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Food Microbiology has been conceived as a means of communication on all microbiological aspects of the food and beverage industries. Primary research reports will form the core of the journal, with additional features such as short communications, reviews, reports of meetings, book reviews, letters and news items. A substantial proportion of published material will relate to food spoilage and safety and to developments arising out of present-day microbiology. Material relating to the developing areas of predictive microbiology, rapid methodology and the application of chemical and physical approaches to food microbiology will also be welcome.

The journal will also publish state-of-the-art reviews on topics of relevance to food and drink microbiology. The reviews will distil and present the essential features of topics and provide the considered opinions of experts, rather than summarizing all past work.

Biotechnology is well established in the beverage industry and is developing rapidly in food processing. *Food Microbiology* will include papers on biotechnological aspects of established processes and on developing topics such as the production of fermented foods and the use of novel microbial processes to produce flavours and food related enzymes.

Whilst maintaining high quality with regard to both the research and the development aspects of food and beverage microbiology, a key aspect of *Food Microbiology* will be its rapid rate of publication.

Utilization of electron acceptors for anaerobic metabolism by *Lactobacillus plantarum*. Enzymes and intermediates in the utilization of citrate†

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Received 3 February 1986.

Evidence was obtained to demonstrate the operation of a pathway for quantitative conversion of citrate to succinate and ethanol by *Lactobacillus plantarum* (WSO) during anaerobic growth on mannitol. With the exception of oxaloacetate reductase, the enzymes required for the proposed pathway were constitutive. Enzyme activity changes during the course of citrate utilization and activity changes when acetate, acetyl phosphate, malate, and fumarate were added as electron acceptors were consistent with the proposed pathway. Acetate, malate and fumarate were found to accumulate in the medium during citrate degradation and then disappear as succinate, and ethanol accumulated, demonstrating that they were intermediates in the pathway of citrate utilization.

Introduction

In our previous paper (McFeeters and Chen 1986) a pathway was proposed to explain the utilization of citrate, malate, fumarate, acetate, acetyl phosphate, and acetaldehyde as electron acceptors during anaerobic metabolism of mannitol. The proposal was based upon the products formed from these compounds and

the ratio of mannitol fermented per mole of each acceptor. The objective of this paper is to provide enzymatic evidence and data on the formation and disappearance of intermediates during the course of citrate reduction which provide further evidence that this sequence of reactions is utilized by *L. plantarum*.

Materials and Methods

Micro-organisms and medium

The strain of *L. plantarum*, designated WSO, and the chemically defined basal medium were the same as described previously (McFeeters and Chen 1986).

The terms glucose/citrate, mannitol/citrate, mannitol/acetate, etc. are used in this paper to designate media which consist of the basal medium with 55.5 mM glucose or mannitol as the carbon source and an acid as the available electron acceptor. Citrate was used at a 10 mM concentration. Acetic acid, acetyl phosphate, L-malic acid, and fumaric acid

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were used at a concentration of 20 mM. All media were sterilized by filtration through 0.22 μ sterile Millipore GS filters (Millipore Corporation, Bedford, MA, USA).

Preparation of enzyme extracts from batch cultures

Lactobacillus plantarum was grown anaerobically at 30°C. Anaerobic conditions were generated by use of BBL Gas paks (BBL Microbiological Systems Cockeysville, MD, USA) in a closed jar. Cells were adapted to each medium by transferring four times at 12-h intervals (10^7 cfu ml⁻¹) and then inoculating into a bottle containing 150 ml of the same medium to which they had been adapted.

During the exponential growth phase 12 to 16 h after inoculation, the cells from 150 ml of medium were harvested by centrifugation and washed twice with pH 6.5, 100 mM potassium phosphate buffer. The washed cells were suspended in the phosphate buffer (5 ml) and then disrupted with a sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY, W-220, USA) for 20 min at a temperature near 0°C. The sonicator was centrifuged at $12\,000 \times g$ for 20 min at 4°C. The supernatant extract was assayed for enzyme activity immediately after preparation. Cells from duplicate fermentations were extracted and analysed separately.

Time course experiments on mannitol/citrate medium

In one experiment, the cells were preadapted to mannitol/citrate medium by transferring five times in that medium before the start of the experiment. In another experiment to observe enzyme adaptation, the cells were transferred in glucose medium and then inoculated into citrate/mannitol medium at the start of the experimental period. In both experiments, the cells were washed twice with sterile saline and transferred to a 2-l fermentor (Multigen convertible bench-top culture apparatus M1072-2000, New Brunswick Scientific Company, Inc, Edison, NJ, USA) which contained 1.5 l of mannitol/citrate medium. Anaerobiosis was maintained in the fermentor by flushing the headspace with a mixture of 5% CO₂, 10% H₂ and 85% N₂ (Air Products and Chemicals Company, Allentown, PA, USA). Before entering the fermentor, the gas mixture was passed through a combined solution (200 ml)

of 25% pyrogallol acid and 40% KOH in the ratio of 1:10, then through water, and finally through a sterilized cotton filter to remove any residual oxygen and to humidify the gas mixture.

Samples were taken under aseptic conditions several times during the course of fermentation. A portion of the sample was filtered through a 0.04 μ filter and frozen for chemical analysis of the fermentation broth. The remainder of the sample was used to prepare an enzyme extract as described above.

Enzyme assays

Enzyme assays were performed on duplicate fermentation samples in all experiments. The variation of enzyme activity of duplicate fermentations within an experiment was 10% or less.

Citrate lyase activity was measured according to the method of Ward and Srere (1965), except that the reaction was monitored at 256 nm instead of 285 nm because the enol form of oxaloacetate absorbs maximally at that wavelength (Clark and Switzer 1977). A molar extinction coefficient, $E_{256} = 1.18 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, was determined. Acetate kinase activity was determined by incubating enzyme extract with acetate and ATP in the presence of hydroxylamine and measuring the acetohydroxamic acid formed with ferric chloride (Rose et al. 1954). Phosphotransacetylase activity was determined by the method described by Stadtman (1955). Fumarase, succinate dehydrogenase, and NADH oxidase activities were assayed by the method of Takahashi and Hino (1968). Fumarate reductase activity was measured according to the method of Massey and Singer (1957). One unit of enzyme activity is defined as 1 μ mol of substrate consumed or product formed per min per ml of reaction mixture.

Qualitative assays to demonstrate that alcohol dehydrogenase was present in *L. plantarum* cell extracts were carried out by measuring ethanol formation from acetaldehyde and NADH catalysed by the extracts. A 1.0 ml reaction mixture contained 60 μ mol potassium phosphate buffer (pH 6.5), 10 μ mol acetaldehyde, 10 μ mol NADH, 5 μ mol cysteine, and 0.1 ml cell free extract. The reaction mixture was incubated for 24 h at room temperature under anaerobic conditions. Ethanol formation was measured at the end of the incubation period. Oxalacetate reductase activity was demonstrated by

measuring fumarate formation catalysed by cell free extract. A 1.0 ml reaction mixture contained 20 μmol potassium phosphate buffer (pH 7.2), 5 μmol MgCl_2 , 10 μmol NADH, 20 μmol oxalacetate, and 0.1 ml of cell free extract. Incubation was done as in the alcohol dehydrogenase assay. Products were determined by HPLC.

Protein determination

Protein concentration of cell free extracts was measured by the biuret method (Gornall et al. 1949).

Analysis of organic acids and ethanol

Quantitative HPLC analyses of mannitol, ethanol and organic acids were done using the procedure of McFeeters et al. (1984). In order to measure all of the organic acids formed and degraded in this study, a C_{18} reversed phase column (Techsil 5 μ C_{18} , Phenomenex, Rancho Palos Verdes, CA, USA) was used. A Bio-Rad HPX87H column (Bio-Rad Labs, Richmond, CA, USA) was used for determining low concentrations of fumarate by monitoring the column effluent at 240 nm with a Varian varichrom UV detector. The column was kept at 45°C in a water bath and eluted with 0.08 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} . Ethanol was determined by gas chromatography using the procedure described by McFeeters and Armstrong (1984) for the analysis of methanol.

NADH purification

NADH (N-6005, Sigma Chemical Company St Louis, MO, USA) contained ethanol which interfered with attempts to demonstrate ethanol formation catalysed by cell free extracts of *L. plantarum*. Ethanol was removed from NADH by passing it through a 40.8 \times 1.6 cm Biogel P-2 column. The NADH eluted from the column was monitored by measuring the absorbance at 340 nm. Ethanol could not be detected by gas chromatography in eluted NADH fractions.

Chemicals

Citrate, oxalacetate, malate, NADH, ATP, coenzyme A, ferric chloride, flavin mononucleotide, hydroxylamine monohydrochloride, MgCl_2 , and potassium arsenate were obtained from Sigma Chemical Company. Acetaldehyde, fumarate, acetic acid, potassium hydroxide, potassium phosphate,

sodium acetate, and Tris buffer were purchased from Fisher Scientific Company (Pittsburgh, PA, USA). Sodium dithionite was obtained from Eastman Kodak Company (Rochester, NY, USA).

Results

Enzyme activity changes with different electron acceptors

The basic experiment conducted with acetate, acetyl phosphate, malate, fumarate, and citrate as electron acceptors was to determine the presence and amounts of activity of the enzymes required to carry out the reactions shown in Fig. 1 in cell free extracts of *L. plantarum*. In each instance cells of *L. plantarum* were grown on media which contained glucose, glucose/acceptor and mannitol/acceptor. Cell free extracts from log phase cells, which had been adapted by several transfers in the growth medium, were assayed for the required enzyme activities. Table 1 shows the results of these experiments.

The same general pattern of enzyme activity was found with each acceptor. A low level of activity was present in glucose-grown cells. Addition of an acceptor to the glucose medium resulted in a small increase in enzyme levels. In the mannitol/acceptor medium where reduction of the acceptor was required for cell growth, a further increase of the enzymes required for acceptor metabolism occurred. For example, acetate kinase activity in cells grown on glucose/acetate was 1.4-fold higher than in glucose-grown cells, while the enzyme in mannitol/acetate-grown cells was 4.1-fold higher. Phosphotransacetylase showed the smallest increases among the enzymes assayed. Fumarase and fumarate reductase increased 14.2- and 9.4-fold, respectively, in cells grown on mannitol/malate, compared to glucose-grown cells. The specificity of the activity increases is indicated by the fact that on

Table 1. Effect of electron acceptors on the induction of enzyme activities in *L. plantarum* grown on glucose and mannitol.

Acceptor	Carbon Source	Citrate lyase	Oxaloacetate reductase (mm) ^b	Fumarase	Fumarate reductase	Acetate kinase	Phosphotrans-acetylase	Alcohol dehydrogenase (mm) ^c
Citrate	Glucose	0.01 ^a	N.D.	0.22	0.17	0.87	0.11	0.44
	Glucose	0.03	N.D.	0.27	0.15	0.74	0.17	0.35
	Mannitol	0.09	1.72	1.46	1.28	3.48	0.37	0.52
Malate	Glucose			0.11	0.11			
	Glucose			0.28	0.19			
Malate	Mannitol			1.56	1.03			
	Glucose			0.13	0.10			
Fumarate	Glucose			0.38	0.23			
	Mannitol			0.52	1.33			
Acetate	Glucose					0.56	0.12	0.26
	Glucose					0.80	0.16	0.32
	Mannitol					2.32	0.21	0.84
Acetyl P	Glucose					0.39	0.12	0.32
	Glucose					0.53	0.25	0.50
Acetyl P	Mannitol					1.90	0.25	1.32

^a Enzyme reaction rates are expressed as $\mu\text{mols min}^{-1} \text{mg}^{-1}$ protein.

^b Fumarate formation after a 24-h incubation period.

^c Ethanol formation after a 24-h incubation period.

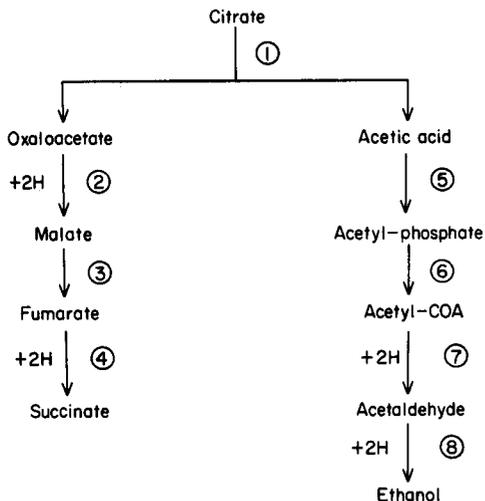


Fig. 1. Pathway for citrate utilization during mannitol fermentation. (1) Citrate lyase; (2) oxaloacetate reductase; (3) fumarase; (4) fumarate reductase; (5) acetate kinase; (6) phosphotransacetylase; (7) acetaldehyde dehydrogenase; (8) alcohol dehydrogenase.

mannitol/fumarate medium fumarase activity, which would not be required to convert fumarate to succinate, increased much less than when malate was the acceptor. On the other hand, fumarate reductase activity increased 13.3-fold in the mannitol/fumarate medium.

Addition of citrate as an electron acceptor resulted in increases in all five of the enzymes in Table 1 which were quantitatively assayed. When citrate was added to glucose medium, a 3.0-fold increase in citrate lyase activity was observed, while there was little or no change in the other enzymes. However, at the time the cells were harvested, no measurable decrease in citrate concentration had occurred in the medium. In contrast, when cells were grown on mannitol/citrate, where citrate metabolism was required for cell growth, 2 mM citrate had been metabolized at the time cells were harvested.

According to the proposed pathway for utilizing citrate as an electron acceptor (Fig. 1), oxaloacetate produced from citrate should be reduced to malate by oxaloacetate reductase. However, quantitative measurement of oxaloacetate reductase activity by measuring the reduction of oxaloacetate by NADH at 340 nm could not be done probably due to spontaneous conversion of oxaloacetate to pyruvate as described by Wilcock and Goldberg (1972). Also, efforts to measure the reverse reaction, i.e. oxidation of malate to oxaloacetate by following the

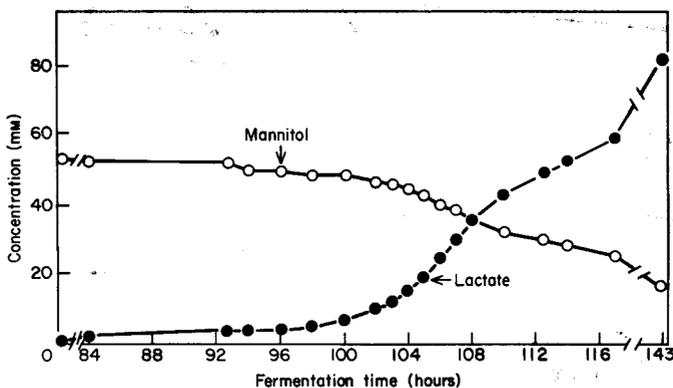


Fig. 2. Mannitol disappearance and lactic acid formation during anaerobic growth of *L. plantarum* with citric acid as the electron acceptor. Cells were grown on glucose medium and then transferred to mannitol/citrate medium as the start of the experiment.

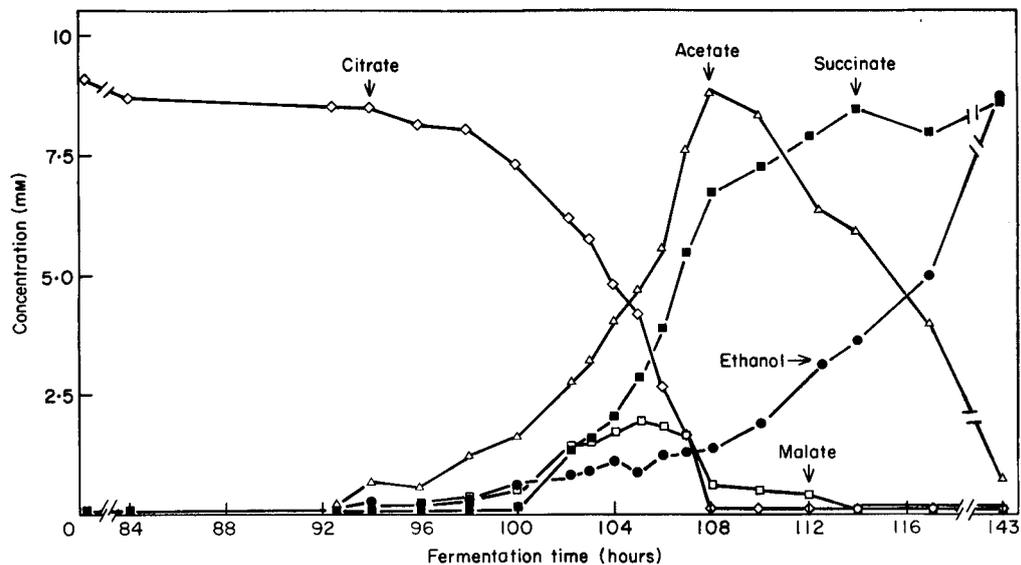


Fig. 3. Metabolism of citrate, acetate, malate, succinate, and ethanol during anaerobic growth of *L. plantarum* with mannitol as the carbon source.

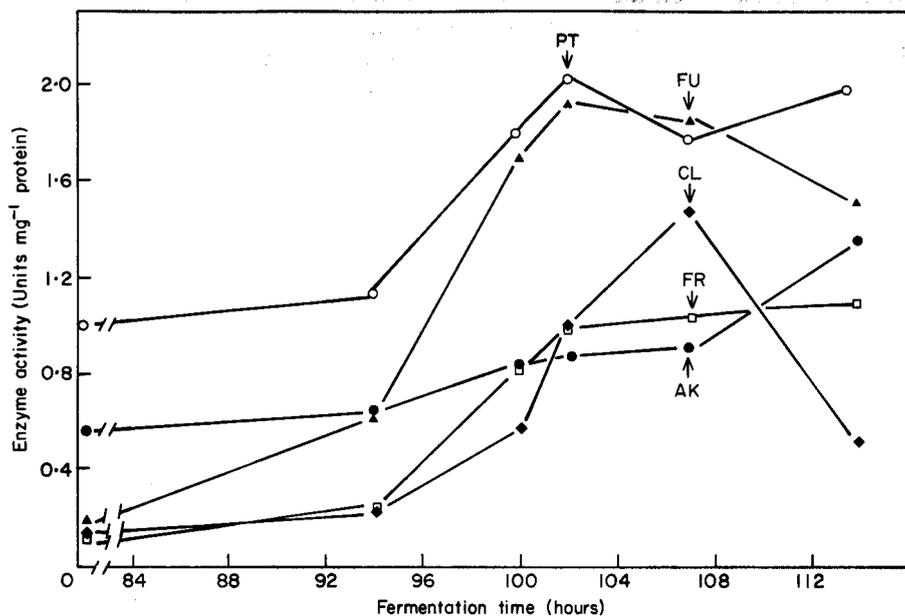


Fig. 4. Changes in enzyme activities during anaerobic growth of *L. plantarum* on mannitol with citrate as the electron acceptor. The activities of citrate lyase and phosphotransacetylase were multiplied by ten to put them on the same scale as the other enzymes. PT = phosphotransacetylase; FU = fumarase; CL = citrate lyase; FR = fumarate reductase; AK = acetate kinase.

reduction of NAD to NADH, were unsuccessful because the enzyme extracts contained considerable NADH oxidase activity (0.7 $\mu\text{mol}/\text{min}/\text{mg}$ protein). To qualitatively demonstrate the presence of the enzyme in cell free extracts, the products formed in cell free extracts with oxaloacetate as substrate and NADH as a cofactor were determined. Fumarate was produced in reactions containing cell-free extract from cells grown on mannitol/citrate medium, but not from cells grown on glucose or glucose/citrate medium (Table 1).

Alcohol dehydrogenase was the other enzyme which we were unable to quantitatively assay in cell-free extracts, presumably due to low activity in the cells coupled with high NADH oxidase activity. The presence of alcohol dehydrogenases was qualitatively demonstrated in *L. plantarum* extracts by showing that they catalysed formation of ethanol in the presence of acetaldehyde and NADH. The data in Table 1 show that ethanol formation was catalysed by cell-free extracts regardless of the carbon source or electron acceptor present in the medium during growth.

Time course of mannitol/citrate fermentations

Changes in fermentation substrates and products were measured both when cells were preadapted to growth on mannitol/citrate and when the cells were transferred directly to mannitol/citrate from glucose medium. Figures 2, 3 and 4 show the changes in substrates, products and enzyme activities when *L. plantarum* was transferred directly from glucose medium to mannitol citrate. A long lag period was required for *L. plantarum* to adapt from glucose medium to mannitol/citrate medium (Fig. 2). Very small amounts of mannitol and citrate were fermented during the lag period (Figs 2

and 3). Thereafter, mannitol and citrate were metabolized at the same time. Two mols of lactate were formed for each mole of mannitol fermented throughout the fermentation.

Analysis of the time course of citrate changes showed the relationship between citrate utilization and formation of intermediates and end products produced from citrate (Fig. 3). By the time all citrate had been utilized 108 h after inoculation, an equimolar amount of acetate had accumulated in the medium. Malate and succinate began to accumulate in the medium as citrate declined. Malate reached a maximum of 2.5 mM and then declined as succinate increased until, at the end of fermentation, a succinate concentration near the initial citrate concentration had accumulated in the medium. Not shown in Fig. 3, small amounts of fumarate (< 0.1 mM) also accumulated and then declined during the fermentation.

The pattern of changes when cells were preadapted to mannitol/citrate medium was similar to those shown in Fig. 3, except that a 12-h instead of a 92-h lag period was observed and malate accumulated to 5.5 mM instead of 2.5 mM before declining with a starting concentration of 10 mM citrate.

Figure 4 shows the pattern of enzyme activity changes in *L. plantarum* cells as they adapted to the utilization of mannitol and citrate. Each of the five enzymes increased during citrate degradation. Citrate lyase activity declined after 108 h when citrate had disappeared from the medium. Fumarase also began to decline after 108 h as the malate reached a very low concentration. Acetate kinase was still increasing after 112 h when acetate was still being converted to ethanol. At the end of fermentation, 4 mols of mannitol had been fermented for each mole of citrate converted to ethanol and succinate.

Discussion

This paper shows that the enzymes required to carry out the degradation of citrate to succinate and ethanol, as proposed in the previous paper (McFeeters and Chen 1986), can be detected in cell-free extracts of *L. plantarum*. In fact, each of the enzymes investigated, except oxaloacetate reductase, appears to be a constitutive enzyme in the cells in that low levels of activity were detected in glucose-grown cells even after several transfers of the cells in a defined medium, which did not contain citrate or any of the intermediates in the pathway to succinate or ethanol. The problems of oxaloacetate instability and the presence of NADH oxidase in the extracts may have made it impossible to detect low constitutive levels of activity.

Activity of each of the five enzymes quantitatively assayed increased over constitutive levels when citrate was used as the electron acceptor for mannitol fermentation. When acceptors such as malate, fumarate, acetate, and acetyl phosphate were added to mannitol medium, the specific enzymes required to convert these compounds to succinate or ethanol also increased. The timing of enzyme activity changes during the transition from growth on glucose to mannitol/citrate was also consistent with the possibility that these enzymes were being used to carry out the steps required for electron acceptor reduction. Finally, the fact that acetate, malate and fumarate accumulated in the medium and then declined during growth on mannitol/citrate medium was additional evidence that the sequence of reactions shown in Fig. 1 were carried out by *L. plantarum* cells during mannitol fermentation.

Citrate utilization is common among lactobacilli, including *L. plantarum* (DuPlessis 1964, Fryer 1970, Thornhill and Cogen 1984). DuPlessis (1964) found

it could be used as a carbon source by *L. leichmannii*, *L. brevis* and *L. hilgardii*. In other cases, a metabolic function for citrate metabolism was not established. This is the first work in which citrate has been found to act as part of an obligatory electron acceptor pathway.

Among the enzymes involved in the reactions which are involved in the citrate degradation, citrate lyase has been investigated in several microorganisms. It has been found to be inducible in *E. coli* (Wheat and Ajl 1955), *Aerobacter aerogenes* (Dagley and Dawes 1953) and *Streptococcus faecalis* (Smith et al. 1956). Harvey and Collins (1961) found that citrate lyase was a constitutive enzyme in *Streptococcus diacetilactis*. Oxaloacetate reductase activity has not been previously reported in lactic acid bacteria. Fumarase was found in *L. plantarum* when cells were grown in a malate-containing medium (Winter and Kandler 1976). Fumarate reductase activity has also been detected in *L. plantarum* (Snoswell 1959).

The steps involved in the reduction of acetate to ethanol are the same as occur in heterofermentative lactic acid bacteria (Gunsalus and Gibbs 1952). These reactions have also been demonstrated in *S. diacetilactis*, a homofermentative organism (Collins and Bruhn 1970). Acetate kinase was the only enzyme involved in these steps which had been previously demonstrated in cell-free extracts of *L. plantarum* (Heath et al. 1958).

In this investigation, malate was quantitatively converted to succinate during mannitol fermentation. However, when grown on glucose and malate, a malolactic enzyme which decarboxylates malate to lactate is induced in *L. plantarum* (Schultz and Radler 1973). In cucumber juice fermentations by *L. plantarum*, malate decarboxylation occurs quite early in the

fermentation (McFeeters et al. 1982). These results suggest that some factor other than just the presence of malate is required for induction of a malolactic enzyme in order to regulate malate utilization to the best advantage of the fermentation organism.

One other point of interest in this study was the long lag period before mannitol fermentation began when citrate was used as an electron acceptor. Pirt (1975) has suggested that long lag periods as organisms adapt to a new medium can be an indication of a

requirement for genetic modification of the organism before it is capable of growth. With a lag period of nearly 4 days for this fermentation, it may be useful to determine if there are genetic differences between the parent organism and the cells which grow in the mannitol/citrate medium.

Acknowledgements

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, IL, USA.

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