**Preparation of marine silage of swordfish, ray and shark
visceral waste by lactic acid bacteria**

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Headline: Bio-silage of fish visceral waste by lactic acid bacteria

ABSTRACT

The goal of the present work was to study the efficacy of several lactic acid bacteria (LAB) as bio-silage inoculants of swordfish, ray and shark viscera by-products. A sterilised medium was initially used as a model system for assessing the potential of these microorganisms in batch and fed-batch cultures with re-neutralisation. In all cases, batch cultivations without re-neutralisation led to the highest production and yields of the main metabolites of LAB fermentation (lactic and acetic acids). The dynamics of these metabolites followed a conversion pattern from lactic to acetic acid with a final joint concentration over 16 g/L and final pH lower than 4.5. Both productions were modelled by means of logistic modified equations. In addition, the capability of LAB to ferment the fish visceral wastes was always high and easily reproducible. Finally, the results obtained for non-sterilised fermentations with *Lactobacillus casei* CECT 4043 were similar to those obtained for sterilised media, and a stable material was obtained after 72 h of culture.

Keywords: visceral waste upgrading; lactic acid bacteria; bio-silage; marine peptones; fish by-products; environmental pollution.

1. INTRODUCTION

Fishing and aquaculture activities play an important economic and social role in the European Atlantic region, in which Port of Vigo (NW, Spain) is the largest fishing port with more than 150,000 tons landed in 2008 (PAV, 2008). Many seafood canning industries and fish processing companies are also located in

the harbour of this city. However, more than 35% of the total weight of captured and cultivated fishes is a waste product that leads to environmental pollution. Excluding fish discards and skeletons, the largest part of this by-product consists of viscera; however, visceral waste has great potential to be used as a protein supplement in animal feeds (Fai et al. 1997). In most cases they are upgraded into fishmeal, but the silage process has been reported to be a feasible, simple and lower-cost alternative (Vidotti et al. 2003). During the last two decades, fish silage has been successfully used as a low cost ingredient in aquaculture diets (Espe et al. 1992).

Fish silage can be defined as a liquid-pasty product generated from fish (dead fish, unused species, marine fishing by-products, commercial fish waste and industrial residues or residual fractions from marine peptone processing) in an acidic medium (Raa and Gildberg 1982). Traditionally, this process has been carried out by adding acids, mainly sulphuric and formic acid, to the wastes, homogenising the bulk and developing acidity to conserve it (Vizcarra-Magaña et al. 1999). Nevertheless, this mixture is not very nutritious, not very acceptable for being used in animal fodders and requires managing strong acids (Raghunath and Gopakumar 2002). However, using other organic acids (acetic, lactic, propionic) involves higher costs and simulates the bio-conversions that could be performed by lactic acid bacteria (LAB) fermentation. This last biological alternative is a microbiological process termed as bio-silage, and has considerable advantages over chemical silage: higher acceptability for being used in animal feed, the probiotic characteristics of the inoculum, and the possibility of promoting and controlling adequate fermentation patterns.

Furthermore, it is a simpler, faster, more environmentally friendly, and cost-efficient process (Ahmed and Mahendrakar 1996; Raghunath and Gopakumar 2002).

Among the LAB previously used for biological silage of marine by-products, we find: *Lactobacillus plantarum* (Raghunath and Gopakumar 2002), *L. brevis* (Uchida et al. 2004), yoghurt-bacteria such as *L. bulgaricus* and *Streptococcus thermophilus* (Yoon et al. 1997), native LAB from digestive tract of freshwater carps (Ganesan et al., 2009), as well as *L. buchneri* and *L. casei* (Vázquez et al. 2008). In all cases, an additional source of carbohydrates had to be added in the form of molasses or dextrose (Hammoumi et al. 1998; Vázquez et al. 2004a).

On the other hand, fish visceral waste are an excellent source of proteolytic activities (Murado et al. 2009), marine peptones for supporting bacteriocin production (Vázquez et al. 2004a), recovery of carotenoid from shrimp biowaste (Bhaskar et al., 2007), lactobacillus growth (Aspmo et al. 2005; Horn et al. 2005), biomass production from marine bacteria (Vázquez et al. 2004b), hyaluronic and lactic acid production (Vázquez et al. 2009), recovery of oil from viscera (Rai et al., 2010) and also microbial enzyme production (Ellouz et al. 2001; Vázquez et al. 2006; Rebah et al. 2008).

The aim of this work is to evaluate the suitability of different LAB for ensilaging three fish viscera substrates supplemented with glucose as carbon source. Cell growth, metabolite productions and substrates uptakes will be measured and

lactic and acetic acids formation will be modelled. Two experimental modalities are performed and compared in all cases: glucose fed-batch and re-neutralised cultures versus batch cultivation without pH-control.

2. MATERIALS AND METHODS

2.1. Preparation of fish viscera

Visceral waste from swordfish (*Xiphias gladius*), thornback ray (*Raja clavata*) and shark (*Isurus oxyrinchus*) were sampled immediately after industrial processing and prepared on the same day. Visceral masses (stomach and intestine) were ground, using meat grinder, and the homogenates were characterised by determining the chemical composition. Swordfish substrates were stored at -80°C until use (no changes in chemical composition were observed in 2 months of storage) and ray wastes were processed directly to avoid the production of ammonium compounds that inhibit the growth of LAB (data not shown).

2.2. Microbiological methods

The microorganisms are shown in Table 1. Stock cultures were stored at -80°C in MRS commercial medium (Pronadisa, Hispanlab S.A., Spain) with 25% glycerol. Inocula (0.5% w/v) consisted of cellular suspensions from 24-h and 12-h (Lc HD1) cultures on MRS medium, concentrated by centrifugation (4000 $\times$ g, 10 min) until the required weight. The initial concentrations of these inocula for the different LAB were established in the following values (as Log (cfu/mL)): 11.2 (Lb 3.04), 11.3 (Lb 8.01), 10.3 (Lb 10.01), 10.6 (Ln 3.04), 10.7 (Pc 1.02) and 11.6 (Lc HD1).

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118 Fermentations were carried out in duplicate using 300 mL Erlenmeyer flasks
119 with 25 g of waste substrate, 8 mL of glucose (175 g/L) and 2 mL of the
120 corresponding inoculum of LAB. This level of glucose (~40 g/L) was formulated
121 in order to duplicate the initial concentration of sugar presents in MRS medium.
122 The experiments were carried out at 25°C and orbital shaking was at 200 rpm
123 (optimal conditions for LAB growth but not excessively difficult of maintaining to
124 an industrial scale). In all cases, the initial pH was adjusted to 7.0 with NaOH 5
125 N and the media were sterilised separately with a steam flow at 101°C for 60
126 min. In fed-batch cultures at 24 h, the glucose was increased to 20 g/L by
127 added of sterile glucose solution of 500 g/L. In addition, in these cultures,
128 neutralisation to pH 7.0 was performed at 24 h with 5N NaOH. Equal volumes of
129 sterile distilled water were added to controls, which had a spontaneous pH
130 evolution.

132 **2.3. Analytical methods**

133 At pre-determined times, each experimental unit was divided into two aliquots.
134 The first aliquot was used for quantifying viable cells by means of a plate count
135 technique on MRS agar medium. Serial tenfold dilutions were prepared in
136 peptone-buffered solutions, and 0.1 mL samples were plated in quadruplicate,
137 incubated at 30°C for 48-72 h, and manually counted. The results were
138 expressed as colony-forming units per mL (cfu/mL). The second aliquot (the
139 rest of the culture) was centrifuged at $6,000 \times g$ for 12 minutes, and the
140 supernatant was used for determining the proportions of proteins, lactic and
141 acetic acid and reducing sugars.

The moisture content was determined by drying the homogenate in an oven at 105°C until a constant weight was obtained (AOAC 1990). The crude protein content was calculated by converting the total nitrogen concentration ($6.25 \times N$) determined with the Kjeldahl procedure (Havilah et al. 1997). Fat was determined by the method described in AOAC (1990), using the Soxhlet system. Ash content was measured by dry ashing in an oven at 550°C for 24 h. Reducing sugars were quantified by means of a 3,5-dinitrosalicylic reaction (Bernfeld 1951). Soluble proteins were obtained using the method of Lowry et al. (1951). Thiobarbituric acid reactive substances (TBARS) were determined according to Ohkawa et al. (1978). Lactic and acetic acids were measured by HPLC, after the samples had been membrane filtered (0.22 µm Millex-GV, Millipore, USA) using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow = 0.4 mL/min) at 65°C and a refractive-index detector.

2.4. Mathematical equations and numerical methods

Although the main objective of our report is to study the ability of LAB to ferment different fish visceral wastes, the most important variable to control for obtaining a material stable and free of opportunistic and pathogen bacteria is the production of lactic and acetic acids (Raghunath and Gopakumar 2002). Thus, the mathematical models used to describe the patterns of lactic and acetic acid production were (Vázquez et al. 2008):

$$L = \frac{L_m}{1 + \exp\left[2 + \frac{4 \cdot v_L}{L_m} \cdot (\lambda_L - t)\right]} \cdot [1 - k_L \cdot A(t)] \quad (1)$$

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$$A = \frac{A_m}{1 + \exp\left[2 + \frac{4 \cdot v_a}{A_m} \cdot (\lambda_a - t)\right]} \cdot [1 + k_A \cdot L(t)] \quad (2)$$

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170 where:

171

172 L : lactic acid concentration (g/L),

173 t : time (h),

174 L_m : mathematical constant (g/L),

175 v_L : apparent maximum lactic acid production rate (g L⁻¹ h⁻¹),

176 λ_L : mathematical constant (h),

177 k_L : parameter of proportionality (L/g of acetic acid),

178 A : acetic acid concentration (g/L),

179 A_m : mathematical constant (g/L),

180 v_a : apparent maximum acetic acid production rate (g L⁻¹ h⁻¹),

181 λ_a : mathematical constant (h),

182 k_A : parameter of proportionality (L/g of lactic acid),

183 $L(t)$: lactic acid concentration at time $-t-$ (g/L),

184 $A(t)$: acetic acid concentration at time $-t-$ (g/L),

185

186 Fitting procedures and parametric estimations calculated from the results were

187 carried out by minimising the sum of quadratic differences between the

observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro-‘Solver’ of the Microsoft Excel XP spreadsheet.

2.5. Statistical analyses

The values of the differences among experimental data at the initial and final time of fermentation for substrate uptakes and cell counts were subjected to one-way analysis of variance (ANOVA). The means were compared using a contrast test at $\alpha = 0.05$. Statistica 8.0 (StatSoft, Inc. 2007) was used for these analyses.

3. RESULTS AND DISCUSSION

The chemical composition as percentages (mean $\pm$ confidence intervals for $n=3$ and $\alpha=0.05$) for swordfish visceral waste was 66.04 ± 0.80 , 26.10 ± 1.04 , 5.46 ± 0.52 and 2.41 ± 0.74 for moisture content, proteins, fat and ash respectively. In the case of ray, these values were 67.21 ± 1.16 , 24.85 ± 0.93 , 4.37 ± 0.39 and 3.57 ± 1.03 . Finally, shark composition was 64.88 ± 2.03 , 25.75 ± 1.08 , 3.87 ± 0.89 and 5.50 ± 1.52 . The LAB used in the present work were selected due to their high fermentative ability in relation to marine peptones from fish by-products and their capability to produce bacteriocins (Vázquez et al. 2008).

3.1. LAB fermentation of swordfish viscera

Figure 1 shows the experimental profiles obtained with LAB fermentations of swordfish viscera. In general, the kinetic trends were very similar for all the

bacteria. The spontaneous evolution of pH followed an exponential pattern and reached final values of up to 4.39. The bacteria responded favourably to the re-neutralisation in the sense that the exponential profile of pH acidification was recovered and had higher final values. Microbial population changes did not show the classical sigmoid profiles (Vázquez and Murado 2008). In our cases, the profiles showed an initial growth and subsequent exponential drop after 24 h. Similar tendencies were also observed by other authors using fish industry by-products as sardine wastes (Fai et al. 1997) and undefined fish viscera (Ahamed et al. 1996) fermented with lactobacilli.

The values of nutrient uptakes and the drop in biomass were also calculated (data not shown). Based on these values, the differences between the decreases in biomass for Lb 8.01, Lb 3.04, Lb 10.01 and Ln 3.04 for the two kinds of fermentations tested were not significant ($P>0.05$). A smaller decrease in cell counts was achieved in the re-neutralised cultures from Pc 1.02 and Lc HD1.

A heterofermentative metabolism was observed in almost all cases when lactic acid was converted to acetic acid from an early culture phase. The high relative concentrations of acetic acid suggest that part of the lactic acid secreted could have been metabolised to an equivalent amount of acetic acid with a reversible lactate dehydrogenase. These experimental profiles were accurately fitted to equations 1 and 2. All the values predicted in the non-linear adjustments produced high linear correlation coefficients among experimental and expected data ($r > 0.90$). These findings were in agreement with previous outcomes and

are considered a response to the stress of experimental conditions (Guerra et al. 2007), culture media formulated with fish peptones (Vázquez et al. 2008) and the aerobic conditions combined with a low glucose concentration (Sedewitz et al. 1984).

Recently, Taniai et al. (2008) reported that lactate productions were also transformed to acetate in aerobic fermentations with *Streptococcus pneumoniae* by means of a sequential pathway catalysed by lactate oxidase, pyruvate oxidase and acetate kinase. Similarly, the lactic acid initially produced from glucose was modified to acetic acid after glucose exhaustion in an early stationary growth phase of *L. mesenteroides* and *L. plantarum* (Wagner et al. 2005; Goffin et al. 2006). In these cases, however, the reversible reaction, lactate to pyruvate, was modulated and controlled by lactate dehydrogenase.

In our results, the highest acetic acid conversion was obtained with *Lactobacillus casei* in the two culture types. Higher yields of metabolite production in relation to glucose consumption were obtained using batch cultivations. This response of decreasing yields of metabolite production by LAB to the stress conditions of pH re-neutralisation has been reported previously (Guerra et al., 2005; Vázquez et al., 2005; Guerra et al., 2007). These authors have showed for different LAB and culture media that repeated re-neutralisations led to lower yield in the production of metabolites as ethanol, lactic and acetic acids per glucose consumed. In addition, more than 45 g/L of soluble protein and less than 20 mg/L of TBARS (data not shown) were observed at the end of the fermentations.

3.2. LAB fermentation of ray viscera

Previous outcomes obtained using ray viscera bulks stored at -20°C for 1 week led to LAB fermentation being inhibited (data not shown). Cultivations were thus carried out immediately after these substrates were collected from fish processing. Figure 2 shows the time course of LAB silage under the two experimental conditions defined. In relation to the biomass decreasing over time, no statistical significance (Lb 3.04 and Lb 10.01: $P>0.05$), low significance (Lb 8.01 and Pc 1.02: $P=0.05$) and high significance (Lc HD1: $P<0.01$) were obtained when the batch and fed-batch procedures were compared.

The production of organic acids by LAB did not follow the common sigmoid profiles that characterise the kinetics of these metabolites (Vázquez et al. 2004a). Nevertheless, acetic acid formation was also in accordance with the lactic acid consumption as shown in the previous waste material assayed. The values simulated by the modified logistic models 1 and 2 were satisfactory in all the cultures. The linear correlation coefficients between the predicted and observed values were ranged between 0.933 and 0.999.

Lower yields of metabolite production in relation to sugar uptake were obtained in fed-batch cultures. Hence, superfluous substrate consumption occurs in this sort of cultivation, which generates an unnecessary cost for this upgrading process. The final pH in the re-neutralised cultures (up to 5.0) also limits their usefulness for preserving fermented material. In all cases, the final

concentration of soluble protein was up to 45 g/L and less than 15 mg/L for TBARS.

3.3. LAB fermentation of shark viscera

Experimental results of these fermentations are depicted in Figure 3. It can be observed that similar experimental trends to those obtained for other viscera were generated for all the variables measured. In the growths of Lb 8.01, Pc 1.02 and Lc HD1, the differences among fed-batch and batch cultures were statistically significant. No significant differences were shown for the other bacteria assayed.

Metabolic profiles with transformation between lactic and acetic acids were also produced in all the microorganisms selected to silage the visceral waste. Moreover, the consumption of glucose was not very effective in the fed-batch fermentations in comparison with conventional cultures.

3.4. Bio-silage of fish viscera without previous sterilisation

In order to simplify the bio-silage process, cultures were carried out without previous sterilisation. Moreover, taking into account the lower efficiency of re-neutralisation and fed-batch studies, fermentations with Lb 3.04 were run in a conventional batch system.

Figure 4 shows experimental data of the fermentations in a non-thermal processing substrate. In both wastes the cultivation dynamics were similar and in agreement with early results in sterilised media. Higher consumptions of

glucose (35.01 g/L) and proteins (7.07 g/L) were achieved in shark compared with ray (34.64 and 6.96 g/L) and swordfish (31.13 and 3.96 g/L) wastes. For metabolite production, a higher final lactic acid concentration and lower acetic acid concentration were obtained with swordfish media. Markedly higher ($P<0.001$) total organic acid production (lactic+acetic) occurred in ray media than the rest of viscera tested. Excellent mathematical fits were achieved by the proposed equations ($r>0.980$). Concentrations of soluble protein up to 50 g/L and TBARS down to 15 mg/L were also generated at 216 h of culture.

Nevertheless, further experiments should be carried out in order to study the possibility of incorporating the fermented material into animal fodder. In addition, another low-cost source of carbohydrates (e.g. mussel processing wastewaters, sugarcane bagasse, wheat flour wastes) should be tested in order to reduce production costs of the bio-silage process.

4. CONCLUSIONS

The most important technological issue in the development of bio-silage with fish by-products is the ability of LAB strains to ferment the waste materials and thus to produce organic acids, basically lactic and acetic acids, in order to preserve and generate ingredients for animal feed. This work shows that all LAB strains used in the two fish viscera substrates led to metabolite productions and pH values suitable for bio-silage fermentation and preservation of the corresponding materials. Furthermore, re-neutralisation and fed-batch cultivation did not lead to a significant improvement in the acid production, as they obtained lower production yields. In non-sterile cultures using *Lactobacillus*

casei as the inoculum, a stable fermented material from swordfish and ray visceral waste could be obtained with only 72 hours of cultivation. Finally, the equations presented in this work fitted the metabolite dynamics well and they could be used to follow and predict the production of organic acids.

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TABLE

Table 1: Lactic acid bacteria used.		
Strains LAB	References	Key IIM
<i>Lactobacillus plantarum</i>	CECT 220	Lb 8.01
<i>Lactobacillus buchneri</i>	CECT 4111	Lb 10.01
<i>Lactobacillus casei ssp. casei</i>	CECT 4043	Lb 3.04
<i>Lactococcus lactis ssp. lactis</i>	HD1-IIM	Lc HD1
<i>Leuconostoc mesenteroides ssp. mesenteroides</i>	CECT 219	Ln 3.04
<i>Pediococcus acidilactici</i>	NRRL B-5627	Pc 1.02
CECT: Spanish Type Culture Collection (University of Valencia, Spain).		
NRRL: Northern Regional Research Laboratory (Peoria, Illinois, USA).		
HD-IIM: Department Animal Science, University of Wyoming (Wyoming, USA)		
Key IIM: Abbreviated notation used in this work.		

FIGURE CAPTIONS

Figure 1: Bio-silage of swordfish visceral waste by LAB (Lb 8.01, Lb 3.04, Lb 10.01, Lc HD1, Pc 1.02 and Ln 3.04) with re-neutralisation and glucose fed-batch (●) and without (○). L: lactic acid, $\log [N \text{ (cfu/mL)}]: \log_{10} \text{ (cfu/mL)}$, G: glucose as reducing sugars quantification, Pr: soluble protein, A: acetic acid. Solid lines show the fits of the experimental data (points) to equations 1 and 2; dotted lines only represent the experimental profiles. Error bars are the confidence intervals for $n=2$ and $\alpha=0.05$.

Figure 2: Bio-silage of thornback ray visceral waste by LAB with re-neutralisation and glucose fed-batch (●) and without (○). Keys and notations as in Figure 1.

Figure 3: Bio-silage of shark visceral waste by LAB with re-neutralisation and glucose fed-batch (●) and without (○). Keys and notations as in Figure 1.

Figure 4: Bio-silage of thornback ray (●), swordfish (○) and shark (△) viscera by Lb 3.04 without previous sterilisation of culture media. Keys and notations as in Figure 1.

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4