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Trophic Relationships between *Saccharomyces cerevisiae* and *Lactobacillus plantarum* and Their Metabolism of Glucose and Citrate

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Glucose and citrate are two major carbon sources in fruits or fruit juices such as orange juice. Their metabolism and the microorganisms involved in their degradation were studied by inoculating with an aliquot of fermented orange juice a synthetic model medium containing glucose and citrate. At pH 3.6, their degradation led, first, to the formation of ethanol due to the activity of yeasts fermenting glucose and, eventually, to the formation of acetate resulting from the activity of lactobacilli. The yeast population always outcompeted the lactobacilli even when the fermented orange juice used as inoculum was mixed with fermented beet leaves containing a wider variety of lactic acid bacteria. The evolution of the medium remained similar between pH 3.3 and 5.0. At pH 3.0 or below, the fermentation of citrate was totally inhibited. *Saccharomyces cerevisiae* and *Lactobacillus plantarum* were identified as the only dominant microorganisms. The evolution of the model medium with the complex microbial community was successfully reconstituted with a defined coculture of *S. cerevisiae* and *L. plantarum*. The study of the fermentation of the defined model medium with a reconstituted microbial community allows us to better understand the behavior not only of fermented orange juice but also of many other fruit fermentations utilized for the production of alcoholic beverages.

Yeasts and lactic acid bacteria are often encountered together in natural ecosystems and may be in competition for the same substrates (1, 4). They appear frequently during spontaneous fermentations of some foods and some beverages such as wine, cider, and kefir (2, 11, 16), which are media characterized by an acidic pH. In these natural ecosystems, yeasts usually ferment the sugars and lactic acid bacteria mainly ferment the acids (2). Under anaerobic conditions, strict aerobes like the acetic acid bacteria do not appear and the only dominant bacterial populations are the lactic acid bacteria.

In fermented beverages made from fruits and fruit juices, the most frequently encountered acids are malic and citric acids. These natural ecosystems also contain various sugars used as carbon and energy sources by the microbial community. The fermentation of sugars by yeasts and by lactic acid bacteria has been studied extensively. Regarding the fermentation of acids, some reports exist on the behavior of pure cultures of lactic acid bacteria with citrate as the sole carbon source or as a cosubstrate in a mixture with glucose (5, 14, 15). No studies exist concerning the behavior of cocultures of yeasts and lactic acid bacteria in a defined medium containing glucose and citrate as a cosubstrate, as they appear together in natural ecosystems.

For those who wish to elucidate the biochemical and microbiological behavior of yeasts and lactic acid bacteria in coculture, the natural ecosystems mentioned above represent a complex subject of study. The main difficulties result probably from the numerous interactions between the environment and the microorganisms and among the microorganisms themselves that hamper a complete interpretation of the behavior of the system. On the contrary, the use of chemically and/or microbiologically defined media (model system), simulating part of the complex natural system, readily allows a reliable interpretation of the reactions that occur and a better understanding of trophic relations between microorganisms. The reproduction of such natural fermentations of complex media in a defined medium with a reconstituted microbial community is difficult, however, and in most cases is not successful (2, 23).

This paper describes the behavior of the two key groups of microorganisms for the food and beverage industries, yeasts and lactic acid bacteria. The fate of two important carbon sources in the production of alcoholic beverages by fermentation of fruits, namely, glucose and citrate, has been studied. The dominant yeasts and lactic acid bacteria fermenting these carbon sources in orange juice have been isolated, identified, and inoculated in mono- or cocultures in a defined model medium containing glucose and citrate, allowing an accurate interpretation and a better understanding of their trophic relations and their metabolic behavior.

MATERIALS AND METHODS

Culture medium and growth conditions. The model medium GC included the following: glucose, 100 mM; citric acid, 10 mM; urea, 10 mM; yeast extract, 200 mg/liter; mineral salts solution, 12.5 ml/liter (12, 21). The pH was adjusted to 3.6 by the addition of NaOH. An 800-ml portion of the medium was dispensed in 1-liter fermentors which were subsequently flushed for a few minutes with dinitrogen to obtain anaerobic conditions. The fermentations were conducted at 35°C.

Inoculum. Inoculum OJ was prepared from fermented

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orange juice. After complete fermentation of the medium, 30 ml was centrifuged at 10,000 × *g* for 5 min, and the pellets were resuspended in 5 ml of water just before use. Inoculum BL, containing a wider variety of lactic acid bacteria, was prepared in the same way from fermented beet leaves in an aqueous suspension. The experiments were started with an initial microbial count of between 10^3 and 10^5 cells per ml.

Isolation of yeasts and lactic acid bacteria. Samples, 1 ml, of medium GC were removed from the fermentor at the end of the glucose fermentation and at the end of the citrate fermentation. After 10-fold serial dilutions, an aliquot of each dilution was spread on Sabouraud agar for yeasts or on MRS agar for lactobacilli (9). The plates were incubated at 30°C for yeasts and at 35°C for lactobacilli under either aerobic or strict anaerobic conditions in a glove bag. From the highest positive dilutions, 25 colonies were isolated and purified from Sabouraud agar and 29 colonies were isolated from MRS agar for taxonomic studies.

Taxonomic studies. The yeasts were identified mainly on the basis of morphological and physiological criteria. Different carbohydrates were tested for their fermentation by using API ATB-32 C galleries (API system, La Balme les Grottes, France). Sensitivity to cycloheximide (Acti-dione) was also assessed. The assimilation of different nitrogen sources was tested with the same medium as for API ATB-32 C galleries, with glucose as the carbon source. The following nitrogen sources were tested at 5 g/liter: ammonium sulfate, sodium nitrate, peptone, and Casamino Acids. The sexual characteristics were studied on RAT medium (24) of the API system as well as with the medium of MacClary and that of Fowell (3). The lactobacilli were identified by classical methods (22). Different carbohydrates were tested on API 50 CHL galleries. The guanine-plus-cytosine content of the DNA was determined by the thermal denaturation procedure (19). The method utilized for DNA-DNA hybridizations was described previously (25) with slight modifications for lactobacilli.

Quantitative evaluation of the dominant microbial populations. The quantitative evolution of each dominant microbial population, characterized by its metabolic function, was estimated by the most-probable-number (MPN) technique, using 25-ml serum vials initially containing 9 ml of medium. The MPN was determined for three vials per 10-fold dilution by referring to existing tables (20). The three populations, or physiological groups, followed were yeasts metabolizing glucose into ethanol and quantified in Sabouraud broth, lactic acid bacteria metabolizing glucose into lactate quantified in MRS broth, and bacteria metabolizing citrate into acetate by using the citrate broth described below. The last group was prepared under anaerobic conditions by flushing the vials with a mixture of N_2-CO_2, 85:15. After testing different media with compositions close to the composition of medium GC, the most adequate medium (citrate broth) for the quantification of the physiological group metabolizing citrate into acetate was composed of the following (in grams per liter): citrate, 2; glucose, 0.8; urea, 0.6; ammonium chloride, 0.5; ethanol, 13; yeast extract, 0.5; and 12.5 ml of a mineral salts solution (12, 21). The pH was adjusted to 4.5 with NaOH. It was shown that this broth was the most appropriate for enumeration of the physiological group of microorganisms degrading citrate in the model medium GC by testing various parameters such as the concentration of the carbon sources and various kinds of nitrogen sources and ethanol at different concentrations, as well as the effect of yeast extract. A pH of 4.5, closer to the original pH of medium GC, gave better results than pHs of 6 to 6.5. A vial was considered positive whenever at least 50% of the citrate was degraded to acetate. No antifungal agent was used in the medium.

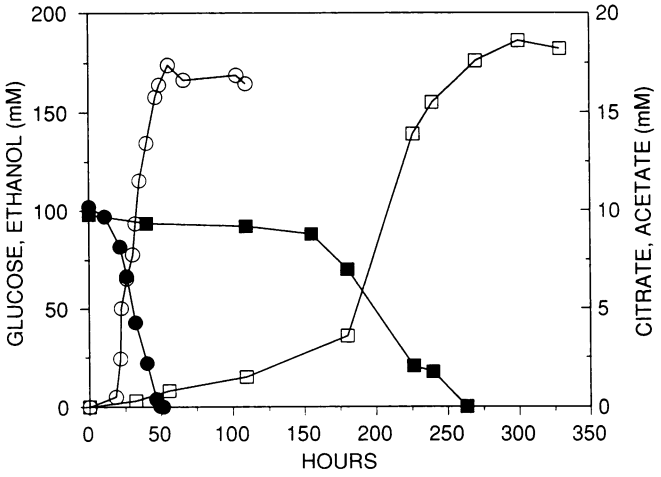

FIG. 1. Kinetics of glucose (●) fermentation into ethanol (○), and citrate (■) fermentation into acetate (□), at initial pH 3.6. A typical fermentation profile is represented. The experiment was repeated at least three times and proved to be highly reproducible.

Analysis of substrates and metabolites. High-performance liquid chromatography was used to determine the concentrations of glucose and citric and lactic acids. Glucose was detected with a Li Chrosorb NH_2 column (Merck, Darmstadt, Germany) and a differential refractometer. The mobile phase was composed of acetonitrile-water (1:1, vol/vol). The acids were detected with an RP18 column (Waters Associates, Milford, Mass.) and a UV detector at 210 nm. The mobile phase was a solution of $(NH_4)_2HPO_4$ at 10 g/liter. Citric acid was also analyzed, using enzymatic kits from Boehringer, Mannheim, Germany. The concentration of acetic acid was determined with a gas chromatograph Intersmat IGC 120 DF 3 equipped with a flame ionization detector. The column was filled with a mixture of 71% Chromosorb W 100 (200 mesh), 20% Carbowax 20 M, and 2% phosphoric acid (85%, vol/vol, in distilled water) (10). The concentration of ethanol was determined by gas chromotagraphy, using an Intersmat IGC 121 DEL equipped with a flame ionization detector. The column was filled with Porapak Q (80/100 mesh).

RESULTS

Metabolic evolution of the defined model medium GC. Inoculum OJ was used to inoculate medium GC to reach a total initial concentration of microorganisms of 10^4 cells per ml. A typical evolution of the substrates and their respective metabolites is given in Fig. 1. Each experiment was repeated at least three times, showing a highly reproducible metabolic evolution. After a lag phase of 10 to 15 h, a yeast population started fermenting glucose (100 mM) into ethanol (approximately 170 mM). The fermentation reached completion in about 24 h. Before the end of that stage, nonsporulated nonmotile rods with a regular shape had also become dominant and converted eventually about 1% of the total initial glucose into small amounts of lactate (about 1 mM). The subsequent fermentation of citrate into acetate appeared a few days later. After a slight pH decrease from 3.6 to 3.55 to 3.50 during the fermentation of glucose, the shift from 10

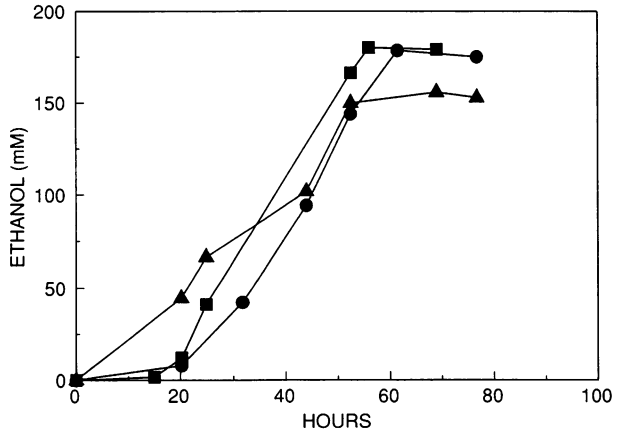

FIG. 2. Kinetics of ethanol formation from glucose at initial pH 3.0 (●), 4.2 (■), and 5.0 (▲).

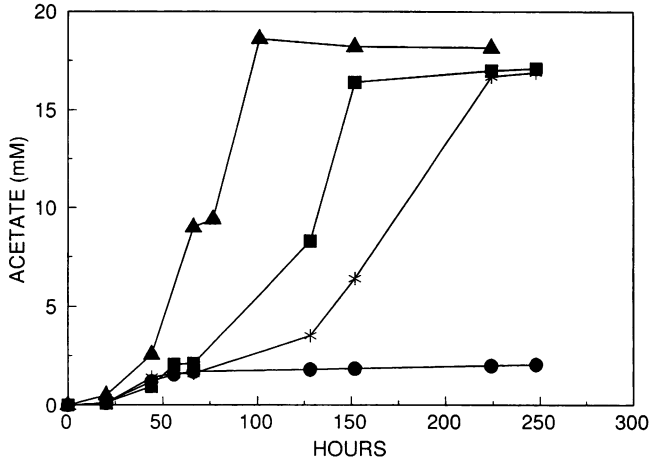

FIG. 3. Kinetics of acetate formation from citrate at initial pH 3.0 (●), 3.3 (*), 3.6 (■), and 5.0 (▲).

mM citric acid-citrate to 17 to 18 mM acetic acid-acetate in a second stage was mainly responsible for the subsequent pH increase from 3.50 to 3.55 to 4.2. During the fermentation of citrate, the lactate concentration in the medium increased slightly by about 1 mM. When inoculum OJ was mixed with an equal volume of inoculum BL, in which lactic acid bacteria formed the dominant population, the same (bio) chemical evolution was obtained. Even in the presence of a bacterium-dominant community, the selective pressure was advantageous to the yeast population and, again, nonsporulated, nonmotile rods grew later.

Effect of initial pH on (bio)chemical and microbial evolutions of medium GC. The evolution of the synthetic medium GC was tested for different initial pH values ranging from 3.0 to 5.0. The use of the mixed inoculum (OJ plus BL) provided a larger variety of bacteria than inoculum OJ alone, especially lactic acid bacteria, which are able to compete with the yeasts for glucose. In all cases, on the basis of morphological characteristics, it was determined that similar yeast and bacterial (non-sporulated immotile rods) populations appeared in the medium. At higher initial pH values, the growth of bacteria took place earlier. Nevertheless, the same two successive metabolic steps appeared for each pH value: glucose fermentation into ethanol (Fig. 2) and citrate fermentation into acetate (Fig. 3), except for initial pH 3.0, at which only traces (<1 mM) of lactate were detected in the medium and citrate was not degraded. For pH 3.3, 3.6, and 3.9, about 1 mM lactate was formed. For a higher initial pH, a slightly more significant amount of lactate was produced during the glucose fermentation. The variation in lactate concentration, however, was not significant in the pH range 3.0 to 4.2, and the difference between pH 3.0 and 4.2 was not higher than 2 mM. The ethanol concentration over that pH range remained essentially the same (Fig. 2). At higher initial pH values, above 4.7, the ethanol production from glucose started earlier but the amount produced was significantly lower. The lower alcohol concentration at that pH was compensated for by greater lactate production, reaching 7 mM. A difference showed up in the kinetics of citrate fermentation. For initial pH values of 4.2 or higher, the degradation of citrate started before the degradation of glucose into ethanol had reached completion.

Physiological characteristics of the dominant species. The experiment at pH 3.6 was repeated for microbiological studies. In all cases, the microorganisms isolated on Sabouraud agar belonged to the single species *Saccharomyces cerevisiae* and to five different biotypes based on physiological characteristics shown in Table 1.

Taxonomic studies performed on 29 purified colonies from the highest positive dilutions obtained on MRS agar showed that they all belonged to the single species *Lactobacillus plantarum*. Two similar biotypes were present, only distinguishable by their ability to ferment either α-methyl-D-glucoside or D-turanose among 49 carbohydrates tested with API 50 CHL galleries. The same samples were also spread on MRS agar in which 10 g of sodium citrate per liter was used as the carbon source instead of glucose. A similar number of colonies as on MRS agar with glucose appeared on this medium, but the size of the colonies was much smaller. The identification of *L. plantarum* was confirmed by the determination of the G+C content of the DNA and by DNA-DNA hybridizations with the type strain *L. plantarum* DSM 20174. The cross-hybridizations between each biotype and with the type strain showed ≥80% homology.

The yeasts and the lactobacilli present in the fermented orange juice were also identified by plating serially diluted samples on Sabouraud agar and MRS agar. The taxonomic studies confirmed the presence in orange juice of *S. cerevisiae* and *L. plantarum* among other unidentified bacteria. In fermented beet leaves, $<10^4$ CFU of yeasts per ml were obtained on Sabouraud agar and they were not identified. *L. plantarum* was present in that medium at 10^7 CFU/ml, but the biotypes were different from those found in medium GC.

TABLE 1. Physiological characteristics of the five biotypes of *S. cerevisiae* isolated from medium GC

Biotype	No. of strains	Growth[a]			
		Galactose	Lactate	α-CH_3-D-Glucoside	Palatinose
LA	2	−	−	+	+
LB	7	−	+	+	+
LC	3	+	±	−	+
LD	12	+	+	−	+
LE	1	+	±	+	−

[a] +, Growth; ±, weak growth; −, no growth.

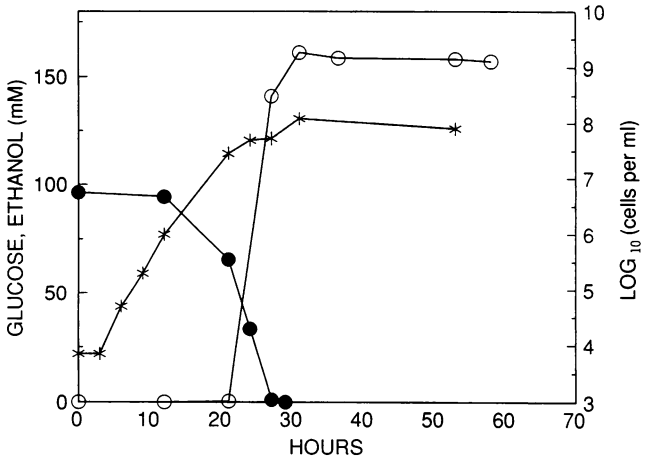

FIG. 4. Evolution of the yeast population (*), glucose (●), and ethanol (○), at initial pH 3.6.

Various other unidentified bacteria present in the fermented beet leaves grew on MRS agar.

Growth kinetics of yeasts and lactobacilli fermenting glucose and citrate in medium GC. The quantitative evolution of yeasts and lactobacilli fermenting glucose or citrate was determined by the MPN technique. In medium GC at an initial pH of 3.6, the yeasts started growing as soon as glucose degradation appeared (Fig. 4). When the first traces of ethanol were detected, the yeasts had nearly reached their maximal cell concentration of 10^8 per ml.

The lactobacilli also initiated their growth during the glucose fermentation but with a higher doubling time than the yeasts. According to the MPN values, the growth of the bacterial populations quantified in MRS broth and that of those quantified in citrate broth followed very similar kinetics (Fig. 5). Observations under the microscope and further taxonomic studies showed that they were identical *L. plantarum* strains. The lower MPN value obtained with citrate broth versus MRS broth must be because the latter is a richer medium. It is interesting that in citrate broth, the presence of ethanol (13 g/liter) increased the MPN value

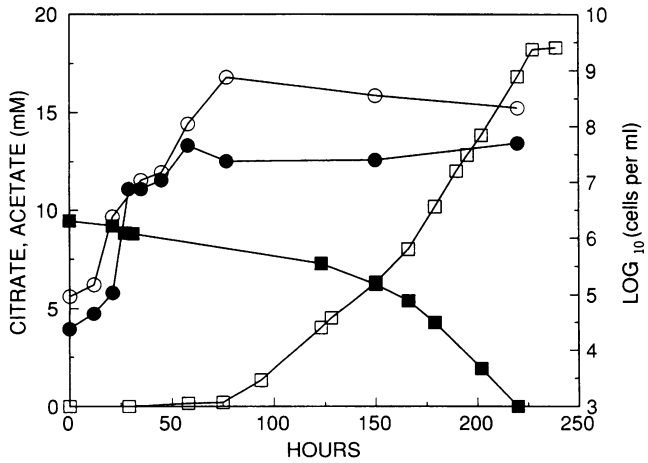

FIG. 5. Evolution of the lactobacilli (MRS broth) (○), the bacteria fermenting citrate (citrate broth) (●), citrate (■), and acetate (□), at initial pH 3.6.

obtained by 5- to 10-fold, for the same incubation period, compared with citrate broth without ethanol. The degradation of citrate was also faster. Acidic pH values of 4.5 proved to be more favorable than values closer to neutrality (pH 6.5). When the lactobacilli stopped growing in medium GC, after having reached 5×10^8 cells per ml, citrate degradation had already begun. No further cell increase took place during the completion of that second stage (Fig. 5).

Pure monoculture studies. When medium GC was inoculated with 10^8 CFU of a monoculture of *S. cerevisiae* LD per ml, 100 mM glucose was fermented, in about 24 h, into approximately 170 mM ethanol. Even after maintaining the medium for 2 more weeks at 35°C, citrate was not degraded and the pH value remained stable at 3.6 ± 0.1.

The same experiment was done with monocultures of each biotype of *L. plantarum*, as well as with a mixture of both. The initial concentration of microorganisms was 10^5 to 10^6 CFU/ml. A similar metabolic evolution was obtained for each of the three cultures. Glucose was rapidly fermented into lactate due to the absence of competition for glucose between the lactic acid bacteria and the yeast and the higher affinity of *L. plantarum* for glucose than for citrate. The bacteria reached maximal concentrations of 2×10^9 to 4×10^9 CFU/ml after 1 to 2 days of fermentation. Since the medium followed a natural (nonregulated) evolution, the pH decreased resulting from the formation of the lactic acid-lactate couple. When the pH reached a value of 2.9, *L. plantarum* became totally inhibited and the glucose fermentation stopped. One mole of glucose at pH 3.6 yielded 0.4 mol of lactate and almost 0.8 mol of residual glucose at pH 2.9. The cultures were maintained at 35°C. After 10 days, new samples were removed from the media. The concentration of viable lactobacilli had dropped, in each case, to 10^2 to 10^3 CFU/ml. Citrate was not degraded.

The behavior of *L. plantarum* was also tested in the same medium without glucose. The medium was inoculated with 10^3 to 10^4 CFU/ml. It was maintained at 35°C for 4 weeks. After that period of time, only traces (<10%) of citrate had disappeared. Nonsignificant amounts of acetate were detected.

Cocultures of *S. cerevisiae* and *L. plantarum*: reconstitution of the fermentation with a defined coculture. The chemical evolution of medium GC was followed for cocultures of the isolated *S. cerevisiae* with each of the *L. plantarum* strains as well as for a mixture of both *Lactobacillus* strains in coculture with the yeast. In the case of the triculture, it was possible to distinguish between the two strains of *L. plantarum* by plating the samples on MRS agar in which glucose was replaced by α-methyl-D-glucoside.

Identical results were obtained for the three cocultures. The media contained 10^4 cells of each microorganism per ml just after inoculation and approximately 10^8 cells per ml at the end of the experiment. Glucose was fermented reproducibly into ethanol (Fig. 6). The maximal yeast concentration was already reached when the first traces of ethanol were detected in the medium. The lactobacilli produced lactate from glucose in amounts slightly higher (5 mM) than with the natural microbial community. Citrate was fermented into acetate (Fig. 6). The phenomenon of competition for glucose between the yeast and the lactobacilli was favorable to the citrate fermentation. The presence of *S. cerevisiae* allowed the growth of *L. plantarum* on traces of glucose but avoided a significant drop in pH that would have resulted otherwise from the glucose fermentation into lactic acid-lactate. When the pH did not drop to a value lower than 3.0, citrate fermentation was possible, as in the present case.

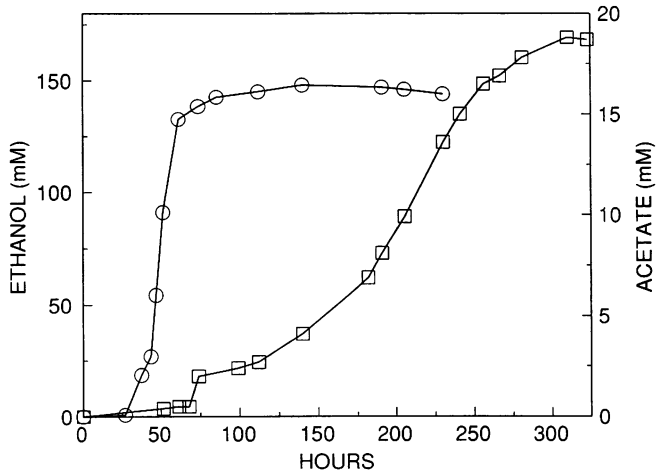

FIG. 6. Ethanol (○) formation from glucose and acetate (□) formation from citrate with a coculture of *S. cerevisiae* and *L. plantarum*.

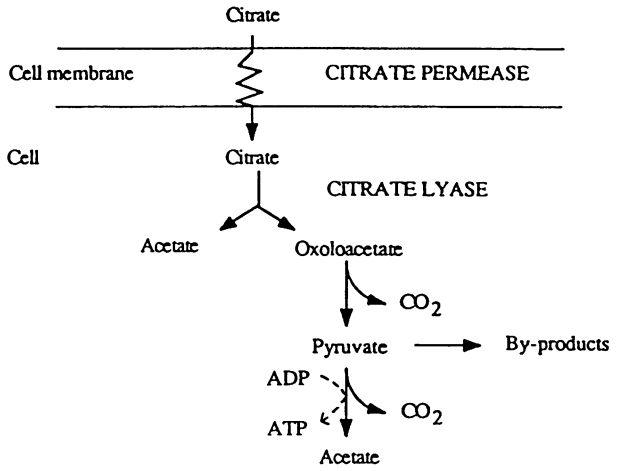

FIG. 7. Metabolic pathway leading from citrate to acetate.

DISCUSSION

The metabolic evolution observed during the fermentation of natural complex systems, such as orange juice, and the role of specific organisms with a given function can accurately be explained by isolating the organisms, reconstituting a community with the isolates, and inoculating the latter consortium in a defined model medium in which all physicochemical and biological parameters can be followed.

The fermentation of fruits and fruit juices such as orange juice under acidic and anaerobic conditions is performed by two groups of microorganisms, namely, yeasts and lactic acid bacteria. These organisms successively metabolize the sugars and organic acids present, such as glucose and citrate among a few others. For a mixture of glucose and citrate used as a defined model medium and inoculated with orange juice, it was demonstrated that yeasts, in this case *S. cerevisiae*, always outcompeted the lactic acid bacteria, in this case *L. plantarum*, for the degradation of glucose in the pH range 3.0 to 5.0. Such competition played an important role in the subsequent fermentation of citrate. The metabolism of glucose by *S. cerevisiae* resulted in the production of ethanol, leaving the pH of the medium almost unchanged. On the contrary, its fermentation into lactic acid-lactate would have resulted in a pH drop to inhibitory values for *L. plantarum*, since the initial pH (3.6) was already low. It must, however, reach values as low as 3.0 or lower before a complete inhibition of the metabolic activity of *L. plantarum* and lysis of the cells. The growth of *L. plantarum* reaching 10^8 cells per ml at a pH as low as 3.6 or 3.3 is unusual and has not been reported so far.

The fermentation of citrate depended on the previous growth of *L. plantarum* on traces of glucose since the organic acid does not provide enough energy to support the growth on its own (15a). When it is used as a cosubstrate with glucose, citrate is supposed to be able to enhance the growth of lactic acid bacteria due to the availability of more ATP (5) (Fig. 7). *L. plantarum* converted the citric acid-citrate buffer into the acetic acid-acetate buffer with a concomitant pH increase. *S. cerevisiae* was unable to ferment citrate.

Besides the influence on the metabolic profile of the growth rates on glucose and the initial pH, the ethanol produced may also play a role on the microbial activity. The latter is usually studied for its inhibitory effect rather than for any favorable effect (17, 18). Regarding citrate broth used to quantify the population fermenting citrate into acetate, it appeared that the presence of ethanol was favorable to the citrate degradation. That phenomenon has not been checked with medium GC. It is possible that in fermented fruits a relatively high concentration of alcohol due to the activity of a yeast population might help the citrate permease activity and facilitate the entry of the organic acid into the cell. By the same token, it has been shown that lactic acid bacteria without any citrate permease activity are able to degrade citrate when they are dried in a solvent such as acetone that alters the membrane structure (7).

The behavior of the defined medium we studied is a model system for many fruit fermentations. Wine production, for instance, is another example in which *Leuconostoc oenos* (6, 8, 13) generally plays the role played by *L. plantarum* in the present case. These natural ecosystems, however, also contain many metabolically active subdominant populations and are characterized by a complex chemical and microbial composition changing or evolving constantly. This makes a completely reliable explanation of the role of each microorganism and of each chemical compound very difficult. If the use of a defined model medium cannot give a complete image of a natural complex ecosystem, it definitely allows us to obtain an easily interpretable simulation of part of it.

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