ISSN : 0974 - 7451 Volume 6 Issue 6

Trade Science Inc.

Environmental Science

An Indian Journal

Current Research Paper

ESAIJ, 6(6), 2011 [285-291]

Microbiological characterization of fish waste and treatment assay by fermentation

B.Salouhi*, E.Berny, H.Z.Sqalli, M.Ouhssine, A.Chaouch, M.Elyachioui, M.Jadal

Laboratory of Biotechnology, Environment and Quality, Faculty of Science, Ibn Tofail University, P.O. Box 133 Kénitra, (MOROCCO)

E-mail : salouhi1975@yahoo.com; hassan_berny@hotmail.com; sqallizineb@yahoo.fr; ouhssine.m40@gmail.com; a_chaouch@hotmail.com; elyachioui@hotmail.com; m.jadal@hotmail.fr

Received: 3rd June, 2011 ; Accepted: 3rd July, 2011

ABSTRACT

Morocco is one of fishing-producing country. Many production companies of fish are set up in Morocco. These manufacturing's represent a risk for the environment and make human health in danger by producing more and more fish waste. In order to estimate the risks, we have implemented a microbiological characterization of these fish by-products in kenitra city, Morocco. Seven samples of 15 kg were used as a microbiological characterization. The results showed that all the samples present an elevated contamination. Whole Standard Plate Count appears in all samples analyzed with an elevated rate that varies between $1,6.10^7$ and $1,2.10^9$, with an average of $6,8.10^8$ CFU/gram. The coliforms appear with an average of $7,5.10^7$ CFU/gram. For the *Enterococcus*, the analysis showed a rate that varies between 110 to 145 CFU/gram. While Clostridium range from 207 to 680. *Staphylococci* were detected in three samples from seven suspects. *Salmonella* strains were isolated, the Lipolytic microorganisms and Proteolytic microorganisms were respectively $1,5.10^6$ CFU/gram and $2,6.10^6$ CFU/gram. This study reveals that the fish by-products shelter many pathogens species such as *Salmonella*, *Clostridium* and *Staphylococcus*. That's why these underproductive constitute a serious problem upon human health and environment. The adoption of a preventive measures reduce this risk, became an obligation. © 2011 Trade Science Inc. - INDIA

KEYWORDS

Characterization;
Fish waste;
Pathogens;
Health;
Environment.

INTRODUCTION

With 3,500 kilometers of coastlines, Morocco has an important fishery sector, and its exclusive economic zone of 200 miles makes it one of the richest fish reservoirs of the world (1st in Africa and 25th in the world). The annual production is 950 000 tones[1]. Morocco is also the leader exporter country of sardines, especially "*Sardina pilchardus*"[2].

Nearly half of production is devoted to industrial processing (canning industry, semi-conserve, freezing, packaging of fresh fish, fishmeal and fish oil), and the other half quantity is devoted to the local consumption[1].

Faid et al.[10] reported that the fish industry as well as the fish markets, produce a large amount of by-products. Drying units use part of the fish waste for fish meal, but this process is expensive, high energy consuming and requires highly qualified engineers and large amounts of fish waste. Therefore, the biotransformation of local fish wastes, which is a low cost process, can be an appropriate method to obtain benefit from fish waste, protecting the environment and balancing the increasing shortage in the agricultural crops used in animal feeding.

Fish waste may consists of the offals in the canning industry (viscera, heads, damaged and small fishes, strange species etc...). Fish waste may include also wastes of fish markets. In this case deteriorated fish may also be considered as well as altered cans and altered frozen fish. Huss[12] reported that fish is composed of 66 to 81 % of water. The dry matter consists of lipids, proteins and sugars with 67%, 28% and 1.5% respectively. Raa et al.[15] and Cheftel et al.[6] showed that the surface of fish skin is composed of mucus (polysaccharides, amino acids and ethylamine oxide 3), which makes the degradation process of fish by-products fast.

Furthermore, in Morocco, fish by-products are disposed of, like the other waste, in landfills without any treatment. Microbial infections remain one of the most important problems for environment and public health.

The objective of this work is the microbiological characterization of fish by-products by determining the standard plate count (SPC), hygienic flora (*Coliform*, *Enterococcus*, *Clostridium*, *Salmonella* and *Staphylococcus*) and the spoilage flora (proteolytic and lipolytic), to show their impact on the population and the environment, and to propose a treatment assay by biological fermentation. This approach clarifies the importance of biotechnology as a tool for biotransformation by fermentation of fish by-products[9].

MATERIALS AND METHODS

Fish by-products collection and preparation

Fish by-products samples were taken from various sites in Kenitra city (Morocco), with *Sardina pilchardus* as the main species and were the subject of a microbiological characterization. Fish waste may consist of the offals in the canning industry (viscera, heads, damaged and small fishes, strange species etc...). Fish waste may include also wastes of fish markets. In this case deteriorated fish may also be considered as well as altered cans and altered frozen fish.

The samples were placed in plastic bags and transported to the laboratory. Our tests include the qualitative and quantitative study the Aerobic Plate Count bacteria (APC), hygienic flora (*Coliform*, *Clostridium*, *Salmonella* and *Staphylococcus*) and the alteration flora (proteolytic and lipolytic).

Fish waste were ground aseptically with an electric leaf shredder type Moulinex. Ten grams of the extract paste is thoroughly mixed up in an Erlenmeyer flask containing 90 ml of sterile saline water in order to prepare a stock solution of 10^{-1}. From this solution, dilutions were performed up to a dilution 10^{-7}. Cultures were performed from each dilution and for each group of microorganisms.

Microbiological determinations

Ten grams of sample were thoroughly homogenized using 90 ml of sterilized physiological saline water (NaCl, 0.85% g/l). From the 10^{-1} dilution, other decimal dilutions up to 10^{-7} were prepared. Appropriate media were used for enumeration and identification of microflora.

Lactic acid bacteria and standard plate count (SPC) were determined respectively by plating appropriate dilutions on MRS-agar and on plate count agar (Difco, USA). Plates were incubated for 48 h at 30°C. Yeasts are determined by spread-plating on Potato Dextrose Agar (PDA, Difco) were incubated for 48 h at 30°C. Staphylococci was determined on Mannitol Salt Agar (Merck, Germany). Plates were incubated at 37ºC for 24 h. Yellow colonies on the medium were checked for Gram and catalase reactions. Coliforms were determined using Mac Conkey agar (Biokar France), the plates were incubated for 24h at 37°C for total coliforms and at 44ºC for fecal coliforms. *Salmonella* was determined in the raw material and in the final product by transferring 25 g portions into 250 ml flasks containing 100 ml of peptone water. The flasks were incubated at 37°C for 18 h and then 1 ml of the culture was transferred to selenite broth (Merk, Germany) and tetrathionate broth (Merk, Germany). Positive tubes were isolated using Shigella-Salmonella agar (SS) and Hektoen agar (Merck, Germany). Non-colored colo-

nies with and without a dark center were purified and streaked on trypticase soya agar (Biokar, France) slants and stored at 4°C. The enumeration of *Enterobacteriaceae* (*Streptococcus faecalis)* is performed on DCL (Deoxycholate Citrate Lactose Agar) after incubation at 37 °C for 24 hours. *Enterococci* are counted by the most probable number method (MPN) on liquid medium, using three tubes per dilution. Cultures are carried out on Azide Dextrose Broth medium and transferred into Ethyl Azide Dextrose Broth after incubation at 37°C for 24 hours. For *Clostridium sp* identification we used Reinforced Clostridium agar. The initial dilution was heat-activated at 80°C for 10 min and immediately cooled in iced water in order to destroy all vegetative forms and to activate spores. Three tubes containing Reinforced Clostridium agar were inoculated respectively with 1, 2 and 5 ml of the dilution 10^{-1} and incubated at 44°C in anaerobic conditions for 48h, and black colonies were counted.

Proteolytic and lipolytic bacteria were determined respectively, on Nutrient Agar (oxoide) + 1% Skim Milk and Tributyrin Agar (oxoid). Duplicates plates were incubated for 3 days at 30°C. After incubation, colonies had been counted. Colonies producing clear zones of casein hydrolysis on (Nutrient Agar + 1% Skim Milk) were recorded as proteolytic[3]. Lipolytic bacteria were determined on Tributyrin Agar, the medium appears opaque but lipolytic colonies were surrounded by zones of clear medium[5].

Microbiological data were transformed into logarithms to assess the number of colony forming units (CFU/gram). All experiments were conducted in triplicate.

Statistical analysis

Statistical analysis of microbiological and chemical determinations was carried out using Microsoft Excel 7.0 and SAS 6.12 Programmes (SAS Institute Inc, 1990). Data were subjected to analysis of variance (ANOVA). The least significant difference (LSD) procedure was used to test the differences between means at 5% significance level.

Treatment of fish by-products by fermentation

Isolation of lactic acid bacteria and yeast strains

Ten strains of lactic bacteria and yeasts are isolated from different biotopes (cane sugar juice, lemon juice and fermented molasses). Four lactic acid bacteria and six yeasts were isolated. Lactic acid bacteria (SBL_1) and yeast (SBY_1) have shown the most powerful acidifying and fermentation activity, are retained to carry out the fermentation of fish by-products. One the most efficient lactic acid bacteria and yeast strains were chosen for this study. The selection of suitable strains was based on acidity and fermentation proprieties.

Preparation of inoculums

An inoculum has been prepared from lactic acid bacteria SBL_1 and yeast SBY_1 on a semi-synthetic medium (4 g sucrose, 4 g yeast extract, 0.5 g MgSO4, 3g KH_2PO_4, 1 g $(NH4)_2SO_4$, 2ml of mineral solution of Cooney and Levine (1972), qs 1 liter distilled water) containing 2% molasses, incubated for 48 h at 30 °C.

Mixing and inoculation

15 kg of fish by-products are ground and mixed with 15% of molasses. After homogenization they are inoculated with starter containing the lactic bacteria and yeast.

Follow-up of fermentation

The evolution of the fermentation was follow-up for 15 days. Samples are taken daily to determine the pH, acidity and enumeration of microbial flora.

RESULTS AND DISCUSSION

Microbiological analysis

The pelagic industry generates a significant amount of fish by-products. These wastes, like other wastes are often dumped haphazardly in landfills without prior treatment with an impact on the environment. The presence of pathogens is often the cause of serious health problems for the population bordering the landfill.

Our research is the evaluation of risks posed by these by-products by studying their microbiological characteristics.

The analysis of different samples taken from incremental sale outlets the city of Kenitra is made. The results are summarized in TABLE 1.

Aerobic plate count bacteria (APC)

The seven tested samples of fish by-product show

Current Research Paper

TABLE 1 : Microbiological analysis of different samples of fish by-products (CFU/gram)

Germs/samples	APC . ufc/g	Colif. ufc/g	Strep F ufc/g	Staphy. ufc/g	Salmon.	Clostufc/g	Lipoly. ufc/g	Proteoly. ufc/g
1	$1,6.10^{+8}$	$3.0.10^5$	110	$5.0.10^4$	+++	670	4.10^6	1.10^6
2	$6,2.10^{+8}$	$3.1.10^5$	110	$3.2.10^4$	-	680	2.10^6	2.10^5
3	7.10^7	$1.1.10^6$	110	$7.0.\ 10^4$	+++	370	1.10^6	3.10^6
4	$5,5.10^{+8}$	$3.0.10^4$	140	$3.0.\ 10^4$	-	520	7.10^6	1.10^6
5	$2,6.10^{+9}$	$4.7.10^4$	45	$2.1.10^4$	+++	670	1.10^5	2.10^6
6	$2,9.10^{+9}$	$3.0.10^5$	140	$8.3.10^4$	-	207	1.10^6	1.10^6
7	$1,8.10^{+8}$	$2.7.10^5$	145	$1.7.10^4$	-	211	2.10^6	8.10^5
Average	$3.86.10^8$	$3.37.10^5$	114,28	$4,33.10^4$	-	475	$2,44.10^6$	$1,29.10^6$

APC : Aerobic plate count bacteria; Colif : Coliforms; Staph : Staphylococci ; Salmon. : Salmonella; Clostr.: Clostridia ; Strep F: Streptococcus feacalis; Lipoly : Lipolytic; Proteo : Proteolytic.

a high level of aerobic plate count bacteria. It varies from 1.6 10^7 CFU/gram to $1,8.10^8$ CFU/gram with an average of $3,86.10^8$ CFU/gram. This rate remains higher than previous work[10, 11].

Coliforms

The results of enumeration of this flora show that most samples show a coliform load greater than 105 CFU/gram, with an overall average of $3,37.10^5$ CFU/gram.

The TABLE 2 shows that in percent terms 54.16% of coliform bacteria identified were *Escherichia coli*, followed respectively by 9.16%, and *Enterobacter Klebsilla pneumonia* 6.66%, *Schigella dysenteria* 5% and *Citrobacter reundi* and *Citrobacter freundi* with 4.16% respectively, while less than 3% was introduced by *Shigella sp* and *Moraxella*.

TABLE 2 : Species of Enterobacteriaceae isolated from fish by-products

Species	number	%
Echerchia coli	64	54.16
Enterobacter	8	6.66
Kluyvera sp	5	4.16
Klebsilla pneumonia	8	6.66
Proteus merabilis	11	9.16
Schigella dysenteria	6	5.00
Shigella sp	3	2.50
Pseudomonas aeroginosa	5	4.16
Citrobacter freundi	5	4.16
Moraxella	2	1.66
Salmonella thyphi	1	0.83
Salmonella parathyphi	1	0.83
Salmonella enteredis	1	0.83
Total	120	100%

Salmonella

Three species of salmonella have been identified, i.e *S. enteritidis. S thyphi. S. typhoidal*.

Enterococcus

The Enterococcus vary from 110 to 145 CFU/gram with an average load of 114 organisms per gram.

Staphylococci

Staphylococcus load shows a quantitative variation from $1,7.10^4$ to $8,3.10^4$ CFU/gram, with an average of $4,33.10^4$ CFU/gram.

The suspected strains have been subject to an identification using specific identification galleries. The results demonstrated that 10 of 20 suspected strains were identified as *Staphylococcus aureus*. This rate remains lower than staph found in poultry manure by other authors[7, 8].

Clostridium

The tested samples show a significant load *Clostridia*, which varies between 207 and 680 CFU/gram, with an average of 475 CFU/gram which is lower than that found by Rahmi et al.[16].

Flora of alteration

Proteolytic

we note that proteolytic bacteria were disproportionately represented in these by-products of fish. They exceed 10^5 CFU/gram in 75% of the samples with an average $1,29.10^6$ CFU/gram.

Lipolytic

samples tested show a variation from 1.10^5 to 7.10^6

CFU/gram with an average of 2,44.10^6 CFU/gram.

The spoilage flora is of paramount importance since it allows follow up the evolution of the hygienic quality of a product. Indeed, the spoilage flora is involved in two types of risk. They produce toxins that are capable of causing fatalities, and are also behind the production of volatile odors. In fact the by-products of tested fish were spiked with proteolytic revealing that the product was in an advanced stage of degradation. The large load of these lipolytic byproducts of fish is mainly due to the fat in the intestines.

To sum up, this study reveals that the fish by-products are home to several species of bacterial pathogens, and thus constitutes a serious public health problem. Hence, there is a need for effective and sustainable treatment of these by-products. For this purpose, we performed tests of the latter by fermentation using bacteria and yeast with a strong fermentation power and acidification.

Fermentation of fish by-products

Preparation of starter cultures

As was described in materials and methods, we prepared a mixed starter consisting of a yeast strain (SBY_1) and a lactic acid bacterium (SBL_1) having power and acidifying fermentation.

Fermentation test

15 kg of fish by-product chopped, supplemented with 15% molasses, are inoculated with mixed starter (SBY_1 + SBL_1) and they are placed in plastic barrels at room temperature. A void volume was maintained in each barrel to facilitate stirring of the contents and prevent a possible overflow due to gas production during fermentation[13].

The evolution of the fermentation was follow up for 15 days and controlled by physico-chemical and microbiological processes.

Physico-chemical and microbiological analysis

physicochemical analysis

The Figure 1 shows that the pH gradually stripped during fermentation to stabilize at 3.57, while the acidity shows a remarkable evolution. It goes from 0.43 to 1.26% after a week of fermentation, which shows a good acidification during fermentation by the mixed starter selected. This can be explained by the timing of acidifying potential with lactic acid bacteria and yeast.

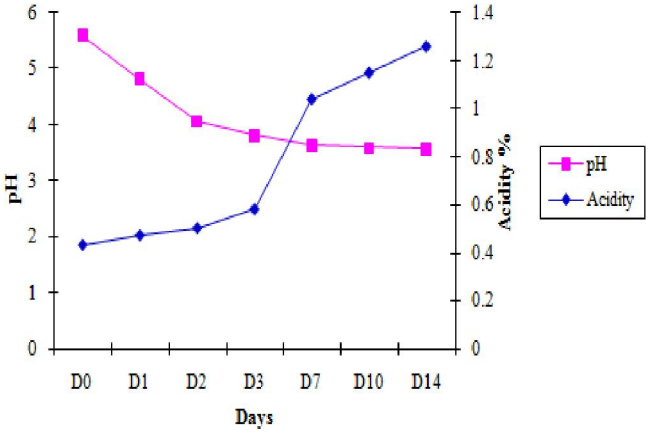

Figure 1 : Evolution of pH and acidity during the fermentation of fish by-products by the mixed starter (SBY_1 + SBL_1)

The results recorded (TABLE 3) show that the rate of dry matter and ash are respectively 20% and 54% while the level of matter of total nitrogen and non-protein nitrogen after stabilizing finished product are significantly higher. The values obtained are respectively 45 and 30% for the finished product.

TABLE 3 : Physico-chemical properties of the obtained product after fermentation of fish by-products

Physico-chemical parametrs	Fish waste before treatment	The obtained product
pH	5.6	3.57
Titratable acidity %	0.43	1.26
Total nitrogen %	3.47	4.73
Organic carbon %	15.60	20.93
Phosphorus %	5.18	8.32
Potassium %	39.12	45.40
Dry matter %	64.07	54.40
Organic matter %	39.20	43.86

Microbiological analysis

The study of the profile of the microbial flora during the fermentation of fish by-products showed a significant reduction of SPC and *streptococci* (Figure 2), and the total elimination of *coliforms* and *Clostridia* at the end of fermentation and stabilizing the pH of the obtained product. Inhibition of plant tissue of interest (Figure 3) as is the case of the spoilage flora are the consequences of the acidification and/or to some inhibitory compounds formed by the lactic acid bacteria. Owens and Mendoza[14] reported that the pathogens (*Salmonella*)

and the toxigenic microorganisms (*Clostridium* and *Staphylococcus*) are sensitive to a low pH. Moreover the fermentation by lactic acid bacteria and yeasts can result in some inhibitors formed by these microorganisms and which may ensure the safety of the product.

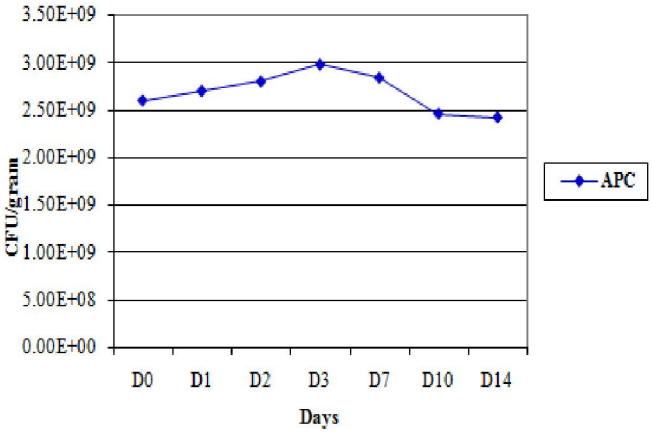

Figure 2 : Evolution of Aerobic plate count bacteria (APC) during the fermentation of fish by-products

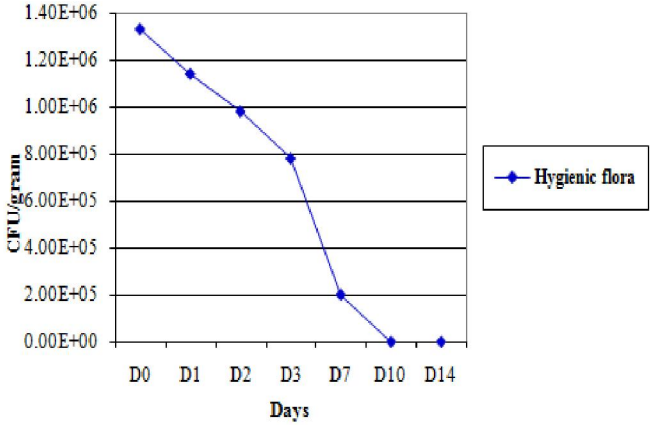

Figure 3 : Evolution of the hygienic flora during the fermentation of fish by-products

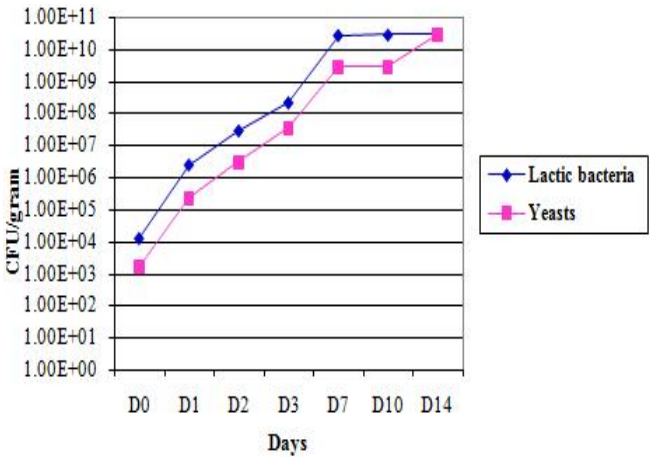

Figure 4 : Evolution of lactic bacteria and yeasts during the fermentation of fish by-products

Moreover, lactic acid bacteria and yeasts have increased substantially (Figure 4). During fermentation, they are rose up from $1,2.10^4$ and $1,5.10^3$ to $2,9.10^{10}$ and $2,98.10^{10}$ respectively, and stabilize on 7th day, which shows the success of the fermentation.

Previous studies have already been conducted in our laboratory in the same way on the Biotransformation of poultry waste and testing of recovery in the animal feed industry[7], municipal solid waste[4], and poultry waste[8] are similar to our results.

CONCLUSION

Fish by-products are home to several species of fish pathogens such as salmonella, staphylococci and clostridia, which poses serious health and environmental problems. The physico-chemical analysis showed that these wastes are rich in minerals and organic matter, hence the need for effective lasting treatment. To do this, we conducted a treatment via fermentation of these sub-products, by using strains of lactic acid bacteria and yeast that have an acidifying and fermenting power.

Our experiments allowed us to obtain a final product rich in proteins and stable in a short time and which has important characteristics. It is free of pathogens, parasites and fungi, and shows a texture, color and remarkable aromatic odor. The latter can be used as an ingredient in food rations (fish farming) or as a biofertilizer for the soil.

ACKNOWLEDGEMENTS

Our sincere thanks to Mr. Boziane.B, for having accepted to test some corn silage by our starter in his farm in El Gara, Morocco.

BIBLIOGRAPHY

[1] O.N.P.Anonymous, U.R.L.Maroc; http://www.onp.co.ma/images/Pdf/direct/Rapport Statistique_jun2006_v.pdf **(2006)**.

[2] O.N.P.Anonymous, U.R.L.Maroc; http://www.maroc-agricole.com. **(2009)**.

[3] APHA (American Public Health Association, Compendium of Methods for Microbiological Examination of Food. 3rd. Edition, Edwards brothers, Washington.D.C., **(1992)**.

[4] A.Benzakour, M Ouhssine, B.EL-Mansouri, M.Elyachioui ; Science Lettrs, **4(1)**, (**2009**)

[5] E.Y.Bridson; The Oxoid Manual, 8th Edition, Oxoid Limited, Basingstoke,UK **(1998)**.

[6] J.C.Cheftel, H.E.T.Cheftel, P.Besançon ; Introduction à la Biochimie et à la Technologie des Aliments. Vol 1. Ed. Technique et Documentation. **20**, 263-283 **(1986)**.

[7] M.H.El Jalil, M.Faid, M.Elyachioui; Cahiers Agricultures, **12(3)**, 175-80 **(2003)**.

[8] L.Elmoualdi, H.Labioui, M.Elyachioui, M.Ouhssine ; Afrique.SCIENCE, **02(1)**, 102-115 **(2006)**.

[9] M Faid, A.Zouiten, Elmarakchi, Achkari-Begdouri; Food Chemistry, **60(1)**, 13-18 **(1997)**.

[10] M.Faid, H.Karani, A.Elmarrakchi, A.Achkari-Begdouri; Cahier Agriculture, **4**, 109-112 **(1995)**.

[11] A.Hammoumi, M.Faid, A.Zouiten, H.Amarouch; Nutrition, **15(1)**, 61-69 **(1997)**.

[12] H.H.Huss; La qualité et son évolution dans le poisson frais. FAO Document technique sur les pêches, Rome, **348**, 198 **(1999)**.

[13] H.Labioui, L Elmoualdi, Y.Benabbou, M.Elyachioui, M.Ouhssine ; Traitement et Valorisation Déchets en Provenance D'abattoirs au Maroc, Agrosolutions juin 2007 **18(1)**, **(2007)**.

[14] J.D.Owens, L.S.Mendoza; J.Food Technol., **20**, 273-293 **(1985)**.

[15] J Raa; A Gildberg; T.Strom; Silage Production-Theory and Practice, Wastes for Feeds and Foods, Nottingham Colloque International, **(1983)**.

[16] M Rahmi, M.Faid, M.Elyachioui, E.Berny, M.Fakir, M.Ouhssine; African Journal of Microbiology Research, **2**, 073-077 **(2008)**.