Journal of Industrial Microbiology, 13 (1994) 335–343

Published by The Macmillan Press Ltd

Aeration-controlled formation of acetic acid in heterolactic fermentations

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(Received 18 February 1994; accepted 1 June 1994)

Key words: Aeration; *Leuconostoc mesenteroides*; Heterolactic fermentation; Acetic acid; Headspace gas chromatography

SUMMARY

Controlled aeration of *Leuconostoc mesenteroides* was studied as a possible mechanism for control of the formation of acetic acid, a metabolite of major influence on the taste of lactic fermented foods. Fermentations were carried out in small scale in a medium in which growth was limited by the buffer capacity only. Ethanol and acetic acid formed during the fermentation were analyzed by rapid head space gas chromatography, and the ratio of the molar concentrations of these two volatiles quantitatively predicted the balance between the formation of acetic acid and lactic acid. The oxygen concentration during the fermentations decreased rapidly to zero, meaning that oxygen transfer was limited by the volumetric oxygen transfer rate, k_1aC^*. A linear correlation between k_1aC^* and the quantity of acetic acid produced was established, and it is suggested that such oxygenated heterolactic fermentation processes should be analyzed as fed-batch fermentations with oxygen as the limiting substrate. Addition of fructose in limited amounts leads to the formation of one half mole of acetic acid for each mole fructose, thus offering an alternative mechanism for controlling acetic acid formation.

INTRODUCTION

Lactic acid fermentation of cereals, legumes and green vegetables is applied throughout the world for the production of a large variety of foods. In Europe, at least 21 different vegetables are fermented, and the total production is around 800 000 tons per year [5]. In many Asian and African countries, lactic acid-fermented legumes, cereals, or legume–cereal blends are important staples or popular beverages. With few exceptions lactic-fermented vegetable foods have been produced for many years by traditional, experience-based processes; however, there is a strong trend to develop some of these into modern food biotechnology processes [20], on a par with the developments years ago in the brewing, dairy, and soy sauce industries.

As part of an international program on the industrialization of lactic acid fermentation technology, an experimental process for producing a lactic acid-fermented beverage from a blend of soymilk and mashed cereals was studied [2]. A key to the process was a particular strain of *Leuconostoc mesenteroides* which had been selected by Lee and co-workers for its ability to produce a unique, pleasant taste of the fermented beverage [14]. This organism is heterofermentative, producing lactic acid, ethanol, acetic acid and CO_2 as the major metabolites (Fig. 1) [9]. The pleasant taste in the fermented beverage is critically dependent on the balance between the concentrations of lactic acid and acetic acid, respectively, in conjunction with the level of sweetness [7,8]. Therefore, control of organic acid formation during fermentation is a paramount objective in the successful industrialization of this process. Furthermore, since *L. mesenteroides* is a ubiquitous organism in fermented vegetable foods [13,19], the issue of controlling the formation

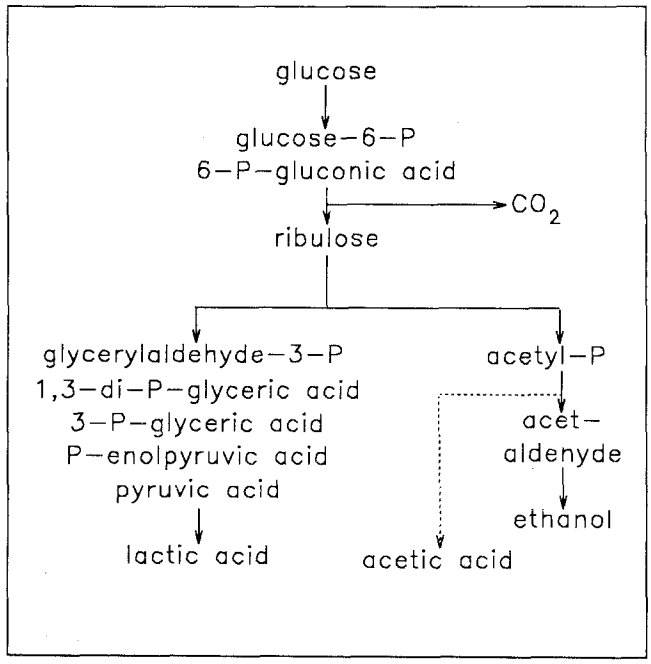

Fig. 1. Carbohydrate metabolism of heterofermentative lactic acid bacteria. Adapted from [9].

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of the two organic acids in a particular balance could be of wider interest.

In his studies of the fermentation process with this strain of *L. mesenteroides*, Chung observed that aeration had a positive effect on flavor by inducing the formation of acetic acid in higher concentrations [7]. This observation complied with previous physiological studies of *Leuconostoc* species which demonstrated the formation of acetic acid in aerated cultures [4,15,18]. The mechanism involves NADH-oxidase activity and utilizes oxygen for regeneration of NAD^+ from NADH [9,15]. This allows the organism to redirect the conversion of acetylphosphate from ethanol to acetic acid, whereby an additional mole of ATP is gained for each mole of glucose metabolized [9].

A particular aspect of the above mechanism for acetic acid formation is that fructose can also serve as an electron acceptor. For each mole of acetic acid formed, two moles of mannitol are produced by reduction of fructose [10]. The fructose moiety in sucrose also exhibits this effect [7].

The effect of oxygen on the carbohydrate metabolism of *L. mesenteroides* thus seems well elucidated, qualitatively. However, it is an open question whether or not this aeration effect can be utilized as an active control mechanism for the final concentration of acetic acid in an industrial food fermentation process. In two studies in which *L. mesenteroides* was grown in aerated fermenters, maximum aeration was maintained, and neither study was concerned with control of acetic acid formation [4,21]. In fact, not much attention has been devoted to the practical use of controlled aeration of heterolactic fermentations. In one study [11], it was found that controlled injection of air in a conventional ten-ton vessel for fermenting olives resulted in a faster fermentation and an improved taste of the olives, and since *Leuconostoc* species (together with yeasts) were among the dominant species in the first phase of the fermentation, this work can be regarded as one of the few published examples of an aeration-controlled, predominantly heterolactic fermentation.

The present study was undertaken to study controlled aeration as a control mechanism for acetic acid production in heterolactic fermentations. An additional objective is to develop and apply analytical methods to assess the effect of oxygenation in the manufacture of a broad range of lactic fermented foods and beverages.

MATERIALS AND METHODS

Organism

The organism was a strain of *Leuconostoc mesenteroides* isolated from *sikhae*, a Korean traditional lactic acid-fermented fish product [14]. The organism was maintained on Lactobacilli MRS (Difco Laboratories, Detroit, MI, USA) agar plates and cultivated in capped test tubes with MRS broth for 24 h at 30 °C. Purity and viability of the strain were checked by plating and recultivation.

Medium

A complex medium, denoted TYM, was developed in which the organism grows rapidly under fermentation conditions (see Results). Growth was not limited by any nutrient, only by the buffering capacity of the medium. The medium had the following composition: 10 g L^{-1} tryptone (Difco), 2 g L^{-1} yeast extract (Difco), 150 mM glucose (unless otherwise specified), 50 mM phosphate buffer (25 mM Na_2HPO_4 + 15 mM NaH_2PO_4 + 10 mM KH_2PO_4), and a mineral base (0.05 g L^{-1} $MnSO_4$ + 0.10 g L^{-1} $MgSO_4$, calculated on the basis of the anhydrous salts). The pH was 6.8.

Seed culture

A seed culture was prepared by inoculating 75 ml TYM medium with 2 ml of a test tube culture and cultivating it under standard aerated fermentation conditions (see below) for 24 h.

Fermentation experiments

Small scale batch fermentations were carried out as duplicate experiments in square 150-ml bottles with screw caps (Corning Glass Works 1367, Corning, NY, USA). Up to ten bottles were shaken simultaneously at 160 r.p.m. in a water bath at 30 °C. The medium was boiled and cooled under cover immediately before transfer with a sterile pipette to the sterilized flasks. Flasks were inoculated with a freshly grown seed culture, and the inoculation volume was 6% based on total fermentation volume unless otherwise specified. Total fermentation volumes were between 25 and 75 ml, with 75 ml as standard. The decrease in volume due to evaporation was less than 0.5 ml.

Standard aeration conditions during fermentations were achieved by loosening the screw caps on the bottles. Different aeration conditions were achieved by flushing the headspace above the fermentation broth with pure nitrogen or mixtures of nitrogen and air. The flushing arrangement was constructed by placing a rubber septum in the neck of the bottle and passing a thin plastic tube through the septum. Inside the bottle, the plastic tube was connected to a 53-mm long, 1.2-mm i.d. stainless steel syringe tube with its lower opening hanging about 5 mm above the surface of the fermentation broth. In a few experiments, air was bubbled through the fermentation broth to increase oxygen transfer; this was achieved by using a longer plastic tube so that the lower end of the syringe tube was placed just above the bottom of the bottles. The flushing equipment was cleaned with a detergent and rinsed with sterile water immediately before use. Gas flow rates were controlled by needle valves and the oxygen content of the flushing gas was measured by passing the gas through a tube with distilled water in which a polarographic oxygen electrode (Jenway 9070 DO_2-meter, Dunmow, UK) was immersed. A few batch fermentations were carried out in an open 1-L beaker with 750 ml medium shaken at 120 r.p.m. to allow continuous measurement of dissolved oxygen during the first few hours of the fermentation.

Determination of growth

Bacterial growth was followed by drawing 3-ml samples and measuring absorbance (OD) at 600 nm. OD increased linearly with bacterial concentration up to an OD of 0.4. All reported absorbance values are in mOD (OD $\times$ 10^{-3}). The standard deviation on OD between samples was calculated from sixteen, randomly chosen, duplicate samples drawn simultaneously from duplicate aerated fermentations. It was found to be 6 mOD (d.f. = 16) which demonstrates a high reproducibility within each series.

Determination of net acidity

The 3-ml sample was subsequently titrated in the test tube with 0.1 N NaOH (standardized against KH-phthalate) using phenolphthalein as indicator. The end point was defined as the point where the color changed from very pale pink to a distinct pink, corresponding to pH 8.0–8.2. Net acidity was defined as the concentration of titratable acid minus the contribution from the medium itself. The standard deviation of the titration was about 0.05 ml (one drop), corresponding to 1.6–1.7 mM acid.

Determination of acetic acid and ethanol

Acetic acid and ethanol were determined by headspace gas chromatography. *n*-Butanol was added as internal standard, and the sample was saturated with $(NH_4)_2SO_4$ to increase the relative vapor pressure of the volatiles [12]. A 0.80-ml sample of the fermentation broth was transferred to a test tube (13 $\times$ 100 mm) containing $(NH_4)_2SO_4$ in excess (1.1–1.5 g); 0.20 ml of acidic *n*-butanol (0.2 ml *n*-butanol in 100 ml 1.5 M H_2SO_4) was added, and the tube was closed with a rubber septum, shaken and placed in a thermostated heating block at 45 °C for 1.5–2.0 h. A 0.2–0.3-ml sample of the headspace air was drawn with a syringe and immediately injected into a Varian 3400 single-column gas chromatograph with a flame ionization detector and a 4400 Integrator unit. The column was a DB Wax Megabore capillary column (0.53 mm $\times$ 15 m) from JW Scientific, Folsom, CA, USA. The chromatograms were run isothermally at 110 °C and were completed in 2 min. Retention times were approximately 0.29 min (ethanol), 0.43 min (*n*-butanol), and 1.35 min (acetic acid), and all three peaks were narrow and well separated. The detector attenuation was changed from 10^{-10} to 10^{-11} at 0.8 min because of the low relative volatility of acetic acid compared to that of ethanol. To convert integrator areas to mM, a standard solution of 25 mM ethanol + 25 mM acetic acid was also analyzed.

The accuracy and reproducibility of the chromatographic analysis is indicated from the following observations: TYM medium gave rise to one small peak at about 0.24 min, which merged with the ethanol peak at high concentrations of ethanol; its contribution to the area of the ethanol peak was, however, insignificant. Repeated analyses of samples to which known amounts of ethanol and acetic acid had been added gave 100% recovery on the average. The standard deviation for a single analysis was determined from double determinations of different samples and found to be 1.3 mM (d.f. = 13) for ethanol. Similar calculations for acetic acid indicated that the variance was constant at low concentrations of acetic acid but increased considerably with the concentration above 20 mM acetic acid. It was therefore concluded that the analytical variation on the acetic acid determination should be expressed as either a standard deviation of 4.8 mM or a variation coefficient of 20% whichever turned out to be the largest. As will be shown later, this relatively high uncertainty is compensated for in the mass balance calculation.

Determination of dissolved oxygen

Dissolved oxygen was generally determined with a polarographic oxygen electrode (Jenway 9070 DO_2-meter, Dunmow, UK). For the evaluation of the ability of the aeration system to establish and maintain a controlled atmospheric composition of the headspace, the electrode could not be used, and dissolved oxygen was determined chemically by the Winkler method [3]. A 60-ml bottle was used instead of the standard 250–300-ml bottles, and the procedure was scaled down accordingly.

Determination of volumetric oxygen transfer rates

The volumetric oxygen transfer rates, k_1aC^*, where C^* is the partial pressure of O_2 under standard atmospheric conditions (0.21 atm), were determined in the 150-ml bottles by the sulfite method [17]. Instead of TYM medium, the bottles contained 0.5 N Na_2SO_3 and 1 mM $CuSO_4$, and the decrease in SO_3^{2-} with time, caused by the absorption of oxygen, was determined by iodometry. In the 1-L beaker experiments, k_1aC^* was determined by static gassing out, i.e. the (uninoculated) medium was flushed with nitrogen until the concentration of dissolved oxygen was reduced to less than 1 mg L^{-1}. The medium was then allowed to absorb air; the initial slope of dissolved oxygen versus time gives an estimate of k_1aC^*.

RESULTS

Development of TYM medium

The soymilk–cereal blend used in the beverage process contains sucrose (this originates from the soybeans which contain sucrose, stachyose and raffinose as the principal sugars), which complicates the study of the effect of aeration *per se* on the formation of acetic acid, cf. the Introduction. The standard commercial medium used for the initial growth studies [7], Lactobacilli MRS broth, contains acetic acid and also could not be used. Thus, it was decided to develop a medium which did not contain fructose or acetic acid.

As a starting point the MRS medium served as model, except that peptone was replaced by tryptone (a casein hydrolysate), and acetic acid was eliminated. The composition of this initial medium was 15 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 100 mM glucose, 0.05 g L^{-1} $MnSO_4$, and 0.10 g L^{-1} $MgSO_4$. Systematic fermentation experiments (standard aeration conditions), in which the concentrations of the components of the medium were varied, showed that:

1) Under standard conditions, the fermentation proceeded

as rapidly as in the original soymilk–cereal beverage [7], being practically completed in less than 24 h.

2) Elimination of Mg and Mn led to a lower final cell density and net acidity.
3) The concentrations of tryptone and yeast extract could be reduced, but the concomitant reduction in buffer capacity of the medium had to be compensated for. When 50 mM phosphate buffer was incorporated, cell density after 24 h was unaffected when the tryptone was reduced to 10 g L^{-1} and yeast extract to 2 g L^{-1}. Thus, these concentrations of tryptone and yeast extract were chosen as standard.
4) There was a slight negative effect of glucose concentration on growth rate during the first hours of fermentation, but net acidity at the end of the fermentation was only marginally affected in the range 75–300 mM glucose. To ascertain that glucose was never limiting, a standard concentration of 150 mM was chosen.
5) The net acidity after 24 h was proportional to the molarity of the phosphate buffer up to 150 mM which strongly suggested that the medium was limited only by the buffering capacity of the medium. 50 mM phosphate buffer gave approximately the same final net acidity as observed in the original fermented soymilk [7].

Mass balance

The theoretical mass balance of the heterolactic fermentation can be summarized: 1 mole of glucose (G) yields 1 mole of lactic acid (L), 1 mole of CO_2 (C), α moles of acetic acid (A), and $(1-\alpha)$ moles of ethanol (E). In the following, α is denoted as the *acetic/lactic ratio*. Furthermore, for each mole of acetic acid formed, one mole of oxygen is consumed (oxygen is reduced to H_2O_2, cf. the observation that H_2O_2 accumulated in cultures with NADH-oxidase-positive strains [15]). Biomass, which is negligible, is produced from the other nutrients and does not enter the mass balance. The relationship among the concentrations of the metabolites, L, A, and E is:

$$[L] = [A] + [E] \quad (1)$$

When inserted in the definition of α, Eqn (1) gives:

$$\alpha = \frac{[A]}{[A] + [E]} = \frac{1}{1 + [E]/[A]} \quad (2)$$

Both equations were used throughout this work for estimating [L] and α = [A]/[L]. Their validity is based both by the information in the literature [15,18], and by empirical testing, using the measurements of net acidity in the present study.

In the determination of net acidity, both lactic acid (pK 3.08) and acetic acid (pK 4.76) were fully titrated, as demonstrated by titration of known standards of these two acids in the medium. Thus, the contributions to the net acidity from these acids can be regarded as quantitative, i.e. net acidity = [L] + [A]. Inserting Eqn (1) gives:

$$\text{Net acidity} = [E] + 2[A] \quad (3)$$

There may, however, also be a contribution to the net acidity from CO_2, and the magnitude of this is estimated theoretically as follows: the pK value of carbonic acid is 6.37 which is below the pH value at the end-point, and CO_2 should, in principle, also contribute significantly to the net acidity. However, during the fermentation, pH drops from about 6.8 to around 4, and at these low pH values, the equilibrium between HCO_3^- and CO_2 is shifted entirely towards the latter. The resulting contribution to the net acidity from the bicarbonate formed during the fermentation is thus determined by the solubility of CO_2 at the fermentation temperature (30 °C) which is 0.126 g per 100 g at a partial pressure of 1 atm. This corresponds to 29 mM, and any CO_2 in excess of that will rapidly disappear. Although the partial pressure of CO_2 in the atmosphere is only 0.0003 atm, the remaining CO_2 will be released quite slowly and at an unknown rate, because CO_2 bubbles can only form when the partial pressure is above 1 atm. Below that value, the rate of CO_2 transfer will be determined by the gas–liquid interphase resistance, just as the transfer of O_2. In the present study, all fermentations yielded more than 29 mM, and since the fermentation times are approximately the same, it can be assumed that under the present circumstances, CO_2 may contribute to the net acidity with a fairly *constant* value anywhere between 0 and 29 mM. Thus, Eqn (3) is modified to:

$$\text{Net acidity} = [E] + 2[A] + C \quad (4)$$

where C is the constant contribution from CO_2.

Figure 2 shows a plot of net acidity against [E] + 2[A] as a test of this mass balance equation, and it is obeyed. The slope of the regression line is not significantly different

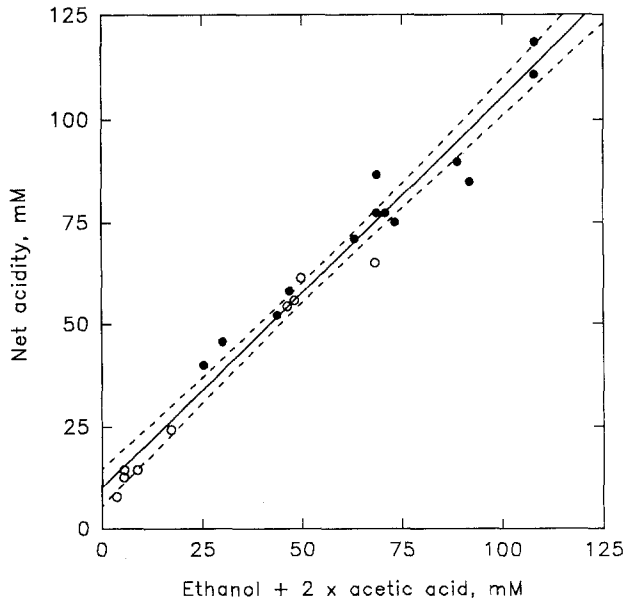

Fig. 2. Verification of the mass balance. (○) Oxygen-free; (●) aerated fermentations. Slope of regression line (solid line) = 0.95, intercept = 10 mM, r = 0.986. Dashed lines indicate the 95% confidence interval around the regression line.

from one, and the line intersects close to (0,0). The intercept of 10 mM is, however, significantly different from zero, and this may be explained as the contribution from CO_2, as argued above.

Although the intercept value lies within the range yielded by the theoretical calculation, the possible role of CO_2 is, of course, not proven by these experiments. The verification of the mass balance equation is the basis for calculating the acetic/lactic ratio from the analyses of ethanol and acetic acid.

Determination of oxygen transfer rates

Volumetric oxygen transfer rates, k_1aC^*, were determined for three different fermentation volumes, 25 ml, 50 ml, and 75 ml (Fig. 3). The k_1aC^* values were all lower than in typical aerobic shake-flask fermentations [6]. In separate experiments, the oxygen transfer rate was measured under bubbling conditions. The results varied, however, much more than the results from headspace aerations, which was due to the obvious difficulties in reproducing the exact bubbling conditions from series to series. In other experiments, both higher and lower oxygen transfer rates were measured for bubbling. Finally, the determination of k_1aC^* in the scaled-up 1-L beaker experiments (fermentation volume = 750 ml) showed that the oxygen transfer rates of the medium and that of water were similar (Fig. 4), thereby validating the rate transfer measurements by the sulfite method. The oxygen transfer rate in the scaled-up experiment was only one-eighth of that of the standard 75-ml aerated fermentation; this demonstrates the pronounced scale-up effect for headspace aeration which is typical for lactic fermentations in the food industry.

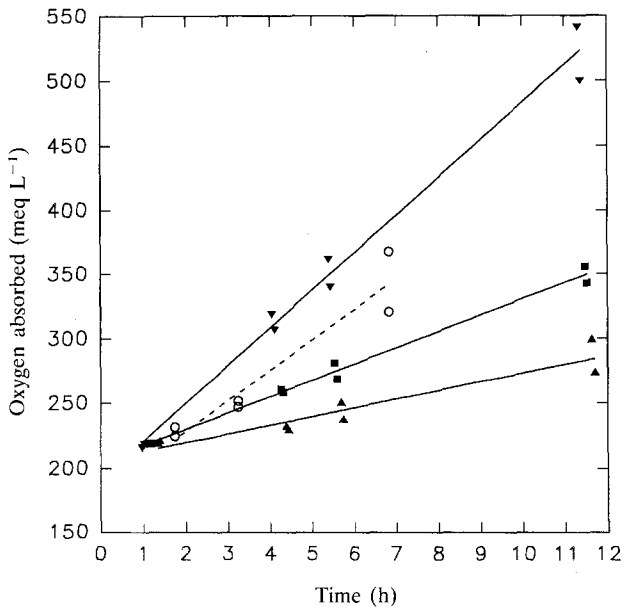

Fig. 3. Determination of the volumetric oxygen transfer rate, k_1aC^*, in the square culture flasks. (▼) 25 ml medium volume; (■) 50 ml; (▲) 75 ml; (○) 75 ml with bubbling. The k_1aC^* values in meq L^{-1} h^{-1} are: 29.2 (25 ml); 12.6 (50 ml); 6.66 (75 ml); 23.5 (75 ml, bubbling). C^* refers to standard atmosphere (0.21 atm O_2).

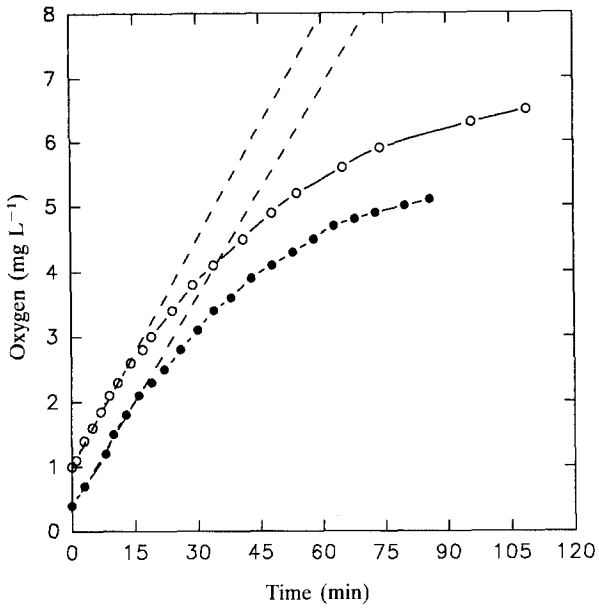

Fig. 4. Determination of the volumetric oxygen transfer rate, k_1aC^*, in the 750 ml experiments. (○) Water; (●) TYM medium. The k_1aC^* values in meq L^{-1} h^{-1} are: 0.87 (water); 0.81 (TYM medium). C^* refers to standard atmosphere (0.21 atm O_2).

In order to establish that the aeration system was also efficient for establishing a volumetric oxygen transfer rate of zero, distilled water was flushed with nitrogen for 1 h and dissolved oxygen was measured by the Winkler method. The concentration was found to be 0.3–0.5 mg L^{-1}, compared to the aerated controls which contained 6.9 mg L^{-1}. This sets an extreme upper value of the volumetric oxygen transfer rate during conditions of nitrogen flushing to about 5% of the standard aeration conditions, which is entirely satisfactory.

Kinetics of growth and stoichiometry of product formation

Figure 5 shows the changes in absorbance, net acidity, acetic acid concentration, and lactic acid concentration (calculated using Eqn (1)) during a typical fermentation experiment (75 ml fermentation volume). Growth and lactic acid formation were much slower under oxygen-free conditions than when the fermentations were aerated. This was also observed in a previous study [15]. However, towards the end of the fermentation, the growth rate accelerated, and the final concentration of lactic acid was not much below that of the aerated fermentations. These observations were confirmed repeatedly. There was no difference between the increase in absorbance nor the increase in lactic acid between the two aeration conditions indicating that even a little aeration is enough to stimulate the growth of the organism. The effect is evidently of great technical significance, even in cases when the formation of acetic acid *per se* is not of importance.

Figure 5 also shows that no acetic acid was formed in the oxygen-free fermentation while it was formed when the fermentation was aerated. Under standard aeration

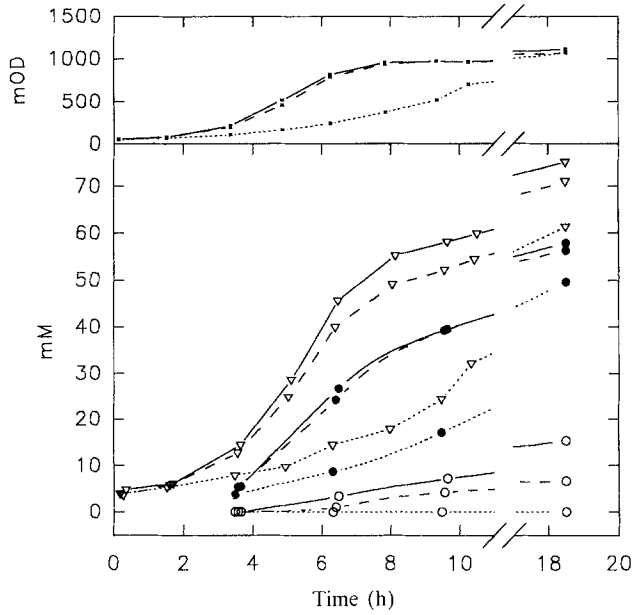

Fig. 5. Growth and product formation under different aeration atmospheres. Standard TYM medium, 75 ml. Upper graph shows absorbance, lower graph formation of acid. (----) 0% air; (– – –) 40% air; (——) 100% air. (○) acetic acid; (●) lactic acid; and (▽) net acidity.

conditions, the final acetic acid concentration corresponded to an acetic/lactic ratio of about 0.25. The experiment in which the fermentation was aerated with a rarified atmosphere (40% air) gave a rate of acetic acid formation which was correspondingly lower. This indicated but did not prove that acetic acid formation was controlled by the transfer of oxygen.

Table 1 summarizes the main results from all the fermentation experiments. Acetic acid concentration increased with increasing aeration, regardless of how the change in aeration condition was achieved, i.e. by the oxygen content of the atmosphere, by the fermentation volume, or by bubbling. In all the oxygen-free fermentations, lactic acid was somewhat lower than in the aerated fermentations. With increasing aeration rate, lactic acid increased only slightly.

The final pH values were all in the range reported as the limiting pH value for termination of growth of *L. mesenteroides* in media containing lactic acid and acetic acid [16]. This is a further confirmation that the fermentations were limited by the buffer capacity and not by any nutrients.

The lower section of Table 1 contains data for fermentation experiments in which fructose was added. As mentioned, for two moles of fructose reduced, one mole of acetic acid is formed. The data show that this stoichiometric ratio was obeyed for both the 25 mM and 50 mM experiments. The experiments with 150 mM fructose gave close to an acetic/lactic ratio of 1.0 and a final concentration of acetic acid a little below the theoretical value of 75 mM, indicating that at this concentration fructose was no longer a limiting factor for the formation of acetic acid.

Statistical analysis of the fermentation data

From the eight aerated duplicate experiments plus the experiment with 150 mM fructose (Table 1), the following standard deviations (all with d.f. = 9) were calculated: 2.1 mM (net acidity) and 2.3 mM (ethanol); for acetic acid the variation coefficient was 8.7%. The first two are larger than the standard deviation of the analyses, reflecting the contribution from batch to batch variation while the variation coefficient for acetic acid was lower than expected for a duplicate analysis (= 14%), but still higher than the variation coefficient of 4–5% which could be calculated for typical values of ethanol. Fortunately, because [A] appears both in the numerator and denominator of Eqn (2), the statistical variation on [A] is partly self-compensating when calculating the standard deviation of α by differentiation of Eqn (2). The details of this will not be given here, but it turns out that for typical values of [A] and [E], the variation coefficient of [A]/[E] is about 10% for a single experiment, and thus 7% for a duplicate experiment. Using this value in Eqn (2), the standard deviation of α is calculated to 0.035 for α = 0.5, where the standard deviation has its theoretical maximum. This is considered satisfactory.

Oxygen concentration during fermentation

The oxygen concentration during a fermentation under standard aerated conditions decreased to zero after 1 h and remained there (Fig. 6); similar results were found in other experiments. Thus, except for the initial fermentation period, the oxygen concentration was zero during the fermentations. These observations were supplemented with studies on a larger scale where dissolved oxygen could be measured continuously and more accurately. It appears (Fig. 6) that the larger the inoculation volume, the shorter was the period before the oxygen level reached zero (actually 0.1 mg L^{-1}). Furthermore, no significant decrease was observed in an uninoculated medium, while the slopes of the portion of the curves between 0.5 and 3.5 mg L^{-1} were found to increase numerically with the inoculation volume (values reported in legend to Fig. 6). This demonstrates that the bacterial culture was the cause of the rapid depletion of free oxygen in the fermentation medium.

Theoretically, in standard aeration experiments, where 8–15 mM acetic acid is formed, a maximum of 15 mM or 60 meq L^{-1} oxygen would be consumed. With a volumetric oxygen transfer rate of 6.7 meq L^{-1} h^{-1}, this amount would be transferred in 9 h which means that on the average, a similar amount of oxygen must be consumed through other reducing reactions. During fastest growth, however, 4 mM acetic acid was formed in 3 h (Fig. 5); this corresponds to a theoretical oxygen transfer rate of 5.3 meq L^{-1} h^{-1}, which is close to the measured value of 6.7 meq L^{-1} h^{-1}. The calculation indicates that at least during the fastest growth phase, the amount of acetic acid formed is quantitatively limited by the oxygen supply.

DISCUSSION

Lactic acid fermentations are usually considered anaerobic and with good reason, because the transfer of oxygen by

TABLE 1

Effect of aeration or fructose addition on product composition at end of fermentation

Aeration conditions	k_1aC^* meq L^{-1} h^{-1}	Time (h)	Measured values for duplicate experiments								Averages	
			Final pH		Net acidity		Ethanol		Acetic acid		L	α
					mM	mM	mM	mM	mM	mM	mM	
0% air, headspace	0.0	18.5	4.01		61		50		0		50	0.00
40% air, headspace	2.7	18.5	3.95	3.95	72	70	48	51	8	6	56	0.12
100% air, headspace	6.7	18.5	3.94	3.93	74	76	45	41	15	16	58	0.26
0% air, headspace	0.0	21.8	4.24		56		48		0		48	0.00
25% air, headspace	1.7	21.8	3.96	3.97	81	74	62	58	3	7	65	0.07
100% air, headspace	6.7	21.8	3.92	3.92	87	87	52	53	8	8	61	0.14
100% air, bubbling	23.5	21.8	3.83	3.82	116	121	28	22	39	44	67	0.62
0% air, headspace	0.0	18.8	4.12	4.33	60	49	52	41	0	0	46	0.00
100% air, do., 75 ml	6.7	18.8	3.96	3.96	77	78	53	56	8	8	63	0.13
100% air, do., 50 ml	12.6	18.8	3.94	3.94	85	85	45	51	20	24	70	0.31
100% air, do., 25 ml	29.2	18.8	3.88	3.86	109	113	23	19	57	47	72	0.71
0% air, headspace:												
25 mM fructose		17.3	3.94		91		31		16		47	0.33
50 mM fructose		17.3	3.95		97		23		28		50	0.55
150 mM fructose		18.5	3.75	3.75	133	134	5	4	71	65	72	0.94

L = lactic acid concentration, calculated as the average sum of ethanol and acetic acid concentrations. α = A/L = acetic/lactic ratio. In the first two series, the aeration system failed for one of the flasks aerated with pure nitrogen.

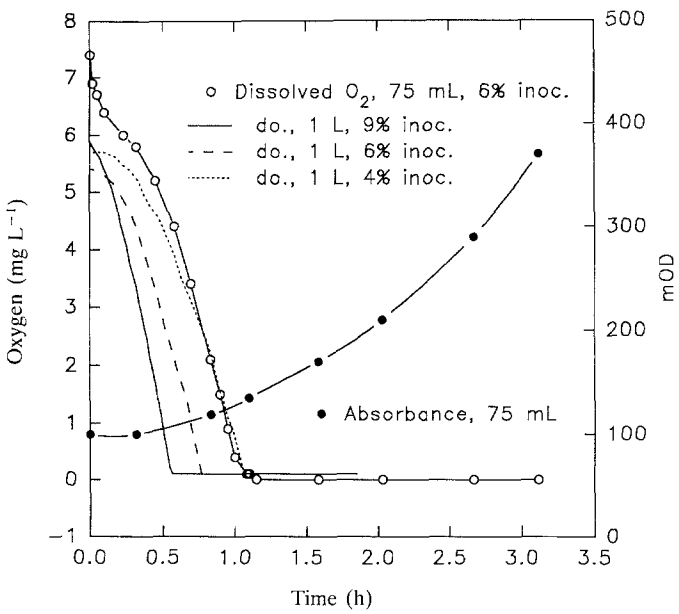

Fig. 6. Initial drop in dissolved oxygen concentration. Symbols refer to 75 ml medium volume (6% inoc. vol.), lines with no symbols to 750 ml medium volume. (○) Dissolved O_2; (●) absorbance. (----) 4% inoc. vol.; (– – –) 6% inoc. vol.; (——) 9% inoc. vol. The slopes of the 750 ml experiments are: −0.97 meq L^{-1} h^{-1} (4%); −1.25 meq L^{-1} h^{-1} (6%); −1.75 meq L^{-1} h^{-1} (9% inoc. vol.). In a control (uninoculated medium), the dissolved oxygen concentration remained constant, around 6 mg L^{-1}.

head space aeration to an industrial size reactor is likely to be insignificant. Lactic acid bacteria rapidly remove oxygen from the fermentation medium [9], giving further support for considering the fermentations anaerobic.

The present study has shown that aeration of a fermentation with a heterolactic organism, *L. mesenteroides*, has a profound influence on the growth rate and product formation, even though the concentration of dissolved oxygen is maintained close to zero. The study not only confirms previously found qualitative effects, i.e. faster growth and the formation of acetic acid instead of ethanol, but also demonstrates that the formation of acetic acid is quantitatively controlled by the volumetric oxygen transfer rate, k_1aC^*. Since the lactic acid concentrations in all the aerated experiments were at approximately the same level, it follows that the acetic/lactic ratio should be actively controlled by k_1aC^*. Figure 7 shows that this is the case, because a linear relationship between k_1aC^* and the acetic/lactic ratio for all the aerated experiments can be established (data taken from Table 1).

The study has also indicated that the final concentration of acetic acid could be controlled by the addition of fructose. However, apart from the fact that this may prove prohibitively costly, the formation of two moles of mannitol for each mole of acetic acid is a consequence which must be considered. For example, a final concentration of acetic acid of 30 mM (0.18%) corresponds to a mannitol concentration of 60 mM, or about 1.1%, which is not negligible in a beverage formulation containing typically 10% dry matter.

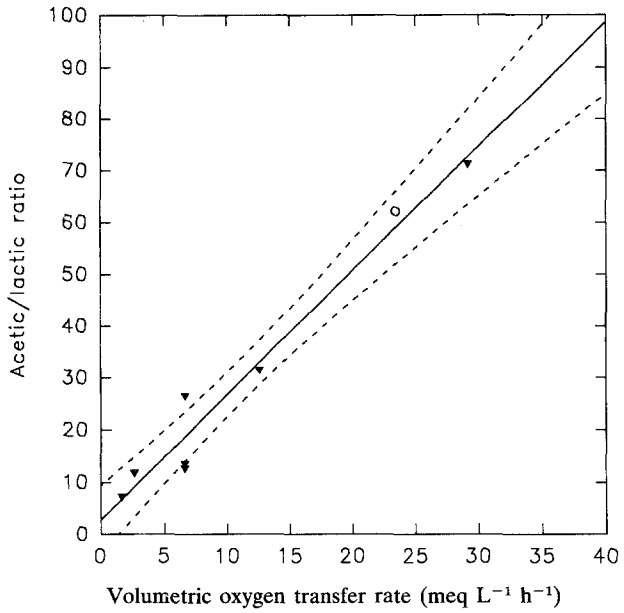

Fig. 7. Correlation between volumetric oxygen transfer rate, k_1aC^*, and acetic/lactic ratio. (▼) Head space aeration; (○) bubbling. r = 0.982. Dashed lines indicate the 95% confidence interval around the regression line (solid line).

The addition of sucrose may be more realistic economically, but the issue of mannitol formation is the same, and the presence of sucrose in the medium may lead *L. mesenteroides* to produce dextran [21] which would alter the rheological properties of the product drastically.

Determination of the acetic/lactic ratio is in principle based only on the gas chromatographic analysis of ethanol and acetic acid, the two metabolites which most directly reflect the extent to which the organism makes use of O_2 for regenerating NAD^+. For the determination of ethanol, the method is undoubtedly unsurpassed in sensitivity and accuracy, while the sensitivity of the determination of acetic acid is about the same as by HPLC [7].

The gas chromatographic method offers certain advantages over other methods, notably HPLC, for studying heterolactic fermentations under pilot plant and industrial conditions, because sampling is easy and uncomplicated and no sample preparation is required. The further reaction is immediately arrested by the lowering of pH so that the sample tubes can be kept and analyzed afterwards. The method without modifications can be directly applied to foods and food emulsions, such as soymilk. It is important that both ethanol and acetic acid are hydrophilic, because the relative volatility of hydrophilic compounds, such as ethanol, is the same in water and in an emulsion, while a hydrophobic volatile, such as *n*-hexanal, is strongly decreased in relative volatility when emulsified oil is present [1].

For scale-up of the fermentations, head space aeration is not feasible, as demonstrated by the very modest scale-up from 75 ml to 750 ml; therefore, a practical system for control of the oxygen transfer in industrial scale will have to be devised. The desired range of k_1aC^* values is, however, considerably below that needed in aerobic, non-food fermentations, and the demands with respect to a high transfer coefficient of the aeration system are much less severe. The use of the highly oxygen-permeable silicone tubing, which is often used for aerating mammalian cell cultures, might be an option which combines a simple control mechanism with the demands for proper process hygiene.

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