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Influence of acetate and CO_2 on the TMAO-reduction reaction by *Shewanella baltica*

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Abstract

In this work, the TMAO-reduction by *Shewanella baltica*, one of the representative spoilage organisms in modified atmosphere packaged marine fish fillets, and the effect of acetate and CO_2 on this reduction were studied in vitro. The growth of *S. baltica* and the corresponding evolution of some compounds (acetate, lactate, pyruvate, glucose and trimethylamine (TMA)) were followed during storage at 4°C in two types of broths. The first medium was a defined medium (pH = 6.8) to which lactate or pyruvate was added as hydrogen donor. Pyruvate showed to be more efficient as H-donor for *S. baltica* than lactate, as growth was much faster when equimolar amounts of pyruvate instead of lactate were present. Although the growth of *S. baltica*, when pyruvate is used as H-donor and no acetate is added, was not much inhibited by the CO_2-atmosphere, CO_2 had a pronounced effect on the studied reactions as it partly inhibited the reduction of pyruvate to acetate. The effect of acetate on this reaction was, on the other hand, not significant.

To simulate the reactions occurring in situ, a buffered fish extract (pH = 6.8) was used. In spite of the neutral pH, the growth of *S. baltica* in this medium was highly inhibited by relatively small concentrations of acetate (< 0.3%). When 0.1% of acetate was added to the fish extract, less acetate was formed and lactate was more slowly consumed in comparison to the experiments without the addition of acetate. The consumption of lactate and the production of acetate were almost completely inhibited when the fish extract contained 0.25% of acetate. Apparently, the addition of acetate inhibited the use of lactate as H-donor. After extended storage times (17 days at 4°C) TMA production started. Most probably, alternative H-donors were used by *S. baltica*, from which the pathway seems to be less energy efficient. This can be deduced from the exceptional growth inhibition of *S. baltica* by small amounts of acetate. However, when practical storage times for fish (e.g. 6 days at 4°C after packaging) are considered, growth and TMAO-reduction by *S. baltica* was completely inhibited during this period by 0.25% of acetate.

Keywords: Fish; *Shewanella baltica*; TMAO; TMA; Acetate; Modified atmosphere packaging

1. Introduction

Spoilage of fish is mainly due to microbial processes. In marine fish, trimethylamine (TMA) is the main component responsible for an unpleasant 'fishy' odour (Dainty, 1996). When oxygen levels are de-

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pleted, TMAO serves as a terminal electron acceptor for anaerobic respiration and is reduced to TMA (Easter et al., 1983). TMAO-respiration is of importance to bacterial growth during spoilage of marine fish, but few details are known about substrate preferences, catabolic processes and energy conservation during TMAO-dependent anaerobic growth of specific spoilage organisms. Carbon sources utilized by *Shewanella* species grown on LB-broth or a minimal salts medium at 20–30°C include glucose, lactate, pyruvate, propionate, ethanol, acetate, formate and a number of carboxylic and amino acids (Scott and Nealson, 1994). Lactate has been suggested to be the natural H-donor for TMAO-reduction during fish spoilage because its concentration is high (about 25 mM) in fish muscle, and it disappears as TMAO is reduced (StrØm and Larsen, 1979, StrØm et al., 1979). The reduction of TMAO in the presence of lactate by *Shewanella* species was summarized as follows (Ruiter, 1971):

$$CH_3CHOHCOOH + (CH_3)_3NO \rightarrow CH_3COCOOH + (CH_3)_3N + H_2O \quad (1)$$

The pyruvate is oxidized according to:

$$CH_3COCOOH + (CH_3)_3NO \rightarrow CH_3COOH + (CH_3)_3N + CO_2 \quad (2)$$

The overall reaction can be written as follows:

$$CH_3CHOHCOOH + 2(CH_3)_3NO \rightarrow CH_3COOH + 2(CH_3)_3N + H_2O + CO_2 \quad (3)$$

Since acetate has been proposed as an end product during the TMAO-dependent respiration of specific spoilage bacteria such as *Shewanella* species (Ruiter, 1971; RingØ et al., 1984), see Venkateswaran et al. (1999) for the taxonomy of the genus *Shewanella*, the addition of acetate in the fish tissue can be expected to inhibit the TMAO-reduction by bacterial trimethylamine oxide reductases. Next to an inhibition of H_2S-producing bacteria, Boskou and Debevere (2000) noticed that the production of total volatile bases and TMA was, indeed, inhibited by a treatment with an acetic acid/Na-acetate buffer (10% (v/v)-spray pH 5.6 of fresh cod fillets (*Gadus morhua*) stored at 7°C, under modified atmosphere. The treatment resulted in a pH-drop from 6.63 to 6.29 and a total volatile acidity in the treated cod fillets of 260 mg acetic acid/100 g). However, the shelf life extending effect on fish of the applied acetic acid/acetate buffer solution could, next to its product inhibitory effect on the TMAO-reduction reaction, possibly also be explained by the known anti-microbial effect of acetate by acidification of the cytoplasma, causing protein denaturation and energy loss due to the activation of ATP-dependent proton pumps located in the cell membrane (Marshall et al., 2000). Therefore, the aim of this work was to study in vitro the biochemical reactions involving the TMAO-reduction by *Shewanella baltica* and the influence of acetate and CO_2 on this reaction. In a defined medium, the effect of CO_2 and acetate on reactions (2) and (3) were investigated separately. The second part of the experiment was performed in a fish extract to better simulate the natural environment of the microorganism.

2. Materials and methods

2.1. Strain

The applied strain was isolated from refrigerated ($T = 4$°C) modified atmosphere packaged (60% CO_2–30% O_2–10% N_2) cod fillets at the end of the shelf life and was previously called *Shewanella*-like (Boskou and Debevere, 1997). Later, the strain was identified by LMG as *S. baltica* using the following reference strains: *S. baltica* NCTC 10735^T (Ziemke et al., 1998) and *S. baltica* LMG 2250^T. The strain was chosen as it was representative for the spoilage flora of modified atmosphere packaged fresh marine fish stored at 4°C (Boskou and Debevere, 1997). The strain was stored on Marine Agar (37.4 g/l Marine Broth 2216, DIFCO 0791-17 and 15 g/l Agar no. 1, OXOID L11) slants at 6°C which were renewed every month.

2.2. Experimental set-up

The growth of *S. baltica* and the corresponding evolution of acetate, lactate, pyruvate, glucose and trimethylamine (TMA) was followed during storage at 4°C in two types of inoculated broths.

The first medium was a defined medium based on that described by Easter et al. (1983) and Graham and Ward (1988). It contained 5 g/l bacteriological peptone (OXOID, L34), 2.5 g/l D-(+)-glucose (SIGMA), 7.2 g/l TMAO $2H_2O$ (SIGMA), 20 g/l NaCl (VEL), 1 g/l K_2HPO_4 (SIGMA), 1 g/l $MgSO_4 \cdot 7H_2O$ (SIGMA) and 1 g/l NH_4Cl (SIGMA). To this medium, 60 mM of lactate (SIGMA) or 65 mM pyruvate (SIGMA) was added as hydrogen donor. For each hydrogen donor, experiments were performed with three different concentrations (v/v) of acetate (0%, 0.5% and 1.0%) and in two gas atmospheres (100% of N_2 or 50% of N_2 and 50% of CO_2). 50% of CO_2, combined with a headspace of 1/1, resulted in 253 mg/l and 263 mg/l dissolved CO_2 in the defined medium when lactate or pyruvate were, respectively, used as H-donor. After the addition of the appropriate amount of acetate, the pH was adjusted to 6.8 by means of 10 N NaOH solution (VEL).

To simulate the reactions occurring in situ, a fish extract was used as the second liquid medium. It was prepared as previously described by Boskou and Debevere (1997). The TMAO-content was, after determination of the initial content, adjusted to a level of 80 mg TMAO-N/100 ml by the addition of $TMAO \cdot 2H_2O$ (SIGMA). Additionally, 7 g/l KH_2PO_4 (SIGMA) and 7 g/l K_2HPO_4 (SIGMA) were added to buffer the medium (Dalgaard, 1995). To estimate the effect of acetate on the growth of *S. baltica* and its production/consumption of the abovementioned chemical compounds, experiments were performed with 0%, 0.1%, 0.25% and 0.5% of added acetate (SIGMA). After the addition of the appropriate amount of acetate, the pH of the medium was also adjusted to 6.8 by means of 10 N NaOH solution (VEL).

Experiments were performed in 600-ml jars provided with a Teflon® valve and a central opening which was closed with a silicone septum (Devlieghere et al. 1998). The glass jars were filled with 300 ml of liquid medium (resulting in a gas/product volume ratio of 1) and autoclaved at 121°C for 15 min. The pH was adjusted to 6.8 after autoclaving with filter sterilised 2 N NaOH or 2 N HCl.

2.3. Inoculation procedure and sampling

The *S. baltica* strain was subcultured in Marine broth 2216 (Difco) for 48 h at 30°C. A second subculture in Marine broth (Difco) (0.1 ml in 10 ml) was incubated for 16 h at 30°C. The inoculum was then stored for 8 h at 4°C to allow the test strain to adapt to the chilling temperature. After the adaptation period, the sterilised glass jars, filled with previously cooled (4°C) liquid medium, were inoculated with *S. baltica* to a level of 10^6 cfu/ml. After inoculation, the jars were immediately gas packaged (100% of N_2 or 50% of N_2 + 50% of CO_2) as described by Devlieghere et al. (1998) and stored at 4°C. To follow the evolution of the chemical and microbial parameters in the broth during time, 4-ml samples were taken at regular time intervals by means of sterile disposable 10-ml syringes.

2.4. Microbial determination

The evolution of the number of *S. baltica* (cfu/ml) was followed during time in the broth, by diluting the samples, if necessary, with Peptone Physiological Salt solution (0.1% peptone, 0.85% NaCl) and plating them in duplicate on Marine Agar with a Spiral Plater (Model D, Spiral Systems, CT, USA). The plates were aerobically incubated for 2 days at 30°C.

2.5. Chemical determinations

Trimethylamine-N (TMA-N) content was determined in duplicate with the spectrophotometric method (Dyer, 1945) as modified by Boskou and Debevere (2000).

The pH was measured in duplicate with an Ingold sharp point electrode and a knick pH-meter.

Concentrations of glucose, acetate, pyruvate and lactate in different media were determined by means of Ion Moderated Partion High Performance Liquid Chromatography (Fernandez-Garcia and McGregor, 1994). Samples were previously heated for 15 min at 80°C and centrifuged (Eppendorf Centrifuge 5415 C) for 10 min at 10,000 rpm. The supernatant was

filtered through a 0.22 μm Millex (Millipore) filter before injection. The HPLC apparatus consisted of a HPLC pump (GILSON 307), a precolumn (Biorad), a separation column (Aminex HPX-87H, Bio-rad) which was thermostatically regulated at 35°C, a Refractive Index detector (GILSON 132) and an integrator (Shimadzu C-R6A chromatopac). The mobile phase was filtered (22 μm) and deaerated 5 mM H_2SO_4 at a flow rate of 0.5 ml/min. Retention times for acetate, glucose, pyruvate and lactate were, 14.7, 8.7, 9.6 and 12.2 min, respectively. Standard curves were derived for every compound that was used for the calculation of the effective concentrations. All analyses were performed in duplicate.

To estimate the precision on the chemical determinations, a sample was independently analysed in duplicate six times. The standard deviation of the chemical determinations amounted to 1.8% for acetate, 3.0% for pyruvate, 6.7% for lactate, 5.9% for glucose and 9.6% for TMA.

3. Results and discussion

3.1. Experiments in the defined medium

Fig. 1 illustrates the effect of CO_2 and acetate on the growth of *S. baltica* at 4°C in defined media

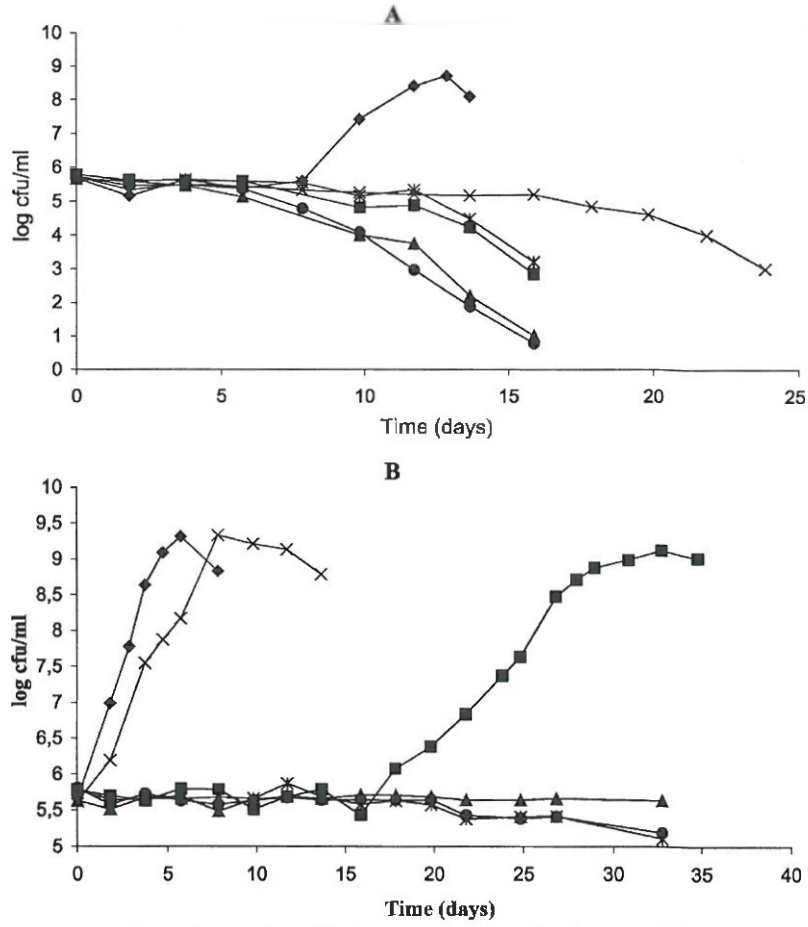

Fig. 1. Influence of acetate concentration and atmosphere (◆ 100% of N_2 and 0% (v/v) acetate, ■ 100% of N_2 and 0.5% (v/v) acetate, ▲ 100% of N_2 and 1% (v/v) acetate, × 50% N_2 + 50% of CO_2 and 0% (v/v) acetate, * 50% N_2 + 50% of CO_2 and 0.5% (v/v) acetate, ● 50% N_2 + 50% of CO_2 and 1% (v/v) acetate) on the growth of *S. baltica* at 4°C in a defined medium (pH = 6.8) with lactate (A) or pyruvate (B) as H-donor.

(pH = 6.8) containing lactate (Fig. 1(A)) or pyruvate (Fig. 1(B)) as H-donor. Pyruvate appeared to be more efficient as H-donor for *S. baltica* than lactate, as growth was much faster when equimolar amounts of pyruvate instead of lactate were present as H-donor. Possibly, this difference in growth rate could also be explained by the anti-microbial effect of lactate, which is, however, very limited at the experimental pH of 6.8. When lactate was present as H-donor, a gas concentration of 50% of CO_2 or the addition of acetate (0.5% or 1.0%) completely inhibited the growth of *S. baltica* for 25 days. This was not the case in the medium containing pyruvate. However, when 1% of acetate was present, no growth was detected within 33 days. The growth-inhibiting effect of acetate and CO_2 on *Shewanella* species was already previously described by Boskou and Debevere (2000).

The evolution of the concentration of TMA-N, acetate, lactate, glucose and pyruvate at experimental conditions where growth of *S. baltica* occurred is shown in Fig. 2. When no acetate or CO_2 was added, and when lactate was added as a H-donor (Fig. 2(A)), 1 mole of lactate was consumed, resulting in 2 moles of TMA and 0.65 moles of acetate. RingØ et al. (1984) also described only a partial accumulation (40 mol%) of acetate when lactate was used by *S. putrefaciens* as substrate. When pyruvate was added as H-donor (Fig. 2(B)), the reaction was more symmetric (1 mole of pyruvate and TMAO reacted, resulting in 1 mole of TMA and acetate). At the same time, small amounts of lactate were accumu-

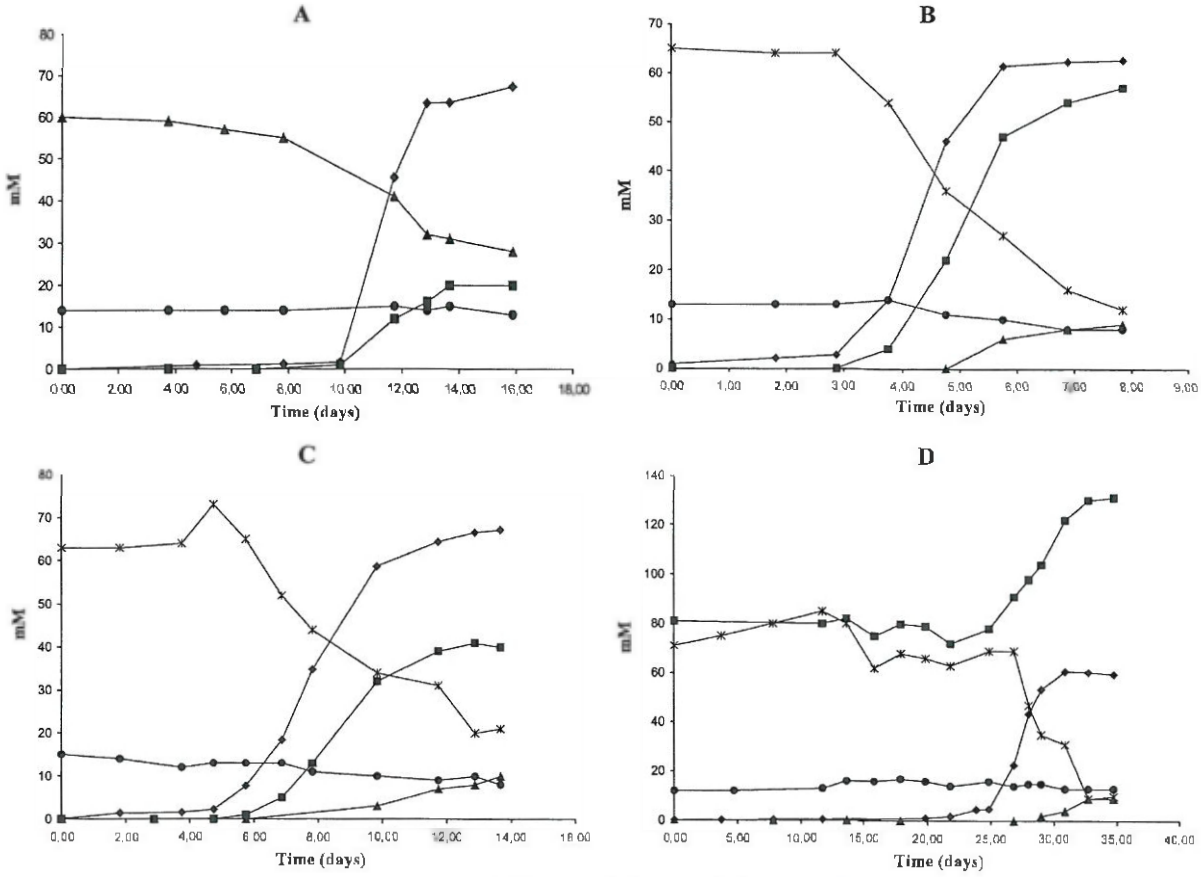

Fig. 2. Evolution of the concentrations (mM) of TMA-N (♦), acetate (■), lactate (▲), glucose (●) and pyruvate (*) in a defined medium at 4°C inoculated with *S. baltica* in 100% of N_2 and 0% (v/v) acetate and lactate as H-donor (A), 100% of N_2 and 0% (v/v) acetate and pyruvate as H-donor (B), 50% N_2 + 50% CO_2 and 0% (v/v) acetate and pyruvate as H-donor (C), 100% of N_2 and 0.5% (v/v) acetate and pyruvate as H-donor (D).

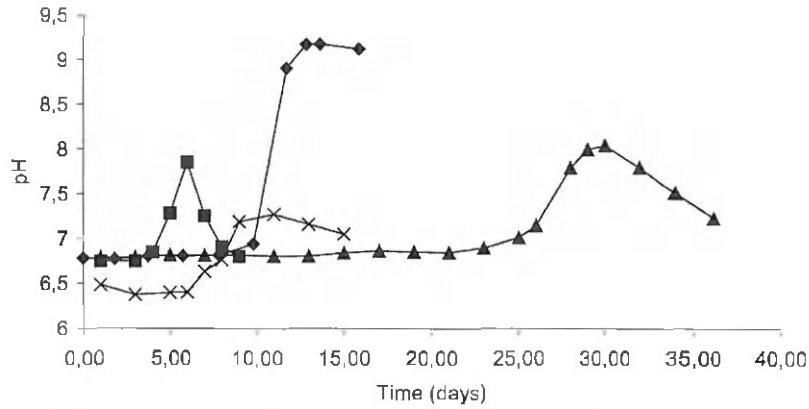

Fig. 3. Influence of acetate concentration and atmosphere (◆ 100% of N_2, 0% (v/v) acetate and lactate as H-donor, ■ 100% of N_2, 0% (v/v) acetate, and pyruvate as H-donor, ▲ 100% of N_2, 0.5% (v/v) acetate and pyruvate as H-donor, × 50% N_2 + 50% of CO_2, 0% (v/v) acetate and pyruvate as H-donor) on the pH evolution at 4°C in a defined medium (pH = 6.8) inoculated with *S. baltica*.

lated (9 mM) probably because of a fermentative metabolism from glucose (5 mM of consumption). This accumulation also illustrates that pyruvate will be preferred over lactate as H-donor by *S. baltica*.

Although the growth of *S. baltica*, with pyruvate as H-donor and no acetate added, was not much inhibited by the CO_2 atmosphere, CO_2 had a pronounced effect on the studied reactions (Fig. 2(C)). CO_2 partly inhibited reaction (2), as at the end of the experiment, only 40 mM of acetate was produced (instead of 58 mM, when no CO_2 was added) and 41 mM of pyruvate was consumed. However, under this condition, H-donors other than pyruvate were apparently used, because similar amounts of TMA were produced (68 mM) than when no CO_2 was present in the headspace. Bacteriological peptone, a source for easily convertible amino acids, can indeed also function as H-donor for the TMAO-reduction reaction, as described by RingØ et al. (1984) for *S. putrefaciens* and by Easter et al. (1982) for *Shewanella* spp. Acetate greatly delayed reaction (2) as the start of the TMA production was postponed from day 3 to day 25 (Fig. 2(D)). However, at the end of the reaction, similar amounts of TMA and acetate were produced as when no acetate was added (Fig. 2(B)). For all tested conditions, the pH started to increase at the moment TMA production was noticed while at the end, the pH decreased (Fig. 3). The pH drop at

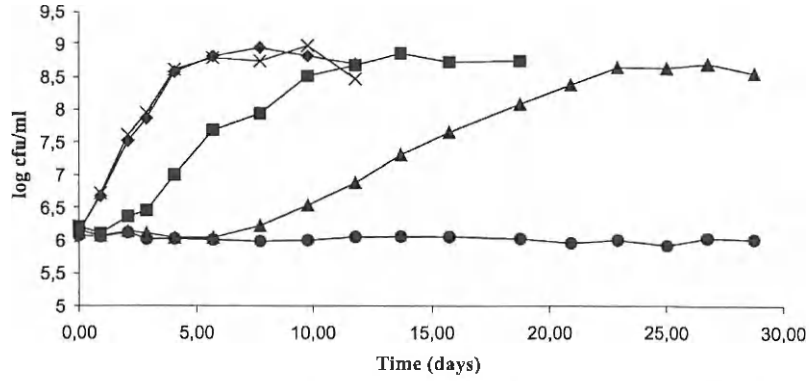

Fig. 4. Influence of acetate concentration and atmosphere (◆ 100% of N_2 and 0% (v/v) acetate, ■ 100% of N_2 and 0.1% (v/v) acetate, ▲ 100% of N_2 and 0.25% (v/v) acetate, × air and 0% (v/v) acetate, ● 100% of N_2 and 0.5% (v/v) acetate) on the growth of *S. baltica* at 4°C in a buffered fish extract (pH = 6.8).

the end of the reaction can be explained by the significant lactate production by *S. baltica* at the end of the experiments.

3.2. Experiments in fish extract

Previous investigations (Kim et al., 1995; Boskou and Debevere, 2000) have demonstrated that dipping in an acetate solution resulted in an extension of the shelf life of refrigerated fish fillets. To investigate the effect of an acetate treatment on the TMAO-reduction reaction by *S. baltica* in fish, experiments were performed in a fish extract. The growth of *S. baltica* in 100% N_2 was highly inhibited by relatively small amounts of acetate (Fig. 4). The time to realize a two log increase at 4°C was prolonged from 3 to 8 days, and 16 days by the addition of 0.1% and 0.25%, respectively, of acetate. At concentrations of 0.5% of acetate, no growth was observed at 4°C within 29 days. At a neutral pH, which is the case in this study (pH = 6.8), the anti-microbial effect of acetate (pK = 4.75) is normally very low due to the low fraction of undissociated molecules. At pH = 6.8 the ratio of undissociated to total acid is 0.0084. It is therefore remarkable that the growth-inhibiting effect is that pronounced at such low concentrations of acetate.

When no acetate was added, using lactate as H-donor, TMAO was reduced, resulting in 65 mol% of acetate (15 mM of lactate resulted in 10 mM of acetate and 30 mM of TMA) (Fig. 5(A) and (B)). These results are in agreement with those obtained in the defined medium. No additional acetate was produced at the moment that all lactate was consumed. However, TMA production continued, suggesting that other H-donors, such as simple amino acids like

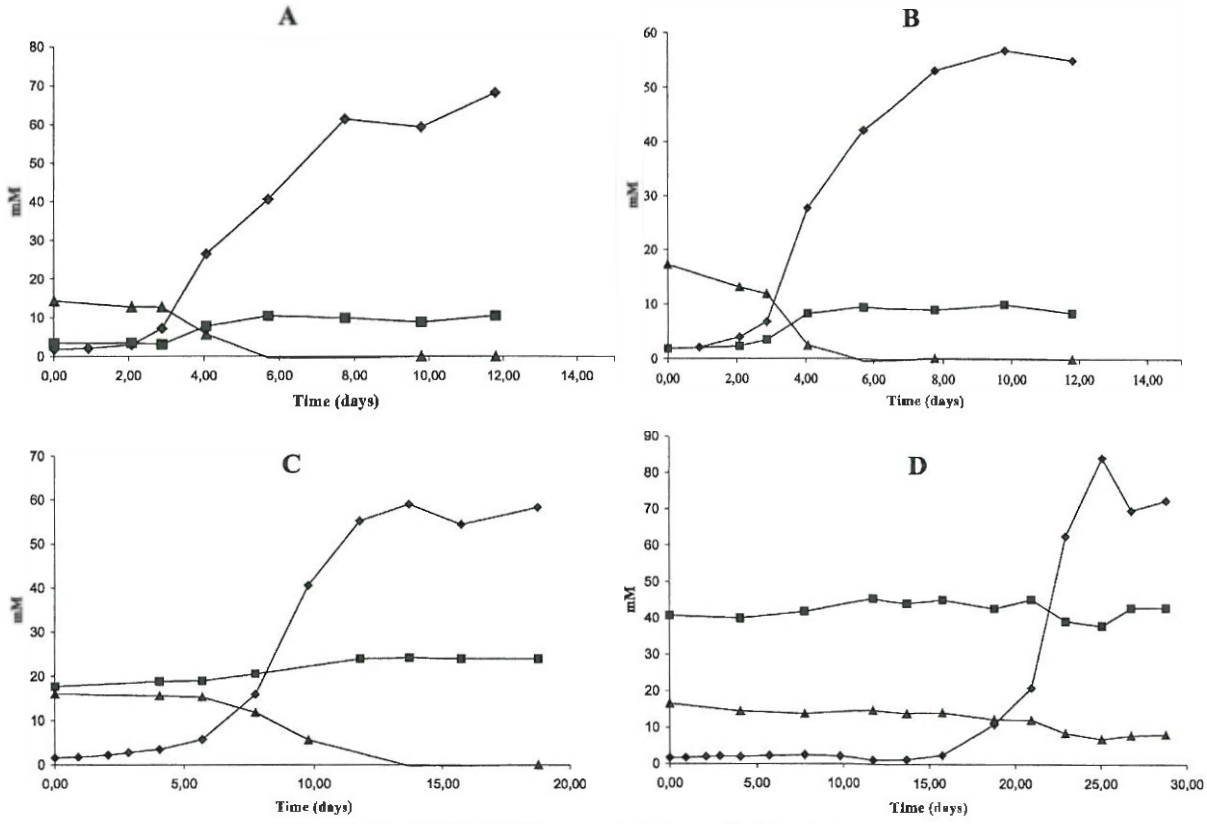

Fig. 5. Evolution of the concentrations (mM) of TMA-N (♦), acetate (■), lactate (▲) in a buffered fish extract at 4°C inoculated with *S. baltica* in 100% of N_2 and 0% (v/v) acetate (A), air and 0% (v/v) acetate (B), 100% of N_2 and 0.1% (v/v) acetate (C) and 100% of N_2 and 0.25% (v/v) acetate (D).

serine and cysteine, which are common extractives of fish, were at that moment used as H-donors for further TMAO-reduction. Amino acids such as serine and cysteine are completely oxidized to CO_2 without the formation of acetate (RingØ et al., 1984). This explains the stop in acetate formation at the moment that all lactate is consumed, which was also reported for *S. putrefaciens* NCMB 1735 in a defined medium by RingØ et al. (1984). However, at the moment that all lactate was consumed, levels of 30 mM TMA-N (corresponding with 42 mg THA-N/100 ml) were already produced, which is well over the maximum limit of 15 mg TMA-N/100 g in marine fish stored on ice (Connell, 1975). Therefore, the TMAO-reduction with H-donors other than lactate is probably only of limited importance in practice. However, to study the mechanism of TMAO-reduction by *S. baltica*, extended experiments with other H-donors (or reaction-intermediates) such as pyruvate, are necessary.

When 0.1% of acetate was added to the fish extract (Fig. 5(C)), less acetate was formed and lactate was more slowly consumed in comparison to the experiments without the addition of acetate. The consumption of lactate and the production of acetate were almost completely inhibited when the fish extract contained 0.25% of acetate (Fig. 5(D)). Apparently, the addition of acetate inhibited the use of lactate as H-donor. However, the addition of acetate did not influence the final concentrations of TMA produced by *S. baltica*. Probably, alternative H-donors were used by *S. baltica*, but this pathway seems to be less energy efficient, which can be deduced from the exceptional growth inhibition of small amounts of acetate.

4. Conclusions

Debevere and Boskou (1996) demonstrated that the shelf life of modified atmosphere packaged fish fillets could be prolonged by spraying fish fillets with a 10% acetic acid/acetate solution (pH = 5.6). Such a treatment resulted in an increased concentration of acetic acid/acetate at the surface of the fish fillets and lowered the surface pH of the fish fillets. The actual paper demonstrated, on the other hand, in situ that even at neutral pH (6.8) low acetate concentrations ($< 0.3\%$) cause a delay of the TMAO-reduction reaction by *S. baltica* in which lactate is used as a H-donor. When practical storage times for fish (e.g. 6 days at 4°C after packaging) are considered, growth and TMAO-reduction by *S. baltica* was completely inhibited during this period when 0.25% of acetate was present in the growth medium. Most probably, in the presence of acetate H-donors other than lactate have to be drawn on by *S. baltica*, resulting in less energy efficient processes and consequently, in the inhibition of growth. Although CO_2 partly inhibited reaction (2), its contribution to the inhibition of the reaction mechanism was of minor importance. On the contrary, as could be derived from Fig. 1, the effect of CO_2 on the growth of *S. baltica* was of major importance when lactate, the natural H-donor for TMAO-reduction during fish spoilage, was available.

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