INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of “sectioning” the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106
Liu, Jim-Wen Robert

EFFECT OF PH, SULFUR DIOXIDE, INSOLUBLE SOLIDS, AND IMMOBILIZED LEUCONOSTOC OENOS PSU-1 ON THE INDUCTION OF MALOLACTIC FERMENTATION IN TABLE WINES

The Ohio State University

University Microfilms International

300 N. Zeeb Road, Ann Arbor, MI 48106
EFFECT OF pH, SULFUR DIOXIDE, INSOLUBLE SOLIDS, AND IMMOLIZED Leuconostoc oenos PSU-1 ON THE INDUCTION OF MALOLACTIC FERMENTATION IN TABLE WINES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Jim-Wen Robert Liu, B.S., M.S.

* * * * *

The Ohio State University
1983

Reading Committee:
Dr. J. F. Gallander
Dr. G. A. Cahoon
Dr. J. R. Geisman
Dr. A. C. Peng

Approved By

James F. Gallander
Adviser
Department of Horticulture
ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. James F. Gallander for his guidance, understanding, and constant support in performing this research and preparing this dissertation.

The author is also grateful to Drs. Robert B. Beelman, Garth A. Cahoon, Jean R. Geisman, Andrew C. Peng, and Robert L. Clements for their time and valuable advice during the course of this research.

Appreciation is extended to Dr. Robert G. Hill, Jr. for his support, and to Drs. Junius F. Snell and David C. Ferree for their constructive criticism of the dissertation.

Appreciation is also extended to Judith Stetson for her friendship and valuable assistance, to Dr. Demetrio G. Ortega, Jr., and many staff members of O.A.R.D.C. for their helpful assistance.

Gratitude is expressed to The George F. Lonz Foundation for financial support.

To my parents for their support and encouragement.

To my wife Ting-Ting, for her support, understanding, and sacrifices over the past years.
VITA

January 5, 1948 . . . .  Born - Taiwan, Republic of China

1972 . . . . . . . . .  B.S., Department of Biology, National Taiwan Normal University, Taipei, Republic of China

1977 . . . . . . . . .  M.S., Department of Horticulture, The Pennsylvania State University, University Park, Pennsylvania

1979 . . . . . . . . .  M.S., Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania

1979 - 1983 . . . . .  Research Associate, Department of Horticulture, The Ohio State University, Columbus, Ohio

PUBLICATIONS


Effect of pH and sulfur dioxide on the rate of malolactic fermentation in red table wines. Am. J. Enol. Vitic. (accepted for publication).
VITA (continued)

FIELD OF STUDY

Major Field: Food Technology


Studies in Food Microbiology. Professor Emil M. Mikolajcik.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>GENERAL REVIEW OF LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>The Microbiology and Biochemical Pathways of Malolactic Fermentation</td>
<td>5</td>
</tr>
<tr>
<td>Factors Affecting Malolactic Fermentation</td>
<td>11</td>
</tr>
<tr>
<td>Wine Deacidification by Malolactic Fermentation</td>
<td>19</td>
</tr>
<tr>
<td>Methods for Inducing Malolactic Fermentation</td>
<td>21</td>
</tr>
<tr>
<td>Immobilized Cell Systems and Their Application to Induction of Malolactic Fermentation</td>
<td>25</td>
</tr>
</tbody>
</table>

**Chapter**

I. EFFECT OF pH AND SULFUR DIOXIDE ON THE RATE OF MALOLACTIC FERMENTATION IN RED TABLE WINES . 29

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>31</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>33</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>43</td>
</tr>
</tbody>
</table>

II. EFFECT OF INSOLUBLE SOLIDS ON THE SULFUR DIOXIDE CONTENT AND RATE OF MALOLACTIC FERMENTATION IN WHITE TABLE WINES . 45

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>46</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>49</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>59</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. CHARACTERISTICS OF INSOLUBLE SOLIDS INVOLVED</td>
<td>61</td>
</tr>
<tr>
<td>IN THE REDUCTION OF SULFUR DIOXIDE CONTENT OF</td>
<td></td>
</tr>
<tr>
<td>WHITE GRAPE JUICES AND TABLE WINES</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>63</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>67</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>80</td>
</tr>
<tr>
<td>IV.  INDUCTION OF MALOLACTIC FERMENTATION IN TABLE WINES BY IMMOLIZED</td>
<td>82</td>
</tr>
<tr>
<td>CELLS OF Leuconostoc oenos PSU-1</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>84</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>88</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>103</td>
</tr>
<tr>
<td>GENERAL DISCUSSION AND CONCLUSION</td>
<td>105</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>109</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Time (weeks) to complete malolactic fermentation in Chancellor wines at different must pH and sulfur dioxide levels.</td>
<td>34</td>
</tr>
<tr>
<td>2.</td>
<td>pH values of fermenting musts at the time of bacterial inoculation</td>
<td>37</td>
</tr>
<tr>
<td>3.</td>
<td>Free, bound, and total sulfur dioxide contents of Vidal blanc and Catawba juices 12 hours after the addition of 30 ppm of sulfur dioxide</td>
<td>50</td>
</tr>
<tr>
<td>4.</td>
<td>Rate of malolactic fermentation in Vidal blanc and Catawba wines with different levels of insoluble solids</td>
<td>55</td>
</tr>
<tr>
<td>5.</td>
<td>Rate of alcoholic fermentation in Vidal blanc and Catawba wines with different levels of insoluble solids</td>
<td>58</td>
</tr>
<tr>
<td>6.</td>
<td>The insoluble solids and total sulfur dioxide contents in three types of juice from five different grape varieties</td>
<td>68</td>
</tr>
<tr>
<td>7.</td>
<td>The insoluble solids content, pH, and total acidity of five grape varieties at harvest.</td>
<td>70</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of sampling time on the sulfur dioxide contents of Cayuga White and Catawba juices with various amounts of insoluble solids.</td>
<td>71</td>
</tr>
<tr>
<td>9.</td>
<td>The effect of initial sulfur dioxide levels on the sulfur dioxide content of Vidal blanc juices with 0.0 and 9.2% of insoluble solids.</td>
<td>73</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>10. Addition of various particulate materials and its effect on the sulfur dioxide content of Aurora juice initially treated with 30 ppm of sulfur dioxide</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>11. Effect of EDTA (5 mM) and ascorbic acid (5 mM) on the sulfur dioxide content of Vidal blanc juices added with 1.0 and 8.0% of insoluble solids and 30 ppm of sulfur dioxide</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>12. Effect of pasteurization and insoluble solids on the total sulfur dioxide content in Seyval blanc juices and wines</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>13. The rate of malolactic fermentation in Chancellor wines without inoculation (A), and with inoculation of free cells (B), free cells plus gel pellets (C), gel pellets (D), and immobilized cells (E) of <em>L. oenos</em> PSU-1</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>14. The rate of malolactic fermentation in Chancellor wines inoculated with free or immobilized cells of <em>L. oenos</em> PSU-1</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>15. Chemical composition of two batches of Chancellor wine before and after malolactic fermentation induced by immobilized cells of <em>L. oenos</em> PSU-1</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>16. Chemical composition of Vidal blanc wine before and after malolactic fermentation induced by immobilized cells of <em>L. oenos</em> PSU-1</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>17. Effect of volume ratio of immobilized cells/wine on the rate of malolactic fermentation in Vidal blanc wines</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>18. Time (days) required for immobilized cells of <em>L. oenos</em> PSU-1 to complete malolactic fermentation in Chancellor wines at different pH and free sulfur dioxide levels</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>19. The concentration of immobilized and leaked cells after malolactic fermentation induced by immobilized cells of <em>L. oenos</em> PSU-1</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.3 must.</td>
<td>38</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.5 must.</td>
<td>39</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.7 must.</td>
<td>40</td>
</tr>
<tr>
<td>4.</td>
<td>Changes in total sulfur dioxide content during the vinification of Vidal blanc (1979) wine with different levels of insoluble solids.</td>
<td>52</td>
</tr>
<tr>
<td>5.</td>
<td>Changes in total sulfur dioxide content during the vinification of Vidal blanc (1980) wine with different levels of insoluble solids.</td>
<td>53</td>
</tr>
<tr>
<td>6.</td>
<td>Changes in total sulfur dioxide content during the vinification of Catawba (1980) wine with different levels of insoluble solids.</td>
<td>54</td>
</tr>
<tr>
<td>7.</td>
<td>Growth curves of Leuconostoc oenos strain PSU-1 in Catawba wines with different levels of insoluble solids.</td>
<td>57</td>
</tr>
</tbody>
</table>
INTRODUCTION

Malolactic fermentation is the process of degrading L-malic acid to L-lactic acid and carbon dioxide by the enzymatic action of certain lactic acid bacteria. The early occurrence and completion of this fermentation is highly desirable in acidic wines produced in relatively cool regions. In these regions, the high malic acid content is responsible for the excess of acidity in table wines. Through malolactic fermentation, wine acidity is reduced to a more desirable level, because lactic acid (monocarboxylic) is a chemically weaker acid and less tart than malic acid (dicarboxylic).

In addition to reducing wine acidity, a complete malolactic fermentation will deplete the malic acid and nutrients in a wine. Thus, malolactic bacteria are not able to grow and cause spoilage in finished wines. In other words, malolactic fermentation increases the bacteriological stability of wines. Without this stability, there is a high risk of having a spontaneous malolactic fermentation after bottling. If this occurs, the wine is considered spoiled because of the turbidity and effervescence produced by the fermentation. For these reasons, it is advantageous to induce an early malolactic fermentation, prior to bottling.
To stimulate malolactic fermentation, there are several practices being used during vinification. The most effective method has been the inoculation of high numbers of malolactic bacteria during alcoholic fermentation. Although this method stimulates malolactic fermentation, it does not guarantee the occurrence of malolactic fermentation. The main reason is that levels of certain chemical constituents vary widely in wine, depending on the grape variety and method of vinification. These variations affect the growth behavior of malolactic bacteria and rate of malolactic fermentation. The chemical constituents that have been found to influence malolactic fermentation include: pH, sulfur dioxide, alcohol, and nutrients. Low pH and high concentration of sulfur dioxide are the most important inhibitors to malolactic bacteria. The inhibitory effect is related to the influence of low pH on the metabolic rate of malolactic bacteria and the antimicrobial activity of sulfur dioxide.

In order to stimulate malolactic fermentation in low-pH wines of eastern United States, it has been recommended that initial addition of sulfur dioxide to musts should be reduced or eliminated. There appears to be a lack of information demonstrating the effect of sulfur dioxide level on the rate of malolactic fermentation in musts with different pH values. Information is needed in estimating the maximum level of sulfur dioxide which could be added to musts at a certain pH without inhibiting malolactic fermentation. With this in mind, a study was initiated to determine the effect
of pH and sulfur dioxide levels on the growth behavior of malolactic bacteria and rate of malolactic fermentation in red wines.

Since sulfur dioxide content is closely related to malolactic fermentation, any vinification method able to cause its change is likely to affect malolactic fermentation. Recently, racking and filtration were found to affect both the insoluble solids and sulfur dioxide contents in white grape juice. Insoluble solids in musts appeared to have an effect on sulfur dioxide content. These substances are the particulate material from grape pulp and skin which suspends in the juice or settles to the bottom of juice upon standing. If the insoluble solids indeed affect sulfur dioxide content, it is possible that they would also influence malolactic fermentation. A study was initiated to determine the effect of insoluble solids on the sulfur dioxide content and the rate of malolactic fermentation in white table wines.

A study was also conducted to assess the variation in insoluble solids content of juices obtained from several grape varieties and its effect on sulfur dioxide content of the juices. Additionally, the mechanism possibly involved in the reduction of sulfur dioxide by insoluble solids was investigated.

Although inoculation of a high number of malolactic bacterial cells is rather effective in stimulating malolactic fermentation, it has not been widely used by
wineries. The reasons for this limited use are the high cost of commercial preparations of malolactic bacterial cultures and the inability of these bacteria to induce malolactic fermentation under inhibitory conditions such as low pH which is often present in the wines of eastern United States. As the third part of this research, a study was conducted to develop a more reliable and economical method for inducing malolactic fermentation. It was thought that by confining or immobilizing a large number of malolactic bacteria in a solid gel matrix, the bacteria could be easily recycled after completing a malolactic fermentation to reduce the cost. Also, the gel matrix may protect malolactic bacteria from the toxic effects of low pH, high ethanol and sulfur dioxide contents to facilitate bacterial growth and malolactic fermentation.

In summary, the objectives of this research were to:

1. Determine the effect of pH and sulfur dioxide on the rate of malolactic fermentation in red table wines.
2. Determine the effect of insoluble solids on the sulfur dioxide content and rate of malolactic fermentation in white table wines.
3. Study the variation in insoluble solids content of white grape juices and the mechanism involved in the reduction of sulfur dioxide by insoluble solids.
4. Develop an immobilized cell system for inducing malolactic fermentation in table wines.
The Microbiology and Biochemical Pathways of Malolactic Fermentation.

Malolactic Bacteria. Malolactic fermentation is the conversion of L-malic acid to L-lactic acid and carbon dioxide. It occurs in wines and ciders. The causative agents are certain strains of lactic acid bacteria. They were first isolated from wine by Koch in 1900 (Kunkee, 1967b). He also demonstrated that malolactic fermentation could be induced by inoculation with these organisms. The lactic acid bacteria that cause malolactic fermentation are known as malolactic bacteria. Like other lactic acid bacteria, malolactic bacteria are Gram positive, non-spore-forming, and carbohydrate-fermenting lactic acid producers (Ingram, 1975). They are acid-tolerant, catalase-negative and facultative anaerobes; typically they are non-motile and do not reduce nitrate. Their temperature requirement is mesophilic.

Since 1900, more malolactic bacteria have been isolated, identified, and classified. For isolation and cultivation, Fornachon's yeast extract agar and a modified Rogosa medium have been proven to be satisfactory (Ingraham,
et al., 1960). Most of the strains belong to three genera: \textit{Lactobacillus}, \textit{Leuconostoc}, and \textit{Pediococcus}. They are usually divided into two physiological responsive groups. One group produces mostly a single product (lactic acid) from glucose, and thus is called homofermentative. The other group, heterofermentative bacteria, converts glucose to lactic acid, carbon dioxide, ethanol, and acetic acid. Under the genus \textit{Lactobacillus}, there are eight species of rod-shaped malolactic bacteria including four homofermentative species (\textit{L. casei}, \textit{L. delbrueckii}, \textit{L. plantarum}, and \textit{L. leichmannii}) and four heterofermentative species (\textit{L. brevis}, \textit{L. buchneri}, \textit{L. hilgardii}, and \textit{L. pastorianus}). The genus \textit{Leuconostoc} has three species which are malolactic: \textit{L. citrovorum (oenos)}, \textit{L. dextranicum}, and \textit{L. mesenteroides}. All three are heterofermentative cocci. \textit{Pediococcus cerevisiae} is a homofermentative coccus. All the bacteria except \textit{L. leichmannii} are described in Bergey's Manual of Determinative Bacteriology (Breed et al., 1957).

The distinction between homofermentative and heterofermentative bacteria resides in the inherent enzyme complements associated with the fermentative degradation of glucose (Stamer, 1979). Homofermentative bacteria are able to utilize glucose via glycolytic pathway. Glucose is degraded to pyruvic acid which in turn is reduced to lactic acid. For each molecule of glucose converted to lactic acid, there are two molecules of adenosine triphosphate (ATP) being formed. In contrast, the heterofermentative
bacteria lack the enzyme fructose diphosphate aldolase (E.C. 4.1.2.13) which is necessary for splitting hexose diphosphate into triose phosphate. Thus, the glycolytic pathway is blocked in heterofermentative bacteria. Instead, glucose is first converted to glucose-6-phosphate by glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), and then oxidized to 6-phospho-gluconate by 6-phospho-gluconate dehydrogenase (E.C. 1.1.1.43). The 6-phospho-gluconate is further converted to ribose-5-phosphate which splits into a 3-carbon unit and 2-carbon unit. The 3-carbon unit is oxidized to pyruvic acid, and then converted to lactic acid. While the 2-carbon compound is reduced to ethanol and/or acetic acid (Pederson, 1971). In addition, heterofermentative bacteria are able to produce mannitol from fructose and dextran from glucose and sucrose. To differentiate the heterofermentative cocci (Leuconostoc) from the heterofermentative rods of Lactobacillus, the stereoisomers of lactic acid (D, L, or DL) produced during fermentation may be used for identification. Leuconostoc species produce the D(-) isomer exclusively, whereas the heterofermentative lactobacilli produce optically inactive or racemic (DL) forms (Stamer, 1979).

The biochemical pathways of organic acids including pyruvic, citric, sorbic, fumaric, and malic acid are important to wine quality. Pyruvic acid is an important intermediate not only because it is extremely reactive, but also it is a precursor to diacetyl (2,3-butandione), acetoin
(3-hydroxy-2-butanone), and butylene glycol (2,3-butandiol). Diacetyl and acetoin are odorous compounds which can be produced by malolactic bacteria. Diacetyl is responsible for the buttery flavor of dairy products. Diacetyl and acetoin are known to affect wine flavor (Kunkee, 1974).

Diacetyl is also produced from the metabolism of citric acid by malolactic bacteria. Citric acid is normally found in grapes at much lower levels than tartaric and malic acids. It is sometimes added to acidify wine. Fermentation of citric acid by malolactic bacteria has been studied by duPlessis (1964). The major end products were lactic acid, acetic acid, and carbon dioxide. Minor amounts of formic acid, ethanol, succinic acid, and acetoin were also found.

Sorbic acid (2,4-hexadienoic acid) is sometimes added to juice or wine to prevent spoilage by yeasts and molds. However, this unsaturated carboxylic acid can be transformed by lactic acid bacteria to yield 2-ethoxyhexa-3,5-diene. This compound gives rise to a highly objectionable geranium-like odor (Crowell and Guyman, 1975).

Fumaric acid has been used as an acidulant in California wines for almost a decade. Recently, fumaric acid was shown to delay or even prevent malolactic fermentation in wines (Cofran and Meyer, 1970; Tchelistcheff et al., 1971). It was possibly due to the bacteriacidal effect of fumaric acid on the wine leuconostoc (L. oenos L-181 and ML-34) (Pilone et al., 1974; Pilone, 1975). The action or activity of fumaric acid on lactobacilli and pediococci is
not known. When malolactic fermentation occurred in the presence of fumaric acid, both L-malic and fumaric acid were degraded. Fumaric acid is first converted to L-malic acid by the fumarase, and then degraded to L-lactic acid through malolactic fermentation.

**Malolactic Fermentation.** Malic acid is utilized by malolactic bacteria through malolactic fermentation. According to Kunkee (1967b), the pathway of malolactic fermentation was first established by Seifert in 1901 as follows:

\[
\text{Malic acid} \rightarrow \text{Lactic acid + CO}_2
\]

The enzyme which catalyzes malolactic fermentation was not known until 1948, when Korkes and Ochoa (1948) first isolated the malic enzyme from *Lactobacillus arabinos (plantarum)*. They suggested that conversion of L-malate to lactate and carbon dioxide was from two reactions,

\[
\begin{align*}
\text{malic enzyme} & : \quad \text{L-malate} + \text{DPN(ox)} \rightarrow \text{pyruvate} + \text{CO}_2 + \text{DPN(red)} \\
\text{lactic dehydrogenase} & : \quad \text{pyruvate} + \text{DPN(red)} \rightarrow \text{lactate} + \text{DPN(ox)}
\end{align*}
\]

It was proven that the malic enzyme (L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.38) brings about exclusively the transformation of malate into pyruvate; the reduction of the pyruvate into D-lactate or L-lactate was dependent upon the presence of the corresponding lactic dehydrogenase. However, in 1968, Peynaud (1968) discovered
that strains of *Leuconostoc* produced only L-lactic acid from L-malic acid, while D- and L-lactic acid was produced from pyruvate. This implied that lactic dehydrogenase was not involved in the conversion of L-malic acid to L-lactic acid. This finding was confirmed by Radler et al. (1970). The conversion of L-malic acid to L-lactic acid and carbon dioxide was found to occur within malolactic bacteria which contained no L-lactic dehydrogenase and did not form L-lactic acid from glucose. Therefore, Radler (1975) suggested that L-malic acid was probably converted directly to L-lactic acid and carbon dioxide without pyruvic acid being an intermediate. The enzyme was different from malic enzyme and should be given the name malolactic enzyme or L-malate carboxy-lyase (Radler, 1975). However, it is also possible that malolactic enzyme may be a bifunctional protein with a sequential mode of action or a multi-enzyme system consisting of two intimately associated proteins. In both instances the malolactic enzyme would ensure the two reactions which are catalyzed by malic enzyme and lactic dehydrogenase, respectively (Lonvaud and Ribereau-Gayon, 1975). In 1982, Lonvaud-Funel and Strasser de Saad (1982) described the purification and properties of a malolactic enzyme from *Leuconostoc mesenteroides*. Their results indicated that malolactic activity was not a combination of malic enzyme and lactic dehydrogenase activities, but was carried out by a complex. It produced the sum of these
reactions without catalyzing the partial reactions. NAD was essential for malolactic activity.

Whiting and Coggins, as cited by Kunkee (1967b), studied the malolactic enzyme induction in several malolactic bacteria isolated from cider. Some of the bacteria were found to have an inducible malolactic enzyme, but in others, the enzyme was constitutive. The studies of Blanchard et al. (1950) indicated that a relatively high concentration (150mM) of DL-malic acid was needed to induce the enzyme. However, other workers induced the enzyme by using concentrations which are normally found in grape juice (30mM) (Nathan, 1961; Flesch and Holbach, 1965). They found that strains of malolactic bacteria that required high concentrations of malic acid for induction of the enzyme would not carry out malolactic fermentation in wines with low malic acid.

Factors Affecting Malolactic Fermentation.

In order to obtain malolactic fermentation in a wine, malolactic bacteria have to be present in the wine. In addition, the bacteria must be able to grow and develop into a large population (1 x 10^6 colony forming units per mL) before the decarboxylation of malic acid can occur (Rice and Mattick, 1970). Hence, factors which affect the growth of malolactic bacteria should be distinguished from those directly involved in the decarboxylation of malic acid.
Many factors have been found to influence malolactic fermentation. Those factors include: nutrients, bacteriophage, pH, sulfur dioxide, alcohol, and temperature. All the above factors except nutrients and bacteriophage can directly affect the decarboxylation of malic acid.

**Nutrients.** Nutritional studies on the growth of malolactic bacteria with synthetic media showed that their growth requirements were similar to those for other lactic acid bacteria. These bacteria require an extensive complement of amino acids, purines, pyrimidines, and vitamins, besides minerals, carbon, and nitrogen sources (Kunkee, 1967b). Yeast extract or yeast autolysate appears to supply nutrients for malolactic bacteria (Luthi and Vetsch, 1959; Fornachon, 1957). Tomato juice contains some growth factors which are required by malolactic bacteria. Stamer et al. (1964) reported that the ash of tomato juice was the active fraction. While Amachi et al. (1971) and Amachi (1975) identified a growth factor in tomato juice as 4'-O-(β-D-glucopyranosyl)-D-(R)-pantothenic acid. The presence of grape skins during alcoholic fermentation has been shown to be stimulatory to malolactic fermentation (Kunkee, 1967a; Beelman and Gallander, 1970). Kunkee (1967a) suggested that some material was extracted from the skins which was stimulatory to malolactic bacteria.

**Bacteriophages.** Recently, bacteriophages have been reported to cause interruptions of malolactic fermentation.
by Sozzi et al. (1982). They found that several strains of Leuconostoc oenos were attacked and destroyed by bacteriophages.

**pH.** The pH has a major influence on malolactic fermentation. The optimum pH for malolactic bacteria is much higher than the pH of wine, but it is generally lower than the optimum pH for other lactic acid bacteria (Ingraham et al., 1960). DeMenezes et al. (1972) reported that the optimum pH for the growth of Leuconostoc oenos ML-34 was between 4.5 and 5.4, but the decarboxylation rate was highest at pH 3.4 to 4.4. Similar findings were also reported by Lafon-Lafourcade (1975). According to Kunkee (1974), the critical pH for malolactic fermentation is 3.3. Above pH 3.3, malolactic fermentation is more likely to occur. Below pH 3.3, a special effort is needed to initiate this secondary fermentation. Castino et al. (1975) analyze 72 wines for the effect of pH, SO₂, and alcohol content on malolactic fermentation. They concluded that low pH alone was a powerful inhibitor of the malolactic fermentation and malolactic fermentation did not occur below pH 3.2. The same conclusion was also made by Fornachon (1957). However, this was not true in New York as pointed out by Rice and Mattick (1970). They reported that malolactic fermentation occurred quite regularly in New York wines as low as pH 3.0 and occasionally below pH 3.0. Despite differences in the lowest pH limit for malolactic fermentation, there is a general agreement that low pH is inhibitory to malolactic
fermentation and the rate of fermentation is directly
related to the pH of wine.

The pH values for musts of vinifera varieties grown in
California were reported in the range of pH 3.1 to 3.9
(Amerine et al., 1967). The majority of pH values were from
(1965) reported that the incidence of malolactic fermenta-
tion in southern California wines was approximately 75%.
Since the survey was conducted for wines made from a warm
region where attempts were not made to inhibit the fermenta-
tion, Kunkee (1974) pointed out that this high incidence
should not be used to represent the incidence of malolactic
fermentation for all California wines. Grapes and wines
produced in eastern United States including Ohio and New
York are usually of low pH. The pH of musts from labrusca
and hybrid varieties grown in New York ranged from pH 2.65
to 3.40, with the majority of pH values ranging from 2.95 to
wines having natural malolactic fermentation was 45%, in-
cluding both white (27%) and red (68%) wines.

Gallander and Stetson (1977) reported that the pH of
grapes grown in southern Ohio varied from 2.99 to 3.74. The
pH of these wines ranged from 2.90 to 3.77. The incidence
of malolactic fermentation in Ohio wines was only 10% ac-
ccording to Graumlich and Gallander (1973). This was lower
than that of southern California and New York. They indi-
cated that low sulfur dioxide level is important in
obtaining a malolactic fermentation. This is especially true with eastern United States wines which normally have low pH values. The low pH not only retards the growth and metabolism of malolactic bacteria, but also enhances the antimicrobial activity of sulfur dioxide.

**Sulfur Dioxide.** Sulfur dioxide has been used in winemaking since ancient times. This compound is used to serve three major functions: control of undesirable microorganisms, inhibition of browning, and antioxidant. Usually 75-150 ppm (mg/L) of SO₂ is added to the must as soon as possible after crushing the grapes to assure proper control of fermentation.

Sulfur dioxide exists in various species in solution: molecular sulfur dioxide (SO₂), bisulfite (HSO₃⁻), sulfite (SO₃⁻), and metabisulfite (S₂O₅²⁻). These species of sulfur dioxide establish equilibriums in a solution as follows:

\[
\begin{align*}
\text{SO}_2(g) & \rightleftharpoons \text{SO}_2(aq) \\
\text{SO}_2(aq) + \text{H}_2\text{O} & \rightleftharpoons \text{H}^+ + \text{HSO}_3^- \\
\text{HSO}_3^- + \text{HSO}_3^- & \rightleftharpoons \text{S}_2\text{O}_5^{\text{=}^-} + \text{H}_2\text{O} \\
\text{HSO}_3^- & \rightleftharpoons \text{SO}_3^{=} + \text{H}^+
\end{align*}
\]

The percentage of each species is dependent upon the solution pH and can be calculated by means of the Henderson-Hasselbach equation

\[
\text{pH} = \text{pK}' + \log \frac{[\text{base}]}{[\text{acid}]}
\]

and using pK'₁ = 1.81 and pK'₂ = 6.91 for sulfurous acid (Macris and Markakis, 1974).
In the range of wine pH, molecular sulfur dioxide, bisulfite, and sulfite are the major species. However, several carbonyl compounds, such as acetaldehyde, exist in wines. They combine with bisulfite to form other compounds known as hydroxysulfonates:

\[
\text{H} \quad \text{R - C = O + KHSO}_3 \quad \text{\rightarrow \quad R - C - SO}_3\text{K} \quad \text{OH}
\]

Aldehyde  "Free"  Hydroxysulfonate
bisulfite  ("bound" bisulfite)

The bisulfite ion combines with carbonyl compounds and is called "bound" sulfur dioxide. The unbound bisulfite and the other three species which do not combine with carbonyl compounds are called "free" sulfur dioxide. Since free sulfur dioxide has the greatest antioxidant and antimicrobial activity, it is the most important factor in controlling malolactic fermentation.

The toxicity of sulfur dioxide towards yeasts and lactic acid bacteria has been related to the concentration of molecular sulfur dioxide (Macris and Markakis, 1974; Carr et al. 1976). By calculating the molecular sulfur dioxide concentration, it is possible to compare the lethal effect of various sulfur dioxide species at different pHs. The formula for calculating molecular sulfur dioxide concentration is as follows:

\[
\text{Molecular SO}_2 \text{ (mg/L)} = \text{Free SO}_2 \text{ (mg/mL)} \times (\% \text{ of Molecular SO}_2 \text{ at a certain pH})
\]
The toxicity of sulfur dioxide (molecular \( \text{SO}_2 \)) is increased by decreasing the pH of a solution. Although the use of molecular sulfur dioxide could provide a basis for comparing the lethal effect of sulfur dioxide at different pHs, it has not been widely accepted. Instead, free and bound sulfur dioxide are commonly used by enologists.

Both free and bound sulfur dioxide have been found to inhibit malolactic bacteria. Rice (1975) reported that the growth of lactic bacteria is inhibited as the levels of free sulfur dioxide approach or exceed 50 ppm. Fornachon (1963) and Lafon-Lafourcade (1975) reported that bound sulfur dioxide is inhibitory to malolactic bacterial growth. Also, Lafon-Lafourcade (1975) showed that the ability of decarboxylating malic acid by resting cells of \( \text{L. oenos} \) ML-34 was inhibited by free and bound sulfur dioxide. The rate of decarboxylation decreased with an increase in free or bound sulfur dioxide content. No decarboxylation was observed in a medium (pH 4.0) containing 100 mg/L of free or bound sulfur dioxide.

**Ethanol.** Peynaud (1967) reported that the effect of ethanol on bacterial growth depends on the strain, nitrogen status, and pH of the culture medium. Lafon-Lafourcade (1975) studied the decarboxylation of malic acid with resting cells of \( \text{L. oenos} \) ML-34 in a synthetic medium. She reported that the rate of decarboxylation was identical in the media containing 0 to 11% ethanol; however, the rate
decreased to only 56% and 16% at 12 and 13% ethanol content, respectively.

DeMenezes et al. (1972) studied the interactions of pH and ethanol on malolactic fermentation. They concluded that when ethanol levels exceed 6% and pH levels are less than 4.0, it is extremely difficult to induce an effective malolactic fermentation.

For winemakers using pure cultures of malolactic bacteria, Kunkee (1974) recommended bacterial inoculation should be carried out during alcoholic fermentation, before the alcohol content reaches too high. However, Gallander (1979) found that bacterial inoculation after alcoholic fermentation was equally favorable in stimulating malolactic fermentation.

Temperature. The optimum temperature for the growth of malolactic bacteria is between 25 to 30°C, and the rate of decarboxylation is highest from 30 to 36°C (DeMenezes et al., 1972; Lafon-Lafourcade, 1975). These temperatures are higher than those recommended for making red and white wines. Low temperature, low pH, and high sulfur dioxide are the main reasons for the low incidence of malolactic fermentation in white wines. Rice (1975) reported that in the range of 60 to 72°F (15.6 to 22.2°C), malolactic fermentation is directly related to the temperature.
Wine Deacidification by Malolactic Fermentation.

Acidity is an important quality factor of wines. It affects the taste, color, clarity, and stability of wines. However, the most apparent aspect of the acidity is its effect on taste. Proper acidity is essential in producing a pleasant, well-balanced wine. A high acid wine will taste too tart while a low acid wine may taste flat or insipid (Beelman, 1973).

In cool viticultural regions such as eastern United States, excessive amounts of acids are normally present in the grapes, and thus cause high acidity in wines. In these regions, it is necessary to employ some deacidification method to reduce wine acidity to a more desirable level. Basically, there are two major types of wine deacidification: physiochemical and biological methods (Beelman and Gallander, 1979). Physiochemical methods include: amelioration with syrup or sugar, neutralization and precipitation with carbonates, and ion exchange. While biological methods include: malolactic fermentation, fission yeast fermentation, and carbonic maceration. Each of the above methods has been reviewed by Beelman and Gallander (1979) for their advantages and disadvantages. Among these methods, malolactic fermentation is considered highly desirable and is often encouraged in cool regions where acidic grapes are grown. This is because malolactic fermentation has three important effects on wine quality. The first immediate effect of malolactic fermentation is deacidification. Since lactic
acid is a chemically weaker acid and less tart than malic acid, malolactic fermentation reduces wine acidity by decreasing titratable acidity and increasing pH. The second effect of malolactic fermentation is bacteriological stability. A complete malolactic fermentation will deplete L-malic acid in a wine. Thus, malolactic bacteria are not able to grow and cause spoilage. Other deacidification methods can not completely remove L-malic acid. A wine treated with these methods is not bacteriological stable, and there is a high risk of having a spontaneous malolactic fermentation after bottling the wine. If malolactic fermentation occurs in the bottle, the wine is considered spoiled because of the turbidity and effervescence produced by the fermentation. Malolactic fermentation is sometimes encouraged even in low acid wines to obtain bacteriological stability (Amerine et al., 1980). The loss of acidity brought about by the fermentation can be adjusted by addition of certain organic acids, within legal limits. Citric acid and D-malic acid are often used, but tartaric acid is preferred for its biological stability. Rankine (1977) stated that the most convenient procedure is to allow the wine to undergo malolactic fermentation and then correct for the resultant deficiency in acidity. The third effect is flavor complexity. Malolactic bacteria produce some flavor compounds such as diacetyl and acetoin. At their threshold levels, these two compounds may add subtle complexity to wine flavor (Kunkee, 1974). There is a controversy as to
whether or not malolactic fermentation actually improves the sensory quality of wine, other than the acidity change. Nevertheless, natural (spontaneous) malolactic fermentation occurs in all the important viticultural areas of the world. It occurs in premium quality wines as well as in wines of lower quality. Malolactic fermentation is considered undesirable in the delicate and light white wines of Germany, because a high level of lactic acid is detrimental to the fruity character of their wines. For this reason, other deacidification methods are used to reduce acidity in many German wines. However, Bordeaux and Burgundy winemakers believe that malolactic fermentation is desirable in making high quality red wines.

Since Ohio wines are generally made from grapes high in malic acid, malolactic fermentation is desirable from the standpoint of reducing acidity and providing bacteriological stability. Although malolactic fermentation may benefit Ohio wines, the incidence of malolactic fermentation in Ohio wines was only 10% in 1973 according to a survey of 30 wines from 15 wineries (Graumlich and Gallander, 1973).

**Methods for Inducing Malolactic Fermentation.**

There are no practical methods for absolute control of malolactic fermentation, but there are several vinification practices which can be employed to increase the induction of this fermentation (Amerine et al., 1980). To stimulate
malolactic fermentation, the following practices have been recommended:

1. Using low levels of SO₂ (less than 50 ppm).
2. Adding SO₂ after alcoholic fermentation.
3. Racking the wine from yeast lees should be delayed.
4. Storing wine at 18 - 21°C or higher.
5. Adjusting the wine pH to 3.3 or above.

However, these above practices will also promote yeast spoilage, especially Brettanomyces or Dekkera yeasts. To prevent yeast spoilage winemakers inoculate with malolactic bacteria and immediately following the fermentation treat with SO₂ (30 ppm, free form).

Inoculation with malolactic bacteria is a new trend in winemaking (Kunkee, 1974). Kunkee (1974) reported the potential usefulness of bacterial inoculation. He recommended that it should be performed by trained personnel using microbiological facilities. The use of bacterial inoculation appears to be the best method for inducing malolactic fermentation. This method has been used commercially in France, Germany, Switzerland, and Portugal (Kunkee, 1967b). Beelman (1976, 1979) found that bacteria-inoculated wines complete malolactic fermentation faster than without bacterial inoculation. Wines which have previously resisted a spontaneous malolactic fermentation can be induced by bacterial inoculation (Beelman, 1976). Similar results were also reported by Gallander (1979).
Inoculation of malolactic bacteria can be accomplished by adding wines which have recently undergone spontaneous malolactic fermentation. Unless the bacteria are isolated, identified, and characterized, it is difficult to predict their behavior and performance. For bacterial inoculation, Kunkee (1974) advocated the use of pure cultures of *Leuconostoc oenos* ML-34. This organism was formerly named *L. citrovorum* ML-34 and is called *L. gracile* Cf 34 by Bordeaux workers. It was isolated from a red wine from a Napa Valley winery by Ingraham and Cooke (1960). This organism was selected, for pure culture inoculation, because it has the capability of growing at a high alcohol and sulfur dioxide concentration, and a low pH, storage temperature, and nutrient supply (Kunkee, 1974). Other strains of malolactic bacteria which have been used for pure culture inoculation include: *L. oenos* PSU-1 (Beelman et al., 1977), *L. oenos* L181 (Rankine, 1977), *L. oenos* B44.40 (Silver and Leighton, 1981), and *Lactobacillus* sp. (Ardin, 1972). They were isolated from different viticultural regions. It has been suggested that the most suitable organism for inducing malolactic fermentation in a certain region would be those isolated from their wines (Beelman et al., 1977). For example, the Pennsylvania strain PSU-1 was able to induce faster or more consistent malolactic fermentation in Pennsylvania wines than the Napa Valley strain ML-34. Gallander (1976) reported similar results with Ohio wines.
Pure culture inoculation of wines with selected strains of bacteria has two important advantages over reliance on natural microflora. It provides a high number of malolactic bacteria which usually does not occur naturally in musts or wines. Furthermore, the selected strains would dominate the fermentation thus preventing possible off-odors and flavors associated with some natural strains (Beelman, 1979).

Although pure culture inoculation is beneficial, it has not been widely used by wineries. Most wineries do not have personnel and facilities to maintain and prepare bacterial cultures. Also, liquid cultures are at maximum efficiency for only a short period of time, and winemakers must know some time in advance when cultures will be required (Rankine, 1977). In order to solve these problems, two freeze-dried cultures of malolactic bacteria have been developed and sold commercially: Equilait in France and LeucoStart in Pennsylvania. Equilait is a pure culture of *Lactobacillus* sp. and LeucoStart is *Leuconostoc oenos* PSU-1. These freeze-dried cultures are very convenient to use, but rather expensive. To reduce the cost of using freeze-dried cultures, studies have been made for expanding these cultures into larger inocula (Beelman, 1979; Krielow, 1981). The results indicated that expanded cultures were effective in stimulating malolactic fermentation (Beelman, 1979; Krielow, 1981).
Immobilized Cell Systems and Their Application to Induction of Malolactic Fermentation

Although pure culture inoculation with malolactic bacteria is rather effective in stimulating malolactic fermentation, there is a general opinion that this method needs improvement. Rankine (1977) reported that inoculation with wine undergoing malolactic fermentation or deposits from wine which has undergone the fermentation has frequently been successful in stimulating malolactic fermentation. However, the most consistent results were found by adding prepared bacterial cultures during or at the end of alcoholic fermentation. He also pointed out that only about 70% of the inoculations were successful in Australia wines, and bacterial inoculation is seldom used. Amerine et al. (1980) reported that it is extremely difficult to induce malolactic fermentation by inoculation with bacteria after dryness. This is similar to the findings of DeMenezes et al. (1972). They reported that malolactic fermentation is extremely difficult to induce in finished wines having ethanol levels exceeding 6.0% and pH levels less than 4.0.

Several explanations have been presented by Amerine et al. (1980) for the difficulty in obtaining a malolactic fermentation under favorable conditions. These include: lack of nutrients for these fastidious bacteria, activity of bound sulfur dioxide as well as free sulfur dioxide, and bacteriophage infection of the bacteria. The inhibitory effect of low pH and nutrients, and high sulfur dioxide and ethanol
contents on malolactic bacteria and its fermentation is important in determining the effectiveness of pure culture inoculation. Although these factors may be controlled to a certain extent to induce malolactic fermentation, practices become laborious and ineffective since chemical constituents vary from wine to wine. Therefore, it appears that a new system which is less influenced by the pH, sulfur dioxide, ethanol, and nutrients would be highly advantageous for inducing malolactic fermentation.

Immobilized enzyme system has been used successfully by the food industry for many years. Immobilized cell or immobilized enzyme reactors for obtaining the malolactic conversion has been suggested by Whiting (1975) and Beelman and Gallander (1979). Since the malolactic enzyme has not been completely purified and characterized, the immobilized malolactic enzyme system can not be developed. The extraction and purification of malolactic enzyme from the bacterial cells and the cofactor (NAD) requirement for malolactic reactions make an immobilized enzyme system rather expensive to use.

Immobilized cell systems have been developed and used by many industries such as pharmaceutical and brewing industries (Chibata et al., 1974; Abbott, 1977; Vieth and Venkatasubramania, 1979; Kolot, 1980). The techniques of cell immobilization have been reviewed by Kolot (1980). There are three basic techniques: (1) adsorption, (2) entrapment, and (3) coupling. Processes using immobilized
microbial cells have several potential advantages as compared to conventional fermentations (Abbott 1977; Vieth and Venkatsubramania, 1979; Kolot 1980). These include:

1. Capability of recycling microorganisms.
2. Ability to conduct continuous operations.
3. Decrease or elimination of lag or growth phases of the microorganisms.
4. Maintain high cell population to achieve a fast reaction rate.
5. Cofactors are regenerated by the cells.

The potential usefulness of immobilized cell systems for the wine industry has been limited. Only a few studies have been conducted concerning the possible application of an immobilized cell system to wine technology. Immobilized yeast cells have been recommended for making sparkling wine (Divies, et al., 1979; Divies, 1981). For decomposition of L-malic acid in red table wines, Totsuka and Hara (1981) developed an immobilized cell system using *Schizosaccharomyces pombe* and *Leuconostoc mesenteroides*. Microbial cells were entrapped in agar or calcium alginate gels having $10^5$ to $10^6$ cells per $\text{cm}^3$ gel. The entrapped cells were then incubated in malt extract or grape juice for *S. pombe* and mandarin orange-apple juice for *L. mesenteroides*. After incubation, the cell number increased to $10^8$ to $10^9$ per $\text{cm}^3$. The gels containing the immobilized cells were then incubated in red wines. They found that the activity of the immobilized cells was more stable under
conditions which inhibit malolactic fermentation. In 1982, Spettoli et al. (1982) immobilized *Leuconostoc oenos* ML-34 cells in an alginate gel. They demonstrated that immobilized cells were able to decompose malic acid in red table wines. The malate-decomposing activity of the immobilized cells was greatest at pH 4.0. The activity declined 43% at pH 3.0. They also observed a gradually decreasing activity in immobilized cells after several experiments. The limitation of gel porosity by condensed tannins and tannin-protein complexes of molecular weight above 3000 was suggested.
CHAPTER I. EFFECT OF pH AND SULFUR DIOXIDE ON THE RATE OF MALOLACTIC FERMENTATION IN RED TABLE WINES

Malolactic fermentation is considered desirable in acidic wines produced in relatively cool regions such as eastern United States. Many factors are known to inhibit malolactic fermentation. Two most important factors are low pH and sulfur dioxide. Generally, special efforts must be made to obtain malolactic fermentation in a wine with a pH below 3.3 (Kunkee, 1974). Castino et al. (1975) found that malolactic fermentation was impossible below pH 3.2. A similar finding was also reported by Fornachon (1957). However, Rice and Mattick (1970) reported that malolactic fermentation occurred regularly in New York wines with pH values of 3.0. Despite the difference in the lowest pH limit for malolactic fermentation, there is an agreement that low pH is inhibitory to the development of malolactic bacteria, and the rate of malolactic fermentation is closely related to the initial pH of the wine (Bousbouras and Kunkee, 1971).

Malolactic fermentation is often difficult to initiate in eastern United States wines. The main reason is the low pHs of the wines produced in these regions. There are also indications that high sulfur contents may add difficulties
to the initiation of malolactic fermentation. For making red wines, the addition of 75 to 150 ppm (mg/L) of sulfur dioxide have been recommended at the time of grape crushing (Amerine et al., 1980). These sulfur dioxide levels appear to be too high, especially for the low-pH grapes of eastern United States if malolactic fermentation is desired. When a low-pH must is treated with sulfur dioxide, the inhibitory effect on malolactic bacteria is related to the influence of low pH on the metabolic rate of the bacteria and the antimicrobial activity of sulfur dioxide (Kunkee, 1967a). In other words, the combined antimicrobial action of low pH and high sulfur dioxide is detrimental to the development of malolactic bacteria. For this reason, Beelman (1976) has recommended that the initial addition of sulfur dioxide to red musts with low pHs should be reduced or eliminated in order to encourage malolactic fermentation.

Although the effect of pH and sulfur dioxide on malolactic fermentation seems to be apparent from the literature, it has not been demonstrated in red musts with different pH values. This information is needed in estimating the maximum level of sulfur dioxide which could be added to musts at a certain pH without inhibiting malolactic fermentation. With this in mind, this study was initiated to determine the effect of pH and sulfur dioxide levels on the growth behavior of malolactic bacteria and rate of malolactic fermentation.
MATERIALS AND METHODS

Must Preparation: In 1980, grapes from a red French hybrid (Chancellor) were harvested from a commercial vineyard in southern Ohio. The grapes were destemmed, crushed, and divided to nine lots (22.7 kg each). The musts were analyzed for pH, soluble solids (°Brix), and total acidity, and ameliorated with sucrose to 20°Brix.

pH Adjustment: The pH of the nine must lots was adjusted with 12 N NaOH to 3.3, 3.5, or 3.7 (three lots for each pH value). Two hours after the initial addition of NaOH, the pH values of the musts were checked and adjusted until all lots were at the specified pH.

Vinification: After pH adjustment, the three must lots within each pH group were treated with sulfur dioxide at 25, 50, and 75 ppm, respectively, by addition of potassium metabisulfite. Approximately twelve hours after the bisulfite treatment, each lot was inoculated with rehydrated active dry yeast, Saccharomyces cerevisiae Montrachet #522 (Universal Foods Corp., Milwaukee, WI) and fermented-on-the-skins at 18°C. The fermenting musts were stirred twice daily and were pressed at 5°Brix. The juice from each lot was then used to prepare four 3-L sublots in 1-gallon glass carboys equipped with water seals. Two of the 4 sublots were inoculated with malolactic bacteria, Leuconostoc oenos PSU-1, to a final concentration of 2 x 10^7 colony forming unit/mL (cfu/mL). The inoculum was a rehydrated
freeze-dried culture of *L. oenos* PSU-1 (Tri-Bio, Inc., State College, PA). The remaining two sublots served as non-inoculated controls. When the wines reached dryness, they were racked without addition of sulfur dioxide. Bacterial populations were determined by plating aliquots of wine on a modified Rogosa Medium at pH 5.5 with 0.01% Actidione (Pilone et al., 1966). The plates were incubated in a desiccator with a lighted candle at 25°C for 7 days. Since leuconostoc bacteria often grow in pairs, chains, or clumps of cells and these units appear only as one single colony on the solid medium, it was difficult to obtain the actual cell number of viable bacteria. For convenience, the number of bacteria was counted as colony forming units (abbreviated as cfu) rather than cells. The degree of malolactic fermentation was determined by paper chromatography (Kunkee, 1968). Results were expressed as number of weeks for the completion of malolactic fermentation (absence of malic acid), beginning with the first day of alcoholic fermentation.
RESULTS AND DISCUSSION

As expected, initial pH and sulfur dioxide levels affected the rate of malolactic fermentation in both non-inoculated (yeast only) and inoculated (yeast and bacteria) wines (Table 1.). The highest and lowest rates of malolactic fermentation in the inoculated wines were 3.0 and 9.5 weeks, respectively. In the non-inoculated wines, the highest rate was 7.5 weeks while no malolactic fermentation was observed for the slowest. The highest rate occurred at the highest initial pH (3.7) and lowest sulfur dioxide (25 ppm). The lowest rate occurred at the lowest initial pH (3.3) and highest sulfur dioxide (75 ppm). The non-inoculated wines showed inconsistent results between duplicates. This is possibly due to an uneven distribution of natural malolactic bacteria in the musts. Nevertheless, malolactic fermentation rate was higher in the inoculated wines than in the non-inoculated wines. Beelman (1976) and Gallander (1979) both have shown that wines inoculated with L. oenos PSU-1 complete malolactic fermentation faster than wines without inoculation. However, it has been reported that a more rapid malolactic fermentation can be observed in non-inoculated wines than those inoculated (Kunkee, 1974). The exact reason is not known.

Since the non-inoculated wines showed inconsistent results between duplicates, no further comparisons were made. In the inoculated wines, it appears that at the same
Table 1. Time (weeks) to complete malolactic fermentation in Chancellor wines at different must pH and sulfur dioxide levels.

<table>
<thead>
<tr>
<th>Must pH</th>
<th>Sulfur Dioxide (ppm)</th>
<th>Yeast + Yeast Bacteria</th>
<th>Yeast + Yeast Bacteria</th>
<th>Yeast + Yeast Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
<td>50</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>9.5</td>
<td>5.0</td>
<td>13.5</td>
<td>8.5</td>
</tr>
<tr>
<td>3.5</td>
<td>9.8</td>
<td>3.8</td>
<td>12.0</td>
<td>4.5</td>
</tr>
<tr>
<td>3.7</td>
<td>7.5</td>
<td>3.0</td>
<td>9.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

a
Malolactic fermentation occurred in one of the duplicates.

b
Malolactic fermentation did not occur.
sulfur dioxide level the malolactic fermentation rate was nearly identical at pH 3.5 and 3.7, and much slower at pH 3.3 (Table 1). For example, at 50 ppm of sulfur dioxide, the rate of malolactic fermentation was 8.5, 4.5, and 4.5 weeks in wines made from the musts with pHs 3.3, 3.5, and 3.7, respectively. The results support the findings of Kunkee (1967a). He indicated that malolactic fermentation was inhibited at pH less than 3.5. He also reported that there was a correlation between malolactic fermentation rate and the natural pH of the musts ranging from 3.35 to 3.54, but little correlation was found at must pHs higher than 3.54. This response was different from that observed by Bousbouras and Kunkee (1971) in a study to determine the effect of pH on the rate of malolactic fermentation. They found that the rate of malolactic fermentation was directly related to the initial pHs of wines ranging from 3.15 to 3.83. It is not known why the effect of wine pH on malolactic fermentation rate is different from that of natural must pH. Recently, van der Westhuizen and Loos (1981) reported that there was no significant difference in the rate of malolactic fermentation between the pH values 3.5 and 3.8. They suggested that the two pH levels might fall within the range where malolactic bacteria could bring about the malolactic fermentation relatively easily. In this study, the analyses of wines pH at the time of bacterial inoculation showed that the pH 3.5 musts had increased their pH values to 3.6 while pH 3.7 musts remained relatively the
same (Table 2). It appears that similar pH values for the pH 3.5 and 3.7 musts may explain the closeness of the malolactic fermentation rates (Table 1).

At a given pH, the rate of malolactic fermentation was highest at the lowest sulfur dioxide level (Table 1). This is in agreement with the findings of other researchers (Fornachon, 1963; Kunkee, 1967a; Van Wyk, 1976; van der Westhuizen and Loos, 1981). It was also noted that at pH 3.3 there was a large difference (2.5 weeks) in malolactic fermentation rate between 25 and 50 ppm of sulfur dioxide. It appears that 50 ppm of sulfur dioxide is a critical level at pH 3.3. Rankine (1977) has recommended that initial sulfur dioxide levels should be kept below 50 ppm in order to encourage malolactic fermentation.

If the results of Table 1 are viewed in conjunction with the growth of the inoculated bacteria (Figures 1, 2, and 3), there seems to be a close relationship between survival rate of bacteria and malolactic fermentation rate. Near the end of the first week of alcoholic fermentation, malolactic bacteria were inoculated to wines to obtain an initial population of about $2 \times 10^7$ cfu/mL. Following the inoculation, bacterial population decreased for two weeks and reached the lowest level by the end of the third week of alcoholic fermentation. The largest decrease in bacterial population was observed at pH 3.3 and 75 ppm of sulfur dioxide (more than four log cycles). The extent of the population decrease was approximately the same at pH 3.5 and
Table 2. pH values of fermenting musts at the time of bacterial inoculation.

<table>
<thead>
<tr>
<th>Initial pH of Must</th>
<th>Sulfur Dioxide (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>3.3</td>
<td>3.46</td>
</tr>
<tr>
<td>3.5</td>
<td>3.60</td>
</tr>
<tr>
<td>3.7</td>
<td>3.72</td>
</tr>
</tbody>
</table>
Figure 1. Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.3 must. Arrows indicate completion of malolactic fermentation (MLF).
Figure 2. Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.5 must. Arrows indicate completion of malolactic fermentation (MLF).
Figure 3. Effect of sulfur dioxide level on the growth of malolactic bacteria during the vinification of Chancellor wines made from pH 3.7 must. Arrows indicate completion of malolactic fermentation (MLF).
3.7. The amount of population decrease was apparently related to the sulfur dioxide level. Beelman et al. (1980) have reported that following inoculation into a fermenting wine, the population of L. oenos PSU-1 decreased for nearly two weeks. In their study, a hot pressed must with an initial pH of 3.57 and 50 ppm of sulfur dioxide was used. The extent of decrease in viable bacteria population was smaller (less than one log cycle) when compared to the results of this study (more than one log cycle). To encourage malolactic fermentation, Beelman (1976) has recommended a maximum of 20 ppm of sulfur dioxide for red musts at pH 3.3. At this pH and sulfur dioxide level the lethal effect would be minimal based on the finding of this study.

Generally, malolactic fermentation was completed 7 to 10 days after the bacterial population had reached a concentration of $1 \times 10^6$ cfu/mL. This was similar to the findings of Rice and Mattick (1970). No apparent growth of malolactic bacteria was observed at pH 3.5 and 25 ppm of sulfur dioxide and at pH 3.7 and 25 ppm of sulfur dioxide. Since the populations were higher than $1 \times 10^6$ cfu/mL, it is reasonable that malolactic fermentation should have occurred.

In summary, the pH and sulfur dioxide levels were found to affect the growth behavior of malolactic bacteria and the rate of malolactic fermentation. Low pH and high sulfur dioxide levels greatly reduced the survival rate of the inoculated bacteria. The malolactic fermentation rate
appeared to be directly related to the survival rate of the bacteria. Therefore, the initial treatment of sulfur dioxide to the must is an important factor to consider when using a pure culture inoculation to induce malolactic fermentation. For the low-pH musts of eastern United States, the initial addition of sulfur dioxide should be less than 50 ppm if malolactic fermentation is to be encouraged.


CHAPTER II. EFFECT OF INSOLUBLE SOLIDS ON THE
SULFUR DIOXIDE CONTENT AND RATE OF MALOLACTIC
FERMENTATION IN WHITE TABLE WINES

Grape insoluble solids are the particulate material
which suspends in the juice or settles to the bottom of
juice upon standing. These substances are the residual from
grape pulp and skin. Their presence in the juice not only
contributes to turbidity, but also affects the yeast ferme­
tation rate (Groat and Ough, 1978; Houtman, et al., 1980)
and quality of wines (Singleton, et al., 1975). During
vinification, the insoluble solids are often removed prior
to yeast fermentation by settling and racking, filtration,
or centrifugation. Wines made from clarified juices are
usually higher in quality than those made from unclarified
juices (Singleton, et al., 1975).

Recently, a variation in the sulfur dioxide content in
a white grape juice sample was noted. This variation ap­
peared to be attributed to the presence of insoluble solids.
If insoluble solids indeed affect sulfur dioxide content, it
is possible that they would also influence malolactic fer­
mentation. This study was initiated to study the effect of
grape insoluble solids on the sulfur dioxide content and the
rate of malolactic fermentation in white table wines.
MATERIALS AND METHODS

Must Preparation: In 1979, grapes from Vidal blanc were harvested from a commercial vineyard in northern Ohio. Again in 1980, Vidal blanc, plus another grape variety, Catawba, were harvested at the same location. After the grapes were destemmed, crushed, and pressed, the juices were analyzed for pH, total acidity, and soluble solids (°Brix). The juices were ameliorated with sucrose to 20°Brix prior to the adjustment of insoluble solids.

Adjustment of Insoluble Solids Content: Insoluble solids content was determined by pipetting a 10-ml juice sample into a calibrated centrifuge tube and centrifuging on a IEC-140 rotor at 2000 rpm for 10 minutes. The amount of insoluble solids was recorded as percent by volume.

Three different ranges of insoluble solids were obtained; 0.0, 1.0, and 2.0% for Vidal blanc, 1979; 0.5, 2.5, and 5.0% for Vidal blanc, 1980; and 1.0, 7.5, and 15.0% for Catawba, 1980. In 1979, the insoluble solids for Vidal blanc were obtained by cooling the juice to 0°C overnight and filtering through Seitz filter pads K5 (Republic Seitz Filter Corp., Milldale, CT). The insoluble solids were collected, resuspended, and added back to the clarified juice to the desired insoluble solids content. In 1980, the juices were settled overnight at 0°C and then racked to separate the clear juice from the sediment. The insoluble
solids were added back to the clear juice to obtain the desired range of insoluble solids.

**pH Adjustment:** Following the adjustment of insoluble solids content, the pH values of 1979 Vidal blanc and 1980 Catawba juices were adjusted with 12 N sodium hydroxide to 3.7 and 3.5, respectively. No pH adjustment was made for the 1980 Vidal blanc juice.

**Addition and Analyses of Sulfur Dioxide:** A total of 30 ppm sulfur dioxide was added to each juice in the form of potassium metabisulfite. Approximately twelve hours later, the juices were analyzed for free and bound sulfur dioxide by the aeration-oxidation method as described by Bueschenstein and Ough (1978).

**Bacterial Inoculation:** Four 8-liter juice lots were prepared at each level of insoluble solids. They were inoculated with rehydrated active dry yeast, Montrachet yeast strain #522 (Universal Foods Corp., Milwaukee, WI) and were fermented at 18°C. When the wines reached dryness, two lots were inoculated with *Leuconostoc oenos* PSU-1 and the other two served as controls. A freeze-dried culture of *L. oenos* PSU-1 was obtained from Tri-Bio Lab (State College, PA) and was rehydrated with freshly double distilled water. The wines were inoculated with the rehydrated bacterial culture to obtain a final bacterial population of approximately $2.5 \times 10^7$ cfu per ml.

Bacterial counts were determined by plating the samples on a modified Rogosa medium (pH 5.5) with 0.01% Actidione.
(Pilone, et al., 1966). Malolactic fermentation was monitored by paper chromatography (Kunkee 1968). The results were expressed as number of weeks for completion of malolactic fermentation, beginning with the first day of alcoholic fermentation.
RESULTS AND DISCUSSION

Effect on the Sulfur Dioxide Content of Juices:
Approximately twelve hours after the addition of 30 ppm of sulfur dioxide, just prior to yeast inoculation, the grape juices were analyzed for free and bound sulfur dioxide. The results indicated that the sulfur dioxide content (free, bound, and total) in the Vidal blanc wines decreased with increasing amounts of insoluble solids (Table 3). No such trend was observed for Catawba juices. This lack of response was probably due to the high level of insoluble solids in the juices. The observed total sulfur dioxide contents were mathematically adjusted according to the volume of insoluble solids added to each sample. These adjusted total sulfur dioxide contents indicated that the decrease in sulfur dioxide content was not only caused by the addition of insoluble solids to the samples, but also by some unknown factor present in the insoluble solids. A decrease of total sulfur dioxide content has been reported by Singleton et al. (1980) in white wines prepared from musts with prolonged pomace contact. Since insoluble solids are derived from pomace, it was reasonable to find a decrease of sulfur dioxide in the presence of insoluble solids. The mechanism may involve oxidation of sulfur dioxide to sulfate. White and Ough (1973) pointed out that oxygen uptake by white juice was affected by clarifying the juice by centrifugation and addition of bentonite with centrifugation. Further
Table 3. Free, bound, and total sulfur dioxide contents of Vidal blanc and Catawba juices 12 hours after the addition of 30 ppm of sulfur dioxide.

<table>
<thead>
<tr>
<th>Juice</th>
<th>Insoluble Solids (%)</th>
<th>pH</th>
<th>Free SO$_2$ (ppm)</th>
<th>Bound SO$_2$ (ppm)</th>
<th>Total SO$_2$ (ppm)</th>
<th>Adjusted Total SO$_2$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vidal(1979)</td>
<td></td>
<td>3.7</td>
<td>$5.2 \pm 0.3$</td>
<td>$16.9 \pm 0.6$</td>
<td>$22.4 \pm 0.8$</td>
<td>$22.4 \pm 0.8$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>$0.0 \pm 0.0$</td>
<td>$16.9 \pm 0.1$</td>
<td>$16.0 \pm 0.1$</td>
<td>$16.2 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>$0.0 \pm 0.0$</td>
<td>$11.9 \pm 0.1$</td>
<td>$11.9 \pm 0.1$</td>
<td>$12.1 \pm 0.1$</td>
</tr>
<tr>
<td>Vidal(1980)</td>
<td></td>
<td>3.2</td>
<td>$4.8 \pm 1.1$</td>
<td>$16.0 \pm 0.0$</td>
<td>$20.8 \pm 1.1$</td>
<td>$20.8 \pm 1.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>$2.4 \pm 0.0$</td>
<td>$13.0 \pm 1.4$</td>
<td>$15.4 \pm 1.4$</td>
<td>$15.8 \pm 1.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>$1.6 \pm 0.0$</td>
<td>$11.4 \pm 0.3$</td>
<td>$13.0 \pm 0.3$</td>
<td>$13.7 \pm 0.3$</td>
</tr>
<tr>
<td>Catawba</td>
<td></td>
<td>3.5</td>
<td>$0.4 \pm 0.0$</td>
<td>$12.8 \pm 0.1$</td>
<td>$13.2 \pm 0.1$</td>
<td>$13.3 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>$0.4 \pm 0.0$</td>
<td>$11.8 \pm 0.3$</td>
<td>$12.2 \pm 0.3$</td>
<td>$13.2 \pm 0.3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.0</td>
<td>$0.4 \pm 0.0$</td>
<td>$11.2 \pm 0.1$</td>
<td>$11.6 \pm 0.1$</td>
<td>$13.6 \pm 0.1$</td>
</tr>
</tbody>
</table>

a Adjusted total SO$_2$ (ppm) = $\frac{\text{total SO}_2 \text{ (ppm)}}{100\% - \text{insoluble solids(\%)}}$

b Means of duplicates + standard error.
study on oxidase activity of insoluble solids may be helpful in understanding this mechanism.

**Effect on the Sulfur Dioxide Content of Wine:** The effect of insoluble solids on sulfur dioxide was also found in the wines. Weekly analyses beginning at dryness showed that sulfur dioxide increased in the wines with musts containing less than 2.0% insoluble solids. No net increase in sulfur dioxide was found in the wines with more than 2.0% insoluble solids (Figures 4, 5, and 6). Therefore, the highest sulfur dioxide content was found at the lowest (0.0%) insoluble solids level. It appeared that during alcoholic fermentation, Montrachet yeast produced a fixed amount of sulfur dioxide which caused an increase in sulfur dioxide content. And, the increase in sulfur dioxide content caused by the yeast was balanced by the decrease in sulfur dioxide content caused by high insoluble solids content. It has been reported that yeasts produce variable amounts of sulfur dioxide, and the largest amount is formed in a filtered pasteurized must (Zang and Franzen, 1967). This suggests that particle removal by filtration and inactivation of oxidative enzyme(s) by pasteurization may affect the sulfur dioxide content of juice and wine.

**Effect on Malolactic Fermentation Rate:** The rate of malolactic fermentation was influenced by the level of insoluble solids (Table 4). Wines prepared from musts having the highest insoluble solids experienced the fastest rate of malolactic fermentation. This is true with both control and
Changes in total sulfur dioxide content during the vinification of Vidal blanc (1979) wine with different levels of insoluble solids. The arrows indicate the inoculation of Leuconostoc oenos strain PSU-1 and completion of malolactic fermentation (MLF), respectively.
Figure 5. Changes in total sulfur dioxide content during the vinification of Vidal blanc (1980) wine with different levels of insoluble solids.
Figure 6. Changes in total sulfur dioxide content during the vinification of Catawba (1980) wine with different levels of insoluble solids. The arrow indicates the inoculation of \textit{Leuconostoc oenos} PSU-1.
Table 4. Rate of malolactic fermentation in Vidal blanc and Catawba wines with different levels of insoluble solids.

<table>
<thead>
<tr>
<th>Wine</th>
<th>Insoluble Solids (%)</th>
<th>Time (weeks) to complete MLF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Vidal blanc (1979)</td>
<td>0.0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Vidal blanc (1980)</td>
<td>0.5</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>--</td>
</tr>
<tr>
<td>Catawba (1980)</td>
<td>1.0</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>7.5</td>
</tr>
</tbody>
</table>

a Malolactic fermentation did not occur in 15 weeks.
b Means of duplicates.
inoculated wines. Results of viable bacterial counts showed that the population of inoculated wines decreased more drastically at a lower insoluble solids level than at a higher insoluble solids level (Figure 7). It appeared that the high sulfur dioxide content (essentially bound form) was responsible for the high death rate of bacteria. Fornachon (1963) and Lafon-Lafourcade (1975) have demonstrated that bound sulfur dioxide was inhibitory to Leuconostoc. However, there is a possibility that certain growth factors may have been extracted from insoluble solids to stimulate yeast and bacterial growth. Growth factor(s) could be more readily available at higher levels of insoluble solids. Another possibility is that multiplication and autolyses of yeast cells may be faster at a higher insoluble solids level, and thus provide necessary nutrients for bacterial growth (Beelman, R. personal communication, 1981). Based on the time required to reach dryness in wines, the rate of yeast fermentation was highest in the presence of high insoluble solids (Table 5).

In summary, the rate of malolactic fermentation was influenced by the level of insoluble solids. Wines with the highest insoluble solids experienced the fastest rate of malolactic fermentation. In addition, the insoluble solids appeared to have an effect upon the sulfur dioxide content. Wines with the highest insoluble solids tended to be lowest in sulfur dioxide. The exact reason for the decreases in sulfur dioxide remains to be investigated.
Figure 7. Growth curves of Leuconostoc oenos strain PSU-1 in Catawba wines with different levels of insoluble solids. Arrows indicate completion of malolactic fermentation (MLF).
Table 5. Rate of alcoholic fermentation in Vidal blanc and Catawba wines with different levels of insoluble solids.

<table>
<thead>
<tr>
<th>Wine</th>
<th>Insoluble Solids (%)</th>
<th>Days to Dryness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vidal blanc</td>
<td>0.0</td>
<td>13</td>
</tr>
<tr>
<td>(1979)</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>8</td>
</tr>
<tr>
<td>Vidal blanc</td>
<td>0.5</td>
<td>7</td>
</tr>
<tr>
<td>(1980)</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6</td>
</tr>
<tr>
<td>Catawba</td>
<td>1.0</td>
<td>9</td>
</tr>
<tr>
<td>(1980)</td>
<td>7.5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>6</td>
</tr>
</tbody>
</table>

\( ^a \) Number of days required to complete alcoholic fermentation (-1.0 Brix).
LITERATURE CITED


CHAPTER III. CHARACTERISTICS OF INSOLUBLE SOLIDS INVOLVED IN THE REDUCTION OF SULFUR DIOXIDE CONTENT OF WHITE GRAPE JUICES AND TABLE WINES

Grape insoluble solids have been shown to influence the rate of alcoholic fermentation (Groat and Ough, 1978; Houtman et al., 1980), production of higher alcohols (Guymon, 1972; Groat and Ough, 1978), and quality of wine (Singleton et al., 1975). Recently, the rate of malolactic fermentation was found to be affected by grape insoluble solids (Liu and Gallander, 1981, 1982a; Beelman and Romberger, 1982).

Although the effect of insoluble solids on wine quality is well known, their characteristics have not been studied much detail. One of the characteristics of insoluble solids is their ability to reduce sulfur dioxide content in grape juices and wines (Liu and Gallander, 1982a). Results indicated that insoluble solids may contain oxidative enzyme(s) (White and Ough, 1973) which oxidize sulfur dioxide to sulfate. Ponting and Johnson (1945) reported that macerated tissues of apples and apricots were able to cause rapid losses of sulfur dioxide. They suggested that polyphenoloxidase in tissues catalyzed the oxidation of sulfur dioxide and thus caused losses of sulfur dioxide.
Whether a similar mechanism is responsible for the loss of sulfur dioxide in grape juices and wines by insoluble solids remains to be studied.

In a previous study (Liu and Gallander, 1982a), only one juice sample from two grape varieties were used for sulfur dioxide analysis. However, the present study was expanded to include several grape varieties, sulfur dioxide levels, and sampling dates. In addition, the effects of particulate materials, inhibitors of polyphenoloxidase and pasteurization were studied to better understand the mechanism involved in the loss of sulfur dioxide by insoluble solids.
MATERIALS AND METHODS

Juice Preparation: In 1981, eight grape varieties were harvested from commercial vineyards in Ohio. These varieties included: Aurora, Catawba, Seyval blanc, Cayuga White, Villard blanc, Vidal blanc, Chardonnay, and White Riesling. After the grapes were destemmed, crushed, and pressed, the juices were analyzed for pH, total acidity and soluble solids (°Brix). All juices except Seyval blanc were not ameliorated. All experiments were conducted with duplicated lots of juice.

Effect of Grape Variety on Insoluble Solids and Sulfur Dioxide Contents: To study the effect of grape variety, three types of juices were prepared for each variety: clarified, non-clarified, and turbid. The clear juice after settling was racked off and was used as the clarified juice. Non-clarified was juice that was not settled or racked. The turbid juice was prepared by adding the sediment after settling to an unsettled and unracked juice. The insoluble solids content of the juices was determined according to the procedure described in the previous study (Liu and Gallander, 1982a).

To each type of the juice, 30 ppm of sulfur dioxide was added in the form of potassium metabisulfite. Approximately 12 hours after the addition, the sulfur dioxide content of
the juices was analyzed as previously described by Buechsenstein and Ough (1978).

**Effect of Sampling Time on Sulfur Dioxide Content:** To determine the effect of sampling time, the sulfur dioxide content of Cayuga White and Catawba juices with various amounts of insoluble solids (0.4 - 11.1%) was analyzed 12 and 16 hours after the initial addition of 30 ppm of sulfur dioxide.

**Effect of Initial Sulfur Dioxide Level:** The effect of insoluble solids on sulfur dioxide was studied in a Vidal blanc juice treated with a sulfur dioxide level other than 30 ppm. Two types of juice were prepared from the Vidal blanc: clear and turbid. The clear juice was obtained by filtration in a filter press equipped with Seitz filter pads (K5 grade) (Republic Seitz Filter Corp., Milldale, CT) to remove particles larger than 2 microns. The turbid juice was prepared by adding the sediment after settling to an unsettled and unracked juice. Both clear and turbid juices were then treated with five different amounts of sulfur dioxide: 0, 30, 60, 120, and 240 ppm. The sulfur dioxide content of each juice was analyzed approximately 12 hours after the addition of sulfur dioxide.

**Addition of Other Particulate Materials:** Particulate materials other than insoluble solids were added to a juice treated with 30 ppm of sulfur dioxide to examine their effect on sulfur dioxide content of the juice. These particulate materials included bentonite, cellulose,
microcrystalline cellulose (Avicel, FMC Corp., Marcus Hook, PA), pectin (citrus), dextran, and algin. Five grams of each particulate material (dry form) were added to a 1-L filtered Aurora juice (0.0% insoluble solids) in one-half gallon glass carboys. In addition, 50 grams of grape skin, pulp, and insoluble solids were also added to the juice for comparison. Berries of Aurora grapes were dissected, and the skin and pulp were separated and collected. The pulp was pressed against a metal screen to remove juice and insoluble solids. Insoluble solids were collected by centrifugation.

Addition of Enzyme Inhibitors: Two inhibitors of polyphenoloxidase, ascorbic acid and ethylene diamine tetraacetic acid (EDTA), were added to a clarified juice (1.0% insoluble solids) and a turbid juice (8.0% insoluble solids) of Vidal blanc. The inhibitors were added to the juices at a concentration of 5 mM immediately after the addition of 30 ppm of sulfur dioxide.

Effect of Pasteurization: To study the effect of pasteurization on the ability of insoluble solids to reduce sulfur dioxide content, a Seyval blanc juice was used. The juice was ameliorated to 20°Brix with sucrose, and was filtered through Seitz filter pads (S grade) with a pore size less than 0.5 μ in diameter to remove yeasts and insoluble solids. Following filtration, the juice was divided into two groups. One group was heated to 80°C, maintained at that temperature for 5 minutes, and cooled to 25°C with cold
tap water bath. The pasteurized juice was then used to prepare four 3-L lots in 1-gallon glass carboys equipped with water seals. Two of the 4 lots were treated with 30 ppm of sulfur dioxide. The remaining two lots were not treated with sulfur dioxide. The other group of juice was not pasteurized, and was used to prepare eight 3-L lots in 1-gallon glass carboys equipped with water seals. Four of the eight lots were treated with 0 or 30 ppm of sulfur dioxide as described for the pasteurized juice. The remaining 4 lots of non-pasteurized juice were assigned to receive 3.0% of insoluble solids, either pasteurized or non-pasteurized, and then treated with 30 ppm of sulfur dioxide.

Approximately 12 hours after the addition of sulfur dioxide, a wine yeast *Saccharomyces cerevisiae* Montrachet strain #522 was inoculated to all the juices of Seyval blanc. The fermentation was carried out at 18°C until the wines reached dryness. The sulfur dioxide content of juices and wines was analyzed 12 hours after SO₂ addition and at dryness.
RESULTS AND DISCUSSION

Effect of Grape Variety: For each grape variety, three types of juice were prepared: clarified, non-clarified, and turbid. The insoluble solids content was determined for each juice type (Table 6). In the clarified juices, the insoluble solids content varied from the 0.1% in White Riesling to the 1.7% in Villard blanc. In contrast, the insoluble solids content of the non-clarified juices ranged from 4.2 to 6.2%. The insoluble solids content of the turbid juices which contained 2.0 to 2.9 times the insoluble solids content of the non-clarified juices, was lowest in Cayuga White with 10.0% and highest in White Riesling with 15.0%. It has been reported that the critical level of insoluble solids in grape juice was between 0.1 and 0.5% (v/v), below which alcoholic fermentation was inhibited and above which it was little affected (Groat and Ough, 1978). Clarification of white musts with pectic enzymes to remove insoluble solids tended to slow yeast fermentation (Ough et al., 1975), increase the chance of a stuck (unfinished) alcoholic fermentation, and tied up fermentation cooperage. Results of this study indicated that the clarified juices of the five grape varieties contained insoluble solids at the critical level as reported by Groat and Ough (1978).

The effect of insoluble solids on the sulfur dioxide content of the juices appeared to vary from one variety to another (Table 6). Approximately 12 hours after the initial
Table 6. The insoluble solids and total sulfur dioxide contents in three types of juice from five different grape varieties.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Type of Juice</th>
<th>Insoluble Solids (%)</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Riesling</td>
<td>C</td>
<td>0.1</td>
<td>30</td>
<td>16.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>6.2</td>
<td>30</td>
<td>16.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>15.0</td>
<td>30</td>
<td>14.1 ± 2.7</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>C</td>
<td>0.2</td>
<td>30</td>
<td>19.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>4.2</td>
<td>30</td>
<td>19.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>12.0</td>
<td>30</td>
<td>17.5 ± 1.6</td>
</tr>
<tr>
<td>Villard blanc</td>
<td>C</td>
<td>1.7</td>
<td>30</td>
<td>22.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>7.0</td>
<td>30</td>
<td>21.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>12.0</td>
<td>30</td>
<td>21.4 ± 0.6</td>
</tr>
<tr>
<td>Cayuga White</td>
<td>C</td>
<td>0.4</td>
<td>30</td>
<td>22.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>5.0</td>
<td>30</td>
<td>17.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>10.0</td>
<td>30</td>
<td>14.5 ± 3.8</td>
</tr>
<tr>
<td>Catawba</td>
<td>C</td>
<td>0.4</td>
<td>30</td>
<td>17.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>5.7</td>
<td>30</td>
<td>14.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>11.1</td>
<td>30</td>
<td>12.6 ± 1.3</td>
</tr>
</tbody>
</table>

\(^a\) Clarified, Non-clarified, Turbid.

\(^b\) Means of duplicates ± standard error.
addition of 30 ppm of sulfur dioxide, the total sulfur dioxide content in each juice was analyzed. For White Riesling, Chardonnay, and Villard blanc, the sulfur dioxide content of each juice was not affected by insoluble solids content. While in Cayuga White and Catawba juices, sulfur dioxide content was lower at higher levels of insoluble solids. Three other varieties used in this study, Aurora, Vidal blanc, and Seyval blanc also showed a decrease in sulfur dioxide content with increasing amounts of insoluble solids (not shown in Table 6). This finding was in agreement with the results of a previous study (Liu and Gallander, 1982a) in which Vidal blanc and Catawba were used. Although there seemed to be a varietal effect on the contents of insoluble solids and sulfur dioxide, other factor(s) may also be involved since the 5 varieties were different in their pH, total acidity and soluble solids content at harvest (Table 7).

**Sampling Time for Sulfur Dioxide Analyses:** As previously described, the sulfur dioxide content of the juices was analyzed approximately 12 hours after the initial addition of 30 ppm of sulfur dioxide. During the course of this study, it was noted that the time of sampling also affected the results of sulfur dioxide analyses. The 16-hour samples appeared to have less sulfur dioxide than the 12-hour samples, although the differences between the two samples were not statistically significant (Table 8).
Table 7. The soluble solids content, pH, and total acidity of five grape varieties at harvest.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Soluble Solids (°Brix)</th>
<th>pH</th>
<th>Total Acidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Riesling</td>
<td>16.5</td>
<td>3.1</td>
<td>1.24</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>19.4</td>
<td>3.2</td>
<td>1.15</td>
</tr>
<tr>
<td>Villard blanc</td>
<td>15.0</td>
<td>2.9</td>
<td>1.26</td>
</tr>
<tr>
<td>Cayuga White</td>
<td>17.2</td>
<td>3.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Catawba</td>
<td>17.4</td>
<td>3.1</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Table 8. Effect of sampling time on the sulfur dioxide contents of Cayuga White and Catawba juices with various amounts of insoluble solids.

<table>
<thead>
<tr>
<th>Insoluble Solids Contents (%)</th>
<th>Total Sulfur Dioxide Content (ppm)</th>
<th>12-hour Sample</th>
<th>16-hour Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cayuga White</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td><em>22.5 ± 0.1</em></td>
<td><em>22.8 ± 0.6</em></td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>17.3 ± 1.8</td>
<td>16.0 ± 3.4</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>14.5 ± 3.8</td>
<td>11.8 ± 1.4</td>
</tr>
<tr>
<td>Catawba</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td></td>
<td>17.1 ± 0.3</td>
<td>12.0 ± 0.1</td>
</tr>
<tr>
<td>5.7</td>
<td></td>
<td>14.5 ± 1.2</td>
<td>10.8 ± 0.3</td>
</tr>
<tr>
<td>11.1</td>
<td></td>
<td>12.6 ± 1.3</td>
<td>8.9 ± 2.3</td>
</tr>
</tbody>
</table>

*a* Means of duplicates ± standard error.
Effect of Initial Sulfur Dioxide Levels: A Vidal blanc juice was treated with various amounts of sulfur dioxide. The effect of insoluble solids on sulfur dioxide was found to be similar to a previous study (Liu and Gallander, 1982a) (Table 9). At 9.2% insoluble solids, the sulfur dioxide content of Vidal blanc juice decreased from the initial 30 ppm to 12.0 ppm. While in the filtered juice (0.0% insoluble solids), the sulfur dioxide content decreased from the initial 30 ppm to 22.0 ppm. In the juices treated with 60, 120, and 240 ppm sulfur dioxide, the final sulfur dioxide contents were also lower in the 9.2% insoluble solids group. It appeared that insoluble solids did affect the sulfur dioxide content of Vidal blanc juices initially treated with different amounts of sulfur dioxide.

Effect of Other Particulate Materials: Since insoluble solids are particulate material from skin and pulp, an experiment was conducted to determine the effect of other particulate materials such as bentonite, cellulose, microcrystalline cellulose, pectin, dextran, and algin on the reduction of SO\textsubscript{2} in grape juices. Equal amount (5 grams) of each particulate material was added to a filtered Aurora juice, and treated with 30 ppm of sulfur dioxide. The total sulfur dioxide content was analyzed 12 hours after the addition of sulfur dioxide and was found to be approximately the same (i.e., 20 ppm) for the juices without any addition and the juices with added bentonite, cellulose, microcrystalline cellulose, pectin, dextran, and algin (Table 10). The
Table 9. The effect of initial sulfur dioxide levels on the sulfur dioxide content of Vidal blanc juices with 0.0 and 9.2% of insoluble solids.

<table>
<thead>
<tr>
<th>Initial SO₂ Content (ppm)</th>
<th>Final Sulfur Dioxide Content (ppm)</th>
<th>In Juice with 0.0% Insoluble Solids</th>
<th>In Juice with 9.2% Insoluble Solids</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>30</td>
<td>22.0 ± 0.6</td>
<td>12.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>49.0 ± 2.5</td>
<td>37.6 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>100.4 ± 0.3</td>
<td>91.0 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>210.0 ± 10.7</td>
<td>198.5 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>

*Means of duplicates ± standard error.*
Table 10. Addition of various particulate materials and its effect on the sulfur dioxide content of Aurora juice initially treated with 30 ppm of sulfur dioxide.

<table>
<thead>
<tr>
<th>Particulate Material</th>
<th>Percent (v/v) of Particulate</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>30</td>
<td>21.0 ± 0.3</td>
</tr>
<tr>
<td>Insoluble Solids</td>
<td>4.8</td>
<td>30</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td>Grape Skin</td>
<td>4.4</td>
<td>30</td>
<td>17.4 ± 0.8</td>
</tr>
<tr>
<td>Grape Pulp</td>
<td>5.3</td>
<td>30</td>
<td>18.4 ± 2.3</td>
</tr>
<tr>
<td>Bentonite</td>
<td>4.6</td>
<td>30</td>
<td>19.6 ± 1.4</td>
</tr>
<tr>
<td>Cellulose</td>
<td>4.1</td>
<td>30</td>
<td>20.7 ± 2.1</td>
</tr>
<tr>
<td>Microcrystalline Cellulose</td>
<td>2.5</td>
<td>30</td>
<td>20.4 ± 0.6</td>
</tr>
<tr>
<td>Pectin</td>
<td>5.9</td>
<td>30</td>
<td>20.8 ± 1.1</td>
</tr>
<tr>
<td>Dextran</td>
<td>0.0</td>
<td>30</td>
<td>19.6 ± 1.7</td>
</tr>
<tr>
<td>Algin</td>
<td>8.0</td>
<td>30</td>
<td>20.8 ± 1.1</td>
</tr>
</tbody>
</table>

\[a\] Means of duplicates ± standard error.
\[b\] Grape pulp without insoluble solids and juice.
particulate material content in these juices varied from 0.0 to 8.0%. In juices with added insoluble solids, grape skin, and grape pulp (without insoluble solids), the sulfur dioxide content was reduced to 16.4, 17.4, and 18.4 ppm, respectively. These sulfur dioxide levels were lower than those of the juices with other added particulate materials. It appeared that the effect of insoluble solids on the sulfur dioxide content could not be found with other particulate materials.

**Effect of Inhibitors and Pasteurization:** Since losses of sulfur dioxide have been related to polyphenoloxidase (Ponting and Johnson, 1945) and insoluble solids were reported to carry the enzyme (White and Ough, 1973), it was assumed that losses of sulfur dioxide may be due to the activity of polyphenoloxidase. Two inhibitors of polyphenoloxidase, EDTA and ascorbic acid, were added to Villard blanc juices with 1.0 and 8.0% insoluble solids. At 1.0% insoluble solids, the total sulfur dioxide content in the juices with no inhibitor, EDTA, and ascorbic acid was 18.4, 18.0, and 14.4 ppm, respectively (Table 11). Similar results were found for the juices with 8.0% insoluble solids. Apparently, EDTA and ascorbic acid were not able to inhibit the reduction of sulfur dioxide. This may indicate that polyphenoloxidase was not responsible for losses of sulfur dioxide.
Table 11. Effect of EDTA (5 mM) and ascorbic acid (5 mM) on the sulfur dioxide content of Vidal blanc juices added with 1.0 and 8.0% of insoluble solids and 30 ppm of sulfur dioxide.

<table>
<thead>
<tr>
<th>Insoluble Solids Content (%)</th>
<th>Inhibitor</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>None</td>
<td>30</td>
<td>$18.4 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>30</td>
<td>$18.0 \pm 2.8$</td>
</tr>
<tr>
<td></td>
<td>Ascorbic Acid</td>
<td>30</td>
<td>$14.4 \pm 0.1$</td>
</tr>
<tr>
<td>8.0</td>
<td>None</td>
<td>30</td>
<td>$12.6 \pm 0.3$</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>30</td>
<td>$14.2 \pm 0.8$</td>
</tr>
<tr>
<td></td>
<td>Ascorbic Acid</td>
<td>30</td>
<td>$12.2 \pm 0.8$</td>
</tr>
</tbody>
</table>

$^a$ Means of duplicates $\pm$ standard error.
In order to further prove that the effect of insoluble solids on sulfur dioxide was not catalyzed by polyphenol-oxidase, the juices and/or insoluble solids were pasteurized by heating at 80°C for 5 minutes. This should be sufficient to denature polyphenoloxidase according to White and Ough (1973). In the absence of insoluble solids, both pasteurized and nonpasteurized Seyval blanc juices showed similar changes in sulfur dioxide content (Table 12). In juices without the initial addition of sulfur dioxide, the amount of sulfur dioxide produced by the yeast *Saccharomyces cerevisiae* Montrachet strain #522 was about 30 ppm (Table 12). For the other juices initially treated with 30 ppm of sulfur dioxide, 25 ppm of sulfur dioxide was found in the 12-hour juice samples (before alcoholic fermentation) and 51 ppm was found in the wine samples (after alcoholic fermentation) (Table 12). Rankine and Pocock (1969) showed that *Saccharomyces cerevisiae* No.729 produced 26 ppm of sulfur dioxide in a white grape juice at pH 3.6. However, *S. cerevisiae* No.275 did not produce sulfur dioxide. Nevertheless, in this study, the increases in sulfur dioxide after alcoholic fermentation were caused by the production of sulfur dioxide by wine yeasts.

After fermentation, increase in sulfur dioxide was less in the juices containing 3.0% of insoluble solids than in juices without insoluble solids (Table 12). This indicated that insoluble solids may exert their effect on sulfur dioxide even during alcoholic fermentation. Similar results
Table 12. Effect of pasteurization and insoluble solids on the total sulfur dioxide content in Seyval blanc juices and wines.

<table>
<thead>
<tr>
<th>Filtered Juice</th>
<th>Addition of Insoluble Solids</th>
<th>Amount of SO₂ Added (ppm)</th>
<th>Total SO₂ Content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>In Juice</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>None</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>30.0</td>
<td>24.0 ± 1.1</td>
</tr>
<tr>
<td>Non-pasteurized</td>
<td>None</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>30.0</td>
<td>25.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>3.0% (Pasteurized)</td>
<td>30.0</td>
<td>20.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3.0% (Non-pasteurized)</td>
<td>30.0</td>
<td>22.5 ± 0.3</td>
</tr>
</tbody>
</table>

Mean of duplicates ± standard error.
were obtained from juices with added pasteurized or nonpasteurized insoluble solids. Thus, pasteurization of insoluble solids did not eliminate the effect of insoluble solids on the reduction of sulfur dioxide. The losses of sulfur dioxide in grape juice in the presence of insoluble solids may be through a mechanism different from that reported by Ponting and Johnson (1945). However, it is possible that a heat-insensitive enzyme may be associated with insoluble solids. Further investigations are needed to clarify the mechanism involved in the losses of sulfur dioxide in the grape juice due to the presence of insoluble solids.
LITERATURE CITED


Pure culture inoculation of wine with malolactic bacteria has been effective for induction of malolactic fermentation. In this method, freely suspended bacterial cells are inoculated into wine and are not recovered after malolactic fermentation. Since pure cultures of malolactic bacteria are rather expensive, the cost could be reduced if the bacterial cells are recycled and reused.

Although centrifugation and filtration can be used to collect and recycle the bacterial cells, immobilization of bacterial cells seems to be more desirable. The reason is that cell immobilization could provide other important advantages. Through immobilization, high numbers of bacterial cells are confined and concentrated in (or on) a solid support. The dense microbial populations and solid supports tend to enhance mass transfer within the system, and thus increase the fermentation rate (Abbott, 1977). This property of immobilized cell systems can not be obtained with conventional fermentation systems which employ freely suspended cells. In addition, immobilization of microbial cells by entrapment in polymeric gels seems to protect organisms from the toxic effect of ethanol (Holeberg and Margalith, 1981).
A recent study reported that under adverse conditions (i.e., low pH, high sulfur dioxide, and high ethanol concentrations) the malolactic activity of *Leuconostoc mesenteroides* in red table wines was higher in immobilized cells than in freely suspended cells (Totsuka and Hara, 1981). The bacteria were immobilized by entrapment in agar and alginate gels.

Recently, malolactic bacteria, *Leuconostoc oenos* ML-34 were immobilized in an alginate gel and were found to be effective in inducing malolactic fermentation in red table wines (Spettoli et al., 1982). It appears that immobilized malolactic bacteria may be particularly useful for inducing malolactic fermentation in the low-pH wines of eastern United States. Since *Leuconostoc oenos* PSU-1 is a more suitable strain than ML-34 for eastern United States wines (Beelman et al., 1977), it may be advantageous to immobilize PSU-1. The purpose of this study was to develop an immobilized cell system with *Leuconostoc oenos* PSU-1 and to test its malolactic activity in red and white table wines. To further characterize the system, the effect of pH and sulfur dioxide on malolactic activity of immobilized cells was determined. In addition, the cell leakage from the gel pellets was also studied. This study may lead to a better method of inducing malolactic fermentation in eastern table wines.
MATERIALS AND METHODS

Cell Immobilization: The cells of *Leuconostoc oenos* PSU-1 were prepared from LeucoStart, a commercial freeze-dried culture (Tri-Bio Lab, State College, PA). The freeze-dried cultures were rehydrated in freshly double distilled water for five to ten minutes. Aliquots of rehydrated bacterial cultures were used for cell immobilization. The procedure for cell immobilization was according to Chua et al. (1980). An 4.5% aqueous solution of carrageenan was prepared from the kappa-carrageenan (FMC Corp., Rockland, MN). The solution temperature was maintained at 43°C in a water bath. Two mL of the bacterial culture and 48 mL of the carrageenan solution were mixed in a 60-mL syringe with a tip having an orifice of 1.5 mm in diameter. To form the gel pellets, the mixture was then extruded as droplets into 500 mL of cold 2% KCl with stirring. Each pellet had a diameter of about 5 mm and a volume of about 50 μL. Gel pellets were then soaked in 4°C KCl solution for 14 hours, rinsed with deionized water, and blotted dry with filter paper.

Induction of Malolactic Fermentation in Red Table Wine: To induce malolactic fermentation in red table wine, 50-mL of gel pellets containing approximately $3.0 \times 10^9$ cfu's of *Leuconostoc oenos* PSU-1 were inoculated into 200 mL of Chancellor wine. The malolactic activity of these immobilized bacterial cells was compared to freely suspended cells by inoculating $3.8 \times 10^9$ cfu's of the same organism to 250 mL
of wine. All fermentations were conducted in 250-mL flasks equipped with water seals. In both instances, the final bacterial concentration was approximately $1.5 \times 10^7$ cfu/mL. In order to eliminate the effect of carrageenan gel, three additional treatments were used. First, 50 ml of gel pellets without bacteria cells were added to 200 mL of the same wine. Second, 50 ml of gel pellets without cells, and $3.0 \times 10^9$ cfu's of free bacterial cell was inoculated to 200 mL of the wine. Third, 250 mL of wine with bacterial inoculation and addition of gel pellets. There were two replications for each treatment.

The malolactic activity of recycled immobilized cells was studied in three consecutive fermentations. Following malolactic fermentation, the wines were decanted for chemical analysis while the immobilized cells were re-inoculated into a new batch of wine. All wines were incubated without stirring at 25°C. The progress of malolactic fermentation was monitored by paper chromatography (Kunkee, 1968). Results were expressed as number of days for completion of malolactic fermentation (absence of malic acid), beginning with the first day of bacterial inoculation.

**Induction of Malolactic Fermentation in White Table Wines:** Before they were inoculated into white wines, the immobilized bacteria were pre-incubated in a grape juice medium at 25°C until malolactic fermentation was completed. The medium was prepared from a white grape juice diluted with an equal volume of distilled water. The diluted juice
was supplemented with 0.05% of Yeastex-61 (Scott Laboratories, San Rafael, CA). The pH of the juice was adjusted to 4.5 with CaCO₃ and sterilized at 252°F (122°C) for 20 minutes. When the medium reached room temperature, it was inoculated with Montrachet yeast strain #522 (Universal Foods Corp., Milwaukee, WI) at the rate of 0.1% (w/w).

Approximately 6 hours after yeast inoculation, the immobilized bacteria were added to the juice medium. When malolactic fermentation was completed, as indicated by the absence of malic acid on a paper chromatogram, the immobilized bacteria were removed, rinsed with deionized water, blotted dry with filter papers, and inoculated into Vidal blanc wine.

This experiment was conducted with two replications in 50-mL test tubes. Two sizes of inoculum of immobilized cells were used: 12.5 and 25.0 mL. The malolactic activity of the immobilized bacteria was determined by paper chromatography (Kunkee, 1968).

**Chemical Analysis of Wine:** The pH, total acidity, volatile acidity (Cash Apparatus), and ethanol (ebulimeter) were determined as described by Amerine and Ough (1974). L-malic acid was determined with malic dehydrogenase (Mayer and Busch, 1963). Free and total sulfur dioxide were analyzed by the aeration-oxidation method (Buechsenstein and Ough, 1978).

**Adjustments of Free Sulfur Dioxide Content and pH:** For the study on the effect of pH and sulfur dioxide on the malolactic activity of immobilized bacteria, a Chancellor
wine was used. Fifteen mL of wine were inoculated with 15 mL of immobilized bacteria in a 30-mL test tube. Two replications were used for this experiment. The free sulfur dioxide content of the wine was adjusted according to the procedure of Gallander et al. (1981). Four levels of free sulfur dioxide were obtained: 5, 10, 15, and 20 ppm. When the free and bound sulfur dioxide had reached an equilibrium in three days, the pH of the wines was adjusted to four different levels: 3.0, 3.2, 3.4, and 3.6. The amount of 4N HCl or 2N NaOH required for the adjustment were pre-determined by titrating 30 mL of wine to the desired pH. The wine pH was adjusted by adding required amount of acid or alkali without stirring to avoid losses of sulfur dioxide. Two hours later, all the wines were inoculated with immobilized bacteria.

**Bacterial Counts:** The procedure used to determine the viable number of freely suspended bacteria in the wines or cultures was the same as described in the Materials and Methods of Chapter I. For determinations of viable bacteria in carrageenan gel, 15 grams of the gel were dissolved in a 1500 mL of sterile distilled water at 40°C. Aliquots were diluted and plated according to the procedure for freely suspended bacterial cells.
Induction of Malolactic Fermentation in Red Table

Wines: The immobilized cells of *Leuconostoc oenos* PSU-1 were found to induce malolactic fermentation in Chancellor wines. Following inoculation of immobilized cells, malolactic fermentation (MLF) was completed in 10 and 3 days for the first and second batches of wine, respectively (Table 13). Wines inoculated with both free cells and gel pellets (without cells) (treatment C) also completed malolactic fermentation in 14 days. In contrast, no malolactic fermentation was observed with free cells alone (treatment B), or gel pellet without cells (treatment D), or control wines without inoculation (treatment A). Thus, immobilized cells or free cells plus gel pellets (without cells) were effective in inducing fermentation while free cells were not. The difference in malolactic activity between free and immobilized cells was verified in the next experiment. The results indicated that immobilized cells completed malolactic fermentation in 13 and 25 days for the first and second incubation batches, respectively (Table 14). The free cells were not able to induce malolactic fermentation. Totsuka and Hara (1981) demonstrated that immobilized cells of *Leuconostoc mesenteroides* contained a higher malolactic activity than free cells. Also, DeMenezes et al. (1972) reported that a large number of free cells, 4.1 to 8.2 x 10^8 cells per mL,
Table 13. The rate of malolactic fermentation in Chancellor wines without inoculation (A), and with inoculation of free cells (B), free cells plus gel pellets (C), gel pellets (D), and immobilized cells (E) of *L. oenos* PSU-1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inoculation of PSU-1 cells and/or gel pellets to wine</th>
<th>Time (days) to complete malolactic fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>first batch</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>--</td>
</tr>
<tr>
<td>B</td>
<td>Free cells</td>
<td>--</td>
</tr>
<tr>
<td>C</td>
<td>Free cells plus gel pellets (without cells)</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>Gel pellets (without cells)</td>
<td>--</td>
</tr>
<tr>
<td>E</td>
<td>Immobilized cells</td>
<td>10</td>
</tr>
</tbody>
</table>

a

No malolactic fermentation occurred.
Table 14. The rate of malolactic fermentation in Chancellor wines inoculated with free or immobilized cell of *L. oenos* PSU-1.

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Time (days) to complete malolactic fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first batch</td>
</tr>
<tr>
<td>Free cells</td>
<td>b</td>
</tr>
<tr>
<td>Immobilized cells</td>
<td>13</td>
</tr>
</tbody>
</table>

a Initial cell concentration was $1.4 \times 10^7$ cfu/mL.

b Malolactic fermentation did not complete in 89 days.
was not effective in inducing malolactic fermentation in finished Seibel 10868 and Concord wines. The results of this study support their findings. The reason free cells of malolactic bacteria do not induce malolactic fermentation in finished wines is not known. The finished wines may contain a substance(s) which is highly inhibitory to bacteria (DeMenezes, et al., 1972).

The malolactic activity of immobilized cells was found to decrease after two wine incubations. Only a slight activity was observed in the third incubation and malolactic fermentation was not completed even after a prolonged incubation. Spettoli et al. (1982) reported that the activity of immobilized cells gradually decreased after several incubations. They suggested that the decrease in activity was due to the reduction of gel porosity by condensed tannins and tannin-protein complexes of molecular weight above 3,000. However, Totsuka and Hara (1981) did not observe any decrease in malolactic activity of immobilized cells during a 60-day continuous reaction. The immobilized cells were packed in a sectional column, and a red wine was passed through the column at an unknown flow rate at 20°C.

The Chancellor wines were analyzed before and after malolactic fermentation. The results showed that malolactic fermentation increased the pH and decreased the titratable acidity and ethanol content in the first and second batches of wine (Table 15). However, the decrease in titratable acidity appeared to be greater than what could be accounted
Table 15. Chemical composition of two batches of Chancellor wine before and after malolactic fermentation induced by immobilized cells of *L. oenos* PSU-1.

<table>
<thead>
<tr>
<th>Chemical (measure)</th>
<th>Before MLF</th>
<th>After MLF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>first batch</td>
</tr>
<tr>
<td>pH</td>
<td>3.31</td>
<td>3.45</td>
</tr>
<tr>
<td>T.A. (%)</td>
<td>0.91</td>
<td>0.54</td>
</tr>
<tr>
<td>V.A. (%)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Malate (%)</td>
<td>0.48</td>
<td>0.00</td>
</tr>
<tr>
<td>Ethanol(%,v/v)</td>
<td>12.4</td>
<td>9.9</td>
</tr>
<tr>
<td>SO₂(mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free</td>
<td>5.0</td>
<td>ND</td>
</tr>
<tr>
<td>total</td>
<td>32.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* Malolactic fermentation.

b Titratable acidity as tartaric acid, g/100mL.

c Volatile acidity as acetic acid, g/100mL.

d Not determined.
by the conversion of malate to lactate. Also, the decrease in ethanol was unusual since *Leuconostoc oenos* does not decompose ethanol. After malolactic fermentation by the immobilized cells, the ethanol content in the first batch of wine decreased from 12.4 to 9.9%. The same amount of ethanol decrease was found in wines inoculated with free cells plus gel pellets (without cells) and with gel pellets (without cells). In wines inoculated with free cells, the ethanol contents decreased slightly to 12.1% (not shown in Table 15). The immobilized cells were found to be soaked with red wine after malolactic fermentation. It was very likely that an equilibrium was established between the wine and gel. Since gels contained approximately 95% water, 50 mL of gel pellets would dilute the ethanol concentration to 10.0% in a 200-mL wine with 12.4% ethanol. The ethanol content in the second wine batch was 11.7% which was much higher than the first batch (Table 15). Although there was no determination of ethanol in the gel, it could be assumed that through equilibrium the gels would contain 9.9% ethanol following incubation in the first wine batch. Consequently, when these gel pellets were re-inoculated into a new batch of wine, less reduction in ethanol concentration in the wine would be expected.

Through the equilibrium, the chemical constituents in the wines including sulfur dioxide, ethanol, and malate were diluted and a more favorable condition was created for the bacteria. DeMenezes et al. (1972) reported that malolactic
fermentation was induced in finished wines when diluted with equal amounts of water and inoculated with a large number of malolactic bacteria. Since malolactic fermentation was not induced without dilution, they concluded that the wines contained a substance(s) that inhibited the bacteria. This dilution effect may explain the induction of malolactic fermentation by immobilized cells and freely suspended cells when gel pellets (without cells) were present (Table 13). Although the dilution effect was smaller during the second incubation, the immobilized cells were still able to complete malolactic fermentation. This indicated that other factors may be involved. These could be enhanced mass transfer by the system (Abbott, 1977) and protection by the carrageenan gel from the toxic effect of ethanol (Holeberg and Margalith, 1981). Studies of Totsuka and Hara (1981) and Spettoli et al. (1982) used alginate gels to immobilize malolactic bacteria. Their wines may have been diluted with water from the gels, although this was not reported by the authors.

Because of the legal aspect of adding water to wine, it will be necessary to study the transport of water from gels to wine. To solve the dilution problem, future research should include immobilized bacteria in dried gel pellets.

**Induction of Malolactic Fermentation in White Table Wines:** Results of this experiment showed that free and immobilized cells were not able to induce malolactic
fermentation in white wines. While wine constituents appeared to be similar, the pH of Vidal blanc wines was lower than the Chancellor wines (Table 16). However, the immobilized cells were not able to induce the fermentation even if the wine pH was adjusted to the same value as the Chancellor wines. The exact reason for the inability of immobilized bacteria to induce malolactic fermentation in the Vidal blanc wines is not known.

White wines are usually less susceptible to malolactic fermentation than red wines (Ingraham and Cooke, 1960; Rice, 1965). This is because of the higher concentrations of sulfur dioxide and acid and low pH values usually found in white wines (Rice, 1965; Kunkee, 1967a). The studies of DeMenezes et al. (1972) showed that it was extremely difficult to induce malolactic fermentation in finished wines by pure culture inoculation, particularly in white wines.

The Chancellor wines were made by fermenting crushed grapes on the skin. Since grape skins were reported to contain a growth factor for malolactic bacteria (Kunkee, 1967a), it appears that Vidal blanc wines lacked sufficient nutrients to support bacterial growth and malolactic fermentation. It is also possible that freeze-dried bacteria used for immobilization in this study may have a low malolactic activity. Freeze-dried PSU-1 cells were found to be more effective in inducing malolactic fermentation if they were incubated in grape juice prior to wine inoculation to wine (Krielow, 1979; Liu and Gallander, 1982b). This is probably
Table 16. Chemical composition of Vidal blanc wines before and after malolactic fermentation induced by immobilized cells of *L. oenos* PSU-1.

<table>
<thead>
<tr>
<th>Chemical (measure)</th>
<th>Before MLF</th>
<th>After MLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.12</td>
<td>3.24</td>
</tr>
<tr>
<td>T.A. (%)</td>
<td>1.10</td>
<td>0.46</td>
</tr>
<tr>
<td>V.A. (%)</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Malate(%)</td>
<td>0.43</td>
<td>0.02</td>
</tr>
<tr>
<td>Ethanol(%, v/v)</td>
<td>10.8</td>
<td>ND</td>
</tr>
<tr>
<td>SO₂ (mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>43.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* Malolactic fermentation.

*b* Titratable acidity as tartaric acid, g/100mL.

*c* Volatile acidity as acetic acid, g/100mL.

*d* Not determined.
because freeze-dried bacteria were provided a favorable condition for growing and recovering from the stress of freeze-drying. Based on the above reasons, the immobilized cells were pre-incubated in a grape juice medium prior to their inoculation into wine. Pre-incubation was found to stimulate malolactic activity of the immobilized cells. In Table 17, wines inoculated with 25 mL of the pre-incubated immobilized cells, completed malolactic fermentation in 5 and 19 days for the first and second batches of wine, respectively. Only a slight malolactic activity remained in the immobilized cells in the third incubation batch. Inoculation of 13 mL of the same immobilized cells was not effective inducing malolactic fermentation. This may indicate that the volume ratio of wine/immobilized cells or the number of immobilized cells was critical to malolactic fermentation. The pre-incubation procedure seems to improve the malolactic activity of immobilized cells.

**Effect of pH and Sulfur Dioxide:** The malolactic activity of immobilized cells was affected by the pH and free sulfur dioxide content of the wines (Table 18). At a given level of sulfur dioxide, malolactic fermentation rate was directly related to the initial pH of the wine. This was similar to the finding of Bousbouras and Kunkee (1971) with free cells of malolactic bacteria. Totsuka and Hara (1981) reported that immobilized cells maintained a high malolactic activity even at a pH value as low as 3.0. The results of
Table 17. Effect of volume ratio of immobilized cells/wine on the rate of malolactic fermentation in Vidal blanc wines.

<table>
<thead>
<tr>
<th>Volume of immobilized cells of PSU-1 (mL)</th>
<th>Volume of Vidal blanc wine (mL)</th>
<th>Time (days) to complete malolactic fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>first batch</td>
</tr>
<tr>
<td>0.0</td>
<td>50.0</td>
<td>a</td>
</tr>
<tr>
<td>12.5</td>
<td>37.5</td>
<td>--</td>
</tr>
<tr>
<td>25.0</td>
<td>25.0</td>
<td>b</td>
</tr>
</tbody>
</table>

a
No malolactic fermentation occurred.
b
Means of duplicates.
Table 18. Time (days) required for immobilized cells of L. oenos PSU-1 to complete malolactic fermentation in Chancellor wines at different pH and free sulfur dioxide levels.

<table>
<thead>
<tr>
<th>pH</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>a</td>
<td></td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>3.4</td>
<td>17</td>
<td>17</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>3.6</td>
<td>11</td>
<td>13</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

a Means of duplicates.
b No malolactic fermentation occurred.
this study seem to support their findings, although fermentation was inhibited by 15 and 20 ppm of free sulfur dioxide at pH 3.0. Therefore, immobilized malolactic bacteria may be useful in inducing malolactic fermentation in low-pH wines of eastern United States.

However, the ethanol content of Chancellor wines was found to be only 6.5% after the malolactic fermentation induced by immobilized PSU-1 cells. This was approximately half as much as the initial ethanol content (12.7%) of the wines. Also, the titratable acidity decreased from 0.94 to 0.38% after malolactic fermentation. Apparently, there was a dilution of wine constituents due to the addition of gel pellets.

Cell Leakage: Five Chancellor wines were used to study the effect of pH and sulfur dioxide on cell leakage from the gel. These five wines had a pH range from 3.2 to 3.6 and a free sulfur dioxide content range from 5 to 20 ppm (Table 19). They completed malolactic fermentation in a time period ranging from 8 to 29 days following the inoculation of immobilized PSU-1 cells. Immediately after malolactic fermentation, bacteria were found in the wines as freely suspended cells. Apparently, they resulted from cell leakage from the gel. The cell concentration varied from $3.5 \times 10^5$ to $2.1 \times 10^7$ cfu's/mL, and appeared to be related to the time required to complete malolactic fermentation (Table 19). Since the conversion of malate to lactate began
Table 19. The concentration of immobilized and leaked cells after malolactic fermentation induced by immobilized cells of *L. oenos* PSU-1.

<table>
<thead>
<tr>
<th>Wine</th>
<th>pH</th>
<th>Free SO₂ conc. (ppm or mg/L)</th>
<th>Time (days) a to complete MLF</th>
<th>Immobilized cell conc. (cfu/mL gel)</th>
<th>Leaked cell conc. (cfu/mL wine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.6</td>
<td>5</td>
<td>8</td>
<td>ND</td>
<td>4.5 x 10</td>
</tr>
<tr>
<td>B</td>
<td>3.6</td>
<td>10</td>
<td>10</td>
<td>ND</td>
<td>3.5 x 10</td>
</tr>
<tr>
<td>C</td>
<td>3.4</td>
<td>10</td>
<td>16</td>
<td>2.4 x 10</td>
<td>1.3 x 10</td>
</tr>
<tr>
<td>D</td>
<td>3.2</td>
<td>15</td>
<td>23</td>
<td>2.3 x 10</td>
<td>3.9 x 10</td>
</tr>
<tr>
<td>E</td>
<td>3.2</td>
<td>20</td>
<td>29</td>
<td>1.8 x 10</td>
<td>2.1 x 10</td>
</tr>
</tbody>
</table>

a  Malolactic fermentation.

b  Initial concentration was 1.2 x 10 cfu/mL gel.

c  Not determined.
at \( 1 \times 10^6 \) cfu's/mL, it is likely that in samples C, D, and E the freely suspended cells may have contributed to malolactic fermentation. While in samples A and B, malolactic fermentation was induced by the immobilized cells. Cell leakage from gels has also been reported by Spettoli et al. (1982). They found \( 2 \times 10^5 \) cfu's/mL bacteria in their wines. This level was smaller than the concentration found in this study. This may be due to the different type of gels and bacteria used in the studies. Another reason may be due to growth of the bacterial cells from a low concentration (3.5 \( \times 10^5 \) cfu's/mL) to a large population.

In summary, immobilized PSU-1 cells in a carrageenan gel were found to induce malolactic fermentation in red and white table wines. However pre-incubation of immobilized cells was necessary for induction of malolactic fermentation in white wines. Additions of gel pellets seemed to dilute wine constituents. The malolactic activity of immobilized cells was affected by pH and free sulfur dioxide in the wines. Low pH and high sulfur dioxide tended to inhibit the activity. Cell leakage from the gel pellets into the wines appeared to be directly related to the time required to complete malolactic fermentation.
LITERATURE CITED


GENERAL DISCUSSION AND CONCLUSION

The inability to consistently stimulate malolactic fermentation, even following pure culture inoculation, still remains as a problem to the enologists (Beelman and Gallander, 1979). A more basic understanding of the factors which control the growth of malolactic bacteria in wine is needed.

Malolactic fermentation is often difficult to initiate in the low-pH wines of eastern United States. This difficulty is also attributed to the addition of sulfur dioxide during vinification. In order to stimulate malolactic fermentation in wines, the initial treatment of musts with sulfur dioxide should be reduced or eliminated. This has been demonstrated in this study. The addition of sulfur dioxide to musts clearly affected the growth of malolactic bacteria and rate of malolactic fermentation in red table wines. Although the initial population of malolactic bacteria was high ($1 \times 10^7$ cfu/mL), it declined continuously for two weeks following the inoculation of the bacteria into wines. The decrease in bacterial population was directly related to the initial sulfur dioxide level. The growth of malolactic bacteria was highly inhibited in the wines prepared from pH 3.3 must with an initial sulfur dioxide level higher than 50
Since the musts and wines produced in the eastern United States are of low pH, the initial sulfur dioxide levels should be kept below 50 ppm in order to encourage malolactic fermentation in these regions.

The growth behavior of malolactic bacteria is also influenced by the insoluble solids content of the grape juice. As demonstrated in this study, the death rate of malolactic bacteria following their inoculation into white table wines was lowest at the highest insoluble solids level. As a result, wines prepared from musts having the highest insoluble solids experienced the fastest rate of malolactic fermentation. Results also indicated that the insoluble solids affected the sulfur dioxide content of white juices and wines. Juices and wines with the highest insoluble solids tended to be lowest in sulfur dioxide. It appeared that the low sulfur dioxide content in wines was responsible for the lower death rate of malolactic bacteria and higher malolactic fermentation rate. However, there is a possibility that certain growth factors may have been extracted from insoluble solids to stimulate the growth of malolactic bacteria. Another possibility is that multiplication and autolyses of yeast cells may be faster at a higher insoluble solids levels, and thus provide necessary nutrients for bacterial growth.

The effect of insoluble solids on the sulfur dioxide content of white juices and wines has been confirmed by this study. Among the eight varieties of grapes analyzed, five
varieties showed decreases in sulfur dioxide in their juices with increasing amounts of insoluble solids. While in the other three varieties, sulfur dioxide content of the juices was not affected by the level of insoluble solids. The study also showed that reduction in sulfur dioxide can not be found by the addition of other particulate materials than grape insoluble solids. There is an indication from the literature that insoluble solids contain polyphenoloxidase which may cause the loss of sulfur dioxide. However, the addition of polyphenoloxidase inhibitors (ascorbic acid and EDTA) and pasteurization of insoluble solids did not prevent the reduction of sulfur dioxide by insoluble solids. This suggests that polyphenoloxidase was not involved in the loss of sulfur dioxide in grape juices and wines. Further investigations are needed to understand the exact mechanism.

For induction of malolactic fermentation in finish table wines, pure culture inoculation is the most effective method available. However, it has not been widely used by wineries. The main reasons for its limited use are the high cost of pure cultures of malolactic bacteria and the inability of these bacteria to grow in certain wines, especially the white wines and the low-pH wines of eastern United States. In an effort to improve this method, a study has been conducted to develop an immobilized cell system of malolactic bacteria. Malolactic bacteria were entrapped in a carrageenan gel, and were inoculated into wines to compare their activity with freely suspended cells. Malolactic
fermentation was induced by the immobilized bacteria in both red and white wines, and even in wines with pH values as low as 3.0. In contrast, no malolactic fermentation was observed in wines inoculated with freely suspended bacteria. Therefore, immobilized malolactic bacteria appeared to be useful for the induction of malolactic fermentation in the low-pH wines of eastern United States. In order to reduce the cost for malolactic bacterial cultures, the immobilized bacteria were recycled and reused several times. Results indicated that the immobilized bacteria could only be used twice and thereafter very little malolactic activity remained. More studies are needed to improve this system. Results also indicated that the alcohol content of wines decreased after malolactic fermentation by immobilized bacteria. This was apparently due to the addition of carrageenan gel. To solve this problem, the use of immobilized bacteria in a dried gel may be helpful. Also, a smaller volume of gel should be used and tested for immobilizing the bacteria. Future research should be directed towards the development of a continuous fermentation reactor with immobilized malolactic bacteria.
BIBLIOGRAPHY


