Revista Mexicana de Ingeniería Química

Vol. 7, No. 3 (2008) 195-204

UTILIZATION OF FISHERIES BY-CATCH AND PROCESSING WASTES FOR LACTIC ACID FERMENTED SILAGE AND EVALUATION OF DEGREE OF PROTEIN HYDROLYSIS AND *in vitro* DIGESTIBILITY

APROVECHAMIENTO DE FAUNA DE ACOMPAÑAMIENTO DEL CAMARÓN Y DESPERDICIOS DE FILETEADO DE PESCADO PARA LA PRODUCCIÓN DE HIDROLIZADOS PROTEICOS Y EVALUACIÓN DEL GRADO DE HIDRÓLISIS Y DIGESTIBILIDAD *in vitro*

J. C. Ramírez- Ramírez[1,3], S. Huerta[1], L. Arias[2], A. Prado[1] and K. Shirai[1*]

[1] *Departamento de Biotecnología, Laboratorio de Biopolímeros*
[2] *Departamento de Biología de la Reproducción*
Universidad Autónoma Metropolitana – Iztapalapa.
San Rafael Atlixco No. 186. Col. Vicentina, C.P. 09340., Mexico D.F.
[3] *Universidad Autónoma de Nayarit Ciudad de la Cultura Amado Nervo, C.P. 63155 Tepic, Nayarit. México.*

Received 7th of May 2008; Accepted 26th of September 2008

Abstract

The purpose of this study was to produce protein hydrolysates from lactic acid fermentation of three sources of fish wastes: Shrimp by catch (SC), *Sphyraena ensis* wastes (SB) and mixture of fisheries processing wastes from several species (MixW). MixW were added with several sugar cane molasses concentrations as the carbon source, 180 $g.kg^{-1}$ of sugar molasses gave the fastest acidification. The maximum concentration of lactic acid (P_{max}) was significantly higher with *Lactobacillus sp.* B2 than that obtained with *Lb. plantarum*. MixW was selected for scaling up and inoculated with *Lactobacillus* sp. B2 due to the enhanced lactic acid production and availability. According to microbiological and chemical analyses, the fermented product was well preserved due to the acid produced and the reduction in a_W (0.94) that inhibit spoilage microorganisms and putrefaction. The coefficient of protein hydrolysis at 144 h of fermentation was significantly higher (0.94) than the obtained with raw MixW (0.12). The coefficient of protein *in vitro* digestibility (CPD) was also determined, which was higher, 0.88, than raw MixW (0.69).

Keywords: fish wastes, *Lactobacillus*, protein hydrolysis, digestibility, lactic acid.

Resumen

El propósito de este estudio fue producir hidrolizados proteicos por fermentación ácido láctica de tres fuentes de desecho de pescado: Fauna de acompañamiento del camarón (SC), desechos de bicuda *Sphyraena ensis* (SB) y una mezcla de desperdicios del fileteado de varias especies de pescado (MixW). MixW fue mezclada con varias concentraciones de melaza de caña de azúcar (60, 120 y 180 $g.kg^{-1}$) como fuente de carbono, 180 $g.kg^{-1}$ fue la cantidad de melaza que produjo acidificación más rápida. Se evalúo la actividad de dos lactobacilos, *Lactobacillus sp.* B2 presento la mayor concentración de ácido láctico (P_{max}) que con *Lb. plantarum.* MixW y *Lactobacillus* sp. B2 fueron seleccionados para el escalamiento. La masa fermentada obtenida de acuerdo a los análisis microbiológicos y químicos realizados fue conservada debido al ácido producido y la reducción en a_W (0.94). El coeficiente de hidrólisis de proteína de este producto a las 144 h de fermentación fue significativamente más alto (0.94) que el obtenido con MixW sin fermentar (0.12). El coeficiente de digestibilidad *in vitro* de proteína (CPD) también fue más alto (0.88) que el de MixW sin fermentar (0.69).

Palabras clave: desperdicios de pescado, *Lactobacillus*, hidrólisis de proteína, digestibilidad, ácido láctico.

1. Introduction

The processing and filleting in the fish industries, as well as the presence of fish species with no commercial value, generate wastes that cause environmental problems mainly due to a non-proper disposal. For instance, Mexico produced approximately 912,562 ton of fisheries by-products

* *Corresponding author. E-mail: smk@xanum.uam.mx*
Tel: +(52) 5558044921; Fax: +(52) 5558044712

Publicado por la Academia Mexicana de Investigación y Docencia en Ingeniería Química A.C.

and 9,000 ton of non commercial species of shrimp by-catch (SC) in 2003 (Anonymous, 2003). These fish wastes can be transformed into fish meal which has an important market for animal feed. Unfortunately, the high production costs and depletion of fish species used as raw mater have caused a significant increase in fish meal prices.

Alternatively, several efforts have been driven to find alternative protein sources throughout a less pollutant and cheaper processes to stabilize the aquatic wastes. The lactic acid fermentation has been traditionally applied as a preservation method, in which the acids produced inhibit the growth of spoilage microorganisms (Arason, 1994). Biological acidification is produced by lactic acid fermentation and it is cheaper with higher nutritional value than the product preserved by chemicals. Besides, offers an alternative and promising process of recovery of value-added compounds from aquatic sources, such as proteins, lipids, minerals and chitin (Cira *et al*., 2002; Plascencia *et al.*, 2002). There are other potential applications such as the use as natural substrate and inducer to produce enzymes (Matsumoto *et al.*, 2004), ingredients in culture media for microorganism growth (Horn *et al*., 2005), for production of lysine (Coello *et al*., 2000), bacteriocins production (Vazquez *et al*., 2005), fish sauce production (Gildberg, 2004) and other compounds such as peptides which are of interest in health and cosmetology (Ravallec *et al*., 2001).

In the lactic acid fermentation (LAF) lower amounts of other compounds, such as diacetyl, hydrogen peroxide and bacteriocins might be produced along with the acid (Ray, 1996). The pH below 4.5 inhibits the growth of harmful microorganisms, thus the shelf life of the silage is increased. Lactic fermentation is susceptible to stabilize the fat of fish silage and this improves animal food acceptability. Furthermore, some lactic acid bacteria are able to degrade biogenic amines by means of amino oxidases (Dapkevicius *et al*., 2000). Moreover, other cheap by-products (such as agricultural wastes) can be used as carbon source for lactic acid bacteria in the fermentation.

During LAF, the lactic bacteria play an important role in the acidification and subsequent activation of digestive endogenous proteases, which participate in the liquefaction (Shirai *et al*., 1997). Consequently, protein hydrolysis is attained and it improves the digestibility due to peptide production, as well as the production of highly soluble free amino acids that exhibit high valuable nutritional properties. Proteins from fermented silages are reported to be more digestible than those from acid addition (Vidotti *et al*., 2002). However, the use of inoculums and the amount of carbon source that guarantees a rapid bacterial growth and acidification are required for the fermentation control.

Therefore, the aim of this work was to apply a traditional method to preserve the fish waste in a controlled lactic acid fermentation at a pilot scale that allow the protein hydrolysis thereby increase the digestibility of fisheries by-catch and fish processing wastes. For this purpose several types of fisheries by-catch and by-products were evaluated in lactic acid fermentation, as well as the establishment of molasses level and selection of a suitable starter. The microbiological counts, acidification profiles, sugar consumption and chemical composition were also determined.

2. Materials and methods

2.1 Materials

2.1.1. Fish wastes

Fresh fish and fish wastes were obtained from the fisheries located in port of San Blas, Nayarit (Mexico) and they were shipped in ice to the laboratory in Mexico City. Fish and by-products were minced through a 5 mm sieve using a meat mincer (Torrey 32-3, Mexico) and stored at -20°C until used. Three types of fish and fish wastes were used in this study and they are described as follows:

i) Shrimp by catch (SC) were whole fish of several non-commercial species caught along with shrimp that were identified according to CONABIO (National Commission for the Knowledge and Use of the Biodiversity, Government of Mexico), as yellow fin menhaden (*Brevoortia smithi*), sardine (*Harengula jaguana*), spotfin mojarra (*Gerres cinereus*), shortfin humpback grunt (*Microlepidotus brevipinnis),* spotted head sargo (*Anisotremus dovi*), brassy grunt *(Orthopristis chalceus),* striped sea chub *(Kyphosus analogus),* blue bobo *(Polydactylus approximans),* yellow bobo *(Polydactylus operculari),* pacific moonfish *(Selene peruviana),* longfin salema *(Xenichthys xanti)* and bigscale goatfish *(Pseudupeneus grandisquamis)*.

ii) Mexican barracuda *(Sphyraena ensis)* wastes (SB) were comprised of heads, viscera, skin, bones and residual meat.

iii) Mixture of fisheries processing wastes (MixW), which contained heads, viscera, skin, bones and residual meat of the following species: salema butterfish (*Peprilus snyderi), Sphyraena ensis,* derbio (*Trachynotus ovatus)*, meagre (*Argyrosomus regius)* and common two-banded seabream (*Diplodus vulgaris)*.

2.1.2. Starters

Lactobacillus plantarum (APG-Eurozym) and *Lactobacillus* sp. B2 (isolated from shrimp wastes) were cultivated in Man Rogosa and Sharpe broth at 30°C during 24 h until a concentration of $1X10^9$ cfu ml^{-1} (Shirai *et al*., 2001).

2.1.3. Carbon source

Sugar cane molasses was supplied by sugar mill "El Molino S.A. de C. V.", Nayarit, (Mexico). The chemical composition was determined according to standard techniques (AOAC 1990): water content 251 $g.kg^{-1}$, ashes 104 $g.kg^{-1}$ (wet basis) and soluble carbohydrates 557 $g.kg^{-1}$ (wet basis).

2.2 Flask scale experiments

2.2.1. Sugar cane molasses added to lactic acid fermentation (LAF) of fish waste

MixW were mixed with sugar cane molasses (60, 120 or 180 $g.kg^{-1}$, wet basis) and 50 $ml.kg^{-1}$ (wet basis) of *Lactobacillus plantarum* as inoculum. 20 g of the mixture was placed into glass flask with 30 g of capacity and incubated at 30 ºC. Fermentations without the addition of inoculum were used as control. pH, lactic acid and total soluble sugars concentrations were determined.

2.2.2. Type of fish waste in LAF

The fermentations were carried out using three sources of fish waste: i) SC, ii) SB and iii) MixW. The sugar cane molasses amount was set at 180 $g.kg^{-1}$, *Lactobacillus plantarum* (50 $ml.kg^{-1}$, wet basis) was added and incubated at 30°C. pH and lactic acid concentrations were determined.

2.2.3. Starter selection

The MixW were mixed with 180 $g.kg^{-1}$ of sugar cane molasses and inoculated with (50 $ml.kg^{-1}$, wet basis) of each of the starters. Fermentations without inoculums were used as control and incubation was realized at 30°C. pH, lactic acid and total soluble sugars concentrations were determined.

2.3. Pilot scale in packed bed column reactor

The fermentation was scaled-up upon the optimum results attained from the flask scale experiments, 50 $ml.kg^{-1}$ of *Lactobacillus* sp. B2 as starter and 180 $g.kg^{-1}$ of sugar cane molasses. The fermentation was carried out in packed bed column reactor which was filled up with 13.6 kg of MixW with added molasses and starter and fermented in a controlled temperature room (30°C) during 240 h. pH, water activity (a_W), lactic acid, total soluble sugars concentrations, microbiological analysis, degree of protein hydrolysis and digestibility were determined.

2.4. Microbiological examination

The microbial growth was determined by colony enumeration of samples from packed bed reactor. Decimal dilutions were prepared by homogenization in saline solution (0.9 $g.l^{-1}$) of NaCl using an Ultraturrax (460003 Sper Scientific) for 3 minutes at 12,000 rpm. The media used to determine total mesophiles, coliforms, lactic acid bacteria, yeast and fungi were the standard methods agar, eosine methylene blue, Man Rogosa Sharpe and potato dextrose agar, respectively (Shirai *et al*., 2001).

2.5. Analyses of samples

The pH of the samples was measured using an electrode (pH 210 HANNA, Italy). The samples were diluted in distilled water (1:10) and the lactic acid concentration was determined by titration with NaOH 0.1N until a final pH of 7.5, and expressed as mmol of lactic acid per gram. The total soluble sugars were determined by the method of phenol sulfuric acid (Dubois *et al*., 1956). a_w was measured using the cooled mirror (dew point) technique (AQUALAB CX-2, Labsen Scientific, Co. Home Word IL). The MixW and samples of silage were analyzed using standard methods (AOAC, 1990). Dry matter (DM) and ash contents were determined by methods 950.46B and 938.08, respectively. Crude fat were determined by the solvent extraction method using ethylic ether during 16 hours of extraction. Nitrogen was determined using the Kjeldahl method to measure crude protein (N x 6.25) (984.13 in AOAC, 1990). All determinations were carried out by triplicates.

2.6. Determination of α-amino acids and coefficient of protein hydrolysis (CPH)

The content of α-amino acids were determined using a methodology based on the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Benjakul and Morrisey, 1997). The samples were dissolved in buffer of phosphates 0.2 M pH 7 in a ratio 2:3 (silage:buffer). The mixture was homogenized and centrifuged at 16,000 x *g* during 10 min (Beckman J2-M, Fullerton USA). The supernatant was used to determine the degree of protein hydrolysis as follow: 64 µl of supernatant, 1 ml of buffer of phosphates (0.2 M, pH 8.2) and 0.5 ml of TNBS solution (0.1 $g.l^{-1}$) were shaken in vortex during 10 seconds and it was placed in water bath at 50ºC for 30 minutes in the dark. The reaction was stopped by the addition of 1 ml of sodium sulfite 0.1 M. The mixture was kept at room temperature during 15 minutes and the absorbance was measured at 420 nm in a spectrophotometer (JENWAY, Ltd. Felsted, U.K). Residual sugars molasses in the samples can react with the TNBS, in order to correct these interferences the corresponding concentrations of sugars were prepared by dilution of sugar cane molasses and the TNBS method was applied. The obtained absorbance was subtracted from the total absorbance in order to avoid overestimation of α-amino acids concentration. The concentration of α-

amino acids was determined with known concentrations of L-leucine and it was expressed as mM of α-amino acids. The coefficient of protein hydrolysis (CPH) was calculated using Eq. (1)

$$CPH = \frac{\left[\left(NH_2\right)_{tx} - \left(NH_2\right)_{t_0}\right]}{\left[\left(NH_2\right)_{HT} - \left(NH_2\right)_{t_0}\right]} \qquad (1)$$

where: $(NH_2)t_x$ is the amount of terminal α-amino groups released at the time of fermentation t_x.
$(NH_2)_{to}$ is the amount of terminal α-amino groups released at the time of fermentation t_0.
$(NH_2)_{HT}$ is the amount of terminal α-amino groups in MixW (without fermenting) obtained after acid hydrolysis.

The acid hydrolysis was carried out according to the method reported by Baek and Cadwallader (1995). The homogenization of mixW in phosphate buffer 0.2 M pH 7 in a ratio 2:3 was carried out using a Waring Blendor and centrifuged. 0.5 ml of the supernatant was placed in a 10 ml glass test tube and mixed with 4.5 ml of HCl 6 N. The oxygen in the mixture was removed by a nitrogen gas stream and the tube was sealed. The hydrolysis was carried out at 100°C for 24 h. Later on the reaction mixture was filtered through paper Whatman No.1 and the supernatant was neutralized by addition of 4.5 ml of NaOH 6 N. The amount of terminal α-amino groups was determined by the TNBS method.

2.7. Determination of protein digestibility in vitro

The *in vitro* protein digestibilities of the samples were determined with pepsin and pancreatin using a modified method reported by Calsamiglia and Stern (1995). The silages were dried and total nitrogen contents were determined by Kjeldahl method (AOAC, 1990). An amount of sample with a content of nitrogen of 15 mg was placed in a centrifuge tube and mixed with 10 ml of (1 $g.l^{-1}$) pepsin solution (Sigma P-7012, Sigma) in 0.1 N of HCl (pH 1.9). The mixture was incubated during 1 h at 38°C with stirring. Further, 0.5 ml of 1 N NaOH and 13.5 ml of 3 $g.l^{-1}$ pancreatin/ phosphates buffer 0.5M pH 7.8 solution (Sigma P-7545, Sigma) were added and incubated at 38°C during 24 h with stirring. After incubation, 3 ml of 6.1 N trichloroacetic acid (TCA) was added to quench the reaction and precipitate insoluble proteins. The samples were vigorously stirred and reacted for 15 min. The digested samples were centrifuged at 10,000 x *g* during 15 min in a refrigerated centrifuge Beckman J2-M1 (Fullerton USA). The total soluble nitrogen was determined in the supernatant using the Kjeldahl method (AOAC, 1990). The protein digested by pepsin and pancreatin was reported as the coefficient of protein digestibility (CPD) using the Eq. (2)

$$\mathrm{CPD} = \frac{\left(\text{soluble nitrogen in TCA}\right)}{\left(\text{total nitrogen in the sample}\right)} \qquad (2)$$

2.8. Statistical analysis

The data obtained were analyzed by one-way variance analysis, with the program NCSS 2001 (NCSS Inc., USA). The variation sources were starters, molasses level and type of fish waste. The difference between the treatments was determined using the test of multiple comparisons of means by Tukey-Kramer with pH, lactic acid, sugar concentrations, water activity (a_w), coefficients of protein hydrolysis and digestibility as response variables at the significant level of $P < 0.05$.

2.9. Estimation of the lactic acid production and sugars consumption with Gompertz model

Gompertz model was applied to determine the constants P_{max}, b and k in the fermentation. These constants were compared in order to select the most efficient starter by the non lineal estimation program STATISTICA (StatSoft, Inc). The product P (lactic acid) is a function of time t according to the Eq. (3)

$$P = P_{max}\, exp\left[-b\, exp\left(kt\right)\right] \qquad (3)$$

where P_{max} is the maximum concentration of product ($t\rightarrow\infty$), b is a constant related to the initial conditions (when t=0, then $P = P_0 = P_{max}\exp(-b)$ and k is the constant of the acidification rate.

The maximum acid production rate ($V_{max,}$) was calculated from the estimated parameters by Gompertz model using the Eq. (4)

$$V_{max} = 0.368\, kP_{max} \qquad (4)$$

Sugar consumption (S) was calculated by the subtraction of initial sugar concentration and sugar concentration at a given time (t). The Gompertz model was also used to estimate the sugars consumption parameters, maximum sugars consumption (S_{max}), b is a constant related to the initial conditions and the sugars consumption rate (k). The maximum sugars consumption rates ($V_{max,}$) were also calculated as it was described above. The yield of lactic acid-sugars was calculated considering the maximum concentration of product (P_{max}) and maximum sugars consumption (S_{max}).

3. Results and discussion

3.1. Effect of sugarcane molasses and type of fish waste in LAF

LAF silage requires the addition of carbohydrates for the growth of the microorganisms due to the low quantities of sugars present in the fish. Among other sources of carbohydrates, the molasses have been extensively used for this purpose due to low cost and availability. The results obtained in this study with LAF silage using the mixture of fisheries processing wastes (MixW) with three levels of molasses 60, 120 and 180 $g.kg^{-1}$ are shown in Fig. 1. The multiple comparisons of means by Tukey-Kramer test

displayed that the LAF with the most relevant acidification profiles were those using inoculum and 180 g.kg^{-1} of sugar cane molasses. The highest acidification was obtained at 48 h when the pH decreased from 6.34 to 4.44, and the maximal production of lactic acid (0.5 mmol.g^{-1}) was attained after 96 h (Fig. 1a and b).

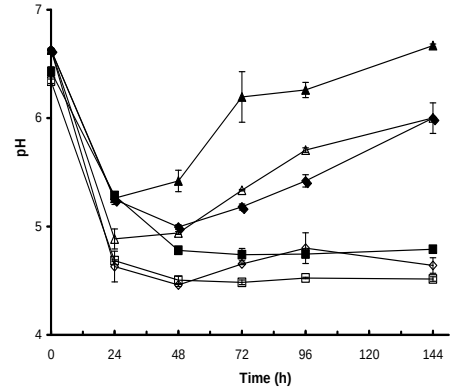
The fermented silage with a level molasses of 60 g.kg^{-1} showed the highest pH values and the lowest acidity. The pH in the control and inoculated samples at 48 h were 5.42 and 4.94, respectively, while lactic acid concentrations were 0.11 and 0.20 mmol.g^{-1}, respectively. A fast pH increase was observed after 48 h (Fig. 1a and b), and the fish silage presented bad odour and rotting. Indeed, fermentations with low levels of molasses showed signs of putrefaction after 48 h, which was caused mainly by the decrement of lactic acid production, as well as the buffer effect by proteins and derived peptides from hydrolysis and the presence of other compounds such as minerals from bones of MixW (Fig. 1b).

The consumption of sugars increased during the first 48 h of fermentation when starter was used, which was in accordance with the maximum lactic acid produced. The carbohydrates were not completely consumed by microorganisms when 180 g.kg^{-1} of sugar cane molasses was used along with the starter. The sugar concentration decreased at 144 h from 88.35 to 12.61 mg.g^{-1} and there was still availability of sugars which promoted bacterial growth and acidification (Fig. 1 c).

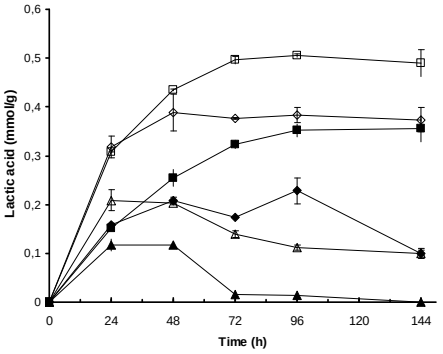
Traditional fermentations usually depend on the microorganisms present in the substrate or in the equipment as well as the treatment used in the process. Herein the use of starter that might ensure a rapid acidification and the prevalence of microorganisms, as well as the adequate sugar cane molasses concentration have been considered, in order to improve the fermentation process even when low quality fish wastes are used.

It has been reported that the amount of sugars and addition of starters are the most important factors for controlling the lactic acid fermentation, however, an extended lag phase was observed in ensilation of shrimp waste due to the decrease in the a_W of the system (e.g. 15% a_W 0.958) when glucose concentration was higher than 10% (Shirai *et al*., 2001). Zahar *et al*., (2002) reported the fermentation of sardine and their wastes without inoculation, where the spoilage microflora was controlled by addition of high levels of molasses with concentrations up to 400 g.kg^{-1}, salt and stirring. Herein, the addition of sugar cane molasses of 180 g.kg^{-1} and starter (50 ml.kg^{-1}) was enough to control the undesirable microflora by driven a lactic acid fermentation of mixture of fisheries processing wastes (MixW).

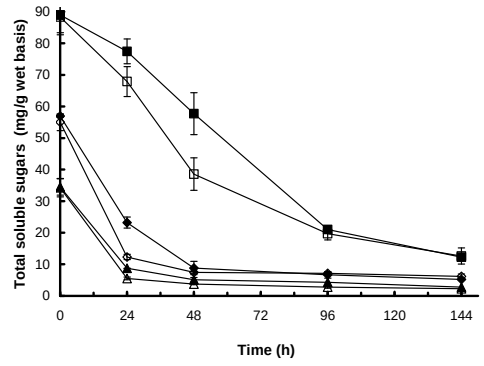
Further experiments were carried out in order to determine the effect of fish waste source. For this purpose molasses level of 180 g.kg^{-1} and *Lactobacillus plantarum* (50 ml.kg^{-1}) were mixed with SC, SB and MixW. The results of pH, lactic acid production and a_W obtained were analyzed by ANOVA and compared by Tukey-Kramer test. The maximum production of lactic acid for the fermentations with different sources of fish waste was estimated by the non lineal model of Gompertz, where the acidification data presented a high correlation coefficient ($R^2 > 0.90$) (Table 1).



a)



b)



c)

Fig. 1. Effect of sugar cane molasses level on fermentation of mixture of fish wastes (MixW) inoculated with 50 g.kg^{-1} (wet basis) of *Lactobacillus plantarum*. a) pH evolution; b) Lactic acid concentration; c) Total soluble sugars. Symbols: Sugar cane molasses level: 60 g.kg^{-1}: ▲ control, △ inoculated; 120 g.kg^{-1}: ◆ control, ◇ inoculated; 180 g.kg^{-1}: ■ control □ inoculated.

Table 1. pH and kinetic constants: *Pmax* (lactic acid), *k* and *Vmax* estimated by Gompertz model of fermentations of fisheries by-catch and processing wastes at 96 h with 180g.kg^{-1} of sugar cane molasses and 50 ml.kg^{-1} (v/w wet basis) of *Lactobacillus plantarum.*

Type of fish waste	*P max* Lactic acid (mmol.g^{-1})	*k*(h^{-1})	V_{max} (mmol.g^{-1}h^{-1})	pH
Mixture of fisheries by-products (MixW)	0.491	0.076	0.01373	4.49 A
Mexican barracuda wastes (SB)	0.414	0.066	0.01006	4.18 B
Shrimp by catch (SC)	0.36	0.3581	0.04757	4.43 A

Values in a column with the same letter are not significantly different ($P < 0.05$).
$R^2 > 0.94$.

Table 2. Kinetic constants estimated by Gompertz model for the acidification and sugar consumption data from fermentations of fish waste (Mix W) with 180 g.kg^{-1} of sugar cane molasses: Control (non inoculated) and 50 ml.kg^{-1} of starters: *Lactobacillus plantarum* and *Lactobacillus* sp. B2.

Condition	*Acid Production*			*Sugar Consumption*			$Y\frac{\text{lactic acid}}{\text{sugars}}$
	P_{max} (mmol.g^{-1})	*k* (h^{-1})	V_{max} (mmol.g^{-1}h^{-1})	S_{max} (mmol.g^{-1})	*k* (h^{-1})	V_{max} (mmol.g^{-1}h^{-1})	
Control	0.34	0.072	0.009	0.43	0.036	0.005	0.792
Lb. plantarum	0.48	0.093	0.016	0.42	0.042	0.006	1.156
Lb. sp. B2	0.54	0.084	0.016	0.37	0.121	0.016	1.437

The lowest pH was attained on fermentations with SB (4.18) and the highest with MixW (4.49) after 96 h fermentation. Despite of higher pH values, silage with the mixture of fisheries processing wastes (MixW) displayed the highest acid production (0.49 mmol.g^{-1}) than the other sources of fish waste (Table 1). This can be explained by the buffering effect of minerals from the bones in the mixture of wastes, since MixW (without fermenting) presented higher minerals content (190 g.kg^{-1} DM), while the minerals content of SC and SB was 180 g and 160 g.kg^{-1} of DM, respectively. Due to abundance and high lactic acid production, the MixW was selected for scaling up experiments.

3.2. Selection of the starter in LAF

Lactobacillus plantarum and *Lactobacillus* sp. B2 were tested in order to select the most efficient starter. The inoculums level was set at 50 ml.kg^{-1} and sugarcane molasses at 180 g.kg^{-1} as carbon source in the mixture of fisheries processing wastes (MixW). The content of lactic acid, and therefore pH among the strains, were significantly different after 48 h of fermentation. The maximum concentration of lactic acid was attained at 96 h.

Lactic acid production and consumption of sugars using both starters and without inoculums were adjusted to the non lineal model Gompertz and they showed correlation coefficients higher than 0.9. P_{max}, V_{max} and *k* values for lactic acid production and sugars consumption increased with inoculum addition (Table 2). $V_{max,}$ of lactic acid were estimated as the same value for both starters (0.016 mmol.g^{-1}h^{-1}). Non significant differences of acidification rates, *k,* were observed. These values were 0.093 h^{-1} and 0.084 h^{-1} for *Lactobacillus plantarum* and *Lactobacillus* sp. B2, respectively.

The control as well as the inoculated silage with *Lactobacillus plantarum* showed similar S_{max} and they were higher than the estimated in the silage with *Lactobacillus* sp. B2 as starter. Nevertheless, P_{max} of lactic acid was higher (0.54 mmol.g^{-1}) using *Lactobacillus* sp. B2 than *Lb. plantarum* (0.48 mmol.g^{-1}). The highest production of lactic acid in silages with *Lb. sp.* B2 was related with the maximum consumption of total soluble sugars. In that sense the yield of lactic acid-sugars was higher with *Lactobacillus* sp. B2 than with *Lactobacillus plantarum* and control. Indeed, these values were 1.437, 1.156 and 0.792, for *Lactobacillus* sp. B2 *Lactobacillus plantarum* and control, respectively (Table 2).

The difference in the consumption of sugars between the control and the starters was observed at 48 h of fermentation when the initial concentration of 0.492 mmol.g^{-1} decreased to 0.317 mmol.g^{-1} for the control, 0.214 mmol.g^{-1} for *Lactobacillus plantarum* and 0.167 mmol.g^{-1} for *Lactobacillus* sp. B2. At the end of the fermentation, the silage inoculated with *Lactobacillus* sp. B2 presented 0.044 mmol.g^{-1} of total soluble sugar with a consumption of 91%. According to these results, *Lactobacillus* sp. B2 was chosen for further scale up.

It has been reported that *Lactobacillus* sp. B2

is a homofermentative microorganism and it acidifies better than other lactic bacteria in shrimp waste silages (Shirai *et al*., 2001). As well, the acid production in the lactic fermentation of fish waste depends on the nature of the substrate, the amount of carbon source and the starter. The parameters of P_{max}, k and V_{max} of lactic acid obtained herein for fish silages were higher to those reported for fermentations of shrimp waste using sugarcane as carbon source (Cira *et al*., 2002).

The inhibitory activity of the lactic acid is important to low the pH by means of their diffusion through the membrane in their not ionized form, and its subsequent dissociation inside the cell causing disturbance in metabolic processes. On the other hand, Gram negative bacteria are more sensitive to low pH values than Gram positive (Jay, 2000). It has also been proved in this work that the time needed to reach the lowest pH in inoculated silages (4.38) were lower than other reports (Zahar *et al*., 2002). These measurements represent an important feature since a fast lactic fermentation avoids undesirable microbial growth due to the inhibition by acid and other compound such as bacteriocins (Vazquez *et al*., 2005).

3.3. Scaling up of the lactic acid fermentation in packed bed column reactor

Once the starter was selected, as well as molasses level, the fermentation of mixture of fish wastes (MixW) was carried out using a packed bed column reactor, which was filled at 13.6 kg scale, added with 180 $g.kg^{-1}$ of sugarcane molasses and 50 $ml.kg^{-1}$ of *Lactobacillus* sp. B2 in wet basis.

The lactic acid production in the silages increased up to 0.46 $mmol.g^{-1}$ at the end of fermentation (240 h). The pH reached the lowest value at 96 h of fermentation (4.30) and it remained with non significant alterations allowing the product conservation (Fig. 2). The sugar consumption was lower in the reactor than in flask cultures, for instance at 96 h in flask the sugar concentration was 19.7 $mg.g^{-1}$ whereas in the reactor was 62.5 $mg.g^{-1}$. Therefore, the lactic acid concentrations in the reactor cultures attained 0.40 $mmol.g^{-1}$, whereas flask cultures attained 0.50 $mmol.g^{-1}$ (Fig. 1b and 2).

3.3.1. Microbiological examination and proximate chemical composition

The microbiological quality of the silage during scaling up was not affected unfavourably, in despite of differences on lactic acid production among flask and reactor cultures as it is shown in Fig. 3. In the microbial counts carried out during fermentation the coliforms decreased significantly, with two logarithmic cycles, during the first 48 h and remained constant until 240 h, whereas the lactic acid bacteria increased 5 logarithmic cycles during 96 h of fermentation (Fig. 3).

The main causes of spoilage of silage is the highly occurrence of fungi during storage. Herein, moulds were not found in the product although the long time of fermentation.

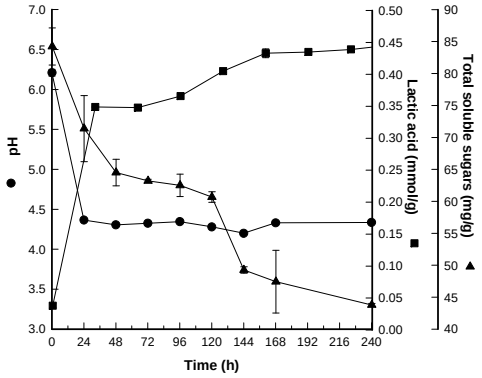


Fig. 2. pH, lactic acid production and sugar consumption during fermentation of MixW with 180 $g.kg^{-1}$ (wet basis) of sugar cane molasses and 50 $g.kg^{-1}$ (wet basis) of *Lactobacillus sp.* B2 as inoculum in 13.6 kg packed bed column reactor.

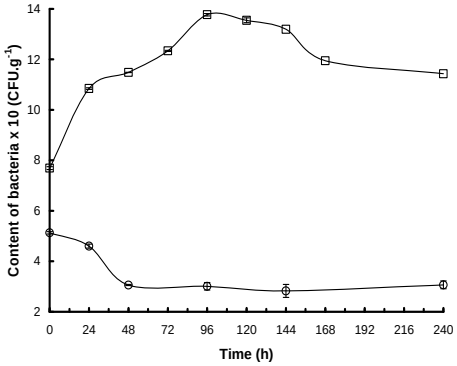


Fig. 3. Coliforms (○) and lactic acid bacteria (□) counts during fermentation of MixW with 180 $g.kg^{-1}$ (wet basis) of sugar cane molasses and 50 $ml.kg^{-1}$ (wet basis) of *Lactobacillus sp.* B2 as inoculum in 13.6 kg packed bed column reactor.

The extension of the shelf life of the fish waste throughout fermentation was due to the acid produced, but also the water content and a_W in the reactor was reduced. In this sense, a drop in a_W was observed from 0.98 to 0.94 at 144 h (Table 3). This can be explained due to proteins hydrolysis during the fermentation, which occurs by the action of endogenous enzymes. This effect increases the concentration of ammonium, amines, amino acids and peptides. These compounds, along with the sugars, suffer from hydration which restricts the water available for biological reactions. Moreover, the counts of lactic acid bacteria increased significantly and the coliforms were diminished thereby fish silage was preserved. In case of shrimp

Table 3. Proximate composition[a] of mixture of fish waste (Mix W): raw and fermented in packed bed column reactor.

Components	MixW	Fermented MixW[c]
Moisture (%)	70.3	65.5
Crude protein (%)	52.4	39.9
Crude lipids (%)	24.5	14.5
Crude fiber (%)	0.51	0.62
Ashes (%)	19	18
N.F.E.[b]	3.6	27
pH	6.49	4.4
Lactic acid (mmol/g)	ND	0.49
Water activity	0.98	0.94

[a]All results are the mean of three determinations, expressed as percentages on dry weight basis.
[b] Nitrogen Free Extract = 100 - (% moisture + % crude protein + % crude fiber + % crude lipids + % ash).
[c] MixW with added 18% (w/w) sugar cane molasses and 5% (v/w) *Lactobacillus* sp. B2 after 144h of fermentation.
ND Non determined

waste silage, which was fermented in similar conditions, the substrate presented a high buffer capacity because of mineral content, thus it reduces the inhibition of the lactic acid bacteria by product accumulation (Cira *et al*., 2002).

The proximal analysis is shown in the table 3. The protein and lipids contents of fermented fish wastes comprised 399 $g.kg^{-1}$ and 140.5 $g.kg^{-1}$ DM, respectively, which means that 76.14% of the total protein and 59.18% of lipids contained in the raw fish waste are recovered throughout the fermentation. Furthermore, no differences were observed in the content of ashes between the fermented and the raw fish waste. Although, the content of carbohydrates was significantly higher in the former due to the addition of molasses. High amount of crude protein and lipids were found in the fermented waste from the raw material. These components can be easily recovered from the wastes to bear an added value product.

3.3.2. Coefficient of protein hydrolysis (CPH) and protein digestibility (CPD)

The protein hydrolysis increased when the time of fermentation increased (Fig. 4). The free α-amino groups reached the maximum content, thus CPH was 0.94 at 192 h, which was significantly higher ($P<0.05$) than the raw mixture of fish processing wastes (0.12) (Fig. 4). The CPH of fish silage was related with the high CPD reached (0.88) and thereby they showed significant differences ($P<0.05$) with the raw MixW (Fig. 4). This value of CPD was obtained at 48 h and it remained almost constant until the end of fermentation.

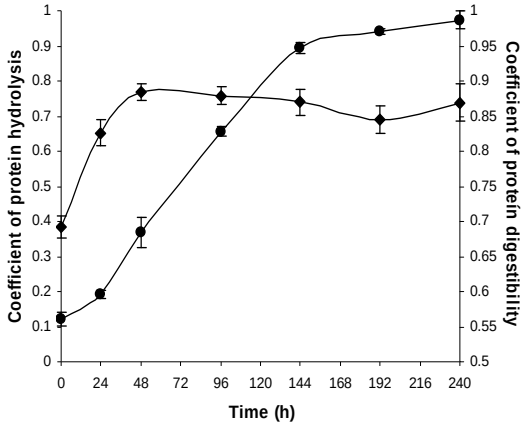


Fig. 4. Coefficient of protein hydrolysis (●) and digestibility (◆) determined with pepsin and pancreatin in fermentation of MixW with 180 $g.kg^{-1}$ (wet basis) of sugar cane molasses and 50 $ml.kg^{-1}$ (wet basis) of *Lactobacillus* sp. B2 in 13.6 kg packed bed column reactor.

The high CPH in the silage obtained is explained by the activity of the endogenous digestive enzymes, especially proteases of the fish that cleave the proteins to low molecular weights (Shirai *et al*, 1997)

Tungkawachara *et al*., (2003) found that CPH was approximately 0.7 in fish sauces using a mixture of hake by-products. Our results were higher, which is attributed to the proteolysis carried out by the endogenous proteases and those secreted by the lactic bacteria during the fermentation (Yin *et al*., 2005).

Analogous reports based on the use of commercial enzymes to produce protein hydrolysates of fish wastes at shorter times have been reported, with CPH comparable to the obtained in this work (Liceaga-Gesualdo and Li Chan, 1999). However, in the fish fermentation the autolysis occurred by the action of its own endogenous enzymes, which has the advantage to avoid the addition of commercial enzymes thus reducing the overall production costs.

The fermented fish waste produced in this work could be used as an alternative as protein source for animal feed, due to its high nutritional value, chemical composition, CPH and CPD. In this sense, feeding assays in several species of monogastric animals have shown the advantage of contained a portion of pre-digested protein in the diet; however there is a limit at which the animals would have difficulties on using the protein absorbed for biosynthesis purposes. High degree of hydrolysis of protein in fish silage might decrease the nutritious value of the food in ruminants, although this problem is not observed in monogastric animals (Espe *et al*., 1992). The determination of protein digestibility *in vitro* allows the estimation of the nutritional value of the ingredients used in the formulation of diets for animals. The protein hydrolysis favors the

digestibility and therefore it is more convenient in the formulation of diets. Also, the release of peptides and free amino acids from the hydrolysis could be potential chemo-attractants as well as nutritious stimulants in carnivorous (Lian *et al*., 2005).

The results allow the estimation of high nutritional value of the by-products from the fishing industry as recovered by means of biological process.

Conclusions

The lactic fermentation of the fish waste was more successful when *Lactobacillus* sp. B2 was used as starter and highly stable product was obtained. The degree of hydrolysis and *in vitro* digestibility of the protein increased when inoculum was added, which means that a product with higher nutritional value than the raw matter was attained.

Acknowledgments

The authors would like to thank to SAGARPA-CONACYT (Mexico) and European Union for research funding No. 2005-1, to PROMEP (Government of Mexico) for scholarship grant to Mr. Ramirez.

References

AOAC (1990). *Methods of Analysis* (15th ed.). Association of Official Analytical Chemist. Washington, D.C.

Anonymous (2003). Anuario estadístico de pesca. SAGARPA. Gobierno de México.

Arason, S. (1994). Production of fish silage. In: *Fisheries processing, Biotechnological applications.* (A.M.Martin, eds.) 244-272. Chapman & Hall, London.

Baek, H.H., Cadwallader, K.R. (1995). Enzymatic hydrolysis of crayfish processing by-products. *Journal of Food Science 60*, 929-935.

Benjakul, S., Morrisey, M.T. (1997). Protein hydrolysates from pacific whiting solid wastes. *Journal of Agriculture and Food Chemistry 45*, 3423-3430.

Calsamiglia, S., Stern, M.D.A. (1995). Three-step *in vitro* procedure for estimating intestinal digestion of protein in ruminant. *Journal of Animal Science 73*, 1459-1465.

Cira, L.A., Huerta, S., Hall, G.M., Shirai, K. (2002). Pilot scale lactic acid fermentation of shrimp wastes for chitin recovery. *Process Biochemistry 37*, 1359-1366.

Coello, N., Brito, L., Nonus, M. (2000). Biosynthesis of L-lysine by *Corynebacterium glutamicum* grown on fish silage. *Bioresource Technology 73*, 221-225.

Dapkevicius, M.L., Nout, M.J., Rombouts, F.M., Houben, J.H., Wymenga, W. (2000). Biogenic amine formation and degradation by potential fish silage starter microorganisms. *International Journal of Food Microbiology 57*, 107-114.

Dubois, M., Gilles, K.A., Hamilton, J.K., Robers, P.A., Smith, F. (1956). Colorimetric method for determination of sugars and related sugars. *Analytical Chemistry 28*, 350-356.

Espe, M.H., Haaland, H., Njaa, L.R. (1992). Substitution of fish silage protein and a free amino acid mixture for fish meal protein in a chicken diet. *Journal of the Science of Food and Agriculture 58*, 315-319.

Gildberg, A. (2004). Enzymes and bioactive peptides from fish waste related to fish silage, fish feed and fish sauce production. *Journal of Aquatic Food Product Technology 13*, 3-11.

Horn, S.J., Aspmo, S.I., Eijsink, V.G.H. (2005). Growth of *Lactobacillus plantarum* in media containing hydrolysates of fish viscera. *Journal of Applied Microbiology 99*, 1082-1089.

Jay, J.M. (2000). *Modern Food Microbiology* (6th ed.). Aspen Publishers, Gaithersburg, Maryland.

Lian, P.Z., Lee, C.M., Park, E. (2005). Characterization of squid-processing byproduct hydrolysate and its potential as aquaculture feed ingredient. *Journal of Agriculture and Food Chemistry 53*, 5587-5592.

Liceaga-Gesualdo, A.M., Li-Chan, E.C.Y. (1999). Functional properties of fish protein hydrolysate from Herring (*Clupea harengus*). *Journal of Food Science 64*, 1000-1004.

Matsumoto, Y., Saucedo-Castañeda, G., Revah, S., Shirai, K. (2004). Production of β-N-acetylhexosaminidase of *Verticillium lecanii* by solid state and submerged fermentations utilizing shrimp waste silage as substrate and inducer. *Process Biochemistry 39*, 665-671.

Plascencia, J.M., Olvera, M.A., Arredondo, J.L.A., Shirai, K. (2002). Feasibility of fishmeal replacement by shrimp head silage protein hydrolysates in Nile Tilapia (*Oreochromis niloticus* L) diets. *Journal of the Science of Food and Agriculture 82*, 753-759.

Ravallec, P.R., Charlot, C., Pires, C., Braga, V., Batista, I., Van, W.A., Le, G.Y., Fouchereau, P.M. (2001). The presence of bioactive peptides in hydrolysates prepared from processing waste of sardine (*Sardine pilchardus*). *Journal of the Science of Food and Agriculture 81*, 1120-1125.

Ray, B. (1996). Probiotics of lactic acid bacteria: Science or myth. En: *Lactic acid bacteria: Current Advances in Metabolism, Genetic and Applications*, (T.F. Bozoglu & B, Ray, eds.), Pp. 100-135. Springer-Verlag, Berlin, Alemania.

Shirai, K., Guerrero, I., Huerta, S., Saucedo, G., Hall, G.M. (1997). Aspects of protein breakdown during the lactic acid fermentation. In: *Advances in Chitin Science*, (A. Domard, F.A.F. Roberts & K.M. Vârum eds.), Pp. 56-63. Jacques André Publisher, Lyon, Francia.

Shirai, K., Guerrero, I., Huerta, S., Saucedo, G., Castillo, A., Gonzalez, R.O., Hall, G.M. (2001). Effect of initial glucose concentration and inoculation level of lactic acid bacteria in shrimp waste ensilation. *Enzyme Microbial Technology 28*, 446-452.

Tungkawachara, S., Park, J.W., Choi, Y.J. (2003). Biochemical properties and consumer acceptance of pacific whiting fish sauce. *Journal of Food Science 68*, 855-860.

Vazquez, J.A., González, M.P., Murado, M.A. (2005). Peptones from autohydrolysed fish viscera for nisin and pediocin production. *Journal of Biotechnology 112,* 299-311.

Vidotti, R.M., Carneiro, D.J., Viegas, E.M. (2002). Acid and fermented silage characterization and determination of apparent digestibility coefficient of crude protein for *Piaractus mesopotamicus. Journal of the World Acuaculture Society 33*, 57- 62.

Yin, L.J., Tong, Y.L., Jiang, S.T. (2005). Improvement of the functionality of minced mackerel by hydrolysis and subsequent lactic acid bacterial fermentation. *Journal of Food Science 70*, 172-178.

Zahar, M., Benkerrow, N., Guerouali, A., Laraki, Y., Yaboudi, K.E. (2002). Effect of temperature, anaerobiosis, stirring and salt addition on natural fermentation silage of sardine and sardine wastes in sugarcane molasses. *Bioresource Technology 82*, 171-176.