

Induction of Red Color Formation in Cabbage Juice by *Lactobacillus brevis* and Its Relationship to Pink Sauerkraut¹

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Membrane-filtered cabbage juice, when fermented by *Lactobacillus brevis* under conditions of controlled pH, frequently produced a water-soluble red pigment. The pigment, presumably responsible for imparting a highly objectionable discoloration to sauerkraut, was formed during the post logarithmic phase of growth. Color development is pH dependent (5.2 to 6.3) and can be suppressed by chemical reductants or anaerobic conditions of growth. The compound responsible for discoloration was purified and partially characterized.

The grade of commercial canned sauerkraut is established by a composite value derived from the singular evaluations of character, cut, defect, flavor, and color. Of these factors, color and flavor are given the highest, but equal, considerations (1). The maintenance of a bright, cream to light straw color is necessary for producing a high-quality fermented product. The inability to conform to the highest color standard results in the downgrading of quality; if discoloration is serious, the product may be rejected. One such serious color defect is the formation of red or pink kraut.

One type of discoloration is a light pink to deep burgundy coloration, which causes considerable economic losses to processors in Holland (10) and the United States. Since climatic conditions and the varieties of cabbage grown for kraut production vary widely within each locale, it would appear that factors related to the fermentation processes are primarily responsible for inducing the objectionable color formation. Furthermore, the red color develops during the course of the fermentation or immediately prior to processing.

The initiation of red color formation in cabbage and sauerkraut apparently proceeds via a series of complex chemical reactions. Recent studies pertaining to the effects of dehydration upon cabbage indicate that ascorbic acid—amino acid interactions are responsible for

producing non-enzymatic discoloration in the dried product (6), whereas in pink kraut the color has been attributed to the formation of a leucoanthocyanidin (4).

Since the kraut fermentation arises as a result of a heterogeneous microbial population, it is difficult to assess the role each species contributes to color formation. Although yeasts are known to impart color to kraut (2, 3, 5, 10; E. Steinbuch, Antonie van Leeuwenhoek J. Microbiol. Serol., Yeast Symposium Suppl. no. 35, F39, 1969), the potential induction of red color by pure cultures of lactic acid bacteria associated with the "normal" fermentation of kraut has, to our knowledge, never been reported. Therefore, this paper describes those conditions which contribute to the onset of discoloration, with particular emphasis on the role of *Lactobacillus brevis*, in inducing a cerise color in filter-sterilized cabbage juice.

MATERIALS AND METHODS

Cultures. *L. brevis* B155, *L. plantarum* B246, *Leuconostoc mesenteroides* C33, *Pediococcus cerevisiae* E66, and *Streptococcus faecalis* 8043 were obtained from the culture collection of this department.

Growth measurements. Fresh cabbage juice (variety Glory) was prepared as previously described (9). The cabbage sera, sterilized by membrane filtration in a glass assembly, were dispensed aseptically in 10-ml volumes (16- by 150-mm test tubes) for growth and color studies. Each sample was inoculated with one loopful (about 1.8×10^4 cells/ml) of a 24-h culture grown previously in cabbage juice. The viable cell counts were taken at 24-h intervals and were esti-

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mated by plating on tryptone-glucose-yeast extract-salts agar medium (8).

Sodium hydroxide (0.5 N) or solid calcium carbonate were used for maintaining preselected pH values. Sterile CaCO_3 , when added to cabbage juice at concentrations of 0.25, 0.50, 0.75, 1.0, and 1.5% (wt/vol), provided final pH values of 4.2, 4.4, 5.2, 5.8, and 6.0, respectively, whereas the maintenance of pH by NaOH was controlled by pH-stat. In addition to using smaller volumes (test tubes) for the studies of color and growth development, larger quantities of juice (40 to 80 ml) were fermented in a sterile fermentation assembly equipped with sampling ports, a magnetic stirring bar, and pH electrodes and maintained at 32 or 22 C by a constant temperature water bath.

Measurement and purification of colored materials. Since it was desirable to establish the chemical composition of the unknown product(s), larger volumes of juice (5 to 10 liters) were fermented in the hope of obtaining increased quantities of red pigment. It was found, however, that in addition to being difficult to filter-sterilize, the fermented juice was extremely vulnerable to extensive browning, a condition which resulted in excessive losses in the yield of red color. It was observed that juice, when prepared in 150-ml quantities and contained in 250-ml Erlenmeyer flasks was more stable, and therefore was used for subsequent fermentation studies.

After fermentation and maximum color production (about 8 days), the juices were centrifuged ($20,000 \times g$) for 20 min. The clear, red juice was extracted four times with 25-ml volumes of diethyl ether. The aqueous phase, containing the red color, was concentrated sixfold under vacuum at 40 C. The red concentrate was treated with acetone (85% saturation) and filtered through paper. The resulting supernatant fraction was evaporated to dryness, dissolved in 10 ml of water, and applied to a polyvinylpyrrolidone (Polyclar AT) column (2.5- by 10-cm) which had been previously equilibrated with water. The column, retaining the adsorbed pigment, was washed with five bed-volumes of water and was subsequently eluted with 900 ml of methanol containing 0.01% hydrochloric acid. After elution, fractions containing the red pigment were concentrated to 0.2 ml, applied as a band on preparative cellulose thin-layer chromatography (TLC) plates, and further purified in the following solvent systems: (i) water-acetic acid-hydrochloric acid (80:25:5); (ii) 2% aqueous acetic acid; and (iii) upper phase of butanol-acetic acid-water (4:1:5). After irrigation, the red band was scraped from the plate, eluted with methanol (50 ml), and concentrated to 10 ml. A sample of the concentrate (4 ml) was subjected to ultraviolet light (UV) and visible spectroscopic analyses. The remainder of the concentrate (5 ml) was evaporated to dryness and the residue was mixed with KBr (0.10 g) and pressed into micropellets for infrared analysis. The sprays used for the qualitative analyses of functional groups included: 2,6-dichloroquinone chlorimide, ferric chloride, and phosphomolybdic-phosphotungstic acid, for phenolic compounds; silver nitrate for reducing materials; and ninhydrin for amino acids.

RESULTS AND DISCUSSION

While studying the effects of pH upon the growth rates of lactic acid bacteria (9), it was observed that *L. brevis*, when grown in cabbage juice containing calcium carbonate, imparted a brilliant red color to the fermented extract. Of the five microorganisms commonly associated with the kraut fermentation, *L. brevis* was the only species which induced color formation in the buffered juice (Table 1). Under these conditions, *L. brevis* produced a fivefold increase in color (A_{558}) within 7 days of incubation at 32 C. In unbuffered juice, the culture produced no apparent changes in color, and the final absorbance values were similar to those displayed by the non-color-forming species. The inability of the cultures, other than *L. brevis*, to produce a red color in buffered juices cannot be attributed to differences in growth-sustaining properties of the buffered and regular extracts. This was confirmed by plate counts; each juice, when inoculated with about 1.8×10^4 cells per ml, provided final populations of 9×10^8 to 1.2×10^9 cells per ml after 3 days of incubation at 32 C.

The rates of color formation as a function of the growth of *L. brevis* at two temperatures (32, and 22 C) in cabbage are shown in Fig. 1. It may be observed that the growth rates at each respective temperature in both buffered and unbuffered extracts were quite similar. After 5 days of incubation at 32 C, the juice containing no CaCO_3 yielded a total viable population of 5×10^8 cells per ml. During this time period the initial pH of the medium was abruptly lowered from 6.2 to 3.8, a value which remained constant throughout the 21-day incubation. No red color was formed under these conditions of growth.

TABLE 1. Comparison of red color development in cabbage juice by lactic acid bacteria associated with the sauerkraut fermentation^a

Culture	Regular juice		Buffered juice ^b	
	Ab-sorbance ^c	pH	Ab-sorbance	pH
<i>L. brevis</i>	0.040	3.8	0.275	6.0
<i>L. plantarum</i>	0.035	3.5	0.060	4.8
<i>L. mesenteroides</i>	0.055	3.7	0.050	6.0
<i>P. cerevisiae</i>	0.030	3.6	0.055	4.9
<i>S. faecalis</i>	0.030	4.2	0.050	6.2
None (control)	0.050	6.2	0.050	6.2

^a Incubated at 32 C for 7 days.

^b Regular cabbage juice containing 1.5% CaCO_3 .

^c 558 nm.

Although CaCO_3 had no apparent effect upon growth, it induced most markedly the development of red color. Color production was initiated at about 5 days of incubation at 32 C and reached its maximum intensity 5 days later (Fig. 1). It appeared that color production occurred during the latter stages of post-logarithmic growth and attained its maximum intensity during the stationary growth phase. Incubation beyond 10 days invariably resulted in a marked decrease in absorbance at 558 nm, and after 21 days of incubation the maximum color intensity was reduced more than 60%. The reduction in absorbance at 558 nm was accompanied by a concomitant increase in the 500-nm region suggesting the onset of browning.

A comparison of color formation as a function of temperature is also shown in Fig. 1. As might be expected, a 10 degree down-shift in temperature, i.e., from 32 to 22 C, reduced both growth and color development rates by nearly 50%. At 32 C, 6 days were required to achieve maximum cell yields and 8 to 10 days were required for the formation of maximum color intensity, whereas at 22 C, 13 and 19 days were required, respectively, to achieve similar results. Again, as in the case of incubation at 32 C, color production was initiated during the latter phases of growth and occurred only in the juice containing CaCO_3 .

To determine if CaCO_3 served as a buffering or chelating agent, the effects of various mono- and divalent ions upon color production were examined. The addition of the chloride or sulfate salts of calcium, magnesium, manganese, iron, sodium, and potassium, when supplied at a concentration of 0.5%, produced

no enhancement in color response. Therefore, it was concluded that CaCO_3 played the role of a buffering agent in invoking the color development.

Additional evidence that pH was involved, in part, in color formation, was established by growing the culture in cabbage juice with varying concentrations of hydrogen ions. The culture, when maintained at constant pH by sterile NaOH (dispensed by pH-stat) provided color intensities similar to those obtained by the CaCO_3 -containing systems. As reflected in the increased absorbance values at 558 nm, pH markedly influenced color formation (Fig. 2). Increased quantities of red color were formed as the pH was raised from 4.5 to 6.0. Approximately equal levels of red color were attained in cultures constantly adjusted to pH values of 6.0 and 6.3; higher pH values were not studied. Since the growth rate of *L. brevis* is suppressed by lowering the pH of cabbage juice (9), the absorbances reported in Fig. 2 represent the maximum values attained as a result of growth at each respective pH. The absorbances observed at pH 5.0 or greater were obtained after 9 days of incubation, whereas those intensities produced at pH 4.5 or less were recorded after 21 days of incubation. These latter prolonged periods of incubation were used to permit the development of maximum color intensities under more acidic conditions. Direct microscopic count showed that each sample reached a minimum population of 9×10^8 cells per ml during the course of the fermentation. The failure of these extended incubations to provide color intensities equal to those observed under the more alkaline conditions show that pH plays a vital role in inducing color formation.

This dependence of maximum color formation upon pH, i.e., >5.2 , appears to be similar to that reported for the induction of color in dried cabbage by chemical mechanisms (6). However, the route of color generation in the cabbage extracts differs from that of the dehydrated product, in that color development in fermented juices is not only pH dependent, but also requires the presence of *L. brevis*.

Further studies concerning the significance of the bacterium in initiating color response and the inability of sterile sera to undergo spontaneous color changes as a result of chemical or inherent enzymatic reactions likewise were investigated. A 48-h, unbuffered cabbage juice culture, containing 5.5×10^8 cells per ml, showed no evidence of color. However, when 25-ml samples of this culture (pH 3.9) were adjusted to pH 5.5 with NaOH (40%) and

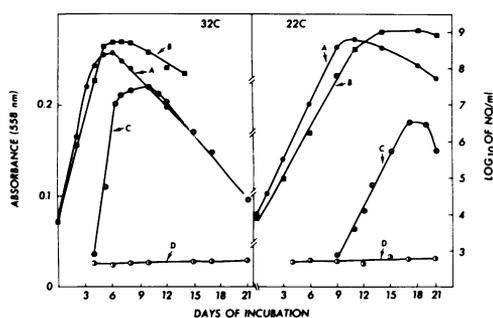


FIG. 1. Effects of calcium carbonate and temperature upon red color formation and growth of *L. brevis* in cabbage juice. A, viable cell count in regular juice; B, viable cell count in juice containing 1.0% CaCO_3 ; C, red color formation in juice containing 1.0% CaCO_3 ; final pH 5.2; D, color production in regular juice, final pH 3.8.

reincubated, color production was initiated within 16 h (Fig. 3). Maximum color intensity was achieved after 4 days of incubation; viable cell numbers remained at about 1.1×10^9 per ml throughout this period. No color development occurred (Fig. 3) when the pH of the juice (3.9) was not altered or when the pH of the juice was adjusted to 5.5 and the juice was sterilized by filtration before it was reincubated.

Juice, when fermented in 10-ml volumes for 8 to 10 days at 32 C, often possessed a reddish hue at the air-liquid interphase. This suggested air-induced color formation. A comparison of

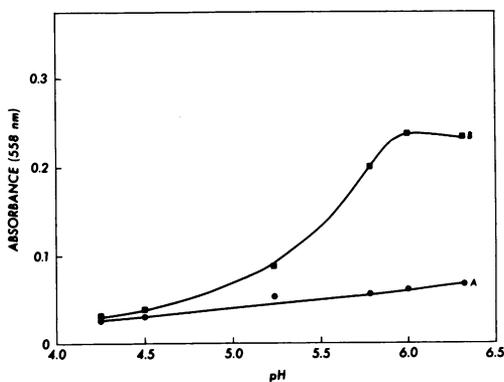


FIG. 2. Effects of pH upon red color production by *L. brevis* grown in buffered cabbage juice at 32 C. A, controls; B, fermented juices. pH maintained by sterile CaCO_3 or NaOH (0.5 N) dispensed by pH-stat. Absorbances represent maximum color production prior to onset of degradation.

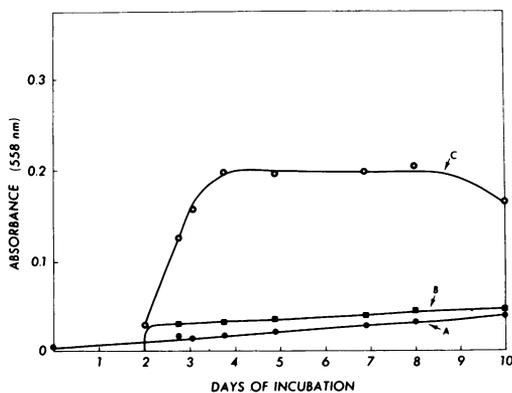


FIG. 3. Effects of pH adjustment upon induction of red color development by *L. brevis* grown in cabbage juice. Cabbage juice (75 ml) was fermented at 32 C for 48 h (to pH 3.9) and divided into three samples: A, no pH adjustment; B, pH adjusted to 5.5, and then the juice was filter-sterilized; and C, pH adjusted and maintained at 5.5 in a pH-stat. All samples were reincubated at 32 C.

absorbances arising as a result of bacterial growth under atmospheres comprised of varying air and nitrogen compositions show that aerobic conditions were most conducive to color formation (Fig. 4). Although each juice, buffered at pH 5.7, showed evidence of color, those extracts incubated in the highest air-nitrogen atmospheres (90 to 100% air, respectively), provided intensities threefold greater than the corresponding extract grown under nitrogen only.

In addition to suppressing color formation, the less aerobic conditions of growth produced lower cell yields in the unbuffered extracts than in the buffered series. As shown in Fig. 4, the total populations in each of the buffered extracts (determined by direct microscopic count) was in excess of 10^9 cells per ml, whereas the cell yields in the unbuffered juice decreased as a function of increasing anaerobiosis. In the case of the unbuffered extracts, incubation in an air atmosphere under stationary conditions provided a cell yield similar to that of the buffered media (2×10^9 cells/ml), whereas under anaerobic conditions (100% nitrogen) the cell yield was only 1.1×10^8 cells per ml. Although the growth yields of *L. brevis* can be increased by aerobic conditions (8), pH also appears to produce vital interactions in limiting growth in cabbage extracts.

Since discoloration was initiated in part by aerobic conditions, the use of chemical reductants as potential suppressors of color formation were investigated. The results of fermenting cabbage juice, pH 5.8, supplemented with ascorbic acid, cysteine, glutathione, and S-methylcysteine (0.1, 0.5, 1.0, and 2.5 mg/ml) are

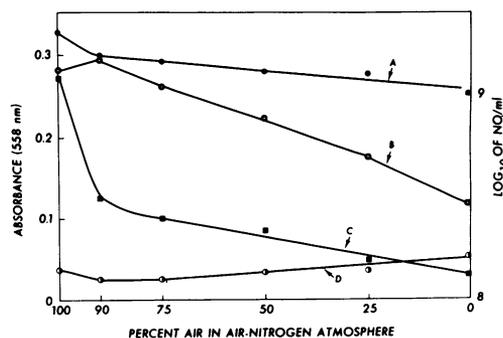


FIG. 4. Effects of air and nitrogen atmospheres upon red color development and growth of *L. brevis* in buffered and unbuffered cabbage juice. A and B, direct microscopic cell counts in buffered (pH 5.2) and unbuffered juices, respectively; C and D show red color formation in buffered and unbuffered juices. Incubated 8 days at 32 C.

shown in Fig. 5. (Although *S*-methyl-cysteine is a nonreducing compound, it is a major sulfur amino acid found in cabbage [11] and its role as potential reductant remains to be clarified.) Of the above compounds examined, ascorbic acid was the more effective color suppressant at lower concentrations (less than 1 mg/ml) than was either cysteine or glutathione, whereas the reducing sulfur materials (cysteine and glutathione) were more effective retardants of color at higher concentrations (2.5 mg/ml). It was also observed that, once the pigment had formed, the color could not be reversed by the addition of the above compounds. Since *S*-methyl-cysteine was without effect, it appears that this compound provides no color-reducing properties in the kraut fermentation.

Attempts to increase the final yield of red pigment by fermenting cabbage juice concentrates consistently resulted in producing a dark brown solution containing little red color. Lyophilized juice, reconstituted to provide 2- to 10-fold increases in soluble solids, or when added to regular juice to provide 2-fold concentrates, resulted in similar failures to produce a bright, red color. This loss in red color also occurred when fermented juices were stored at -50°C for 3 days. Not only is the red color unstable under conditions of rapid freezing and thawing, but it is labile to heat treatment. For example, the color intensities of two fermented juices, pH 3.5 and 5.5, decreased 35% when immersed in boiling water for 30 min. The effects of heat shifted the 558/500 nm ratio from 1.78 to 1.13 and produced browning. This suggests that temperatures used for processing

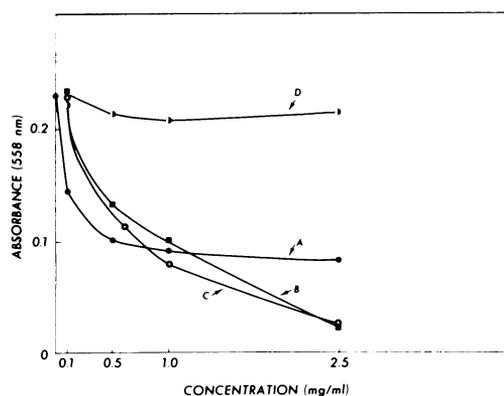


FIG. 5. Influence of reducing agents upon the suppression of red color development in buffered cabbage juice fermented by *L. brevis*. A, ascorbic acid; B, glutathione; C, cysteine; D, *S*-methyl-L-cysteine. Incubated 9 days at 32°C , pH 5.2.

commercial kraut (75°C) cannot be used advantageously for eliminating red color formation without imparting deleterious discolorations.

At room temperature, the pigment is more stable under acidic than alkaline conditions. The adjustment of pH from 4.5 to 1.0 produced no significant change in absorbance at 558 to 568 nm (Fig. 6). At pH 8.5 the extract showed no visible absorption. Upon acidification with hydrochloride (concentrated) the alkaline solution regained its original red color. Although this pH-dependent color response is reversible, the yield, as measured by peak areas, decreased nearly 50%.

The inability to extract the red color with ethers (petroleum or diethyl), amyl acetate,

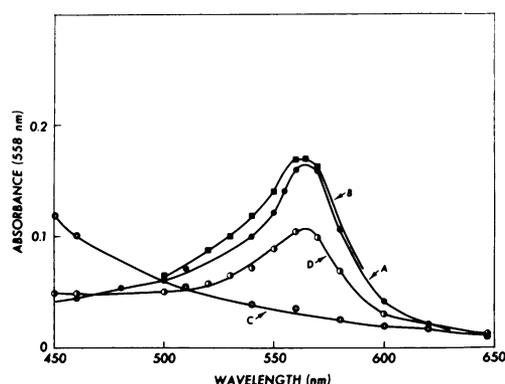


FIG. 6. Effect of pH upon the stability of red pigment produced in buffered cabbage juice by *L. brevis*. Juice (pH 5.2) fermented 8 days at 32°C , centrifuged, and pH adjusted to: A, 4.5 (concentrated hydrochloric acid); B, 1.0 (concentrated hydrochloric acid); C, 8.5 (40% NaOH); D, (C) acidified to pH 1.0 (concentrated hydrochloride) and absorbances were read immediately.

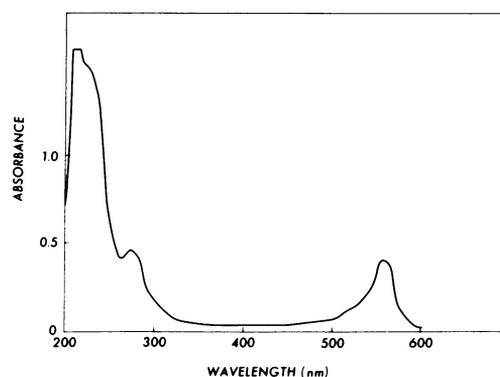


FIG. 7. Spectrum of purified red pigment produced in buffered cabbage juice by *L. brevis*.

chloroform, or *n*-butanol, but its complete solubility in water-miscible reagents, such as methanol, ethanol, and acetone, show that the pigment is an extremely hydrophilic compound. This pigment, purified by column chromatography and then applied to TLC cellulose plates and irrigated in three solvent systems, provided a visible, singular, red band with the following R_f values: water:acetic acid:concentrated hydrochloric acid (80:20:5), 0.09; water:acetic acid (98:2), 0.11; butanol:acetic acid:water (4:1:5), 0.38. The above pigment, when subjected to various spray reagents, failed to show the presence of amino acids, reducing, aromatic, and indole constituents.

Spectral analyses of the acidified methanolic elute (Fig. 7) show that the purified pigment possessed three absorbance peaks, at 558, 272, and 226 nm, respectively. Although these UV absorbances show similarities to the spectral properties assigned to the red constituents of kraut by Gorin and Jans (4), the visible absorbance of the pigment produced by pure culture fermentation occurred at a wavelength considerably higher than they had reported (558 versus 540 nm).

Furthermore, the infrared spectrum of the pigment (in KBr) showed absorption bands at the following frequencies: 675 (w); 746 (m); 1,045 (w); 1,075 (m); 1,125 (s); 1,283 (s); 1,387 (s); 1,470 (m); 1,610 (s); 1,737 (s); 2,870 (s); 2,940 (s); 2,960 (s); and 3,500 (m) cm^{-1} . (Abbreviations [absorption intensities]: w, weak; m, medium; s, strong.)

A correlation between the above band positions and types of grouping present indicates that the pigment (i) is aliphatic in character (strong signals in the 2,960-2,870 cm^{-1} range), (ii) contains a carbonyl group(s) (1,737 cm^{-1}), (iii) contains a methyl group(s) (1,387 cm^{-1}),

(iv) contains a number of hydroxyl groups (3,500 cm^{-1}). Therefore, these data suggest that the red discoloration produced by *L. brevis* is not a flavonoid, anthocyanin, or anthocyanidin, but rather that the pigment is a saturated aliphatic ester, aldehyde, or diketone, or contains a five-membered ring ketone within its structure. Further determinations of the exact structure of the cabbage pigment were hampered by the difficulties encountered in producing large quantities of the red cabbage juice and by the general lability of the compound responsible for this discoloration.

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