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Production of *Leuconostoc oenos* Biomass under pH Control†

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***Leuconostoc oenos* was grown on apple juice-based media. The effect of pH control on metabolism and biomass production was studied. Without pH control, *L. oenos* acidified the apple juice media to approximately pH 3.6. More than 75% of the malic acid was used under these conditions, but less than half of the carbohydrates was assimilated. Under pH control, biomass yields increased by 60%; most of the malic acid was used, but high levels of unfermented carbohydrates remained. The addition of tomato juice, vitamins, nucleotides, Mn^+, and malic acid did not permit further increases in the cell counts; however, malic acid did induce further acidification. Growth without pH control favored a more homofermentative metabolism. Biomass production was higher in filter-sterilized apple juice media compared with that in the autoclaved media.**

The inoculation of pasteurized milk with starter cultures is a well-developed practice in the dairy industry (15). To answer the needs of the industry, specialized companies produce concentrated suspensions of lactic acid bacteria which can be added to large culture vessels or directly to the production vats. Thus, many studies have been performed on the biomass production of lactic acid bacteria (10).

In enology, inoculation of selected cultures of lactic acid bacteria in musts or wine in order to promote the malo-lactic fermentation is a more recent occurrence (4). A growing number of culture suppliers now provide malo-lactic bacteria to the wine industry in frozen (13) or freeze-dried form (7). Malo-lactic fermentation can be performed by various species of *Lactobacillus*, *Pediococcus*, and *Leuconostoc* (5, 14, 16). *Leuconostoc oenos* generally has been preferred over other lactic acid bacteria for vat inoculation, since this species is the most tolerant to the high acidity of wines (5, 16).

Very few studies have been performed on the biomass production of malo-lactic cultures compared with that of lactic acid bacterial cultures used in milk fermentations (23). Although complex media have been proposed for biomass production of *L. oenos*, diluted grape juice is the most widely used (4). It is generally supplemented with yeast extracts, tomato juice, Tween, peptone, or other compounds (4). Studies on the influence of pH (5, 7) and temperature (16) on growth were mostly performed in wines and were not aimed at optimizing the growth rate or biomass production. *L. oenos* can grow at pH 3.2 (5), but its optimum pH is 4.8 in the presence of malic acid and 5.4 in the absence of this substrate (18). Maret et al. (16) also reported variations in optimal pH values in different media. Lactic acid bacteria acidify the medium when they are growing on carbohydrates. The lowering of pH which thus occurs inhibits their growth (22). At low pH values, uncoupling of growth and acid production has also been reported (24). By controlling the pH of the medium, it was possible to multiply the biomass obtained for certain strains of streptococci by a factor of 10 (23). Since *L. oenos* also produces acid from carbohydrates (9), it was expected that pH control of the growth medium would promote increased biomass yield.

There are very few reports of growth of *L. oenos* in apple juice (6). Fresh apple juice contains approximately 15.2% solids (6), of which 14.1% are carbohydrates. Fructose (6%), sucrose (2.5 to 3.5%), and glucose (1.5 to 2.0%) are the major components of these carbohydrates (6). Complete fermentation of these sugars, even under pH control, would potentially produce inhibitory levels of lactate (20) and acetate. We therefore studied biomass production on diluted apple juices, as is generally done with grape juice (4). The use of apple juice as a growth medium has the advantage of providing higher levels of malic acid than those in grape juice.

Our aim was thus to study the effect of pH control and mode of medium sterilization on biomass production of *L. oenos* in apple juice-based media.

MATERIALS AND METHODS

Organisms and growth conditions. *L. oenos* 2001 was obtained from the Rosell Institute Inc. (Montreal, Quebec, Canada) collection. This is one of the cultures marketed by the company for vat inoculation in order to perform the malo-lactic fermentation of wines. The cultures were maintained and transferred monthly on tomato juice agar (Difco Laboratories, Detroit, Mich.) slants in which the pH was adjusted to 4.7 with 5 N H_2SO_4 before steam sterilization (121°C, 10 min). Weekly transfers were performed by inoculating tomato juice broth cultures (pH 4.7; Difco) in screw-cap culture tubes at a level of 1% (vol/vol) and incubating them at 22 to 24°C for 5 days. Cultures were kept at 4°C between transfers.

***L. oenos* production.** Two media were used to produce a biomass of *L. oenos*. Both media contained the following, per liter: 5.0 g of yeast extracts (Difco), 5.0 g of Bacto-Peptone (Difco), and 5 or 10% (wt/wt) apple solids from concentrated juice (68% [wt/wt] solids; Lassonde et Fils, Rougemont, Quebec, Canada). Broth cultures were sterilized either by autoclaving them at 121°C for 10 min or filter sterilizing them through a 0.45-μm-pore-size membrane filter (Millipore Corp., Montreal, Quebec, Canada). A total of 1 liter of medium was then aseptically transferred into sterile 2-liter fermentors (Biostat M; B Braun Instruments, Melsun-

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TABLE 1. Preparation of supplements added to prefermented apple juice media

Supplement	Method of sterilization	Level of addition (% [vol/vol])
1. Vitamins[a]	Filtration[b]	1.0
2. Tomato juice (adjusted to pH 4.7 with 5 N NaOH)	Filtration[b]	2.5
3. $MnSO_4 \cdot 2H_2O$ (500 mg/liter)	121°C, 10 min	1.0
4. Nucleotides[c]	121°C, 10 min	1.0
5. Malic acid (40% solution neutralized to pH 4.7 with 5 N NaOH)	121°C, 10 min	1.0

[a] Vitamins were added as described by Barnett et al. (1). The following vitamins were added, per liter: *p*-aminobenzoic acid, 200 μg; biotin, 20 μg; folic acid, 2 μg; myoinositol, 10 mg; nicotinic acid, 400 μg; calcium pantothenate, 2 mg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; thiamine, 400 μg.

[b] Filtration was done through a 0.22-μm-pore-size filter.

[c] Nucleotides were added as described by Garvie (11). The following nucleotides were added, per liter: adenine, 200 mg; guanine, 100 mg; and uracil, 200 mg.

gen, Federal Republic of Germany), and the pH was adjusted to 4.7 with sterile 5 N NaOH. Fermentors were inoculated with 1% (vol/vol) of the stock culture. Strain 2001 was grown at 28°C with agitation at 60 rpm for 4 to 5 days. In some fermentations, the pH was monitored and maintained at pH 4.7 with 5 N NaOH by using a pH stat (Braun).

Addition of supplements. A medium containing 5% apple solids, 0.5% yeast extracts, and 0.5% peptone was separated into three parts: two were heat sterilized (121°C, 10 min) and one was filter sterilized. All media were then inoculated with *L. oenos*, and fermentation was allowed to proceed for 96 h. In one heat-sterilized medium, no pH control was performed during the course of the primary fermentation, while in the two other media, the pH was maintained at 4.7 with 5 N NaOH. At the end of this prefermentation, each of the media was divided into six 100-ml volumes. One was kept as a control, and each of the five others was supplemented with various growth factors (Table 1). The prefermented media were then incubated for 24 h at 30°C without pH control; CFU as well as pH and direct microscopic counts were determined prior to and following this 24-h incubation.

Analytical methods. Samples were withdrawn aseptically with a syringe through the fermentor sampling port. CFU were obtained daily by plating samples on MRS agar (pH 4.7; Difco) and by incubating the cultures for 96 h at 25°C. The direct microscopic counts were obtained by dyeing the smear of the suspension, which was diluted in 1% skim milk, with methylene blue by standard methods of the American Public Health Association (19). *L. oenos* can form chains. Thus, in performing direct microscopic counts, chains were counted as units, whatever their lengths. This was done with the purpose of more closely approximating the CFU. For chemical analysis, the samples were centrifuged at 3,000 rpm for 20 min (Damon centrifuge; Damon, Needham Heights, Mass.), and 10 ml of the supernatants was filtered through a cartridge (C-18 SepPak; Waters Associates, Inc., Milford, Mass.) to clarify the sample and was then filtered through a 0.45-μm-pore-size membrane filter (Millipore). The first 5 ml of the sample was removed and discarded, while the remaining volume was distributed in cap-sealed vials. Sugars and fermentation acids were quantified by high-pressure liquid chromatography. A data acquisition system (Expert Software; Waters) was used to analyze the data and plot the chromatograms. An autosampler (Wisp 710 B; Waters) was used to inject 15-μl portions into a column (Ion 300; Mandel

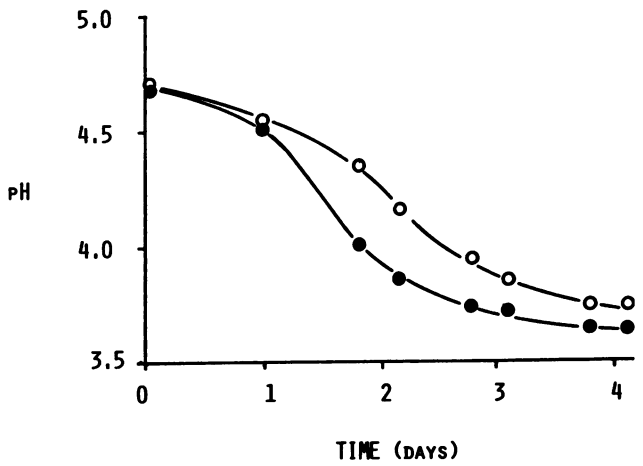

FIG. 1. Effect of apple juice solids on acidification (without pH control) of apple juice media by *L. oenos* 2001. Symbols: ○, 10% apple juice solids; ●, 5% apple juice solids.

Scientific Cie, Rockwood, Ontario, Canada). The column temperature was maintained at 25°C. The mobile phase was 0.008 N H_2SO_4 at a flow rate of 0.4 ml/min. The sample passed through a dual UV detector (490; Waters) at 210 nm and a refractometer (R401; Waters) set to an attenuation of 8. External standard mixtures of pyruvic acid, L-malic acid, L-lactic acid, acetic acid, sucrose, glucose, and fructose were used. All samples were cooled to approximately 4°C before injection.

Statistical analysis (Duncan multiple range variance) was performed by using Statistical Analysis Systems software (SAS Institute Inc., Cary, N.C.). Results were the averages of three separate fermentations that were performed sequentially.

RESULTS AND DISCUSSION

Effect of apple juice solids and pH control on growth. Since apple juice contains 15.2% solids, the 5 and 10% apple juice solids media we used represented apple juice diluted 66 and 33%, respectively. When no pH control was performed during growth of *L. oenos* on apple juice media, acidification occurred and the pH was reduced to 3.6 within 4 days (Fig. 1). The decrease in pH was faster on 5% apple juice solids medium compared to that on 10% apple juice solids medium. This may have been related to the greater buffering capacity of the 10% medium and to the faster initial growth observed in the 5% medium with or without pH control (Fig. 2). Our results thus suggest that inhibitory compounds are present in apple juice and their dilution promotes a faster growth rate. Malic acid could be partially responsible for this effect, since the addition of 0.2% malic acid to the 5% apple juice medium inhibited growth (Fig. 3). Dilution also reduced the osmotic pressure, which could have stimulated *L. oenos*. Although the growth rate was initially slower in 10% apple juice solids, the final CFU that was obtained was higher in this medium (Fig. 2). The effect of a pH control on biomass production was more pronounced in the 10% apple solids medium. There was a 44% increase in CFU when the pH was controlled to 4.7 compared with that in the noncontrolled sample. This did not appear to be related to higher levels of malic acid, since the addition of 0.2% malic acid to the 5% apple juice medium did not result in an increased population.

Effect of mode of sterilization on growth. Heating of the 5% apple juice medium for purposes of sterilization reduced

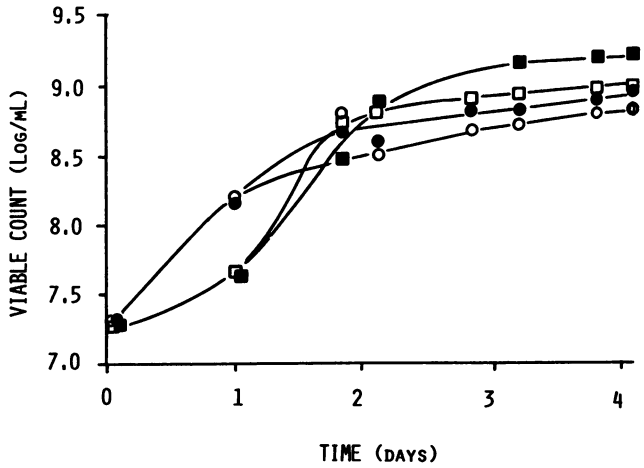

FIG. 2. Effect of apple juice solids content and effect of pH control on growth of *L. oenos* 2001. Symbols: ○, 5% solids with no pH control; ●, 5% solids with pH control (pH 4.7); □, 10% solids with no pH control; ■, 10% solids with pH control (pH 4.7).

growth and the final CFU of *L. oenos*; the filtered media containing 5% solids yielded 52% more viable cells than the autoclaved media did. *L. oenos* has complex nutritional needs; many vitamins, nucleotides, and amino acids are required for its growth (8, 12). Heating may denature some of these compounds, particularly vitamins, or stimulate reactions, such as Maillard-type reactions, that reduce their bioavailability or produce inhibitory compounds. Use of large-scale microfiltration units for medium sterilization could thus be of advantage in this process. High levels of postfiltration sanitation would, however, be required, since *L. oenos* does not grow very fast, and industrial production could be subject to contamination. Microfiltration would have the added disadvantage of not eliminating bacteriophages that would infect the culture (11); ultrafiltration would be required for this purpose.

Effect of pH control on metabolism. Substrates for growth were mostly glucose, sucrose, and malic acid (Table 2). Very little fructose was assimilated. Strain 2001 thus appears atypical in that respect, since most *L. oenos* strains produce

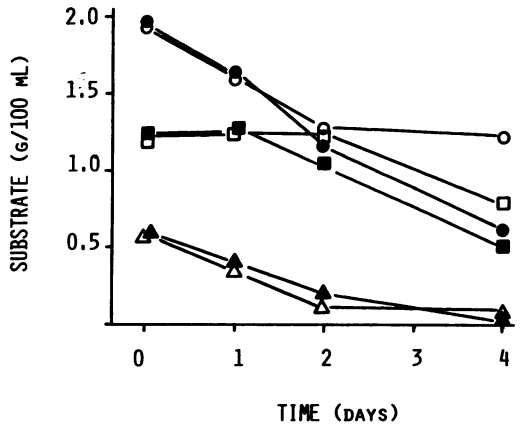

FIG. 3. Effect of pH control on substrate utilization during growth of *L. oenos* 2001 on apple juice media (10% apple solids). Symbols for substrate utilization without pH control: ○, glucose; □, sucrose; △, malic acid. Symbols for fermentation under pH control: ●, glucose; ■, sucrose; ▲, malic acid.

TABLE 2. Effect of pH control and method of medium sterilization on substrate utilization and fermentation end products of *L. oenos* 2001 in apple juice medium (5% solids)

Substrate or end product	Concn (g/100 ml)			
	Unfermented raw medium	No pH control, autoclaved	Fermentation under pH control (4 days)	
			Autoclaved	Filtered
Glucose	0.974	0.447	0.127	0.061
Sucrose	0.591	0.340	0.289	0.301
Fructose	1.938	1.936	1.853	1.836
Malic acid	0.290	0.069	0.020	0.005
Lactic acid	0.043	0.604	0.756	0.789
Pyruvic acid	0.035	0.017	0.004	0.021
Acetic acid	0.002	0.230	0.425	0.457

acid from fructose but not from sucrose (9). Utilization of sucrose by *L. oenos* has, however, been reported (2, 16). When no pH control was performed, less than half of the available carbohydrates were used. More than 75% of the malic acid, however, was assimilated. Under pH control, a higher portion of the carbohydrates were metabolized (Table 2), as was almost all of the malic acid. This increase in substrate utilization under pH control provided for higher biomass yields (Fig. 2). Our results thus suggest that as the pH decreased to 3.6, limited growth of *L. oenos* occurred and that pH control allowed continued growth. In this respect, *L. oenos* behaves like lactic acid streptococci (10). It is probable that growth at pH 3.6 could continue, although at a very slow rate (18), since it is well established that *L. oenos* can grow at pH values under 3.6 (5). Even under pH control, all available substrates were not metabolized in either 5% (Table 2) or 10% (Fig. 3) apple juice medium. In both cases, high levels of carbohydrates remained. Malic acid, however, was almost completely used in all cases. It appears that our culture media lacked nutrients that would permit further utilization of carbohydrates by *L. oenos*.

We attempted to determine the lactate yields. Since four substrates were metabolized, theoretical yields were calculated and compared with the actual results. Malate is converted to lactate and CO_2 by *L. oenos* (3, 9). The pathway of glucose fermentation in *L. oenos* has not been fully confirmed, but *Leuconostoc* species ferment glucose by a combination of the hexose monophosphate and phosphoketolase pathways (9). Thus, lactate is formed in equimolar ratios from hexoses.

Since malic acid assimilation was similar under all conditions and carbohydrate utilization was greater under conditions of pH control, we expected a higher lactate:acetate ratio without pH control. Our results showed that this was the case (Table 3).

TABLE 3. Effect of pH control and method of medium sterilization on end products

Fermented medium (4 days)	Ratio of lactic acid/ acetic acid	Lactic acid yield (%)[a]
Autoclaved, without pH control	2.46	103
Autoclaved, with pH control	1.69	89
Filtered, with pH control	1.63	86

[a] Yields are expressed as follows: (concentration of lactic acid obtained/ theoretical concentration of end product) × 100.

TABLE 4. Acidification of various fermented apple juice media (5% solids) following supplementation

Supplement	pH of the following prefermented media[a]		
	Autoclaved, no pH control	Autoclaved with pH control	Filtered with pH control
None (control)	3.67[b]	4.61[c,d]	4.72[e]
Vitamins	3.64[b,f]	4.61[c,d]	4.72[e]
$MnSO_4$	3.61[b,f]	4.61[c,d]	4.73[e]
Tomato juice	3.66[b]	4.57[d]	4.68[c,e]
Nucleotides	3.67[b]	4.62[c,d]	4.73[e]
Malic acid	3.56[f]	4.40[g]	4.45[g]

[a] pH control was only performed during 4 days of prefermentation but not following supplementation. Values with the same letter are not significantly different ($P < 0.05$) by the Duncan multiple range test.

We did not obtain as much lactate under pH control (≤89%) as was theoretically possible (Table 3). This was not related to excretion of pyruvate in the medium (Table 2). The lower lactate yields under pH control might be related to increased utilization of the carbohydrates. Chauvet et al. (3) have reported that glucose is incorporated into biomass. It could also be related to a pH effect. Increased homofermentative metabolism under low pH conditions has been reported (21). *Lactobacillus bulgaricus* produces substantially more lactic acid from glucose at low pH values than it does at pHs in the alkaline region. This has been explained by the fact that there is a greater level of synthesis of lactate dehydrogenase under acidic conditions (21).

Effect of additional supplementation. Carbohydrate assimilation was incomplete, even under pH-controlled conditions, suggesting that a nutrient requirement was not met. Although the apple juice media were initially supplemented with yeast extracts and peptones, various substances (Table 1) were added at the end of 4 days of fermentation to stimulate the growth of *L. oenos*. Following the addition of these supplements, the pH was not controlled. We thought that a further uptake of the carbohydrates would result in acidification. A significant pH drop only occurred when malic acid was added (Table 4). Since the metabolism of malic acid by *L. oenos* resulted in a pH increase, the decrease in pH observed in our media suggests all the more that carbohydrate fermentation occurred. It has been reported that malic acid stimulates growth and glucose utilization in *L. oenos* (3). Tomato juice induced a small reacidification which was not judged to be significant (Table 4). The relatively small amount of malic acid present in tomato juice, 0.4 to 2.1 g/liter, was insufficient to provide enough malic acid for significant stimulation.

Although acidification occurred in some samples, none of these growth factors had a significant effect on biomass production, which suggests that acid production could occur without growth. Such uncoupling between acid production and growth is common for stressed lactic acid bacteria (24). However, after 4 days, even after the addition of a supplement, considerable mortality was observed. Direct microscopic counts were 1.9×10^9/ml in the malic acid-supplemented media, of which 5.4×10^8 were CFU. The control presented, respectively, 1.4×10^9 total and 5.2×10^8 CFU/ml. Thus, some growth appears to have occurred following malic acid addition, confirming the observations of Pilone and Kunkee (17), who found that there can be increases in cell yields from malic acid, but only when the acid is metabolized in the presence of fermentable carbohydrates.

Biomass production of *L. oenos* under pH control permitted increased yields. However, the coupling of malic acid and carbohydrate utilization under these conditions should be more thoroughly investigated in order to increase yields further.

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