Food Chemistry **13** (1984) 181–192

Physical, Chemical and Microbiological Changes During Green Plant Juice Fermentation

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(Received: 7 September, 1983)

ABSTRACT

Protein was precipitated from alfalfa juice by fermentation at 30°C, 35°C and 40°C.

During fermentation there was a decrease in the pH, the total non-structural carbohydrate and the solid content while there was an increase in the lactic acid concentration and the population of the lactic acid bacteria in the juice. The lactic acid bacteria, which were initially only about 0·1% to 1·0% of the total bacteria count in the juice, predominated after a period of fermentation, producing mostly lactic acid and considerable amounts of acetic acid but only trace amounts of other volatile fatty acids.

The addition of some lactic acid bacteria cultures—Lactobacillus plantarum *and* Pediococcus cereviseae—*to the juice at the beginning of fermentation substantially reduced the lag phase and the final pH of the juice.*

INTRODUCTION

Alfalfa (*Medicago sativa*) is regarded as one of the crops that will be best adapted to green plant juice protein concentrate production, because it has low toxicity, high palatability and high yield of total protein. It also has the advantage of having wide adaptability.

Work at the University of Wisconsin-Madison has been oriented

Food Chemistry 0308-8146/84/$03·00

towards developing a single-pass, weather-independent forage handling system. This will allow the farmer to harvest green alfalfa when it is at peak protein level and process the plant material by mechanical dewatering to produce a high quality forage of proper moisture content for ensiling for animal feed. Concurrent with the mechanical dewatering process, approximately 25 % of the total plant protein is removed with the excess moisture. Using heat coagulation (Pirie, 1971), fermentation (Stahmann, 1978), acid addition (Miller *et al.*, 1975) or any other separation procedure, the plant juice protein is concentrated into high value protein with a high protein quality and low fibre content. Of the separation techniques known, fermentation appears most attractive because of its low energy requirements, and the simple equipment required (Koegel & Bruhn, 1978).

The path of fermentation is determined mainly by the constituents of the food and the bacterial flora present on it. When the natural raw materials are acidic and contain free sugars, yeasts develop readily and the alcohol which they produce restricts the activities of most other naturally contaminating organisms (as in the production of wines from fruits). If, on the other hand, the acidity of a plant product permits good bacterial growth and, at the same time, the product is high in simple sugars, lactic acid bacteria may be expected to develop, as in sauerkraut fermentation (Jay, 1978).

An understanding of the changes that take place during alfalfa juice fermentation will allow for the optimization of the process and the development of a technique for the production of protein precipitate of high quality. This paper is on the physical, chemical and microbiological changes that take place during alfalfa juice fermentation in preparation for the optimization.

MATERIALS AND METHODS

The fermentation was carried out in cast acrylic resin tubes, 0·05 m in diameter and about 0·6 m long. Measured amounts of alfalfa juice were placed in the tubes and these were placed in heated and insulated chambers. The desired temperature in each chamber was maintained by a 100 W bulb switched on and off by a thermistemp temperature controller YSI Model 73ATC. A thermistor type temperature sensor was immersed in one of the tubes containing alfalfa juice in the chamber. This system

provided very precise control as the thermistor probe and controller unit are sensitive to a temperature change of 0·05 °C. A fan was placed in each chamber to aid air circulation and ensure uniform temperature throughout the chamber. The temperatures of the juice samples in the tubes were checked at intervals using the thermometer. The fermentation temperatures used were 30 °C, 35 °C and 40 °C. The pH of the juice was continuously monitored and recorded using an apparatus that combines the Orion gel-filled combination pH electrode Model 90-05 with the Honeywell multi-point millivolt recorder and an amplifier circuit (D. E. Zentner, personal communication). The amplifier circuit and the Honeywell recorder served as a recording pH meter. The unit was balanced at pH values of 6·86 and 4·01 using buffer solutions.

Additional carbohydrate in the form of sucrose (granulated pure cane sugar) was added to the juice during some experiments and its effects on fermentation rate and final pH of the product were observed. Sucrose was used because it has been shown to be the most effective form of carbohydrate in assisting fermentation (Acton *et al.*, 1977). Anaerobic conditions were maintained in some experiments by fitting a stopper with a hole to the top of the acrylic tube. The hole served as a passage to a water trap which allowed gas produced during fermentation to escape without allowing entry of air to the fermentation tube.

Cultures of *Lactobacillus plantarum* and *Pediococcus cereviseae* were cultivated by incubation at 30 °C in all-purpose Tween (APT) broth for about 48 h. Some juice samples were inoculated with these cultures and their effects on the fermentation process were noted.

Samples of the juice were taken for the determination of the solid content and the carbohydrate level (in terms of per cent total non-structural carbohydrate) before and during the fermentation. The total non-structural carbohydrate (TNC) was expected to indicate the total fermentable carbohydrate in the juice. Samples were also taken for volatile fatty acid and lactic acid analysis. At the end of the experiments, volume ratio and solid content of both supernatant and precipitate were determined. Replicates of each experiment were run.

Solid contents of juice, supernatant and precipitate samples were determined by taking samples of about 10–20 g and drying in a controlled oven at 103 °C for about 24 h. Masses of these samples were noted before and after drying in the oven and the solid content was determined from these values.

The procedure used for the determination of the total non-structural

carbohydrate (TNC) was the modified Weinmann method (Smith, 1980).

The method used for the lactic acid determination is one that depends upon the reaction by which lactic acid is quantitatively converted to acetaldehyde. The procedure is detailed in Barker & Summerson (1941) and Waldo (1955).

The volatile fatty acids were analysed by gas–liquid chromatography as described by Baumagardt (1964). The results were plotted on graph sheets and the values were calculated automatically by a computer attached to the chromatograph.

Samples of juice were taken at the beginning, during and at the end of each fermentation run for microbiological analysis. Serial dilutions were made with sterile 0·1 % Proteose Peptone (Difco) solution and were plated for:

(a) Microbial count: Plate agar (Difco), incubation at 30 °C for about 48 h.
(b) Gram positive bacteria: APT agar (Difco), incubation at 30 °C for about 48 h.
(c) Lactic acid bacteria: APT agar (Difco) + 0·02 % sodium azide, incubation at 30 °C for about 48 h.
(d) Detection of coliform: Streaks of the cultures picked from plate count agar were made on eosin methylene blue (EMB) agar and incubated at 30 °C for about 48 h.

RESULTS AND DISCUSSION

A number of factors, including the abundance of rain, weather factors, plant-related factors and soil fertility are known to affect such properties of alfalfa as pH, solid content, carbohydrate level, protein content, chemical composition and the microbial population. Thus there was substantial variability in the properties of juice obtained from plants from different plots or at different times.

The alfalfa juice was found to have a density of about 1·0 kg/litre with few variations; however, the solid content varied greatly from values as low as 4·5 % to values as high as 17·0 %. The pH varied from 5·6 to about 6·1 but usually fresh alfalfa juice had pH values between 5·8 and 6·0. The carbohydrate level, measured as per cent total non-structural carbohydrate, varied between 0·31 and 3·37. The total non-structural carbohydrate, measured in terms of percentage weight of juice, is an

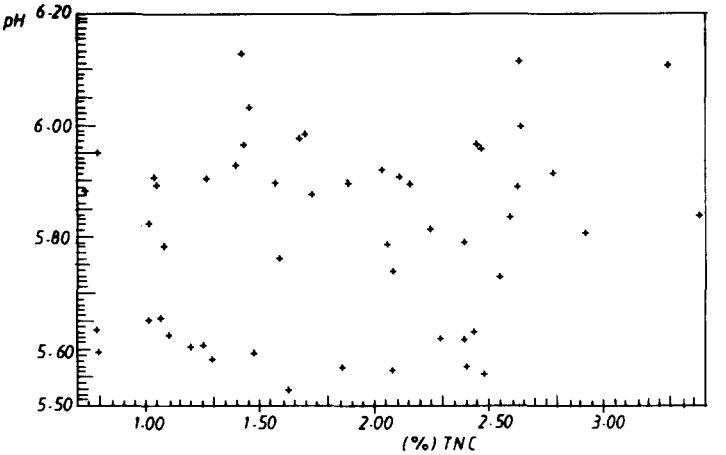

Fig. 1. Variation of juice pH with carbohydrate level.

estimate of the carbohydrate energy that can be made readily available for microbial digestion.

An attempt to interrelate some of the properties of the juice was made by plotting variables against each other. Plots of the pH of the juice against %TNC (Fig. 1) and pH against solid content (Fig. 2) show no relationships. These Figures show random variations. However, a plot of the %TNC against solid content (Fig. 3) shows a trend of increase in %TNC with increase in solid content of the juice.

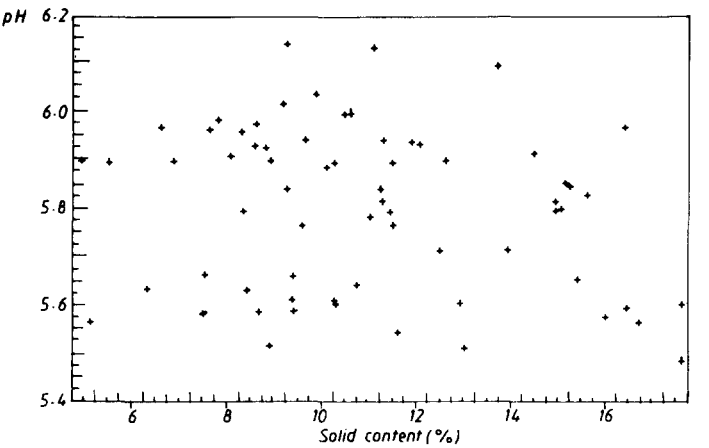

Fig. 2. Variation of juice pH with solid content.

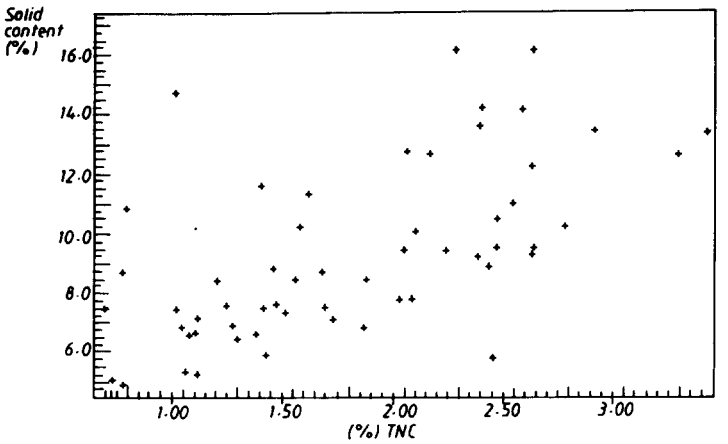

Fig. 3. Variation of juice solid content with carbohydrate level.

Generally there were two phases in the fermentation of alfalfa juice. The first phase (the lag phase) was one in which the pH was relatively constant, usually after an initial pH drop of about 0·2 to 0·3 units during the first hour. In the second phase (the rapid depression phase) there was a rapid and continuous pH drop which continued until further fermentation and pH drop was inhibited.

As the fermentation progressed, there was some kind of layering observed. Large solid particles were noticed to precipitate first at the bottom of the fermentation tube while the top half of the juice was observed to be lighter but still containing green protein material.

Gradually the top part of the juice lost the green colour and reduced in solid content as fermentation continued. Finally, at a pH level of about 4·5, a definitive distinction could be made in the juice. At this point there were two different products with a characteristic acidic smell: the supernatant (the brown juice) and the precipitate (deep green in colour).

The precipitate, which always had a higher solid content, was found in the lower one-third of the fermentation tube with the supernatant above it. Both the supernatant and the precipitate were found to have densities of about 1·0 kg/litre. The solid content of the precipitate has been shown to be related to the solid content of the original juice (Ajibola *et al.*, 1981).

In cases where the pH of the juice dropped to about 3·6 a thin layer of light yellow precipitate (possibly white protein material) was noticed on the top layer of the green precipitate.

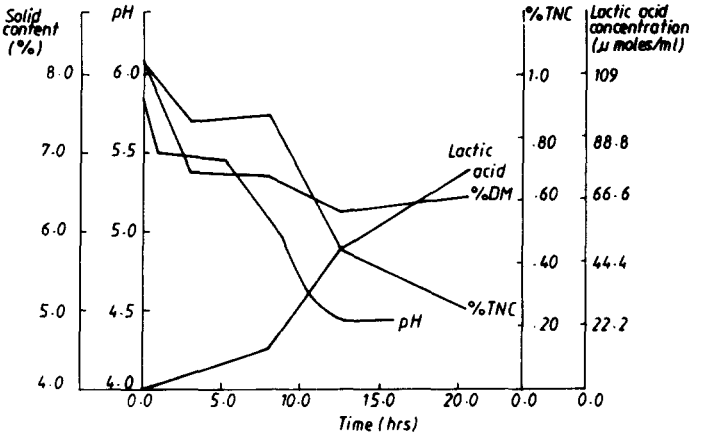

Fig. 4. Changes in juice properties during fermentation.

No effect of the conditions of fermentation such as temperature or the maintenance of anaerobic conditions were observed on the properties of either the supernatant or the precipitate.

The addition of sucrose, while not affecting the extent of the lag phase and the pH depression rate, did consistently result in the lowering of the final pH of the juice after fermentation.

It was noted, however, that, after a point, the addition of more carbohydrate did not result in lower final pH values. On no occasion was the pH of the juice reduced below 3·5 by the fermentation process.

During fermentation the concentration of the total non-structural carbohydrate (TNC) decreased gradually; however, in a few cases, an initial increase in the TNC was noticed before a decrease in its value. The TNC are the carbohydrates that can be accumulated and readily mobilized for metabolism. They do not include cellulose and other structural carbohydrates that will definitely be part of the solids in the juice. It is then quite possible that some of these structural carbohydrates are broken down or hydrolysed enzymatically during the initial period of the fermentation into dimeric and monomeric sugar molecules. As shown in Fig. 4, typically, as fermentation progressed, there was a reduction in %TNC, pH and solid content with a concurrent increase in lactic acid concentration. The reduction in solid content of the juice with time during fermentation indicates that the precipitation of the protein from alfalfa juice by fermentation is gradual.

For all the data collected, a correlation coefficient of equal to, or

greater than, 0·96 was obtained between pH reduction and lactic acid concentration during fermentation. These results suggest a great influence of lactic acid production on the pH depression of alfalfa juice during fermentation.

The production of butyric acid during fermentation is known to indicate the existence of harmful fermentation and the accompanying changes of a proteolytic and putrefactive nature (Watson & Nash, 1960). The presence of this acid in the product is also believed to affect adversely the palatability of the product. The analysis of fermented juice sample (supernatant) for volatile fatty acids (VFA) indicates that the major VFA in fermented alfalfa juice is acetic acid with almost immeasurable amounts of other fatty acids. A typical output from the VFA analysis is shown in Fig. 5. That butyric acid was not produced significantly indicates that undesirable fermentation did not occur in any appreciable form.

The results show that there were appreciable quantities of lactic and acetic acids produced during the fermentation of alfalfa juice, with lactic acid predominating. Ratios of lactic acid concentration to acetic acid concentration varied widely; however, in almost all cases the lactic acid was higher in concentration than the acetic acid (Table 1). The dissociation constant of lactic acid is eight times as much as that of acetic acid. It is, therefore, safe to conclude that the pH depression is primarily

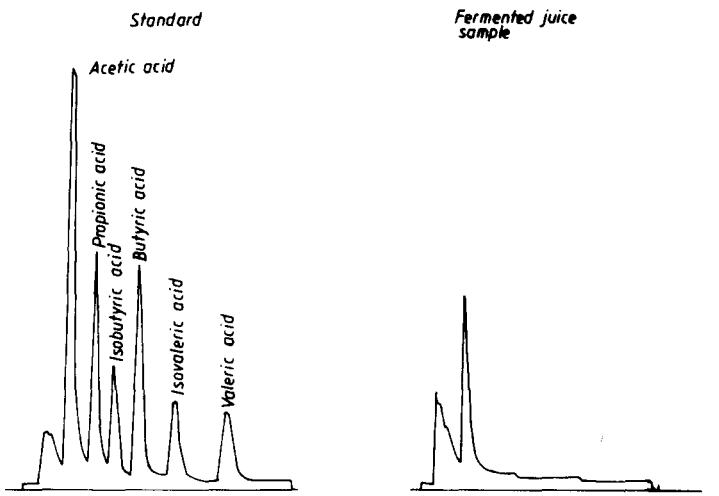

Fig. 5. Print out of chromatograph and computer on VFA analysis.

TABLE 1
Relative Amounts of Organic Acids Produced During Fermentation (Samples Obtained at the End of Fermentation)

Lactic acid (μmoles/ml)	*Acetic acid (μmoles/ml)*	*Lactic/Acetic acid*
123	66·4	1·85
135	63·4	2·13
137	81·6	1·67
74·4	45·3	1·64
96·6	63·9	1·51
124	63·9	1·94
101	52·1	1·94
114	53·5	2·14
190	50·6	3·75
133	68·6	1·94
129	60·3	2·14
225	57·9	3·89
136	50·6	2·68
139	45·8	3·03
140	26·3	5·32
178	34·8	5·10
117	7·99	14·6
118	29·3	4·03

determined by the lactic acid produced in the juice during fermentation. The presence of substantial amounts of acetic acid in the products of fermentation and the fact that during fermentation a lot of gas bubbling through the juice was noticed, indicate clearly the activities of heterofermentative organisms in the juice.

The wide variation in the lactic/acetic acid ratios is believed to be due to the different fermentation patterns possible in the juice, which lead to different proportions of lactic and acetic acids being produced.

Alfalfa juice showed the presence of various types of bacteria at the beginning of the fermentation with the lactic acid bacteria making up only about 0·1 to 1·0% of the total bacterial count which was in the range of 10^8 to 10^9 cells per millilitre.

Usually about one-third of the initial microbial population that showed on plate count agar were gram-negative, yellow pigmented cultures that grew on EMB agar, indicating that they were coliforms. The

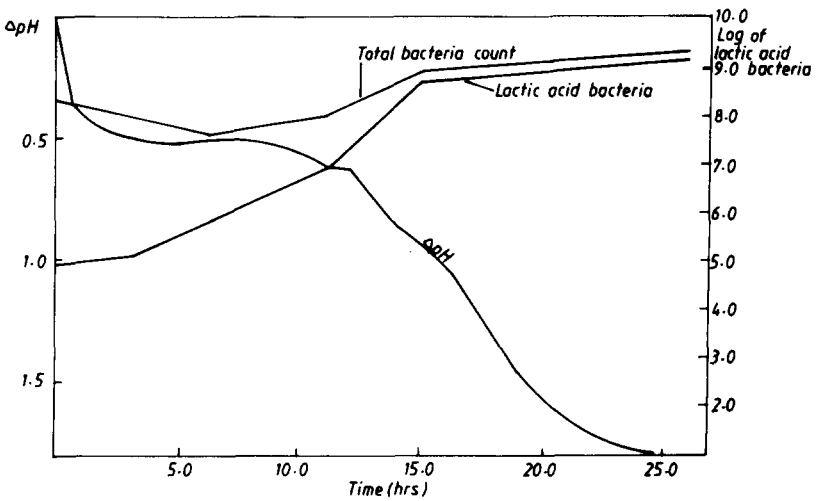

Fig. 6. Growth of bacteria during fermentation.

initial bacterial count varied greatly in the juice from one juice sample to the other and so did the ratio of the lactic acid bacteria to the total count.

During the lag period it was observed that the lactic acid bacteria grew rapidly, as shown in Fig. 6, while the total count decreased slightly. It was noticed that the yellow pigmented coliforms died off gradually during the lag phase and became almost non-existent at the beginning of the rapid pH depression phase. The lag phase was observed to end in all experiments when the lactic acid bacteria count reached the 10^7 to 10^8 cells per millilitre level. At this point the lactics had become almost 90% of the total bacterial population. As the pH depression progressed, the lactic acid bacterial count continued to increase, reaching levels of about 10^9 cells per millilitre of juice by the end of the fermentation. Whenever the juice was allowed to stand after fermentation had ceased and no further pH depression was observed, the lactic acid bacteria count was found to decrease slightly.

From the results obtained it is believed that the lactic acid bacteria were responsible for the fermentation of alfalfa juice, thus producing lactic and acetic acids in quantities that were enough to effect pH drop in the juice. Some juice samples were inoculated with lactic acid bacteria cultures that are known to be good acid producers, namely, *Lactobacillus plantarum* and *Pediococcus cereviseae*. As shown in Fig. 7, the addition of these lactic cultures showed a substantial effect of reducing the lag period and

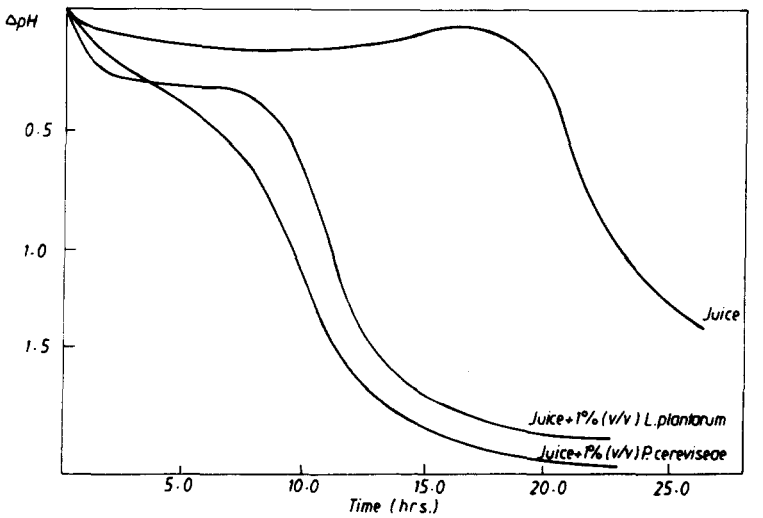

Fig. 7. Effect of addition of lactic acid bacteria culture to alfalfa juice on fermentation.

eventually giving a lower final pH but the rate of fermentation (in terms of pH/time in the pH depression phase) was not altered. This indicates that the lag period was caused mainly by an inadequate number of lactic acid bacteria at the beginning of fermentation. The lowering of the final pH could be due to the better efficiency of the cultures introduced in comparison with the mixed culture developed in the juice during natural fermentation in the conversion of carbohydrate to lactic acid and other organic acids.

REFERENCES

Acton, J. C., Dick, R. L. & Norris, E. L. (1977). Utilization of various carbohydrates in fermented sausage. *J. Food Sci.*, **42**, 174–8.

Ajibola, O. O., Straub, R. J., Bruhn, H. D. & Koegel, R. G. (1981). Fermentation of plant juice as a protein separation technique. ASAE paper 81-1528. ASAE St. Joseph, Michigan, 49085.

Barker, S. B. & Summerson, W. H. (1941). The coulometric determination of lactic acid in biological material. *J. Biol. Chem.*, **138**, 535.

Baumagardt, B. R. (1964). *Practical observations on quantitative analysis of free volatile fatty acid (VFA) in aqueous solution by gas–liquid chromatography*. Dept. Bull. No. 1. Department of Dairy Sci., Univ. of Wisconsin, Madison.

Jay, J. M. (1978). *Modern food microbiology*. D. Van Nostrand Co., New York.

Koegel, R. G. & Bruhn, H. D. (1978). Energy economics of alfalfa juice protein. *Trans. of the ASAE*, **21**(4), 605–9.

Miller, R. E., de Fremery, D., Bickoff, E. M. & Kohler, G. O. (1975). Soluble protein concentrates from alfalfa juice by low temperature acid precipitation. *J. Agric. Food Chem.*, **23**(6), 1177–9.

Pirie, N. W. (1971). *Leaf protein: Its agronomy, preparation, quality and use*. IBP Handbook No. 20. Blackwell Scientific, Oxford.

Smith, Dale (1980). *Removing and analyzing total non-structural carbohydrates from plant tissue*. Research Report No. 41. Research Division, College of Agric. and Life Sciences, Univ. of Wisconsin, Madison.

Stahmann, M. A. (1978). Anaerobic fermentation for coagulation of plant juice protein. Paper presented at the CENTO Symposium on Biological Conversion of Agro-Industrial Wastes into Feed. Lahore, Pakistan.

Waldo, D. R. (1955). Unpublished MS thesis, Cornell Univ., Ithaca, New York.

Watson, S. J. & Nash, M. J. (1960). *The conservation of grass and forage crops*. Oliver and Boyd, Edinburgh and London.