (Dunathan and Voet 1974, John 1995). According to John (1995), a Schiff base (Fig. 5) is formed from an aldehyde group of pyridoxal and an amine group of an amino acid. This compound can undergo reactions with amino acids including transaminations, racemizations, and decarboxylations.

PLP is also required as a cofactor in carbohydrate and sulfur metabolism (Koser 1968). The overall thiamine and pyridoxine biochemical roles related to glycolysis, TCA cycle and nitrogen metabolism are shown in Figure 6.

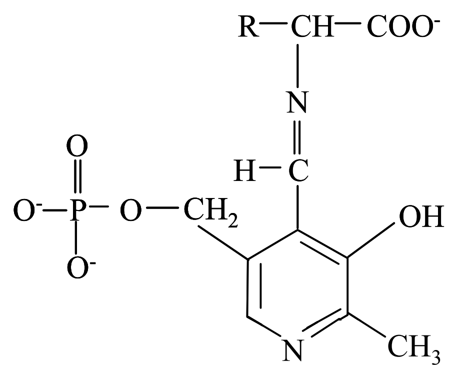


Figure 5. Schiff base formed by an amino acid and pyridoxal phosphate (PLP).

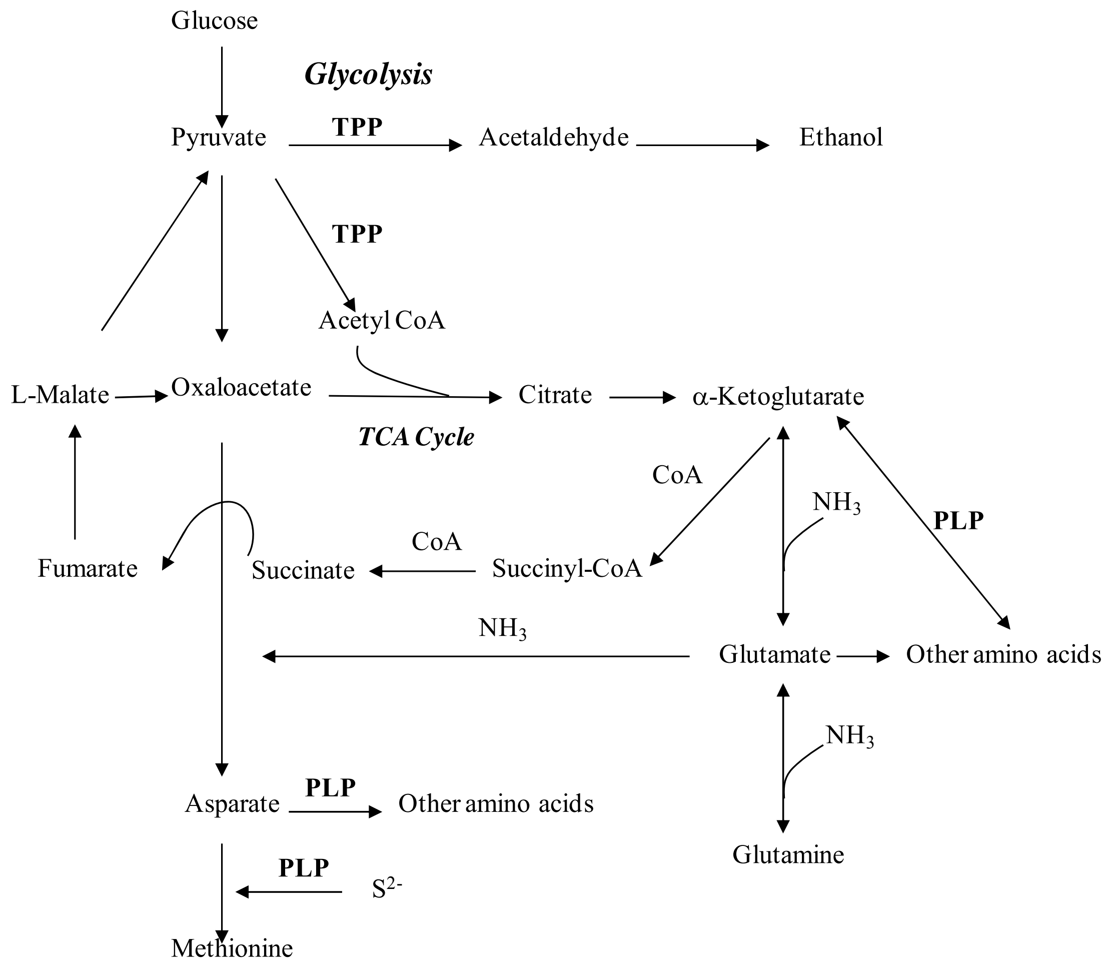


Figure 6. Biochemical roles of thiamine phosphate (TPP) and pyridoxal phosphate (PLP) involved in glycolysis, TCA cycle and nitrogen metabolism.

Impact on fermentation and wine quality

The influence of pyridoxine on yeast growth and metabolism is highly related to thiamine and the interactions between thiamine and pyridoxine have been studied (Chiao and Peterson 1956, Harris 1952, 1956, Haskell and Snell 1970, Lewin and Brown 1963, Nakamura et al. 1981, Rabinowitz and Snell 1951, Rogosa 1944, Schultz and Atkin 1947, Schultz et al. 1940). It is known that thiamine addition exhibits an inhibition effect on some *S. cerevisiae* yeast growth in pyridoxine-free medium. However, the addition of pyridoxine alleviates the inhibition effect of thiamine (Rogosa 1944, Schultz and Atkin 1947, Schultz et al. 1940). On the other hand, Harris (1952, 1956) found that excess pyridoxine inhibited yeast growth of *Neurospora* and this inhibition was effectively reduced by addition of thiamine. Chiao and Peterson (1956) stated the most acceptable explanation was that these vitamins interact with each other at some point, where both are factors in a common phenomenon. One possible point is that both of these vitamins must be phosphorylated before they can serve as coenzymes. Another point is their competition for pyruvic acid. Thiamine pyrophosphate is the coenzyme for decarboxylation of pyruvic acid, which is also a key compound in transamination reactions that require pyridoxal phosphate.

Thiamine and pyridoxine can substitute or assist synthesizing each other during yeast metabolism (Chiao and Peterson 1956, Leonian and Lilly 1942). Some strains of *S. cerevisiae* require either thiamine or pyridoxine for their growth and they seem to help each other in promoting yeast growth (Leonian and Lilly 1942, Schultz and Atkin 1947). Moses and Joslyn (1953) stated that the explanations for this effect could be: (1) either

thiamine or pyridoxine may serve as the precursor of an intermediate from which the other may be synthesized, or otherwise catalyze the synthesis of the other, thus allowing the reversible interconversion of thiamine and pyridoxine; (2) one might serve as a functional substitute for the other in the synthesis of some vital intermediate produced through the influence of either.

Problems during Alcoholic Fermentation

Sluggish/stuck fermentation

Sluggish fermentations are those fermentations where metabolism of sugars prematurely slows, while stuck fermentations imply no apparent activity (Bisson and Butzke 2000, Kunkee 1991). Fermentations last longer than 30 days are normally considered sluggish/stuck fermentations (Munoz and Ingledew 1989). One concern of sluggish/stuck fermentation is that high levels of residual sugars can be utilized by spoilage microorganisms (Bisson 1999). In addition, sweetness and low alcohol is undesirable in many finished wines (Varela et al. 2004). It is currently a serious problem in the wine industry since it is directly related to wine quality and economic issues (Bisson 1999, Kunkee 1991).

The factors that cause sluggish/stuck fermentation have been discussed by others (Bisson 1999, Bisson and Butzke 2000, Kunkee 1991, Varela et al. 2004). Sablayrolles et al. (1996) summarized that the principal mechanisms involved in sluggish fermentations are: (1) nitrogen deficiency, (2) vitamin deficiencies, (3) lack of oxygen, (4) excessive clarification of the must, (5) inhibition effect of fermentation by-products on yeast, and (6) pesticides.

Nitrogen deficiency is the most important factors related to sluggish /stuck fermentations (Alexandre and Charpentier 1998, Bely et al. 1990, 1994, Bisson 1999, Boulton et al. 1999, Cramer et al. 2002, Fleet and Heard 1993, Ingledew and Kunkee 1985, Jiranek et al. 1990, 1995a, Kunkee 1991, Monteiro and Bisson 1991, Sablayrolles et al. 1996, Sablayrolles 1996, Spayd et al. 1995, Varela et al. 2004). The amount of nitrogen in the must is directly correlated with yeast growth and its level of ethanol resistance (Boulton et al. 1999, Kunkee 1991). Nitrogen provides yeast with amino acid precursors for biosynthesis of proteins through the glycolytic pathway. Nitrogen deficiency results either in accelerated turnover of glucose permeases, thus reducing fermentative capacity, or in high residual sugar, which leads to sluggish or incomplete fermentation (Fleet and Heard 1993).

Besides nitrogen deficiency, vitamin deficiencies also can cause sluggish/stuck fermentation (Bohlscheid 2005, Nakamura et al. 1981, Wang et al. 2003). Some yeast strains have an absolute requirement for certain vitamins, such as biotin and inositol (Dixon and Rose 1964, 1966, Ough et al. 1989). Although most of the vitamins contained in the grape must are sufficient for yeast growth, they may not be optimal for the whole fermentation process (Ribéreau-Gayon et al. 2000). Furthermore, some vitamins can be destroyed, for instance thiamine can be cleaved by sulfur dioxide (Alexandre and Charpentier 1998, Boulton et al. 1999). As microbial infection of grapes can also lower the vitamin content, supplements may be required to reinitiate fermentation (Jackson 2000, Ough et al. 1989).

Sluggish /stuck fermentations can be minimized in different ways, such as by using yeast strains with good fermentation ability, aerating fermenting red grape must during yeast growth, adding nutrients, and controlling fermentation temperature (Bisson and Butzke 2000). As to nutrient deficiencies, adding certain amounts of nutrients required by yeasts are needed (Monk 1982). Addition of nitrogen sources, such as diammonium phosphate (DAP), has been widely studied and applied in wineries to prevent nitrogen deficiency (Bely et al. 1994, Jiranek et al. 1990, Julien et al. 2000, Malherbe et al. 2003, Manginot et al. 1997, 1998, Monk and Costello 1984, Monteiro and Bisson 1991, Sablayrolles 1996). Salmon (1989) concluded that addition of ammonium ions during the stationary phase leads to partial reactivation of the hexose transport systems, which increases the fermentation rate. However, it is difficult to define the optimal nitrogen concentration for different grape musts to eliminate stuck fermentation.

Hydrogen sulfide

Hydrogen sulfide (H_2S) is characterized by a rotten egg odor and is one of the most undesirable metabolites of alcoholic fermentation (Eschenbruch 1974, Eschenbruch et al. 1978, 1986, Jackson 2000, Moreira et al. 2002). H_2S leaves a “rubbery” or “sulfide-like” aroma in the finished wine (Rankine 1963), which reduces quality. Major biochemical sources of H_2S include inorganic sulfur, such as sulfite or elemental sulfur derived from vineyard sprays, and organic sulfur compounds, such as sulfur-containing amino acids and vitamins (Acree et al. 1972, Eschenbruch 1974, Henschke and Jiranek 1991, Lawrence and Cole 1968, Maw 1965, Rankine 1963, Schutz and Kunkee 1977).

Sulfur metabolism involves the reduction of inorganic sulfur by fermentation yeast strains. Figure 7 shows the overall sulfur metabolism of *Saccharomyces* during fermentation based on Wang et al. (2003). The principal inorganic sulfur compound is sulfate, which is present in grape must (Henschke and Jiranek 1991). ATP-sulfrylase and APS-kinase are used in the reduction of sulfate to sulfite and then to adenosine-5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-PAPS reductase then reduces PAPS to sulfite. This pathway, called sulfate reduction sequence (SRS), involves large amounts of energy (ATP) consumption.

The nitrogen containing compounds, *O*-acetylserine (OAS) and *O*-acetylhomoserine (OAH), react with sulfide to generate cysteine and homocysteine, catalyzed by the enzyme OAS-OAH (Henschke and Jiranek 1991). Homocysteine, obtains a methyl group from N^5-methyltetradydrafolate to form methionine in the presence of homocysteine methyltransferase. Cysteine and methionine are required by yeast for protein synthesis. Methionine can also be adenylated to form *S*-adenosylmethionine (SAM), a methyl group donor in yeast metabolism. Cysteine can also be involved in formation of glutathione (Henschke and Jiranek 1991). When the nitrogen content is limiting in grape musts, H_2S will accumulate because of inadequate amount of *O*-acetyl-homoserine and *O*-acetyl-serine that combine with sulfide from the SRS pathway.

Wiebers and Garner (1967) showed that PLP was the cofactor for condensation of

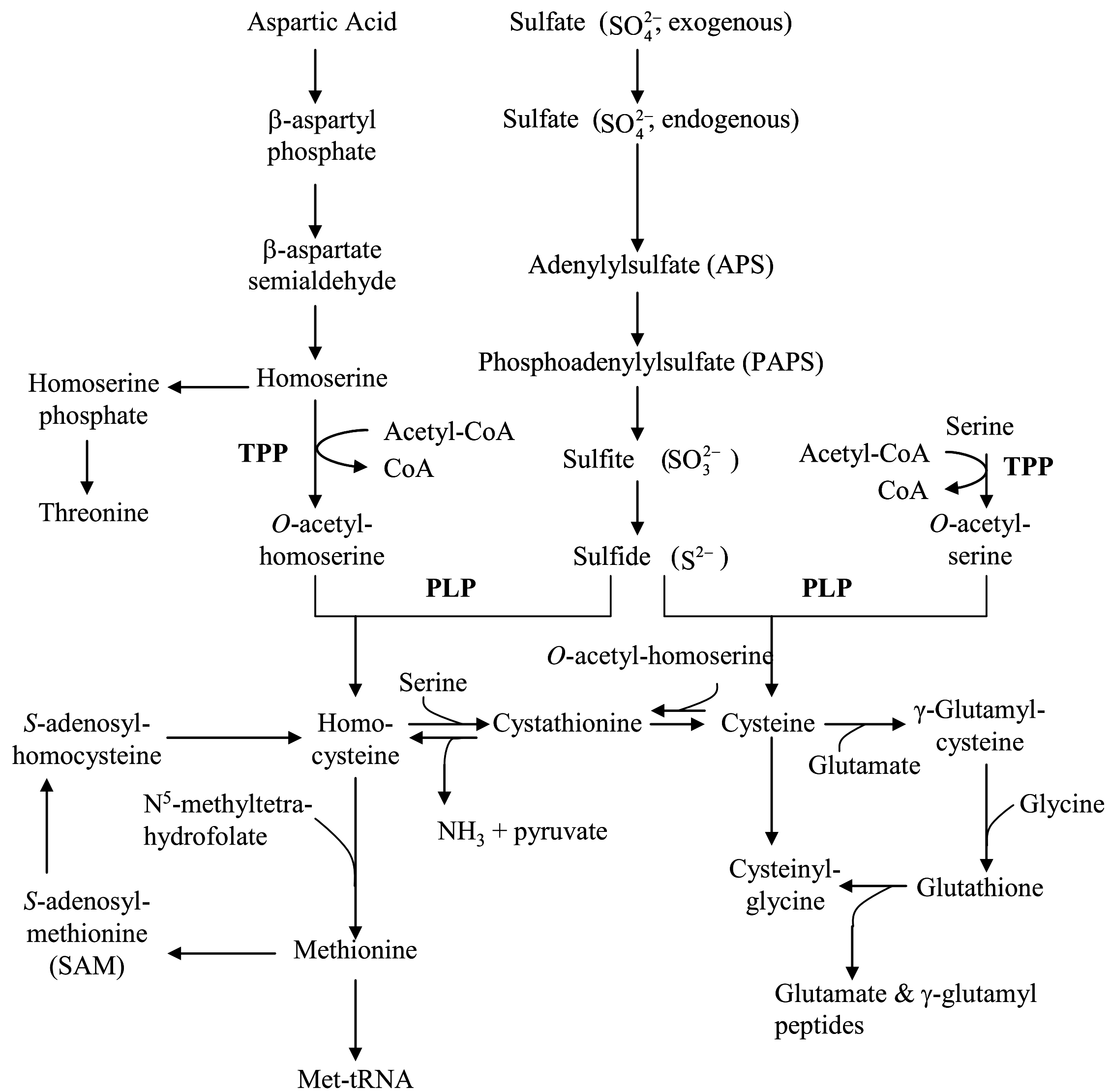


Figure 7. Thiamine phosphate (TPP) and pyridoxal phosphate (PLP) involved in sulfur metabolism of *Saccharomyces* based on Wang et al. (2003).

O-acetyl-homoserine and sulfide to form homocysteine. Similarly, the condensation of *O*-acetyl-serine and sulfide to form cysteine also requires PLP (Botsford and Parks 1969, Wiebers and Garner 1967). Thus, a limitation of pyridoxine could theoretically result in an increase in H_2S exportation. Wainwright (1970) found that *O*-acetyl-homoserine, homoserine, homocysteine, and methionine inhibited H_2S formation, but this effect depended on concentrations and yeast strains.

Factors that affect H_2S formation are classified as being either yeast strain-related or environmental factors. The strain-related aspects include differences in metabolism and genetic constitution (Acree et al. 1972, Eschenbruch 1974, Henschke and Jiranek 1991, Tamayo et al. 1999). For example, Montrachet is widely known for high H_2S production compared to other strains (Acree et al. 1972, Rupela and Tauro 1984). Environmental factors include temperature, oxidation-reduction potential, sulfur and nitrogen sources, and vitamin deficiencies (Henschke and Jiranek 1991, Thomas et al. 1993). In addition, researchers concluded that the H_2S formation is reduced at low temperatures (Rankine 1963, Schutz and Kunkee 1977).

Nitrogen and sulfur compounds content in grape juice has a strong relationship with H_2S production (Giudici and Kunkee 1994, Hallinan et al. 1999, Park et al. 2000, Thomas et al. 1993). Nitrogen deficiency can lead to a higher production of H_2S because of changes in the sulfur metabolism pathway (Boulton et al. 1999, Henschke and Jiranek 1991, Jiranek et al. 1995b, Stratford and Rose 1985, Vos and Gray 1979). For instance, Stratford and Rose (1985) found that when nitrogen supply of ammonia and/or glutamate is limited, fewer sulfur-containing amino acid precursors, OAS and OAH, are produced.

Since sulfite is continuously reduced by sulfite reductase into sulfide from SRS reduction pathway, there is not enough OAS and OAH present to combine with sulfide to form homocysteine and cysteine for further amino acid synthesis. As a result, excessive sulfide will be produced (Henschke and Jiranek 1991, Jiranek et al. 1995b, Stratford and Rose 1985).

Some vitamin deficiencies can also cause H_2S production (Eschenbruch 1974, Spiropoulos et al. 2000, Stratford and Rose 1985, Wainwright 1971). Pantothenate deficiencies may cause low levels of methionine production in the cells, which results in H_2S formation (Eschenbruch et al. 1978, Wainwright 1970). Interactions of certain vitamins and nitrogen on hydrogen sulfide production have been investigated. For instance, Wang et al. (2003) studied nitrogen and pantothenic acid effect on H_2S formation and found that pantothenic acid deficiency affected H_2S production. Opposite H_2S production trends were presented between fermentations with and without pantothenic acid when nitrogen was increased. Bohlscheid (2005) examined nitrogen and biotin effects on H_2S production and reported that nitrogen and biotin interactions influence the H_2S formation. The maximum H_2S production occurred in the medium containing 60 mg/L yeast assimilable nitrogen and 1 μg/L biotin (Bohlscheid et al. 2007).

Many strategies have been proposed to control H_2S, such as using suitable yeast strains, avoidance of excessive quantities of copper, and elimination of elemental sulfur (Acree et al. 1972, Eschenbruch 1974, Giudici and Kunkee 1994, Henschke and Jiranek 1991, Jiranek et al. 1995b, Mendes-Ferreira et al. 2002, Schutz and Kunkee 1977, Spiropoulos et al. 2000, Tamayo et al. 1999, Thomas et al. 1993, Vos et al. 1980, Vos

and Gray 1979). Clarification of grape musts can reduce elemental sulfur in the musts, therefore it can also minimize H_2S formation (Thomas et al. 1993). Eschenbruch proposed that increasing concentrations of methionine or cysteine to grape juice would lower the formation of hydrogen sulfide (Eschenbruch 1972, Eschenbruch et al. 1973). Direct addition of DAP to the must is also used based on the nitrogen deficiency leading to H_2S production. DAP could supply enough ammonia to continue the SRS pathway in sulfur metabolism, thus reducing H_2S production (Henschke and Jiranek 1991).

MATERIALS AND METHODS

Yeast Selection

Sixteen commercial yeast strains were obtained for this research. Fifteen were strains of *S. cerevisiae* including UCD 522 (Montrachet), EC1118, Epernay 2, CY3079, D254, Syrah, UCD 679 (Assmannshausen), 71B, BM45, Steinberg Lager, D47, and BDX obtained from Lallemand Inc. (Montreal, Quebec, Canada), Chasson and UCD 904 (Pasteur Red) from Red Star (Milwaukee, WI, USA), and CH05 from Chr Hansen (Milwaukee, WI, USA). In addition, one strain of *S. uvarum* S6U was obtained from Lallemand Inc. (Montreal, Quebec, Canada). All strains were maintained on potato dextrose agar (Difco, Detroit, MI, USA) stored at $4^{o}C$.

Thiamine / Pyridoxine Requirement

Thiamine hydrochloride (0 and 0.05 mg/L) and/or pyridoxine hydrochloride (0 and 0.2 mg/L) were added to a culture medium containing vitamin-free yeast nitrogen base (16.7 g/L), glucose (20 g/L), myo-inositol (0.2 g/L), nicotinic acid 4 (mg/L), pantothenic acid (2 mg/L), riboflavin 0.4 (mg/L), biotin (10 μg/L), and folic acid (0.2 mg/L). After incubation at $24^{o}C$ for 48 hr on wort agar, a single colony was inoculated into the culture medium. Optical density was measured using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., NY, USA) at wavelength of 600 nm.

Synthetic Grape Juice Fermentations

Starter culture preparation

A single colony of *S. cerevisiae* UCD 522 was inoculated into 10 mL of 0.67% w/v yeast nitrogen base (Difco, Detroit, MI, USA) containing 5% w/v glucose. After incubation at 24°C for 48 hr, the culture was centrifuged (2,000 × g for 30 min) and repeatedly washed and centrifuged (3 ×) with 10 mL 0.2 M phosphate buffer (pH 7). After the last washing/centrifuging step, the culture was suspended in 10 mL 0.2 M phosphate buffer (pH 7), and inoculated into a starter culture medium. This medium was prepared with vitamin-free yeast nitrogen base (16.7 g/L), glucose (20 g/L), myo-inosital (0.2 g/L), nicotinic acid 4 (mg/L), pantothenic acid (2 mg/L), riboflavin 0.4 (mg/L), biotin (10 μg/L), and folic acid (0.2 mg/L).

To prepare the starter culture, the yeast was depleted of thiamine, pyridoxine, or both by sequential transfer in the medium described above. When the yeast was to be depleted by thiamine, 2 mg/L pyridoxine hydrochloride was added to the starter medium. Conversely, when pyridoxine was to be depleted, 0.5 mg/L thiamine hydrochloride was added. For depleting both thiamine and pyridoxine, neither was added to the starter medium. Yeast culture (1 mL) from the first depletion was inoculated into each of 18 flasks containing 100 mL starter medium and incubated at 24°C for another 48 hr. After centrifuging (2,000 × g for 30 min) and washing for three times, the culture was suspended in 100 ml 0.2 M phosphate buffer (pH 7).

Fermentations

Three fermentations were conducted: (1) a 2 × 3 factorial design with nitrogen (60 and 250 mg/L) and thiamine (0, 0.2, and 0.5 mg/L) as variables containing pyridoxine (2 mg/L), (2) a 2 × 3 factorial design with nitrogen (60 and 250 mg/L) and pyridoxine (0, 0.25, and 0.5 mg/L) as variables and containing thiamine (0.5 mg/L), and (3) a 3 × 3 factorial design with thiamine (0, 0.2, and 0.5 mg/L) and pyridoxine (0, 0.25, and 0.5 mg/L) as variables at the low concentration of nitrogen (60 mg/L YAN).

Synthetic grape juice was prepared based on Wang et al. (2003) with a sufficient concentration of pantothenic acid (0.25 mg/L). Three replicates were used for fermentations with thiamine x nitrogen as variables and pyridoxine x nitrogen as variables. Two replicates were used for thiamine x pyridoxine as variables because of the 3 x 3 factorial design. Each individual fermentor (Wheaton Science Products, Millville, NJ, USA) contained 3 L of synthetic grape juice, which was previously filtered through 0.22 μm Millipore Stericup filters (Millipore Corporation, Billerica, MA, USA). SigmaCell® (Sigma-Aldrich, Inc., St. Louis, MO, USA) was suspended in 0.2 M phosphate buffer (pH=7) and autoclaved before being added at 0.1% (w/v).

Yeast starter culture (10 mL) was added into each fermentor to yield an initial viable cell population of 10^5 CFU/mL. Fermentations were conducted at 22°C with stirring for 5 min prior to sampling. A syringe-type sampling system (Model M1230-6000, New Brunswick Scientific Co., Edison, NJ, USA) was utilized for sampling. An H_2S trapping system was conducted following Wang et al. (2003).

Enumeration and Analytical Methods

Samples were regularly obtained for microbiological and chemical analyses. An Autoplate® 4000 spiral plating device (Spiral Biotech, Bethesda, MD, USA) was used for viable yeast cell population enumerations by plating on wort agar and incubation at 24°C for 48 hr. Soluble solids were measured using the method of Ingledew and Kunkee (1985). Fermentations were considered to be finished when the concentration of reducing sugars were less than 2 g/L, following the Clinitest test (Edwards 1990). H_2S was analyzed based on the method described by Jiranek et al. (1995b) using a Genesys™ 10 Series Spectrophotometer (Thermo Spectronic, Rochester, NY, USA) with the cumulative amount evolved calculated for each of the 3 L fermentations.

Sensory Evaluation

Overall difference test

The sensory panel was composed of 30 participants, all between the ages of 21 and 65 and evenly distributed between male and female. The panelists were recruited from Washington State University faculty, staff and students. All participants signed an Informed Consent Form and the project was approved by the Washington State University Institutional Review Board for participation by human subjects.

An overall difference test was used to determine if aroma differences existed between the four synthetic wine samples fermented with different thiamine (0 and 0.5 mg/L) and pyridoxine (0 and 0.5 mg/L) concentrations. Specifically, a triangle test was used with an incomplete balanced block design and replication (Meilgaard et al. 1999). Synthetic wine samples from these four fermentation combinations were marked as A

(fermented with 0 mg/L thiamine and 0 mg/L pyridoxine), B (0 mg/L thiamine and 0.5 mg/L pyridoxine), C (0.5 mg/L thiamine and 0 mg/L pyridoxine) and D (0.5 mg/L thiamine and 0.5 mg/L pyridoxine) with six pairs of the wines (AB, AC, AD, BC, BD, and CD) tested. For each pair, three wine samples were prepared, two of which were the same. They were coded with a three-digit code and presented to each panelist in randomized serving order. Synthetic wine samples (30 mL) were presented in 50 mL beakers covered with a plastic lid, and panelists were asked to select the different sample. Red lights were used to mask potential differences in wine color. Number of correct responses was compared to the critical correct number for triangle test to determine if there were significant differences existed among the samples.

Aroma attribute rating test

Following the triangle test, an experienced panel (n = 5) was assembled to determine appropriate descriptors for aroma differences between the synthetic wines. From this panel, the aroma attributes of rotten egg, yeasty, floral, and mushroom-musty were determined to be the attributes of interest.

An untrained panelists consists of 11 males and 13 females were recruited from Washington State University faculty, staff and students. Four synthetic wine samples, each assigned a three-digit code, were presented (30 mL) in a randomized serving order one at a time for panelists to sniff. Red lights were used to mask visual differences. Aroma attribute rating test using a complete block design were conducted (Meilgaard et al., 1999). A 9-point scale was used for intensity evaluation, anchored with the terms from “very low”, “medium low”, “low”, “below moderate”, “moderate”, “above

moderate", "high", "medium high", and "very high". Panelists were invited to familiarize themselves with the appropriate aromas through the evaluation of standard aroma solutions for rotten egg, yeasty, floral, and mushroom/musty. Standard solutions were prepared as follows. For the floral standard, linalool was added to 100 mL base white wine (Noble et al. 1987). For the hydrogen sulfide standard, boiled egg (yolk only) was added to the base wine. To prepare the mushroom standard, fresh mushrooms were soaked in a based white wine for 24 hrs and a previously prepared, lab fermented synthetic wine sample with a strong yeasty aroma was used as the yeasty standard for panelists to sniff.

Statistical Analysis

Statistical analyses of all data were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). Normality analysis was performed to determine if data were normally distributed before running the multi-comparison tests. Significance was established at $p \leq 0.05$ and ANOVA with Fisher's LSD was conducted for mean separation. Means separation was carried out on sensory evaluation data with Compusense 5.0 software (Compusense, Guelph, Canada).

RESULTS

Initial Screening

Media with or without thiamine or pyridoxine inoculated with different yeast strains showed vigorous growth within 48 hr based on optical density. Although strains such as Epernay 2 and Chasson exhibited less growth in the early stage (24 hr) than others, all of the sixteen yeast strains exhibited visible growth at 48 hr (Appendix A).

Synthetic Grape Juice Fermentations

Thiamine × nitrogen

Yeast in the synthetic grape juice with selected concentrations of thiamine (0, 0.2, and 0.5 mg/L) and YAN (60 and 250 mg/L) achieved populations greater than 5.7×10^7 CFU/mL within 4 days (Figure 8). After 15 days, viability decreased to 1.5×10^7 CFU/mL in fermentations containing high YAN and any thiamine. Media high in YAN but without thiamine were able to maintain yeast viability above 6.9×10^7 CFU/mL 22 days after inoculation. Yeast growing in media low in YAN with thiamine remained viable above 2.4×10^7 CFU/mL until 47 days.

Under conditions of high YAN (250 mg/L), the decreases in soluble solids were faster than those containing low YAN (60 mg/L) at any concentration of thiamine (Figure 9). Maximum fermentation rates presented in Table 1 show that within each YAN level, there were significant increases ($p \leq 0.05$) when thiamine was added. Fermentations containing thiamine that were high in YAN reached dryness (< 2 g/L reducing sugars), 17 days earlier than fermentations low in YAN.

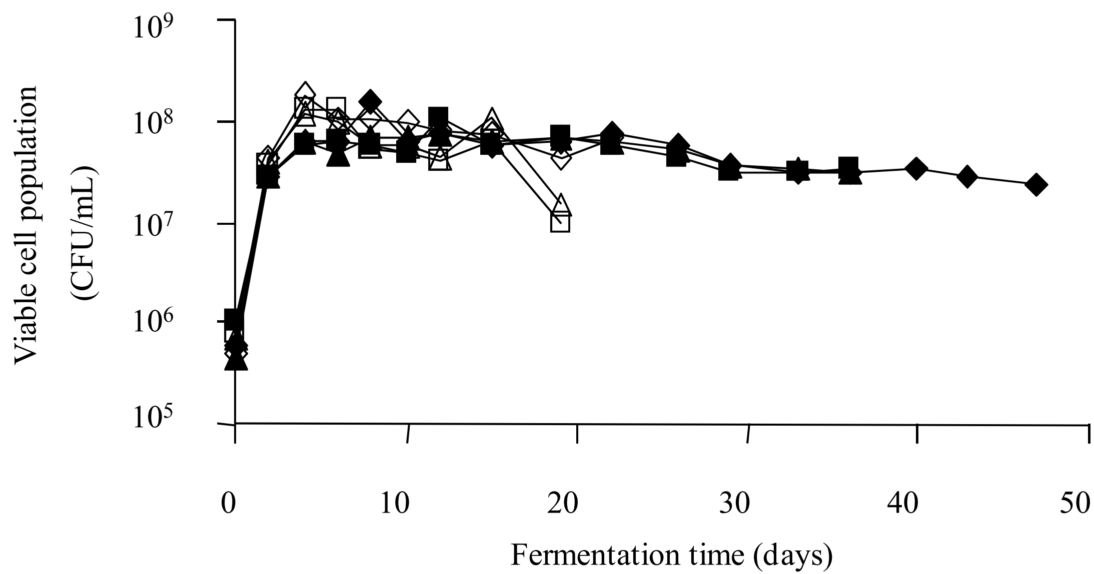


Figure 8. Yeast growth during fermentation with low (◆, ■, ▲) or high (◇, □, △) amounts of YAN and 0 (◆, ◇), 0.2 (■, □), or 0.5 (▲, △) mg/L thiamine with 2 mg/L pyridoxine (mean values of three replicates).

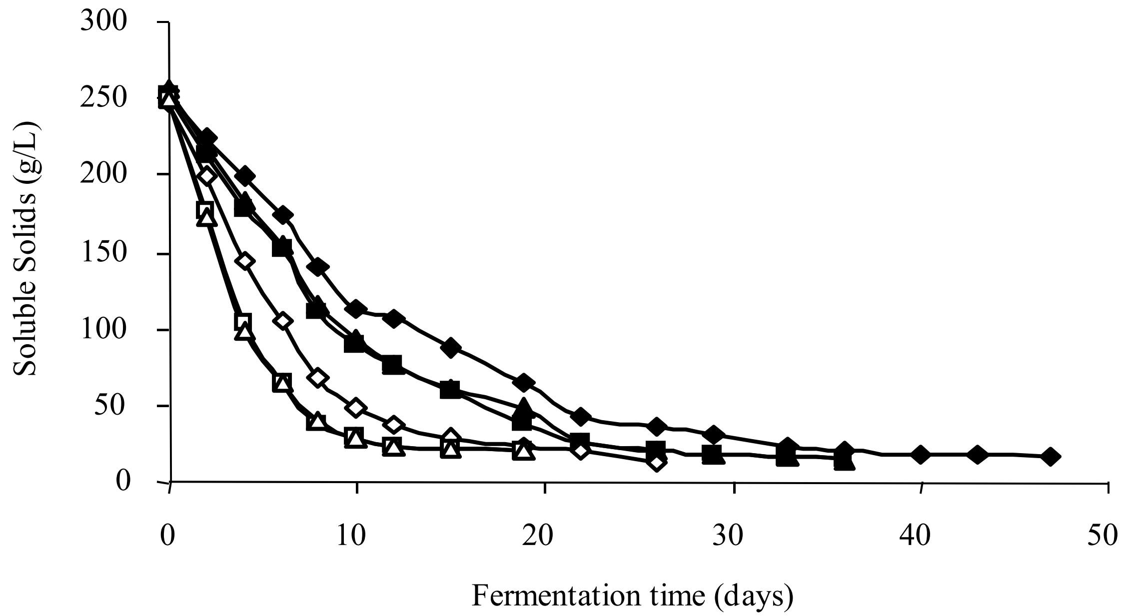


Figure 9. Soluble solids changes during fermentation with low (◆, ■, ▲) or high (◇, □, △) amounts of YAN and 0 (◆, ◇), 0.2 (■, □), or 0.5 (▲, △) mg/L thiamine with 2 mg/L pyridoxine (mean values of three replicates).

Table 1. Maximum fermentation rates for fermentations with thiamine and nitrogen as variables.

YAN (mg/L)	Thiamine (mg/L)	Maximum fermentation rate (g soluble solids/L/day)
60	0	14[c]
60	0.2	17[c]
60	0.5	17[c]
250	0	23[b]
250	0.2	27[a]
250	0.5	26[a]

*Mean values (three replicates) with different letters are significantly different at $p \leq 0.05$.

Without thiamine, fermentations required additional time to reach dryness at both low and high YAN levels.

H_2S production was higher in the medium containing low nitrogen (60 mg/L) than that with high nitrogen (250 mg/L) at selected concentrations of thiamine (Figure 10). At 0.5 mg/L thiamine, there was only a difference of 39 μg/L in H_2S production between two YAN levels, whereas without thiamine, there was 99 μg/L difference. At low YAN, H_2S decreased as the concentration of thiamine increased, however, at high YAN, H_2S increased with increases in thiamine. Fermentations high in YAN without thiamine produced the lowest H_2S (18 μg/L). Two-way ANOVA test showed that nitrogen and thiamine both significantly ($p \leq 0.05$) affected H_2S production and the interaction effects between thiamine and nitrogen were also significant ($p \leq 0.05$).

Pyridoxine × nitrogen

When the concentration of thiamine was held constant (0.5 mg/L) with pyridoxine and nitrogen as variables, the initial population of yeast reached growth peak after 12 days, compared to previous fermentations, which peaked much sooner (Figure 11). At high YAN (250 mg/L), the additions of pyridoxine led to a one log reduction of yeast viability after 15 days. Figure 11 shows that yeast in media low in YAN maintained a high population (above 10^8 CFU/mL) until 36 days, whereas yeast without pyridoxine sustained growth up to 40 days.

The soluble solids curve presented similar trends (Figure 12) to fermentations with thiamine and nitrogen as variables (Figure 9). Under conditions of high YAN (250 mg/L),

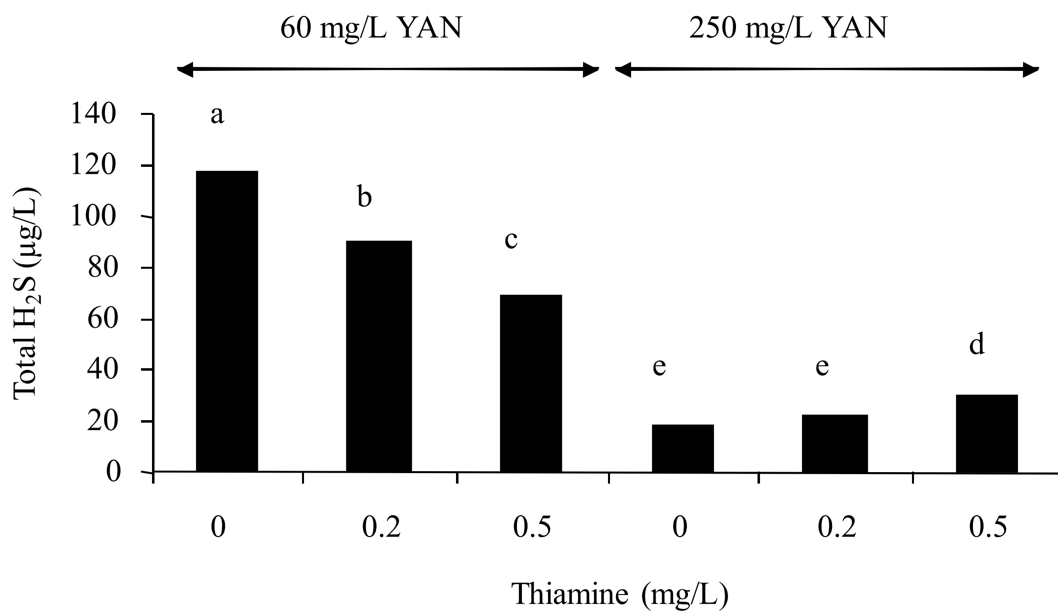


Figure 10. Total H_2S evolution in synthetic grape juice fermented by UCD 522 with initial nitrogen concentrations of 60 and 250 mg/L YAN and 0, 0.2, or 0.5 mg/L thiamine with 2 mg/L pyridoxine. Mean values (three replicates) with different letters are significantly different at $p \leq 0.05$.

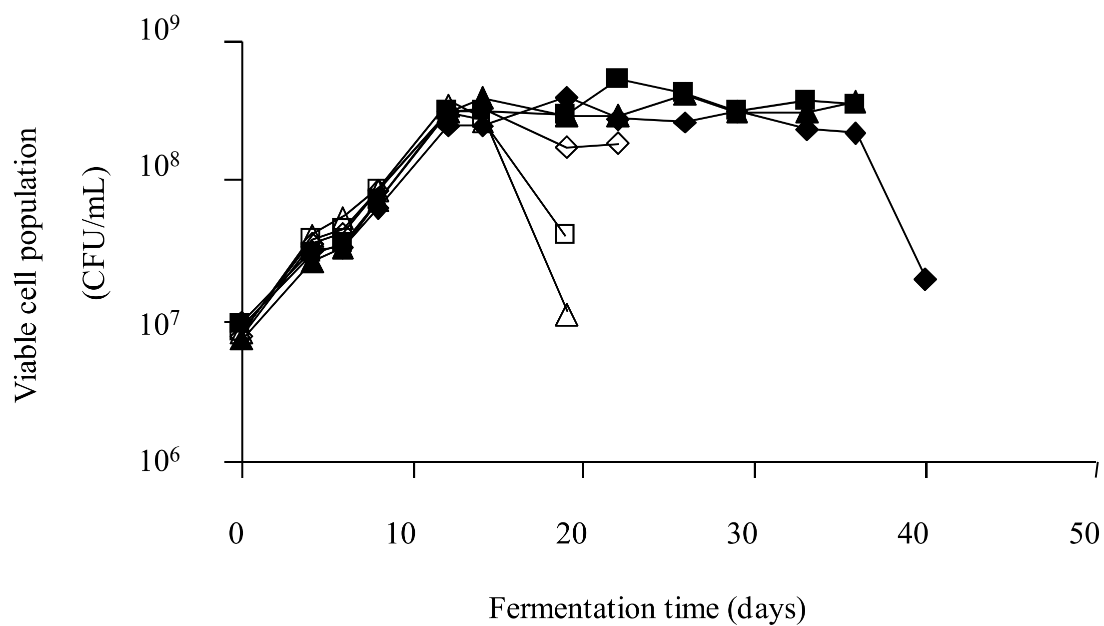


Figure 11. Yeast growth during fermentation with low (◆ , ■ , ▲) or high (◇ ,□ ,△) amounts of YAN and 0 (◆ ,◇), 0.25 (■ , □), or 0.5 (▲ , △) mg/L pyridoxine with 0.5 mg/L thiamine (mean values of three replicates).

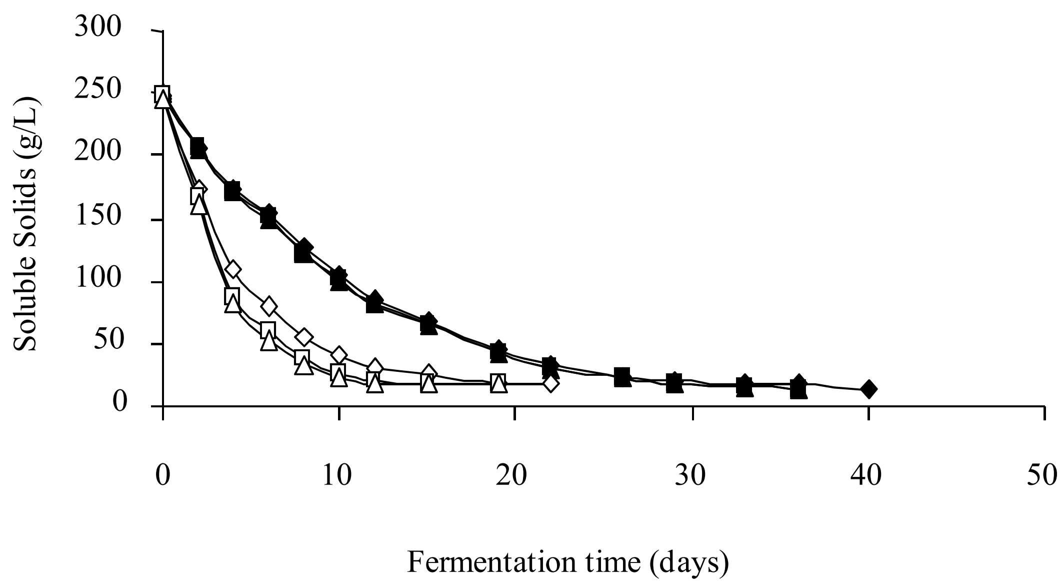


Figure 12. Soluble solids changes during fermentation with low (◆, ■, ▲) or high (◇, □, △) amounts of YAN and 0 (◆ , ◇), 0.25 (■ , □), or 0.5 (▲ , △) mg/L pyridoxine with 0.5 mg/L thiamine (mean values of three replicates).

the decreases in soluble solids were faster than those containing low YAN (60 mg/L) at any concentration of added pyridoxine. Maximum fermentation rates presented in Table 2 show that within each YAN level, there were significant increases ($p \leq 0.05$) in maximum fermentation rate when pyridoxine was added. Like those containing thiamine, fermentations containing pyridoxine that were high in YAN reached dryness (< 2 g/L reducing sugars) 17 days earlier than fermentations low in YAN. Without pyridoxine, fermentations required additional days to reach dryness at both low and high YAN levels.

H_2S production varied depending on pyridoxine and nitrogen concentrations (Figure 13). Here, the amount of H_2S increased with an increase of YAN when pyridoxine was not present. In contrast, H_2S production decreased from high YAN to low YAN with pyridoxine present (0.25 and 0.5 mg/L). Based on two-way ANOVA analysis, nitrogen and pyridoxine significantly ($p \leq 0.05$) affected H_2S production, and pyridoxine and nitrogen interaction effect was also significantly ($p \leq 0.05$). However, at high YAN, there were no significant differences in H_2S production between the fermentations containing pyridoxine (0.25 and 0.5 mg/L). The lowest H_2S (13 μg/L) was produced with fermentation high in YAN at 0.25 mg/L pyridoxine.

<u>Thiamine × pyridoxine</u>

YAN was maintained at 60 mg/L for the fermentations with thiamine (0, 0.2, 0.5 mg/L) and pyridoxine (0, 0.25, 0.5 mg/L) as variables. Yeast population had an initial increase of more than one log from 10^5 CFU/mL within two days of fermentation, and remained > 10^6 CFU/mL during 42 days of fermentation (Figures 14 A, B, and C). Without

Table 2. Maximum fermentation rates for fermentations with pyridoxine and nitrogen as variables.

YAN (mg/L)	Pyridoxine (mg/L)	Maximum fermentation rate (g soluble solids/L/day)
60	0	15[c]
60	0.25	15[c]
60	0.5	16[c]
250	0	24[b]
250	0.25	26[a]
250	0.5	27[a]

*Mean values (three replicates) with different letters are significantly different at $p \leq 0.05$.

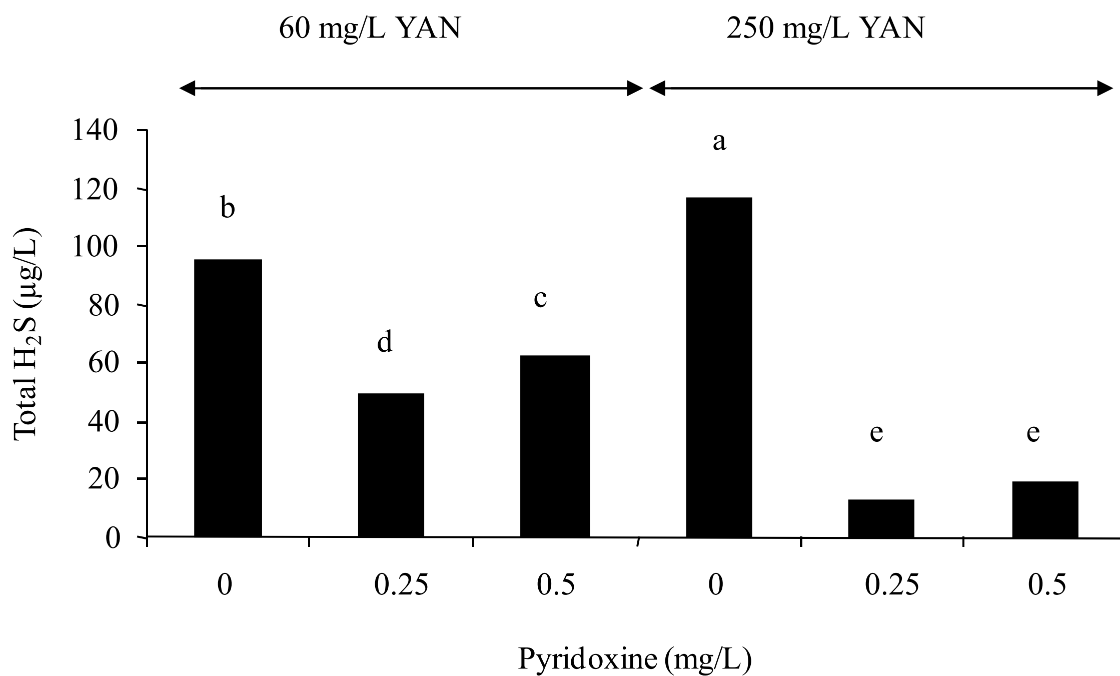


Figure 13. Total H_2S evolution in synthetic grape juice fermented by UCD 522 with initial nitrogen concentrations of 60 and 250 mg/L YAN and 0, 0.25, or 0.5 mg/L pyridoxine with 0.5 mg/L thiamine. Mean values (three replicates) with different letters are significantly different at $p \leq 0.05$.

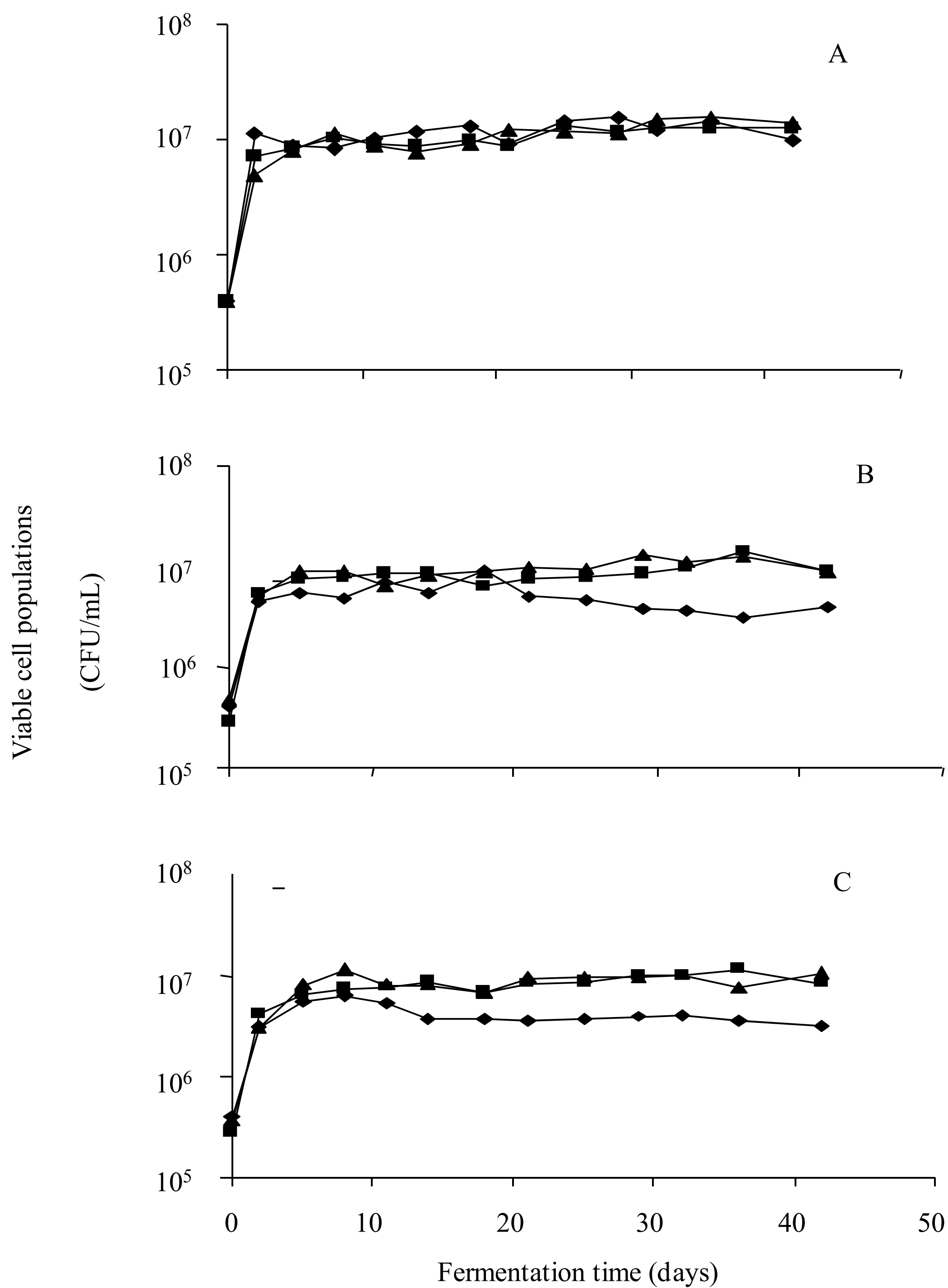


Figure 14. Yeast growth during fermentation with no thiamine (A), 0.2 mg/L thiamine (B), 0.5 mg/L thiamine (C) and 0 (◆), 0.25 (■), or 0.5 (▲) mg/L pyridoxine at low YAN (60 mg/L) (mean values of two replicates).

pyridoxine, yeast began to decrease in numbers after 21 (Figure 14 B) and 11 (Figure 14 C) days at thiamine levels of 0.2 mg/L and 0.5 mg/L, respectively.

Fermentations showed that the decreases in soluble solids without thiamine did not change with the addition of pyridoxine (Figure 15 A). However, addition of pyridoxine showed a faster decrease in soluble solids change with thiamine contained in the media (0.2 mg/L and 0.5 mg/L) (Figures 15 B and C). Adding thiamine (0.2 and 0.5 mg/L) in the media, with or without pyridoxine, significantly ($p \leq 0.05$) increased the maximum fermentation rates (Table 3). However, adding pyridoxine (0.25 and 0.5 mg/L) in the media only significantly ($p \leq 0.05$) increased the maximum fermentation rates for the media with thiamine (0.2 and 0.5 mg/L) (Table 3). All fermentations at low YAN with any thiamine and pyridoxine level did not reach dryness (< 2 g/L reducing sugars) after 42 days, which exhibited sluggish/stuck fermentations. However, fermentations containing pyridoxine only had lower soluble solids left (< 26 g/L) than those without pyridoxine (> 55 g/L) at 42 days.

High amount of H_2S was produced without pyridoxine, whereas increasing pyridoxine concentration from 0.25 to 0.5 mg/L caused different changes in H_2S production (Figure 16). At 0.2 mg/L thiamine, the amount of H_2S trapped decreased from 49 to 44 μg/L as the concentration of pyridoxine increased from 0.25 to 0.5 mg/L. However, increases in H_2S production present at both 0 and 0.5 mg/L thiamine levels with pyridoxine changed from 0.25 to 0.5 mg/L. Thiamine, pyridoxine, and their interactions affected H_2S production significantly at $p \leq 0.05$.

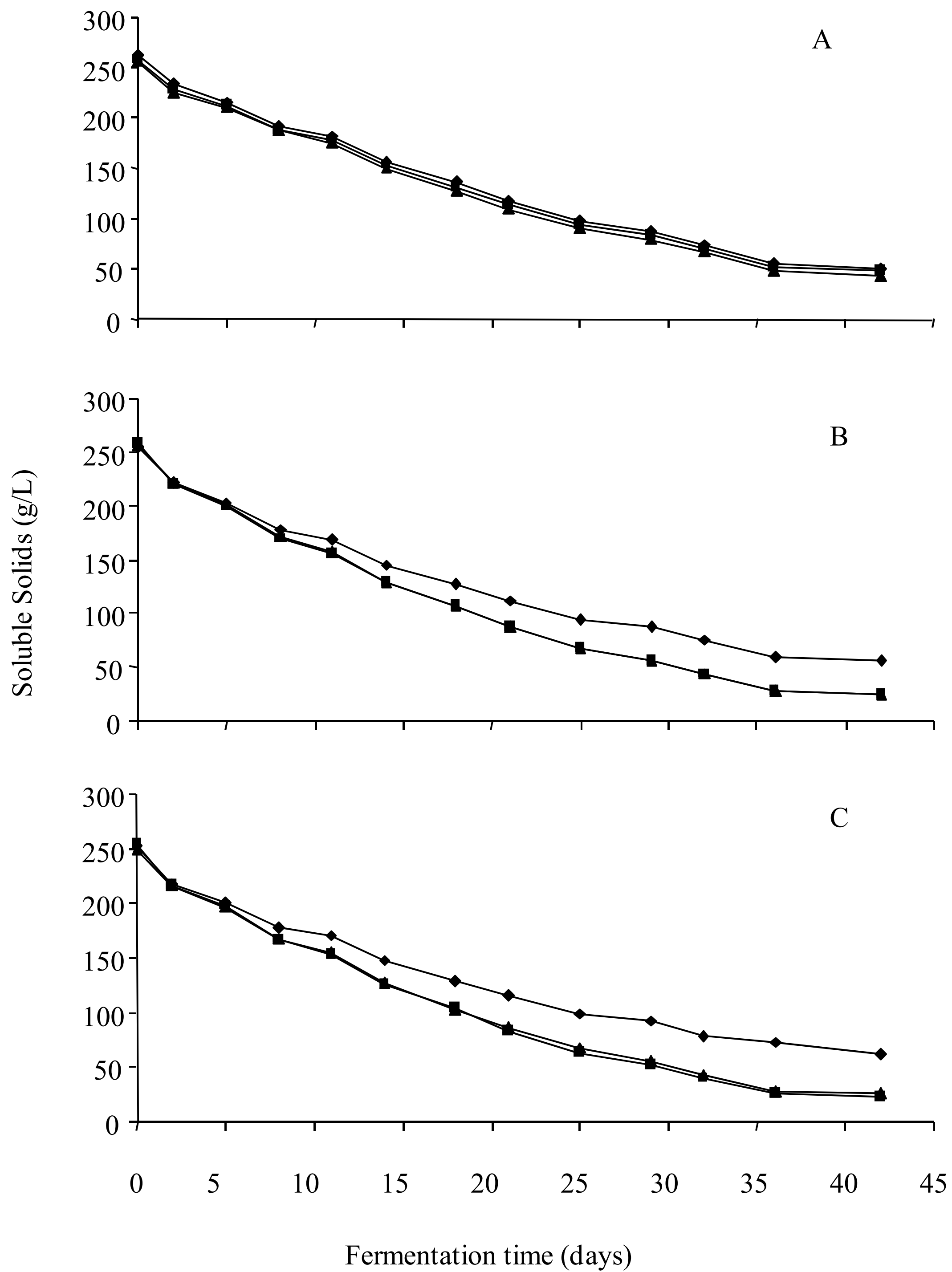


Figure 15. Soluble solids changes during fermentation with no thiamine (A), 0.2 mg/L thiamine (B), 0.5 mg/L thiamine (C) and 0 (◆), 0.25 (■), or 0.5 (▲) mg/L pyridoxine with low YAN (60 mg/L) (mean values of two replicates).

Table 3. Maximum fermentation rates for fermentations with thiamine and pyridoxine as variables (YAN = 60 mg/L).

Thiamine (mg/L)	Pyridoxine (mg/L)	Maximum fermentation rate (g soluble solids/L/day)
0	0	8[d]
0	0.25	8[d]
0	0.5	8[d]
0.2	0	9[b]
0.2	0.25	10[a]
0.2	0.5	10[a]
0.5	0	9[bc]
0.5	0.25	10[a]
0.5	0.5	10[a]

*Mean values (two replicates) with different letters are significantly different at $p \leq 0.05$.

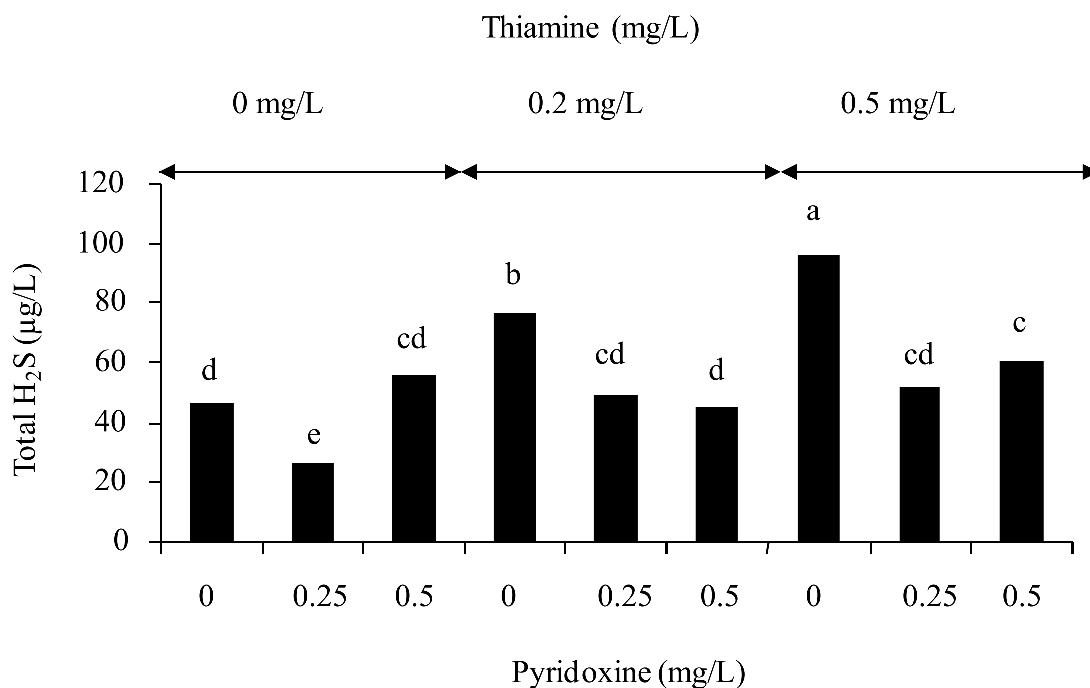


Figure 16. Total H_2S evolution in synthetic grape juice fermented by UCD 522 with initial thiamine concentrations of 0, 0.2, or 0.5 mg/L and 0, 0.25, or 0.5 mg/L pyridoxine at low YAN (60 mg/L). Mean values (two replicates) with different letters are significantly different at $p \leq 0.05$.

Sensory Evaluation

The triangle test showed that panelists could notice significant differences ($p \leq 0.05$) between four pairs (AB, AD, BC, and CD) of the six pairs (AB, AC, AD, BC, BD, and CD) of wine samples based on overall aroma (data not shown). The four synthetic wine samples A, B, C, and D stand for A: fermented with 0 mg/L thiamine and 0 mg/L pyridoxine, B: fermented with 0 mg/L thiamine and 0.5 mg/L pyridoxine, C: fermented with 0.5 mg/L thiamine and 0 mg/L pyridoxine, and D: fermented with 0.5 mg/L thiamine and 0.5 mg/L pyridoxine. At both 0 and 0.5 mg/L pyridoxine levels, the pairs containing 0 and 0.5 mg/L thiamine were not perceived to be different by the panelists.

Four aroma attributes, rotten egg, yeasty, floral, and mushroom-musty, determined to be appropriate descriptors by an experienced panel from the synthetic wine samples, were used in the aroma attribute rating test. For all the synthetic wine samples, panelists rated low to moderate values for yeasty, mushroom-musty, and floral; and very low to above moderate for rotten egg. The mean rating values compared by Tukey's HSD showed that panelists could tell significant differences ($p \leq 0.05$) between samples with rotten egg aroma and they were not able to tell significant differences ($p \leq 0.05$) between samples for the yeasty, mushroom-musty, and floral aromas (Table 4). The rotten egg aroma was the smell from hydrogen sulfide produced during wine fermentation. Based on the fermentation results, fermentations without thiamine and pyridoxine produced the lowest H_2S; however fermentations without pyridoxine, but with 0.5 mg/L thiamine, had the highest H_2S produced as determined by sensory evaluation.

Table 4. Aroma attribute rating for fermentations with different thiamine (0 and 0.5 mg/L) and pyridoxine (0 and 0.5 mg/L) concentrations at significance level of 5% using Tukey's HSD.

Thiamine	Pyridoxine	Sensory Attribute*			
(mg/L)	(mg/L)	Rotten egg	Yeasty	Mushroom-musty	Floral
0	0	5.29[a]	3.58[a]	4.00[a]	3.46[a]
0	0.5	4.42[a]	3.96[a]	4.33[a]	2.67[a]
0.5	0	3.96[ab]	4.79[a]	4.08[a]	3.79[a]
0.5	0.5	2.88[b]	4.54[a]	3.33[a]	4.00[a]

*Rating scale ranged from 1 to 9, with 1 indicating extreme low intensity and 9 indicating extreme high intensity for all the attributes.
Mean values (two replicates) with different letters are significantly different at $p \leq 0.05$.

DISCUSSION

Yeast Screening

Optical density measurements showed that the sixteen yeast strains tested in this study exhibited visible growth with or without thiamine or pyridoxine in the medium. One possible explanation could be that yeast strains synthesized thiamine and pyridoxine by themselves without thiamine or pyridoxine contained in the medium. This finding is in agreement with previous research that many *S. cerevisiae* strains are able to synthesize thiamine or pyridoxine by themselves (Bataillon et al. 1996, Dong et al. 2004, Kawasaki et al. 1990, Kondo et al. 2004, Nishimura et al. 1991, Nosaka et al. 1994, Park et al. 2003, Reddick et al. 2001, Singleton 1997, Tazuya et al. 1995). Leonian and Lilly (1942) studied the effect of vitamins on ten strains of *S. cerevisiae* and found that the yeast strains were able to synthesize thiamine in the presence of pyridoxine or synthesize pyridoxine in the presence of thiamine.

This screening procedure was used to determine whether these sixteen yeast stains had different requirements for thiamine or pyridoxine during growth. Fermentation conditions can also affect thiamine requirement by yeast strains. Laser (1941) studied the effect of thiamine on yeast fermentation under anaerobic and aerobic conditions. It was found that different thiamine concentrations highly influence fermentation rate under anaerobic condition, however, fermentation rate did not differ by changing thiamine concentrations under aerobic condition.

Since all the sixteen strains showed similar requirements for thiamine and pyridoxine during growth, one of the strains, *S. cerevisiae* UCD522 (Montrachet), was

selected for later synthetic grape juice fermentations. *S. cerevisiae* UCD522 was chosen for fermentations is firstly because this strain is widely used in wineries for producing vigorous fermentations at high sugar content for both red and white wines (Porter and Ough 1982, Spiropoulos et al. 2000). In addition, UCD522 often produces excessive quantities of H_2S and requires more nutrients than other yeast strains for fermentation (Acree et al. 1972, Mendes-Ferreira et al. 2002, Wang et al. 2003).

Nitrogen Effect

<u>Yeast growth and fermentation rate</u>

Synthetic grape juice with high YAN (250 mg/L) exhibited faster maximum fermentation rate (Table 1 and 3) and soluble solids changes than those with low YAN (60 mg/L) (Figures 9 and 12). This was because 60 mg/L YAN in the fermentation was lower than the normal nitrogen requirement of 140-150 mg/L YAN for yeast metabolism, thus yeast fermentation capacity was decreased (Bely et al. 1990). Fermentations with low YAN took more than 36 days to complete fermentation, whereas those containing high YAN finished fermentation within 22 days (Figures 8 and 9). These trends were consistent with the previous results that nitrogen was one of the most important factors affecting yeast metabolism and fermentation rate. Nitrogen metabolism influenced fermentation rate by affecting the availability of amino acid precursors for the biosynthesis of proteins, yeast cell biomass, and the glycolysis pathway (Boulton et al. 1999, Schulze et al. 1996, Varela et al. 2004).

Sluggish/stuck fermentation

Nitrogen deficiency was one of the main factors that cause sluggish/stuck fermentation (Bely et al. 1990, Cramer et al. 2002, Jiranek et al. 1990, Lagunas 1979, Salmon 1989, Varela et al. 2004). In the current study, the slow fermentation rate could be due to the slow protein synthesis caused by insufficient nitrogen source, which led to sugar transport inactivation. This explanation was proposed by previous research (Salmon 1989, Schulze et al. 1996). On the other hand, research has also shown that ammonia is an activator of phosphofructokinase, which is the key enzyme for fructose metabolism in glycolysis (Alexandre and Charpentier 1998, Ramaiah 1974). The limited amount of ammonia may have caused inactivation of phosphofructokinase, and therefore slowed down the whole glycolysis pathway.

H_2S production

H_2S has the characteristic of rotten egg flavor, which decreases wine quality. Research has been conducted on the factors that affect H_2S production, such as sulfur and nitrogen deficiency, and how these factors affect H_2S formation. In this study, nitrogen deficiency in the synthetic grape juice caused higher H_2S production (Figures 10 and 13), which is in agreement with previous studies that nitrogen deficiency was the key factor to excessive H_2S production (Giudici and Kunkee 1994, Henschke and Jiranek 1991, Vos and Gray 1979). When nitrogen compounds, especially sulfur-containing amino acid precursors (OAS and OAH) are limited, there are not enough OAS and OAH present to combine with sulfide from the SRS reduction to form homocysteine and cysteine for

further amino acid synthesis (Figure 7), resulting in excessive sulfide produced (Henschke and Jiranek 1991, Jiranek et al. 1995b, Stratford and Rose 1985).

Thiamine Effects

Yeast growth and fermentation rate

Slower fermentation rates were exhibited without thiamine at both low and high nitrogen levels (Figure 9 and Table 1), in agreement with the results of Bataillon et al. (1996). Their results showed that *S. cerevisiae* was able to synthesize thiamine in thiamine-depleted culture medium; however, fermentation with thiamine-depleted synthetic grape juice exhibited a very slow fermentation rate. This indicates that thiamine as one of the nutrients for yeast growth and metabolism plays an important role during fermentation.

As an activator for fermentation, thiamine improves yeast cell growth and stimulates fermentation rate (Bugajewska and Wzorek 1995, Kotarska et al. 2006, Schultz et al. 1937a). Thiamine was synthesized into the biochemically active form, thiamine pyrophosphate (TPP), by yeast either inside or outside of the cell before being involved in to the metabolism pathways. In glycolysis, TPP serves as cofactor for the conversion of acetaldehyde to coenzyme A (CoA) and ethanol by alcohol dehydrogenase, named non-oxidative decarboxylation (Cooper and Benedict 1966, Hohmann and Meacock 1998, Laser 1941, Muller et al. 1999, O'Fallon 1975, Park et al. 2003, Schenk et al. 1998, Schneider and Lindqvist 1993, Singleton 1997, Williams et al. 1941, Zeidler et al. 2002). In the current study, synthetic grape juice without thiamine might not have

provided sufficient TPP to serve as a cofactor during fermentation. Therefore glycolysis was partially slowed down and less pyruvate was converted into alcohol.

TPP is also the coenzyme for oxidative decarboxylation of pyruvate catalyzed by pyruvate dehydrogenase complex (PDHC) (Cooper and Benedict 1966, Koser 1968):

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{TPP}} \text{acetyl CoA} + CO_2 + \text{NADH} + H^+$$

Acetyl CoA and CoA are both involved in amino acids metabolism and other pathways, such as H_2S formation, therefore TPP can also indirectly influence these metabolism.

<u>Sluggish/stuck fermentation</u>

Synthetic grape juice without thiamine took longer time to complete compared to the one containing thiamine at both low and high nitrogen (Figures 8 and 9). However, thiamine deficiency was only a trace factor that affected sluggish fermentation compared to nitrogen deficiency. This may be because that the requirement of thiamine by yeast during fermentation is very low and also yeast can synthesize thiamine itself at thiamine depleted condition. If thiamine is deficient in the grape juice, it is allowed to be added to grape must for wine fermentation in the United State to reduce the sluggish effect that caused by thiamine deficiency (Ribéreau-Gayon et al. 2000).

Thiamine × Nitrogen Effect

<u>Yeast growth and fermentation rate</u>

Bataillon et al. (1996) studied the nitrogen and thiamine interaction effect on fermentation using a synthetic medium culture and found that the effect of thiamine on fermentation kinetics depended on nitrogen concentrations. Thiamine did not show an

effect on fermentations at a high nitrogen level, whereas it influenced fermentation rate at low nitrogen concentration. In the current study, soluble solids change did not show much difference between 0.2 and 0.5 mg/L thiamine at both low and high nitrogen concentrations (Figure 9), which indicated that 0.2 mg/L thiamine is a sufficient concentration for yeast growth and other metabolism needs during fermentation. Thiamine concentration at 0.5 mg/L was also in the sufficient range, but not too much to cause changes in yeast metabolism.

H_2S production

Different H_2S production trends were exhibited at low and high nitrogen concentrations with increase in thiamine, indicating an interaction impact of thiamine and nitrogen (Figure 10). In the H_2S formation pathway, known as sulfate reduction sequences (SRS) (Figure 7), coenzyme A (CoA) and acetyl-CoA are the two compounds playing important roles in converting homoserine to O-acetyl-homoserine and converting serine to O-acetyl-serine. The biologically active form of thiamine, thiamine phosphate (TPP), serves as coenzyme for both non-oxidative decarboxylation and oxidative decarboxylation, with final products of CoA and acetyl-CoA. When thiamine is limited, less acetyl-CoA is available for sulfur metabolism, meanwhile less OAS and OAH are produced to combine with sulfide for amino acid synthesis, therefore excess H_2S is formed. On the other hand, methionine was found to inhibit H_2S formation (Eschenbruch et al. 1973, Lawrence and Cole 1968, Wainwright 1971). With increases of thiamine concentration, more acetyl-CoA is available for sulfur metabolism synthesizing more methionine. Methionine inhibits H_2S formation; thus fermentation with higher thiamine

concentration produces less H_2S compared to fermentations with low or no thiamine. These could be possible explanations for the decrease of H_2S production with increasing thiamine concentration under nitrogen deficiency conditions.

However, the addition of thiamine increased the H_2S production when nitrogen was in excess (Figure 10). At this point, nitrogen supply is sufficient for sulfur metabolism and variations in thiamine concentration become the factors affecting sulfur metabolism. H_2S production increases with increases in thiamine concentration. This may be because at sufficient thiamine levels, more acetyl-CoA is available for converting homoserine to *O*-acetyl-homoserine and then to homocysteine. Since homocyeteine was found to encourage H_2S production by Wainwright (1970), it could explain why H_2S production increased when thiamine concentration was increased. Although *O*-acetyl-homoserine, homoserine, homocysteine, and methionine were also found to inhibit H_2S formation in the same study, these inhibition effects could be surmounted by the encouragement of H_2S production from homocysteine.

Pyridoxine Effect

<u>Yeast growth and fermentation rate</u>

Trends in yeast growth and fermentation rate with pyridoxine as variables (Figures 11 and 12) were similar to those with thiamine as variables (Figures 8 and 9). At both low and high nitrogen levels, addition of pyridoxine increased the fermentation rates indicated by faster decrease rates in soluble solids changes (Table 2). Therefore, pyridoxine itself also affected yeast growth and fermentation rate. Absence of pyridoxine in the medium caused slow yeast growth and fermentation rate. Rogosa (1944) showed

this result in the growth curve of lactose-fermenting yeast, *Saccharomyces fragilis* #15. The biochemically active form, pyridoxal phosphate (PLP), is the coenzyme for several reactions by forming a Schiff base (Dunathan and Voet 1974, John 1995). This compound can undergo reactions with amino acids including transaminations, racemizations, and decarboxylations. α-Ketoglutarate is the major compound in transamination to produce different amino acids and is also the product converted from pyruvate, which is derived from glycolysis. Thus, PLP, to some extent, indirectly affected several related pathways and then influenced yeast metabolism and fermentation rate.

Sluggish/stuck fermentation

Similar to thiamine deficiency, pyridoxine deficiency was also a trace factor that impacted sluggish/stuck fermentation. Synthetic grape juice without pyridoxine took several more days to complete compared to the one containing pyridoxine at both low and high nitrogen (Figures 11 and 12). This may also be because that the requirement of pyridoxine by yeast during fermentation is very low and yeast can also synthesize pyridoxine at pyridoxine depleted condition.

Pyridoxine × Nitrogen Effect

Yeast growth and fermentation rate

Although nitrogen highly affected yeast growth and fermentation rate (Figures 8, 9, 11 and 12), more interest was focus on the interaction impact of pyridoxine and nitrogen on fermentation in the current study. The influence of pyridoxine on fermentation was obvious at high nitrogen level, whereas no effect was shown at low nitrogen concentration (Figure 12). This was similar to the effect of thiamine on

fermentation rate as reported by Bataillon et al. (1996). This may be because nitrogen deficiency was dominant on fermentation. Compared to nitrogen contained in grape juice, pyridoxine content was much less. Therefore, nitrogen deficiency might mask the pyridoxine effect and show fewer differences among the fermentations with different pyridoxine concentrations.

H_2S production

The impact of pyridoxine and nitrogen on H_2S production was demonstrated in Figure 13. In addition to thiamine, pyridoxine is also involved in H_2S formation pathway (Figure 7). Pyridoxal phosphate (PLP), as the biologically active form of pyridoxine, is required for the condensation of *O*-acetyl-homoserine and H_2S to form homocysteine, and the condensation of *O*-acetyl-serine and sulfide to form cysteine in the sulfur metabolism pathway (Botsford and Parks 1969, Wiebers and Garner 1967). Thus, pyridoxine deficiencies in many *S. cerevisiae* strains may cause low levels of methionine production resulting in H_2S formation (Eschenbruch 1974, Jiranek et al. 1995b, Lawrence and Cole 1968, Maw 1965, Wainwright 1970). As shown in Figure 13, at both low and high nitrogen levels, higher amounts of H_2S were produced without pyridoxine in the synthetic grape juice. This indicated that pyridoxine or PLP played an important role in affecting H_2S production. With pyridoxine in the synthetic grape juice, less H_2S was formed. However, the higher pyridoxine concentration (0.5 mg/L) resulted in higher H_2S production than the lower pyridoxine added (0.25 mg/L). The biochemical changes based on this condition were not clear.

Thiamine × Pyridoxine Effect

Yeast growth and fermentation rate

Thiamine and pyridoxine, individually and together, affected yeast growth and fermentation rate. Yeast were able to reach high cell population at any level of thiamine without pyridoxine, any level of pyridoxine without thiamine, or no thiamine and pyridoxine. This is likely because this *S. cerevisiae* strain synthesized sufficient thiamine and pyridoxine by itself, under depleting conditions, or some other nutriments may have served as precursors in the medium to assist synthesizing thiamine and pyridoxine by the yeast. This was in agreement with previous results that thiamine and pyridoxine were found to support the synthesis of each other in many *S. cerevisiae* strains (Bataillon et al. 1996, Chiao and Peterson 1956, Leonian and Lilly 1942, Schultz and Atkin 1947, Tanphaichitr 2001). The possible explanation proposed by Moses and Joslyn (1953) stated that either thiamine or pyridoxine may serve as a precursor of an intermediate form from which the other may be synthesized, or one might serve as a functional substitute for the other in the synthesis of some vital intermediates produced through the influence of either of them. Later on, more research was conducted on thiamine and pyridoxine synthesis. Zeidler et al. (2003) showed that pyridoxine is an intermediate in the synthesis of thiamine especially for *S. cerevisiae* cells, where the pyrimidine unit of thiamine is synthesized from histidine and pyridoxine. Therefore, thiamine and pyridoxine are highly related to each other in affecting yeast growth and fermentation rate.

An inhibitory effect on yeast growth was found when increasing thiamine concentration in pyridoxine-free medium and this effect could be solved by adding pyridoxine into the medium (Chiao and Peterson 1956, Nakamura et al. 1981, Schultz and Atkin 1947). In the current study, increasing thiamine concentration inhibited yeast growth for fermentations without pyridoxine (Figure 14). For example, in Fig. 14A, yeast reached 10^7 CFU/mL in the medium with and without pyridoxine. However, in Figures 14B and 14C, yeast in medium without pyridoxine only reached 10^6 CFU/mL, which indicated an inhibitory effect of thiamine in pyridoxine-free medium. By adding pyridoxine to pyridoxine-free medium (0.25 and 0.5 mg/L), the effect of thiamine inhibition on yeast was stopped, which agreed with previous research results by Nakamura et al. (1981).

Highly related interactions between thiamine and pyridoxine may be due to their similar functions in yeast metabolism (Chiao and Pererson 1956). Before serving as coenzymes in yeast metabolism, both thiamine and pyridoxine are required to be phosphorylated to phosphorylated forms, thiamine pyrophosphate (TPP) and pyridoxal-5-phosphate (PLP). On the other hand, TPP and PLP compete for pyruvic acid during fermentation. TPP is the coenzyme for decarboxylating pyruvic acid to acetaldehyde, and PLP is the coenzyme for the transamination reaction involving pyruvic acids as the substrates to form α-keto compounds. Based on these similarities, thiamine and pyridoxine therefore highly interact or affect each other in yeast metabolism during fermentation.

H_2S production

When nitrogen concentration was controlled at 60 mg/L, thiamine and pyridoxine effects on H_2S production were present in Figure 16. Thiamine, pyridoxine and their interactions significantly ($p \leq 0.05$) affected H_2S production because these two vitamins are coenzymes involved in sulfur metabolism. Both involved in the SRS metabolism pathway (Fig. 7), similar functions of thiamine and pyridoxine and their interactions in yeast metabolism may cause variation of H_2S formation. In addition, the nitrogen ring of pyridoxine was found to be derived from the amide group of glutamine in *S. cerevisiae* (Tazuya et al. 1995). Glutamine and glutamate are convertible in nitrogen metabolism and glutamate is involved in H_2S formation. Therefore, complicated relations among thiamine, pyridoxine and H_2S formation may also cause H_2S variation. Since no trend was exhibited in the H_2S production with increase of thiamine or pyridoxine concentrations, more complicated metabolism pathways may be carried by the yeast due to variations in concentration of these two vitamins.

Sensory Evaluation

Results from the triangle test showed that panelists were able to notice overall aroma differences among the wines fermented under different thiamine and pyridoxine combinations except for two pairs (wines fermented with 0 and 0.5 mg/L thiamine at both levels of pyridoxine). This indicated that differences in thiamine and pyridoxine concentration led to the formation of different aroma compounds, and variations in H_2S production may have been the factor that caused the differences. The yeast metabolism pathway may be altered to generate various volatile compounds, such as some higher

alcohols, which normally present pungent aromas. Thiamine was found to influence higher alcohols formation during fermentation (Jackson 2000). Panelists could not tell aroma differences between the two thiamine concentrations (0 and 0.5 mg/L) with or without pyridoxine, which may be because the different pungent compounds produced due to different metabolic pathways caused by thiamine and pyridoxine variations, therefore it was more difficult for people to distinguish differences.

Among the aroma attributes selected by the experienced panel, the aroma attribute rating test showed that panelists only found significant differences in rotten egg aroma and they could not distinguish any differences from the other three attributes, yeasty, floral, and mushroom-musty, in the synthetic wines. This indicated that the variation in H_2S highly affected the wine aromas.

CONCLUSIONS

Nitrogen was one of the key factors that affected fermentation rate and H_2S production, which was consistent with previous research. Thiamine and pyridoxine influenced yeast growth and fermentation rate, although their impacts were smaller than nitrogen.

Although H_2S production was significantly affected by thiamine, overall H_2S production was very low ($\leq$ 30 µg/L) at any thiamine levels when sufficient nitrogen was available in the medium. However, media with sufficient thiamine produced less H_2S compared to the ones with insufficient thiamine when nitrogen was deficient in the media. Pyridoxine showed significant effect ($p \leq 0.05$) on H_2S production where high amounts ($\geq$ 80 µg/L) of H_2S were produced at both low and high nitrogen levels without pyridoxine. Interactions between thiamine and pyridoxine highly affected H_2S production, which may be due to their similar roles and interactions in yeast metabolism, especially sulfur metabolism. Since thiamine is legally allowed to be added in the grape must in the United State, wineries can adjust thiamine to help reduce H_2S production when the grape juice has insufficient nitrogen.

The significant difference in rotten egg aroma detected by panelists from the sensory aroma evaluation test indicated that perhaps H_2S produced during fermentation highly affected final wine aromas. Besides H_2S, other volatile compounds produced due to different thiamine and pyridoxine combinations may also affect the aroma differences.

FUTURE RESEARCH

Yeast strain *S. cerevisiae* UCD 522 was utilized in this study to determine how thiamine and pyridoxine, together with nitrogen affect fermentation rate and H_2S production. Other yeast strains, especially the ones exhibited different growth in various thiamine and pyridoxine media as shown in optical density reading, need to be tested using the same test design. This will help determine how these two vitamins affect the fermentation with different yeast strains and if different yeast strains show various influences on fermentation rate and H_2S production. In addition, gene technologies could be applied to find out how genes control yeast metabolism pathways and synthesis of these two vitamins, to help understand thiamine and pyridoxine effects on yeast metabolism.

The sensory evaluation results in this study showed significant difference in H_2S aroma, but not other aromas. Different volatile compounds produced due to the variation of thiamine and pyridoxine concentrations may also affect the sensory characteristics of the finished wine. Further tests using gas chromatography, to determine the volatile compounds produced under different thiamine and pyridoxine combinations, will help understand the change of biosynthetic pathway due to thiamine, pyridoxine, and nitrogen variations.

Finally, elemental sulfur contained or added to grape juice is a precursor to H_2S production during fermentation. In this study, 30 ppm SO_2 was added to all three fermentations, therefore the original elemental sulfur contained in the synthetic grape juice were all same for all these three fermentations. This is not considered as a factor

that affected the H_2S production variations. However, with different nitrogen, thiamine and pyridoxine combinations, elemental sulfur may influence H_2S production through different yeast metabolism pathways, therefore more research needs to be conducted to consider the elemental sulfur effects.

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APPENDIX

Appendix A. Optical Density measurement for yeast strains growing in different depleting media.

Yeast Strain	O. D. (24 hr)				O. D. (48 hr)			
	S	S+T	S+P	S+T+P	S	S+T	S+P	S+T+P
UCD522	0.226	0.23	0.208	0.222	0.295	0.254	0.27	0.286
EC1118	0.252	0.214	0.22	0.222	0.335	0.32	0.278	0.305
Chos	0.134	0.136	0.156	0.162	0.29	0.264	0.286	0.296
Epernay 2	0.078	0.096	0.092	0.18	0.218	0.218	0.222	0.232
CY3079	0.136	0.184	0.17	0.168	0.274	0.27	0.28	0.268
D254	0.2	0.19	0.19	0.204	0.246	0.296	0.272	0.296
Syrah	0.166	0.184	0.192	0.192	0.294	0.26	0.304	0.282
UCD679	0.048	0.058	0.052	0.056	0.238	0.284	0.244	0.264
71B	0.136	0.11	0.128	0.118	0.31	0.304	0.316	0.306
BM45	0.13	0.138	0.156	0.126	0.254	0.28	0.294	0.29
Pasteur Red	0.08	0.122	0.172	0.16	0.278	0.27	0.312	0.338
Chasson	0.062	0.07	0.052	0.038	0.254	0.268	0.288	0.34
Steinberg Lager	0.044	0.138	0.074	0.084	0.18	0.14	0.216	0.276
D47	0.096	0.16	0.17	0.16	0.31	0.308	0.338	0.322
BDX	0.108	0.142	0.076	0.136	0.268	0.288	0.282	0.196
S6U	0.066	0.14	0.242	0.216	0.29	0.314	0.342	0.318

*S, starter media without thiamine & pyridoxine; T, thiamine (0.05 mg/L); P, pyridoxine (0.2 mg/L).