**Food Microbiology,** 1997, **14**, 543–553

ORIGINAL ARTICLE

# Reduction of trimethylamine oxide by *Shewanella* spp. under modified atmospheres *in vitro*

**G. Boskou and J. Debevere***

*Two strains of* Shewanella *spp. were isolated from cod fillets packed in modified atmosphere (60% $CO_2$, 30% $O_2$, 10% $N_2$). One of the strains was identified as* Shewanella putrefaciens. *The other strain could not be fully identified but was determined as a* Shewanella *spp. different from* S. putrefaciens. *The effect of modified atmosphere ($CO_2$, $O_2$) on the growth of the two strains and on the reduction of TMAO to TMA was studied using solid medium from fish extract packed under variable mixtures of $CO_2$, $O_2$ and $N_2$. All the samples were incubated at 7°C for 7 days. The* Shewanella*-like strain was shown to be a stronger TMAO reducer and was more resistant to $CO_2$ than* S. putrefaciens per se. *Modified atmosphere packaging of marine fish can inhibit the growth and TMAO-reducing activity of* S. putrefaciens *when 50% of $CO_2$ together with 10% of $O_2$ are introduced into the packaging atmosphere. The growth and TMAO-reducing activity of the* Shewanella*-like strain can be inhibited when higher proportions of $CO_2$ together with as high as possible proportions of $O_2$ are introduced into the packaging atmosphere. It is suggested that a combination of 60–70% $CO_2$ and 30–40% $O_2$ is introduced into the packaging atmosphere in order to prevent TMA production by* Shewanella *spp.*


## Introduction

Among the bacteria responsible for the spoilage of marine fish are a group of facultative anaerobic, Gram-negative, psychrotrophic rods classified as *Shewanella putrefaciens* (previously called *Alteromonas putrefaciens*, McDonnel and Colwell 1985). These bacteria can proliferate under chilled conditions, and by the time the food is unacceptable for human consumption they often account for more than 30% of the total bacterial population. Compared to other species of the marine flora, the generation time of these bacteria can become shorter as temperature decreases (Shewan 1977). The reason for these bacteria spoiling seafood seems to be the production of off-flavouring volatile compounds such as ammonia, trimethylamine (TMA) and sulphides (Lee 1979, Shewan and Murray 1979, Gill 1992). Trimethylamine is the main component responsible for an unpleasant 'fishy' odour (Dainty 1996).

Trimethylamine oxide (TMAO) is a major constituent of the non-protein nitrogen fraction in marine teleosts with osmoregulatory function (Agustsson and Strøm 1981, Huss 1988, Gill 1992). The ability of bacteria to reduce TMAO to TMA is used as a taxonomic criterion for the non-fermentative *S. putrefaciens* (Lee et al. 1977). TMAO is found to pro-

*Corresponding author.

*Received: 10 November 1996*

*Department of Food Technology and Nutrition, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Coupure Links 653, 9000 Ghent, Belgium*

0740-0020/97/060543+11 $25.00/0/fd970132

mote micro-aerophillic or anaerobic growth (Easter et al. 1982). When oxygen levels are depleted, TMAO serves as a terminal electron acceptor for anaerobic respiration and is reduced to TMA (Easter et al. 1983). TMAO respiration in anaerobic growth is linked with an energy conservation mechanism (Ringo et al. 1984). Enzymological data presented by Scott and Nealson (1994) indicates that *S. putrefaciens* uses the Entner-Doudoroff pathway to produce pyruvate from glucose and enter a course of anaerobic carbon assimilation with methlytrophic growth on formate. Electrons transferred through the serine pathway find TMAO as the final electron acceptor and the liberated energy is used for the formation of energy rich phosphates (Huss 1988).

*Shewanella putrefaciens*, apart from being the main spoilage micro-organism in ice stored fish, is also responsible for the spoilage of fish in modified atmosphere packaging (Shewan 1977, Huss 1988, Stammen et al 1990). In atmospheres containing 50% and higher $CO_2$, *S. putrefaciens* has been reported to be part of the dominant flora of cod and sole together with *Lactobacillus* spp. (Lee 1981, Stenstrom 1985). In general, Gram-negative, psychrotrophic bacteria, including some common spoilage bacteria, are susceptible to $CO_2$ (Gill and Tan 1980). Nevertheless, there is no complete growth inhibition but a slower development of the spoilage bacteria in gas packed fish than in ice stored fish. The growth and activity of an important spoilage bacterium such as *S. putrefaciens* merits further investigation.

The aim of this study was to examine *in vitro* the influence of modified atmosphere packaging on the spoilage capacity of two *Shewanella* spp. strains that were isolated from fish packed in modified atmosphere and spoiled by TMA. The effect of modified atmosphere ($CO_2$, $O_2$) on the growth of the two strains and on the reduction of TMAO to TMA was studied using solid medium from fish extract packed under variable mixtures of $CO_2$, $O_2$ and $N_2$. All the samples were incubated at 7°C. The temperature selected is a conventional storage temperature for prepacked seafood in the retail market.

## Materials and Methods

### *Bacterial strains*

Two bacterial strains were isolated from cod fillets packed in modified atmosphere (60% $CO_2$, 30% $O_2$, 10% $N_2$) after 4 days of storage at 7°C, when the levels of TMA were already high. The isolated strains were some of the brownish colonies developing on streak plates of Marine Agar (Marine Broth, Difco and 1·6% Agar No. 1, Oxoid Unipath Ltd., Hampshire, England) after 3 days incubation at 20°C. The above mentioned brownish colonies accounted for *c.* 60% of the total number of colonies counted on each plate. The colonies were picked off and enriched for 48 h in Marine Broth (Difco Laboratories, Detroit, MI, USA) at 20°C before streaking on plates of Marine Agar. The strains were tested for Gram colour, morphology, oxidase activity (Dryslide™, Difco), catalase activity (with 3% $H_2O_2$), TMAO reduction (Debevere and Voets 1974) and TMAO reduction with $H_2S$ production (Gram et al. 1987). One of the isolates could be well identified with triplicate API-2ONE tests (bioMerieux s.a., Marcy-l'Etoile, France) as *Shewanella putrefaciens*.

The second isolate could not be identified with multiple API-2ONE tests so was further identified by the Identification Service of the LMG Culture Collection (Laboratory of Microbiology, University of Ghent). LMG Identification Service carried out: (a) fatty acid analysis using the commercial MIDI System (Microbial Identification System, Inc., Delaware, USA); (b) determination of the gel-electrophoretic protein pattern (Pot et al. 1994a, b); and (c) cluster analysis of the pattern with micro-organisms from the LMG reference database. From here on this strain will be referred in the text as '*Shewanella*-like strain'.

A summary of the tests performed is given in Table 1.

Stock cultures were prepared on slants of Marine Agar and kept at 6°C.

### *Preparation of the fish extract*

Cod fillets (*Gadus morhua*) were divided into portions of 200 g. Each portion was blended for 1 min with 400 ml deionized water in a

Waring Commercial Blender. The blended mixture was placed in a water bath at 80°C for 1 h, to precipitate the major structural proteins. After cooling down, the mixture was squeezed through a bag of cheesecloth to remove the precipitate. The filtrate was centrifuged for 10 min at 16 000×*g* (4°C) with a Sorval RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments). The supernatant was filtered through S&S 597½ filter paper. The overall yield was 2 l of fish extract starting with 1 kg of cod and 2 l deionized water. After the determination of TMAO, the extract was supplemented with TMAO · $2H_2O$ (Sigma) to obtain a final concentration of ±70 mg N/100 g. The pH of the extract was 6·6.

### *Preparation of the solid medium*

Several litres of fish extract were produced and distributed in bottles of 500 ml. In each bottle 200 ml of the fish extract and 3 g Technical Agar (Agar No. 3, Oxoid) was added. The bottles were heated carefully on a hot plate with a magnetic stirrer to melt and dissolve the agar. Sterilization was applied on this medium by autoclaving at 121°C for 15 min. After sterilization the medium was

**Table 1.** Identification tests for isolated strains

| Test | *Shewanella putrefaciens* | *Shewanella*-like strain |
|---|---|---|
| Gram | −(3/3)[a] | −(17/17)[a] |
| Morphology | rods (3/3) | rods (17/17) |
| Motility | +(3/3) | +(17/17) |
| Oxidase | +(3/3) | +(17/17) |
| Catalase | +(3/3) | +(17/17) |
| TMAO reduction (Debevere and Voets 1974) | +(3/3) | +(17/17) |
| TMAO reduction (Gram et al. 1987) | +(3/3) | +(17/17) |
| $H_2S$ production (Gram et al. 1987) | +(3/3) | +(17/17) |
| API-20NE: | | |
| Nitrate (reduction) | +(3/3) | +(15/15) |
| Indole (production) | −(3/3) | −(15/15) |
| Glucose (acidification) | −(3/3) | −(15/15) |
| Arginine (argininedehydrolase) | −(3/3) | −(15/15) |
| Urea (urease) | −(3/3) | −(15/15) |
| Esculine (β-glucosidase) | +(3/3) | +(15/15) |
| Gelatine (gelatinase) | −(3/3) | +(15/15) |
| p-nitro-phenyl-βD-galactopyranoside (β-galactosidase) | −(3/3) | −(15/15) |
| Glucose (assimilation) | −(3/3) | +(8/15) |
| Arabinose (assimilation) | −(3/3) | +(11/15) |
| Mannose (assimilation) | −(3/3) | −(14/15) |
| Mannitol (assimilation) | −(3/3) | −(15/15) |
| N-acetyl-glucosamine (assimilation) | +(3/3) | +(15/15) |
| Maltose (assimilation) | −(3/3) | +(15/15) |
| Gluconate (assimilation) | −(3/3) | +(15/15) |
| Caprate (assimilation) | −(3/3) | −(14/15) |
| Adipate (assimilation) | −(3/3) | −(15/15) |
| Malate (assimilation) | +(3/3) | +(14/15) |
| Citrate (assimilation) | −(3/3) | +(13/15) |
| Phenylacetate (assimilation) | −(3/3) | −(15/15) |
| reference profile | *Shewanella putrefaciens* | No matching reference |
| LMG Identification: | | |
| Fatty acid analysis | | *Shewanella putrefaciens* (2/2) |
| Gel-electrophoretic protein pattern | | 29% homology with *Shewanella putrefaciens* (<50%) (2/2) |

[a]The figures in parentheses show the frequency of the score.

left to cool until 45°C before it was poured into petri plates of 14 cm diameter and 2 cm depth. While pouring the medium the plates were standing on a balance to make sure that *c.*50 g was added. After the solidification of the medium, the plates were stored at 3°C.

### *Inoculation of the fish extract*

Three tubes containing Marine Broth (10 ml/tube) were inoculated with *S. putrefaciens* and the *Shewanella*-like strain from the slant cultures with an inoculation loop. After 48 h incubation the content of the tubes was poured into bottles with 300 ml sterile Marine Broth. Before pouring, the culture was tested for purity by Gram staining, morphology, oxidase and catalase test. The inoculated Marine Broth was incubated for 72 h at 20°C. Then each plate of solid fish extract was inoculated with 2 ml of the Marine Broth culture that was spread all over the surface with a drigalski spatula. The plates were left for 10 min in the laminar flow to dry and then were packed in modified atmosphere. All the plates were incubated at 7°C and 95% RH for 7 days. Microbiological and chemical analysis were carried out on the day of inoculation (day 0) as well as after 3, 4, 5, 6 and 7 days incubation.

### *Modified atmosphere packaging*

The inoculated plates were introduced in 25× 35 cm high oxygen barrier plastic bags (Sidamil, UCB Transpac, Belgium). One plate was introduced in each bag. The plastic bags were made of PVdC, laminated with polyester, and its gas-permeability was: 6 cc/$m^2$/24 h for $O_2$, 2 cc/$m^2$/24 h for $N_2$ and 15 cc/$m^2$/24 h for $CO_2$ at 25°C and 100% RH. The bags were filled with the appropriate gas mixture and sealed in a MULTIVAC A300/42 packaging set (Sepp. Haggenmuller KG, Germany). Four volumes of gas mixture were introduced into each bag (200 cc gas/50 g fish extract). Duplicate bags were filled with the gas mixtures for each day of the charted sampling. The composition of the gas mixtures is shown in Table 2. For the *Shewanella*-like strain a more extended variety of gas mixtures was used.

### *Microbiological analysis*

From a plate with solid fish extract 10 g was aseptically collected and diluted 10 times with physiological saline-peptone solution (PPS, 0·85% NaCl, 0·1% peptone, pH 7) in a Stomacher bag. The mixture was homogenized with a Colworth Stomacher 400 (Seward Laboratory, London, UK). Tenfold dilution series were made in PPS for plating. The viable count of *Shewanella* spp. was determined as streak plate counts on Marine Agar (Marine Broth, Difco with 1·6% Agar No. 1, Oxoid). The plates were incubated for 3 days at room temperature (20°C).

### *Chemical analysis*

The composition of the gas mixtures used for modified atmosphere packaging was monitored with a Servomex gas analyser (Food Package Analyser, Series 1400).

The initial content of the fish extract in trimethylamine oxide was determined as described by Wekell and Barnett (1991).

Trimethylamine was determined as follows:

*Reagents:* (i) A 2% picric acid stock solution in toluene was prepared, which was filtered through Whatman No. 1 paper and dried with $Na_2SO_4$. From this stock a 0·2% work solution in toluene was prepared daily. (ii) A standard solution was prepared by diluting TMA · HCl (Fluka, purum) in 6% trichloroacetic acid (UCB).

*Procedure:* Ten grams of solid fish extract was diluted 10 times with 6% TCA (trichloroacetic acid) in a Stomacher bag and blended for 1 min with a Colworth Stomacher 400 (Seward Laboratory). One millilitre of the above suspension was placed in tubes with aluminium screw caps containing 3 ml toluene (UCB, p.a.). In every tube 200 µl of 40% formol and 500 µl of 90% KOH was added. The tubes were twice mildly vortexed (to avoid formation of foam) and left to stand for 15 min. Aliquots of 1 ml from the toluene layer were transferred in dry tubes with 80–100 mg anhydrous $Na_2SO_4$. In each tube,

3 ml of the picric acid work solution was added. The tubes were vortexed and left to stand for 5 min. The absorbance of the picrate salt at 410 nm was measured with a double beam DMS-300, UV-Vis., Varian Spectrophotometer. Sample, standard and blank were run in duplicate.

When the absorbance of the picrate salt was higher than 1 a 30:1 dilution, instead of 10:1, was used.

The pH was measured with an Ingold sharp point electrode connected to a Knick pH-meter.

## Results

Examination of the *Shewanella*-like strain by fatty acid analysis identified the micro-organism as *S. putrefaciens* but cluster analysis of the gel-electrophoretic pattern gave less than 50% homology with *Shewanella putrefaciens*. To resume, the second isolated strain was considered to be a member of the *Shewanella* spp. but not identical to *S. putrefaciens*.

When subjected to a modified atmosphere of 30% $CO_2$ and 70% $O_2$, inhibition on the growth of *S. putrefaciens* for the first 5 days of incubation could be noticed, but afterwards outgrowth could no longer be delayed (Fig. 1a). Other gas mixtures, including 30% $CO_2$ and a lower concentration of oxygen, did not prevent the growth of the spoilage bacterium. Carbon dioxide seems to be completely inhibitory at concentration 50% or higher, while in an atmosphere of >96% $N_2$ an outgrowth of *S. putrefaciens* appears after 3 days incubation (Fig. 1b).

Production of TMA is inhibited by the presence of $O_2$ in the packaging atmosphere (Fig. 2). Concentration of $O_2$ as low as 10% is sufficient to inhibit the reduction of TMAO by *S. putrefaciens*. In modified atmospheres with more than 10% $O_2$ no production of TMA was observed (results not illustrated in Fig. 2). In an atmosphere of >96% $N_2$, where there is absence of either oxygen or carbon dioxide, the production of TMA is enhanced, while it was retarded when 30% $CO_2$ was included.

**Table 2.** Code numbers of the gas mixtures applied for modified atmosphere packaging of fish extract inoculated with *Shewanella* spp.

| Gas mixture | %$CO_2$ | %$O_2$ | %$N_2$ |
|---|---|---|---|
| GN2[a,b] | 0 | 0 | >96 |
| GCO2[a,b] | >96 | 0 | 0 |
| G802[b] | 80 | 0 | 20 |
| G703[b] | 70 | 0 | 30 |
| G604[b] | 60 | 0 | 40 |
| G505[b] | 50 | 0 | 50 |
| G406[b] | 40 | 0 | 60 |
| G307[a,b] | 30 | 0 | 70 |
| G316[a] | 30 | 10 | 60 |
| G334[a] | 30 | 30 | 40 |
| G352[a] | 30 | 50 | 20 |
| G028[b] | 0 | 20 | 80 |
| G037[b] | 0 | 30 | 70 |
| G046[b] | 0 | 40 | 60 |
| G055[b] | 0 | 50 | 50 |
| G064[b] | 0 | 60 | 40 |
| G073[b] | 0 | 70 | 30 |
| G820[b] | 80 | 20 | 0 |
| G730[a,b] | 70 | 30 | 0 |
| G640[b] | 60 | 40 | 0 |
| G550[a,b] | 50 | 50 | 0 |
| G460[b] | 40 | 60 | 0 |
| G370[a,b] | 30 | 70 | 0 |

[a]Gas mixtures used for modified atmosphere packaging of fish extract inoculated with *Shewanella putrefaciens*.
[b]Gas mixtures used for modified atmosphere packaging of fish extract inoculated with the *Shewanella*-like strain.

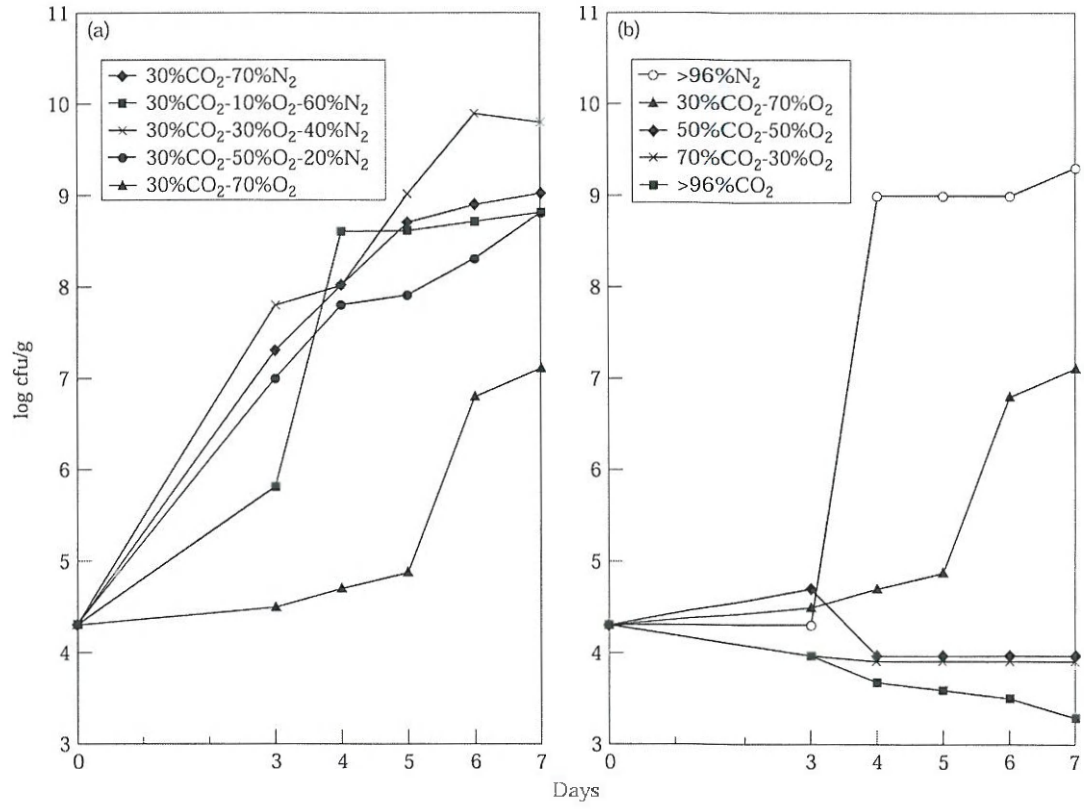


**Figure 1.** Growth of *Shewanella putrefaciens* in fish extract packed in modified atmospheres and stored for 7 days at 7°C.

No production of TMA was observed in an atmosphere with >96% $CO_2$ (results not illustrated in Fig. 2).

The pH of the plates with solid fish extract in modified atmosphere packaging did not notably change throughout the storage period except for the cases where an atmosphere of >96% $N_2$ and >96% $CO_2$ was applied. In the former the pH increased from 6·6 to 7·0, due to the production of TMA, and in the latter the pH decreased to 6·2, due to the diffusion of $CO_2$ in the media.

Figure 3a shows the growth of the *Shewanella*-like strain in an oxygen free atmosphere with increasing concentration of $CO_2$. The figure shows that the development of the strain was slowed down as the concentration of $CO_2$ in the atmosphere increased. Nevertheless, there was outgrowth (3–5 log units) of the *Shewanella*-like strain even at high $CO_2$ concentrations (i.e. 80% $CO_2$ and >96% $CO_2$). The production of TMA was not inhibited at $CO_2$ concentrations less than 50% (Fig. 4a). At $CO_2$ concentrations of 50% or more, inhibition of TMA production was noticed. However, after 4 days of incubation, an increase in TMA production was observed, which finally crossed the threshold of 35 mg N/100 g at the end of 7 days storage. Only for an atmosphere of >96% $CO_2$ was there a minimal production of TMA, remaining beneath 10 mg N/100 g for the whole period of storage (Fig. 4a).

There was no effect of $O_2$ concentration on the growth of the *Shewanella*-like strain (Fig. 3b). The production rate of TMA decreased as the $O_2$ concentration in the packaging atmosphere increased (Fig. 4b). At concentrations of 40 and 50% $O_2$, TMA reached the unacceptable level of 35 mg N/100 g after 3 days, and, after 4 days of storage, TMA production rose to 60–70 mg N/100 g. At concentrations of 60

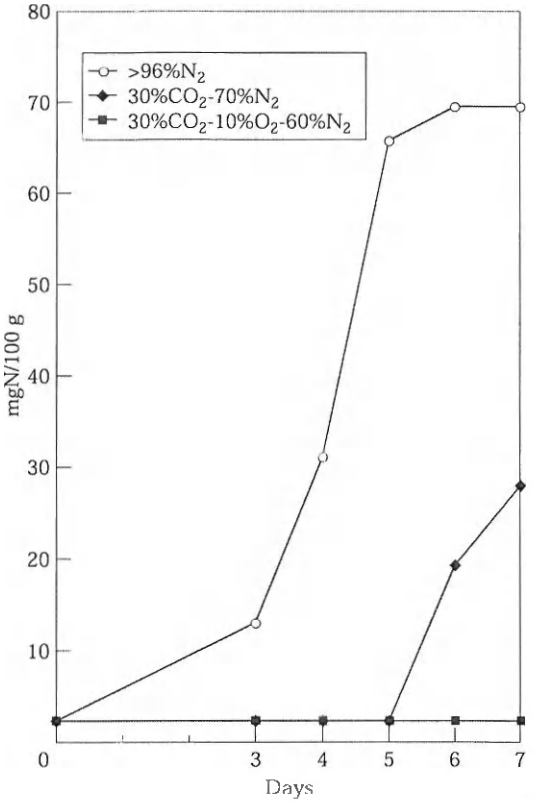


**Figure 2.** TMA production by *Shewanella putrefaciens* in fish extract packed in modified atmospheres and stored for 7 days at 7°C.

and 70% $O_2$ the TMA production was inhibited for 4 days, although this was only a delay, and after 4 days, TMA was detected at relatively high levels. Best results for the inhibition of TMA production were obtained with a 70% $O_2$ concentration (Fig. 4b).

When both $CO_2$ and $O_2$ were used in the modified atmosphere there was a combined effect on the growth and TMAO reducing activity of the *Shewanella*-like strain. Figure 3c shows in general lower outgrowth (1·5–2·5 log cfu/g) of the strain if compared to the results shown in Figs 3a and 3b (3–5 log cfu/g), where only one of the two gases was applied. Nevertheless, the effect of $CO_2$ on the growth rate prevails over the effect of $O_2$ (Fig. 3c). The combined effect of the two gases is better seen in Fig. 4c. No differences are observed between the efficacy of the different gas mixtures for the reduction of TMAO to TMA.

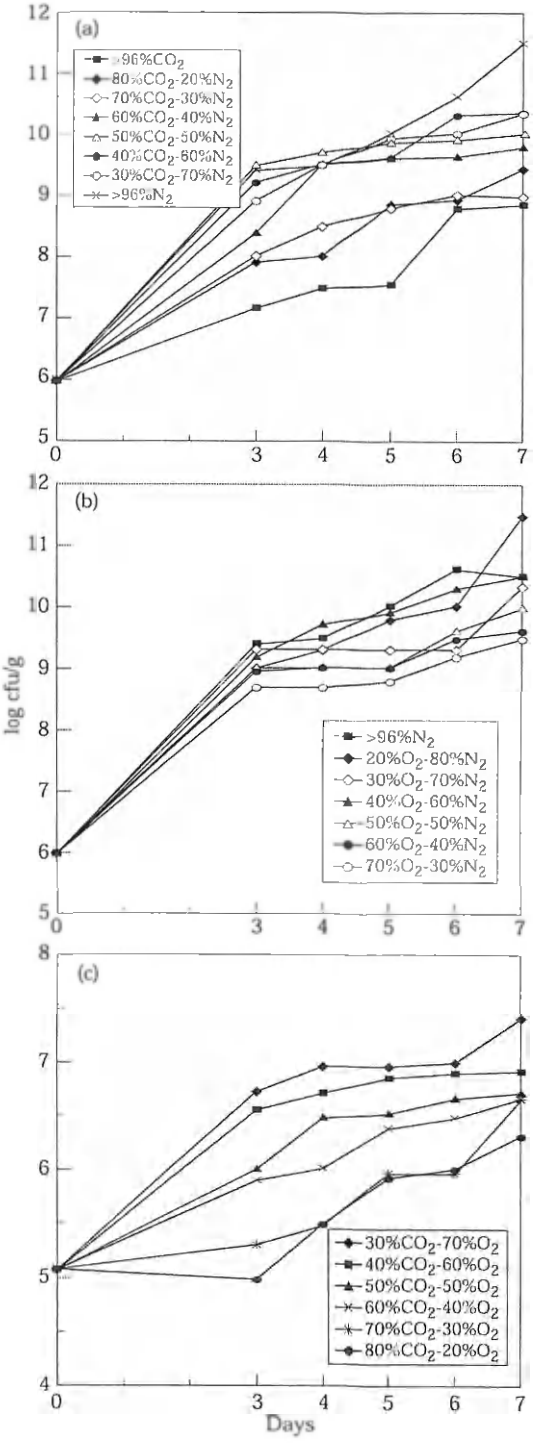


**Figure 3.** Growth of the *Shewanella*-like strain in fish extract packed in modified atmospheres with: variable $CO_2$ concentrations (a); variable $O_2$ concentrations (b); or variable $CO_2$ and $O_2$ concentrations (c) and stored for 7 days at 7°C.

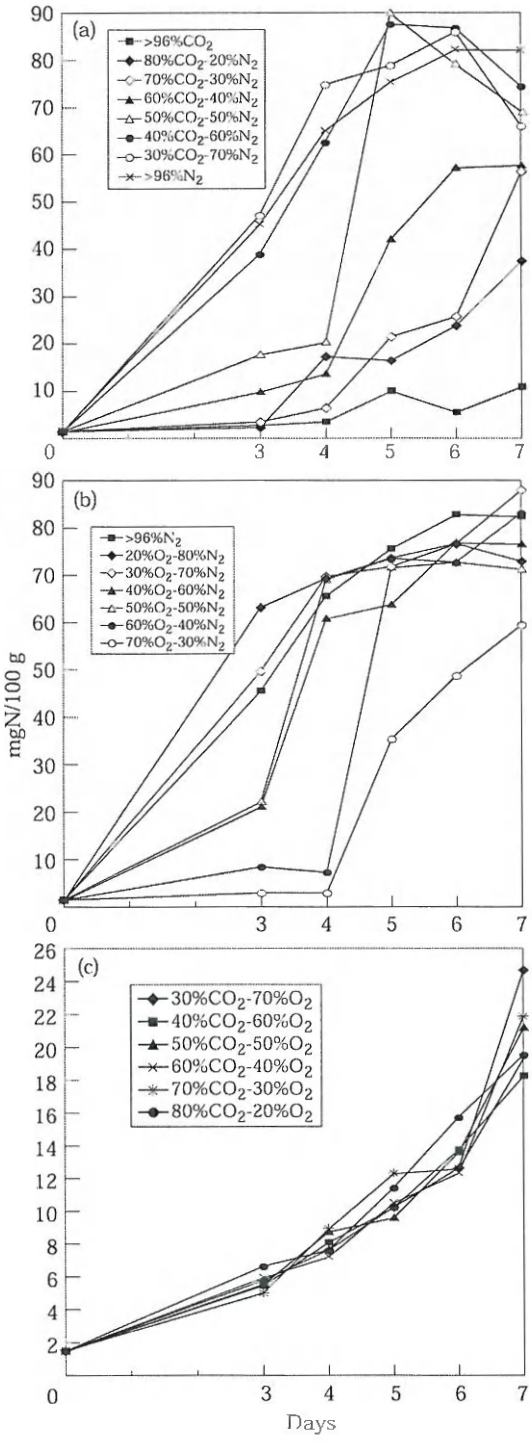


**Figure 4.** TMA production by the *Shewanella*-like strain in fish extract packed in modified atmospheres with: variable $CO_2$ concentrations (a); variable $O_2$ concentrations (b); or variable $CO_2$ and $O_2$ concentrations (c) and stored for 7 days at 7°C.

The initial pH of the plates with solid fish extract was 6·6. This value increased up to between 6·8 and 7·0 when there was production of TMA. Only in the cases where 90% or >96% $CO_2$ was inserted, did the pH decrease to c. 6·1 during the first 3 days of incubation and increased later on due to TMA production.

The initial headspace concentration of $CO_2$ in the modified atmospheres using high $CO_2$ levels decreased up to 50% during the first 3 days of incubation due to diffusion in the media. On the contrary, for these atmospheres, the concentrations of $N_2$ or $O_2$ increased taking over the space becoming available because of $CO_2$ diffusion. The increase in $N_2$ concentration in these cases was higher since it is less soluble than $O_2$. At the onset of bacterial growth, $CO_2$ concentration increased and $O_2$ concentration decreased due to metabolic activity. If no $N_2$ was included in the modified atmosphere, $CO_2$ diffusion in the sample caused collapsing of the package, resulting to an outlook of the gas-packed sample being similar to vacuum-packaging.

## Discussion

Stenstrom and Molin (1990) studied several strains of *S. putrefaciens* isolated from fish products and found heterogeneous phenotypes among them. Gram and Huss (1996) suggest that there can be clonal selection of *S. putrefaciens* strains during a storage trial. However, there is distinct difference between the phenotypes and genotypes of *Shewanella* spp. (Jensen et al. 1980, van Landschoot and De Ley 1983). The reduction of TMAO and production of $H_2S$ by *S. putrefaciens* in the fish muscle has been extensively studied by several researchers but there is limited data for other *Shewanella* spp. (e.g. *S. alga, S. benthica, S. colwelliana, S. hanedai*). An extensive bacteriological analysis is necessary to determine the homology of the *Shewanella*-like strain with all the members of *Shewanella* spp.

*Shewanella putrefaciens* was unable to develop if high concentrations of $CO_2$ were applied (>50%, Fig. 1b). On the contrary, the

*Shewanella*-like strain is more resistant to $CO_2$ (Fig. 3a). Nevertheless, the resistance to $CO_2$ and the growth rate of this bacterium decreased as the $CO_2$ concentration increased in the modified atmosphere (Fig. 3a). Lee (1981) examined the growth of *S. putrefaciens* in air and in atmospheres including 20 to 60% $CO_2$, and found that the generation time of the strain was increased while $CO_2$ concentration increased in the atmosphere. The details of the inhibition mechanism are not known, but it is likely that $CO_2$ affects certain enzyme systems. Since anaerobic growth of facultative anaerobic bacteria is only slightly affected and since *Lactobacillus* spp., using a fermentative metabolism, are resistant to $CO_2$, this indicates that the main site of action is connected with oxidative metabolism (Huss 1988). According to Scott and Nealson (1994) *S. putrefaciens* uses an incomplete tricarboxylic acid cycle during anaerobiosis, while methylotrophic growth by the serine pathway could utilize $CO_2$ as a carbon source.

High concentrations of oxygen (70%) seem to have some inhibitory effect on the growth of *S. putrefaciens* but not on the growth of the *Shewanella*-like strain (Figs. 1a, 3b). Oxygen is known to be toxic in high concentrations for most bacteria due to various mechanisms (i.e. inactivation of enzymes, formation of $H_2O_2$ and free radicals) (Huss 1988).

No production of trimethylamine is observed when there is sufficient oxygen for aerobic respiration of *S. putrefaciens*. This was achieved even at low concentrations of oxygen (10% $O_2$, Fig. 2). Figure 4b shows that the *Shewanella*-like strain, unlike *S. putrefaciens*, could produce TMA at $O_2$ concentrations higher than 10%. Nevertheless, the production rate of TMA, by the *Shewanella*-like strain, decreased as $O_2$ concentration in the modified atmosphere increased. Inhibition of TMA production by the *Shewanella*-like strain was observed only with 60 and 70% $O_2$ and then only for the period of the first 4 days.

According to Easter (1982) oxygen exerts an inhibitory effect of the TMAO-reductase of *Alteromonas* spp., while high $CO_2$ concentration has an indirect inhibitory effect on the same enzyme by reducing the pH. Compared to an atmosphere of >96% $N_2$, a concentration of 30% $CO_2$ resulted in a much lower production of TMA by *S. putrefaciens* (Fig. 2). High $CO_2$ concentrations (>90%) inhibited the production of TMA by the *Shewanella*-like strain but for only 4 days storage (Fig. 4a). While packaging under elevated $CO_2$ concentrations could reduce the pH of the fillets, production of TMA counteracts this by causing the pH to rise, thus enhancing more TMA production. TMAO-reductases of *Alteromonas* spp. have optimum activity at pH 6·8 (Easter et al. 1982).

According to Jorgensen and Huss (1989), Dalgaard (1995a, b) and Dalgaard et al. (1996), a concentration of more than $10^8$ cfu/g for *S. putrefaciens* is required to produce TMA at a concentration of 30 mgN/100 g and have perceptible spoilage. This was the case with >96% $N_2$ and 30% $CO_2$–70% $N_2$ where there was an absence of oxygen (Figs 1a, 1b, 2). On the contrary, in 30% $CO_2$–10% $O_2$–60% $N_2$, 30% $CO_2$–30% $O_2$–40% $N_2$ and 30% $CO_2$–50% $O_2$–20% $N_2$ the growth of *S. putrefaciens* reached and passed the level of $10^8$ cfu/g but no production of TMA was detected due to the presence of oxygen in the modified atmosphere (Figs 1a, 2). The *Shewanella*-like strain demonstrated high production of TMA when its population was at the level of $10^7$ cfu/g or more (Figs 3a, b, c, 4a, b, c,).

When $CO_2$ and $O_2$ were combined in the same modified atmosphere there was inhibition on both the growth and TMA production of the *Shewanella*-like strain (Figs 3c, 4c). These two gases were complementary to each other. Combination of the two gases resulted in lower growth rates and even lower TMA production than a single application of each gas. Although $CO_2$ is mainly responsible for the growth rate restriction there is a joint effect of the two gases on the TMA production. When $O_2$ concentration was low, $CO_2$ could inhibit the generation of the cells and hence delay TMA production. On the other hand, when $CO_2$ concentration was not sufficient to inhibit the bacterial growth, enough $O_2$ was present in the atmosphere to prevent TMAO reduction. The concentration of TMA in the fish muscle never crossed the threshold of 30 mgN/100 g.

lated from spoiling fish. *Int. J. Food Microbiol.* **9,** 51–62.

van Landschoot, A. and De Ley, J. (1983) Intra- and intergeneric similarities of the rRNA cistrons of *Alteromonas, Marinomonas* (gen. nov.) and some other Gram negative bacteria. *J. Gen. Microbiol.* **129,** 3057–3074.

Lee, J. V. (1979) *Alteromonas (Pseudomonas) putrefaciens. Soc. Appl. Bacteriol. Tech. Ser.* **13,** 59–65.

Lee, J. S. (1981) Selection and growth of seafood under $CO_2$ environment. In *Proc. First Nat. Conf. Modified and Controlled Atmosphere Packaging of Seafood Products* (Eds R. E. Martin) p. 59. National Fisheries Institute, Washington D.C.

Lee, J. V., Gibson, D. M. and Shewan, J. M. (1977) A numerical taxonomic study of some pseudomonas-like marine bacteria. *J. Gen. Microbiol.* **98,** 439–451.

McDonnell, M. T. and Colwell, R. R. (1985) Phylogeny of the *Vibrionaceae*, and recommendation for two new genera, *Listonella* and *Shewanella*. *System. Appl. Microbiol.* **6,** 171–172.

Pot, B., Vandamme, P. and Kersters, K. (1994a) Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Bacterial Systematics* (Eds M. Goodfellow, A. G. O'Donnell) Chichester, Wiley.

Pot, B., Ludwig, W., Kersters, K. and Shleifer, K. H. (1994b) The taxonomy of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications* (Eds L. De Vuyst, E. J. Vandamme) London, Blakie Academic & Professional.

Ringo, E., Stenberg, E. and Strom, A. R. (1984) Amino acid and lactate catabolism in trimethylamine oxide respiration of *Alteromonas putrefaciens* NCMB 1735. *Appl. Environ. Microbiol.* **47,** 1084–1089.

Scott, J. H. and Nealson, K. H. (1994) A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. *J. Bacteriol.* **176,** 3408–3411.

Shewan, J. M. (1977) The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. *Conference on the Handling, Processing and Marketing of Tropical Fish.* Tropical Products Institute, London, proceedings pp. 51–66.

Shewan, J. M. and Murray, C. K. (1979) The microbial spoilage of fish with special reference to the role of psychrofiles. *Soc. Appl. Bacteriol. Tech. Ser.* **13,** 117–136.

Stammen, K., Gerdes, D. and Caporaso, F. (1990) Modified atmosphere packaging of seafood. CRC Rev. *Food Sc. & Nutrit.* **29**(5), 301–303.

Stenstrom, I. M. (1985) Microbial flora of cod fillets (*Gadus morhua*) stored at 2°C in different mixtures of carbon dioxide and nitrogen/oxygen. *J. Food Prot.* **48,** 585–589.

Stenstrom, I. M. and Molin, G. (1990) Classification of the spoilage flora of fish, with special reference to *Shewanella putrefaciens*. *J. Appl. Bacteriol.* **68,** 601–618.

Wekell, J. C. and Barnett, H. (1991) New method for analysis of trimethylamine oxide using ferrous sulphate and EDTA. *J. Food Science* **56,** 132–138.