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Biogenic amine formation and degradation by potential fish silage starter microorganisms

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Abstract

Fish waste can be advantageously upgraded into animal feed by fermentation with lactic acid bacteria (LAB). This procedure is safe, economically advantageous and environment friendly. The pH value of the fish pastes decreases to below 4.5 during ensilage. This pH decrease is partly responsible for preservation. Decreased pH values and relatively low oxygen concentrations within the silage facilitate decarboxylase activity. Biogenic amines may constitute a potential risk in this kind of product since their precursor amino acids are present in fish silage. It is of great importance to ensure that the LAB strains chosen for starters do not produce biogenic amines. Some bacteria, among which some LAB species, are able to degrade these metabolites by means of amino oxidases. This could be of interest for fish silage production, to control biogenic amine build-up in this product. Seventy-seven LAB cultures isolated from fish pastes submitted to natural fermentation at two temperatures (15 and 22°C) and selected combinations of these isolates were examined for histamine, tyramine, cadaverine and putrescine production. Of the isolates tested, 17% were found to produce one or more of these biogenic amines. The behaviour of diamine oxidase was tested under the conditions present in fish silage. Addition of 12% sucrose or 2% NaCl did not affect histamine degradation. Addition of 0.05% cysteine decreased histamine degradation. Degradation occurred at all temperatures tested (15, 22 and 30°C), but not at pH 4.5. Forty-eight potential fish silage starters were tested for histamine degradation in MRS broth containing 0.005 g l⁻¹ histamine and incubated at 30°C. Indications were found that five of these isolates could degrade as much as 20–56% of the histamine added to the medium within 30 h, when used as pure cultures. No histamine degradation was observed with combinations of cultures. Histamine degradation (50–54%) by two of these isolates was also observed in ensiled fish slurry. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Biogenic amines; Histamine degradation; Diamine oxidase; Lactic acid bacteria; Fish silage

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1. Introduction

Industrial processing of fish yields approximately 60% waste (Raa and Gildberg, 1982). Fish waste is a valuable source of proteins and lipids that can be used for human and animal nutrition. These by-products can be upgraded in a cheap, environment friendly way by fermentation with lactic acid bacteria (LAB), after adding a fermentable carbohydrate (Raa and Gildberg, 1982). Due to acidification and enzymatic activities, LAB can play a considerable role in improving the organoleptic, hygienic and nutritional quality of fish by-products (Raa and Gildberg, 1982). Further research is needed to ensure that this promising preservation method will yield safe, high quality products.

Ensiled fish contains a considerable amount of the free amino acids that constitute the precursors for biogenic amines such as histamine, tyramine, putrescine and cadaverine (Haard et al., 1985). Biogenic amines are formed by decarboxylation of their precursor amino acids, as a result of the action of either endogenous amino acid decarboxylase activity (Halasz et al., 1994) or by the growth of decarboxylase positive microorganisms (Halasz et al., 1994; Silla Santos, 1996). The low pH (below 4.5 after 2 days of fermentation) and the physical characteristics of fish silage that lead to a low oxygen concentration within the ensiled fish are favourable for the action of amino acid decarboxylases (Beutling, 1992). Thus, biogenic amines may pose a potential risk in fish silage. These compounds are toxic for livestock, causing liver damage and decreasing the performance of the animals (Krzek, 1991). In susceptible humans, they can lead to a variety of cutaneous, gastrointestinal, haemodynamic and neurological symptoms (Taylor, 1986).

Biogenic amines can be degraded by amine oxidases. Diamines, such as histamine and putrescine, are degraded by diamine oxidase (DAO). The activity of these enzymes is maximum under neutral to alkaline conditions, and oxygen is necessary for their action (Beutling, 1992). DAO can provide a means of controlling histamine accumulation during the first days of ensilage, while the pH is high enough and some oxygen is still available within the product. Little is known about histamine degradation by LAB, but Voigt and Eitenmiller (1978) and Leuschner et al. (1998) found DAO activity among some dairy and meat isolates, respectively.

The aims of the present work were to select a starter culture for the production of safe fish silage and to assess the ability of diamine oxidase to degrade histamine under the conditions prevailing in this product. LAB isolates were screened for the production of biogenic amines. The effect of different conditions on the amount of histamine degraded by DAO was studied and LAB isolates were tested for DAO activity in broth and in ensiled fish slurry.

2. Materials and methods

2.1. Biogenic amine production by LAB

Seventy-seven LAB isolates obtained from naturally fermented pastes prepared from mackerel (*Scomber scombrus* Linnaeus 1785) and sucrose (22:3, w/w) incubated at 15 and 22°C were screened for their ability to produce biogenic amines by decarboxylation of the corresponding amino acids (histidine, tyrosine, lysine and ornithine). Histidine, tyrosine, lysine and ornithine degradation were tested in a pH-based differential medium for the detection of biogenic amine formation, the modified Moeller broth (Joosten and Northolt, 1987). Histamine formation was confirmed by qualitative thin-layer chromatography, according to Lieber and Taylor (1978), using silica-gel plates (F_{254} , 20 × 20 cm, thickness of layer: 0.25 mm; Merck no. 5715, Darmstadt, Germany). Aliquots of 10 µl of modified Moeller broth inoculated with the bacterial cultures under study and incubated at 30°C for 48 h were spotted onto the plates. Samples of modified Moeller broth containing added histamine were also spotted as a control. The samples were eluted with methanol–ammonia (20:1). After drying, the plates were developed with ninhydrine spray reagent (300 mg of ninhydrine, 100 ml of *n*-butanol, 3 ml of glacial acetic acid). Histamine was detected as violet-grey spots ($R_f = 0.74$). The detection level for this method was 0.01 g l⁻¹.

2.2. Functionality of diamine oxidase

The functionality of this enzyme was assessed in a model system consisting of a solution of 0.05 g l⁻¹ histamine dihydrochloride (Acros Organics, 15062-0050, Geel, Belgium) in phosphate buffer (0.5 M, pH 7) to which 633.5 units l⁻¹ of DAO (EC 1.4.3.6,

from porcine liver, 0.14 units/mg, Sigma, D-7867, St. Louis, USA) were added. Unless stated otherwise, the reaction mixture was incubated at 37°C. Several conditions were tested: (a) pH 4.5, achieved by the addition of lactic acid (racemic mixture); (b) micro-aerobic conditions, achieved by adding 0.05 g l⁻¹ cysteine to the culture medium prior to sterilisation and overlaying the reaction mixture with sterile paraffin oil; (c) effect of the usual additives intended for use in fish silage: sucrose (12%, w/w) and NaCl (2%, w/w); (d) different temperatures, which were 37, 30, 22 and 15°C).

Samples were taken at 0, 0.5, 1.0, 3.0 and 5.0 h and the reaction mixture was rapidly heated in a boiling water bath for 30 min to inactivate the enzyme. The inactivated samples were submitted to an extraction procedure according to Shore (1971) and histamine was quantified by a spectrofluorimetric method based on the reaction of histamine with *o*-phthalaldehyde (Shore, 1971).

2.3. Degradation of histamine by LAB in a model system

Forty-eight LAB cultures isolated from naturally fermented fish pastes were tested for their ability to degrade histamine in a model system, diamine oxidase assay broth, consisting of 1 g l⁻¹ glucose, 3 g l⁻¹ yeast extract, 3 g l⁻¹ tryptone, 5 g l⁻¹ NaCl, 1 g l⁻¹ Tween 80, 0.25 g l⁻¹ MgSO₄ · 7H₂O, 0.038 g l⁻¹ MnSO₄ · H₂O, 0.08 g l⁻¹ FeSO₄ · 7H₂O and 0.05 g l⁻¹ histamine dihydrochloride, pH 6.0. The isolates tested had been selected for their good growth, acidification and antimicrobial properties in previous studies (unpublished data). Mixed cultures consisting of *Lactobacillus curvatus* 22.24 and various isolates of *Lactobacillus sakei* (15.05, 15.18, 15.36 and 15.39) were also tested for histamine degradation in diamine oxidase assay broth. The histamine concentration was determined by the spectrofluorimetric method of Shore (1971). Samples were taken at time 0 and after 30 h of incubation at 30°C. When apparent, histamine degradation was confirmed by quantitative HPLC analysis of the histamine concentration. The model system and incubation conditions were the same as above, with the exception of added histamine dihydrochloride (0.075 g l⁻¹). The method for HPLC determination of histamine was based on Luten et al. (1991) and Gouygou et al. (1987).

Sample aliquots of 159 µl were derivatised in a plastic tube protected from light by adding 1.9 ml of distilled water and 0.4 ml of 1 N NaOH. This mixture was allowed to stand for 1 min and then 100 µl of an *o*-phthalaldehyde methanolic solution (1 g of *o*-phthalaldehyde l⁻¹ in methanol) was added. The tube was shaken vigorously, the reaction was allowed to proceed for 4 min and then stopped by adding 0.2 ml of 3 N HCl. The derivatised sample solution was centrifuged for 15 min at 8000 rpm, filtered through 0.2-µm millipore syringe filters (Schleicher and Schuell, FP 030/AS, Ref. 462960, 's Hertogensbosch, The Netherlands) and analysed by reversed phase-HPLC (RP-HPLC). Separation was carried out on a C₁₈ column (Spherisorb 2.5 µm ODS; 25 cm × 4.6 mm internal diameter; Chrompack, Middelburg, The Netherlands). A 20-µl aliquot of injected sample mixture was eluted isocratically over 20 min with a mixture of acetonitrile (40%, HPLC-grade, LAB-Scan, C2502, Dublin, Ireland) and 50 mM NaH₂PO₄ solution in millipore water (60%). The flow-rate was 0.70 ml min⁻¹. Detection was done with a continuous flow fluorescence meter (Spectra System Series, Thermo Separation Products, FL 3000, Riviera Beach, Florida, USA) with excitation at λ = 350 nm and emission at λ = 450 nm. All determinations were carried out in triplicate.

2.4. Degradation of histamine by LAB in ensiled fish slurry

Atlantic mackerel was purchased from a local retailer (Wageningen, The Netherlands) and ground in a meat grinder with a 4-mm die. Each batch of silage consisted of 100 g of ground mackerel to which 100 ml of an aqueous solution containing 2.4 g l⁻¹ sucrose, 0.4 g l⁻¹ NaCl and 0.2 g l⁻¹ histamine dihydrochloride were added. The resulting mixture was thoroughly homogenised using a spatula and 2% (v/w) of an 18-h MRS broth (Oxoid, CM 359, Basingstoke, UK) culture of the designated LAB isolate was added. For the DAO experiment, 10 780 units kg⁻¹ DAO were added as well. Cultures 15.18 and 15.36 (both *Lb. sakei*) were used as an inoculum in the fish experiment, since they had been found to degrade histamine in the model system. Fish slurries with added DAO were inoculated with a non-histamine degrading bacterium (*Lb. curvatus* 22.24). A control slurry was inoculated with isolate 22.24 only, to determine histamine autodegradation during ensil-

age. After thorough mixing with a spatula, the fish pastes were distributed in glass jars closed with a screw cap fitted with a water-column type air lock. Incubation was at 30°C for 4 days. Samples were aseptically withdrawn for pH measurement and spectrofluorimetric histamine determination.

2.5. Identification of LAB isolates

LAB isolates that were found to degrade histamine and some of the biogenic amine producing isolates were identified to the species level on the basis of cell morphology, Gram stain, motility, spore formation, catalase activity, gas production from gluconate, ability to grow at 15 and 45°C and acid production from ribose, arabinose, xylose, rhamnose, mannitol, sorbitol, ribitol, glycerol, fructose, mannose, galactose, glucose, lactose, maltose, sucrose, trehalose, cellobiose, raffinose, melibiose, salicin and gluconate and NH₃ production from arginine. Further tests (configuration of lactic acid, presence of *meso*-diaminopimelic acid in cell hydrolysate, peptidoglycan type and 16S rDNA sequencing) were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Darmstadt, Germany).

3. Results and discussion

The results of the tests for amino acid decarboxylation are summarised in Table 1. The production of more than one of the assessed biogenic amines was common among the isolates tested. Of the screened isolates under study, 17% produced one or more of the biogenic amines under study. This shows that attention must be paid to biogenic amine production when selecting bacterial strains for fish silage production. Most of the biogenic amine positive isolates were obligatory heterofermentative lactobacilli belonging to the species *Lb. sakei* and *Lb. curvatus* (data not shown).

To obtain a deeper insight into its potential for histamine degradation in fish silage, the effect of added DAO was investigated in a buffered model system simulating the main conditions prevailing in this product. Estimations of DAO activity in the literature have been based on determinations of oxygen and histamine consumption, histamine consumption, aldehyde, ammonia or hydrogen peroxide

Table 1

Amino acid degradation by 13 lactic acid bacteria isolated from naturally fermented mackerel–sucrose (88:12, w/w) pastes^a

Code and identity of the isolates	His	Lys	Orn	Tyr
15.07 (<i>Leuconostoc</i> or related genus)	–	+	–	–
15.09 (<i>Lactobacillus</i> sp., FH)	+	–	–	–
15.19 (<i>Lb. sakei</i>)	+	–	–	+
15.22 (<i>Leuconostoc</i> or related genus)	–	–	+	+
15.23 (<i>Lactobacillus</i> sp., FH)	–	–	–	+
15.31 (<i>Lactobacillus</i> sp., FH)	+	+	–	–
15.32 (<i>Weissella hellenica</i>)	+	–	–	–
22.04 (<i>Leuconostoc</i> or related genus)	+	–	–	+
22.07 (<i>Lactobacillus</i> sp., FH)	–	+	+	–
22.12 (<i>E. faecium</i> species group)	+	–	+	+
22.15 (<i>Lactobacillus</i> sp., OH)	+	+	–	–
22.19 (<i>Leuconostoc mesenteroides</i>)	+	–	–	+
22.36 (<i>Leuconostoc</i> or related genus)	–	–	–	+

^a His = Histidine, histamine precursor; Lys = lysine, cadaverine precursor; Orn = ornithine, putrescine precursor; Tyr = tyrosine, tyramine precursor; FH = facultative heterofermenter; OH = obligatory heterofermenter.

formation (Roscoe and Kupfer, 1972). In this experiment, histamine degradation resulting from DAO activity was determined by measuring the decrease in histamine content.

The effect of temperature on histamine degradation was noticeable (Fig. 1). The highest degradation rate was observed at 37°C, but at 22 and 15°C degradation was still considerable. These results are in agreement with the characteristics described for DAO (Schomburg and Stephan, 1993). DAO has its temperature optimum at 37°C and is active within a range of 20–63°C, retaining about 50% of its maximum activity at 20°C.

The pH of the model system was adjusted to 4.5 to assess the pH sensitivity of DAO. This pH value corresponds to the final pH achieved during the ensilage of fish. Oxygen diffusion to the interior of the fermenting fish pastes is difficult, resulting in reduced oxygen availability within the fish mass. To simulate micro-aerobic conditions, cysteine was added to the test solution and the reaction mixture was covered with sterile paraffin oil before incubating. NaCl and sucrose were chosen as additives for the production of fish silage. Model systems with adequate amounts of these compounds were also prepared to determine whether their presence interferes with DAO. The results of this set of experiments are shown in Fig. 2.

Decreasing the pH to 4.5 proved to be the most

Fig. 1. Effect of temperature on the degradation of histamine by diamine oxidase in phosphate buffer (pH 7.0). The bars represent the standard deviation ($N = 3$).

Fig. 2. Histamine degradation by diamine oxidase in phosphate buffer (pH 7.0). Effect of pH 4.5, micro-aerobic conditions (pH 7.0) and the addition of 2% NaCl or 12% sucrose (pH 7.0). The bars represent the standard deviation ($N = 3$).

limiting factor. This was not unexpected, since DAO is known to have a pH optimum of 7.0 and a pH range of 5–10 (Schomburg and Stephan, 1993). Furthermore, a precipitate was formed in the reaction mixture at pH 4.5, indicating denaturation of the enzyme. However, fish silage acidifies gradually, and will only reach pH 4.5 after 2 days of fermentation. Initially, the pH is within the range that facilitates DAO activity. During this initial period, bacterial contaminants present in the fish are still active and their decarboxylating activity can take place. Al-

though decarboxylase enzymes have their optimum activity at low pH values, they can also be active at higher pHs. The pH optimum of the L-histidine decarboxylase from one strain of *Leuconostoc oenos* was found to be 4.8, both for cell suspensions and cell-free extracts. The enzyme still showed activity at pH 7.2 and at pH values close to 3.5 (Rollan et al., 1995). Thus, the presence of DAO could be of value to prevent build-up of histamine during the early stages of ensilage. During fermentation, the majority of Gram negative bacteria disappear due to microbial

competition and acidity. In silages that reached a pH below 4.5, such bacteria would thus not contribute to biogenic amine production. However, non-starter LAB can become a problem at the later stages of ensilage, since the low pH attained will not inhibit their growth completely. In this case, another approach should be used. The use of starter cultures that have antimicrobial properties against such contaminants could reduce the risk of biogenic amine formation during the later stages of ensilage. Micro-aerobic conditions also had a limiting effect on the degradation of histamine. However, it is possible that the little amount of oxygen left within the fish mass during ensilage would be enough to allow for some decrease in the level of histamine in the silage. As it can be seen in Fig. 2, almost one half of the histamine added was degraded within a 5-h period under micro-aerobic conditions. The addition of NaCl or sucrose showed only a minor effect on the functionality of DAO. Initially, histamine degradation was hindered by the presence of NaCl.

Little is known about histamine degradation by bacteria. Kumagai et al. (1969) described some monoamine oxidase activity in the species *Sarcina lutea*. Voigt and Eitenmiller (1978), Beutling (1992) and Leuschner et al. (1998) investigated bacterial DAO activity in a rich medium containing several

nitrogen sources. In the present study, histamine degradation was initially investigated in modified MRS medium. Further experiments were performed in ensiled mackerel slurry. The results of these experiments are shown in Fig. 3 and Table 2. Histamine autodegraded in the ensiled fish slurry as it did in the model system. The histamine concentration remained stable for the first 72 h and decreased to 80% of the initial concentration after 96 h of ensilage (Fig. 3). DAO degraded histamine to about 20% of its initial concentration. Its inactivation after 24 h may be related to the decrease in pH values within the ensiled fish slurries. Fig. 3 shows that DAO was active during the early stages of ensilage and that it can be used to reduce the histamine content in fish pastes.

The effect of different LAB isolates on the histamine concentration and pH in the model system and in fish slurry silage are shown in Table 2. Four isolates (15.05, 15.18, 15.36 and 15.39, all *Lb. sakei*) were found to degrade histamine in the model system. These results were confirmed when monitoring histamine degradation by RP-HPLC. Additionally, RP-HPLC assessment indicated that one more isolate, *Lb. curvatus* 15.35, was able to degrade histamine (Fig. 4). Although it showed good results in the model system, *Lb. sakei* 15.05 was not also

Fig. 3. Histamine degradation by diamine oxidase in a fish slurry (pH 6.40) incubated at 30°C. The bars represent the standard deviation ($N = 3$).

Table 2

Degraded histamine and pH values in MRS broth with 50 mg l⁻¹ histamine (starting pH 6.49) and in ensiled fish slurry with 0.01 g l⁻¹ histamine (starting pH 6.40) after 30 and 96 h, respectively, of incubation at 30°C with lactic acid bacteria isolates or diamine oxidase^a

	Model system		Ensiled fish slurry	
	Degraded histamine (g 100 g ⁻¹)	Final pH	Degraded histamine (g 100 g ⁻¹)	Final pH
<i>Lb. sakei</i> 15.05	56.18±4.28	3.72	ND	ND
<i>Lb. sakei</i> 15.18	35.81±4.53	3.75	49.85±3.60	4.35
<i>Lb. sakei</i> 15.36	28.67±4.75	3.78	53.63±2.54	4.42
<i>Lb. sakei</i> 15.39	19.61±3.15	3.74	ND	ND
DAO	99.49±0.01	7.00	86.20±5.99	4.50
Control	16.36±3.15	6.49	33.64±1.81	4.51

^a The results of isolates that were not found to degrade histamine are not shown. The numbers represent the average±S.D. of three values. ND = Not determined.

Fig. 4. Histamine degradation measured by RP-HPLC in diamine oxidase assay broth with added histamine (750 mg ml⁻¹, pH 6.49) after 24 h of incubation at 30°C with different lactic acid bacteria isolates and diamine oxidase. The bars represent the standard deviation ($N = 3$).

used for the degradation experiment in ensiled fish slurry because it does not yield stable silages. *Lb. sakei* 15.39 was not used because it gave similar results to 15.36 in the model system experiment. None of the combinations of isolates tested showed histamine degradation under the conditions of our experiment.

The pH decrease in fish slurry silage is much slower than in the buffered model system, therefore, more time was allowed in the fish experiment than in the model system (96 and 30 h, respectively). Nevertheless, the pH of the ensiled fish slurries did not reach the very low values achieved in the model system (Table 2). This may be due to the difference

in buffering capacity between both systems. DAO was less efficient in the fish slurry silage than in the model system (Table 2) perhaps due to the gradual decrease in pH in the first. However, a higher degradation could be observed for the LAB in the silage than in the model system. The different pH decrease patterns might provide an explanation for this. Histamine autodegradation was comparable in both cases.

In conclusion, attention should be paid to biogenic amine production by LAB intended for fish silage starters. A considerable proportion of the LAB isolated from fish display such specific decarboxylase activity and may cause problems in the later

stages of the ensilage. Ideally, fish silage starters should be able to inhibit undesirable non-starter LAB.

The results obtained showed that DAO is able to degrade histamine during the early stages of fish silage fermentation, when the pH is still above 4.5 and there is some oxygen left within the fish mass. Indications were found that four of the 48 LAB isolates tested could degrade histamine in a model system. Histamine degradation by two of these isolates was also observed in the ensiled fish slurry. Their use for fish silage preparation could reduce the risks posed by this amine.

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