Growth Stimulants in Plant Extracts for
Leuconostoc citrovorum

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The growth of Leuconostoc citrovorum ML 34, an isolate associated with the malo-lactic fermentation of wine, was stimulated in part by grape, orange, cabbage, and tomato juices. The stimulatory activity of tomato serum was associated with the carbohydrate fraction. Further purification of the fraction showed that fructose was the factor responsible for initiating growth. In addition to fructose, the organism required CO₂ for establishing growth. Saturated CO₂ atmosphere and catalytic amounts of fructose served as substitutes for plant extracts in a complex glucose medium.

The addition of plant extracts to a medium for the subsequent enhancement of growth of many lactic acid bacteria has been routinely employed in laboratories for many years. Tomato juice, an ingredient commonly added to media for cultivating lactic acid bacteria, serves as an essential adjunct for the propagation of the fastidious bacteria associated with the malo-lactic fermentation of wines (6, 13, 17). These gram-positive, heterofermentative cocci, in addition to requiring unknown growth factors present in tomato juice, produce no slime, initiate growth at low pH (3.7), and are more tolerant to ethanol (10%) than are other leuconostocs. Based upon these and other discerning physiological properties, a new species Leuconostoc oenos has been proposed (5).

L. citrovorum ML 34, a wine isolate possessing many of the taxonomic characteristics of this newly proposed species, was used to induce the malo-lactic fermentations successfully in California (8). This microorganism, likewise, is difficult to cultivate in complex media and requires unknown growth factors present in tomato and grape juices (9). Therefore, the present study was undertaken to define the conditions essential for obtaining maximum growth yields, and to determine the nature of the plant materials responsible for promoting such growth enhancement activities in L. citrovorum ML 34.

MATERIALS AND METHODS

Culture. L. citrovorum ML 34 was obtained from Ralph E. Kunkee, Department of Enology, University of California, Davis. The culture was maintained in a tryptone-glucone-yeast extract-salts medium (TGYE) supplemented with tomato juice (10%) as previously described (15).

Preparation of plant extracts. Fresh tomatoes (New Yorker variety) were washed well and the juice was expressed in a paddle-finisher. The juice, pH 4.4, was centrifuged (20,000 × g) for 20 min. The tomato serum was concentrated 12-fold by vacuum distillation at 40 C. The concentrate containing 897.8 g (total solids) was adjusted to pH 7.0 with granular calcium carbonate and treated with ethanol (80% saturation). After 18 hr of standing at 4 C, the alcohol-soluble fraction (411.5 g) was decanted and further treated with acetone (38% final). The alcohol-acetone-soluble fraction (334.2 g) was obtained by decantation after 3 days of standing at 4 C. Organic solvents were removed by vacuum distillation, and the various fractions were stored at −20 C.

Ion-exchange chromatography. A portion of the alcohol-acetone-soluble fraction (31.8 g, pH 6.0) was passed through a column (4.5 by 42.5 cm) of the cation-exchange resin, Dowex 50 (H⁺). The effluent (29.2 g, pH 2.3) was passed immediately through a Dowex 3 (OH⁻) column of similar capacity (yield: 27.1 g, pH 6.7). A portion of this latter fraction (11.1 g) was passed through an activated charcoal (Darco, 80 mesh) column similar to the above. The clear, decolorized, aqueous effluent provided 9.6 g of material. The columns were washed with water until no sugar was detected in the effluent by anthrone reagent (12).

All fractions were then adjusted to pH 5.2 with phosphoric acid (2 N) and made to a volume equiva-
lent to that concentration of the original serum (29 mg/ml, dry weight). The independent fractions were sterilized by filtration and added to 5-ml quantities of double-strength TGYE broth (final volumes of 10 ml).

**Paper chromatography.** Samples (200 mg) were spotted as compact bands on sheets (46 by 57 cm) of Whatman no. 1 high-capacity chromatography paper. Descending chromatography employing butanol-acetic acid-water (5:1:4) or butanol-ethyl alcohol-water (2:1:1) solvent systems were used for irradiation. After 96 hr of development, marker strips were sprayed with silver nitrate or p-anisidine (2). The areas corresponding to the color-developed marker strips were eluted with distilled water, concentrated under vacuum, filter sterilized, and added to the TGYE assay broth. Turbidometric measurements (660 nm) served as a means of determining the biologically active areas.

**Other natural sources of the stimulatory factor.** Frozen orange juice concentrate, fresh grape, and cabbage juices were centrifuged, filter sterilized, and added as 10% supplements to the TGYE basal medium, pH 5.2.

** Destruction of the stimulatory factor.** Each filter-sterilized plant extract (100 ml) was fermented by *Saccharomyces cerevisiae* 223 for 21 days at room temperature. After completion of the yeast fermentation, as evidenced by the cessation of bubbling activity, the broths were clarified by centrifugation (20,000 × g, 20 min). The ethanol was removed by vacuum distillation at 40 C. The concentrates were reconstituted with distilled water to the original volumes and assayed as described above.

**Carbon dioxide atmospheres.** The media (10-ml samples) were added to sterile colorimeter tubes (16 by 145 mm) and inoculated with one loop of an actively growing culture of *L. citrovorum* ML 34. The tubes, fitted with sterile rubber stoppers and equipped with venting ports containing glass wool, were placed in anaerobic jars for evacuation. Prior to closure of the pinch clamps, the atmospheres were displaced three times with the respective CO₂ gas concentrations. All assays were run in triplicate and incubated at 32 C.

The various CO₂-air mixtures were obtained by metering compressed CO₂ and CO₂-free air into 20-liter water-filled carboys. The volumes of water displaced by the compressed gases served to establish the ratios of final gas concentrations used.

**RESULTS AND DISCUSSION**

The addition of filter-sterilized plant extracts to a TGYE basal medium produced a 12- to 30-fold increase in the final optical density (OD) values (Table 1). The cell yields indicate that the growth-enhancing properties of the juices were not unique to any particular plant extract. The rather universal occurrence of stimulatory activity suggested that the factor(s) was different from that described by Garvie and Mabbitt (6). However, there are apparent differences between grape varieties (red versus white) and the state of maturity of the tomato extracts (mature versus green). The red juice, Seibel, provided 45% greater growth response than that obtained with the white juice, Ravat 34, and the mature tomato extract produced a twofold greater yield than its immature counterpart. It appears, therefore, that variety and state of maturity contributed significantly to the observed stimulatory effects.

Since *L. citrovorum* ML 34 was used successfully for the induction of the malo-lactic fermentation of California wines (9), the effects of the yeast-fermented extracts were also investigated. Alcohol-free fermented extracts, when added as 10% supplements to the TGYE base, produced no significant stimulatory effects (Table 1). Results suggested that the yeast had either utilized the factor or had produced an inhibitory substance that masked its effect. As later data will show, the former appeared to be the explanation.

In an attempt to identify the nature of the stimulatory factor, various fractionation steps were employed. Those fractions obtained as a result of organic solvent extraction and column chromatography were analyzed with ninhydrin and anthrone reagents, and ultraviolet absorption properties (280:260 nm ratios). It is apparent (Table 2) that each successive fractionation step increased the cell yields and that the greatest specific activity (OD per milligram, dry weight, of tomato serum) was observed in the charcoal effluent fraction. This latter fraction, in addition to showing a threefold increase in specific activity, was ninhydrin-negative and contained less than 0.28% protein. Those factors responsible for inducing growth were best correlated to the carbohydrate-enriched fractions. Nearly 98.5%

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**Table 1. Growth response of Leuconostoc citrovorum ML 34 to fresh and yeast-fermented plant extracts**

| Supplement | Fresh juice | Fermented juice |
|------------|-------------|-----------------|
| Grape      |             |                 |
| White (Ravat 34) | 1,640       | 119             |
| Red (Seibel)  | 2,390       | 143             |
| Cabbage     | 1,875       | 137             |
| Orange juice (coned) | 2,485     |                 |
| Tomato (New Yorker) | 2,220     | 187             |
| Mature      | 2,220       |                 |
| Green       | 1,075       |                 |
| None—TGYE broth | 71         | 71              |

* a Added to TGYE broth as 10% (v/v), pH 5.2. Incubation 9 days at 32 C.

* b Alcohol removed.
TABLE 2. Effects of tomato serum fractions upon the growth of Leuconostoc citrovorum ML 34a

| Fractionb | ODc | SAC | Carbohydrate contentd |
|-----------|-----|-----|------------------------|
|           |     |     | Total | Reducing |
| Fresh serum | 831 | 28.8 | 20.1 | 16.7 |
| Acetone-ethanol soluble | 1,590 | 55.0 | 26.0 | 24.4 |
| Dowex-50 effluent | 1,940 | 67.0 | 28.2 | 26.1 |
| Dowex-3 effluent | 2,160 | 72.0 | 27.6 | 26.4 |
| Charcoal, effluent | 2,460 | 85.0 | 28.5 | 27.5 |
| TYGE broth (no additions) | 77 |     |       |       |

a Tryptone, glucose, yeast extract broth, pH 5.2 (final volume 10 ml).
b Added as 10% supplements (v/v) of tomato juice, equivalent to 29 mg (dry weight).
c OD × 1,000, 660 nm after 9 days of incubation at 32 C.
d Specific activity: OD per milligram (dry weight).

* Expressed as milligrams of glucose.

of this latter fraction was comprised of carbohydrate, expressed as glucose, and more than 96% of the total carbohydrate content was composed of reducing carbohydrate material. Paper chromatography of the latter fraction in neutral and acidic solvent systems, followed by color development with ninhydrin, silver nitrate, p-anisidine, and ultraviolet absorption, indicated the presence of only two distinct reducing substances. Elution of the bands and subsequent rechromatography indicated that glucose and fructose were the only detectable constituents present. Hydrolysis of the latter fraction with hydrochloric acid (2 N) in a boiling-water bath for 30 min failed to produce additional substances.

The aqueous eluate corresponding to fructose and fructose standards (range: 0.25 to 2.0%) were compared for growth-enhancing properties in the filter-sterilized TGYE medium incubated under stationary conditions (air). The TGYE medium devoid of fructose produced no visible growth within 14 days, whereas the paper chromatographic eluate and the fructose standards produced OD values in excess of 1.80 within 9 days. It was observed, however, that 6 days of incubation were required before the onset of measurable turbidity. This extended lag period suggested that factors other than fructose, or in addition to fructose, were essential for initiating early growth.

Since the original culture was isolated from a wine fermentation, and the latter fermentation under commercial conditions produces an atmosphere high in CO₂ (1), the effects of CO₂ concentrations upon the growth in a TGYE autoclaved medium were investigated.

Increased CO₂ tensions markedly decreased the lag phase (Fig. 1). The culture incubated in an air atmosphere under stationary conditions required more than 240 hr of incubation to reach an OD value of 0.10, whereas, under CO₂ saturated atmosphere, the lag phase was decreased to less than 24 hr. Reductions in lag phases appear to be related to the concentration of CO₂. Also, when aerobically grown cultures reached an OD of 0.030, and were then supplied with a saturated CO₂ atmosphere, less than 21 hr was required to induce accelerated growth rates. The similarities in the slopes of the growth rates during the accelerated growth phase indicate that, once active growth is initiated (OD greater than 0.10), the generation times are quite similar.

The above growth responses were measured in an autoclaved TGYE medium. The effects of autoclaving upon complex media and its inhibitory or enhancing properties, or both, were well documented by many investigators. It was previously reported (16) that fructose is generated by the temperatures used for sterilization of this

![FIG. 1. Effects of CO₂-air mixtures upon the growth of Leuconostoc citrovorum (ML 34). Autoclaved basal medium, pH 5.2. Cultures incubated at 32 C.](image-url)
complex medium. Thus it appears that catalytic amounts of fructose, arising as the result of autoclaving, and the elevated CO₂ tensions were responsible for the observed growth responses.

The actions and interactions of fructose, glucose, tomato serum, and CO₂ upon growth are summarized in Fig. 2. *L. citrovorum* was able to initiate only very limited growth on either fructose or glucose in the presence of air or saturated CO₂. However, the additions of varying amounts of fructose (0.025 to 1.0%) or tomato serum (2.5 to 15%) to a TGYE filtered medium, incubated under a saturated CO₂ atmosphere, produced marked stimulatory responses within 4 days. When grown under CO₂, the addition of 0.25% fructose produced a growth response value similar to that provided by the 3% level of tomato serum grown in the absence of CO₂. Those cultures grown in air produced less than 20% of the growth observed in CO₂.

It has been known for many years that certain heterotrophic microorganisms require CO₂ for growth (14), and many of these CO₂-mediated reactions were extensively reviewed by Wood (19). Although high concentrations of CO₂ (50% v/v) were shown to be inhibitory (A. D. King, Jr., Ph.D. thesis, Washington State Univ., 1966) and stimulatory (3) to growth and enzymatic activity, *L. citrovorum* responded most favorably to an atmosphere comprised solely of CO₂. The CO₂ served a vital function in initiating growth; however, its exact role remains to be elucidated. Since hydrogen and nitrogen atmospheres were no more conducive to establishing good growth than an atmosphere of air, it appears unlikely that anaerobiosis is a requirement for growth.

In many cases the exogenous CO₂ requirement may be eliminated by its replacement with an essential growth factor. Among such effective replacements are the following: oleic acid, Tween 80, (polyoxyethyleneorbitanmonoooleate), aspartate, oxalacetate, (10); nucleic acid derivatives (11, 4); dicarboxylic acids (7); and vitamins (19). To determine whether known growth factors would enhance early growth in an air atmosphere, compounds, added singly and as complete mixtures, were tested and found to be ineffective.

Acids (sodium salts) were acetate, L-aspartate, fumarate, β-hydroxybutyrate, malate, oleate, oxalacetate, and succinate. Amines were asparagine, glucosamine, and glutamine. Amino acids were cysteine, cystine, and methionine. Purines were adenine, 2,6 diaminopurine, guanine, hypoxanthine, uric acid, and xanthine. Pyrimidines were thymine, cytosine, and uracil. Ribonucleosides were adenosine, cytidine, deoxyadenosine, deoxyguanosine, deoxyinosine, inosine, thymidine, uridine, and xanthosine. Ribonucleotides were 3'-adenylc, 5'-adenylc, cytidylic, guanylic, and uridylic acids. Miscellaneous compounds were biotin, glutathione, and vitamin B₁₂.

Fructose is known to serve as a hydrogen acceptor in the respiratory pathway of some heterolactic members of the family *Lactobacil*-
lacae (18). Therefore, the small amounts of fructose required for growth suggest that this carbohydrate may serve as an essential reductant or catalyst to initiate the onset of vital growth processes. The requirements for fructose appear to be quite specific in that L-sorbose, the keto analogue of fructose, was inactive. The addition of nonphosphorylated trioses, namely dihydroxyacetone, glycerate, and pyruvate to the medium did not enhance early growth. Thus it appears that tomato serum, when added to TGYE broth, provides fructose, an essential constituent for inducing early growth of L. citrovorum.

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