Chapter 4

VALORIZATION OF MOROCCAN OLIVE CAKE USING SOLID STATE FERMENTATION

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Abstract

Three kinds of olive cakes from Moroccan area were used as substrates for solid state fermentation: 1) Crude Maasra Cake (CMaC) from a traditional unit of olive oil extraction (maasra), 2) Crude Exhausted Cake (CEC) from a semi-traditional unit, 3) Crude Mill Cake (CMC) collected from an industrial unit of olive oil extraction. Biochemical characterization of these olive cakes showed that these raw materials were rich in fibers (cellulose, hemi-cellulose and lignin), 46.8-55.8%. Fat content in CEC was lower (6.9%) than in CMaC (18.4%) and CMC (17.0%). Sixteen fungal strains were isolated from samples collected from olive cakes mainly from CMaC (11 strains). Solid state fermentation was used as a tool to screen these strains in terms of protein enrichment and fiber degradation. The best performances were obtained by the strain *Aspergillus niger* S13. After 48h of fermentation, the maximal real proteins increase (RPI), actual loss in cellulose, actual loss in hemi-cellulose and actual loss in lignin were 9.3%, 19.4%, 22.1% and 4.3%, respectively.

Keywords: Solid state fermentation, olive cake, fungal strains, *Aspergillus niger*, protein enrichment.

Introduction

Solid state fermentation is defined as any fermentation that takes place on solid or semi solid substrate or that occurs in a nutritionally inert solid support which provides to the microorganism access to nutrients, and other microbial products (**1**). Such processes have been employed in the production of useful metabolites such as enzymes (**2**), aroma (**3**), preservation of food (**4**), and proteins (**5**). Protein enrichment of agro-industrial residues makes them suitable for animal feed. Many

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studies were reported on protein enrichment of different agro-industrial residues, which were well documented by Pandey (**6**).

Morocco has an olive oil industry that generates important quantities of olive cake rich in organic matter. The by-products of the olive industry are estimated to be 200,000 tons/year (**7**). A large part of these raw materials are scarcely exploited if at all, and their elimination in nature is a threat to the environment.

Cordova *et al.* (**7**) studied the lipase production by thermophilic fungal strains grown on olive cake. To our knowledge, protein enrichment of this raw matter has not been yet studied.

In this work, we report on the characterization of three kinds of olive cakes, the isolation of fungal strains from natural biotopes and their culture on olive cakes for protein enrichment.

Materials and Methods

Substrates

Three kinds of olive cakes from Moroccan area were used as substrates for solid state fermentation: 1) Crude maasra cake (CMaC) from a traditional unit of olive extraction (maasra). 2) Crude exhausted cake (CEC) from a semi-traditional unit. 3) Crude mill cake (CMC) collected from an industrial unit of olive oil extraction.

Biochemical characterization

Biochemical characterization of olive cakes was determined according to Cordova *et al.* (**7**). The dry matter and relative humidity were determined by incubating the substrate at 105°C for 24 hours. The pH value of ten-fold diluted samples in distilled water was assayed by a digital pH model 215 (Denver Instrument Company Ltd, Norfolk, UK). The Kjeldhal method according to AFNOR Norms (**8**) was used for the determination of total and mineral nitrogen, respectively. For the chemical and physical characterization, the olive cake samples were crushed and sieved to a particle size lower than 1mm. Ashes were determined according to the AOAC method (**9**). Fat was determined according to Soxhlet method (**10**). The parietal constituents, particularly the total fibers (NDF), the ligno-cellulosical fibers (ADF) and the lignin (ADL) were determined by the Van Soest method (**11**). Each analysis was done in triplicate.

Isolation of fungal strains

The isolation of filamentous fungi strains was done from three kinds of olive cakes. Ten grams of each type of olive cake were diluted in 90 ml distilled water with two drops of Tween 80. The solution was shaken for 10 minutes. Then, 0.1 ml was spread on a Petri plate containing Potato Dextrose Agar (PDA) medium (Difco Laboratories, Detroit, USA) supplemented with chloramphenicol (1g/l of medium). Plates were incubated at 25°C. Mycelium morphology and spores color were observed directly under optical microscope. Strains that presented pure mycelia

were isolated in PDA medium. The isolated strains were stored as spore suspensions on PDA medium or on sand.

Culture medium and solid state fermentation

The culture medium used contained in g/l Na_2HPO_4, 3.59, KH_2PO_4, 3.59, $(NH4)_2SO_4$, 6.66, Mg SO_4, 2.9, $CaCl_2$, 2.9 and was sterilized at 121°C for 15 min. Inoculum was produced from purified strains in Petri dishes containing 30 ml of PDA medium and incubated at 28°C for 7 to 10 days. The spores were harvested using a platinum loop in 10 ml sterile distilled water containing 2 drops of tween 80. After dilution, spores were counted using Malassez cell. Solid state fermentation was performed in laboratory columns (4 cm in diameter and 20cm in length with an effective reaction volume of 250 mL) as described by Raimbault (**12**). Columns contained two different compartments, one serving as a humidifying chamber and one reactor column. The substrate previously crushed and sieved was supplemented with culture medium allowing 35% as moisture content and sterilization was done at 121°C for 15 min. The moisture content was then adjusted to 65-70 % by addition of spore suspension containing $2x10^7$ spores/g dry weight of substrate. The inoculated substrate was transferred (30 g per unit) into sterile reactor columns. The whole device was immersed in a steam bath adjusted at the incubating temperature of 27°C. The air-flow was 15 ml/min/column. Respiratory activity was assessed by estimating O_2 consumption and CO_2 production using a chrompack CP 9001 equipped with an electrovalve and a catharometric detector. It was used as an indirect measure of the growth of different strains.

Performance indexes

Four performance indexes were used to evaluate olive cake fermentation: real protein increase (RPI), the actual loss in cellulose, the actual loss in hemi-cellulose and the actual loss in lignin.

Real protein increase (RPI) was calculated according to Durand and Chereau (**13**). This method takes weight loss into account during the culture. The RPI expresses the amount of protein (g) produced per 100 g of initial dry mater.

$$RPI = \frac{M(t) \times Pn(t) - M(i)Pn(i) \times 100}{Mi}$$

On the other hand, the actual loss in cellulose, the actual loss in hemi-cellulose and actual loss in lignin were determined by the gravimetric method of Van Soest (**11**) taking into the count the weight loss during the culture.

$$\text{actual loss in hemi-cellulose} = \frac{M(i)HC(i) - M(t) \times HC(t) \times 100}{Mi \times HC(i)}$$

$$\text{actual loss in lignin} = \frac{M(i)ADL(i) - M(t) \times ADL(t) \times 100}{Mi \times ADL(i)}$$

Where: M(i) is the weight (kg of dry mater) at the beginning of culture; Pn(i) is the initial protein content (% of dry matter); M(t) is the weight (kg of dry matter) at

time t ; Pn(t) is the protein content at time t (% of dry matter); HC(i) is the initial hemi-cellulose content (% of dry matter); HC(t) is the hemi-cellulose content (% of dry matter) at time t ; ADL(i) is the initial lignin content (% of dry matter); ADL (t) is the lignin content (% of dry matter) at time t.

Results and discussion

Biochemical characterization of the olive cakes

Biochemical composition of olive cakes was studied before the fermentation to assess their potential as fermentation substrates and to check the difference between the three kinds of cakes. Dry, organic and nitrogenous matter, crude ashes, fat, hemi-cellulose, cellulose and lignin contents were determined.

All the cakes tested contained about 91% of organic matter and 5% of nitrogenous matters (Table 1). Lignin content in all cakes was high (24-29%). This result showed that olive cake is a high lignin by-product. Fat content of Maasra cake (CMaC) and Mill cake (CMC) were 18.4% and 17% respectively while Exhausted cake (CEC) contained only 7%. This low value is explained by the fact that Exhausted cake is obtained after a second oil extraction from Maasra cake with organic solvent (usually hexane). Thus, the high fat content of CMaC and CMC makes these cakes suitable substrates for the culture of lipase/esterase producing microorganisms.

Table 1. Biochemical composition of the three kinds of olive cakes.

Parameters	Maasra cake CMaC	Mill cake CMC	Exhausted cake CEC
Dry matter	94.70	93.02	97.10
Components		(%) Dry Matter	
Crude ashes	9.05	7.97	8.90
Organic matter	90.95	92.03	91.10
Nitrogenous matter	5.25	5.02	5.18
Fat	18.41	16.98	6.88
Hemi-cellulose	14.53	15.74	17.08
Cellulose	8	10.58	9.78
Lignin	24.25	25.48	28.93

In a previous work, Cordova *et al.* (**7**) showed the potent role of olive cake as a substrate for lipase production. High content of fibers showed that, like many agricultural residues (**14**), olive cake is not attractive as an animal feed.

Isolation of fungal strains

To isolate fungal strains, samples from all cakes were diluted in sterile water containing Tween 80 and the spores collected were spread on Petri PDA medium containing Chloramphenicol to prevent a bacterial growth.

Sixteen non-identified strains were isolated from 30 samples tested. Eleven strains (S3, S4, S10, S11, S12, S13, S14, S15, S16, S17, S18) were isolated from maasra cake and five other (S5, S6, S7, S8, S9) were isolated from the exhausted cake. However, no fungal strain was isolated from the mill cake. The results suggested that the maasra cake was more favorable to contamination by microorganisms than the other olive cakes, probably due to the outdoor storage of this kind of cake.

Olive cake fermentation

The cakes were soaked with culture medium and inoculated with spores from the isolated strains. The inoculated cakes were transferred into columns and incubated at 27°C for 48 hours. Dry matter and pH were measured before and at the end of fermentation and the performance indexes were calculated.

Dry matter evolution

The loss in dry weight at the end of fermentation depended on the strains and the substrate tested. When the strain S13 was grown on the maasra cake dry weight decreased from 94.7 to 85.1 %. The growth of strain S10 on exhausted cakes resulted in the lowest dry weight loss (from 97.1 to 93.6%).

pH evolution

The pH decreased from 6.0 to 4.0 at the end of fermentation for all the strains tested. This acidification may be explained by the production of organic acids during the fermentation (**15**).

Real protein increase (RPI)

The RPI (real protein increase) represents the protein quantity produced in grams per 100 g of substrates. This parameter takes into account the initial protein content and the loss in dry matter during fermentation. The RPI obtained on all cakes tested ranged from 0.9% (strain S16 on CEC) to 9.3% (strain S13 on CmaC) (Fig. 1A). RPI values obtained on all cakes tested were slightly different except for the strains S10, S17 and S18 whose RPI values obtained on CMaC were much higher than those on CEC and CMC. The RPI obtained by the strain S13 was similar to that obtained by *Aspergillus terreus* grown on sugarcane trash (**5**) and that obtained when *Rhizopus oryzae* was grown on cassava (**16**). *Neurospora sitophila* increased the protein content of wheat bran from 13% to 30% (**17**).

Among all the strains tested, 13 strains showed a loss in hemi-cellulose lower or equal to 10% (Fig. 1B). Strains S8, S13 and S18 exhibited high values of hemi-cellulose loss i.e. 20.3%, 22.1% and 23.2% respectively on CEC.

The highest loss in cellulose observed was obtained by strains S8 and S13 on CMaC, 17.1% and 19.4%, respectively (Fig. 1C).

The results suggested that these strains produced an appreciable amount of cellulolytic enzymes. A good degradation of lignin was observed only in cultures of strains S8 and S13: 3.1% and 4.3%, respectively (Fig. 1D). For all other strains, the loss in lignin was very low. Probably these strains did not produce ligninolytic

enzymes. The performance of strains S8 and S13 was lower than that of strain *Polyporus* sp. which degraded 25% of lignin content in bagasse (**18**). In conclusion, the present work clearly indicated that olive cake was a suitable substrate for protein enrichment. Also, cellulose and hemi-cellulose contents in this substrate were reduced by the culture of strains S8 and S13.

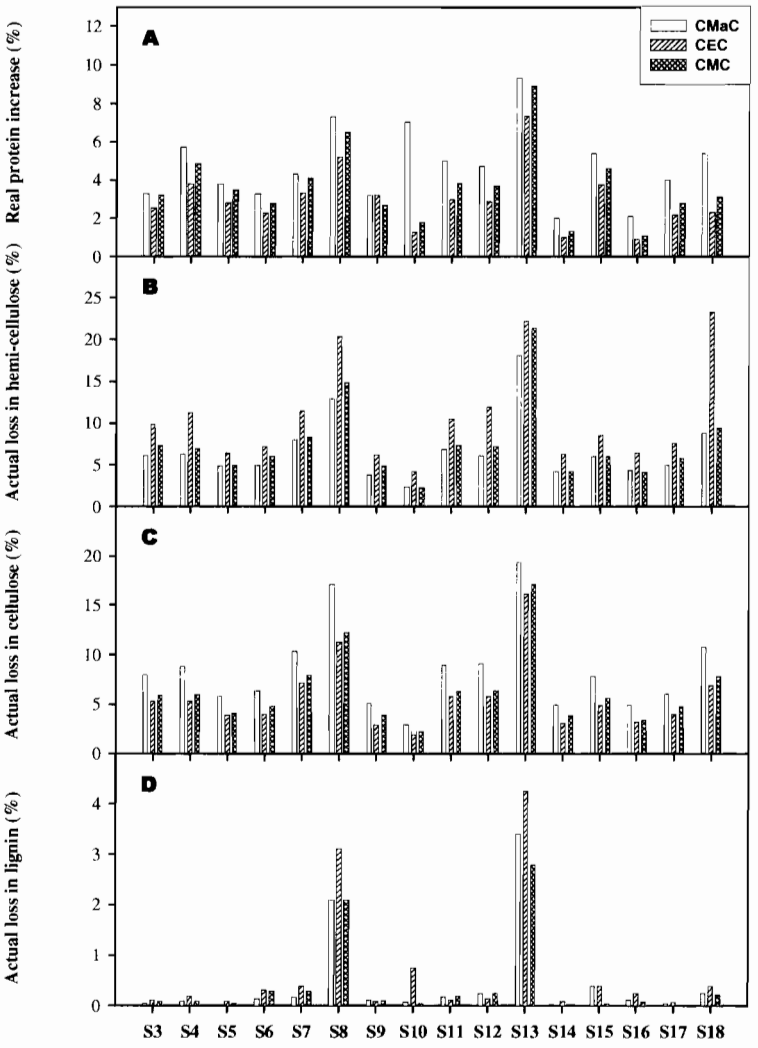

Figure 1. Real Protein Increase (A), actual loss in hemi-cellulose (B), actual loss in cellulose (C) and actual loss in lignin (D) obtained by fungal strains grown on olive cakes.

The strain S13 was identified as *Aspergillus niger*. Digestibility estimated *in sacco* incubation in the rumen of sheep was very high (unpublished data). The results suggested that olive cake enriched with microbial proteins could be a good alternative for production of animal feed. It would therefore be interesting to optimize the protein enrichment of olive cake and to use the potential of the S13 strain to produce other secondary metabolites like aroma compounds and organic acids.

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