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Production of formate, acetate, and succinate by anaerobic fermentation of *Lactobacillus pentosus* in the presence of citrate

Jörg Cselovszky, Gudrun Wolf, and Walter P. Hammes

Institut für Lebensmitteltechnologie, Universität Hohenheim, Garbenstrasse 25, W-7000 Stuttgart 70, Federal Republic of Germany

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Summary. The formation of acetate, formate and succinate was studied in *Lactobacillus pentosus*. These compounds were produced in addition to lactic acid when cells were exposed to anaerobic growth conditions with limited carbohydrates and in the presence of citrate. Citrate was metabolised via oxalacetate serving as an H-acceptor in a joint process together with lactate. The metabolism of citrate resulted in stoichiometric amounts of succinate and acetate. Lactate was degraded to formate and acetate in a reaction catalysed by pyruvate formate lyase. These fermentation products can potentially affect the flavour of fermented food but ecological factors in fermenting meat, e.g. the presence of glucose, nitrate or nitrite prevent this reaction.

Introduction

The formation of products other than lactic acid by homofermentative lactic acid bacteria is well documented (Kandler 1983). Homolactic fermentation is generally found under anaerobic conditions when glucose is present at concentrations allowing high growth rates (Fordyce et al. 1984). However, when *Lactococcus lactis* is grown under aerobic conditions, at a rate of 5% of maximum lactose utilization, the fermentation products change and glycolysis can even result in the formation of acetate as the major product (Smart and Thomas 1987). This also takes place with glucose under anaerobic conditions, when nitrate or nitrite are simultaneously reduced in the presence of haem by *L. pentosus* (Wolf et al. 1991). The conversion of carbohydrates to acetate leads to the generation of an additional molecule of ATP by substrate level phosphorylation via acetate kinase.

When glucose becomes exploited under anaerobic growth conditions and a reducible substrate is available, acetate, ethanol and formate are formed by *L. lactis* in addition to lactate (Thomas 1976). The production of formate was also reported for the obligate homofermentative *L. bulgaricus* (Rhee and Pack 1980) and the facultative heterofermentative *L. casei* (de Vries et al. 1970). Lindgren et al. (1990) described the production of formate, acetate and succinate by *L. plantarum* after addition of citrate and prolonged incubation (7–30 days). These metabolites strongly affect the flavour of fermented food. Thus, it is important to obtain insight into the conditions permitting their formation. We studied the physiological regulation of their formation with *L. pentosus* under the special ecological conditions prevailing during fermentation of meat. It will be shown that the presence of nitrate and nitrite, of fermentable sugars, and access of oxygen exert a regulatory influence on the products of fermentation.

Materials and methods

Organism and culture conditions. L. pentosus DSM 20314 was grown in a medium containing the following substances per litre: tryptone, 10 g; yeast extract, 5 g; tryptose, 3 g; K_2HPO_4, 3 g; KH_2PO_4, 3 g; Tween 80, 1 g; D-glucose, 1.8 g; L-cysteine, 0.2 g: 5 ml of a salt solution were added to the medium, consisting of $MgSO_4 \cdot 7H_2O$, 11.5 g; $FeSO_4 \cdot 7H_2O$, 1.7 g; $MnSO_4 \cdot 2H_2O$, 2.4 g; distilled water to 100 ml. Where indicated 2.3 g diammonium citrate was added. Sterile 50-ml erlenmeyer flasks were filled to the top with autoclaved medium, inoculated and sealed with serum stoppers. Cultures were placed in a shaking incubator and incubated at 130 rpm and 30° C. Aliquots of 2 ml were taken with sterile syringes and stored at −28° C until analysis. Where indicated, additions were made through the serum stoppers with sterile syringes.

For aerobic culture, bacteria were grown at 30° C in 250-ml erlenmeyer flasks with 100 ml medium in a shaking incubator (130 rpm).

Determination of metabolites. The concentrations of lactate, citrate, succinate, acetate, ethanol and glucose were determined enzymatically with test kits from Boehringer (Mannheim, FRG).

Offprint requests to: G. Wolf

Results

Time course of the formation of metabolites

L. pentosus was grown anaerobically with glucose as the fermentable sugar. In accordance with the homolactic nature of the organism, lactate was the main product with acetate, formate and ethanol as minor by-products (Fig. 1).

The time course of the formation of products in the presence of 9.5 mmol/l of citrate is illustrated in Fig. 2A and B. Under these conditions acetate was the main product. A maximum of 27 mmol acetate was attained after 38 h incubation. This event coincided with complete consumption of citrate and no further decrease in lactate concentration. The concentration of lactate began to decrease after 17 h, at which time glucose was used up. Concomitantly the production of formate and succinate commenced and the concentration of citrate decreased. The turnover of citrate is virtually stoichiometric with the formation of succinate and nearly the same holds true for the consumption of lactate (9 mmol) and the formation of formate (9 mmol).

Ecological factors affecting the production of formate

Effect of oxygen. As illustrated in Fig. 3, the production of formate takes place only under anaerobic conditions in the presence of citrate (10 mmol/l). Under aerobic growth conditions, formate production is strongly inhibited. The aeration of the culture after 24 h incubation caused the cessation of formate production.

Effect of nitrate or nitrite. As demonstrated in Fig. 4 both nitrate and nitrite inhibit the production of formate in *L. pentosus* in such a way that in the presence of these compounds the production of formate stagnated at a low level. When haematin was added to the culture, nitrite reduction took place in *L. pentosus* (Wolf and Hammes 1988) and the inhibitory effect of the compound was abolished.

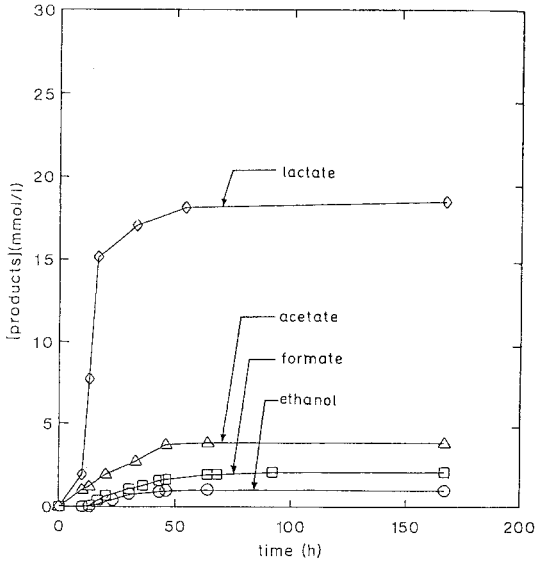

Fig. 1. Kinetics of product formation by *Lactobacillus pentosus*

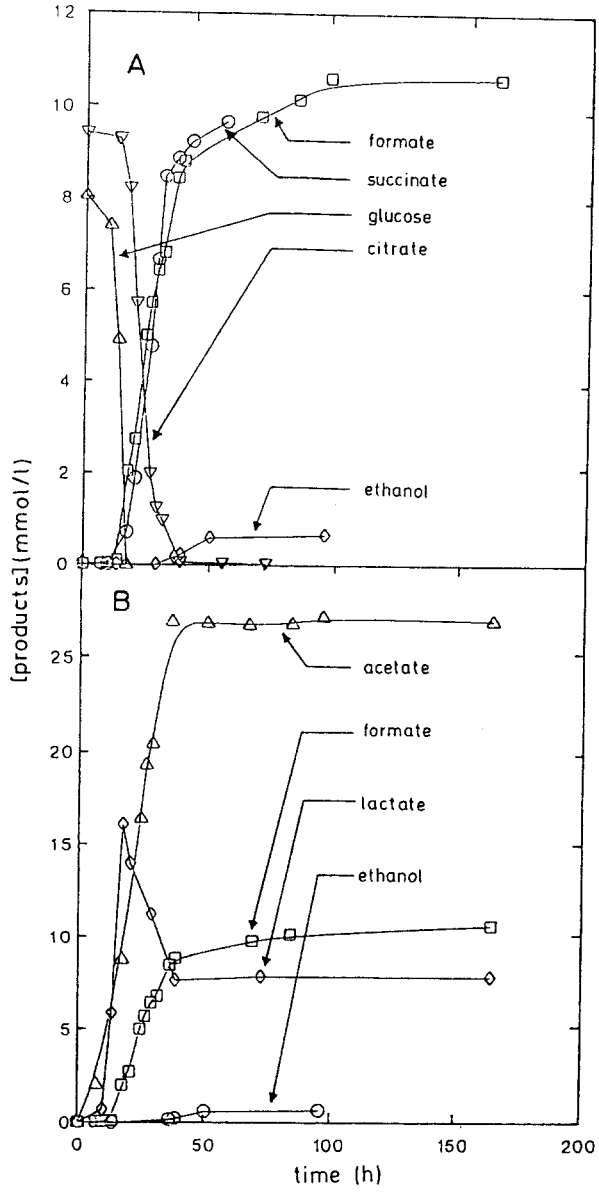

Fig. 2A, B. Kinetics of product formation by *L. pentosus* in the presence of 10 mmol/l of citrate. The curves were obtained in two experiments. **A** Consumption of glucose and citrate and production of formate, succinate and ethanol. **B** Production of acetate, formate, lactate, and ethanol

Effect of fermentable carbohydrates. As demonstrated in Fig. 5, the addition of 10 mmol/l of glucose after 24 or 30 h inhibits the formation of formate. The same effect was observed after adding 10 mmol/l of fructose and galactose (data not shown). In contrast to these effects the addition of 10 mmol/l of gluconate stimulated the production of formate. The same was observed after addition of lactate (data not shown).

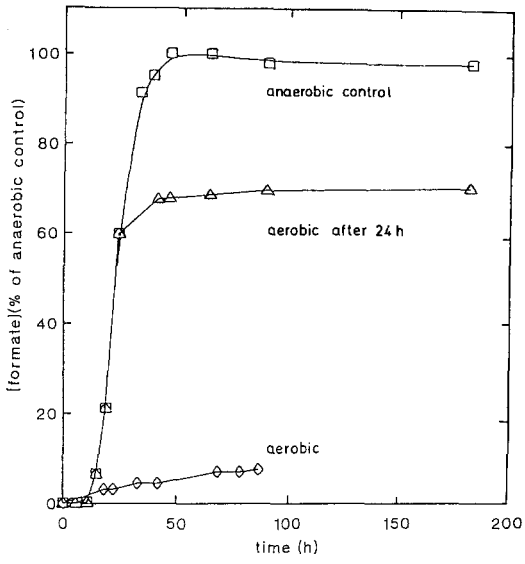

Fig. 3. Effect of oxygen on synthesis of formate by *L. pentosus* in the presence of 10 mmol/l of citrate: 100% corresponds to 4 mmol/l of formate. Cells were grown under anaerobic conditions (□) or aerobic conditions (◇). In a parallel experiment the anaerobic culture was aerated after 24 h (△)

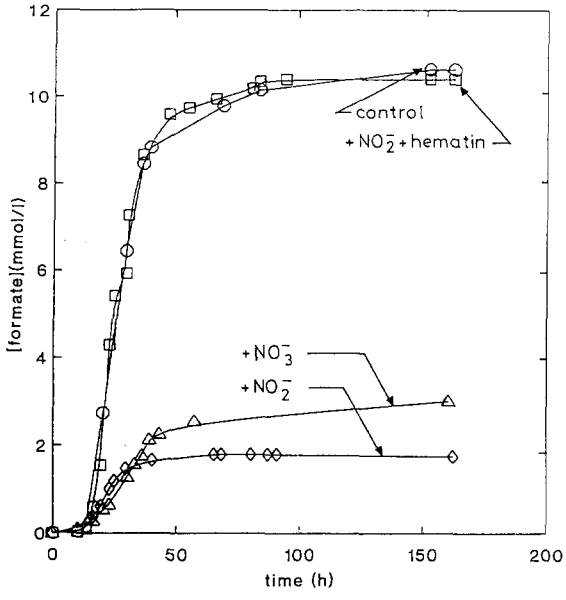

Fig. 4. Kinetics of synthesis of formate by *L. pentosus* in the presence of nitrate (2 g/l), nitrite (20 mg/l) or nitrite plus haematin (30 µmol/l). The medium contained 10 mmol/l of citrate

Discussion

The regulatory effects of the ecological factors present during fermentation of meat ensure a "clean" fermentation with mainly lactic acid as the end-product. Both nitrate and nitrite are traditionally used as curing agents, which in their turn exert effects on hygienic safety and sensory quality (Leistner 1986). It is remarkable that after consumption of nitrite by *L. pentosus* metabolic routes lead to formation of formic and succinic

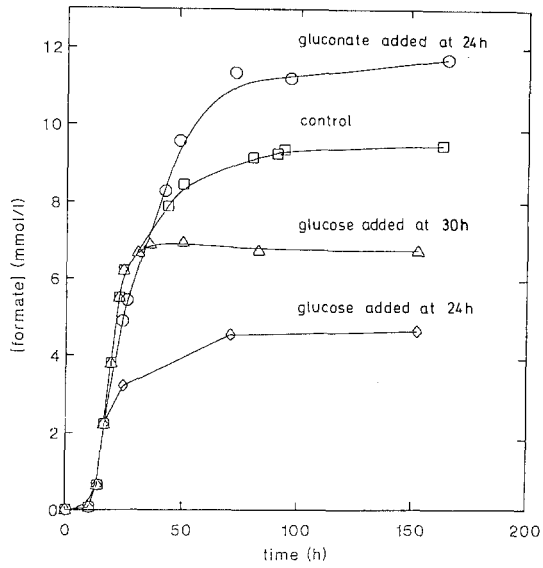

Fig. 5. Effect of the addition of glucose or gluconate on the formation of formate by *L. pentosus* in the presence of 10 mmol/l of citrate

acid. The additional presence of fermentable sugar is a further factor supporting the effect of the curing aids by inhibiting formate formation. These regulatory factors are effective at least during the early phase of meat fermentation and may disappear in later phases. However, during the ripening process the water activity of the fermenting meat mixture decreases gradually until a range is obtained at which the metabolism of lactic acid bacteria is restricted.

The results obtained also contribute to an improved knowledge of the basic metabolism of lactobacilli. As demonstrated by Thomas et al. (1979) in lactococci, formate and acetate are the end-products of the pyruvate-formate pathway. This pathway is an alternative to the lactate dehydrogenase pathway and operates under anaerobic conditions and glucose limitation. These conditions result in low levels of fructose 1,6-diphosphate and glyceraldehyde-3-phosphate (GAP). The pyruvate formate lyase has been detected in Enterobacteriaceae, clostridia, some streptococci and lactobacilli (Lindgren et al. 1990). In *Escherichia coli* this enzyme is regulated post-translation by anaerobiosis, and additionally at the level of gene expression (Knappe and Sawers 1990). On this level also nitrate inhibits expression of the pyruvate formate lyase (Iuchi and Lin 1987).

In contrast to *E. coli,* in *L. pentosus* and *L. lactis* the pyruvate formate lyase is inhibited by the addition of fermentable carbohydrates. In *L. lactis* the effect of the hexoses is mediated by fructose-1,6-diphosphate and GAP (Fordyce et al. 1984). However, the effect of gluconate should be mediated by GAP only. This raises the question of why GAP is not effective with this compound.

In *L. pentosus* the pyruvate formate lyase becomes active when glucose is used up. Thereafter lactate is utilized in the presence of a reducible substrate, e.g. ci-

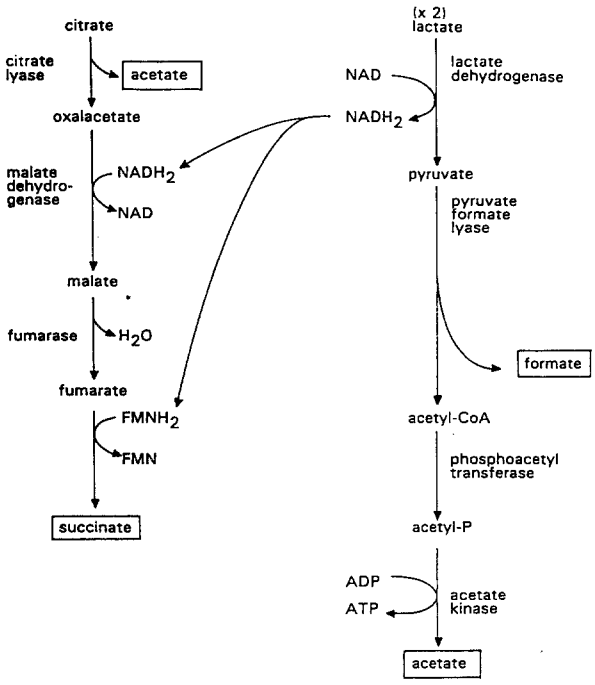

Fig. 6. Joint metabolism of lactate and citrate by *L. pentosus*

trate. We suggest a metabolic pathway as illustrated in Fig. 6. Citrate is split into acetate and oxalacetate by citrate lyase. The latter compound is reduced to malate with regeneration of NAD. By further reduction succinate is formed as the end-product via fumarate. The scheme in Fig. 6 is consistent with reaction 1:

2 lactate + 1 citrate → 3 acetate + 2 formate + 1 succinate.

The results demonstrated in Fig. 2 show that 9.5 mmol citrate and 8 mmol glucose were transformed into 27 mmol acetate and 9 mmol succinate. However, the ratio of the turnover of lactate (9 mmol) and citrate (9.5 mmol) does not obey reaction 1. This may be explained by the metabolic reactions required for cell growth. Further work with non-growing cells in defined medium is therefore required.

Since citric acid is found in fermentable plant materials and milk, and is also used as an additive for the production of fermented sausages, the formation of succinic, formic and acetic acids in fermented food may become detrimental because of the pungent and penetrating odour of formic acid, the strong and characteristic odour of acetic acid and the salty and bitter flavour of succinic acid. In fermented food, which does not contain nitrate or nitrite, the activity of pyruvate formate lyase may give rise to these undesirable flavours. The application of appropriate starter organisms, formula and fermentation technology are means of preventing this risk.

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