AN ABSTRACT OF THE THESIS OF

Visith Chavasit for the degree of Doctor of Philosophy in Food Science and Technology presented on June 16, 1989.

Title: Studies in Food Science for Industrial Applications: Chemical and Sensory Analysis of Fermented Cucumbers; Insoluble Chitosan-Polyacrylic Acid Complexes

Abstract approved:

/ J. Michael Hudson

Chemical and Sensory Analysis of Cucumber Juice Brine Fermented by Propionic and Lactic Acid Bacteria

_Pediococcus cerevisiae, Lactobacillus casei, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactococcus diacetylactis Bifidobacterium bifidum, Leuconostoc oenos_, and mixed cultures of Propionibacterium shermanii and _P. cerevisiae_ were used to ferment cucumber juice brine (CJB) at 22-26°C for 1.5 months. Sugar utilization ranged from 14.6 to 86.1%. pH of the fermented CJB ranged from 3.24 to 4.12 and titratable acidity ranged from 0.30 to 0.93%. All strains tested degraded malic acid and citric acid. _Leu. mesenteroides_ and _Leu. oenos_ did not utilize citric acid for diacetyl-acetoin production. The concentration of acetic, propionic and lactic acids varied among the fermentation treatments. The heterofermenters produced high concentrations of CO₂, ethanol and mannitol and CJB with high volatile/nonvolatile acid ratios. The fermentation balance indicated that sugars had been used to produce compounds not measured in this study.
Twelve aroma and six flavor by mouth descriptors were used to describe flavor of the final products in the trained panel descriptive analysis. Sourness intensity was the only sensory descriptor that correlated with the chemical analysis data. The intensities of seven aroma and three flavor by mouth descriptors were significantly different ($p < 0.05$) among treatments. Aroma preference mean scores from 9-point hedonic scale ranged from 4.69-5.39; they were not significantly different ($p > 0.05$).

**Formation and Potential Industrial Applications of An Insoluble Polyelectrolyte Complex: Chitosan-Polyacrylic Acid**

Chitosan and polyacrylic acid mixtures were prepared in different mole ratios and at different ionic strengths (0.025-0.300). No insoluble complex formation at pH=2 was detected. In the 3 to 6 pH range, the maximum complex formation occurred at different mole ratios. The complex composition is affected by pH but not by ionic strength. An electrostatic interaction between $-\text{COO}^-$ and $-\text{NH}_3^+$ groups was involved in complex formation. This study suggests that process recommendations for industrial application of chitosan as a coagulating agent can be made based on the ionic strength, pH and charge group concentration of the fluid to be treated.
Studies in Food Science for Industrial Applications:
Chemical and Sensory Analysis of Fermented Cucumbers;
Insoluble Chitosan-Polyacrylic Acid Complexes

by

Visith Chavasit

A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June 16, 1989
Commencement June, 1990
APPROVED:

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Date thesis is presented June 16, 1989
ACKNOWLEDGEMENTS

I want to express my appreciation to Dr. J. Michael Hudson for his guidance during the development of cucumber pickles project, his friendliness and his sincerity. To Dr. Mina McDaniel for the best guidance in sensory evaluation and serving as a major advisor during the last part of the cucumber pickles project. To Dr. J. Antonio Torres for his guidance in the chitosan project and his best concern as a major advisor during the last part of the cucumber pickles project. To Dr. & Mrs. Carlos A. Kienzle-Sterzer for their guidance during the development of the chitosan project. To Dr. W.E. Sandine for his valuable advice on microbiology problems and his support. To Dr. W.D. Loomis on his advice on biochemistry problems. To Dr. Keith L. Levien for serving in my committee. To Dr. David R. Thomas and Dr. Lyle Calvin for their helpful advices on statistics. To Dr. Mark A. Daeschel for his valuable advices during the development of the cucumber pickles project. To the sensory group for their friendliness and helpfulness during the sensory evaluation part of the cucumber pickles project. To members of the pickle panel for their time and effort. To Mr. Jim Barbour for his help in gas chromatography and many other things.

I wish to express my thanks to Steinfeld's Products Company at Portland for contribution of fresh cucumbers used in this research.
Acknowledgement is also made to faculties, staffs and fellow graduate students at the Department of Food Science and Technology for their helpfulness and friendliness. I also express my appreciation to Chun-Tien Yeh, Victor Hong, San Land Young, Ruud Valyasevi, Sam Beattie, Boontee Kruetrachue, Peggy Baier and Seksom Attamangkune for their friendliness and best concerns.

Very special appreciation goes to my parents for their love and unfailing support.
TABLE OF CONTENTS

Chemical and Sensory Analysis of Cucumber Juice Brine Fermented by Lactic and Propionic Acid Bacteria.

INTRODUCTION 1

1. LITERATURE REVIEW 3
   Cucumber fermentation 3
   Lactic acid bacteria 8
   Propionic acid bacteria 18
   Diacetyl and acetoin formation 21
   Sensory descriptive analysis 26
   Sensory studies of cucumber pickles 29

2. Lactate and Propionate Fermentation of Cucumber Juice Brine 31
   ABSTRACT 31
   INTRODUCTION 31
   MATERIALS & METHODS 36
   RESULTS & DISCUSSION 44
   CONCLUSIONS 59
   REFERENCES 61

3. Descriptive Analysis and Consumer Testing of Cucumber Juice Brine Fermented by Lactic and Propionic Acid Bacteria 64
   ABSTRACT 64
   INTRODUCTION 64
   MATERIALS & METHODS 67
   RESULTS & DISCUSSION 75
   CONCLUSIONS 94
   REFERENCES 95

Formation and Potential Industrial Applications of An Insoluble Polyelectrolyte Complex: Chitosan-Polyacrylic Acid.

INTRODUCTION 97

4. LITERATURE REVIEW 99
   Chitin and chitosan 99
   Polyelectrolyte complexes 105
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Formation of lactate from glucose by the homofermentative pathway</td>
<td>11</td>
</tr>
<tr>
<td>1.2</td>
<td>Formation of CO₂, lactate, and ethanol from glucose by the heterofermentative pathway</td>
<td>13</td>
</tr>
<tr>
<td>1.3</td>
<td>Formation of acetate and lactate from glucose by the bifidum pathway</td>
<td>16</td>
</tr>
<tr>
<td>1.4</td>
<td>Formation of acetate, CO₂, propionate, and ATP during propionic acid fermentation</td>
<td>19</td>
</tr>
<tr>
<td>1.5</td>
<td>Formation of acetoin and 2,3 butanediol during growth of bacilli on glucose</td>
<td>23</td>
</tr>
<tr>
<td>1.6</td>
<td>Biosynthetic pathway among dairy lactic acid streptococci for the production of diacetyl and its reduction products from citric acid</td>
<td>25</td>
</tr>
<tr>
<td>1.7</td>
<td>Butanediol fermentation</td>
<td>27</td>
</tr>
<tr>
<td>2.1</td>
<td>Fermentation bottle with attachments</td>
<td>40</td>
</tr>
<tr>
<td>2.2</td>
<td>Microbial counts during fermentation</td>
<td>45</td>
</tr>
<tr>
<td>2.3</td>
<td>pH of cucumber juice brine during fermentation</td>
<td>48</td>
</tr>
<tr>
<td>2.4</td>
<td>Acid production during fermentation</td>
<td>49</td>
</tr>
<tr>
<td>2.5</td>
<td>Carbon dioxide production during fermentation</td>
<td>51</td>
</tr>
<tr>
<td>3.1</td>
<td>Quantitative Descriptive Analysis (QDA) configurations for the fermented CJB</td>
<td>93</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1</td>
<td>Molecular structures of chitin and chitosan</td>
<td>100</td>
</tr>
<tr>
<td>4.2</td>
<td>Flow diagram of chitin and chitosan processing</td>
<td>101</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of mixing ratio on complex formation: turbidity measurements.</td>
<td>113</td>
</tr>
<tr>
<td>5.2</td>
<td>Effect of mixing ratio on complex formation: pellet weight determinations.</td>
<td>114</td>
</tr>
<tr>
<td>5.3</td>
<td>Effect of ionic strength on complex formation</td>
<td>115</td>
</tr>
<tr>
<td>5.4</td>
<td>Effect of complex formation on supernatant pH.</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>a. initial pH = 3</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>b. initial pH = 4</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>c. initial pH = 5</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>d. initial pH = 6</td>
<td>120</td>
</tr>
<tr>
<td>5.5</td>
<td>Effect of mixing ratio and initial pH on supernatant composition</td>
<td>122</td>
</tr>
<tr>
<td>5.6</td>
<td>IR analysis of a mixture of chitosan and polyacrylic acid and of a complex</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>formed at initial pH = 3 and mixing ratio = 0.122</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Molecular structures of chitin, chitosan and polyacrylic acid</td>
<td>129</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex formation as a function of polymer mixing ratio and initial pH (ionic strength = 0.3) a. Turbidity measurements (420 nm) b. Insoluble complex weight</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex formation as a function of polymer mixing ratio, initial pH and various ionic strengths</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirmation of complex formation mechanism: supernatant pH measurements</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confirmation of complex formation mechanism: analysis of supernatant composition</td>
<td>140</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 pH, acid and alcohol concentrations in fermented cucumber juice brine (CJB) after 1.5 months.</td>
<td>47</td>
</tr>
<tr>
<td>2.2 Sugar contents, sugar utilization in fermented cucumber juice brine (CJB) after 1.5 months.</td>
<td>52</td>
</tr>
<tr>
<td>2.3 Sugar fermentation profiles after 48 hours.</td>
<td>54</td>
</tr>
<tr>
<td>2.4 Percent carbon recovery after 1.5 month fermentation.</td>
<td>55</td>
</tr>
<tr>
<td>2.5 Citric acid, diacetyl and acetoin concentrations in unfermented and fermented cucumber juice brine (CJB) after 1.5 months.</td>
<td>58</td>
</tr>
<tr>
<td>3.1 Standards used to represent each aromatic descriptor during panelist training for descriptive analysis.</td>
<td>73</td>
</tr>
<tr>
<td>3.2 F-values for each source of variation of each sensory descriptor rated by the trained panel.</td>
<td>76</td>
</tr>
<tr>
<td>3.3 Means and standard deviations of trained panel aroma descriptors for eight treatments of cucumber juice brine (CJB) fermented by different microorganisms.</td>
<td>78</td>
</tr>
<tr>
<td>3.4 Chemical composition of cucumber juice brine (CJB) fermented by different microorganisms.</td>
<td>80</td>
</tr>
<tr>
<td>3.5 Means and standard deviations of trained panel flavor by mouth descriptors for eight treatments of cucumber juice brine (CJB) fermented by different microorganisms.</td>
<td>82</td>
</tr>
<tr>
<td>3.6 Aftertaste comments during descriptive analysis</td>
<td>84</td>
</tr>
<tr>
<td>3.7 Analysis of variance of doubly balanced incomplete block design for each replication of fermented cucumber juice brine (CJB) for Consumer testing.</td>
<td>90</td>
</tr>
<tr>
<td>3.8 Analysis of variance for fermented cucumber juice brine (CJB) using balanced incomplete block design for Consumer testing.</td>
<td>91</td>
</tr>
<tr>
<td>3.9 Adjusted means for Consumer testing scores.</td>
<td>92</td>
</tr>
</tbody>
</table>
INTRODUCTION

The controlled fermentation of cucumbers allows a consistent and predictable fermentation and thus yield a high quality product (Etchells et al., 1973). By adding CaCl2 at the beginning of the controlled fermentation, it is also possible to ferment and store cucumbers in low salt concentration, and the storage brine may be able to use as a packing brine as well. Therefore, flavor of the end product is mainly based on products produced by microorganisms during the fermentation.

Homofermenters such as Lactobacillus plantarum and Pediococcus cerevisiae have been used as an inoculum for the controlled fermentation of vegetables such as cucumbers because of their low carbon dioxide production and high sugar utilization ability. Chen et al. (1983) suggested that the lactic acid flavor developed by these homofermentative organisms may be too strong to be desirable for many people. Moreover, some studies have shown that the volatile/nonvolatile acid ratio rather than the total acid concentration affect more significantly the flavor of fermented vegetables (Juhasz et al., 1974).
Heterofermenters, Bifidobacteria and Propionibacteria have been used in many fermented food products but their use in cucumber fermentation has not been studied extensively. Some of these bacteria might be able to produce final products with different or better flavor qualities.

Also, quantitative measurements are needed to describe product quality to facilitate product development and quality control procedures. These measurements should include sensory descriptive analysis of fermented cucumbers. However, no study has been published on the full scale descriptor analysis of cucumber pickles. Therefore, the goal of this research effort was to evaluate the use of different bacteria for the controlled fermentation of cucumbers and to produce a sensory profile (descriptive analysis) of fermented cucumbers. Of particular interest is their sugar utilizing ability and carbon dioxide and organic acid production. Cucumber juice brine (CJB) was used as a model system in this study.
1. LITERATURE REVIEW

Cucumber fermentation

Cucumber pickles are manufactured either directly from fresh cucumbers (fresh pack) or from cucumbers that have been fermented in salt-brine (fermented pickles). The salt-brined fermentation remains an important method of preservation for several reasons. Fermented pickles have desirable flavor and texture characteristics. In addition, fermentation in bulk containers offers important economic advantages: (i) large volume of cucumbers can be preserved quickly during the hectic harvest season; (ii) the product can be removed from storage at various times during the year for manufacturing into desired products, thus distributing labor and equipment needs throughout the year; (iii) bulk storage allows for market hedging; (iv) fermentation offers the potential for energy saving since pasteurization or refrigeration may not be required in properly fermented products (Fleming, 1984).

Traditional (natural) fermentation and controlled fermentation are two different techniques currently used in commercial cucumber fermentation. In the traditional process, cucumbers are fermented in large open top wooden or fiber glass tanks. Cucumbers are brined in a 5-8% NaCl solution with dry salt added during the fermentation to maintain this concentration constant. The fermented cucumbers are stored in a 8-16% NaCl solution until packing. The high salt concentration used during fermentation provides a selective environment for the growth of natural lactic acid bacteria and helps preserve
textural quality during storage. Fermented cucumbers are usually rinsed and repacked in a freshly prepared brine. The 'spent' brine remaining in the storage tank has a low pH and a high salt concentration (8-18%) which makes its disposal difficult and expensive. In addition, the natural flavors, acids, pigments and nutrients produced during the fermentation are discarded.

*Lactobacillus plantarum, Lactobacillus brevis, Pediococcus cerevisiae, Leuconostoc mesenteroides, Streptococcus faecalis,* and aerobic species i.e. Pseudomonas, Flavobacterium and Achromobacter are frequently found in natural fermentations (Pederson and Ward, 1949). The natural fermentation of cucumbers can be divided into four distinct stages which are initiation, primary fermentation, secondary fermentation, and post fermentation (Fleming, 1982). Various gram positive and gram negative bacteria are prevalent microorganisms during the initiation stage while lactic acid bacteria and yeasts grow during the primary fermentation. During the initiation stage, the growth of Pseudomonas, Flavobacterium and coliform bacteria can impart undesirable flavors to pickles. *Bacillus* sp. produce *H₂S* which causes an undesirable blackening of pickles (Frazier and Westhoff, 1978). *Leuconostoc mesenteroides, Streptococcus faecalis, Pediococcus cerevisiae, Lactobacillus plantarum, Lactobacillus brevis* and fermentative yeasts are found during the primary fermentation stage. The fermentative yeasts continue to grow during the secondary fermentation stage if there are sugars still available. During the primary and secondary fermentation stages, heterofermentative lactic acid bacteria and fermentative yeasts produce large amounts of *CO₂* which can cause bloater damage to the cucumbers. During the post fermentation stage, oxidative yeasts (film yeasts) oxidize acids produced by
lactic acid bacteria which lowers the brine acidity and allows the growth of other spoilage microorganisms (Etchells et al., 1975). Molds with pectolytic enzymes such as Penicillium, Fusarium, Ascochyta, Cladosporium and Alternaria degrade pectin and result in undesirably soft pickles. Frazier and Westhoff (1978) concluded that the traditional fermentation of cucumbers is a variable, complicated and unpredictable process.

The controlled fermentation of cucumbers was introduced in the United States in 1973 (Etchells et al., 1973). The purpose of the controlled fermentation is to obtain a high quality product by means of consistent and predictable fermentations. The initiation and secondary fermentation stages of natural fermentation must be eliminated and undesirable damage such as bloater damage must be prevented. Therefore, the natural flora present in cucumbers must be removed or destroyed. Cucumbers are then inoculated with a desirable pure culture and the fermentation conditions are adjusted to optimize the growth of the inoculated culture.

In 1964, Etchells et al. suggested hot water blanching or gamma ray irradiation of cucumbers as treatment alternatives before pure culture inoculation. Neither technique has been used commercially (Daeschel and Fleming, 1988). The controlled fermentation procedure suggested by Etchells et al. in 1973 is a more practical technique for commercial use. This procedure includes thorough washing of the cucumbers with water, in-container sanitizing with chlorine solution, acidification with vinegar, buffering with either NaOH or sodium acetate, purging with nitrogen to reduce the CO2 content in the brine, and inoculation with species of lactic acid bacteria with
rapid, vigorous growth and acid production (Etchells et al., 1973; Lingle, 1975; Andres, 1977; Wallace and Andres, 1977). Buffering allows the fermentation of all sugars and prevents the secondary fermentation by yeasts. This procedure does not result in a pure culture fermentation, but serves to set the environment to favor growth of the starter culture (Daeschel and Fleming, 1987). While washing removes most of the bacteria adhering to fresh cucumbers and acidification suppresses the growth of the natural microflora during the initiation stage of the natural fermentation, chlorination is not an efficient way to sanitize cucumbers because chlorine activity is reduced by the high concentration of organic compounds (Fleming, 1988).

In 1983, Fleming et al. used a closed-top anaerobic tank and the procedures developed by Etchells et al. (1973) for the fermentation of cucumbers in a closely controlled environment. A closed-top anaerobic tank eliminates the problems caused by the post fermentation stage, since anaerobiosis (achieved by N₂ purging) inhibited the growth of oxidative yeasts and molds. Fleming et al. (1988) found that when cucumbers were inoculated with Lactobacillus plantarum, fermentations in closed-top anaerobic tanks were predominantly homofermentative. Also, fermented cucumbers could be stored at low salt concentrations (2.7 or 4.6%) in a closed-top anaerobic tank for 1 year and still retain an acceptable firmness when Ca salt was added at the beginning of the fermentation (Fleming et al., 1988).

The use of CaCl₂ in cucumber fermentation and storage brine provides for the use of low salt levels (2-5%) by preventing enzymatic and non-enzymatic softening of pickle tissue (Buescher et al., 1979; 1981a,b; Buescher
Calcium binds to pectic substances in the cell wall (Doesburg, 1965; Grant et al., 1973) forming a complex of Ca-pectate that retard softening due to hydrolases (Buescher and Hudson, 1984; Buescher et al., 1979). However, the mechanism of firming does not appear to be completely based on the amount of Ca\(^{++}\) bound to the pectic substances of cell wall material (Buescher and Hudson, 1986). Rather, addition of Ca\(^{++}\) to fresh cucumber tissue was shown to protect against excessive pectin demethylation and subsequent tissue softening of cucumber pickles stored in low salt brines (Buescher et al., 1981a; Hudson and Buescher, 1986). Therefore, the environmental problem caused the spent brine can be reduced and it is also possible to use storage brine as a packing brine for the finished products.

Daeschel et al. (1988) used a cucumber juice brine as a model to simulate the fermentation of cucumbers under controlled conditions. Fresh cucumbers were frozen overnight, partially thawed, homogenized in a blender, boiled, and then rapidly cooled to approximately 25\(\degree\)C. The cucumber juice obtained was sterilized using a 0.2 micron Millipore\textsuperscript{TM} filter (Millipore Corporation, Bedford, MA) and then mixed with a sterilized buffered salt solution. The resulting cucumber juice brine is a good model to simulate the controlled fermentation of cucumbers since it is an homogenous solution free of the natural flora. The fermentation period in cucumber juice brine is also shortened because nutrients are readily available in the solution. The cucumber juice brine is also a useful model to study the flavor quality of fermented cucumbers. Some microorganisms cause physical damage to the fermented fruits and may bias the panelist sensory response to flavor. However, cucumber juice brine is not a perfect model. During the preparation
process, nutrients such as reducing sugars and amino acids may be lost due to the Maillard reaction. The extraction process disintegrates the fruit tissue and might release phenolic compounds that are inhibitory to microbial growth. Niwa et al. (1987) found that fruit juices i.e. orange and grape juices contain a polyphenol, a bacteriostatic substance, which inhibits lactic acid bacteria. The extracted phenolic compounds may also interact with proteins and amino acids (Loomis, 1974) and thus reduce nutrient availability. In addition, the growth ability of some bacteria in cucumber juice brine which provides immediate nutrients might be different from the growth in brined cucumbers in which nutrients need to diffuse out from the fruits.

Lactic acid bacteria

The lactic acid bacteria are immobile, rod-shaped or spherical organisms. The name derives from the fact that ATP is synthesized through fermentations of carbohydrates, which yield lactic acid as a major (and sometimes as virtually the sole) end product (Stanier et al.,1976). Since lactic acid bacteria exhibit very complex nutritional requirements, they are found in environments such as plant materials, milk, and the intestinal tract of animals (Gottschalk, 1979).

Most lactic acid bacteria are strictly fermentative, catalase negative and aerotolerant (facultative anacrobes) which grow readily on the surface of solid media exposed to air. However, they are unable to synthesize ATP by respiratory means, a reflection of their failure to synthesize cytochromes and the other heme-containing enzymes. Although, they can perform limited
oxidations of a few organic compounds, mediated by flavoprotein enzymes, either oxidases or peroxidases, these oxidations are not accompanied by ATP formation (Stanier et al., 1976; Fleming et al., 1985). A number of oxidative reactions, usually catalyzed by flavin enzymes have been found in different species of lactic acid bacteria. Gotz et al. (1980a) found that Lactobacillus plantarum (ATCC 8014) cells consume molecular oxygen when incubated with either glucose, D/L-lactate or pyruvate as substrate. The reactions involved are shown below:

\[
\begin{align*}
3- & \text{ Pyruvate oxidase} \\
\text{Pyruvate} + O_2 + PO_3 & \rightarrow \text{Acetyl phosphate} + CO_2 + H_2O_2 \\
\text{NADH oxidase} & \\
\text{NADH} + H^+ + O_2 & \rightarrow \text{NAD} + H_2O_2 \\
\text{NADH peroxidase} & \\
\text{NADH} + H_2O_2 + H^+ & \rightarrow \text{NAD}^+ + 2H_2O.
\end{align*}
\]

In 1983, Kandler summarized two groups of enzymes responsible for lactate oxidations. The mechanisms for these enzyme groups are:

\[
\begin{align*}
\text{L-lactate oxidase} & \\
\text{Lactate} + O_2 & \rightarrow \text{Pyruvate} + H_2O_2 \\
\text{NAD-independent D-lactate dehydrogenase} & \\
\text{Lactate} + O_2 & \rightarrow \text{Pyruvate} + H_2O_2
\end{align*}
\]

However, the protective mechanisms against oxygen toxicity in lactic acid bacteria is not entirely due to enzymes. Gotz et al. (1980b) found that scavenging of the superoxide radical is performed by manganous ion which is shown in the reaction below:

\[
\begin{align*}
\text{nonenzymatic superoxide reaction} & \\
O_2^- + 2H^+ + Mn^{2+} & \rightarrow H_2O_2 + Mn^{2+}
\end{align*}
\]
Lactic acid bacteria include species from the genera Lactobacillus, Lactococcus, Sporolactobacillus, Streptococcus, Leuconostoc, Pediococcus, and Bifidobacterium. The homofermentative, heterofermentative or the bifidum pathway is employed by these microorganisms for the fermentation of carbohydrates to lactate (Gottschalk, 1979).

**Homofermentative pathway**

Homofermenters convert glucose almost quantitatively to lactic acid. The homofermentative pathway yields 2 moles lactate per mol of glucose. The pathway is illustrated in Figure 1.1. Glucose is degraded via the Embden-Meyerhof pathway to pyruvate which is then used as the H-acceptor. The ATP yield is 2 moles of ATP per mol of glucose (Gottschalk, 1979). Lactic acid bacteria in the genera Sporolactobacillus, Streptococcus, Pediococcus, and some species of the genus Lactobacillus are homofermenters. Homofermenters such as *Lactobacillus plantarum* and *Pediococcus cerevisiae* are always found in the natural fermentation of vegetables. They are very acid-tolerant and are used for pure culture inoculation in many fermented vegetable products such as fermented olives and cucumber pickles. However, Chen et al. (1983) suggested that one potential problem with these fermentations is that the lactic acid flavor developed by homofermentative organisms may be too strong and thus unacceptable to many consumers.
Figure 1.1. Formation of lactate from glucose by the homofermentative pathway. 1, enzymes of the Embden-Meyerhof pathway; 2, lactate dehydrogenase. (from Gottschalk, 1979)
Heterofermentative pathway

Heterofermenters convert glucose to an equimolar mixture of lactic acid, ethanol and CO₂ (Fig. 1.2). Heterofermenters cannot utilize the Embden-Meyerhof pathway since they lack a key enzyme, fructose-diphosphate aldolase, which mediates cleavage of the sugar-phosphate bond (Stanier et al., 1976). As in the oxidative pentose phosphate cycle, ribulose-5-phosphate is formed via 6-phosphogluconate. Epimerization yields xylulose-5-phosphate, which is cleaved by phosphoketolase into glyceraldehyde-3-phosphate and acetyl phosphate. Acetyl phosphate is converted into acetyl-CoA by phosphotransacetylase. Subsequent reduction by acetaldehyde and alcohol dehydrogenase yields ethanol. The glyceraldehyde-3-phosphate formed in the phosphoketolase reaction is converted to lactate as in the homofermentative pathway (Gottschalk, 1979). In this fermentation, 2 NADH₂ are formed and consumed, the ATP yield is one per mole of glucose. The formation of ethanol by enzymes 6, 7, and 8 in Figure 1.2 regenerates 2 NAD⁺ and balances the redox reaction. In some cases, NADH₂ can be oxidized by other oxidizing agents, then acetyl phosphate may be transformed to acetic acid by the enzyme acetate kinase. This reaction yields one more ATP per mole of glucose (Gottschalk, 1979).

Some heterofermentative lactobacilli can ferment glucose aerobically, reoxidizing NADH₂ at the expense of oxygen by means of a flavoprotein enzyme. The overall reaction for glucose fermentation under these conditions
Figure 1.2. Formation of CO₂, lactate, and ethanol from glucose by the heterofermentative pathway. 1, hexokinase; 2, glucose-6-phosphate dehydrogenase; 3, 6-phosphogluconate dehydrogenase; 4, ribulose-5-phosphate 3-epimerase; 5, phosphoketolase. The cleavage reaction yields glyceraldehyde-3-phosphate and enzyme-bound alpha, beta-dihydroxyethylthiamin pyrophosphate. This is converted to acetyl-TPP-E via the alpha-hydroxyvinyl derivative; phosphorylic cleavage results in acetyl phosphate formation. 6, phosphotransacetylase; 7, acetaldehyde dehydrogenase; 8, alcohol dehydrogenase; 9. enzymes as in homofermentative pathway. (from Gottschalk, 1979)
becomes:

\[
\text{Glucose} + \text{O}_2 \rightarrow \text{Lactate} + \text{Acetate} + \text{CO}_2
\]

(Stanier et al., 1976)

Many other heterofermenters of the genera Lactobacillus and Leuconostoc contain mannitol dehydrogenase and produce mannitol as a product of fructose. In this reaction, fructose is reduced to mannitol, NADH₂ is oxidized to NAD⁺, and acetyl phosphate is converted to acetate. The overall equation for the fructose fermentation is:

\[
3 \text{Fructose} \rightarrow \text{Lactate} + \text{Acetate} + \text{CO}_2 + 2 \text{Mannitol}
\]

(Stanier et al., 1976)

Lactic acid bacteria from the genus Leuconostoc and some species in the genus Lactobacillus are heterofermenters. *Leu. mesenteroides* is a heterofermenter frequently found in natural vegetable fermentations. However, its presence is not desirable in some products because of gas damage (e.g. in cucumber pickles, fermented olives and fermented turnips) (Fleming, 1982). However, *Leu. mesenteroides* contributes a desirable flavor in sauerkraut since it produces a high concentration of volatile compounds (Pederson and Albury, 1969). Juhasz et al. (1974) found that cucumbers fermented by *Lactobacillus brevis* had the best flavor among the 52 strains of lactic acid bacteria included in their test. They concluded that the flavor depends more significantly on the ratio of volatile to non-volatile acids, rather than on the total organic acid concentration, with a high ratio being preferred.
Bifidum pathway

The bifidum pathway is found in bacteria of the genus Bifidobacterium. Bifidobacterium bacteria resemble other lactic acid bacteria in several aspects. They are catalase negative and have complex nutritional requirements. They also ferment sugars with the formation of lactic acid as a major end product (Stanier et al., 1976).

The bifidum pathway is shown in Figure 1.3. Two phosphoketolasces are involved in the glucose breakdown process: one specific for fructose-6-phosphate and one specific for xylulose-5-phosphate. Fructose-6-phosphate phosphoketolase splits fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate. Without the participation of hydrogenation and dehydrogenation reactions, 2 moles of glucose are converted into 3 moles of acetate and 2 moles of glyceraldehyde-3-phosphate. The latter is converted to lactate as in the homofermentative pathway. The formation of acetate from acetyl phosphate is coupled to the formation of ATP from ADP which is catalyzed by acetate kinase. The bifidum pathway yields 2.5 moles of ATP per mole of glucose, i.e. a higher ratio than the homo- and heterofermentative pathways.

As compared to other lactic acid bacteria, Bifidobacteria are very new to the fermented vegetable industry. These organisms are present in the intestine of man and of various animals and in honey bees. They are also found in sewage and human clinical material (Scardovi, 1974). Several milk products containing viable cells of Bifidobacteria have been produced (Mutai
Figure 1.3. Formation of acetate and lactate from glucose by the bifidum pathway. 1, hexokinase and glucose-6-phosphate isomerase; 2, fructose-6-phosphate phosphoketolase; 3, transaldolase; 4, transketolase; 5, ribose-5-phosphate isomerase; 6, ribulose-5-phosphate 3-epimerase; 7, xylulose-5-phosphate phosphoketolase; 8, acetate kinase; 9, enzymes as in homofermentative pathway. (from Gottschalk, 1979)
et al., 1978; Kisza et al., 1978; Lang and Lang, 1978; Mutai et al., 1980; Borivosa, 1987; Taylor, 1987). *Lactobacillus bifidus* (now known as *Bifidobacterium bifidum*) was used to ferment fruits and vegetables, which were then freeze-dried for use as a food ingredient (Anonymous, 1971). Schuler (1971) inoculated a nutrient solution with Bifidobacteria culture and suggested its use as an inoculum to ferment cabbage, herrings, or gherkins.

**Malo-lactic fermentation**

Many lactic acid bacteria can ferment malic acid to L-lactic acid. The end product of this pathway is always L-lactate regardless of the lactic acid isomcr produced by the bacteria from the fermentation of carbohydrates (Fleming et al., 1985). Korkes et al. (1950) found an enzyme which transformed malic acid into lactic acid and CO₂. Schultz and Radler (1973) suggested that malate can be converted directly to L-lactate as shown below:

\[
\text{malo-lactic enzyme} \\
\text{L-malic acid} \rightarrow \text{L-lactic acid} + \text{CO}_2 \\
\text{Mn,NAD}
\]

(Schultz and Radler, 1973; Fleming et al., 1985)

This reaction proceeds in the absence of L-lactate dehydrogenase and without free carbonyl compounds acting as intermediates. Enzyme-bound oxaloacetate and pyruvate are probable reaction intermediates (Alizade and Simon, 1973).

The decomposition of malate by malo-lactic enzyme does not yield utilizable energy. However, at low pH when the removal of hydrogen ions is
important for bacterial growth, the decomposition of malic acid to lactic acid enables the bacteria to utilize more sugar and thus favors microbial growth (Radler, 1975). Malo-lactic fermentation is not a necessary pathway for the fermentation of vegetables. Actually, this fermentation may damage some fermented vegetable products, e.g. CO₂ production causes bloater damage in fermented cucumbers (Fleming et al., 1985). Daeschel et al. (1985) found that it is possible to remove the malo-lactic acid pathway from lactic acid bacteria by the use of genetic engineering techniques.

Propionic acid bacteria

In general, Propionibacteria are gram-positive, catalase-positive, non spore-forming, non motile, facultative anaerobic rod-shaped bacteria (Hettinga and Reinbold, 1972a). The nutritional requirements of propionibacteria are very complex. Vitamins, minerals and unknown constituents of yeast extract are required for their growth and metabolism (Hettinga and Reinbold, 1972a). As shown in Figure 1.4, sugars enter the Embden-Meyerhof pathway leading to the formation of propionate, acetate, and CO₂ accompanied by some succinate. Approximately 6 moles of ATP are formed per 1.5 moles of glucose. The formation of succinate is strongly influenced by the content of CO₂ in the growth medium. The glycolytic intermediate, phosphoenol pyruvate, is carboxylated to yield oxaloacetate
Figure 1.4. Reactions of the propionic acid fermentation and the formation of acetate, CO₂, propionate, and ATP. Me-malonyl-CoA is methylmalonyl-CoA and (a) and (b) are the two isomers. FP is flavoprotein and FPH₂ is reduced flavoprotein. (from Allen et al., 1964)
which is subsequently reduced to succinate:

\[
\begin{align*}
\text{CH}_2 & \quad \text{COOH} \\
\text{C}-\text{O}(\text{P}) + \text{CO}_2 & \longrightarrow \text{C}=\text{O} \quad \text{CH}_2 \\
\text{COOH} & \quad \text{COOH} - \text{H}_2\text{O}
\end{align*}
\]

(Stanier et al., 1976).

A preferred substrate of propionibacteria is lactate (Gottschalk, 1979). The production of propionic acid from starch-based media is possible by the use of a mixed culture of Propionibacterium freudenreichii sub sp. shermanii and Lactobacillus amylophilus (Border, 1987). Lactate is initially oxidized to pyruvate which follows the pathway shown in Figure 1.4 to form propionate, acetate and CO\(_2\). The reaction yields only 1 mole of ATP per mole of lactate. The overall fermentation equation is:

\[
3 \text{ Lactate} \longrightarrow 2 \text{ Propionate} + \text{ Acetate} + \text{ CO}_2
\]

(Gottschalk, 1979).

The effect of salt on the growth of Propionibacteria in a lactate substrate seem to be strain specific. At pH 7.0, a 6% salt concentration was required to impede the growth while only 3% was required at pH 5.2. On the other hand, a slow growing strain had greater salt tolerance at pH 5.2 than at 7.0 (Rollman and Sjostrom, 1946).

Propionibacteria play important roles in several industrial processes. They are critical in the development of the characteristic flavor and eye formation in Swiss-type cheeses (Ayres et al., 1980). Propionic acid is a well-known mycostatic agent and plays an important role in extending the shelf
life of dairy and bakery products (Hittinga and Reinbold, 1972a). Propionibacteria are also used to ferment moist grain sorghum and high-moisture corn to yield long-term storage products (Flores-Galarza et al., 1985; Rangaswamy et al., 1974). *Propionibacterium shermanii* is recommended for the desaccharification of egg white. Since it has no proteolytic activity, it does not utilize the egg white, and enriches egg white with vitamin B12 (Stoyanova et al., 1976).

Propionic acid is sometimes mentioned as an undesirable constituent in fermented vegetables. Pederson and Albury (1969) lists n-propionic acid as one of the lower molecular weight fatty acids that cause cheese-like off flavor in sauerkraut. Propionic acid produced by Propionibacteria is also undesirable in green table olives (Rejano Navarro et al., 1978; Gonzalez Cancho et al., 1980). However, Ro et al. (1979) found that the secondary fermentation of fermented kimchi by *Propionibacterium freudenreichii* subsp. *shermanii* produces a good quality kimchi with high vitamin B12. Czarnocka-Roczniakowa et al. (1981) found that inoculation with *Propionibacterium jensenii* increases the concentration of vitamin B12 and folacin in sauerkraut and improve its sensory properties.

Diacetyl and acetoin formation

Diacetyl is best known as the compound responsible for the characteristic flavor of butter (Vedamuthu, 1979). It is produced by some strains of the genera Streptococcus, Lactococcus, Leuconostoc, Lactobacillus, and Pediococcus, as well as by other organisms (Gottschalk, 1979). *Lactococcus*
*lactis* subsp.*lactis* biovar. *diacetylactis* (previously known as *Streptococcus diacetilactis*) is well known for its high production of diacetyl in several milk products (Ayres et al., 1980). A diacetyl concentration level close to 1 mg/kg of butter (1 ppm) is sufficient to obtain good quality products (Oberman et al., 1982). However, Golovnya et al. (1986) recommended that the concentration of diacetyl in distilled water used for the selection of panelists with an ability to recognize the aroma should be 0.001% (10 ppm) which, however, was found too high by panelists.

Not much research has been done on the effect of diacetyl on the quality of fermented vegetables. Horubala (1955) could not increase the diacetyl level in sauerkraut by adding citrate. It has been suggested that some off-flavors detected in orange juice concentrate are due to diacetyl produced by bacteria of the genera *Lactobacillus* and *Leuconostoc* (Murdock et al., 1952).

Diacetyl and acetoin can be produced by three different pathways which are the incomplete oxidation of glucose, citrate degradation, and the butanediol fermentation (Gottschalk, 1979). The pathway for the incomplete oxidation of glucose (Figure 1.5a) is active in most bacilli growing under aerobic conditions with carbohydrates as a substrate. Acetoin and 2,3-butanediol are formed from pyruvate via alpha-acetolactate. During sporulation, acetate is formed from these C4-compounds by the 2,3-butanediol cycle (Figure 1.5b). The acetate thus produced is fed into the tricarboxylic acid cycle.
Figure 1.5. Formation of acetoin and 2,3-butanediol during growth of bacilli on glucose (a) and acetate formation by the 2,3-butanediol cycle during sporulation (b). 1, alpha-acetolactate synthase, a thiamin pyrophosphate-containing enzyme; 2, alpha-acetolactate decarboxylase; 3, 2,3-butanediol dehydrogenase; 4, acetoin dehydrogenase; 5, diacetylcarbinol synthase; 6, diacetylcarbinol reductase; 7, acetylbutanediol hydrolase. (from Gottschalk, 1979)
The citrate degradation pathway for the anaerobic breakdown of citrate involves the citrate lyase enzyme present in enterobacteria and in lactic acid bacteria. The acetate formed by the citrate lyase reaction is excreted, and oxaloacetate is decarboxylated to yield pyruvate (Figure 1.6). Diacetyl synthesis requires the conversion of pyruvate into C2-compounds. Diacetyl synthesis is accomplished by the reaction of acetyl-CoA with 'active acetaldehyde' (enzyme-bound hydroxy ethyl-TPP) (Jonsson and Pettersson, 1977; Gottschalk, 1979). Lactic acid bacteria with pyruvate dehydrogenase multienzyme complex can synthesize acetyl-CoA from pyruvate, while lactic acid bacteria with pyruvate oxidase and lactate oxidase enzymes produce acetyl phosphate and lactate from pyruvate. Lactic acid bacteria with the pyruvate-formate lyase system produce ethanol and formate from pyruvate. The intermediate for diacetyl formation has not been fully identified (Vedamuthu, 1979; Cogan, 1985). In 1963, Seitz et al. suggested alpha-acetolactate as the precursor for diacetyl formation. However, Speckman and Collins (1968) found that only acetyl-CoA intermediate can be used for diacetyl biosynthesis in Streptococcus diacetilactis and Leuconostoc citrovorum. The work by Jonsson and Pettersson (1977) who studied the metabolic pathway for diacetyl production in Streptococcus diacetilactis and Lactobacillus cremoris tend to support the finding by Speckman and Collins (1968).

The retention of synthesized diacetyl is difficult because bacteria reduce diacetyl to acetoin, a flavorless compound (Vedamuthu, 1979). Acetoin dehydrogenase (diacetyl reductase) reduce diacetyl to acetoin which is then reduced to 2,3-butanediol by the enzyme 2,3-butanediol dehydrogenase. The
Figure 1.6. Biosynthetic pathway among dairy lactic acid streptococci for the production of diacetyl and its reduction products from citric acid.

A - Citratase; B - Oxaloacetate decarboxylase;
C - Pyruvate decarboxylase; D - Alpha acetate synthetase;
E - Diacetyl reductase; F - Alpha acetate decarboxylase;
G - 2,3 butanediol dehydrogenase.

Broken line represents the step on which disagreement exists in the literature. It is thought of either as a nonenzymatic reaction or an enzymatic step catalyzed by alpha-acetolactate oxidase.

(from Vedamuthu, 1979)
selection of diacetyl reductase-negative mutants or variants with low reductase activity is a possible but tedious process (Vedamuthu, 1979).

The butanediol fermentation is usually found in species of the genera Enterobacter, Serratia and Erwinia. In this pathway, hexose is broken down to pyruvate via the Embden-Meyerhof pathway (Figure 1.7). In the presence of pyruvate-formate lyase, ethanol and formate are formed from pyruvate. In the presence of alpha-acetolactate synthase, pyruvate is decarboxylated to form alpha-acetolactate, which is then decarboxylated to yield acetoin. In addition, a small amount of lactate can be formed from pyruvate.

Unfortunately, acetoin can not be oxidized back to diacetyl, therefore only the citrate degradation pathway will yield diacetyl. Furthermore, citrate is a good substrate for diacetyl production because it yields pyruvate without the production of NADH. The pyruvate biosynthesis from hexose produces NADH which is then oxidized in other reactions not leading to diacetyl formation. Montville et al. (1987) reported that glucose and lactose lower diacetyl-acetoin synthesis in many strains of Lactobacillus plantarum. However, Drinan et al. (1976) found that some strains of Lactobacillus plantarum and Streptococcus diacetilactis can produce acetoin in a modified MRS medium in the absence of citrate. Furthermore, one Lactobacillus plantarum strain produced diacetyl in the same medium.

Sensory descriptive analysis

Descriptive analysis is a method of sensory evaluation which utilizes a highly trained panel to identify, describe and quantitate the sensory attributes
Figure 1.7. Butanediol fermentation. 1, enzymes of the Embden-Meyerhof pathway; 2, lactate dehydrogenase; 3, pyruvate-formate lyase; 4, formate-hydrogen lyase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, alpha-acetolactate synthase; 8, alpha-acetolactate decarboxylase; 9, 2,3 butanediol dehydrogenase. (adapted from Gottschalk, 1979)
of a product (Stone and Sidel, 1985). Descriptive data can be used in several applications: (i) interpretation of other sensory data, (ii) correlation with instrumental measurements, (iii) quality monitoring, (iv) product development and maintenance (Civilie and Lawless, 1986). The main components of descriptive analysis includes: (i) characteristics - the qualitative aspect, (ii) intensity - the quantitative aspect, (iii) order of appearance - the time aspect, (iv) overall impression - the integrated aspect (Meilgaard et al., 1987). The most commonly used descriptive test methods are the Flavor Profile (Cairncross and Sjostorm, 1950; Caul, 1957), the Texture Profile (Brandt et al., 1963), the Quantitative Descriptive Analysis (QDA) (Stone et al., 1974; Stone and Sidel, 1985), time-intensity descriptive analysis (Neilson, 1957) and the SPECTRUM™ method (Meilgaard et al., 1987). Some of these methods have gained and maintained popularity as standard methods. One should review several methods and combinations of methods before selecting the descriptive analysis system which can provide the most comprehensive, accurate, and reproducible description of each product and the best discrimination between products (Meilgaard et al., 1987).

Traditionally, descriptive analysis methods utilize a small panel of 5 -10 highly trained subjects (Meilgaard et al., 1987). Panelists must be able to detect and describe the perceived qualitative sensory attributes of a sample. These qualitative aspects of a product combine to define the product and include all of the appearance, aroma, flavor, texture, or sound properties of a product which differentiate it from others (Meilgaard et al., 1987). The terminology used in describing perception of character notes must permit (i) differentiation from similar sensation, (ii) identification of the object it
describes, (iii) recognition of the object by others seeing the term and (iv) validation by convergence (e.g. when different scientists in different laboratories converge in similar descriptive systems, one can infer a high degree of validity in the system) (Civille and Lawless, 1986). In addition, panelists must learn to differentiate and rate the quantitative or intensity aspects of a sample and to define to what degree each characteristic or qualitative note is present in that sample. Order of appearance and overall impression may be noted in some descriptive test methods (Meilgaard et al., 1987).

Sensory studies of cucumber pickles

Most sensory studies of pickle cucumbers involve rating of properties such as shape and texture. Ennis and O'Sullivan (1979) developed multiple regression equations for the overall qualities of cucumbers before and after brining by considering the sensory scores of some characteristics such as color, shape, seed size, firmness, etc. as independent variables for the equations; they used six experienced judges to evaluate some characteristics such as color appeal on 12.7 cm (5 in) line scale. They found that it is impossible to predict the quality of brined cucumbers from the quality of the fresh cucumbers by considering only these parameters. Fleming et al. (1978) used a taste panel of five members to evaluate physical properties of sliced fermented cucumbers including texture, seed area and color. The texture was evaluated quantitatively by using a 10-point scale (9-10 = excellent; 1-2 = unacceptable). The seed area and color are qualitatively described by using different terms such as soft and firm for seed area, and straw, light, raw and
pale for color. The relationship between sensory and instrumental textural measurements in different kinds of cucumber pickles has been studied by several researchers (Jeon et al., 1975a; Jeon et al., 1975b; Rodrigo and Alvarruiz, 1988).

Relatively, little research has been done on the sensory flavor quality of fermented cucumbers. Most sensory studies on the flavor of cucumber pickles have been done by expert panels using terms that are sometimes either hard to recognize or too subjective. Aurand et al. (1965) used an experienced panel (number was not mentioned) to describe flavor and odor characteristics of pure-culture fermented cucumbers by different descriptors such as clean, raw cucumber, acid, aromatic, musty, hay-like, fruity, pleasant, etc. Etchells et al. (1968) used a panel of 6-29 judges to qualitatively evaluate cucumber pickles fermented by different microorganisms; the judges used descriptors such as good, fair or poor in their evaluation. Terms such as hay-like off-flavor and acid were used to qualitatively described the quality of sliced, fermented cucumbers by a taste panel of five (Fleming et al., 1978).

By using 315 panelists, James and Buescher (1983) conducted a preference test using whole dill pickles obtained from 15 different companies (9 point hedonic scalar technique where 1=dislike extremely, 5=neither like nor dislike, 9=like extremely). The mean preference scores were in the range of 4.6-6.7 and there were significant differences in preference among the samples. They also found that the mean preference scores were not significantly correlated with NaCl levels, acetic acid levels or texture measurements.
2. Lactate and Propionate Fermentation of Cucumber Juice Brine

ABSTRACT

_Pediococcus cerevisiae, Lactobacillus casei, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactococcus diacetylactis, Bifidobacterium bifidum, Leuconostoc oenos_, and a mixed of _Propionibacterium shermanii_ and _P. cerevisiae_ culture were used to ferment cucumber juice brine (CJB) at 22-26°C for 1.5 months. Sugar utilization levels were 58%, 24%, 86%, 83%, 15%, 28%, 82% and 64%, respectively. Fermentation balances accounted for 64 to 105% of available carbon. CO₂ production ranged from 40 to 60 mg/100ml CJB except for Leuconostoc genera which produced from 120 to 180 mg/100ml CJB. Final pH values were in the 3.24-4.12 range and titratable acidity ranged from 0.30 to 0.93%. All the microorganisms tested utilized malic and citric acids; however, _Leu. mesenteroides_ and _Leu. oenos_ did not utilize citric acid for diacetyl-acetoin production. The final fermentation broths contained different concentrations of acetic, propionic and lactic acids. Heterofermenters produced end products with a high volatile/nonvolatile acid ratio and high ethanol and mannitol concentrations.

INTRODUCTION

The natural fermentation of cucumbers (_Cucumis sativus_) can be divided into four distinct stages: initiation, primary fermentation, secondary fermentation and post fermentation (Fleming, 1982). The natural
fermentation of cucumbers is a variable, complicated and unpredictable process (Frazier and Westhoff, 1978). Several defects are found in fermented cucumbers produced by natural fermentations. Undesirable flavor and color are caused by gram-negative and gram-positive bacteria during the initiation stage (Frazier and Westhoff, 1978). Residual sugars remaining after the primary stage are fermented by yeasts during the secondary stage which results in CO2 production and subsequent bloater damage (Etchells et al., 1973). Bloater damage can also be caused by lactic acid bacteria with heterofermentative and malo-lactate pathways. During the post fermentation stage, growth of molds and film yeasts results in fruit softening and acid oxidation, respectively (Etchells et al., 1973). These defects have been minimized by the use of high salt concentrations during cucumber fermentation (5-8%) and storage of fermented cucumbers (8-18%). The primary function of high salt storage was to retard softening of pickles during brine storage (salt stock). Processing into a finished product required disposal of the storage brine (spent brine) and desalting of the salt stock pickles by holding and washing in several volumes of fresh water. This process results in a serious problem of disposal of large quantities of high chloride waste water. In addition, the natural flavors, acids, pigments and nutrients produced during the fermentation are discarded.

The use of CaCl2 in cucumber fermentation and storage brines provides for the use of low salt levels (2-5%) by preventing enzymatic and non-enzymatic softening of pickle tissue (Buescher et al., 1979, 1981a,b; Hudson and Buescher, 1980, 1984). Calcium binds to pectic substances in the cell wall (Doesburg, 1965; Grant et al., 1973) forming a complex of Ca-pectates
that retard softening due to hydrolases (Buescher and Hudson, 1984; Buescher et al., 1979). However, the mechanism of firming does not appear to be completely based on the amount of Ca\(^{++}\) bound to the pectin substances of cell wall material (Buescher and Hudson, 1986). Rather, addition of Ca\(^{++}\) to fresh cucumber tissue was shown to protect against excessive pectin demethylation and subsequent tissue softening of cucumber pickles stored in low salt brines (Hudson and Buescher, 1985; 1986).

The controlled fermentation of cucumbers using closed tank and pure culture inoculation techniques tends to prevent the initiation, secondary and post fermentation stages and provide maximum control of the primary fermentation. The initiation stage can be minimized by prewashing the fruits before brining. The secondary fermentation is minimized when the inoculated pure culture utilizes all available sugar during the primary fermentation stage. The post fermentation stage is eliminated when fermentation and storage occur in a closed top tank. Nitrogen-purging, used during the fermentation, removes carbon dioxide from the brine and prevents bloater damage. Daeschel and Fleming (1987) have shown that even though controlled fermentation techniques do not result in a pure culture fermentation, they serve to set the environment to favor the growth of the starter culture.

When 0.2-0.5% CaCl\(_2\) is added to the brine at the beginning of the fermentation, it is possible to ferment and store cucumbers in lower salt concentration (3-5%) under the controlled fermentation in a closed-top anaerobic tank (Buescher et al., 1981a,b; Fleming et al., 1988). It is also
possible to use the same brine as a packing brine for the fermented cucumbers; this can reduce the environmental problems caused by the disposal of the spent brine. Since the packing brine is also consumed by consumers, it is, therefore, necessary to investigate the impact of the composition of fermentation brines on the flavor of the finished pickle product. Where in the past, the only concern about lactic acid bacteria was production of high acid for the sole purpose of preservation, now the ability of selected bacteria to produce specific chemical profiles in the finished product needs to be investigated.

Homofermenters such as *Lactobacillus plantarum*, *Pediococcus cerevisiae* have been used for the controlled fermentation of vegetables such as cucumbers because of their low carbon dioxide production and high sugar utilization ability. Chen et al. (1983) suggested that the lactic acid flavor developed by these homofermentative organisms may be too strong to be desirable to a large number of consumers. Furthermore, the ratio of volatile to non-volatile acids has been shown to affect the flavor of fermented vegetables more significantly than the total acid concentration. For example, Juhasz et al. (1974) found pickles with the best flavor quality contained a higher ratio of volatile to non-volatile acids.

Many strains of the genera Streptococcus, Leuconostoc and Pediococcus produce diacetyl. Furthermore, it has been suggested that some off-flavors detected in orange juice concentrate are due to diacetyl produced by lactic acid bacteria (Murdock et al., 1952). Diacetyl and acetoin can be produced by three different pathways which are the incomplete oxidation of
glucose, citrate degradation and the butanediol fermentation (Gottschalk, 1979). Not much research had been done on the concentration of diacetyl in fermented vegetables and its effect on product quality. In an earlier report, Horubala (1955) showed that diacetyl levels in sauerkraut were not increased by citric acid addition.

Metabolic pathways found in bacteria not currently being used for the fermentation of cucumbers may lead to end products with different or better flavor quality. Heterofermenters, bifidobacteria and propionibacteria are acid-producing bacteria found or used in many fermented food products (Pederson and Albury, 1969; Mutai et al., 1978; Ro et al., 1979; Czarnocka-Roczniakowa et al., 1981). In addition to lactic acid, heterofermenters produce acetic acid, ethanol, mannitol and CO₂ (Gottschalk, 1979). On the other hand, bifidobacteria produce lactic acid and acetic acid from glucose (Gottschalk, 1979) while propionibacteria produce CO₂, acetate and propionate from either glucose or lactic acid (Allen et al., 1964) with the latter being a preferred substrate (Gottschalk, 1979). Since information on the use of these bacteria for the fermentation of cucumber pickles is still limited, the goal of this study was to evaluate their utilization for the controlled fermentation of cucumbers. Pure culture fermentation conditions were facilitated by the use of a cucumber juice brine (CJB) as a model system for cucumber fermentations (Daeschel et al., 1988). This approach allows a more uniform and shorter fermentation because CJB is an homogeneous system. The fermentation parameters analyzed in this study were microbial counts, sugar utilization, acid production and CO₂ production. The final
fermentation broth was further analyzed to determine its sugar and organic acid composition as well as its concentration of diacetyl, acetoin and ethanol.

MATERIALS & METHODS

Cultures and inoculum preparation

*Propionibacterium freudenreichii* sub sp. *shermanii* (ATCC 13673) (*Prop. shermanii*), *Bifidobacterium bifidum* (ATCC 11147) (*B. bifidum*), *Leuconostoc oenos* (ATCC 27307) (*Leu. oenos*) were obtained from the American Type Culture Collection (Rockville, MD). *Lactobacillus plantarum* sub sp. *arabinosus* (Midwest 220) (*L. plantarum*) was obtained from the Midwest Culture Service (Terre Haute, IN). *Pediococcus cerevisiae* (AFERM 772) (*P. cerevisiae*) and *Lactobacillus casei* (AFERM 771) (*L. casei*) are cultures currently being used for pickled vegetables (Microlife Technics, Sarasota, FL). *Leuconostoc mesenteroides* 98 (*Leu. mesenteroides*) and *Lactococcus lactis* sub sp. *lactis* bio var. *diacetylactis* 1816 (*Lac. diacetylactis*) (previously known as *Streptococcus diacetilactis*) were obtained from the Dept. of Microbiology at Oregon State University (Corvallis, OR).

*P. cerevisiae* and *L. casei* cultures were received in frozen concentrate form. These cultures were then thawed, weighed, diluted with 10 ml of 0.85% NaCl solution and used directly as inocula. The initial inoculum was at least $10^6$ cfu/ml CJB.
*L. plantarum, Leu. mesenteroides* and *Lac. diacetylactis* cultures were received as streak cultures on agar slants. *Prop. shermanii, B. bifidum* and *Leu. oenos* cultures were received in freeze-dried form. All cultures were transferred to MRS broth (Difco, Detroit, MI) except for *Prop. shermanii* which was transferred to sodium lactate broth prepared as described by Schwab et al. (1984) and then incubated at 30°C for 24-48 h before being transferred to a 60% glycerol solution for storage at -40°C.

*L. plantarum, Leu. mesenteroides, Lac. diacetylactis, B. bifidum* cultures were transferred to MRS broth and incubated at 30°C for 24 h. A 48 h incubation period at 30°C was used for *Leu. oenos*. *Prop. shermanii* was grown in sodium lactate broth at 30°C for 72 h. All broths were then centrifuged at 2603 x g for 10 minutes, rinsed twice with a 0.85% NaCl solution, centrifuged and finally diluted with 10 ml of the salt solution before inoculation. The initial inoculum for each microorganism was at least 10⁶ cfu/ml CJB.

Cucumber juice brine (CJB) preparation

The CJB preparation method was a modification of the procedure described by Daeschel et al. (1988). Fresh pickling cucumbers (Pioneer variety) were obtained from Steinfeld’s Products Co., Portland, OR. Defect-free fruits were washed with tap water, stored overnight at -23°C in a covered 18 liter (5 gal) plastic pail and then partially thawed at room temperature for 4-5 h. The partially thawed cucumbers were disintegrated to a homogeneous slurry in a hammermill (Model D Comminuting Machine, The
W.J. Fitzpatrick Company, Chicago, IL) using a solid screen size 0.42 cm (3/8 in) diameter. Juice was extracted from the slurry in a 15 cm (6 in) diameter fruit finisher with a solid screen size 0.05 cm (0.02 in) diameter. The extracted juice was brought to boiling in a 74 liter (20 gal) steam jacket kettle and cooled down in a 3°C cold room in a covered 37 liter (10 gal) stainless steel pail (6 h, final temperature ca. 5°C). The juice was then filtered through cheese cloth and diluted with a buffered salt brine (1:1 ratio). The buffered brine contained NaCl, CaCl₂, acetic acid and NaOH to yield after dilution a CJB with 2.5% NaCl, 0.2% CaCl₂, 0.1% acetic acid and pH 5.0. CJB was then filtered through VWR qualitative filter paper grade 617 (VWR Scientific, San Francisco, CA) and sterilized using a hollow fiber ultrafiltration cartridge (Romicon PM 500) with an effective area of 4500 sq cm (5 sq ft) and a 500,000 MW cut off (Romicon, Woburn, MA). The filtration process was done following recommended operating instructions (Anonymous, 1983).

Fermentation and chemical analysis

Each microorganism was first tested for its ability to ferment glucose, fructose and mannitol in 48 h using the API™ CHL kit (Analytab Products, Plainview, NY). The test was performed by following the recommended procedure (Anonymous, 1987a) at the recommended temperature of 37°C and also at 30°C.

All microorganisms were used as a pure culture except for a mixed culture of Prop. shermanii and P. cerevisiae. CJB (8.5 liter) was fermented
at room temperature (22-26°C) for 1.5 months in a sterile 10-liter screw cap glass bottle (Pyrex Corning™). A rubber septum and a swinnex disc filter holder with a 0.45 micron Millipore™ filter (diameter 13 mm) (Millipore Corporation, Bedford, MA) were attached to the screw cap (Figure 2.1). The screw cap and attachments were sterile. The filter holder was connected to a Teflon tubing (ca. 7.5 cm long) which was normally kept close with a host clamp. During the fermentation, the Teflon tubing was opened to allow sterile air ventilation while 20 ml CJB samples were removed through the rubber septum. These samples were immediately used for the determination of microbial counts, pH, titratable acidity and CO2 concentration.

*P. cerevisiae, L. casei, L. plantarum, Leu. mesenteroides, Lac. diacetylactis* and *B. bifidum* were enumerated on MRS agar at 30°C after a 48 h incubation period except for *Leu. oenos* which required 6 days. Preliminary tests showed that both *Prop. shermanii* and *P. cerevisiae* grew on sodium lactate agar; however, only *P. cerevisiae* grew on MRS agar. Therefore, in the case of mixed cultures of these two microorganisms, the counts on sodium lactate agar incubated anaerobically (Gas Pak, BBL) (BBL, Cockeysville, MD) for 5 days at 30°C represented total counts, while *P. cerevisiae* counts were obtained from MRS agar incubated aerobically at 30°C for 48 h. pH was measured using a microprocessor pH/mV meter (Orion model 811) equipped with a combination pH electrode (Ross model 81550). Titratable acidity was determined as percentage lactic acid following the
Figure 2.1. Fermentation bottle with attachments
method described by Fleming (1984). Carbon dioxide was analyzed using the microdiffusion method (Fleming et al., 1974).

At the end of the fermentation, bacterial cells in the CJB were removed by vacuum-filtration through a 2.5 cm thick layer of Celite (Manville Products Corporation, Lompoc, CA) placed in between 2 pieces of filter paper (VWR 617) on a 30 cm diameter Buchner funnel. The filtered samples were kept frozen at -40°C.

Sugar and alcohol analysis

Reducing sugars were determined using the colorimetric method described by Nelson (1944). Glucose, fructose and mannitol were analyzed by high performance liquid chromatography (HPLC) using an Aminex HPX-87C monosaccharide analysis column as described by Pilando (1986). The analysis and quantitation was based on a modification of the procedure described by Hong and Wrolstad (1986). Samples were adjusted to pH 7 using NaOH, passed through a C18 SEP-PAK column (Waters Associates Milford, MA), and then through an AG1-X8 anion exchange resin column (Bio-Rad, Richmond, CA). Finally, samples were mixed with an internal standard (0.1% sorbitol) and then filtered through a 0.45 m Millipore filter prior to HPLC injection.

Ethanol was analyzed enzymatically using an enzymatic assay kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The analysis was done following the recommended procedure (Anonymous, 1987c).
Volatile/nonvolatile acids ratio

Total volatile acidity was analyzed using a cash electric still (Gowans, 1964). The total volatile acidity was determined as acetic acid. Total nonvolatile acidity, determined as lactic acid, was obtained by subtracting volatile acidity from titratable acidity. It should be noted that the volatile/nonvolatile acids ratio may be affected by the sample pH and the pKa of each acid in the fermentation broth.

Organic acid analysis

Five ml of fermented CJB was passed through a C18 SEP-PAK column (Waters Associates Milford, MA) and then filtered through 0.45 m Millipore® filter before enzymatic analysis of malic and citric acid using enzymatic assay kits (Boehringer Mannheim Biochemicals, Indianapolis, IN) and following recommended procedures (Anonymous, 1986; Anonymous, 1987b).

Other organic acids were analyzed by Gas Chromatography using a hydrogen flame ionization detector (FID). Samples were prepared as described in Supelco GC Bulletin 748 H (Anonymous, 1985). Volatile acids were directly extracted with ethyl ether while nonvolatile acids were esterified with methanol before being extracted with chloroform. A 1.8 m x 0.3 cm (6' x 1/8") OD nickel column was packed with GP 10% SP-1000/1% H₃PO₄ on 100/120 Chromosorb. GC operating conditions were: column
temperature 85-160°C increasing at 4°C/min for volatile acid analysis and at 2°C/min for nonvolatile acid analysis; injector, 180°C; detector, 220°C; and, He gas flow rate, 25 ml/min. Under these operating conditions, detectable volatile acids included formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and heptanoic acid; while nonvolatile acids included pyruvic, lactic, oxaloacetic, oxalic, methylmalonic, malonic, fumaric and succinic acid. A preliminary qualitative test on all fermentation broths indicated that butyric acid could be used as an internal standard for volatile acid analysis while malonic acid could be used as an internal standard for nonvolatile acid analysis.

Diacetyl-Acetoin analysis

Diacetyl-acetoin was analyzed using the colorimetric method described by Westerfeld (1944). Diacetyl was analyzed by a method described by Byer (1954).

Carbon recovery

Carbon recoveries were calculated from modifications of the formula obtained by Chen et al. (1983):

\[
\text{Homofermentative and heterofermentative pathways:}
\]

\[
\text{Carbon recovery } = \frac{(L_f-L_i) + (A_f-A_i) + (E_f-E_i) + 2(M_f-M_i)}{2(R_i-R_f) + (M_A-M_f)} \times 100
\]
Bifidum pathway:

Carbon recovery = \frac{(L_f - L_i) + (A_f - A_i)}{2.5(R_i - R_f) + (MA_i - MA_f)} \times 100

Mixed cultures:

Carbon recovery = \frac{(L_f - L_i) + (A_f - A_i) + (E_f - E_i) + 2(M_f - M_i) + (P_f - P_i)}{2(R_i - R_f) + (MA_i - MA_f)} \times 100

where \( L \) = lactic acid, \( A \) = acetic acid, \( E \) = ethanol, \( M \) = mannitol, \( P \) = propionic acid, \( R \) = reducing sugar and \( MA \) = malic acid. The subscripts \( i \) and \( f \) indicate the initial and final molar concentrations of each compound.

Statistical analysis

The difference between the means of chemical analysis data were tested for their significance at \( p \leq 0.05 \) using a Fisher's Protected Least Significant Difference (FPLSD) procedure (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

Fermentation analysis

Microbial counts for the various fermentations are shown in Figure 2.2. The microbial counts for CJB fermented by a mixed culture of Prop. shermanii and P. cerevisiae is shown as a plot for the growth of both bacteria (total counts) and a plot of P. cerevisiae. Preliminary studies
Figure 2.2 Microbial counts during fermentation
Note: Total count = Count for Prop. shermanii and P. cerevisiae
showed that a pure culture of *Prop. shermanii* could not grow in CJB (data not shown). However, as a mixed culture with *P. cerevisiae*, *Prop. shermanii* grew in CJB producing low level of propionic acid utilizing lactate produced by *P. cerevisiae* as the probable substrate (Fig. 2.2; Table 2.1) (Gottschalk, 1979). However, this observation is not supported by a significant decrease in lactic acid as compared to the *P. cerevisiae* treatment (Table 2.1). Furthermore, *Prop. shermanii* counts are not detected after the 15th day of fermentation (Fig. 2.2) when the medium pH reached 3.5 (Fig. 2.3).

Therefore, if this bacterium is found to be desirable for the fermentation of cucumbers, it would need to be used either very early during the fermentation or the fermentation broth would need to be pH-adjusted. *L. casei*, *Leu. mesenteroides* and *Lac. diacetylactis* counts drop drastically after 5-10 days of fermentation (Fig. 2.2). Fermentations by these organisms also stop around that time as shown by pH measurements (Fig. 2.3) and acid production (Fig. 2.4). This could reflect low acid tolerance of these bacteria. Although *L. casei* (AFERM 771) is the recommended strain for the production of pickled vegetables, its main function in commercial fermentations could be rapid reduction of the fermentation pH and thus establishing selective conditions for the growth of desirable natural flora. *Leu. mesenteroides* is found in most fermented vegetables at the beginning of the fermentation (Pederson and Albury, 1954; Stamer, 1988). *Lac. diacetylactis*, a bacterium not usually found in vegetable fermentations, is used in many dairy products as an aroma producer and not as an acid producer (Ayres et al., 1980).
Table 2.1: pH, acid and alcohol concentrations in fermented cucumber juice brine (CJB)\(^1,2\) after 1.5 months

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Titratable pH acidity(%)(^3)</th>
<th>Acetic acid(%)(^4)</th>
<th>Proplonic acid(%)</th>
<th>Lactic acid(%)</th>
<th>Vol/Nonvol acids(^5)</th>
<th>Malic acid(%)</th>
<th>Mannitol (%)</th>
<th>Ethanol(^6) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture(^7)</td>
<td>3.48e 0.67b</td>
<td>0.016c</td>
<td>0.016a</td>
<td>0.69b</td>
<td>0.20e</td>
<td>ND</td>
<td>ND</td>
<td>0d</td>
</tr>
<tr>
<td>(P.) cerevisiae</td>
<td>3.45e 0.66b</td>
<td>0.013cd</td>
<td>ND</td>
<td>0.65b</td>
<td>0.16f</td>
<td>ND</td>
<td>ND</td>
<td>0d</td>
</tr>
<tr>
<td>(L.) casei</td>
<td>3.98c 0.35f</td>
<td>0.004e</td>
<td>ND</td>
<td>0.36cd</td>
<td>0.39c</td>
<td>ND</td>
<td>ND</td>
<td>0d</td>
</tr>
<tr>
<td>(L.) plantarum</td>
<td>3.24f 0.93a</td>
<td>0.007de</td>
<td>ND</td>
<td>0.92a</td>
<td>0.11g</td>
<td>ND</td>
<td>ND</td>
<td>0.0073c</td>
</tr>
<tr>
<td>(Leu.) mesenteroides</td>
<td>3.82d 0.50c</td>
<td>0.048b</td>
<td>ND</td>
<td>0.40c</td>
<td>0.52b</td>
<td>ND</td>
<td>0.29a</td>
<td>0.0940a</td>
</tr>
<tr>
<td>(Lac.) diacetylactis</td>
<td>4.12a 0.30g</td>
<td>0.008de</td>
<td>ND</td>
<td>0.28e</td>
<td>0.50b</td>
<td>ND</td>
<td>ND</td>
<td>0d</td>
</tr>
<tr>
<td>(B.) bifidum</td>
<td>3.84d 0.39e</td>
<td>0.0012cd</td>
<td>ND</td>
<td>0.39c</td>
<td>0.32d</td>
<td>ND</td>
<td>ND</td>
<td>0d</td>
</tr>
<tr>
<td>(Leu.) oenos</td>
<td>4.07b 0.43d</td>
<td>0.089a</td>
<td>ND</td>
<td>0.31de</td>
<td>0.94a</td>
<td>ND</td>
<td>0.25b</td>
<td>0.0820b</td>
</tr>
</tbody>
</table>

\(^1\) Mean of two replications; means within columns followed by the same letter are not significantly different (\(p > 0.05\))

\(^2\) Unfermented CJB (pH 5.02) contained: titratable acidity 0.14%; malic acid 0.14%; ethanol 0.0024%.

\(^3\) As lactic acid

\(^4\) Value of acetic acid was adjusted by subtracting the value of added acetic acid in unfermented CJB (0.082%).

\(^5\) Value of volatile acid was adjusted by subtracting the value of volatile acid in unfermented CJB (= 0.041) before calculating the vol/nonvol acids ratio.

\(^6\) Value was adjusted with percent ethanol found in unfermented CJB.

\(^7\) \(Prop.\) shermanii and \(P.\) cerevisiae

\(^8\) Not-detected
Figure 2.3  pH of cucumber juice brine during fermentation
Figure 2.4 Acid production during fermentation
P. cerevisiae, L. plantarum, B. bifidum and Leu. oenos counts do not drop drastically during fermentation (Fig. 2.2) which indicated that these bacteria are more acid tolerant. P. cerevisiae and L. plantarum have been found in many fermented vegetables with L. plantarum being responsible for completing most fermentations (Pederson and Albury, 1954; 1956). B. bifidum has been used in cultured milk products as an acid producer (Kosikowska, 1978). Lafon-Lafourcade et al. (1983) attributed the presence of Leu. oenos in wine fermentations to its high acid tolerance.

Carbon dioxide production in homofermenters and bifidobacteria ranged from 40 to 60 mg/100ml CJB (Fig. 2.5). The CO2 production could be explained by the malic acid degradation ability of these bacteria (Table 2.1). The disappearance of malic acid initially present in CJB (0.14%) suggests that the malo-lactic fermentation pathway was used by these microorganisms to produce CO2 (Table 2.1). CO2 produced by Leu. mesenteroides and Leu. oenos might be attributed to the malo-lactic fermentation and the heterofermentative pathways. This would explain the high levels of CO2, 120 to 180 mg CO2 /100 ml CJB, measured in these samples (Fig. 2.5). Microbial production of high CO2 levels in fermented cucumbers has been related to bloater damage. N2 purging during the controlled fermentation of cucumbers should be able to overcome this problem (Etchells et al., 1965).

None of the bacteria investigated in this study utilized all the sugars available in CJB (Table 2.2). Only L. plantarum, Leu. mesenteroides and Leu. oenos utilized more than 80% of the available sugars. Glucose was a
Figure 2.5 Carbon dioxide production during fermentation
Note: Mixed culture = *Prop. shermanii* and *P. cerevisiae*
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Reducing sugar(%)</th>
<th>% sugar utilization</th>
<th>Glucose (%)</th>
<th>Fructose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture(^4)</td>
<td>0.46e</td>
<td>63.5c</td>
<td>ND(^5)</td>
<td>0.41c</td>
</tr>
<tr>
<td><em>P. cerevisiae</em></td>
<td>0.53d</td>
<td>57.9d</td>
<td>ND</td>
<td>0.47b</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>0.96b</td>
<td>24.0f</td>
<td>0.40b</td>
<td>0.59a</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>0.18g</td>
<td>86.1a</td>
<td>ND</td>
<td>0.16e</td>
</tr>
<tr>
<td><em>Leu. mesenteroides</em></td>
<td>0.22f</td>
<td>83.0ab</td>
<td>ND</td>
<td>0.24d</td>
</tr>
<tr>
<td><em>Lac. diacetylactis</em></td>
<td>1.08a</td>
<td>14.6g</td>
<td>0.52a</td>
<td>0.58a</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>0.91c</td>
<td>28.0e</td>
<td>0.46ab</td>
<td>0.49b</td>
</tr>
<tr>
<td><em>Leu. oenos</em></td>
<td>0.23f</td>
<td>82.0b</td>
<td>0.25c</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\)Mean of two replications; means within columns followed by the same letter are not significantly different (p > 0.05)

\(^2\)Sugar content of unfermented CJB were:
- reducing sugar 1.27%; sucrose 0%; glucose 0.70%; fructose 0.61%.

\(^3\)Calculation was based on % reducing sugar of unfermented CJB.

\(^4\) *Prop. shermanii* and *P. cerevisiae*.

\(^5\)Not-detected
preferred carbohydrate source for *P. cerevisiae, L. plantarum,* and *Leu. mesenteroides* while fructose was a better source for *Leu. oenos* (Table 2.2).

A sugar fermentation profile at 48 h showed that *Leu. oenos* utilized only fructose (Table 2.3). Based on the concentration of available sugars in the final fermentation broth, cucumbers fermented by *L. plantarum, Leu. mesenteroides* and *Leu. oenos* should be the most microbiologically stable products while cucumbers fermented by *Lac. diacetylactis* should be the least stable (Table 2.2).

Carbon recovery from hexose fermentation ranges from 64 to 105% (Table 2.4). A carbon recovery lower than 100% suggests that hexose sugars were used to produce compounds not measured in this study. For example, Crow (1988) found that the carbon recovery percentage in fermentation of lactose by propionibacteria is also affected by the production of polysaccharides. These other compounds may also affect the flavor quality of fermented cucumbers and their identification should be included in future studies. Another source for low carbon recovery could be a loss of some volatile compounds such as acetic acid and ethanol during fermentation and sample handling.

Characterization of fermented CJB

The organic acids detected in fermented CJB are lactic, acetic and propionic acids (Table 2.1). Homofermenters, i.e. *L. plantarum, P. cerevisiae, L. casei* and *Lac. diacetylactis* produce lactic acid as a major fermentation product (Table 2.1). As shown in Table 2.1, the propionic acid level in the
Table 2.3: Sugar fermentation profiles after 48 hours

<table>
<thead>
<tr>
<th>Culture</th>
<th>Glucose</th>
<th>Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prop. shermanii&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P. cerevisiae&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. casei&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. plantarum&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leu. mesenteroides&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lac. diacetylactis&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. bifidum&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leu. oenos&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>1</sup>Using the API<sup>TM</sup> CHL kit.

"+": the bacteria can ferment the sugar in 48 h.

"-": the bacteria cannot ferment the sugar in 48 h.

<sup>2</sup>Test at 30°C

<sup>3</sup>Test at 37°C
Table 2.4: Percent carbon recovery after 1.5 month fermentation.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture²</td>
<td>81bc</td>
</tr>
<tr>
<td><em>P. cerevisiae</em></td>
<td>81bc</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>92b</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>79c</td>
</tr>
<tr>
<td><em>Leu. mesenteroides</em></td>
<td>71cd</td>
</tr>
<tr>
<td><em>Lac. diacetylactis</em></td>
<td>105a</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>74cd</td>
</tr>
<tr>
<td><em>Leu. oenos</em></td>
<td>64d</td>
</tr>
</tbody>
</table>

¹ Mean of two replications; means within columns followed by the same letter are not significantly different (p > 0.05)

² *Prop. shermanii* and *P. cerevisiae*
mixed culture treatment containing *P. cerevisiae* and *Prop. shermanii* might not be high enough to cause a significant decrease in the lactic acid level. Propionic acid at the level found in this treatment (0.016%) could affect flavor quality. The recognition threshold concentration for this acid has been shown to be as low as 0.01% (Golovnya et al., 1986). Acetic acid and CO₂ can also be produced from lactic acid by the propionic acid pathway (Gottschalk, 1979).

Lactic acid was the main acid produced by *B. bifidum* (Table 2.1), even though acetic acid and lactic acid are supposed to be produced by the bifidum pathway in a 1:1 ratio (w/w) (Gottschalk, 1979). Kosikowska (1978) observed a similar situation in milk fermented by bifidobacteria. In addition to lactic acid, *Leu. mesenteroides* and *Leu. oenos* produced acetic acid, mannitol and ethanol as end products (Table 2.1). The mannitol dehydrogenase enzyme present in these bacteria could have reduced fructose to mannitol while oxidizing NADH₂ to NAD⁺, and thus produce acetic acid instead of ethanol (Stanier et al., 1976). Mannitol, a sugar alcohol produced by heterofermenters cannot be fermented anaerobically by yeasts (Suomalainen and Oura, 1971). Therefore, it should not affect product stability.

The ratio of volatile/nonvolatile acids varied for the different bacteria used in this study (Table 2.1). Heterofermenters produced a high ratio of volatile/nonvolatile acids. *Lac. diacetylactis* also resulted in a high volatile/nonvolatile acid ratio. It should be noted, however, that the volatile/nonvolatile acid ratio can be affected by factors such as sample pH,
and the pKa of each acid (esp. lactic acid) in the fermentation broth. Volatile acids, i.e. acetic and propionic acids, might affect the flavor quality of the final product as suggested by Pederson and Albury (1969). A higher ratio of volatile/nonvolatile acids might result in better flavor quality. Juhasz (1974) observed that a high volatile/nonvolatile acid ratio was found in fermented cucumbers with better flavor.

*L. plantarum* and *P. cerevisiae* fermentation broths are high in acid and low in pH (Table 2.1). In the case of the mixed *Prop. shermanii* and *P. cerevisiae* culture fermentation, high level of titratable acidity was probably due to *P. cerevisiae*. As compared to *L. plantarum* and *P. cerevisiae*, *Leu. mesenteroides* and *L. oenos* did not produce high acid and low pH products reflecting the difference in their fermentation pathway. After fermentation of the same amount of sugar, homofermenters and bifidobacteria should produce higher acid concentrations than heterofermenters since heterofermenters produce compounds other than acids (Gottschalk, 1979). However, *Lac. diacetylactis*, *L. casei* and *B. bifidum* did not yield high acid concentrations because they did not survive low pH conditions (Fig. 2.2). *B. bifidum* can produce high acid concentrations only under the high buffering condition such as existing in milk.

The small amount of citric acid (62 ppm) initially present in CJB was not found in the final fermentation broth (Table 2.5). Many lactic acid bacteria can produce diacetyl, acetate and CO₂ from citric acid (Gottschalk, 1979). Diacetyl, a flavorful buttery-type compound, could affect the flavor
Table 2.5: Citric acid, diacetyl and acetoin concentrations in unfermented and fermented cucumber juice brine (CJB) after 1.5 months.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Citric acid (ppm)</th>
<th>Diacetyl (ppm)</th>
<th>Acetoin (ppm)</th>
<th>Citrate needed for diacetyl+acetoin production (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented CJB</td>
<td>62</td>
<td>ND4</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Mixed culture³</td>
<td>ND</td>
<td>ND</td>
<td>24b</td>
<td>158</td>
</tr>
<tr>
<td>P. cerevisiae</td>
<td>ND</td>
<td>ND</td>
<td>8d</td>
<td>54</td>
</tr>
<tr>
<td>L. casei</td>
<td>ND</td>
<td>ND</td>
<td>3f</td>
<td>19</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>ND</td>
<td>ND</td>
<td>34b</td>
<td>226</td>
</tr>
<tr>
<td>Leu. mesenteroides</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Lac. diacetylactis</td>
<td>ND</td>
<td>ND</td>
<td>9c</td>
<td>62</td>
</tr>
<tr>
<td>B. bifidum</td>
<td>ND</td>
<td>ND</td>
<td>6e</td>
<td>41</td>
</tr>
<tr>
<td>Leu. oenos</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Mean of two replications; means within columns followed by the same letter are not significantly different (p > 0.05)
2 Citrate needed = \(\frac{3 \times \text{MW of citric acid}}{\text{MW of acetoin}} \times (\text{ppm of diacetyl} + \text{acetoin})\)
3 Prop. shermanii and P. cerevisiae.
4 Not-detected
quality of fermented cucumbers. However, diacetyl was absent in the final fermentation broths and only acetoin, a flavorless compound, was detected (Table 2.5). Many lactic acid bacteria contain diacetyl reductase and 2,3 butanediol dehydrogenase enzymes which reduce diacetyl first to acetoin and then to 2,3 butanediol, respectively (Vedamuthu, 1979). However, acetoin in the mixed Prop. shermanii and P. cerevisiae culture fermentation and L. plantarum fermentation broths were too high to be accounted for by the amount of available citric acid in unfermented CJB. Diacetyl and acetoin can be produced by Prop. shermanii from citrate, glucose or lactate (Hettinga and Reinbold, 1972). Also, Drinan et al. (1976) reported that some strains of L. plantarum produced acetoin from glucose. Leu. mesenteroides and Leu. oenos did not produce diacetyl nor acetoin (Table 2.5). Citric acid may have been degraded by citrate lyase, pyruvate oxidase and lactate oxidase enzymes to form acetate, acetyl phosphate and lactate; or, by a citrate lyase and pyruvate-formate lyase system to form acetate, ethanol and formate (Gottschalk, 1979).

CONCLUSIONS

None of the fermentation treatments utilized available sugars to completion. The chemical components produced by L. casei and B. bifidum were most similar. The chemical composition of the fermented broths produced by all other bacterial species were significantly different. Therefore, it should be possible to select the bacterial species for production of specific chemical profiles in fermented cucumber products. Carbon recovery percentages indicated that available sugars were utilized to produce
fermentation products not measured in this study. Citric acid was degraded by all bacterial species investigated in this study. However, diacetyl, a flavorful compound, was not found in any of the fermentation broths. Most of the bacteria included in this study preferred glucose as a carbohydrate source except Leu. oenos which preferred fructose. The preference of Leu. oenos for fructose may be beneficial for future sugar utilization studies in mixed culture fermentations.
REFERENCES


Fleming, H.P. 1988. Private communication, Department of Food Science, North Carolina State University, Raleigh, NC.


3. Descriptive Analysis and Consumer Testing of Cucumber Juice Brine Fermented by Lactic and Propionic Acid Bacteria

ABSTRACT

Eight treatments of fermented cucumber juice brine (CJB) were produced by using eight species of acid-producing bacteria. The fermented CJB was analyzed by sensory descriptive analysis for aroma and taste characteristics using a nine member trained panel. Twelve descriptors were used to describe aroma and six descriptors were used to describe flavor by mouth by descriptive analysis. Intensities of seven aroma descriptors and three flavor by mouth descriptors were significantly different among treatments. Most descriptors, except for sourness, could not be explained by the chemical analysis data. A sensory consumer test for aroma was conducted by using a doubly balanced incomplete block design. A nine-point hedonic scale (1=dislike extremely, 5=neither like nor dislike, 9=like extremely) was used for rating degree of liking. The scores for aroma ranged from 4.69-5.39 for CJB fermented by the eight different microorganisms. However, there were no significant differences (p = 0.05) in these scores between any of the treatments.

INTRODUCTION

Even though flavor quality measurement can be used as an effective tool for quality control and product development in the cucumber (Cucumis sativus) pickle industry, few studies have been conducted on the development of the terms or descriptors used to describe pickle flavor or the
chemical compounds responsible for that flavor. Aurand et al. (1965) used a high-vacuum distillation method with liquid-nitrogen trapping to separate the volatile components present in pure-culture fermentations of cucumbers and then confirmed their identity by gas-liquid chromatography. They identified formaldehyde, acetaldehyde, acetone, ethyl alcohol, propionaldehyde, and butyraldehyde in the fermented brine, however they could not relate the results of their chemical analyses to the sensory observation provided by an experienced panel. These investigators concluded that the flavor of pickles was due to a blend of volatile components rather than due to the presence or absence of a single component (Aurand et al., 1965). James and Buescher (1983) could not correlate mean preference scores of commercial dill pickles with simple chemical or instrumental analytical data such as NaCl levels, acetic acid levels or texture measurements.

Sensory descriptive analysis can be used in several applications such as quality monitoring, product development and product maintenance (Civille and Lawless, 1986); it has proven most useful in assessing the quality attributes of different foods and beverages (Meilgaard et al., 1987). Sensory descriptive analysis could be an effective choice for the study of flavor quality of cucumber pickles. In 1965, Aurand et al. used selected sensory terms in an effort to describe flavor characteristics of fermented cucumbers; descriptors such as clean, raw cucumber, acid, aromatic, pleasant, etc. These terms were developed by an "experienced panel," but the investigators did not use any standards to represent these sensory terms.
In this study, a small trained panel developed sensory flavor profiles for cucumber juice brine fermented by different acid producing bacteria. Standard substances or solutions were selected to represent each sensory descriptor. A consumer panel was utilized to measure acceptability of the aroma of the brine. Cucumber juice brine (CJB) was used in this study to simulate cucumber fermentation under controlled pure culture fermentation. By using CJB, bias factors caused by other quality factors such as physical damage or textural defect could be avoided. As compared to the whole fruit, CJB was easily sterilized; therefore, the flavor of the finished products was solely derived from the microorganisms introduced. The bacteria used in this study included homofermentative and heterofermentative lactic acid bacteria, bifidobacteria, and propionibacteria. Some of these bacteria flora (i.e. *Lactobacillus plantarum*, *Pediococcus cerevisiae*, *Lactobacillus casei*) have been recommended generally for use in cucumber fermentations, whereas other bacterial genera or species (the heterofermenters: *Leuconostoc mesenteroides*, *Leuconostoc oenos*) have been considered as undesirable due to their tendency to cause physical damage. The other microflora (i.e. *Streptococcus lactis* (now known as *Lactococcus lactis sub sp. lactis* biovar. *diacetylactis*), *Bifidobacterium bifidum*, *Propionibacterium freudenreichii*) are known to produce desirable flavor quality in other products such as milk and sauerkraut.
 MATERIALS & METHODS

Cucumber juice brine (CJB) preparation

The CJB preparation method was a modification of the procedure described by Daeschel et al. (1988). Fresh pickling cucumbers (Pioneer variety) were obtained from Steinfeld's Products Co., Portland, OR. Defect-free fruits were washed with tap water, stored overnight at -23°C in a covered 18 liter (5 gal) plastic pail and then partially thawed at room temperature for 4-5 h. The partially thawed cucumbers were disintegrated to a homogeneous slurry in a hammermill (Model D Comminuting Machine, The W.J. Fitzpatrick Company, Chicago, IL) using a solid screen size 0.42 cm (3/8 in) diameter. Juice was extracted from the slurry in a 15 cm (6 in) diameter fruit finisher with a solid screen size 0.05 cm (0.02 in) diameter. The extracted juice was brought to boiling in a 74 liter (20 gal) steam jacketed kettle and cooled in a 3°C cold room in a covered 37 liter (10 gal) stainless steel pail (6 h, final temperature ca. 5°C). The juice was then filtered through cheese cloth and diluted with a buffered salt brine (1:1 ratio). The buffered brine contained NaCl, CaCl₂, acetic acid and NaOH to yield after dilution a CJB with 2.5% NaCl, 0.2% CaCl₂, 0.1% acetic acid and pH 5.0. CJB was then filtered through qualitative filter paper grade 617 (VWR Scientific, San Francisco, CA) and sterilized using a hollow fiber ultrafiltration cartridge (Romicon PM 500) with an effective area of 4500 sq cm (5 sq ft) and a 500,000 MW cut off (Romicon, Woburn, MA). The filtration process was done by following Romicon operating instructions (Anonymous, 1983). Some of the
prepared CJB was frozen at -40°C for use as a standard for subsequent descriptive analysis.

**Cultures and inoculum preparation**

*Propionibacterium freudenreichii* sub sp. *shermanii* (ATCC 13673), *Bifidobacterium bifidum* (ATCC 11147) (*B. bifidum*), *Leuconostoc oenos* (ATCC 27307) (*Leu. oenos*), *Propionibacterium shermanii*, and *Bifidobacterium bifidum* (ATCC 11147) (*B. bifidum*) were obtained from the American Type Culture Collection (Rockville, MD). *Lactobacillus plantarum* sub sp. *arabinosus* (Midwest 220) (*L. plantarum*) was obtained from the Midwest Culture Service (Terre Haute, IN). *Pediococcus cerevisiae* (AFERM 772) (*P. cerevisiae*) and *Lactobacillus casei* (AFERM 771) (*L. casei*) are cultures currently being used for pickled vegetables (Microlife Technics, Sarasota, FL). *Leuconostoc mesenteroides* 98 (*Leu. mesenteroides*) and *Lactococcus lactis* sub sp. *lactis* biovar. *diacetylactis* 1816 (previously known as *Streptococcus diacetilactis*) (*Lac. diacetylactis*) were obtained from the Dept. of Microbiology at Oregon State University (Corvallis, OR).

The *P. cerevisiae* and *L. casei* cultures were received as a frozen concentrate form. These cultures were thawed, weighed, diluted with 10 ml of 0.85% NaCl solution and used directly as inocula. The rate of initial inoculum was at least 10^6 cfu/ml CJB.

*L. plantarum*, *Leu. mesenteroides*, and *Lac. diacetylactis* cultures were received as streak cultures on agar slants. The *Prop. shermanii*, *B. bifidum* and *Leu. oenos* cultures were received in freeze-dried form. All cultures
were transferred to either MRS broth (Difco, Detroit, MI) or sodium lactate broth (for Prop. shermanii) prepared as described by Schwab et al. (1984) and then incubated at 30°C for 24-48 h before being transferred to a 60% glycerol solution at -40°C for storage.

*L. plantarum, Leu. mesenteroides, Lac. diacetylactis, and B. bifidum* cultures were then transferred to MRS broth and incubated at 30°C for 24 h. A 48 h incubation period at 30°C was used for *Leu. oenos*. *Prop. shermanii* was grown in sodium lactate broth at 30°C for 72 h (Schwab et al., 1984). Next, all broths were centrifuged at 2603 x g for 10 minutes, rinsed twice with a 0.85% NaCl solution, centrifuged and diluted with 10 ml of the salt solution before inoculation. The initial inoculum for each microorganism was at least 10⁶ cfu/ml CJB.

**Treatments and fermentation**

All strains were used as a pure culture except for mixed cultures of *Pro. shermanii* and *P. cerevisiae*. CJB was fermented in duplicate batches in a sterile 10-liter screw cap glass bottle (Pyrex Corning®) at room temperature (22-26°C) for 1.5 months. The fermented CJB was filtered through a layer of Celite (Manville Products Corporation, Lompoc, CA) to remove bacterial cells before freezing the samples at -23°C prior to further analysis.
Chemical analysis

All pH measurements were conducted on a microprocessor pH/mV meter (Orion model 811) equipped with a combination pH electrode (Ross model 81550). Titratable acidity was determined as percent lactic acid. Reducing sugar was determined by using a colorimetric method as described by Nelson (1944). Diacetyl was analyzed by a modified Westerfeld's method as described by Byer (1954). Ethanol was analyzed by using an enzyme assay kit available from Boehringer Mannheim Biochemicals (Indianapolis, IN) based on their recommended procedure (Anonymous, 1987).

Volatile and nonvolatile acids were analyzed by gas chromatography with a hydrogen flame ionization detector (FID). The sample preparation procedure described in Supelco GC Bulletin 748 H (Anonymous, 1985) was followed. Volatile acids were directly extracted with ethyl ether, while nonvolatile acids were esterified with methanol before being extracted with chloroform. The packing material GP 10% SP-1000/1% H₃PO₄ on 100/120 Chromosorb was packed in a 1.8 m x 0.3 cm (6' x 1/8") OD nickel column, and the operating conditions were: column temperature 85-160°C at 4°C/min for volatile acid analysis and at 2°C/min for nonvolatile acid analysis; injector 180°C; detector 220°C; He gas flow rate 25 ml/min. Under these operating conditions, volatile acids including formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and heptanoic acids, and nonvolatile acids including pyruvic, lactic, oxaloacetic, oxalic, methylmalonic, malonic, fumaric and succinic acids could be identified. A preliminary qualitative test on all fermentation broths indicated that butyric acid could be used as an
internal standard for volatile acid analysis while malonic acid could be used as an internal standard for nonvolatile acid analysis.

Statistical analysis of chemical data

Chemical data was subjected to an analysis of variance (ANOVA), and then differences between the means of the chemical data from each treatment were tested for their significance at $p \leq 0.05$ using a Fisher's Protected Least Significant Difference (FPLSD) procedure (Snedecor and Cochran, 1980). The relationship between some chemical analysis data and sensory intensity scores were reported as a Coefficient of Correlation ($R$) using the StatWorks™ program (Cricket Software Inc., Philadelphia, PA).

Serving and testing conditions

Ten ml samples were served in covered 227 ml (8 oz) wine glasses at room temperature. Each sample was coded with a three-digit random number.

Training sessions for descriptive analysis were conducted in the Sensory Science Laboratory in the Department of Food Science and Technology, Oregon State University. Sample testing for both descriptive analysis and consumer testing were performed in individual testing booths under white incandescent light.
Descriptive analysis

Nine panelists including faculty, staff members and graduate students of the Department of Food Science and Technology, Oregon State University were trained for 10 h (1 h/session) before performing the test. During the training sessions, panelists developed character notes for aroma and selected standards to represent those character notes (listed in Table 3.1). Aroma intensity was rated by using a 15-point intensity scale which ranged from "none"(0) to "moderate"(7) to "extreme"(15). The standards for aroma intensity rating included: cooking oil (Wesson, Beatrice/Hunt-Wesson Inc., Fullerton, CA) for a 3 rating; canned orange juice (Hi-CTM Orange Drink, Coca-Cola Foods, Houston, TX) for a 7 rating, grape juice (Welch's 100% Pure Grape Juice, Welch Foods Inc., Concord, MA) for an 11 rating, artificially flavored cinnamon gum (Wrigley's Big Red, W.M. Wrigler J.R. Company, Chicago, IL) for a 15 rating. Character note and intensity standards were both served in covered 227 ml (8 oz) wine glasses.

Character notes for taste and mouthfeel included sweetness, saltiness, sourness, astringency, bitterness and aftertaste. The character note "aftertaste" also included personal comments from each panelist. Any term that was mentioned more than 3 times in each treatment was reported. Flavor by mouth intensity was rated on a 15 cm line scale which ranged from "none"(at 0 cm) to "moderate"(at 7.5 cm) to "extreme"(at 15 cm).

All eight samples were presented in a balanced complete block design within each day of testing. Four samples were presented first, the judge took
Table 3.1: Standards used to represent each aromatic descriptor during panelist training for descriptive analysis.

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Standard¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Intensity</td>
<td>No standard</td>
</tr>
<tr>
<td>Floral</td>
<td>Geraniol (Sigma St. Louis, MO) on the tip of a filter paper (Whatman no. 1) strip (0.5 cm x 3 cm)</td>
</tr>
<tr>
<td>Fruity</td>
<td>20 ml of Muller Thurgau wine (Tualatin Vineyards, Forest Grove, OR)</td>
</tr>
<tr>
<td>Woody/smokey</td>
<td>15 ml of 0.07% V/V Wright's Natural Hickory Seasoning-Liquid Smoke (Nabisco Brand Inc. East Hanover, NJ) in distilled water</td>
</tr>
<tr>
<td>Vegetative</td>
<td>Canned asparagus (3-4 pieces with 10 ml brine) Thawed frozen green bean (15 gm)</td>
</tr>
<tr>
<td>Cucumber juice</td>
<td>5 gm of French's dried dill weed (The R.T. French Co. Rochester, NY)</td>
</tr>
<tr>
<td>Herbal</td>
<td>15 ml of unfermented CJB²</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>15 ml of 0.5% V/V glacial acetic acid (Aldrich Milwaukee, WI) in distilled water³</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>15 ml of 0.01% V/V butyric acid (Aldrich Milwaukee, WI) in distilled water³</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>15 ml of 0.01% V/V propionic acid (Aldrich Milwaukee, WI) in distilled water</td>
</tr>
<tr>
<td>Buttery</td>
<td>15 ml of 0.001% V/V diacetyl (Sigma St. Louis, MO) in distilled water³</td>
</tr>
<tr>
<td>Sweet</td>
<td>5 gm of dried malt grain with 10 ml of boiling water</td>
</tr>
</tbody>
</table>

¹Standards were served in a covered 227 ml (8 oz) wine glass at room temperature.
²Cucumber juice brine
³As recommended by Golovnya et al., 1986.
a 15-20 minute break, and then the final four samples were evaluated. The order of sample presentation was randomized for each judge. Three panel replications were conducted for each batch replication of each treatment. Analysis of variance with LSD comparisons at \( p \leq 0.05 \) was used to determine intensity difference of each descriptor by using SAS/STAT (SAS Institute Inc. Cary, NC) software. However, F-values for panelist (Pan), batch (Bat) and treatment (Trt) were treated as random effects and they were calculated by using the following formulas:

\[
F(Pan) = \frac{MS(Pan) + MS(error)}{MS(Pan \times Trt) + MS(Pan \times Bat)}
\]

\[
F(Bat) = \frac{MS(Bat) + MS(error)}{MS(Pan \times Bat) + MS(Bat \times Trt)}
\]

\[
F(Trt) = \frac{MS(Trt) + MS(error)}{MS(Pan \times Trt) + MS(Bat \times Trt)}
\]

(Cochran, 1951; Anderson and Bancroft, 1952; Lundahl and McDaniel, 1988). The degrees of freedom of each F-value were estimated as described by Cochran, 1951.

**Consumer test**

The degree of liking for aroma was measured by use of the 9-point hedonic scalar technique (Larmond, 1977). The scale ranged from "dislike extremely"(1) to "neither like nor dislike"(5) to "like extremely"(9). A total of 140 pickle consumers, who were students and staff members of Oregon State University or people in the Corvallis community, were used in the test. Seventy panelists were used to test each replication of the treatments.
A doubly balanced incomplete block design was used for the design of sample presentation (Calvin, 1954). Four different samples were served to each panelist at each setting. From this design, each treatment of each batch replication was tested by 35 panelists. Each pair of treatments occurred together fifteen times; and each triplet of treatments occurred together five times.

Data of each replication were tested if the correlation effect was significant as described by Calvin (1954). Then, the data were analyzed by analysis of variance and means were adjusted by combining intrablock and interblock estimates (Yates, 1940; Gacula and Singh, 1984).

RESULTS & DISCUSSION

Descriptive analysis

The trained panel selected twelve descriptors to describe aroma characteristics of the fermented cucumber juice brine. The descriptors and the standards used during training sessions are shown in Table 3.1.

Six sources of variation (SOV) needed to be considered in the experimental design used in this study (Table 3.2). Panelist, batch and treatment SOV were treated as random effects. Panelist SOV was significant for many descriptors, meaning only that different panelists used different parts of the scale. Lundahl and McDaniel (1988) have suggested that
Table 3.2: F-values for each source of variation of each sensory descriptor rated by the trained panel.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Panellist (Pan)</th>
<th>Batch (Bat)</th>
<th>Treatment (Trt)</th>
<th>PanxBat</th>
<th>PanxTrt</th>
<th>BatxTrt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees of freedom</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>8</td>
<td>56</td>
<td>7</td>
</tr>
</tbody>
</table>

**Descriptor**

**Aroma:**

- **Overall Intensity**: 8.73***, 0.80NS, 7.89***, 2.64**, 1.60**, 0.14NS
- **Floral**: 2.54*, 0.42NS, 1.42NS, 0.84NS, 3.23**, 1.75NS
- **Fruity**: 5.93***, 2.91NS, 2.90**, 0.86NS, 2.43***, 0.93NS
- **Woody/Smokey**: 4.59***, 0.80NS, 2.43*, 0.87NS, 2.64***, 0.97NS
- **Vegetative**: 3.46*, 0.18NS, 0.76NS, 4.32***, 2.10***, 1.34NS
- **Cucumber Juice**: 26.54***, 0.55NS, 2.08*, 1.54NS, 1.40*, 0.65NS
- **Herbal**: 13.09***, 0.35NS, 0.75NS, 1.70NS, 2.03***, 1.61NS
- **Acetic acid**: 8.86***, 0.43NS, 4.13***, 1.85NS, 2.40***, 0.47NS
- **Butyric acid**: 1.41NS, 1.05NS, 4.06***, 0.44NS, 3.71***, 1.18NS
- **Propionic acid**: 11.09***, 0.41NS, 0.58NS, 1.22NS, 3.89***, 2.51*
- **Buttery**: 4.33**, 1.24NS, 5.61***, 1.80NS, 2.08***, 1.21NS
- **Sweet**: 6.40***, 0.93NS, 1.94NS, 1.91NS, 2.98***, 1.34NS

**Flavor by mouth:**

- **Sweetness**: 18.48***, 6.37*, 1.28NS, 6.51***, 2.13***, 0.54NS
- **Saltiness**: 7.36***, 1.85NS, 1.22NS, 2.91**, 1.65**, 0.78NS
- **Bitterness**: 10.64***, 4.85*, 2.30*, 0.78NS, 2.88***, 0.36NS
- **Astringency**: 47.61***, 0.27NS, 2.18NS, 2.17*, 2.84***, 2.45*
- **Sourness**: 6.29***, 0.72NS, 25.62***, 2.27*, 1.48*, 0.39NS
- **Aftertaste**: 2.73*, 0.46NS, 3.47**, 1.66NS, 2.95***, 1.03NS

Note: NS: Nonsignificant difference at p = 0.05.
  *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001
panelists, selected from a population, naturally differ in their susceptibilities to various factors that contribute to response variation. Screening and training methods may reduce, but cannot eliminate all sources of variation attributable to panelists.

The F-values for batch in most descriptors were not significantly different, thus implying that differences in sensory quality between batches within each treatment were not found in this study (Table 3.2). This demonstrated the consistency of flavor quality of fermented CJB within each fermentation batch. BatxTrt interaction (for most descriptors) were also not significant (Table 3.2); the panelists did not detect any significant difference for these descriptors among batches of each treatment.

Of the twelve aroma descriptors and six flavor by mouth descriptors, seven and three were found to be significant, respectively. Significant PanxTrt interactions for the significant aroma or flavor by mouth descriptors are discussed in a later section.

Treatment effects

Aroma. Seven of the twelve descriptors for aroma, overall intensity, fruity, woody/smokey, cucumber juice, acetic acid, butyric acid and buttery, were significantly different among treatments (Table 3.2). Table 3.3 shows that fermented CJB from different microorganisms contained different aroma characters at different intensities. The mixed cultures of Prop. freudenreichii and P. cerevisiae resulted in a product with high butyric
Table 3.3: Means and standard deviations\(^1\) of trained panel aroma descriptors for eight treatments of cucumber juice brine (CJB) fermented by different microorganisms.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MC(^3)</th>
<th>Pc(^4)</th>
<th>Lc(^5)</th>
<th>Lp(^6)</th>
<th>Lm(^7)</th>
<th>Ld(^8)</th>
<th>Bd(^9)</th>
<th>Lo(^{10})</th>
<th>LSD(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Intensity</td>
<td>9.24cd</td>
<td>8.89d</td>
<td>9.54bc</td>
<td>10.33a</td>
<td>9.89ab</td>
<td>9.28cd</td>
<td>8.98d</td>
<td>10.00ab</td>
<td>0.51</td>
</tr>
<tr>
<td>Floral</td>
<td>(1.92)</td>
<td>(1.74)</td>
<td>(1.66)</td>
<td>(1.46)</td>
<td>(1.62)</td>
<td>(1.51)</td>
<td>(2.06)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruity</td>
<td>1.09a</td>
<td>2.74a</td>
<td>3.06a</td>
<td>3.39a</td>
<td>2.54a</td>
<td>2.61a</td>
<td>2.83a</td>
<td>3.30a</td>
<td></td>
</tr>
<tr>
<td>(1.93)</td>
<td>(2.27)</td>
<td>(2.37)</td>
<td>(3.10)</td>
<td>(2.58)</td>
<td>(2.70)</td>
<td>(2.51)</td>
<td>(3.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Woody/Smokey</td>
<td>1.63b</td>
<td>2.13b</td>
<td>1.46b</td>
<td>4.11a</td>
<td>1.80b</td>
<td>1.57b</td>
<td>1.87b</td>
<td>2.41b</td>
<td>1.30</td>
</tr>
<tr>
<td>(2.64)</td>
<td>(2.67)</td>
<td>(2.13)</td>
<td>(3.49)</td>
<td>(2.50)</td>
<td>(2.27)</td>
<td>(2.29)</td>
<td>(2.88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative</td>
<td>4.35a</td>
<td>3.65a</td>
<td>4.28a</td>
<td>4.13a</td>
<td>4.54a</td>
<td>4.74a</td>
<td>3.78a</td>
<td>3.85a</td>
<td></td>
</tr>
<tr>
<td>(3.02)</td>
<td>(2.69)</td>
<td>(2.62)</td>
<td>(3.30)</td>
<td>(2.81)</td>
<td>(2.52)</td>
<td>(2.52)</td>
<td>(2.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cucumber Juice</td>
<td>4.04c</td>
<td>4.89ab</td>
<td>5.48a</td>
<td>4.61bc</td>
<td>4.54bc</td>
<td>5.00ab</td>
<td>5.24ab</td>
<td>4.70abc</td>
<td>0.79</td>
</tr>
<tr>
<td>(2.71)</td>
<td>(2.49)</td>
<td>(3.11)</td>
<td>(3.13)</td>
<td>(3.15)</td>
<td>(2.69)</td>
<td>(2.52)</td>
<td>(3.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herbal</td>
<td>1.41a</td>
<td>1.56a</td>
<td>1.70a</td>
<td>2.04a</td>
<td>2.20a</td>
<td>1.57a</td>
<td>1.35a</td>
<td>2.17a</td>
<td></td>
</tr>
<tr>
<td>(2.20)</td>
<td>(2.19)</td>
<td>(2.68)</td>
<td>(2.99)</td>
<td>(3.25)</td>
<td>(2.97)</td>
<td>(2.38)</td>
<td>(2.87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.74c</td>
<td>1.65c</td>
<td>2.28c</td>
<td>3.89a</td>
<td>2.59bc</td>
<td>1.54c</td>
<td>2.00c</td>
<td>3.57ab</td>
<td>1.08</td>
</tr>
<tr>
<td>(2.32)</td>
<td>(2.12)</td>
<td>(2.74)</td>
<td>(2.79)</td>
<td>(3.11)</td>
<td>(2.55)</td>
<td>(2.46)</td>
<td>(3.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyric acid</td>
<td>3.74a</td>
<td>0.72b</td>
<td>0.80b</td>
<td>1.35b</td>
<td>1.59b</td>
<td>0.94b</td>
<td>0.81b</td>
<td>1.17b</td>
<td>1.30</td>
</tr>
<tr>
<td>(2.78)</td>
<td>(1.58)</td>
<td>(1.59)</td>
<td>(2.25)</td>
<td>(2.27)</td>
<td>(1.80)</td>
<td>(1.60)</td>
<td>(1.89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionic acid</td>
<td>3.28a</td>
<td>2.57a</td>
<td>3.20a</td>
<td>3.44a</td>
<td>4.00a</td>
<td>3.04a</td>
<td>2.93a</td>
<td>3.59a</td>
<td></td>
</tr>
<tr>
<td>(2.72)</td>
<td>(2.47)</td>
<td>(2.99)</td>
<td>(3.37)</td>
<td>(3.23)</td>
<td>(2.81)</td>
<td>(2.93)</td>
<td>(3.53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buttery</td>
<td>1.65cd</td>
<td>3.04b</td>
<td>3.44ab</td>
<td>1.11d</td>
<td>2.42bc</td>
<td>4.54a</td>
<td>3.39ab</td>
<td>1.46cd</td>
<td>1.27</td>
</tr>
<tr>
<td>(2.16)</td>
<td>(2.71)</td>
<td>(2.73)</td>
<td>(2.06)</td>
<td>(2.65)</td>
<td>(2.49)</td>
<td>(2.62)</td>
<td>(2.42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet</td>
<td>1.04a</td>
<td>2.48a</td>
<td>2.37a</td>
<td>1.33a</td>
<td>2.07a</td>
<td>2.72a</td>
<td>3.11a</td>
<td>1.50a</td>
<td></td>
</tr>
<tr>
<td>(2.06)</td>
<td>(2.82)</td>
<td>(2.64)</td>
<td>(2.90)</td>
<td>(2.49)</td>
<td>(2.72)</td>
<td>(2.84)</td>
<td>(2.77)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{abcd}\)Means within rows followed by the same letter are not significantly different (p > 0.05).

\(^1\)Standard deviation is shown in parenthesis under mean value.

\(^2\)Least significant difference at p = 0.05.

\(^3\)MC: Mixed cultures: Prop. shermanii and P. cerevisiae; \(^4\)Pc: P. cerevisiae; \(^5\)Lc: L. casei; \(^6\)Lp: L. plantarum; \(^7\)Lm: Leu mesenteroides; \(^8\)Ld: Lac. diacetylactis; \(^9\)Bb: B. bifidum; \(^10\)Lo: Leu oenos.
acid character. Ironically, butyric acid was not actually found as a CJB component in this study (Table 3.4). Propionic acid was found in the mixed culture treatment at the level of 0.016%, which is higher than the recognition threshold for this acid (Golovnya et al., 1986). However, the propionic acid character was not significantly different among treatments. The panel may have been confused by the similar sensory nature of these acids, and they may have responded to other compounds present which exhibited similar aroma character.

The fermented CJB produced by \textit{L. casei}, \textit{S. diacetilactis} and \textit{B. bifidum} were high in buttery and cucumber juice characteristics. The buttery character in dairy products is primarily due to diacetyl compounds, however, diacetyl was not found in any of the fermented CJB (Table 3.4).

\textit{L. plantarum}, \textit{Leu. mesenteroides} and \textit{Leu. oenos} produced fermented CJB which was high in overall intensity and woody/smokey characteristics. Table 3.4 shows that CJB fermented by heterofermenters, i.e. \textit{Leu mesenteroides} and \textit{Leu. oenos}, contained the highest levels of volatile compounds such as acetic acid and ethanol (Table 3.4) which are usually produced from their main fermentation pathway (Gottschalk, 1979). This might result in high overall aroma intensity. However, \textit{L. plantarum} produced quite low levels of volatile compounds in this study (Table 3.4). It is not known to produce any volatile compounds from its main fermentation pathway (Gottschalk, 1979). Therefore, more than just the products from the main fermentation pathways have contributed aroma characteristics to the treatments. \textit{Leu. oenos} and \textit{L. plantarum} were also rated high in acetic acid
Table 3.4: Chemical composition\(^1\) of cucumber juice brine (CJB) fermented by different microorganisms.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>pH</th>
<th>Acetic acid(%)</th>
<th>Propionic acid(%)</th>
<th>Lactic acid(%)</th>
<th>Diacetyl (ppm)</th>
<th>Reducing sugar (%)</th>
<th>Ethanol(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture(^2)</td>
<td>3.48e 0.67b</td>
<td>0.098c</td>
<td>0.016a</td>
<td>0.689b</td>
<td>ND(^4)</td>
<td>0.46e</td>
<td>0.0026d</td>
</tr>
<tr>
<td>P. cerevisiae</td>
<td>3.45e 0.66b</td>
<td>0.095cd</td>
<td>ND</td>
<td>0.650b</td>
<td>ND</td>
<td>0.53d</td>
<td>0.0022d</td>
</tr>
<tr>
<td>L. casei</td>
<td>3.98c 0.35f</td>
<td>0.086e</td>
<td>ND</td>
<td>0.363cd</td>
<td>ND</td>
<td>0.96b</td>
<td>0.0025d</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>3.24f 0.93a</td>
<td>0.089d</td>
<td>ND</td>
<td>0.916a</td>
<td>ND</td>
<td>0.18g</td>
<td>0.0097c</td>
</tr>
<tr>
<td>Leu. mesenteroides</td>
<td>3.82d 0.50c</td>
<td>0.130b</td>
<td>ND</td>
<td>0.404c</td>
<td>ND</td>
<td>0.22f</td>
<td>0.0960a</td>
</tr>
<tr>
<td>Lac. diacetylactis</td>
<td>4.12a 0.30g</td>
<td>0.090de</td>
<td>ND</td>
<td>0.281e</td>
<td>ND</td>
<td>1.08a</td>
<td>0.0024d</td>
</tr>
<tr>
<td>B. bifidum</td>
<td>3.84d 0.39e</td>
<td>0.094cd</td>
<td>ND</td>
<td>0.393c</td>
<td>ND</td>
<td>0.91c</td>
<td>0.0025d</td>
</tr>
<tr>
<td>Leu. oenos</td>
<td>4.07b 0.43d</td>
<td>0.171a</td>
<td>ND</td>
<td>0.310de</td>
<td>ND</td>
<td>0.23f</td>
<td>0.0840b</td>
</tr>
</tbody>
</table>

1 Mean of two replications; means within columns followed by the same letter are not significantly different (p > 0.05).
2 Unfermented CJB contained 0.082% acetic acid and 0.0024% ethanol.
3 Prop. shermanii and P. cerevisiae.
4 Not-detected.
character. Acetic acid was found in all treatments since it was added as a buffering agent prior to fermentation at a level of 0.1%. However, acetic acid was highest in the samples fermented by Leu. mesenteroides and Leu. oenos (Table 3.4). The L. plantarum treatment, which was rated the highest by the panel, was relatively low in acetic acid. The level of acetic acid found in these treatments may not be high enough to be recognized since Golovnya et al. (1986) recommended acetic acid at 0.5% as a recognition threshold concentration.

The fruity character was noted only in the CJB fermented by L. plantarum. Table 3.1 shows that the fruity note in this study was represented by a Muller Thurgau wine standard; Niwa et al. (1987) also made mention of a wine-like flavor in fruit juices fermented by Lactobacillus sp.

Even though some aroma descriptors such as acetic acid, propionic acid, butyric acid and buttery, may be considered to be represented by chemical analysis results of these compounds, they were not. The sensory aroma descriptor results of this study were not in agreement with the data obtained from chemical analysis (Table 3.4).

Flavor by mouth. Three of the taste descriptors, bitterness, sourness and aftertaste, were significantly different among treatments (Table 3.2). The bitterness intensity was rated quite low in all treatments (Table 3.5) but it was still significantly different among samples; Leu. oenos and L. casei treatments led to the highest degree of bitterness. However, an examination
Table 3.5: Means and standard deviations\(^1\) of trained panel flavor by mouth descriptors for eight treatments of cucumber juice brine (CJB) fermented by different microorganisms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MC(^3)</th>
<th>PC(^4)</th>
<th>Lc(^5)</th>
<th>Lp(^6)</th>
<th>Lm(^7)</th>
<th>Ld(^8)</th>
<th>Bb(^9)</th>
<th>Lo(^{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavor by mouth:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweetness</td>
<td>2.31a</td>
<td>2.32a</td>
<td>2.86a</td>
<td>2.06a</td>
<td>2.18a</td>
<td>2.40a</td>
<td>2.28a</td>
<td>1.93a</td>
</tr>
<tr>
<td>(2.77)</td>
<td>(2.93)</td>
<td>(3.07)</td>
<td>(2.74)</td>
<td>(2.41)</td>
<td>(2.62)</td>
<td>(2.62)</td>
<td>(2.48)</td>
<td></td>
</tr>
<tr>
<td>Saltiness</td>
<td>8.30a</td>
<td>8.70a</td>
<td>7.91a</td>
<td>8.82a</td>
<td>8.51a</td>
<td>7.74a</td>
<td>8.23a</td>
<td>8.52a</td>
</tr>
<tr>
<td>(2.67)</td>
<td>(2.67)</td>
<td>(2.21)</td>
<td>(2.96)</td>
<td>(2.35)</td>
<td>(3.04)</td>
<td>(2.06)</td>
<td>(2.53)</td>
<td></td>
</tr>
<tr>
<td>Bitterness</td>
<td>1.49c</td>
<td>1.52bc</td>
<td>2.47a</td>
<td>1.65bc</td>
<td>2.18abc</td>
<td>2.20ab</td>
<td>1.83abc</td>
<td>2.47a</td>
</tr>
<tr>
<td>(2.95)</td>
<td>(3.03)</td>
<td>(3.94)</td>
<td>(2.66)</td>
<td>(3.15)</td>
<td>(3.85)</td>
<td>(3.40)</td>
<td>(4.01)</td>
<td></td>
</tr>
<tr>
<td>Astringency</td>
<td>3.00a</td>
<td>3.13a</td>
<td>2.03a</td>
<td>3.92a</td>
<td>2.52a</td>
<td>1.96a</td>
<td>2.45a</td>
<td>2.41a</td>
</tr>
<tr>
<td>(3.77)</td>
<td>(4.00)</td>
<td>(2.82)</td>
<td>(4.46)</td>
<td>(3.46)</td>
<td>(2.87)</td>
<td>(3.12)</td>
<td>(3.29)</td>
<td></td>
</tr>
<tr>
<td>Soursness</td>
<td>9.80b</td>
<td>9.43b</td>
<td>6.05d</td>
<td>11.55a</td>
<td>7.75c</td>
<td>5.78d</td>
<td>7.18c</td>
<td>7.46c</td>
</tr>
<tr>
<td>(2.80)</td>
<td>(2.74)</td>
<td>(2.47)</td>
<td>(2.24)</td>
<td>(2.74)</td>
<td>(2.31)</td>
<td>(2.66)</td>
<td>(2.74)</td>
<td></td>
</tr>
<tr>
<td>Aftertaste</td>
<td>1.65bc</td>
<td>1.16c</td>
<td>1.24bc</td>
<td>2.85ab</td>
<td>4.45a</td>
<td>1.29bc</td>
<td>1.20c</td>
<td>2.04bc</td>
</tr>
<tr>
<td>(2.60)</td>
<td>(2.36)</td>
<td>(2.08)</td>
<td>(4.06)</td>
<td>(4.11)</td>
<td>(2.28)</td>
<td>(2.08)</td>
<td>(2.45)</td>
<td></td>
</tr>
</tbody>
</table>

Means within rows followed by the same letter are not significantly different (p > 0.05).

\(^1\)Standard deviation is shown in parenthesis under mean value.

\(^2\)Least significant difference at p = 0.05.

\(^3\)MC: Mixed cultures: Prop. shermanii and P. cerevisiae; \(^4\)PC: P. cerevisiae; \(^5\)Lc: L. casei; \(^6\)Lp: L. plantarum;

\(^7\)Lm: Leu. mesenteroides; \(^8\)Ld: Lac. diacetylactis; \(^9\)Bb: B. bifidum; \(^10\)Lo: Leu. oenos.
of the individual panelist's rating (Appendix 1) revealed that only 3 of the 9 panelists detected bitterness in fermented CJB.

*L. plantarum* produced the product with the most marked degree of sourness, while the products fermented by *S. diacetilactis* and *L. casei* were the least sour (Table 3.5). The homofermenters with high acid tolerance i.e. *L. plantarum* and *P. cerevisiae* (in both pure culture and mixed culture treatments) produced strong sourness. Sourness perception correlated well with chemical analysis data i.e. pH (R = 0.952) and titratable acidity (R = 0.988) (Table 3.4, 3.5). Based on these results, either pH or titratable acidity might be a good indicator for sourness in cucumber pickles.

Fleming (1984) stated that cucumber pickles with reduced sugar levels are more resistant to the occurrence of spoilage by other microorganisms within the fermentation system. In view of the common amount of reducing sugars in each treatment (Table 3.4), the heterofermenters i.e. *Leu. mesenteroides* and *Leu. oenos* tend to produce a less sour taste due to utilization of the same or more sugars. Hence, the aforementioned microorganisms may be useful for producing a more stable pickle; one lower in sour taste.

The products fermented by *Leu. mesenteroides* and *L. plantarum* exhibited the highest extent of aftertaste (Table 3.5). The volunteered written comments associated the aftertaste descriptor (Table 3.6) were quite subjective, furthermore, these observations could not be representative of the population, since the panel size was so small. However, the comments
Table 3.6: Aftertaste comments during descriptive analysis

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture&lt;sup&gt;1&lt;/sup&gt;</td>
<td>sour</td>
</tr>
<tr>
<td><em>P. cerevisiae</em></td>
<td>sour</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>pumpkin</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>sour, straight vinegar</td>
</tr>
<tr>
<td><em>Leu. mesenteroides</em></td>
<td>smokey, nutty, rotten, putrid, burnt, perfumery, undesirable</td>
</tr>
<tr>
<td><em>Lac. diacetylactis</em></td>
<td>none</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>none</td>
</tr>
<tr>
<td><em>Leu. oenos</em></td>
<td>slight smokey, burnt</td>
</tr>
</tbody>
</table>

<sup>1 Prop. shermanii and P. cerevisiae.</sup>
obtained perhaps suggest that some bacteria may produce potential aftertastes which could affect final product acceptability. The trained panelists mentioned that the CJB fermented by high acid-tolerant homofermenters i.e. *L. plantarum* and *P. cerevisiae* manifested a sour aftertaste; this strong acid character (produced by homofermenters) could be undesirable to many consumers (Chen et al., 1983). The *Leu. mesenteroides* treatment was rated high in aromatic character and low sourness; and this treatment also fermented nearly all of the available sugar (Table 3.3, 3.4 and 3.5). However, some panelists reported an undesirable (rotten, putrid, burnt) aftertaste associated with this treatment. These aftertaste observations may serve as precautions or limitations to the application of pure cultures in the pickle industry.

**Pan x Trt interaction**

Interactions between panelist and treatment were also significant for each descriptor (Table 3.2); thus indicating that a descriptor intensity rating (for a specific treatment) was rated high by one or more panelists and rated low by other panelists, as compared to intensity scores of that descriptor for the other treatments. Appendix I summarizes the plots of means of intensity scores of each treatment as rated by each panelist; the arrangement of the treatments (on X-axis) is in order of low to high (left to right) for the overall mean of each treatment.

The PanxTrt interaction for the overall intensity descriptor was significant at *p* < 0.01. Disagreements could be mainly found in the
microorganisms MC and Lo, wherein panelists A, C and H generated low ratings as compared to the other treatments, while the remaining panelists rated them higher. The interaction became more significant since panelist B rated Lo quite high as compared to the other treatments (Appendix I). The overall intensity descriptor rating was only based on the intensity standards for aroma. In rating overall intensity, the judges had no specific or common character on which to focus. Also, these disagreements could have been caused by the phenomenon of panelist variation that is normally expected from panelists selected from a population (Lundahl and McDaniel, 1988).

The PanxTrt interaction for cucumber juice and sourness descriptors were significant at \( p < 0.05 \); they were the least significant as compared to the degree of significance for other descriptors. All panelists agreed that Lp was the most sour treatment and Ld was the least sour, however several panelist disagreements occurred with the treatments that resulted in medium intensities (Appendix I). The panelists seemed to become confused when the descriptor intensities were less readily differentiated. In the case of the cucumber juice descriptor, marked disagreements were found in intensity ratings for treatments MC, Lm, Lp, and Lo (the stronger and more flavorful samples); however the panel was in good agreement for treatments Pc, Ld, Bb and Lc which led to higher cucumber juice intensity (Appendix I). Some panelists expressed opinions that the cucumber juice character was masked by the other strong aroma notes, while other panelists did not. Panelist C rated the highly flavored Lm and Lp samples highest in cucumber juice character.
The panelist and treatment interactions were more significant \((p<0.001)\) for the remaining descriptors i.e. fruity, acetic acid, butyric acid, buttery, woody/smokey, bitter and aftertaste. Several disagreements among panelists were also found in the plot for the fruity descriptor (Appendix I). The fruity intensity for Lm was rated high by panelists E, while other panelists rated it low as compared to that descriptor intensity score in other treatments (Appendix I); the same situation occurred in treatment MC for panelists C and F. However, most panelists tended to rate Lp as the highest in fruity intensity as compared to the other treatments.

For the butyric acid descriptor, most panelists tended to rate MC as exhibiting the highest intensity, except for panelists A and E who rated Lp the highest and C who rated Lm the highest; this disagreement was also noted for the Lo treatment where panelist B rated Lo as high intensity compared with the other treatments.

Most panelists agreed that treatment Lp exhibited a high degree of an acetic acid character. Panelists B and G, however, found Lo to manifest even higher acetic note while the remainder of the panel rated it lower. Many panelist disagreements were observed for the treatments that resulted in lower descriptor intensities; consequently, no significant differences were found.

For buttery character, the highest three samples (Bb, Lc and Ld) were clearly higher in intensity than the lowest three samples (Lp, Lo and MC) (Table 3.3 and Appendix I). However, within those groupings, panelists
obviously did not agree on which samples led the highest buttery intensity, thus creating the significant interaction.

For the woody/smokey descriptor, there was fairly good agreement for the microorganisms rated high in this character, but agreement was poor for those rated lower. For example, panelists I and G rated Ld and MC very high in woody/smokey character, while the remaining panelists rated them quite low, thus causing significant interaction.

The sensory examination by the panelists for bitterness was quite different from the situation just described. The majority of the panelists did not perceive bitterness in the samples; hence, the results reported here are based on the ratings by only three panelists. Because of this, the sensory results pertaining to bitterness should be disregarded.

Aftertaste was rated for intensity and with a personal comment as to character by each panelist. The disagreements were high in the treatments which contained higher aftertaste i.e. treatments Lm and Lp. This descriptor was more subjective than others; some panelists might be more influenced by an aftertaste in one treatment than by a different aftertaste of another treatment. Therefore, the treatments with high aftertaste tended to be rated based on each panelist's impression. For example, panelists B, E, F and G might object to the sour aftertaste in treatment Lp but not to the nutty, putrid or burnt character associated with treatment Lm, while the reverse situation may have occurred for panelists C, D, H and I. This suffices to create the significant interaction for this sensory descriptor.
The disagreements found for given descriptor intensity ratings might have been caused by (i) not having a good standard, (ii) not having enough training for the panelists and (iii) normal panelist variability.

**Consumer test**

The consumer test on CJB was conducted by using a balanced incomplete block design. Calvin (1954) suggested that the effect of dependence on or correlation with other samples in the same serving may be found in this design, and this effect was tested. The results presented in Table 3.7 shows that this effect was not significant at the 95% confidence level for both replications. The data were then combined and analyzed as a balanced incomplete block design with replication. The F-values presented in Table 3.8 indicate that consumer scores rating the degree of liking among treatments were not significantly different at the $p = 0.05$ level. The difference was significant at only the $p < 0.15$ level. The adjusted means for the 9-pt hedonic scale data ranged from 4.69-5.39 (Table 3.9). From the hedonic scores, the aroma of the *L. casei* treatment received the highest rating while the aroma of the *L. plantarum* treatment was rated the lowest (Table 3.9). Figure 3.1 shows Quantitative Descriptive Analysis (QDA) configurations of both the *L. plantarum* and *L. casei* treatments. The QDA configurations present the aroma quality differences that might have affected the consumer hedonic scores. *L. casei* was high in sweet, buttery, and cucumber juice aroma, while in contrast *L. plantarum* was high in fruity, woody/smokey and acetic acid characters.
Table 3.7: Analysis of variance of doubly balanced incomplete block design for each replication of fermented cucumber juice brine (CJB) for Consumer testing

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom</th>
<th>Sum Square</th>
<th>Mean Square</th>
<th>F-value $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{st} replication:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>279</td>
<td>1019.00</td>
<td>3.65</td>
<td></td>
</tr>
<tr>
<td>Blocks</td>
<td>69</td>
<td>267.90</td>
<td>3.88</td>
<td></td>
</tr>
<tr>
<td>Treatment(adjusted)</td>
<td>7</td>
<td>48.84</td>
<td>6.98</td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>21</td>
<td>99.33</td>
<td>4.73</td>
<td>1.43NS</td>
</tr>
<tr>
<td>Error</td>
<td>182</td>
<td>602.60</td>
<td>3.31</td>
<td></td>
</tr>
<tr>
<td>2\textsuperscript{nd} replication:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>279</td>
<td>1076.00</td>
<td>3.86</td>
<td></td>
</tr>
<tr>
<td>Block</td>
<td>69</td>
<td>355.00</td>
<td>5.14</td>
<td></td>
</tr>
<tr>
<td>Treatment(adjusted)</td>
<td>7</td>
<td>13.80</td>
<td>1.97</td>
<td></td>
</tr>
<tr>
<td>Correlation</td>
<td>21</td>
<td>54.13</td>
<td>2.58</td>
<td>0.72NS</td>
</tr>
<tr>
<td>Error</td>
<td>182</td>
<td>653.28</td>
<td>3.59</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ NS: Nonsignificant difference at p = 0.05
Table 3.8: Analysis of variance for fermented cucumber juice brine (CJB) using balanced incomplete block design for Consumer testing

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom</th>
<th>Sum Square</th>
<th>Mean Square</th>
<th>F-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>559</td>
<td>2106.00</td>
<td>3.77</td>
<td></td>
</tr>
<tr>
<td>Treatments (unadjusted)</td>
<td>7</td>
<td>36.80</td>
<td>5.26</td>
<td></td>
</tr>
<tr>
<td>Blocks (adjusted)</td>
<td>139</td>
<td>636.00</td>
<td>4.57</td>
<td></td>
</tr>
<tr>
<td>Replications</td>
<td>1</td>
<td>11.43</td>
<td>11.43</td>
<td>3.29NS</td>
</tr>
<tr>
<td>Treatments (adjusted)</td>
<td>7</td>
<td>38.50</td>
<td>5.50</td>
<td>1.59NS</td>
</tr>
<tr>
<td>Blocks (unadjusted)</td>
<td>139</td>
<td>634.00</td>
<td>4.56</td>
<td></td>
</tr>
<tr>
<td>Intrablock error</td>
<td>413</td>
<td>1433.00</td>
<td>3.47</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ NS: Nonsignificant difference at $p = 0.05$. 
Table 3.9: Adjusted means\(^1\) for Consumer testing scores.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Intrablock</th>
<th>Interblock</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Culture(^3)</td>
<td>5.04</td>
<td>3.84</td>
<td>4.91</td>
</tr>
<tr>
<td><em>P. cerevisiae</em></td>
<td>5.30</td>
<td>4.99</td>
<td>5.27</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>5.42</td>
<td>5.19</td>
<td>5.39</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>4.70</td>
<td>4.62</td>
<td>4.69</td>
</tr>
<tr>
<td><em>Leu. mesenteroides</em></td>
<td>5.28</td>
<td>5.72</td>
<td>5.33</td>
</tr>
<tr>
<td><em>Lac. diacetylactis</em></td>
<td>4.64</td>
<td>5.67</td>
<td>4.76</td>
</tr>
<tr>
<td><em>B. bifidum</em></td>
<td>4.73</td>
<td>5.34</td>
<td>4.80</td>
</tr>
<tr>
<td><em>Leu. oenos</em></td>
<td>5.04</td>
<td>4.77</td>
<td>5.01</td>
</tr>
</tbody>
</table>

\(^1\)Original mean scores were from 9-point hedonic scale (1: dislike extremely, 9: like extremely)

\(^2\)Grand mean = 5.02

\(^3\) *Prop. shermanii* and *P. cerevisiae*.
Figure 3.1. Quantitative Descriptive Analysis (QDA) configurations for the fermented cucumber juice brine. *L. plantarum* (——) and *L. casei* (----).
Statistically, the difference in aroma quality, however, did not have much effect on consumer acceptability of the products. This may have been due to the somewhat unusual form of the test, i.e. the evaluation of the aroma of the CJB rather than the evaluation of the pickles themselves.

CONCLUSIONS

The sensory panel employed in this study were able to differentiate the CJB fermented by different microorganisms based on seven aroma and three flavor by mouth descriptors; thus indicating that the selected microorganisms did indeed produce different flavor profiles. Sourness descriptors was positively correlated with the pH and titratable acidity of the fermented CJB; however, the other descriptors were not found to be related to the chemical indices included in this study. In order to better explain these treatment differences, a thorough flavor chemistry analysis, which was not conducted in this study, might be required.

The aroma differences among treatments did not significantly affect consumer ratings of degree of liking of the fermented cucumber juice brine. A further study on consumer testing of fermented cucumber pickles (on both aroma and taste) would be required before making any final conclusion on the effect of the flavor differences on consumer acceptability.
REFERENCES


Calvin, L.D. 1954. Doubly balanced incomplete block designs for experiments in which the treatment effects are correlated. Biometrics 10: 61.


Formation and Potential Industrial Applications of An Insoluble Polyelectrolyte Complex: Chitosan-Polyacrylic Acid.

INTRODUCTION

Chitin is the second most abundant organic compound on earth (Ruiz-Herrera, 1976; Tracey, 1957). Chitin is found especially in marine invertebrates, insects, fungi, and yeast (Austin et al., 1981; Rudall, 1969). On the basis of shrimp, crab and krill processing waste alone, the global estimate of accessible chitin amounts to $150 \times 10^6$ kg/year (Allan et al., 1978; Revah-Moiseev and Carroad, 1981).

Chitosan, which consists of unbranched chains of $\beta$ (1-4) 2-amino-2-deoxy-D-glucan residues, is the best known chitin derivative. Both chitin and chitosan are obtained industrially from shell fish processing waste; chitosan is obtained by deacetylation of chitin.

Chitosan has great potential in food industry and biotechnology applications because of its unique cationic character. As reviewed by Knorr (1984), the three key future applications of chitosan in the food industry are its use: (1) as a flocculating agent; (2) as a functional food ingredient; and (3) as a new polymer for polymer technology applications.

The formation of polyelectrolyte complexes between chitosan and other polyanions such as sodium carboxymethylcellulose (Fukuda, 1979), heparin (Kikuchi and Noda, 1976), acidic glycosaminoglycans (Hirano et al.,
1978) have been also reported. The formation and potential industrial applications of chitosan-alginate coacervate capsules has been recently reviewed by Daly and Knorr (1988). Researchers have also attempted to use chitosan as a water clarifying agent (Prabhu et al., 1976) and as a flocculating agent in some industrial effluent treatment applications (Milazzo, 1982; Bough et al., 1976).

The purpose of this study is to elucidate the insoluble complex formation mechanism between chitosan and a polyanion with a very simple structure, polyacrylic acid. A thorough understanding of interpolymer complex formation should lead to the control of complex formation and facilitate industrial chitosan applications by allowing the preparation of interpolymer complexes with specific functional properties.

In our model studies we considered the effect on complex formation of pH, ionic strength and mixing ratio of chitosan and polyacrylic acid. Turbidity was used as an index of insoluble complex formation. pH measurement and IR analysis were used to investigate the complex formation mechanism. Quantitative analysis of supernatants were done to determine the composition of complexes at different pH values. The potential applications of this model study in industry were also discussed.
4. LITERATURE REVIEW

Chitin and chitosan

Chitin, poly-β (1-4)-N-acetyl-D-glucosamine, a cellulose-like biopolymer (Fig. 4.1), is the second most abundant organic compound on earth (Ruiz-Herrera, 1978; Tracey, 1957). Chitin is found especially in marine invertebrates, insects, fungi, and yeast (Austin et al., 1981; Rudall, 1969). Knorr (1984) noted that chitin is a growing waste disposal problem. On the basis of shrimp, crab and krill processing waste alone, the global estimate of accessible chitin amounts to 150 x 10^6 kg/year (Allan et al., 1978; Revah-Moiseev and Carroad, 1981).

Chitosan, poly-β (1-4) 2-amino-2-deoxy-D-glucan (Fig. 4.1), is commercially produced by deacetylation of chitin (e.g. BioShell Products, Albany, Oregon, USA; Kyowa Oil and Fat, Tokyo, Japan; Kyokuyo Co., Tokyo, Japan). Shrimp and crab processing shell waste contain on a dry weight basis 14-27% and 13-15% chitin, respectively, is used for the production of chitin and chitosan as shown in Fig. 4.2 (Ashford et al., 1977). The crustacean shell waste is usually ground and mixed with a dilute aqueous sodium hydroxide solution to dissolve the protein. The residual material is then treated with diluted aqueous hydrochloric acid to dissolve the calcium carbonate as calcium chloride. Chitin thus obtained is deacetylated using a hot concentrated sodium hydroxide solution (40-50%) (Muzzarelli, 1977). Chitosan could also be produced from many fungal fermentations (Davis and Bartnicki-Garcia, 1984; McGahren et al., 1984).
Figure 4.1: Molecular structures of chitin and chitosan
Figure 4.2: Flow diagram of chitin and chitosan processing
Potential industrial applications of chitosan

Chitosan is the only commercially available cation polyelectrolytes produced from a natural source. Kienzle-Sterzer et al. (1982c) found that chitosan is soluble in dilute hydrochloric acid or organic acid but insoluble in neutral or alkaline aqueous solvent. However, a water soluble chitosan has also been produced (Protan Labs, Redmond, WA).

Chitin and chitosan are, at least to a small extent, already part of our food supply. This observation should facilitate the approval by regulatory agencies of chitosan uses by the food industry. Chitosan could be used as a functional ingredient, as a coagulating agent for treatment of aqueous systems, and as a new polymer for polymer technology applications (Knorr, 1984).

a. Chitosan as a functional ingredient

Chitosan has been suggested as a gluten replacement in bakery products (Anon., 1981). It could also be used to adjust the viscosity of liquid ingredients and to improve the flowability of dry ingredients (Knorr, 1984). The film-forming properties of chitosan have been studied by many researchers (Rigby, 1936; McNeely, 1959; Muzzarelli, 1976). The relationship between the mechanical properties of films and the chitosan structure were investigated by Kienzle-Sterzer et al. (1982a, 1982b). Vojadani and Torres
(1988) evaluated the use of chitosan to prepare edible coatings to control the diffusion of preservatives applied on food surfaces.

Several studies have shown that chitosan applied on plant surfaces has fungicidal action (Stoessel and Leuba, 1984; Hadwiger, 1988). Chitosan sprayed on fresh fruit surfaces prolong shelf life by slowing down the fruit metabolism. Yang and Zall (1984) evaluated the use of chitosan films for reverse osmosis applications.

b. Chitosan as a coagulating agent

The application of chitosan as an agent for the treatment of aqueous systems include the reduction of total solids and the recovery of proteins and polysaccharides from food processing waste streams, the purification of processing and drinking water, as well as the removal of undesirable substances from beverages (e.g. clarification and acid removal). Chitosan has been approved for potable water purification by the U.S. Environmental Protection Agency (Knorr, 1984). Coagulated by products produced from the treatment of food processing waste streams could be used as animal feed (Bough and Landes, 1978). Suspended solids have been removed from waste generated from vegetable processing (Bough, 1975a), poultry processing (Muzzarelli, 1977; Bough, 1975b, 1976), egg breaking operation (Bough, 1975b), meat processing (Bough, 1976), shrimp processing (Bough, 1976), cheese processing (Bough and Landes, 1976; Wu et al., 1978) and fruit cake processing (Bough, 1976). These studies have shown that chitosan can reduce the suspended solids by 70 to 98%. Bough and Landes (1978) found
that the percentage of protein recovered in the coagulated solids ranges from 13-68%.

Chitosan can also be used to deacidify coffee extracts by reducing their chlorogenic acid content as well as by removing other acids such as oxalic, citric, fumaric, malic, pyruvic, quinic and caffeic acids (Magnolato, 1978). The use of chitosan as a clarification agent for apple juice has also been investigated by Sato-Peralta et al. (1989).

Many undesirable metals such as mercury, lead, zinc, copper, chromium, plutonium, uranium and cadmium can be removed from an aqueous system after treatment with chitosan (Eiden et al., 1980; Galun et al., 1983; Marsi et al., 1974; Silver, 1978; Jha et al., 1988). Chitosan has also been used to purify PCB (polychlorinated biphenyls) contaminated water (Van Daele and Thome, 1986).

c. Chitosan for new polymer technology application

Polymer applications for chitosan include: (i) the use of chitosan as carriers; (ii) the use of chitosan for encapsulation/entrapment; and, (iii) the use of chitosan for controlled release systems (Knorr, 1984). Chitosan may be used in the future as a nonabsorbable carrier for concentrated ingredients such as flavors and nutrients (Knorr, 1984). A matrix formed by crosslinking chitosan with multivalent anions as counterions can be used in an entrapment process for immobilizing whole microorganism cells,
enzymes or concentrated ingredients (Knorr, 1984). A modified chitosan has also been used to immobilize enzyme β-D-galactosidase (Lionil et al., 1984).

The formation of polyelectrolyte complexes between chitosan and other polyanions such as sodium carboxymethylcellulose (Fukuda, 1979), heparin (Kikuchi and Noda, 1976), acidic glycosaminoglycans (Hirano et al., 1978) also have been reported. The formation and potential industrial applications of chitosan-alginate coacervate capsules has been recently reviewed by Daly and Knorr (1988). Such capsules are mechanically strong and stable in a wide pH range (Daly and Knorr, 1988).

Limitations of the use of chitinous polymers by the food industry include (Knorr, 1984): (i) the properties of chitin/chitosan vary with source and method of preparation; (ii) chitin has not yet been petitioned to the FDA for food use although an approval for chitosan as a feed additive has been granted; and, (iii) there is very little information on the metabolism of chitin and chitosan in humans.

Polypelectrolyte complexes

On the basis of the main interaction forces, intermacromolecular complexes are divided into four classes: (i) polyelectrolyte complexes; (ii) hydrogen-bonding complexes; (iii) stereocomplexes; and, (iv) charge transfer complexes (Tsuchida and Abe, 1982; Hiemenz, 1986). The polyelectrolyte complexes are formed by electrostatic interaction forces which are much stronger than other secondary binding forces so that the
products obtained are expected to display specific physico-chemical properties.

Polyelectrolyte complexes are formed by mixing oppositely charged polyelectrolyte i.e. polyanions and polycations. Microions are released almost quantitatively as shown in the equation below:

\[ \text{Polyanion} + \text{Polycation} \rightarrow \text{Complex} + \text{Microions} \]

(Tsuchida and Abe, 1982).

Polyelectrolyte complexes can be further classified on the basis of strong and weak polyelectrolytes. In general, the composition of the polyelectrolyte complexes depends on the degree of dissociation of the polyelectrolyte components. In the case of a strong polybase-strong polyacid complexes, the composition of the obtained complexes is unity, while, in the case of weak polyelectrolytes, the composition of the complex depends markedly on their degree of dissociation (Tsuchida and Abe, 1982). The degree of dissociation or degree of ionization (\( \alpha \)) is defined as:

\[
\alpha = \frac{[\text{R-COO}^-]}{[\text{R-COO}^-] + [\text{R-COOH}]} = \frac{[\text{R-COO}^-]}{[\text{R-COOH}]_{\text{total}}}
\]

where \([\text{R-COOH}]_{\text{total}}\) is the total monomer concentration of the polyelectrolyte in solution. The degree of dissociation (\( \alpha \)) of a weak polyelectrolyte is affected by its dissociation constant (\( K_0 \)) and the
solution pH according to the following relationship:

$$\text{pH} = \text{pKo} - \log \left[ \frac{(1-\alpha)/\alpha}{1-\alpha} \right]$$

(Nagasawa et al., 1965; Nagasawa, 1971).

The Ko of polyion can be affected by the polyion concentration, ionic strength and temperature of the solution (Nagasawa, 1971; Abe et al., 1977).

An increase in polyelectrolyte concentration leads to a suppression of the dissociation of the polyelectrolyte due to a rise in the inter- and intra macromolecular electrostatic repulsion and to the interpenetrating of polymer chains (Tsuchida and Abe, 1982).

Increasing the ionic strength of the solution causes: (i) a reduction of electrostatic interactions due to the screening effect of salts; (ii) an acceleration of the dissociation of the weak polyelectrolyte because of a decrease in the intramolecular electrostatic repulsion; and, (iii) an increase of hydrophobicity caused by the contraction of polyelectrolyte chains (Tsuchida and Abe, 1982).
5. Formation and Characterization of an Insoluble Polyelectrolyte Complex: Chitosan-Polyacrylic Acid

ABSTRACT

Chitosan and polyacrylic acid mixtures were prepared in different mole ratios and at different pH values and ionic strengths (0.025-0.300). Complex formation was detected by turbidity measurement and quantified by weighing the freeze dried pellet recovered by centrifugation. No insoluble complex formation at pH = 2 was detected. In the 3 to 6 pH range, the maximum complex formation occurred at different mole ratios. Quantitative analysis of the supernatant showed that pH affects the complex composition. Solution ionic strength in the 0.025-0.300 range did not affect complex formation.

Supernatant pH measurement showed that in the 3 to 5 pH range, the pH of the mixture decreased as the complex was formed. At pH = 6, the opposite behavior was observed. This information was used to propose a mechanism for complex formation which was confirmed by quantitative analysis of the supernatant and IR analysis of the insoluble complex. These studies showed that an electrostatic interaction between -COO" and -NH3"+ groups was involved in complex formation.
INTRODUCTION

Chitosan, the best known chitin derivative, is obtained by deacetylation of chitin. It consists of unbranched chains of \( \beta(1-4)2 \)-amino-2-deoxy-D-glucan residues. Chitosan has great potential in food industry and biotechnology applications because of its unique cationic character. An understanding of the complex formation between chitosan and polyanions could be used to design improved systems for the recovery of proteins and other bioproducts.

Polyelectrolyte complex formation between chitosan and other polyanions such as sodium carboxymethylcellulose (Fukuda, 1979), heparin (Kikuchi and Noda, 1976), and acidic glycosaminoglycans (Hirano et al., 1978) have been previously reported. Researchers have also attempted to use chitosan as a water clarifying agent (Prabhu et al., 1976), and as a floculating agent in some industrial effluent treatment applications (Milazzo, 1982; Bough et al., 1976). In this paper we present work on the elucidation of the insoluble complex formation mechanism between chitosan and a polyanion with a very simple structure, polyacrylic acid. A thorough understanding of interpolymer complex formation should lead to the control of complex formation and facilitate industrial chitosan applications by allowing the preparation of interpolymer complexes with specific functional properties.

In our model studies we considered the effect on complex formation of pH, ionic strength and mixing ratio of chitosan and polyacrylic acid.
Turbidity was used as an index of insoluble complex formation. pH measurement and IR analysis were used to investigate the complex formation mechanism. Quantitative analysis of supernatants were done to determine the composition of complexes at different pH values. The properties of the complex itself such as solubility, porosity, charge density, etc. will not be discussed in this paper.

MATERIALS & METHODS

Materials

Chitosan (CHI, Lot: 5112A) was purchased from Bioshell Inc., Albany, Oregon and was purified by dissolving it in 0.1N HCl, filtering through a medium porosity fritted disk Buchner type filtration funnel, reprecipitating with NaOH, rinsing with deionized water and then freeze-drying. The molecular weight of CHI (220,000) was determined at 25°C using a Cannon-Fenske viscometer with 27.5 g NaCl in 1000 ml of 1% acetic acid as the solvent. Polyacrylic acid (PAA) was purchased from Aldrich (Milwaukee, Wisconsin). Using dioxane as a solvent, its molecular weight was estimated to be 202,000 (Sutterlin, 1975).

Complex formation

0.1 g CHI and PAA were dissolved in 100 ml hydrochloric acid and NaCl solutions, respectively. The ionic strength, 0.025 to 0.300, was controlled by adjusting the concentration of the hydrochloric acid and NaCl solutions. The
pH of both reactants, 2.000 to 6.000, was adjusted by using hydrochloric acid or sodium hydroxide solutions. CHI was insoluble at pH values greater than 6.3. pH was measured with a combination pH electrode (Ross model 81550) and read to 0.001 pH units on a microprocessor pH/mV meter (Orion model 811). The amount of added pH adjusting solutions were recorded to calculate the final concentration of each reactant.

Reactant solutions with equal pH values were mixed in the following volumetric proportions (ml CHI:ml PAA): 0:40, 5:35, 10:30, 15:25, 20:20, 25:15, 30:10, 35:5, 40:0. A mixing ratio was defined as $A/(A+B)$ where:

$$A = \frac{\text{weight of chitosan}}{\text{m.w. of monomer of chitosan}}$$

and

$$B = \frac{\text{weight of polyacrylic acid}}{\text{m.w. of monomer of polyacrylic acid}}$$

The mixture was shaken vigorously and left for 15 minutes before measuring turbidity in a Varian DMS 80 U.V./Visible Spectrophotometer (absorbancy at 420 nm). The insoluble complex was separated by centrifugation at 34,800 g for 40 minutes. The pellet was twice resuspended in distilled water and then centrifuged again. The washed complex was finally freeze dried, weighed and analyzed by IR using the KBr pellet technique (Fukuda, 1979). The pH of the supernatant was recorded and the CHI concentration was measured using the Nessler reagent method (Lang, 1958). By knowing the weight of the complex, the original reactants mixture and the CHI which was left in the supernatant, it was possible to calculate the amount of PAA left in the supernatant.
RESULTS AND DISCUSSION

At pH = 2 and at all ionic strengths tested, there was no insoluble complex formation. At this pH the PAA carboxylic groups do not have a charge density sufficiently high to form a complex with CHI (Nagasawa et al., 1965). The insoluble complex formation occurred only in the pH 3 to 6 range. Although turbidity was a good indicator for complex formation, under some experimental conditions it was not directly related to the amount of complex formed. This occurred when extensive complex formation resulted in sedimentation and lowered the turbidity of the mixture. For example, measurements of mixtures at pH 5 and ionic strength = 0.3 showed two turbidity maxima at 0.56 and 0.30 mixing ratio (Fig. 5.1) while missing the true maximum at 0.41 (Fig. 5.1). Note also that the mixing ratio for maximum insoluble conformation increased with the initial pH of the solution (Fig. 5.2).

The ionic strength range covered in this study, 0.025 to 0.300, did not affect the amount of complex formation. Fig. 5.3 shows complex formation at pH 3 and 6 and only minor differences can be seen between curves at different ionic strengths at these two pH conditions even though the ionic strength changed more than one order of magnitude. The rest of this paper will refer specifically to experiments at ionic strength =0.300.

At pH 3, 4 and 5, the degree of ionization of chitosan was about 1.0, 0.95 and 0.85, respectively (Kienzle-Sterzer, 1984). At the same conditions the
Figure 5.1: Effect of mixing ratio on complex formation: turbidity measurements.
Figure 5.2: Effect of mixing ratio on complex formation: pellet weight determinations.

\[ \text{orH} = 3, \text{DrH} = 4, \text{npH} = 5 \text{ and spH} = 6. \]
Figure 5.3: Effect of ionic strength on complex formation
degree of ionization of polyacrylic acid was about 0.1, 0.2 and 0.5, respectively (Nagasawa, et al., 1965). In other words, in the 3 to 5 pH range, most of the CHI amine groups are in the NH$_3^+$ form while most of the PAA carboxyl groups are in the COOH form. This suggests the following complex formation mechanism:

\[
\text{NH}_3^+ + \text{HOOC} \rightarrow \text{NH}_3^+ \cdot \text{OOC} + \text{H}^+ \quad (1)
\]

Eq. (1) suggests that complex formation should lower the supernatant pH. The relationship between the supernatant pH value and complex weight for mixtures at initial pH 3, 4 and 5 is shown in Figs. 5.4a, 5.4b and 5.4c, respectively. These figures show that indeed, the pH of the mixtures decreased as the amount of complex increased.

At pH = 6, the degree of ionization of chitosan is reduced to about 0.6 (Kienzle-Sterzer, 1984) while that of PAA is about 0.8 (Nagasawa, 1965); i.e. most of the amine groups are in the NH$_2$ form while most of the PAA carboxyl groups are in the COO$^-$ form. This suggests the following complex formation mechanism:

\[
\text{NH}_2 + \cdot \text{OOC} \rightarrow \text{NH}_3^+ \cdot \text{OOC} \quad (2)
\]

Eq. (2) suggests that complex formation should decrease the concentration of free H$^+$, i.e. increase the pH of the supernatant. Fig. 5.4d shows the relationship between supernatant pH and amount of complex formed for mixtures with initial pH 6. It shows that the supernatant pH behaved as predicted by Eq. (2). The effect of pH on the mixing ratio for maximum
Figure 5.4a: Effect of complex formation on supernatant pH
initial pH = 4
$\Delta = \text{pH}$
$\alpha = \text{insoluble complex weight}$

Figure 5.4b: Effect of complex formation on supernatant pH
Figure 5.4c: Effect of complex formation on supernatant pH
Figure 5.4d: Effect of complex formation on supernatant pH

\[ \Delta = pH \]

\[ a = \text{insoluble complex weight} \]

initial pH = 6
insoluble complex formation noticed in Fig. 5.2 has been analyzed in more details in Fig. 5.5. We have incorporated information on the analysis of the supernatant fraction to indicate which reactant was present in trace amounts and which one was found in excess. The arrows indicate the direction in which the excess reactant supernatant concentration increases. The supernatant pH changes are not only a function of the initial pH conditions and the amount of complex formed, but also of the buffering properties of the excess reactant present in the supernatant. Due to the CHI and PAA buffering capacities, the existence or disappearance of them in the supernatant should affect the change in the supernatant pH. For example, in Fig. 5.4a, when the amount of complex formed was 27.5 mg (points 1 and 3), the supernatant pH values (points 2 and 4) were different. This was due to the difference in the amount of excess reactant remaining in the supernatant (Fig. 5.5).

The maximum amount of complex formed, occurred at different mixing ratios depending upon the initial pH conditions. It should be noted that both reactants had different charge densities at different pH values. At low pH values, CHI had a high charge density while PAA had a low charge density. Therefore, complex formation at low pH values required a large amount of PAA to neutralize small amounts of CHI and form the complex. At higher pH values, CHI had a lower charge density and PAA had a higher charge density, therefore the complex formation needed more CHI and less PAA. When the maximum amount of complex was formed, all of the reactants were present in the complex and none left in the supernatant. Therefore, it was possible to assume that the mixing ratio at that point represents the composition of the
Figure 5.5: Effect of mixing ratio and initial pH on supernatant composition
complex formed at each pH value. The result shows that the higher the pH value, the higher the CHI ratio in the complex.

An example of IR analysis is shown in Fig. 5.6 and indicates that the main differences, between the IR spectra of a reactants mixture in the same proportion found in the complex and the complex itself, occurred at 1410 cm\(^{-1}\) and around 1520-1600 cm\(^{-1}\). The wavelengths of 1410 cm\(^{-1}\) and 1580 cm\(^{-1}\) correspond to the antisymmetrical and symmetrical valency vibration of the carboxylate anion present in the complex (Zezin et al., 1975). The absorbancy at 1520 cm\(^{-1}\) has been reported to correspond to NH\(_3^+\) groups present in the complex (Nagasawa et al., 1965). These observations confirm that ionic bonding was involved in the complex formation reaction.

CONCLUSIONS

Our experimental evidence indicates that the insoluble complex formed by reacting chitosan and polyacrylic acid are polyelectrolyte complexes. We have also shown that their composition is a function of the initial pH of the reaction mixture. This finding suggests that it is possible to prepare chitosan-polyacrylic acid complexes with specific and controlled properties.
Figure 5.6: IR analysis of a mixture of chitosan and polyacrylic acid and of a complex formed at initial pH = 3 and mixing ratio = 0.122
REFERENCES


ABSTRACT

This paper discusses applications of recent findings on polymer complex formation obtained with a chitosan-polyacrylic acid model system. This information should aid the optimization of several potential industrial applications. An area of particular importance in the food industry and which is receiving increased attention is the use of poly-electrolytic coagulants of natural origin to facilitate the clarification of food beverages and the recovery of colloidal and dispersed particles from food processing waste streams. Chitosan is a cationic polyelectrolyte and differs from current commercial coagulating agents which are mostly neutral or polyanionic in nature.

The present study suggests that process recommendations can be made based on the ionic strength, pH and charged group concentration of the fluid to be treated. In addition, information on the mechanism of complex formation indicates that pH measurements can be used to monitor the coagulation process. Finally, it shows that the ratio of chitosan to polyacrylic acid in the complex formed is controlled solely by the solution pH. Moreover, when chitosan is added to the solution with that pH controlled chitosan to
polyacrylic acid ratio, both reactants are totally removed from the solution as an insoluble complex.

INTRODUCTION

Chitosan, the best known chitin derivative, is obtained by deacetylation of chitin. Both chitin and chitosan are obtained industrially from shellfish processing waste, e.g., Bioshell Products, Albany, Oregon, U.S.A.; Kyowa Oil and Fat, Tokyo, Japan; Kyokuyo Co., Tokyo, Japan. Despite the quantitative importance of chitin, only limited attention has been given to its applications. This is especially true for food applications (Knorr, 1984).

Chitin is present in marine invertebrates, insects, fungi, and yeast, and wholly deacetylated chitin (i.e. chitosan) has been found in various fungi (Rudall, 1969; Austin et al., 1981). Thus chitin and chitosan are, at least to a small extent, already part of our food supply. It is also a readily available material and currently constitutes a serious waste problem. This can be illustrated by noting that the solid waste fraction of the average U.S. landing of shellfish ranges from 50 to 90% (Swanson et al., 1980; Revah-Moiseev and Carroad, 1981). Annually this results in an estimated $5.3 \times 10^6$ kg to $7.8 \times 10^6$ kg of chitin (Knorr, 1984). Total annual global estimates of accessible chitin amounts to $150 \times 10^6$ kg (Allan et al., 1978; Revah-Moisee and Carroad, 1981). However, collection of wastes for centralized processing remains a problem (Knorr, 1984).
Chitosan consists of unbranched chains of β(1-->4)2-amino-2-deoxy-D-glucan residues (Fig. 6.1). Chitosan toxicity studies with animal models have shown no physiological effects (Arai et al., 1968; Landes and Bough, 1976). For example, chitosan-protein complexes containing up to 5% chitosan fed to rats for six weeks resulted in insignificant differences in growth rate, blood, or liver compared to control groups (Bough and Landes, 1978). Kay (1982) estimated that the use of chitosan as a protein coagulating aid to recover proteins from food processing wastes would result in very low chitosan concentrations in the recovered product.

As reviewed by Knorr (1984), the three key future applications of chitosan in the food industry are its use: (1) as a flocculation agent; (2) as a functional food ingredient; and (3) as a new polymer for the formation of a matrix with unique properties. The use of chitosan to prepare edible coatings which control diffusion of preservatives applied on food surfaces has recently been examined in our laboratory (Vojdani and Torres, 1988). The complex formation process between chitosan and polyanions, which could be used to design improved systems for the recovery of proteins and other bioproducts has also been the subject of research in our laboratory (Chavasit et al., 1988).

During the past decade increasing attention has been given to poly-electrolytic coagulants of natural origin to aid the separation of colloidal and dispersed particles from food processing wastes (Green and Framer, 1979; Kargi and Shuler, 1980). Chitosan, the polycationic carbohydrate polymer
Figure 6.1: Molecular structures of chitin, chitosan and polyacrylic acid
has been found to be particularly effective in aiding the coagulation of protein from food processing waste (Fugita, 1972; Bough, 1976). Examples reported in the literature of biomass recovery from food processing waste have ranged from 70 to 97% (Knorr, 1984). Undoubtedly, it is possible to find synthetic polymers that perform as well or better than chitosan. The difference is that toxicological studies suggest that it should be possible to obtain FDA and USDA approval to use chitosan-coagulated by-products recovered from food processing waste as a feed ingredient (Bough and Landes, 1978).

Polyelectrolyte complex formation between chitosan and other polyanions such as alginates (Daly and Knorr, 1988), esterified alginates (Daly and Knorr, 1988) sodium carboxymethylcellulose (Fukuda, 1979), heparin (Kikuchi and Noda, 1976) and acidic glycosaminoglycans (Hirano, et al., 1978) have been previously reported, and have contributed to an understanding of the insoluble complex formation mechanism between chitosan and polyanions. The long term goal is to establish strategies to control the complex formation process and thus facilitate industrial chitosan applications.

In this paper we review the potential applications of model studies conducted in our laboratory to characterize the effect of pH, ionic strength and mixing ratio on chitosan-polyacrylic acid complex formation. Polyacrylic acid has the experimental advantage of its very simple structure (Fig. 6.1). Particular attention is given to industrial food processes such as
beverage clarification, waste water treatment and biomass recovery from food processing waste. The formation and potential industrial applications of chitosan-alginate coacervate capsules has been recently reviewed by Daly and Knorr (1988). Such capsules are mechanically strong and stable in a wide pH range (Daly and Knorr, 1988). Information on the mechanism formation process for chitosan-polyacrylic acid complexes show that chitosan-polyacrylic acid complexes could also be used for microencapsulation purposes as well.

MATERIALS & METHODS

Materials

Chitosan (CHI, Lot: 5112A) was purchased from Bioshell Inc., Albany, OR. To obtain a higher purity material, it was first dissolved in 0.1 N HCl, then filtered through a medium porosity fritted disk Buchner type filtration funnel, reprecipitated with NaOH, rinsed with deionized water and finally freeze-dried. The molecular weight of CHI (220,000) was determined at 25°C using a Cannon-Fenske viscometer and following the procedures reviewed by Kienzle-Sterzer (1984). CHI was dissolved in a solution of 27.5 g NaCl in 1000 ml of 1% acetic acid. The molecular weight of polyacrylic acid (PAA, Aldrich, Milwaukee, WI) was estimated to be 202,000 using dioxane as the solvent (Sutterlin, 1975).
Complex formation

0.1 g CHI and 0.1 g PAA were dissolved in 100 ml HCl and 100 ml NaCl solutions, respectively. The ionic strength, 0.025 to 0.300, was varied by adjusting the concentration of the HCl and NaCl solutions. No complex can be formed at pH 2 (Chavasit et al., 1988). Nagasawa et al., (1965) have shown that at this pH the PAA does not have a charge density sufficiently high to form a complex with chitosan. Since chitosan is insoluble at pH values higher than 6, experiments could be conducted only in the pH 3 to 6 range. The pH of both reactants was adjusted using HCl or NaOH solutions. The pH was measured with a combination pH electrode (Ross model 81550) and read to 0.001 pH units on a microprocessor pH/mV meter (Orion model 811). The amounts of added pH adjusting solutions were recorded to determine the final reactant concentrations.

Reactant solutions with equal pH values were mixed in 5 ml increments in volumetric proportions (ml CHI:ml PAA) ranging from 0:40 to 40:0. A mixing ratio (MR) was defined as:

\[
MR = \frac{A}{A + B}
\]

where:

\[
A = \frac{\text{weight of chitosan}}{\text{m.w. of chitosan monomer}}
\]
and

\[ B = \frac{\text{weight of polyacrylic acid}}{\text{m.w. of polyacrylic acid monomer}} \]

The mixture was shaken vigorously and left for 15 minutes before measuring turbidity in a Varian DMS 80 U.V./Visible Spectrophotometer (absorbancy at 420 nm).

Complex characterization

The insoluble complex was separated by centrifugation at 34,800 x g for 40 minutes. The pellet was twice resuspended in distilled water and then recentrifuged. The washed complex was finally freeze dried and weighed. The pH of the supernatant was recorded and the CHI concentration was measured using the Nessler reagent method (Lang, 1958). A material balance was used to calculate the amount of PAA left in the supernatant.

RESULTS AND DISCUSSION

Although turbidity is a simple indicator for complex formation it cannot always be used to quantitate the amount of complex formed. Some complex formation conditions can result in sedimentation and lower the expected turbidity of the mixture. For instance, measurements of mixtures at pH 5 (ionic strength = 0.3) show two turbidity maxima (MR = 0.56 and MR =
0.30, Fig. 6.2a) while missing the true maximum (MR = 0.41, Fig. 6.2b). This observation highlights how easily a complex can be removed from the solution and explains why one of the most promising chitosan industrial applications is its use as a natural flocculating agent. However, as noted by Chavasit et al. (1988) future model studies are needed to characterize these chitosan-polyacrylic acid complexes.

Another problem of turbidity determinations is that they are affected by particle size. As will be shown later the complex composition (chitosan to polyacrylic acid ratio) is a function of the pH of the solution. Thus, it can be expected that the complex size will depend upon solution pH.

The amount of complex formed at a given initial pH was the same for all ionic strength values (0.025 to 0.300) used in this study (Fig. 6.3). This finding has practical value since the ionic concentration of industrial waste streams can vary widely.

pH measurements have been used to investigate the complex formation mechanism and confirmed by quantitative and IR analysis (Chavasit et al., 1988). At pH 3, 4 and 5, the degree of ionization of chitosan is about 1.0, 0.95 and 0.85, respectively (Kienzle-Sterzer, 1984). At the same conditions, the degree of ionization of polyacrylic acid is about 0.1, 0.2 and 0.5, respectively (Nagasawa, et al., 1965). In other words, in the 3 to 5 pH range, most of the CHI amine groups are in the NH$_3^+$ form while most of the PAA
Figure 6.2: Complex formation as a function of polymer mixing ratio and initial pH (ionic strength = 0.3)

a. Turbidity measurements (420 nm)
b. Insoluble complex weight
Figure 6.3: Complex formation as a function of polymer mixing ratio, initial pH and various ionic strengths
carboxyl groups are in the COOH form. This suggested the following complex formation mechanism (Chavasit et al., 1988):

\[
\text{NH}_3^+ + \text{HOOC} \rightarrow \text{NH}_3^+ \cdot \text{OOC} + \text{H}^+ \quad \text{(1)}
\]

\[(\text{CHI}) + \ (\text{PAA}) \ (\text{complex}) \ (\text{pH decrease})\]

At pH = 6, the degree of ionization of chitosan is reduced to about 0.6 (Kienzle-Sterzer, 1984) while that of PAA is about 0.8 (Nagasawa et al., 1965); i.e. most of the amine groups are in the \(\text{NH}_2\) form while most of the PAA carboxyl groups are in the \(\text{COO}^-\) form. This suggested the following complex formation mechanism (Chavasit et al., 1988):

\[
\text{NH}_2^- + \text{OOC}^- \rightarrow \text{NH}_3^+ \cdot \text{OOC}^- \quad \text{(2)}
\]

\[(\text{CHI}) + \ (\text{PAA}) \ (\text{complex}) \ (\text{pH increase})\]

Eq.(1) suggests that complex formation at low initial pH values, should lower the supernatant pH while Eq. (2) suggests that the opposite behavior should be observed at high initial pH values. Supernatant pH determinations were consistent with this expected behavior (Fig. 6.4). The complex formation effect on supernatant pH suggests that pH measurements could be used in industrial processes to monitor flocculation rate.

The supernatant pH changes are not only a function of the initial pH conditions and the amount of complex formed, but also of the buffering properties of the excess reactant present in the supernatant. Due to the CHI and PAA buffering capacities, the existence or disappearance of them in the
Figure 6.4: Confirmation of complex formation mechanism supernatant pH measurements
supernatant should affect the change in the supernatant pH. For example, in Fig. 6.4a, when the initial pH was 4 and the amount of complex formed was 20 mg, the supernatant pH values after complex formation were different depending upon the mixing ratio condition (pH=3.95 for MR~0.08 and pH=3.6 for MR~0.58). This was due to the difference in the amount of excess reactant remaining in the supernatant (Fig. 6.5). A similar situation was observed for initial pH 6 conditions.

The effect of pH on the mixing ratio for maximum insoluble complex formation was analyzed in more details using analysis data of the supernatant fraction (Fig. 6.5). The arrows indicate the direction in which the excess reactant supernatant concentration increases. Fig. 6.5 confirmed that the mixing ratio for maximum insoluble complex formation depends upon the initial pH of the solution (Fig. 6.3). Furthermore, it indicates that at the mixing ratio for maximum insoluble complex formation, there were no excess reactants left in the supernatant. This observation has particular significance for applications such as beverage clarification. It would facilitate the approval of regulatory agencies since only trace amounts would be left in the solution while achieving a high level of clarification.

Further analysis of Fig. 6.5 suggests that the complex composition at a given pH is constant. Excess reactants remain in solution. This finding suggests that pH adjustment could be used to control the chitosan concentration of the coagulated by-products to be recovered from food
Figure 6.5: Confirmation of complex formation mechanism: analysis of supernatant composition
processing wastes. This would be particularly valuable if the objective is to use these recovered by-products as an animal feed ingredient.

CONCLUSIONS

Poly-electrolytic coagulants of natural origin, such as chitosan, should facilitate beverage clarification processes and the recovery of colloidal and dispersed particles from processing waste streams. Furthermore, an understanding of the complex formation process can be used to identify process control strategies (e.g. monitoring supernatant pH values).

Initial pH conditions determine the composition of the recovered by-products. This information could be used to obtain by-products with desirable properties.

Future studies will be conducted to further characterize the chitosan-polyacrylic acid complex. The physical and chemical stability, the rheological properties and the charge density of chitosan-polyanionic complexes needs to be quantified. Of particular interest would be the analysis of the interaction of these complexes with proteins and polysaccharides of industrial interest.
REFERENCES


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APPENDIX A

Intensity mean scores of each descriptor rated by each panelist

Data from "OVERALL INTENSITY"
Data from "BITTER"

Data from "AFTERTASTE"