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Characterization and valorization of olive pomace for production of cellulase from *Trichoderma reesei* RUT C30 in solid-state fermentation

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ABSTRACT/RESUME

Abstract: *The aim of the present work is to characterize the olive pomace (physico-chemical and microbiological properties) of four regions known by their high production of olive oil in the north of Algeria and to valorize the lignocellulosic biomass as culture substrate for cellulases production using Trichoderma reesei RUT C-30. The results obtained show that the olive pomace is a favorable environment for the growth of fungi. The load of total aerobic mesophilic flora, yeasts and molds varies from 0.45×10^{6} to 1.36×10^{8} CFU/g DM and from 2×10^{6} to 4.76×10^{7} CFU/g DM, respectively. Physico-chemical parameters of the four regions revealed significant differences ($p<0.05$). The determination of filter paper activities of enzymatic extracts reveals that olive pomace from Jijel presented the best activity (0.78 U/gds).*

I. Introduction

World production of olive oil is estimated to be more than 18 million tones / year [1, 2]. In Algeria, the olive grove constitutes about 1.5% of this production [2]. The olive growing is the first national arboreal wealth. It is a source of livelihood for many families. The olive grove occupies 45% of the total arboreal orchard and has 35 million trees of which 80% are intended for the production of olive oil which is estimated between 55000 and 70000 tons/year [3,4] .Waste derived from olive trees and olive oil extractions are generally known as "by-products of the olive", this later is considered as renewable source as patented by Prior Pinto Oliveira et al. [5].

The production of olive oil can generate between 30 and 40% of olive cake depending on the grinding process used [6, 7]. This waste contains an average of 28.5% water, 41.5% solid waste, 21.5% pulp and 8.5% oil. These by-products are stored in the mill's immediate environment before being burned and the vegetable waters are often released into the environment without prior treatment. However, they are toxic and can have a significant impact on terrestrial and aquatic environments due to its high phytotoxicity [8, 9]. This toxicity is explained by the fact that the phenols and lipids contained in these residues decompose at much slower reaction rates compared to other constituents such as sugars and short-chain volatile acids [3]. As a result, these wastes are recalcitrant compounds for natural degradation [9]. However, a large amount of its solid olive oil by-products remains unimplemented because only small amounts are used as natural fertilizers, fuel and feed additives [7]. Therefore, there is a need for guidelines to manage this waste through technologies that minimize environmental problems, improve the economic situation of olive oil producers and lead to sustainable use of resources [8].

Filamentous fungi species are known for their high capacity to secrete large amount of enzymes into their culture medium, making them very attractive

for industrial enzyme production [10, 11]. In general, *Trichoderma* and *Aspergillus* sp have been the most widely used for the production of carbohydrase enzymes, such as cellulases and xylanases [10, 11]. However, a new thermal resistant fungal strain *Lecythophora* sp with excellent cellulases activity has been isolated and patented by Kim et al. [12]. Moreover, cellulases enzymes are widely used in different industries such as textile industry, detergents and laundries, paper and pulp industry and biofuel production [13]. In addition, fungal cellulases also have an interesting role in the food industry, mainly in the production of peptides for the liquefaction of plant cell material, as patented by Van Den Broeck et al. [14].

The present study aims to find a way to make more profitable use of olive cake, using it as a natural fermentation substrate available and abundant for the microorganisms producing enzymes. This work has the following aims: the physico-chemical characterization of olive pomace from four regions and the selection of the region of provenance of the pomace which gives a better production of cellulases.

II. Materials and methods

II.1. Raw material

The raw material used in this study was Olive pomace (OP) provided by four oil factories of Algeria (Tazmalt, Ahnif, Tahir and Tadmait) located in the regions of Bejaia, Bouira, Jijel and Tizi-Ouzou respectively. After the pressing operation, fresh samples were immediately collected. A part of the samples was stored at 4°C for microbial analysis and the remaining quantities were packed in polyethylene bags and kept at -20°C for physico-chemical analysis.

II.2. Microbial characterization of OP

Culturable microorganisms were enumerated by traditional viable cell counts. Ten grams of fresh olive cake were suspended in 90 mL of physiological solution. Serial dilutions of each suspension (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were inoculated on plate count agar (PCA, Laboratorios Conda-Pronadisa, Madrid, Spain) (for isolation of total aerobic mesophilic flora), on Man Rogosa and Sharpe (MRS, Merck KGaA, Darmstadt, Germany) (for isolation lactic bacteria), and on Chloramphenicol Sabouraud Agar at 0.5g/L (Merck KGaA, Darmstadt, Germany) (for microfungi and yeasts isolation) as described by [15]. Data were expressed as colony forming units (CFU)/g of dry matter (DM).

II.3. Physico-chemical characterization of OP

The samples were analyzed for pH in aqueous extracts (1:10 w/v). Dry matter and moisture content were determined by drying OP samples at 105°C for 24 h until reaching constant weight. The crude ash content was estimated by incineration in a muffle furnace (Nabertherm GmbH, Germany) at 550°C for 24h. Total nitrogen was analyzed using the Kjeldhal method. The crude protein contents of substrates were determined by multiplying the nitrogen content by 6.25, according to the method AOAC [16]. Fat content of the OP was determined by Soxtec^{TM}2043 (FOSS, Sweden) Apparatus. The lipid content was determined by weighing the extracts after solvent evaporation**.** Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), and Acid Detergent Lignin (ADL) were assessed using Fibertec 2010 (FOSS, Sweden) apparatus, according to the methods of Van Soest and Robertson [17].Total sugar content was determined using the phenol-sulfuric acid method of Dubois *et al*. [18]. Reducing sugar content was determined using the dinitrosalicylic acid (DNS) method Miller [19]. All measurements were done in triplicate.

II.4. Fungal strain and spore suspension preparation

Trichoderma reesei RUT C30 (a high cellulase producer strain) was procured from the Industrial Microbiology Laboratory of the University of Reims Champagne-Ardenne (France).

The spore suspension was prepared by incubating the cultures on PDA (Potato Dextrose Agar) plates at 30 °C for about 7 days and stored at 4°C. The spores were harvested by washing the Petri plate with 10 mL of sterile water containing 0.1% (v/v) Tween 80 and the obtained suspension was adjusted to the desired concentration 3×10^{7} spores/mL after counting directly with a microscope by using a hemocytometer.

II.5. Enzymes production under solid-state fermentation (SSF)

The SSF process was carried out in 250 mL Erlenmeyer flasks, each containing 5 g of fresh OP the initial moisture content of the media was adjusted to ratio of 1:1 with distilled water and autoclaved at 121°C for 20 min. After cooling, contents of the flasks were inoculated with spore suspension and incubated at 30°C under static conditions.

II.5.1. Extraction of crude enzyme

The enzymes were extracted with distilled water 1:10 (w/v) and homogenized by the Ultra-Turrax

(IKA, T25digital, Germany) for 1 min. The contents of the flasks were then filtered through a metallic sieve, centrifuged (8500 g, 4°C) for 20 min and the clear supernatant was analyzed for cellulases enzyme complex, pH and protein content.

II.5.2. Determination of total cellulase activity

The determination of total cellulase activity *(FPase)* against Whatman N°1 filter paper (W1FP) was based on the I.U.P.A.C. method Ghose [20] as reported by Silveira *et al.* [21]. Total cellulase activity against W1FP was determined by mixing 0.5 ml of 0.05 M citrate-phosphate buffer pH 4.8 and 0.5 ml of the enzyme extract. After 10 min at 50°C, rolled strips of W1FP weighing approximately 50 mg (1.0×6.0cm) used as substrate was added vertically to the test tubes. The assay mixture was incubated without agitation in a water bath for 60 min. Reducing sugars were assayed by 1.5 mL of DNS reagent acid (DNS, Alfa Aesar G.m.b.H., Karlsruhe, Germany) and absorbance was read at 540 nm by UV/visible spectro-photometer (Agilent Technologies Cary 60 UV-Vis, Germany) after placing tubes in a boiling water bath for 5 min. A standard curve of D-glucose was used as reference. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose, from the appropriate substrate, per ml per min under the assay conditions.

II.5.3. Protein assay

Bradford's method [22] was used to determine the protein content in solutions. The enzyme concentrations were determined by comparing against a standard curve. Coomassie brilliant blue (Biochem Chemopharma, Cosne/loire, France) solution was used as a dye reagent. Bovine serum albumin (BSA) (VWR Chemicals Prolabo, Oud-Heverlee, Belgium) was used as a standard to construct the calibration curve.

II.6. Statistical analysis

The physico-chemical results obtained from the four regions were analyzed by ANOVA followed by a multiple comparison of the means using STATISTICA software version 5.5. Chemical analyzes were carried out in three trials and all results were expressed as mean± standard deviation.

III. Results and discussion

III.1. Microbiological characterization of OP

The fresh olive pomace was mainly colonized by the total aerobic mesophilic flora, including lactic bacteria (mesophilic and thermophilic), fungi and yeasts. The results of microbiological analysis of the samples are grouped in Table 1.
This study reveals that this waste contains a fairly large microbial load. This microbial community has a similar trend to that reported by Mennane *et al.* [15]. The total aerobic mesophilic flora of the samples has reached high values (case of Bouira region in this study). According to the authors, It is probably favored by the storage extended at the level of orchards, in contact with the soil and in the exhibition of the microbial contaminants, as well as in the multiplication of microorganisms at the level of the operations of trituration and pressing of olives and prolonged storage in the open air of the pomace in the oil mills [15]. They also found that the olives are loaded with microorganisms such as: yeasts and lactic bacteria (especially lactobacilli) which are of technological interest. Baffi *et al.* [23] found that there is a wide variety of filamentous fungi in substrates that are poorly analyzed at the present time, such as olive fruit, paste and olive pomace. They succeeded for the first time to isolate from the olive pomace species like *Rhizomucor variabilis var. variabilis. Rhizomucor* spp. is often isolated from fermenting and composting organic matter.

III.2. Physico-chemical characterization of OP

The results of the chemical composition of the olive pomace samples show that the average contents found for the four regions are significantly different ($p < 0.05$). Fresh olive pomace from all regions has low protein content and a high fat content (Table 2). It is the OP of Jijel region that has the largest protein and lipid contents (4.31% and 12.33%, respectively). Roussos *et al.* [24] explain that residual fats from OP promote the high production of biomass and enzymes such as lipases. The substrates of the different regions are rich in NDF, ADF and ADL which are approximately of 80%, 70% and 50%, respectively. These results are higher than those reported by Yansari *et al.* [25] (68.9%, 51.2% and 31.3%), and Neifar *et al.* [26] (59 %, 45% and 31%) respectively for NDF, ADF and ADL, in the residues of olive cakes, which they analyzed. The proteins have a low degradability, because 75 to 90% of the nitrogen in the by-products of the olive is linked to the ADF fraction. Olive pomace is particularly rich in lignin, which protects the carbohydrates bound to ADF and lignin [25, 26]. This last limits the rate and extend of enzymatic hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be

hydrolyzed [27]. Analysis of variance showed a significant effect *(P <0.05)* of the region factor on the NDF, ADF, ADL, cellulose and hemicellulose content. It is the Bouira region which displays the highest proportion of Cellulose (28.55%). On the other hand, the Jijel region has the lowest Hemicellulose content.

Table 1. Microbiological characterization of fresh olive pomace from Jijel, Bejaia, Bouira and Tizi-Ouzou regions expressed by CFU/g DM

	Jijel	**Bejaia**	**Bouira**	**Tizi-Ouzou**
Total aerobic mesophilic flora	1.36×10^8	1.50×10^7	Unc	0.45×10^6
Mesophilic lactic Bacteria	3.80×10^7	4.41×10^5	3.00×10^8	2.53×10^6
Thermophilic lactic Bacteria	3.00×10^5	0.70×10^4	1.00×10^5	0.13×10^4
Yeast and Mould	2.25×10^7	4.76×10^7	7.30×10^6	2.00×10^6

Unc: Uncountable.

Table 2. Chemical Composition of the Fresh OP of the Different Regions as % of Dry Matter

Component (% DM)	**Jijel**	**Bejaia**	**Bouira**	**Tizi-Ouzou**
Moisture	37.64 ± 1.47^b	29.24 ± 0.28^c	43.99 ± 0.99^a	44.27 ± 2.13^a
Dry Matter	62.36 ± 1.47^b	70.76 ± 0.28^a	56.01 ± 0.99^c	55.73 ± 2.13^c
pH	6.38 ± 0.10^a	4.57 ± 0.05^c	4.69 ± 0.03^c	5.01 ± 0.05^b
Ash	1.77 ± 0.08^c	2.76 ± 0.22^a	2.21 ± 0.06^b	1.67 ± 0.11^c
Lipids	12.33 ± 0.78^a	7.74 ± 0.48^b	4.54 ± 0.82^d	6.28 ± 0.77^c
Total Nitrogen	0.69 ± 0.05^a	0.43 ± 0.04^b	0.43 ± 0.01^b	0.50 ± 0.05^b
Protein	4.31 ± 0.31^a	2.85 ± 0.36^b	2.71 ± 0.07^b	2.76 ± 0.45^b
Reducingsugar	1.09 ± 0.11^c	2.27 ± 0.05^a	1.89 ± 0.02^b	1.71 ± 0.29^b
Total Carbohydratyes	1.38 ± 0.15^b	2.65 ± 0.30^a	2.50 ± 0.04^a	2.16 ± 0.08^a
Neutral Detergent Fiber (NDF)	78.35 ± 1.29^b	79.06 ± 5.94^b	82.73 ± 1.72^{ab}	87.61 ± 2.80^a
Acid Detergent Fiber (ADF)	72.21 ± 3.12^{ab}	69.72 ± 1.62^{ab}	75.05 ± 6.54^a	65.29 ± 6.26^b
Acid Detergent Lignin(ADL)	53.52 ± 4.45^a	47.58 ± 3.24^{ab}	46.50 ± 7.28^{ab}	42.49 ± 4.44^b
Cellulose	18.69 ± 3.11^b	22.14 ± 1.65^b	28.55 ± 1.34^a	22.80 ± 2.97^b
Hemicellulose	6.18 ± 0.80^c	11.79 ± 1.23^b	9.87 ± 1.77^b	22.42 ± 2.82^a

Values with different letters in the same row (a-d) are significantly different (P<0.05) from each other.

III.3. Enzyme activity *FPase*

Rapid and remarkable growth of the mycelium of *T. reesei* RUT C30 was observed from the first day of fermentation for all regions. In contrast to the total cellulolytic *FPase* activity shown in Figure1, the maximum activity was 0.78 U/gds for the Jijel region followed by Tizi-Ouzou, Bouira and Béjaia (0.39, 0.37 and 0.33 U/gds, respectively). These results allowed us to select the best region to be used later in the rest of the study. This difference in activity between the region of Jijel and the other regions can be explained by its specific chemical composition or by the proportions of remnant olive pulp noted in this residue compared to the residues of the other regions. Given that the chemical composition of olive residue is very variable and depends on factors such as olive variety, maturity stage, soil type, climatic conditions, oil extraction process and solvent depletion [28]. Hendriks and Zeeman [27] mention also that cellulases can get trapped in the pores if the internal area is much larger than the external area, which is the case for many lignocellulosic biomasses, this can be the third cause of this difference of activities.

The production of cellulolytic *FPase* enzymes by *T. reesei* RUT C30 has been the subject of several studies using different substrates [29-31]. However, there are works on the production of enzymes (laccase, xynalase and cellulase) where the substrate used was fresh olive pomace but with another fungus (*Fomesfomentarius)* [20]. Moreover, it is difficult to compare the results of cellulase activities reported by different authors in the absence of standard conditions. Indeed the production depends on several factors, the microorganism in question, the conditions of culture, the dosing methods, etc) [32].

The soluble protein content of enzymatic extracts in the Jijel region increases when the cellulolytic activity rise to reach a maximum value of 5.21 mg/gds at 14 days (Figure 2). The pH evolution shows a stable profile from the 4th day to the last day of fermentation (Figure3).

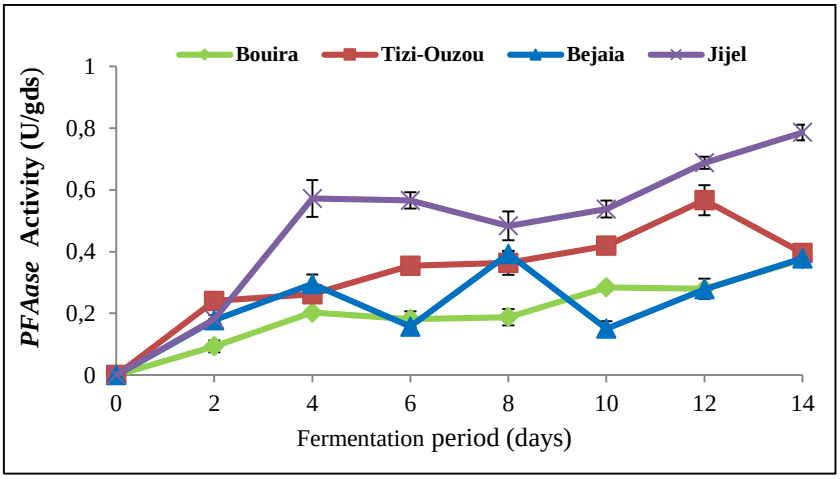

Figure 1. Evolution of *FPase* activity in the culture of *T. reesei* RUT C30 during 14 days of cultivation on fresh olive pomace from different regions

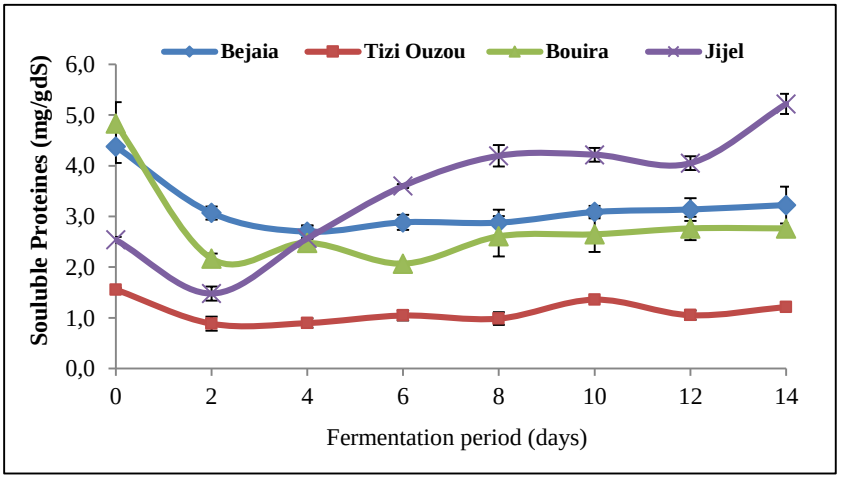

Figure 2. Evolution of soluble proteins in the culture of *T. reesei* RUT C30 during 14 days of cultivation on fresh olive pomace from different regions

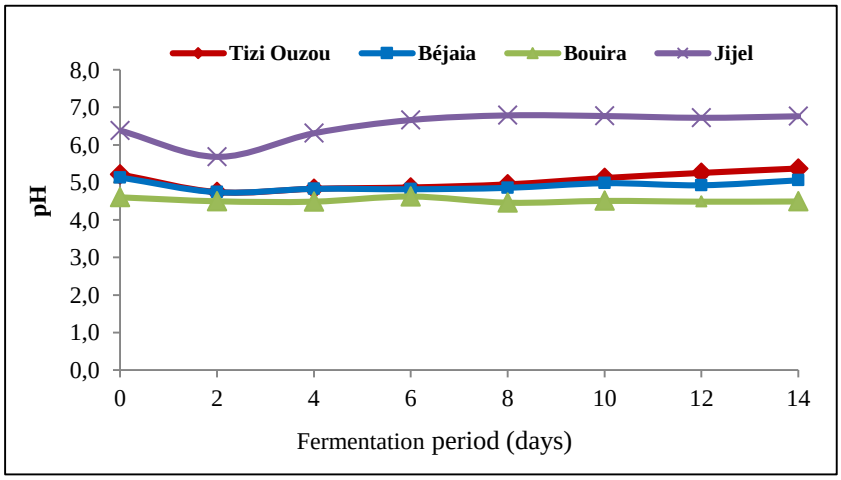

Figure 3. Evolution of pH values in the culture of *T. reesei* RUT C30 during 14 days of cultivation on fresh olive pomace from different regions.

IV. Conclusion

The study carried out on olive pomace, a by-product of the olive transformation industry, has enabled us to develop the following conclusion. The microbiological and physico-chemical characterization of raw olive pomace without pretreatment from different regions of the country is interesting. It can therefore be used as a favorable medium for the development of lignocellulolytic fungi such as *T. reesei* RUT C30 and also as a fermentation substrate for the production of cellulases.

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V. References

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